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Exploring RNA interference of TMEM97 as a potential treatment option for Niemann-Pick type C disease

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Niemann-Pick type C disease (NP-C) is a hereditary lipid storage disorder. Mutations in both copies of either the *Niemann-Pick C1* (*NPC1)* or the *Niemann-Pick C2* (*NPC2)* gene result in a progressive accumulation of free cholesterol and further lipids in late endosomes and lysosomes of patient cells. A clinical consequence of this imbalance in subcellular distribution of lipids is neuronal degeneration and premature death due to a progressive organ failure. Until today there is no causative treatment for NP-C. Previous work in the Runz laboratory using tissue culture models could identify the uncharacterized membrane protein TMEM97 as the first NPC1-interacting protein and show that a knockdown of the TMEM97 protein in NPC1-deficient cells counteracts cholesterol enrichment in lysosomes. Aim of this study was to establish efficient RNA interference (RNAi)-based strategies that would allow to specifically reduce TMEM97 protein levels *in vivo* and that could be applied to restore NP-C pathological features in NPC1 mouse models.

In order to translate *in vitro* results to an *in vivo* system, the first step was the design and production of short hairpin RNA (shRNA) oligonucleotides targeting human and murine TMEM97 and NPC1. Based on the reference sequences and by applying bioinformatic tools, sequences considered as a suitable core for efficient shRNA-binding were identified. For each gene six shRNA oligonucleotides were prioritized and designed to match the target sequence with either 19 or 21 nucleotides overlap. The oligonucleotides were subcloned into a modified adeno-associated viral (AAV) vector as a backbone, amplified in *E. coli* and subsequently purified to high yields.

Knockdown efficiencies of the newly designed shRNA oligonucleotides were first tested in different cell culture models using a lipid-based transfection method: 1) two murine cell models, the hepatocyte-like cell line Hepa 1.6 and NIH3T3 murine fibroblasts and 2) selected primary skin fibroblast cultures obtained from NPC-patients and a healthy control. The efficiency of RNAi-based gene silencing was monitored on a transcriptional level by determining target gene mRNA levels using quantitative polymerase chain reaction (qPCR) and on a gene product level via Western Blot analysis.

As expected and as proof-of-concept for the RNAi-strategy chosen, knockdown of *NPC1* with the newly-generated RNAi reagents reconstituted characteristic features of NP-C in the tissue culture cells. Conversely, simultaneous transfection of shRNAs targeting *TMEM97* resulted in a pronounced increase of the NPC1 protein. This reproduced previous findings of the Runz laboratory, yet now with RNAi-reagents applicable for *in vivo* studies.

For safe delivery into a host organism, the most efficient TMEM97 shRNA was transferred into both, an adeno-associated virus (AAV) 2 and AAV8 context, tested again for impact on NPC1 levels in murine and human cells, and purified to the required amounts for subsequent animal studies by external collaborators.

In summary, this work has generated and ensured the efficiency of tools for a safe and persistent reduction of TMEM97 mRNA and protein levels in animal models and primary human tissues. With this, this study paves the way for an exploration of the potential of TMEM97-antisense strategies to increase NPC1 levels *in vivo* and to possibly serve as a basis for future causative therapies of NP-C.