Cholestasis-induced bile acid elevates estrogen level via farnesoid X receptor–mediated suppression of the estrogen sulfotransferase SULT1E1

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The liver is the main site of estrogen metabolism, and liver disease is usually associated with an abnormal estrogen status. However, little is known about the mechanism underlying this connection. Here, we investigated the effects of bile acid (BA)-activated farnesoid X receptor (FXR) on the metabolism of 17β-estradiol (E2) during blockage of bile flow (cholestasis). Correlations between BA levels and E2 concentrations were established in patients with cholestasis, and hepatic expression profiles of key genes involved in estrogen metabolism were investigated in both WT and FXR$^{-/-}$ mice. We found that the elevated E2 level positively correlated with BA concentrations in the patients with cholestasis. We further observed that bile duct ligation (BDL) increases E2 levels in mouse serum, and this elevation effect was alleviated by deleting the FXR gene. Of note, FXR down-regulated the expression of hepatic sulfotransferase SULT1E1, the primary enzyme responsible for metabolic estrogen inactivation. At the molecular level, we found that FXR competes with the protein acetylase CREB-binding protein (CBP) for binding to the transcription factor hepatocyte nuclear factor 4α (HNF4α). This competition decreased HNF4α acetylation and nuclear retention, which, in turn, repressed HNF4α-dependent *SULT1E1* gene transcription. These findings suggest that cholestasis induces BA-activated FXR activity, leading to downstream inhibition of SULT1E1 and hence impeding hepatic degradation of estrogen.

Estrogens are key regulators of growth, differentiation, and metabolism in a wide array of target tissues, including the female reproductive tract, mammary gland, skeletal and cardiovascular systems (1). The most potent and dominant estrogen in humans is 17β-estradiol (E2),[4] along with lower level of estrone (E1) and estriol (E3) (2). In terms of estrogenic effect, E2 is about 10-fold as potent as E1 and about 80-fold as potent as E3. In postmenopausal women, the serum E2 level (<130 pmol/ml) is roughly comparable with that in men (50~200 pmol/ml) (3). Normal estrogen level is necessary for optimal bone density, cognitive function, cardiovascular integrity, and sexual function. Estrogen imbalance is a critical inducer of a variety of diseases (4–8). Estrogen is metabolized in several organs, especially the liver, which accounts for more than 50% of the catabolism and conjugation of estrogens (9). Prospective and epidemiological studies have shown that liver disease is correlated with abnormal estrogen status (10–12). However, little is known about the mechanism underlying such correlation.

Bile acids (BAs), important liver products, are substantially increased in cholestatic liver disease. BAs are endogenous ligands for the Farnesoid X receptor (FXR) (13, 14), which is a nuclear receptor highly expressed in the liver (14). FXR regulates genes involved in bile acid synthesis, lipid and lipoprotein metabolism, including small heterodimer partner (*SHP*), cholesterol 7α-hydroxylase (*CYP7A1*), sterol 12α-hydroxylase (*CYP8B1*), bile salt export pump (*BSEP*), apolipoprotein (*Apo*) AI, *ApoC*-II, *ApoC*-III, and the phospholipid transfer protein (PLTP) (15, 16). In addition, FXR plays a critical role in glucose metabolism, insulin sensitivity, and atherosclerosis (17). FXR has been extensively studied as a new therapeutic target in numerous metabolic disorders (17–19). However, how activated FXR regulates estrogen metabolism in the liver remains unknown.

In this study, we reported that the elevated BA increases the E2 level via FXR activation during cholestasis. FXR competed with CREB-binding protein (CBP) for HNF4α binding, and hence, decreased HNF4α acetylation and nuclear retention,

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This article contains Figs. S1–S9, Tables S1–S6, and Methods.

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[4] The abbreviations used are: E2, 17β-estradiol; FXR, farnesoid X receptor; SULT1E1, sulfotransferase *1E1*; HNF4α, hepatocyte nuclear factor 4α; ER, estrogen receptor; BA, bile acids; SHP, small heterodimer partner; CYP7A1, cholesterol 7α-hydroxylase; CYP8B1, sterol 12α-hydroxylase; BSEP, bile salt export pump; Apo, apolipoprotein AI; Alt, alanine aminotransferase; AST, aspartate transaminase (AST); γ-GT, γ-glutamyltransferase; BDL, bile duct ligation; CREB, cAMP-response element-binding protein; PBC, primary biliary cirrhosis; CA, cholic acid; DCA, deoxycholic acid; qPCR, quantitative PCR; EMSA, electrophoretic mobility shift assay; shRNA, short hairpin RNA; GFP, green fluorescent protein; PXR, pregnane X receptor.

which in turn, led to the repression of HNF4α-dependent sulfotransferase 1E1 (*SULT1E1*) transcription.

Results

Elevated bile acids drastically increased E2 level in patients with obstructive cholestasis or primary biliary cirrhosis (PBC)

It is consistently noticed that patients suffering from obstructive cholestasis exhibited clinically very high levels of estrogen. We conducted a systematic analysis of the molecules, such as BA, bilirubin, and γ-GT, which might correlate with the estrogen levels in these patients. Table S1 lists the results from 40 postmenopausal female patients suffering from biliary obstruction due to pancreatic, gallbladder, bile duct cancer, or choledocholithiasis. All patients showed elevated serum bilirubin concentrations (201 ± 88 μmol/liter) and raised γ-GT level (195 ± 69 μmol/liter). After successful surgical or endoscopic treatment of biliary obstruction, bilirubin, γ-GT, and total BA levels returned to normal. Notably, the serum total bile acid levels were 125.2 ± 47.3 μmol/liter before therapy and 16.8 ± 18.4 μmol/liter after therapy (Fig. 1*A*, *left panel*). Interestingly, the serum E2 level in these patients also decreased about 5-fold after therapy from 738.7 ± 222.4 to 130.2 ± 69.8 pmol/liter, which were reduced following therapy (Fig. 1*A*, *right panel*). The serum estrogen concentrations were positively correlated with serum BA levels before therapy ($R^2 = 0.2402$, $p = 0.0013$) (Fig. 1*B*).

We next investigated the effect of BA on the E2 level in patients with PBC. The baseline patient characteristics are summarized in Table S2. Thirty-six postmenopausal female patients with PBC were divided in two groups: patients with normal serum BA level (low BA group, $n = 22$) and patients with high serum BA level (high BA group, $n = 14$) (Fig. 1*C*, *left panel*). The high BA group was associated with significantly elevated E2 level (454.5 ± 151.8 pmol/liter), whereas the low group is associated with low E2 level (91 ± 41.5 pmol/liter) (Fig. 1*C*, *right panel*). After appropriate treatment, the BA concentrations of 8 patients from the high BA group returned to normal levels (Fig. 1*D*, *left panel*). Interestingly, serum E2 levels in those 8 patients also dropped from 492.9 ± 159 to 185.5 ± 86.5 pmol/liter (Fig. 1*D*, *right panel*). Moreover, the serum E2 level is positively correlated with serum BA concentrations in the high BA group ($R^2 = 0.317$, $p = 0.0361$) (Fig. 1*E*). Overall, these data suggested that BA might increase the E2 level.

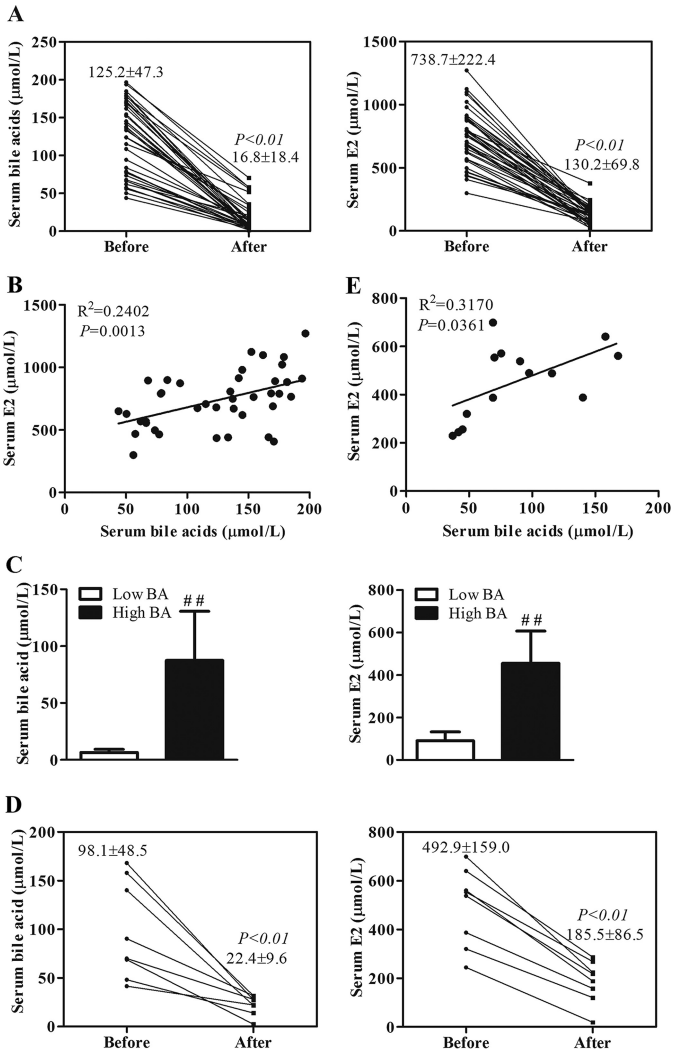

Figure 1. High serum E2 level in patients with obstructive jaundice or PBC. *A,* serum samples from 40 postmenopausal female patients suffering from obstructive jaundice were assayed for total bile acid (*left panel*) and E2 levels (*right panel*), before and after surgical or endoscopic treatment. Values are expressed as mean ± S.D. See Table S1 for details. *B,* correlation between serum bile acid and serum E2 in the patients investigated before therapy. *C,* postmenopausal female patients with PBC were divided into two groups: patients with normal serum BA level (low BA group, $n = 22$) and patients with high serum BA level (high BA group, $n = 14$). Serum total bile acid (*left panel*) and serum E2 levels (*right panel*) were assayed for these two groups. *D,* after appropriate treatment, the BA concentrations returned to normal level in 8 patients from the high BA group. Serum total bile acid (*left panel*) and serum E2 levels (*right panel*) were compared in these 8 patients, before and after appropriate treatment. Values are expressed as mean ± S.D. *E,* correlation between serum bile acid and serum E2 in the high BA group before therapy. ##, $p < 0.01$ *versus* low BA.

Bile acid-induced E2 level increase is mediated by FXR in a cholestatic mouse model

To determine the effects of obstructive cholestasis on serum E2 level, wildtype (WT) and $FXR^{-/-}$ female mice were subjected to biliary obstruction by common bile duct ligation (BDL) for 3 days. BDL resulted in significantly elevated serum liver enzymes, bilirubin (Fig. S1, *A–C*, hepatic and serum total bile acids (Fig. 2, *A*, *B*, *D*, and *E*) in WT and $FXR^{-/-}$ mice. The accumulation of endogenous BA in WT mice led to a significant elevation of serum E2 by 143% (Fig. 2*C*), compared with the 31% increase in $FXR^{-/-}$ mice (Fig. 2*F*), which might be due to inflammation and hepatic injury. Likewise, BDL-induced hepatic and serum total bile acids increase was also accompanied with an elevation of serum E2 in ovariectomized female mice (Fig. 2, *A–C*). In conclusion, in the BDL mice model, high BA is associated with a high E2 level, an effect that can be dampened by the loss of FXR.

FXR deletion up-regulated the expression of SULT1E1 in liver

To investigate the role of FXR in regulating E2 metabolism *in vivo*, we first studied the BA and E2 concentrations in WT and $FXR^{-/-}$ female mice. Compared with WT mice, $FXR^{-/-}$ mice showed elevated serum BA (Fig. 3*A*, *left panel*), which is consistent with a previous report that FXR deficiency leads to cholestasis (20). Surprisingly, a decreased E2 level was observed

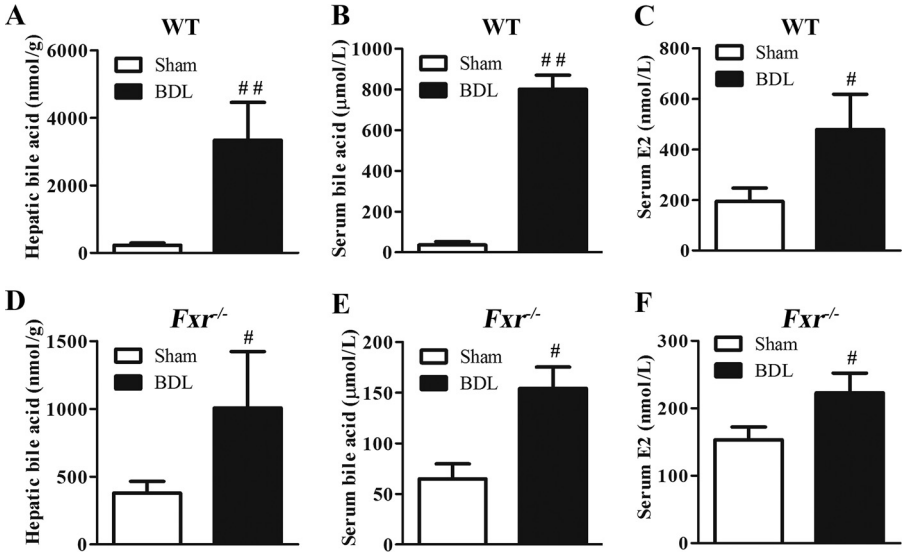

Figure 2. Elevated serum E2 level in a mouse cholestatic model. Liver and serum samples were collected 3 days after BDL or Sham operation in WT and *FXR*$^{-/-}$ female mice. *A–C,* hepatic bile acid, serum bile acid, and E2 level was assayed in WT mice. *D–F,* hepatic bile acid, serum bile acid, and E2 levels were also assayed in *FXR*$^{-/-}$ mice. All data represent mean ± S.D. of $n = 8$; #, $p < 0.05$ *versus* Sham; ##, $p < 0.01$ *versus* sham.

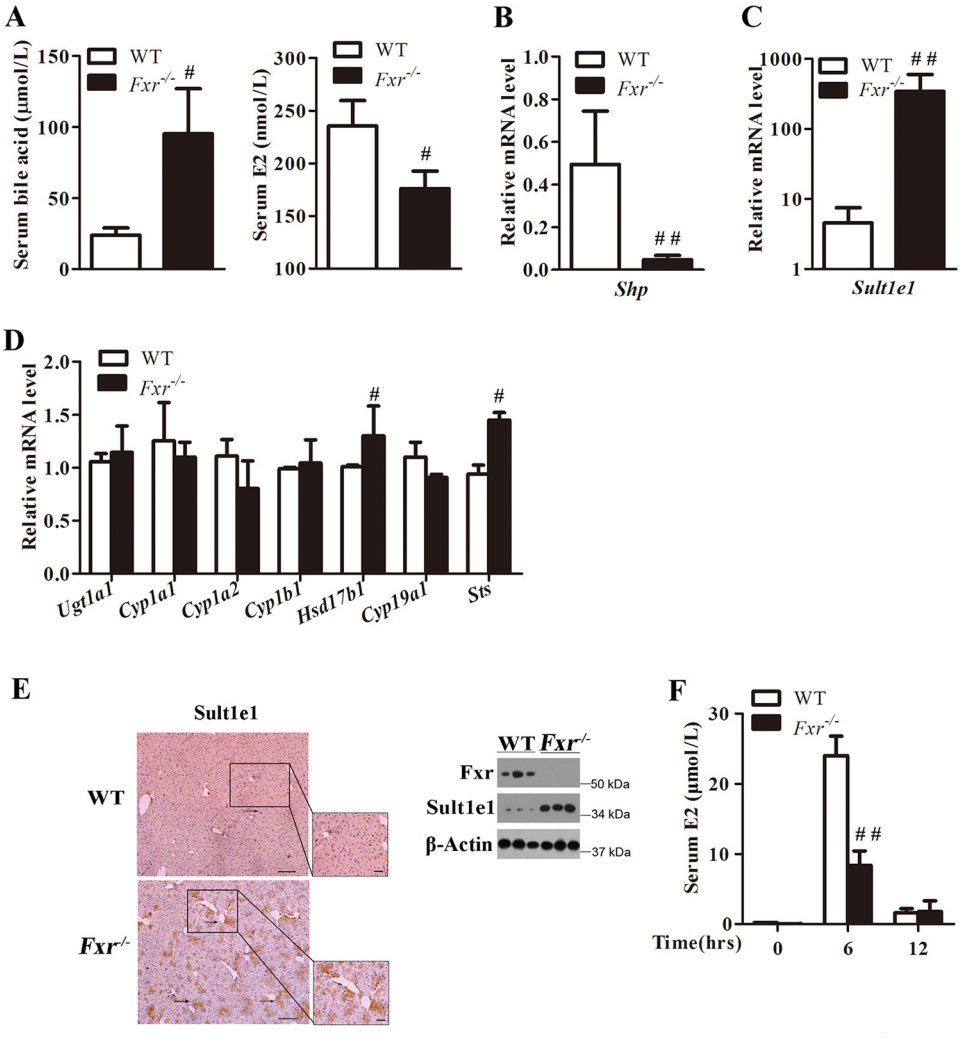

Figure 3. Hepatic *SULT1E1* level in wildtype (WT) and *FXR*-deficient mice. *A,* serum bile acid and E2 levels in WT and *FXR*$^{-/-}$ female mice. *B–D,* quantitative RT-PCR analysis of mRNA expression of FXR target gene *Shp* and estrogen metabolism genes including *SULT1E1 Ugt1a1*, *Cyp1a1*, *Cyp1a2*, *Cyp1b1*, *Hsd17b1*, *Cyp19a1*, and *Sts* in livers from WT and *FXR*$^{-/-}$ female mice. *E,* representative immunohistochemical analysis and Western blotting of SULT1E1 from livers of WT and *FXR*$^{-/-}$ female mice. *Scale bar*, 100 μm. *F,* clearance of exogenously administered E2 from serum was analyzed in WT and *FXR*$^{-/-}$ ovariectomized female mice. All data represent mean ± S.D. of $n = 6–8$; #, $p < 0.05$ *versus* sham; ##, $p < 0.01$ *versus* sham.

in $FXR^{-/-}$ mice (Fig. 3*A*, *right panel*). We next scanned the expression of key genes involved in estrogen metabolism in hepatic cells. Among the 8 primary estrogen-metabolizing enzymes, the transcription level of *SULT1E1* in $FXR^{-/-}$ mice increased dramatically by about 500-fold compared with that in WT mice (Fig. 3*C*). Aside for a slightly higher expression of *Hsd17b1* and *Sts* in $FXR^{-/-}$ mice, no difference in hepatic expression of *Ugt1a1*, *Cyp1a1*, *Cyp1a2*, *Cyp1b1*, and *Cyp19a1* between $FXR^{-/-}$ mice and WT mice was observed (Fig. 3*D*). The observed decrease of the small heterodimer partner (*Shp*, −94%), a known FXR target gene, in $FXR^{-/-}$ mice confirmed FXR deficiency (Fig. 3*B*). Immunohistochemistry and Western blotting confirmed the protein level increase of SULT1E1 in $FXR^{-/-}$ mice compared with that in WT mice (Fig. 3*E*). Interestingly, no difference was observed between $FXR^{-/-}$ mice and WT mice in Hsd17b1 and Sts protein expression levels (data not shown). SULT1E1 is a key enzyme in estrogen metabolism, and is responsible for the inactivation and elimination of E2 at physiological concentrations (21). We subsequently examined the SULT1E1 protein level in a panel of tissues from female mice. The SULT1E1 protein level was significantly increased in the kidney, liver, and colon from $FXR^{-/-}$ mice compared with that from WT mice (Fig. S3). We also measured FXR function in the clearance of exogenously administrated E2 in female mice. At 6 h after E2 administration, the E2 level in $FXR^{-/-}$ mice was significantly lower than that in the WT mice, whereas at 12 h no difference was observed (Fig. 3*F*).

FXR agonists reduced SULT1E1 expression in vitro and in vivo

We studied the effect of FXR agonists on *SULT1E1* expression in human liver carcinoma Huh7 cells. Real-time quantitative PCR and Western blotting revealed a significant decrease of SULT1E1 in both transcript and protein levels upon FXR agonist treatment (Fig. 4*A*). In contrast, FXR knockdown in Huh7 cells led to a significant increase of SULT1E1 at both mRNA and protein levels (Fig. 4*B*).

We further studied the regulation of the *SULT1E1* by bile acids in a noncholestatic model, WT and $FXR^{-/-}$ mice were fed for 5 days with either a normal chow diet (control) or a chow diet supplemented with 0.2% cholic acid (CA) (w/w). Upon CA feeding, reduced serum total cholesterol and triglyceride levels were observed in WT mice but not in $FXR^{-/-}$ mice (Fig. S4, *A–D*). A 0.2% CA supplementation led to a significant increase in serum E2 level and decrease in the hepatic *Su1t1e1* level in WT mice (Fig. 4*C*, *left panel*), but not in $FXR^{-/-}$ mice (Fig. 4*D*, *left panel*). No difference in hepatic expression of *Ugt1a1*, *Cyp1a1*, *Cyp1a2*, *Cyp1b1*, *Hsd17b1*, *Cyp19a1*, and *Sts* was observed between these two groups (Fig. 4, *C* and *D*, *right panels*). Similar findings were observed when WT and $FXR^{-/-}$ female mice were treated with either vehicle or WAY-362450 (30 mg/kg/day), a synthetic nonsteriodal FXR agonist (Fig. S5, *A–D*) (21). We further transduced the SULT1E1-expressing recombinant adenovirus (Ad-SULT1E1), which primarily targets the liver, into mouse through tail vein injection (22). Our results suggested that liver-specific *SULT1E1* overexpression abolished the 0.2% CA supplementation-induced E2 increase (Fig. 4*E*) and facilitated the clearance of exogenously administrated E2 in mice (Fig. 4*F*). Taken together, these data indicated that the FXR-induced E2 elevation is mediated through suppression of hepatic *SULT1E1*.

FXR repressed SULT1E1 by inhibiting HNF4α transactivation activity in vitro

We next searched for the putative FXR-binding sites in the intragenic regions upstream of the transcriptional start site of the *SULT1E1* gene in the human and mouse genome. No conservative FXR response element was present in the *SULT1E1* promoter. We then searched for the occurrence of putative FXR response elements of the *SULT1E1* promoter using the genome wide ChIP-seq datasets published by Thomas *et al.* (23) and Boergesen *et al.* (24). The FXR UCSC genome browser tracks did not show any FXR-binding signal in the intragenic regions upstream of the transcriptional start site of the *SULT1E1* gene. Kodama *et al.* (25) found that knockdown of hepatocyte nuclear factor 4α (HNF4α) reduced the SULT1E1 mRNA levels by 90%. Our results indicated that FXR activation can suppress SULT1E1 expression, an effect that cannot be reversed by co-treatment with the protein synthesis inhibitor cycloheximide (Fig. 5*A*). This indicated that mRNA synthesis, but not protein synthesis, was required for FXR-mediated repression of the *SULT1E1* gene. Interestingly, we also found that FXR strongly repressed HNF4α-stimulated *SULT1E1* promoter activity in a dose-dependent manner (Fig. 5*B*). Surprisingly, qChIP assay demonstrated that the occupancies of FXR were not changed at the *SULT1E1* promoter after treatment with WAY-362450 (Fig. 5*C*, *upper panel*). However, agonist-activated FXR remarkably reduced the binding of HNF4α to *SULT1E1* promoter (Fig. 5*C*, *middle panel*). Moreover, activated FXR enriched the H3K27me3 repressive mark in the *SULT1E1* promoter (Fig. 5*C*, *lower panel*). In contrast, FXR knockdown augmented the binding of HNF4α to the *SULT1E1* promoter and removed the H3K27me3 repressive mark in the *SULT1E1* promoter (Fig. 5*D*). The repressive function of agonist-activated FXR on *SULT1E1* promoter activity was abolished by deletion of the direct repeat half-sites (DRs), which were composed of three direct repeats of the motif GGACC and referred to as an HNF4α-response element (Fig. 5*E*)(25). EMSA experiments, using nuclear extract from FXR-transfected or FXR-activated Huh7 cells, demonstrated decreased binding capacity of HNF4α to the *SULT1E1* promoter via the HNF4α-response element (Fig. 6*A*). Interestingly, despite the observed direct interaction between FXR and HNF4α, we found that FXR did not inhibit the binding of HNF4a with HNF4α-response element *in vitro* (Fig. 6, *B* and *C*), a phenomenon that prompted us to examine whether FXR inhibited the HNF4α transactivation activity in an indirect way.

Acetylation is a key posttranslational modification that affects HNF4α transactivation activity. CBP-mediated acetylation is required for the proper nuclear retention of HNF-4α (26). In an attempt to identify the molecular basis of FXR-mediated inhibition of HNF4α transactivation activity, we examined the effect of FXR on the status of HNF4α acetylation in Huh7 cells. FXR activation decreased HNF4α acetylation (Fig. 6*D*, *left panel*). This event was not reversed by Sirtinol, an inhibitor of the Sirtuin class of deacetylases (data not shown), suggesting that deacetylation of HNF4α elicited by FXR activa-

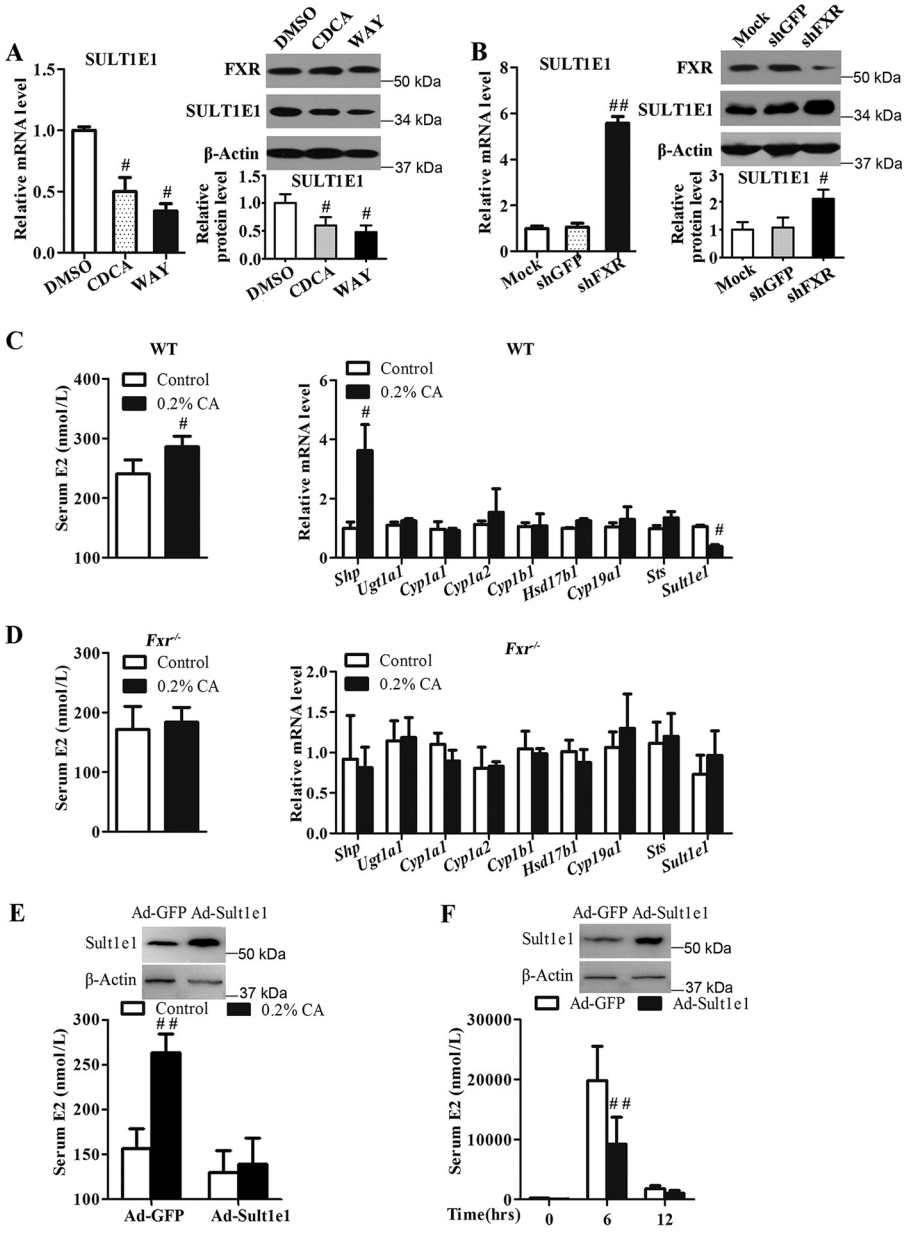

Figure 4. FXR activation reduced *SULT1E1* expression and increased E2 level. *A,* quantitative RT-PCR analysis (*left panel*) and Western blotting (*right panel*) of the SULT1E1 level in Huh7 cells treated with vehicle, CDCA (50 μM), and WAY-362450 (*WAY*, 3 μM) for 24 h. *B,* quantitative RT-PCR analysis (*left panel*) and Western blotting (*right panel*) of the SULT1E1 level in Huh7 cells following FXR shRNA transfection. *C,* WT and *FXR*$^{-/-}$ female mice were fed with 0.2% CA (w/w) mixed with normal chow for 5 days. Control female mice received normal rodent chow. Serum E2 level (*left panel*) and hepatic expression of the FXR target gene *Shp* and estrogen metabolism genes including *Ugt1a1*, *Cyp1a1*, *Cyp1a2*, *Cyp1b1*, *Hsd17b1*, *Cyp19a1*, *Sts,* and *SULT1E1* (*right panel*) were determined in WT mice. *D,* serum E2 level (*left panel*) and hepatic expression of FXR target gene *Shp* and estrogen metabolism genes (*right panel*) were determined in *FXR*$^{-/-}$ mice. *E, Sult1e1* overexpression was verified by Western blotting. Liver-specific *SULT1E1* overexpression abolished the 0.2% CA-induced E2 increase in female mice. Data are expressed as mean ± S.D. of $n = 14–18$. *F, Sult1e1* overexpression was verified by Western blotting. Liver-specific *SULT1E1* overexpression facilitated the clearance of exogenously administrated E2 in ovariectomized female mice. Data are expressed as mean ± S.D. of $n = 6–8$; #, $p < 0.05$ *versus* DMSO, Mock or control; ##, $p < 0.01$ *versus* Mock or adenovirus-green fluorescent protein (*Ad-GFP*).

tion might not result from an increase in deacetylase activity. Because both FXR and CBP can interact with HNF4α, we examined whether FXR competed with CBP for HNF4α binding. As expected, FXR activation increased its association with HNF4α, and prevented the interaction between CBP and HNF4α (Fig. 6*D*, *right panel*). Because the sites of acetylation are located at the HNF4α nuclear localization sequence region, we tested the role of FXR in HNF4α nuclear localization sequence. *In situ* immunofluorescence and biochemical fractionation experiments showed that FXR activation resulted in the translocation of HNF4α from the nucleus to cytoplasm in Huh7 cells (Fig. 6, *E* and *F*). In addition, FXR activation led to the translocation of HNF4α from the nucleus to cytoplasm in primary hepatocytes from WT mice, but not that from *FXR*$^{-/-}$ mice (Fig. S6).

Elevated bile acids provoked HNF4α translocation to cytoplasm and repressed SULT1E1 expression in cholestatic patients

Based on the previous studies, we hypothesized that FXR-mediated *SULT1E1* suppression might be involved in BA-in-

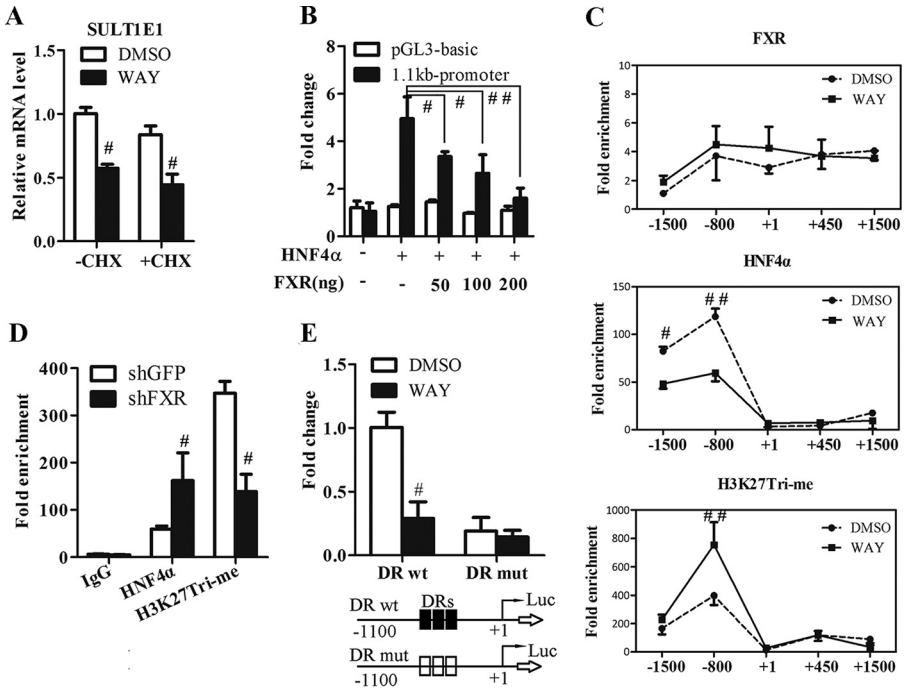

Figure 5. FXR disrupted the binding of HNF4α to *SULT1E1* promoter, and thereby repressed SULT1E1 transcription. *A, left panel*, quantitative RT-PCR analysis of *SULT1E1* level in Huh7 cells treated with vehicle or WAY-362450 in the presence or absence of CHX for 24 h. *B,* HEK293T cells were co-transfected with the 1.1-kb h*SULT1E1* promoter or pGL3-Basic reporter in the presence or absence of increasing amounts of FXR expression vector for 36 h. Luc activity was determined. *C,* Huh7 cells were treated with DMSO or WAY-363450 (3 μM), then subjected to qChIP assays using anti-FXR, anti-HNF4α, and anti-H3K27Tri-me antibodies. *D,* qChIP analysis of *SULT1E1* promoter in the Huh7 cells after transfection with control shRNA and shRNA targeting FXR using the indicated antibodies. *E,* Huh7 cells were transiently transfected with the 1.1-kb h*SULT1E1* promoter or its indicated mutant constructs and then treated with vehicle and WAY-362450 for 24 h. Luc activity was determined. All data are expressed mean ± S.D. of $n = 3–6$; #, $p < 0.05$ *versus* DMSO or shGFP; ##, $p < 0.01$ *versus* DMSO.

duced E2 levels of elevation during cholestasis. To test this hypothesis, *SULT1E1* expression was examined in liver tissues from patients suffering from biliary obstructions. In line with the increased level of total bile acids, the level of γ-GT, bilirubin, and E2 was also significantly higher in patients with cholestasis (Fig. 7*A* and Table S3). The levels of estrone sulfate, a SULT1E1 substrate, were also decreased in patients with cholestasis *versus* controls (Fig. 7*B*). In cholestasis patients, the *SULT1E1* mRNA level decreased by 89%, compared with controls (Fig. 7*C*, *left panel*). Western blot analysis and immunohistochemical detection revealed reduced protein levels of SULT1E1 in cholestatic patients *versus* controls (Fig. 7*D*, Fig. S7). Meanwhile, the expression of *CYP7A1* and *NTCP,* genes negatively regulated by FXR, decreased by 75 and 74%, respectively, in cholestatic patients. Furthermore, the expression of *BSEP,* a gene positively regulated by FXR, increased by 59% in cholestatic patients (Fig. 7*C*, *right panel*). These results indicated that FXR was activated in cholestatic patients. Moreover, EMSA demonstrated that the binding of HNF4α to the SULT1E1 promoter in liver samples from cholestatic patients was decreased compared with that from control livers (Fig. 7*E*, *upper panel*). Finally we demonstrated that obstructive cholestasis led to impaired HNF4α acetylation and the reduced HNF4α nuclear retention in liver, which was consistent with our *in vitro* studies (Fig. 7*E*, *middle* and *lower panels*).

Discussion

In this study, we identified the bile acid-activated nuclear receptor FXR as a major regulator of estrogen metabolism in liver. An elevated serum E2 level was observed in patients with high bile acid level. Notably, AST and ALT enzymes, which are specific markers for liver cell injury, were within normal range in 8 of 40 cholestatic patients who have elevated BA and E2 levels. In addition, no statistically significant difference of AST and ALT was observed between two groups of PBC patients, although their level of BA and E2 were significantly different. These results suggest that a high E2 level in cholestasis might be mainly due to a bile acid-activated regulatory cascade rather than due to liver cell injury. Indeed, therapeutic normalization of bile acid concentrations led to decreased serum estrogen levels. In addition, cholestatic patients' bile acid concentrations positively correlated with E2 levels. Moreover, BDL-treated mice showed an increased E2 level, and this effect was alleviated by FXR deletion. Because toxic bile acids may induce hepatocyte necrosis and apoptosis, we cannot exclude the possibility that, in addition to the primary effect of FXR, cholestatic hepatocellular injury could partially contribute to the E2 up-regulation during cholestasis. And the small increase of serum E2 level in BDL-treated $FXR^{-/-}$ mice might be due to toxicity of bile acid, which could directly kill hepatocytes, induce hepatic injury, and consequently inhibit hepatic metabolism of E2. It is noteworthy that E2 has been classified as a carcinogen by the International Agency for Research on Cancer, primarily based on its association with breast and endometrial carcinoma (4, 5, 27). Recently, an interesting case-control study by Costarelli *et al.* (28) showed that the plasma bile acids deoxycholic acid (DCA) level was 52% higher in postmenopausal patients with

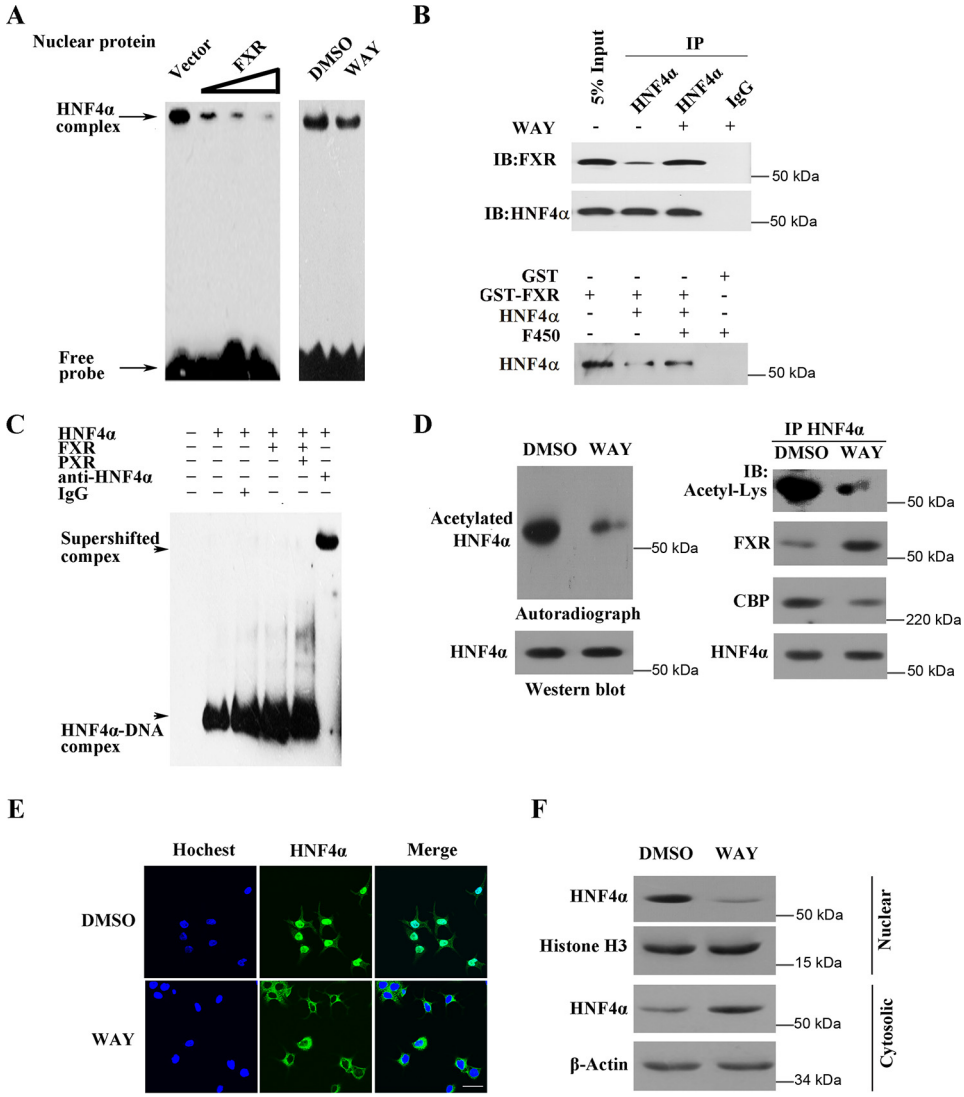

Figure 6. FXR competed with CBP for HNF4α binding, decreased HNF4α acetylation level, and increased its translocation to cytoplasm. *A,* EMSA demonstrated decreased HNF4α-binding activity to the HNF4α-response element in the *SULT1E1* promoter after FXR overexpression or WAY-362450 (*WAY*, 3 μM) treatment. *B,* co-immunoprecipitation in Huh7 cells and glutathione *S*-transferase–pulldown assay demonstrated the interaction of FXR and HNF4α. *C,* EMSA demonstrated that FXR or the FXR-RXR heterodimer did not interfere with the binding of HNF4α to the HNF4α-response element in the *SULT1E1* promoter. Normal IgG or anti-HNF4α antibody were used to verify the specific formation of the DNA–protein complex as indicated. *D,* decrease in the HNF4α/CBP interaction and HNF4α acetylation by the binding of FXR with HNF4α. Acetylation assay demonstrated the decreased HNF4α acetylation after treatment with DMSO or WAY-362450 (*left panel*). Huh7 cells were metabolically labeled with [^{3}H]acetate (1 mCi/ml), and whole cell extracts were pre-prepared, immunoprecipitated with HNF-4α antibody, and analyzed by SDS-PAGE (autoradiograph). Parts of the extracts were used for Western blotting with the same antibody (Western blotting). Immunoblottings (*IB*) for acetylated lysine, FXR, or CBP were performed on FXR immunoprecipitates prepared from Huh7 cells treated with DMSO or WAY-362450 (*right panel*). *E* and *F*, immunofluorescence (*E*) and Western blotting (*F*) demonstrated the translocation of HNF4α from nucleus to cytoplasm after WAY-362450 treatment. *Scale bar*, 20 μm.

breast cancer compared with healthy controls, supporting the concept of a correlation between bile acid disorders and estrogen-related disease.

Currently, attention has focused on the effect of bile acid-activated FXR in steroid hormone metabolism. Bile acids were shown to inhibit 5β-reduction of glucocorticoids and aldosterone through FXR-dependent 5β-reductase repression (29, 30). In addition, FXR was reported to up-regulate *HSD3B2,* which is a critical enzyme in the synthesis of aldosterone and cortisol (31). In our study, *SULT1E1*, an enzyme critical for metabolic estrogen deactivation, was found to be up-regulated by FXR. Indeed, deletion of FXR increased the hepatic *SULT1E1* level and accelerated the metabolic clearance of exogenous estrogen in female mice. In addition, CA or WAY-362450 treatment suppressed hepatic *SULT1E1* expression and increased the serum E2 levels in WT mice but not in *FXR*$^{-/-}$ female mice. Consistent with the *in vivo* data, our study showed that activation of FXR with CDCA or WAY-362450 potently reduced *SULT1E1* expression *in vitro*.

SULT1E1 is the primary sulfotransferase involved in estrogen sulfation at physiological concentrations (<10 nM) due to its low K_m value for E2 (21). Although other SULTs such as SULT1A1 and SULT2A1 only exhibit high activity toward E2 at nonphysiological concentrations. Consistent with the notion that SULT1E1 plays a critical role in modulating estrogen metabolism, disruption of SULT1E1 in female mice resulted in both local and systemic estrogen excess, leading to placental thrombosis and fetal loss (32). In our studies, liver-specific

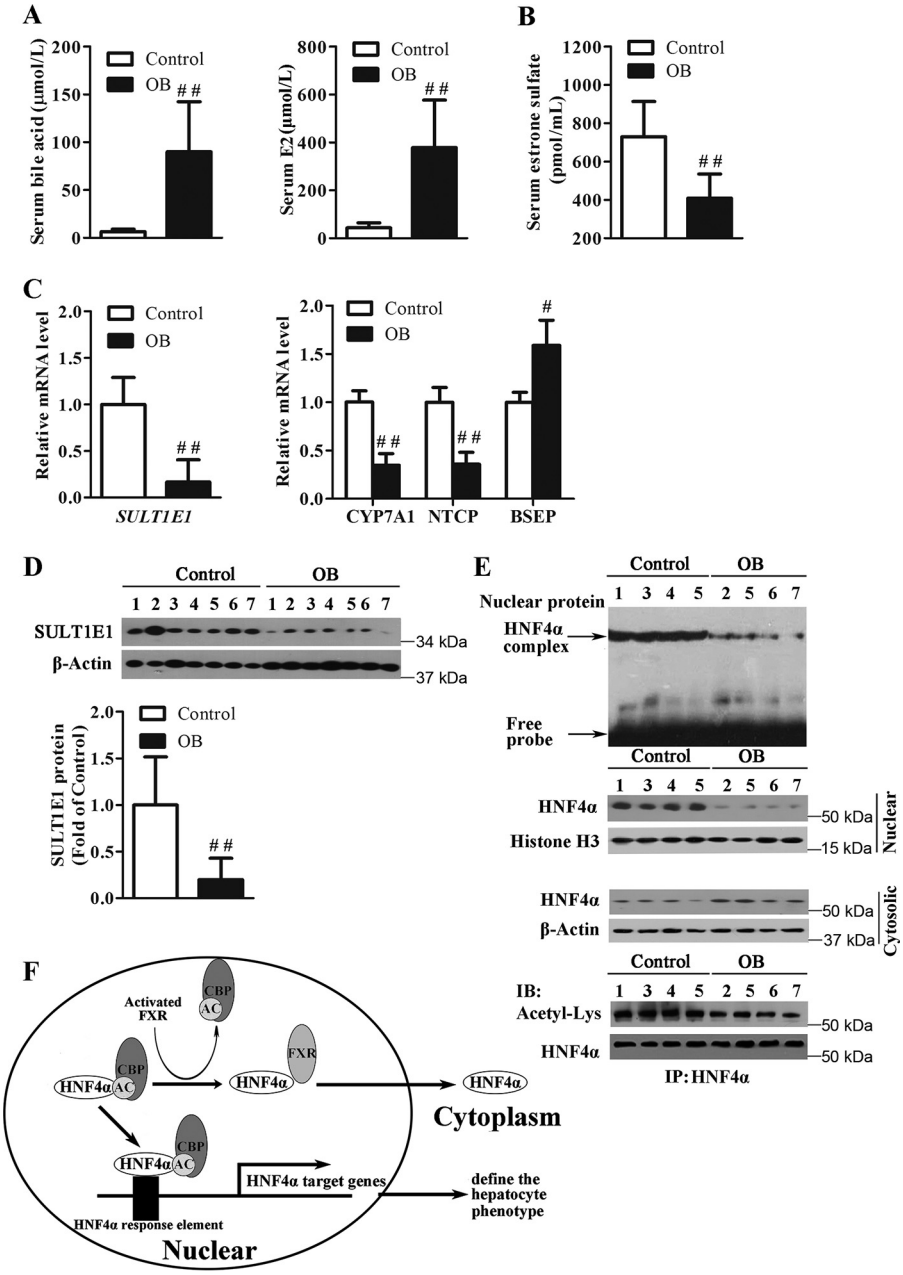

Figure 7. Increased expression of *SULT1E1* in obstructive cholestasis (*OB*) patient. *A* and *B,* serum bile acid, E2, and estrone sulfate concentrations in patients (control group, $n = 17$; OB group, $n = 14$). *C,* hepatic mRNA expression of *SULT1E1*, *CYP7A1*, *NTCP*, and *BSEP*. *D,* representative Western blotting and corresponding densitometry of hepatic SULT1E1. *E,* representative EMSA of HNF4α response elements in *SULT1E1* promoter using the nuclear extract of patient liver samples (*upper panel*). Representative Western blotting of nuclear and cytoplasmic HNF4α (*middle panel*) and HNF4α acetylation (*lower panel*). *F,* a proposed model of the nongenomic effect of FXR on the HNF4α transactivation activity. All data are expressed mean ± S.D.; #, $p < 0.05$ *versus* control; ##, $p < 0.01$ *versus* control.

SULT1E1 overexpression abolished the regulatory effect of CA feeding on E2 levels in female mice. This finding confirmed that the effect of FXR on E2 was SULT1E1-dependent. In patients with obstructive cholestasis, the accumulation of bile acids led to reduced mRNA and protein expression of hepatic SULT1E1, increased serum E2 levels, and decreased serum estrone sulfate concentration. Taken together, these data indicated that FXR-mediated *SULT1E1* suppression was involved in BA-associated E2 level increasing.

Previously, we reported that FXR activation attenuated liver inflammation and fibrosis via its genomic activities (14, 33–35). This study provides a novel mechanism by which FXR interacts and modulates the other transcription factor activity via its nongenomic activities (Fig. 7*F*). FXR competes with CBP for HNF4α binding and then inhibits HNF4α acetylation in hepatocytes, an effect that leads to the translocation of HNF4α from the nucleus to cytoplasm. HNF4α plays a central role in the coordination of the complex transcription factor network that defines the hepatocyte phenotype. Altered nuclear localization of HNF4α would have enormous consequences on most hepatic function, suggesting that potential mechanisms regulating HNF4α translocation are of great biological importance.

Pregnane X receptor (PXR), a closely related nuclear xenobiotic-activated receptor activated by a diverse array of lipophilic chemicals, was reported to repress the *SULT1E1* gene in human primary hepatocytes *in vitro* (25). Unlike FXR, PXR was not activated efficiently by primary bile acids, cholic acid, and chenodeoxycholic acid. Alternatively, PXR were activated efficiently by the secondary bile acid lithocholic acid (36). Notably, in a clinical cholestasis patient or BDL mouse, multiple primary bile acids accumulate, without a significant lithocholic acid increase (37). Consistent with a previous report, we found that liver-specific PXR knockdown did not prevent BDL-induced E2 elevation in mice (Fig. S8, *A–G*). BDL in adenoviral control shRNA-treated mice increased the E2 level by 133%, whereas BDL in adenoviral PXR shRNA#1- and shRNA#2-treated mice increased the E2 level by 107 and 116%, respectively. Thus, in cholestasis, FXR, but not PXR, appeared to play a critical role in the regulation of E2 level.

On the other hand, estrogens have been observed to alter bile acid homeostasis in human liver and to unmask cholestasis in asymptomatic individuals at risk (38). Consistent with a previous report, we observed a significant increase in hepatic and serum concentrations of BA after estrogen treatment (Fig. S9, *A* and *B*). Importantly, the elevated hepatic bile acid concentrations seemed to be caused by estrogen-related impairment in FXR activity. We observed a significant inhibition of FXR target gene transcription in mouse primary hepatocytes treated with estrogen (Fig. S9*D*). Specifically, we observed reduced expression of the transporter gene *Oatp2*, which is involved in import, and *Bsep*, which is involved in export, in bile acid homeostasis. In addition, we detected increased expression of bile acid synthesis genes *Cyp7b1* and *Cyp8b1*. Furthermore, we found that EE2 treatment facilitated the interaction between ERα and FXR (Fig. S9*C*). Exogenous E2 overrode the effect of FXR activation-induced *SULT1E1* suppression (Fig. S9*E*). These results are consistent with previous reports that ERα-mediated repression of FXR signaling may contribute to development of the estrogen-induced hepatotoxicity (38, 39).

Based on these findings, we propose the existence of an important cross-talk between bile acid/FXR and estrogen/Erα, a signaling pathway that may play a crucial role in the regulation of bile acid homeostasis and estrogen metabolism (Fig. S9*F*). These findings also suggest a novel role for elevated bile acids on liver dysfunction during cholestasis through inhibition of HNF4α signaling via the nongenomic effect of FXR.

Experimental procedures

Human samples

We conducted serum estrogen studies in postmenopausal female patients suffering from obstructive jaundice due to gallstones or malignancy (Table S1), by analyzing the serum level of E2, bilirubin, total bile acids, ALT, AST, and γ-GT in postmenopausal patients referred to for surgery or endoscopy. We measured the serum E2 levels again after successful treatment for jaundice and normalization of serum bilirubin. Serum estrogen studies in patients with PBC were conducted in postmenopausal females that are clinically and histologically defined as PBC (Table S2). Hepatic SULT1E1 studies in cholestatic patients were conducted using control liver samples obtained during resections for liver hemangioma, and liver metastases without cholestasis (Table S3). Cholestatic liver samples were surgically resected from patients with obstructive cholestasis caused by pancreas carcinoma, gallbladder carcinoma, and bile duct carcinoma. All human studies were approved by the Ethics Committee of Fudan University and Zhongshan Hospital, and were performed in accordance with the Helsinki Declaration. Informed consent was obtained from all participants. Subjects were excluded from the study if they manifested severe obesity (>100 kg) or ongoing alcohol consumption.

Serum parameters

Human serum bilirubin, ALT, AST, and γ-GT were assayed on a Roche Hitachi 917 analyzer (Roche Diagnostic GmbH). Serum E2 was determined using an ELISA kit (Cayman Chemical). Serum estrone sulfate was determined using an ELISA kit (Endocrine Technologies Inc., Newark, CA). Serum bile acid content was measured was measured enzymatically (14).

Animal studies

FXR null mice were purchased from The Jackson Laboratory. FXR null mice and all other mice were established on a C57BL/6 genetic background. All animal procedures were performed according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86–23, revised 1985). Studies were approved by the Shanghai Medical Experimental Animal Care Commission. See supporting Methods for details.

Construct generation

Human *SULT1E1* (h*SULT1E1*) promoter (GenBank™ accession number NM_005420.2, 1–1100 bp) containing an HNF4α-binding site was subcloned into pGL3-basic-luc (Promega) as described previously (25), The primers for 1.1-kb h*SULT1E1* promoter or its mutant construct were listed in Table S4. GFP-tagged murine SULT1E1 constructs were made as described previously (40). FXR and PXR knockdown studies were carried out using shRNA plasmids purchased from Abmgood Inc. and Santa Cruz Biotechology, respectively. Adenoviruses containing shRNAs were subcloned into and packaged with the pAD-Track/pAd-Easy system.

RNA extraction and quantitative real-time PCR (qPCR)

The qPCR was performed using primers described in Table S5.

qChIP assay

The qChIP assay was performed using primers described in Table S6. Huh7 cells were treated with vehicle or WAY-362450 (WAY, 3 μM) for 24 h. Cells were fixed in formaldehyde for 10 min, lysed, and sonicated to yield 500–1,000–bp DNA fragments. Sheared chromatin was immunoprecipitated using 2 μg of anti-FXR, anti-HNF4α, and anti-H3K27Tri-me antibodies. Protein A-Sepharose beads (Roche Applied Science) were used to capture the antibody–chromatin complex and washed with low salt, LiCl, and TE buffers. The enriched DNA was quanti-

fied by real-time PCR using human *SULT1E1* promoter qChIP primer. The amount of immunoprecipitated DNA is represented as signal relative to the unprecipitated DNA (input) chromatin.

Statistics

Statistical analysis was performed by two-tailed, unpaired Student's *t* test for comparison between 2 groups. Data are expressed as the mean ± S.D. Linear regression analysis was performed using the SPSS software (PASW Statistics 18, IBM, Chicago, IL). *p* values of less than 0.05 were considered significant. Additional materials and methods are shown in the supporting Methods.

Author contributions—X. L. and R. X. data curation; X. L., R. X., C. Y., S. C., and S. Z. formal analysis; X. L. methodology; X. L. and S. Z. project administration; R. X., S. C., and S. Z. funding acquisition; R. X. and C. Y. investigation; J. G. and S. Z. supervision; J. G. validation; J. G., S. C., and S. Z. writing-review and editing; S. C. visualization; S. C. and S. Z. writing-original draft; S. Z. resources.

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