Estrogen-dependent gallbladder carcinogenesis in LXRβ$^{-/-}$ female mice

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Gallbladder cancer is a highly aggressive disease with poor prognosis that is two to six times more frequent in women than men. The development of gallbladder cancer occurs over a long time (more than 15 y) and evolves from chronic inflammation to dysplasia/metaplasia, carcinoma in situ, and invasive carcinoma. In the present study we found that, in female mice in which the oxysterol receptor liver X receptor–β (LXRβ) has been inactivated, preneoplastic lesions of the gallbladder developed and evolved to cancer in old animals. LXRβ is a nuclear receptor involved in the control of lipid homeostasis, glucose metabolism, inflammation, proliferation, and CNS development. LXRβ$^{-/-}$ female gallbladders were severely inflamed, with regions of dysplasia and high cell density, hyperchromasia, metaplasia, and adenomas. No abnormalities were evident in male mice, nor in LXRα$^{-/-}$ or LXRα$^{-/-}$β$^{-/-}$ animals of either sex. Interestingly, the elimination of estrogens with ovariectomy prevented development of preneoplastic lesions in LXRβ$^{-/-}$ mice. The etiopathological mechanism seems to involve TGF-β signaling, as the precancerous lesions were characterized by strong nuclear reactivity of phospho-SMAD-2 and SMAD-4 and loss of E-cadherin expression. Upon ovariectomy, E-cadherin was reexpressed on the cell membranes and immunoreactivity of pSMAD-2 in the nuclei was reduced. These findings suggest that LXRβ in a complex interplay with estrogens and TGF-β could play a crucial role in the malignant transformation of the gallbladder epithelium.

cancer | hormone | oxysterols | SMAD | E-cadherin

Liver X receptors (LXRs) α (NR1H3) and β (NR1H2) are ligand-activated transcription factors belonging to the super family of nuclear receptors. As obligate heterodimerization partners with the retinoid X receptor (1, 2), LXRs bind to LXR responsive elements (LXREs) consisting of a direct repeat (DR) sequence (5′-AGGTCA-3′) separated by four nucleotides in the promoter of target genes (3). Oxidized metabolites of cholesterol such as 22-hydroxycholesterol, 24(S)-hydroxycholesterol, 24(S), 25-epoxycholesterol, and 27-hydroxycholesterol are natural agonists of LXRs (4). Upon activation, LXRs promote cholesterol catabolism into bile acids by enhancing the rate-limiting enzyme CYP7A1 (5) and by inducing canalicular membrane transporters ABCG5 and ABCG8 (6), which increase cholesterol secretion into bile. In addition, LXR activity in the liver is associated with induction of lipogenesis (7, 8), inhibition of gluconeogenesis (9), and control of hepatocyte proliferation (10). During liver regeneration, LXR signaling is down-regulated and treatment with LXR agonist decreases hepatocyte proliferation (10). Effects of LXR on regulation of cell cycle have been described in other tissues including the prostate (11), breast cancer cells (12), pancreatic β cells (13, 14), and T and B lymphocytes (15). Indeed, the ventral prostate of LXRα$^{-/-}$ mice is affected by a pathological stromal overgrowth positive for pSMAD-2/3 and SNAIL, markers of enhanced TGF-β signaling (16). Moreover, LXR agonist inhibits the growth of prostate tumors in athymic mice inoculated with prostate cancer cells (11) and inhibits estrogen-dependent proliferation of uterine epithelial cells and breast cancer-xenograph growth in nude mice (17).

Carcinoma of the gallbladder is a highly fatal and aggressive disease with a poor prognosis. It is the most common malignant tumor of the biliary tract, with 5,000 estimated new cases per year in the United States (18). Incidence of gallbladder carcinoma varies with sex and ethnicity. Women are affected two to six times more than men and the highest incidences are reported in Native Americans, South American populations, and people from Poland and North of India (19). The etiology of gallbladder carcinoma involves a complex interplay between hormones, metabolic alterations, infections, and even anatomical anomalies (20). Epidemiological studies have shown a strong association of this tumor (in particular the squamous and adenosquamous variants) with cholesterol gallstone disease (21) and with many of its risk factors like obesity, high carbohydrate intake, and female sex (22). The strong female incidence has raised the possibility that estrogens could play a key pathophysiological role in the development of gallbladder cancer. It has been shown that hormone replacement therapy in postmenopausal women significantly increases the risk of gallbladder diseases (23, 24) that becomes even higher with oral versus transdermal therapy (25). Gallbladder cancer is preceded by a sequence of molecular and histopathological alterations evolving to cancer over a long period, estimated to be approximately 15 y (26). This multistage pathogenesis starts from chronic inflammation, often associated with cholesterol gallstones, and progresses to dysplastic flat-epithelial changes characterized by high cell density, hyperchromasia, and elongated nuclei (27, 28). Subsequently, the atypical epithelium undergoes increased stratification leading to the high-grade premalignant lesion called carcinoma in situ (27, 28). Besides this most frequent evolution, a small number of gallbladder carcinomas seems to evolve from preexisting adenomas (29).

In the present study we show that the gallbadder of 11-mo-old LXRβ$^{-/-}$ female mice is affected by chronic cholecystitis, dysplasia, metaplasia, and adenoma, characteristic preneoplastic lesions leading to gallbladder cancer that becomes evident in old mice. No alterations are detected in male LXRβ$^{-/-}$ mice and none in LXRα$^{-/-}$ or LXRα$^{-/-}$β$^{-/-}$ mice of either sex. Surprisingly, the elimination of estrogens by ovariectomy prevents the development of preneoplastic/neoplastic lesions in LXRβ$^{-/-}$ mice. The molecular pathogenesis seems to involve TGF-β signaling that is upregulated in LXRβ$^{-/-}$ mice and becomes less pronounced after ovariectomy.

Results

LXRα and LXRβ Are Expressed in Gallbladder Cholangiocytes. Anti-LXRα and anti-LXRβ antibodies were developed in our laboratory (16, 30) and used in immunohistochemical studies to investigate expression and localization of LXRα and LXRβ in the gallbladders of WT, LXRα$^{-/-}$, and LXRβ$^{-/-}$ mice. Positive immunoreactivity both for LXRα (Fig. 1*A*) and LXRβ (Fig. 1*B*) was

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detectable in the nuclei of gallbladder cholangiocytes, mainly in those located in the body-fundus of the gallbladder. No staining was detected in KO mice used as controls.

Preneoplastic and Neoplastic Lesions in the Gallbladders of LXRβ$^{-/-}$ Female Mice. Histopathological study of the gallbladder of 11-mo-old LXRβ$^{-/-}$ female mice compared with WT mice (Fig. 2*A*) showed the presence of severe chronic cholecystitis with a large amount of immune cells infiltrating the entire gallbladder wall (Fig. 2*B*).

In association with this inflammatory condition, preneoplastic lesions like dysplasia, hyperplasia, metaplasia, or adenomas were seen in all 12 LXRβ$^{-/-}$ female mice studied (Fig. 2 *B–E*).

An important change in the maturation of the gallbladder epithelium of LXRβ$^{-/-}$ female mice was evident (Fig. 2 *B* and *C*) with nuclear irregularity and pseudostratification, increased nucleus/cytoplasm ratio, loss of cell polarization, and hyperchromatism. Areas of inflammation with edema of the mucosa (Fig. 2*D*) were also detected; 16% of KO animals presented adenomas (Fig. 2*E*) with polypoid epithelial proliferation mainly of tubulopapillary type. At the age of 19 mo, 30% of LXRβ$^{-/-}$ female gallbladders showed the presence of carcinoma in situ with malignant epithelial cells invading the lamina propria of the gallbladder mucosa (Fig. 2*F*).

No signs of inflammation were detected in the gallbladders of female LXRα$^{-/-}$ (Fig. 2*G*) or LXRα$^{-/-}$β$^{-/-}$ (Fig. 2*H*) mice at the age of 11 mo. All the described preneoplastic/neoplastic lesions were evident exclusively in female LXRβ$^{-/-}$ mice.

No abnormalities, except for a moderate atrophy, were observed in the gallbladders of male LXRβ$^{-/-}$ mice (Fig. 3*C*) compared with WT mice (Fig. 3*A*). At the same time, neither LXRα$^{-/-}$ (Fig. 3*B*) nor LXRα$^{-/-}$β$^{-/-}$ (Fig. 3*D*) male gallbladders showed any inflammatory alterations.

Increased Proliferation of Epithelial Cells in the Gallbladders of 11-mo-Old Female LXRβ$^{-/-}$ Mice. Proliferative activity of gallbladder epithelium was studied by measuring the percentage of proliferating cell nuclear antigen (PCNA)–positive cells in WT and LXRβ$^{-/-}$ female mice at 4 and 11 mo of age. At the age of 4 mo, LXRβ$^{-/-}$ gallbladders did not exhibit any morphological abnormalities, but compared with WT mice (Fig. 4*A*), there was a significant ($P < 0.01$) increase in the percentage of PCNA-positive epithelial cells (Fig. 4 *B–E*). In WT mice, PCNA-positive cells were extremely rare (Fig. 4*A*).

At the age of 11 mo, PCNA-positive cells were predominant in the inflammatory, dysplastic, metaplastic, and adenomatous lesions of the LXRβ$^{-/-}$ gallbladder (Fig. 4 *D* and *E*). The proliferation marker PCNA remained very low in WT mice (Fig. 4 *C–E*).

Reduced Cell Death of Epithelial Cells in the Gallbladders of 11-mo-Old Female LXRβ$^{-/-}$ Mice. The rate of cell death was studied with TUNEL staining. Whereas WT mice exhibited an average of 2% to 5% positive cells both at the age of 4 and 11 mo (Fig. 4*F*), LXRβ$^{-/-}$ mice exhibit increased cell death at 4 mo (Fig. 4*G*) but almost completely absent reactivity at 11 mo (Fig. 4*H*).

Loss of E-Cadherin and Increased TGF-β Signaling in the Gallbladder of LXRβ$^{-/-}$ Female Mice. E-cadherin is a cell surface transmembrane glycoprotein with a key role in not only calcium-dependent intracellular adhesion but also cell polarity, growth, and differentiation (31). Loss of or reduced membrane positivity of E-cadherin has been described in biliary tract cancer (32, 33) as well as in breast (34) and colon cancer (35). E-cadherin expression was studied with immunohistochemistry. In the gallbladders of WT mice, E-cadherin was clearly detected on the plasma membrane and in the cytoplasmic region (Fig. 5*A*) of epithelial cells. In LXRβ$^{-/-}$ female gallbladders, E-cadherin was not evident on the plasma membrane, but there was a weak cytoplasmic staining in few epithelial cells (Fig. 5 *B* and *C*). E-cadherin expression is known to be negatively regulated by TGF-β (36) during epithelial-to-mesenchymal transition (EMT). Phospho-SMAD-2 (pSMAD-2) and SMAD-4 are intracellular mediators of the TGF-β cascade. In the gallbladders of WT mice, pSMAD-2 (Fig. 5 *D–F*) and SMAD-4 (Fig. 5 *D–G*) were expressed in the nuclei of epithelial cells. There was a markedly increased expression of these two transcription factors in the adenomas (Fig. 5*E*) and in the inflammatory/metaplastic/dysplastic areas (Fig. 5*H*) of LXRβ$^{-/-}$ female mouse gallbladders. pSMAD-2–positive cells were counted

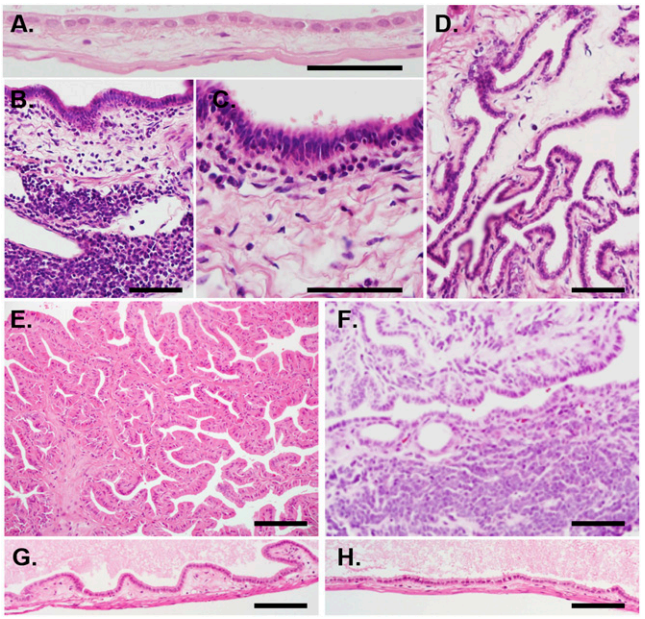

Fig. 2. Morphological study of 11- and 19-mo-old female mouse gallbladders. H&E staining in LXRβ$^{-/-}$ mice shows chronic cholecystitis (*B*) with severe immune cells infiltration compared with WT (*A*). (*B* and *C*) Dysplastic flat epithelia in which nuclear irregularities, pseudostratification, increased nucleus/cytoplasm ratio, loss of cell polarization, and hyperchromatism are evident. Cholecystitis (*D*) and adenoma (*E*) also appear in LXRβ$^{-/-}$ mice. (*F*) Carcinoma in situ with epithelial cells invading the entire gallbladder wall of 19-mo-old LXRβ$^{-/-}$ female mouse. Representative sections from the body of the gallbladder of LXRα$^{-/-}$ (*G*) and LXRα$^{-/-}$β$^{-/-}$ mice (*H*) do not demonstrate any inflammatory alterations with H&E staining. (Scale bars: 50 μm in *A–F*; 100 μm in *G* and *H*.)

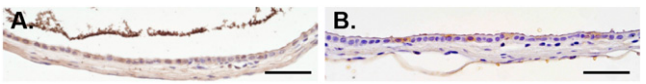

Fig. 1. Positive immunoreactivity of LXRα (*A*) and LXRβ (*B*) in the cell nuclei of gallbladder cholangiocytes. Representative sections from the body of the gallbladder of 11-mo-old WT female mice are shown. (Scale bars: 100 μm.)

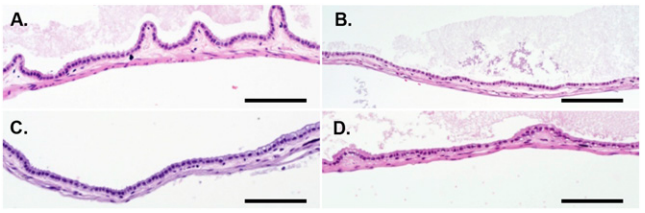

Fig. 3. Morphological study of 11-mo-old male mouse gallbladder. No inflammatory alterations are evident in LXRα$^{-/-}$ (*B*), LXRβ$^{-/-}$ (*C*), or LXRα$^{-/-}$β$^{-/-}$ (*D*) male gallbladders compared with WT (*A*). H&E staining of representative sections from the body of the gallbladder is shown. (Scale bar: 100 μm.)

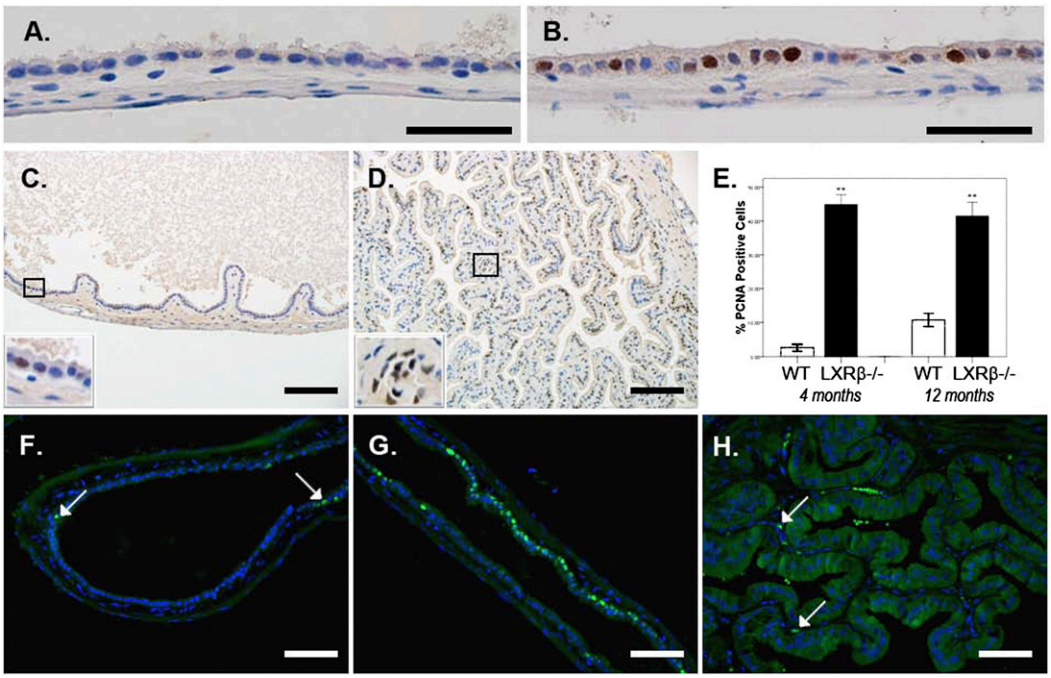

Fig. 4. Proliferation and cell death study in 4- and 11-mo-old female gallbladders. PCNA-positive immunoreactivity in LXRβ$^{-/-}$ gallbladder (*B*) is significantly increased compared with WT (*A*) at 4 mo of age. In the adenoma (*D*) of LXRβ$^{-/-}$ 11-mo-old gallbladder, PCNA-positive staining is enhanced compared with age-matched WT (*C*). PCNA-positive cells were counted. A statistically significant increase is shown in *E*. Data represent the mean ± SEM; $^{**}P < 0.01$. TUNEL staining shows an increased reactivity of LXRβ$^{-/-}$ gallbladders at 4 mo (*G*) compared with WT (*F*). At 11 mo (*H*), the positive reactivity was detected in only some stromal cells (arrows) but was almost completely absent in epithelial cells. (Scale bars: 50 μm in *A* and *B*; 100 μm in *C–H*.) *Inset*: Magnifications of selected areas.

in all the LXRβ$^{-/-}$ gallbladders, in which a significant increase ($P < 0.01$) was evident compared with WT (Fig. 5*F*).

Serum concentrations of TGF-β were measured by ELISA. Interestingly, compared with WT mice, levels of TGF-β were significantly ($P < 0.05$) higher in LXRβ$^{-/-}$ female mice affected by gallbladder disease (Fig. 5*I*).

Prevention of Gallbladder Preneoplastic Lesions by Ovariectomy in LXRβ$^{-/-}$ Mice. Because of the exclusive phenotype of cholecystitis/dysplasia/metaplasia/adenoma in only female mice and because of the higher prevalence of gallbladder diseases in women than in men, we ovariectomized LXRβ$^{-/-}$ mice at the age of 3 mo. Mice were then killed at 12 mo and compared with sham-operated littermates. Sham-operated LXRβ$^{-/-}$ mice showed chronic cholecystitis with dysplastic and metaplastic changes in their gallbladders (Fig. 6*A*). Interestingly, ovariectomized animals did not have any signs of inflammation, dysplasia, or metaplasia. The gallbladders of ovariectomized LXRβ$^{-/-}$ mice appeared to be atrophic with a very thin wall (Fig. 6*B*).

In sharp contrast to the sham-operated mice in which E-cadherin was not detectable on the plasma membrane (Fig. 6*C*), after ovariectomy, E-cadherin was expressed on the plasma membrane in a pattern similar to that of WT mice (Fig. 6*D*). Likewise, the immunoreactivity for pSMAD-2 was significantly ($P < 0.01$) reduced after ovariectomy (Fig. 6 *F* and *G*) compared with sham-operated animals (Fig. 6*E*).

Discussion

In this study we show with specific antibodies that LXRα and LXRβ are expressed in the nuclei of gallbladder cholangiocytes (Fig. 1). Whereas LXRα$^{-/-}$ and LXRα$^{-/-}$β$^{-/-}$ mice do not show any gallbladder abnormalities, the absence of LXRβ in female mice leads to a wide range of preneoplastic lesions, such as dysplasia, metaplasia, hyperplasia, and adenomas on a background of chronic cholecystitis (Fig. 2) at the age of 11 mo. Indeed, LXRβ seems to have an antiproliferative and proapoptotic role in the gallbladder cholangiocytes. As early as 4 mo of age, most of the epithelial cells of LXRβ$^{-/-}$ female mice are PCNA-positive (Fig. 3), which, at this age, is not associated with any morphological alterations, probably because of a slight increase in cell death (Fig. 4). The results of an early-age hyperproliferation is evident only after months in adult mice, as it is in the human, in which the carcinogenic process of the gallbladder takes as long as 15 y (26).

Interestingly, LXRα$^{-/-}$β$^{-/-}$ mice do not show any pathological alteration of the gallbladder. In a speculative view, LXRα may have a proproliferative activity in cholangiocytes, which is capable of balancing the action of LXRβ.

Treatment with LXR agonist has an antiproliferative effect in vitro and in vivo (11–15) with a mechanism that seems to involve an increase of the kinase inhibitor p27 (37), suppression of the oncogenic β-catenin (38), and an interplay with TGF-β signaling (16). Indeed, in the ventral prostate of LXRα$^{-/-}$ mice, an abnormal stromal overgrowth is accompanied by high expression of SNAIL and SMAD-2/3, markers of an enhanced TGF-β activity (16). A cross-talk between LXRα and TGF-β has been previously hypothesized but still remains poorly understood. This interplay seems to involve the nuclear receptor coactivator RAP250 that has the capacity to bind to LXRα and LXR-β (39) and to SMAD-2/3 (40). As a result of this interaction, TGF-β has synergistic effects with LXR agonists (40) on the transcription of LXR's target genes. TGF-β signaling is also affected in the gallbladder of LXRβ$^{-/-}$ mice, in which an increased nuclear expression of pSMAD-2 and SMAD-4 has been detected (Fig. 5). The consequence of a strong TGF-β activity is the loss of E-cadherin immunoreactivity from the membrane of epithelial cells (Fig. 5). The analogy with human gallbladder carcinogenesis is quite strong. In human gallbladder cancers and in precursor lesions, E-cadherin expression is significantly reduced (41) and correlates with poor

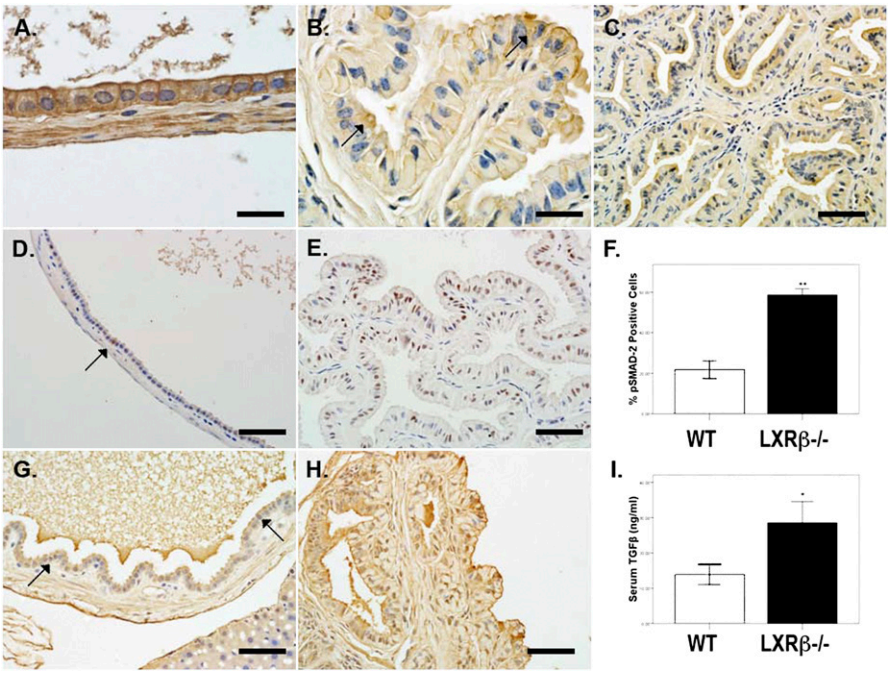

Fig. 5. Loss of E-cadherin and increased TGF-β signaling in 11-mo-old LXRβ$^{-/-}$ mice. (*A*) E-cadherin–positive immunoreactivity is seen mainly on the membrane of gallbladder epithelial cells in WT mice. The membrane positivity is lost in LXRβ$^{-/-}$ gallbladders (*B* and *C*), in which only a weak cytoplasmatic staining is seen in few epithelial cells (arrows). (*D*) pSMAD-2 nuclear immunoreactivity is evident in nuclei of epithelial cells (arrow) of WT mice. A larger number of positive cells is seen in the adenoma (*E*) of LXRβ$^{-/-}$ mice. (*F*) Statistical analysis of pSMAD-2–positive cells shows a significant increase in the KO animals. Data represent the mean ± SEM; $^{**}P < 0.01$. (*G*) SMAD-4 nuclear immunoreactivity is seen in nuclei of epithelial cells (arrows) of WT mice. A larger number of positive cells is seen in the gallbladder of LXRβ$^{-/-}$ mice (*H*). (*I*) TGF-β levels in serum of 11-mo-old LXRβ$^{-/-}$ female mice are significantly increased compared with WT. Data represent mean ± SEM; $^{*}P < 0.05$; WT mice, $n = 10$; LXRβ$^{-/-}$, $n = 12$. (Scale bar: 50 μm.)

prognosis (33, 42). TGF-β signaling is affected as well. Strong TGF-β immunoreactivity is detected in human gallbladder tumors independently of the histological type, as well as in premalignant lesions (43). Specific polymorphism of TGF-β1 has been associated with an increased susceptibility to gallbladder cancers without gallstones (44), and SMAD-4 mutations have been described in biliary tract cancer (45). Interestingly, mice with orthotopic transplantation of human gallbladder carcinoma cell line into the gallbladder wall exhibit 300% higher plasma TGF-β than control mice (43).

Accordingly, we measured serum TGF-β in our transgenic animals and found that LXRβ$^{-/-}$ female mice have significantly higher levels of TGF-β compared with WT mice (Fig. 5). We may therefore speculate that TGF-β hyperproduction and secretion by LXRβ$^{-/-}$ premalignant epithelia may be responsible for an autocrine mechanism that results in a loss of E-cadherin and in a further malignant transformation.

Female sex (19), use of contraceptive pills (46), and oral hormonal replacement therapy (25) are associated with high risk of gallbladder cancer, indicating a strong role for estrogens in the carcinogenesis process of this organ. Only female LXRβ$^{-/-}$ animals demonstrate preneoplastic lesions and the elimination of estrogens with ovariectomy prevents the formation of these lesions (Fig. 6). Interestingly, after ovariectomy, E-cadherin expression returns to normal on membrane of epithelial cells (Fig. 6).

Our results indicate that LXRβ may act as an antiproliferative agent capable to oppose the proliferative action of estrogens on the gallbladder epithelium. In conclusion, the gallbladder preneoplastic lesions in LXRβ mice are the effect of a complex interplay between the absence of the antiproliferative and antiinflammatory action of LXRβ as well as the hyperactivation of TGF-β signaling and estrogen action.

More studies are required to clarify this cross-talk, especially in women, in whom the combination of LXR agonist and antiestrogenic drugs could be a promising treatment for gallbladder cancer.

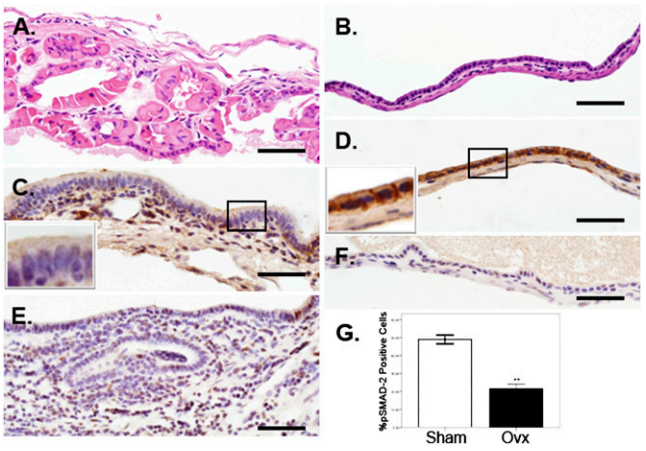

Fig. 6. Normal gallbladder phenotype in ovariectomized LXRβ$^{-/-}$ mice. H&E staining shows cholecystitis in LXRβ$^{-/-}$ sham-operated female mice (*A*). No inflammatory alterations are seen in gallbladders from ovariectomized LXRβ$^{-/-}$ mice (*B*). In sham LXRβ$^{-/-}$ mice, E-cadherin immunoreactivity is lost from the membrane of gallbladder epithelial cells (*C*), whereas it is expressed in ovariectomized LXRβ$^{-/-}$ mice (*D*). (*Inset*) Higher magnifications of selected areas. pSMAD2 is highly expressed in the gallbladder of sham-operated LXRβ$^{-/-}$ mice (*E*), whereas only few nuclei show pSMAD-2 expression in ovariectomized LXRβ$^{-/-}$ mice (*F*). (*G*) Statistical study of pSMAD-2–positive cells shows a significantly decreased expression after ovariectomy. Data represent mean ± SEM; $^{**}P < 0.01$; LXRβ$^{-/-}$ ovariectomized, $n = 10$; LXRβ$^{-/-}$ sham, $n = 10$. (Scale bar: 50 μm.)

Materials and Methods

Animals. WT, LXR$\alpha^{-/-}$, LXR$\beta^{-/-}$, and LXR$\alpha^{-/-}\beta^{-/-}$ mice were generated as previously described (47, 48). All mice were backcrossed to C57BL/6 mice for at least 10 generations. Animals were housed on a 12-h light/dark cycle under controlled temperature (20–22 °C) and humidity (50–65%) in the Karolinska University Hospital Animal Facility (Huddinge, Sweden). Mice were given free access to tap water and standard chow diet (RM3; Scanbur).

Experiments were approved by the local ethical committee for animal experiments and the guidelines for the care and use of laboratory animals were followed.

Ovariectomy. At 3 mo of age, 10 LXR$\beta^{-/-}$ female mice were ovariectomized and 10 mice were sham-operated as controls. Anesthesia was performed with an i.p. injection of midazolam (0.1 mg/10 g of body weight), medetomidine hydrochloride (0.01 mg per 10 g body weight) and fentanyl (0.5 μg per 10 g body weight). Mice were bilaterally ovariectomized through a single dorsal midline incision across the lumbar region, making both ovaries accessible. The ovary-attached fat pad was gently grasped to lift and exteriorize the ovary. Subsequently, the periovarian sac was peeled back over the surface of the ovary, allowing removal of the whole ovary. Both ovaries of the ovariectomized group mice were removed, whereas ovaries of the sham-operated group were left in situ.

Tissues Processing. Mice were killed by CO_2 asphyxiation. Blood was collected by intracardiac puncture for serum collection. Gallbladders were dissected and fixed overnight in 4% paraformaldehyde at 4 °C for immunohistochemical studies.

Antibodies. Goat polyclonal IgG against LXRα and against LXRβ were prepared in our laboratory as previously described (16, 30). The following antibodies were used: rabbit polyclonal to PCNA (GTX22426; GeneTex) at 1:200 dilution; rabbit polyclonal anti–E-cadherin (H-108, sc-7870; Santa Cruz Biotechnology) at 1:200; rabbit anti–pSMAD-2 (AB3849; Chemicon) at 1:100; and rabbit monoclonal to SMAD-4 (Ab40759; Abcam) at 1:100. Secondary antibodies—goat antirabbit (65-6140) and rabbit antigoat (81-1640)—were purchased from Zymed and used at 1:200 dilutions.

Immunohistochemistry. Paraffin sections (4 μm thickness) were dewaxed in xylene and rehydrated through graded ethanol. Antigens were retrieved by boiling 10 mM citrate buffer (pH 6.0) for 5 min. Cooled sections were incubated in 3% H_2O_2 in 50% methanol for 30 min at room temperature to quench endogenous peroxidase, and then incubated in 0.5% Triton X-100 in PBS solution for 10 min. Nonspecific binding was blocked with PBS solution containing 1% BSA and 0.1% Nonidet P-40 for 1 h at 4 °C. Sections were then immunostained with primary antibodies in PBS solution containing 1% BSA and 0.1% Nonidet P-40 overnight at 4 °C. PBS solution containing 1% BSA and 0.1% Nonidet P-40 replaced the primary antibodies as negative control. After washing, sections were incubated with the corresponding secondary antibodies at room temperature for 1 h. The Vectastain ABC kit (Vector Laboratories) was used for avidin–biotin complex method to visualize the signal, according to the manufacturer's instructions. After washing in PBS solution, sections were developed with 3,3′-diaminobenzidine tetrahydrochloride substrate (Dako) and lightly counterstained with Mayer hematoxylin. Sections were then dehydrated through a graded ethanol series and xylene and mounted.

The histopathological analysis was performed by an experienced pathologist blinded to the groups.

Proliferation Study and Evaluation of Cell Death. PCNA staining was specific for the nuclei. Although the nuclear staining had different intensity, all detectable staining was considered positive. Three random sections were studied for each sample at a magnification 40× under an optical microscope (BX-51; Olympus). One hundred and 500 cell nuclei were counted in each section of WT and LXR$\beta^{-/-}$ mice, respectively.

To identify cell death, TUNEL was performed using an in situ cell death detection kit (Roche) according to the manufacturer's instructions for paraffin-embedded tissues.

Serum TGF-β Assay. Levels of TGF-β in serum were measured using the Quantikine ELISA kit (MB100B; R&D Systems) according to the manufacturer's instructions.

Statistical Analyses. Data were expressed as mean ± SE and the Student *t* test was used to analyze individual differences. A value of $P < 0.05$ was considered to be statistically significant. Statistical analysis was performed with the aid of SPSS statistical software (version 17.0 for Windows).

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1. Apfel R, et al. (1994) A novel orphan receptor specific for a subset of thyroid hormone-responsive elements and its interaction with the retinoid/thyroid hormone receptor subfamily. *Mol Cell Biol* 14:7025–7035.
2. Teboul M, et al. (1995) OR-1, a member of the nuclear receptor superfamily that interacts with the 9-cis-retinoic acid receptor. *Proc Natl Acad Sci USA* 92:2096–2100.
3. Willy PJ, Mangelsdorf DJ (1997) Unique requirements for retinoid-dependent transcriptional activation by the orphan receptor LXR. *Genes Dev* 11:289–298.
4. Janowski BA, et al. (1999) Structural requirements of ligands for the oxysterol liver X receptors LXRalpha and LXRbeta. *Proc Natl Acad Sci USA* 96:266–271.
5. Peet DJ, et al. (1998) Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXR alpha. *Cell* 93:693–704.
6. Yu L, et al. (2005) Expression of ABCG5 and ABCG8 is required for regulation of biliary cholesterol secretion. *J Biol Chem* 280:8742–8747.
7. Repa JJ, et al. (2000) Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta. *Genes Dev* 14: 2819–2830.
8. Joseph SB, et al. (2002) Direct and indirect mechanisms for regulation of fatty acid synthase gene expression by liver X receptors. *J Biol Chem* 277:11019–11025.
9. Cao G, et al. (2003) Antidiabetic action of a liver x receptor agonist mediated by inhibition of hepatic gluconeogenesis. *J Biol Chem* 278:1131–1136.
10. Lo Sasso G, et al. (2010) Down-regulation of the LXR transcriptome provides the requisite cholesterol levels to proliferating hepatocytes. *Hepatology* 51:1334–1344.
11. Chuu CP, et al. (2006) Inhibition of tumor growth and progression of LNCaP prostate cancer cells in athymic mice by androgen and liver X receptor agonist. *Cancer Res* 66: 6482–6486.
12. Vedin LL, Lewandowski SA, Parini P, Gustafsson JA, Steffensen KR (2009) The oxysterol receptor LXR inhibits proliferation of human breast cancer cells. *Carcinogenesis* 30: 575–579.
13. Wente W, Brenner MB, Zitzer H, Gromada J, Efanov AM (2007) Activation of liver X receptors and retinoid X receptors induces growth arrest and apoptosis in insulin-secreting cells. *Endocrinology* 148:1843–1849.
14. Meng ZX, et al. (2009) Activation of liver X receptors inhibits pancreatic islet beta cell proliferation through cell cycle arrest. *Diabetologia* 52:125–135.
15. Geyeregger R, et al. (2009) Liver X receptors interfere with cytokine-induced proliferation and cell survival in normal and leukemic lymphocytes. *J Leukoc Biol* 86: 1039–1048.
16. Kim HJ, Andersson LC, Bouton D, Warner M, Gustafsson JA (2009) Stromal growth and epithelial cell proliferation in ventral prostates of liver X receptor knockout mice. *Proc Natl Acad Sci USA* 106:558–563.
17. Gong H, et al. (2007) Estrogen deprivation and inhibition of breast cancer growth in vivo through activation of the orphan nuclear receptor liver X receptor. *Mol Endocrinol* 21:1781–1790.
18. Landis SH, Murray T, Bolden S, Wingo PA (1998) Cancer statistics, 1998. *CA Cancer J Clin* 48:6–29.
19. Lazcano-Ponce EC, et al. (2001) Epidemiology and molecular pathology of gallbladder cancer. *CA Cancer J Clin* 51:349–364.
20. Misra S, Chaturvedi A, Misra NC, Sharma ID (2003) Carcinoma of the gallbladder. *Lancet Oncol* 4:167–176.
21. Henson DE, Albores-Saavedra J, Corle D (1992) Carcinoma of the gallbladder. Histologic types, stage of disease, grade, and survival rates. *Cancer* 70:1493–1497.
22. Lowenfels AB, Walker AM, Althaus DP, Townsend G, Domellöf L (1989) Gallstone growth, size, and risk of gallbladder cancer: An interracial study. *Int J Epidemiol* 18: 50–54.
23. Farquhar C, Marjoribanks J, Lethaby A, Suckling JA, Lamberts Q (2009) Long term hormone therapy for perimenopausal and postmenopausal women. *Cochrane Database Syst Rev* 2:CD004143.
24. Gallus S, et al. (2002) Post-menopausal hormonal therapy and gallbladder cancer risk. *Int J Cancer* 99:762–763.
25. Liu B, et al.; Million Women Study Collaborators (2008) Gallbladder disease and use of transdermal versus oral hormone replacement therapy in postmenopausal women: prospective cohort study. *Br Med J* 337:a386.
26. Roa I, et al. (1996) Preneoplastic lesions and gallbladder cancer: An estimate of the period required for progression. *Gastroenterology* 111:232–236.
27. Roa I, de Aretxabala X, Araya JC, Roa J (2006) Preneoplastic lesions in gallbladder cancer. *J Surg Oncol* 93:615–623.
28. Wistuba II, Gazdar AF (2004) Gallbladder cancer: lessons from a rare tumour. *Nat Rev Cancer* 4:695–706.
29. Kozuka S, Tsubone N, Yasui A, Hachisuka K (1982) Relation of adenoma to carcinoma in the gallbladder. *Cancer* 50:2226–2234.
30. Gabbi C, et al. (2008) Pancreatic exocrine insufficiency in LXRbeta-/- mice is associated with a reduction in aquaporin-1 expression. *Proc Natl Acad Sci USA* 105:15052–15057.

31. Ivanov DB, Philippova MP, Tkachuk VA (2001) Structure and functions of classical cadherins. *Biochemistry (Mosc)* 66:1174–1186.
32. Németh Z, et al. (2009) Zonula occludens-1, occludin, and E-cadherin protein expression in biliary tract cancers. *Pathol Oncol Res* 15:533–539.
33. Hirata K, et al. (2006) Frequent occurrence of abnormal E-cadherin/beta-catenin protein expression in advanced gallbladder cancers and its association with decreased apoptosis. *Oncology* 71:102–110.
34. Berx G, Van Roy F (2001) The E-cadherin/catenin complex: an important gatekeeper in breast cancer tumorigenesis and malignant progression. *Breast Cancer Res* 3: 289–293.
35. Hugh TJ, et al. (1999) Cadherin-catenin expression in primary colorectal cancer: a survival analysis. *Br J Cancer* 80:1046–1051.
36. Cano A, et al. (2000) The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat Cell Biol* 2:76–83.
37. Fukuchi J, Kokontis JM, Hiipakka RA, Chuu CP, Liao S (2004) Antiproliferative effect of liver X receptor agonists on LNCaP human prostate cancer cells. *Cancer Res* 64: 7686–7689.
38. Uno S, et al. (2009) Suppression of beta-catenin signaling by liver X receptor ligands. *Biochem Pharmacol* 77:186–195.
39. Lee SK, et al. (2001) Two distinct nuclear receptor-interaction domains and CREB-binding protein-dependent transactivation function of activating signal cointegrator-2. *Mol Endocrinol* 15:241–254.
40. Antonson P, et al. (2008) RAP250 is a coactivator in the transforming growth factor beta signaling pathway that interacts with Smad2 and Smad3. *J Biol Chem* 283:8995–9001.
41. Choi YL, et al. (2004) An immunohistochemical study of the expression of adhesion molecules in gallbladder lesions. *J Histochem Cytochem* 52:591–601.
42. Mori M, Miyazaki K (2000) Factors affecting morphogenesis of rabbit gallbladder epithelial cells cultured in collagen gels. *Cell Tissue Res* 300:331–344.
43. Gohongi T, et al. (1999) Tumor-host interactions in the gallbladder suppress distal angiogenesis and tumor growth: Involvement of transforming growth factor beta1. *Nat Med* 5:1203–1208.
44. Vishnoi M, Pandey SN, Modi DR, Kumar A, Mittal B (2008) Genetic susceptibility of epidermal growth factor +61A>G and transforming growth factor beta1 -509C>T gene polymorphisms with gallbladder cancer. *Hum Immunol* 69:360–367.
45. Hahn SA, et al. (1998) Mutations of the DPC4/Smad4 gene in biliary tract carcinoma. *Cancer Res* 58:1124–1126.
46. Dorjgochoo T, et al. (2009) Use of oral contraceptives, intrauterine devices and tubal sterilization and cancer risk in a large prospective study, from 1996 to 2006. *Int J Cancer* 124:2442–2449.
47. Schuster GU, et al. (2002) Accumulation of foam cells in liver X receptor-deficient mice. *Circulation* 106:1147–1153.
48. Alberti S, et al. (2001) Hepatic cholesterol metabolism and resistance to dietary cholesterol in LXRbeta-deficient mice. *J Clin Invest* 107:565–573.