Chapter 9

Summary
Pompe disease is an inherited metabolic disorder caused by a mutation in the gene of the enzyme acid-α-glucosidase (GAA). This enzyme is responsible for the breakdown of glycogen into glucose in the lysosomal compartment. Deficiency of GAA leads to massive accumulation of glycogen in the lysosomes mainly in the muscle cells. Accumulation of glycogen causes destruction of the muscle cell, ultimately leading to loss of muscle function and finally death of the patient. The nature of the mutation in the gene encoding GAA largely determines the residual enzyme activity and the severity of the phenotype of the disease.

The only available therapy for Pompe disease is enzyme replacement therapy (ERT). This therapy compensates for the missing enzyme by intravenously administration of recombinant produced enzyme. Although ERT extends the lives of patients, the therapy is still hampered by inefficient uptake, high production costs and induction of an immune response. Moreover, ERT does not cure the genetic defect in Pompe disease. An overview of the disease and the treatment options is given in chapter 2.

In this thesis we focused on novel strategies to address Pompe disease in order to circumvent the existing drawbacks of ERT. The strategies presented in this thesis are divided in two groups:

1. Improving the current ERT using the cationic lipid SAINT as delivery vehicle.
2. Correction of the mutation in the acid-α-glucosidase gene using targeted nucleases.

The first strategy has been explored in chapters 3 - 5. In chapter 3 of this thesis, we examined the delivery of different lysosomal enzymes in primary fibroblasts derived from patients with different lysosomal storage disorders. We hypothesized that packaging of the enzyme, using SAINT as carrier, can improve the uptake of the enzyme in the cell. For this purpose, three different lysosomal enzymes were used: acid-α-glucosidase (GAA), α-L-iduronidase and β-galactosidase. Deficiencies of acid-α-glucosidase, α-L-iduronidase and β-galactosidase are involved in Pompe disease, Mucopolysaccharidosis type I, and GM1 gangliosidosis respectively. The biochemical properties of the enzymes with or without SAINT were first examined. Although differences in size and zetapotential were observed between the different lysosomal enzymes, all enzymes could be delivered into the primary fibroblasts as demonstrated by immunohistochemistry or by enzyme activity analysis. For the delivery of GAA, the protein involved in Pompe disease where we were most interested in, we observed more SAINT-mediated GAA delivery in deficient primary skin fibroblasts compared to GAA alone, which was most pronounced 72 h after delivery. Based on this pilot-study we decided to further investigate the ability of SAINT to deliver proteins in other cell types.

In chapter 4, we analyzed the delivery of proteins, antibodies, and restriction endonucleases with or without SAINT in human kidney cells and in mouse fibroblasts cells. We found that purified proteins such as β-galactosidase could be delivered into kidney cells. The delivered β-galactosidase enzyme was properly folded and was able to cleave its substrate beta-gal in kidney cells demonstrating functional delivery. Also proteins such as IgG antibodies could be efficiently delivered into the kidney cells as demonstrated by immunohistochemistry. In addition, mouse
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Fibroblasts cells were used to study the delivery of the restriction endonuclease MunI. For the cells treated with MunI in complex with SAINT, we observed an increase in DNA degradation 48 h after delivery. Treatment of the cells with MunI or SAINT alone did not cause any DNA degradation. Overall, the data presented in chapter 3 and chapter 4 confirmed the versatility of SAINT to deliver its cargo in a variety of cell types in vitro.

In chapter 5, we expanded our research to in vivo studies to further investigate the biodistribution properties of SAINT. We hypothesized that SAINT could deliver its cargo to cells of interest for Pompe disease in mice. For this purpose, small interfering RNA (siRNA) targeted against GAA mRNA were designed and were intravenously injected in complex with SAINT. Lowered GAA plasma levels were observed 14 days after the first injection, indicating the ability of SAINT to deliver siRNA to GAA secreting cells. Biodistribution studies using radioactive labeled SAINT showed that SAINT mainly localized in the liver, suggesting that the liver is involved in GAA production. Unfortunately, we could not identify which liver subtypes were the main GAA producing- and secreting cells. We can conclude from this study that SAINT is able to deliver therapeutic agents in an in vivo setting. This suggests that SAINT is a promising non-viral delivery tool for enhancement of ERT in Pompe disease.

The first strategy described in this thesis, enhancement of ERT using a non-viral delivery system, has great potential for clinical application in the very near future. However, this strategy does not address the genetic defect. In the second part of this thesis, we set out to investigate whether gene correction can correct the single base pair mutation in the GAA gene. Correction of the genetic mutation would lead to a restored production of GAA protein and to secretion of the GAA protein for uptake in neighboring cells. Gene correction aims at permanently modifying the genome via the process of homologous recombination (HR). In mammalian cells, HR occurs at a very low frequency. Induction of a double strand break (DSB) in the DNA can increase this frequency dramatically. Double strand breaks can be induced by restriction endonucleases, which recognize short stretches of DNA sequences. As these recognition sites are abundantly present in the genome, we hypothesized that restriction endonucleases could induce apoptosis after delivery. In chapter 6, we explored the delivery of different types of restriction endonucleases in ovarian cancer cells and analyzed their ability to induce apoptosis. First, we demonstrated nuclear localization by cleavage of genomic DNA after delivery of the restriction endonucleases NucA, BfiI and PvuII in both cell lines. Next, we investigated induction of apoptosis by analyzing the cells for loss of mitochondrial-membrane-potential. From the three restriction endonucleases used in this study, we found that PvuII was the most promising candidate for apoptosis induction in both cell lines. We thus investigated the PvuII-induced apoptosis in more detail. Confocal microscopy confirmed nuclear localization of PvuII after delivery with SAINT. Moreover, Western Blot analysis and apoptosis experiments using a caspase inhibitor further proved that the observed apoptosis is the result of direct delivery of the enzyme into the nucleus and not the result of a secondary process (the caspase pathway). Our data indicate that restriction endonucleases could be efficiently delivered in cancer cell lines using
SAINT and hence looks promising as novel therapeutic tool for the treatment of ovarian carcinomas. As we observed toxic effects in normal cells after delivery of untargeted restriction endonucleases, it is necessary to create GAA specificity using DNA binding domains such as Triple helix Forming Oligonucleotides (TFO) or Zinc Finger Proteins in order to use them for the gene correction strategy described in chapter 7. In this thesis, we used TFOs to target restriction endonucleases to GAA and explored their ability to stimulate HR (chapter 7). TFOs are short synthetic single stranded oligodeoxyribonucleotides that recognize oligopurine•oligopyrimidine regions in double stranded DNA and bind their target DNA in the major groove via Hoogsteen base pairs. In chapter 7, we designed human- and mouse TFOs for acid-α-glucosidase (GAA) and fused the TFOs to camptothecin or the Munl restriction endonuclease. We analyzed these fusions for target binding affinity, for DNA damage, and for HR induction. We found that the human TFO had the highest binding affinity and when fused to camptothecin, less genome wide DNA damage occurred compared to the positive control PvuII, suggesting increased specificity. Using promoterless reporter plasmids as a model system, we could induce promoter activity up to 24% using TFO nuclease compared to promoter containing reporter plasmids. These data indicate that TFO-fusions are promising candidates for gene correction approaches for the monogenetic disorder Pompe disease.

Conclusion
For Pompe disease it is of great importance that new treatment options are being developed for at least two reasons 1) the current ERT is inefficient and 2) ERT provides no cure for Pompe disease. In this thesis we focused on those aspects and developed new strategies to improve Pompe patients by 1) more efficient cellular delivery of ERT by SAINT and 2) development of tools for gene correction of the Pompe mutation. We found that SAINT is able to deliver its cargo in a variety of cell types both in vitro and in vivo and thus is a promising candidate for the enhancement strategy of ERT as well as for the delivery of the gene correction tools. The development of a novel class of targeted nucleases, described in this thesis might open new doors to actually cure the genetic defect in Pompe disease. It is however necessary to further develop both strategies and future research should indicate if the strategies are feasible in a clinical set-up.