Gastrointestinal, Hepatobiliary and Pancreatic Pathology

Ezrin-Radixin-Moesin-Binding Phosphoprotein (EBP50), an Estrogen-Inducible Scaffold Protein, Contributes to Biliary Epithelial Cell Proliferation

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Ezrin-radixin-moesin-binding phosphoprotein 50 (EBP50) anchors and regulates apical membrane proteins in epithelia. EBP50 is inducible by estrogen and may affect cell proliferation, although this latter function remains unclear. The goal of this study was to determine whether EBP50 was implicated in the ductular reaction that occurs in liver disease. EBP50 expression was examined in normal human liver, in human cholangiopathies (ie, cystic fibrosis, primary biliary cirrhosis, and primary sclerosing cholangitis), and in rats subjected to bile-duct ligation. The regulation of EBP50 by estrogens and its impact on proliferation were assessed in both bile duct-ligated rats and Mz-Cha-1 human biliary epithelial cells. Analyses of cell isolates and immunohistochemical studies showed that in normal human liver, EBP50 is expressed in the canalicular membranes of hepatocytes and, together with ezrin and cystic fibrosis transmembrane conductance regulator, in the apical domains of cholangiocytes. In both human cholangiopathies and bile duct-ligated rats, EBP50 was redistributed to the cytoplasmic and nuclear compartments. EBP50 underwent a transient increase in rat

cholangiocytes after bile-duct ligation, whereas such expression was down-regulated in ovariectomized rats. In addition, in Mz-Cha-1 cells, EBP50 underwent up-regulation and intracellular redistribution in response to 17 β -estradiol, whereas its proliferation was inhibited by siRNA-mediated EBP50 knockdown. These results indicate that both the expression and distribution of EBP50 are regulated by estrogens and contribute to the proliferative response in biliary epithelial cells. (*Am J Pathol* 2009, 174:869–880; DOI: 10.2353/ajpath.2009.080079)

Ezrin-radixin-moesin (ERM) binding phosphoprotein 50 (EBP50) is an adapter protein, normally localized in the apical region of epithelial cells.¹ This protein contains two postsynaptic density 95/disc-large/zona occludens (PDZ) domains that can bind integral membrane proteins such as transporters, and an ERM-binding domain. EBP50 is required for the maintenance of active ERM proteins at the cortical brush border membranes of polarized epithelia.² In addition, EBP50 assembles multiprotein complexes that anchor transporters to the apical actin cytoskeleton and facilitate their regulation. The liver is the tissue that displays the highest level of EBP50 expression.^{3,4} We previously showed that in rat liver, EBP50 is expressed both in hepatocytes and in cholangiocytes, and that, in the latter, the interaction of cystic fibrosis transmembrane conductance regulator (CFTR) with the PDZ1 domain of EBP50 is required for cAMP-

Supported by the Vaincre la Mucoviscidose, the Agence Nationale de la Recherche (program PHYSIO 2006, LIFR-PP), the Institut National du Cancer (grant PL027), the Swedish Research Council (grant 9127), the Karolinska Institute, the Swedish Society of Medicine (Bengt Ihre's fund), and an unrestricted grant from the Lundin family.

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Accepted for publication November 24, 2008.

Supplemental material for this article can be found on <http://ajp.amjpathol.org>.

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dependent chloride secretion,⁵ suggesting an important role of EBP50 in bile secretory functions.

Different lines of evidence suggest that EBP50 has other regulatory functions that potentially include the modulation of cell proliferation.⁶ In tumors, ie, in hepatocellular or breast carcinomas, and in non tumor proliferative tissue such as the endometrium, EBP50 can be overexpressed and redistributed to the cytoplasm and/or nucleus of epithelial cells.^{7–10} In addition, some EBP50 binding partners, eg, platelet-derived growth factor or epidermal growth factor receptors, PTEN, β -catenin, and Pin1 signaling molecules, are directly involved in cell proliferation.^{8,11–15} However, the exact impact of EBP50 on cell proliferation remains unclear. Whereas *in vitro* experiments have suggested anti-proliferative functions of EBP50,^{14–16} a positive correlation between EBP50 expression, estrogen receptor status, and tumor progression has been found in human breast cancer.^{9,10} Of particular interest with respect to biliary pathophysiology, the major regulators of EBP50 expression are estrogens,^{4,17} which are known to target the biliary tree, where they modulate the proliferative activities of cholangiocytes.^{18–20}

In the present study, we examined the expression of EBP50 and binding partners in the human liver, under normal conditions and in the setting of biliary disorders, ie, in different types of human cholangiopathies and after bile duct ligation (BDL) in rats. We tested the hypothesis that EBP50 could be regulated by estrogens, control cell proliferation in cholangiocytes, and thereby participate to ductular reactions in biliary disorders.

Materials and Methods

Patients and Human Tissue Samples

Human tissue samples were used with informed consent of the patients on approval by the Regional Ethical Committees at Karolinska and Göteborg University Hospitals. Liver tissue was obtained by liver biopsy from 10 patients with cystic fibrosis (CF) liver disease (4 were females; mean age, 15.3 years; range, 3.6 to 38 years), 5 with primary biliary cirrhosis (PBC) (all females; mean age, 60.8 years; range, 45 to 68 years), 3 with primary sclerosing cholangitis (PSC) (all females; mean age, 51.3 years; range, 42 to 58 years), and 1 (male) who had bile stone obstruction for 7 months. Most of the patients had early-stage liver disease, with the exception of one CF patient having extensive fibrosis, one PBC, and one PSC patient, having cirrhosis. All CF patients were either homozygous or compound heterozygous for the $\Delta F508$ CFTR mutation. Normal liver and gallbladder tissue specimens (ie, with no histological abnormality) were obtained from patients who underwent cholecystectomy or liver surgery for focal lesion(s). Immediately after tissue collection, part of the sample was snap-frozen in liquid nitrogen and stored at -70°C .

Animal Model

BDL was performed by double ligation and section of the common bile duct in male and female Sprague-Dawley rats (Janvier, Le Genest Saint-Isle, France) 10 to 12 weeks of age, as reported.²¹ Sham operation consisted in laparotomy and bile duct exposure without ligation. To assess the impact of estrogens, female rats were ovariectomized 3 to 5 weeks before BDL was performed, as previously described.¹⁹ The time course of cholangiocyte proliferation including after ovariectomy, was previously established.^{19,22,23} Experiments were conducted in compliance with the national ethical guidelines for the care and use of laboratory animals. The animals were anesthetized with a subcutaneous injection of chlorpromazine (2 mg/kg) and ketamine (20 mg/kg). Investigations were performed on postoperative days 1, 2, and 7. For immunofluorescence analyses, the liver was perfused *in situ* with 4% paraformaldehyde, cut in small pieces, postfixed in 4% paraformaldehyde for 1 hour at 4°C , and stored in 1% paraformaldehyde overnight at 4°C .

Cell Isolation and Culture

Human hepatocytes, intrahepatic bile ducts, and gallbladder epithelial cells were isolated from samples of normal human liver and gallbladder, using established methods.²⁴ Isolation of intrahepatic bile ducts from rat liver was performed as described.²⁵ More than 90% of the cells in bile duct preparations were cholangiocytes, as ascertained by cytokeratin 19 and γ -glutamyltransferase staining.²⁵ The human biliary epithelial cell line Mz-Cha-1²⁶ was provided by Alexander Knuth, (Zurich University Hospital, Zurich, Switzerland). Mz-Cha-1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% Hepes. To test the effect of estrogens, Mz-Cha-1 cells were placed in serum- and phenol red-free Dulbecco's modified Eagle's medium/1% Hepes for 24 hours before treatment with 17β -estradiol (Sigma-Aldrich Chemie S.a.r.l., L'Isle d'Abeau Chesnes, France) at 10^{-10} or 10^{-7} mol/L for 6 to 24 hours. Alternatively, to completely deprive Mz-Cha-1 cells of estrogen receptor stimulation, the cells were maintained in phenol red-free Dulbecco's modified Eagle's medium/1% Hepes supplemented with 10% charcoal-stripped serum for 2 to 3 weeks.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from liver tissue and cell preparations using RNA lysis solution (Quantum, Montreuil-sous-Bois, France). Complementary DNA was synthesized from 1 μg of total RNA using pd(N)₆ primers (Amersham, GE Health Care Europe GmbH, Saclay, France) and the Moloney murine leukemia virus reverse transcriptase (Invitrogen, Cergy-Pontoise, France).

EBP50, ezrin, CFTR, and β -actin transcripts were detected by conventional PCR, using the following primers: 5'-GATCGCATTGTGGAGGTGAA-3' (forward) and 5'-

GGAGATGTTGAAGTCTAGGA-3' (reverse) to amplify a 389-bp fragment of EBP50 cDNA; 5'-GCAGGACTATGAGGAGAAGAC-3' (forward) and 5'-GTGATGCGCTTCTCCTCATTG-3' (reverse) to amplify a 503-bp fragment of ezrin cDNA; 5'-AACTGCTGAACGAGAGGAGC-3' (forward) and 5'-TTGACTATTGCCAGGAAGCC-3' (reverse) to amplify a 367-bp fragment of CFTR cDNA; 5'-CCTCATGAAGATCCTCACCG-3' (forward) and 5'-CAGTGATCTCCTTCTGCATCC-3' (reverse) to amplify a 660-bp fragment of β -actin cDNA. PCR products obtained after completion of 28 cycles were separated by electrophoresis through a 2% agarose gel stained with ethidium bromide.

Quantitative real-time PCR was performed with the TaqMan system, using the SYBR green master mix (Applied Biosystems, Courtaboeuf, France). The primers were designed according to published human cDNA sequences in GenBank database using the Primer Express software v1.5 (Applied Biosystems). EBP50 (*SLC9A3R1*, accession no. NM_004252), 5'-CCAGGATCGCATTGTGGAG-3' (forward) and 5'-CCATTGGTGAAGGGCACAG-3' (reverse); ezrin (*VIL2*, accession no. NM_003379), 5'-CTAGAGGCTGACCGTATGGCTG-3' (forward) and 5'-GAGGGCAATCTTGGCAGTGT-3' (reverse); CFTR (*ABCC7*, accession no. NM_000492), 5'-CCATCAGCCCCCTCCGAC-3' (forward) and 5'-AAAGCCTTGTATCTTGCACCTCT-3' (reverse); 18S rRNA (accession no. NM_002801), 5'-GAGCGAAAGCATTTGCCAAG-3' (forward) and 5'-GGCATCGTTTATGGTCGGAA-3' (reverse). 18S rRNA TaqMan assay reagent was used for internal control. One-step RT-PCR was performed for both target gene and endogenous controls. Duplicate C_T values were analyzed in Microsoft Excel (Microsoft Corp., Redmond, WA) using the comparative C_T ($\Delta\Delta C_T$) method as described by the manufacturer (Applied Biosystems). The amount of target ($2^{-\Delta\Delta C_T}$) was obtained as normalized to 18S.

Immunohisto(cyto)chemical Analyses

Human liver cryosections (6 μ m) were subjected to immunolabeling with anti-EBP50 rabbit polyclonal antibody (catalog no. 324620; Calbiochem, Fontenay sous Bois, France) at a dilution of 1:300; or mouse monoclonal antibodies raised against EBP50 (catalog no. 611160; R&D Systems Europe Ltd., Lille, France) at 1:50; ezrin (catalog no. MS-661-P1; NeoMarker, Montluçon, France) at 1:40; CFTR (catalog no. MAB-25031; R&D Systems Europe Ltd., Abingdon, UK) at 1:100, or Ki-67 (catalog no. dia607; Dianova, Hamburg, Germany) at 1:100. Except for ezrin detection by an alkaline phosphatase anti-alkaline phosphatase/Fast Red protocol (Vector Laboratories, Paris, France), immunolabeling was performed using an avidin-biotin method. For the latter, the sections were fixed in 4% paraformaldehyde and were incubated subsequently with serum and avidin-biotin blocking reagents (Vectastain ABC, Vector Laboratories), with the primary antibody overnight at 4°C, with a biotinylated secondary antibody (Vector Laboratories) for 30 minutes at room temperature and with Cy3-streptavidin (catalog no. S6402, Sigma-Aldrich Chemie S.a.r.l.) for 60 minutes at

room temperature. For double immunofluorescence, secondary antibodies were conjugated with tetramethylrhodamine isothiocyanate (Jackson ImmunoResearch Europe Ltd., Monlutcon, France) or Alexa 488 (Invitrogen Molecular Probes, Cergy-Pontoise, Paris); 4',6-diamidino-2-phenylindole (Sigma-Aldrich Chemie S.a.r.l.) at a dilution of 1:10,000 or SYTO16 (Invitrogen Molecular Probes) at 1:25,000 were added to the second-last wash for nuclear staining. The samples were mounted with an anti-fading medium (Vectashield, Vector Laboratories). The slides were examined with a Nikon Eclipse E800 microscope connected to a Nikon DXM1200 digital camera and images were acquired with ACT-1 software (Nikon, Tokyo, Japan). Laser confocal microscopy was performed with a Leica TCS SP confocal laser-scanning microscope and images were analyzed with Leica confocal software (Leica, Wetzlar, Germany).

Triple staining of EBP50, actin, and DNA was achieved on thick sections (50 μ m) of rat liver, obtained with a vibrating blade microtome (VT1000E, Leica Microsystems) and permeabilized in saponin 0.1% (Sigma-Aldrich Chemie S.a.r.l.) for 1 hour. All incubations were performed on floating sections at room temperature (unless otherwise stated), under gentle rocking. After quenching of the aldehydes [30 minutes in phosphate-buffered saline (PBS)/NH₄Cl 50 mmol/L], the sections were incubated for 2 hours in blocking buffer (PBS with bovine serum albumin 1%, goat serum 10% and saponin 0.1%). All subsequent incubations were performed in PBS, saponin 1%. For EBP50 staining, the sections were incubated successively with anti-EBP50 rabbit polyclonal antibody (Calbiochem) (2 μ g/ml, overnight at 4°C) and Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen Molecular Probes) (10 μ g/ml, 2 hours). Filamentous actin was stained with Alexa Fluor 555 phalloidin (5 U/ml for 40 minutes) and eventually cell nuclei were counterstained with TO-PRO-3 (1 μ mol/L for 20 minutes). Sections were mounted in glycerol/PBS (90/10:v/v). Images were recorded on a Leica TCS SP2 confocal microscope (Leica Microsystems) equipped with a $\times 63$ oil-immersion objective (NA = 1.32). The three channels were acquired sequentially with the following excitation and emission parameters: 488 nm, 500 to 540 nm, for Alexa 488; 543 nm, 555 to 615 nm, for Alexa 555; and 633 nm, 645 to 750 nm, for TO-PRO-3. Gains were adjusted to avoid saturation in pixels intensity.

The same procedure was used to perform triple staining in Mz-Cha-1 cells, except that incubation times were reduced to 15 minutes for fixation, 10 minutes for permeabilization, 1 hour for each antibody, 20 minutes for phalloidin, and 10 minutes for TO-PRO-3. β -Catenin immunostaining was performed with an anti- β -catenin polyclonal antibody (Cell Signaling, Ozyme, Saint Quentin en Yvelines, France). Ki-67 immunolabeling of Mz-Cha-1 cells was analyzed using an anti-Ki-67 polyclonal antibody coupled with fluorescein isothiocyanate (dilution 1:100; Abcam, Paris, France). Nuclei were stained with TO-PRO-3, and the percentage of Ki-67-labeled nuclei was determined.

SiRNA Stable Transfection

Mz-Cha-1 cells were stably transfected with a plasmid encoding human EBP50 siRNA (provided by Brian R. Doctor, University of Colorado Health Sciences Center, Denver, CO) or scrambled siRNA by incubation in the presence of Lipofectamine 2000 (Invitrogen) for 2 days. Transfected cells were then 10-fold serially diluted into 10-cm Petri dishes and incubated in the absence of selection for 2 additional days. Puromycin was added at a final concentration of 1 μ g/ml to select cells that had acquired the plasmids. Culture medium containing puromycin was changed every 2 to 3 days and when colonies appeared, generally 12 to 14 days later, individual puromycin-resistant colonies were selected and cultured in 12-well culture dishes.

BrdU Proliferation Assay

Mz-Cha-1 cells were cultured in 96-wells plates (10,000 cells/well) in serum-deprived medium for 3 days and then incubated with 10 μ mol/L BrdU in the presence of serum for 1 hour. BrdU incorporation was measured in triplicate, using a cell proliferation enzyme-linked immunosorbent assay kit (Roche Applied Science, Meylan, France).

Immunoblotting

Immunoblotting was performed with mouse monoclonal antibodies raised against proliferating cell nuclear antigen (PCNA) (clone PC10, Cell Signaling) at a dilution of 1:2000, EBP50 (clone 6; BD Biosciences, Le Pont-De-Claix, France) at 1:250, α -tubulin (clone DM1A; Abcam, Paris, France) at 1:5000, lamin A/C (clone JOL2; Millipore Chemicon, Paris, France) at 1:500 or β -actin (Sigma-Aldrich Chimie S.a.r.l.) at 1:10,000. Proteins were extracted from Mz-Cha-1 cells using a lysis buffer composed of 150 mmol/L NaCl, 2 mmol/L phenylmethyl sulfonyl fluoride, 1% Nonidet P-40, 0.5% deoxycholate, 1 mg/L aprotinin, 0.1% sodium dodecyl sulfate, and 50 mmol/L Tris-HCl, pH 7.5. Lysates were precleared by centrifugation at 13,000 $\times$ g for 30 minutes at 4°C. Subcellular fractions were prepared with the NE-PER nuclear and cytoplasmic extraction reagents kit (Pierce, Perbio Science France SAS, Brebières, France). Protein concentration was determined by the bicinchoninic acid-based BCA protein assay kit (Pierce, Perbio Science France SAS). Proteins (5 to 20 μ g) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were incubated with the primary antibodies overnight and with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling) at 1:2000, for 1 hour. Immunoreactive bands were detected by enhanced chemiluminescence using an enhanced chemiluminescence kit (Amersham, GE Health Care Europe GmbH). After exposure to X-OMAT film, the autoradiographic bands were scanned and

quantified with Chemilmager 4400 (Astec Co. Ltd., Osaka, Japan).

Statistical Analyses

Comparisons between pairs were made using the Mann-Whitney *U*-test. Comparisons between multiple groups were made using two-way analysis of variance with repeated measures (Statview, Abacus Concept, CA) followed by pairwise comparison. Differences of $P < 0.05$ were considered statistically significant.

Results

Normal Expression of EBP50 in the Human Biliary Tree

The expressions of EBP50 and its binding partners, ezrin and CFTR, were examined in isolated human hepatocytes, bile duct epithelial cells (cholangiocytes), and gallbladder epithelial cells. EBP50 transcripts were detected by RT-PCR in all epithelial cell types, whereas CFTR and ezrin transcripts were detected only in biliary epithelial cells (Figure 1A). qRT-PCR analyses showed that EBP50 is expressed at similar levels in bile ducts and in hepatocytes, whereas in gallbladder epithelial cells, the amount of EBP50 transcripts is $\sim$ 40-fold higher, along with a higher expression of ezrin (by twofold) and of CFTR (by eightfold) in the gallbladder compared with bile duct epithelial cells (Figure 1B). Immunohistochemical analyses showed that in normal conditions, EBP50 protein is localized in the canalicular/apical domains of human hepatocytes, bile duct, and gallbladder epithelial cells (Figure 1C, middle and right). In hepatocytes, EBP50 immunostaining decorated juxta canalicular vesicles in addition to canalicular membranes (Figure 1C, left), suggesting a possible link between this protein and canalicular transporter(s). In the liver, ezrin immunostaining was confined to the apical domain of bile ducts (Figure 1D), and double staining showed an overlapping of EBP50 with CFTR in this region (Figure 1E), consistent with the view that EBP50 acts as a linker between ezrin and CFTR in cholangiocytes.

Changes of EBP50 Expression in Human Cholangiopathies

Next, the pattern of EBP50 expression was examined by immunohistochemistry in the liver of patients with cholangiopathies, ie, with CF liver disease, PBC, PSC, or bile stone-induced obstruction. The CF patients were either homozygous or heterozygous for Δ F508, a mutation that impairs the trafficking of CFTR to the cell surface. In their liver specimens, EBP50 was detected not only in hepatocytes and in native bile ducts, but also in cells of the ductular reaction. A basolateral and intracellular distribution of EBP50 was detected selectively in these cells (Figure 2A), as opposed to native bile duct cells (not

Figure 1. Normal hepatobiliary expression of EBP50 and binding partners. Detection of EBP50, ezrin, and CFTR in normal human liver (L), hepatocytes (H), intrahepatic bile ducts (BDs), and gallbladder epithelial cells (G) by conventional RT-PCR (representative of preparations from three patients) (**A**) and by qRT-PCR (**B**). Results of qRT-PCR are expressed as mRNA levels normalized to 18S rRNA and relative to the mean bile duct value (means $\pm$ SEM of three preparations; * $P < 0.05$ versus bile ducts). Immunostaining of normal human liver cryosections for EBP50 (**C**, red), which is detected in the canalicular domain (**left** and **middle, arrowheads**), in juxtacanalicular vesicles (**left, arrow**) in hepatocytes, and in the apical domain of bile ducts (**middle, arrow**) and of gallbladder epithelial cells (**right**); ezrin, detected in the apical domain of bile ducts (**D**); EBP50 (red, **arrow**) and CFTR (green, **arrow**), which are shown to co-localize (yellow, **arrow**) in the apical domain of bile ducts by double staining and confocal microscopy (**E**). An avidin-biotin immunofluorescence method was used except for ezrin, which was detected by alkaline phosphatase anti-alkaline phosphatase/Fast Red. L, Lumen.

shown). Ezrin was also detected but remained strictly apical in the ductular reactive cells (Figure 2A). In addition, the immunostaining of EBP50 only partly overlapped with that of CFTR mutant protein in these cells (Figure 2B). Both a cytoplasmic and a nuclear localization of EBP50 was detected in the ductular cells after double staining with a nuclear marker, at confocal microscopy (Figure 2C). From these results, we inferred that the delocalization of EBP50 was probably unrelated to CFTR

abnormal trafficking, which was further supported by the fact that EBP50 aberrant distribution was also detected within cells of the ductular reaction in the liver from patients with PBC or PSC (Figure 3, A and B), and from a patient with bile stone-induced obstruction (not shown). A possible relationship with cell proliferation was suggested by the immunodetection of Ki-67 in ductular cells that displayed aberrant distribution of EBP50, in CF liver specimens (Figure 3C).

Figure 2. Distribution of EBP50 and binding partners in CF liver disease. Cryosections of liver tissue from CF patients underwent EBP50 or ezrin immunofluorescent staining (**A**), showing that in cells of the ductular reaction, EBP50 (red) has a basolateral and intracellular distribution (**top**, **arrowheads**), whereas ezrin (red) is strictly apical (**bottom**, **arrowheads**); EBP50 (red, **arrow**) and CFTR (green, **arrow**) double-immunofluorescent staining showing that both proteins partly co-localize (yellow, **arrow**) within cells of the ductular reaction via confocal microscopy (**B**); double-EBP50 immunostaining (red) and nuclear staining with SYTO16 (green) (**C**). The analysis via confocal microscopy shows that, compared with apical localization in bile ducts of normal liver (**left**), in ductular cells of CF liver (**right**), EBP50 immunoreactivity is distributed throughout the cytoplasm (red spots) and in the nucleus (yellow spots) of ductular cells.

Changes of EBP50 Expression in Proliferative Biliary Epithelial Cells

Because it has been previously established that the ductular reaction results from the proliferation of pre-existing cholangiocytes in the animal model of BDL, we examined the potential changes of EBP50 expression induced by BDL in these cells. EBP50 expression was analyzed in the liver of BDL rats, during the onset of ductular reaction, ie, 1, 2, and 7 days after BDL. In bile

ducts from normal rats, EBP50 was localized together with actin, next to the apical plasma membrane of cholangiocytes (Figure 4A) and of hepatocytes (not shown). After BDL, the intensity of EBP50 immunostaining progressively decreased in the apical region of proliferative cholangiocytes while an intracellular redistribution of the protein occurred, leading to a cytoplasmic and nuclear pattern of expression after 7 days, as shown by confocal microscopy (Figure 4A). These changes were accompanied by a transient increase in the EBP50 protein content

Figure 3. Distribution of EBP50 within cells of the ductular reaction in human cholangiopathies. Immunofluorescent staining of EBP50 was performed in liver cryosections from patients with PBC (**A**), PSC (**B**), or CF liver disease (**C**). **A** and **B**: In the ductular reaction of patients with PBC or PSC, EBP50 (red) is detected in basolateral and intracellular localizations (**arrows**), in addition to the apical staining. **C**: In CF liver, active proliferation of cells in a ductular reaction with EBP50 redistribution is shown by double staining of EBP50 (red) and Ki-67 (turquoise blue).

Figure 4. Changes of EBP50 expression in rat proliferative cholangiocytes. **A:** EBP50 expression was analyzed in normal and BDL rats on postoperative days 1, 2, and 7 by examination via confocal microscopy of liver sections subjected to EBP50 immunostaining (yellow) combined with actin staining by Alexa Fluor 488-phalloidin (purple) and nuclear staining by TO-PRO-3 (blue). In normal rat liver, EBP50 immunostaining decorates the apical domain of bile ducts; 1, 2, and 7 days after BDL, EBP50 is delocalized to multiple spots in the cytoplasm and nuclei of ductular cells. Representative of three animals in each group. Size bar represents 10 μ m. **B:** Immunoblot analyses of isolated bile ducts. A representative blot and the means $\pm$ SEM of three experiments are shown; * $P < 0.05$ versus normal.

of cholangiocytes. Western blot analyses of bile duct preparations isolated from normal and BDL rats indicated that the levels of EBP50 increased after 24 hours and returned to basal levels after 48 hours (Figure 4B).

Estrogen-Mediated Regulation of EBP50 in Biliary Epithelial Cells

Because estrogens are known both as inducers of EBP50 expression and as regulators of cholangiocyte proliferation, we tested their ability to induce changes of EBP50 expression in cholangiocytes. Ovariectomy, which was previously shown to reduce BDL-induced proliferation of rat cholangiocytes,¹⁹ caused a significant decrease in

EBP50 expression in cholangiocytes, both under basal and BDL-induced proliferative conditions (Figure 5A). *In vitro*, Mz-Ch-A1 human biliary epithelial cells that were completely deprived of estrogen receptor stimulation for 2 to 3 weeks, displayed a major decrease in EBP50 content (Figure 5B) together with a marked decrease in their proliferative activity, as assessed by Ki-67 and PCNA cell cycle markers (Figure 5C). Conversely, the incubation of Mz-Ch-A1 cells with 17 β -estradiol caused an increase in EBP50 protein levels (Figure 5D). In addition, although in control cells, EBP50 was mainly localized next to the plasma membrane at the periphery of the cells, in cells exposed to 17 β -estradiol, EBP50 was redistributed to intracellular including nuclear localizations

Figure 5. EBP50 regulation by estrogens in proliferative biliary epithelial cells. **A:** Bile ducts isolated from control female rats (Non-Ov) and from ovariectomized female rats (Ov) that were sham-operated or bile duct-ligated for 1 or 7 days were analyzed by immunoblot for EBP50 expression. A representative blot and the means $\pm$ SEM of four to six experiments are shown; * P < 0.05 versus Non-Ov controls, * P < 0.05. **B** and **C:** Effect of estrogen deprivation on EBP50 expression and proliferation in Mz-Ch-A1

(Figure 5E). We concluded from these results that EBP50 is together with proliferation, regulated by estrogens in biliary epithelial cells.

Impact of EBP50 on Biliary Epithelial Cell Proliferation

To test the hypothesis that EBP50 may control the proliferation of cholangiocytes, EBP50 expression was down-regulated by siRNA in Mz-Ch-A1 biliary epithelial cells. In the presence of serum including estrogens, the cells were proliferating and showed the presence of EBP50 in their cytoplasm and nucleus as evaluated by immunofluorescence and Western blot (Figure 6A). In these conditions, β -catenin, an EBP50 binding partner, which regulates cell proliferation was also detected in both cytoplasm and nucleus of the cells. Transfection of the cells with a vector encoding an EBP50 siRNA compared with a scrambled siRNA, caused a 90% reduction in EBP50 protein content (Figure 6B). EBP50 remained detectable only at low levels in the nucleus in EBP50-inhibited cells (Figure 6B). In these cells compared with controls, PCNA expression and BrdU incorporation were reduced by 90% and 70%, respectively (Figure 6C), indicating that decrease in EBP50 expression caused an inhibition of proliferation in biliary epithelial cells.

Discussion

In the present study, we show that EBP50 is expressed all along the human biliary tree, and is concentrated in the apical domain of hepatocytes and cholangiocytes. We previously showed that EBP50 controls the activity of CFTR in cholangiocytes, presumably via the formation of a multiprotein complex with ezrin/protein kinase A.⁵ In agreement with this model, the three binding partners CFTR, EBP50, and ezrin, had the same apical localization in human cholangiocytes. The complete overlap of CFTR with EBP50 in the luminal aspects of the cells (Figure 1E, right) further supports the assumption that CFTR interacts with EBP50 at this site. The three partners were ex-

human biliary epithelial cells. **B:** Cells were cultured in phenol red-free medium supplemented with 10% charcoal-stripped serum for 2 to 3 weeks, before EBP50 expression was analyzed by immunoblotting. A representative blot and the means $\pm$ SEM of three experiments are shown; * P < 0.05 versus cells cultured in the presence of estrogens, in medium containing phenol red and 10% serum. **C:** Cell cycling was analyzed by Ki-67 immunostaining (**left**) and by PCNA immunoblotting (**right**). A representative blot and the means $\pm$ SEM of three experiments are shown; * P < 0.05 versus cells cultured in presence of estrogens. **D:** Effect of estrogen stimulation on EBP50 expression in Mz-Ch-A1 cells. Cells were placed in serum- and phenol red-free medium for 24 hours and then were incubated with or without 17 β -estradiol (10^{-7} and 10^{-10} mol/L) for 6 to 24 hours. EBP50 expression was analyzed by immunoblotting after 6 hours; a representative blot and the means $\pm$ SEM of three experiments are shown; * P < 0.05 versus normal. **E:** Effect of estrogen stimulation on EBP50 localization in Mz-Ch-A1 cells. Cells were cultured in phenol red-free medium supplemented with 10% charcoal-stripped serum for 3 weeks, then treated with 17 β -estradiol (10^{-7} mol/L) in the absence of serum for 72 hours. EBP50 localization was analyzed by immunocytochemical staining; confocal analysis of middle sections shows that after 72 hours, compared to controls, in which EBP50 (purple) is mainly localized next to the membrane (arrows), EBP50 (purple) is redistributed to intracellular including cytoplasmic (asterisk) and nuclear (arrowhead) localizations in cells exposed to 17 β -estradiol (representative of three experiments).

pressed at higher levels in the gallbladder than in intrahepatic bile ducts. Although the same was previously reported regarding the expression of the vasoactive intestinal peptide receptor,²⁴ these findings point to the gallbladder as the predominant site of bile volume regulation in humans. Whereas EBP50 was also detected in human hepatocytes, ezrin was not, in agreement with previous studies showing that radixin is the dominant ERM protein in hepatocytes.^{5,27} It was recently shown that radixin is essential for maintaining the canalicular membrane structure and function in hepatocytes by interacting with canalicular transporters, in particular with multidrug-resistance-associated protein 2 (MRP2).^{27,28} Although radixin is able to associate directly with the carboxy-terminal domain of MRP2 *in vitro*, there is evidence to indicate that MRP2 also binds radixin indirectly through a PDZ-containing protein,²⁸ and that EBP50 may act as the cross-linker between radixin and MRP2²⁹ or other transporters in hepatocytes. Consistent with this possibility, EBP50 staining in normal human liver (Figure 1C, left) mimicked MRP2 staining,³⁰ and outlined not only the canalicular membranes, but also juxta canalicular vesicles, from which transporters may undergo insertion to (and retrieval from) the plasma membrane.^{31,32}

The major contribution of this work is the demonstration that EBP50 undergoes up-regulation and intracellular redistribution in proliferative biliary epithelial cells, and controls positively proliferation in these cells. It was also previously reported that EBP50 is overexpressed and redistributed to the cytoplasm and/or nucleus of proliferative cells in hepatocellular carcinoma⁸ and in estrogen-stimulated-tissues, ie, in endometrium and breast tumors.^{7,9,10} Although EBP50 was first postulated as a mitogenic factor,⁶ it was subsequently shown to act either as an oncogene^{8,9,11,13} or as a tumor suppressor,^{14–16,33,34} and so far its actual impact on cell proliferation has been unclear. Here, on the basis of gene silencing, we could clearly show that EBP50 controls proliferation positively in biliary epithelial cells. A delocalization of EBP50 in proliferative biliary epithelial cells was demonstrated in these cells both *in vitro*, and *in vivo*, in the ductular reaction of BDL rats, a model of intense cholangiocyte proliferation.^{35,36} EBP50 was also delocalized within cells of the ductular reaction, in patients with different types of cholangiopathies. The biliary lineage of these cells was indicated by the fact that they expressed CFTR (a very specific marker of cholangiocytes in the liver) in CF (Figure 2B) as well as in PBC and PSC (not shown). A contribution of hepatocytic or stem cell lineages to the ductular reaction was previously reported mainly in severe and end-stage liver disease,^{37,38} and was less likely in these patients who, in majority, had early-stage disease. Ki-67 positivity in ductular cells was detected essentially in patients with CF liver disease, suggesting that cholangiocytes proliferate more actively in this disease than in PBC or PSC. Whether EBP50 undergoes cytoplasmic delocalization in homozygous $\Delta F508$ CFTR bronchial epithelial cells is a matter of debate.^{39,40} Here we show that $\Delta F508$ CFTR mutation is neither sufficient nor necessary for EBP50 to undergo delocalization in cholangiocytes. Accordingly, delocal-

ization of EBP50 was absent in the native bile ducts from $\Delta F508$ CF patients and present in the ductular cells from non CF patients.

Both *in vitro* studies performed in Mz-Cha-1 cells²⁰ and *in vivo* data from PBC patients⁴¹ or from the BDL animal model^{18,19} suggest that estrogens play a pivotal role in cholangiocyte proliferation. In addition, during cholestasis, estrogen serum levels are increased.⁴² Therefore, it is of particular interest that the effect of 17β -estradiol on Mz-Cha-1 cells, reproduced the changes in EBP50 expression and localization observed in proliferative cholangiocytes *in vivo*. Conversely, long-term estrogen depletion achieved by culture conditions *in vitro* and by ovariectomy *in vivo*, caused EBP50 down-regulation together with reduced proliferation in biliary epithelial cells. The EBP50 gene possesses 13 half-estrogen responsive elements and is overexpressed in response to estrogens in proliferative mammary and endometrial cells.^{4,7,9,10,43} Notably, 17β -estradiol was recently shown to increase the amount of EBP50 in the nuclear fraction of human airway epithelial cells.⁴⁴ In addition, cholangiocyte proliferation is regulated by cyclic AMP,⁴⁵ which has also been shown to induce an intracellular redistribution of EBP50 in renal epithelial cells.⁴⁶

Present and previous data^{7–9,47} raise a number of questions regarding the significance of EBP50 distribution with respect to cellular proliferative status. EBP50 may act as a tumor suppressor, eg, by forming a ternary complex with platelet-derived growth factor receptor and PTEN at the plasma membrane, and thereby exerting an inhibitory action on PI3K signaling^{14,48} or by interacting with β -catenin and stabilizing adherent junctions at the plasma membrane. As proposed by Georgescu,³⁴ overexpression and intracellular delocalization of EBP50 in proliferative cells may not only disrupt complexes with PTEN or β -catenin, normally localized beneath the plasma membrane of epithelial cells, but also scaffold complexes in the cytoplasm and/or nucleus, thus sequestering signaling molecules away from the plasma membrane. Consistent with this possibility, we herein found that β -catenin was redistributed together with EBP50 in the cytoplasm and nucleus of proliferative Mz-Cha-1 cells. In addition, Khundmiri and colleagues⁴⁹ recently raised the possibility that EBP50 interacts with two transcription factors, TAZ and SRY, suggesting that EBP50 may regulate gene transcription.

We conclude that in the liver, EBP50 contributes not only to bile secretory functions, but also to estrogen response and proliferation in biliary epithelial cells.

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