Peribiliary Gland Niche Participates in Biliary Tree Regeneration in Mouse and in Human Primary Sclerosing Cholangitis

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BACKGROUND AND AIMs: Mechanisms underlying the repair of extrahepatic biliary tree (EHBT) after injury have been scarcely explored. The aims of this study were to evaluate, by using a lineage tracing approach, the contribution of peribiliary gland (PBG) niche in the regeneration of EHBT after damage and to evaluate, in vivo and in vitro, the signaling pathways involved.

APPROACH AND RESULTS: Bile duct injury was induced by the administration of 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet for 14 days to Krt19CreERT2;R26rtomato mice. Human biliary tree stem/progenitor cells (BTSC) within PBGs were isolated from EHBT obtained from liver donors. Hepatic duct samples (n = 10) were obtained from patients affected by primary sclerosing cholangitis (PSC). Samples were analyzed by histology, immunohistochemistry, western blotting, and polymerase chain reaction. DDC administration causes hyperplasia of PBGs and periductal fibrosis in EHBT. A PBG cell population (Cytokeratin19/Sox9) is involved in the renewal of surface epithelium in injured EHBT. The Wnt signaling pathway triggers human BTSC proliferation in vitro and influences PBG hyperplasia in vivo in the DDC-mediated mouse biliary injury model. The Notch signaling pathway activation induces BTSC differentiation in vitro toward mature cholangiocytes and is associated with PBG activation in the DDC model. In human PSC, inflammatory and stromal cells trigger PBG activation through the up-regulation of the Wnt and Notch signaling pathways.

CONCLUSIONS: We demonstrated the involvement of PBG cells in regenerating the injured biliary epithelium and identified the signaling pathways driving BTSC activation. These results could have relevant implications on the pathophysiology and treatment of cholangiopathies. (HEPATOL 2020;71:972-989).

The biliary tree is a system of interconnected ducts comprising intrahepatic bile ducts (IHBDs) and extrahepatic bile ducts (EHBDs). Bile ducts are lined by specialized epithelial cells named cholangiocytes. Cholangiocytes are involved in the modification of bile composition, and their proliferation is responsible for the turnover of the biliary epithelium. However, human diseases affecting the biliary tree (i.e., cholangiopathies) determine an impairment of cholangiocyte proliferative capabilities. In such conditions, the regeneration of interlobular bile duct epithelium is supported by hepatic stem/progenitor cells within bile ductules. Moreover, peribiliary glands (PBGs) within large IHBDs and EHBDs contain a

Abbreviations: 2-wkREC, 2-week recovery; 4-wkREC, 4-week recovery; AE2, anion exchanger 2; BTSC, biliary tree stem/progenitor cell; CFTR, cystic fibrosis transmembrane conductance regulator; CK, cytokeratin; CTR, control mice; DDC, 3,5-dietethoxycarbonyl-1,4-dihydrocollidine; DLL, delta-like ligand; EHBD, extrahepatic bile duct; EHBT, extrahepatic biliary tree; h, human; IHBD, intrahepatic bile duct; Jag, Jagged; KM, Kubota’s medium; L, bile duct lumen; LPS, lipopolysaccharide; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; NICD, Notch intracellular domain; non p, nonphosphorylated; Notch-M, Notch pathway-stimulation medium; OM, original magnification; PAS, periodic acid–Schiff; PBG, peribiliary gland; PCNA, proliferating cell nuclear antigen; PDT, population doubling time; PSC, primary sclerosing cholangitis; S100A9, S100 calcium-binding protein A9; SCTR, secretin receptor; SE, surface epithelium; SOX, sex-determining region Y-box; SQ, semiquantitative; SR, Sirius red; td-Tom, td-Tomato; WB, western blot; Wnt-M, Wnt pathway-stimulation medium; Wnt-M+Block, Wnt-M plus Wnt inhibitor; Wnt1, Wnt family member 1; Wnt family member 3a; α-SMA, α-smooth muscle actin; β-cat, β-catenin.

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unique stem cell niche. (8) Biliary tree stem/progenitor cells (BTSCs) in PBGs possess high self-renewal and proliferative capabilities. (9,10) However, their role in biliary regeneration has not been precisely addressed. In primary sclerosing cholangitis (PSC), which involves large IHBDs and EHBDs, cholangiocyte senescence and apoptosis are paralleled by BTSC activation followed by PBG hyperplasia in affected ducts. (11) These aspects suggest a putative contribution of BTSCs in the regenerative processes involved in pathologies affecting these portions of the biliary tree when cholangiocyte proliferation is impaired.

The aims of the present study are i) to evaluate the contribution of BTSCs in the repair of damaged EHBDs by using a mouse lineage tracing model, ii) to evaluate the signaling pathways involved in BTSC activation and differentiation both in vitro and in vivo, and iii) to study the expression of activated signaling pathways within PBGs in human PSC samples.

Materials and Methods

MURINE MODEL

The animals in this study were on a C57BL6/J background. Both male and female mice were used. All animal experiments were carried out under procedural guidelines and severity protocols and with ethical permission from the University of Edinburgh Animal Welfare and Ethical Review Body and the Home Office (UK). The Krt19CreTdTomatoLSL mice was induced by three individual intraperitoneal injections of tamoxifen (20 mg/mL; Sigma-Aldrich UK) at a dose of 4 mg during the light cycle. Animals received 2 weeks of normal diet after the last tamoxifen injection before commencing the diet regime (T0).

To induce bile duct injury, mice were given 0.1% 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) mixed with Rat and Mouse No. 1 Maintenance diet (Special Diet Services) for 14 days. After DDC diet, mice were given normal chow and drinking water for the successive 14 and 28 days (recovery period). Mice were euthanized i) after 14 days of DDC diet (DDC group: n = 5), ii) after 14 days of recovery period (2-week recovery [2-wkREC] group: n = 5), and iii) after 28 days of recovery period (4-week recovery [4-wkREC] group: n = 5). As control, mice (n = 15) were maintained under a normal chow diet for the entire experimental period and sacrificed at the same time as mice fed with DDC diet (14, 28, 42 days after T0).

The murine extrahepatic biliary tree (EHBT) was dissected and fixed en bloc, as described. (12)
LIGHT MICROSCOPY, HISTOPATHOLOGY, AND IMMUNOHISTOCHEMISTRY

For immunohistochemistry, sections were incubated overnight at 4°C with primary antibodies (Supporting Table S1). Samples were rinsed twice with phosphate-buffered saline and incubated at room temperature with secondary biotinylated antibody and then with Streptavidin-HRP (LSAB+ System-HRP, code K0690, Dako, Glostrup, Denmark). Diaminobenzidine (Dako, Glostrup, Denmark) was used as substrate, and sections were counterstained with hematoxylin. For immunofluorescence, labeled isotype-specific secondary antibodies were used (Alexa Fluor; Invitrogen, Life Technologies Ltd., Paisley, UK), and samples were counterstained with 4,6-diamidino-2-phenylindole for visualization of cell nuclei. For all immunoreactions, negative controls (primary antibodies were replaced with preimmune serum) were included. Sections were examined in a coded fashion by Leica Microsystems DM4500 B Light and Fluorescence Microscopy (Leica Microsystems, Wetzlar, Germany) equipped with a Jenoptik ProgRes C10 Plus Videocam (Jena, Germany). Immunofluorescence stains were also analyzed by Confocal Microscopy (Leica TCS-SP2). Slides were further processed with an Image Analysis System (IAS; Delta Sistemi, Roma, Italy) and were independently evaluated by two researchers in a blind fashion.

The thickness of bile duct walls was measured by an IAS (Delta Sistemi, Rome, Italy). The extension of fibrosis was evaluated in sirius red (SR) stains. The volume occupied by PBGs was expressed as the percentage with respect to the total duct wall. For immunoreactions, the number of positive cells was automatically calculated by an algorithm on the entire section, and then a semiquantitative (SQ) scoring system was applied (0 = <5%; 1 = 6%-10%; 2 = 11%-30%; 3 = 31%-50%; 4 = >50%). The number of sex-determining region Y-box (SOX9)+ cells per field at 20× was calculated in six nonoverlapping fields.

HUMAN TISSUE SOURCING

Human EHBT samples were obtained from the “Paride Stefanini” Department of General Surgery and Organ Transplantation, Sapienza University of Rome, Rome, Italy. Written informed consent to use tissues for research was obtained from our transplant program. All samples were derived from adults between the ages of 19 and 73 years. Protocols received the approval of our Institutional Review Board, and processing was compliant with current Good Manufacturing Practice. The study protocol conformed to the Ethical Guidelines of the 1975 Declaration of Helsinki. The research protocol was reviewed and approved by the Ethic Committees of Policlinico Umberto I of Rome, Italy. No donor organs were obtained from executed prisoners or other institutionalized individuals.

BTSC ISOLATION, CELL CULTURES, MEDIA, AND SOLUTIONS

BTSCs were isolated from human organs. As control conditions, human (h)BTSC were cultured in a self-replication medium (i.e., Kubota’s medium [KM]). The in vitro effects of signaling pathways on hBTSC were evaluated by supplementing the KM with specific Notch and Wnt activators and inhibitors. The following conditions have been tested:

- Notch pathway-stimulation medium (Notch-M)
- Wnt pathway-stimulation medium (Wnt-M)
- Wnt-M plus Wnt inhibitor (Wnt-M+Block)
- KM supplemented with lipopolysaccharides (LPS)

The exact composition of analyzed conditions is provided in the Supporting Information. Cell proliferation was assessed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay, DNA Quantification (see the Supporting Information), and population doubling time (PDT). Cell migration has been assessed by a scratch test.

QUANTITATIVE REVERSE-TRANSCRIPTION POLYMERASE CHAIN REACTION ANALYSIS

Total RNA was extracted from cell cultures by the procedures of Chomczynski and Sacchi. Subsequently, the mRNA levels were analyzed by quantitative reverse-transcription polymerase chain reaction. Glyceraldehyde 3-phosphate dehydrogenase was used as an in vitro reference gene. The primer sequences are reported in Supporting Table S2.
PROTEIN EXTRACTION AND WESTERN BLOT ANALYSIS

Proteins were extracted by radioimmunoprecipitation assay buffer added with protease inhibitor cocktail 1:100 (Sigma-Aldrich #P8340) and phosphatase inhibitor cocktail 1:100 (Sigma-Aldrich #P5726). The proteins were quantized by Bradford assay using the Protein Assay Dye Reagent concentrate (Bio-Rad #500-0006). The protein extracts were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 4%-20% polyacrylamide gel (Mini-PROTEAN TGX Bio-Rad #4561021) under reducing conditions and subsequently transferred on a nitrocellulose filter by 0.2 μm (Bio-Rad #1620146). Membranes were blocked overnight at 4°C with 5% Marvel skimmed milk in 1× trishydroxymethylaminomethane-buffered saline Tween 20 (TBST; 20 mM Tris hydrochloride; pH = 8.300 mM NaCl; 0.1% Tween 20) and were incubated for 1 hour at room temperature with primary antibody. Subsequently, membranes were rinsed with TBST and incubated for 1 hour at room temperature with conjugated secondary antibody peroxidase. The LiteAblot Plus (#EMP011005, EuroClone, Pero, Milan, Italy) chemiluminescence system was used for detection. The antibodies used are described in Supporting Table S3.

STATISTICAL ANALYSIS

Data are indicated as mean ± standard deviation. The Student t test or Mann-Whitney U test was used to determine differences between groups for normally or not normally distributed data, respectively. The Pearson correlation coefficient or the Spearman non-parametric correlation was used. A P value of <0.05 was considered statistically significant. Analyses were performed using SPSS software (IBM, Milan, Italy).

Results

DDC ADMINISTRATION CAUSES PBG HYPERPLASIA AND PERIDUCTAL FIBROSIS IN THE EHBDs

DDC mice (Fig. 1) showed focal disruption of the surface epithelium (SE) and a marked increase of the wall area fraction occupied by PBGs (PBG area = 15.4% ± 1.9%) with respect to normal ducts (3.6% ± 2.7%; P < 0.001). After 2-wkREC from DDC injury, the SE was mostly restored and continuous without disruption. After 4-wkREC, the PBG area was significantly reduced (9.49% ± 1.83%) compared with DDC mice (P < 0.01). As far as mucinous cells are concerned, DDC-injured ducts presented a marked increase in the number of periodic acid–Schiff (PAS)+ cells within PBGs, which returned to control value after the recovery period (histogram in Fig. 1B).

The examination of proliferating cell nuclear antigen (PCNA) expression (a marker of proliferation; Fig. 1C) indicated a marked increase in the number of PCNA+ cells within PBGs in DDC mice (30.7% ± 4.0%) compared with controls (12.7% ± 2.08%; P < 0.01). On the other hand, PCNA expression by SE cells (i.e., mature cholangiocytes) did not significantly increase in DDC mice (3.3% ± 1.5%). After recovery from DDC injury, the number of proliferating PBG cells returned to control values (P < 0.02 versus DDC).

Furthermore, DDC administration induced periductal fibrosis in EHBD walls (Fig. 2); the wall thickness of EHBD walls in DDC mice was greatly increased (118.1 ± 7.1 μm) compared with control mice (CTR; 76.9 ± 15.1 μm; P < 0.01; Fig. 2A); moreover, the extent of SR-stained fibers was increased in EHBD walls of DDC mice (0.043 ± 0.018 μm²) compared with controls (0.0018 ± 0.0009 μm²; P < 0.01); finally, the area fraction of the duct wall occupied by Collagen I was increased in DDC mice compared with controls (Fig. 2B). When α smooth muscle actin-positive and desmin-positive myofibroblasts were counted, DDC mice showed an increase in the number of α smooth muscle actin+ and desmin+ myofibroblasts within EHBD walls compared with controls (Fig. 2C,D). EHBD wall thickness (r = 0.76; P < 0.01) and fibrillar collagen extent (r = 0.60; P < 0.05) were correlated with the PBG area.

The thickness of EHBD walls significantly reduced both after 2-wkREC and 4-wkREC (108.2 ± 4.3 μm and 106.9 ± 2.9 μm; P < 0.05 versus DDC mice). SR-stained fiber extent and Collagen I area fraction showed a significant reduction after 4-wkREC (0.017 ± 0.009 μm²; P < 0.05 compared with DDC mice). Similarly, in the recovery period, the number of α smooth muscle actin+ and desmin+ myofibroblasts within EHBD walls (Fig. 2C,D) was reduced compared with DDC mice (P < 0.02).
The expression and distribution of the stem cell marker SOX9 and mature cholangiocyte markers (K19; anion exchanger 2 [AE2]; cystic fibrosis transmembrane conductance regulator [CFTR]) were studied in EHBD. In controls (Fig. 3), virtually all cholangiocytes lining the SE were K19+, CFTR+, AE2+ but SOX9-. Most but not all PBG cells expressed K19, and the percentage of K19- cells accounted for nearly 5%. Rare PBG cells expressed AE2 and CFTR. PBG contained SOX9+ cells that accounted for nearly 10% (8.7% ± 3.2%); a Sox9+ PBG cell subpopulation was K19- and located at the bottom of PBGs.

In DDC mice (Fig. 3), the percentage of SOX9+ cells increased both in PBGs (48.0% ± 5.6%) and in SE (15.3% ± 6.3%) compared with controls (P < 0.05); interestingly, SE cells in DDC mice were K19+ but almost all were negative for CFTR and AE2.

To investigate the possible role of K19+/SOX9+ PBG cell population in the renewal of SE and mature cholangiocyte turnover, we analyzed a lineage tracing system in the DDC model. To label cells, we used the Krt19CreERT2LSLtdTom mouse and found labeling (td-Tomato [td-Tom] positivity) strictly specific to the epithelial cells within the EHBD. In controls (Fig. 4), the percentage of td-Tom- cells in the SE was low (5.4% ± 2.9%), and td-Tom+ cells lining EHBDs represented >90% of cells; cholangiocytes lining EHBD in normal conditions were constantly K19+ and SOX9-, as demonstrated in Fig. 3. These observations confirmed that the slow physiological turnover of biliary epithelium was achieved by the proliferation of mature K19+ cholangiocytes. In DDC mice (Fig. 4A), the number of K19+/td-Tom- cells within the
SE greatly increased to 34% ± 8.21% (P < 0.001 versus controls). In DDC mice, some td-Tom\(^{-}\) cells within the SE were SOX9 positive (Fig. 4C); contrarily, these cells were AE2/CFTR negative, given the demonstrated lack of AE2/CFTR expression in SE of DDC mice (Fig. 3).

During recovery phase, the percentage of td-Tom\(^{-}\) cells within the SE progressively decreased at 2 and 4 weeks (P < 0.05 versus DDC mice) but remained significantly higher compared with controls (P < 0.05; Fig. 4B). td-Tom\(^{-}\) cells within SE progressively showed phenotypic features of mature cholangiocyte differentiation, such as the loss of SOX9 positivity at 2 weeks (not shown) and the appearance of CFTR expression at 4 weeks (Fig. 4D).
FIG. 3. Biliary damage in rodent EHBDs is characterized by the loss of mature cholangiocytes in SE. (A) Double immunofluorescence for cytokeratin (CK) 19 and SOX9 in CTR and DDC-fed mice. Separate channels are provided. In CTR, virtually all cholangiocytes lining the SE were K19+/SOX9− (green arrowheads); PBGs were composed of K19+/SOX9+ cells (yellow arrows) and SOX9+/K19− cells located at the bottom of PBGs (red arrows). In DDC-injured ducts, the SE was characterized by the presence of K19+/SOX9+ cells (yellow arrowheads). Nuclei are displayed in blue. Original magnification (OM): 40×. (B) Double immunofluorescence for AE2 and CFTR in CTR, and for CFTR and SOX9 in DDC-fed mice. Separate channels are provided. SE in CTR ducts was composed of AE2 and CFTR positive cholangiocytes (yellow arrowheads). Contrarily, in DDC-injured ducts, SE cells were negative for CFTR but SOX9 positive (red arrowhead). Nuclei are displayed in blue. OM: 40×. (C) Immunofluorescence for SOX9 in CTR and DDC mice. In DDC mice, the percentage of SOX9+ cells increased in PBGs compared with CTR (red arrows). Cells lining SE (i.e., cholangiocytes) were SOX9+ in CTR (blue arrowhead) but SOX9− in DDC (red arrowhead). Nuclei are displayed in blue. OM: 10×. The histogram shows mean and standard deviation for the percentage of SOX9+ cells within PBGs and SE. *P < 0.05 versus CTR; §P < 0.05 versus SE in CTR; ^P < 0.05 versus SE in DDC. Abbreviation: L, bile duct lumen.
FIG. 4. PBG cells participate in the renewal of SE in injured EHBT in mice. (A) Double immunofluorescence for td-Tom and CK19 in control (CTR), DDC-fed 2-wkREC, and 4-wkREC Krt19CreTdTOMatoLSL mice. Separate channels are provided. In CTR mice, most K19⁺ SE cells (>90%) were lineage tracked and coexpressed td-Tom; only a minority of K19⁺ cells were not tracked (green arrow). In DDC-injured ducts, the number of td-Tom cells within the K19⁺ SE greatly increased (green arrows); in DDC, some td-Tom⁻/K19⁺ cells were present within SE (black arrows). During the recovery period, td-Tom⁻/K19⁺ cells (green arrows) were present within SE. Dotted line delimits bile duct lumen (L). OM: 20×. (B) The histogram shows mean and standard deviation for the percentage of td-Tom⁻ cells within SE. *P < 0.05 versus other groups. (C) Double immunofluorescence for td-Tom (in green) and SOX9 (in red) in DDC mice. Separate channels are provided. In DDC-injured ducts, td-Tom⁻/SOX9⁺ were distributed throughout SE (red arrows). Nuclei are displayed in blue. OM: 40×. (D) Double immunofluorescence for CFTR and td-Tom in 4-wkREC mice. Separate channels are provided. Only in 4-wkREC mice did td-Tom⁻ cells within SE show the appearance of CFTR expression (green arrows). Nuclei are displayed in blue. OM: 40×.
Wnt PATHWAY TRIGGERS hBTSC PROLIFERATION IN VITRO AND INFLUENCES PBG HYPERPLASIA IN DDC-MEDIATED DUCT INJURY IN MICE

The *in vitro* administration of R-spondin 1 (Wnt activator) significantly increased BTSC proliferation as demonstrated by MTS assay, PDT, DNA concentration, cyclin D1 expression, and PCNA gene expression (Fig. 5A; Supporting Fig. S1); R-spondin 1 also increased cell migration as demonstrated by the scratch test (Supporting Fig. S1). The administration of a Wnt inhibitor prevented these effects. Interestingly, R-spondin 1 administration did not change the expression levels of genes related to stemness (Supporting Fig. S1). The stimulation of the Wnt pathway in BTSCs determined the increased expression of nonphosphorylated (non p) β-catenin (β-cat; active) with respect to total protein amount as demonstrated by western blot (WB) analysis and by immunofluorescence (Fig. 5B-D). In turn, the expression of active β-cat was also increased by stimulating BTSC with LPS (Fig. 5B-D; Supporting Fig. S1), a substance proved to have proliferative effects on BTSCs. (20)

The stimulation of the Notch pathway by the administration of recombinant human soluble delta-like ligand (DLL) 1 determined in BTSCs the increase of Notch intracellular domain (NICD) by WB analysis and NOTCH1 expression by immunofluorescence compared with control conditions (*P* < 0.05; Fig. 6A). The activation of the Notch pathway in BTSCs significantly reduced cell proliferation and stem cell gene expression (Fig. 6B; Supporting Fig. S2). Remarkably, the Notch pathway activator specifically triggered cholangiocyte differentiation, as shown by i) the up-regulation of mature cholangiocyte genes (i.e., CFTR, secretin receptor [SCTR], apical sodium-dependent bile acid transporter [ASBT]) compared with control conditions (Fig. 6B) and ii) the expression of mature cholangiocyte marker demonstrated by WB analysis (i.e., CFTR, AE2; Fig. 6C) and immunofluorescence (i.e., SCTR and presence of primary cilia; Fig. 6D). No sign of differentiation toward hepatocyte or endocrine pancreatic lineages was detected when cells were stimulated by the Notch activator (not shown).

The expression of Notch pathway elements was further studied *in vivo* in the DDC mouse model. In controls, NOTCH1 (Fig. 6E) and NOTCH2 (Supporting Fig. S2), but not NOTCH3 (Supporting Fig. S2), were expressed by a few cells within PBGs. DDC injury determined a significant increase in the percentage of PBG cells expressing NOTCH1, NOTCH2, and NOTCH3 compared with controls (*P* < 0.05). After the recovery period, this significant increase was partially reduced but still higher compared with controls (*P* < 0.05). In parallel, DDC injury increased Jagged (Jag) 1 (a Notch ligand) expression by both PBGs and stromal cells (*P* < 0.05 versus controls), which returned to control levels after the recovery period.

HUMAN PSC IS CHARACTERIZED BY MODIFICATION OF WNT AND NOTCH PATHWAY EXPRESSION IN PBGs

In human PSC samples, PBG area was increased compared with control subjects (i.e., liver donors); furthermore, PBGs showed higher percentages of PAS⁺ (mucinous), SOX9⁺ (stem/progenitor), and PCNA⁺ (proliferating) cells compared with controls (Fig. 7A; Supporting Fig. S3).

As regards the Wnt pathway, the ratio between active β-cat with respect to total β-cat expression,
FIG. 5. Wnt pathway triggers BTSC activation. (A) Proliferation assays in hBTSCs cultured in KM, in Wnt-M, and in Wnt-M+Block. Wnt pathway stimulation significantly increased BTSC proliferation as demonstrated by PDT, MTS assay, cyclin D1 expression by WB analysis, DNA concentration, and PCNA4 gene expression. The administration of Wnt-M+Block prevented this effect. (B) WB analysis for non p-β-catenin and total β-catenin showed an increased expression of non p (active) form of β-catenin in hBTSCs cultured in Wnt-M compared with control conditions (KM). (C, D) Immunofluorescence and WB analyses for non p-β-catenin expression by hBTSCs cultured in KM, in Wnt-M, and in KM + LPS. Non p-β-catenin expression was enhanced in cells under Wnt-M and in cells stimulated with LPS (a condition proven to induce BTSC proliferation). Nuclei in panel C are displayed in blue. OM: 20×. (E) Double immunofluorescence for CK19, Wnt3a (upper panels), and non p-β-catenin (lower panels) in controls (CTR), DDC-fed mice, and 4-wkREC mice. Compared with controls, DDC-injured ducts displayed an increased expression of Wnt3a (a Wnt ligand) in stromal cells (red arrows) around PBGs, which declined after the recovery period. In parallel, DDC injury increased non p-β-catenin expression by PBGs (yellow arrows), which returned to CTR levels after the recovery period. Nuclei are displayed in blue. OM: 20×. (F) Double immunofluorescence for CK19, Wnt3a (upper panels), and non p-β-catenin (lower panels) in controls (CTR), DDC-fed mice, and 4-wkREC mice. Compared with controls, DDC-injured ducts displayed an increased expression of Wnt3a (a Wnt ligand) in stromal cells (red arrows) around PBGs, which declined after the recovery period. In parallel, DDC injury increased non p-β-catenin expression by PBGs (yellow arrows), which returned to CTR levels after the recovery period. Nuclei are displayed in blue. OM: 20×. (G) Double immunofluorescence for CK19, Wnt3a (upper panels), and non p-β-catenin (lower panels) in controls (CTR), DDC-fed mice, and 4-wkREC mice. Compared with controls, DDC-injured ducts displayed an increased expression of Wnt3a (a Wnt ligand) in stromal cells (red arrows) around PBGs, which declined after the recovery period. In parallel, DDC injury increased non p-β-catenin expression by PBGs (yellow arrows), which returned to CTR levels after the recovery period. Nuclei are displayed in blue. OM: 20×. (H) Double immunofluorescence for CK19, Wnt3a (upper panels), and non p-β-catenin (lower panels) in controls (CTR), DDC-fed mice, and 4-wkREC mice. Compared with controls, DDC-injured ducts displayed an increased expression of Wnt3a (a Wnt ligand) in stromal cells (red arrows) around PBGs, which declined after the recovery period. In parallel, DDC injury increased non p-β-catenin expression by PBGs (yellow arrows), which returned to CTR levels after the recovery period. Nuclei are displayed in blue. OM: 20×.

Discussion

The present study demonstrated that i) DDC injury in mice mimics typical PSC histopathological lesions, including PBG hyperplasia, mucinous metaplasia, and periductal fibrosis; ii) SOX9⁺ cells within PBGs proliferate and participate in the regeneration of cholangiocytes lining biliary epithelium after DDC-induced damage; iii) Wnt triggers BTSC proliferation and migration, whereas the Notch pathway induces their differentiation toward a mature phenotype; iv) in patients with PSC, PBG proliferation is associated with sustained activation of Wnt and Notch pathways by inflammatory and stromal cells. Altogether, our results support the involvement of BTSCs located in PBGs in the regeneration of the biliary epithelium after injury and in disease progression (Fig. 8D).

In humans, PBGs represent the niche of BTSCs, a stem/progenitor cell population with multipotent capabilities. Both in vitro and in vivo, BTSCs can mature toward hepatocyte, cholangiocyte, and pancreatic lineages. PBGs are not distributed homogeneously along the entire biliary tree; they are present in the EHBT and in larger intrahepatic (i.e., segmental and area) bile ducts. The involvement of PBGs and BTSCs in biliary tract pathologies has been described in experimental models and human...
cholangiopathies. In ischemic biliary lesions, an increased number of proliferating progenitor cells have been demonstrated in PBGs, the PBG injury caused by ischemia is associated with the occurrence of biliary strictures after liver transplantation. Interestingly, PBGs are also involved in the pathogenesis of biliary strictures in patients with PSC; in this disease, chronic biliary inflammation stimulates PBG stem cell niche with subsequent myofibroblast activation, leading to biliary fibrosis and strictures. Furthermore, PBG stem cell niche gives rise to a secondary regenerative response, leading to biliary carcinogenesis in human patients with PSC and in experimental genetically induced biliary injury. Generally, the emerging concept is that PBG stem cell niche can be activated in the context of pathologies affecting larger IHBDs and EHBDs despite the well-recognized proliferative capability of mature cholangiocytes. Thus, the actual contribution of BTSCs in the bile duct regeneration remains an open question.

In this study, biliary injury was experimentally induced in a lineage tracing model. Most of the experimental model of biliary injury mainly focused on IHBD and liver fibrosis. In contrast, few studies investigated histological damage of extrahepatic ducts and the development of biliary concentric fibrosis (i.e., strictures). Here, we observed that DDC administration was able to induce damage and fibrosis in EHBDs with histological depicts mimicking PSC lesions, including SE destruction, PBG proliferation and mucinous metaplasia, myofibroblast activation, and concentric fibrosis. Other experimental models have been proved to mimic PSC (e.g., multidrug resistance protein 2 knockout mice); however, the aims of the present study led us to opt for the chemically induced DDC model instead of a genetic one given the possibility to remove the toxic agent and observe the recovery. Interestingly, DDC administration induced the expansion of the SOX9 cell population in PBGs. The SOX9 cell compartment extended toward the SE, which became devoid of AE2 and CFTR (i.e., mature) cholangiocytes. The interruption of the DDC diet (recovery period) determined a progressive restoration of the bile duct histology. Our lineage tracing system tracked K19 cholangiocytes with an efficiency around 90% in EHBD SE; following DDC administration, not-tracked cells replenished the SE, thus indicating a substantial participation of K19 cells in the regenerative response. In EHBDs, K19 cells were only localized within PBGs and corresponded to the SOX9 cell population. In keeping with that, td-Tom cells within the SE of injured ducts were SOX9 and CFTR/AE2, thus confirming the PBG compartment as a source of SE cells in injured ducts. Interestingly, some SOX9/td-Tom cells within SE showed positivity for K19, indicating signs of initial maturation; this feature was followed by the progressive loss of SOX9 expression and the appearance of functional cholangiocyte markers (i.e., CFTR) during the recovery period. The choice to focus on EHBDs excludes the eventual interference of small SOX9 cholangiocytes in our model; small cholangiocytes have proliferative and regenerative capabilities.
however, this cell compartment is anatomically distinct from the EHBT because it corresponds to the bile ductules inside the liver parenchyma; moreover, small cholangiocytes express K19, thus phenotypically differing from K19+ PBG subpopulation investigated in this study. Taken together, our data indicate an active contribution by SOX9+ PBG cells in the restoration of SE after injury and their capability to differentiate into mature cholangiocytes. Our findings are in keeping with the evidence that the regeneration of ischemic injured SE is ensured by BTSC activation in a human ex vivo model.\(^{(34)}\)

Stem cell activation is precisely modulated by molecular signals furnished by a specialized niche. In the liver, the hepatic progenitor cell niche is well characterized, and the contribution of Notch and Wnt pathways has been thoroughly demonstrated.\(^{(19,23)}\) On the contrary, no information is present regarding the signaling pathways that drive BTSC activation. Therefore, the present study further evaluated the activation of Wnt and Notch pathways, indicating their role in BTSC proliferation (Wnt/β-cat) or cholangiocyte fate choice (Notch). In DDC, Wnt and Notch ligands are furnished by ductal (myo-)fibroblasts around PBGs, thus indicating a putative role of these cells in the niche composition. In this experimental model, further studies based on direct in vivo inhibition/stimulation of signaling pathways would be compelling. However, we directed our attention on the role of these signals in the context of human cholangiopathies. Previous studies demonstrated a divergent activation of the PBG niche in PSC compared with primary biliary cholangitis; in primary biliary cholangitis, PBG hyperplasia does not take place because the chronic inflammation primarily involves the interlobular bile duct, mostly sparing large IHBDs and EHBD.\(^{(11)}\) On the other hand, in PSC, the PBG niche is activated, especially in ducts with high fibrosis and inflammation.\(^{(11)}\) The present study further describes a prominent activation of Wnt and Notch pathways, potentially supported by ligands from infiltrating inflammatory cells. PBGs can produce Notch ligands themselves, thus suggesting an autocrine effect and a further paracrine effect on neighboring (myo-)fibroblasts. Interestingly, previous evidence indicated that, in human biliary disease, PBGs can secrete vascular endothelial growth factors (VEGFs), influencing the modification of peribiliary vascular plexus in PSC\(^{(20)}\) and hypoxic conditions.\(^{(34,35)}\) Taken together with previous studies,\(^{(11,20)}\) our results indicate that the activation of PBG niche represents a consequence of toxic or inflammatory biliary damage (Fig. 8). In the DDC model, Notch and Wnt signaling pathways are turned off once the toxic agent is interrupted, thus determining a progressive restoration to the normal condition. In PSC, chronic inflammation continuously triggers PBG niche activation; in turn, chronically activated PBGs start to produce growth factors (VEGFs),\(^{(20,34,35)}\) signals (sonic hedgehog, Notch ligands),\(^{(11)}\) and inflammatory cytokines (interleukin-6, transforming growth factor-β), which further stimulate fibrogenetic and inflammatory cells. Therefore, this secretory phenotype\(^{(36)}\) of PBGs in PSC configures a sort of vicious cycle.
that is more prone to developing concentric fibrosis than furnishing coordinated signals for *restitutio ad integrum*. Our results further support the role of PBGs in biliary carcinogenesis in patients with PSC; the effects on BTSC proliferation and migration by β-catenin activation and the evidence of prolonged Wnt ligand production by chronic inflammation are in touch with the pathogenetic role of the Wnt pathway in cholangiocarcinoma.\(^{37,38}\)

In conclusion, the present study furnishes evidence on the direct role of BTSCs in the regeneration of injured biliary epithelium and identifies Wnt and Notch as key signaling pathways driving BTSC activation, with relevant implications in the pathophysiology and clinical management of cholangiopathies.

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