Pompe disease
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Chapter 1

Introduction
Chapter 1

POMPE DISEASE: A SEVERE LYSOSOMAL STORAGE DISEASE

Lysosomal storage diseases form a group of more than 50 clinically distinct inherited disorders caused by a deficiency of lysosomal enzymes. Loss of enzyme activity results in accumulation of undegradable substrates in the lysosomal compartment leading to cellular and tissue damage, organ dysfunction and in some cases to death of the patient in an early phase $^{1-4}$. In this thesis, we focus on the autosomal recessive lysosomal storage disease Pompe disease (incidence 1:40,000) which is caused by a genetic mutation in the gene encoding $^{5,6}$ GAA. The disease is characterized by glycogen accumulation in cardiac and skeletal muscle leading to muscle weakness combined with cardiomyopathy resulting in early death of the patient. The disease can be divided in two different phenotypes. The infantile-form is the most severe form having complete deficiency of enzyme activity (< 1% of normal controls). The late-onset form, divided in juvenile and adult forms, are milder variants having partial enzyme deficiency (2-25% of normal controls). The severity of the disease is well correlated with the amount of residual enzyme activity.

PATHOPHYSIOLOGY OF POMPE DISEASE

Acid-α-glucosidase is processed and targeted to the lysosome through several steps. The 110 kDa precursor form of acid-α-glucosidase is posttranslationally modified by glycosylation in the Endoplasmatic Reticulum and subsequent phosphorylation of mannose-6 residues in the Golgi compartment. Mannose-6-phosphate (M6P) residues are required for transport from the Golgi compartment, via the trans Golgi network, to the late endosomes. Finally, the precursor form of acid-α-glucosidase ends up in the lysosome where it undergoes proteolytic cleavage resulting in the 70- and 76 kDa active forms. However, a portion of the precursor form enters the secretory pathway and can be taken up by other cell types via the M6P receptor. This indicates the importance of the M6P residues present on the precursor form and the M6P receptor present on the cell surface for re-uptake and transportation to the lysosome $^7$.

In the lysosomal compartment, acid-α-glucosidase is responsible for the degradation of glycogen into glucose. This degradation takes place when energy is required to maintain blood glucose levels (by liver cells) or to serve as an energy source (by muscle cells). Deficiency of acid-α-glucosidase leads to a massive accumulation of glycogen (Figure 1) mainly in cardiac and skeletal muscle, resulting in severe muscle damage. The progression of the disease was divided in five stages by Thurberg $^8$.

ENZYME REPLACEMENT THERAPY AND ANIMAL MODELS

After characterization of acid-α-glucosidase and the importance of mannose-6-phosphate modifications, researchers focused on obtaining recombinant acid-α-glucosidase with M6P residues via transgenic rabbit milk $^9$ or via production in CHO cells $^{10}$. By intravenously administration of recombinantly produced enzyme (rhGAA) compensation for the deficient GAA enzyme is achieved. The safety and effects of ERT were preclinically tested in several knock-out animal models $^{11-13}$. After
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The promising results, ERT was further tested in a clinical setting. The knock-out animal models developed for investigation of ERT and the clinical studies performed with rhGAA produced in milk of transgenic rabbits and CHO cells have been described in detail in chapter 2.

Although ERT is able to prolong lives of the patients thereby diminishing severeness, some drawbacks hampers the success dramatically. These drawbacks include requirement of high levels of therapeutic enzyme, high costs, low uptake leading to variability of response, life-long treatment to continue effectiveness and formation of antibodies.

GENE THERAPY

To improve the limitations of ERT described above, researchers focused on alternative therapies. Most of the research groups concentrated on gene therapy, which focuses on the delivery of a functional gene to the host cells to produce the functional enzyme. With this approach, a longer survival of the patients is expected compared to ERT. Several research groups studied gene therapy using vehicles such as adenoviral, adenoviral adeno-associated vectors and lentiviral vectors. The therapy appeared to be very promising as an increase in enzymatic activity could be observed in vivo. Moreover, the precursor form of acid-α-glucosidase could be secreted by the liver and could be taken up by skeletal and cardiac muscle, leading to reversal of glycogen accumulation in these cells. As of today, several drawbacks hinders the success of gene therapy, such as long-term expression of the gene, antibody formation against the viral vector and/or acid-α-glucosidase and the safety of the vector.

Figure 1. A. In a normal individual, acid-α-glucosidase (GAA) is synthesized and transported to the lysosomes (red marks) where it is responsible for the degradation of glycogen (blue circles) into glucose (green circles). A portion of acid-α-glucosidase is excreted but can be taken up via the Mannose-6-Phoshate receptor. B. In a Pompe disease affected patient, GAA is absent or non-functional due to a mutation in the gene encoding GAA which leads to lysosomal glycogen accumulation. Enzyme Replacement Therapy (ERT) is the only available therapeutic option (yellow marks) in which recombinant GAA is given to the patients intravenously in order to prevent the process of glycogen accumulation and the subsequent processes of cellular damage and organ dysfunction. (see for color image page 153)
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GENE CORRECTION USING TARGETED NUCLEASES

To truly affect the underlying molecular mechanism in Pompe disease, gene correction therapy might offer novel opportunities. Correction of the mutation can be achieved by the process of homologous recombination, in which the mutated DNA is replaced by correct wild-type DNA. In mammalian cells, this recombination occurs at a very low frequency (1 event per $10^5$ to $10^7$ treated cells). Previously, research groups reported that induction of double strands break at a specific site in the genome, using the homing endonuclease I-SceI, activates the cellular repair system and stimulates the homologous recombination frequency by 1000-fold. A drawback of this approach is, however, that the recognition site of such homing endonucleases needs to be uniquely present in the genome at the site of interest, which is hardly the case. Further research was performed to develop methods in order to create site-specific double strand breaks. Researchers succeeded in the engineering of three so-called targeted nucleases to study the approach of homologous recombination and gene modification at a specific target sequence (Figure 2).

First, Triple helix Forming Oligonucleotides (TFO) coupled to DNA damaging agents or restriction endonucleases can be used as targeted nucleases. TFOs are short synthetic single stranded DNA stretches (20-25 bp) that recognize polypurine:polypyrimidine regions in double stranded DNA. TFOs bind the DNA in the major groove via Hoogsteen hydrogen bonds and always bind to the purine strand of their target. TFOs have been extensively studied for gene targeting approaches using different types of DNA damaging agents to introduce double strand breaks.

Secondly, Zinc Finger Nucleases (ZFNs) can be used for specific gene targeting. Zinc Finger proteins (ZFP) consists of two β sheets and one α helix, which is responsible for binding the DNA in the major groove. One ZFP recognizes 3 bp in the genome, engineering a six-ZFP array (recognizing

![Figure 2. Approaches for specific gene targeting and homologous recombination using targeted nucleases: TFO-nuclease, Zinc Finger Nuclease and Meganuclease. This figure is adapted from 33.](see for color image page 153)
18 bp) has increased affinity and binds unique target sites in the human genome. So far, researchers focused on FokI as restriction endonuclease to induce specific double strand break and studied the use of ZFN for targeted gene correction. At this moment patients are recruited for a clinical trial to investigate the potential of a ZFN to disrupt the CCR5 gene in CD4+ T cells as treatment for HIV/AIDS (http://clinicaltrials.gov).

Meganucleases are also under investigation to study homologous recombination and gene correction. Meganucleases recognize large DNA sequences and induce double strand break at specific sites in the cell. Because the chance of finding a naturally occurring cleavage site in a chosen gene is low, artificial meganucleases are engineered with desired specificities and their ability to induce efficient and specific gene correction was demonstrated in vitro.

Improvements in the three fields of targeted nucleases show great promise to induce site-specific double strand breaks and stimulate homologous recombination for future therapeutic purposes, especially for monogenetic disorders like Pompe Disease.

AIM OF THIS THESIS
The general aim of this thesis is to investigate novel strategies to better treat Pompe disease. First, we review the literature regarding the autosomal recessive lysosomal storage disorder Pompe Disease in chapter 2. It gives an overview of the available knock-out mouse models, the experimental studies performed by researchers using recombinant produced enzyme, the current therapy (ERT) available in the clinic and future therapies like gene correction therapy.

As ERT is hampered by inefficient targeting and uptake, we first aimed to enhance the therapy using the cationic lipid compound SAINT as delivery vehicle (Figure 3). We hypothesized that

**Figure 3.** A. Pompe disease affected cell, GAA is absent or non-functional due to a mutation in the gene encoding GAA which leads to lysosomal glycogen accumulation. Two novel strategies are being investigated in this thesis in order to increase therapeutic options for Pompe Disease. B. The first strategy aims at enhancing the current ERT using the delivery vehicle SAINT. C. The second strategy aims at correcting the genetic mutation using targeted nucleases. (see for color image page 154)
recombinant produced lysosomal enzymes could be more efficiently delivered in patient derived deficient fibroblast cells using SAINT compared to enzyme alone (chapter 3). In chapter 4, we investigated the versatility of SAINT to deliver different proteins to different cell types. To evaluate whether SAINT can deliver its cargo to GAA secreting cells *in vivo*, we explored the delivery of siRNA in order to modulate GAA expression and activity (chapter 5) (Figure 3).

To analyze future gene correction strategies for Pompe disease, several hurdles need to be faced such as the delivery of both the targeted nucleases and homologous recombination fragments and their specificity. As the material of TFO conjugates is scarce, we first investigated nuclear and functional delivery of untargeted restriction endonucleases in several cell lines using SAINT (chapter 4 and chapter 6). In chapter 7 we aimed at investigating the delivery of targeted nucleases and their ability to induce homologous recombination on plasmid level. We set out to demonstrate that delivery of targeted nucleases and a donor fragment leads to efficient homologous recombination events (Figure 3).

Chapter 8 provides a general discussion of the results presented in this thesis and suggestions for future research.
Reference List


