# Hepatic Uptake of Conjugated Bile Acids Is Mediated by Both Sodium Taurocholate Cotransporting Polypeptide and Organic Anion Transporting Polypeptides and Modulated by Intestinal Sensing of Plasma Bile Acid Levels in Mice

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The Na$^+$-taurocholate cotransporting polypeptide (NTCP/SLC10A1) is believed to be pivotal for hepatic uptake of conjugated bile acids. However, plasma bile acid levels are normal in a subset of NTCP knockout mice and in mice treated with myrcludex B, a specific NTCP inhibitor. Here, we elucidated which transport proteins mediate the hepatic uptake of conjugated bile acids and demonstrated intestinal sensing of elevated bile acid levels in plasma in mice. Mice or healthy volunteers were treated with myrcludex B. Hepatic bile acid uptake kinetics were determined in wild-type (WT), organic anion transporting polypeptide (OATP) knockout mice (lacking *Slco1a/1b* isoforms), and human OATP1B1-transgenic mice. Effects of fibroblast growth factor 19 (FGF19) on hepatic transporter mRNA levels were assessed in rat hepatoma cells and in mice by peptide injection or adeno-associated virus–mediated overexpression. NTCP inhibition using myrcludex B had only moderate effects on bile acid kinetics in WT mice, but completely inhibited active transport of conjugated bile acid species in OATP knockout mice. Cholesterol 7α-hydroxylase *Cyp7a1* expression was strongly down-regulated upon prolonged inhibition of hepatic uptake of conjugated bile acids. *Fgf15* (mouse counterpart of *FGF19*) expression was induced in hypercholanemic OATP and NTCP knockout mice, as well as in myrcludex B–treated cholestatic mice, whereas plasma FGF19 was not induced in humans treated with myrcludex B. *Fgf15/FGF19* expression was induced in polarized human enterocyte-models and mouse organoids by basolateral incubation with a high concentration (1 mM) of conjugated bile acids. *Conclusion*: NTCP and OATPs contribute to hepatic uptake of conjugated bile acids in mice, whereas the predominant uptake in humans is NTCP mediated. Enterocytes sense highly elevated levels of (conjugated) bile acids in the systemic circulation to induce FGF15/19, which modulates hepatic bile acid synthesis and uptake. (HEPATOLOGY 2017;66:1631-1643).

Bile acids are amphipathic molecules that solubilize lipids in the intestinal tract, and are potent systemic signaling hormones, acting through farnesoid X receptor (FXR) and transmembrane G protein-coupled receptor 5 (TGR5) in various organs.[1] Bile acids undergo effective (re)uptake from the portal blood, limiting escape of bile acids to the systemic circulation. The sodium-dependent taurocholate cotransporting polypeptide (*Slc10a1*/NTCP) extracts the majority of conjugated bile acids at the basolateral membrane of the liver.[2] In 2012, NTCP was found to be the main receptor for the hepatitis B virus (HBV).[3] The hepatitis B/D virus (HBV/HDV) entry inhibitor, myrcludex B, interferes with viral entry into the hepatocyte[4,5] and is currently being tested in HBV/HDV clinical trials.[6,7] Notably, myrcludex B is a specific NTCP inhibitor *in vivo*,[8] and administration of myrcludex B in healthy volunteers and mutations in the

*SLC10A1* gene result in increased plasma conjugated bile acid levels (A. Blank, personal communication[9,10]).

The role of NTCP as the major hepatic uptake transporter for conjugated bile acids *in vivo* was further corroborated in NTCP knockout mice.[8] More than half of the adult NTCP knockout mice maintain normal levels of (conjugated) bile acids in plasma, although hepatic bile acid uptake is delayed. NTCP knockout mice that show elevated plasma levels of conjugated bile acids display altered hepatic expression of members of the sodium-independent organic anion transporting polypeptide (*Slco*/OATP) family. OATP1A4 was strongly up-regulated and OATP1A1 was completely down-regulated. It was previously demonstrated that members of the OATP family are essential for efficient hepatic uptake of unconjugated bile acids. Total *Slco1a/1b*-family knockout mice showed a marked conjugated hyperbilirubinemia and elevated unconjugated bile acid levels in blood,[11] and the latter phenotype was also present in *Slco1b2*

knockout mice, suggesting that this isoform is most critical for unconjugated bile acid uptake.[12] The three most abundant hepatic OATP isoforms in rodents are OATP1B2, OATP1A1, and OATP1A4.[13] Here, we investigated whether expression of OATP isoforms is sufficient to maintain hepatic uptake of *conjugated* bile acids in adult mice, even in the absence of functional NTCP. To this end, we used *Slco1a/1b* knockout mice injected with myrcludex B to inhibit NTCP. We also investigated whether functional expression of human OATP1B1 in mice compensates for loss of mouse OATPs and NTCP regarding transport of conjugated bile acids. Finally, while searching for an explanation for the two distinct phenotypical presentations of NTCP-deficient mice, we found that enterocytes can sense highly elevated basolateral levels of bile acids, to induce fibroblast growth factor (FGF) 15/FGF19, which eventually modifies bile acid synthesis as well as the hepatic bile acid uptake machinery in mice.

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# Materials and Methods

Detailed information on materials and methods can be found in the Supporting Information.

## MOUSE STUDIES

Male *Slc10a1* knockout mice (C57Bl6/J background) were housed and bred in the Academic Medical Center, Amsterdam.[8] Control wild-type (WT) mice were purchased from Envigo, Horst, the Netherlands. Male *Slco1a/1b* knockouts mice (lacking *Slco1b2* and *Slco1a1* to *Slco1a5*) and *Slco1a/1b* knockouts reconstituted with liver-specific expression of human OATP1B1 (FVB/N background) and WT littermates were housed and bred in the Netherlands Cancer Institute, Amsterdam[11] or obtained from Taconic, Silkeborg, Denmark. A single dose of myrcludex B (intravenously, 5 μg/g body weight [BW]) was administered in acute taurocholate (TCA) clearance studies to block NTCP-mediated TCA uptake. Male WT mice were subjected to common bile duct ligation (BDL) and cholecystectomy, as described.[14] Treatment with myrcludex B (5-day subcutaneously, 2.5 μg/g BW) was performed in male (OATP1B1-humanized) *Slco1a/1b* knockouts and in BDL mice. To investigate acute regulation of hepatic bile acid uptake transporters, male WT and *Fgf15* knockout mice (3-4 months of age, described in an earlier work[15]) were injected intraperitoneally with recombinant human FGF19 (hFGF19; 1 mg/kg) or saline at 3 AM. Between 8:00 and 9:00 AM, mice were killed by isofluorane overdosage. *Db/db* male mice (11-week-old, #000642, BKS background) were purchased from The Jackson Laboratory (Ben Harbor, ME) and received a single intravenous dose of $3 \times 10^{11}$ vector genomes (v.g.) of adeno-associated virus (AAV) encoding either FGF19 or a control protein (GFP). *Db/db m*ice were euthanized 24 weeks post-AAV administration to examine long-term effects of FGF19. The study design and animal care and handling were approved by the Institutional Animal Care and Use Committee of the University of Amsterdam (Amsterdam, The Netherlands) and the University of Texas Southwestern Medical Center of Dallas (Dallas, TX).

## HUMAN STUDY

The Clinical Research Unit of the Department of Clinical Pharmacology and Pharmacoepidemiology at the Heidelberg University Hospital (EN ISO-standard 9001) conducted a clinical study, which followed the guideline of good clinical practice, the ethical principles expressed in the Declaration of Helsinki, and all legal requirements for clinical studies in Germany. The responsible ethics committee of the Medical Faculty of Heidelberg University (Heidelberg, Germany) and the competent national authority approved the study (BfArM, Germany, EudraCT: 2014-003289-26). Each participant provided written informed consent before any study-related procedure was conducted. Further details are described in Blank et al. (2017; manuscript submitted).

## STATISTICAL ANALYSIS

Data are provided as the mean ± SEM. Differences between groups were analyzed using the Mann-Whitney U test or Kruskal-Wallis test. Statistical significance was considered at $P < 0.05$, and calculations and graphs were generated using GraphPad Prism software (version 6.0; GraphPad Software Inc.).

# Results

## TCA TRANSPORT IN Slco1a/1b KNOCKOUT MICE AFTER MYRCLUDEX B

Despite delayed bile acid uptake, only a subset of adult NTCP knockout mice showed elevated plasma bile acid levels.[8] In these mice, OATP1A1 was completely absent in the liver (Fig. 1A). In contrast, OATP1A4 expression was up-regulated, in particular in the periportal area (Fig. 1B). Expression of both OATP isoforms was unaffected in normocholanemic NTCP knockout mice (data not shown). To investigate the contribution of the OATP isoforms to hepatic uptake of conjugated bile acids, TCA clearance was determined in WT and *Slco1a/1b* knockout mice. Although plasma TCA elimination of WT mice injected with myrcludex B was slightly reduced during the first 5 minutes of clearance compared to vehicle injections, eventually all TCA was effectively cleared, suggesting significant NTCP-independent TCA uptake in mouse liver (Fig. 1C; red line, open dots). Plasma TCA clearance was not reduced in *Slco1a/1b* knockout mice treated with vehicle compared to WT mice. Strikingly, plasma TCA clearance in *Slco1a/1b* knockout mice injected with myrcludex B (Fig. 1C, red line, open squares) was completely abrogated.
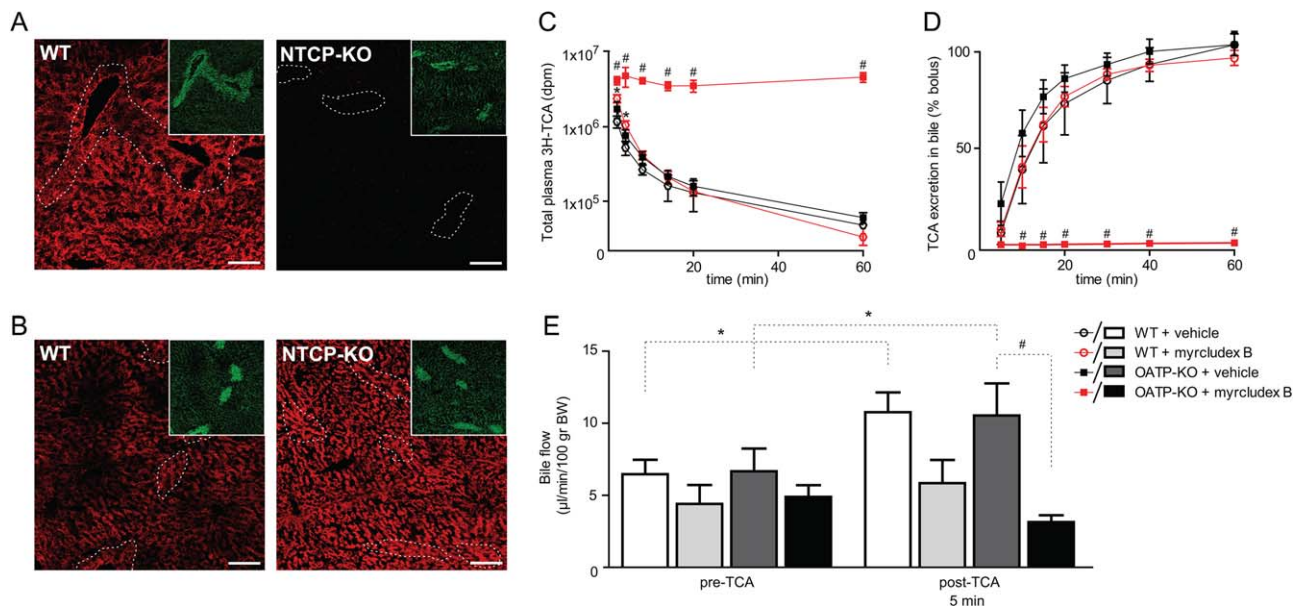
**FIG. 1.** TCA kinetics in WT and *Slco1a/1b* (OATP1A/1B) knockout mice. (A,B) Immunofluorescent visualization of mouse Oatp1a1 (A) or −Oatp1a4 (B) in male WT and *Slc10a1* knockout (NTCP-KO) mice with high plasma bile acid levels. Pericentral hepatocytes were visualized by costaining of glutamine synthetase (green, as insert). Scale bar is 40 $\mu$m. (C) Plasma TCA clearance in WT and OATP1A/1B knockout mice after myrcludex B or vehicle administration. TCA with tracer amounts of [$^3$H]TCA was injected into the tail vein of mice and tritium activity (dpm) was measured in plasma (C) or bile (D) at indicated time points. (E) Hepatic bile flow pre-TCA and post-TCA injection in WT and OATP1A/1B knockout mice injected with myrcludex B or vehicle. (C-E) N = 5/6 mice per group. Asterisk indicates significant difference between vehicle and myrcludex B in WT mice, and hash indicates significant difference between vehicle and myrcludex B in OATP1A/1B knockout mice ($P < 0.05$; Mann-Whitney U test).

Biliary TCA excretion matched these results, because labeled TCA was hardly detectable (<1%) in bile of *Slco1a/1b* knockout mice after myrcludex B treatment, even after 60 minutes. In contrast, 38% and 56% of the labeled TCA was recovered over a 10-minute period in bile of vehicle-injected WT or *Slco1a/1b* knockout mice versus 41% recovery in bile of WT mice injected with myrcludex B (Fig. 1D). This shows that NTCP and OATPs together mediate all hepatic uptake of conjugated bile acid.

To ensure that these effects were not attributed to defective apical bile acid excretion, hepatic bile flow was determined after myrcludex B injections. WT mice showed similar bile flow as *Slco1a/1b* knockout mice, 10 minutes after vehicle injections (Fig. 1E). Myrcludex B treatment did not block bile flow, although there was a tendency to a reduction in both genotypes, even before TCA administration (~30%; not significant). Upon intravenous administration of TCA, bile flow transiently increased in WT and *Slco1a/1b* knockout mice. This increase was less prominent in WT mice after myrcludex B injections, and bile flow did not increase at all upon TCA administration in mice that functionally lack both uptake pathways.

# ELEVATED LEVELS OF CONJUGATED BILE ACIDS IN Slco1a/1b KNOCKOUT MICE INJECTED WITH MYRCLUDEX B

The TCA kinetics data raised the question as to whether myrcludex B causes elevated conjugated bile acid levels in blood of *Slco1a/1b* knockout mice. Measurement of individual plasma bile acid species by high-performance liquid chromatography (HPLC) showed increased conjugated bile acid levels 3 hours after the last myrcludex B injection in *Slco1a/1b* knockout mice, reaching a concentration of ~1 mM and mainly composed of TCA (904.4 ± 108.6 $\mu$M) and tauro-$\beta$-muricholic acid (T$\beta$MCA; 181.6 ± 18.7 $\mu$M; Fig. 2A). Plasma unconjugated bile acids, such as cholate (CA), were modestly elevated as well (Fig. 2B). Similar results were obtained with myrcludex B
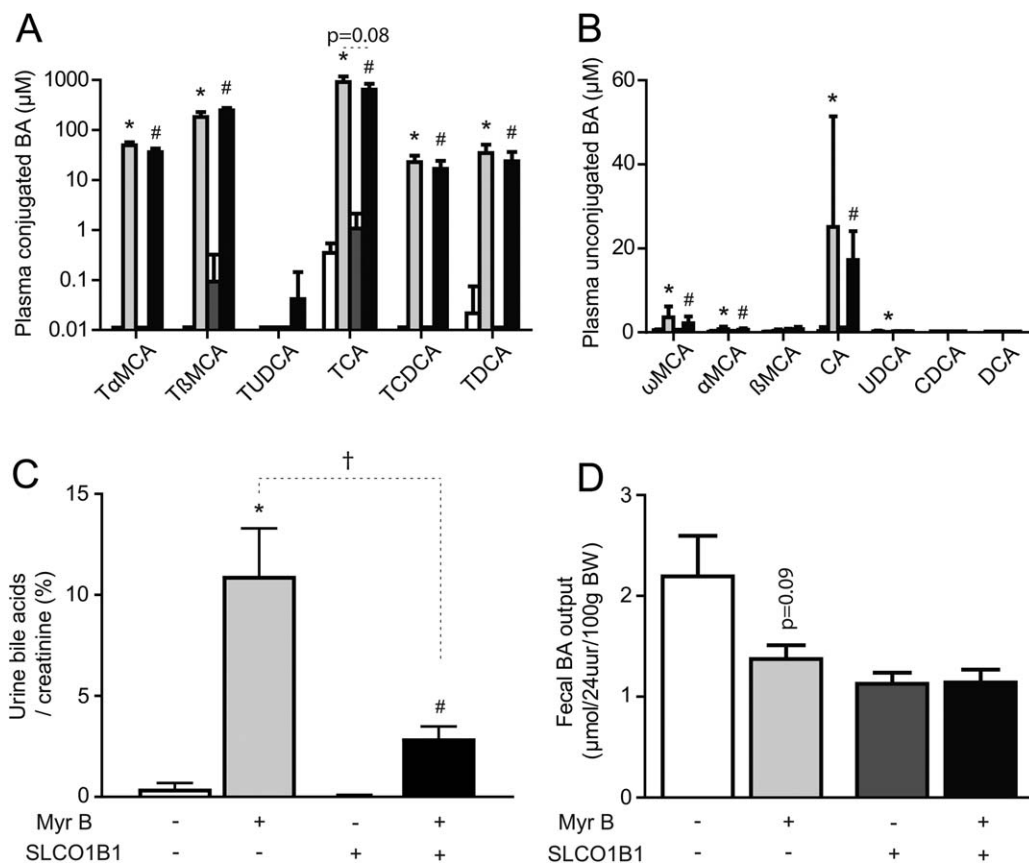
**FIG. 2.** Myrcludex B increases bile acid levels in plasma and urine, but not in feces, of (OATP1B1-humanized) *Slco1a/1b* knockout mice. Myrcludex B or vehicle was injected for 5 days. Plasma composition of (A) conjugated and (B) unconjugated bile acid species as quantified by HPLC. Concentrations ($\mu$M) are given on a $^{10}$log scale for 6 mice/group. (C) Urinary bile acid concentrations corrected for creatinine concentrations in each mouse ($\mu$mol/mmol). (D) Total fecal bile acid excretion ($\mu$mol/24 hours/100 g BW). White and light-gray bars indicate OATP-KO mice, injected with vehicle or myrcludex B, respectively. Dark-gray or black bars indicate OATP1B1-humanized OATP-KO mice, injected with vehicle or mycludex B, respectively. Asterisk indicates significant change for OATP-KO mice, and hash indicates significant change for OATP1B1-humanized OATP-KO mice. Dagger indicates significant change between genotypes upon myrcludex B treatment ($P < 0.05$; Mann-Whitney U test). Abbreviations: BA, bile acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; KO, knockout; MCA, muricholic acid; Myr B, myrcludex B; TDCA, taurodeoxycholic acid; TUDCA, tauroursodeoxycholic acid.

treatment in *Slco1a/1b* knockout mice that express the human OATP1B1 specifically in the liver.[16]

Total plasma bile acid concentrations were virtually normalized in both mouse models 24 hours after myrcludex B injection (Supporting Fig. S1A), indicating only transiently inhibited hepatic bile acid uptake upon daily myrcludex B injection in mice. This is consistent with studies of myrcludex B in humans, rats, and dogs, reporting a rapid distribution to the liver (maximum hepatic binding 4 hours postinjection) and an elimination half-life of ~12 hours.[7,17]

Renal bile acid elimination mirrored plasma bile acid levels, because urinary concentrations in *Slco1a/1b* knockout and OATP1B1-humanized *Slco1a/1b*

knockout mice were low after vehicle injections and massively increased 3 hours after myrcludex B in both genotypes (Fig. 2C). OATP1B1-humanized *Slco1a/1b* knockout mice had reduced renal bile acid excretion compared to *Slco1a/1b* knockout mice. Also, plasma TCA levels showed a trend to decrease in OATP1B1-humanized *Slco1a/1b* knockouts after myrcludex B administration. Functionality of human OATP1B1 was demonstrated by normalization of plasma bilirubin levels, which were elevated in *Slco1a/1b* knockout mice (Supporting Table S2). The renal bile acid transporter, organic solute transporter alpha/beta (*Ostα/β*), was induced by myrcludex B treatment (269% in *Slco1a/1b* knockout and 178% in OATP1B1-humanized *Slco1a/*
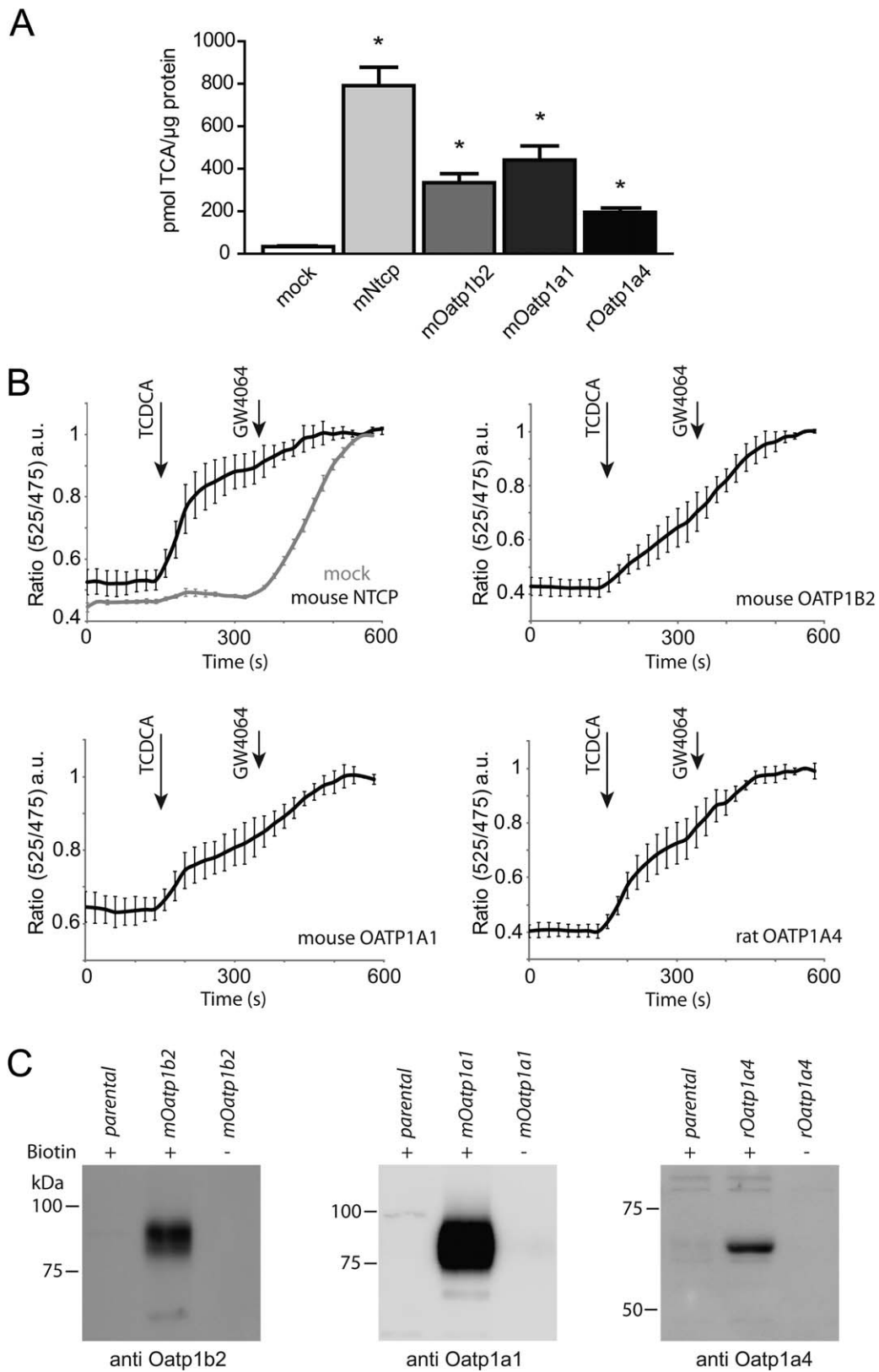
FIG. 3

*1b* knockout mice; Supporting Fig. S1B). The same figure shows that expression of apical sodium-dependent bile acid transporter (*Asbt*), multidrug resistance-associated protein 2 (*Mrp2*), and multidrug resistance-associated protein 4 (*Mrp4*) was unaffected by myrcludex B. Fecal bile acid excretion was not significantly affected in *Slco1a/1b* knockouts upon myrcludex B treatment (Fig. 2D). In both *Slco1a/1b* models, we observed no signs of hepatobiliary damage after myrcludex B treatment, because plasma aspartate aminotransferase/alanine aminotransferase and alkaline phosphatase were comparable and not elevated in any group (Supporting Table S2). Conjugated bilirubin levels were higher in *Slco1a/1b* knockout mice compared to OATP1B1-humanized *Slco1a/1b* knockout mice, in line with OATP-mediated bilirubin transport,[18] and myrcludex B treatment did not affect plasma levels of (conjugated) bilirubin in either genotype (Supporting Table S2). Histological examination of hematoxylin and eosin–stained liver sections showed no abnormalities in (OATP1B1-humanized) *Slco1a/1b* knockouts after 5-day myrcludex B administration (Supporting Fig. S2).

## THREE MURINE HEPATIC OATP ISOFORMS TRANSPORT CONJUGATED BILE ACIDS *IN VITRO*

Bile acid uptake experiments were performed *in vitro* to investigate whether the three liver-specific OATPs can mediate conjugated bile acid transport. All three OATP isoforms were capable of significant TCA uptake compared to untransfected U2OS cells (mock; Fig. 3A). In line with a previous study of Leuthold et al.,[19] mouse OATP1A1 had the highest conjugated TCA uptake velocity (after NTCP, the positive control of this experiment). OATP-mediated uptake of conjugated bile acids was confirmed using the Förster resonance energy transfer (FRET)-based bile acid sensor we previously developed to assess real-time intracellular bile acid uptake dynamics.[20] FRET

ratio increased upon addition of taurochenodeoxycholic acid (TCDCA) when OATP1B2, OATP1A1, OATP1A4, or NTCP were transiently expressed in U2OS cells, whereas mock-transfected U2OS cells did not show bile acid uptake (Fig. 3B). Expression of OATP1B2, OATP1A1, and OATP1A4 at the plasma membrane was confirmed by cell-surface biotinylation and western blotting analysis (Fig. 3C).

## OATP1A1 DOWN-REGULATION IS NOT ATTRIBUTED TO INFLAMMATION

We aimed to uncover the mechanism underlying the reduced OATP1A1 expression, associated with strongly elevated levels of conjugated bile acids in NTCP knockout mice. Inflammation has previously been described to down-regulate *Oatp* expression in a cytokine-mediated manner.[21] We could indeed confirm that inflammation significantly reduces hepatic expression of *Oatp1a1* in a sterile inflammation model (T-cell transfer; Supporting Fig. S3A). However, cytokine levels in the blood of *Slco1a/1b* and *Slc10a1* knockout mice were below detection levels (Supporting Fig. S3B), even when bile acid levels were highly elevated. Given that histology also did not show any hepatic recruitment of leukocytes (Supporting Fig. S2), this suggests that inflammation does not underlie the observed changes in *Oatp* expression. Fortunately, analysis of *Cyp7a1* transcriptional dynamics pointed us to an intestine-derived signal that may modulate expression of hepatic bile acid transporters, including OATPs.

## CHOLESTEROL 7α-HYDROXYLASE DOWN-REGULATION POINTS TO INDUCTION OF ILEAL FGF15 WHEN BILE ACIDS LEVELS ARE ELEVATED

Cholesterol 7α-hydroxylase (CYP7A1) mediates the first and rate-limiting step in bile acid production. We

**FIG. 3.** OATP isoforms mediate uptake of conjugated bile acids *in vitro*. (A) [³H]TCA uptake in U2OS transiently overexpressing mouse *Ntcp*, *Oatp1b2*, *Oatp1a1*, and rat *Oatp1a4*. N = 6 wells/group from two independent experiments. NTCP- and OATP-mediated transport was compared to parental cells (mock), and asterisk indicates significant difference (*P* < 0.05). (B) FRET-based sensing of NTCP- and OATP-mediated TCDCA influx. The nuclear FRET sensor for bile acids (NucleoBAS) was cotransfected with empty plasmid (mock) or each individual bile acid uptake transporter in U2OS cells. The emission ratio of citrin (525 nm)/cerulean (475 nm) is plotted in time. Compound addition is indicated by arrows. To fully activate NucleoBAS, each experiment ended with addition of 5 μM of GW4064. N = 6 cells/group, from two independent experiments. (C) Evident plasma membrane expression of mouse OATP1B2, OATP1A1, and rat OATP1A4 was demonstrated by cell surface biotinylation. (+) indicates with biotin. (–) indicates without biotin.

first determined hepatic *Cyp7a1* mRNA expression in mice that received myrcludex B once 1.5 hours before sacrifice. *Cyp7a1* increased in *Slco1a/1b* knockout mice, but not in WT mice (Fig. 4A). This matches the



previously described CYP7A1 induction in human liver chimeric mice upon myrcludex B treatment.[22] Remarkably, and in contrast to this acute upregulation, 5-day myrcludex B treatment caused significant down-regulation of *Cyp7a1* mRNA levels, in both *Slco1a/1b* knockout mice and OATP1B1-humanized *Slco1a/1b* knockout mice (Fig. 4B). Similarly, reduced bile acid synthesis was functionally demonstrated in NTCP knockout mice with elevated bile acid levels, because 7α-hydroxy-4-cholesten-3-one (C4) levels were significantly lower in plasma (Fig. 4C). Down-regulation of *Cyp7a1* could indicate increased intestinal FXR-FGF15 signaling.[23,24] Several ileal FXR target genes were tested to see whether intestinal FXR is activated by 5-day myrcludex B treatment in *Slco1a/1b* knockout mice (Fig. 5A). Both small heterodimer partner (*Shp*) and *Fgf15* mRNA strongly increased in *Slco1a/1b* knockout mice and in OATP1B1-humanized *Slco1a/1b* knockout mice after myrcludex B treatment. Ileal bile acid binding protein (*Ibabp*) mRNA expression moderately increased, but only in total *Slco1a/1b* knockout mice. To confirm induction of the ileal FXR-FGF15 axis when bile acid levels are elevated in the systemic circulation, we also analyzed ileal mRNA expression in WT and NTCP knockout mice with high plasma bile acid levels (Fig. 5B). NTCP knockout mice display clearly increased *Shp* and *Fgf15* mRNA levels.
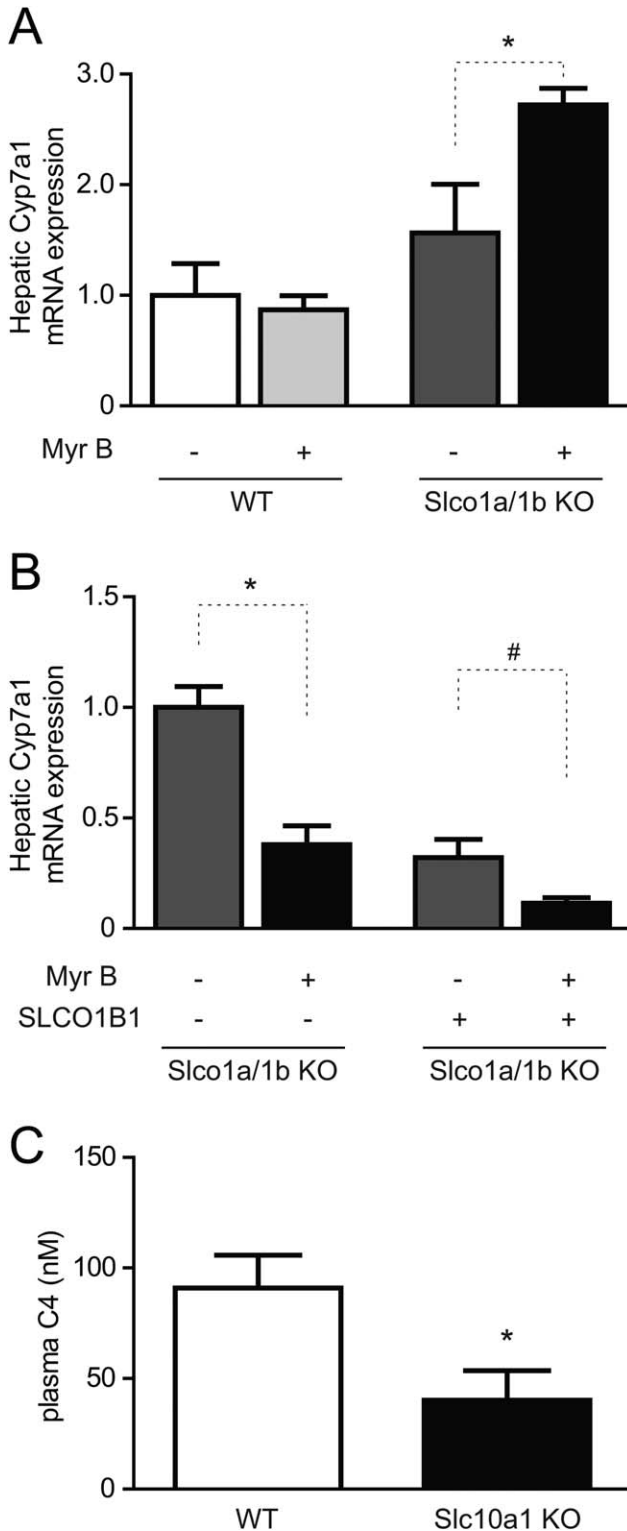
## ENTEROCYTES SENSE BASOLATERAL ELEVATIONS IN BILE ACID LEVELS TO INDUCE Fgf15/FGF19

To verify whether high concentrations of conjugated bile acids in plasma activate intestinal FXR-FGF15

FIG. 4. Cyp7a1 expression after acute or prolonged myrcludex B treatment. (A) Hepatic mRNA expression of the rate-limiting bile acid synthesis enzyme, *Cyp7a1*, shortly (1.5 hours) after vehicle or myrcludex B treatment in WT or *Slco1a/1b* knockout (KO) mice. Values are normalized to vehicle-treated WT mice. Asterisk indicates significant increase ($P < 0.05$) between vehicle and myrcludex B injection in *Slco1a/1b* knockout mice. (B) Hepatic *Cyp7a1* mRNA expression after 5 daily injections with vehicle or myrcludex B in (OATP1B1-humanized) *Slco1a/1b* knockout mice. Asterisk or hash symbols indicate significant decrease ($P < 0.05$; 5-6 fasted male mice/group) compared to vehicle controls. Data are calculated using the geometric mean of reference genes *Rplp0* and *Tbp* and normalized to vehicle-treated *Slco1a/1b* knockout mice. (C) C4 levels, a marker for bile acid synthesis, were reduced in plasma of *Slc10a1* knockout compared to WT mice. Data from 5-6 fasted female mice/group. Abbreviations: KO, knockout; Myr B, myrcludex B.
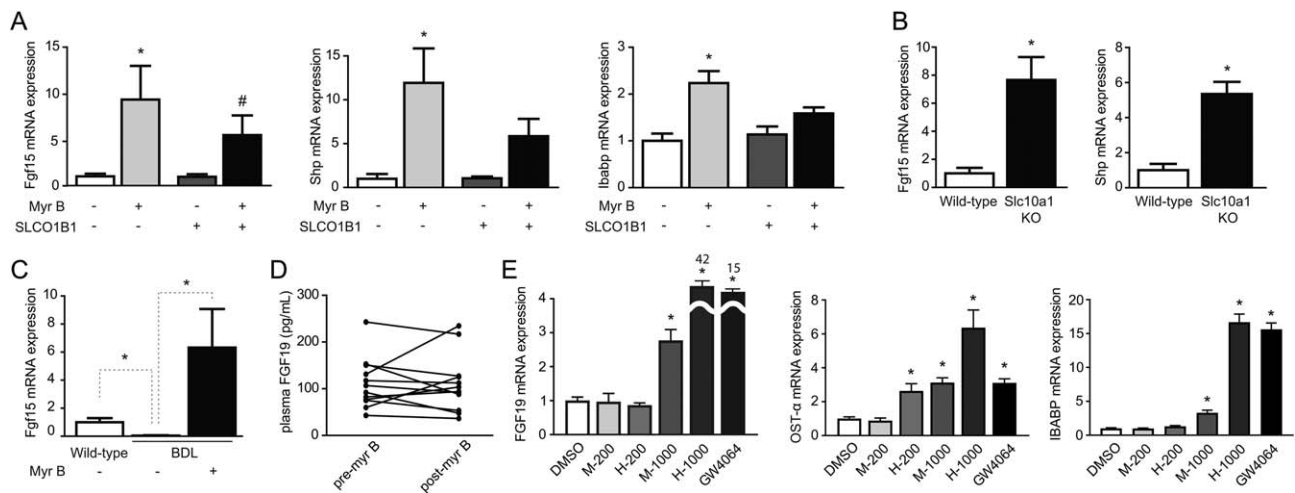
**FIG. 5.** Millimolar concentrations of conjugated bile acids, present in blood after myrcludex B treatment, induce intestinal FXR-FGF15/19 signaling. (A) Relative mRNA expression of FXR target genes *Shp* and *Fgf15* in the distal ileum in (OATP1B1-humanized) *Slco1a/1b* knockout mice after 5-day injections with vehicle or myrcludex B. (B) Relative mRNA expression of *Shp* and *Fgf15* in the distal ileum for WT and *Slc10a1* knockout mice with high plasma bile acid levels. Data are calculated using the geometric mean of reference genes *Hprt* and *Ppib* and normalized to vehicle-treated *Slco1a/1b* KO mice or *Slc10a1* KO mice. Asterisk and hash symbols indicate significant changes ($P < 0.05$; 6 fasted male mice/group). (C) Relative *Fgf15* mRNA expression in the distal ileum after 5-day BDL (5-6 fasted male mice/group), normalized to vehicle-treated WT mice. (D) Effects of myrcludex B on human plasma FGF19 levels. Blood samples were drawn at 9:45 AM (fasting), immediately before myrcludex B administration. After 3 hours, a consecutive blood sample was taken. FGF19 levels were measured by enzyme-linked immunosorbent assay in 12 individuals. (E) Mouse and human conjugated bile acids (200 $\mu$M and 1 mM) were added to the basolateral compartment of filter-grown T84 cells. mRNA expression of FXR target genes *FGF19*, *OST$\alpha$*, and *IBABP* was determined after 24-hour incubation. GW4064 (5 $\mu$M) was used as a positive control. Data are calculated using the geometric mean of reference genes *Ppib* and *Hprt* and normalized to the vehicle-treated condition. Asterisk indicates significant change ($P < 0.05$; 5-6 replicates/group). Abbreviations: DMSO, dimethyl sulfoxide; KO, knockout; Myr B, myrcludex B.

from the basolateral side of the enterocyte, mice underwent 5-day BDL. In this model, bile acids are essentially absent from the intestinal lumen. Plasma bile acid concentrations were elevated in BDL mice, and this further increased (reaching 3-4 mM) upon daily myrcludex B treatment. Plasma bile acids mostly consist of TCA ($\sim$78%) during myrcludex B treatment, whereas plasma of vehicle-treated BDL mice is mainly the more hydrophilic T$\beta$MCA ($\sim$72%; Supporting Fig. S4A). Ileal *Fgf15* mRNA expression significantly decreased in vehicle-treated BDL mice compared to WTs. However, myrcludex B treatment in BDL mice strongly induced *Fgf15* expression (Fig. 5C), supporting the notion that bile acids can enter enterocytes from the basolateral side at highly elevated concentrations ($\geq$1 mM). Subsequently, we determined systemic FGF19 release in 12 healthy human volunteers with elevated bile acid levels in blood after myrcludex B treatment. All individuals displayed an increase of bile acid levels in blood 3 hours after myrcludex B injection (range 70-550 $\mu$M; Supporting Fig. S4B). Plasma

FGF19 levels did not increase after myrcludex B treatment (Fig. 5D). Additionally, we determined plasma FGF19 kinetics in 4 individuals and showed that diurnal variation of plasma FGF19 was not affected by myrcludex B treatment (Supporting Fig. S4C).

Murine bile acids (TCA > tauromuricholic acid > TCDCA) or human bile acids (glycocholic acid [GCA] = glycochenodeoxycholic acid [GCDCA] >> TCA) were added to the basolateral compartment of polarized cells in increasing concentrations, to investigate the discrepancy in FGF15/19 induction between mice and humans upon myrcludex B treatment. Of note, bile acid concentrations in myrcludex B–treated mice are substantially higher than in myrcludex B–treated humans (1,000 vs. 200 $\mu$M). One-millimolar mouse bile acid mixture induced significantly more *FGF19* compared to 200 $\mu$M of human bile acids in filter-grown T84 cells (Fig. 5E). Human conjugated bile acid species are slightly more effective than murine ones in inducing *FGF19* at 1,000 $\mu$M. *OST$\alpha$* and *IBABP* mRNA responded in similar fashion as
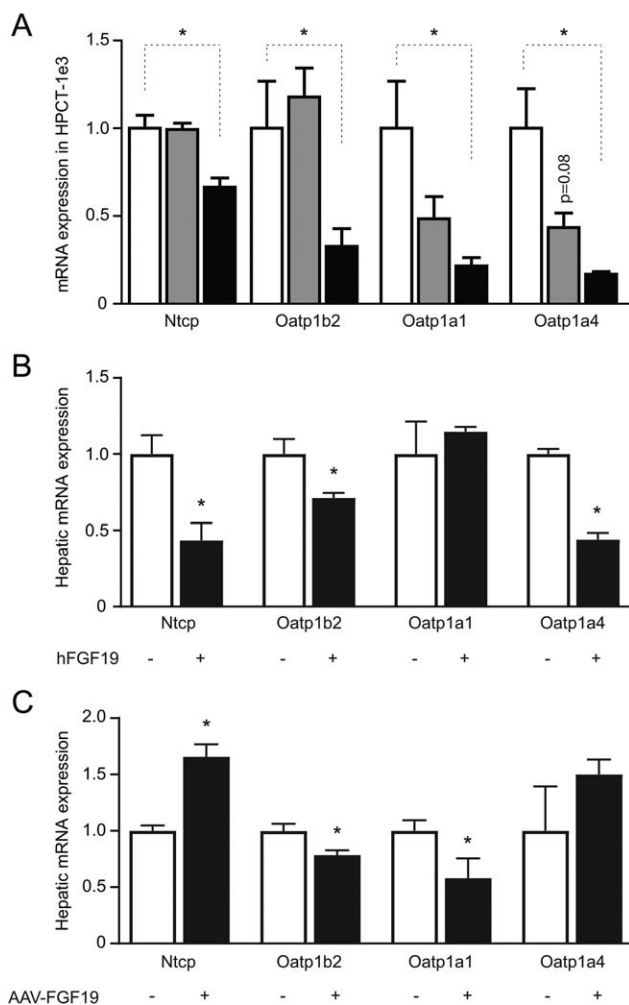
**FIG. 6.** FGF19 modulates the hepatic bile acid uptake machinery in rat hepatocytes (HPCT-1e3) and mice upon peptide injection or AAV-mediated overexpression. (A) Effects of TCA (100 $\mu$M) or hFGF19 (40 ng/mL) on *Ntcp, Oatp1b2, Oatp1a1,* and *Oatp1a4* expression in rat hepatoma cells (HPCT-1e3) cells was determined by qRT-PCR using the geometric mean of *Rplp0* and *Tbp* as a reference. Data are normalized to vehicle (6 replicates/group). Asterisk indicates significant change ($P < 0.05$). (B) Relative hepatic mRNA expression of *Ntcp* and *Oatp* isoforms was determined 6 hours (acutely) after a single injection of hFGF19 (1 mg/kg intraperitoneally) or vehicle in male WT mice, using the housekeeping gene *Rplp0* as a reference. Data are normalized to vehicle-treated WT mice. Asterisk symbols indicate significant ($P < 0.05$; 5 fasted male mice/group). (C) Relative hepatic mRNA expression of *Ntcp* and *Oatp* isoforms after 24 weeks of AAV-induced (3 $\times$ $10^{11}$ v.g.) FGF19 overexpression in *db/db* male mice, using the housekeeping gene, *Rplp0,* as a reference. Data are normalized to AAV-GFP treatment. Asterisk indicates significant change ($P < 0.05$; 8-10 fasted male mice/group).

FGF19, in line with potent FXR activation by conjugated bile acids. Cell viability and epithelial integrity were not affected by this treatment

(Supporting Fig. S5A). A similar induction of *Fgf15* mRNA was demonstrated in mouse organoids upon basolateral exposure to millimolar levels of conjugated bile acid, with preserved organoid morphology (Supporting Fig. S5B,C).

## FGF19-TREATMENT MODULATES HEPATIC BILE ACID UPTAKE TRANSPORTERS

We subsequently tested whether FGF15/19 induction affects the hepatic bile acid uptake machinery. *Ntcp* and *Oatp1b2, Oatp1a1,* and *Oatp1a4* are all significantly down-regulated by FGF19 in a rat hepatoma cell line (HPCT-1e3), which expressed *Fgfr4* and *β-klotho,* but not *Fgf15* mRNA (Fig. 6A). High concentrations of the bile acid, TCA (100 $\mu$M), did not significantly affect mRNA expression of *Ntcp* or *Oatp* isoforms, and similar lack of effect was found upon incubation with CA (data not shown). Subsequently, we investigated the regulation of *Ntcp* and *Oatp* 6 hours after administration of recombinant hFGF19 in mice. hFGF19 injections down-regulated *Ntcp* mRNA by ~50% in WT mice (Figure 6B). Furthermore, *Oatp1b2* and *Oatp1a4* were down-regulated by FGF19 as well to a similar extent, indicating that acute FGF19-mediated repression of hepatic bile acid uptake transporters also occurs *in vivo.* Similar results were obtained in FGF15 knockout mice (Supporting Fig. S6A). Finally, we investigated the effects of continuous FGF19 exposure on hepatic bile acid uptake transporters *in vivo.* FGF19 overexpression caused significant down-regulation of *Oatp1a1* and *Oatp1b2,* whereas *Oatp1a4* showed a trend to increase (Fig. 6C). In diet-induced obese mice, *Oatp1a4* was significantly induced (Supporting Fig. S6B). *Ntcp* expression was up-regulated in long-term FGF19 overexpression mouse models.

## Discussion

This study provides evidence that OATP1A/1B isoforms control hepatic conjugated bile acid uptake during (transient) NTCP deficiency in mice. Myrcludex B, the first specific NTCP inhibitor[25] currently used in human HBV/HDV studies, totally blocks plasma TCA clearance and increases plasma conjugated bile acids in *Slco1a/1b* knockout, but not in WT mice. Therefore, the first major finding is that the activity of OATP isoforms establishes systemic bile acid clearance

during NTCP deficiency, which explains the physiological systemic bile acid levels in many NTCP-deficient mice.

Furthermore, our findings indicate that microsomal epoxide hydrolase 1[26] does not mediate conjugated bile acid transport, as supported by a previous study.[27] The strongly reduced OATP1A1 expression in NTCP knockout mice appears a relevant causative factor for hypercholanemia. This isoform is not present in humans, which might contribute to the differential phenotype between humans and mice upon myrcludex B treatment. Similarly, we can refute other OATP proteins (e.g., OATP2B1) as hepatic transporters for conjugated bile acids in mice. Mice lacking only *Slco1b2* or the total *Slco1a/1b* family display reduced uptake of unconjugated bile acids, and 10- to 13-fold elevated levels of unconjugated bile acids in plasma,[11,12] whereas conjugated bile acid concentrations remain mostly unchanged. Systemic conjugated bile acid concentrations in the *Slco1a/1b* knockout mouse model treated with myrcludex B evidently increase, to an even higher level than in humans treated with myrcludex B.[6,7,9] Humanizing the *Slco1a/1b* knockout mouse with OATP1B1 (one of the human isoforms) still results in high plasma-conjugated bile acid levels, whereas plasma bilirubin levels normalize. These mice do show reduced urinary bile acid excretion upon myrcludex B treatment. Nevertheless, human OATP1B1 alone is unable to effectively clear circulating conjugated bile acids. Our study indicates that NTCP dominates hepatic uptake of conjugated bile acids in humans, whereas the activity of the OATP1A/1B family members can functionally complement NTCP in mice. However, loss of function of NTCP in mice results in an unstable bile acid balance that can easily tip toward hypercholanemia. Using mathematical simulation of bile acid transport, Cravetto et al. have demonstrated that the plasma bile acid concentration is very sensitive to isolated defects of hepatic bile acid uptake, but only at largely reduced fractional hepatic extraction ($>70\%$ reduced uptake rate).[28] We have previously shown such a reduction in NTCP-deficient normocholanemic mice, which need 3.5 times more time to clear a fixed amount of conjugated cholate.[8]

In the quest for cellular regulators of the hepatic bile acid uptake machinery, systemic inflammation was proven absent in *Slc10a1* and *Slco1a/1b* knockout mice (undetectable cytokine levels, normal liver histology) and is therefore unlikely to regulate hepatic transporter expression. Remarkably, hepatic CYP7A1 levels,

regulating the size of the bile acid pool,[29] acutely increased after myrcludex B treatment in *Slco1a/1b* knockout mice. Immediate sensing of decreased hepatic bile acid uptake seems to occur, and this may increase bile acid synthesis. Such a process is in line with the study of Oehler et al.,[22] describing increased CYP7A1 upon HBV/myrcludex B binding to NTCP in humanized mouse livers. No clear bile acid concentration differences among the different groups were found in this study. Here, we show that elevated levels of conjugated bile acids in blood (as observed in *Slc10a1* and myrcludex B–treated *Slco1a/1b* knockout or BDL mice) reduce *Cyp7a1* expression. This is likely explained by activation of the intestinal FXR-FGF15/19-hepatic FGFR4/$\beta$-Klotho axis.[23,24] Previously, Tomiyama et al. demonstrated acute, Klotho-dependent down-regulation of *Oatp1a1* upon FGF19 injection,[30] in line with our *in vitro* results (Fig. 6A). However, *in vivo*, we detected *Oatp1a1* down-regulation (and *Oatp1a4* up-regulation) only upon prolonged FGF19 treatment, not following single FGF19 injections. Therefore, we cannot conclude whether the effects of FGF15/19 on expression of hepatic bile acid uptake transporters is direct or a consequence of FGF15/19 effects on bile acid pool size or composition.[23]

For intestinal FXR-FGF15/19 signaling to occur, we hypothesize that OST$\alpha$/$\beta$ mediates uptake of conjugated bile acid at the basolateral side of enterocytes. In physiological situations, the heterodimer, OST$\alpha$/$\beta$, is a bile acid export transporter in the small intestine.[31] Initial studies described OST$\alpha$/$\beta$ as a bile acid transporter that could mediate both efflux and uptake,[32] and we could confirm this using the FRET sensor for bile acids.[20] Our data provide mechanistic evidence for *Fgf15/19* induction by systemic bile acids, shown by selective basolateral exposure of filter-grown T84 cells and mouse organoids to (millimolar levels of) conjugated bile acids. Also, BDL-mice treated with myrcludex B show highly increased ($\sim$3-4 mM) plasma bile acid concentrations (mostly TCA) and *Fgf15*, whereas vehicle-injected BDL mice predominantly display T$\beta$MCA, a weak FXR agonist (or even antagonist),[33] at around 300 $\mu$M. Low FGF15 levels in vehicle-injected BDL mice could also be attributed to up-regulation of cytosolic bile acids buffers or extrusion mechanisms pumping out bile acids sufficiently given that Ibabp and Mrp2/3 are increased upon BDL in mice.[34] Plasma FGF19 levels were unaltered after myrcludex B treatment in (healthy) humans, suggesting that (local) intestinal bile acid levels are

insufficient to induce FGF signaling, whereas this threshold is reached in mice. Notably, the first described NTCP-deficient (p.R252H) individual displayed highly elevated plasma bile acid levels, accompanied by plasma FGF19 levels close to the upper limit of normal.[10] Plasma C4 levels, a quantitative indicator of bile acid synthesis, were below the lower limit of normal in this patient (12.8 nmol/L; normal, 18-134).[35]

In summary, we conclude that (1) NTCP and OATP isoforms cooperatively form a stable bile acid uptake machinery in mice, whereas NTCP is predominant in humans, and (2) intestinal sensing of highly elevated levels of conjugated bile acids in blood promotes FGF15/FGF19 signaling, reducing hepatic bile acid synthesis and modulating bile acid transporters.

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# Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.29251/suppinfo.