Hepatic Over-expression of Murine Abcb11 Increases Hepatobiliary Lipid Secretion and Reduces Hepatic Steatosis

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Summary

*Abcb11* encodes for the liver bile salt export pump (BSEP), which is rate limiting for hepatobiliary bile salt secretion. We employed transthyretin (TTR)-*Abcb11* and BAC-*Abcb11* transgenes to develop mice over-expressing BSEP in the liver. The mice manifest increases in bile flow and biliary secretion of bile salts, phosphatidylcholine, and cholesterol. Hepatic gene expression of cholesterol 7α-hydroxylase and ileal expression of the apical sodium bile salt transporter are markedly reduced, while gene expression of targets of the nuclear bile salt receptor FXR (ileal lipid binding protein, short heterodimer partner 1/SHP1) is increased. Because these changes in gene expression are associated with an increased overall hydrophobicity of the bile salt pool and a four-fold increase of the FXR ligand taurodeoxycholate, they reflect bile salt-mediated regulation of FXR and SHP target genes. Despite the increased biliary secretion of bile salts, fecal bile salt excretion is unchanged, suggestive of an enhanced enterohepatic cycling of bile salts. *Abcb11* transgenic mice fed a lithogenic (high-cholesterol/fat/cholic acid) diet display markedly reduced hepatic steatosis compared to wild-type controls. We conclude that mice over-expressing *Abcb11* display an increase in biliary bile salt secretion and taurodeoxycholate content, which is associated with FXR/SHP-mediated changes in hepatic and ileal gene expression. Because these mice are resistant to hepatic lipid accumulation, regulation of *Abcb11* may be important for the pathogenesis and treatment of steatohepatitis.
**Introduction**

Bile formation and secretion are essential functions of the mammalian liver. The rate-limiting step in hepatobiliary bile salt secretion is the ATP-dependent canalicular secretion of bile salts (1). The \textit{Abcb11} gene encodes for a P-glycoprotein that is responsible for ATP-dependent canalicular bile salt secretion [also termed sister of P-glycoprotein (SPGP) or bile salt export pump (BSEP)] (2-4). Although this protein was demonstrated to represent the major transport protein responsible for hepatobiliary secretion of bile salts (3), its specific roles in regulating the enterohepatic circulation and hepatobiliary lipid metabolism remain poorly understood. Defects in the human \textit{ABCB11} gene are responsible for progressive familial intrahepatic cholestasis type 2 (PFIC2), a chronic cholestatic disorder that leads to liver cirrhosis in early childhood (5). However, a recently described \textit{Abcb11}-null mouse manifests a much less cholestatic phenotype compared to PFIC2 patients (6). Although prior studies have evaluated and characterized the kinetics of ABCB11 utilizing \textit{in vitro} systems or by administering cholestatic stimuli, the effects of increased function on hepatobiliary lipid metabolism and secretion have not been explored.

The hepatobiliary secretion of bile salts is the major stimulus for bile formation. In addition, bile salts transcriptionally regulate many liver-specific genes, including \textit{Cyp7a1}, which encodes for cholesterol 7α-hydroxylase, the rate-limiting enzyme for the “classic” or neutral bile salt synthetic pathway (7,8). \textit{Cyp7a1} expression is down-regulated \textit{in vitro}, when hepatocytes are cultured in the presence of bile salts, or \textit{in vivo}, when the bile salt pool is expanded by bile salt feeding (8). Similarly, expression of the ileal apical sodium bile salt transporter (ASBT) is also transcriptionally regulated by bile salts (9). These effects have been demonstrated by bile salt feeding or infusing animals, and considerable evidence has demonstrated that they occur due to
increases in the bile salt ligands of the nuclear bile salt receptor FXR, in particular chenodeoxycholic acid and deoxycholic acid, as well as their glyco- and tauroconjugates (10). However, exploration of physiologic mechanisms responsible for the regulation of many of these genes has been limited, in part, due to the lack of animal models in which the bile salt pool and content, as well as enterohepatic cycling of bile salts has be manipulate without exogenous bile salt administration or sequestration.

In the current study, we have employed two distinct transgenes in order to develop transgenic mice that functionally over-express Abcb11 in the liver. Enhanced canalicular expression of Abcb11 resulted in a marked increase of both bile flow and biliary lipid secretion. The increased bile salt secretion was not accompanied by increased fecal bile salt excretion, but was associated with increases in SHP expression, with the resultant decreases in expression of Cyp7a1 and Asbt. Unfortunately, when placed on a lithogenic (high-cholesterol/fat/cholic acid) diet for 4 to 8 weeks, mice over-expressing Abcb11 mice in a 129S1/SvImJ background do not manifest a marked difference in gallstone-susceptibility. However, Abcb11 over-expressing mice displayed a profound resistance to the development of hepatic steatosis. These phenotypes will be important in enhancing our understanding of the pathophysiologic role of Abcb11 in regulating gene expression throughout the enterohepatic circulation, with resultant changes in hepatobiliary lipid metabolism and secretion.
**Experimental Procedures**

**Vector constructs and generation of transgenic mice**

*Transthyretin (TTR)-Abcb11 transgenic mice*

A transthyretin (TTR) promoter-*Abcb11* construct was utilized for the development of TTR-*Abcb11* transgenic mice. The construct consists of -3 kB of the TTR promoter region with the first and second exons (including the first intron) fused to the SV40 3' UT and the polyadenylation tail (vector kindly provided by Dr. Robert Costa, University of Illinois, Chicago, IL). The full-length *Abcb11* coding region was inserted at the *StuI* site in the second TTR exon. Previous studies demonstrate that the *Abcb11* cDNA encodes for a functional bile salt transporter (4). Transgenic mice were generated using standard microinjection techniques at the University of Illinois Transgenic Facility (Chicago, IL). Founder lines were screened for the presence of the transgene by performing PCR analysis and Southern blot analysis of genomic DNA extracted from mouse tails using DNeasy Tissue Kits (Qiagen, Hilden, Germany). PCR was performed using primer pairs encoding for the SV40 region with sense (5'-AAAGTCCTGGATGCTGTCCGAG-3') and antisense (5'-CAGACATGATAAGATACATTGATG-3') primers, and Southern blotting was performed using *DraI* digested genomic DNA. The correct orientation of the *Abcb11* gene was also confirmed using PCR primers corresponding to the coding region and the SV40 3' primer and by nucleotide sequencing. Initial genomic screening revealed 5 founder lines containing the transgene construct. All TTR-*Abcb11* transgenic mice were derived in an FVB/NJ background, with all control experiments performed on littermate mice of the same background strain.
Bacterial artificial chromosome (BAC) transgenic mice

Fourteen bacterial artificial chromosomes (BACs) containing the whole Abcb11 gene were identified by screening a genomic BAC library (RPCI-23) from the gallstone-susceptible inbred mouse strain C57BL/6J (BACPAC Resources, Roswell Park Cancer Institute, Buffalo, NY). As DNA probes, we used 5' and 3' fragments of the gene, radioactively labeled with $[\alpha-^{32}\text{P}]$-dCTP (Random Primer Labeling System, Gibco BRL, Gaithersbrug, MD). The hybridization of membranes was carried out at 65 °C overnight in Church buffer (1 M NaHPO$_4$ [pH 7.2], 1 % BSA, 0.5 M EDTA, 20 % SDS). Small amounts of BAC DNA were isolated by alkaline lysis; preparative BAC DNA isolation was carried out using the Nucleobond AX kit (Macherey-Nagel, Düren, Germany). Isolated BAC DNA was digested with NotI and size-fractionated on a 1 % agarose gel by pulsed-field gel electrophoresis (CHEF-DR III system, Biorad, Hercules, CA). For BAC sequencing, 800 - 1,000 ng BAC DNA were incubated for 30 min at 60 °C, denatured at 95 °C for 5 min with 12 µl Big Dye Terminator Reaction Mix (Applera, Norwalk, CT) and 0.5 µM primer, and amplified for 100 cycles as follows: 95 °C / 30 sec, 50 °C / 20 sec, 60 °C / 4 min. Products were purified on Centrisep columns (Emp Biotech, Berlin) and separated by capillary electrophoresis on an ABI 310 automated sequencer (Applera). The smallest BAC clones (RP23-291P1) containing the complete Abcb11 gene was chosen for transgenesis and submitted to the Trans-NIH BAC Sequencing Program (http://www.nih.gov/science/models/bacsequencing/) for full sequencing. The 182-kb insert contains 33 kb of flanking sequence at the 5' end, and 44 kb of flanking sequence at the 3' end of Abcb11, as well as the complete gene encoding the glucose-6-phosphatase catalytic related protein (G6pc-rs) 5' of Abcb11; a gene of unknown function expressed primarily in the pancreas (11).
The purified BAC DNA was dissolved in microinjection buffer (10 mM Tris-HCl [pH 7.5], 0.1 mM EDTA, 30 µM spermine, 70 µM spermidine, 100 mM NaCl) at a concentration of about 2 - 4 ng/µl and microinjected into pronuclei of fertilized eggs from gallstone-resistant strains AKR/J, CBA/J, and 129S1/SvImJ (Mouse Phenome Database, http://www.jax.org/phenome/). Genomic DNA was extracted from tails tips, as described above. Founder mice were screened for transgene integration by PCR amplification using T7 and SP6 primers in combination with oligonucleotides specific for the BAC insert (5'-CTGGTGTCACGGTCCATCTTG-3' and 5'-CCAATTCAGACCAGCTGAGAGC-3', respectively), and the results were confirmed by Southern blotting. Two of 72 offspring from 129S1/SvImJ contained at least one copy of the transgene, with transgene copy number measured by comparing band intensities in Southern blot analysis to those of standard amounts of BAC DNA. Two lines of Abcb11-BAC transgenic mice (F-46, official name 129S1/SvImJ- Tg(RP23-291P1)1f1p and F-60, 129S1/SvImJ-Tg(RP23-291P1)2f1p) were established by mating transgenic founder mice to 129S1/SvImJ inbred mice.

**Mouse husbandry and diets**

All mice were housed in a temperature-controlled room (22 °C) with 12-hour-light/12-hour-dark cycling and fed Purina or Altromin chow, which contains < 0.02% (wt/wt) cholesterol, as determined by HPLC (12). At 6 - 8 weeks of age, the mice were switched to a lithogenic diet containing 1.25 % cholesterol, 0.5 % cholic acid, and 15 % dairy fat (13). Unless otherwise stated, male mice were used for the phenotypic characterization. Protocols were approved by the Institutional Animal Care and Use Committees and euthanasia was consistent with recommendations of the American Veterinary Medical Association.
Biliary lipid analysis

Biliary lipid secretion was determined following gallbladder puncture and collection of bile. PE-10 tubing was inserted into the gallbladder, and the bile aspirate was removed and analyzed as gallbladder bile. For analysis of hepatic bile, the gallbladder was cannulated with PE-10 tubing and secured with a silk suture. The common bile duct was ligated and bile collection was performed gravimetrically. The initial 15 min of biliary secretion that contained concentrated gallbladder bile were not used for analysis, and bile collection continued for up to one hour and was constant over this time period. All procedures were performed using xylazine and ketamine anesthesia. The mice had free access to food and water prior to all experiments and body temperature was maintained within 0.25°C throughout the experiment. The biliary concentrations of cholesterol, phospholipids, and bile salts were measured in gallbladder bile from transgenic and wild-type animals (n = 5) (12,13). Molecular bile salt species were determined by HPLC as described (13). Bile salt hydrophobicity index was calculated according to Heuman (14). Concentrations and molecular species of phosphatidylcholine in bile were quantified by electrospray injection mass spectrometry, using 15:0-15:0 phosphatidylcholine as an internal standard (15).

Bile salt pool sizes were determined following bile salt extraction of liver, small intestine, and gallbladder. Tracer amounts of 3H-taurocholate or glycocholate were used as internal standards. Bile salts were extracted by incubation in 50 ml methanol for 24 hours at 60°C. Following extraction, 10 ml-aliquots were dried down and resuspended in 1 ml methanol. The suspension was centrifuged for 15 min at 14,000 g, and the supernatant was filtered through PVDE acrodisc filters. The filtrate was dried down, resuspended in methanol-water (3:1,v/v), and re-centrifuged at 14,000 g for 15 min. The supernatant was analyzed by both HPLC using
glycocholate as an internal standard, or a spectrophotometric assay employing the 3α-hydroxysteroid dehydrogenase method (Sigma, St. Louis, MO) with 3H-taurocholate as internal standard. Parallel experiments were performed with transgenic and wild-type mice at all times.

Stool fecal collections were performed for 24 hours utilizing metabolic cages to prevent coprophagy. The stool was dried *in vacuo*, pulverized, and 0.2g samples of dry feces were added to 4 ml of t-butanol/H$_2$O (1:1, v/v) and mixed at 37 °C for 15 min. The suspension was centrifuged at 3,000 g for 10 min and the supernatant was assayed enzymatically for total bile salt content as described above.

**Biliary and hepatic phenotypes**

After feeding the lithogenic diet for up to 8 weeks, mice were fasted for 4 hours and anesthetized, and cholecystectomy was performed after euthanasia with isoflurane and ligation of the cystic duct. Gallbladder volumes were determined gravimetrically, assuming a bile density of 1 g/ml. Fresh gallbladder biles were examined by polarizing light microscopy. Liquid crystals, solid crystals and stones were scored semi-quantitatively according to Wang et al. (13). Gallstone composition was determined by infrared spectroscopy (16).

Liver and ileum were excised after euthanasia and either utilized immediately or snap-frozen in liquid nitrogen and stored at -70 °C. Hepatic cholesterol and triglyceride contents were obtained employing spectrophotometric kits according to the manufacturer's instructions (Sigma, St. Louis, MO). Histological analysis was performed using hematoxylin and eosin, and Oil Red O staining.

**RNA expression analysis**
For all expression analyses, tissues were harvested from mice fasted for 4 hours, starting between 8 and 9 am. Northern blotting was performed using liver or ileum RNA from individual mice as previously described (4,17). Briefly, 15 µg of total RNA were isolated using phenol-chloroform extraction (Biotecx, Houston, TX), electrophoresed on a 1% denaturing formaldehyde agarose gel, transferred to a nylon membrane (Dupont NEN, Boston, MA), and hybridized with [α-32P]-dCTP-labeled cDNA probes for Abcb11, Abcb4, Abcg5, Abcg8, Cyp7a1, Cyp27a1, ASBT (Slc10a2), ILBP (Fabp6), actin and ubiquitin at 42 °C. cDNA probes for Abcg5/Abcg8 and Cyp7a1 were kindly provided by Dr. Helen Hobbs and Dr. David Russell, respectively (University of Texas Southwestern Medical Center, Dallas, TX). The other probes were cloned from the corresponding cDNAs after RT-PCR or derived from EST clones with >95% homology to the previously identified genes. After stringent washing, membranes were exposed to either photographic film (Eastman Kodak Co., Rochester, NY) or a phosphor screen, and densitometry was performed using the Fluor S Multiimager CCD camera system and Quantity One software (Biorad).

Quantitative RT-PCR to examine hepatic RNA expression of Abcb11, Cyp7a1, FXR (Nr1h4), SHP (Nr0b2), and the scavenger receptor BI (SR-BI, Scarb1) was performed utilizing the GeneAmp 5700 Sequence Detection System (Applera, Norwalk, CT). Data were normalized using actin primers. Preliminary experiments confirmed that actin RNA was constitutively expressed in the mouse strains under the utilized conditions. Primer sequences were Cyp7a1 for 5'-AGCAACTAAACAACCTGCCAGTACTA-3', rev 5'-GTCCGGATATTCAAGGATGCA-3'; SHP for 5'-GGAGGCCTTGGATGTCCTAG-3', rev 5'-AGCCTCCTGTTGCAGGTGTG-3'; and SR-BI for 5'-TTTCAGCAGGATCCATCTGGTGGA-3', rev 5'-AGTTCATGGGGATCCACGACTGAC-3', for 5'-TCGTCAGATAAGGAAAATGAGGAAA-3',

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Immunoblot analysis

Canalicular liver membranes (cLPM) which were 20-fold enriched in canalicular domain markers were prepared as previously described (4). Protein concentrations were determined using a Bradford Kit (Biorad). Equal amounts of protein were loaded onto 10 % sodium dodecyl sulfate–polyacrylamide gels and subjected to electrophoresis (17). Equal protein loading was confirmed by Coomassie staining of gels and Ponceau S staining of membranes after transfer. After electrotransfer onto nitrocellulose membranes (Biorad), the blots were blocked with Tris-buffered saline containing 0.1 % Tween and 10 % bovine serum albumin for 30 min and incubated for 1 hour at room temperature with a polyclonal antiserum directed against the carboxy-terminus of mouse BSEP (dilution 1:2,500) (4,17). Immune complexes were detected using horseradish-conjugated goat antirabbit or sheep antimouse IgG F(ab')2 fragments (dilution 1:1,000; Dako, Glastrup, Denmark) according to the ECL Western blotting detection system (Amersham, Buckinghamshire, UK). Densitometry was performed using the CCD camera system described above.

Statistical analysis

All data are reported as the mean ± SEM. The differences between the mean values were tested for statistical significance by the two-tailed Student's t test.
**GenBank and Mouse Genome Database accession**

BAC clone RP23-291P1 AC084429. *Abcb11*-BAC transgenic mice MGI:2384356 and MGI:2386568.
Results

Generation of *Abcb11* transgenic mice

Five founder lines of transgenic mice were obtained containing the TTR-*Abcb11* transgene in a FVB/NJ background. The F51 founder line had the highest level of expression and was utilized for the majority of the experiments characterizing the phenotype. In addition, the F21 founder line was analyzed and demonstrated similar phenotype effects, excluding the possibility that phenotypic expression is due to a gene insertional effect. Figure 1A demonstrates the increased expression of the *Abcb11* gene in the livers of the TTR-*Abcb11* transgenic mice. Gene expression is increased three-fold compared to littermate controls.

Transgenic mice containing a bacterial artificial chromosome (BAC) encompassing the *Abcb11* gene under the control of its own regulatory sequences were generated in a 129S1/SvImJ background. Figure 1B displays a Southern Blot analysis demonstrating that both *Abcb11*-BAC transgenic lines F46 and F60 possess one to two additional intact copies of the *Abcb11* gene in comparison to wild-type control, and gene expression is also increased approximately two-fold higher in the transgenic progeny (Fig. 1A).

Western blot analysis was performed using purified canalicular liver membranes (cLPM) to confirm that the transgene results in ABCB11 protein over-expression in the liver. Figure 1C shows that similar to *Abcb11* mRNA levels, hepatic canalicular membrane expression of ABCB11 protein is increased to a comparable degree. These findings indicate that the transgenes over-express and target the ABCB11 protein to the liver canalicular membrane.

All transgenic mice appeared healthy and grossly normal at baseline. They exhibited a normal reproductive rate and gender distribution. In addition, the mice were born in a normal
Mendelian distribution, excluding a lethal developmental abnormality. Both the TTR-Abcb11 and Abcb11-BAC transgenic mice had food consumption identical to their background strain controls. The transgenic mice also did not demonstrate any difference in longevity with observation of one year.

**Expression of bile salt and cholesterol metabolic and transporter genes**

We initially determined the hepatic expression of selected bile salt and cholesterol metabolic and transporter genes. Figure 2A displays that there is a decreased gene expression of hepatic Cyp7a1 in both TTR-Abcb11 and Abcb11-BAC transgenic mice fed chow. Northern blotting with densitometry analysis indicates that Cyp7a1 expression is 35 ± 14 % of wild-type controls. This enzyme initiates, and is likely rate-limiting for the "classic" (a.k.a. neutral) pathway of hepatic bile salt synthesis and for the conversion of cholesterol into bile salts (8). In contrast, there is no diminished expression of Cyp27a1, the gene that encodes a key enzyme of an alternative pathway in bile salt synthesis via the acidic pathway.

Figure 3 describes quantitative RT-PCR that was utilized to further confirm the decreased expression of Cyp7a1 in Abcb11 transgenic mice. These studies also determined that it is associated with a 4-fold increase in hepatic expression of SHP (Fig. 3C); SHP is the nuclear receptor that mediates FXR:RXR transcriptional regulation by hydrophobic bile salts (18,19).

Transgenic and wild-type mice display similar hepatic mRNA levels of Abcb4 (Fig. 2B) and Abcg5/Abcg8 (Fig. 2C) or SR-BI, which encode for membrane proteins reported to be involved in the hepatobiliary secretion of the major biliary phospholipid (phosphatidylcholine) and cholesterol. Figure 2D also illustrates that in wild-type and transgenic mice, a lithogenic (high-cholesterol/fat/cholic acid) diet increases the hepatic expression levels of the Abcg5/Abcg8...
genes 5.5- and 4.4-fold, respectively (p < 0.01). These data are consistent with previous studies demonstrating \textit{Abcg5/Abcg8} induction by cholesterol via the nuclear hormone receptor LXR\(\alpha\) (20,21). The hepatic expression of the SR-BI gene is also increased by lithogenic diet feeding. Figure 3D illustrates that the increase (2.4-fold) of SR-BI expression in \textit{Abcb11} transgenic mice is 60\% greater than the increase of SR-BI expression in wild-type mice (p < 0.01). These data are also consistent with previous data reporting a significant hepatic induction of the SR-BI receptor in response to the lithogenic diet (22). In contrast, the expression levels of \textit{Abcb11} (Fig. 2C) and \textit{Abcb4} are not induced by the lithogenic diet.

In order to confirm the functional consequence of over-expressing \textit{Abcb11}, we assayed bile flow by collecting hepatic bile from 8 - 10 weeks old mice. Table 1 describes the biliary lipid secretion rates in TTR-\textit{Abcb11} transgenic mice. Bile flow and bile salt secretion are increased by 48\% and 85\%, respectively, compared to control mice (p < 0.01). The bile-salt independent component of bile flow is similar in TTR-\textit{Abcb11} transgenic mice and control mice of the identical background strain. These data indicate that the choleretic effect observed in the TTR-\textit{Abcb11} transgenic mice is due to the hypersecretion of bile salts. Biliary phospholipid and cholesterol secretion are also increased by 32\% and 69\% in the TTR-\textit{Abcb11} transgenic mice compared to wild-type controls, respectively (p < 0.01). These data are consistent with coupling of biliary phospholipid and cholesterol secretion to the increase in biliary bile salt secretion.

We next examined the bile salt pool and composition in the transgenic mice. Bile salts were extracted from the gallbladder, liver and small intestine, and the bile salt content was analyzed using two different methods. Figure 4A demonstrates the daily fecal excretion of bile salts from transgenic mice. Despite a significant 47\% increase in the biliary secretion of bile salts, there is no significant alteration in the quantity of bile salts excreted in the feces of the
Abcb11 transgenic compared to controls. Figures 4B and 4C describe the total bile salt pool, employing two distinct analytical methodologies from different cohorts of mice. Both experiments demonstrate that the total bile salt pool is similar in Abcb11 transgenic and wild-type mice. The significantly increased bile salt secretion in the setting of constant pool size and no increase of fecal excretion indicates an increase in the rate of bile salt cycling within the enterohepatic circulation. The bile salt content, however, was significantly more hydrophobic in Abcb11 transgenic mice than in wild-type controls (p < 0.001) (Figure 4D).

Figure 5A demonstrates that bile obtained from transgenic mice had a highly significant four-fold increase in the FXR ligand taurodeoxycholate (TDC), compared to wild-type mice. The less abundant bile salt taurochenodeoxycholate (TCDC) is unchanged. Figure 5B further demonstrates that the total amount of FXR ligands (TDC and TCDC) are increased three-fold in the Abcb11 transgenic mice. The major phosphatidylcholine specie in gallbladder bile (16:0-18:2) is significantly reduced in TTR-Abcb11 mice from 40.4 ± 2.2% to 36.7 ± 1.7% (p < 0.05), while the two other major species (16:0-18:1 and 16:0-20:4) are identical. In addition, there are significant increases in 18:0-18:1 (3.1 ± 1.0 vs 1.8 ± 0.1%), 16:0-22:6 (7.6 ± 0.7 vs. 6.1 ± 0.5%) and a decrease in 16:1-18:2 (1.7 ± 0.3 vs. 2.3 ± 0.1%) in the TTR-Abcb11 compared to wild-type mice.

Figure 6 demonstrates that in the ileum, the expression of the apical sodium bile salt transporter (ASBT) is down regulated in the Abcb11 transgenic mice, whereas mRNA levels of the ileal lipid binding protein (ILBP) are markedly increased. SHP is again markedly increased, consistent with FXR ligand activation via the SHP pathway.

**Cholesterol gallstone formation in Abcb11 transgenic mice**
Abcb11 is one of the candidate genes for the major gallstone gene locus Lith1 (24-26). Abcb11 steady-state mRNA and protein levels have been reported to be up to two times higher in gallstone-susceptible (C57L/J) compared to resistant (AKR/J) inbred strains of mice (27,28). We therefore investigated the influence of increased Abcb11 expression on cholesterol gallstone formation by feeding them a lithogenic diet for 2-8 weeks (29). However, rapid cholesterol precipitation and mucin accumulation in gallbladders from both TTR-Abcb11 transgenic and FVB/NJ wild-type controls precluded further analysis of stone formation and crystallization sequences in this model system. To elucidate the consequences of over-expressing Abcb11 on cholesterol gallstone formation, we therefore employed our second transgenic model that was bred in a different genetic background (129S1/SvImJ). These mice are transgenic for a BAC that contains the whole Abcb11 gene together with its 5'-upstream regulatory elements.

Figure 7 shows that feeding the lithogenic diet for 2 weeks results in the formation of small liquid crystals and cholesterol monohydrate crystals in both Abcb11-BAC transgenic lines and 129S1/SvImJ wild-type controls. After 4 weeks of lithogenic diet, the mice display cholesterol monohydrate (ChM) crystals, sandy stones, and true gallstones. By infrared spectroscopy, all stones are composed principally of cholesterol. There was a trend, although no significant differences between transgenic and wild-type mice with respect to semi-quantitative scores for ChM crystals (1.4 ± 0.6 vs. 1.1 ± 0.6) and sandy stones (0.5 ± 0.3 vs. 0.4 ± 0.3) or prevalence of true gallstones (25% vs. 14%) (Fig. 7B). Furthermore, the phenotypes of transgenic and wild-type mice are similar after 8 weeks of lithogenic diet feeding. Gallbladder volumes do not differ significantly between Abcb11-BAC transgenic and wild-type mice both before (17 ± 2 vs. 20 ± 4 µl) and after lithogenic diet feeding for 8 weeks (21 ± 4 vs. 15 ± 2 µl). These findings...
do not indicate that physiologically increased \textit{Abcb11} expression levels markedly affect cholesterol gallstone phenotypes, at least in the 129S1/SvImJ background used in this study.

\textbf{Reduced hepatic steatosis in \textit{Abcb11} transgenic mice}

When challenged with the lithogenic diet (containing 1.25\% cholesterol, 0.5\% cholic acid and 15\% fat) for 6 weeks, the TTR-\textit{Abcb11} transgenic mice display a profound resistance in hepatic lipid accumulation. Figure 8\textit{A} demonstrates that FVB/NJ control mice have marked hepatic steatosis, consistent with other wild-type strains of inbred mice (Mouse Phenome Database, \url{http://www.jax.org/phenome/}). However, the transgenic mice fed the high-fat diet for 6 weeks have grossly normal appearing livers. Figure 8\textit{B} displays the representative hepatic histology, revealing marked differences in fat accumulation. These histologic findings are confirmed with Oil Red O staining (Fig. 8\textit{C}).

In line with these observations, hepatic cholesterol contents are similar in both strains of mice on chow. However, the cholesterol content is significantly lower in the TTR-\textit{Abcb11} transgenic mice compared to the wild-type controls, when these mice are placed on the lithogenic diet for 6 weeks (p < 0.05) (Fig. 9\textit{A}). Figure 9\textit{B} shows that the hepatic triglyceride contents do not differ between both strains fed chow, although there is a trend towards a lower triglyceride content in the transgenic mice. As is noted with the hepatic cholesterol analysis, the hepatic triglyceride contents are also significantly (p < 0.05) higher in the wild-type compared to the transgenic mice after 6 weeks on the diet.
Discussion

The Abcb11 gene encodes for the liver bile salt export pump (BSEP), which is the major canalicular bile salt transporter (30,31). However, little is known about the in vivo regulation of Abcb11, as well as its mechanisms of regulation in normal physiology and pathophysiologic states. Therefore, we developed transgenic mice that over-express murine Abcb11 and found that it was targeted it to the canalicular domain. Furthermore, to confirm that the phenotypes of these mice was due to Abcb11 over-expression, rather than a gene insertional effects of the transgene, we utilized two distinct transgenes to over-express Abcb11 in the mouse and developed these mice from two genetically distinct background strains.

We detected a significant reduction in gene expression of both hepatic Cyp7a1 and the ileal sodium-dependent bile salt transporter (ASBT or Slc10a2), accompanied by an increased expression of the ileal bile acid binding protein (ILBP or Fabp6). This occurs with the concomitantly enhanced expression of short heterodimer partner (SHP or Nr0b2) expression in both the ileum and liver. Recent data demonstrates the importance of selective bile salts, acting as ligands of the nuclear receptor FXR and via SHP, as transcriptional regulators of these hepatic and ileal genes (9,18,32,33). The bile salt pool of TTR-Abcb11 transgenic mice displayed a significant increase of the hydrophobic bile salt TDC (and total content of TDC and TCDC), and an overall increase in hydrophobicity. TDC has been shown to be an important endogenous ligands for the nuclear receptor FXR (Nr1h4) and to strongly activate transcription of SHP. SHP, in turn, suppresses Cyp7a1 and ASBT transcription (9,18,33). This regulatory cascade fully explains the expression pattern of the FXR responsive genes Cyp7a1, ASBT, ILBP, and SHP, which was observed in the Abcb11 transgenic mice. Although data prior to the discovery of FXR largely focused on the importance of total bile salt pools on the regulation of bile salt-responsive
genes (8), these effects may in fact have been due to increases in the hydrophobic bile salts which act as strong FXR ligands and activators, rather than the bile salt pool size or its hydrophobicity per se.

The enhanced biliary bile salt secretion of Abcb11 transgenic mice, without changes of fecal bile salt excretion or bile salt pool size, suggests the presence of an increased frequency of bile salt recycling in the enterohepatic circulation (1). An increased flux of bile salts across the hepatocyte and ileal mucosa could act in an additive or synergistic manner to facilitate FXR-bile salt ligand binding and transcriptional activation. Nonetheless, the increase in TDC, in and of itself, explains the enhanced regulation of FXR responsive genes. Interestingly, the inbred mouse strain C57L/J mice manifests increased expression of Abcb11, hypersecretion of bile salts, reduced Cyp7a1 activity without reductions in the total bile salt pool, an expanded TDC pool, and repressed bile salt synthesis; thus manifesting alterations of bile salt metabolism that are similar to Abcb11 transgenic mice (12,34-36). We conclude that the increased TDC content (potentially in concert with enhanced enterohepatic cycling of the bile salt pool) in Abcb11 over-expressing mice leads to enhanced ligand-receptor binding and thus transcriptionally regulates the bile-salt responsive genes in the liver and ileum that we have examined.

The majority of hepatobiliary bile salt secretion occurs due to recycling of bile salts via the enterohepatic circulation, rather than by de novo synthesis by the liver. In light of suppressed Cyp7a1 expression found in both transgenic strains of mice, and retention of the total bile salt pool size with unchanged fecal excretion, bile salt synthesis must be compensated by alternative synthetic pathways, including Cyp7b1 and Cyp27. Hepatic Cyp27 expression is preserved in Abcb11 transgenic mice. It is interesting to note that C57L/J mice, which manifest bile salt metabolism similar to those noted in the Abcb11 transgenic mice, have reduced hepatic Cyp7a1
activity without reduced (and actually expanded) total bile salt pool size, relative to AKR/J mice (12, 34). The increased biliary bile salt secretion, without increased bile salt pool size or fecal bile salt output, suggests the presence of an increased enterohepatic recycling frequency of the bile salt pool, yet the ASBT expression is decreased in Abcb11 transgenic mice. However, it remains unclear if ileal bile salt transport mechanisms in rodents operate near maximal capacity. In fact, FXR (-/-) mice exhibit over 2-fold increases in biliary bile salt output and intestinal cholate reabsorption, while ASBT expression remains unchanged and ILBP is markedly reduced (37). This suggests that mice possess a significant excess capacity for intestinal bile salt absorption and that the reduced ASBT expression may not interfere with increased enterohepatic bile salt cycling. FXR-dependent activation of SHP is, however, markedly induced in ileal enterocytes from Abcb11 transgenic mice (9). In fact, the increase in TDC in the Abcb11 mice may be caused by enhanced biliary bile salt secretion combined with the diminished ileal expression of ASBT. This may lead to increased bile salt exposure to colonic bacteria that synthesize TDC, allowing colonic absorption of TDC and its return to the liver via the enterohepatic circulation, where it subsequently can exert an FXR-mediated effect on SHP and Cyp7a1.

As expected from previous studies (1,34), the increased bile salt secretion rates were associated with increased biliary secretion of both cholesterol and phospholipids. In contrast, transgenic mice over-expressing the cholesterol transporter Abcg5/g8 display no significant changes in bile salt secretion compared to controls (20). We did not detect any alterations of the biliary cholesterol transporters Abcg5 and Abcg8, despite an increased biliary cholesterol secretion. Similarly, hepatic expression of the phosphatidylcholine "flippase" Abcb4 and the scavenger receptor SR-B1 (Scarb1), which is another membrane protein reported to be localized
in the canalicular membrane and to promote the excretion of phospholipid and cholesterol into the bile (38,39), were also unchanged. These data suggest that the increased cholesterol and phosphatidylcholine secretion into bile is coupled quantitatively to bile salt secretion in our model, but is not due to transcriptional regulators of hepatic canalicular ABC transporters.

Inbred mouse strains that differ in their genetic susceptibility for cholesterol gallstone formation have been employed in genome-wide scans to identify a major lithogenic locus \((Lith1)\) on mouse chromosome 2 (26). Interestingly, the \(Abcb11\) gene maps to the \(Lith1\) locus (24,25) and given its physiologic function and the physical-chemical mechanisms of cholesterol gallstone formation, has been a candidate gene for \(Lith1\) (40). \(Abcb11\) transgenic mice display a characteristic lithogenic phenotype: including rapidly recycling of bile salts, a more hydrophobic bile salt pool, and enrichment with TDC, the secondary bile salt metabolized from cholate by the colonic flora (41,42). Cholesterol gallstone patients usually display higher biliary TDC concentrations compared with stone-free controls (43), and increased TDC levels promote cholesterol crystallization in model and human biles \textit{in vitro} (13,44). Also of note, a recent genome-wide analysis in inbred mice identified higher hepatic gene expression of both FXR and SHP in mice carrying gallstone susceptibility alleles at these loci (45), as was observed in our transgenic mice. However, in the murine background strain examined, and under the utilized conditions, enhanced gallstone susceptibility was not noted. Nonetheless, genetic or dietary modification of the current model might provide additional insights.

When fed a high fat, lithogenic diet for 6 weeks, the TTR-\(Abcb11\) transgenic mice developed significantly less hepatic steatosis than wild-type controls. Grossly appearing fatty livers are observed in virtually all mouse strains that are placed on this diet for several weeks (46). Although there is some hepatic lipid accumulation in the livers of TTR-\(Abcb11\) mice fed
The lithogenic diet (compared to chow fed mice), hepatic cholesterol and triglyceride levels are significantly reduced compared to wild-type mice. TTR-Abcb11 mice placed on a high-fat diet gain more weight than wild-type controls (data not shown), making fat malabsorption unlikely to account for the difference in the phenotype of hepatic steatosis. Unfortunately, mouse gallbladders were completely occluded with gallstone and mucin after six weeks with the lithogenic diet, and thus it was not technically possible to characterize biliary lipid secretion. Abcb11-BAC transgenic mice in the 129S1/SvImJ strain background fed a lithogenic diet did not have appreciable reductions in hepatic steatosis compared to controls. This may have been due to strain-specific changes in hepatic lipid metabolism or potentially due to differences in the functional expression of Abcb11 in the mice. Abcb11-BAC transgenic mice retain many of the cis-regulatory elements present in the native Abcb11 gene (47) and strain-specific changes in hepatic lipid metabolism (46) likely influence the progression of hepatic steatosis, as expected for a multifactorial, complex trait (48,49).

We have employed two distinct transgenes to develop transgenic mice functionally over-expressing Abcb11 in the mouse liver canalicular membrane. Both strains of mice manifest a phenotype that indicates the presence of an enhanced FXR mediated gene regulation. The mice display significant changes in hepatic Cyp7A1 and ileal ASBT and ILBP gene expression, associated with changes in SHP expression, indicating that bile salt responsive elements in these genes are responding to the increased content of the FXR ligand TDC. In addition, the TTR-Abcb11 transgenic mice show a marked difference in the development of hepatic steatosis, including triglyceride accumulation, in response to the lithogenic diet. Non-alcoholic steatohepatitis (NASH) and non-alcoholic fatty liver disease (NAFLD) represent the most common form of liver disease in the United States (50). Our findings suggest that pharmacologic
(or other) manipulation of Abcb11 function may be a novel therapeutic target for treating this highly prevalent disease.
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Footnotes

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Abbreviations: Abc, ATP-binding cassette transporter gene; ASBT, apical sodium dependent bile salt transporter; BAC, bacterial artificial chromosome; BSEP, bile salt export pump; cLPM, canalicular liver plasma membranes; ChM, cholesterol monohydrate; CSI, cholesterol saturation index; Cyp, cytochrome P450 gene; ILBP, ileal lipid binding protein; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PFIC, progressive familial intrahepatic cholestasis; SHP, short heterodimer partner; TC, taurocholate; T-β-MC, tauro-β-muricholate; TCDC, taurochenodeoxycholate; TDC, taurodeoxycholate; TUDC, tauroursodeoxycholate; TTR, transthyretin.
Figure Legends

Figure 1

A) Northern blot analysis of Abcb11 mRNA expression in wild-type (wt), TTR-Abcb11 and Abcb11-BAC transgenic mice on chow. Northern blot analysis was performed with total liver RNA from individual mice of the indicated genotypes, using a [α-32P]-dCTP-labeled 3' Abcb11 cDNA probe (upper line). Expression levels were normalized, employing an ubiquitin control (lower line). The transgenic lines display a 1.5 to 3-fold increase of Abcb11 expression in comparison to wt controls.

B) Southern blot analysis of HindIII-digested genomic DNA from Abcb11-BAC transgenic mice. For testing of Abcb11 integrity, a 4 KB long [α-32P]-dCTP-labeled cDNA probe that covers the 5' and 3' ends of the cDNA was used (left panel). The band patterns for the transgenic lines F46 and F60 are identical to that produced by the wild-type strain (wt) or the injected BAC (RP23-291P1). Using a shorter 3' probe, we quantitated the number of extra copies of the Abcb11 gene in the transgenic lines (right panel). The signal was compared to standards (1, 3 or 5 copies of BAC RP23-291P1 added to wt DNA) and normalized using ubiquitin by densitometry. Both transgenic lines have at least one extra copy apart from their endogenous Abcb11 alleles.

C) Immunoblot analysis of mouse ABCB11 protein expression in TTR-Abcb11 and Abcb11-BAC transgenic lines compared to wt mice. Western blots of canalicular liver membranes (cLPM) from individual mice were incubated with a polyclonal antiserum directed against the carboxy-terminus of ABCB11 (dilution 1:2,500) (4), and immune complexes were detected using the ECL detection system. Each panel represents an individual experiment including the
three indicated mouse lines. TTR-Abcb11 transgenic mice (lines F4, F21, F44, F51) and Abcb11-BAC transgenic mice (lines F46 and F60) showed marked increases of ABCB11 protein levels.

Figure 2

Northern blot analysis of wild-type (wt), TTR-Abcb11 and Abcb11-BAC transgenic mice. Total liver RNA isolated from individual mice of the indicated genotypes was utilized and expression levels were normalized, employing ubiquitin or actin.

A) The mRNA expression of Cyp7a1, but not Cyp 27, is significantly decreased in TTR-Abcb11 mice compared to wt controls (p < 0.01).

B) The mRNA expression of Cyp7a1, but not Abcb4 is significantly decreased in Abcb11-BAC transgenic mice compared to wt controls (p < 0.01).

C) Abcb11-BAC transgenic and wt mice display similar hepatic Abcg5 and Abcg8 mRNA levels. In contrast to Abcb11 expression, Abcg5 and Abcg8 expression is induced by the lithogenic diet in all mice. Two major mRNAs for both Abcg5 (~2.3 and 3.3 kb) and Abcg8 (~2.6 and 3.7 kb) are detected (arrows), as reported previously [41]. (-) indicates gene expression on chow, (+) indicates expression after a lithogenic diet for 8 weeks. Abbreviations and gene names: Abcb4, ABC transporter B4 (phosphatidylcholine flippase); Abcg5/g8, ABC transporter G5/G8 (heterodimeric cholesterol export pump); Cyp27a1, sterol 27-hydroxylase; Cyp7a1, cholesterol 7α-hydroxylase.

Figure 3

Quantitative RT-PCR of hepatic RNA isolated from wild-type and Abcb11 transgenic mice (n = 5). Total RNA was isolated from the livers and RT-PCR was performed using gene-specific
primers and actin primers. All data is normalized for actin expression. Black boxes represent wild-type mice; gray boxes represent Abcb11 transgenic mice.

A) Abcb11 expression is increased 2.5-fold in Abcb11 transgenic mice compared to wild-type (p < 0.001).

B) Cyp7a1 expression is reduced by 68% in Abcb11 transgenic mice compared to wild-type (p < 0.01).

C) SHP expression is increased 4-fold in Abcb11 transgenic mice compared to wild-type (p < 0.001).

D) Scarb1 (SR-BI) expression is increased 1.6-fold greater in Abcb11 transgenic mice fed the lithogenic diet, compared to wild-type mice. (p < 0.01).

**Figure 4**

Fecal and total salt pool of individual wild-type FVB/NJ (wt) and TTR-Abcb11 transgenic mice, A) Feces were collected over 24 hours, bile salts were extracted and analyzed using the 3α-hydroxysteroid dehydrogenase method. Fecal bile salt excretion was similar between TTR-Abcb11 and wild-type mice.

B). Total bile salt pool was assayed employing HPLC. Bile salt pool sizes (n = 9) are similar between wild-type and TTR-Abcb11 mice.

C) Total bile salt pool was assayed employing the 3α-hydroxysteroid dehydrogenase method. Bile salt pool sizes (n = 5) are similar between wild-type and TTR-Abcb11 mice.
D) Bile salt content was analyzed by HPLC and hydrophobicity was measured using the method of Heumann [15]. The bile salt pool from TTR-Abcb11 mice were significantly more hydrophobic than wild-type controls (p < 0.001). Black boxes represent wild-type mice, gray boxes represent TTR-Abcb11 transgenic mice.

**Figure 5**

Taurochenodeoxycholate and total taurodeoxycholate/taurochenodeoxycholate content in wild-type and TTR-Abcb11 mice. The bile salt pool was extracted after excision of the gallbladder, liver and small intestine and analyzed by HPLC. A) TDC content in TTR-Abcb11 mice is increased 4-fold compare to wild-type mice (p < 0.001) B) The total content of TDC + TCDC is increased 3-fold compare to wild-type mice (p < 0.02). Black boxes represent wild-type mice, gray boxes represent TTR-Abcb11 transgenic mice.

**Figure 6**

Northern blot analysis of wild-type (wt) and TTR-Abcb11 transgenic mice. Total RNA was isolated from the ileum of individual chow fed mice, and radiolabelled cDNA probes corresponding to the apical sodium bile salt transporter (ASBT), the ileal lipid binding protein (ILBT) and the nuclear receptor SHP were utilized for Northern blot analysis. Equal amounts of ileal RNA from wt (odd numbered lanes) or TTR-Abcb11 (even numbered lanes) mice were analyzed. ASBT expression is markedly down regulated in the transgenic mice, whereas ILBP levels are markedly increased.

**Figure 7**
A) Crystal and stone phenotypes observed in gallbladder bile after lithogenic diet feeding. Polarizing light microscopy of cholesterol monohydrate (ChM) crystals (upper panel, magnification 200×), agglomerated ChM crystals and sandy stones (middle panel, magnification 100×), and a true gallstone exhibiting rounded contour and black center from light scattering/absorption (lower panel, magnification 200×).

B) Semi quantitative scores for liquid crystals (range 0 - 4), ChM crystals (range 0 – 4) and sandy stones (range 0 - 2) as well as gallstone prevalence (in %). Data are given for wild-type (wt) and Abcb11-BAC transgenic mice after 4 weeks (gray bars) and 8 weeks (black bars) of lithogenic diet feeding (means ± SEM, n = 4 - 8).

Figure 8

A) Macroscopic appearance of livers after feeding 1.25% cholesterol, 0.5% cholic acid and 15% fat for 6 weeks. FVB/NJ wild-type mice display marked hepatic steatosis, whereas the liver of TTR-Abcb11 transgenic mice appears grossly normal.

B) Representative liver histology after hematoxylin-eosine staining. Wild-type mice but not transgenic mice show macrovesicular steatosis.

C) Liver histology after Oil Red O staining confirms prominent hepatic steatosis in wt but not TTR-Abcb11 transgenic mice.

Figure 9

A) Hepatic cholesterol contents in wild-type (wt) and TTR-Abcb11 transgenic mice on high-fat diet (black bars) and chow (gray bars). Cholesterol concentrations are similar on chow, but are
significantly (*, p < 0.05) reduced in TTR-Abcb11 transgenic mice compared to wild-type controls, when placed on the diet for 6 weeks.

_B) Hepatic triglyceride contents in wild-type (wt) and TTR-Abcb11 transgenic mice on high-fat diet (black bars) and chow (gray bars). There is a trend (p = 0.06) towards lower triglyceride contents in the transgenic mice, and the two lines differ significantly (*, p < 0.05) upon dietary challenge._
Table 1: Bile flow and biliary lipid secretion rates in TTR-Abcb11 transgenic mice and wild-type mice

|                      | TTR-Abcb11    | Wild-type |
|----------------------|--------------|-----------|
| Bile flow (μl/min/kg)| 63 ± 11*     | 48 ± 13   |
| Bile salt secretion (μmol/hr/kg) | 378 ± 66* | 204 ± 8   |
| Phospholipid secretion (μmol/hr/kg) | 51.2 ± 11.4* | 38.8 ± 8.7 |
| Cholesterol secretion (μmol/hr/kg) | 3.66 ± 1.26* | 2.16 ± 0.84 |

Data represent means ± SD (n = 5). *p < 0.01.
Figure 1

A) TTR-Abcb11

|          | wt | TTR-Abcb11 |
|----------|----|------------|
| mBsep    |    |            |
| Ubiquitin|    |            |

B) wt BAC F46 F60

|          | wt BAC F46 F60 +1 +3 +5 |
|----------|--------------------------|
| mBsep    |                          |
| Ubiquitin|                          |

C) F44 F51 wt

|          | F21 F4 wt |
|----------|----------|
| Abcb11   | ABCB11   |
| F46 F60  | ABCB11   |
Figure 2

A)  

|         | wt                  | TTR-Abcb11       |
|---------|---------------------|------------------|
| Cyp7a1  |                     |                  |
| Cyp27a1 |                     |                  |
| Ubiquitin |                   |                  |

B)  

|         | wt                  | Abcb11-BAC       |
|---------|---------------------|------------------|
| Cyp7a1  |                     |                  |
| Abcb4   |                     |                  |
| Actin   |                     |                  |

C)  

|         | wt                  | Abcb11-BAC       |
|---------|---------------------|------------------|
| Abcg5   |                     |                  |
| Abcg8   |                     |                  |
| Abcb11  |                     |                  |
| Ubiquitin |                   |                  |
Figure 3

A) Abcb11 RNA Expression

B) Cyp7a1 RNA Expression

C) SHP RNA Expression

D) Scarb1 RNA Expression
Figure 4

A) TDC (µmoles/100g bw)

B) TDC+TCDC (µmoles/100g bw)
Figure 5

A) Fecal Bile Salts (µmoles/100g bw)

B) Total Bile Salt Pool (µmol/100g bw)

C) Total Bile Salt Pool (µmol/100g bw)

D) Bile Salt Hydrophobicity
Figure 6

|   | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| ASBT |   |   |   |   |   |   |   |   |   |
| ILBP |   |   |   |   |   |   |   |   |   |
| SHP  |   |   |   |   |   |   |   |   |   |
| 28S rRNA |   |   |   |   |   |   |   |   |   |
Figure 7

A) Liquid crystals / score
B) ChM crystals / score
C) Sandy stones / score
D) Gallstone Prevalence / %
Figure 8

A) 

B) 

C)
Figure 9

A) Liver cholesterol (µg chol/mg protein)

B) Hepatic triglyceride (µg trig/mg protein)

- Wild-type
- TTR-Abcb11

* indicates significant difference.
Hepatic over-expression of murine Abcb11 increases hepatobiliary lipid secretion and reduces hepatic steatosis
Anne Figge, Frank Lammert, Beverly Paigen, Anne Henkel, Siegfried Matern, Ron Korstanje, Benjamin L. Schneider, Frank Chen, Erik Stoltenberg, Kathryn Spatz, Farzana Hoda, David E. Cohen and Richard M. Green

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