ADDENDUM

Summary in English

The liver plays a major role in the metabolism and has a number of functions in the body, including glycogen storage, decomposition of red blood cells, plasma protein synthesis, hormone production, and detoxification. Familial hypercholesterolemia (FH) is a genetic disorder characterized by high levels of low density lipoprotein cholesterol (LDL-C) and increasing the risk of cardio vascular diseases. FH and many other liver diseases can possibly be treated with RNA interference (RNAi). RNAi is a natural process of regulation of gene expression by binding of small RNA molecules to complementary sequences in the mRNA of a gene and hence inducing its degradation or translational repression. In this thesis, we aimed at developing a safe and robust RNAi-based therapy for FH by inhibiting Apolipoprotein B100 (ApoB). ApoB is a primary component of the low density lipoprotein (LDL), a lipoprotein that transports "bad" cholesterol LDL-C.

In **chapter 2** we tested the inhibitory potential of adeno-associated virus (AAV) delivered shRNA targeting ApoB (shApoB). AAV-shApoB induced a strong knockdown of ApoB mRNA and protein levels which correlated with a reduction in total cholesterol levels and elevated lipid accumulation in the liver. In this study, shApoB was transcribed by the polymerase III (pol III) promoter, which provides high expression levels. This probably resulted in (mild) hepatotoxity, when high doses of AAV-shRNA were used.

Therefore, in **chapter 3**, we explored the possibility of using the weaker pol II promoters to express shRNA. Pol II promoters can also be tissue specific or regulated, which would provide additional safety level for gene therapy applications. However pol II expression of shRNA was not optimal, therefore we developed artificial miRNA as an alternative to shRNA expression. Artificial miRNA demonstrated target knockdown efficiency similar to that of shRNA, while expressing less siRNA molecules.

In **chapter 4,** the long-term efficacy of AAV delivered shRNA and artificial miRNA targeting ApoB (shApoB and miApoB, respectively) *in vivo* was investigated. As additional safety measure miApoB was expressed from the liver specific LP1 promoter. AAV-shApoB and AAV-miApoB provided potent gene knockdown, which resulted in a clear phenotypic effect of cholesterol reduction. Interestingly, the strong ApoB knockdown effect was quickly lost over the time for AAV-shApoB while AAV-miApoB had a more sustained effect. Analysis of shApoB and miApoB revealed differential processing that may have implications for their off-targeting and toxic effects *in vivo*.

The consequences of the differential functioning and processing of shApoB and miApoB were further investigated in **chapter 5** by histological, transcriptome and pathway prediction analysis. Interestingly, AAV-shApoB but not AAV-miApoB increased hepatocytes proliferation and affected genes involved in cell proliferation and death as well as immune response. Additionally, endogenous miRNA profile was differentially affected by both molecules *in vitro* and *in vivo*. In **chapter 6**, inducible

RNAi has been designed and tested as a further step towards safer and robust RNAi gene therapy. The mifepristone-inducible GeneSwitch system was used to express several artificial miRNAs. We optimized the system components in order to minimize basal expression and maximize gene induction rate. *In vitro*, GeneSwitch expressed miRNA demonstrated sequence specific and dose dependent target knockdown indicating that the system can be applied in the future for regulated RNAi expression.

In conclusion, we have shown that RNAi therapy using AAV-delivered artificial miRNA is a promising approach for treatment of FH and possibly other liver diseases. Our mechanistic studies revealed differences in shRNA and miRNA processing and functioning *in vivo.* Finally, our findings have significant impact on understanding and overcoming toxicity and off-targeting related problems of RNAi based gene therapy in the liver using AAV vectors.