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Modulation of gene expression in the liver: towards targeted correction of hyperlipidemia

Vrins, C.

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**Modulation of gene expression in the liver:
towards targeted correction of hyperlipidemia**

Modulation of gene expression in the liver: towards targeted correction of hyperlipidemia

Proefschrift

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“I love deadlines. I like the whooshing sound they make as they fly by.”

-Douglas Adams

Aan mijn familie

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Vrins, Carlos Lambert Juan

Modulation of gene expression in the liver: towards targeted correction of hyperlipidemia

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General Introduction

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1.1 Atherosclerosis.

The prime cause of mortality in the Western world involves cardiovascular diseases (CVD) such as myocardial or cerebral infarction. Illustratively, in the Netherlands, CVD account for 34% of all deaths (Statistics Netherlands CBS, 2002). The underlying process leading to these disorders is the development of atherosclerosis which is characterized by endothelial dysfunction, vascular inflammation, and the progressive accumulation of lipids, cholesterol, calcium, and cellular debris in the intima of the vessel wall (“the plaque”). Atherosclerosis occurs mainly in large and medium sized elastic and muscular arteries and may result in vascular remodeling, acute and chronic luminal obstruction, abnormalities of blood flow and diminished oxygen supply to distal target organs. The processes that contribute to atherosclerosis appear to be multifactorial. At present, the response-to-injury theory in which endothelial injury causes a vascular inflammatory and ensuing fibroproliferative response is most widely accepted ^{1,2}. Identified risk factors that can inflict such an injury include hypertension, elevated blood cholesterol levels, high stress levels, diabetes mellitus, cigarette smoking, obesity, and lack of exercise. Endothelial injury often is accompanied by permeability to lipoproteins and migration of monocytes and T-lymphocytes to the site of injury. The extravasated monocytes differentiate into macrophages and subsequently, start to accumulate lipids from circulating lipoproteins leading to the development of so-called foam cells, a hallmark event in atherosclerosis. With regard to atherosclerosis, high serum lipid levels contribute considerably to the rate and extent of subendothelial retention of lipids and hyperlipidemic patients thus form a high-risk group for cardiovascular diseases.

1.2 Lipid Metabolism

Cholesterol and triglycerides are the most common dietary lipids and essential for membrane structure, synthesis of steroid hormones, vitamins, bile acids, and energy-storage. Although cholesterol is absorbed from the diet, the body itself can produce sufficient amounts of cholesterol and normally contributes over 50% of daily required cholesterol ³. Cholesterol is a vital component of the plasma membranes as it determines the fluidity of lipid bilayers. Cholesterol is also essential in the biosynthesis of vitamin D, bile acids, and steroid hormones

such as testosterone, estradiol, and cortisol. Triglycerides play a central role as energy supply for skeletal and cardiac muscles.

Lipids are transported through the body via blood and lymph. To this end the hydrophobic cholesterol (-esters) and triglycerides are assembled in lipoprotein particles, which are macromolecular complexes with a hydrophobic core containing the cholesterol esters and triglycerides. To render it soluble in an aqueous environment, the lipid core is surrounded by a monolayer of polar phospholipids which also contains unesterified cholesterol and proteins called apolipoproteins (table I). Apolipoproteins serve as ligands for cell-surface receptors and co-factors/inhibitors of lipases and other enzymes.

Table1. Apolipoproteins associated with lipoproteins

apolipoprotein	Lipoprotein association	Comments
apoA1	Chylomicron, HDL	Major protein of HDL, activates LCAT
apoA2	Chylomicron, HDL	Enhances hepatic lipase activity
apoA4	Chylomicron, HDL	Co-factor for LPL activation, may have a role in chylomicron and VLDL secretion and catabolism, activates LCAT
apoA5	HDL	Activates LPL
apoB48	Chylomicron	Exclusively found in chylomicrons, alternative splice product of the apoB100 gene thereby lacking the LDL receptor-binding domain
apoB100	VLDL, IDL, LDL	Ligand for the LDL receptor
apoC1	Chylomicron, VLDL, IDL, HDL	Appears to modulate the interaction of apoE with β -VLDL and inhibit binding of β -VLDL to LRP
apoC2	Chylomicron, VLDL, IDL, HDL	Activates LPL
apoC3	Chylomicron, VLDL, IDL, HDL	Inhibits LPL
apoE	Chylomicron, VLDL, IDL, HDL	Ligand for LDL receptor and LRP

Based on their size, and lipid and apolipoprotein composition, lipoproteins can be categorized into five classes: chylomicrons, very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL), and the high density lipoprotein (HDL). However, heterogeneity exists within each class because of the constant remodeling of their composition, shape, size, and surface charge, and exchange of protein and lipid constituents.

The liver plays a central role in lipid metabolism. Lipoprotein trafficking from and to the liver proceeds along two pathways: the endogenous and the exogenous pathway. Cholesterol transport from peripheral tissues to the liver is often considered as the third pathway.

1.2.1 Exogenous and Endogenous pathway

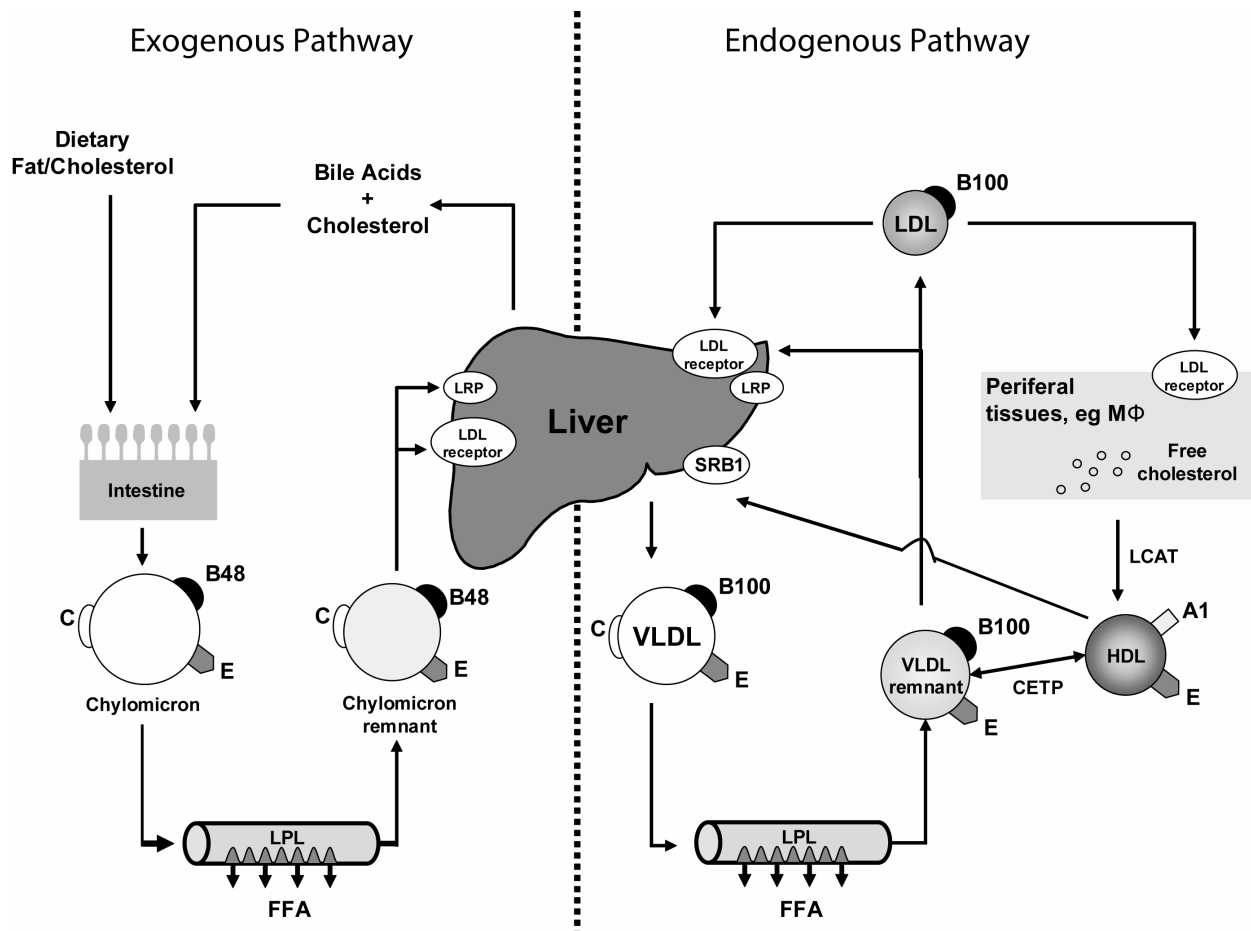


Figure 1. Schematic illustration of metabolic pathways of lipid metabolism. Adapted from Dr. K. Willems van Dijk.

The exogenous pathway involves the uptake of dietary lipids by the intestine followed by their transport through the body via lipoproteins (Fig.1). After the lipid intake, the triglycerides and the cholesteryl esters will be hydrolyzed in the intestine and subsequently assembled into chylomicrons. These large triglyceride-rich chylomicrons containing apoA1, apoA2, apoA4, and apoB48 are secreted into the lymph in order to transport entrapped lipids to the circulation. In the

bloodstream, the chylomicrons exchange apoA1 and associated apoA4 for apoC1, apoC2, apoC3, and apoE with HDL which subsequently results in the formation of the more cholesterol-rich chylomicron remnants due to loss of triglycerides to peripheral tissue. ApoC2 is an activator of the lipolytic enzyme lipoprotein lipase (LPL), which is associated with the capillary endothelium of skeletal muscle, cardiac muscle, and adipose tissue ⁴. ApoC2 aided interaction of chylomicrons with LPL allows the lipolysis of its core triglycerides leading to the formation of free fatty acids which are taken up by surrounding tissue for further use. Lipolysis gradually becomes less efficient due to depletion of the LPL co-activator apoC2. Eventually the chylomicron remnants, enriched in cholesteryl esters, apoB48, and apoE, are eliminated from the circulation by the liver mainly via the uptake by the LDL-receptor which specifically recognizes apoE and apoB100, and by the LDL-receptor related protein (LRP) ^{5,6}.

The liver produces the triglyceride-rich lipoprotein, VLDL, which is introduced into the endogenous pathway. Some of the VLDL contained triglycerides will be derived from internalized chylomicron remnants, but the bulk of the VLDL triglycerides are synthesized de novo in the liver from fatty acids produced from dietary carbohydrate derived Acetyl-CoA ⁷. The formation of VLDL starts with the transfer of lipids towards the major structural protein of VLDL, apolipoprotein B100 (apoB100). This transfer is catalyzed by the microsomal triglyceride transfer protein (MTP), making this enzyme a key factor in the assembly of VLDL. Subsequently before secretion into the blood circulation, the pre-VLDL particle acquires apoC1, apoC2, apoC3, and ApoE. ApoE plays a critical role when it comes to secretion of VLDL and clearance of VLDL remnants, ⁸. Similar to chylomicrons, VLDL transports triglycerides to skeletal muscle, cardiac muscle, and adipose tissue and also serves as substrate for LPL lipolysis in the circulation. As a result, VLDL particles gradually reduce in size due to hydrolysis of the triglycerides leading to the formation of the more cholesterol-rich IDL. Small sized VLDL and IDL are also referred to as VLDL remnants. These remnants are partially cleared by the liver through an apoE-mediated process while the remainder can be converted to LDL ⁹⁻¹¹. The conversion to LDL is accompanied not only by a further loss in triglycerides but also by the depletion of apoE and the apoC's. With apoB100 as sole apolipoprotein constituent of LDL, LDL can be cleared by the liver or taken up by peripheral tissue via the LDL receptor. High levels of LDL and/or VLDL remnants are considered as important risk factors for atherosclerosis ¹², and are therefore also termed atherogenic lipoproteins.

1.2.2 Reverse cholesterol transport

While the first two pathways focus mainly on the transport of triglycerides from intestine and liver to peripheral tissue via apoB-containing lipoproteins followed by the clearance of cholesterol-rich lipoprotein remnants, the third pathway involves the net transport of (excess) cholesterol from peripheral tissue back to the liver where it can be removed from the body via the bile by conversion to bile acids or as biliary cholesterol (Fig 1). The reverse transport of cholesterol is mediated by the lipoprotein HDL, of which the metabolism is complex and only partially understood. HDL is a heterogeneous class of lipoprotein particles existing of different subfractions that differ in e.g. apolipoprotein and lipid composition, size, density, and even physiologic function. However, apoA1 plays a key role in the biogenesis and function of all HDL subclasses^{13,14}. Generation of HDL starts with the intestinal and hepatic synthesis and secretion of lipid-poor apoA1. This ApoA1 ‘particle’ acquires phospholipids and unesterified cholesterol from cell membranes and plasma lipoprotein to convert into a discoidal pre β -HDL particle. Both nascent pre-HDL particles are potent cholesterol acceptors and take up free cholesterol from peripheral cells and, importantly from an cardiovascular point of view, macrophages. This cholesterol efflux is mediated by the integral membrane protein ATP-binding cassette transporter A-1 (ABCA1), which actively transports cellular cholesterol (and phospholipids) to the nascent HDL^{15,16}. The importance of ABCA1 in reverse cholesterol was established in patients with Tangier disease, who are characterized by HDL deficiency. This disease is caused by a dysfunctional ABCA1 and results in massive accumulation of cholesterol in tissue macrophages and prevalent atherosclerosis¹⁷⁻¹⁹. Cholesterol taken up by the nascent HDL particles will be esterified by the enzyme lecithin:cholesterol acyltransferase (LCAT), transforming nascent pre β -HDL into larger spherical HDL-particles. Subsequently, the latter HDL particles are subjected to remodeling after interaction with other plasma lipoproteins or tissues. For instance, the cholesteryl ester transfer protein (CETP) catalyzes the exchange of cholesteryl esters for triglycerides with apoB-containing lipoproteins. The uptake of HDL cholesterol by the liver is mainly mediated by the scavenger receptor class B1 (SR-B1). Once in the liver, cholesterol can be re-used for lipoprotein assembly, used as substrate for bile acid synthesis, or secreted directly as free cholesterol into the bile.

Although during the last years classifications of the different stages of HDL have become as intricate as the particle itself due to the various compositions, sizes, functions, ect., it is becoming increasingly clear that HDL plays a significant role in cholesterol homeostasis. Particularly the role of HDL in reverse cholesterol transport and the athero-protective characteristics of this particle are widely accepted ²⁰.

1.3 Genetic factors in dyslipidemia

The expression of lipid-related genes under normal conditions is tightly coordinated in order to maintain lipid homeostasis. Unfortunately, in western societies and in emerging economies a large part of the population suffer from a perturbed lipid homeostasis resulting in unbalanced plasma levels of the different lipoproteins and an increased incidence of coronary events and cardiovascular deaths. Although factors such as a high cholesterol consumption and increasing age are important, undesirable lipoprotein profiles are also determined by genetic factors such as dysfunctional genes.

Familial hypercholesterolemia (FH), one of the most common causes of hypercholesterolemia, was the first genetic disease of lipid metabolism to be characterized ^{21,22}. Patients suffer from elevated plasma levels of LDL-cholesterol caused by mutations in the LDL-receptor gene. To date, well over 500 mutations of the LDL-receptor have been identified ²³. The severity of the hypercholesterolemia depends on the nature of the molecular defect and whether the mutation is homo- or heterozygous. Patients with homozygous FH develop significant atherosclerotic heart disease at a relative early age ²⁴. The clinical prognosis of patients with heterozygous FH is related not only to the magnitude of the elevation in plasma LDL-cholesterol levels, but also to the presence of other cardiovascular risk factors ²⁵.

A disease that shows similarity with FH in that it also results in a reduced uptake of LDL by the LDL-receptor is familial defective apoB100 (FDB). This disease is caused by several mutations in apoB100 at the postulated binding site to the LDL-receptor ²⁶⁻²⁸. However, the phenotype of patients homozygous for FDB generally is less severe than that of FH patients ²⁹. The latter is the result of a reduced formation rate of LDL particles in the plasma, since FDB patients are still able to clear the LDL precursor particles (VLDL, IDL, and their remnants) via apoE mediated uptake ³⁰.

Polymorphisms in lipid related genes, such as apoE, also predispose to cardiovascular diseases. Three major apoE isoforms, E2, E3, and E4, are encoded by three common alleles at the apoE gene locus: $\epsilon 2$, $\epsilon 3$, and $\epsilon 4$ ^{31,32}. The apoE2 isoform is associated with lower and that of apoE4 with elevated total plasma- and LDL-cholesterol levels than the more common apoE3 isoform. These three isoforms differ in their affinity for the LDL receptor which can influence the serum profile and levels of apoE containing lipoproteins. For example, the majority of patients with type III hyperlipidemia, which is characterized by elevated levels of cholesteryl ester enriched remnant particles, appear to be homozygous for apoE2 ³³. The apoE4 allele, which is receiving great attention due to its linkage to Alzheimer's disease, is associated with an increased prevalence for cardiovascular disease especially in populations consuming diets rich in saturated fat and cholesterol ³⁴.

Many other, mostly rare, single lipid-related gene mutations such as the ABCA1 mutations found in patients with Tangier disease discussed earlier, have been described. However, these and the aforementioned examples are of monogenic nature and, with the exception of FH, show a low frequency. Dyslipidemia being a multifactorial process, the major portion of this predisposition is polygenic, reflecting cumulative effects of multiple genetic sequence variants. These hereditary factors are difficult to identify since possible dyslipidemia phenotypes are the result of complex interactions between genetic and environmental factors (ref, Corella D and Ordovas JM, 2004).

1.4 Central role of the liver in lipid metabolism

As indicated above, maintenance of lipid homeostasis is the sum of numerous complex physiological processes both at an organ, and at a cellular level. The organ that plays a key role in the synthesis and catabolism of lipids is the liver (Fig.2). It expresses enzymes that are responsible for synthesis (e.g. fatty acid synthetase and HMG-CoA reductase) of triglycerides and cholesterol (esters). Excess of cholesterol can be metabolized in the liver via enzymes such as cholesterol-7- α -hydroxylase and sterol 27-hydroxylase (cyp7A1 and cyp27, respectively), and removed from the body via bile acid synthesis. Further, it is involved in assembly and secretion of the main lipoproteins, i.e. VLDL and HDL, and the elimination of serum lipoproteins via lipoprotein receptors. Most, if not all, apolipoproteins involved in the metabolism of lipoproteins

are expressed in the liver. In order to prevent high lipid levels, which correlate with increased risk of cardiovascular disorders, it is essential that the expression of lipid-related genes in the liver is well orchestrated. Several aspects and processes will be highlighted below.

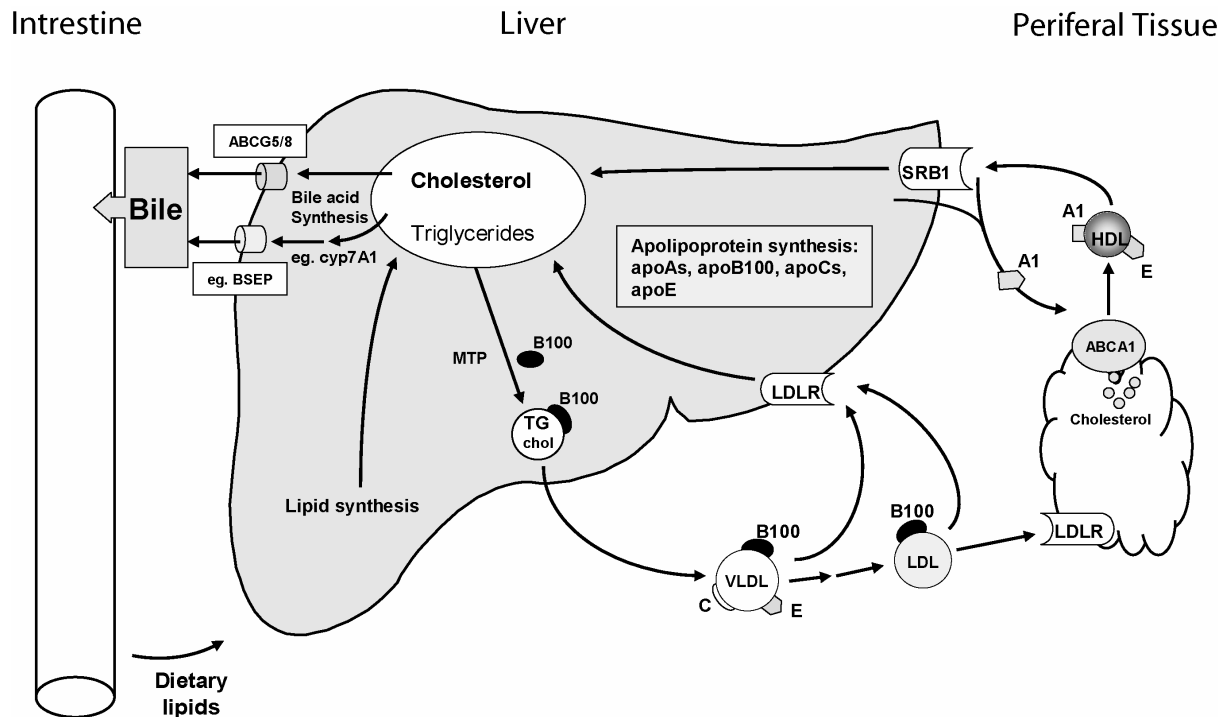


Figure 2. The liver plays a central role in maintaining lipid homeostasis.

1.4.1 Lipoprotein synthesis

Via the formation of triglycerides-rich lipoproteins in the liver, lipids are distributed throughout the body. Hepatic assembly and secretion of apoB-containing VLDL requires a temporary pool of lipids to form the monolayer surface (mainly phosphatidyl choline and cholesterol) and the neutral lipid core, the production of the structural protein apoB, and the rate limiting enzyme MTP^{35,36}. The lipid pool used for VLDL synthesis is fed by two sources: uptake of dietary lipids and de novo synthesis.

Important rate limiting enzymes for the synthesis of fatty acids are acetyl-CoA carboxylase and fatty acid synthase, while the enzymes HMG-CoA synthase and HMG-CoA

reductase are major check points in cholesterol synthesis^{37,38}. The availability of lipids and rate of lipid biosynthesis, lipogenesis, influence the rate of VLDL assembly and secretion.

The structural protein of VLDL is apoB. ApoB exists in two forms: the full length apoB100 and a truncated protein that contains only 48% of the full length version, apoB48. ApoB48 is the result of post-transcriptional modification of apoB100 mRNA by a cytidine deaminase (apobec1) containing enzyme complex^{36,39,40}. In humans, apobec1 is solely active in the intestine, and unlike rodents, no hepatic apobec1 expression can be found⁴¹. Hence, VLDL particles synthesized in human liver only contain apoB100. Although apoB100 is the major apolipoprotein of VLDL, a significant portion of newly synthesized apoB is degraded intracellular via several possible proteasomal and non-proteasomal processes prior to secretion⁴²⁻⁴⁴. Studies showing that an increased apoB100 secretion is not accompanied by a detectable change in apoB mRNA, support the notion that apoB100 production is regulated at a post-transcriptional level by means of degradation⁽⁴⁴⁻⁴⁶⁾. The underlying mechanisms of degradation and its regulatory control have not been completely elaborated, but it has become clear that premature degradation can be prevented by translocation across the endoplasmic reticulum (ER) and subsequent lipidation of the nascent apoB100. The protein crucial for these processes is MTP, which resides in the lumen of the ER as a heterodimer with the ubiquitous ER-enzyme, protein-disulfide isomerase. The latter seems to be required by MTP for its solubility, ER retention, and for lipid-transfer-activity of MTP^{47,48}. In the ER, MTP binds to newly synthesized lipid-poor apoB and facilitates the transfer of triglycerides, cholesterol esters, and phospholipids. Recent studies indicate that during the lipidation process the affinity of MTP for apoB decreases, allowing at a certain point the dissociation of MTP from ApoB, and the formation of a secretion-competent primordial VLDL⁴⁹. Based on findings with conditional liver-specific MTP gene knockout mice, specific MTP inhibitors, and hepatic MTP overexpression in mice, MTP is considered to be the rate-limiting enzyme in the VLDL assembly/secretion pathway⁵⁰⁻⁵³.

1.4.2 Gene clusters of essential apolipoproteins

After lipidation and upon secretion, the nascent VLDL acquires apoEs and apoCs in the bloodstream and matures into an apoE, apoC1, apoC2, apoC3 containing VLDL with a single copy of apoB100. Interestingly, all of these apolipoproteins, except for apoB100, are genetically

located in two gene clusters: the apoE/C1/C4/C2 and the apoA1/C3/A4/A5 gene cluster (Fig. 3). Besides having their own proximal promoters the genes in each cluster share common regulatory regions and therefore are under a tight transcriptional control^{54,55}.

apoE/C1/C4/C2 cluster



apoA1/C3/A4/A5 cluster

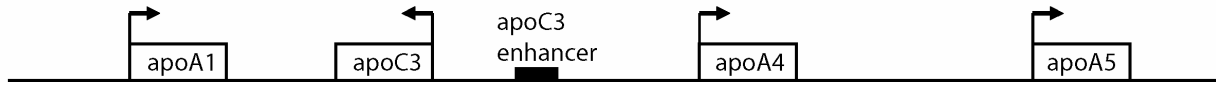


Figure 3. A schematic of the apoE/C1/C4/C2 and the apoA1/C3/A4/A5 gene clusters. The genomic organization of the apoE/C1/C4/C2 gene cluster is largely conserved between mouse and human with the exception that in mouse there is no duplication of the genomic region containing ME.1, apoC1, and HCR.1. In humans, this duplication gives rise to ME.2, the pseudogene apoC1', and the HCR.2.

In the human apoE/apoC1/apoC4/apoC2 gene cluster common enhancer regions called hepatic control regions-1 and -2 (HCR-1 and HCR-2) have been identified, which appear to be necessary for a coordinate liver-specific expression⁵⁶. The HCR-2 has 85% sequence identity to the HCR-1, and is likely the result of an evolutionary duplication of the genomic region containing apoC1 and HCR-1 since the conserved gene cluster in mice lacks HCR-2^{57,58}. Although each HCR can individually orchestrate the expression of all four genes, it seems that HCR-1 has a dominant effect on the expression of apoE and apoC1, while vice versa HCR-2 controls the expression of apoC4 and apoC2. Removal of both control regions, however, almost completely ablates the expression of all four genes⁵⁶. Recently two homologous enhancers, distinct from the HCRs and designated multienhancer-1 and -2 (ME.1 and ME.2), have been identified in the apoE/apoC1/apoC4/apoC2 cluster⁵⁹. Both enhancers were found to be involved in the control of gene expression in non-hepatic cells such as macrophages and adipocytes^{60,61}.

For the apoA1/C3/A4/A5 gene cluster coordinated expression is more complicated, but several studies have established that the hepatic transcription of the first three genes in the gene

cluster are regulated by a common enhancer located upstream of the apoC3^{54,62}. Although the first characteristics of the proximal promoter of the most recently identified member of the gene cluster, apoA5, have been described, its control has to date not been linked to the upstream members of the gene cluster⁶³

1.4.3 Hepatic uptake and removal of cholesterol

As described above, cholesterol-rich remnants and HDL are cleared from the plasma via hepatic receptors. The main receptors responsible for the uptake of chylomicron- and VLDL-remnants, and LDL are the LDL receptor and LRP^{64,65}. Given the importance of the reverse cholesterol pathway in lipid homeostasis, the identification of the HDL-receptor, scavenger receptor class B type 1 (SR-B1), was a breakthrough. SR-B1 is a cell-surface transmembrane protein which functions as a high affinity receptor for HDL⁶⁶, but also can bind a wide variety of other ligands including LDL and VLDL⁶⁷. Not only does SR-B1 bind HDL, but it also mediates selective lipoprotein cholesterol uptake⁶⁸. This selective uptake involving cholesterol delivery from plasma HDL to the liver without particle degradation does not require co-factors^{69,70}. Upon cholesterol uptake, the cholesterol can be stored in the liver after conversion to cholesteryl esters by acyl-CoA:cholesterol acyltransferase (ACAT). The intracellular cholesterol pool can be re-used for assembly of VLDL or eliminated from the liver via bile directly or via bile acid synthesis.

Direct secretion of cholesterol into the bile proceeds through two ATP-binding cassette (ABC) transporters, ABCG5 and ABCG8⁷¹⁻⁷³. These ATP dependent sterol transporters function as obligate heterodimers to promote sterol excretion into the bile⁷⁴. Conversion of cholesterol into bile acid involves a cascade of reactions and almost 20 different enzymes have been identified. Basically, at present two pathways exist for bile acid synthesis⁷⁵. The classical neutral pathway, which is the main route, involves the rate-limiting enzyme cholesterol 7 α -hydroxylase (cyp7A1), and leads to the production of mainly cholic acid. The alternative pathway or acidic pathway is initiated by the enzyme sterol 27-hydroxylase (cyp27) which preferentially produces chenodeoxycholic acid. Both bile acids can be transported into the bile after which the bile acids are secreted into the intestine to leave the body or to be re-absorbed.

1.4.4 Regulators of lipid-related genes

With the identification of the hepatic enzymes and the structural components involved in lipid metabolism, the attention of scientific research has shifted towards the regulatory control of lipid metabolism. The last years more insight has been obtained in the direct effect that several lipid-related processes have on hepatic gene transcription. Especially studies demonstrating that lipids and their metabolites can act as ligands for transcriptional regulators have become of great interest.

One of the first transcription factors which was found to have a central role in controlling hepatic gene expression was the hepatic nuclear receptor HNF-4⁷⁶. Studies with conditional HNF-4 knockout mice showed that this transcription factor is involved in the control of a wide array of genes⁷⁷. These knockout mice showed reduced VLDL secretion due to decreased expression of MTP and apoB100 and an increased uptake of HDL due to increased expression of SR-B1. In addition, also the apolipoprotein genes found in the two gene clusters were seen to be HNF-4 responsive⁵⁵. Interestingly, the transcriptional activity of HNF-4 is modulated via binding of fatty acids^{78,79}.

Transcription of genes involved in the synthesis of fatty acids, triglycerides, and cholesterol are in part orchestrated by the sterol regulatory element-binding proteins (SREBPs)⁸⁰. SREBP-1c is involved in fatty acid metabolism, whereas SREBP-2 plays a major role in regulation of cholesterol synthesis. The SREBPs are synthesized as precursor proteins bound to the ER membrane and nuclear envelope. The transcriptionally active N-terminal domain SREBP must be released from the membrane to enter the nucleus and transactivate response genes. This release is mediated by the SREBP chaperone, cleavage-activating protein (SCAP) which acts as a sterol sensor. When cells become depleted in cholesterol, SCAP will escort the SREBP to the two proteases that are responsible for cleavage. Conversely, this proteolytic process is blocked when the cells are overloaded with sterols.

Recently, a specific group of nuclear receptors, the orphan receptors, has received great attention. Prior to DNA binding and gene activation, these receptors need to form heterodimeric complexes with retinoid X receptor (RXR). Members of this group include the peroxisome proliferator-activated receptors (PPARs), the liver X receptors (LXRs), and the farnesoid C receptors (FXRs) (Fig. 4).

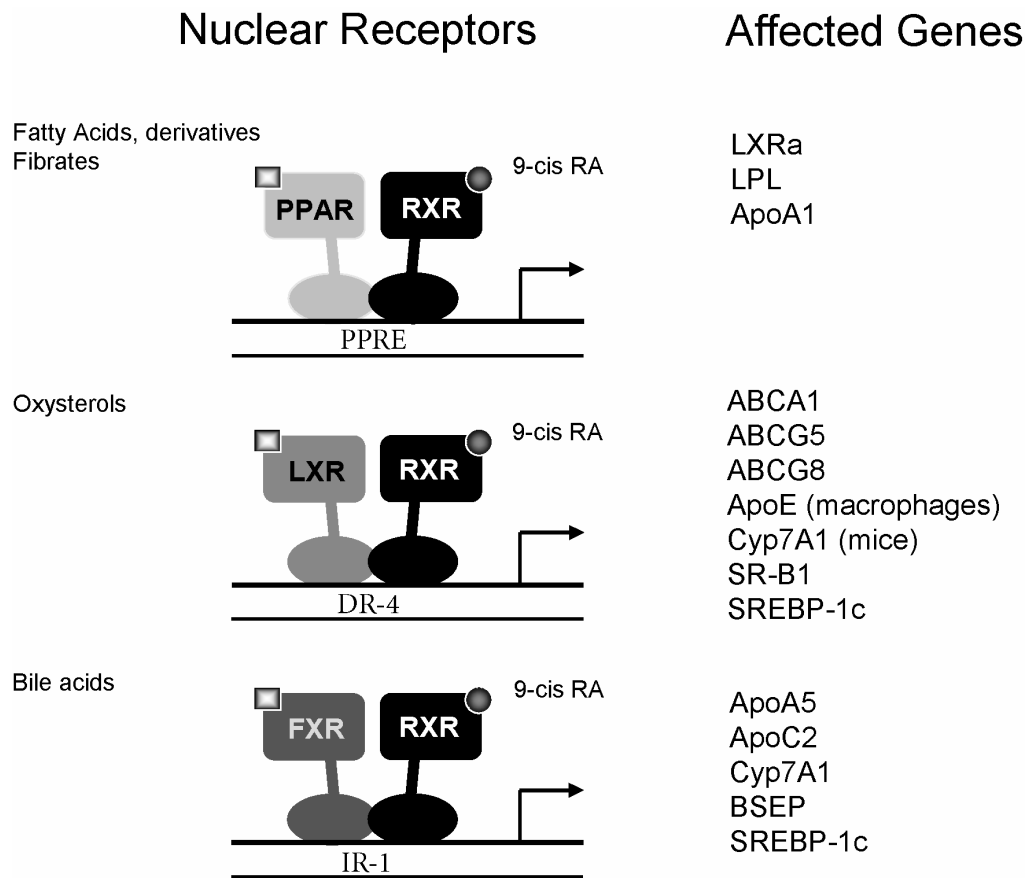


Figure 4. Nuclear receptors PPAR, LXR, and FXR form heterodimeric complexes with RXR and affect lipid related genes. The ligands that activate the indicated nuclear receptor are shown in the cartoon on the left. The natural ligand for RXR is 9-*cis* retinoic acid (9-*cis* RA). Some of the genes involved in lipid metabolism affected by these nuclear receptors are listed on the right.

The PPAR family consists of three members: PPAR α , PPAR γ , and PPAR δ (also called PPAR β). These three subtypes bind fatty acids and fatty acid metabolites and regulate the expression of genes involved in the transport, metabolism and storage of these ligands within cells⁸¹. Whereas PPAR δ is abundantly expressed throughout the body, PPAR γ and PPAR α are mainly expressed in adipose tissue and in liver, respectively⁸¹. The initial finding that PPARs can be activated by fibrates, a class of cholesterol lowering agents, gave more insight into the function of these nuclear receptors and helped to identify target genes⁸²⁻⁸⁴. In the liver, PPAR α plays a key role in the uptake of free fatty acids and in fatty acid catabolism. Triglyceride clearance by the liver is increased by the PPAR α dependent activation of genes such as LPL⁸⁵. Furthermore, PPAR α leads to an inhibition of apoC3, a natural inhibitor of LPL activity, and an

induction of apoA5, an inducer of LPL activity, resulting in a further decrease in serum triglycerides^{63,86-90}. Through the PPAR α induced upregulation of genes involved in β -oxidation of fatty acids, the pool of fatty acids available for triglyceride synthesis and incorporation into VLDL will be further diminished^{84,91}. PPAR α also influences reverse cholesterol transport at different levels in a species-specific manner. In humans for instance, PPAR α enhances the expression of apoA1, which leads to increased plasma HDL levels, while in mice the opposite seems to be the case⁹².

LXR plays an essential role in cholesterol homeostasis throughout the whole body and serves as a cholesterol sensor that stimulates the expression of multiple genes involved in the efflux, reverse transport, and excretion of cholesterol⁹³. Hence, LXR is considered a potentially important therapeutic target to prevent atherosclerosis. There are two highly conserved isoforms of LXR, LXR α and LXR β ⁹⁴, which are both activated by mono-oxidized derivatives of cholesterol called oxysterols^{95,96}. LXR α is highly expressed in the liver and, based on the effects seen in LXR α -null and LXR β -null mice, it appears to be the most crucial regulatory factor⁹⁷⁻⁹⁹. Among the hepatic LXR target genes that have a beneficial effect on cholesterol metabolism are ABCG5, ABCG8, and SR-B1^{71,100}. Furthermore, LXR reduces cholesterol synthesis by repressing the expression of the enzymes HMG-CoA reductase and synthase and their transcriptional regulator SREBP-2^{98,101}. In rodents, LXR α also promotes the conversion of cholesterol to bile acids due to enhanced expression and activity of the cyp7A1 gene^{96,98}. Interestingly, activation of LXR α does not lead to enhanced cyp7A1 activity in humans^{102,103}. Besides cholesterol metabolism, LXR also regulates fatty acid synthesis. In fact, the synthesis of fatty acids is induced by LXR via the activation of SREBP-1c^{101,104}.

The third member of this nuclear receptor family, FXR, acts as a bile acid sensor that protects the body from cytotoxic levels of bile acids and is activated by bile acids such as chenodeoxycholic acid and cholic acids¹⁰⁵. Upon activation, FXR upregulates genes involved in the transport of bile acids to the intestine such as ABCB11 (bile salt efflux pump, BSEP)¹⁰⁶. FXR also affects the bile acid synthesis by repressing cyp7A1 and thus prevents a further build up of bile acids¹⁰⁷⁻¹⁰⁹. Besides its effect on bile acid metabolism, FXR activation lowers serum triglyceride levels by attenuating SREBP-1c expression¹¹⁰. A recent report has also suggested the involvement of FXR in the repression of MTP¹¹¹. Like PPAR α , FXR stimulates lipolysis by

affecting the expression of apoC2, apoC3, and apoA5^{63,112,113}. Finally, down-regulation of apoA1 expression by FXR demonstrates that FXR also modulates HDL metabolism¹¹⁴.

The identification of the aforementioned transcription factors and their target genes contributes to a better understanding of the different hepatic and non-hepatic processes involved in lipid metabolism and their regulatory control. However, with the completion of the human genome project and subsequent genetic studies, even more genes with possible involvement in lipid metabolism are being identified. Among these novel genes are new putative regulators of transcription. One such factor is the zinc finger protein 202 (ZNF202), which was identified in the locus linked to heritable hypoalphalipoproteinemia in Utah pedigrees¹¹⁵. Biochemical analysis showed that ZNF202 functions as a transcriptional repressor of target genes from both apolipoprotein gene clusters, and of ABCA1¹¹⁶. In vitro studies confirmed the repressive effect of ZNF202 on the expression of apoE, apoA4, and ABCA1, and established its assumed role in HDL-metabolism by showing that ZNF202 is inversely regulated in macrophages with apoE and ABCA1 expression¹¹⁶⁻¹¹⁸. How ZNF202 affects lipid homeostasis and its specific role in metabolic disorders remains to be clarified.

1.5 Current treatment of hyperlipidemia

A reduction in plasma lipid levels is generally thought to be associated with a substantial reduction in cardiovascular events. The treatment of hyperlipidemia can roughly be divided into two strategies: reduction of the atherogenic apoB-containing lipoproteins or induction of the reverse cholesterol transport.

The former can be achieved by lifestyle changes such as a change of diet. However for patients with inherited hyperlipidemia these measures might not be adequate. With our increasing knowledge of the molecular mechanisms underlying cardiovascular disease, specific lipid-lowering agents have been introduced. Notably the statins, potent HMG-CoA reductase inhibitors which lower hyperlipidemia by upregulating LDL receptors, have demonstrated their success and are to date the most commonly prescribed drugs against hyperlipidemia^{119,120}. A number of drugs have been designed that can increase HDL levels and thus stimulate reverse cholesterol transport such as fibric acid derivatives (PPAR agonists) or nicotinic acid¹²¹.

MTP inhibitors that will directly influence the production of apoB-containing lipoproteins are under investigation at a pre-clinical level ^{51,52,122,123}. However, several studies with MTP inhibitors have shown that their application may incidentally be accompanied by steatosis and deficiency of lipid soluble vitamins ^{50,124,125}. LXR agonists are also being explored at present due to their athero-protective and possibly lipid regulating activity ¹²⁶. Yet, these agonists are not without side-effects, such as an up-regulation of fatty acid synthesis leading to increased plasma triglyceride levels ^{104,127,128}.

Apart from this, the effectiveness of the current approaches for the treatment of lipid disorders vary per patient which can in part be attributed to interindividual genetic variation ^{129,130}. The remarkable advance in genomics has opened new entries for therapeutic approaches to interfere with hyperlipidemia in a more specific manner and it is expected that this might reduce the variable outcome of lipid lowering therapy.

1.6 Modulation of Gene Expression in the liver as a therapeutic approach.

Due to better understanding of lipid-related processes and the identification of genes with pro- or anti-atherogenic properties, potential new targets for gene and drug therapy are emerging and are currently being explored in appropriate in vitro and in vivo models. With the liver being the central organ in lipid metabolism, liver-directed gene therapy is certainly a primary target for future clinical treatment. Deficient or dysfunctional gene products such as apoE leading to elevated serum levels of apoB-containing lipoproteins could be replaced by transferring genes expressing those proteins ^{131,132}. In other cases, protective genes such as apoA1 could be overexpressed for therapeutic purposes to favor HDL-metabolism ¹³³. In some cases, proteins that are normally expressed at extrahepatic sites could be expressed in the liver for specific purposes. For instance, the introduction of APOBEC-1 to the liver to switch the hepatic apolipoprotein production from apo B100 to apo B48, thereby reducing the production of LDL, has been studied ^{134,135}.

Instead of replacing or supplementing genes, nucleic acids may be used to attenuate or even inhibit the expression of genes such as MTP. In this regard, new gene silencing tools which include antisense oligonucleotides, ribozymes or more recently inhibitory double-stranded RNA (RNA interference, RNAi) have received great attention ^{136,137}. These inhibitors of gene

expression can be introduced as synthetic oligonucleotides, but ribozymes and RNAi drugs can also be transferred into the liver via expression constructs and subsequently expressed via the endogenous transcription machinery to accomplish sustained inhibition.

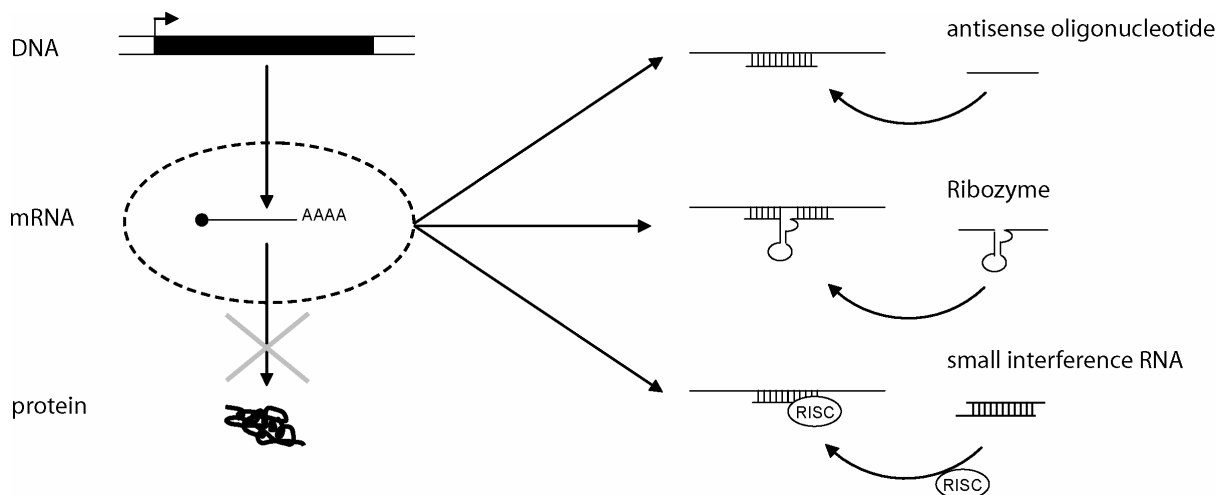


Figure 5. Antisense based strategies to knockdown expression of target genes. Gene silencing tools such as antisense oligonucleotides, ribozymes, and RNA interference prevent the translation of transcribed mRNA to proteins via Watson-Crick based hybridization of short complementary sequences to their target mRNA.

Although numerous targets and approaches have been proposed, a great concern is the tissue specificity and therapeutic control of gene therapy. To increase the tissue specificity of gene delivery, targeting of viral and non-viral carriers to cell specific surface receptors is being investigated¹³⁸⁻¹⁴⁰. Once arrived in the appropriate cells, it is preferable that these genes are under tight control. Moreover, the use of tissue specific and inducible promoters will reduce undesirable effects of long-term expression of transgenic genes¹⁴¹.

In conclusion, modulation of hepatic gene expression can be a powerful therapeutic tool to treat lipid disorders. However, despite the increased knowledge of the molecular mechanism of lipid metabolism, the effects of gene therapy-based intervention should be carefully monitored, in particular when aiming at a modulation of regulatory factors with a broad activity profile.

1.7 Outline of this thesis

In this thesis we have evaluated the potential of two gene therapeutic targets for lipid lowering. First, we describe the development of antisense-based drugs against MTP, the rate limiting enzyme in VLDL synthesis. Antisense based knock-down of this gene should lead to a reduction of apoB-containing lipoproteins and a correction of the hyperlipidemic phenotype. In the second part of this thesis, we will address the role of the recently discovered Znf202 in lipid metabolism and its potential as therapeutic target *in vivo*.

The pro-atherogenic nature of elevated serum levels of LDL is well established ^{1,12}. Interfering with the hepatic apoB-containing lipoprotein synthesis will result in a reduction of plasma LDL levels. The fact that MTP is a rate-limiting factor in the VLDL-assembly makes it an attractive target for lipid lowering therapy ¹⁴². In this study we envisioned an antisense (**chapter 2**) as well as a ribozyme (**chapter 3**) directed genetic approach to downregulate MTP mRNA levels. Both strategies are based on the specific recognition of the target mRNA by short complementary oligonucleotide sequences and can thus be valuable tools to investigate the effect of specific inhibition of VLDL production in mouse models and to explore the therapeutic potential of gene therapy directed against MTP.

ZNF202 does affect a variety of genes involved in cellular transport of lipids and lipid metabolism in general ¹⁴³. Especially its apparent linkage to HDL metabolism has drawn considerable interest. To address the perspectives of ZNF202 as therapeutic target in the correction of hyperlipidemia, we aimed to modulate hepatic ZNF202 expression via adenovirus (Ad) mediated gene transfer. First the murine ZNF202 gene was cloned from liver cDNA of C57BL/6J mice and Ad.CMV-ZNF202 was produced (**chapter 4**). To investigate the mechanism of ZNF202 repression deletion analysis of the mouse apoE promoter was performed. Via adenoviral mediated gene transfer we analyzed the effect of ZNF202 overexpression in a mouse hepatoma cell line on the expression of apolipoprotein gene clusters. Next the overall effect of ZNF202 in lipid metabolism was mapped *in vivo* in hyperlipidemic (LDLR^{-/-}) and normolipidemic (C57Bl/6JICO) mice (**chapter 5**). The mice were injected intravenously with Ad.CMV.ZNF202 resulting in significant hepatic overexpression of ZNF202. After several days, hepatic and serum lipid levels were analyzed together with hepatic mRNA levels of relevant lipid-related genes. Finally the results presented in this thesis will be discussed (**chapter 6**).

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2

Rational Design of Potent Antisense Drugs for Inhibiting Hepatic Microsomal Triglyceride Transfer Protein Expression

Carlos L.J. Vrans^{#,§}, Peter A.Chr. 't Hoen[#], Perry Prince[#], Peter Van Santbrink[#], Ko Willems van Dijk[§], Theo J.C. Van Berkel[#], Martin K. Bijsterbosch[#], and Erik A.L. Biessen[#]

[#] Division of Biopharmaceutics, Leiden/Amsterdam Center for Drug Research, Leiden University, P.O. Box 9502, 2300 RA Leiden.

[§] Department of Human and Clinical Genetics, LUMC, Leiden University, P.O. Box 9503, 2300 RA, Leiden, The Netherlands.

Abstract

In this study we describe the design and screening of antisense drugs against mouse Microsomal Triglyceride transfer Protein (muMTP) mRNA, which is a key enzyme in the assembly and secretion of atherogenic apolipoprotein B (apoB) containing lipoproteins in parenchymal liver cells. As the identification of suitable target sites accessible to antisense hybridization and the effectiveness of potent antisense drugs in a cellular context are rather unpredictable, we have pursued the an in vitro strategy. Based on the predicted secondary structure of muMTP mRNA we designed 12 18-mer antisense oligodeoxynucleotides (AS-ODNs). Evaluation of these AS-ODNs for their capacity to inhibit muMTP expression in a cell-free transcription/translation assay revealed that 6 out of 12 antisense drugs (50%) led to more than 80% reduction of muMTP expression at which the most potent one (AS569) displayed an IC₅₀ of 15 nM. Screening of AS569 for its ability to quench green fluorescent protein (GFP) derived fluorescence in GFP-muMTP transgenic COS-7 cells revealed a sequence specific and dose-dependent reduction of muMTP expression (IC₅₀=50 nM). In conclusion, rational antisense drug design combined with a fast high throughput screening method was used to identify potent antisense drugs for muMTP. We anticipate that the most potent AS-ODN, AS569, is a promising lead in the development of potent antisense drugs for treating hyperlipidemia.

Introduction

Antisense based gene inhibition continues to be an important approach in genomics-based research to study gene function and has led to the identification of novel drug targets. In fact, several antisense based therapies have been approved for clinical trials and are currently underway ¹⁻³. Even though the use of antisense oligodeoxynucleotides (AS-ODNs) has helped considerably in the validation of gene function, the design of potent antisense drugs remains a challenge. A major obstacle is the ability of an antisense drug to engage in Watson-Crick hybridization with its target mRNA, which depends on the accessibility of the complementary target, and thus on its secondary and tertiary structure ⁴⁻⁶. Several strategies to identify accessible sites on the target mRNA have been pursued ⁷. Most straightforward is the screening of large

libraries of randomly selected antisense sequences targeted against various regions of the mRNA, albeit the success rate of this approach is less than 10%^{8,9}. To increase the success rate of antisense design a pre-selection step has been proposed, at which suitable target regions are defined on the basis of the secondary mRNA structure¹⁰. Several computerized algorithms, such as Mfold, have been developed to predict the secondary structure of target mRNAs^{11,12}.

After design of potential antisense drugs directed against the target mRNA a next barrier involves the rapid screening of the drugs for intrinsic antisense activity. The uptake and intracellular kinetics of AS-ODNs may be sequence dependent and this may considerably perturb the interpretation of the data. A cell-free assay to monitor AS-ODN activity will circumvent this setback⁷. In particular assays for coupled transcription/translation, which cover antisense effects from transcription to translation show great potential in this regard¹³. As these assays are not necessarily reflective of the AS-ODN activity at a cellular level, it will be essential to reconfirm the in vitro data in a cellular context. We have previously described a sensitive and reproducible method to quantify AS-ODN efficacy that is not perturbed by differences in stability or cellular uptake¹⁴. This method utilizes fusion constructs consisting of the target gene with the gene encoding enhanced green fluorescent protein (EGFP) as reporter and links the antisense activity directly to the decrease in EGFP expression.

In this report we describe the rational design and screening of antisense drugs against mouse microsomal triglyceride transfer protein (muMTP), a rate limiting key enzyme in the synthesis of atherogenic apolipoprotein B100 (apoB100) containing lipoproteins¹⁵⁻¹⁷ and promising target for intervention in hyperlipidemia¹⁸⁻²⁰. A two step strategy is pursued for the screening of antisense drugs involving a cell-free assay and a subsequent cellular fluorescence assay. From the predicted secondary structure of muMTP we have selected 9 regions that are not involved in Watson-Crick base-pairing and designed a set of 12 AS-ODNs targeting these epitopes. Using a cell-free combined transcription and translation assay those AS-ODNs were evaluated for their ability to specifically inhibit the expression of muMTP. The most effective AS-ODN was able to selectively inhibit the expression of muMTP at a cellular level.

Materials and Methods

Materials

Primers, phosphodiester and phosphothioate oligonucleotides (PS-ODN and PO-ODN), and Goldstar DNA polymerase were from Eurogentec (Seraing, Belgium). The pEGFP-C1 plasmid and the rabbit anti-EGFP Living Colors Peptide antibody were obtained from Clontech (Palo Alto, CA.). Vent[®] DNA Polymerase was from New England Biolabs (Beverly, MA). The TNT T7 coupled Reticulocyte Lysate System was from Promega (Leiden, The Netherlands). Exgen500 was obtained from Fermentas (St. Leon-Rot, Germany). Propidium iodide was purchased from Sigma (St. Louis, MO). Tetramethylrhodamine isothiocyanate 5,6-mixed isomers (TRITC) was from Molecular Probes (Eugene, OR). Cell culture agents were obtained from BioWhittaker (Verviers, Belgium). All other chemicals were of analytical grade.

Table 1: Nucleotide sequences of the antisense drugs

Name	Sequence	Target site*
AS014	5' -GGATCATGCTGGCTCCCT	14-31
AS176	5' -AGCCACGCTGTCTTGCG	176-193
AS344	5' -TCCCTATGATCTTAGGTG	344-361
AS559	5' -TTTGTCTTGTGGGCCTG	559-576
AS569	5' -TTTTGACCACTTTGTCTT	569-586
AS579	5' -AGAGCCTTAATTTGACC	579-596
AS1084	5' -CAGCTGAGGGAGCACTTC	1084-1101
AS1414	5' -GTCTTCTTTCTTCTCTGG	1414-1431
AS1767	5' -GGCATTTCAAATGCAGG	1767-1784
AS1785	5' -CGGATCATTTTGCTTGCA	1785-1802
AS1979	5' -GCTCTGTTCTTTGATGT	1979-1996
AS2791	5' -CATCTTAAATACAGGTAA	2791-2808
SC569A	5' -TTTCTTCCATGATGTCTT	
SC560B	5' -TTTCCATGACTTCTTTGT	

* nucleotide numbers based on Gene Bank Accession nr. L47970

Cloning of muMTP cDNA into pEGFP-C1

Full-length cDNA coding for muMTP (cloned in pBLUEscriptII-KS; pMTP) was kindly provided by Dr L. Chan (Houston, USA). A 2.8 kb fragment (nt 25 to 2860, entailing the coding region and a part of the untranslated 3' prime end) was PCR amplified from MuMTP using Vent DNA polymerase with extended primers containing *Kpn-I* and *Bgl-II* restriction sites (Forward: *GGT.ACC.ATG.ATC.CTC.TTG.GCA.GTG*; Reverse: *AGA.TCT.CCT.GAA.TAG.GTT.CAA.CTT*) and was subsequently cloned into the PME-1 site of pcDNA3.1 (Clontech, Palo Alto, CA). The muMTP fragment was then excised by *Kpn-I* and *Bgl-II* digestion and the fragment ligated into *Kpn-I/BamHI* digested pEGFP-C1 plasmid to generate the C-terminal fusion construct pEGFP-muMTP. Sequencing of the plasmids confirmed the in-frame ligation of the muMTP cDNA.

Cell-Free Assay for Coupled Transcription/Translation (TnT).

TnT assays on T7 promoter containing pBluescriptII-muMTP were performed according to the manufacturer's protocol with or without antisense ODNs (without the RNasin Ribonuclease inhibitor). After incubation for 60 min at

30°C protein sample buffer (62 mM Tris, 12.5 % glycerol, (v/v), 1.25 % SDS (w/v), 2.5% β -mercaptoethanol (v/v), and 0.25% bromophenol blue at pH 6.8) was added to the reaction mixtures, and the mixtures were denatured for 5 min. at 95°C. Samples were then loaded on 15% denaturing SDS-polyacrylamide gels, and the gels were run in 0.02 M Tris, 0.16 M Glycine, and 0.1% SDS (w/v) at 100 V (30 min) and subsequently at 200 V (1 h). After fixation in a solution of 45% methanol (v/v) and 0.5 M acetic acid the gels were visualized and the synthesis of ^{35}S -methionine labeled muMTP was quantified with a phosphor-imager (Molecular Dynamics, Sunnyvale, CA).

Cell culture and transfection

COS-7 cells (European Collection of Cell Cultures, Salisbury, UK) were seeded in 12-wells plates and grown at 37 °C under a 5 % CO₂ atmosphere in Dulbecco's modified Eagle's medium (DMEM) containing 10 % (v/v) fetal calf serum, 2 mM L-glutamine, 100 U ml⁻¹ penicillin and 100 $\mu\text{g ml}^{-1}$ streptomycin to a confluency of approximately 50 %. Cotransfections of plasmid and phosphorothioate-modified ODN were performed with Exgen 500 (Fermentas) according to the manufacturer's protocol. After 24 hours cells were detached from the culture plates with trypsin, centrifuged for 5 min at 400 g, washed once with 1 ml of phosphate buffered saline (PBS), and dispersed in 1 ml of PBS for flow cytometric analysis.

Flow cytometry

Immediately before FACS analysis, 3 μl of 1 μM propidium iodide was added. Cellular fluorescence of approximately 3,000 cells was determined in a Becton Dickinson FACS Calibur flow cytometer. Only single cells were gated in forward / sideward scatter plots; dead cells were excluded from the analysis by gating of propidium iodide-positive cells.

Data processing - Data were analysed statistically for significance with two-tailed student t-test. GraphPad Prism Software (Graphpad Software Inc., San Diego, CA) was used for this purpose.

Results

Antisense Design

The first step towards the design of an effective muMTP mRNA reducing antisense drug involved the identification of domains within the muMTP mRNA that are not involved in base pairing reactions and that thus are open for hybridization with the antisense drug. To this end we have determined a single strand frequency plot for muMTP from the 25 energetically most favourable RNA configurations as calculated by the M-Fold algorithm ¹². From this plot, 9 domains throughout the muMTP mRNA could be identified with an increased single strand frequency (fig.1), and we designed 12 18-mer oligodeoxynucleotide sequences complementary to

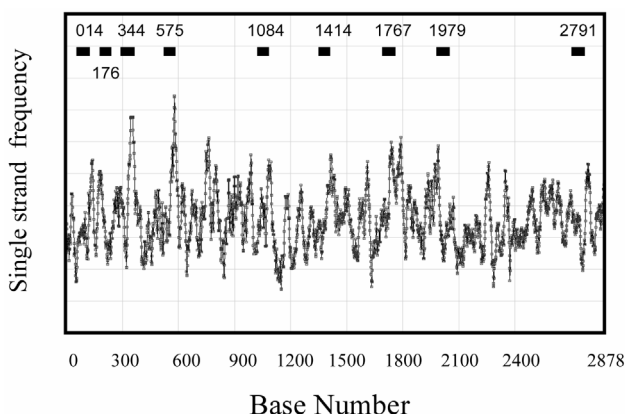


Figure 1: Folding pattern of muMTP RNA. Plotted is the frequency of nucleotides within the muMTP mRNA to be involved in base-pairing or hairpin formation obtained from the 25 energetically most favourable secondary structures as calculated via the Mfold algorithm. The numbered bars refer to the mRNA regions targeted by the antisense drugs.

these domains (table 1). Subsequently, we have screened the 18-mer AS-ODNs for their ability to interdict muMTP synthesis in a rabbit reticulocyte lysate based system for coupled transcription/translation, which is RNaseH competent (fig.2A). In the presence of muMTP cDNA in vitro transcription/translation led to the formation of a [^{35} S]methionine labeled 97 kD protein which corresponded with full length muMTP. As a control, in vitro transcription/translation was also tested for luciferase, by adding pLuc to the mixture. This gave an intense band of full length luciferase at 47 kD (data not shown).

Formation of full length muMTP was significantly quenched (>65%) in the presence of only 150 nM of AS-ODNs, targeting domains 344, 575, 579, 1084, 1414, and 1767 (fig. 2B). In particular antisense drugs targeting site 569 and 1414 appeared to be very effective with >95% inhibition ($p < 0.001$). To verify that this inhibition was not caused by a sequence-dependent artifact, also AS-ODNs which target sites flanking the AS569 stretch were tested for their

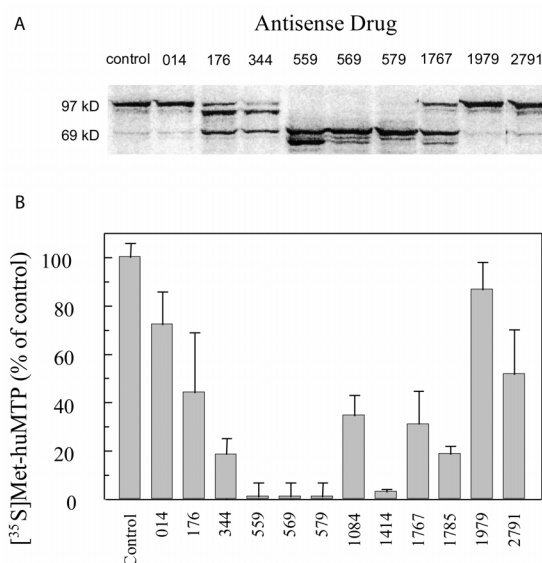


Figure 2: Effect of antisense drugs on [^{35} S]Met-muMTP formation in a reticulocyte lysate system for coupled transcription/translation. Rabbit reticulocyte lysate was incubated for 60 min at 30°C with RNA-T7 polymerase, methionine deficient amino acid mix, [^{35} S]Methionine, and pMTP (200 ng) in the absence (control) or presence of phosphodiester ODN (150 nM). The mixture was applied to SDS-PAGE gel-electrophoresis and analyzed on the PhosphorImager for the intensity of the muMTP (A). The intensity of the 97 kD MTP protein bands were quantified and the control was set at 100% (B). Values are means $\pm$ SD of three independent experiments.

muMTP translation inhibitory potency. As these AS559 and AS579 were equally effective inhibitors but structurally different, we can assume that the inhibition is achieved through domain specific interaction.

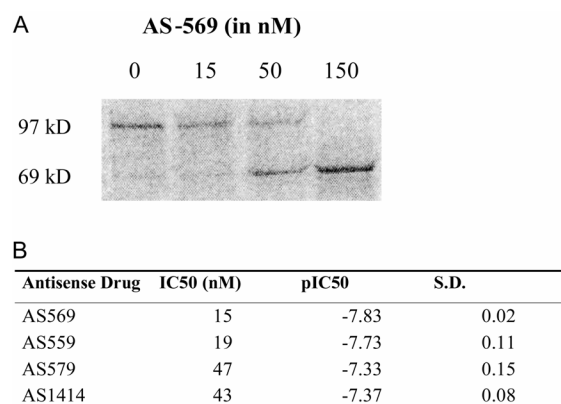


Figure 3 Dosis-effect study of the most potent antisense drugs. Rabbit reticulocyte lysate was incubated for 60 min at 30°C with RNA-T7 polymerase, methionine-deficient amino acid mix, [³⁵S]Methionine, and pMTP (200 ng) in the presence of an increasing concentration of antisense drugs (0-150 nM). The mixture was applied to SDS-PAGE gel-electrophoresis and analyzed on the PhosphoImager for the intensity of the muMTP protein bands (A). Intensities of the 97 kD bands were calculated by image analysis and IC₅₀ values were calculated from sigmoidal concentration effect curves using Graphpad Prism Software (B). Values are means ± SD of three independent experiments.

In vitro dose effect studies confirmed that the AS-ODNs AS559/579 and AS1412 were already able to reduce muMTP formation at nanomolar concentrations (fig.3). IC₅₀ values calculated from the dose-effect studies ranged from 38 nM for AS1412 to only 15 nM for AS569. The inhibitory activity of AS569 was sequence specific as [³⁵S]-muMTP synthesis was not affected by the presence of two scrambled ODN sequences, while only AS569 gave a significant >80% reduction (P<0.001), indicating that the inhibitory effect of AS569 can be attributed to a antisense specific mechanism (fig.4).

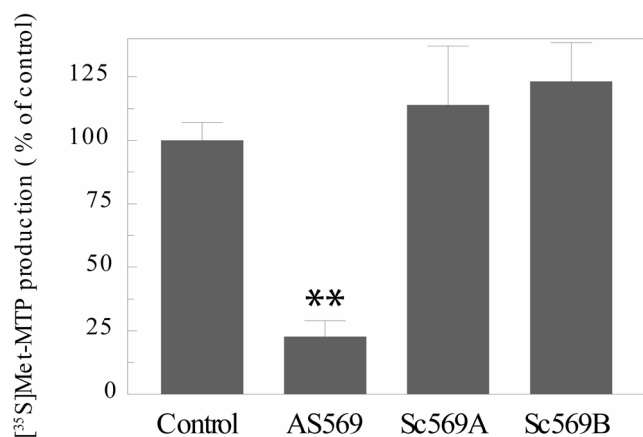


Figure 4: Specificity of AS569 on [³⁵S]Met-muMTP formation in a reticulocyte lysate system for coupled transcription/translation. Rabbit reticulocyte lysate was incubated for 60 min at 30°C with RNA-T7 polymerase, methionine deficient amino acid mix, [³⁵S]Methionine, and pMTP (200 ng) in the presence of phosphodiester ODNs (100 nM). The mixture was applied to SDS-PAGE gel-electrophoresis and analyzed on the PhosphoImager for the intensity of the muMTP. The intensity of the 97 kD MTP protein bands were quantified and the control was set at 100%. Values are means ±SD (n=3) and ** indicates p<0.001.

Activity of the antisense drugs in a cellular context.

To establish antisense activity of AS569 in a cellular setting, we have set-up a flow cytometry based read-out system for muMTP. C-terminal fusion constructs of muMTP with EGFP (pEGFP-muMTP) were made by ligating PCR-amplified cDNA, encoding muMTP, into the multiple-cloning site of the pEGFP-C1 fusion vector. Sequence analysis confirmed a correct in-frame ligation of the cDNA and the absence of any sequence errors in the inserts. By flow cytometric analysis, it was shown that transfection of COS-7 cells with pEGFP and pEGFP-muMTP proceeded at equal efficiency, albeit that the average fluorescence intensity of EGFP-muMTP transfected cells was lower than that of EGFP itself (data not shown). This indicated that the EGFP moiety of the fusion protein retained its activity. Fluorescent microscopy revealed that EGFP and the EGFP-muMTP fusion proteins were both localized in the cytosol.

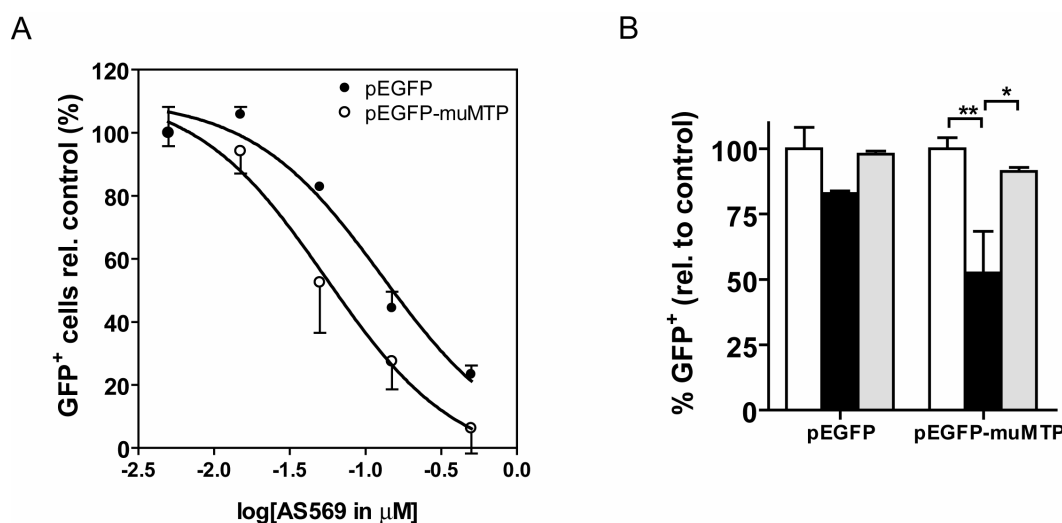


Figure 5: Specificity of antisense activity of AS569 in a EGFP-muMTP fusion protein-based cellular read-out. COS-7 cells were transfected with 2 μ g of pEGFP (●) or pEGFP-muMTP (○), together with the indicated concentrations of phosphodiester AS569 using Exgen500 (charge ratio 1:3). After 24 hrs cells were analyzed for GFP expression by flow cytometry. Percentage of GFP positive cells transfected with plasmid but without AS-ODN was set at 100% (A). Under similar conditions COS-7 cells expressing either EGFP or EGFP-muMTP (white bars) were transfected with 50nM of AS569 (black bars) or scrambled ODN (Sc569; grey bars) (B) Values are means $\pm$ SD (n=3).

Next, we have optimized the transfection conditions for COS-7 cells. Fluorescent microscopy showed that while EGFP-muMTP was localized mainly in cytosolic vesicles (endosomes) as well as in the nucleus in 30-40% of cells, the TRITC labeled PS-ODN was predominantly found in the latter organelle (data not shown). To determine the potency and specificity of AS569 to inhibit the expression of muMTP RNA we transfected COS-7 cells with

different concentrations of TRITC labeled AS569 in the presence of either pEGFP-muMTP or pEGFP (fig. 5A). Our results show that AS569 is also a potent inhibitor of EGFP-muMTP at a cellular level and that the antisense effect is concentration-dependent ($IC_{50} = 54$ nM). However, at high concentrations (> 0.05 μ M) the oligonucleotide also affected the expression of EGFP, although to a lesser extent ($p < 0.05$). The sequence specificity of EGFP-muMTP inhibition by AS569 at low concentration was further corroborated by the lack of effect of scrambled ODN (50 nM) on the expression of EGFP-muMTP (fig. 5B). The expression of EGFP in COS-7 cells transfected with our control pEGFP also remained unaffected by Sc569.

Discussion

This study describes the rational design and characterisation of antisense drugs that target the gene encoding murine MTP, which catalyzes a rate-limiting step in the assembly of pro-atherogenic apoB100 containing lipoproteins, *i.e.* VLDL and LDL¹⁵⁻¹⁷. From secondary mRNA structure analysis for muMTP mRNA using the Zuker algorithm (Mfold version 3.0) we have selected domains within the muMTP mRNA, which are not involved in base-pairing reactions (hairpin or pseudoknot formation) and thus are accessible to antisense drug hybridisation¹¹. Antisense drugs targeting these domains were tested for their ability to down-regulate muMTP mRNA levels in a two step-strategy involving rapid prescreening under cell free conditions and subsequent verification of potentially active antisense drugs in a cellular read-out system.

For the former we have used a T7 RNA polymerase promoter coupled reticulocyte lysate system. We found that 6 out of 12 (50%) of the designed antisense drugs resulted in $>80\%$ inhibition of MTP expression. Although the reliability of computer predictions for folded RNA structures is debatable^{21,22}, the success rate of identification of effective antisense sequences in this study is considerably higher than the 2-10% normally reported for unbiased antisense design^{8,9}. For further characterization we selected the most potent antisense sequences that resulted in almost complete inhibition of MTP expression: AS559/AS579 and AS1414. Dose-effect studies showed that each of these 4 antisense drugs displayed IC_{50} values in the nanomolar range (47 to 15 nM). In particular nucleotide strand 559-599 appeared to be sensitive to antisense drug hybridization. Antisense specificity of AS569 was confirmed by the lack of effect of a scrambled ODN.

In the second step the most potent antisense drugs were evaluated for their antisense effects in a cellular assay. For this purpose we used the phosphorothioate-modified AS569, since PS-ODNs are less sensitive to nucleolytic degradation²³. In a previous report we have described a novel cellular screening assay for the selection of effective AS-ODNs with a sequence-specific mode of action¹⁴. In analogy to this study, we have used COS-7 cells transfected with muMTP/EGFP fusion protein to directly correlate antisense activity with EGFP-derived fluorescence by flow cytometry. This sensitive assay appeared to be quantitative and obviated the need of specific antibodies or isoform-specific activity assays. The high reproducibility of this assay is probably due to elimination of variable transfection efficiencies as complicating factor in the assessment of antisense effectiveness. We had already shown in a previous study that after cotransfection of plasmid and fluorescently labeled ODNs¹⁴, all EGFP-positive cells had taken up ODNs and fluorescence microscopy showed that AS-ODNs are predominantly present in the nucleus. Therefore, in this assay antisense effects will not be masked by a low transfection.

In our cellular assay AS569 displayed an IC₅₀ value of 54 nM, which is comparable to IC₅₀ values of other potent PS-ODNs tested in a cellular environment^{9,24}. True antisense effect by AS569 was found to be concentration dependent. At higher concentrations of AS569 also non-target specific effects could be detected as judged by the reduced EGFP expression, although to a lesser extent with 77% reduction of EGFP expression compared to almost 95% reduction of EGFP-muEGFP expression at the highest AS569 concentration. Nonspecific antisense effects at high concentrations by more nuclease tolerant PS-ODNs have been reported earlier^{25,26}. It is believed that PS-ODNs may engage in sequence-dependent and -independent interactions, largely of electrostatic and induced dipole nature, with cellular proteins^{27,28}. In particular key regulatory proteins such as transcription factors are notorious PS-ODN binders²⁹. With the recent advance in antisense drug design (such as the development of peptide nucleic acids, PNAs) the efficacy of antisense drugs has been improved and unfavorable side effects attenuated^{2,30,31}. Specific delivery to tissue by conjugating ODNs to ligands of targeted receptors or liposome mediated targeting of ODNs may further improve the effectiveness of the antisense drugs^{32,33}. Indeed we have observed a marked inhibition of muMTP in parenchymal liver cells by targeting glycoconjugated PNA569 (which targets the same mRNA region as AS569) against the asialoglycoprotein receptor³⁴.

In conclusion, the use of rational antisense drug design combined with a two step evaluation strategy involving a cell free assay and a subsequent cellular read-out assay based on an EGFP fusion construct appears to be a very effective strategy in the design of potent antisense drugs. A specific AS-ODN targeted against muMTP was obtained and shows therapeutic potential in treatment of hyperlipidemia. Moreover, we identified a potential region within the muMTP mRNA that is accessible to other antisense and RNA interference based therapies.

Acknowledgements:

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3

Development of Hammerhead Ribozymes Targeted Against Mouse Microsomal Triglyceride Transfer Protein

Carlos L.J. Vrans^{#§}, Peter Van Santbrink[#], Theo J.C. Van Berkel[#], Ko Willems van Dijk^{§‡}, and Erik A.L. Biessen[#]

[#] Division of Biopharmaceutics, Leiden/Amsterdam Center for Drug Research, Leiden University, Leiden, The Netherlands.

[§] Department of Human Genetics, LUMC, Leiden University, Leiden, The Netherlands.

[‡] Department of General Internal Medicine, LUMC, Leiden University, Leiden, The Netherlands.

Abstract

Therapeutic reduction of very low-density lipoprotein (VLDL) levels by intervention in its biosynthesis in the liver is considered a very legitimate approach to lower the risk of cardiovascular diseases (CVD). Microsomal triglyceride transfer protein (MTP), catalyzes a rate limiting step in the assembly of VLDL and thus, constitutes an attractive target for VLDL lowering. In this study we present a new gene therapeutic strategy to stably reduce MTP levels involving the use of ribozyme vectors. Three hammerhead ribozymes were designed against mouse MTP, targeting regions within the MTP mRNA that appeared accessible for ribozyme hybridisation from RNA folding analysis and that encompassed a GU↓X cleavage site: Rz342, Rz581 and Rz1802. The intrinsic activity of the ribozymes was assessed under cell free conditions after incubation of [³²P]-labeled MTP RNA (0.5 μM) with *in vitro* transcribed ribozymes (2.0 μM). For all ribozymes, endonuclease activity was demonstrated and the size of the cleavage products concurred with their predicted fragment length. Kinetic analysis gave V_{max} values ranging from 12 to 140 nM/ minute at a K_m of approximately 7 μM, at which the tandem repeat RzT581 appeared to be most potent. Adenoviral expression vectors were generated for Rz581 and RzT581 under control of different polymerase-II and III dependent promoters (i.e. CMV and tRNA or U6+27, respectively). MTP cDNA transfected Hela and mouse hepatoma mAT3F2 cells were transduced with the adenoviral ribozymes. Ribozyme based reduction of MTP mRNA levels was observed in both cell lines but depended on the type of expression cassette used. In conclusion, ribozyme vectors were generated that affect a significant downregulation of MTP expression *in vitro*. The most potent ribozyme vector could not only be a powerful mechanistic tool for future studies on hepatic MTP, but could form a starting point for the development of a novel targeted therapy for reducing MTP activity and thus levels of the atherogenic VLDL.

Introduction

Elevated levels of apolipoprotein B100 (apoB100), the structural protein of circulating lipoproteins carrying triglycerides and cholesterol¹, are a risk factor for cardiovascular diseases.

A key enzyme in the assembly and secretion of apoB containing lipoproteins is microsomal triglyceride transfer protein (MTP)^{2,3}. This makes MTP a potential target in search of therapeutic agents that are able to reduce VLDL and LDL. Several synthetic MTP inhibitors have already been described, but their action is not limited to the liver and may cause adverse effects including deficiency of lipid soluble vitamins⁴⁻⁶. A straightforward strategy to reduce MTP is to intervene in its biosynthesis, which could amongst others be effected by hammerhead ribozyme therapy.

Ribozymes are small RNA sequences with endonuclease activity⁷⁻⁹ and can be rendered substrate specific by flanking the catalytic domain with short sequences complementary to the target RNA. However, the successful application of ribozymes depends on the target site accessibility, efficient delivery, and stability.

In search for suitable sites, the RNA cleavage site not only needs to be accessible but should also contain a specific triplet sequence. The specificity to ribozyme hybridization is determined by the secondary RNA structure and possible association with cellular proteins and is difficult to predict. Various algorithms have been developed to calculate secondary structures of RNA such as Mfold which can be helpful but have proven not to be fully reliable^{10,11}. Secondly, the preferred triplet for cleavage was suggested to be 5'-GU↓X where X is either C, A or U, as most of the naturally occurring ribozymes targeted this triplet¹²⁻¹⁴.

There are two ways to introduce ribozymes to target cells. Ribozymes can be delivered to the aimed target exogenously as chemically synthesized oligodeoxynucleotides potentially equipped with phosphothioate or inverted nucleotide groups to confer stability, or they can be synthesized endogenously upon transfer of ribozyme expression vectors to the target cells¹⁵. For the latter strategy the efficacy of ribozyme vectors will depend on the expression level and intracellular distribution which are governed by the choice between RNA-polymerase III (pol III) dependent promoters (such as the U6 snRNA and tRNA promoter) which generally are fit for driving transcription of small RNAs, and RNA-polymerase II (pol II) dependent viral promoters (like CMV)^{16,17}. Furthermore, the enzymatic stability of the ribozymes and proper folding of the catalytic domain can be improved by introducing flanking sequences and/or hairpins. Illustratively, introduction of artificial stem-loops was reported to considerably enhance the stability of hammerhead ribozymes and to promote a catalytically active configuration^{18,19}.

In this study we describe the development of a mouse MTP (muMTP) specific hammerhead ribozymes for ribozyme based gene therapy to lower VLDL production by the liver.

site of pBluescript II KS (Stratagene). Orientation of the insert was determined by restriction analysis and the constructs were sequence verified (LGTC, Leiden, The Netherlands). Constructs with reverse orientation of the ribozymes were used as negative control. To obtain RNA substrates for the in vitro ribozyme cleavage reactions, two PCR fragments of muMTP were generated using the following two primer pairs: MTP1 forward 5'CAGAGGGAGCCAGCATGATC and MTP1 reverse 5' TCCTGCTATGGTTTGTGGA and MTP2 forward 5'AGAGATACGATGTCTCCTTC and MTP2 reverse 5'AGAATAAGCCCTTTCACCAC. The MTP1 amplicon spanned nucleotides 12 to 768 (nucleotide numbers are based on GeneBank accession number L47970) and contained the target site for Rz342 and Rz581, while that of MTP2, spanning nt 1550 to 2219, contained the target site for Rz1802. Both PCR products were cloned into pGEM-T (Promega) and verified by sequencing.

Construction of Adenoviral Vectors

The construction of pTZU6+27 and pTZ18U containing respectively the U6+27 and the tRNA^{met} expression cassette was described by Good et al.¹⁹. Rz581 was excised from the pBluescript backbone as a SalI-XbaI fragment. The shuttle vector pAdTrack-cmvRz581 was generated by inserting the Rz581 fragment in a SalI and XbaI digested pAdTrackCMV. To obtain the shuttle vectors pAdTrack-U6.Rz581 and pAdTrack-tRNA.Rz581, Rz581 was first inserted into the SalI and XbaI sites of respectively the U6+27 and the tRNA^{met} expression cassette. The expression cassettes were then cut out with KpnI and HindIII and cloned into pAdTrack. RzT581 was cloned into pAdTrackCMV via restriction sites NotI and HindIII. Recombinant adenoviral plasmids was generated by homologous recombination with pAdEasy1 in BJ5183 cells as described previously²⁴. Correct clones were propagated in STBL2 cells (Life Technologies). For the production of adenoviral ribozymes Per.C6 cells were transfected with 3 µg Pac-I-linearized adenoviral construct on 35 mm dishes using LipofectAMINE PLUS kit as described by the manufacturer (Life Technologies). After 16 hours transfection medium was replaced by growth medium. Transfected cells were harvested at day 7 post-transfection and after three cycles of freeze-thaw the lysate was used for large-scale production of adenoviral ribozymes in Per.C6 cells. Virus was purified by CsCl centrifugation steps and dialysis. Final yields were assessed via a plaque assay on 911 cells.

In Vitro Transcription and Ribozyme Cleavage Reaction

For in vitro transcription the T7 promoter based RiboMAX system was used according to the manufacturer's protocol (Promega, Leiden, the Netherlands). Ribozyme constructs in pBluescript II KS and target sequences in pGEM-T were linearized with *EcoRI* and *NotI*, respectively. Target RNA sequences and ribozymes were radiolabeled by the incorporation of [³²P_α]UTP (Amersham Biosciences, Buckinghamshire, England) during in vitro transcription. After the in vitro transcription reaction, the reaction mixture was treated with RQ1 RNase-free DNase (Promega) and the unincorporated nucleotides were removed by isopropanol precipitation. RNA concentrations were determined by measuring optical density at 260 nm. For the cleavage reaction substrate (10 pmol) substrate and ribozyme RNA (40 pmol) were mixed in 18 µl of Tris buffer (50 mM; pH 7.5). The mixture was heated at 85°C for 2 min followed by a 10 min incubation at 37°C. MgCl₂ (20 mM) was added to a final volume of 20 µl and the mixture was incubated for up to 60 min at 37°C. The reaction was stopped by the addition of an equal volume of loading buffer (90% formamide, 20 mM EDTA, 0.03% bromophenol blue, 0.03% xylene cyanol). This mixture was heated at

95°C for 2 min and the samples were run on a 6% polyacrylamide-8 M urea gel in Tris-borate EDTA buffer. Cleavage products were visualized and quantified with a phosphorimager (Molecular Dynamics, Sunnyvale, CA). To determine the cleavage kinetics of the four ribozymes, 0.1 uM of ribozyme was incubated different concentrations of substrate (0.25, 0.5, 1, 1.5 uM) and the reaction was monitored over a period of 10 min. Reaction velocities were determined and K_m and V_{max} were calculated by non-linear regression analysis ($V=(V_{max} \times [\text{substrate}]) / (K_m + [\text{substrate}])$).

Transfection and Infection

Hela cells were seeded in a 12-wells plate and grown to a confluency of 50%. Cells were transfected with pEGFP-muMTP by lipofection (FUGENE). At 24 hours post-transfection cells were infected with either AdGFP-Rz581, AdGFP-RzT581, AdGFP-U6.Rz581, AdGFP-tRNA.Rz581, or control AdGFP (MOI=100). Cells were harvested and lysed with Trizol 48 hours after transfection. To address ribozyme effects on endogenous muMTP expression, mhAT3F2 cells were seeded in a 12-wells plate and grown to a confluency of 60-70% before infection. Infection with recombinant adenoviruses and harvesting of the cells was performed under similar conditions as described for Hela cells.

Quantitative Real Time PCR analysis

Total RNA from Hela and AT3F2 infected cells was isolated with Trizol according to the manufacturer's protocol (Eurogentec SA, Seraing, Belgium). Purified RNA was treated with RQ1 RNase-free DNase (Promega, 1 units/ 2 µg of total RNA) and reverse transcribed with SuperScript II Reverse Transcriptase (Invitrogen) according to the protocols supplied by the manufacturers. Quantitative gene expression analysis was performed on an ABI prism7700 Sequence Detection System (Applied Biosystems, Foster City, CA) using SYBR Green technology as described earlier²⁵. For design of the PCR primers (table 2) Primer Express 1.7 software was used with the manufacturer's default settings (Applied Biosystems) and primer sets were validated for amplification efficiency. The absence of genomic DNA contamination in the RNA preparations was confirmed by using total RNA samples that had not been subjected to reverse transcription. Hypoxanthine-guanine phosphoribosyl transferase (HPRT) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as the standard housekeeping genes.

Data Analysis—The significance of differences in relative gene expression numbers C_t ($C_{t(HPRT)} - C_{t(\text{target gene})}$) measured by real time quantitative PCR was calculated using a two-tailed Student's *t* test. Probability values less than 0.05 were considered significant.

Table 2. PCR Primers

Gene	GenBank Acces.	Forward Primer	Reverse Primer
huGAPDH	BC029618	CAGAACATCATCCCTGCCTCTA	CCAGTGAGCTTCCCGTTCA
huHPRT	NM_000194	TGACACTGGCAAAACAATGCA	AGCTTGCGACCTTGACCATCT
muHPRT	J00423	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG
muMTP	L47970	TCAAGAGAGGCTTGGCTAGCTT	GGCCTGGTAGGTCACTTTACAATC

Results

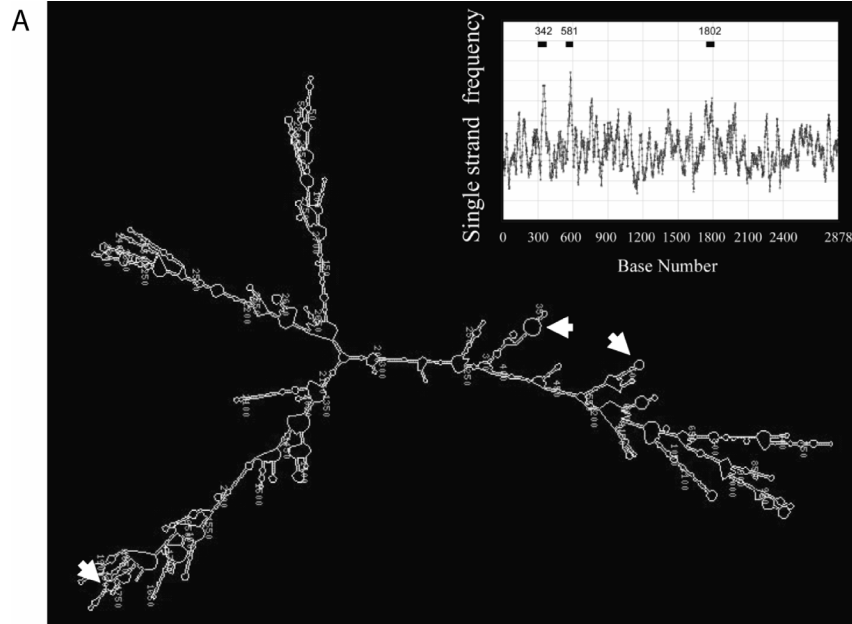
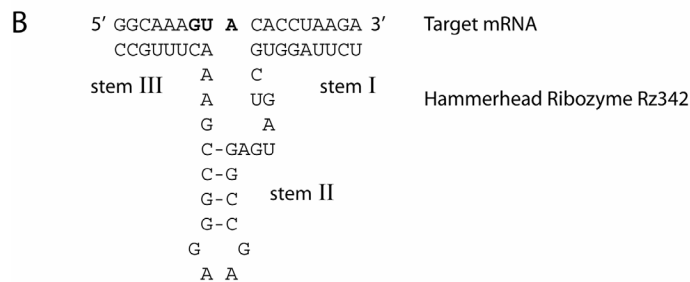


Figure 1. Identification of potential target sites for hammerhead ribozymes against muMTP. Accessibility for ribozymes was determined by mapping of the secondary structure of muMTP mRNA using Mfold algorithm. Based on the single strand frequency and the presence of a cleavable triplet according to the GUX-rule three target sites were identified for ribozyme(A). The general architecture of a ribozyme, at which the catalytic domain of the ribozyme, stem II, is flanked by two hybridizing arms that form stem I and III, respectively (B).



Design of Ribozymes targeted against muMTP

In a previous study we have identified several domains throughout the muMTP RNA with an increased average single strand frequency that appeared to be accessible for antisense hybridization and thus capable of inhibiting muMTP expression (Vrins et al. manuscript in preparation). Three of these domains contained a GUX triplet and were selected as potential

ribozyme target sites (fig. 1A). A schematic diagram of the secondary structure of one the three hammerhead ribozymes is shown in figure 1B. The catalytic domain (stem II) is flanked by 8-9 nucleotide long hybridizing arms complementary to muMTP mRNA (stem I and III). The ribozymes Rz342, Rz581, and Rz1802 are defined by their respective target site of hydrolysis.

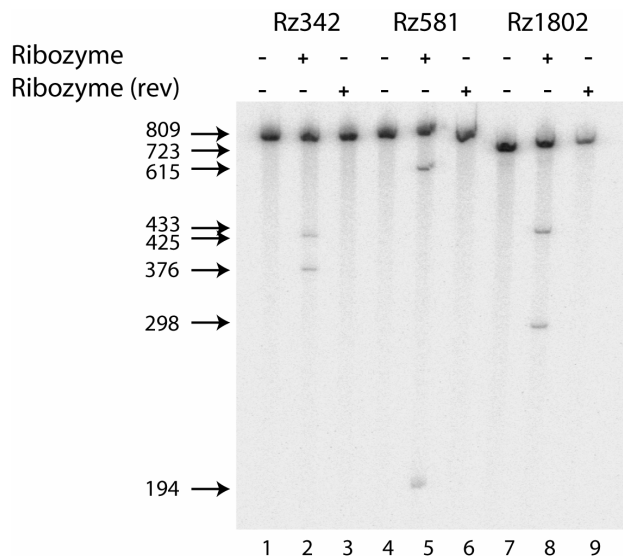


Figure 2. Cleavage of muMTP RNA by ribozymes in cell free assay system. [^{32} P]UTP-labeled muMTP RNA (10 pmol) was incubated with the RNA ribozyme (40 pmol) for 60 minutes at 37 °C. Control experiments were carried out using antisense ribozyme sequences of R342, Rz581, or Rz1802. Reaction mixtures were applied to 6% polyacrylamide urea gel electrophoresis and gels analyzed by autoradiography. The sizes of the RNA fragments are indicated on the left.

Cell free testing of ribozyme activity

To test the ability of the ribozymes to cleave their target, constructs encoding the ribozyme and the ribozyme target sequences were cloned into a T7 promoter containing vector pBluescript. Each ribozyme was able to cleave its substrate and to produce two fragments (fig. 2, lane 2, 5, 8) with the predicted fragment size. Control transcripts from the minus strand of the ribozyme constructs did not show any activity (lane 3, 6, 9). The catalytic activity of all three ribozymes was Mg^{2+} dependent (data not shown). To assess the intrinsic activity of the ribozymes we monitored product formation in time (fig. 3). In the process of cloning the Rz581 construct in pBluescript we obtained a clone containing Rz581 in tandem formation (RzT581). This tandem ribozyme construct encoded an active ribozyme (data not shown), at which the 3' Rz581 had a

Table 3. Kinetics of ribozyme mediated target RNA cleavage

	Km (μ M)	Vmax (nmolar/min)
Rz342	8	12.5
Rz581	5	14.2
Rz1802	7.5	12.5
RzT581	5.7	187.4

single nucleotide deletion within the stem-I hybridizing arm. Although Rz581 and RzT581 share the same target site the latter proved to be considerably more active (Fig. 3A). Moreover as shown in figure 3B, RzT581 was also found to be more efficient than Rz342 and Rz1802. Next, we analyzed the cleavage kinetics of the four ribozymes under substrate excess conditions. For each ribozyme the cleavage reactions with different substrate concentrations was followed over a time period of 10 minutes. As shown in table 3, all four ribozymes have similar K_m -values. While the maximum cleavage rates (V_{max}) of Rz581, Rz342, and Rz1802 were identical, that of RzT581 was found to be more than 10-fold higher compared to the other ribozymes.

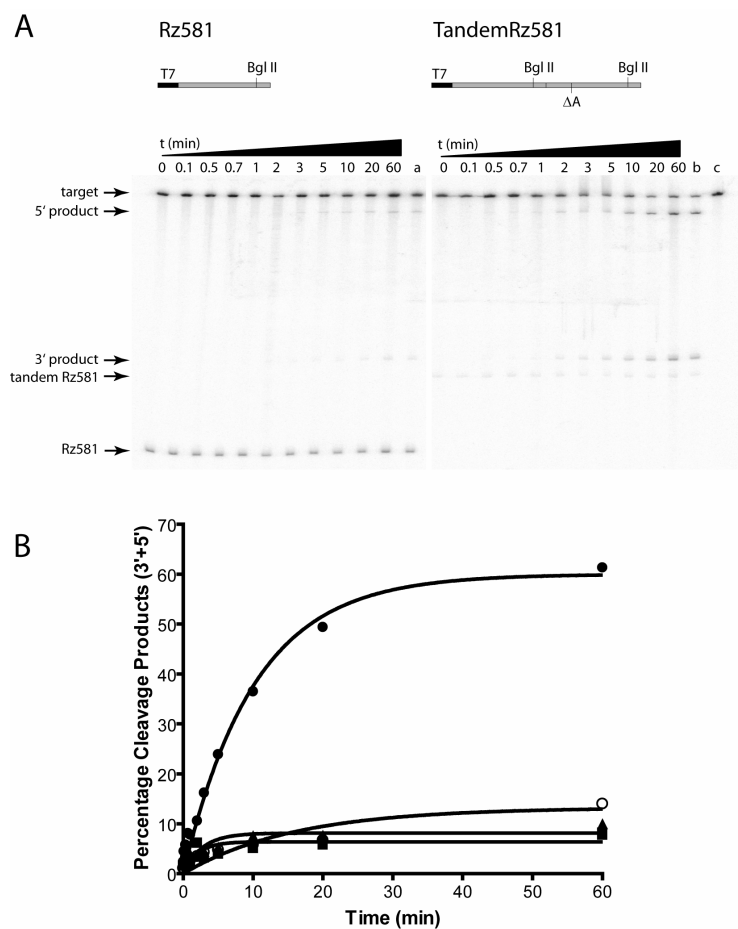


Figure 3. Time curves of ribozyme mediated cleavage reactions in cell free system. [^{32} P]UTP-labeled muMTP RNA (10 pmol) was incubated with the ribozymes RNA (40 pmol) at 37 °C and samples were taken at several time points. Reaction mixtures were applied to 6% polyacrylamide urea gel electrophoresis and subjected to autoradiography (A). The intensity of the cleavage products (3' and 5' products) was analyzed on the phosphoImager. Product formation is plotted vs time for Rz342 (▲), Rz581 (■), Rz1802 (○), and RzT581 (●) (B).

Effect of Adenovirus-mediated Rz581 expression on muMTP RNA levels in Hela cells

To evaluate the mRNA cleaving capacity of the muMTP ribozymes at a cellular level we have generated recombinant adenoviral vectors. Although all muMTP targeting hammerhead ribozymes demonstrated efficient endonuclease activity, Rz581 appeared to be the most

Table 4. Effect of adenoviral ribozyme transfer to EGFP-muMTP transfected Hela cells on the relative EGFP-muMTP expression

	EGFP-muMTP relative to HPRT	EGFP-muMTP relative to GAPDH	GAPDH relative to HPRT
Ad-GFP	100.0	100.0	100.0
AdGFP-CMV.Rz581	165.3	170.5	96.6
AdGFP-CMV.RzT581	55.9*	55.9*	104.1
AdGFP-U6.Rz581	64.3*	58.7*	100.0
AdGFP-tRNA.Rz581	52.4*	65.4*	83.5

* p<0.05

Note: No muMTP RNA levels detected in untransfected cells (Ct-values 38.4 compared to 19)

promising under cell free conditions. Therefore we choose this target construct to generate a series of recombinant adenoviral vectors. In earlier *in vitro* studies it was already suggested that transcript stability, expression level and transcript location critically depends on the promotor used to drive ribozyme transcription¹⁶⁻¹⁹, and this seemed to be corroborated by our finding that RzT581 was more active than Rz581 itself. Therefore we have in this study generated different adenoviral vectors at which ribozyme expression was under the control of the CMV, the U6+27 or the tRNA^{met} promoter, respectively, termed AdGFP-CMV.Rz581, AdGFP-U6.Rz581, and AdGFP-tRNA.581. With RzT581 being the most potent ribozyme against muMTP mRNA, we also generated recombinant adenovirus AdGFP-CMV.RzT581. MuMTP deficient Hela cells were transfected with pEGFP-muMTP carrying the muMTP gene. After pre-transfection, Hela cells were infected with one of the adenoviral ribozymes or with control AdGFP. MuMTP mRNA levels were analyzed by quantitative real time PCR at which the amplicon produced by the MTP primers encompassed the putative ribozyme target site GUC⁵⁸¹ (table 4). Except from AdGFP-CMV.Rz581, all adenoviral based ribozymes expressions led to a significant 40% reduction of muMTP expression.

Effect of adenoviral ribozymes on endogenous muMTP expression *in vitro*

Next, we have examined to what extent adenoviral ribozyme activity is influenced by the expression cassette *in vitro*. The various ribozyme vectors were tested for their ability to inhibit endogenous muMTP expression in the mouse hepatoma cell line mhAT3F2. MhAT3F2 cells were transduced at almost 100% efficiency as judged from the percentage of GFP positive cells at

24 hours post-infection. Relative muMTP mRNA levels were analyzed 24 hours after infection and compared with those of mock infected controls (fig. 4). Both CMV driven ribozyme vectors, AdGFP-CMV.Rz581 and AdGFP-CMV.RzT581, inhibited muMTP expression, albeit that the reduction did not reach significance. AdGFP-U6.Rz581 had no apparent effect on muMTP expression either. In contrast, infection with AdGFP-tRNA.Rz581 resulted in a significant reduction of muMTP mRNA levels (55%, $p < 0.05$).

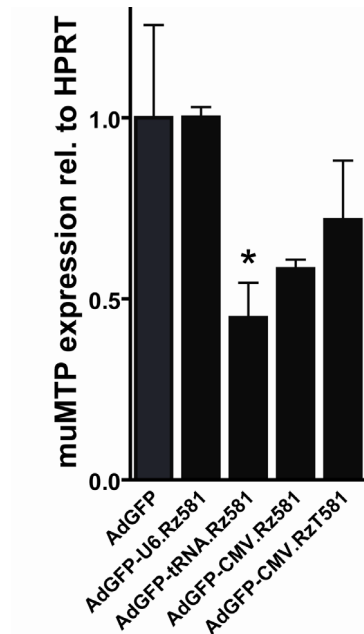


Figure 4. Effect of adenoviral ribozyme transfer to mhAT3F2 hepatoma cells on endogenous muMTP expression ($\pm$ SEM). Mouse hepatoma AT3/F2 cells were seeded in a 12-wells plate and grown to a confluency of 60-70% before infection. Cells were infected with AdGFP, AdGFP-CMV.Rz, AdGFP-CMV.RzT581, AdGFP-U6.Rz581, or AdGFP-tRNA.Rz581 (MOI=100) and 24 hours post-infection cells harvested for RNA isolation. The muMTP mRNA levels were assessed and normalized to HPRT levels using quantitative real time PCR analysis. Values are means $\pm$ S.D. of a quadruplicate determination ($P < 0.05$ is indicated by an asterix).

Discussion

On the basis of RNA folding analysis for antisense drug development (Vrins et al. manuscript in preparation) we have defined three GU $\downarrow$ X candidate sites (GUA³⁴², GUC⁵⁸¹, and GUC¹⁸⁰²) for ribozyme design. A cell free screening assay revealed that all ribozymes were able to cleave muMTP RNA, at which the cleavage products possessed the expected product lengths indicating that the ribozymes hydrolyzed their RNA substrates specifically at the aimed cleavage site. A tandem Rz581 repeat (RzT581) bearing a single nucleotide deletion at the stem I hybridizing arm of the 3' ribozyme unit, which incidentally was generated during ribozyme cloning, proved to be approximately 10-fold more efficient in cleaving its substrate than its single ribozyme counterpart and the other ribozymes. Although it cannot be excluded that RzT581

contains two fully functioning muMTP cleaving domains ²⁷, the mutation in the 3' ribozyme is likely to attenuate hybridization with its target RNA. In view of the unexpectedly high (10-fold) increase in $V_{\max}$, it is however more probable that the extra stem loop favors a catalytically active configuration of the ribozyme. Moreover, flanking hairpins will also provide further protection against exonucleases thus increasing its half life ²⁸.

The high efficacy of RzT581 underscores the importance of ribozyme stability and conformation which both dictate ribozyme levels that can be achieved intracellularly. To improve the stability and expression level of Rz581 we generated a series of adenoviral vectors based on Rz581, at which the promoter and flanking sequences was varied. Expression of adenoviral ribozymes is often driven by a pol II dependent promoter of viral origin, eg. CMV and RSV ²⁹⁻³², generating 5' capped and polyadenylated transcripts. By contrast, pol-III dependent promoters, such as U6snRNA or tRNA^{met}, are eminently equipped for mediating high expression of small RNA transcripts in eukaryotic cells ¹⁹. Moreover, U6snRNA and tRNA^{met} derived transcripts will be provided with stabilizing 3' stem loops. For comparison we have also generated a CMV driven adenovirus for the tandem repeat ribozyme RzT581 (Ad-CMV.RzT581), arguing that the CMV expression cassette would benefit most from the higher stability of the tandem ribozyme. Except for the CMV.Rz581 adenovirus, all of the GUC⁵⁸¹ targeted ribozyme vectors were demonstrated to be effective *in vitro* in GFP-muMTP expressing Hela cells. Both RzT581 and the Rz581 transcripts expressed from the RNA-polymerase III driven cassettes effected a significant reduction of GFP-muMTP expression, supporting our initial notion that indeed the addition of stabilizing sequences including artificial stem loops or an extra ribozyme sequence is a key factor in increasing ribozyme efficacy.

Surprisingly, only AdGFP-tRNA.Rz581 was able to significantly lower endogenous muMTP mRNA levels in mhAT3F2 cells. CMV driven expression of ribozymes tended to reduce muMTP expression albeit not significant, while the U6 transcript appeared to be completely ineffective. The observed differential effect may be attributable to differences in the folding of endogenous versus cDNA construct derived muMTP transcripts. Alternatively, promoter- or cell-dependent differences in ribozyme expression, stability or intracellular location may underlie the observed effects ¹⁸. In regard to the latter it is important to note that eukaryotic pol II driven transcripts, being guanosine capped and polyadenylated, are generally exported to the cytoplasm, while U6+27 and tRNA derived transcripts remain primarily located in the nucleus ^{18,19}.

Although this explain the inability of U6+27 generated ribozymes to quench muMTP expression in mhAT3F2 cells, it leaves unanswered the high activity of the tRNA^{met}-promoter based construct. Conceivably, the high intracellular levels of tRNA driven Rz581 may compensate for the suboptimal location of the transcript. In that case, the use of a conventional tRNA promoter/terminator, favoring cytosolic translocalisation of derived transcripts, could further improve the inhibitory potential of the ribozyme^{33,34}.

Although a number of MTP-inhibitors already were shown to have therapeutic potential^{6,35}, these inhibitors interfere with hepatic and intestinal MTP activity which may be accompanied by side effects including deficiency of lipid soluble vitamins⁵. Targeted ribozyme therapy as proposed in this study may, with its liver and MTP specific mode of action, perform better in this regard, while its sustained inhibition also favorably contrasts with that of previously described antisense drugs³⁶.

In conclusion, a ribozyme has been designed which specifically targets muMTP and effects cleavage of MTP mRNA at the predicted site. While a CMV driven adenovirus construct was ineffective, a tRNA^{met} promoter construct appeared to be capable of lowering muMTP expression in vitro by >50%. This ribozyme construct may thus proof a valuable tool for future mechanistic studies on MTP but also as starting point for gene therapy strategies aiming at MTP lowering

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4

Repression of mouse Apolipoprotein E Transcription by Zinc Finger Protein Znf202

Carlos L.J. Vrans^{1,2}, André van der Zee², Peter van Santbrink¹, Tokameh Mahmoudi³, Christiaan van der Lans¹, Theo J.C. van Berkel¹, Louis M. Havekes^{4,5,6}, Ko Willems van Dijk^{2,5}, and Erik A.L. Biessen¹

¹ Division of Biopharmaceutics, Leiden/Amsterdam Center for Drug Research, Leiden University, Leiden, The Netherlands.

² Department of Human Genetics, LUMC, Leiden University, Leiden, The Netherlands.

³ Department of Molecular and Cell Biology, LUMC, Leiden University, Leiden, The Netherlands.

⁴ TNO Prevention and Health, Gaubius Laboratory, Leiden, The Netherlands.

⁵ Department of General Internal Medicine, LUMC, Leiden University, Leiden, The Netherlands.

⁶ Department of Cardiology, LUMC, Leiden University, Leiden, The Netherlands.

Abstract

Genetic studies have indicated that in humans *znf202* is functionally linked to hypoalphalipoproteinemia and recent in vitro studies have shown that the zinc finger protein Znf202 binds to and represses the promoter activity of lipid related genes such as *ABCA1*, *apoA4*, and *apoE*. However, the net effect of modulation of Znf202 expression on lipid metabolism remains unclear. In this study we have verified whether interspecies differences exist in Znf202 regulation that preclude the mice as testing species for Znf202 function. Infection of the murine hepatoma cell line, mhAT3F2, with Ad-Znf202 led to a significant up to 50% reduction in the expression of the *apoE/C1/C2* and the *apoA1/C3/A4/A5* gene clusters. Promoter deletion analysis of the mouse apoE promoter revealed that Znf202 caused a more than two-fold reduction in apoE promoter activity. The repressive activity could be localized to a region spanning nucleotides –705 and –362, which includes two putative Znf202 response elements. The Znf202 response elements are considerably more upstream than that in the human *apoE* gene, whereas similar repressive effect of Znf202 on the mouse *apoE* gene was observed. These findings support that Znf202 governs the transcription repression of key genes in lipid metabolism and that mouse models might be a valuable tool to assess the role of Znf202 in lipid metabolism.

Introduction

To date, several genes have been identified that transcriptionally regulate the expression of lipid related genes. The gene of one such transcription factor, the zinc finger protein *znf202*, was found in the locus linked to heritable hypoalphalipoproteinemia in Utah pedigrees ^{1,2}. It encodes a protein belonging to the subfamily of Cys₂-His₂ zinc-finger proteins which contain a Leucine-rich domain known as SCAN and Krueppel-associated box (KRAB) ^{1,3}. Although the function of the individual domains are still under investigation, the presence of a KRAB domain in Znf202 indicates that it may function as a transcriptional repressor ⁴. The consensus DNA motif of the Znf202 response element has been characterized as GGGGT (GnT) ⁵. Based on presence of this consensus site in various promoters, Znf202 was suggested to have a broad range of target genes involved in lipid metabolism. In vitro studies have confirmed that Znf202 overexpression is accompanied by a repression of apoE, apoA4, ABCA1, and ABCG1 promoter

activity and that during monocyte differentiation and foam cell formation Znf202 expression is inversely correlated with apoE and ABCA1 expression⁵⁻⁷. These findings provide insight in the important role of Znf202 in lipid metabolism, but the overall phenotypic effect of modified Znf202 expression is still hard to predict. Since *znf202* and their target genes are strongly conserved between mice and humans, mouse models could be a valuable tool to investigate the putative role of Znf202 in lipid metabolism. The different response of cholesterol 7 α -hydroxylase (*cyp7A1*) expression upon liver X receptor (LXR) activation between both species has illustrated that before extrapolating transcriptional regulatory pathways between species the regulatory control in both species should first be firmly established^{8,9}.

Therefore, in this study we set out to investigate the transcriptional regulation of murine *apoE* by Znf202. Since promoter-reporter assays have demonstrated the control of Znf202 over the human apoE promoter and an inverse correlation between endogenous Znf202 and apoE expression during monocyte differentiation was shown, we analyzed the mouse apoE promoter for Znf202 consensus sites. We found a strongly conserved region of 200 bp upstream of the transcription initiation site, which included a TATA box, a GC box, and a Znf202 binding motif. Our results show that the activity of the apoE promoter is reduced in the presence of Znf202 in a hepatic related cell type. The region of the apoE promoter ranging from nucleotides -705 to -362, was required for repressive action by Znf202 and the transcription factor co-localized with the Znf202 consensus sites within that region. Although this report confirms repressive activity of mouse Znf202, we found a species-specific difference in the promoter region of apoE.

Material and Methods

Cell Culture

Mouse hepatoma mhAT3F2 cells¹⁰ were cultured in Dulbecco's Modified Eagle's Medium with F-12 (cat. no. 31331, Gibco) supplemented with 10% fetal calf serum (Gibco), 100 IU/ml Penicillin, 100 μ g/ml Streptomycin, 20 mM GlutaMAX I, 400 nM Insulin (Sigma), 10 nM Dexamethasone (Sigma). Per.C6 and 911^{11,12} cells were both cultured in Dulbecco's Modified Eagle's Medium (cat. no. 41966, Gibco), supplemented with 10% fetal calf serum (Gibco), 100 IU/ml Penicillin, 100 μ g/ml Streptomycin, 2 mM GlutaMAX I (Gibco), with the difference that for Per.C6 cells 10 mM MgCl₂ was added. For large scale production of recombinant adenovirus in Per.C6 cells the 10% fetal calf serum was substituted with 2% horse serum (Gibco).

Construction of expression constructs

Mouse Znf202m1 was obtained from liver cDNA of C57Bl/6Jlco by PCR (nucleotide positions 186 to 2208, GenBank accession number AF292648), with forward primer 5' -GGTACCATAACACCCAAGAGCCAGGA -3' containing a KpnI site and a reverse primer 5' -TCTAGACAGAACCCATCCGTCTCAGT -3' containing a XbaI site. The amplicon Znf202 was cloned into pShuttleCMV and pAdTrackCMV via these restriction sites¹³. Sequences were verified by sequence analysis using ABI 3700 (LGTC, Leiden).

Construction of reporter gene constructs

The apolipoprotein E promoter (GenBank accession number D00466) was cloned from cosmid mAPOE/B1 cos27 (C2RB) by PCR. Two reverse primers were designed, one targeting nt 1020 of exon 1, and one targeting nt 1801 of exon 2, immediately upstream of start codon, because intron 1 contains an Intron Regulatory Element 1 (IRE 1). The mouse promoter was sub-divided in 6 sections and forward primers were chosen accordingly (table 1).

The amplicons were cloned into the pCR.2.1 TOPO vector of the TOPO TA Cloning kit (Invitrogen). Subsequently, KpnI-HindIII fragments were cut out of the pCR.2.1 vector and inserted into KpnI-HindIII restricted pGL3-Basic (Promega). All promoter fragments in the resulting reporter gene constructs were verified by sequencing (LGTC, Leiden).

Table 1. primers used to generate promoter constructs

primer	Sequence
mApoEp-705FW	5' CCGGTACCGGCTTGAGAGTGGAAAATGC
mApoEp-362FW	5' CCGGTACCAGGGATCCCAGAACAACGAT
mApoEp-218FW	5' CCGGTACCCCAAGGCCTGGATTATTCAA
mApoEp-90FW	5' CCGGTACCTCGTGACAGCGTGACAG
mApoEp-34FW	5' CCGGTACCCCTATAATTGGACCGGTCTGG
mApoEp-9FW	5' CCGGTACCCGATCCCCTGCTCAGACC
	KpnI
mApoEpEx1RV	5' GGGAAGCTTGTCTTAGCCTCCAGGGTCT
mApoEpEx2RV	5' GGGAAGCTTCGCAATTGTGATTGGCC
	HindIII

Luciferase reporter assays

Transient transfections in mhAT3F2 cells were performed in triplicate in 6-well plates using Fugene 6 Transfection Reagent (Roche Molecular Biochemicals). Luciferase activity of the different promoter-reporter constructs and a promoterless pGL3-basis serving as control was measured by co-transfecting the cells with 200 ng of a reporter gene construct and 100 ng of pCMV-LacZ. After 24 h, the cells were lysed after 24 h with reporter lysis buffer (Promega) and after centrifugation of 2 min, supernatant was used for determining β -galactosidase normalized luciferase activity by adding 100 μ l luciferyl-CoA (Promega) to 20 μ l of cell extract in a monolight luminometer (BD Biosciences). β -galactosidase was measured in a 96-well microtiter plate using the β -Galactosidase Enzyme Assay System in reporter lysis buffer (Promega). Absorbance at 405 nm was determined in a microplate reader. The effect of Znf202 on transcription regulation of the reporter gene constructs was determined by co-transfecting the cells with

100 ng of a reporter gene construct and 1900 ng of the expression vector pShuttleCMV-Znf202 or an empty pShuttleCMV control vector. Luciferase activities were measured as described above and normalized for protein concentrations using BCA System (Pierce).

Whole cell extract preparation and electrophoretic mobility shift assays

911 cells were transfected with 5 µg pAdTrackCMV-Znf202 or control plasmid on 10 cm dishes using LipofectAMINE plus kit. After 40 hours cells were harvested, and cell pellets were resuspended in equal volumes of a lysis buffer (20 mM Hepes, 600 mM KCl, 0.2 mM EDTA, 1mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM leupeptine, 0.5 mM Aprotinin, 0.5 mM pepstatinA, 0.5 mM 4-(2-amino ethyl)benzene sulfonyl fluoride (ABSF)). This mixture was agitated gently for 30 minutes at 4°C and centrifuged for 10 minutes at 4°C. Supernatant was collected and an equal volume of a glycerol buffer was added (1 (20 mM Hepes, 40% glycerol, 0.2 mM EDTA, 1mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM leupeptine, 0.5 mM Aprotinin, 0.5 mM pepstatinA, 0.5 mM 4-(2-amino ethyl)benzene sulfonyl fluoride (ABSF)). Protein concentrations were determined using Bradford assay (BioRad). The DNA-binding activity of mouse Znf202 in whole cell extracts was studied by means of EMSA. Double-stranded DNA probes containing the Znf202 binding sites, the consensus GnT oligonucleotide, the putative -564 and -678 Znf202 binding oligonucleotides, as well as a control unrelated fragment containing the pleiohomeotic (PHO) consensus binding site were used for gel shift analyses after end-labeling of each probe with [γ -³²P]ATP and T₄ polynucleotide kinase. The sequences of the GnT, -564, -678, and PHO oligonucleotides were 5'- GTGGGGTGGGGGTGGGGGGTCC-3', 5'- GCTCTGTAGGGGTAGCTCTTCC-3', 5'- TGCACATGCAGGGGTGCACACC -3' and 5'- CCGGCGCAGCCATTAAGGAGG -3' respectively. ³²P labeled oligonucleotide probes (~20 fmol) were incubated with 0.1 – 0.6 µg of whole cell extract of control or Znf202 overexpressing cells for 30 min at room temperature in 25 µl of reaction mixture containing 20 mM HEPES–KOH pH 7.9, 5 mM MgCl₂, 75 mM KCl, 1 mM DTT, 0.1 mM EDTA, 2 µg of BSA and 2 µg of poly(dI–dC)·poly(dI–dC) as competitor DNA. Specificity of binding was determined by competition experiments in the presence of a 50-fold excess of unlabeled GnT, -564, or -678 oligonucleotide. Reaction mixtures were loaded on native 6% polyacrylamide gels, run in 0.5x Tris-Glycine at 200 V at 4°C, and visualized by autoradiography.

Adenoviral vectors

Recombinant adenoviral plasmids were generated by homologous recombination with pAdEasy1 in BJ5183 cells as described previously¹³. Correct clones were propagated in STBL2 cells (Life Technologies). For the production of Ad-Znf202 Per.C6 cells were transfected with 3 µg Pac-I-linearized adenoviral construct on 35 mm dishes using LipofectAMINE PLUS kit as described by the manufacturer (Life Technologies). After 16 hours transfection medium was replaced by growth medium. Transfected cells were harvested at day 7 post-transfection and after three cycles of freeze-thaw the lysate was used for large-scale production of Ad-Znf202 in Per.C6 cells. Virus was purified by CsCl centrifugation and subsequently dialyzed. Final yields as assessed by plaque assay in 911 cells were approximately 2×10^{10} plaque forming units (pfu)/ml. Control virus Ad-LacZ was produced as described previously¹⁴. MhAT3F2 cells were seeded in a 12-wells plate and grown to a confluency of 70%. Cells were infected with either Ad-Znf202 or control Ad-LacZ (MOI=100).

Real time quantitative PCR analysis

Total RNA from mhAT3F2 infected cells was isolated by using the **Trizol** method according to the manufacturer's protocol (Eurogentec). Purified RNA was treated with RQ1 RNase-free DNase (Promega, 1 units/ 2 µg of total RNA) and reverse transcribed with SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. Quantitative gene expression analysis was performed on an ABI prism7700 Sequence Detection System (Applied Biosystems) using SYBR Green as described earlier ¹⁵. PCR primer sets (table 2) were designed via Primer Express 1.7 software with the manufacturer's default settings (Applied Biosystems) and were validated for amplification efficiency. The absence of genomic DNA contamination in the RNA preparations was confirmed in a separate PCR reaction on total RNA samples which were not reverse transcribed. Hypoxanthine-guanine phosphoribosyl transferase (HPRT) was used as the standard housekeeping gene.

Data Analysis—The significance of differences in relative gene expression numbers C_t ($C_{t(HPRT)} - C_{t(target\ gene)}$) measured by real time quantitative PCR was calculated using a two-tailed Student's *t* test. Probability values less than 0.05 were considered significant.

Table 2. Primers used for gene expression analysis

Gene	GenBank Acces.	Forward Primer	Reverse Primer	Size
36B4	X03672	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG	85
ApoA1	NM_009692	GGCAGAGACTATGTGTCCCAGTT	CCCAGTTTTCCAGGAGATTTCAG	97
ApoA4	XM_134754	GGAGGCTGTAGAACAGTTTCAGAAG	TCCCAAGTTTGTCTGGAAC	94
ApoA5	BC011198	GAGCAAAGGCGTGATGGG	TGCTCGAAGCTGCCTTTCA	66
ApoC1	BC019398	CGGGCAGCCATTGAACATA	TTGCCAAATGCCTCTGAGAAC	77
ApoC2	NM_009695	TTGCCAAAGACCTGTACCAGAA	GCCGAGCTTTTGTGTACATG	78
ApoC3	NM_023114	CATCTGCCCGAGCTGAAGA	GCTTGTTCCATGTAGCCCTGTAC	93
apoE	M12414	AGCCAATAGTGGAAGACATGCA	GCAGGACAGGAGAAGGATACTCAT	130
Cyclophilin	M60456	TCGGAGCGCAATATGAAGGT	AAAAGGAAGACGACGGAGCC	65
GAPDH	NM_008084	TCCATGACAACTTTGGCATTG	TCACGCCACAGCTTTCCA	103
HPRT	J00423	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG	91
Znf202	AF292648	GTGTGGCCGCTCCTTCAG	GGGCGATGATCCTGTACTGG	79

Results

Genes within the apoE/C1/C2 and apoA1/C3/A4/A5 clusters are downregulated upon Znf202 overexpression.

We analyzed the effect of Znf202 overexpression on the *apoE/C1/C2* and *apoA1/C3/A4/A5* gene clusters in the mouse hepatoma cell line mhAT3F2. To study the effect of Znf202 overexpression in cells we constructed a recombinant adenovirus containing the mouse Znf202 gene. MhAT3F2 cells were transduced with Ad-Znf202 at a multiplicity of infection of 100 (MOI=100). After 24 hours the cells were harvested and total RNA was isolated for

quantitative real time PCR analysis (Fig. 1). GAPDH, cyclophilin, or 36B4 expression remained unaffected by Znf202 overexpression. However, Znf202 overexpression did result in a significant downregulation of apolipoprotein genes from both clusters. RNA levels were reduced up to 50% (apoC2 and apoC3) and reached significance for all genes ($p < 0.05$) except *apoA5*.

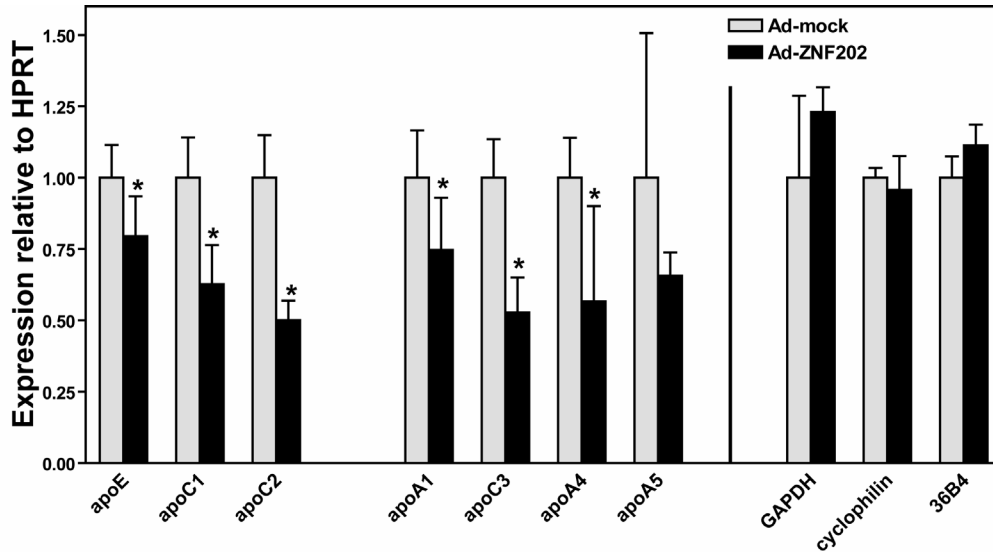


Figure 1. Overexpression of Znf202 in mhAT3F2 cells leads to repression of genes that are situated within the apoE/C1/C2 and apoA1/C3/A4/A5 gene clusters. MhAT3F2 cells were transduced with Ad-Znf202 (black bars) or Ad -LacZ (grey bars)(MOI=100) and RNA levels were measured via quantitative real time PCR at 24 hours post-transfection. Data represent average of four transductions for each group (mean $\pm$ S.D) and expressions are relative to HPRT.

Localisation of repressive effect of Znf202 on mouse apoE promoter to -705/-362 region.

Truncation of the upstream promoter fragment was based on the position of putative Znf202 responsive GnT sites. As a measure of the promoter activity we determined luciferase activity of the various apoE promoter-reporter constructs in mhAT3F2 cells at 24 hours post-transfection (Fig. 2). Expression values were normalized for transfection efficiency, by co-transfection with pCMV-LacZ and β -Galactosidase assay serving as an internal reference. Comparison of the relative luciferase activity of the reporter constructs revealed that the presence of intron-1 had a repressive effect on the apoE promoter activity. In fact, the reporter construct containing the promoter sequence -90/+821 completely lacked activity, whereas activity was found to be 40-fold increased in comparison with the promoterless pGL3-basic upon deletion of intron 1. Further, the presence of nucleotide sequences spanning -362 to -90 was seen to enhance promoter activity, while the addition of region -705/-362 attenuated the enhanced activity to up to

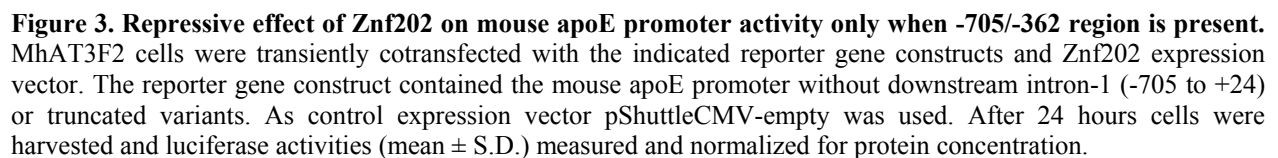
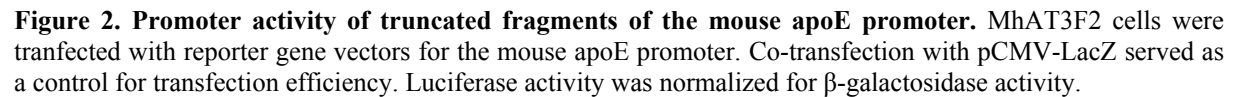


Figure 4. The Znf202 specifically binds to the -678 and -564 sites within the -705/-362 region of the mouse apoE promoter. DNA fragments used in this study containing the consensus or putative Znf202 binding sequence. The putative consensus sequences are shown underlined (A). EMSA with labeled DNA fragments GnT (lanes 1-10), -678 (lanes 11-20), -564 (lanes 21-30), and PHO (lanes 31-35) described above (B). Reactions contained the indicated amount (0.1-0.6 μ g) of whole cell extract made from either control 911 cells (lanes 2, 5, 8, 12, 15, 18, 22, 25, 28, 32, and 34), or from 911 cells overexpressing Znf202 protein (lanes 3, 4, 6, 7, 9, 10, 13, 14, 16, 17, 19, 20, 23, 24, 26, 27, 29, 30, 33, and 35). Competition experiments using 50-fold excess of unlabeled GnT oligonucleotide (lanes 4,7 and 10), -678 oligonucleotide (lanes 14,17 and 20), or -564 oligonucleotide (lanes 24,27 and 30) confirmed the specificity of Znf202 binding. Arrowheads indicate the mobility of unbound DNA and Znf202 protein bound DNA.

We demonstrated above that Znf202 has a repressive effect on apoE expression and that this activity is restricted to the -705/-362 region. This region contained two putative G_nT sites at positions -678 and -564, and we have examined these consensus sites for their capacity to bind Znf202 by EMSA. Thus, 911 cells were transfected with pAdTrackCMV-Znf202 or pAdTrackCMV (empty vector). Since these plasmids also carry the enhanced GFP reporter gene, the transfection efficiency could be easily assessed by fluorescent microscopy showing that 50% of the cells were GFP positive. Whole cell extracts were prepared at 40 hours post-transfection and Znf202 binding to the two ³²P-labeled double-stranded oligonucleotide probes -678 and -564 (-689/-668 and -573/-552) mimicking the alleged binding sites, was assayed (Fig 4A). As

controls we used the Znf202 binding fragment of human ApoA4 promoter containing three consecutive G_nT sites ⁷ and an unrelated probe containing a PHO consensus site. The G_nT control probe (Fig. 4B, lanes 1-10) induced a clear mobility shift indicative of the formation DNA-protein complexes in extracts from Znf202 overexpressing 911 cells (lanes 3, 6, 9). The binding to the probe was specifically competed by 50-fold excess of unlabeled G_nT probe (lane 4, 7, 10). Similar mobility shifts were observed for the ³²P-labeled -678 (lanes 11-20) or -564 probe (lanes 21-30) after Znf202 overexpression.. In analogy to the reference probe, the specificity of binding was confirmed by displacement by the 50-fold excess of either unlabeled -678 probe or -564 probe. Finally, the extract was unable to form DNA-protein complexes with an irrelevant PHO consensus probe, even at high concentrations (lanes 32 and 33). These results clearly show that Znf202 specifically binds to its predicted consensus sites at nucleotide -678 and -564 within the -705/-362 fragment of the apoE promoter.

Discussion

The linkage of the *znf202* gene to familial hypoalphalipoproteinemia together with the identification of several lipid related target genes of Znf202, illustrate the important role of Znf202 in lipid and, in particular, HDL-metabolism ^{1,2}. Previous reports have demonstrated that Znf202 represses the promoter activity of apoE and apoA4 ^{5,7}, two members of the *apoE/C1/C2* and *apoA1/C3/A4/A5* gene cluster, respectively. As transcriptional regulation may differ among species, and as these gene clusters show a strong resemblance with their murine counterparts, we analyzed the effect of mouse Znf202 on the expression of both gene clusters in a mouse hepatoma cell line. We demonstrate that all gene members found in the two gene clusters except apoA5 were repressed in mhAT3F2 cells upon infection with Ad-Znf202.

To investigate the mechanism of transcriptional repression by Znf202 we performed a promoter deletion analysis of the murine *apoE* gene. The repressive effect of Znf202 was previously demonstrated in the -322 to +18 region of the human *apoE* gene and a putative Znf202 consensus site was identified at position -177 ⁵. Screening of the -705/+821 proximal region of the mouse ApoE gene for potential Znf202 binding sites revealed five G_nT consensus sites (positions -678, -564, -338, -315, -160). A series of promoter luciferase constructs were generated at which truncation of the mouse ApoE promoter was based on the position of these

GnT sites, and either contained or lacked intron 1 (+42 to +801), This intron was reported to encompass regulatory elements that may influence Znf202 mediated repression¹⁶.

While the presence of intron 1 led to a 2-fold reduction in ApoE promoter activity in mhAT3F2 cells, it did not affect the overall pattern of expressional regulation. A sharp reduction in promoter activity was observed upon deletion of the -362/-90 region, which may be attributed to enhancer elements located in these region^{16,17}. , Truncation of -705 to -362 was accompanied by a strong 3-fold increase in promoter activity suggesting the presence of suppressor elements in that region. More relevant, Znf202 responsiveness of the mouse apoE promoter activity was ablated upon removal of nt -705/-362. Further analysis established that Znf202 binds specifically to the two GnT sites within that region, to repress mouse apoE expression, we demonstrate here that the Znf202 responsive elements of the mouse ApoE promoter are located far more upstream than that of the human counterpart. Species specific differences in promoter set-up translating into an altered promoter function or responsiveness to modulators of gene expression have been described for several genes such as the osteocalcin, toll-like receptor 3 and *cyp7A1*^{9,18,19}.

In conclusion, although the promoter architecture of the apoE gene, and in particular the location of the Znf202 response elements, appears to differ between mice and men , our data show that mouse apoE expression is regulated by Znf202 as well. In addition, we demonstrate that Znf202 overexpression affects the endogenous expression level of lipid related genes in mice to a similar extent as in men, establishing the mouse as an adequate in vivo model for mechanistic studies on Znf202 regulation of lipid metabolism.

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Hepatic overexpression of Znf202 induces hypotriglyceridemia and steatosis in LDLr^{-/-} but not C57Bl/6 mice.

Carlos L.J. Vrans^{1,2}, Peter van Santbrink¹, André van der Zee², Ruud Out¹, Martine Groenendijk³, Louis M. Havekes^{3,4,5}, Theo J.C. van Berkel¹, Ko Willems van Dijk^{2,4}, and Erik A.L.Biessen¹

¹ Division of Biopharmaceutics, Leiden/Amsterdam Center for Drug Research, Leiden University, P.O.Box 9502, 2300 RA Leiden, The Netherlands.

² Department of Human Genetics, LUMC, Leiden University, Leiden, The Netherlands.

³ TNO Prevention and Health, Gaubius Laboratory, Leiden, The Netherlands.

⁴ Department of General Internal Medicine, LUMC, Leiden University, Leiden, The Netherlands.

⁵ Department of Cardiology, LUMC, Leiden University, Leiden, The Netherlands.

Abstract

Background/Aims: The zinc finger protein Znf202 has been described as a negative transcriptional regulator of lipid related genes and has been linked with hypoalphalipoproteinemia. In this study we are the first to map Znf202 function in lipid metabolism *in vivo*. **Methods:** Hepatic overexpression of *Znf202* in wild type and *Ldlr*^{-/-} mice was achieved by adenovirus gene transfer. Blood samples were taken at day 4 and 5 post-infection, mice were sacrificed and liver biopsies obtained at day 5. Sera were analyzed for lipid and lipoprotein profile, while liver biopsies were used for histological, gene expression, and lipid analysis. **Results:** Znf202 gene transfer to *Ldlr*^{-/-} mice resulted in marked hypercholesterolemia (>2-fold increase of serum total cholesterol) and hypotriglyceridemia (4-fold reduction in serum triglycerides) ($P < 0.05$), which was attributable to an increase in cholesterol-rich, triglyceride-poor VLDL and a concomitant drop in HDL levels. Furthermore, Znf202 overexpression caused severe steatosis and led to a surprising 3-fold increase in hepatic *Apoc2* and *Apoa5* expression ($P < 0.05$), whereas that of other genes with Znf202 responsive elements remained unaffected. As *Apoc2* and *Apoa5* overexpression is associated with hypotriglyceridemia, this could at least partly explain the observed phenotype. While Znf202 overexpression in wild type mice effected a similar increase in *Apoc2* and *Apoa5* expression and drop in HDL levels, this was not accompanied by changes in serum VLDL nor by steatosis. In wild type both not in *Ldlr*^{-/-} mice, Znf202 overexpression sharply increased cholesterol 7 α -hydroxylase expression ($P < 0.05$) and activity ($P < 0.001$), which raises the intriguing premise that this gene may be instrumental in Znf202 induced responses. **Conclusion:** Znf202 overexpression *in vivo* reveals an important role of this transcriptional regulator in liver lipid homeostasis, while establishing the proposed key function in the control of HDL homeostasis.

Introduction

Hypoalphalipoproteinemia, characterized by subnormal levels of serum HDL, is the most common lipoprotein abnormality in patients with coronary heart disease (CHD) ¹. HDL could be atheroprotective on its own account by acting as lipid acceptor in reverse cholesterol transport from the periphery to the liver and by its anti-inflammatory properties. In addition, low HDL levels often are causally linked to other risk factors including diabetes and hyperlipidemia ². Hypoalphalipoproteinemia has been associated in part with an impaired cellular cholesterol efflux caused by mutations in the *ABCA1* gene ³.

The *Znf202* gene has been identified in a chromosomal region linked to heritable hypoalphalipoproteinemia in Utah pedigrees ^{4,5}. The Znf202 protein is a Cys₂-His₂ zinc-finger protein family member and contains a leucine-rich domain known as SCAN and a Krueppel-associated box (KRAB) ^{6,7}. Although the specific function of these domains is still under investigation, the presence of a KRAB domain in Znf202 is suggestive of transcriptional repressor activity ⁸. This is corroborated by *in vitro* findings showing repressional activity of Znf202 on a range of target genes involved in lipid metabolism including *ApoE*, *ApoA4*, *ABCA1*, and *ABCG1* in hepatoma cells ⁵ as well as monocytes ⁹. It was shown that Znf202 interacts with GnT response motifs in the respective promoter regions of these genes. Despite the relatively detailed insight in the range of Znf202 responsive genes *in vitro*, little is known of Znf202 mediated gene regulation *in vivo* in specific tissues. Moreover, the contribution of Znf202 to the regulation of serum lipid and lipoprotein metabolism is up till now unclear.

In the present study, we have investigated the effect of Znf202 overexpression *in vivo* both in normo- (C57Bl/6) as well as in hyperlipidemic (*Ldlr*^{-/-}) mice. To this end, we have generated an adenovirus vector carrying the mouse *Znf202* gene (Ad.Znf202) and assessed the effect of hepatic gene overexpression in both mouse strains on serum lipid and lipoprotein levels and on the hepatic gene expression profile. A marked difference was found between the *in vitro* versus *in vivo* effect of Znf202 overexpression on the mRNA levels of key genes involved in lipid metabolism. While Znf202 overexpression *in vivo* reduced HDL levels in hyperlipidemic *Ldlr*^{-/-} as well as wild type mice, this was accompanied by perturbed lipid homeostasis in hyperlipidemic mice only. The changes in expression of specific genes differed between *Ldlr*^{-/-} and C57Bl/6 mice providing a potential explanation for the differences in serum cholesterol and

TG responses. Our data clearly demonstrate that the transcription factor ZNF202 can act as a key regulator in lipoprotein metabolism.

Material and Methods

Generation of Adenoviral Constructs

Mouse *Znf202m1* (nucleotide positions 186-2208; GenBank accession number AF292648) was obtained from liver cDNA of C57Bl/6Jlco by PCR. Forward (5' GGT.ACC.ATA.ACA.CCC.AAG.AGC.CAG.GA) and reverse primers (5'-TCT.AGA.CAG.AAC.CCA.TCC.GTC.TCA.GT) contained a KpnI and XbaI site, respectively, and *Znf202* amplicon was cloned into pShuttleCMV via these restriction sites. Recombinant adenoviral plasmids by homologous recombination with pAdEasy1 were generated in BJ5183 cells as described by He et al.¹⁰. Correct clones were propagated in STBL2 cells (Invitrogen Life Technologies, Breda, The Netherlands). For the production of Ad.*Znf202* Per.C6 cells (Crucell, Leiden, NL) were transfected with 3 µg Pac-I-linearized adenoviral construct on 35 mm dishes using LipofectAMINE PLUS kit according to the manufacturer's protocol (Life Technologies). After 16 hours transfection medium was replaced by growth medium. Transfected cells were harvested at day 7 post-transfection and after three freeze-thaw cycles the lysate was used for large-scale production of Ad.*Znf202* in Per.C6 cells. Virus was purified by CsCl centrifugation steps and dialysis. Final yields were assessed via a plaque assay in 911 cells and titers were obtained of approximately 2×10^{10} plaque forming units (pfu)/ml. The construction of control virus Ad-mock (Ad-LacZ) has been described previously¹¹.

Animals and Adenovirus Injection

All animal work was approved by the Ethics Committee for Animal Experiments of the Leiden University and the experimental protocols complied with the national guidelines for use of experimental animals. Male C57Bl/6Jlco (Sylvius laboratoria, Leiden, The Netherlands) and *Ldlr*^{-/-} mice on a C57Bl/6Jlco genetic background (TNO-PG, Leiden, The Netherlands) were given a standard mouse diet Chow (Hope Farms, Woerden, NL) and housed under standard conditions in conventional cages with free access to water and food.

Recombinant adenovirus, 2×10^9 pfu in 200 µl of PBS, were administered by injection into the tail vein of mice at the age of 28-32 weeks. Blood samples were taken via tail bleeding 3 days prior to infection and at day 4 and 5 post-infection. At day 5 after bleeding, mice were sacrificed, liver sections were removed and immediately deep-frozen in liquid nitrogen and stored at -80°C. Cryosections were prepared from liver samples and stained with Oil Red-O (Sigma Diagnostics, St. Louis, MO) and hematoxylin (Sigma Diagnostics).

Determination of mRNA Levels

Total RNA was isolated from the caudate liver lobes by the RNA Instapure method according to the manufacturer's protocol (Eurogentec SA, Seraing, Belgium). Purified RNA was treated with RQ1 RNase-free DNase (Promega, 1 units/ 2 µg of total RNA) and reverse transcribed with SuperScript II Reverse Transcriptase (Invitrogen, Breda, The

Table-1: Primers sets used for quantitative real-time RT-PCR

Gene	GenB. Acces.	Forward primer	Reverse primer	size
36B4	X03672	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG	85
ABCA1	NM_013454	GGTTTGGAGATGGTTATACAATAGTTGT	TTCCCGGAAACGCAAGTC	96
ABCG1	NM_009593	AGGTCTCAGCCTTCTAAAGTTCTC	TCTCTCGAAGTGAATGAAATTTATCG	85
ABCG5	NM_031884	TGGCCCTGCTCAGCATCT	ATTTTAAAGGAATGGGCATCTCTT	82
ABCG8	NM_026180	CCGTCGTCAGATTTCCAATGA	GGCTTCGACCCCATGAATG	64
ApoA1	NM_009692	GGCAGAGACTATGTGTCCAGTT	CCCAGTTTTCCAGGAGATTCAG	97
ApoAIV	XM_134754	GGAGGCTGTAGAACAGTTTCAGAAAG	TCCCAAGTTTGTCTGGAA	94
ApoAV	BC011198	GAGCAAAGGCGTGATGGG	TGCTCGAAGCTGCCTTTCA	66
apoB100	XM_137955	GCCCCATTGTGGACAAGTTGATC	CCAGGACTTGGAGGTCTTGGA	122
ApoC1	BC019398	CGGGCAGCCATTGAACATA	TTGCCAAATGCCTCTGAGAAC	77
ApoCII	NM_009695	TTGCCAAAGACCTGTACCAGAA	GCCGAGCTTTGTGTACATG	78
ApoCIII	NM_023114	CATCTGCCCCGAGCTGAAGA	GCTTGTTCATGTAGCCCTGTAC	93
apoE	M12414	AGCCAATAGTGGAAGACATGCA	GCAGGACAGGAGAAGGATACAT	130
CD68	NM_009853	CCTCCACCCTCGCCTAGTC	TTGGGTATAGGATTCGGATTGGA	118
CYP27	BC002183	GTGTCCCGGGATCCCAGTGT	CTTCTCAGCCATCGGTGA	66
CYP7A1	NM_007824	CTGTACATACCACAAAGTCTTATGTCA	ATGCTTCTGTGTCCAAATGCC	75
FXR	XM122102	ACGAAGATCAGATTGCTTTGCTC	CTCCGCCGAACGAAGAAC	65
HMGCR	M62766	TCTGGCAGTCAGTGGGAATATT	CCTCGTCTTCGATCCAATTT	69
HNFB	NM_008261	TGACCCGGGCAAGATCAA	TTGATGTAATCCTCCAGGCTCACT	64
HPRT	J00423	TTGCTCGAGATGTCTATGAAGGA	AGCAGGTCAGCAAGAAGCTTATAG	91
LDL-RECEPTOR	Z19521	CTGTGGGCTCCATAGGCTATCT	GCGGTCCAGGGTCACTTTC	68
LRP	X67469	TGGGTCTCCCGAAATCTGTT	ACCACCGCATTCCTTGAAGGA	95
LXR(a)	AF085745	TCAGCATCTTCTATGCAGACCCGG	TCATTAGCATCCGTGGGAACA	144
LXR(b)	NM_009473	AAGCTGGTGAGCCTGCGC	CGGCAGCTTCTTGTCTG	81
MARCO	MMU18424	AAAGGGTCAAAAAGGCGAATCT	AACCTCAGCTCGGCCTCTGTT	73
MTP	L47970	TCAAGAGAGGCTTGGCTAGCTT	GGCCTGGTAGGTCACTTTACAATC	121
PPAR(a)	NM_011144	TGAACAAAGACGGGATG	TCAAACCTGGGTCCATGAT	106
PPAR(d)	U10375	GAGGGGTGCAAGGGCTTCTT	CACTTGTTCGGTCTTCTTCTG	101
SR-BI	NM_016741	GGCTGCTGTTGTGTCGC	GCTGCTTGATGAGGGAGGG	63
SREBP-1	AB017337	GACCTGGTGGTGGCACTGA	AAGCGGATGTAGTCGATGGC	74
SREBP-2	AF374267	TGAAGCTGGCCAATCAGAAAA	ACATCACTGTCCACCAGACTGC	70
ZNF202	AF292648	GTGTGGCCGCTCCTTCAG	GGGCGATGATCCTGTACTGG	79
Cyclophilin	M60456	TCGAGCGCAATATGAAGGT	AAAAGGAAGACGACGGAGCC	65

Netherlands) according to the protocols supplied by the manufacturers. Quantitative gene expression analysis was performed on an ABI prism7700 Sequence Detection System (Applied Biosystems, Foster City, CA) by SYBR Green as described previously¹². PCR primers (table I) were designed on the basis of Primer Express 1.7 software with the manufacturer's default settings (Applied Biosystems) and validated for identical efficiencies. The absence of genomic DNA contamination in the RNA preparations was verified by PCR of total RNA samples that had not been subjected to reverse transcription. Hypoxanthine-guanine phosphoribosyl transferase (HPRT) and acidic ribosomal phosphoprotein P0 (36B4) were used as standard housekeeping genes. Ratios of target to control gene expression (relative gene expression numbers) were calculated by subtracting the C_t of the target gene from the C_t of HPRT and raising 2 to the power of this difference. C_t values are defined as the number of PCR cycles at which the fluorescent signal during the PCR reaches a fixed threshold. Target gene mRNA expressions are thus expressed relative to HPRT expression.

Lipid and lipoprotein analysis

Blood was collected from each individual mouse through tail bleeding in diethyl-p-nitro phenyl phosphate (paraoxon) coated capillary tubes, after a 4-hour fasting period¹³. Total serum cholesterol (kit No.236691, Boehringer-Mannheim) and triglycerides (kit No. 337-B, Sigma Chemical Co) were measured enzymatically. Lipoprotein distribution was determined by fast performance liquid chromatography (FPLC). A plasma pool was obtained from each group of mice before adenovirus injection and 4 days after adenovirus injection and was diluted 2 to 5-fold with

PBS. A volume of 70 μ l was injected onto a Superose6 column (3.2 x 30 mm, AKTA-system, Pharmacia, Uppsala, Sweden). Elution fractions of 50 μ l were collected and assayed for cholesterol and triglyceride levels as described above. For measurement liver lipids, frozen (N_2) liver samples were homogenized and samples were taken for measurement of protein content (Lowry). Lipids were extracted from the homogenate according to Bligh and Dyer. After evaporation, lipids were dissolved in a 2% Triton-X-100 solution and cholesterol and triglyceride levels were determined as described above.

Cholesterol 7 α -hydroxylase enzyme activity assay

Cyp7A1 enzyme activities in isolated liver microsomes were determined essentially according to Chiang ¹⁴ by measuring the conversion of cholesterol into 7 α -hydroxycholesterol. In short, liver homogenate (750 μ g protein) was incubated in 1 ml of buffer containing 0.1 M potassium phosphate, pH 7.2, 50 mM NaF, 5 mM DTT, 1 mM EDTA, 20% glycerol (w/v), and 0.015% (w/w) CHAPS. Twenty μ l of 1 mg cholesterol in 45% (w/v) hydroxypropyl- β -cyclodextrin was added and the mixture was incubated for 10 min at 37°C under gentle agitation. Regenerating buffer (200 μ l) was added, containing 10 mM sodium isocitrate, 10 mM $MgCl_2$, 1 mM NADPH, and 0.15 U isocitrate-dehydrogenase at 37 °C. After 20 min of incubation, a stop solution (60 μ l) containing 20% sodium cholate and 1 μ g 20 α -hydroxycholesterol, which served as an internal standard, was added. Steroid products were oxidized for 45 min in 100 μ l buffer containing 0.1% cholesterol oxidase, 10 mM K_2HPO_4 , 1 mM DTT, and 20% glycerol at 37 °C, and the reaction was stopped by addition of 2 ml ethanol. Cholesterol metabolites from this reaction mixture were extracted in petroleum ether and the ether layer was evaporated under a stream of nitrogen. Residues resuspended in a mixture of 60% acetonitril, 30% methanol and 10% chloroform (v/v) were analyzed by using C-18 reverse phase HPLC on a Tosohaas TSK gel-ODS 80TM column equilibrated with 70% acetonitrile and 30% methanol at a flow rate of 0.8 ml/min. The amount of products formed was determined by monitoring the absorbance at 240 nm. Peaks were integrated using Data Control software (Cecil Instruments, UK).

Data Analysis– The significance of differences in relative gene expression numbers among the different liver cell types, from three different cell isolations, measured by real time quantitative PCR was calculated using a two-tailed Student's *t* test on the differences in C_t ($C_{t(HPRT)} - C_{t(target\ gene)}$). Differences in lipid, lipoprotein and enzyme activity levels were analyzed statistically by Mann-Whitney-U test. Probability values less than 0.05 were considered significant.

Results

Lipid and lipoprotein levels after adenovirus mediated gene transfer of ZNF202

To address the role of Znf202 in lipid metabolism *in vivo*, we have overexpressed the *Znf202* gene in two mouse strains: the normolipidemic C57Bl/6 (WT) and the hyperlipidemic

Ldlr^{-/-} mouse by adenovirus mediated gene transfer. Injection of Ad.Znf202 in *Ldlr*^{-/-} and WT mice resulted in a significant hepatic overexpression of the transgene from $\Delta C_T = -4$ and -3 to $\Delta C_T = 5$ and 6 , respectively, and corresponding to a 2⁹-fold induction of gene expression (relative expression compared to HPRT, using real-time PCR analysis). Absolute expression levels of the housekeeping genes remained unaltered after Ad.Znf202 treatment.

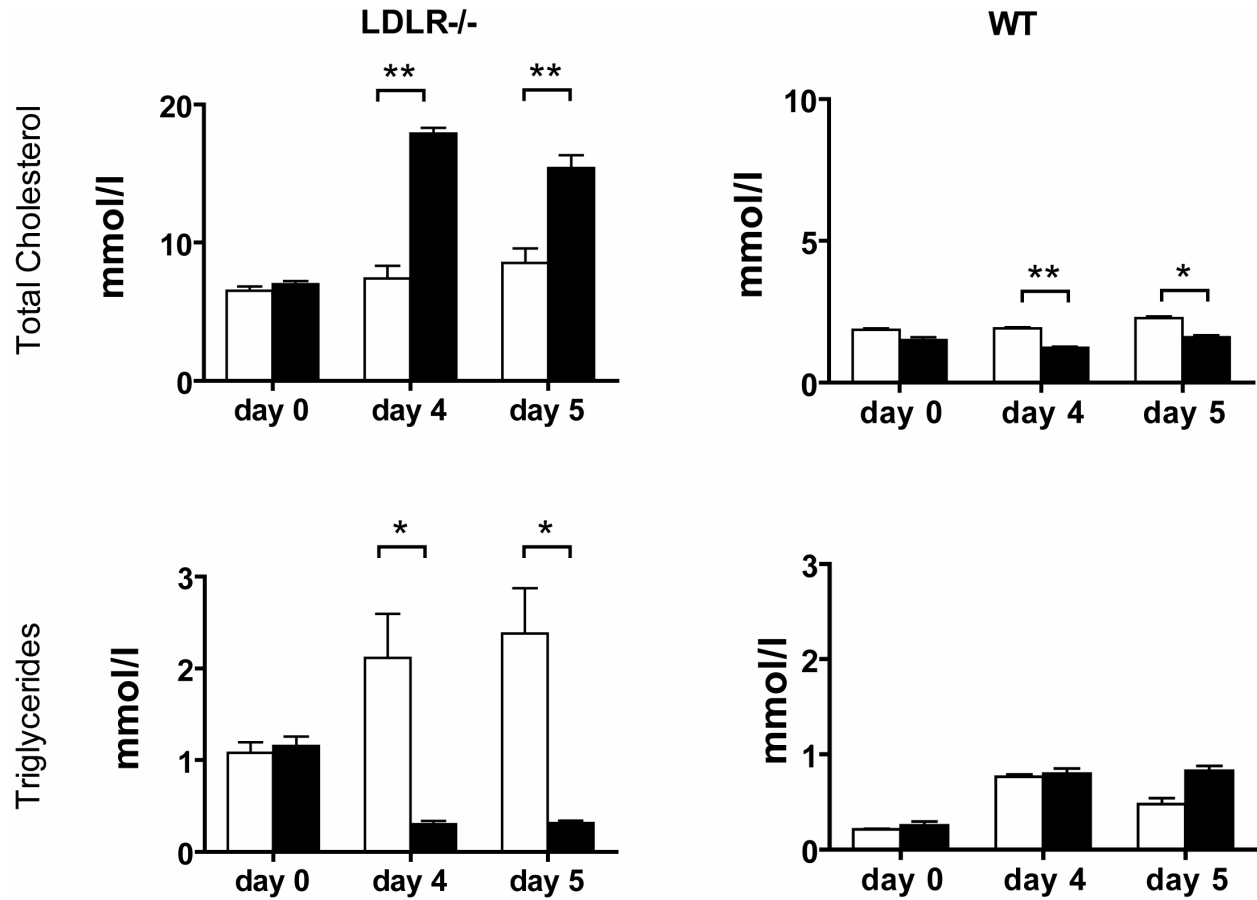


Figure 1: Hepatic Znf202 overexpression specifically alters serum cholesterol and triglyceride content in *Ldlr*^{-/-} mice leaving WT mice unaffected. Blood samples were drawn from *Ldlr*^{-/-} (n=4; left panels) and WT mice (n=4; right panels) 4 or 5 days after injection with 2.10⁹ pfu of Ad.Znf202 (filled bars) or with Ad-mock (open bars) and derived serum was analyzed for triglyceride and total cholesterol content. * and ** indicates P<0.05 and P<0.001, respectively.

Serum lipid analysis revealed a significant 2-fold rise in serum total cholesterol levels in Ad.Znf202 treated *Ldlr*^{-/-} mice on day 4 and 5 after gene transfer (P<0.001), whereas in WT mice Znf202 overexpression did not affect serum TC levels (Fig.1). Surprisingly, the triglyceride levels were dramatically reduced by 4-fold (P<0.05) in *Ldlr*^{-/-} mice only. Subsequent analysis of

the lipoprotein profile revealed that the elevated serum TC level in *Ldlr*^{-/-} mice overexpressing Znf202 was attributable to an increased cholesterol content of the VLDL pool and a concomitant drop in cholesterol in the HDL fraction. Similar to the *Ldlr*^{-/-} mice, WT mice displayed a marked reduction in HDL-cholesterol upon Znf202 overexpression (2.7 mg/ml for Ad-mock treated mice versus 0.7 mg/ml for Ad-Znf202 treated mice) (Fig. 2).

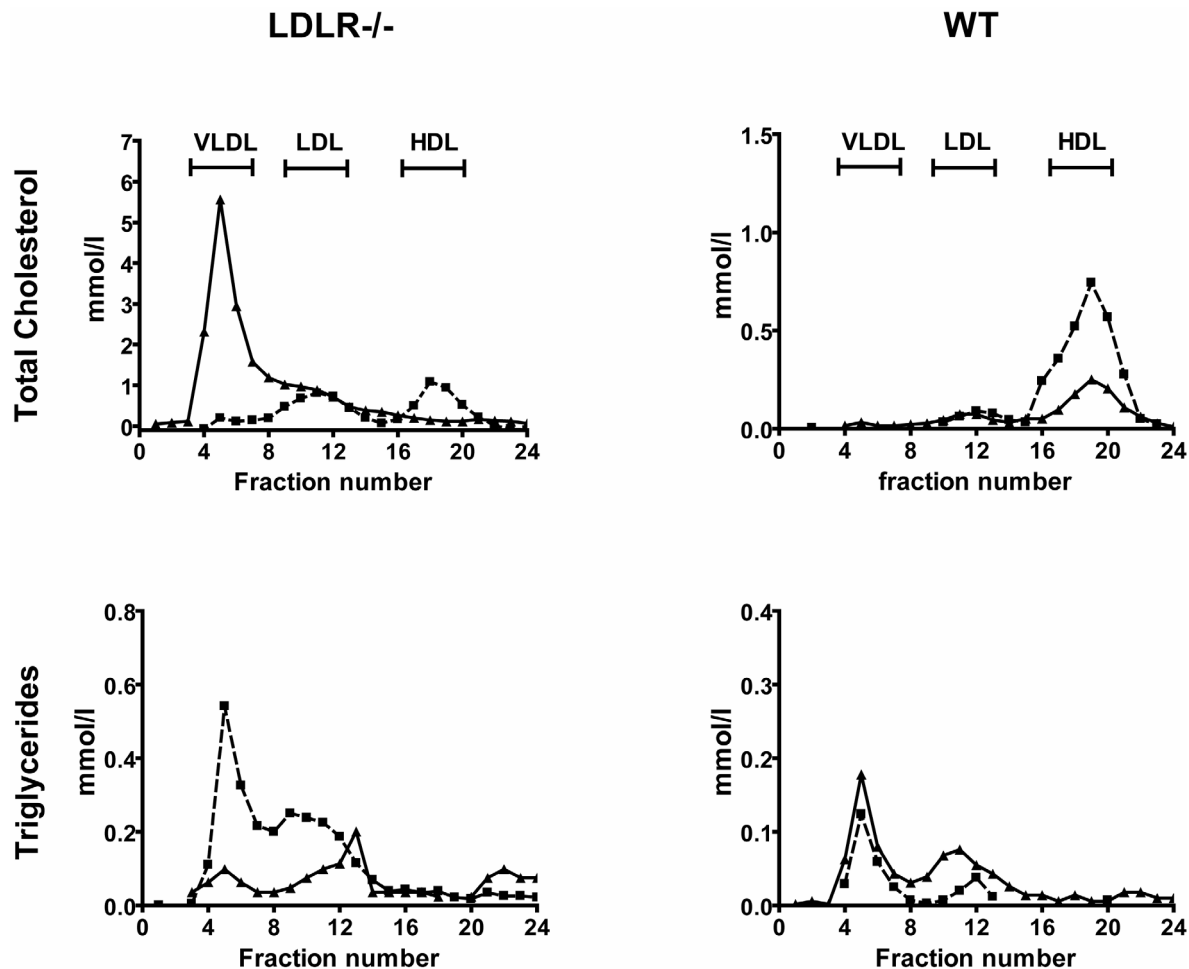


Figure 2: Znf202 overexpression severely affects serum lipoprotein profiles in LDLR^{-/-} mice and WT mice. Blood samples were drawn from *Ldlr*^{-/-} (n=4; left panels) and WT mice (n=4; right panels) 4 days after injection with 2.10^9 pfu of Ad.Znf202 (triangles) or with Ad-mock (squares) and derived serum was analyzed for lipoprotein profile by FPLC and the elution fractions were tested for triglyceride and total cholesterol content.

Hepatic morphology and lipid content after adenovirus mediated gene transfer of ZNF202

Histological analysis of livers isolated at day 5 post transduction from Ad.Znf202 and Ad.Mock treated *Ldlr*^{-/-} and WT mice revealed some striking differences. Oil-red O staining of

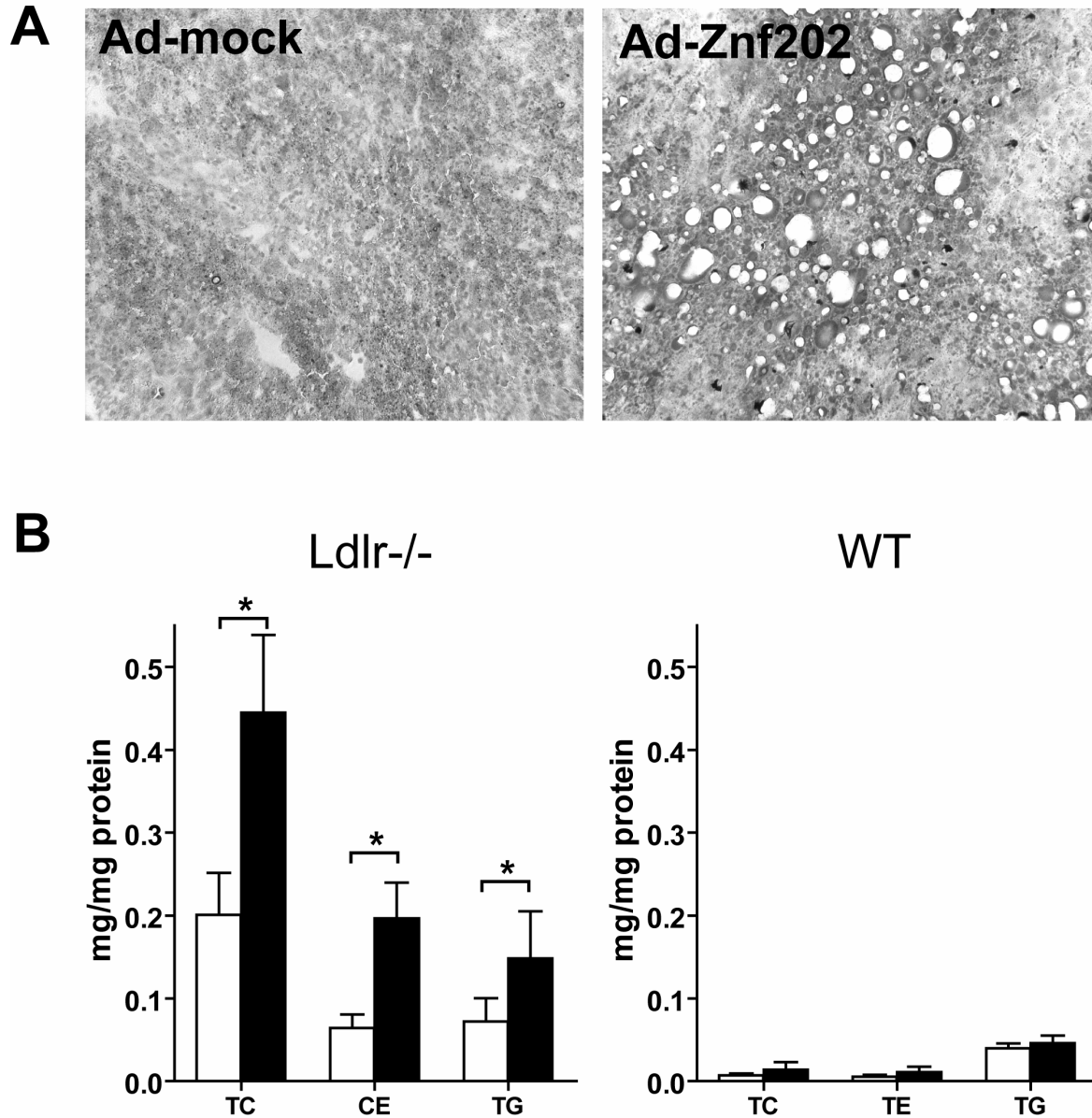


Figure 3: Znf202 overexpression caused steatosis in *Ldlr*^{-/-} mice only with an 2-fold increase of hepatic total cholesterol and triglycerides. Livers were isolated from *Ldlr*^{-/-} and WT mice 5 days after injection with $2 \cdot 10^9$ pfu of Ad.Znf202 (filled bars) or Ad-mock (empty bars). Cryosections were prepared from *Ldlr*^{-/-} liver samples and stained with Oil Red-O (A). Hepatic lipids were extracted from homogenized liver samples and cholesterol and TG concentrations were determined (B). Values are expressed as μg lipid per mg tissue protein and are means $\pm$ SD (N=4). * indicates $p < 0.05$.

liver sections demonstrated that Znf202 overexpressing livers obtained from *Ldlr*^{-/-} mice were characterized by massive lipid deposition in intracellular vacuoles mainly (Fig.3A). As the liver morphology of Ad.Znf202 treated mice was highly reminiscent of steatosis, we assayed the liver lipid content quantitatively. As can be appreciated from figure 3B, Znf202 overexpressing livers

from *Ldlr*^{-/-} mice contained >2-fold higher total cholesterol, cholesteryl esters, and triglyceride levels. In line with the normal liver morphology of WT mice injected with Ad.Znf202, no changes in the hepatic lipid content were found in the Znf202 overexpressing WT mice (Figure-3B).

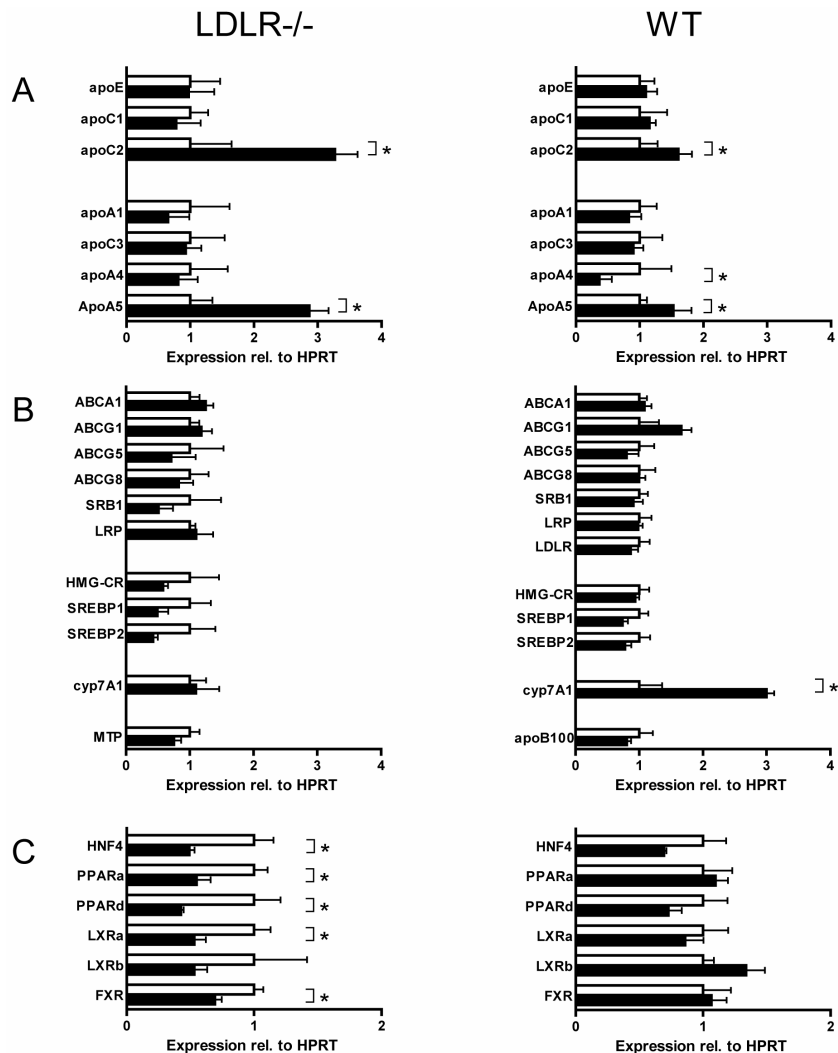


Figure 4: Hepatic Znf202 overexpression is accompanied by a changed expression of ApoA5 and ApoC2 mRNA in the liver. Liver was excised from *Ldlr*^{-/-} (left panels) and WT mice (right panels) 5 days after injection with 2.10^9 pfu of Ad.Znf202 (filled bars) or Ad-mock (empty bars) and total mRNAs was isolated and used for expression profiling by quantitative real time PCR. Gene expression is expressed relative to that of HPRT. Data are means $\pm$ S.D. of N=4 determinations. Asteric indicates $P < 0.05$.

Gene expression profile after adenovirus mediated gene transfer of ZNF202

As Znf202 was reported to be a potent transcriptional repressor of various key genes in lipoprotein metabolism⁵, we have mapped the hepatic expression profile of a range of potentially responsive genes at day 5 after Ad.Znf202 or Ad-mock treatment in *Ldlr*^{-/-} and WT mice. Analysis of the *ApoE/c1/c2* and *ApoA1/c3/a4/a5* gene clusters showed that ZNF202 overexpression increased the expression of *ApoC2* and *ApoA5* by 1.5 fold in WT mice and 3-fold

in *Ldlr*^{-/-} mice ($P < 0.05$), but did not affect the expression of *ApoE* and *ApoA1* both suggested to be Znf202 responsive in previous *in vitro* studies (Fig. 4A). Only *ApoA4* expression was found to be significantly reduced upon ZNF202 overexpression in WT mice.

In search of a possible explanation for the elevated intrahepatic lipid levels, we determined gene expression levels of ABC family members, lipoprotein receptors (e.g. LRP, LDLr, SR-BI), and genes that are related to lipoprotein assembly (e.g. ApoB100, MTP) and cholesterol synthesis and degradation (e.g. HMG-CoA reductase, Cyp7A1, Cyp27, SREBPs) (Fig. 4B). Surprisingly, virtually none of the investigated genes displayed an altered expression level upon Znf202 overexpression, regardless whether obtained from *Ldlr*^{-/-} or from WT mice. Cholesterol 7 α -hydroxylase (*Cyp7A1*) was the only effector gene tested to display a differential effect in WT and *Ldlr*^{-/-} mice upon Znf202 expression. In WT mice Znf202 increased Cyp7A1 expression by >3-fold, but it did not change the expression in *Ldlr*^{-/-} mice.

Hepatic Znf202 overexpression also lead to transcriptional changes in nuclear receptor family members in *Ldlr*^{-/-} mice but not in WT mice, although it remains to be determined whether this effect is causal in or secondary to the Znf202 induced intrahepatic lipid accumulation. The transcription factors HNF-4, PPAR α , PPAR δ , LXRA/b and FXR were downregulated in *Ldlr*^{-/-} mice by 25 to 60% ($P < 0.05$) (Fig. 4C), while no effect was found in WT mice.

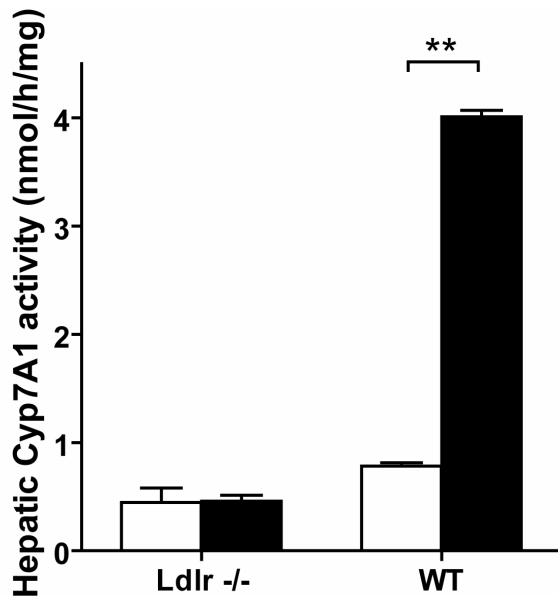


Figure 5: Liver analysis for Cyp7A1 activity in *Ldlr*^{-/-} mice revealed a significant Cyp7A1 induction in WT mice but not in *Ldlr*^{-/-} mice. Liver was excised from *Ldlr*^{-/-} and WT mice 5 days after injection with 2.10^9 pfu of Ad.Znf202 (filled bars) or Ad-mock (empty bars) and cyp7A1 activity was measured. Data are means $\pm$ S.D. of N=4 determinations and ** indicates $p < 0.001$.

As a control for inflammatory responses inflicted by adenoviral gene transfer we have measured MARCO and CD68 mRNA levels in liver. The expression of both genes remained essentially unaffected upon Znf202 treatment (1.0 ± 0.8 and 1.0 ± 0.6 for Ad-mock versus 0.7 ± 0.4 and 1.2 ± 0.2 for Ad.Znf202 treated *Ldlr*^{-/-} mice), thus confirming the histological finding that Znf202 did not promote massive leukocyte recruitment to the liver and excluding that an acute adenovirus elicited inflammatory response is underlying the observed phenotype in *Ldlr*^{-/-} mice.

The elevated hepatic Cyp7A1 mRNA levels in Znf202 overexpressing WT mice prompted us to measure hepatic Cyp7A1 activity. The effect of Znf202 treatment on Cyp7A1 activity was even more pronounced and the activity was increased almost 6-fold from 0.78 ± 0.03 to 4.0 ± 0.06 mmol/mg/h in WT mice ($P < 0.001$) (Fig. 5).

Discussion

From genetic and *in vitro* studies, the transcriptional repressor Znf202 has been proposed as a key regulator of lipid homeostasis and in particular of lipid efflux¹⁵. While its repressor function *in vitro* is well established, the precise role of Znf202 in lipid metabolism *in vivo* remains to be addressed. Using a gain of function approach involving adenoviral *Znf202* gene transfer, we show in this study that hepatic Znf202 overexpression is accompanied by hypoalphalipoproteinemia both in WT and *Ldlr*^{-/-} mice and by hypercholesterolemia and hypotriglyceridemia in *Ldlr*^{-/-} only. Furthermore, in *Ldlr*^{-/-} mice but not WT mice Znf202 overexpression was found to promote steatosis.

Previous genetic studies have linked Znf202 to hypoalphalipoproteinemia in Utah pedigrees^{4,7} while in *in vitro* studies Znf202 was reported to repress the expression of individual genes in the *ApoE/c1/c2* and *ApoA1/c3/a4/a5* cluster and ABC family members⁵, suggesting a critical role of this protein in the regulatory control of HDL levels. Indeed, we show in this study that Znf202 overexpression *in vivo* does induce a large reduction of HDL levels both under normo- and hyper-lipidemic conditions.

In vitro, we did find reduced expression of the apolipoprotein gene clusters by 30-50% by Ad-Znf202 in murine hepatoma mhAT3F2 cells (Vrins et al., manuscript in preparation), while Porsch-Ozcurumez et al.¹⁶ found a reduction in human HepG2 cells of ABCA1 expression. Our

in vivo data, however, clearly indicate that no repression of the *apoe/c1/c2* and *Apoa1/c3/a4/a5* and ABC family members is observed.

The lack of correlation between the in vitro data and the gene expression profiles at 4 or 5 days after adenoviral Znf202 administration might be caused by the induced initial changes in lipid homeostasis leading to secondary changes in lipid/fatty acid driven transcriptional factors like PPARs, LXRs, and FXR. Our aim was exactly to analyze the metabolic effect of an intervention in Znf202 expression and these secondary changes are thus the consequence of such a primary intervention.

Our gene expression analysis revealed that at 4 and 5 days of Znf202 overexpression, an increased expression of *Apoa5* and *Apoc2* in both *Ldlr*^{-/-} and WT mice was noticed. Both ApoA5 and ApoC2 are intimately implicated in TG metabolism and specifically triacylglycerol hydrolysis (lipolysis). Studies by Schaap et al.¹⁷, and by Pennacchio et al.¹⁸, have demonstrated a clear inverse relationship between serum ApoA5 and VLDL-TG levels. Similarly, ApoC2 functions as cofactor in LPL-mediated TG hydrolysis^{19,20} and ApoC2 deficiency in humans is associated with severe hypertriglyceridemia²¹. In line with these findings, Znf202 overexpression in *Ldlr*^{-/-} mice indeed resulted in sharply reduced triglyceride levels compared to Ad-mock treated controls. Simultaneously, serum cholesterol levels increased 2-fold in *LDLr*^{-/-} mice and this cholesterol was predominantly associated with the VLDL-sized lipoprotein fraction. A possible explanation for this phenomenon is that the increased rate of lipolysis results in an enhanced conversion rate of TG-rich VLDL to cholesterol-rich remnant particles, which can not be cleared in the absence of the *LDLr* and thus accumulate in the serum. The lack of this phenomenon in WT mice may stem from the fact that VLDL conversion and clearance are both operative and that additional effects of a further increased VLDL-TG lipolysis are not observed. Interestingly, previous studies with *Apoa5* overexpression in WT mice showed a significant and dose-dependent decrease in serum HDL levels¹⁷, which was also found in the present studies in both WT and *LDLr*^{-/-} mice.

In addition to the difference in serum lipids between both mouse strains, we observed an overt panlobular accumulation of TGs and cholesterol in Znf202 overexpressing livers of *Ldlr*^{-/-} but not WT mice. Interestingly, the only effector gene to be differentially affected upon Znf202 overexpression in WT versus *LDLr*^{-/-} mice was *Cyp7A1*, which encodes a rate-limiting enzyme in bile acid synthesis. The selective *Cyp7A1* upregulation in WT mice raises the intriguing

premise that Cyp7A1 may be instrumental in Znf202 induced responses. However, the mechanism of this confinement to WT mice and the regulatory pathway for the observed Cyp7A1 upregulation remains to be elucidated.

In conclusion we show for the first time that hepatic Znf202 overexpression affects lipid homeostasis *in vivo*, manifesting in hypoalphalipoproteinemia on the one hand, and, in the absence of the LDLr, in hypotriglyceridemia and steatosis on the other hand. The unaffected expression of the presumed Znf202 responsive genes at 4 and 5 days after adenoviral administration is unexpected given the demonstrated repressor function of Znf202 in *in vitro* studies in the literature ^{5,16} and also observed by our group (Vrins et al., manuscript in preparation). However, the induced gene expression of *Apoa5* and *Apoc2* can explain the observed changes in serum lipid profiles after Znf202 overexpression and thus form the final outcome of the increased Znf202 expression. This study thus establishes *in vivo* a key role of Znf202 in HDL metabolism and reveals Znf202 as novel target in maintaining lipid homeostasis.

Acknowledgements

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6

Summary and Perspectives

Summary and Perspectives

The liver plays a crucial role in the maintenance of serum lipoprotein levels. Therefore, treatment of hyperlipidemia via drug-induced alteration of lipid related gene expression in the liver is a rational approach. In this thesis the efficacy of influencing two new therapeutic targets was evaluated: the microsomal triglyceride transfer protein (MTP) for which the potential of inhibition by antisense based drugs was assessed and a new transcription factor Znf202 which is suggested to possess a widely applicable therapeutic potential.

Antisense based drugs: specific targeting of MTP

Since hepatic MTP is a rate-limiting enzyme in the production of the pro-atherogenic apoB100 containing lipoproteins such as VLDL and LDL (Hussain, 2003), the reduction of MTP expression via gene silencing could be an effective strategy to reduce elevated serum levels of VLDL and consequently LDL. In **chapter 2** the design of a potent AS-ODN targeting muMTP is described. Although the reliability of computer-assisted secondary mRNA structure calculation is subject to debate, the use of such a method for finding suitable antisense sequences against muMTP was found to be successful with 50% of the designed AS-ODNs being effective in a cell-free system. The use of a two-step evaluation strategy involving a cell free assay and a subsequent cellular read-out system based on an EGFP fusion construct, proved very effective and revealed AS569 as most potent antisense drug of the tested series with an IC₅₀ of 15 nM.

To increase the stability of AS-569 in a cellular environment phosphorothioate modified AS-ODN were used. Although this modification is widely applied, in agreement with previous studies we found that the administration of high concentrations of phosphorothioate modified ODNs is accompanied by non-target effects probably through aptameric interactions with cellular proteins^{1,2}. For example, non-sequence specific interactions with ribosomal proteins may affect mRNA translation. Recent advances in antisense drug design (e.g. PNAs) may improve the efficacy of antisense drugs and attenuate unfavorable side effects³⁻⁵. In addition, conjugation of AS-ODNs to cell-surface receptor ligands or encapsulation/incorporation in (targeted) liposomes will help to further increase the specific delivery to target tissue⁶⁻⁸. Regardless of their chemistry, AS-ODNs will require repeated administration for sustained therapeutic activity due to their short-termed effect.

Amongst others, the advantage of ribozymes over AS-ODNs is that they are better fit for sustained intracellular production when introduced as ribozyme vectors and that they exhibit intrinsic endonuclease activity. When using ribozyme vectors time- and site-controlled expression and even co-localization of the ribozyme with its target mRNA can be effected by the choice of the right expression cassette. The cell-free screening assay developed in chapter 2 also proved useful in the design of ribozymes against muMTP described in **chapter 3**, as it allowed the identification of accessible regions within the muMTP mRNA. Based on the presence of cleavable GUX triplets we were able to track three possible targets within the muMTP mRNA: GUA³⁴², GUC⁵⁸¹, and GUC¹⁸⁰². During the development of the ribozyme constructs, a fourth, tandem ribozyme emerged as a result of head-to-tail ligation of two Rz581 units during the cloning process, RzT581. For in vitro analysis, the above mentioned two-step strategy was pursued. Target specific cleavage could be demonstrated for all four ribozymes in a cell-free system at which RzT581 proved to be considerably more potent than the other ribozymes. Conceivably this is caused by the presence of an extra stem loop which on the one hand constrains the ribozyme transcript into a catalytically active configuration and on the other hand may render the ribozyme more stable against enzymatic degradation. These results and the finding that the targeted region (nt 581) was seen to be accessible for antisense based inhibition at a cellular level (chapter 2), led us to select Rz581 and RzT581 for subsequent adenovirus-mediated ribozyme transfer.

Cellular stability, the expression level, and intracellular distribution, which are governed by the promoter (pol II or pol III) and the ribozyme flanking sequences, are generally deemed to determine the efficacy of the ribozyme⁹⁻¹². Therefore, we generated a panel of adenoviral vectors at which the ribozyme is expressed from different expression cassettes. Indeed, our data demonstrate that the ribozyme-mediated reduction of cellular muMTP mRNA depends on the expression cassette used, and that the tRNA^{met}-expression cassette appears to be most suitable for further exploration of Rz581s potential *in vivo*.

Znf202: a noval factor in lipid metabolism

The search for promising therapeutic gene targets for hyperlipidemia has been aided considerably by investigations of the role of transcriptional regulators in lipid disorders. As discussed in chapter 1, nuclear receptors such as LXR, and the PPARs have received much

attention in this regard ^{13,14}. Although its precise role in lipid metabolism has not yet been fully established, a new gene target in the regulation of lipid homeostasis is Znf202, a transcription factor that has recently been linked to hypoalphalipoproteinemia. In this thesis we first have characterized the repressive effect of Znf202 *in vitro* (**chapter 4**). After having demonstrated that the *apoE/C1/C2* and *apoA1/C3/A4/A5* gene clusters were affected by Znf202 overexpression in cultured mouse cells, we have investigated the Znf202-mediated downregulation of mouse apoE expression. ApoE is an important protein in lipoprotein metabolism as it forms the recognition marker for chylomicron- and VLDL-remnant uptake from the blood circulation by the liver ¹⁵. The apoE promoter region directly upstream of the transcription initiation site, which is strongly conserved between mouse and human, was sufficient for repression of human apoE promoter activity by Znf202 ¹⁶. While the extent of Znf202 repression of apoE expression between mouse and human was very similar, our studies revealed a species specific difference in the genetic architecture of the ApoE promoter with a Znf202 response region situated in a more upstream region containing two separate Znf202 binding motifs. Even though the *in vitro* data confirm the role of Znf202 as transcriptional regulator of essential lipid related genes, the latter finding can not rule out that species-specific differences exist in responsiveness to Znf202.

Nevertheless, we clearly show in **chapter 5** that Znf202 expression is linked with HDL serum levels and that Znf202 overexpression affects lipid metabolism. Furthermore, Znf202 overexpression *in vivo* gave some surprising results in that it led to upregulation of two genes involved in lipolysis (*Apoa5* and *Apoc2*) in both *Ldlr*^{-/-} and wild type mice instead of the expected downregulation of several genes reported to be Znf202 responsive *in vitro*. The observed hypotriglyceridemia and increased levels of cholesterol-rich VLDL in *Ldlr*^{-/-} overexpressing Znf202 can be attributed to an induced ApoA5 and ApoC2 expression ^{17,18}. The findings of Schaap et al. that HDL-levels were also lowered upon ApoA5 overexpression ¹⁸ may also point to a direct role of these two genes in Znf202 induced hypoalphalipoproteinemia in both mouse strains. Additionally, Znf202 overexpression was accompanied by induced expression of Cyp7A1, a key enzyme involved in bile acid synthesis, in wild type but not hyperlipidemic *LDLr*^{-/-} mice. As Znf202 overexpression induced steatosis in *Ldlr*^{-/-} but not WT mice, the differential Cyp7A1 upregulation could be instrumental in intrahepatic lipid deposition after Znf202 overexpression. Although the altered gene expression profile caused by Znf202 overexpression could at least partly explain the observed phenotypes, it still remains to be

clarified by which mechanism Znf202 is mediating these responses. In this respect the analysis of the time dependent changes in liver gene expression upon adenoviral Znf202 administration may be informative.

Conclusions and Perspectives

Now the therapeutic potential of reducing MTP activity for the treatment of hyperlipidemia has been established^{19,20} several MTP inhibitors have recently entered phase-II clinical trials. In one study, administration of an MTP inhibitor, CP-346086, resulted in a significant reduction of total cholesterol and triglycerides in serum of human subjects²¹. However, studies with these MTP inhibitors, liver specific knock-out mice, and findings observed in patients with abetalipoproteinemia have revealed potential adverse effects. These effects include fat accumulation in the liver and since current inhibitors do not discriminate between MTP function in intestine and liver, fat-soluble vitamin malabsorption, and steatorrhea^{22,23}. Thus, sequence specific MTP mRNA knockdown agents, delivered via liver specific vectors, could be a very attractive alternative approach to specifically lower hepatic MTP levels^{6,8,24}. In this regard, the MTP mRNA reducing potential of the conventional antisense based AS569 (chapter 2) and ribozyme Rz581 (chapter 3) described in this thesis offer great promise in specific lowering of atherogenic apoB-containing lipoproteins. In addition, the identification of an effective target site within MTP mRNA also will facilitate the design of future short interfering RNA drugs, as also the latter drugs rely heavily on target RNA hybridization to be effective. The latter approach to knockdown gene expression has proven to be rather successful in vitro and in vivo. In fact, most, if not all, of the current attempts to reduce gene expression at a mRNA level are based on RNA interference (RNAi)²⁵. This is further illustrated by a recent report that describes the RNAi-based intervention of the expression of apoB100 *in vivo* resulting in the reduction of apoB-containing serum lipoproteins²⁶.

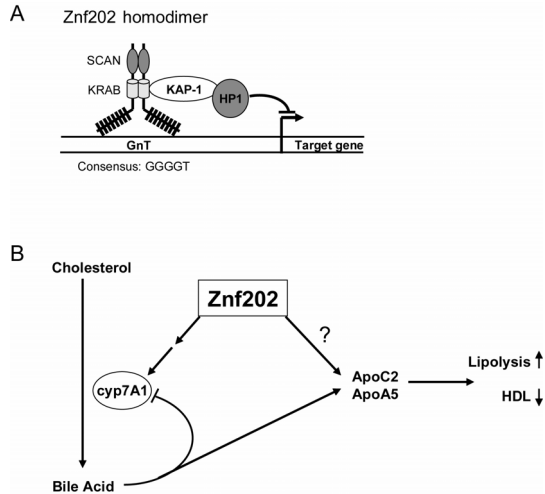
However, even though RNAi has rapidly evolved into the most common tool for target validation and therapeutic applications, this gene silencing technique also has its application problems. The major obstacle shared by all of the aforementioned strategies continues to be their effective targeting to the desired site of action *in vivo*. This is illustrated by a plethora of in vitro studies on antisense and gene silencing technology while only a limited number describe the

successful application *in vivo* ^{5,27}. The poor applicability *in vivo* is partly attributable to low stability of these oligonucleotide drugs, to off-target effects and to unfavourable kinetics ²⁸. As these hurdles are fundamentally similar to those encountered with antisense therapy, progress in RNAi drug design will certainly benefit from earlier experiences with the chemically related antisense drugs (e.g. stabilizing RNA chemistry, improvement of *in vivo* biodistribution and specific targeting adjustments).

In conclusion, these antisense based methods for target validation have demonstrated potential effectivity and several antisense drugs already have entered clinical trials. Before widespread clinical application is possible the further development of effective site specific delivery will form a major challenge.

In contrast to MTP, the therapeutic potential of the transcription factor Znf202 has to be further elucidated. In this thesis we have established that Znf202 expression is linked to hypoalphalipoproteinemia *in vivo* but also that Znf202 is involved in the regulatory control of hepatic lipid homeostasis and, possibly, in hyperlipidemia associated steatosis. While it is tempting to regard Znf202 as an attractive target for therapeutic intervention in hypoalphalipoproteinemia and, more in general, in hyperlipidemia, one should be cautious with extrapolating the data to clinical practice. Further investigation will be needed to fully comprehend the regulatory pathways induced by Znf202 expression modulation and the interaction of Znf202 with other hepatic transcription factors. In this regard, it will certainly be relevant to compare phenotypic effects of Znf202 overexpression in LDLr^{-/-} mice (chapter 5) with that of ApoA5, thus establishing that ApoA5 links Znf202 with hypoalphalipoproteinemia.

A second important actor in Znf202 induced responses *in vivo* is Cyp7A1 and it will be of interest to investigate the relation between Znf202 and bile acid related pathways. Recent reports have pointed out that activation of FXR-mediated pathways is accompanied by an upregulation of lipolysis related genes such as *Apoc2* ^{30,31} and *Apoa5* ³². Under hyperlipidemic conditions Znf202 may influence bile acid synthesis indirectly through cyp7A1 leading to excessive hepatic bile acid accumulation which, eventually, via FXR mediated compensatory feedback regulation of cyp7A1, culminates in lowered serum TG levels and steatosis ³³. However, this hypothesis needs to be further explored and further work is needed to fully understand the phenotypic effects observed by Znf202 expression modulation.



Transcription factor Znf202. Znf202 homodimers bind to the GnT consensus site in the promoter region and initiate transcriptional silencing mechanisms.²⁹ In vivo experiments show that hepatic overexpression of Znf202 results in reduced HDL-levels and affects genes involved in bile acid synthesis (cyp7A1) and lipolysis (apoA5 and apoC2).

In addition, the relevance of the naturally occurring splice variant of Znf202 (M3), containing only the SCAN oligomerization domain needs to be addressed¹⁶. Evidence for SCAN-mediated interaction between SCAN-containing proteins has been presented³⁴ and other SCAN-only proteins such as the PPAR γ co-activator SDP-1 have been characterized³⁵. The finding that SDP-1 not only is involved in the PPAR γ pathway, but also binds Znf202 strongly suggests a tight link between these two transcription factors. As for now the actual function of M3 (i.e. antagonist versus inert decoy) remains unclear. However, our preliminary results with M3 adenovectors did not reveal any effect of M3 on the activity of full length Znf202 or its downstream target genes, pleading for the option that M3 is an inert decoy.

Because of the apparently broad role of Znf202 a subtle therapeutic approach based on modulation Znf202 expression might be difficult to achieve. However, better understanding of the Znf202 pathway will contribute to more insight in its potential and might lead to the identification of new targets for therapeutic gene modulation.

In general, modulation of hepatic gene expression holds great promise as therapeutic approach to restore/correct lipid homeostasis. However, therapeutic interference in gene expression levels requires a thorough understanding of the impact of the change in expression of the primary gene on total metabolism. The aim of therapeutic gene expression either by antisense oligonucleotides, ribozymes, or other antisense based strategies requires effective delivery to the specific intracellular action site. These are essential steps needed for future applications of the gene targeted correction of hyperlipidemia in clinical settings.

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7

Samenvatting voor niet-ingewijden

Samenvatting voor niet-ingewijden

Momenteel vormen hart- en vaatziekten de belangrijkste doodsoorzaak in de westerse samenleving. Voor een groot deel is dit een gevolg van een sterke vernauwing van de bloedvaten, ook bekend onder de naam atherosclerose. Diverse risikofactoren zijn geïdentificeerd die bijdragen tot een versnelde vernauwing van de bloedvaten, zoals een verhoogd cholesterol- en triglyceridgehalte in het bloed.

Vetten (lipiden) zoals cholesterol en triglyceriden zijn belangrijk voor het normaal functioneren van ons lichaam. Ze vormen een noodzakelijke grondstof voor de energievoorziening en zijn belangrijk voor de aanmaak van hormonen en de opbouw van celmembranen. Naast de opname van lipiden via voeding, is ons lichaam ook zelf in staat om lipiden aan te maken (biosynthese) en dit vindt voornamelijk plaats in de lever. Een overgroot deel van de dagelijks benodigde hoeveelheid cholesterol is zelfs afkomstig uit de biosynthese.

Omdat lipiden slecht in een waterig milieu, zoals bloed, oplossen, worden ze naar de verschillende weefsels en organen van het lichaam getransporteerd via bolvormige deeltjes – de lipoproteïnen. Deze lipoproteïnen bestaan naast lipiden tevens uit eiwitten genaamd apolipoproteïnen. Op basis van de samenstelling van lipiden en apolipoproteïnen kunnen er verschillende soorten lipoproteïnen onderscheiden worden: chylomicronen, zeer lage dichtheids- (VLDL), lage dichtheids- (LDL) en hoge dichtheids-lipoproteïnen (HDL). Over het algemeen kan men stellen dat chylomicronen, VLDL en LDL lipiden transporteren naar alle delen van het lichaam, terwijl HDL het overtollige cholesterol opneemt en vervolgens transporteert naar de lever.

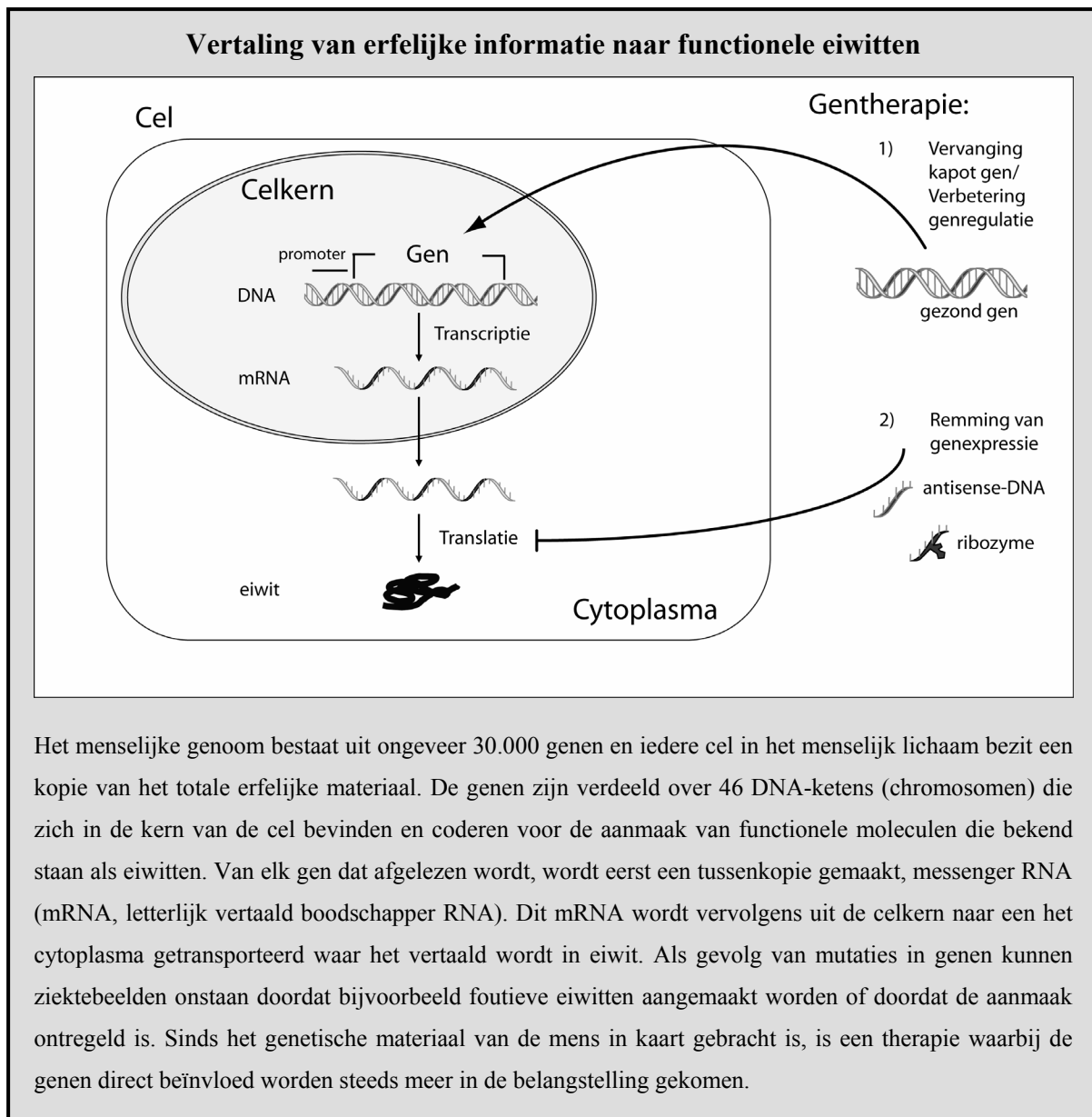
Onder normale omstandigheden bestaat er een evenwicht tussen de aanmaak en opname van lipiden enerzijds en de hoeveelheid die verbruikt of verwijderd wordt anderzijds. Dit evenwicht is bepalend voor de totale concentratie lipiden in het bloed en de verdeling hiervan over de verschillende lipoproteïnen. De lever speelt in dit proces van lipide-stofwisseling (-metabolisme) een belangrijke rol. Een verstoring van het evenwicht in het lipidemetabolisme kan leiden tot een verhoogde bloedspiegel van lipiden genaamd: hyperlipidemia. Een verhoging van de lipide-transporterende deeltjes VLDL en LDL, kan de atherosclerotische vaatvernauwing aanzienlijk versnellen en deze lipoproteïnen worden daarom dan ook vaak aangeduid als het ‘slechte cholesterol’. De oorzaak voor dit verstoorde evenwicht is deels een gevolg van de

westerse levensstijl die in het algemeen gepaard gaat met een te vetrijk dieet en/of te weinig lichaamsbeweging. Echter, mensen kunnen ook een erfelijke aanleg hebben voor hyperlipidemia. In **hoofdstuk 1** wordt een overzicht gegeven van het lipidemetabolisme en de centrale rol die de lever hierin speelt.

Dit project beoogde gen-therapeutische strategieën te ontwikkelen teneinde de bloedspiegelniveau's van de vet-transporterende deeltjes gunstig te beïnvloeden en daarmee de ontwikkeling van hart- en vaatziekten af te remmen. Hierbij hebben we ons onderzoek gericht op twee sleutelmoleculen: het microsomale triglyceride transfer eiwit (MTP) dat verantwoordelijk is voor de aanmaak van schadelijke lipoproteïnen (VLDL) in de lever en een recent ontdekte genregulerende factor Znf202, waarvan onderzoek heeft aangetoond dat het de bloedspiegel van het beschermende HDL beïnvloedt.

In eerste instantie is getracht een nieuwe vorm van gentherapie uit te werken, waarbij het mRNA van het MTP-gen afgebroken wordt teneinde de aanmaak van MTP te verlagen. In **hoofdstuk 2** wordt de ontwikkeling van MTP antisense moleculen beschreven. Antisense-DNA is een klein stukje complementair DNA dat selectief bindt aan het mRNA van een bepaald gen en daarmee de vertaling van dit gen naar het corresponderende eiwit blokkeert (zie tekstbox). Hoewel we theoretisch tegen elke mRNA antisense-DNA kunnen ontwerpen dat de vertaling zal blokkeren, blijkt het ontwerp van een effectief antisense-DNA in de praktijk erg lastig. Dit komt met name omdat mRNA moleculen zijn opgevouwen tot een complexe kluwen, zodat sommige delen van mRNA niet toegankelijk zijn voor binding van het antisense-DNA. Er bestaan wel computerprogramma's waarmee de opvouwing van het RNA en daarmee de domeinen die beschikbaar zijn voor binding, voorspeld kunnen worden, maar dergelijke berekeningen zijn niet altijd volledig betrouwbaar. Daarom worden vaak meerdere antisense-DNAs ontworpen die elk op verschillende plekken van het mRNA aangrijpen. Deze antisense-DNAs moeten dan vervolgens getest worden op hun effectiviteit. Omdat dit laatste een tijdrovend proces kan zijn, hebben wij een nieuwe doeltreffende selectiemethode ontwikkeld en getoetst.

Nadat er via de voorspelde MTP mRNA-opvouwing twaalf antisense-DNA kandidaten waren onworpen, werden deze antisense-DNAs op hun activiteit getest in een zogeheten celvrij transcriptie-translatie systeem. Middels dit systeem, dat de vertalingsreactie van het MTP-gen via mRNA naar eiwit kunstmatig nabootst, kon snel gescreend worden welke MTP antisense-DNAs



functioneel actief waren. Uit dit experiment bleek dat zes van de twaalf antisense-DNAs (50%) in staat waren om de expressie van het MTP te remmen. Echter, een potentiële kandidaat voor gentherapie moet tevens werkzaam zijn in het meer complexe milieu van een cel. Om evaluatie van de therapeutische activiteit van het geselecteerde MTP antisense-DNA in de cel te vereenvoudigen, werd gebruik gemaakt van een nieuw uitleessysteem op basis van een fluorescerend eiwit uit de kwal (green fluorescent protein, GFP). Door het MTP-gen te koppelen aan het GFP-gen en het fusie-eiwit MTP-GFP vervolgens in de testcel tot expressie te brengen, is het mogelijk de intensiteit van de groene fluorescentie in de cel als maat te gebruiken voor de

aanwezige hoeveelheid MTP. Met deze proefopzet konden we aantonen dat het MTP antisense-DNA, AS-569, ook in een cellulair milieu condities effectief in staat was om de MTP expressie te remmen.

Naast deze antisense techniek hebben we in **hoofdstuk 3** ook een tweede strategie bestudeerd om de expressie van MTP te verlagen. In ribozym therapie wordt gebruik gemaakt van een klein, enzymatisch actief RNA fragment, dat niet vertaald wordt naar eiwit, en dat hecht aan een gedefiniëerd gebiedje op het mRNA volgens dezelfde beginselen als antisense-DNA. Echter, door de aanwezigheid van het enzymatisch domain, is het ribozym in staat om het mRNA op de plek van binding in tweeën te knippen en het mRNA vervolgens weer los te laten. Een ribozym biedt dus het voordeel dat het meerdere mRNA kopiën van een gen onwerkzaam kan maken. Omdat de ribozym-binding ook berust op de interactie met toegankelijke regionen binnen het mRNA, konden we voor het ontwerp van het MTP ribozym gebruik maken van de resultaten uit hoofdstuk 2. Er is een reeks ribozymen gemaakt en getest op de capaciteit om MTP mRNA te splitsen in een celvrij systeem. Het MTP specifieke ribozyme Rz581 en met name een tandem versie van dit Rz581 (twee Rz581 aan elkaar gekoppeld) bleken daarbij de hoogste activiteit te bezitten. Omdat ribozymen bestaan uit RNA (vergelijkbaar met mRNA), kunnen ze vanuit een ‘ribozym-gen’ door de cel zelf geproduceerd worden. Het meest actieve ribozym-gen werd ingebouwd in een defect adenovirus (een verkoudheidsvirus dat niet meer in staat is om zich te vermenigvuldigen), dat de capaciteit bezit om ingebouwde genen, dus ook ribozym-genen, heel efficiënt aan levercellen af te leveren. Daarbij is een serie adenovirussen geconstrueerd waarbij de aanmaak van het ribozym gereguleerd werd door diverse promotoren (CMV, snRNA-U6 en tRNA^{met}). Een promotor, die aan het begin van een gen gelegen is (zie tekstbox), is de belangrijkste regulator van de gen-activiteit. Elke promotor bevat eigenschappen die nodig zijn om de expressie van zijn bijbehorende gen te controleren. Sommige promotoren (zoals de van een virus afkomstige CMV promotor) staan vaker ‘aan’ dan andere, enkele promotoren (zoals die voor snRNA-U6 en tRNA^{met}) zijn speciaal toegerust voor de transcriptie van kleine RNA-fragmenten zoals ribozymen, en promotoren kunnen mede bepalen in welk deel van de cel (kern of cytoplasma) het mRNA getransporteerd moet worden. In levercellen bleek uiteindelijk dat Rz581-adenovirussen voorzien van een tRNA^{met} promotor effectief in staat waren de synthese van het MTP te remmen.

De veelbelovende resultaten met zowel het antisense-DNA (AS-569) als het ribozym (Rz581) tegen MTP bieden mogelijkheden om een gerichte therapie te ontwikkelen die de bloedspiegel van VLDL en LDL doen verlagen. Evaluatie van deze kandidaat geneesmiddelen in diermodellen zal zijn nu nodig zijn om hun medicinale werking en eventuele bijwerking te toetsen.

In een tweede onderzoekslijn is de rol van het regulator sleuteleiwit Znf202 in het lipide-metabolisme en zijn potentiële waarde als aangrijppunt voor (gen)therapie bestudeerd. Recent is met behulp van genetisch onderzoek een verband gevonden tussen een tot dan toe nog onbekend gen Znf202 en een specifieke groep patiënten die gekenmerkt worden door een te lage bloedspiegel van HDL en daarmee een verhoogde kans hebben op hart- en vaatziekten. HDL is een essentieel lipoproteïne dat er voor zorgt dat een teveel aan cholesterol afgevoerd kan worden naar de lever waarna het eventueel afgebroken en verwijderd kan worden en wordt daarom ook vaak aangeduid als het ‘goede cholesterol’. Uit vervolgonderzoek bleek dat Znf202 codeert voor een transcriptie factor die door binding aan de promotor van andere genen de expressie van deze genen kan verlagen. Interessant was hierbij dat de genen die mogelijk gecontroleerd worden door Znf202 allen betrokken zijn bij het lipide-metabolisme. Echter, vanwege de verschillende functies die de genen hebben in het lipide-metabolisme, is het moeilijk in te schatten wat het algehele effect van modulatie van de Znf202 activiteit is.

Om antwoord te vinden op de bovengenoemde vraag hebben we het effect van overproductie van Znf202 zowel *in vitro* (levercellen) als *in vivo* (overproductie in de lever van muizen) onderzocht. Hiertoe is er een adenovirus geproduceerd dat het Znf202 gen bevat – Ad-Znf202. In **hoofdstuk 4** wordt beschreven dat Ad-Znf202 in staat is de expressie van een groot aantal belangrijke apolipoproteïne-genen in levercellen te verlagen, waaronder het apolipoproteïne E (apoE) gen. Met behulp van promoter-studies voor apoE expressie kon aangetoond worden dat Znf202 aangrijpt op de expressie door binding aan specifieke domeinen in het promotergeedeelte, waarbij de expressie wordt geremd.

Vervolgens werd *in vivo* het effect bestudeerd van de expressie van Znf202 op het lipide-metabolisme (**Hoofdstuk 5**). Adenoviraal gemedieerde expressie leidt tot effectieve expressie in de lever. Hierbij werden zowel muizen met een normale lipide-bloedspiegel, als genetisch

gemodificeerde muizen (Ldlr^{-/-} muizen) met een verhoogde lipide-bloedspiegel en gevoeligheid voor atherosclerose gebruikt.

De overproductie van Znf202 in de Ldlr^{-/-} muizen resulteerde in een sterke verandering in de totale lipide-bloedspiegel en de verdeling van deze lipiden over de verschillende lipoproteïnen; corresponderend met een sterke toename van het ‘slechte cholesterol’. Tevens leidde overproductie tot een dramatische vervetting van de lever (steatose). Op het gebied van genactiviteit in de lever werd een verhoging in expressie van een aantal apolipoproteïne genen geïnduceerd. Dat laatste was zeer verrassend, aangezien onderzoek *in vitro*, zoals beschreven in hoofdstuk 4, een remmend effect van Znf202 op genactiviteit laat zien. Het effect van Znf202 overproductie bij controle muizen was duidelijk milder met nagenoeg geen veranderingen in de lipide-bloedspiegel en geen waarneembare steatose. Wel werd een duidelijke opregulatie van dezelfde apolipoproteïne genen als in Ldlr^{-/-} muizen waargenomen. Daarnaast werd een zeer sterke verhoging van een eiwit dat betrokken is bij de afbraak van cholesterol in de lever aangetroffen, wat misschien tevens een verklaring is voor de mildere effecten in controle muizen. Een belangrijke waarneming in beide muizen was dat als gevolg van Znf202 overproductie, de HDL-bloedspiegels sterk afnamen. Dit bevestigt dat Znf202 de HDL-bloedspiegels reguleert en dat verlaging van HDL het gevolg kan zijn van verhoogde activiteit van Znf202.

Deze *in vivo* studie toont voor het eerst aan dat Znf202 in de lever vooral onder condities van een verstoorde lipidemetabolisme (Ldlr^{-/-} muizen) een zeer belangrijke regulerende factor betreft. Met betrekking tot het verbeteren van HDL-bloedspiegels lijkt remming van Znf202 dan ook een relevante en veelbelovende therapeutische ingang ter behandeling van patiënten met een verstoorde lipidehuishouding. Echter, in hoeverre remming van Znf202 klinisch perspectiefvol is, moet blijken uit nieuwe experimenten met bijvoorbeeld Znf202-specifieke antisense-DNA of ribozymen.

De identificatie van genen in de lever die betrokken zijn bij de regulatie van het lipide-metabolisme, geven een beter inzicht in de oorzaken en mechanismen van hart-en vaatziekten. Dit onderzoek leidt daarmee tot nieuwe therapeutisch mogelijkheden om deze ziekten te behandelen en te voorkomen. In dit proefschrift worden twee fundamentele trajecten beschreven in het onderzoek naar de perspectieven van levergerichte gentherapie ter correctie van hyperlipidemia: een gentherapeutische strategie ter modulatie van de expressie van een overactief

en/of ziektebevorderend gen (MTP) en de verkenning naar therapeutische mogelijkheden van een recent geïdentificeerde genregulator (Znf202). Van het MTP-gen heeft eerder onderzoek al laten zien dat het een cruciale rol heeft in de aanmaak van slecht cholesterol. Met behulp van antisense technieken zoals onder andere beschreven in hoofdstuk 2 en 3 kunnen we heel specifiek het gen (tijdelijk) uit schakelen om zo weer een gunstig evenwicht te creëren in de lipide-bloedspiegel. In de hoofdstukken 4 en 5 laten we zien dat Znf202 een grote rol speelt in lipidehuishouding en de HDL-niveaus in het bloed reguleert. Toekomstig onderzoek zal moeten uitwijzen of correcties in Znf202 activiteit een gunstig effect kan hebben op patienten met een verstoord vet-metabolisme.

Omdat genen tot de fundamentele bouwstenen van ons lichaam behoren, is het natuurlijk vanzelfsprekend dat elke ingreep op genniveau goed bestudeerd moet worden alvorens van klinische toepassing sprake kan zijn. Echter, samen met de ontwikkeling van strategieën voor de lokale toediening van medicijnen, kan een dergelijke therapeutische benadering binnen de geneeskunde leiden tot een nieuwe generatie geneesmiddelen ter voorkoming en/of bestrijding van hart- en vaatziekten.

Curriculum Vitae

De auteur van dit proefschrift werd op 25 oktober 1971 geboren te Papendrecht. Na het behalen van het VWO diploma aan het Thuredrecht college te Dordrecht, werd in 1991 begonnen met de studie Biologie aan de Universiteit Utrecht. In het kader van de doctoraal fase van de studie werd eerst een stage gedaan bij de afdeling Moleculaire Cel Biologie van de Universiteit Utrecht onder supervisie van Dr. M. Kleijn. Hierbij werd het effect onderzocht van virale infecties op mRNA translatie. De tweede stage werd verricht bij de Divisie Immunologie van het Nederlands Kanker Instituut onder supervisie van Dr. H. Spits, waarbij signaaltransductie werd bestudeerd die leidt tot T Cel Receptor geïnduceerde IL-2 expressie. Tijdens de studie werd gedurende een jaar het voorzitterschap bekleed van het bestuur van de Utrechtse Biologen Vereniging. In 1999 werd een aanvang gemaakt met het promotieonderzoek op de afdelingen Biofarmacie van het Leiden/Amsterdam Center for Drug Research, onder leiding van Dr. E.A.L. Biessen en Prof. Dr. Th.J.C. van Berkel, en het Centrum voor Humane en Klinische Genetica van het Leids Universitair Medisch Centrum, onder leiding van Dr. K. Willems van Dijk. De resultaten van dit door de Nederlandse Hartstichting gesubsidieerde onderzoek, staan beschreven in dit proefschrift.

List of Publications

Carlos L.J. Vrans, Ruud Out, Peter van Santbrink, André van der Zee, Martine Groenendijk, Louis M. Havekes, Theo J.C. van Berkel, Ko Willems van Dijk, and Erik A.L. Biessen, Hepatic overexpression of ZNF202 promotes hypotriglyceridemia and steatosis in LDLr^{-/-} but not C57Bl/6 mice. (submitted)

Carlos L.J. Vrans, Peter van Santbrink, André van der Zee, Tokameh Mahmoudi, Louis M. Havekes, Theo J.C. van Berkel, Erik A.L. Biessen, and Ko Willems van Dijk, Repression of mouse Apolipoprotein E Transcription by Zinc Finger Protein ZNF202. (manuscript in preparation)

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Frank G. Schaap, Patrick C.N. Rensen, Peter J. Voshol, **Carlos Vrans**, Hendrik N. van der Vliet, Robert A.F.M. Chamuleau, Louis M. Havekes, Albert K. Groen, Ko Willems Van Dijk, ApoAV reduces plasma triglycerides by inhibiting very low density lipoprotein-triglyceride (VLDL-TG) production and stimulating lipoprotein lipase-mediated VLDL-TG hydrolysis. J Biol Chem, 279: 27941-7, 2004

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