ApoB siRNA-induced Liver Steatosis is Resistant to Clearance by the Loss of Fatty Acid Transport Protein 5 (Fatp5)

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Abstract The association between hypercholesterolemia and elevated serum apolipoprotein B (APOB) has generated interest in APOB as a therapeutic target for patients at risk of developing cardiovascular disease. In the clinic, mipomersen, an antisense oligonucleotide (ASO) APOB inhibitor, was associated with a trend toward increased hepatic triglycerides, and liver steatosis remains a concern. We found that siRNA-mediated knockdown of ApoB led to elevated hepatic triglycerides and liver steatosis in mice engineered to exhibit a human-like lipid profile. Many genes required for fatty acid synthesis were reduced, suggesting that the observed elevation in hepatic triglycerides is maintained by the cell through fatty acid uptake as opposed to fatty acid synthesis. Fatty acid transport protein 5 (Fatp5/Slc27a5) is required for long chain fatty acid (LCFA) uptake and bile acid reconjugation by the liver. Fatp5 knockout mice exhibited lower levels of hepatic triglycerides due to decreased fatty acid uptake, and shRNA-mediated knockdown of Fatp5 protected mice from diet-induced liver steatosis. Here, we evaluated if siRNA-mediated knockdown of Fatp5 was sufficient to alleviate ApoB knockdown-induced steatosis. We determined that, although Fatp5 siRNA treatment was sufficient to increase the proportion of unconjugated bile acids 100-fold, consistent with FATP5’s role in bile acid reconjugation, Fatp5 knockdown failed to influence the degree, zonal distribution, or composition of the hepatic triglycerides that accumulated following ApoB siRNA treatment.

Keywords APOB · Liver steatosis · siRNA · FATP5 · Slc27a5 · siRNA combinations

Abbreviations

APOB Apolipoprotein B
ASO Antisense oligonucleotide
CLinDMA 2-{4-[(3b)-cholest-5-en-3-yloxy]-butoxy}-N,N-dimethyl-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-1-amine
d Deoxy
f 2’ fluoro
FATP5 Fatty acid transport protein 5
H & E Hematoxylin and eosin
HTG Intra-hepatic triglyceride
LCFA Long chain fatty acid uptake
LDL-c LDL cholesterol
LNP Lipid nanoparticle
MTP Microsomal transfer protein
PEG-DMG Monomethoxy(polyethyleneglycol)-1,2-dimyristoylglycerol
siRNAs: Small interfering RNAs
TCA: Taurocholic acid
o: 2′ O-methyl (o)
VLDL: Very low density lipoprotein

**Introduction**

Elevated LDL cholesterol (LDL-c) promotes atherosclerosis, and it is well established that reducing LDL-c helps to mitigate the risk of developing cardiovascular disease in patients with hypercholesterolemia [1–7]. LDL-c consists of a single apolipoprotein B-100 (APOB-100) molecule, cholesterol, cholesterol-esters and triacylglycerols that are comprised of various dietary and de novo synthesized fatty acids [8]. In the liver, APOB is required for very low density lipoprotein (VLDL) formation and serves as the scaffold to solubilize cholesterol and fatty acids (in the form of triglycerides) for secretion into the blood for circulation [8]. An association between hypercholesterolemia and increased APOB protein levels, together with the observation that reductions in ApoB synthesis reduce LDL-c and the incidence of atherosclerosis, has generated interest in APOB as a therapeutic target [9–13]. Both antisense oligonucleotides (ASOs) and small interfering RNAs (siRNAs) targeting ApoB reduce LDL-c in mice and in non-human primates [14–18]. In mice, ApoB ASOs reduced LDL-c without inducing hepatic steatosis, a liability of microsomal transfer protein (MTP) inhibitors that block triglyceride-rich lipoprotein assembly and secretion [19]. In patients, mipomersen, an ApoB targeting ASO, reduces both LDL-c and APOB, demonstrating the potential for an ApoB-targeted therapeutic [20–24]. Liver steatosis induced by inhibiting ApoB, however, remains an important concern. Recently, mipomersen administration at a sub-maximum efficacious dose was shown to be associated with a trend toward increased intra-hepatic triglyceride (IHTG) content for mipomersen-treated patients with one of the ten patients developing mild steatosis [20]. In addition, mice harboring a base-pair deletion in the coding region of ApoB (ApoB-38.9) exhibited hepatic triglyceride accumulation [25]. In order to attenuate the risk of liver steatosis associated with an ApoB-targeted therapeutic, one approach would be to combine an ApoB ASO or siRNA with another therapeutic that increases the clearance of hepatic triglycerides.

Fatty acid transport protein 5 (Fatp5/Slc27a5) mediates the uptake of long chain fatty acids (LCFAs) to the liver and is involved in bile acid reconjugation during enterohepatic recirculation [26, 27]. Fatp5 knockout mice exhibit lower levels of hepatic triglycerides and free fatty acids due to decreased liver fatty acid uptake [27]. Furthermore, Doege et al. [25] recently showed that adenovirus-shRNA-mediated silencing of Fatp5 mRNA not only protected mice from high fat diet-induced liver steatosis but also reversed steatosis once it was established. This suggested that a FATP5 inhibitor may be an attractive combination therapy with an APOB-targeted therapeutic.

Besides of its role in free fatty acid uptake, FATP5 also plays a critical role in reconjugation of bile acids during enterohepatic recirculation to the liver, and complete deletion of Fatp5 resulted in a significant increase in unconjugated bile acids in both bile and serum [26]. Activation of FXR, a bile acid nuclear receptor, with bile acids or synthetic activators has been shown to reduce the secretion of triglyceride-rich VLDL from the liver in mice, which was associated with a decrease in Srebp1 and 2 pathway genes [28]. The involvement of FATP5 in bile acid metabolism suggests that it too may play a role in hepatic triglyceride metabolism via FXR. However, the contributions of the bile acid reconjugation activity of FATP5 on hepatic steatosis or the contribution of FATP5 on APOB-induced steatosis are currently unknown. By utilizing two siRNAs specifically targeting Fatp5 and ApoB, we evaluated the ability of Fatp5 siRNA treatment to alleviate ApoB siRNA-induced liver steatosis.

**Materials and Methods**

**siRNA Design**

siRNAs were designed to the mRNA transcripts using a previously published design algorithm [34]. siRNA sequences contained the following chemical modifications added to the 2’ position of the ribose sugar when indicated: deoxy (d), 2’ fluoro (f), or 2’ O-methyl (o) [34]. Modification abbreviations are given immediately preceding the base to which they were applied. Passenger strands were capped with an inverted abasic nucleotide on the 5’ and 3’ ends. The control siRNA sequence (Cntrl siRNA) consists of: iB;fluU;fluC;fluU;fluU;fluU;fluU;fluU;fluU;fluU;fluU;fluC;fluU;fluC;fluU;fluC;fluU;fluC;da;dg;dt;dt;iB passenger strand and fluC;fluU;omeG;omeA;omeA;omeG;omeA;omeA;omeA;omeG;omeA;omeG;fluU;fluU;omeA;omeA;omeA;omeA;omeA;omeA;omeA;omeA;omeA;omeA guide strand sequences. The Fatp5(951) siRNA sequence consists of: iB;fluC;fluU;dG;fluC;fluU;da;fluU;fluU;fluU;fluU;fluC;da;fluU;fluC;fluU;fluU;fluU;fluC;dt;dt;iB passenger strand and
rG; rU; rA; omeA; omeA; omeG; omeA; fluU; omeG; omeA; omeA; fluU; omeA; fluU; omeG; fluC; omeG; fluC; omeA; omeG; omeU; omeU guide strand sequences.

The Apob(10168) siRNA sequence consists of:

ib; fluU; fluC; daA; fluU; fluC; daA; daA; fluC; fluC; daA; fluU; daA; fluU; daA; fluC; daA; daA; daA; tT; iB

passenger strand and
rU; rU; rG; omeG; fluU; omeA; fluU; fluU; fluC; omeA; omeG; fluU; omeG; omeA; fluU; omeG; omeA; omeU; omeU guide strand sequences.

siRNA Synthesis

siRNAs were synthesized by methods previously described [35]. For each siRNA, the two individual strands were synthesized separately using solid phase synthesis, and purified by ion-exchange chromatography. The complementary strands were annealed to form the duplex siRNA.

The duplex was then ultrafiltered and lyophilized to form the dry siRNA. Duplex purity was monitored by LC/MS and tested for the presence of endotoxin by standard methods.

Preparation of siRNA-Lipid Nanoparticle (LNP) Complex

LNPs were made as described previously [36]. The encapsulation efficiency of the particles was determined using a SYBR Gold fluorescence assay in the absence and presence of triton, and the particle size measurements were performed using a Wyatt DynaPro plate reader. The siRNA and lipid concentrations in the LNP were quantified by a HPLC method, developed in house, using a PDA detector.

In Vivo siRNAs Treatments

All in vivo work was performed according to an approved animal protocol as set by the Institutional American Association for the Accreditation of Laboratory Animal Care. C57Bl/6 mice engineered to be hemizygous for a knockout of the LDL receptor and hemizygous for the overexpression of the human cholesterol ester transfer protein (CETP) driven by the endogenous apoA1 promoter within a C57Bl/6 background [B6-Ldlr<tm1>Tg(apoA1-CETP); Taconic] were used for these studies. Mice ~16–20 weeks of age were fed Lab Diets (5020 9F) starting 2 weeks prior to the start of the study. siRNAs were administered by intravenous (i.v.) injection. Animals were dosed on days 0 and 14 with either 3 mg/kg of a single LNP formulated siRNA or 1.5 mg/kg of two LNP formulated siRNAs for a 3 mg/kg total siRNA combination dose. For siRNA combinations, siRNA were formulated individually and hand-mixed immediately prior to injections.

Animals were euthanized by CO2 inhalation. Immediately after euthanasia, serum was collected using serum separator tubes and allowed to clot at room temperature for 30 min. Liver sections were excised, placed in either RNA Later (Qiagen) (right medial lobe), 10% buffered formalin (10% NBF, left medial lobe), or flash frozen (the remainder), and stored until further use.

RNA Isolation and qRT-PCR

RNA was isolated using an RNeasy96 Universal Tissue Kit (Qiagen) according to the supplied product protocol and as described previously [37]. TaqMan Gene Expression Assays (Applied Biosystems) were performed as described within the product protocol using the following primer probes, Mm00447768_m1 for Fatp5, Mm01545154_g1 for ApoB, and Cat# 4352339E for the reference, Gapdh. All reactions were performed in duplicate, and data were analyzed using the ddCt method with Gapdh serving as in [38]. Data represented as the log2-fold change (ddCt) relative to the control siRNA. For analysis of the selected Srebp1c and Srebp2 pathway genes and Scd1, expression was normalized to an average of that of mouse β-actin (Actb), Glyceraldehyde 3-phosphate dehydrogenase (Gapdh), Beta-glucuronidase (Gusb), Hypoxanthine–guanine phosphoribosyltransferase (Hprt1), Peptidylprolyl isomerase A (Cyclophilin AlPpia) and ribosomal protein 113a (Rp113a) in each sample. Expression levels of all genes analyzed were normalized to an average of the housekeeping genes (listed above) to obtain dCt. Fold regulation is calculated as: ddCt of gene in treatment group/ddCt of gene in control group. Significance (p value) was calculated from a two-tailed t test between control and treatment groups. Accession numbers for the primer/probes used are listed in Supplemental Table 1.

Cholesterol and Triglyceride Analysis

Serum for cholesterol and triglyceride analysis was analyzed using Wako’s total and HDL cholesterol kits according to the supplied product protocol and as described previously [37]. Non-HDL, which serves as an approximation for LDL, was calculated by subtracting HDL from total cholesterol measurements. The percent difference relative to the control siRNA was calculated using the following equation {100 × [1−(experimental/control)]}.

Histology and Hematology

Mouse livers were fixed with 10% neutral buffered formalin. One hepatic lobe was treated with osmium tetroxide solution, to visualize lipids, overnight at room temperature prior to paraffin embedding and processing. The other
haptic lobe was embedded and processed in paraffin and hematoxylin and eosin (H&E) stained. Samples were sectioned at a thickness of 5 μm. The osmium-stained samples were digitalized using an Aperio ScanScope XT. Percent area (positive pixel count) was calculated using the positive pixel count algorithm (MAN-0024) supplied with the imaging software (Aperio). Samples were also reviewed by a board-certified veterinary pathologist and scored for inflammation and lipidosis using a semi-quantitative score.

Measurement of Serum APOB

The APOB levels in serum were measured by LC/MS as described previously [29]. The concentration of APOB peptide was calculated by dividing the area under the curve for the analyte by the area of its internal standard and multiplying by the internal standard concentration. The concentration of APOB was then converted to and reported as mg/dL.

LC/MS Sample Preparation and Analysis for Bile Acid Conjugation and Hepatic Triglycerides

Terminal bile samples from each group were collected using the stick and pull method. Samples were diluted 1:1,000 v/v in 50% acetonitrile + 0.1% formic acid/50% water + 0.1% formic acid, followed by the addition of 1 μM total internal standard solution (D₄-TCA, D₄-CA, D₄-GCA) (Sigma-Isotec, St. Louis, MO, USA). The mixture was vortexed for 10 s, centrifuged for 10 min at 15,000g, and then stored at −20 °C until LC/MS analysis. Supernatant was injected (10 μL) directly onto the LC/MS system.

A 50-mg slice of frozen liver from each animal was homogenized in 2-mL polypropylene tubes containing a 14-mm ceramic bead using a Precellys 24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). A non-naturally occurring internal standard solution (20 μL) containing TG 51:0 (Sigma Aldrich, St Louis, MO, USA) 0.8 mg/mL along with dichloromethane/methanol (2:1 v/v) was added to each sample prior to homogenization [39]. Samples were homogenized at 5,500 RPM, 2 × 30 s, with a 15 s pause between cycles. In order to generate a two layer liquid separation, 200 μL of distilled water was added, vortexed for 30 s, followed by centrifugation at 20,000g at 5 °C for 10 min. 10 μL of the lower layer, containing the lipids, was removed without disrupting the liver tissue homogenate disk. This was followed by dilution of the extracted lipid sample 1/50 in a solvent mixture (65% ACN, 30% IPA, 5% H₂O).

External endogenous calibration standards in buffer solution were used to cover the endogenous concentrations present in the bile and liver tissues. The inlet system was comprised of an Acquity UPLC (Waters, Milford, MA, USA). Bile and lipid extracts were injected (2 μL) onto a 1.8-μm particle 100 × 2.1 mm id Waters Acquity HSS T3 column (Waters). The column was maintained at 55 °C with a 0.4-mL/min flow rate for the lipid analysis and 65 °C with a 0.7-mL/min flow rate for the bile analysis. A binary gradient system was utilized for the analysis of both sample sets. Two different gradient conditions were used. For the lipid analysis, the same conditions were used as previously described [40]. For the bile acid analysis, water + 0.1% formic acid was used as eluent A. Eluent B consisted of acetonitrile + 0.1% formic acid (Burdick & Jackson, USA). A linear gradient (curve 6) was performed over a 13-min total run time. During the initial portion of the gradient, it was held at 80% A and 20% B. For the next 10 min, the gradient was ramped in a stepped linear fashion to 35% B (curve 5) in 4 min, 45% B in 7.5 min and 99% B in 9.5 min and held at this composition for 1.6 min. Hereafter, the system was switched back to 80% B and 20% A and equilibrated for an additional 2.9 min.

The inlet system described was directly coupled to a hybrid quadrupole orthogonal time of flight mass spectrometer (SYNAPT G2 HDMS; Waters MS Technologies, Manchester, UK). Electrospray (ESI) positive and negative ion ionization modes were used. In both ESI modes, a capillary voltage and cone voltage of ±2 kV and ±30 V, respectively, were used. The desolvation source conditions were as follows: for the desolvation gas 700 L/h was used and the desolvation temperature was kept at 450 °C. Data were acquired over the mass range of 50–1,200 Da.

The LC/MS data acquired was processed by the use of a quantitative data deconvolution package (Positive software by MassLynx; Waters, MA, USA). Data are presented as ± standard error of the mean (SEM). Differences between groups were computed by either Student’s t test or by two-way ANOVA (GraphPad Prism, La Jolla, CA, USA). Post-test analysis for quantifiable variables was conducted using either Bonferroni or Mann–Whitney U non-parametric test with two-tailed p values. Values of p < 0.05 were considered statistically significant.

Lipid Nomenclature

The nomenclature utilized in this manuscript is in accordance with Lipidmaps (http://www.lipidmaps.org). In brief, a triglyceride described as TG 52:2 is interpreted as a triglyceride containing 52 carbons attached to the glycerol back-bone and two double bonds in the fatty acyl chain as described by Fahy et al. [41].
Results

Sustained Knockdown of Both ApoB and Fatp5 mRNA Transcripts was Achieved with siRNAs Administered Either Alone or in Combination

To investigate if we can simultaneously silence both ApoB and Fatp5, we administered siRNAs specifically targeting ApoB and Fatp5, alone and in combination, to CETP/LDLR hemizygous female mice (mice exhibiting a human-like lipid profile) fed a low-fat western diet. Changes to the lipid profile were obtained through a hemizygous mutation of the LDL receptor (±LDLR) and the hemizygous overexpression of a mouse apoA1 promoter-driven human CETP transgene (±apoA1-hCETP). This led to an elevation in LDL and a cholesterol profile that more closely resembled the HDL–LDL ratio observed in humans [29]. Female mice were fed a low-fat western diet ad libitum and treated with siRNAs formulated in a lipid nanoparticle (LNP) to achieve delivery to the liver [30]. Animals were dosed on days 0 and 14 with either 3 mg/kg of a single siRNA or 1.5 mg/kg of two siRNAs for a 3 mg/kg total siRNA combination dose. Efficacy was analyzed using qRT-PCR on liver samples collected on day 21. Analysis of mRNA expression levels revealed no appreciable difference in knockdown (KD) between ApoB siRNA treatments administered either alone or in combination with the Fatp5 siRNA (Fig. 1a; ≥90% KD across all groups). Similar results were observed for the Fatp5 siRNA, where ≥89% knockdown of Fatp5 was observed (Fig. 1b).

Fatp5 Knockdown Impaired Bile Acid Reconjugation

The ratio of unconjugated/conjugated bile acid in the bile was used as a biological indicator for the loss of FATP5 activity following siRNA treatment to female mice fed a low-fat western diet ad libitum [26]. A significant increase in the proportion of unconjugated bile acids was observed for all Fatp5 siRNA treatment groups, indicative of the loss of FATP5 activity (Fig. 2a; \( p = 0.0003 \), t test, Mann–Whitney post-test, cntrl siRNA groups vs. Fatp5 siRNA groups). The unconjugated/conjugated ratio for the cntrl/cntrl siRNA group and the Fatp5/Fatp5 siRNA group was 0.004 and 2.2, respectively. The ratio was lower for some of the Fatp5 siRNA treatment groups when combined with the ApoB siRNA, but nevertheless exhibited a significant level of target engagement when compared with the control siRNA. Cholic acid in the Fatp5 groups was the most predominant unconjugated bile acid found in bile (Fig. 2b), which showed a dramatic increase in concentration (0.77 mM in control siRNA vs. 77.95 mM in Fatp5) after knockdown of Fatp5 \( (p = 0.0003, \text{t test, Mann–Whitney post-test}) \). Unconjugated bile acids, specifically taurocholic acid (TCA), showed the reverse effect following Fatp5 knockdown \( (p = 0.1304, \text{t test, Mann–Whitney post-test}) \). The levels of TCA decreased to 43.2 mM in comparison with the cntrl siRNA group 69.7 mM (data not shown). The ratio of unconjugated/conjugated bile acid in the serum reflected that of the bile, with a significant increase in the unconjugated levels (data not shown).

![Fig. 1](image-url) Similar levels of ApoB and Fatp5 mRNAs were observed for all groups containing siRNAs targeting these genes, either alone or in combination. Mice \( (n = 8/\text{group}) \) were treated with siRNAs targeting ApoB and Fatp5 alone or in combination \( (\text{ApoB, Fatp5, Fatp5/ApoB}) \) at days 0 and 14. Gene expression was analyzed using qRT-PCR (TaqMan) on day 21 \( (a, b) \) post the initial dose. PBS and a control siRNA \( (\text{cntrl siRNA}) \) served as negative controls. Data represented as the log2 fold change \( (\text{ddCt}) \) relative to the cntrl siRNA treatment of individual animals \( (\text{circles}) \) and the group means \( (\text{bars}) \) are shown.
ApoB siRNA Treatment Led to Significant Reductions in Serum APOB Protein, Cholesterol and Triglyceride Levels Alone and in Combination with a Fatp5-Targeting siRNA

ApoB siRNA treatment, either alone or in combination with a siRNA targeting Fatp5, caused a significant reduction (p ≤ 0.001) in serum APOB levels in female mice fed a low-fat western diet ad libitum (Fig. 3). APOB protein reductions were consistent with the reductions in hepatic ApoB mRNA levels (Fig 1a). This led to reductions in circulating cholesterol and triglyceride levels. On day 21, similar reductions in total (76–84%), HDL (67–78%), and non-HDL (79–90%) cholesterol were observed across all ApoB siRNA treatment groups (Fig. 4a–c). No significant decrease in cholesterol levels were observed for the ApoB ? Fatp5 siRNA combination group relative to the ApoB siRNA individual treatment group.

Serum triglycerides were also significantly reduced for ApoB siRNA treatments either alone or in combination on day 21 (Fig. 4d). Taken together, these data point to similar and significant changes in serum cholesterol across ApoB siRNA treatment groups that correlated with the observed reductions in ApoB mRNA and serum protein levels.

Fatp5 siRNA Treatment Failed to Alleviate ApoB siRNA-induced Liver Steatosis

To determine if Fatp5 siRNA treatment was sufficient to alleviate ApoB siRNA-induced liver steatosis, liver sections were processed, sectioned, and stained with either osmium or H&E. Image analysis of the osmium stained slides revealed similar levels of significant lipid accumulation across all ApoB siRNA treatment groups relative to the PBS, Fatp5, or control siRNA groups (Fig. 5a, b). These data indicated that Fatp5 siRNA treatment failed to alleviate ApoB siRNA-induced liver steatosis in female mice fed a low-fat western diet ad libitum. For the PBS, Fatp5, and control siRNA groups, there was some evidence of a periportal to midzonal (zones 1 and 2) distribution of lipid droplets within hepatocytes (Fig. 5b). This contrasted the lipid distribution following ApoB siRNA treatment,
where diffuse infiltration (all zones) was observed (Fig. 5b). The ApoB-treated groups displayed lipid droplets that were smaller and more evenly distributed, while fewer but larger lipid droplets were observed for the remaining groups in many instances and as shown in Fig. 5b. By H&E staining, hepatocytes in the ApoB siRNA treatment groups appeared diffusely swollen, with granular to vacuolated cytoplasmic spaces (data not shown).

Fatp5 Knockdown Failed to Alter ApoB siRNA-induced Liver Triglyceride levels or Composition

Total triglyceride composition analysis by LC/MS revealed a significant increase in the level of triglycerides found in the liver following treatment with ApoB siRNA when compared with siRNA control (Fig. 6a; Ctrl siRNA 7.5 µg/mg of tissue vs. ApoB siRNA 35.5 µg/mg of tissue, p = 0.0002, t test with Mann–Whitney post-test). Comparative measurements between the ApoB/ApoB and the Fatp5/Fatp5 + ApoB groups demonstrated that there was no protection from triglyceride accumulation in female mice fed a low-fat western diet ad libitum. LC/MS indicated that triglycerides 52:2 and 52:3 were significantly higher (p ≤ 0.0001, two-way ANOVA with Bonferroni post-test) following Fatp5/Fatp5 + ApoB treatment relative to ApoB/ApoB treatment (Fig. 6b). Triglycerides 52:4 and 54:3, although not statistically significant, also showed an increase in the Fatp5/Fatp5 + ApoB cohort in

Fig. 4 Comparable levels of serum cholesterol and triglycerides were observed for ApoB siRNA treatments either alone or in combination with a siRNA-targeting Fatp5. Total cholesterol (a), HDL cholesterol (b), and triglycerides (d) were measured on day 21 following days 0 and 14 doses as indicated on the x-axis. Non-HDL cholesterol (c) was calculated by subtracting the HDL cholesterol value from the total cholesterol value. Data represented as group means (bars) ± SD (n = 8/group). The percent difference relative to the control siRNA is shown. Significance (***p ≤ 0.0001) was calculated using a one-way ANOVA, Tukey post-test, and reported for treated groups relative to the siRNA control (Ctrl siRNA) treatment.
comparison with the ApoB/ApoB cohort. Structure elucidation by collision-induced dissociation MS/MS provided information about the possible fatty acid composition for each triglyceride. Triglyceride 52:2 contained 16:0/18:1/18:1, triglyceride 52:3 contained 16:0/18:1/18:2, triglyceride 54:3 contained 18:1/18:1/18:1 and triglyceride 54:3 contained 18:1/18:2/18:1. These triglycerides were confirmed by accurate mass and the use of external standards (data not shown). All of these significantly relevant triglycerides have constituent fatty acids which are non-essential, suggesting that they may be derived from de novo synthesis.

The Hepatic Sterol Response Differed After ApoB and Fatp5 siRNA Treatments

To investigate the hepatic gene response to ApoB, Fatp5 or combination treatments, we performed qRT-PCR analysis on genes involved in the sterol response element binding protein 1 and 2 pathways (Srebp1 and Srebp2). Interestingly, although ApoB knockdown induced steatosis, it resulted in a significant decrease in many genes involved in both pathways (Table 1), including genes that are key regulators of fatty acid and triglyceride synthesis (Fasn, Sed, Fads1/2, Acsl3/5). As previously described, deletion...
Fig. 6  Fatp5 siRNA treatment fails to alter ApoB siRNA-induced liver triglyceride levels or composition. The total triglyceride pool size and composition was measured for different siRNA administrations (Cntrl siRNA/Cntrl siRNA, ApoBl/ApoB, Fatp5/Fatp5 and Fatp5/Fatp5 + ApoB). a A large increase in liver triglycerides was observed for the ApoBl/ApoB group relative to the control siRNA (**p = 0.0002, t test with Mann–Whitney post-test). Fatp5 siRNA treatment (Fatp5/Fatp5 + ApoB) did not show protection from ApoB siRNA-mediated liver steatosis. b Specific triglycerides TG 52:2 and TG 52:3 showed an increase in their concentrations in both the ApoBl/ApoB and Fatp5/Fatp5 + ApoB groups suggesting that Fatp5 knockdown fails to alter the triglyceride composition induced by ApoB knockdown (**p ≤ 0.0001, two-way ANOVA with Bonferroni post-test).

[31] or silencing [25] of Fatp5 caused a reduced uptake of hepatic free fatty acids from serum, which caused a subsequent increase in genes involved in fatty acid synthesis. In contrast to ApoB siRNA treatment, Table 1 shows that Fatp5 siRNA treatment resulted in a significant increase in both Srebp1 and Srebp2 pathways. Although bile acid conjugation levels changed and there was a significant increase in cholic acid in bile (Fig 3b), transcription of FXR or key regulators of FXR did not change (data not shown). ApoB siRNA treatment appeared to have a stronger effect on the Srebp1/2 pathways than Fatp5 siRNA since the simultaneous reductions of both resulted in a hepatic gene signature more similar to that of ApoB siRNA treatment alone.

Discussion

In this report, we found that siRNA-mediated knockdown of ApoB led to a significant reduction in serum lipids, including HDL, which was associated with an increase in hepatic triglycerides leading to liver steatosis in CETP/LDLR hemizygous mice (a mouse model having a human-like lipid profile). It is worth noting that similar results were observed on day 28 for all end points measured [mRNA knockdown, serum lipids, and histological analysis of hepatic lipid levels (data not shown)].

ApoB siRNA-mediated steatosis contrasts with results reported for ASO-mediated ApoB knockdown where only modest but not significant increases in hepatic triglycerides were observed following 6 weeks of biweekly (25 mg/kg) treatment that resolved by week 20 [14]. Crooke et al. reasoned that a compensatory mechanism, which included the activation of AMPK leading to increased fatty acid oxidation and the down-regulation of genes involved in fatty acid synthesis and transport, led to the resolution of hepatic triglyceride accumulation [14]. However, we also observed decreased gene expression within these pathways following the knockdown of ApoB. In addition, we observed increased serum ketone levels following ApoB siRNA treatment, which is indicative of increased fatty acid oxidation (data not shown). Furthermore, mice harboring a base-pair deletion in the coding region of ApoB (ApoB-38.9) also exhibited hepatic triglyceride accumulation and decreased expression of genes involved in fatty acid synthesis [25]. Taken together, these data suggest that an alternative mechanism may explain the lack of ASO-mediated steatosis and the discrepancy between ApoB
siRNA and ASO treatments, which could be explained, at least in part, by the fact that we are observing slightly greater ApoB knockdown (≈90 vs. ≈75%) and cholesterol lowering [≈80 (non-HDL) vs. 66% (LDL)] relative to the ASO-mediated changes.

From our results, HDL appears lower and serum triglycerides higher than one would initially expect for ApoB knockdown. While the cause of the decrease in HDL following ApoB siRNA treatment is unknown, it is likely that the HDL components provided by APOB-containing particles are reduced following ApoB knockdown leading to a reduction HDL levels, which is consistent with findings by Tadin-Strapps et al. [29, 31]. In addition, while serum triglycerides appear disproportionately higher relative to serum APOB for ApoB siRNA-treated groups (Fig. 3 relative to Fig. 4d), some of this apparent discrepancy could be due to the production of larger, more triglyceride-rich, non-HDL particles for the limited number of particles produced. It also remains likely that serum triglycerides are artificially elevated relative to other endpoints, since serum triglycerides are measured enzymatically and glycerol is a reaction intermediate. Thus, any glycerol within the serum would lead to a higher reading than would otherwise be expected.

Fatty acids are either transported into the liver or synthesized de novo. Since fatty acids are required for triglyceride synthesis and ApoB knockdown reduced the expression of many of the genes involved in de novo hepatic fatty acid synthesis, we rationalized that liver steatosis induced by ApoB knockdown could be maintained, at least in part, by the accumulation of fatty acids taken up by hepatocytes [29]. FATP5 is a transporter of long chain fatty acids (LCFAs) into hepatocytes [27, 32], and we reasoned that reducing LCFA uptake by knocking-down Fatp5 would reduce hepatic triglyceride levels induced by ApoB knockdown. We found that Fatp5 knockdown does not influence the size, composition, or zonal distribution of the hepatic triglyceride pool generated by ApoB siRNA treatment. Lipid levels for the Fatp5 siRNA treatment alone were too low to reliably assess differences in the hepatic triglyceride population relative to the control siRNA group, although results reported by Doege et al. [27] would

| Table 1 Srebp1c and Srebp2 gene expression changes observed following ApoB, Fatp5, and combination siRNA treatments |
|-----------------|-----------------|-----------------|-----------------|
| Gene            | ApoB            | Fatp5           | Fatp5/Fatp5/ApoB |
|                 | Fold regulation | p value         | Fold regulation | p value         | Fold regulation | p value         |
| Srebp1c pathway | Acaca           | 1.46            | 0.0705          | 1.78<sup>a</sup> | 0.0156          | 1.86<sup>b</sup> | 0.0266          |
|                 | Acacb           | 1.69<sup>b</sup> | 0.0084          | 1.61<sup>a</sup> | 0.0070          | 5.40<sup>b</sup> | 0.0098          |
|                 | Fasn            | –2.99<sup>b</sup> | 0.0026          | 2.05<sup>a</sup> | 0.0203          |          | 2.03           | 0.0830          |
|                 | Scd             | –3.62<sup>b</sup> | 0.0009          | 1.95<sup>a</sup> | 0.0007          |          | 2.91<sup>b</sup> | 0.0104          |
|                 | Fads1           | –2.18<sup>b</sup> | 0.0000          | 1.13           | 0.0514          |          | 2.18<sup>b</sup> | 0.0000          |
|                 | Fads2           | –2.39<sup>b</sup> | 0.0000          | 1.24           | 0.0705          |          | 2.36<sup>b</sup> | 0.0001          |
|                 | Acsl2           | 1.36<sup>a</sup>  | 0.0075          | 1.12           | 0.0792          |          | 1.28           | 0.0074          |
|                 | Acsl1           | 1.19             | 0.1635          | –1.18          | 0.3571          |          | 1.06           | 0.6852          |
|                 | Acsl3           | –2.16<sup>b</sup> | 0.0002          | 1.05           | 0.8005          |          | –1.76          | 0.0202          |
|                 | Acsl4           | –1.24            | 0.1148          | –1.10          | 0.3702          |          | –1.25          | 0.0948          |
|                 | Acsl5           | –1.28<sup>b</sup> | 0.0405          | 1.40<sup>a</sup> | 0.0020          |          | –1.40          | 0.0475          |
| Srebp2 pathway  | Hmgcs1          | –1.60<sup>b</sup> | 0.0238          | 1.93<sup>a</sup> | 0.0020          |          | –1.24          | 0.3368          |
|                 | Hmger           | –2.23<sup>b</sup> | 0.0034          | 1.72           | 0.0740          |          | –1.30          | 0.5900          |
|                 | Mvk             | –1.11            | 0.3513          | 1.51<sup>a</sup> | 0.0086          |          | –1.02          | 0.8580          |
|                 | Pmvk            | –2.30<sup>b</sup> | 0.0004          | 1.52<sup>a</sup> | 0.0068          |          | –2.33          | 0.0033          |
|                 | Mvd             | –3.26<sup>b</sup> | 0.0033          | 1.94<sup>a</sup> | 0.0072          |          | –1.98          | 0.0528          |
|                 | Id1             | –1.63<sup>b</sup> | 0.0212          | 1.99<sup>a</sup> | 0.0017          |          | –1.24          | 0.3330          |
|                 | Fdps            | –2.60<sup>b</sup> | 0.0022          | 1.88<sup>a</sup> | 0.0039          |          | –2.20<sup>b</sup> | 0.0237          |
|                 | Fdf1            | –1.43<sup>b</sup> | 0.0095          | 1.72<sup>a</sup> | 0.0011          |          | –1.10          | 0.6770          |
|                 | Cyp51a1         | –1.75<sup>b</sup> | 0.0058          | 1.81<sup>a</sup> | 0.0010          |          | –1.40          | 0.1238          |
|                 | Dhcr7           | –1.65<sup>b</sup> | 0.0065          | 1.65<sup>a</sup> | 0.0007          |          | –1.54          | 0.1193          |

Selected Srebp1 and Srebp2 pathway genes were analyzed after siRNA treatments. Mice were treated with siRNAs targeting ApoB and Fatp5 alone or in combination (ApoB, Fatp5, Fatp5/ApoB) at days 0 and 14. Gene expression was analyzed using qRT-PCR (TaqMan) on day 21. Fold regulation was calculated for the treatment group relative to the control siRNA group.

<sup>a</sup> Indicates a significant induction (p < 0.05) and <sup>b</sup>a significant reduction (p < 0.05) in expression relative to the control siRNA group. Significance (p value) was calculated using a two-tailed t test between control and treatment groups.
suggest that reductions in FATP5 would disproportionately decrease the saturated and polyunsaturated fatty acid containing triglycerides relative to monounsaturated fatty acid containing triglycerides.

It remains possible that FATP5 may not function as a transporter of fatty acids under our conditions. In the studies reported here, animals were fed a chow diet containing 9% fat ad libitum, whereas Fatp5 knockout mice fed a similar diet (a chow diet containing 5% fat) exhibited the most dramatic changes in hepatic lipid accumulation under prolonged fasting conditions [32]. One could also argue that Fatp5 knockdown may induce the expression of other Fatp family members, which could compensate for the loss of FATP5 activity under our conditions. However, this is unlikely, since Fatp5 knockout mice did not exhibit altered expression of Fatp1-4 or Fatp6 [32], and we did not observe a change in the expression of either of the two Fatps evaluated (Fatp2 or Fatp4, data not shown). Alternatively, both Fatp2 and Fatp5 are highly expressed in liver, and Fatp2 knockdown has also been reported to decrease hepatic fatty acid uptake in mice [33]. Therefore, it remains possible that FATP2 is sufficient to mediate hepatic fatty acid uptake in the absence of FATP5 under our conditions.

Even though FATP5 knockdown failed to influence the hepatic triglyceride pool generated following ApoB knockdown, it did lead to a 100-fold increase in ratio of unconjugated to conjugated bile acids. The magnitude of the shift within the bile acid pool, from predominately conjugated to predominately unconjugated bile acids, is consistent with FATP5 being required for the majority of bile acid reconjugation during enterohepatic recirculation. Our results are inline with the results reported for Fatp5 knockout mice and suggest that Fatp5 siRNA treatment is sufficient to influence FATP5 activity [26]. We expect these changes to have only a minor effect on fat absorption, since, consistent with Hubbard et al. [26], we observed that unconjugated bile acids are found within gall bladder bile. Thus, unconjugated bile acids can be efficiently secreted into bile following Fatp5 knockdown and should therefore be available for fat absorption. Fatp5 knockout mice also exhibited secondary metabolic changes leading to decreased food intake. Although, it is unclear if this is due to altered bile acid metabolism via the inhibition of bile acid reconjugation, altered lipid metabolism via the inhibition of fatty acid uptake, or both. While outside the scope of this work, it remains possible to use siRNAs targeting Fatp5 to address this question, since we observed the inhibition of bile acid reconjugation but not fatty acid uptake under these conditions.

Finally, we have shown that Fatp5 knockdown resulted in a significant increase in genes involved in hepatic cholesterol biosynthesis (Srebp2 pathway) and fatty acid synthesis (Srebp1 pathway). This is consistent with the increase in fatty acid synthase expression and defects in bile acid reconjugation reported for the loss of FATP5 activity [26, 27].

One could, therefore, speculate that changes in bile acid conjugation levels would result in an increase in de novo cholesterol synthesis, requiring more acetyl-CoA, which may promote fatty acid synthesis.

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