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# The deletion of the estrogen receptor α gene reduces susceptibility to estrogen-induced cholesterol cholelithiasis in female mice

**Ornella de Bari**[1], **Helen H. Wang**[1], **Piero Portincasa**[2], **Min Liu**[3], and **David Q.-H. Wang**[1,4]

[1]Department of Internal Medicine, Division of Gastroenterology and Hepatology, Saint Louis University School of Medicine, St. Louis, MO 63104, USA

[2]Clinica Medica "A. Murri", Department of Biomedical Sciences and Human Oncology, University of Bari Medical School, Bari, Italy

[3]Department of Pathology and Laboratory Medicine, University of Cincinnati College of Medicine, Cincinnati, OH 45237, USA

## Abstract

Compelling evidence has demonstrated that estrogen is a critical risk factor for gallstone formation and enhances cholesterol cholelithogenesis through the hepatic estrogen receptor α (ERα), but not ERβ. To study the lithogenic mechanisms of estrogen through ERα, we investigated whether the deletion of *Erα* protects against gallstone formation in ovariectomized (OVX) female mice fed a lithogenic diet and treated with 17β-estradiol ($E_2$) at 0 or 6 μg/day for 56 days. Our results showed that the prevalence of gallstones was reduced from 100% in OVX ERα (+/+) mice to 30% in OVX ERα (−/−) mice in response to high doses of $E_2$ and the lithogenic diet for 56 days. Hepatic cholesterol secretion was significantly diminished in OVX ERα (−/−) mice compared to OVX ERα (+/+) mice even fed the lithogenic diet and treated with $E_2$ for 56 days. These alterations decreased bile lithogenicity by reducing cholesterol saturation index of gallbladder bile. Immunohistochemical studies revealed that ERα was expressed mainly in the gallbladder smooth muscle cells. High levels of $E_2$ impaired gallbladder emptying function mostly through the ERα and cholecystokinin-1 receptor pathway, leading to gallbladder stasis in OVX ERα (+/+) mice. By contrast, gallbladder emptying function was greatly improved in OVX ERα (−/−) mice. This markedly retarded cholesterol crystallization and the growth and agglomeration of solid cholesterol crystals into microlithiasis and stones. In conclusion, the deletion of *Erα* reduces susceptibility to the formation of $E_2$-induced gallstones by diminishing hepatic cholesterol secretion, desaturating gallbladder bile, and improving gallbladder contraction function in female mice.

[4]Address correspondence to: David Q.-H. Wang, M.D., Ph.D., Department of Internal Medicine, Division of Gastroenterology and Hepatology, Saint Louis University School of Medicine, St. Louis, MO 63104. Phone: (314) 977-8737, Fax: (314) 977-9909, dwang15@slu.edu.

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**Keywords**

bile flow; bile salts; biliary secretion; cholesterol crystallization; gallbladder motility; *Lith* gene

## INTRODUCTION

Epidemiological and clinical studies have clearly shown that women are twice as likely as men to suffer from cholesterol cholelithiasis at all ages in every population investigated. This gender difference begins since puberty and continues through the childbearing years. Most, but not all, studies have found that the use of oral contraceptive steroids and conjugated estrogens markedly increases cholelithogenesis in premenopausal women, leading to hepatic cholesterol hypersecretion and biliary lithogenicity [1–4]. Because plasma concentrations of female sex hormones, especially estrogen, increase positively and linearly with the duration of gestation, the risk of gallstone formation becomes higher in the third trimester of pregnancy. Increasing parity is also a risk factor for gallstones, especially in younger women. Notably, parity and length of the fertility period increase the incidence of gallstones [5], as well as both the number and frequency of pregnancy are important risk factors for gallstone formation [6–10]. Thus, elevated estrogen levels are a critical risk factor for the formation of cholesterol gallstones during pregnancy. Moreover, biliary sludge (a precursor to gallstones) and microlithiasis are observed to disappear spontaneously after parturition in ~60% of cases mostly because of a significant reduction in estrogen concentrations [11]. Similar lithogenic effects are also found during estrogen therapy in postmenopausal women and in men with prostatic carcinoma [12–16]. All of these findings underscore the importance of estrogen on the pathogenesis of gallstones.

As found from human and animal studies, high levels of estrogen enhance susceptibility to cholesterol cholelithiasis by promoting hepatic hypersecretion of biliary cholesterol and increasing bile lithogenicity [17–22]. Such alterations lead to a dramatic increment in cholesterol saturation of bile, thereby increasing risk of developing gallstones [1, 23–25]. It has been established that estrogen plays a critical role in enhancing cholelithogenesis by activating the hepatic estrogen receptor α (ERα), but not ERβ [26]. While ERα is activated by $E_2$, mice still keep synthesizing cholesterol in the liver even though excess amounts of cholesterol are available from a high-cholesterol diet. These metabolic abnormalities indicate a disruption in the negative feedback regulation of cholesterol synthesis in the liver, which is determined by an “estrogen-ERα-SREBP-2” pathway [27]. As a result, this induces excess amounts of newly synthesized cholesterol available for biliary hypersecretion, thus leading to cholesterol-supersaturated bile, as well as rapid cholesterol crystallization and gallstone formation in mice treated with high doses of $E_2$ [26]. Based on these observations, a novel concept has been proposed that estrogen enhances cholesterol cholelithogenesis through the ERα signaling pathway in the liver and that higher risks for gallstone formation in women than in men are related to differences in how cholesterol is handled in the liver under conditions of high estrogen levels [28].

Because ERα has a crucial effect on the formation of $E_2$-induced cholesterol gallstones, in the current study, we studied whether the targeted disruption of the *Erα* gene protects

against the formation of gallstones in mice treated with high doses of $E_2$ and fed a lithogenic diet for 56 days. Our results show that the susceptibility to $E_2$-induced cholesterol cholelithiasis is significantly reduced through the ERα-mediating pathways in the liver and gallbladder in ovariectomized (OVX) ERα (−/−) mice even treated with $E_2$ and fed the lithogenic diet for 56 days.

## MATERIALS AND METHODS

### Animals and diets

The inbred AKR/J mice of both genders from the Jackson Laboratory (Bar Harbor, ME) were bred to generate female mice for the studies. Although AKR/J mice are a gallstone-resistant strain, they are still susceptible to estrogen-induced cholesterol gallstones [26]. AKR/J mice have intact expression of the *Erα* and *Erβ* genes; however, expression levels of *Erβ* are 50-fold lower than those of *Erα* in the liver [26]. Thus, the AKR/J strain was used as control mice, i.e., ERα (+/+) mice. The ERα (+/−) mice of both genders on a C57BL/6J genetic background (the Jackson Laboratory) were crossed with AKR/J mice for ten generations to produce a new strain of ERα (+/−) mice on an AKR/J genetic background. We have established breeding colonies of these mice in-house. Of note, ERα (+/−) heterozygotes are healthy and fertile, and they exhibit no obvious phenotypes in association with the disrupted *Erα* genotype. A cross between heterozygous ERα (+/−) mice resulted in the live birth of normal litter sizes of homozygous ERα (−/−) mice.

To exclude possible interindividual differences in endogenous estrogen concentrations, at 4 weeks of age, all female mice were ovariectomized (OVX). At 8 weeks of age, the mice were implanted subcutaneously with pellets releasing $E_2$ at 0 or 6 μg/day for 56 days (Innovative Research of America, Sarasota, FL) according to our published methods [26]. All animals were maintained in a temperature-controlled room (22±1°C) with a 12-hour day cycle (0600 h – 1800 h) and were provided free access to water and normal mouse chow containing trace cholesterol (<0.02%) (Lab Rodent Diet, St. Louis, MO). For gallstone studies, mice at 8 weeks old were fed a lithogenic diet containing 1% cholesterol, 15% butter fat, and 0.5% cholic acid for 56 days. All procedures were in accordance with current NIH guidelines and were approved by the Institutional Animal Care and Use Committee of Saint Louis University (St. Louis, MO).

### Microscopic studies of gallbladder bile and gallstones

After anesthetization with pentobarbital, a cholecystectomy was performed on fasted mice. Gallbladder bile was collected before (day 0, on chow) and at frequent intervals after feeding the lithogenic diet for 3, 6, 9, and 12 days for cholesterol crystallization studies (n=5 per group for each time point), as well as for 56 days for gallstone studies (n=20 per group). The entire gallbladder bile was studied by phase contrast and polarized light microscopy for observing the presence of mucin gel, liquid crystals, solid cholesterol crystals, sandy stones, and gallstones [29]. The images of cholesterol monohydrate crystals and gallstones were analyzed by a Carl Zeiss Imaging System with an AxioVision Rel 4.6 software (Carl Zeiss Microimaging GmbH, Göttingen, Germany). After microscopic analysis, gallbladder bile was frozen and stored at −20°C for lipid analyses.

### Measurement of biliary lipid output

During laparotomy, the common bile duct was cannulated with a PE-10 polyethylene catheter. After successful catheterization and flow of fistula bile, cholecystectomy was performed. The first hour sample of hepatic bile was collected by gravity in mice (n=5 per group) [30]. Subsequently, hepatic bile was examined by polarizing light microscopy and the volume of each bile sample was measured. During surgery and hepatic bile collection, mouse body temperature was kept at 37 ± 0.5°C with a heating lamp and monitored with a thermometer. All bile samples were frozen and stored at −20°C for further lipid studies.

### Dynamic measurement of gallbladder emptying function

To explore whether the activation of ERα by $E_2$ impairs gallbladder contractility, a dynamic measurement of gallbladder motility function was performed in mice in response to a high-fat meal or to exogenously administered sulfated cholecystokinin octapeptide (CCK-8) at 56 days on the lithogenic diet [31]. After mice (n=5 per group) were fasted overnight but had free access to water, they were anesthetized with pentobarbital. During laparotomy, a PE-10 catheter was inserted into the duodenum. It was externalized through the left abdominal wall and connected to an infusion pump for infusing corn oil. The right jugular vein was cannulated with a PE-10 catheter for intravenous CCK-8 injection. After all surgical procedures were completed, the gallbladder was clearly exposed and its volume was measured with a micro-caliper according to previously published methods [31].

After the first group of mice was intraduodenally infused with corn oil (i.e., a high-fat meal) or 0.9% NaCl (as a control) at 40 μL/minute for 5 minutes, postprandial gallbladder volume was determined at 30 minutes after the infusion. The second group of mice was intravenously injected through the jugular vein with exactly 17 nmol/kg body weight of CCK-8 dissolved in 100 μL of phosphate buffered saline (PBS) solution, or 100 μL of only PBS solution (as a control). Following that, gallbladder volume was measured every 10 minutes for 30 minutes. Gallbladder emptying rate was determined by a difference in gallbladder size before and after the duodenal infusion of corn oil or the intravenous injection of CCK-8.

### Biliary lipid analysis

Biliary phospholipids were determined as inorganic phosphorus by the method of Bartlett [32]. Biliary cholesterol was measured using an enzymatic assay [33]. Total bile salt concentration was enzymatically determined by the 3α-hydroxysteroid dehydrogenase method [34]. Individual bile salt species were analyzed by high-performance liquid chromatography (HPLC) [35] and hydrophobicity index of bile samples was calculated according to Heuman's method [36]. Cholesterol saturation index (CSI) of pooled gallbladder bile was calculated from critical tables [37]. Relative lipid composition of pooled gallbladder bile was plotted on a condensed phased diagram. For graphic analysis, the phase limits of the micellar zone and the crystallization pathways were extrapolated from model bile systems developed for taurocholate at 37°C and at a total lipid concentration of 9 g/dL [38].

### Immunohistochemical staining

The blocks of paraffin-embedded gallbladder tissues were cut at 4-μm thickness, dewaxed, and rehydrated. For ERα staining, antigen retrieval was carried out by boiling in 10 mM citrate buffer (pH 7.0) for 1 minute. All the staining processes were performed by using Histostain Plus 3rd Gen IHC Detection Kit according to the manufacturer's instructions (Invitrogen, Camarillo, CA). The sections were incubated with anti-ERα antibody (Santa Cruz, Dallas, TX) at a dilution of 1:100. The primary antibody was replaced by the blocking solution containing 10% non-immune goat serum for negative control slides. After washing, the sections were incubated with the corresponding secondary antibody for 30 minutes at room temperature. Subsequently, the sections were counterstained with hematoxylin, dehydrated through an alcohol series to xylene, and mounted.

### Quantitative real-time PCR assay

Total RNA was extracted from mouse liver and gallbladder tissues (n=4 per group) using RNeasy Midi (Qiagen, Valencia, CA). Reverse-transcription reaction was performed using the iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Hercules, CA) with 1 μg of total RNA and random hexamers to generate cDNA. Quantitative real-time PCR assays were performed in triplicate for the *Erα* gene and the genes involved in the regulation of hepatic lipid metabolism, including ATP-binding cassette transporter G5 (*Abcg5*), *Abcg8*, *Abcb4*, *Abcb11*, cholesterol 7α-hydroxylase (*Cyp7a1*), sterol 27-hydroxylase (*Cyp27a1*), acyl-CoA:cholesterol acyltransferase, isoform 2 (*Acat2*), sterol-regulatory element binding protein-2 (*Srebp-2*), 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (*Hmgcr*), farnesyl diphosphate synthase (*Fdps*), lathosterol synthase (*Lss*), squalene synthase (*Sqs*), HMG-CoA synthase, isoform 1 (*Hmgcs1*), *Hmgcs2*, and low-density lipoprotein receptor (*Ldlr*), as well as for gallbladder *Erα*, cholecystokinin-1 receptor (*Cck-1r*), *Acat2*, *Abcg5*, *Abcg8*, and Niemann-Pick C1-like 1 (*Npc1l1*). The sequences of the primers for these genes are listed in Table 1. Relative mRNA levels were calculated using the threshold cycle of an unknown sample against a standard curve with known copy numbers. To obtain a normalized target value, the target amount was divided by the endogenous reference amount of mouse *β-Actin* as internal control.

### Statistical method

All data are expressed as mean ± SD. Statistically significant differences among groups of mice were assessed by Student's *t*-test, Mann-Whitney U-tests, or Chi-square tests. If the F-value was significant, comparisons among groups of mice were further analyzed by a multiple comparison test. Analyses were performed with *SuperANOVA* software (Abacus Concepts, Berkeley, CA). Statistical significance was defined as a two-tailed probability of less than 0.05.

## RESULTS

### Prevalence and characteristic of gallstones

Figure 1A shows representative photomicrographs of amorphous mucin gel, liquid crystals, solid cholesterol crystals, and gallstones as observed by polarizing light microscopy in

gallbladder bile of OVX ERα (+/+) and (−/−) mice. At 56 day on the lithogenic diet, gallbladders of OVX ERα (+/+) mice formed a thick layer of sticky mucin gel (100%) interspersed with solid plate-like cholesterol monohydrate crystals (90%) and aggregated and fused liquid crystals (50%). In contrast, gallbladders of OVX ERα (−/−) mice contained a thin layer of mucin gel (100%), aggregated and fused liquid crystals (60%), and cholesterol monohydrate crystals (30%). As shown in Figure 1B, gallstone prevalence in OVX ERα (+/+) mice (100%) is significantly higher than that in OVX ERα (−/−) mice (30%). Assessment of gallstone frequency in OVX ERα (+/+) mice found that the number distribution fell between 1 and 3 in 50% of the mice and between 4 and 6 in 40% of the mice, while 10% of the mice formed more than 10 stones. Moreover, 20% of OVX ERα (−/−) mice had 1–3 stones and 10% of them harbored 4–6 stones. Of special note, 70% of OVX ERα (−/−) did not have gallstones in the gallbladder.

## Lipid composition and CSI values of gallbladder bile

Table 2 lists relative biliary lipid composition of pooled gallbladder bile in mice fed chow (day 0) and the lithogenic diet at different time points. At 6 days on the lithogenic diet, the CSI value reached supersaturated in OVX ERα (+/+) mice. By contrast, gallbladder bile was unsaturated at 6 days in OVX ERα (−/−) mice. Figure 1C shows that during the 56-day period of the lithogenic diet feeding, relative lipid composition of gallbladder bile gradually moves upward and to the right of the phase diagram in both OVX ERα (+/+) and (−/−) mice. Such a change was induced by a relative increase in cholesterol and phospholipid concentrations and relatively unchanged bile salt concentrations. Because relative lipid composition of bile passed through crystallization region B in OVX ERα (+/+) mice between 6 and 12 days on the lithogenic diet, tubular crystals (i.e., anhydrous cholesterol crystals) were found first, followed by classical plate-like cholesterol monohydrate crystals (Figure 1A). By contrast, relative lipid composition of bile in OVX ERα (−/−) mice directly entered crystallization region C from the micellar zone during lithogenesis. In the region C, liquid crystals always appeared before cholesterol monohydrate crystals.

When gallbladder bile from lithogenic diet-fed mice was analyzed by HPLC, OVX ERα (+/+) and (−/−) mice showed similar distributions of the molecular species of bile salts. Moreover, all bile salts were taurine conjugated. Because 0.5% cholic acid in the lithogenic diet was fed to mice, taurocholate became the major bile salt (54.8±5.0% in ERα (+/+) mice vs 50.5±6.3% in ERα (−/−) mice), followed by diminished proportions of taurochenodeoxycholate (24.5±4.3% vs 32.4±7.3%) and taurodeoxycholate (9.6±1.6% vs 8.5±1.1%). Low concentrations of tauro-β-muricholate (5.3±0.9% vs 4.5±0.4%), tauroursodeoxycholate (4.9±2.3% vs 3.2±0.7%), and tauro-ω-muricholate (0.9±0.2% vs 0.9±0.3%) were also found in two groups of mice. Hydrophobicity indexes of gallbladder bile specimens (0.10±0.03 vs 0.14±0.03) were comparable between OVX ERα (+/+) and (−/−) mice.

## Biliary lipid output and bile flow

Figure 2 exhibits biliary lipid output in OVX ERα (+/+) and (−/−) mice during the first hour after interruption of the enterohepatic circulation. Biliary cholesterol and phospholipid output was significantly lower in OVX ERα (−/−) mice (10.9±2.7 μmol/h/kg and 35.8±7.5

μmol/h/kg) than in OVX ERα (+/+) mice (24.6±8.0 μmol/h/kg and 58.6±11.0 μmol/h/kg). Biliary bile acid output was also reduced in OVX ERα (−/−) mice (217.0±69.6 μmol/h/kg) than in OVX ERα (+/+) mice (279.7±59.9 μmol/h/kg), but a significantly statistical difference was not appreciated. The ratios of cholesterol/phospholipid and cholesterol/bile salts were significantly reduced in OVX ERα (−/−) mice than those in OVX ERα (+/+) mice, indicating that bile cholesterol saturation was significantly lower in the former than in the latter. At 56 days on the lithogenic diet, bile flow rate was determined during the first hour of hepatic bile collection, thus avoiding appreciable perturbation of the enterohepatic circulation of bile salts. Bile flow rates were comparable between OVX ERα (+/+) and (−/−) mice.

### Effect of $E_2$ on expression of the genes involved in the regulation of lipid metabolism in the liver

In Figure 3, we focused mainly on the comparison of mRNA levels of these genes between OVX ERα (+/+) and (−/−) mice treated with $E_2$ at 0 or 6 μg/day and fed the lithogenic diet for 56 days. Of special note is that no significant differences in expression of these genes were found between OVX ERα (+/+) and (−/−) mice receiving no $E_2$ and fed the lithogenic diet for 56 days.

It has been reported that $E_2$ binds with high affinity to ERα and regulates its expression. Compared to OVX ERα (+/+) mice receiving no $E_2$, hepatic expression of the *Erα* gene significantly increased in OVX ERα (+/+) mice. As expected, expression of *Erα* in the liver was not detected in OVX ERα (−/−) mice, regardless of whether or not these mice were treated with $E_2$ for 56 days.

In the lithogenic state, $E_2$ significantly increased hepatic expression of *Abcg5* and *Abcg8* in OVX ERα (+/+) mice compared to OVX ERα (+/+) mice receiving no $E_2$. However, mRNA levels of *Abcg5/g8* were significantly reduced in OVX ERα (−/−) mice compared to those in OVX ERα (+/+) mice. Moreover, changes in hepatic expression of *Abcb4* and *Abcb11* displayed the same trend as that of *Abcg5/g8* for OVX ERα (+/+) and (−/−) mice. It has been recognized that ABCG5/G8, ABCB4, and ABCB11 are the transporters on the canalicular membrane of hepatocytes for hepatic secretion of biliary cholesterol, phospholipids, and bile salts, respectively. Furthermore, CYP7A1 and CYP27A1 are two rate-limiting enzymes regulating the classical and the alternative pathways of bile salt synthesis in the liver. Hepatic expression of *Cyp7a1*, but not *Cyp27a1* was significantly reduced only in OVX ERα (−/−) mice. At 56 days on the lithogenic diet, expression of *Srebp2* and *Ldlr* was significantly increased in OVX ERα (+/+), but not (−/−) mice compared to those in OVX ERα (+/+) mice receiving no $E_2$. Moreover, mRNA levels of *Hmgcs1*, *Hmgcs2*, *Hmgcr*, *Fdps*, *Sqs*, and *Lss*, which are involved in cholesterol synthesis, were significantly higher in OVX ERα (+/+) mice than in OVX ERα (−/−) mice. Notably, hepatic expression of *Acat2* remained the same in these groups of OVX mice.

### Effect of $E_2$ on gallbladder contract function

Figure 4A shows fasting gallbladder volumes as functions of days on the lithogenic diet for 56 days. At day 0 on chow, fasting gallbladder volumes were basically the same between

OVX ERα (+/+) and (−/−) mice. However, after 3 days on the lithogenic diet, the gallbladder volume began to increase in OVX ERα (+/+) mice and this persisted thereafter. Obviously, this increase in gallbladder volumes paralleled an increment of CSI values in bile. Overall, in the lithogenic state, gallbladder volumes were dramatically enlarged in OVX ERα (+/+) mice compared to those in OVX ERα (−/−) mice. Figure 4B shows changes in postprandial residual gallbladder volumes in response to the fatty meal at 56 days on the lithogenic diet. Notably, a significant portion of gallbladder bile was emptied out in OVX ERα (−/−), but not (+/+) mice. Postprandial residual gallbladder volumes were significantly larger in OVX ERα (+/+) mice compared to OVX ERα (−/−) mice. These results showed that gallbladder emptying function was impaired in $E_2$-treated OVX ERα (+/+) mice.

Figure 5A, exhibits that gallbladder emptying rate is reduced in OVX ERα (+/+) mice in response to intravenous administration of CCK-8. In contrast, a large portion of gallbladder bile was excreted in OVX ERα (−/−) mice. As a result, at 30 min after CCK-8 administration, residual gallbladder volumes were significantly smaller in OVX ERα (−/−) mice than in OVX ERα (+/+) mice. Furthermore, the concentrations of cholesterol and cholesteryl esters in the gallbladder wall were significantly lower in the former than in the latter (Figure 5B). Of special note, at 56 days of $E_2$ treatment and feeding the lithogenic diet, mRNA levels of *Cck-1r* in the gallbladder were significantly lower in OVX ERα (+/+) mice than in OVX ERα (−/−) mice (Figure 5C). The mRNA levels of *Npc1l1*, *Abcg5* and *Abcg8* (the gallbladder lipid transporters) and *Acat2* (for cholesterol esterification) in the gallbladder were significantly higher in OVX ERα (+/+) mice than those in OVX ERα (−/−) mice. High expression of *Abcg5* and *Abcg8* in OVX ERα (+/+) mice may be secondary to increased accumulation of cholesterol in the gallbladder.

### Expression of ERα in the gallbladder by immunohistochemistry

As shown in Figure 6, ERα is expressed predominately in the gallbladder smooth muscle cells by immunohistochemical staining in chow-fed female ERα (+/+) mice with intact ovaries. In contrast, the expression of ERα was not detected in the gallbladder in chow-fed female ERα (−/−) mice with intact ovaries (data not shown).

## DISCUSSION

A large number of human and animal studies have found that $E_2$ plays a critical role in promoting cholesterol gallstone formation. Although $E_2$ has a crucial effect on the transcriptional modulation of target genes through two classical ERs (i.e., ERα and ERβ), ERα is the key steroid hormone receptor exerting the biological effect of $E_2$ in the liver [26]. The important findings of the present study are summarized as follows: (i) the targeted disruption of the *Erα* gene reduces susceptibility to $E_2$-induced gallstones by diminishing hepatic synthesis and biliary secretion of cholesterol, thus desaturating bile and reducing CSI values; and (ii) the deletion of the *Erα* gene prevents the inhibitory effect of $E_2$ on gallbladder emptying function mostly through the ERα and CCK-1R pathway, thereby deterring cholesterol crystallization and the growth and agglomeration of solid cholesterol crystals into microlithiasis and stones.

One of the mechanisms underlying the lithogenic effect of $E_2$ on hepatic cholesterol hypersecretion is that the activity of HMG-CoA reductase, the rate-limiting enzyme in hepatic cholesterol synthesis, is increased, leading to excess amount of cholesterol from hepatic *de novo* synthesis for biliary hypersecretion [1, 27, 39, 40]. It is well known that the high-cholesterol diet inhibits hepatic cholesterol synthesis by a negative feedback regulation pathway through the SREBP-2 signaling cascade. However, even under a high-cholesterol diet, expression of *Srebp-2* and the SREBP-2-responsive genes is still significantly higher in $E_2$-treated OVX ERα (+/+) mice. These findings indicate an "$E_2$-ERα-SREBP-2" pathway for enhancing cholesterol synthesis in the liver [27]. Therefore, in the face of high $E_2$ levels, there is a continuous cholesterol synthesis in the liver because the negative feedback regulation of its synthesis determined by the SREBP-2 signaling pathway is interrupted by $E_2$ through the hepatic ERα [27]. In addition, $E_2$ increases the delivery of intestinal cholesterol to the liver for biliary hypersecretion by promoting intestinal cholesterol absorption mostly through the intestinal ERα [1, 41]. These metabolic abnormalities in the liver and small intestine lead to hepatic cholesterol hypersecretion and supersaturated gallbladder bile that predisposes to rapid cholesterol crystallization and precipitation of solid cholesterol monohydrate crystals. Our published results have shown that the activation of ERα by $E_2$ enhances hepatic cholesterogensis in OVX ERα (+/+) mice, regardless of whether the chow or the high (1%) cholesterol diet is fed [27]. In the current study, we found that hepatic cholesterol output and CSI values are significantly reduced in OVX ERα (−/−) mice compared to OVX ERα (+/+) mice even in response to high doses of $E_2$ and the lithogenic diet. These results indicate that ERα is crucial to the negative feedback regulation of cholesterol synthesis in the liver and plays a key role in connecting $E_2$ with the SREBP-2 signaling cascade for regulating cholesterol synthesis. Furthermore, our findings, for the first time, show the importance of newly-synthesized cholesterol as a key source for hepatic hypersecretion in $E_2$-treated OVX ERα (+/+) mice.

The *Abcg5/g8* has already been identified as a candidate gene in the mouse *Lith9* locus by quantitative trait locus (QTL) linkage analysis [42]. Subsequently, the *ABCG5/G8* gene was proved to be associated with gallstones in patients (i.e., human *LITH9*) [43]. Moreover, two gallstone-associated variants in *ABCG5/G8* (*ABCG5*-R50C and *ABCG8*-D19H) were identified not only in Germans and Chileans, but also in Chinese and Indians [43–48]. We found that expression of *Abcg5/g8* in the liver is significantly increased in $E_2$-treated OVX ERα (+/+) mice, coupled with hepatic hypersecretion of biliary cholesterol. In contrast, the deletion of *Erα* disrupts $E_2$-mediating up-regulation of hepatic *Abcg5/g8* so that biliary cholesterol secretion is significantly reduced in $E_2$-treated OVX ERα (−/−) mice even fed the lithogenic diet for 56 days. This shows that the activation of ERα by $E_2$ has a role in the transcriptional regulation of *Abcg5/g8*, thus influencing hepatic secretion of biliary cholesterol. We also observed that expression of *Abcb4* was reduced in $E_2$-treated OVX ERα (−/−) mice, coupled with a diminished hepatic output of biliary phospholipids. This shows that ERα activated by $E_2$ may stimulate ABCB4 for regulating biliary phospholipid secretion.

It is widely appreciated that the gallbladder also plays a pivotal role in the pathogenesis of gallstones [49]. However, the mechanisms underlying the lithogenic effect of $E_2$ on

impairing gallbladder motility function are not fully understood. Clinically, real-time ultrasonography has found that fasting gallbladder volumes and postprandial residual volumes are increased by two-fold in women after the first trimester of pregnancy compared to control healthy subjects, a condition pointing to gallbladder stasis [50]. In early pregnancy, there is a 30% decrease in gallbladder emptying rate, and in late pregnancy, incomplete gallbladder emptying leads to a large residual volume that causes biliary sludge and the retention of solid cholesterol crystals [25]. Gallbladder stasis progresses during the first 20 weeks of pregnancy and its severity is directly related to the duration of gestation. Gallbladder volumes quickly return to control values after delivery [12]. However, it is still unclear whether high levels of $E_2$ are involved in gallbladder stasis through ERα.

In the present study, we observed that ERα is expressed mainly in the gallbladder smooth muscle cells by immunohistochemical staining. Moreover, fasting gallbladder volumes are increased and postprandial gallbladder emptying rates are reduced in OVX ERα (+/+) mice in response to the high-fat diet. Gallbladder volumes are comparable before and after exogenous CCK-8 stimulation in $E_2$-treated OVX ERα (+/+) mice, indicating that gallbladder contractility is impaired. In contrast, the deletion of *Erα* protects against the inhibitory effect of $E_2$ on gallbladder contraction function because CCK-8 administration significantly leads to almost complete gallbladder emptying in OVX ERα (−/−) mice even treated with $E_2$. It is highly likely that the activation of ERα by $E_2$ impairs gallbladder emptying function by inhibiting expression of *Cck-1r* in the gallbladder, or interrupting the binding of CCK-8 to CCK-1R, or both. In addition, we observed that the concentrations of cholesterol and cholesteryl esters in the gallbladder tissues are significantly higher in OVX ERα (+/+) mice compared to OVX ERα (−/−) mice. The gallbladder epithelial cells have been found to actively modify biliary lipid composition by absorbing and secreting biliary lipids [49]. When bile is supersaturated with cholesterol, gallbladder cholesterol absorption is enhanced, thus leading to the accumulation of excess cholesterol in the gallbladder wall because gallbladder epithelial cells cannot assemble lipoproteins for lipid transport into plasma. In the lithogenic state, the absorbed cholesterol has to be converted to cholesteryl esters and stored in the mucosa and lamina propria. As a result, the accumulation of excess cholesterol in the gallbladder smooth muscle cells could stiffen sarcolemmal membranes and decouple the G-protein-mediated signal transduction. These abnormalities also impair the binding ability of CCK-8 to its receptor, thereby paralyzing gallbladder contractile function and accordingly inhibiting gallbladder emptying. This change could be responsible for signal-transduction decoupling and for impaired gallbladder contractility to CCK-8 [51].

It is hallmarked by the early appearance of biliary sludge in pregnant women and in patients exposed to high levels of $E_2$, showing that solid cholesterol monohydrate crystals and microlithiasis are trapped within mucin gel in the gallbladder. We found that the impaired gallbladder emptying function promotes cholesterol crystallization and cholesterol crystal growth and agglomeration into microlithiasis and stones in $E_2$-treated OVX ERα (+/+) mice. In contrast, this lithogenic effect of $E_2$, through the gallbladder ERα, on cholesterol crystallization is markedly suppressed in OVX ERα (−/−) mice because gallbladder contractility is protected and bile stasis is prevented.

We conclude that the deletion of *Erα* reduces susceptibility to the formation of $E_2$-induced gallstones by diminishing hepatic cholesterol secretion, desaturating gallbladder bile, and improving gallbladder contraction function in female mice (Figure 7). Although ERα plays a critical role in the pathogenesis of $E_2$-induced gallstones, the deletion of the *Erα* gene alone cannot completely protect against gallstone formation in mice treated with high doses of $E_2$ and fed the lithogenic diet. Our results provide an important clue that the G protein-coupled receptor 30 (GPR30), a novel estrogen receptor, could also have a crucial effect on the formation of $E_2$-induced gallstones in OVX ERα (−/−) mice. Moreover, genetic analysis in inbred mice supports the candidacy of *GPR30* for a new gallstone gene *Lith18* [52–54]. Thus, identifying the lithogenic mechanisms of GPR30 has been a focal point of interest because it is still unknown whether GPR30 acts independently of or in conjunction with ERα on inducing gallstones. The findings from the current studies will provide a rigorous experimental framework for further dissecting the lithogenic mechanisms between ERα and GPR30 on the pathogenesis of $E_2$-induced cholesterol gallstones in female mice.

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## Abbreviations

| | |
|---|---|
| **ABC** | ATP-binding cassette (transporter) |
| **CCK** | cholecystokinin |
| **CSI** | cholesterol saturation index |
| **$E_2$** | 17β-estradiol |
| **ER** | estrogen receptor |
| **ERα** | ER subtype α |
| **ERβ** | ER subtype β |
| **GPR30** | the G protein-coupled receptor 30 |
| **OVX** | ovariectomized |

## References

1. Everson GT, McKinley C, Kern F Jr. Mechanisms of gallstone formation in women. Effects of exogenous estrogen (Premarin) and dietary cholesterol on hepatic lipid metabolism. J Clin Invest. 1991; 87:237–246. [PubMed: 1845870]
2. Cirillo DJ, Wallace RB, Rodabough RJ, Greenland P, LaCroix AZ, Limacher MC, Larson JC. Effect of estrogen therapy on gallbladder disease. JAMA. 2005; 293:330–339. [PubMed: 15657326]
3. Honore LH. Increased incidence of symptomatic cholesterol cholelithiasis in perimenopausal women receiving estrogen replacement therapy: a retrospective study. J Reprod Med. 1980; 25:187–190. [PubMed: 6253639]
4. Thijs C, Knipschild P. Oral contraceptives and the risk of gallbladder disease: a meta-analysis. Am J Public Health. 1993; 83:1113–1120. [PubMed: 8342719]

5. Van Bodegraven AA, Bohmer CJ, Manoliu RA, Paalman E, Van der Klis AH, Roex AJ, Kruishoop AM, Deville WL, Lourens J. Gallbladder contents and fasting gallbladder volumes during and after pregnancy. Scand J Gastroenterol. 1998; 33:993–997. [PubMed: 9759958]

6. Everhart JE, Khare M, Hill M, Maurer KR. Prevalence and ethnic differences in gallbladder disease in the United States. Gastroenterology. 1999; 117:632–639. [PubMed: 10464139]

7. Thijs C, Knipschild P, Leffers P. Pregnancy and gallstone disease: an empiric demonstration of the importance of specification of risk periods. Am J Epidemiol. 1991; 134:186–195. [PubMed: 1862802]

8. Barbara L, Sama C, Morselli Labate AM, Taroni F, Rusticali AG, Festi D, Sapio C, Roda E, Banterle C, Puci A, et al. A population study on the prevalence of gallstone disease: the Sirmione Study. Hepatology. 1987; 7:913–917. [PubMed: 3653855]

9. The Rome Group for Epidemiology and Prevention of Cholelithiasis (GREPCO). The epidemiology of gallstone disease in Rome, Italy. Part II. Factors associated with the disease. Hepatology. 1988; 8:907–913. [PubMed: 3391520]

10. Lindseth G, Bird-Baker MY. Risk factors for cholelithiasis in pregnancy. Res Nurs Health. 2004; 27:382–391. [PubMed: 15514955]

11. Maringhini A, Ciambra M, Baccelliere P, Raimondo M, Orlando A, Tine F, Grasso R, Randazzo MA, Barresi L, Gullo D, Musico M, Pagliaro L. Biliary sludge and gallstones in pregnancy: incidence, risk factors, and natural history. Ann Intern Med. 1993; 119:116–120. [PubMed: 8512160]

12. Henriksson P, Einarsson K, Eriksson A, Kelter U, Angelin B. Estrogen-induced gallstone formation in males. Relation to changes in serum and biliary lipids during hormonal treatment of prostatic carcinoma. J Clin Invest. 1989; 84:811–816. [PubMed: 2760214]

13. Grodstein F, Colditz GA, Stampfer MJ. Postmenopausal hormone use and cholecystectomy in a large prospective study. Obstet Gynecol. 1994; 83:5–11. [PubMed: 8272307]

14. Wang HH, Liu M, Clegg DJ, Portincasa P, Wang DQ. New insights into the molecular mechanisms underlying effects of estrogen on cholesterol gallstone formation. Biochim Biophys Acta. 2009; 1791:1037–1047. [PubMed: 19589396]

15. Angelin B, Olivecrona H, Reihner E, Rudling M, Stahlberg D, Eriksson M, Ewerth S, Henriksson P, Einarsson K. Hepatic cholesterol metabolism in estrogen-treated men. Gastroenterology. 1992; 103:1657–1663. [PubMed: 1426886]

16. Diehl AK. Epidemiology and natural history of gallstone disease. Gastroenterol Clin North Am. 1991; 20:1–19. [PubMed: 2022415]

17. Kern F Jr, Everson GT, DeMark B, McKinley C, Showalter R, Braverman DZ, Szczepanik-Van Leeuwen P, Klein PD. Biliary lipids, bile acids, and gallbladder function in the human female: effects of contraceptive steroids. J Lab Clin Med. 1982; 99:798–805. [PubMed: 7077124]

18. Bennion LJ, Ginsberg RL, Gernick MB, Bennett PH. Effects of oral contraceptives on the gallbladder bile of normal women. N Engl J Med. 1976; 294:189–192. [PubMed: 1244533]

19. Lynn J, Williams L, O'Brien J, Wittenberg J, Egdahl RH. Effects of estrogen upon bile: implications with respect to gallstone formation. Ann Surg. 1973; 178:514–524. [PubMed: 4200628]

20. Kern F Jr, Everson GT. Contraceptive steroids increase cholesterol in bile: mechanisms of action. J Lipid Res. 1987; 28:828–839. [PubMed: 3625039]

21. Bennion LJ, Mott DM, Howard BV. Oral contraceptives raise the cholesterol saturation of bile by increasing biliary cholesterol secretion. Metabolism. 1980; 29:18–22. [PubMed: 7351873]

22. van der Werf SD, van Berge Henegouwen GP, Ruben AT, Palsma DM. Biliary lipids, bile acid metabolism, gallbladder motor function and small intestinal transit during ingestion of a sub-fifty oral contraceptive. J Hepatol. 1987; 4:318–326. [PubMed: 2955033]

23. Everson GT. Pregnancy and gallstones. Hepatology. 1993; 17:159–161. [PubMed: 8423037]

24. Everson GT, McKinley C, Lawson M, Johnson M, Kern F Jr. Gallbladder function in the human female: effect of the ovulatory cycle, pregnancy, and contraceptive steroids. Gastroenterology. 1982; 82:711–719. [PubMed: 7060890]

25. Braverman DZ, Johnson ML, Kern F Jr. Effects of pregnancy and contraceptive steroids on gallbladder function. N Engl J Med. 1980; 302:362–364. [PubMed: 7351927]

26. Wang HH, Afdhal NH, Wang DQ. Estrogen receptor α, but not β, plays a major role in 17β-estradiol-induced murine cholesterol gallstones. Gastroenterology. 2004; 127:239–249. [PubMed: 15236189]
27. Wang HH, Afdhal NH, Wang DQ. Overexpression of estrogen receptor α increases hepatic cholesterogenesis, leading to biliary hypersecretion in mice. J Lipid Res. 2006; 47:778–786. [PubMed: 16380638]
28. Wang HH, Portincasa P, Wang DQ. Molecular pathophysiology and physical chemistry of cholesterol gallstones. Front Biosci. 2008; 13:401–423. [PubMed: 17981556]
29. Wang DQ, Paigen B, Carey MC. Phenotypic characterization of *Lith* genes that determine susceptibility to cholesterol cholelithiasis in inbred mice: physical-chemistry of gallbladder bile. J Lipid Res. 1997; 38:1395–1411. [PubMed: 9254065]
30. Wang DQ, Lammert F, Paigen B, Carey MC. Phenotypic characterization of *Lith* genes that determine susceptibility to cholesterol cholelithiasis in inbred mice. Pathophysiology Of biliary lipid secretion. J Lipid Res. 1999; 40:2066–2079. [PubMed: 10553010]
31. Wang HH, Portincasa P, Liu M, Tso P, Samuelson LC, Wang DQ. Effect of gallbladder hypomotility on cholesterol crystallization and growth in CCK-deficient mice. Biochim Biophys Acta. 2010; 1801:138–146. [PubMed: 19836465]
32. Bartlett GR. Phosphorus assay in column chromatography. J Biol Chem. 1959; 234:466–468. [PubMed: 13641241]
33. Fromm H, Amin P, Klein H, Kupke I. Use of a simple enzymatic assay for cholesterol analysis in human bile. J Lipid Res. 1980; 21:259–261. [PubMed: 7373165]
34. Turley SD, Dietschy JM. Re-evaluation of the 3α-hydroxysteroid dehydrogenase assay for total bile acids in bile. J Lipid Res. 1978; 19:924–928. [PubMed: 712252]
35. Rossi SS, Converse JL, Hofmann AF. High pressure liquid chromatographic analysis of conjugated bile acids in human bile: simultaneous resolution of sulfated and unsulfated lithocholyl amidates and the common conjugated bile acids. J Lipid Res. 1987; 28:589–595. [PubMed: 3598401]
36. Heuman DM. Quantitative estimation of the hydrophilic-hydrophobic balance of mixed bile salt solutions. J Lipid Res. 1989; 30:719–730. [PubMed: 2760545]
37. Carey MC. Critical tables for calculating the cholesterol saturation of native bile. J Lipid Res. 1978; 19:945–955. [PubMed: 731129]
38. Wang DQ, Carey MC. Complete mapping of crystallization pathways during cholesterol precipitation from model bile: influence of physical-chemical variables of pathophysiologic relevance and identification of a stable liquid crystalline state in cold, dilute and hydrophilic bile salt-containing systems. J Lipid Res. 1996; 37:606–630. [PubMed: 8728323]
39. Coyne MJ, Bonorris GG, Chung A, Winchester R, Schoenfield LJ. Estrogen enhances dietary cholesterol induction of saturated bile in the hamster. Gastroenterology. 1978; 75:76–79. [PubMed: 401100]
40. Maclure KM, Hayes KC, Colditz GA, Stampfer MJ, Speizer FE, Willett WC. Weight, diet, and the risk of symptomatic gallstones in middle-aged women. N Engl J Med. 1989; 321:563–569. [PubMed: 2761600]
41. Duan LP, Wang HH, Ohashi A, Wang DQ. Role of intestinal sterol transporters Abcg5, Abcg8, and Npc1l1 in cholesterol absorption in mice: gender and age effects. Am J Physiol. 2006; 290:G269–276.
42. Wittenburg H, Lyons MA, Li R, Churchill GA, Carey MC, Paigen B. FXR and ABCG5/ABCG8 as determinants of cholesterol gallstone formation from quantitative trait locus mapping in mice. Gastroenterology. 2003; 125:868–881. [PubMed: 12949731]
43. Grunhage F, Acalovschi M, Tirziu S, Walier M, Wienker TF, Ciocan A, Mosteanu O, Sauerbruch T, Lammert F. Increased gallstone risk in humans conferred by common variant of hepatic ATP-binding cassette transporter for cholesterol. Hepatology. 2007; 46:793–801. [PubMed: 17626266]
44. Wang Y, Jiang ZY, Fei J, Xin L, Cai Q, Jiang ZH, Zhu ZG, Han TQ, Zhang SD. ATP binding cassette G8 T400K polymorphism may affect the risk of gallstone disease among Chinese males. Clin Chim Acta. 2007; 384:80–85. [PubMed: 17612515]

45. Kuo KK, Shin SJ, Chen ZC, Yang YH, Yang JF, Hsiao PJ. Significant association of ABCG5 604Q and ABCG8 D19H polymorphisms with gallstone disease. Br J Surg. 2008; 95:1005–1011. [PubMed: 18457353]

46. Rudkowska I, Jones PJ. Polymorphisms in ABCG5/G8 transporters linked to hypercholesterolemia and gallstone disease. Nutr Rev. 2008; 66:343–348. [PubMed: 18522623]

47. Katsika D, Magnusson P, Krawczyk M, Grunhage F, Lichtenstein P, Einarsson C, Lammert F, Marschall HU. Gallstone disease in Swedish twins: risk is associated with ABCG8 D19H genotype. J Intern Med. 2010; 268:279–285. [PubMed: 20497293]

48. von Kampen O, Buch S, Nothnagel M, Azocar L, Molina H, Brosch M, Erhart W, von Schonfels W, Egberts J, Seeger M, Arlt A, Balschun T, Franke A, Lerch MM, Mayerle J, Kratzer W, Boehm BO, Huse K, Schniewind B, Tiemann K, Jiang ZY, Han TQ, Mittal B, Srivastava A, Fenger M, Jorgensen T, Schirin-Sokhan R, Tonjes A, Wittenburg H, Stumvoll M, Kalthoff H, Lammert F, Tepel J, Puschel K, Becker T, Schreiber S, Platzer M, Volzke H, Krawczak M, Miquel JF, Schafmayer C, Hampe J. Genetic and functional identification of the likely causative variant for cholesterol gallstone disease at the ABCG5/8 lithogenic locus. Hepatology. 2013; 57:2407–2417. [PubMed: 22898925]

49. Portincasa P, Di Ciaula A, Wang HH, Palasciano G, van Erpecum KJ, Moschetta A, Wang DQ. Coordinate regulation of gallbladder motor function in the gut-liver axis. Hepatology. 2008; 47:2112–2126. [PubMed: 18506897]

50. de Bari O, Wang TY, Liu M, Paik CN, Portincasa P, Wang DQ. Cholesterol cholelithiasis in pregnant women: pathogenesis, prevention and treatment. Ann Hepatol. 2014; 13:728–745. [PubMed: 25332259]

51. Chen Q, Chitinavis V, Xiao Z, Yu P, Oh S, Biancani P, Behar J. Impaired G protein function in gallbladder muscle from progesterone-treated guinea pigs. Am J Physiol. 1998; 274:G283–289. [PubMed: 9486181]

52. Lyons MA, Korstanje R, Li R, Sheehan SM, Walsh KA, Rollins JA, Carey MC, Paigen B, Churchill GA. Single and interacting QTLs for cholesterol gallstones revealed in an intercross between mouse strains NZB and SM. Mamm Genome. 2005; 16:152–163. [PubMed: 15834632]

53. Lyons MA, Wittenburg H. Cholesterol gallstone susceptibility loci: a mouse map, candidate gene evaluation, and guide to human *LITH* genes. Gastroenterology. 2006; 131:1943–1970. [PubMed: 17087948]

54. Wang HH, Portincasa P, Afdhal NH, Wang DQ. *Lith* genes and genetic analysis of cholesterol gallstone formation. Gastroenterol Clin North Am. 2010; 39:185–207. vii–viii. [PubMed: 20478482]

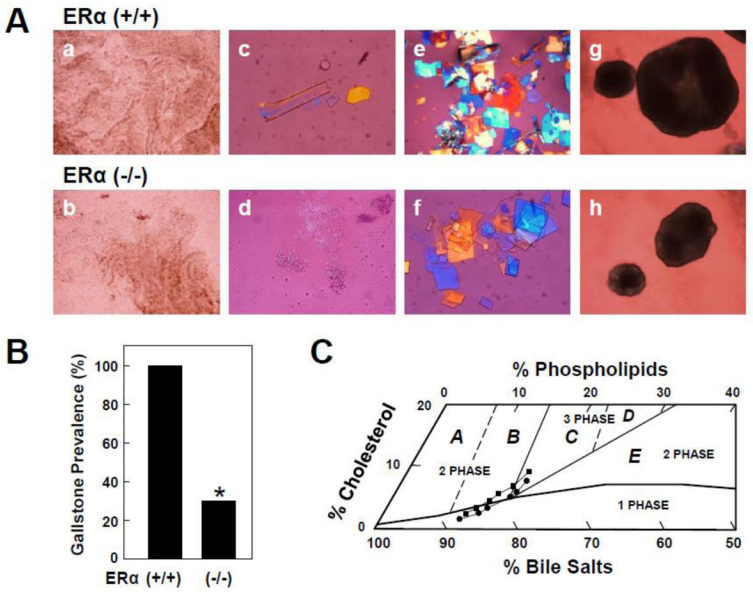


**Figure 1.**

(**A**) Representative photomicrographs of cholesterol crystallization and gallstone formation as observed by polarizing light microscopy in gallbladder bile of $E_2$-treated OVX ERα (+/+) and (−/−) mice during the 56-day period of the lithogenic diet feeding: (a and b) non-birefringent amorphous mucin gel; (c) tubular (possible anhydrous cholesterol) crystal; (d) numerous non-birefringent aggregated liquid crystals and few fused liquid crystals within mucin gel; (e and f) agglomerates of typical cholesterol monohydrate crystals, with 79.2° and 100.8° angles, and often a notched corner; and (g and h) gallstones displaying rounded contours and black centers from light scattering/absorption. All magnifications are ×800, and ×200 for far right two panels. (**B**) At 56 day on the lithogenic diet, the prevalence of gallstones is 100% in OVX ERα (+/+) mice, being significantly ($P<0.05$) higher than that (30%) in OVX ERα (−/−) mice treated with $E_2$ at 6 μg/day. (**C**) The relative lipid composition of pooled gallbladder bile from OVX ERα (+/+) and (−/−) mice at each time point (see Table 2) is plotted on a condensed phase diagram. The one-phase micellar zone at the bottom is enclosed by a solid curved line. Above the micellar zone, two solid lines divide the two-phase zones from a central three-phase zone, showing five cholesterol crystallization pathways A to E. Symbols ● represent OVX ERα (+/+) mice and ■ for OVX ERα (−/−) mice.

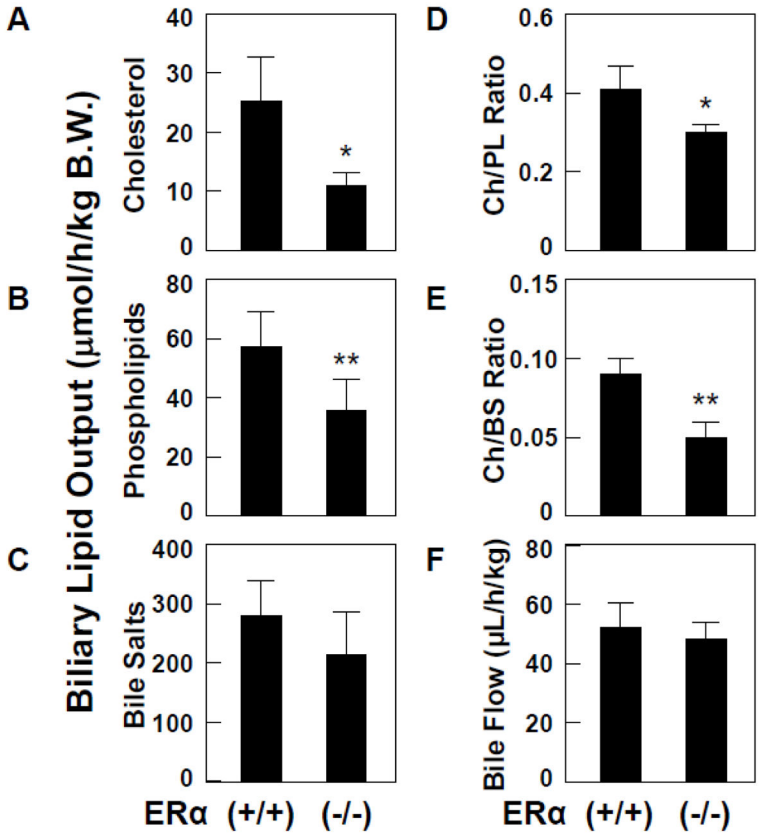


**Figure 2.**

Hepatic output of (**A**) biliary cholesterol, (**B**) phospholipids, and (**C**) bile salts, as well as (**D**) the ratios of Ch/PL and (**E**) Ch/BS, and (**F**) bile flow rates during the first hour of biliary secretion in OVX ERα (+/+) and (−/−) mice treated with $E_2$ at 6 µg/day and fed the lithogenic diet for 56 days. *P<0.01 and **P<0.05, compared with OVX ERα (+/+) mice. Abbreviations: Ch, cholesterol; PL, phospholipids; BS, bile salts.

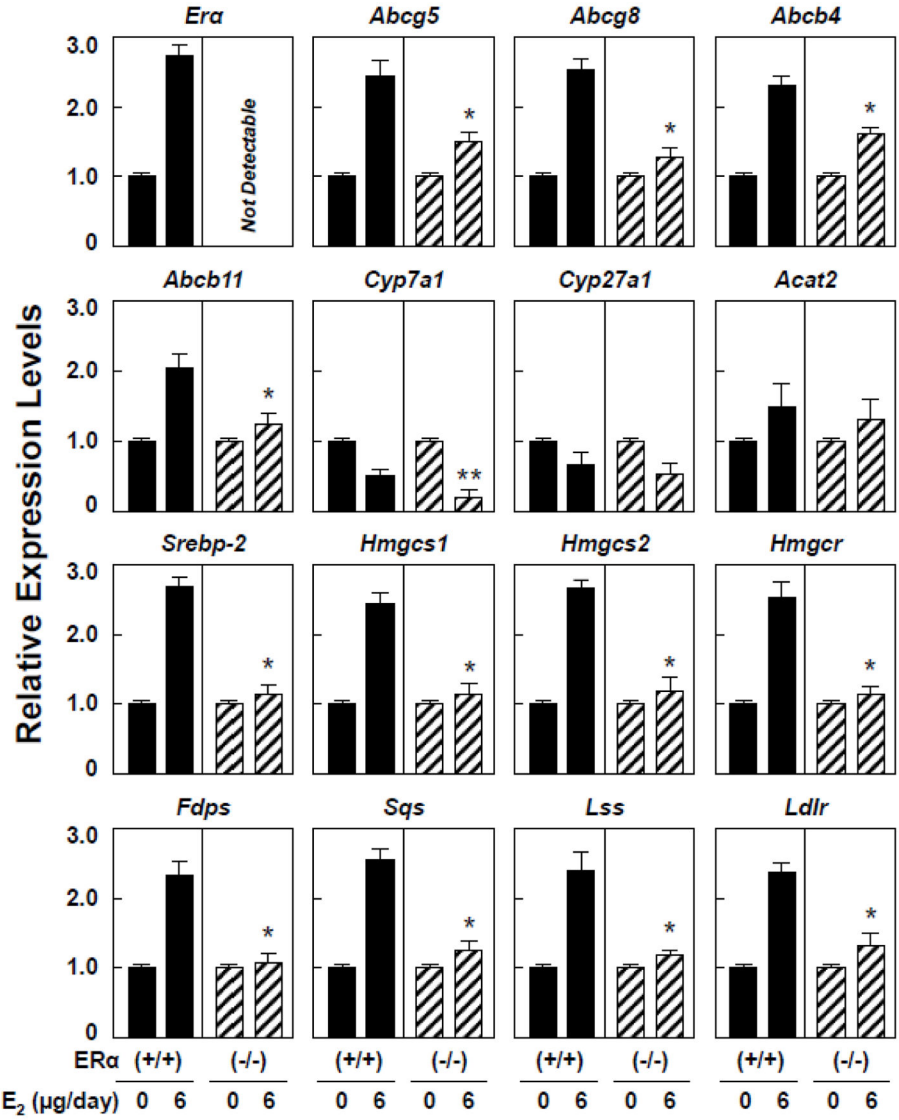


**Figure 3.**

Effect of $E_2$ on mRNA levels of the genes involved in the regulation of hepatic lipid metabolism. The mRNA levels of genes in OVX ERα (+/+) and (−/−) mice receiving no $E_2$ and fed the lithogenic diet for 56 days are set at 1. Notably, there are no significant differences in expression of these genes between OVX ERα (+/+) and (−/−) mice receiving no $E_2$. *P<0.01 and **P<0.05, compared to OVX ERα (+/+) mice treated with $E_2$ at 6 μg/day and fed the lithogenic diet for 56 days. See text for further description and the Methods for abbreviations.

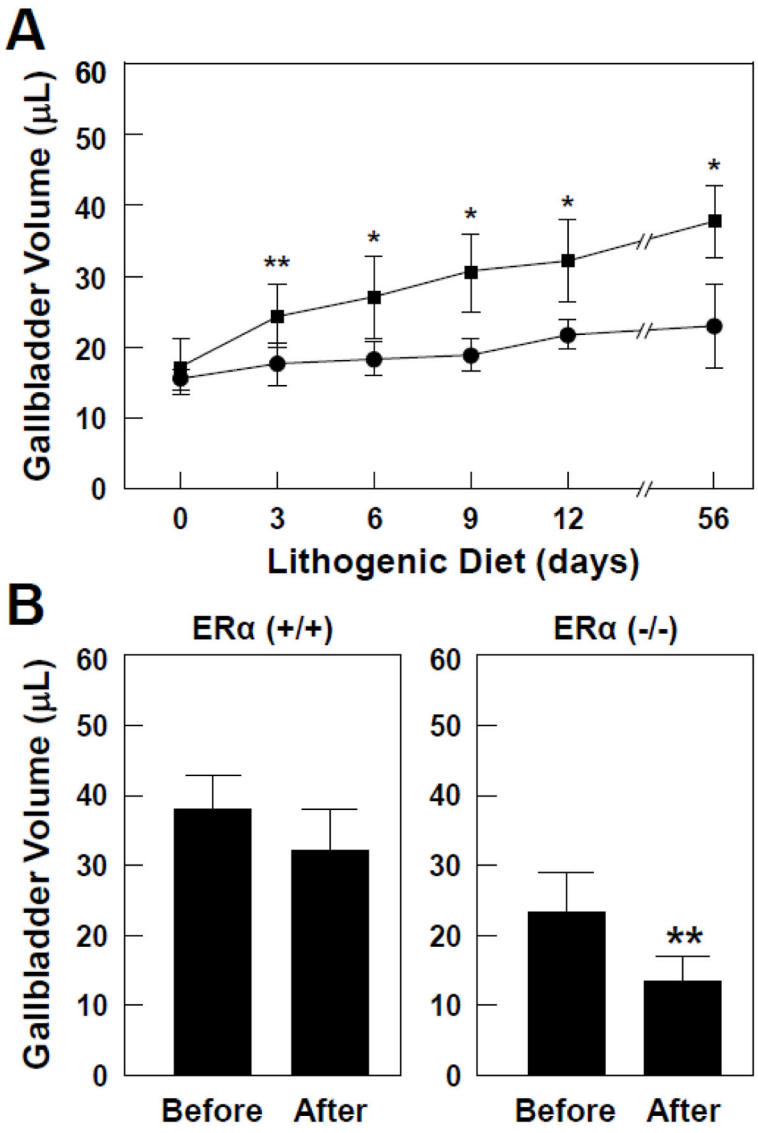


**Figure 4.**

(**A**) Fasting gallbladder volumes as functions of time on the lithogenic diet and (**B**) postprandial gallbladder volumes in response to a fatty meal in $E_2$-treated OVX ERα (+/+) and (−/−) mice fed the lithogenic diet for 56 days. *P<0.01 and **P<0.05, compared to OVX ERα (+/+) mice treated with $E_2$ at 6 μg/day and fed the lithogenic diet for 56 days.

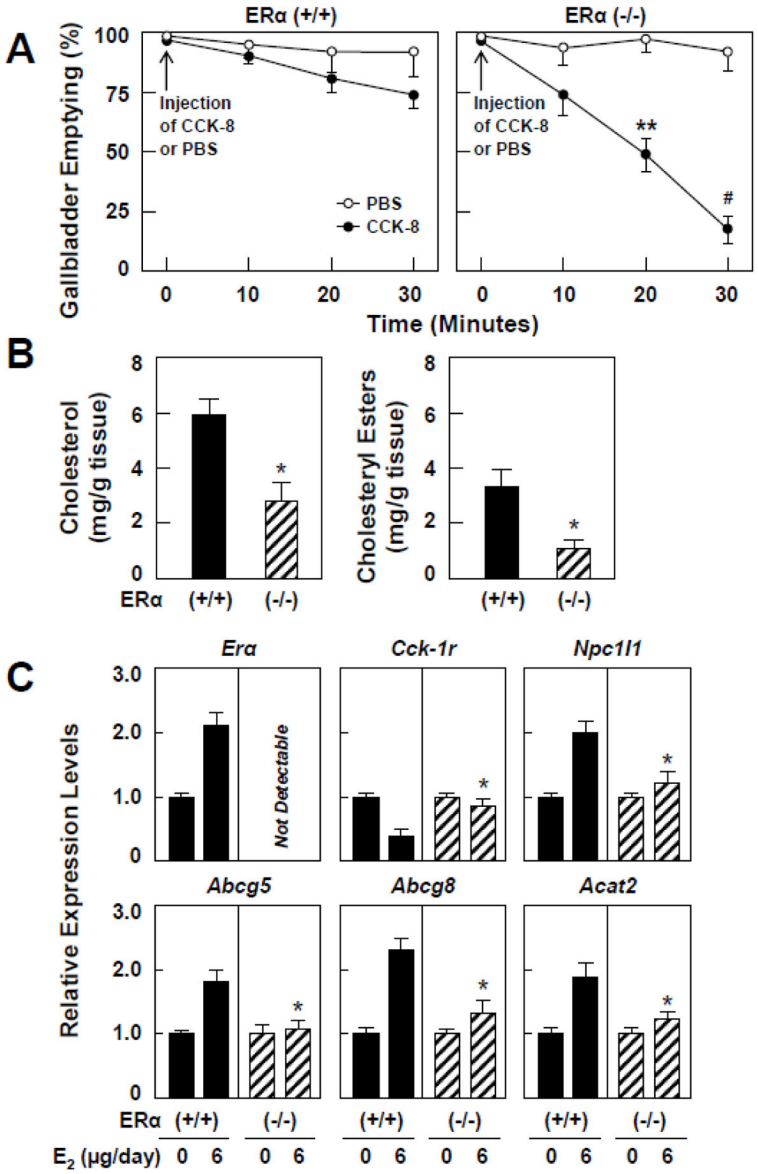


**Figure 5.**

(**A**) Gallbladder emptying rates in response to an exogenous CCK-8 stimulation; (**B**) the concentrations of cholesterol and cholesteryl esters in gallbladder tissues; and (**C**) mRNA levels of the genes encoding gallbladder lipid transporters and involved in the regulation of lipid metabolism in OVX ERα (+/+) and (−/−) mice treated with $E_2$ at 6 μg/day and fed the lithogenic diet for 56 days. The experimental conditions are the same as described in Figure 3. *P<0.01 and #P<0.001, compared with PBS solution, or OVX ERα (+/+) mice treated with $E_2$ at 6 μg/day and fed the lithogenic diet for 56 days.

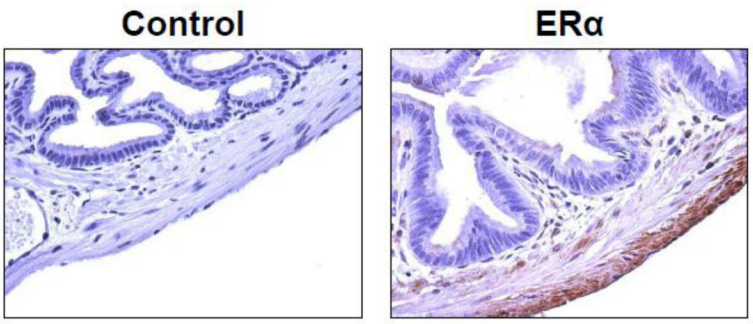


**Figure 6.**

Immunohistochemical studies reveal that ERα is expressed mainly in the gallbladder smooth muscle cells in chow-fed female ERα (+/+) mice with intact ovaries. All magnifications are ×400.

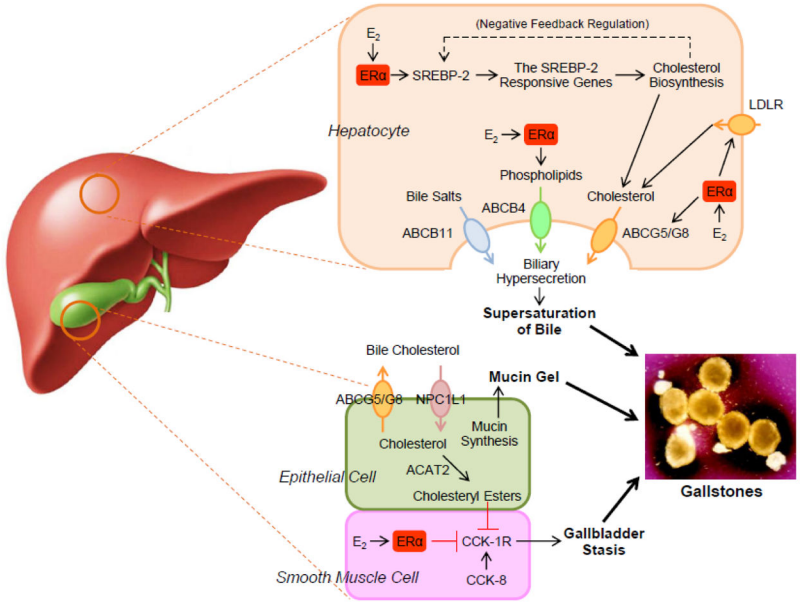


**Figure 7.**

Proposed model for a critical role of ERα in $E_2$-induced cholesterol gallstones. In the liver, $E_2$ activation of ERα interferes with the negative feedback regulation (as shown by the dashed line) of cholesterol synthesis by stimulating *Srebp-2*, with the resulting up-regulation of the SREBP-2-responsive genes for cholesterol synthesis. In addition, ERα increases expression of *Abcg5/g8* and *Ldlr* in the liver through the transcriptional regulation. As a result, hepatic cholesterol secretion is significantly increased, thus leading to supersaturation of bile. In the gallbladder, ERα activated by $E_2$ impairs gallbladder emptying function through the *Cck-1r* pathway, inducing bile stasis and accumulation of mucin gel in the gallbladder lumen. All of these changes promote cholesterol crystallization and solid cholesterol crystal growth and agglomeration into microlithiasis and stones. Of special note is that the deletion of the *Erα* gene reduces susceptibility to $E_2$-induced cholesterol cholelithiasis in female mice.

**Table 1**

Primer and probe sequences in mRNA quantification by real-time PCR

| Gene | Accession Number | Forward | Reverse | Probe |
|---|---|---|---|---|
| *Abca1* | AK019548 | 5′-TTGAAATCTCTCGGATACAATCGA-3′ | 5′-AAGCCCATTGTTTGCCCAT-3′ | 5′-CGTCTGACCTTTGGGATCCTCACCA-3′ |
| *Abcb4* | NM_008830 | 5′-GGAAATCATTGGTGTGGTAAGTCA-3′ | 5′-CACGGCCATAGCGGATATTTT-3′ | 5′-AGCCCGTGCTGTTCTCTACTACGATCGC-3′ |
| *Abcb11* | NM_021022 | 5′-TCTGGTACGGCTCCAGACTTG-3′ | 5′-GCTGCTATTATGACACAGAGGAAAAT-3′ | 5′-AAGGCGAGTACACACCAGGGACACTGATC-3′ |
| *Abcg5* | AF312713 | 5′-CCTGCAGAGCGACGTTTTTC-3′ | 5′-GCATCGCTGTGTATCGCAAC-3′ | 5′-AGCAGCCTCACTGTGCGCGAGA-3′ |
| *Abcg8* | AF324495 | 5′-TGGATAGTGCCTGCATGGATC-3′ | 5′-AATTGAATCTGCATCAGCCCC-3′ | 5′-CAAGCTGTCGTTCCTCCGGTGGTG-3′ |
| *Acat2* | AF078751 | 5′-TTTGCTCTATGCCTGCTTCATC-3′ | 5′-GGTTCCCGGCTCATGTTG-3′ | 5′-TGGGCCGCCTCTGTGTCCCT-3′ |
| *Cck-1r* | NM_009827 | 5′-CAACCTGCTCAAGGATTTCATCT-3′ | 5′-CACGGAAGTGCCCATGAAGT-3′ | 5′-CGGAAGTGCCGTGTGCAAGACTACC-3′ |
| *Cyp7a1* | NM_007824 | 5′-TCGTGATCCTCTGGGCATCT-3′ | 5′-CCAAGTGCATTAACTGTGGGTAAA-3′ | 5′-AGGAGGCTCTGCGGCTCTCC-3′ |
| *Cyp27a1* | NM_024264 | 5′-TGACATGGGCCCTGTACCA-3′ | 5′-TCCCAGGGTTATCAGCCTCTT-3′ | 5′-CAGAGATCCAGGAGGCCTTGCAC-3′ |
| *Erα* | NM_007956 | 5′-CCAGCAGTAACGAGAAAGGAAAC-3′ | 5′-TCATTGCACACGGCACAGT-3′ | 5′-TCATGGAGTCTGCCAAGGAGACTCGC-3′ |
| *Fdps* | BC048497 | 5′-CGGGCAGACTCTAGACCTCATG-3′ | 5′-CGATTTGTACCTCTTTTCAGTGTATCTAC-3′ | 5′-AGCACCCCAGGGCCATGTGGA-3′ |
| *Hmgcr* | M62766 | 5′-ATTCTGGCAGTCAGTGGGAACT-3′ | 5′-CCTCGTCCTTCGATCCAATTT-3′ | 5′-CACCGACAAGAAGCCTGCTGCCA-3′ |
| *Hmgcs1* | NM_145942 | 5′-CCACAGGAAATGCCAGACCTA-3′ | 5′-GGAGCGTTTGGCCCAATTA-3′ | 5′-AGGTGGAGTTGGAGCTGTGGCCCT-3′ |
| *Hmgcs2* | NM_008256 | 5′-TACCTGCGGGCCTTGGAT-3′ | 5′-GGTGAAAGGCTGGTTGTTTCC-3′ | 5′-ATGCTATGCAGCCTACCGCAAGAAGATCC-3′ |
| *Ldlr* | NM_010700 | 5′-GCTCCATAGGCTATCTGCTCTTCA-3′ | 5′-TGCGGTCCAGGGTCATCT-3′ | 5′-CAACCGCCACGAGGTCCGG-3′ |
| *Lss* | NM_146006 | 5′-GCACACCACAGACCTGAGTTTC-3′ | 5′-GGACAGTTTTCTGGAACCTGTGA-3′ | 5′-CTGCAGAAGGCTCACGAGTTCCTGC-3′ |
| *Sqs* | NM_010191 | 5′-GGAAGTGTTAATCCGTGTTGTTCTG-3′ | 5′-GCTCCCCCTGGGTGAGA-3′ | 5′-TGTGAGCTCCCGAGACTGCAGGCT-3′ |
| *Srebp2* | AF374267 | 5′-TGAAGCTGGCCAATCAGAAAA-3′ | 5′-CCACATCACTGTCCACCAGACT-3′ | 5′-CAAGCTCCTGAAGGGCATCGACCTG-3′ |
| *β-Actin* | NM_007393 | 5′-GCTCTGGCTCCTAGCACCAT-3′ | 5′-GCCACCATCCACACAGAGT-3′ | 5′-AAGATCATTGCTCCTCCTGAGCGCAA-3′ |

See text for abbreviations.

**Table 2**

Biliary lipid composition of gallbladder bile during gallstone formation

| Day | Ch (mM) | PL (mM) | BS (mM) | Ch/PL Ratio | Ch/BS Ratio | [TL] (*g/dL*) | CSI |
|---|---|---|---|---|---|---|---|
| ERα (+/+) | | | | | | | |
| 0 | 4.24 | 19.73 | 147.56 | 0.215 | 0.029 | 8.94 | 0.56 |
| 3 | 5.50 | 21.25 | 140.79 | 0.259 | 0.039 | 8.77 | 0.70 |
| 6 | 9.08 | 24.02 | 147.34 | 0.378 | 0.062 | 9.45 | 1.01 |
| 9 | 10.28 | 27.14 | 141.76 | 0.379 | 0.073 | 9.46 | 1.05 |
| 12 | 12.68 | 30.20 | 142.79 | 0.420 | 0.089 | 9.84 | 1.17 |
| 56 | 16.23 | 33.16 | 141.25 | 0.489 | 0.115 | 10.13 | 1.37 |
| ERα (−/−) | | | | | | | |
| 0 | 3.17 | 18.14 | 148.51 | 0.175 | 0.021 | 8.82 | 0.44 |
| 3 | 4.32 | 22.13 | 143.82 | 0.195 | 0.030 | 8.94 | 0.53 |
| 6 | 5.89 | 21.87 | 138.65 | 0.269 | 0.042 | 8.73 | 0.73 |
| 9 | 8.36 | 24.35 | 118.68 | 0.343 | 0.070 | 8.04 | 1.00 |
| 12 | 11.04 | 30.12 | 144.71 | 0.367 | 0.076 | 9.84 | 1.02 |
| 56 | 14.61 | 32.14 | 142.83 | 0.455 | 0.102 | 10.07 | 1.27 |

Values were determined from pooled gallbladder bile (n=5 per group).

Abbreviation: BS, bile salts; Ch, cholesterol; CSI, cholesterol saturation index; PL, phospholipids; [TL], total lipid concentration.