Znf202 Affects High Density Lipoprotein Cholesterol Levels and Promotes Hepatosteatosis in Hyperlipidemic Mice

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Abstract

Background: The zinc finger protein Znf202 is a transcriptional suppressor of lipid related genes and has been linked to hypoalphalipoproteinemia. A functional role of Znf202 in lipid metabolism in vivo still remains to be established.

Methodology and Principal Findings: We generated mouse Znf202 expression vectors, the functionality of which was established in several in vitro systems. Next, effects of adenoviral znf202 overexpression in vivo were determined in normo- as well as hyperlipidemic mouse models. Znf202 overexpression in mouse hepatoma cells mhAT3F2 resulted in downregulation of members of the Apoe/c1/c2 and Apoal/c3/a4 gene cluster. The repressive activity of Znf202 was firmly confirmed in an apoE reporter assay and Znf202 responsive elements within the ApoE promoter were identified. Adenoviral Znf202 transfer to Ldlr−/− mice resulted in downregulation of apoe, apoc1, apoa1, and apoc3 within 24 h after gene transfer. Interestingly, key genes in bile flux (abcg5/8 and bsep) and in bile acid synthesis (cyp7a1) were also downregulated. At 5 days post-infection, the expression of the aforementioned genes was normalized, but mice had developed severe hepatosteatosis accompanied by hypercholesterolemia and hypoalphalipoproteinemia. A much milder phenotype was observed in wildtype mice after 5 days of hepatic Znf202 overexpression. Interestingly and similar to Ldlr−/− mice, HDL cholesterol levels in wildtype mice were lowered after hepatic Znf202 overexpression.

Conclusion/Significance: Znf202 overexpression in vivo reveals an important role of this transcriptional regulator in liver lipid homeostasis, while firmly establishing the proposed key role in the control of HDL levels.

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Introduction

Hypoalphalipoproteinemia is characterized by subnormal levels of serum high density lipoprotein (HDL) irrespective of other lipoprotein or lipid levels. It is a common lipoprotein abnormality in patients with coronary heart disease and low HDL levels were shown in various studies to be linked to risk factors like diabetes and hyperlipidemia [1,2]. By acting as a lipid acceptor in the reverse cholesterol transport and by its anti-inflammatory properties, HDL is considered to be atheroprotective [3–5]. However, a causal link between genetically decreased HDL levels and an increased risk in coronary heart disease has not been established yet and the therapeutic potential of HDL is still to be determined [6–10].

The zinc finger protein 202 (Znf202) gene has been identified in the chromosomal region 11q23 that is linked to heritable hypoalphalipoproteinemia in Utah pedigrees and distinct from an apolipoprotein gene cluster ApoAI/CHI/AIV/AV [11,12]. In contrast, a recent publication failed to confirm a correlation between genetic variations in Znf202 and HDL levels [13]. However, a sequence variation in the promoter of Znf202 did predict atherosclerosis and Ischemic Heart Disease [14]. Znf202 is a Cys2-His2 zinc-finger protein family member containing a leucine-rich SCAN domain and a Krueppel-associated box...
(KRAB) [15,16]. 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 [17]. This is corroborated by in vitro findings showing repressional activity of Znf202 on a range of target genes involved in lipid metabolism including ATP-binding cassette (ABC) transporters Abat1, Abat2 and apolipoproteins Apoe and Apos4, in hepatoma cells [12] as well as monocytes [18]. It was shown that Znf202 interacts with GnT response motifs in the respective promoter regions of these genes. Despite the detailed insights in vitro, very little is known of Znf202 mediated gene regulation in vivo. Moreover, the contribution of Znf202 to the regulation of serum lipids and lipoprotein metabolism is up till now unclear. But even with the contrasting findings in epidemiology studies, its activity pattern in vitro fuels the notion that Znf202 plays an important role in lipid homeostasis and is a potential candidate for a targeted therapy in cardiovascular diseases.

Considering the repressive nature of the transcription factor and, more specifically, its negative effect on the expression levels of HDL-related genes, the observed heritable low HDL cholesterol levels in those aforementioned Utah families are most likely the result of elevated activity of Znf202. Hence, we have investigated ZNF202 overexpression in vivo in both normo- (C57Bl/6J) as well as in hyperlipidemic (low density lipoprotein receptor knockout; Ldlr−/−) mice. To this end, we have generated an adenovirus vector carrying the mouse Znf202 gene. After in vitro analysis for its repressive activity, we assessed effects of hepatic Znf202 overexpression on serum lipid and lipoprotein levels as well as on the hepatic gene expression profile. Along with the suppressive effect on HDL cholesterol levels observed upon Znf202 overexpression in mice, these data clearly demonstrate that the transcription factor Znf202 can act as a key regulator in lipid metabolism.

Materials and Methods

Cell Culture

Mouse hepatoma mhAT3F2 cells [19] were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) with F-12 (Gibco) supplemented with 10% fetal calf serum (Gibco), 100 IU/ml Penicillin, 100 μg/ml Streptomycin, 20 mM GlutaMAX I, 400 nM Insulin (Sigma), 10 nM Dexamethasone (Sigma). Per.C6 and 911 cells [20,21] were both cultured in DMEM (Gibco), supplemented with 10% fetal calf serum (Gibco), 100 IU/ml Penicillin, 100 μg/ml Streptomycin, 2 mM GlutaMAX I (Gibco), at which medium for Per.C6 cells was also supplemented with 10 mM MgCl2. For large scale production of recombinant adenovirus in Per.C6 cells the 10% fetal calf serum was replaced by 2% horse serum (Gibco).

Construction of Expression and Reporter Gene Constructs

Mouse Znf202 was obtained from liver cDNA of C57Bl/6J mice by PCR (nucleotide positions 186 to 2208, GenBank accession number AF292648), with forward primer 5′-GGTACCATAA-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 sites [22]. The ApoE promoter (GenBank accession number D00466) was cloned from cosmid mAPOE/B1 cos27 (C2RB) by PCR. Four different promoter constructs were generated based on the absence or presence of GnT motifs, and forward primers were chosen accordingly and elongated at the 5′ with a KpnI restriction site to facilitate cloning. The promoter sequences were amplified by PCR with a common exon-1 targeted reverse primer carrying a 5′ HindIII site (table S1) and amplicons 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 sequences were verified by sequencing (LGTC, Leiden).

Luciferase Reporter Assays

Transient transfections in mhAT3F2 cells were performed in 6-well plates using Eugene 6 (Roche Molecular Biochemicals). Luciferase activity of 200 ng transfected promoter-reporter constructs, with promoterless pGL3-basic serving as control, was measured by co-transfecting the cells with 100 ng of pCMV-LacZ. After 24 h, the cells were lysed with reporter lysis buffer (Promega) and luciferase activity was determined according manufacturer’s protocol (Promega) in a monolight luminometer (BD Biosciences). β-galactosidase was measured using the β-Galactosidase Enzyme Assay System in reporter lysis buffer (Promega). The effect of Znf202 overexpression on transcription regulation of the reporter gene constructs was determined by co-transfecting mhAT3F2 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, whole cell extract were obtained, and protein concentrations were determined by a Bradford assay (BioRad). Double-stranded [γ-32P]-labeled 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 prepared. The DNA-binding activity of mouse Znf202 in whole cell extracts to these probes was studied by means of electrophoretic mobility shift assay (EMSA). A detailed description of these techniques is provided in supplementary Materials and Methods (Text S1).

Generation of Adenoviral Constructs

Recombinant adenoviral plasmids by homologous recombination of pShuttleCMV-Znf202 with pAdEasy1 were generated in BJ5183 cells (Stratagene) as described by He et al. [22]. Ad.Znf202 was produced in Per.C6 cells (Crucell) and after purification via CsCl centrifugation, the yield was assessed via a plaque assay in 911 cells. A detailed description of these techniques is provided in supplementary Materials and Methods (Text S1). The construction of control virus Ad.lacZ (Ad-lacZ) has been described previously [23].

Animals

All animal work was approved by the Ethics Committee for Animal Experiments of the Leiden University (approval ID: ADEC 03054) and the experimental protocols complied with the national guidelines for use of experimental animals. Male C57Bl/6J mice and Ldlr−/− mice on a C57Bl/6J background were given a standard mouse diet Chow (Hope Farms, Nether-
Figure 1. Znf202 overexpression leads to repression of members of the apoe/c1/c2 and apoa1/c3/a4/a5 gene clusters in mhAT3F2 and inhibits mouse apoE promoter activity. (A) MhAT3F2 cells were transduced with Ad.Znf202 (black bars) or Ad.LacZ (grey bars)(MOI = 100) and mRNA levels were measured via quantitative real time PCR at 24 hours post-infection. Data represent average of four transductions for each group (mean ± S.D) and expressions are relative to HPRT. (B) MhAT3F2 cells were transfected with mouse apoE promoter-reporter constructs, carrying a 729 bp fragment of the mouse apoE promoter lacking the downstream intron-1 (−705 to +24) or truncated variants thereof. Co-transfection with pCMV-LacZ served as a control for transfection efficiency. Luciferase activity was normalized for β-galactosidase activity (n = 4, mean ± S.D.). (C) MhAT3F2 cells were transiently cotransfected with the indicated reporter constructs and Znf202 expression vector (black bars). As control expression vector pShuttleCMV-empty was used (grey bars). After 24 hours cells were harvested and luciferase activities (n = 4, mean ± S.D.) measured and normalized for protein concentration.

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lands) and housed in conventional cages with free access to water and food.

Treatment with Recombinant Adenovirus

In vitro – Murine hAT3F2 hepatoma cells were seeded in a 12-wells plate and grown to 70% confluency. Cells were infected with either Ad.Znf202 or control Ad-mock (MOI = 100). After 24 hours the cells were harvested and total RNA was isolated. In vivo - Recombinant adenovirus, \(2 \times 10^9\) pfu in 200 \(\mu\)l of PBS, was administered by injection into the tail vein of mice at the age of 28–32 weeks. Blood samples were taken via tail bleeding as indicated in the legends. After 24 hrs or 5 days post-infection, mice were sacrificed, liver sections were removed, snap-frozen in liquid nitrogen and stored at \(-80^\circ\)C. Liver cryosections were stained with Oil Red-O (Sigma Diagnostics) and hematoxylin (Sigma Diagnostics).

Determination of mRNA Levels

Total RNA was isolated from treated AT3F2 and liver samples using Trizol according to the manufacturer’s protocol (Invitrogen). Purified RNA was treated with RQ1 RNase-free DNase (Promega, 1 units/2 \(\mu\)g 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 using SYBR green (Applied Biosystems). PCR primers (table S2) were designed on the basis of Primer Express software with the manufacturer’s default settings (Applied Biosystems) and validated for identical efficiencies. Cyclophilin, hypoxanthine-guanine phosphoribosyl transferase (HPRT) and acidic ribosomal phosphoprotein P0 (36B4) were used as control genes.

Lipid and Lipoprotein Analysis

Blood was collected from each individual mouse before (day 0; baseline samples) and at day 1 or at day 4 and 5 after adenovirus injection through tail bleeding in diethyl-p-nitro phenyl phosphate (paraoxon) coated capillary tubes, after a 4-hour fasting period [24]. Total plasma cholesterol (Boehringer-Mannheim) and triglycerides (Sigma Chemical Co) were measured enzymatically. From pooled plasma samples per treatment group, obtained at day 4, lipoprotein distribution was determined by fast performance liquid chromatography (FPLC). A volume of 70 \(\mu\)l was injected onto a Superose6 column (3.2\times30 mm, AKTA-system, Pharmacia). Elution fractions of 30 \(\mu\)l were collected and assayed for cholesterol and triglyceride levels as described above. For measurement of liver lipids, frozen (\(N_2\)) liver samples were

Figure 2. The Znf202 specifically binds to the alleged response elements \(-678\) and \(-564\) within the \(-705/-362\) region of the mouse apoE promoter. (A) DNA fragments used in this study containing the putative Znf202 binding sequence. The putative consensus sequences are underlined. (B) EMSA with labeled DNA fragments GnT (lanes 1–10), \(-678\) (lanes 11–20), \(-564\) (lanes 21–30), and PHO (lanes 31–35) described above. Reactions contained the indicated amount (0.1–0.6 \(\mu\)g) of whole cell extract made from either control 911 cells, or from 911 cells overexpressing Znf202. Competition experiments using 50-fold excess of unlabeled GnT oligonucleotide (lanes 4 and 10), \(-678\) oligonucleotide (lanes 14,17 and 20), or \(-564\) oligonucleotide (lanes 24,27 and 30) confirmed the specificity of Znf202 binding to both GnT elements. Arrowheads indicate the mobility of unbound DNA and Znf202 protein bound DNA. doi:10.1371/journal.pone.0057492.g002
Figure 3. Hepatic Znf202 overexpression reduces HDL-cholesterol in Ldlr−/− and WT mice. Blood samples were drawn from Ldlr−/− (n = 4; left panels) and WT mice (n = 4; right panels) 5 days after injection with 2.10⁷ pfu of Ad.Znf202 (filled bars) or with Ad-mock (open bars) and derived plasma was analyzed for triglyceride and total cholesterol content (A). Lipoprotein profiles were determined from Ldlr−/− (left panels) and WT mice (right panels) 5 days after injection with Ad.Znf202 (triangles) or with Ad-Mock (squares). The elution fractions were tested for triglyceride and total cholesterol content (B). * and ** indicates p < 0.05 and p < 0.001, respectively.

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Figure 4. Hepatic Znf202 overexpression causes hepatosteatosis in Ldlr−/− mice only. Livers were isolated from Ldlr−/− and WT mice 5 days after injection with 2.10⁷ 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 ± SD (n = 4). * indicates p < 0.05.

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Table 1. Relative gene expression in livers 5 days after infection with Ad-mock or Ad-Znf202 in Ldlr−/− and wild type mice.

|                | Ldlr−/−          | Wild Type        |
|----------------|------------------|------------------|
|                | Ad-mock         | Ad-Znf202        | Ad-mock         | Ad-Znf202        |
| ApoE           | 1.00±0.47       | 0.97±0.40        | 1.00±0.23       | 1.10±0.18        |
| ApoC1          | 1.00±0.28       | 0.78±0.39        | 1.00±0.43       | 1.15±0.10        |
| ApoC2          | 1.00±0.65       | 3.27±0.36*       | 1.00±0.28       | 1.61±0.21*       |
| ApoA1          | 1.00±0.62       | 0.65±0.33        | 1.00±0.27       | 0.83±0.20        |
| ApoC3          | 1.00±0.54       | 0.93±0.25        | 1.00±0.35       | 0.90±0.15        |
| ApoA4          | 1.00±0.57       | 0.81±0.30        | 1.00±0.50       | 0.37±0.19*       |
| ApoA5          | 1.00±0.35       | 2.87±0.31*       | 1.00±0.11       | 1.53±0.29*       |
| Abca1          | 1.00±0.31       | 1.25±0.21        | 1.00±0.24       | 1.08±0.22        |
| Abcg5          | 1.00±1.06       | 0.71±0.67        | 1.00±0.47       | 0.80±0.37        |
| Abcg8          | 1.00±0.59       | 0.83±0.40        | 1.00±0.50       | 0.99±0.21        |
| Ldrf           | -                | -                | 1.00±0.28       | 0.88±0.22        |
| Sirt1          | 1.00±0.98       | 0.51±0.39        | 1.00±0.27       | 0.91±0.29        |
| Hmg-CoA red.   | 1.00±0.92       | 0.58±0.13        | 1.00±0.31       | 0.93±0.13        |
| Cyp7a1         | 1.00±0.51       | 1.09±0.73        | 1.00±0.72       | 3.00±0.24*       |

Values are expressed as means ± SD. *Indicates a significant difference (p<0.05) between Ad.Znf202 treated animals and their corresponding Ad.mock treated controls.

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Znf202 Affects Lipid Metabolism

Znf202 Repressed the Expression of Apolipoprotein Genes in vitro

The functionality of the generated recombinant plasmids and adenovirus carrying containing the Znf202 gene construct was verified in vitro. As readout, we analyzed the effect of Znf202 overexpression on the Apoe/c1/c2 and Apoai/c3a14/a3 gene clusters in mAhAT3F2 cells. Cyclophilin or 36B4 expression by transduced mAhAT3F2 cells was not affected by Ad.Znf202 transduction (Fig.1A). 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.

The repressive effect of Znf202 on the expression of one member of investigated apolipoprotein gene clusters, Apoe, has already been demonstrated and was therefore used to address the regulatory capacity of Znf202 [12,18] and to identify its interaction with the mouse Apoe promoter sequence in a reporter assay. The presence of nucleotide sequences spanning −362 to −90 was seen to enhance promoter activity in mAhAT3F2 cells as expected [25,26], while the addition of region −705/−362 attenuated the enhanced activity to up to 4-fold (Fig.1B). To investigate the repressive effect of Znf202 on the Apoe promoter activity, we co-transfected various promoter-reporter constructs and Znf202 expression vector. Znf202 appeared to repress transcriptional activity of the ApoE promoter only when the complete −705/−24 region was present (Fig.1C). Deletion of the 5′ end (−705/−362) abolished the repressive effect of Znf202 on the ApoE promoter activity.

With the Znf202 repressive effect on ApoE promoter activity restricted to the −705/−362 region, we studied the interaction of Znf202 with the two putative GnT sites within this domain at positions −678 and −564 (Fig. 2A) by EMSA. The GnT control probe (Fig. 2B, lanes 1–10) induced a clear mobility shift indicative of the formation of DNA-protein complexes in extracts from Znf202 overexpressing 911 cells (lanes 3, 6, 9). Probe binding was specifically competed by 50-fold excess of unlabeled GnT probe (lane 4, 7, 10). Similar mobility shifts were observed for the [32P]−678 (lanes 11–20) or [32P]−564 probe (lanes 21–30) after Znf202 overexpression. In analogy to the reference probe, the specificity of binding was confirmed by displacement by a 50-fold excess of either unlabeled −678 or −564 probe. Finally, the extracts were unable to form DNA-protein complexes with an irrelevant PHO consensus probe, even at high concentrations (lanes 32 and 33). These in vitro results confirm the repressive role of Znf202 and establish the functionality of our murine Znf202 constructs.

HDL Cholesterol Levels Markedly Reduced after Five Days of Hepatic Znf202 Overexpression

To address the role of Znf202 in lipid metabolism in vivo, we have overexpressed Znf202 in two mouse strains: normolipidemic C57Bl/6j (WT) and hyperlipidemic Ldlr−/− by adenoviral gene transfer. Relative baseline expression of endogenous hepatic Znf202 showed no difference between both mouse models (data not shown). The expression level of Znf202 in WT mice is relatively low (CT 4 and 5), while liver morphology of Znf202 overexpressing livers was characterized by massive oil-red-O stained lipid deposition in intracellular vacuoles mainly (Fig. 4A), while liver morphology of Znf202 transduced WT mice

homogenized and protein content was determined by a Lowry assay using BSA as calibration standard. 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.

Data Analysis

All results are presented as means ± SD. The significance of differences in relative gene expression was calculated using a two-tailed Student’s t test. 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

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was normal (data not shown). As 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 4B, Znf202 overexpressing livers from Ldlr\(^{2/2}\) mice contained over 2-fold higher TC, cholesteryl esters (CE), and TG. In line with their normal liver morphology, no changes in hepatic lipid content were observed in Znf202 transduced WT mice.

Gene Expression Profile After Adenovirus Mediated Gene Transfer of Znf202

Since Znf202 has been reported to be a potent transcriptional repressor of various key genes in lipoprotein metabolism [12], we mapped the hepatic expression profile of a range of putative Znf202 responsive genes at day 5 after Ad.Znf202 or Ad.mock treatment of Ldlr\(^{-/-}\) and WT mice. Unexpectedly, analysis of the Apoe/c1/c2 and Apoa1/c3/e4/a5 gene clusters showed that
Znf202 overexpression even increased the expression of Apoc2 and Apoa5 in WT (1.5-fold) and Ldlr−/− mice (3-fold) (both P<0.05) (table 1). In contrast, Znf202 overexpression did not change Apoa, Apoa1, and Apoa3 expression, even though these genes were seen to be Znf202 responsive in previous in vitro studies [5,11]. 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 cholesterol levels, we determined gene expression levels of cholesterol related genes. Znf202 overexpression did not alter gene expression of major cholesterol transporters Abca1, Abcg5, Abcg8, Sf-b1, and Ldlr (in WT). The gene expressions of Abeg1 and LDL receptor related protein (Lrpg) were unaffected as well (table S3). While similar to cholesterol synthesis, no change in the expression of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGC-CoA reductase) was observed. Interestingly, the bile acid synthesis gene cholesterol 7α-hydroxylase (Cyp7A1) was differentially affected between WT and Ldlr−/− mice upon Znf202 expression. In Ldlr−/− it was unchanged while it was increased in WT (>3-fold). The increased cyp7A1 expression was confirmed at a protein level, as liver extracts from Ad.Znf202 treated WT but not Ldlr−/− mice showed sharply enhanced cyp7A1 activity (Fig. S2).

Lipid homeostasis is under tight control of nuclear receptors [27]. Next, we investigated whether Znf202 could have influenced apolipoprotein and lipid flux gene expression in an indirect manner by modulating nuclear receptor dependent regulatory pathways. The transcription factors Hnf-4, Ppara, Ppard, LXRα/β and FXR were downregulated in Ldlr−/− mice by 25 to 60% (P<0.05) (table S3), while surprisingly no effect was found in WT mice.

As a measure of inflammatory responses potentially inflicted by adenoviral gene transfer we assessed hepatic MARCO and CD68 mRNA levels. The expression of both genes remained essentially unaltered by 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 adenovirus elicited inflammatory responses are underlying the observed phenotype in Ldlr−/− mice.

**Bile Flux Genes Repressed After 24 hrs of Hepatic Znf202 Overexpression in vivo**

Despite hypoalphalipoproteinemia and the dramatic changes in lipid levels, especially in Ldlr−/−, we did not observe any of the expected, repressive effects of Znf202 on hepatic expression of target genes in vivo at 5 day post-transduction. In addition, several lipid related nuclear receptors were dysregulated in Znf202 overexpressing Ldlr−/− mice, although none had been reported to be Znf202 responsive. Conceivably, the initial and suppressive effects of Znf202 overexpression are overruled by secondary effects, which may underly the severe phenotype observed in Ldlr−/− mice. To address this notion, we have monitored hepatic gene expression patterns and lipid homeostasis in the mouse model that was mostly affected, the Ldlr−/−, at 24 hours after transduction. In line with the in vitro results, this increase in hepatic Znf202 gene expression (Fig. S1; from ΔCt = -14 to ΔCt = -11) resulted in the reduced expression of several genes within both apolipoprotein gene clusters in the liver (Fig 5A). The mRNA levels of Apoa1, Apoa3, Apoa1, and Apoa were approximately 2-fold reduced. Interestingly, we also observed a lowered expression (>70%) of the sterol transporter genes Abeg5 and Abeg8 and the bile salt efflux protein (Bsep). Most notably, the expression of the bile acid synthesis gene cholesterol 7α-hydroxylase (Cyp7a1) was more than 1000-fold reduced upon treatment with Ad.Znf202 (P<0.001). The attenuated expression of these bile flux genes suggests that hepatic overexpression of Znf202 affects the biliary secretion. The expression of several lipid related transcription factors such as Fox, Ppara, and Step1 was also reduced by hepatic Znf202 overexpression (table S4). However, a well described target gene of Znf202, Apea1, was unaffected. Additionally, the expression of HMG-CoA reductase, an important enzyme in cholesterol synthesis, was increased.

![Figure 6. Proposed mechanism for Znf202.](image-url)
Znf202 Affects Lipid Metabolism

Together with the altered hepatic gene expression pattern, hepatic Znf202 overexpression had an effect on lipid metabolism already at 24 h post infection. Total cholesterol and triglycerides (TG) levels were markedly elevated (Fig. 5B). These changes in plasma lipids could mainly be ascribed to increased very low density lipoprotein (VLDL). The accumulation of intrahepatic lipids as manifested after 5 days of hepatic Znf202 overexpression, could not be detected at this early time point (data not shown).

Discussion

Based on genetic and in vitro studies, the transcriptional repressor Znf202 has been proposed as a key regulator of lipid homeostasis in particular of lipid efflux [28]. However, while its repressor function in vitro is well established, the precise role of Znf202 in lipid metabolism in vivo remains to be addressed. Despite these initial studies that link Znf202 to HDL, Stene and colleagues were unable to demonstrate a direct correlation between genetic variation within the znf202 gene and HDL cholesterol levels [13]. However, at least one genetic variant was seen to be predictive of a high risk for cardiovascular diseases [29]. In this study we are the first to show, through a gain of function approach involving adenoviral Znf202 gene transfer, a role of Znf202 in lipid metabolism in mice.

To confirm the repressive effect of our Znf202 constructs, we overexpressed murine Znf202 in a mouse hepatoma cell line and showed reduction of expression of the majority of genes within both apolipoprotein gene clusters. The repressive nature of this transcription factor was further established in studies with the murine ApoE promoter. Screening of the proximal region of the murine ApoE gene for potential Znf202 binding sites revealed five Gnt consensus sites (positions −678, −564, −338, −315, −160), of which the first two were shown to carry functional suppressor elements. Removal of these sites ablated Znf202 responsiveness. In addition, Znf202 was seen to specifically interact with the Gnt sites within the responsive −705/−362 region, confirming the specific DNA binding characteristics of Znf202. Because the intron regulatory element 1 (IRE1), situated between exon 1 and exon 2 of the ApoE gene, also contains regulatory elements, we tested the effect of IRE1 [25]. Even though its mere presence reduced promoter activity 2-fold, the overall pattern of expression regulation by Znf202 was not affected (data not shown).

To investigate the role of Znf202 in vivo, the effect of hepatic znf202 overexpression on lipid homeostasis was assessed in hyper- and normolipidemic mice 5 days after adenoviral Znf202 gene transfer. In keeping with earlier genetic and in vitro studies hepatic Znf202 overexpression was accompanied by hypoalphalipoproteinemia both under normolipidemic (WT mice) and hyperlipidemic conditions (Ldlr−/− mice). Furthermore, it was seen to cause hypercholesterolemia and hypertriglyceridemia with concommitant steatosis in Ldlr−/− but not WT mice. Hepatic lipid accumulation was not accompanied by hepatic inflammation excluding that our findings reflect an inflammatory response to adenoviral infection. However, except for apoA4 in WT, we did not observe overt repression of members of the Apoe/c1/c2 and Apoa1/c31/a41/a5 gene clusters in liver of both normo- and hyperlipidemic mice. Also the expression of other lipid related genes including the znf202 target genes Abca1 and Abcg1 [30] was not affected by hepatic Znf202 overexpression.

Although these in vivo data clearly link znf202 activity to hypoalphalipoproteinemia, the apparent lack of correlation between the in vitro data of us and others and the hepatic gene expression profiles at 5 days after adenoviral Znf202 administration was a surprise. This discrepancy might be caused by changes in lipid driven transcriptional factors like Ppar, Lxr, and Fxr in response to Znf202 induced dyslipidemia. For this reason, we analyzed the immediate response in the mouse model that was most affected, the Ldlr−/−. After only 24 hours of hepatic Znf202 overexpression, we did observe lowered expression of members of both apolipoprotein gene clusters. Interestingly, several key genes involved in bile flux and most notably Cyp7a1 were profoundly repressed by Znf202 as well. Closer analysis of the promoter sequence of murine Cyp7a1, expression revealed a putative Znf202 binding (Gnt) site at position −862 bp. Surprisingly, this effect on Cyp7a1 expression coincided with an increase in Hmg-CoA reductase expression suggestive of augmented de novo hepatic cholesterol synthesis. A similar compensatory response to decreased hepatic cholesterol input was seen in studies on the inhibition of sterol absorption by ezetimibe [31,32]. Conceivably, one of the initial responses to Znf202 overexpression may involve the reduced hepatic cholesterol uptake. Nonetheless, the suppressive effect of Znf202 overexpression on bile flux genes such as Cyp7a1 and the concomitant Hmg-CoA reductase upregulation, may explain the strong hepatic lipid accumulation in Ldlr−/− apparent at day 5 post infection [33,34].

Znf202 overexpression led to changed expression of the transcriptional regulators of lipid metabolism Fxr, Srebp1, and Ppar. Fxr, a negative regulator of Cyp7a1 expression, was downand not upregulated. Upregulation of Srebp1 and Pparz has been associated with lipogenesis and hepatic accumulation of lipids, respectively [35]. It remains to be determined whether and how the observed reduced expressions can contribute to the hepatosteatosis in Znf202-treated Ldlr−/− mice.

In response to hepatic Znf202 overexpression in hyperlipidemic Ldlr−/− mice, both VLDL-cholesterol and VLDL-triglycerides were increased within 24 hours. Although potentially reflecting a direct effect of Znf202, the increased VLDL secretion could well be the result of increased hepatic lipids as described previously [36,37]. While the increase in VLDL-cholesterol persisted for at least 4 days, triglyceride levels were sharply reduced in the same time span. Our gene expression analysis revealed increased expression of Apoa5 and Apoc2 at 5 days of Znf202 overexpression both in Ldlr−/− and WT mice. Both ApoA5 and ApoC2 have been implicated in TG metabolism and in particular TG hydrolysis. Studies by Schaap et al. [38], and by Pennacchio et al. [39], already demonstrated a clear inverse relationship between serum ApoA5 and VLDL-TG levels. Similarly, ApoC2 functions as cofactor in LPL-mediated TG hydrolysis [40] and ApoC2 deficiency in humans is associated with severe hypertriglyceridemia [41]. In line with these findings, chronic Znf202 overexpression in Ldlr−/− mice indeed sharply reduced serum triglyceride levels compared to Ad-mock treated controls.

Whereas lipid homeostasis in hyperlipidemic Ldlr−/− mice was strongly affected by Znf202 overexpression, normolipidemic mice only showed mild effects. The moderate effect on total serum lipid levels in WT mice could be attributed in part to clearance via the LDLR. Moreover, additional secondary effects due to a progressively increased VLDL-TG lipolysis and panlobular lipid accumulation are not opportune in WT mice. In contrast to Ldlr−/−, the livers of WT mice show no accumulation of lipids upon Znf202 overexpression at day 5. Interestingly, while in Ldlr−/− the gene expression of Cyp7a1, a key enzyme in bile acid synthesis [42], was apparently normalized after an initial strong Znf202 induced downregulation, it was strongly upregulated in normolipidemic mice. The latter response may underly the mild phenotype seen in WT mice which leads us to propose that the in vivo impact of Znf202 expression depends on the hyperlipidemic status (Fig. 6). Znf202 overexpression directly modulates bile
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Supporting Information

Figure S1 Increased hepatic Znf202 mRNA levels after injection with Ad.Znf202 compared to Ad.mock. Znf202 mRNA levels relative to control genes in livers were determined 24 hrs (Ldlr−/− mice; n = 5) and 5 days (WT and Ldlr−/− mice; n = 4) after injection with Ad.Znf202 (filled bars) or Ad-mock (empty bars). Data are means ± S.D.

Figure S2 Liver analysis for Cyp7A1 activity revealed a significant Cyp7A1 induction in WT mice but not in Ldlr−/− mice at 5 days post-infection. Liver was excised from Ldlr−/− and WT mice 5 days after injection with 2.10⁹ pfu of Ad.Znf202 (filled bars) or Ad-mock (empty bars) and cyp7A1 activity was measured. Data are means ± S.D. of N = 4 determinations and ** indicates p<0.001.

Table S1 Primers used to generate the apoE promoter constructs.

Table S2 Primers sets used for quantitative real-time PCR.

Table S3 Relative gene expression in livers 5 days after infection with Ad-mock or Ad-Znf202 in Ldlr−/− and wild type mice. Values are expressed as means ± SD.

Table S4 Relative gene expression in livers 24 hours after infection with Ad-mock or Ad-Znf202 in Ldlr−/−. Values are expressed as means ± SD.

Text S1 Supplementary material and methods.

Author Contributions

Conceived and designed the experiments: CV KWD EB. Performed the experiments: CV RO PS AZ TM MG. Analyzed the data: CV RO TM LH TB KWD EB. Contributed reagents/materials/analysis tools: RO PS AZ LH. Wrote the paper: CV KWD EB.

References

1. Vega GL, Grundy SM (1996) Hypoalphalipoproteinemia (low high-density lipoprotein) as a risk factor for coronary heart disease. Curr Opin Lipidol 7: 209-216.
2. Di AE, Sarsar N, Perry P, Kaptego S, Ray KK, et al. (2009) Major lipids, apolipoproteins, and risk of vascular disease. JAMA 302: 1993-2000.
3. Schmitz G, Kaminski WE, Porsch-Ozcurumez M, Klacken J, Orso E, et al. (1999) ATP-binding cassette transporter A1 (ABCA1) in macrophages: a dual function in inflammation and lipid metabolism? Pathobiology 67: 236-240.
4. Tall AR (2008) Cholesterol efflux pathways and other potential mechanisms involved in the athero-protective effect of high density lipoproteins. J Intern Med 263: 256-275.
5. Rader DJ (2007) Mechanisms of disease: HDL metabolism as a target for novel therapies. Nat Clin Pract Cardiovasc Med 4: 102-109.
6. Frièke-Schmidt R, Nordestgaard BG, Stene MC, Sethi AA, Remaley AT, et al. (2008) Association of loss-of-function mutations in the ABCA1 gene with high-density lipoprotein cholesterol levels and risk of ischemic heart disease. JAMA 299: 2524-2532.
7. Teslovich TM, Musunuru K, Smith AV, Edmondson AC, Stylianou IM, et al. (2010) Biological, clinical and population relevance of 95 loci for blood lipids. Nature 466: 707-713.
8. Haase CL, Tybjærg-Hansen A, Grande P, Frièke-Schmidt R (2010) Genetically elevated apolipoprotein A1, high-density lipoprotein cholesterol levels, and risk of ischemic heart disease. J Clin Endocrinol Metab 95: E500-E510.
9. Haase CL, Tybjærg-Hansen A, Qvysum AA, Schou J, Nordestgaard BG, et al. (2012) LCAT, HDL cholesterol and ischemic cardiovascular disease: a Mendelian randomization study of HDL cholesterol in 54,500 individuals. J Clin Endocrinol Metab 97: E248-E256.
10. Vaght BF, Pelosi GM, Orho-Melander M, Frièke-Schmidt R, Barbalic M, et al. (2012) Plasma HDL cholesterol and risk of myocardial infarction: a mendelian randomisation study. Lancet 380: 572-580.
11. Kort EN, Ballinger DG, Ding W, Hunt SC, Boren BR, et al. (2000) Evidence of linkage of familial hypoalphalipoproteinemia to a novel locus on chromosome 11q23. Am J Hum Genet 66: 1845-1856.
12. Wagner S, Hess MA, Ormoadn-Hansen P, Malandro J, Hu H, et al. (2000) A broad role for the zinc finger protein ZNF202 in human lipid metabolism. J Biol Chem 275: 15685-15690.
13. Stene MC, Frièke-Schmidt R, Nordestgaard BG, Tybjerg-Hansen A (2006) Zinc Finger Protein 202, genetic variation, and HDL cholesterol in the general population. J Lipid Res 47: 941-952.
14. Stone MC, Frièke-Schmidt R, Nordestgaard BG, Grande P, Schouler P, et al. (2008) Functional promoter variant in zinc finger protein 202 predicts severe atherosclerosis and ischemic heart disease. J Am Coll Cardiol 52: 369-377.
15. Honer C, Chen P, Toth MJ, Schumacher C (2001) Identification of SCAN dimerization domains in four gene families. Biochim Biophys Acta 1517: 441-449.
16. Monaco C, Helmer CM, Caprini E, Vorovichky I, Russo G, et al. (1998) Molecular cloning and characterization of ZNF202: a new gene at 11q23.3 encoding testis-specific zinc finger genes. Genomics 52: 338–362.

17. Lechner MS, Bogg GE, Speicher DW, Rauscher FJ, III (2000) Molecular determinants for targeting heterochromatin protein 1-mediated gene silencing: direct chromoshadow domain-KAP-1 corepressor interaction is essential. Mol Cell Biol 20: 6449–6465.

18. Langmann T, Schumacher C, Morham SG, Honer C, Heimerl S, et al. (2003) ZNF202 is inversely regulated with its target genes ABCA1 and ApoE during macrophage differentiation and foam cell formation. J Lipid Res 44: 968–977.

19. Antoine B, Levrat F, Vallet V, Berbar T, Cartier N, et al. (1992) Gene expression in hepatocyte-like lines established by targeted carcinogenesis in transgenic mice. Exp Cell Res 200: 175–183.

20. Fallaux FJ, Kranenburg O, Cramer SJ, Houweling A, van OH, et al. (1996) Characterization of NIH: a new helper cell line for the titration and propagation of early region 1-deleted adenovirus vectors. Hum Gene Ther 9: 215–222.

21. Fallaux FJ, Bost A, van der Velde I, Van den Wolfenberg DJ, Heliz KM, et al. (1998) New helper cells and matched early region 1-deleted adenovirus vectors prevent generation of replication-competent adenoviruses. Hum Gene Ther 9: 1909–1917.

22. He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, et al. (1998) A simplified system for generating recombinant adenoviruses. Proc Natl Acad Sci U S A 95: 2509–2514.

23. Kobayashi K, Oka K, Forte T, Ishida B, Teng B, et al. (1996) Identification and characterization of transcriptional regulatory regions associated with expression of the human apolipoprotein E gene. J Biol Chem 271: 6852–6860.

24. Zambon A, Brunzell JD (1993) Analysis of techniques to obtain plasma for measurement of levels of free fatty acids. J Lipid Res 34: 1021–1028.

25. Paik YK, Chang DJ, Keay GA, Liu HC, et al. (1998) Identification and characterization of transcriptional regulatory regions associated with expression of the human apolipoprotein E gene. J Biol Chem 263: 13340–13349.

26. Chang DJ, Paik YK, Leren TP, Walker DW, Howlett GT, et al. (1999) Characterization of a human apolipoprotein E gene enhancer element and its associated protein factors. J Biol Chem 265: 9406–9409.

27. Chawla A, Repa JJ, Evans RM, Mangelsdorf DJ (2002) Nuclear receptors and a modulator of cellular lipid efflux. J Biol Chem 276: 12427–12433.

28. Schmitz G, Heimerl S, Langmann T, Morham SG, Honer C, Heimerl S, et al. (2004) Zinc finger protein ZNF202 and apoliprotein influencing triglycerides in humans and mice revealed by comparative sequencing. Science 294: 169–173.

29. LaRosa JC, Levy RI, Herbert P, Levy S. The familial chylomicronemia syndrome. Endocrinol Metab Clin North Am 27: 551–67, viii.

30. Russell DW, Setchell KD (1992) Bile acid biosynthesis. Biochemistry 31: 4737–4749.

31. Van der Vliet HN, Schaap FG, Levels JJ, Ottenhoff R, Losie JG, et al. (2002) Adenoviral overexpression of apolipoprotein A-V reduces serum levels of triglycerides and cholesterol in mice. Biochim Biophys Acta 1586: 129–145.

32. Weinstock PH, Bisgaier CL, alto-Setala K, Radner H, Ramakrishnan R, et al. (1995) Severe hypertriglyceridaemia, reduced high density lipoprotein and very low density lipoprotein secretion: the involvement of apolipoprotein E. J Hepatol 35: 816–822.

33. Zhang DX, Liao CP, Westerterp M, Senokuchi T, Welch CL, et al. (2009) Hepatic insulin signaling regulates VLDL secretion and atherogenesis in mice. J Clin Invest 119: 1029–1041.

34. Schaap FG, Rensen PC, Voshol PJ, Vrijs C, van der Vliet HN, et al. (2004) 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: 27941–27947.

35. Pennacchio LA, Olivier M, Hubacek JA, Cohen JC, Cox DR, et al. (2001) An apolipoprotein influencing triglycerides in humans and mice revealed by comparative sequencing. Science 294: 169–173.

36. Mensenkamp AR, Haverk L, Romijn JA, Kuipers F (2001) Hepatic steatosis and very low density lipoprotein secretion: the involvement of apolipoprotein E. J Hepatol 35: 816–822.

37. Santamarina-Fojo S (1998) The familial chylomicronemia syndrome. Endocrinol Metab Clin North Am 27: 551–67, viii.

38. Russell DW, Setchell KD (1992) Bile acid biosynthesis. Biochemistry 31: 4737–4749.

39. Van der Vliet HN, Schaap FG, Levels JJ, Ottenhoff R, Losie JG, et al. (2002) Adenoviral overexpression of apolipoprotein A-V reduces serum levels of triglycerides and cholesterol in mice. Biochim Biophys Acta 1586: 129–145.