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Ost alpha-Ost beta: A key membrane transporter of bile acids and conjugated steroids

Nazzareno Ballatori[1], **Na Li**[1], **Fang Fang**[1], **James L. Boyer**[2], **Whitney V. Christian**[1], and **Christine L. Hammond**[1]

[1] Department of Environmental Medicine, University of Rochester School of Medicine, Rochester, NY 14642

[2] Department of Medicine and Liver Center, Yale University School of Medicine, New Haven, CT 06520

Abstract

The organic solute and steroid transporter, Ost alpha-Ost beta, is an unusual heteromeric carrier that appears to play a central role in the transport of bile acids, conjugated steroids, and structurally-related molecules across the basolateral membrane of many epithelial cells. The transporter's substrate specificity, transport mechanism, tissue distribution, subcellular localization, transcriptional regulation, as well as the phenotype of the recently characterized *Ost alpha*-deficient mice all strongly support this model. Ost alpha-Ost beta is composed of a predicted 340-amino acid, 7-transmembrane (TM) domain protein (Ost alpha) and a putative 128-amino acid, single-TM domain polypeptide (Ost beta). Heterodimerization of the two subunits increases the stability of the individual proteins, facilitates their post-translational modifications, and is required for delivery of the functional transport complex to the plasma membrane. Ost alpha and Ost beta are expressed in nearly all human tissues that have been examined, but are most abundant in the small intestine, kidney, liver, testis, adrenal gland and other steroidogenic tissues. Ost alpha-Ost beta substrates include bile acids, steroids (estrone 3-sulfate, dehydroepiandrosterone 3-sulfate, and digoxin), and prostaglandin E_2, indicating a role of Ost alpha-Ost beta in the disposition of key cellular metabolites and signaling molecules. Transport occurs by a facilitated diffusion mechanism, and thus Ost alpha-Ost beta can mediate cellular efflux or uptake depending on that substrate's electrochemical gradient. Additional strong evidence for a role of Ost alpha-Ost beta in sterol homeostasis was provided by recent studies in *Ost alpha*-deficient mice. These mice display a marked defect in intestinal bile acid and conjugated steroid absorption; a decrease in bile acid pool size and serum bile acid levels; altered intestinal, hepatic and renal disposition of known substrates of the transporter; and altered serum triglyceride, cholesterol, and glucose levels. Taken together, these observations indicate that Ost alpha-Ost beta is essential for bile acid and sterol disposition, and suggest that the carrier may be involved in human conditions related to imbalances in bile acid or lipid homeostasis.

Correspondence address: Ned Ballatori, Ph.D., Department of Environmental Medicine, Box EHSC, University of Rochester School of Medicine, 575 Elmwood Avenue, Rochester, NY 14642, T: 585-275-0262; F: 585-256-2591; Ned_Ballatori@urmc.rochester.edu.

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2. Introduction

The steroid-derived class of compounds, including the bile acids, steroid hormones, and other cholesterol metabolites, play critical roles in human physiology; however, relatively little is known about the transport proteins that mediate cellular import and export of these molecules. Although it is often assumed that transport of steroid hormones occurs by simple diffusion, this mode of transport would largely preclude the ability to regulate intracellular concentrations of these important bioactive and signaling molecules, and is therefore unlikely to play a significant role in their disposition. The present report summarizes the evidence for an important role of the organic solute and steroid transporter, Ost alpha-Ost beta, in the disposition of sterols, and focuses on the contribution of Ost alpha-Ost beta to the enterohepatic circulation of bile acids.

3. Bile acid synthesis and disposition

Bile acids are the major products of cholesterol catabolism, and they regulate a multitude of biological processes, including hepatic bile secretion and the intestinal absorption of fat and fat-soluble vitamins (1–3). Bile acids also modulate triglyceride, cholesterol, energy, and glucose homeostasis through their activation of specific receptors and signaling pathways (4).

The liver is the only organ that synthesizes bile acids. Approximately 5–10% of the bile acids made in the liver originate from peripherally obtained precursors and around 90–95% directly from cholesterol within hepatocytes (5). Specific hepatic enzymes oxidize cholesterol, and these initial oxidized products are further metabolized to bile acids, which are then secreted into bile and delivered into the intestine (6). Bile acids are defined as primary bile acids when they are sythesized in liver, or as secondary bile acids when they are made by bacteria in the intestine. The classical or neutral pathway and the acidic pathway are responsible for the production of at least 95% of the bile acids, and these two pathways contribute about equally to bile acid synthesis in humans (5). In the neutral pathway, the rate limiting step in bile acid synthesis is the addition of a hydroxyl group on position 7 of the steroid nucleus by the enzyme cholesterol 7 alpha-hydroxylase (CYP7A1). Likewise in the acidic pathway, sterol 27-hydroxylase, CYP27A1, is the rate limiting enzyme that introduces a hydroxy group to the carbon at position 27 of cholesterol. In an alternative pathway, cholesterol 24-hydroxylase, CYP46A1, initiates synthesis by converting cholesterol to 24S-hydroxycholesterol in the brain, which then travels to the liver for its ultimate conversion to bile acids. However, CYP46A1 only contributes about 1% to the overall synthesis of bile acids (5,7,8).

Of note, the transcription of *CYP7A1* is subject to both feedforward and feedback regulation (9). The liver X receptor alpha (LXR alpha), whose ligands include hydroxylated cholesterol metabolites (oxysterols), dimerizes with the retinoid X receptor (RXR), and binds to its response element on the *CYP7A1* promoter, thereby mediating transcriptional activation. Transcription of *CYP7A1* is downregulated by the bile acid-activated farnesoid X receptor (FXR) *via* a complex molecular mechanism that involves the coordinated regulation of several liver-enriched nuclear receptors (10,11). Bile acid-activated FXR dimerizes with RXR and transcriptionally activates its target nuclear receptor, the short heterodimer partner (*SHP*). SHP represses *CYP7A1* indirectly through its association with yet another nuclear receptor, the liver receptor homolog-1 (LRH-1) (12). Another major regulator of *CYP7A1*

transcription is fibroblast growth factor 19 (FGF19 in humans, and the mouse homologue Fgf15). FGF19/Fgf15 originates from ileum and is delivered to liver via the portal circulation with a pronounced diurnal pattern. When bile acid levels in ileocytes are elevated, this leads to the FXR-mediated induction of FGF19/Fgf15. This growth factor is secreted into portal blood, delivered to the liver where it binds to the hepatic FGFR4/Fgfr4 receptor, and represses *CYP7A1* transcription through a mechanism that involves the orphan nuclear receptor SHP (13).

In humans, the most important bile acids are cholic acid, deoxycholic acid, and chenodeoxycholic acid, which differ on the sites of hydroxylation (14). Bile acids may be conjugated with either glycine or taurine, a process that increases water solubility, preventing passive re-absorption once secreted into the small intestine. As a result, the concentration of bile acids in the small intestine can stay high enough to form micelles and solubilize lipids (3).

In the intestine, the detergent properties of bile acids aid in the solubilization of dietary lipids (especially fats and lipid soluble vitamins), lipophilic drugs, and electrolytes. Bile acids also regulate pancreatic secretions and the release of gastrointestinal peptides (15,16), activate carboxyl ester lipase (17), and promote the propulsive motility of the muscle layer in colon (18,19). In the liver, bile acids induce bile flow and biliary lipid secretion, and promote mitosis during hepatic regeneration (3,20). In the biliary tract, bile acids solubilize cholesterol, trap cholephilic xenobiotics in micelles, stimulate bicarbonate secretion via cystic fibrosis transmembrane conductance regulator (CFTR) and anion exchanger AE2, and promote proliferation when bile duct secretion is obstructed (21,22). In brown adipose tissue, bile acids stimulate thermogenesis by thyroid hormone (23). Bile acids also play a role in regulating gene expression via nuclear receptors and triggering various signal pathways impinging on numerous cellular processes, including apoptosis (24,25).

About 90% of the bile acids secreted into the intestine are reabsorbed into portal blood and are then efficiently taken back up by hepatocytes. The ileum is the major site of reabsorption. Bile acids continue to cycle between the intestine and the liver, creating an enterohepatic cycle. Because the majority of the circulating bile acids are conjugated and/or charged, they are unlikely to cross the plasma membrane by simple diffusion. Thus, the transcellular movement of the bile aids is mediated by specialized transporters.

4. Hepatic bile acid transporters

Hepatocytes import bile acids and other organic solutes from the sinusoidal blood and can transport them into bile canaliculi (Fig. 1). Hepatic bile acid uptake is mediated predominantly (>80%) by the Na^+-taurocholate co-transporting polypeptide (NTCP/SLC10A1) (2,25–28). NTCP mediates both conjugated and unconjugated bile acid uptake in a sodium dependent manner with a stoichiometry of 2 Na^+ ions for one taurocholate molecule (29). NTCP is predicted to have 7 transmembrane (TM) domains with an intracellular C-terminus and an extracellular N-terminus. *NTCP* gene expression is suppressed by high levels of bile acids as an adaptive response to reduce bile acid entry into the hepatocytes (30). In hepatocytes, bile acids activate FXR, which induces the expression of SHP, a blocker of the stimulating effect of retinoic acid receptor and retinoid X receptor RAR/RXR heterodimer on the *NTCP* promoter (31).

The sodium-independent organic anion transporting polypeptides, OATPs (e.g., OATP1A2/SLC1A2, OATP1B1/SLC1B1, OATP1B3/SLC1B3) also contribute to hepatic bile acid uptake, particularly for unconjugated bile acids (Fig. 1) (32,33). OATPs are glycoproteins with a common structure consisting of predicted 12 TM domains where both the N- and C-termini face intracellularly. Some OATP proteins mediate bile acid uptake by exchanging

them with the HCO_3^- or reduced glutathione (GSH) (34,35). Other major hepatic organic solute transporters include organic anion transporter 1 (OAT1) and the organic cation transporters (OCTs/SLC22A) (33,36), although these membrane proteins appear to play relatively minor roles in bile acid transport.

Hepatic export of bile acids into canalicular bile is mediated largely by members of the ATP-binding cassette (ABC) superfamily, principally the bile salt export pump (BSEP/ABCB11) and the multidrug resistance associated protein 2 (MRP2) (2,37) (Fig. 1). BSEP is predicted to contain 12 TM domains with two typical intracellular nucleotide-binding domains for binding and hydrolysis of ATP, whereas MRP2 has 17 TM domains (38,39). BSEP preferentially mediates the transport of conjugated monovalent bile acids whereas MRP2 exports divalent bile acids. Both BSEP and MRP2 expression undergo a positive feed-forward regulation by bile acids via the FXR receptor (40,41).

As the bile acids travel along the biliary tree, a small amount may be reabsorbed by the cholangiocytes, which express the apical sodium bile acid cotransporter (Asbt/Slc10a2) on their lumenal membranes (42), and Ost alpha-Ost beta on their basolateral membranes (43). The bile acids that are reabsorbed by cholangiocytes recycle via the peribiliary plexus back to hepatocytes for re-secretion into bile, a process that is referred to as the cholehepatic shunt pathway. It is not clear to what extent the shunt pathway contributes to the overall hepatobiliary transport of bile acids, or to the adaptation to chronic cholestasis due to extrahepatic obstruction (42).

Under normal conditions, the efflux of bile acids across the sinusoidal membrane of hepatocyte is negligible; however, in cholestatic conditions, OST alpha-OST beta and the multidrug-resistance associated proteins MRP3 and MRP4 are upregulated, and may mediate the export of bile acids into the blood plasma, although their functions are not well defined (Fig. 1) (2,27,44–46).

5. Intestinal bile acid transporters

Once bile reaches the small intestine, most (>85%) of the bile acids are reabsorbed and returned to the liver via portal blood. Bile acid uptake from the intestinal lumen is mediated largely by ASBT/SLC10A2 (Fig. 1). ASBT represents a highly efficient bile acid uptake transporter that prefers conjugated bile acids (28). Similar to NTCP, it mediates bile acid uptake coupled with Na^+ at a ratio of 2:1 (47), and bile acids induce a negative feedback regulation of *Asbt* gene expression (48). Bile acids induce FXR-dependent SHP activation, and SHP in turn represses LRH-1 dependent activation of *ASBT* expression (49). In addition to ASBT, Oatp3/Slc21A7 may also contribute to bile acid uptake in the small intestine (50). When ileal bile acid uptake is inhibited, as is the case in $Asbt^{-/-}$ mice (51), bile acid levels within the ileocytes decrease, and this represses the expression of *FGF19/Fgf15*. The lower FGF19/Fgf15 levels relieve the inhibition of Cyp7a1 transcription, and thus bile acid synthesis rate is increased under these conditions.

In contrast to the uptake step, the mechanism of export from enterocytes into the splanchnic circulation was undefined until the recent discovery of OST alpha-OST beta, as described in more detail below. Mrp3 is another putative basolateral bile acid exporter in the enterocytes, but this protein appears to play a small role in the efflux of bile acids into mesenteric venous blood (2).

6. Identification of Ost alpha-Ost beta

In 2001, our laboratory identified a novel organic solute and steroid transporter (Ost) from the little skate *Leucoraja erinacea* using expression cloning in *Xenopus laevis* oocytes (52).

Unlike mammalian hepatocytes, which uptake bile acids largely via Ntcp, skate liver does not appear to express an Ntcp transporter. In addition, the little skate synthesizes and excretes sulfated bile alcohols instead of bile acids, and therefore these animals were thought to have an additional transport mechanism for steroid derived molecules.

In order to identify a possible sterol transporter, total mRNA of skate liver was screened for bile acid (taurocholate) transport activity in *Xenopus* oocytes, and a cDNA library that included two positive mRNA size fractions (0.6–1.5 kb and 1.2–2.3 kb) was constructed (52,53). Using this approach, two distinct genes were identified and denoted as organic solute and steroid transporter alpha and beta (Ost alpha and Ost beta). These two genes must be co-expressed in order to elicit transport activity (52).

In 2003, using an enlarged gene and protein sequence database, human and mouse orthologues of skate Ost alpha and Ost beta were identified (54). The amino acid sequence alignments of OST alpha and OST beta from different species are shown in figures 2 and 3. The predicted human OST alpha protein and the predicted mouse Ost alpha protein each exhibit 41% amino acid identity with skate Ost alpha, and they also exhibit 83% amino acid identity with each other. Human OST alpha and mouse Ost alpha are predicted to have 340 amino acids and a molecular mass of ~ 37kDa. This protein is predicted to have 7 TM domains in all species (Fig. 2). Likewise, the predicted human OST beta and mouse Ost beta proteins exhibit 25% and 29% amino acid identity with skate Ost beta, and they also exhibit 63% amino acid identity with each other (Fig. 3). Human OST beta and mouse Ost beta have 128 amino acids and the predicted molecular mass is ~19 kDa. OST beta proteins are predicted to have a single TM domain (Fig. 3).

7. Ost alpha and Ost beta are expressed in most tissues, but are most abundant in tissues involved in bile acid and steroid homeostasis

Using quantitative real-time PCR analysis, Seward and colleagues (54) demonstrated that *OST alpha* and *OST beta* mRNA is widely expressed in human tissues, with the highest expression in small intestine, liver, colon, kidney, testes, ovary, and the adrenal gland. The distribution was also confirmed by Northern blotting. Additional studies revealed significant species differences in OST protein expression. For example, *OST alpha* and *OST beta* mRNA are relatively abundant in human liver, whereas, both mouse and rat liver have very low levels of these transcripts. Human OST alpha protein is detected in hepatocytes and cholangiocytes, whereas mouse hepatocytes have no detectable Ost alpha protein and mouse cholangiocytes have only moderate expression of this protein (43). Relatively high levels of *Ost alpha* and *Ost beta* message and protein are found in mouse small intestine, colon, and kidney, and the expression is especially high in ileum (55). Tissues with high levels of *OST alpha* generally also have high levels of *OST beta*, indicating co-expression of these genes (43,54). Both transcripts are relatively abundant in tissues that also express *ASBT/Asbt*.

The tissue distribution of Ost alpha and Ost beta mRNA and protein are also supported by expressed sequenced tag (EST) counts (Table 1). Human *OST alpha* and *OST beta* are most abundant in liver, intestine, kidney, testis, mammary gland, uterus, prostate, and thyroid, which are all steroid rich organs (Table 1). This expression pattern suggests that OST alpha-OST beta may transport steroid-derived compounds in these tissues. In mouse, *Ost alpha* and *Ost beta* tissue distribution parallels that of *Asbt* (Table 1).

In support of a role of the transporter in basolateral efflux, both Ost proteins are localized to the basolateral membrane of key epithelial cells, including enterocytes, renal tubular cells, and cholangiocytes (43,55).

8. Ost alpha-Ost beta mediates the transport of bile acids, conjugated steroids, and structurally-related molecules: transport occurs by a facilitated diffusion mechanism

The mechanism of Ost alpha-Ost beta mediated transport was identified by Ballatori *et al.* in 2005 (43). Ost alpha-Ost beta mediated uptake of estrone 3-sulfate was measured in *Xenopus laevis* oocytes. Transport was shown to be bidirectional and unaffected by depletion of intracellular ATP, changes in transmembrane electrolyte concentration gradients, or changes in the pH gradient. Ost alpha-Ost beta mediated transport of estrone 3-sulfate was *trans*-stimulated by known substrates. In addition, direct efflux of substrates was observed in oocytes expressing Ost alpha and Ost beta. These results indicate that Ost alpha-Ost beta mediates transport through facilitated diffusion, and thus can mediate either efflux or uptake depending on the particular substrate's electrochemical gradient (43).

In addition to bile acids, Ost alpha-Ost beta also transports steroids such as estrone 3-sulfate, dehydroepiandrosterone 3-sulfate (DHEAS), and digoxin, as well as the eicosanoid PGE_2 (43,54). Additional studies are needed to further define the substrate selectivity of Ost alpha-Ost beta.

9. Heterodimerization of Ost alpha and Ost beta increases the stability of the individual proteins, facilitates their post-translational modifications, and is required for delivery of the Ost alpha-Ost beta complex to the plasma membrane

As noted above, *in vitro* transfection studies showed that co-expression of Ost alpha and Ost beta is required for delivery of the individual proteins to the plasma membrane (52,54). Dawson et al. (55) demonstrated that co-expression is also required to convert the Ost alpha subunit to a mature N-glycosylated, Endo H-resistant form, suggesting that co-expression facilitates the movement of Ost alpha through the Golgi apparatus. Li et al. (56) extended these findings by showing that an Ost alpha-Ost beta heteromeric complex is apparently formed in the ER, is modified as it transits through the Golgi apparatus, and is then targeted to the plasma membrane. In contrast, when Ost alpha and Ost beta are expressed individually, the proteins appear to be targeted for degradation (56).

Evidence for a direct interaction between Ost alpha and Ost beta was obtained from co-immunoprecipitation and bimolecular fluorescence complementation (BiFC) analyses, two powerful approaches for examining protein-protein interactions (56). In BiFC analysis, two fragments of a fluorescence protein, which are non-fluorescent when separated, are fused to the putative interacting protein partners (57–59). When the partners interact, the two fragments of the fluorescence protein may associate with each other to regenerate a functional fluorophore. BiFC has several features that make it useful to detect protein-protein interactions, including direct visualization of interaction and its subcellular localization in living cells (57,60).

Co-immunoprecipitation studies using mouse ileal proteins and transfected HEK293 cells revealed that the two proteins that generate the organic solute transporter are able to immunoprecipitate each other, indicating formation of a heteromeric complex (56). Mouse ileal Ost alpha protein appeared on Western blots largely as bands of 40 and 80 kDa, the latter band is consistent with an Ost alpha homodimer, and both of these bands were sensitive to digestion by the glycosidase PNGase F. Ost beta appeared as bands of 17 and 19 kDa, and these bands were not sensitive to PNGase F. Both the 40 and 80 kDa forms of Ost

alpha and only the 19 kDa form of Ost beta were detected among the immunoprecipitated proteins, indicating that the interaction between Ost alpha and Ost beta is associated with specific post-translational processing (56). Co-immunoprecipitation of human OST alpha and OST beta also supported the formation of a heterodimeric complex (61).

Additional evidence for homodimerization of Ost alpha and for a direct interaction between Ost alpha and Ost beta was provided by BiFC analysis of HEK293 cells transfected with *Ost alpha* and *Ost beta* tagged with YFP fragments (56). BiFC analysis and surface immunolabeling of transfected HEK293 cells also indicated that the carboxyl termini of both Ost alpha and Ost beta are facing the intracellular space. The interaction between Ost alpha and Ost beta was required not only for delivery of the proteins to the plasma membrane, but it increased their stability, as noted in transfected HEK293 cells and in tissues from *Ost alpha*-deficient mice. In *Ost alpha*$^{-/-}$ mice, *Ost beta* mRNA levels were maintained, yet Ost beta protein was not detectable, indicating that Ost beta protein is not stable in the absence of Ost alpha. Overall, these findings identify the membrane topology of Ost alpha and Ost beta, demonstrate that these proteins are present as heterodimers and/or heteromultimers, and indicate that the interaction between Ost alpha and Ost beta increases the stability of the proteins and is required for delivery of the heteromeric complex to the plasma membrane (56).

10. Expression of both *Ost* genes is positively regulated by bile acids through the bile acid-activated farnesoid X receptor, Fxr

The regulation of OSTs at the cellular and molecular level has been investigated in several studies, and emerging evidence indicates that both subunits are regulated by bile acids through specific transcription factors (Fig. 4). Evidence for a critical role of Fxr in the transcriptional regulation of human *OST alpha* and *OST beta* and mouse *Ost alpha* and *Ost beta* has been provided by studies using transgenic animals, *in vitro* transcription of the genes, electrophoretic mobility shift assays, and promoter analysis (46,62–65). In particular, *Fxr*$^{-/-}$ mice have lower basal levels of Ost alpha and Ost beta expression in ileum, indicating that *Ost alpha* and *Ost beta* gene expression is dependent on Fxr 65). Both the biological Fxr agonist, CDCA and the synthetic Fxr agonist, GW4064 induce OST alpha-OST beta mRNA and protein expression in many cell lines, including two human liver cell lines, HepG2 and Huh7, a human adrenal carcinoma cell line, H295R, and a murine colon adenocarcinoma cell line, CT26 (46,62–64). Reducing FXR expression by transfecting Huh7 cells with a pool of four FXR-specific siRNAs led to the abolition of CDCA and GW4064 induced *OST alpha* and *OST beta* mRNA expression (63). GW4064 induces *Ost alpha* and *Ost beta* mRNAs in wild-type mouse adrenal gland organ culture, but not in the *Fxr*$^{-/-}$ mice organ culture (65). These findings establish that Fxr is an essential regulator for Ost alpha and Ost beta expression.

Two groups have reported that farnesoid X receptor elements (FXRE) are found in human *OST alpha* and *OST beta* promoters by using different computer based algorithms (63,64). Two putative IR-1/FXREs were identified within the *OST alpha* promoter and one within the *OST beta* promoter (Fig. 5). In the mouse, one potential FXRE sequence within the *Ost alpha* promoter and one within the *Ost beta* promoter has also been reported (62) (Fig. 5). These studies reveal that the induction of *OST alpha* and *OST beta* mRNA expression by FXR is ligand dependent and requires the heterodimerization of FXR with RXR alpha. FXR-RXR alpha heterodimers bind to the putative IR-1/FXREs and induce *OST* gene expression when bile acids and other potential FXR ligands are present (Fig. 4).

Although Fxr is the essential regulator, *Ost alpha-Ost beta* expression is fine-tuned by other transcription factors, including small heterodimer partner (Shp) and liver receptor

homolog-1 (Lrh-1) (62). One functional Lrh-1 element was identified in the mouse *Ost alpha* promoter region and two were identified in the mouse *Ost beta* promoter region (Fig. 5). Functional analysis of Lrh-1 in mouse *Ost alpha* and *Ost beta* promoter regions suggests that mouse *Ost alpha* and *Ost beta* expression is negatively regulated by bile acids via the Fxr-Shp-Lrh-1 pathway even though Lrh-1 exerts constitutively positive regulation on mouse *Ost alpha* and *Ost beta* expression (Fig. 4). The same mechanism is used to regulate Cyp7a1, the rate-limiting enzyme for bile acids synthesis. The Fxr-Shp-Lrh-1 pathway is thought to be subordinate under basal conditions in the ileum in $Shp^{-/-}$ mice, where the basal expression level of mouse *Ost alpha* and *Ost beta* is unchanged compared to the wild-type mouse, but *Ost alpha* and *Ost beta* are lower in $Fxr^{-/-}$ mice (62). These findings reveal an elaborate dual regulatory system mediated by nuclear receptors for more precise regulation of mouse *Ost alpha* and *Ost beta* gene expression. They also suggest a potential critical role of Ost alpha-Ost beta in bile acid homeostasis.

Interestingly, mouse *Ost alpha* and *Ost beta* may also be coordinately regulated by Fxr/Rxr and Lxr/Rxr (66). Lxr alpha was found to bind to the same IR-1/FxrEs that Fxr/Rxr alpha binds to activate transcription of the murine organic solute transporter. The physiological implication of the regulation by Lxr alpha is unknown, although the oxysterol nuclear receptor is known to dimerize with Rxr alpha and function in cholesterol and bile acid homeostasis (67). Only a few cases of FXREs functioning as LXREs are known; one being the FXRE of the ileal bile acid binding protein (I-BABP), which is an interesting coincidence since I-BABP and the organic solute transporter are regulated by the same pathways (68). Okuwaki and colleagues (66) also elucidated a role for hepatic nuclear factor-4 alpha (HNF-4 alpha), a regulator of lipid and bile acid homeostasis, in transcriptional activation of the alpha subunit of the murine and human transporter, but not the beta subunit.

Examination into the mechanism of pregnane X receptor (Pxr) activation on bile acid toxicity attenuation revealed a possible novel negative feedback loop for Fxr regulated genes (69). Pxr is known to regulate the expression of several enzymes and transporters involved in bile acid homeostasis of which Cyp7a1 and Mrp3 are of note. Teng and Piquette-Miller (69) observed an increase in expression of *Ost alpha* and *Ost beta* as well as *Mrp3* in Pxr-null livers as compared to WT counterparts, and the Pxr-null mice were less sensitive to cholic acid-induced hepatotoxicity. In cholic acid-fed mice, administration of 5-pregnen-3beta-ol-20-one-16alpha-carbonitrile (PCN), a Pxr activator, caused a down-regulation of *Ost alpha* and *Ost beta* and up-regulation of *Mrp3* in WT mice, but not in Pxr-null mice. In the same mice, Fxr levels were roughly 50% lower upon PCN supplementation (69). Cholic acid and its main conjugate, taurocholic acid, are substrates of rodent Mrp3 (70,71), and sulfated sterol conjugates such as dehydroepiandrosterone sulfate (DHEAS) and estrone 3-sulfate are known substrates of Ost alpha and Ost beta (43). Interestingly, sulfation is not a major metabolic pathway in mice (72), therefore the up-regulation of *Mrp3* rather than *Ost alpha* and *Ost beta* and the subsequent efflux of cholic acid and its conjugates in response to induced hepatotoxicty following PCN treatment could be indicative of preferential regulation due to substrate specificity. In mice and probably in humans, Mrp4 may be more important as it prefers sulfated conjugates, while Mrp3 prefers glucuronide conjugates (45). A hypothesized schematic for this novel negative feedback mechanism is illustrated in figure 6.

Boyer and colleague (46) found that both OST alpha and OST beta mRNA and protein expression are upregulated in human livers from primary biliary cirrhosis (PBC) patients. PBC is a chronic cholestatic disease characterized by inflammatory destruction of the small bile ducts within the liver (73,74) resulting in the accumulation of hepatoxic bile acids. Because OST alpha-OST beta is an important player in bile acid enterohepatic circulation, it

might undergo adaptive regulation to protect the liver from tissue injury. In the mouse, the first indication that Ost alpha-Ost beta is regulated by bile acids was suggested in *Asbt*$^{-/-}$ mice (55). In the cecum and proximal colon of the *Asbt*$^{-/-}$ mouse, Ost alpha and Ost beta are up-regulated; however, they are decreased in the ileum (55). The same expression pattern of Ost alpha-Ost beta is also observed in male C57BL/6J mice fed with 0.2% dietary cholic acid for 14 days (62). The decreased ileal expression of Ost alpha-Ost beta in both models can be explained by reduced uptake of bile acids into the ileal enterocytes.

A recent study demonstrates that arachidonic acid, the precursor to eicosanoids, strongly induces Ost alpha-Ost beta expression in a new cell line derived from an early embryo of *Leucoraja erinacea* (75). Although the function of this transporter in embryo-derived cells is unknown, it may play a role in the disposition of eicosanoids or steroid-derived molecules. This conclusion is supported by the observation that prostaglandin E_2 is a substrate for this transporter (52).

Although relatively little information is available on ontogenic changes in *Ost alpha* and *Ost beta* expression, the available evidence indicates that *Ost alpha* and *Ost beta* expression is quite low in neonatal rats and rabbits and increases to adult levels shortly after weaning (76–78). These changes parallel those for many of the genes involved in bile acid homeostasis including Fxr (79).

11. Ost alpha-Ost beta is required for bile acid and conjugated steroid disposition in the intestine, kidney, and liver

As described above, several lines of evidence support the hypothesis that Ost alpha-Ost beta is the key basolateral exporter of bile acids and related molecules, including its substrate specificity, transport mechanism, tissue distribution, subcellular localization, and transcriptional regulation. To more directly test the functional role of Ost alpha-Ost beta, *Ost alpha*$^{-/-}$ mice have recently been generated and characterized from two different laboratories, and they have provided important insights into the function of this transporter (80,81). As demonstrated by Li *et al.* (56), *Ost alpha*–/– mice lack both Ost alpha and Ost beta proteins because the individual subunits of this heteromeric transport complex are not stable in the absence of their obligate heterodimerization partner. The knockout mice are viable and fertile but exhibit early growth retardation. Pre-weanling *Ost alpha*$^{-/-}$ mice of both sexes are 25% smaller than heterozygous littermates (80) although this difference disappears after the mice reach adulthood. In addition, both pre-weanling and adult *Ost alpha*-deficient mice display intestinal hypertrophy. As expected, *Ost alpha* knockout mice display impaired trans-ileal taurocholate transport, significantly decreased bile acid pool size, and altered hepatic, renal and intestinal disposition of bile acids (80,91). These data provide evidence that Ost alpha-Ost beta is critical for bile acid and steroid homeostasis.

The *Ost alpha* null mice also provide important insight into mechanisms of bile acid homeostasis, and identify Ost alpha-Ost beta as a potential target for interrupting the enterohepatic circulation of bile acids. Although there is a significant decrease of bile acid absorption in *Ost alpha* null mice, the fecal excretion of bile acids is unchanged. This is due in part to a decrease in hepatic bile acid synthesis and the resulting marked decrease in the bile acid pool size, as illustrated schematically in figure 7. Interestingly, cholesterol absorption is less in Ost alpha knockout mice (81). Fecal neutral sterol excretion in the knockout mice is more than the wild type and also the serum cholesterol and triglycerides are lower in knockout mice. Thus, OST alpha-OST beta might be a potential therapeutic target for altering lipid levels.

In addition, in contrast to the increase in hepatic bile acid synthesis seen when intestinal bile acid uptake is interrupted with either bile acid sequestrants or with Asbt inhibitors, hepatic Cyp7a1 expression is decreased in the *Ost alpha*$^{-/-}$ mice indicating a decrease in bile acid synthesis (Fig. 7). As described in more detail below, these contrasting effects are most likely explained by the enhanced intestinal expression of Fgf15, an important negative regulator of hepatic bile acid synthesis (13), when Ost alpha-Ost beta function is abrogated.

As noted above, bile acid concentrations are normally controlled by a feedback regulatory mechanism, whereby bile acid activation of Fxr represses hepatic transcription of Cyp7a1 levels, and thus leads to a decrease in bile acid synthesis (Fig. 7). Bile acid activation of Fxr also leads to decreased expression of the bile acid uptake transporters Asbt and Ntcp, and to increased expression of the bile acid exporters Bsep and Ost alpha-Ost beta. Collectively, these transport proteins, along with the enzyme Cyp7a1, mediate a decrease in intracellular bile acid concentrations back to basal levels. Ileocytes, however, also express Fgf15, another key regulator of bile acid synthesis (13). When intracellular bile acid levels in ileocytes are elevated (as they are expected to be in the *Ost alpha*$^{-/-}$ mice), this leads to the Fxr-mediated induction of Fgf15 (13). Fgf15 is then delivered to the liver, where it represses Cyp7a1 expression through a mechanism that involves Fgf receptor 4 (Fgfr4). Intestinal expression of *Fgf15* was higher and hepatic expression of *Cyp7a1* was lower in *Ost alpha*$^{-/-}$ mice, as predicted by this model (Fig. 7).

These observations in *Ost alpha–/–* mice contrast with those seen in *Asbt*$^{-/-}$ mice (51). The absence of Asbt leads to a diminished ability of the enterocytes to take up bile acids across their apical membranes, and thus, to relatively low intracellular bile acid levels. These low bile acid levels down-regulate Fgf15 expression, and thus relieve Fgf15-mediated repression of Cyp7a1 transcription. In addition, the low hepatocellular bile acid levels in *Asbt*$^{-/-}$ mice also relieve the Fxr- and Shp-mediated repression of hepatic Cyp7a1 activity, with the net result being a 5-fold increase in Cyp7a1 expression and in bile acid synthesis rate in *Asbt*$^{-/-}$ mice (51).

12. Implications for human diseases

Currently, there is no disease that is directly associated with OST alpha-OST beta. However, given the central role of OST alpha-OST beta in bile acid homeostasis, this transporter may be involved in the progression of diseases related to bile acid malabsorption, cholestasis, or cholelithiasis. In support of this hypothesis, changes in *OST alpha* and *OST beta* gene expression have already been reported in some of these conditions (82–85). Bile acid malabsorption is observed in a number of human conditions, including intractable diarrhea, irritable bowel syndrome, immunodeficiency virus (HIV) enteropathy, and cystic fibrosis (86–89). On the other hand, too much bile acid reabsorption is the cause for constipation (90). It will clearly be of interest to examine the possible contribution of OST alpha-OST beta to the etiology and/or progression of these human conditions.

Although bile acids play important roles in many physiological processes, they can also be toxic when present at high levels. Retention of bile acids in cholestatic liver diseases results in progressive liver injury that may ultimately result in organ failure and the need for liver transplantation. The secondary hydrophobic bile acids are more toxic than primary bile acids but all can result in apoptosis and or necrosis of liver tissue (91). Bile acids can also induce oxidative stress, with secondary consequences of inflammation and fibrosis. The rationale for therapy with ursodeoxycholic acid in cholestatic diseases is to reduce the hydrophobicity of the circulating bile acid pool. Another disease that may be caused by elevated bile acid concentrations is necrotizing enterocolitis (NEC), a disease that affects thousands of newborns each year in the United States alone, and with a mortality rate ranging from 10%

to 50% (92–94). NEC remains a major cause of morbidity and mortality in prematurely born infants. Bile acids accumulate in both the ileal lumen and enterocytes of neonatal animals with NEC and the increased bile acid levels are positively correlated with disease severity (76,95). Halpern et al. (76) found that Asbt was up-regulated at the site of injury in rats with experimentally-induced NEC and decreased after EGF treatment; however, the ileal bile acid binding protein was up-regulated only in the NEC and EGF group, and Ost alpha and Ost beta expression was low in all groups, and only slightly increased in the NEC group. The inability to upregulate Ost alpha and Ost beta expression may have contributed to the accumulation of toxic levels of bile acids in the ileocytes. These results suggest that bile acids play a role in the development of ileal damage in experimental NEC and that alterations in bile acid transport in the neonatal ileum may contribute to disease development.

Another disease related to the concentration of bile acids is colon cancer. Epidemiological studies have shown that the concentration of fecal bile acids is positively correlated with the colorectal cancer incidence (96). In animal models, diversion of bile ducts, small bowel resection, or direct installation of bile acids in the large bowel can be tumor promoting (97). Because OST alpha-OST beta regulates enterocyte and intestinal lumen bile aids concentrations, altered levels or function of OST alpha-OST beta may be a risk factor for these diseases, although there is no evidence for this hypothesis as yet.

In addition, bile acids are required for intestinal lipid absorption. Thus, OST alpha-OST beta may also be involved in a variety of human conditions related to imbalances in sterol or lipid homeostasis, although this has not yet been established.

13. Summary

Recent studies indicate that Ost alpha-Ost beta is a major basolateral transporter of bile acids and conjugated steroids in the intestine, kidney, and liver. Crosstalk between multiple signaling pathways and nuclear receptors reveals a more intricate means of regulation for OST alpha-OST beta than initially thought. The involvement of several transcription factors, including FXR, RXR alpha, SHP, LRH-1, LXR alpha, HNF-4 alpha, and PXR, paints a complicated regulatory mechanism that needs further study. *Ost alpha*-deficient mice display impaired trans-ileal taurocholate transport, significantly decreased bile acid pool size, and altered hepatic, renal and intestinal disposition of bile acids (80,81). These data provide evidence that Ost alpha-Ost beta is critical for bile acid and steroid homeostasis, and suggest that the resulting changes in bile acid levels have an effect on serum cholesterol, triglyceride, and glucose levels. In addition, the results indicate that targeted inhibition of Ost alpha-Ost beta may have advantages over other maneuvers that have been used to interrupt the enterohepatic circulation of bile acids.

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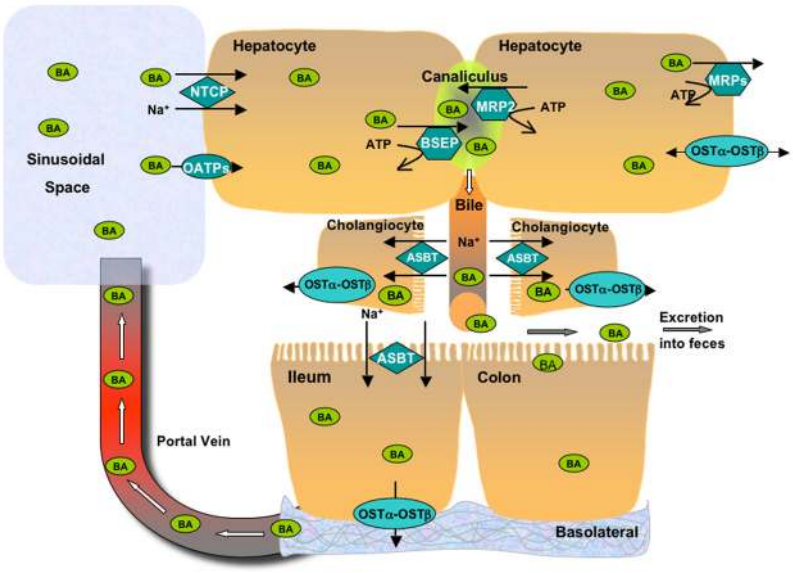

Figure 1. The major bile acid transporters in the enterohepatic circulation

Bile acid transport across the basolateral membrane of the hepatocytes is mediated mainly by the Na+-dependent taurocholate cotransporting polypeptide (NTCP) and organic anion transporting polypeptides (OATPs). Bile acids efflux across the basolateral membrane of hepatocytes may occur via the organic solute and steroid transporter (OST alpha-OST beta) and/or the multidrug resistance-associated proteins 3 and 4 (MRP3 and MRP4). The secretion of bile acids across the canalicular membrane into bile occurs via two members of the ATP-binding cassette transporters: the bile acid export pump (BSEP) and MRP2. Bile acids are delivered to the intestinal lumen through bile duct where they aid in emulsifying dietary lipids. Bile acids are actively re-absorbed in the distal ileum (and in cholangiocytes) via Na+-dependent apical sodium dependent bile acid transporter (ASBT), and are effluxed through OST alpha-OST beta.

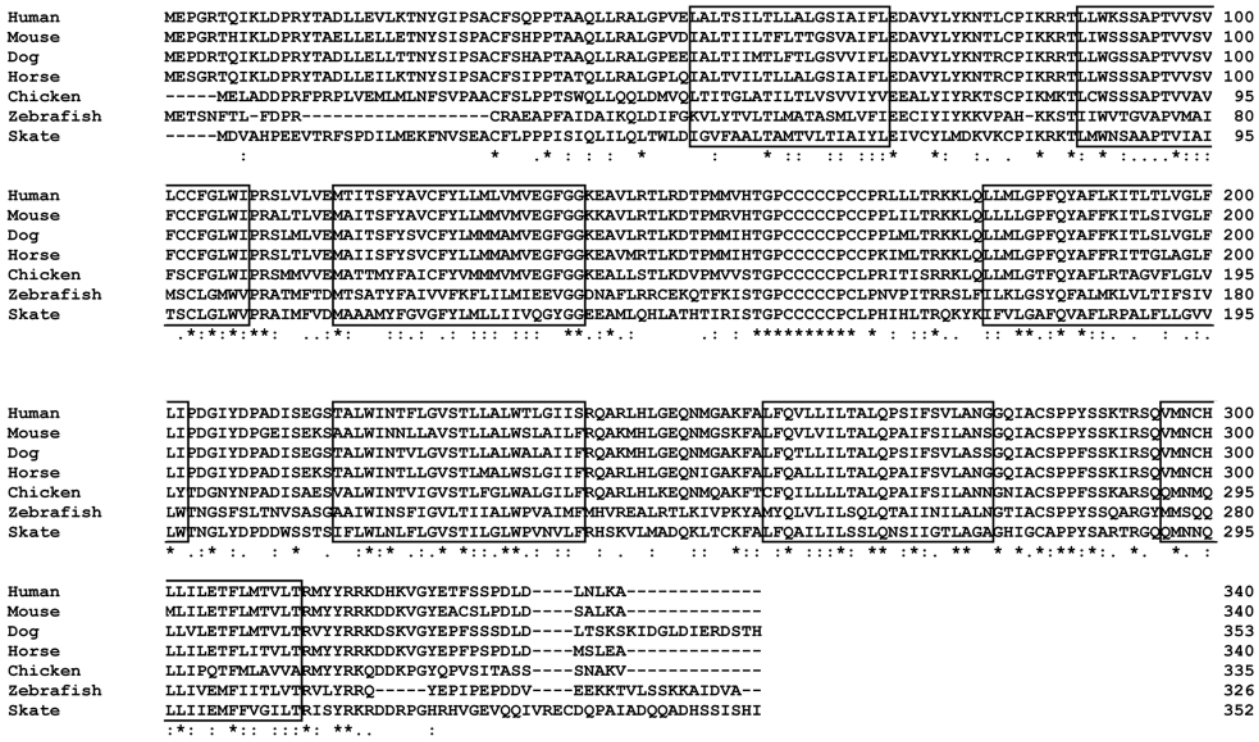

Figure 2. Amino acid alignments of the deduced OST alpha sequences from multiple species

Reference sequences for the human, mouse, dog, horse, chicken, zebrafish, and skate OST alpha proteins were obtained from the NCBI database and were aligned using the MUSCLE program (multiple sequence comparison by log-expectation; http://www.ebi.ac.uk/Tools/muscle/index.html). The predicted TM domain is boxed. "*" denotes the amino acids that are conserved among the seven species; ":" denotes that conserved substitutions are observed; "." denotes that semi-conserved substitutions are observed.

Figure 3. Amino acid alignments of the deduced OST beta sequences from multiple species

Reference sequences for the human, mouse, dog, horse, chicken, zebrafish, and skate OST beta proteins were obtained from the NCBI database and were aligned using the MUSCLE program (multiple sequence comparison by log-expectation; http://www.ebi.ac.uk/Tools/muscle/index.html). The predicted TM domain is boxed. "*" denotes the amino acids that are conserved among the seven species; ":" denotes that conserved substitutions are observed; "." denotes that semi-conserved substitutions are observed.

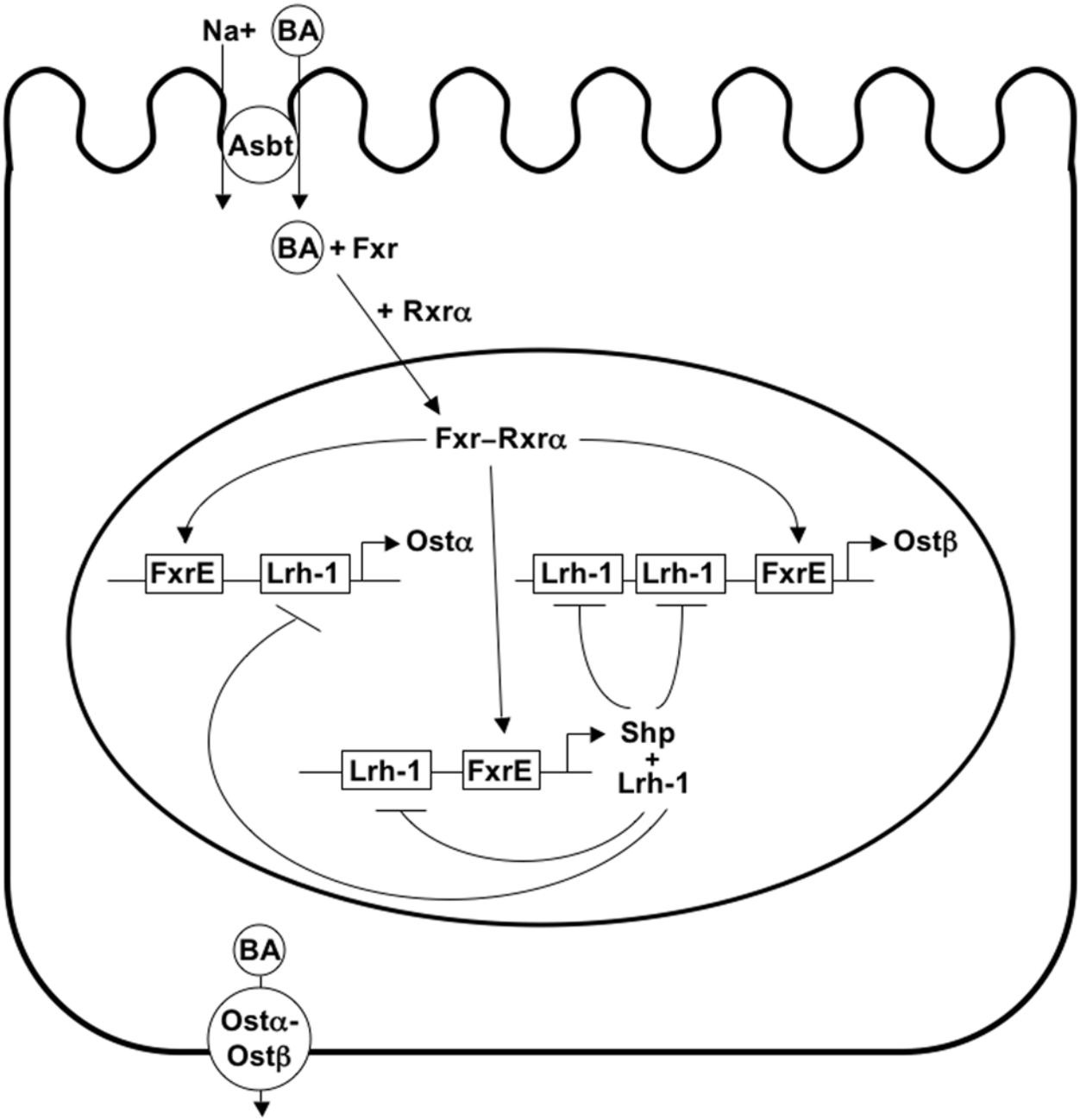

Figure 4. Ost alpha and Ost beta are dynamically regulated via the Fxr-Shp-Lrh-1 pathway

Bile acids are actively transported into enterocytes by Asbt, where they bind to and activate Fxr. Fxr forms a heterodimer with Rxr alpha, which translocates to the nucleus, binds to FxrEs thereby priming expression of target genes Ost alpha, Ost beta, and Shp in a positive feedback manner (62,98). Upon accumulation of Shp, a non-DNA binding orphan nuclear receptor viewed as a transcription inactivator, Shp forms a heterodimer with Lrh-1, an obligatory factor for transcriptional activation, thereby antagonizing Lrh-1 and ultimately leading to down-regulation of Ost alpha, Ost beta, and Shp (62,99). Thus, negative regulation is a subsidiary mechanism to positive feedback via bile acids.

A

Mouse FxrE

	Binding Site	Core Sequence
Ostα	-1221 to -1209	AGGTCAcTGACCT
Ostβ	-5 to +7	GGGTCAtTCACCC
FxrE Consensus		AGGTCAnTGACCT

Mouse Lrh-1

	Binding Site	Core Sequence
Ostα	-55 to +63	ACAAGGTTG
Ostβ	-240 to -232	CCAAGGTCT
	-393 to -401	CCAAGGTCC
Lrh-1 Consensus		YCAAGGYCR

B

Human FXRE

	Binding Site	Core Sequence
OSTα	-1375 to -1363	GGGTGAaTGACCT
	-1295 to -1283	AGGCCAgTGACCC
OSTβ	-134 to -122	AGGTCAgTCACCC
FXRE consensus		AGGTCAnTGACCT

Figure 5. Functional FxrEs and Lrh-1 binding sites in the proximal promoter of murine *Ost alpha* and *Ost beta*, and functional FXREs in the proximal promoter of human *OST alpha* and *OST beta*

After heterodimerization with RXR alpha, FXR binds to the FXRE *cis*-acting element with an idealized sequence of an inverted hexameric nucleotide repeat consisting of minor variants of two AGGTCA half-sites separated by one nucleotide (IR-1) (100–102). LRH-1 binds to a consensus sequence of YCAAGGYCR where Y is any pyridine and R is any purine (103). A: position of functional FxrEs and Lrh-1 binding sites in the promoters of murine *Ost alpha* and *Ost beta* relative to putative transcription start sites and sequence comparison of the functional *cis*-acting elements to optimal binding sequences (62). B: location of human *OST alpha* and *OST beta* functional FXREs with sequence comparison to

an idealized binding sequence (64). Conservation of the murine Lrh-1 binding sites in the promoters of their human counterparts has been indicated although the exact position and sequence of the *cis*-acting elements have not been published (62). Therefore, *OST alpha* and *OST beta* are assumed to be negatively regulated in a similar fashion as their murine orthologues.

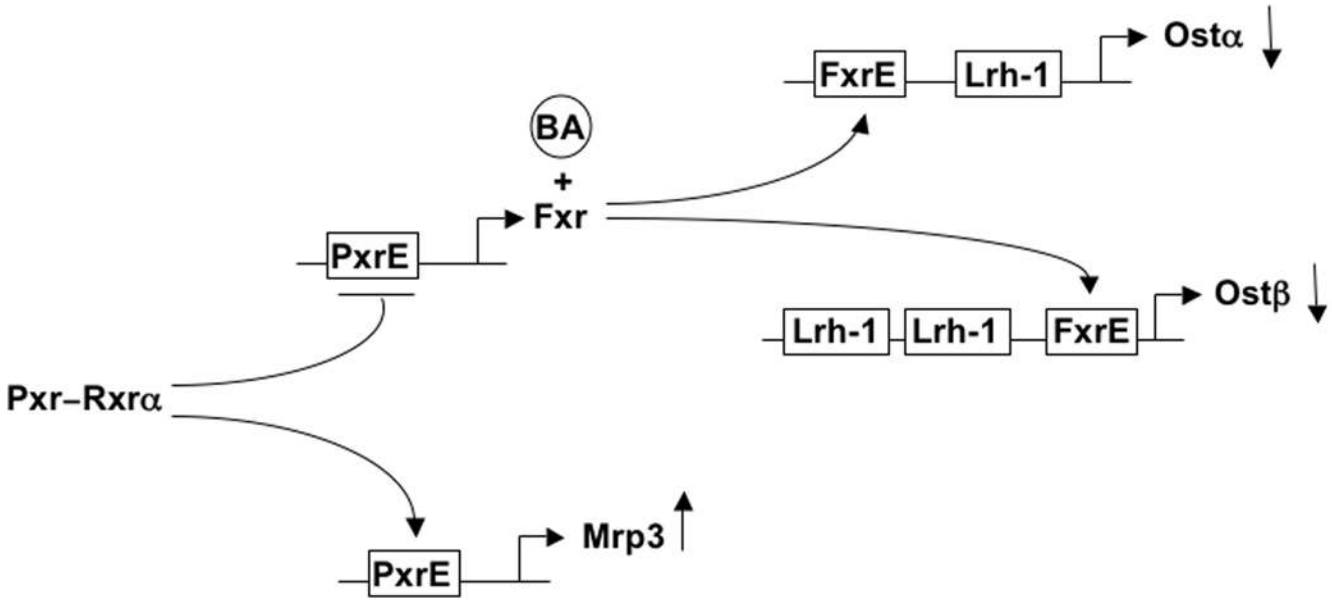

Figure 6. Proposed mechanism of a novel negative feedback pathway for *Ost alpha* and *Ost beta* by Pxr

Ligand activation of Pxr, which dimerizes with Rxr alpha, seemingly reduces Fxr levels ultimately down-regulating *Ost alpha* and *Ost beta*, but simultaneously inducing expression of *Mrp3*. Mrp3 protein can then mediate bile acid efflux. This suggests a negative feedback loop that is absent in Pxr-deficient mice thereby explaining the higher expression levels of *Ost alpha* and *Ost beta* under control conditions as compared to wild type animals (69).

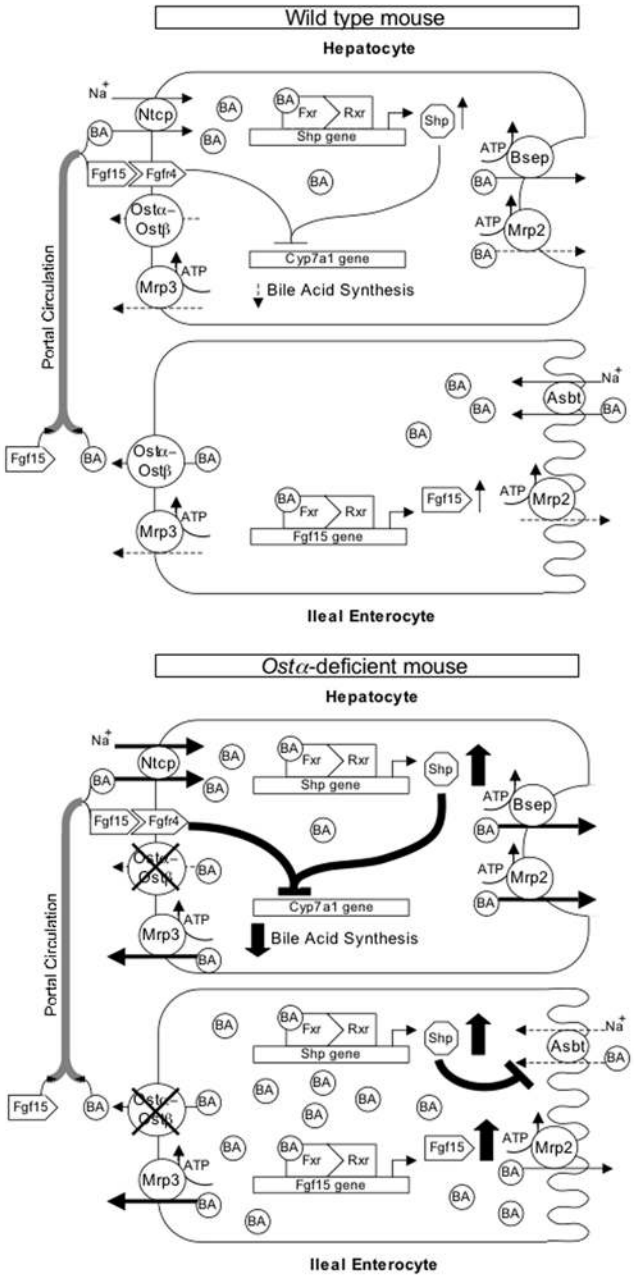

Figure 7. Comparison of the enterohepatic circulation of bile acid in *Ost alpha*$^{+/+}$ and *Ost alpha*$^{-/-}$ mice

Bile acids (BA) are taken up from the lumen of the small intestine into ileal enterocytes via Asbt. Once inside the cell, BA bind to Fxr and mediate transcription of genes such as *Fgf15*. Fgf15 is delivered via portal blood to the hepatocytes where it can bind to Fgfr4. In *Ost alpha*$^{+/+}$ mice, Ost alpha-Ost beta effluxes BA from the enterocyte into blood on the basolateral side, and the BA return to the liver via portal circulation. Also depicted are the apically-localized Mrp2 and basolaterally-localized Mrp3 proteins that may also contribute to BA export. BA enter the hepatocyte from the blood via Ntcp, at which point they may bind Fxr and initiate transcription of Shp, which along with Fgf15-Fgfr4 synergistically mediate transcriptional repression of *Cyp7a1*. Also depicted are Ost alpha-Ost beta and

Mrp3 on the sinusoidal membrane mediating low-level transport of their respective substrates back into blood. BA are in turn actively transported across the canalicular membrane by Bsep, or to an extent by Mrp2, directly into bile where they eventually re-enter the intestines thereby completing enterohepatic circulation. In *Ost alpha*$^{-/-}$ mice, the uptake of BA into the ileal enterocyte results in their accumulation because of the eliminated basolateral efflux mediated by Ost alpha-Ost beta (80,81). The elevated BA levels activate Fxr, leading to a marked up-regulation of Shp, which results in the subsequent down-regulation of Asbt, and Fgf15. Fgf15 in turn down-regulates *Cyp7a1* in the liver after binding Fgfr4. The compensatory BA transporters Mrp3 and Mrp2 are up-regulated, which may help eliminate BA from the enterocytes. In the hepatocyte, an up-regulation of Ntcp, Mrp3, Bsep, and Mrp2 is observed in an attempt to escalate cycling of BA due to their increased loss evident from the decreased bile acid pool size coupled with the unchanged fecal excretion rate as compared to wild type. The decreased bile acid pool size is not only due to intestinal malabsorption of BA, but also to the substantial down-regulation of Cyp7a1, a rate-limiting enzyme in bile acid synthesis (80).

Table 1

Tissue expression of human OST alpha and OST beta and mouse Ost alpha and Ost beta as indicated by analysis of expressed sequence tag (EST) counts

	Transcripts per million					
	Human			Mouse		
Tissue	OSTalpha	OSTbeta	ASBT	Ostalpha	Ostbeta	Asbt
Blood	8	0	0	0	0	0
Lung	11	0	0	0	0	0
Liver	28	4	0	9	0	0
Kidney	14	37	61	185	24	24
Intestine	25	25	8	139	442	23
Mammary Gland	6	0	0	0	0	0
Uterus	4	0	0	0	0	0
Testis	54	3	0	0	0	0
Prostate	10	0	0	0	0	0
Thyroid	20	0	0	0	0	0
Thymus	0	0	0	0	24	0

Data were obtained from the UniGene EST Profile Viewer Web site of the National Center for Biotechnology Information in April, 2008 (http://www.ncbi.nlm.nih.gov/UniGene/)