GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Characterization of Peribiliary Gland—Constituting Cells Based on Differential Expression of Trophoblast Cell Surface Protein 2 in Biliary Tract

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Peribiliary glands (PBGs) are accessory glands with mucinous and serous acini in the biliary tree. The PBG is composed of a heterogeneous cell population, such as mucus- and pancreatic enzyme-producing epithelial cells, whereas it constitutes niches for multipotential stem/progenitor cells in the human extrahepatic bile duct (EHBD). By contrast, the nature of PBGs in the mouse EHBD remains unclear. Our aim was to establish a method for isolating and characterizing PBG-constituting cells in the mouse EHBD. We found that trophoblast cell surface protein 2 (Trop2) was expressed in the luminal epithelium of mouse EHBD exclusively, but not in the PBG. On the basis of the differential expression profile of Trop2, lumen-forming biliary epithelial cells (LBECs) and PBG-constituting epithelial cells (PBECs) were separately isolated for further characterization. Gene expression analysis revealed that the isolated mouse PBECs expressed several marker genes related to human PBGs. In the colony formation assay, PBECs showed significantly higher colony formation capacity than LBECs. In the organoid formation assay, PBECs formed cystic organoid with LBEC-like phenotype. Interestingly, PBECs proliferated, accompanied by reexpression of Trop2 *in vivo* after bile duct ligation. Furthermore, the unique expression profile of Trop2 was conserved in human EHBD. Our findings indicate that Trop2 is a useful marker in investigating the pathophysiological roles and characteristics of mouse and human PBGs in biliary diseases. (*Am J Pathol* 2018, 188: 2059–2073; <https://doi.org/10.1016/j.ajpath.2018.05.016>)

The bile duct is a tubular epithelial tissue, which plays an important role in the transport of bile produced by hepatocytes into the duodenum. The bile duct is grossly classified into the intrahepatic bile duct (IHBD) and the extrahepatic bile duct (EHBD), which are lined with mature biliary epithelial cells (BECs; alias cholangiocytes). The biliary tree starts at the canals of Hering, the junctional structure connecting hepatocytes and the IHBD, and leads to the EHBD through bile ductules, the interlobular duct, and septal and segmental ducts. The EHBD, consisting of the common hepatic duct, gallbladder, cystic duct, and common bile duct (CBD), delivers bile from the IHBD to the gallbladder and then to the intestine. The BECs have never been reported to show a morphologic and functional heterogeneity at the

different levels of the biliary tree, although many recent works have highlighted the property of the biliary system as a stem/progenitor cell compartment.^{1,2} The liver stem/progenitor cell (LPC; alias oval cell in rodents) was proposed as a facultative stem cell, which has bipotential capacity for differentiation into hepatocytes and BECs, contributing to regeneration under severe liver injuries.¹ The LPC has been

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postulated to be located at the canal of Hering in IHBD, although the origin and the substantial role of LPC in liver regeneration are still under intensive debate. By contrast, it has been reported that the multipotent stem/progenitor cell, which can differentiate into hepatocytes, BECs, and pancreatic islets, is present in human EHBD.³ The cell named biliary tree stem/progenitor cell (BTSC) shows a similar gene expression profile to LPCs and pancreatic progenitor cells,^{2,4} and the unique location of niche for BTSC has been proposed as follows. In humans and mice, EHBD is known to accompany many accessory glands with mucinous and serous acini, called peribiliary glands (PBGs), which form the intramural and extramural networks deep within the fibromuscular layer.^{5–7} It has been reported that the PBG contains the cells expressing BTSC markers [eg, epithelial cell adhesion molecule (EpCAM), pancreatic duodenal homeobox 1 (PDX1), sex-determining region Y-box 17, and leucine rich repeat-containing G-protein-coupled receptor 5 (LGR5)] in humans,^{2–4} implicating its role as a reservoir of BTSC. In line with the notion, the possible involvement of PBG in the development of biliary tree disorders, including cholangiocarcinogenesis, is now being studied extensively.^{8–10} Although there are several lines of anatomic and/or immunohistochemical evidence supporting the localization of BTSC in PBG, the pathophysiological role of PBG in biliary diseases and regeneration is poorly understood. In particular, the potential of PBG-constituting cells remains to be elucidated at a single-cell level because a robust method for isolating these cells is currently unavailable.

The objective of this study is to establish a method to characterize PBGs in EHBD. We have previously identified EpCAM and trophoblast cell surface protein 2 (Trop2; alias tumor-associated calcium signal transducer 1 and 2) as LPC markers in 3,5-diethoxycarbonyl-1,4-dihydro-collidine-fed mice,¹¹ a model of cholangiopathy. Trop2 was expressed predominantly in LPCs under the 3,5-diethoxycarbonyl-1,4-dihydro-collidine-induced injury condition, but not in the IHBD of the normal liver. However, the expression of Trop2 in EHBD has not been investigated.

Herein, we show the unique expression profile of Trop2 in mouse EHBD. It is known that the PBG is composed of heterogeneous cellular compartments, including mucus- and pancreatic enzyme-producing epithelial cells,^{12,13} connecting with the lumen of the EHBD. Taking advantage of differential expression of Trop2, PBG-constituting epithelial cells (PBECs) and lumen-forming biliary epithelial cells (LBECs) were isolated by fluorescence-activated cell sorting (FACS). In addition, we examined the property of purified PBECs and LBECs by gene expression analysis, colony formation assay, and three-dimensional (3D) organoid culture.

Furthermore, we investigated the expression profile of Trop2 *in vivo* after bile duct ligation (BDL), a severe biliary injury model. From these results, we demonstrated that Trop2 is a beneficial marker for investigating the homeostatic and pathophysiological role of PBG in the biliary tree.

Materials and Methods

Animal Models

C57BL/6J mice (CLEA Japan, Tokyo, Japan) were housed in a specific pathogen-free animal facility under a 12-hour dark/light cycle and provided with food and water *ad libitum*. Both male and female mice were used for this work. For BDL, mice at 8 weeks of age underwent anesthesia by isoflurane (AbbVie, Tokyo, Japan) inhalation and subcostal incision. The CBD was ligated with a suture thread (4-0 VICRYL; Ethicon, Somerville, NJ) at the distal end of the CBD. After abdominal closure by suture, mice were placed in a recovery cage until they regained consciousness. For 5-bromo-2'-deoxyuridine incorporation assay, 3.75 mg of 5-bromo-2'-deoxyuridine (Sigma-Aldrich, St. Louis, MO) was injected into the peritoneal cavity 2 hours before sampling. In all experiments, an appropriate number of mice was determined on the basis of the preliminary experiment. Basically, freshly isolated cells derived from 5 to 12 mice as $n = 1$ were used for gene expression analysis; and cells derived from 1 to 10 mice as $n = 1$ were used for *in vitro* experiments. Finally, the total number of 350 mice was sacrificed for this study.

Visualization of Mouse Biliary Tree

The whole mouse biliary system was visualized as previously described.¹⁴

Immunofluorescence Staining

Adult mouse tissue was mounted in OCT compound (Sakura Finetek Japan, Tokyo, Japan) directly or after fixation with phosphate-buffered saline (PBS) containing 4% paraformaldehyde overnight at 4°C. In the case of 4% paraformaldehyde fixation, the sample was washed with PBS for 10 minutes at room temperature, and then placed in a series of sucrose dilutions at 4°C. The concentration of sucrose in PBS was gradually elevated. Finally, the tissue was mounted in OCT compound. Frozen sections (8 μ m thick) were permeabilized with 0.2% Triton X-100 in PBS. In case of a section of unfixed sample, 4% paraformaldehyde fixation was performed at room temperature before permeabilization. Subsequently, sections were blocked with blocking buffer (5% skim milk in PBS) for 1 hour at room temperature and incubated with diluted primary antibody overnight at 4°C. Then, the sample was incubated with secondary antibody for 2 hours at room temperature. In case of visualization of 5-bromo-2'-deoxyuridine signal, sections were autoclaved in tris-EDTA buffer (pH 8.0) for 5 minutes before permeabilization. After autoclave treatment, sections were stained as described above. For whole-mount immunostaining, CBD was fixed with 4% paraformaldehyde overnight at 4°C and immersed in blocking buffer (10% bovine serum albumin and 1% Triton X-100 in PBS) overnight at 4°C. Subsequently, the sample was incubated with diluted primary

Table 1 Antibodies Used in This Study

Protein	Host animal	Conjugate	Company/source	Experiment	Dilution
CK19	Rabbit		Tanimizu et al ¹⁶	IHC, ICC	1:1000
Sox9	Rabbit		Merck Millipore (AB5535; Burlington, MA)	IHC, ICC	1:1000
EpCAM	Rat		BD Biosciences (552370; Franklin Lakes, NJ)	IHC, ICC	1:100
EpCAM	Rat	FITC and biotin	Okabe et al ¹¹	FCM	1:50 and 1:100
Trop2	Goat	Biotin	R&D Systems (BAF1122; Minneapolis, MN)	IHC, ICC, FCM	1:100
CD49f	Rat		BD Biosciences (555734)	ICC	1:200
Opn	Goat		R&D Systems (AF808)	IHC	1:200
Pdx1	Guinea pig		Abcam (ab47308; Cambridge, MA)	IHC, ICC	1:200
Cftr	Rabbit		Abcam (ab59394)	IHC	1:50
Ki-67	Rat		eBioscience (14-5698-82; San Diego, CA)	IHC, ICC	1:200
BrdU	Rat		Abcam (ab6326)	IHC	1:100
Phalloidin		Alexa Fluor 555	Thermo Fisher Scientific (A34055; Waltham, MA)	ICC	1:200
FcR blocker	Purified anti-CD16/CD32 antibody		Hybridoma 2.4G2 clone	FCM	1:100
			Biolegend (101302; San Diego, CA)	FCM	1:500

BrdU, 5-bromo-2'-deoxyuridine; Cftr, cystic fibrosis transmembrane conductance regulator; CK19, cytokeratin 19; FCM, flow cytometric; FITC, fluorescein isothiocyanate; ICC, immunocytochemistry; IHC, immunohistochemistry; Opn, osteopontin; Pdx1, pancreatic duodenal homeobox 1; Sox9, sex-determining region Y-box 9.

and secondary antibody overnight at 4°C. Finally, optical clearing was performed by SeeDB overnight at room temperature.¹⁵ For the immunostaining of *in vitro* samples, the same protocol as performed on frozen sections was applied. In case of staining cystic organoids, the same reagents as used in whole-mount immunostaining were used. In all cases, nuclei were stained with Hoechst 33342 (Sigma-Aldrich). Images were observed and captured using Axio observer Z.1 with AxioCam HRc (Carl Zeiss, Jena, Germany), FV1200 and FV3000 (Olympus, Tokyo, Japan). Antibodies are listed in [Table 1](#).

Human Tissue and Immunohistochemistry

Four individual surgical specimens of EHBD were obtained from the patients with pancreatic cancer or extrahepatic

biliary cancer. The deparaffinized and rehydrated sections were microwaved in EDTA buffer (pH 9.0) for 20 minutes in a microwave oven. After endogenous peroxidase blocking, these sections were incubated at 4°C overnight with monoclonal antibody against human TROP2 (rabbit IgG, 1 µg/mL; ab214488; Abcam Japan, Tokyo, Japan) and then at room temperature for 1 hour with goat anti-rabbit Igs conjugated to peroxidase-labeled dextran polymer (Envision; Dako, Tokyo, Japan). After benzidine reaction, sections were lightly counterstained with hematoxylin.

FCM Analysis and Cell Isolation

For FACS analysis of IHBD, liver cells were prepared by collagenase digestion, as previously described.¹¹ For preparation of EHBD cells, mouse CBD ([Supplemental](#)

Table 2 Medium Composition Used for Isolated PBEC and LBEC Culture

Reagent	Company/source	Final concentration
Williams' medium E	Thermo Fisher Scientific (Waltham, MA)	
BSA (fatty acid free)	Sigma-Aldrich (St. Louis, MO)	0.1%
L-Glutamine	Thermo Fisher Scientific	2 mmol/L
Glucose	Wako Pure Chemical Industries (Osaka, Japan)	14 mmol/L
HEPES	Thermo Fisher Scientific	20 mmol/L
Sodium pyruvate	Thermo Fisher Scientific	1 mmol/L
Sodium hydrogen carbonate	Wako Pure Chemical Industries	0.15%
Nicotinamide	Sigma-Aldrich	10 mmol/L
Ascorbic acid	Sigma-Aldrich	0.2 mmol/L
B27	Thermo Fisher Scientific	2%
ITS-X	Thermo Fisher Scientific	1%
Recombinant mouse EGF	PeproTech (London, UK)	10 ng/mL
Recombinant human HGF	PeproTech	10 ng/mL
Dexamethasone	Wako Pure Chemical Industries	0.1 µmol/L
Gentamicin	Wako Pure Chemical Industries	50 µg/mL

BSA, bovine serum albumin; EGF, epidermal growth factor; HGF, hepatocyte growth factor; ITS-X, insulin transferrin selenium ethanolamine solution; LBEC, lumen-forming biliary epithelial cell; PBEC, peribiliary gland—constituting epithelial cell.

Table 3 Primers and Probes Used for RT-qPCR

Gene	Probe no.	Orientation	Primer sequence
<i>Ae2</i>	1	Forward	5'-ATGTGGCCTCACTGTCCTTC-3'
		Reverse	5'-GCTGATCGAGGTCTAAGAGCA-3'
<i>Amy2a5</i>	7	Forward	5'-AGTGAATGGCGAGAAGATG-3'
		Reverse	5'-CTGTCAAGAGGCACCAACC-3'
<i>Cftr</i>	51	Forward	5'-CAGCAGCTCAACAACCTGGA-3'
		Reverse	5'-TGTCACAAGGTGGGTGAAAA-3'
<i>Chga</i>	58	Forward	5'-AGGCTACAAAGCGATCCAGA-3'
		Reverse	5'-CGGAAGCCTCTGTCTTTCC-3'
<i>CK19</i>	97	Forward	5'-AGTCCCAGCTCAGCATGAA-3'
		Reverse	5'-TAACGGGCCTCCGTCTCT-3'
<i>Epcam</i>	52	Forward	5'-AGAATACTGTCAATTGCTCCAACT-3'
		Reverse	5'-GTTCTGGATCGCCCCTTC-3'
<i>Lgr5</i>	38	Forward	5'-CTACCCGCCAGTCTCCTACA-3'
		Reverse	5'-AAAGCATTTCAGCAAGACG-3'
<i>Muc6</i>	89	Forward	5'-GGATGTCTACCAGCCAAGGT-3'
		Reverse	5'-AACGATGTGGACTGATGCTG-3'
<i>Pdx1</i>	51	Forward	5'-GAAATCCACCAAGCTCACG-3'
		Reverse	5'-CGGGTTCCGCTGTGTAAG-3'
<i>Pnlip</i>	32	Forward	5'-CTGTGGACATTTGCAGTGCT-3'
		Reverse	5'-TGAAGCAGCCGAGTTTGTC-3'
<i>Sst</i>	53	Forward	5'-CCCAGACTCCGTCAGTTTCT-3'
		Reverse	5'-GGGCATCATTTCTGTCTGG-3'
<i>Trop2</i>	17	Forward	5'-CGGGCAAATACAAAAGGTG-3'
		Reverse	5'-ACAAGCTAGGTTTCGCTTCTCA-3'

RT-qPCR, real-time quantitative RT-PCR.

Figure S1A) was surgically resected and incubated in the liver perfusion medium (Thermo Fisher Scientific, Waltham, MA) for 10 minutes at 37°C after rinsing with 2% or 3% fetal bovine serum—containing ice-cold PBS. After centrifugation at 310 × g for 3 minutes, the pellet was incubated in digestion medium [0.5 mg/mL collagenase (Sigma-Aldrich), 0.5 mg/mL pronase (Roche, Basel, Switzerland), and 0.25 mg/mL DNase1 (Sigma-Aldrich) in liver digestion medium] for 30 to 50 minutes at 37°C. The dissociated cells were passed through a 70-μm cell strainer (Corning, Corning, NY) and centrifuged at 485 × g for 5 minutes. The cell pellet was resuspended with 2% or 3% fetal bovine serum—containing PBS and incubated with anti-Fc receptor antibody for 20 minutes on ice, followed by the addition of primary antibodies. After incubation for 30 minutes on ice, primary antibodies were washed out with 2% or 3% fetal bovine serum—containing PBS. After centrifugation at 485 × g for 5 minutes, the cell pellet was incubated with secondary antibodies for 20 minutes on ice. To exclude dead cells, propidium iodide was added before flow cytometric (FCM) analysis. For cultured cells, the dissociated cells after incubation with Accutase (Thermo Fisher Scientific) for 40 minutes were stained similarly. The MEBCYTO Apoptosis Kit (MBL, Aichi, Japan) was used for analysis of apoptotic and necrotic cell death, according to the manufacturer’s protocol, after washing secondary antibody away. The stained cells were analyzed by BD FACSCantoll (BD Biosciences, Franklin Lakes, NJ) or

sorted by Moflo XDP (Beckman Coulter, Brea, CA). Antibodies are listed in Table 1.

Cell Culture

For the two-dimensional culture, the isolated LBECs and PBECs were cultured on a type I collagen—coated cell culture dish (Corning). The used medium is described in Table 2. Medium was changed every 3 days. For cyst formation, isolated LBECs and PBECs were cultured as previously described.¹⁷ For organoid culture, isolated PBECs were suspended in 50 μL growth factor—reduced Matrigel (BD Biosciences) and cultured as previously reported.¹⁸

Rhodamine 123 Incorporation Assay

The incorporation of rhodamine 123 into cystic organoid at culture day 11 was evaluated, as previously reported.¹⁹ In this study, 100 μmol/L rhodamine 123 (Sigma-Aldrich) was used for incorporation. For inhibition of multidrug resistance protein (Mdr), the cystic organoid was pretreated with 100 μmol/L R-(+)-verapamil (Sigma-Aldrich) before rhodamine 123 incubation. Time-lapse imaging was performed by FV3000 with a stage top CO₂ incubator (TOKAI HIT, Shizuoka, Japan). Images were acquired every 2 minutes for 90 minutes.

Figure 1 Expression of liver stem/progenitor cell and pancreatic progenitor cell markers in adult mouse extrahepatic bile duct (EHBD). **A–C:** Immunohistochemical images of transverse sections of EHBD. Representative images stained with anti-EpCAM and anti-cytokeratin 19 (CK19; **A**), anti-osteopontin (Opn; **B**), or anti-cystic fibrosis transmembrane conductance regulator (Cftr; **C**) antibodies are shown. **Arrowheads:** Peribiliary glands (PBGs). **D:** Gene expression analysis of *Epcam* and *Pdx1* in biliary epithelial cells derived from intrahepatic bile duct (IHBD) and EHBD by real-time quantitative RT-PCR. Normalized values against *Actb* expression are shown. **E:** Experimental procedure of whole-mount immunostaining of EHBD. **F and G:** Whole-mount immunostaining of EHBD. Representative sagittal images stained with anti-EpCAM and anti-pancreatic duodenal homeobox 1 (Pdx1; **F**) or anti-sex-determining region Y-box 9 (Sox9; **G**) are shown. **Arrowheads:** PBGs. Data are expressed as means $\pm$ SEM (**D**). $n = 3$ (**D**). Scale bar = 100 μ m (**A–C**, **F**, and **G**).

Figure 2 Differential expression of Trop2 in extrahepatic bile duct (EHBD) is applicable to separation of peribiliary gland—constituting epithelial cells and lumen-forming biliary epithelial cells. **A:** Gene expression analysis of *Trop2* in EpCAM⁺ biliary epithelial cells of intrahepatic bile duct (IHBD) and EHBD by real-time quantitative RT-PCR. Normalized values against *Actb* expression are shown. **B:** Flow cytometric analysis of EHBD with anti-EpCAM and anti-Trop2 antibodies. **C:** Whole-mount immunostaining of EHBD using anti-EpCAM and anti-Trop2 antibodies. **Arrowheads:** Peribiliary glands. Data are expressed as means $\pm$ SEM (**A**). $n = 3$ (**A**). $*P < 0.05$. Scale bars: 200 μ m (**C**, top panels); 100 μ m (**C**, bottom panels). APC, allophycocyanin; FITC, fluorescein isothiocyanate.

Colony and Cyst Formation Assay

For colony formation assay, 1000 or 3000 isolated LBECs and PBECs were cultured for 6 or 8 days on the type I collagen-coated dishes (Corning) with a diameter of 60 mm. For cyst formation assay, 3000 isolated LBECs and PBECs were cultured for 7 days on the 8-well coverglass chamber (Thermo Fisher Scientific). After culture, the colonies were stained with Giemsa's solution (Merck Millipore, Burlington, MA) and imaged by IX-83 with DP80 (Olympus). The number of colonies and cysts was counted with the use of the ImageJ software version 1.51a (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>) cell counter tool.

Real-Time Quantitative RT-PCR

Extraction of total RNA from each sample was performed by using TRIzol reagent (Thermo Fisher Scientific) or RNeasy microkit (Qiagen, Hilden, Germany). Genomic DNA was degraded with DNase I (Thermo Fisher Scientific and Qiagen) during an extraction step. The extracted total RNA was reverse transcribed into cDNA with Primescript RT master mix (Takara Bio, Shiga, Japan). The cDNA sample was subjected to RNaseH (Thermo Fisher Scientific) to degrade residual template RNA. Gene expression analysis was performed with the TaqMan universal probe system (Roche). Measurement of the signal was performed by LC96 (Roche). β -Actin was used as an internal control

(Roche, number 05046190001). The information on primer and probe is listed in [Table 3](#).

Study Approval

All mouse studies were conducted in accordance with institutional procedures and approved by the Animal Care and Use Committee of the Institute of Molecular and Cellular Biosciences, The University of Tokyo (Tokyo, Japan; approval numbers 2609, 2706, 2804, and 2904), and the National Center for Global Health and Medicine Research Institute (approval number 17086). The study using human samples was approved by the Kanazawa University Ethics Committee (approval number 305-4), and all of the analyzed samples are derived from patients who provided informed written consent for the use of their tissue samples in research.

Statistical Analysis and Graphing

The statistical difference between two groups was compared by *t*-test, Welch's *t*-test, or *U*-test. When comparing three groups, Kruskal-Wallis test was used, with Dunn's multiple comparison test. Statistical significance was set at two-tailed $P < 0.05$. Determination of the statistical test was based on the result of Shapiro-Wilk normality test and variance analysis. Statistical test and graphing were performed by R version 3.4.1 (R Foundation for Statistical Computing,

Figure 3 Characterization of isolated peribiliary gland-constituting epithelial cells (PBECS) and lumen-forming biliary epithelial cells (LBECs) by gene expression analysis and colony formation assay. **A:** Experimental design for characterization of LBECs (EpCAM⁺Trop2⁺) and PBECS (EpCAM⁺Trop2⁻). **B:** Gene expression profiles of several peribiliary gland- and tissue stem/progenitor-related genes in LBECs and PBECS by real-time quantitative RT-PCR. Normalized values against *Actb* expression are shown. **C:** Colony formation assay of isolated LBECs and PBECS. Representative images of colonies after Giemsa staining are shown. **D:** Measurement of colony number per 3000 cells of isolated LBECs and PBECS. Data are expressed as means $\pm$ SEM (**B** and **D**). $n = 6$ (**B**); $n = 3$ (**D**). $*P < 0.05$, $**P < 0.01$. Scale bar = 5 mm (**C**).

Vienna, Austria; <http://www.R-project.org>) and Prism6 software version 6.0c (GraphPad Software, La Jolla, CA).

Results

Most Canonical Stem/Progenitor Cell Markers Are Unable to Distinguish Mouse PBG from EHBD

To identify PBG in mouse EHBD, the expression profiles of several candidate molecules, which make it possible to distinguish between PBECS and LBECs, were examined. Because it is well known that cytokeratin 19 (CK19), EpCAM, osteopontin, and cystic fibrosis transmembrane conductance regulator are expressed in mouse IHBD, the expression of these markers in EHBD was first investigated. The

immunohistochemical analysis of cross sections demonstrated that all of these markers were expressed in both the lumen and PBG of EHBD, indicating that EpCAM is applicable for purifying both PBECS and LBECs from the tissue of EHBD (Figure 1, A–C). However, another marker was required to distinguish between PBECS and LBECs. Because PDX1 is a marker characteristic of human BTSC but not LPC, the expression of Pdx1 was compared in mouse EHBD as well as IHBD. To avoid the contamination of IHBD to EHBD, a portion of the CBD as an EHBD sample was used for further analysis (Supplemental Figure S1A). First, BECs were isolated from liver and EHBD by FACS using an anti-EpCAM antibody (Supplemental Figure S1B). Gene expression analysis revealed that *Pdx1* was predominantly expressed in BECs isolated from EHBD, but not detected in those from IHBD (Figure 1D).

Figure 4 Cultured peribiliary gland-constituting epithelial cells (PBEs) exhibit characteristics of lumen-forming biliary epithelial cells. **A:** Immunocytochemistry of cultured PBEs. The expressions of EpCAM, cytokeratin 19 (CK19), sex-determining region Y-box 9 (Sox9), and pancreatic duodenal homeobox 1 (Pdx1) were maintained in cultured PBEs. **B:** Expression of Trop2 in cultured PBEs by immunostaining after expansion. **C:** Flow cytometric analysis of freshly isolated and cultured PBEs with anti-EpCAM and anti-Trop2 antibodies. **D:** Comparison of gene expression of *Trop2* and peribiliary gland-related genes between freshly isolated and cultured PBEs by real-time quantitative RT-PCR. Normalized values against *Actb* expression are shown. Data are expressed as means $\pm$ SEM (**D**). $n = 3$ to 4 (**D**). $*P < 0.05$, $***P < 0.001$. Scale bar = 200 μ m (**A** and **B**).

Consistently, immunohistochemical analysis of liver sections showed no expression of Pdx1 in IHBD (Supplemental Figure S2). To determine the localization of Pdx1 in EHBD, bile ducts were visualized by whole-mount immunostaining after optical clearing (Figure 1E). The sagittal images of EHBD, stained with anti-EpCAM and anti-Pdx1 antibodies by confocal microscopy, showed that Pdx1 was expressed in both the luminal epithelium and PBG of EHBD (Figure 1F), which is consistent with previously reported expression patterns.^{13,20} Similarly, the staining of sex-determining region Y-box 9 (Figure 1G), a transcription factor for LPC,²¹ was unable to discriminate PBG from EHBD. Therefore, these molecules are insufficient to define the identity of PBEs in mouse EHBD.

Trop2 Is Predominantly Expressed in LBEs, But Not in PBEs

Trop2 has been previously identified as a stem/progenitor cell marker for the liver as well as the prostate.^{11,22} However, the expression profile of Trop2 in EHBD has not been reported

previously. *Trop2* expression was, therefore, analyzed in EpCAM⁺ BECs isolated from EHBD and IHBD by using real-time quantitative RT-PCR. Strikingly, *Trop2* was expressed in BECs derived from EHBD but not in those from IHBD (Figure 2A). Further FCM analysis revealed that EHBD-derived EpCAM⁺ BECs could be subdivided into Trop2⁺ and Trop2⁻ fractions (Figure 2B). To clarify which type of EpCAM⁺ BECs express Trop2 in EHBD, whole-mount immunostaining was performed using antibodies against Trop2 and EpCAM. Surprisingly, Trop2 was predominantly expressed in the lumen of EHBD, but not in PBG (Figure 2C). From these results, it was hypothesized that Trop2 is a specific cell surface marker to identify the lumen of EHBD, and that EpCAM⁺Trop2⁺ and EpCAM⁺Trop2⁻ BECs correspond to LBEs and PBEs, respectively. To confirm this hypothesis, gene expression related to PBGs and/or BTSCs was compared between freshly isolated Trop2⁺ and Trop2⁻ BECs (Figure 3A). It has been reported that chromogranin A (Chga) and LGR5 are expressed in the mouse and human PBG, respectively.^{2,4,13,23} In addition, it has been

Figure 5 Characterization of isolated peribiliary gland—constituting epithelial cells (PBEs) by the three-dimensional (3D) organoid culture. **A:** Growth of cystic organoid derived from PBEs after the 3D organoid culture. **B:** Immunostaining of cystic organoid with anti-cytokeratin 19 (CK19) and Ki-67 antibodies. **C:** Immunostaining of cystic organoid with anti-CD49f antibody and phalloidin. The magnified images of **dotted boxed areas** (top panels) are shown in higher magnification below (**bottom panels**). **D:** Immunostaining of cystic organoid with anti-EpCAM and anti-Trop2 antibodies. **E:** Comparison of gene expressions of *Trop2* and peribiliary gland—related genes between freshly isolated and organoid-forming PBEs by real-time quantitative RT-PCR. Normalized values against *Actb* expression are shown. **F: Top panel:** Confocal microscopic images of rhodamine 123 incorporation in cystic organoid. Rhodamine 123 gradually accumulates in the luminal space of cystic organoid in a time-dependent manner. **Bottom panel:** Rhodamine 123 accumulation is blocked by pretreatment of cystic organoid with verapamil. Data are expressed as means $\pm$ SEM (**E**). $n = 4$ to 6 (**E**). $**P < 0.01$. Scale bars: 200 μ m (**A**, **B**, **C**, top panel, and **D**); 30 μ m (**C**, bottom panel); 100 μ m (**F**). ND, not detected.

Figure 6 Bile duct ligation (BDL) induces epithelial cell damage and tissue regeneration of extrahepatic bile duct (EHBD). **A:** Hematoxylin and eosin staining of non-treated (NT) and injured EHBD after BDL. The detachment of injured luminal epithelium is denoted with **arrowheads**. **B:** Expression analysis of Trop2 in NT and injured EHBD by immunostaining. Transverse sections of EHBD were stained with anti-EpCAM and anti-Trop2 antibodies. Trop2 expression was induced in peribiliary glands (PBGs) on biliary injury. PBGs are denoted with **arrows**, and the luminal epithelia are denoted with **arrowheads**. **C:** Flow cytometric analysis of NT and injured EHBD with anti-EpCAM and Trop2 antibodies. **D:** Colony formation assay of Trop2⁺ and Trop2⁻ biliary epithelial cells (BECs) isolated from NT and injured EHBD. Representative images of colonies after Giemsa staining are shown. **E:** Colony formation assay of Trop2⁺ and Trop2⁻ BECs isolated from NT and injured EHBD. The colony number per 1000 cells is shown. Data are expressed as means $\pm$ SEM (**E**). $n = 3$ to 5 (**E**). $*P < 0.05$. Scale bars: 200 μ m (**A**); 100 μ m (**B**); 5 mm (**D**). APC, allophycocyanin; FITC, fluorescein isothiocyanate.

reported that human PBGs contain epithelial cells secreting several pancreatic enzymes, such as α -amylase isozymes, trypsin, and pancreatic lipase.¹² Consistent with these previous reports, both *Chga* and *Lgr5* were expressed in Trop2⁻ BECs, whereas the expression of these genes was negligible in Trop2⁺ BECs (Figure 3B). Notably, several pancreatic

molecules, such as somatostatin (*Sst*), pancreatic lipase (*Pnlip*), and amylase 2a5 (*Amy2a5*), were detected in Trop2⁻ BECs, although their expression levels were negligible in Trop2⁺ BECs (Figure 3B). Consistent with the notion that PBGs include mucinous acini, high expression of mucin 6, gastric (*Muc6*), was also detected in Trop2⁻ BECs compared

Figure 7 Expression profile of TROP2 in human common bile duct (CBD). Immunohistochemical images for TROP2 in human CBD are shown. Surgical specimens of extrahepatic bile ducts were obtained from the patients with pancreatic cancer (A), pancreas head cancer (B and C), or extrahepatic biliary cancer (D). All sections are noncancerous part of CBD. Peribiliary glands are denoted with arrows, and the luminal epithelium is denoted with arrowheads.

with Trop2⁺ BECs (Figure 3B). In contrast, most of conventional BEC markers, such as CK19 (*Krt19*), *Epcam*, *Cftr*, and anion exchanger protein 2, were equally expressed between Trop2[−] and Trop2⁺ BECs (Supplemental Figure S3), consistent with the immunohistological data of mouse EHBD (Figure 1, A and C) and rat EHBD.²⁴ These results supported the hypothesis that Trop2 is a valuable cell surface marker to separate the luminal epithelium and PBG of EHBD (ie, LBECs and PBECs).

PBECs Show Higher Capacity for Colony Formation than LBECs in the Primary Culture

To investigate the characteristic difference between LBECs and PBECs, each cell fraction was isolated by FACS, and *in vitro* colony formation assay was performed (Figure 3A). Interestingly, PBECs showed significantly higher colony formation capacity than LBECs in the primary culture on the dish (Figure 3, C and D), suggesting that PBGs contain more stem/progenitor-like cells. To further characterize the expanded cells derived from PBECs, the expression of several EHBD markers was investigated by immunocytochemical and FCM analyses. Immunostaining of cultured PBECs revealed that the expression of EpCAM, CK19, sex-determining region Y-box 9, and Pdx1 was maintained after 6 days of culture (Figure 4A). More interestingly, most cultured PBECs expressed Trop2, although PBECs hardly expressed Trop2 at the beginning of culture (Figure 4B and Supplemental Figure S4). The induction of Trop2 expression in cultured PBECs was confirmed by FCM analysis and real-time quantitative RT-PCR (Figure 4, C and D). In contrast to Trop2 expression, marked down-regulation of *Chga*, *Sst*, *Muc6*, and *Lgr5* was observed (Figure 4D),

suggesting that PBECs produced the cells characteristic of LBECs concomitantly with the expansion on the dish.

PBECs Form Luminal Structure in 3D Culture Condition

Because PBECs showed high potential for the colony formation composed of LBEC-like cells, the bile duct-forming capacity of PBECs was next examined. As previously reported, the potential of bile duct differentiation can be evaluated by the formation of cysts with luminal epithelial polarity in the 3D culture.¹⁷ Cyst formation capacity between PBECs and LBECs was, therefore, compared. Similar to colony formation assay, PBECs showed significantly higher cyst formation capacity than LBECs (Supplemental Figure S5), suggesting that PBECs contain more progenitor-like cells, which have a potential for bile duct formation. However, this culture condition seemed to not be suitable for further analysis of PBECs, because it was not adapted for a long-term culture of PBECs and the growth of cyst had shortly stopped. Therefore, another 3D organoid culture system previously reported by Clevers' group¹⁸ was applied to the isolated PBECs. After 5 days of culture, PBECs formed apparent spherical cystic organoid, growing progressively (Figure 5A). Costaining for CK19 and Ki-67 revealed that the cystic organoid was formed mainly by the proliferation of PBECs rather than their aggregation (Figure 5B). After 10 days of culture, it was investigated whether the cystic organoid exhibited epithelial cell polarity by evaluating the localization of the apical (F-actin) and basolateral (CD49f) markers. Expectedly, F-actin was localized in the apical membrane of the organoid-forming cells, whereas CD49f was localized in the basolateral compartment (Figure 5C), indicating the proper

Figure 8 Schematic summary of the present study. The characteristics of the lumen-forming biliary epithelial cells (LBECS) and peribiliary gland-constituting epithelial cells (PBECS) at steady state. The differential expression profiles of Trop2 in extrahepatic bile duct (EHBD) makes it possible to discriminate between LBECS and PBECS by fluorescence-activated cell sorting in *in vitro* culture. The isolated PBECS show higher colony and cyst formation capacity than LBECS *in vitro*. The expanded PBECS have a potential for forming cystic organoid composed of LBECS-like Trop2⁺ cells with luminal epithelial polarity and secretory function in the three-dimensional culture. Model of biliary regeneration by PBECS after EHBD injury. By bile duct ligation, Trop2 expression is rapidly induced in peribiliary glands *in vivo* and PBECS are proliferated, producing transit-amplifying (TA) cells for the luminal epithelium regeneration.

polarity of luminal epithelium. In addition, immunostaining of Trop2 demonstrated that cystic organoid was composed of Trop2⁺ cells similarly to the luminal epithelium of EHBD *in vivo* (Figure 5D). Consistently, gene expression analysis showed the up-regulation of *Trop2* and the down-regulation of *Chga*, *Sst*, and *Muc6* in the proliferating organoid-forming cells by real-time quantitative RT-PCR (Figure 5E), suggesting that PBECS have a potential for producing LBECS-like cells with luminal structure. In contrast, *Lgr5* was not down-regulated under this 3D culture condition, unlike the plate culture condition (Figure 5E). Furthermore, it was investigated whether the formed luminal structure is functionally similar to *in vivo* bile duct. One of the known functions of bile duct is to modulate bile homeostasis by transportation of water and ions via transmembrane channels, such as Mdr1.²⁵ Recently, Sampaziotis and colleagues²⁶ have reported that human EHBD-derived organoid shows Mdr1-dependent secretory function. Therefore, it was examined whether PBECS-derived cystic organoid has Mdr1-dependent secretory capacity by the incorporation of rhodamine 123, a substrate of Mdr1. As a result, the gradual accumulation of rhodamine 123 was observed in the luminal space of cystic organoid, whereas it was blocked in the presence of verapamil, an Mdr antagonist (Figure 5F). From these results, it was hypothesized that PBECS have a potential to differentiate into functional LBECS.

Trop2 Expression Dramatically Changes in PBGs along with Biliary Injury

It has been previously reported that severe damage to the biliary tree, such as by BDL, induces the proliferation of EHBD.¹³ However, the nature of PBG during regeneration

remains unclear. Hematoxylin and eosin staining of EHBD after BDL showed remarkable dilation of the lumen, accompanied by the detachment of epithelium after 7 days of BDL (Figure 6A). Although EHBD did not exhibit apparent cellular damage 2 days after BDL by hematoxylin and eosin staining, FCM analysis of EHBD using annexin V and propidium iodide revealed that apoptosis and necrosis of EHBD increased remarkably at this time point (Supplemental Figure S6). Next, dual staining of Ki-67 and CK19 was performed in EHBD to investigate the extent of regeneration in LBECS and PBECS (Supplemental Figure S7A). Although intense signal of Ki-67 was detected in PBG before biliary injury, it was observed at the apical membrane of PBECS rather than nucleus (Supplemental Figure S7B), suggesting that this staining is nonspecific for Ki-67. The idea was supported by no incorporation of 5-bromo-2'-deoxyuridine in PBG before injury (Supplemental Figure S7C). In contrast, many LBECS and PBECS showed nuclear staining of Ki-67 after BDL, and then the ratio of Ki-67⁺ cells in the luminal epithelium and PBG gradually decreased, suggesting that LBECS and PBECS are capable of proliferating on biliary insult (Supplemental Figure S7A). More interestingly, Trop2 was expressed in most of PBECS 2 days after BDL (Figure 6B). Consistent with the immunohistochemical data, FCM analysis showed that the ratio of Trop2⁺ cells in EpCAM⁺ BECs rapidly increased after BDL (Figure 6C). These results suggested that PBECS activated by biliary damage may produce the luminal Trop2⁺ BECs for bile duct regeneration, as observed by using *in vitro* culture and organoid formation assay (Figures 4 and 5). Trop2⁺ and Trop2⁻ BECs were, therefore, isolated after BDL (Supplemental Figure S8) to compare their colony formation capacity. The capacity for colony formation in Trop2⁺

BECs was dramatically increased after BDL (Figure 6, D and E). Considering that Trop2⁺ PBECs exhibited higher colony formation capacity than Trop2⁺ LBECs isolated from non-treated EHBD (Figure 3, C and D) and that Trop2 expression was rapidly induced in PBGs after BDL, PBEC-derived Trop2⁺ cells may account for the remarkable increase of colony formation capacity in Trop2⁺ BECs after BDL, rather than the phenotypic change of Trop2⁺ LBECs. In contrast, a small population of Trop2⁻ BECs remained in EHBD, even after BDL (Figure 6, B and C). However, the colony formation capacity of Trop2⁻ BECs was decreased after 7 days of BDL, presumably because of exhaustion of the stem/progenitor cell compartment or fatal damage by prolonged injury (Figure 6, D and E).

Expression Profile of Trop2 Is Conserved in Human EHBD

Although the unique expression profile of Trop2 provided a clue to exploring the nature of PBG in mouse EHBD, it remains unknown in human EHBD. To investigate whether our finding and method in mice is applicable to human study in the future, the expression of TROP2 was examined in four individual human CBD tissues by immunohistochemical analysis. Intriguingly, the luminal biliary epithelium of CBD showed striking staining of TROP2 (Figure 7). In contrast, TROP2 was faintly or not stained in PBGs. More interestingly, the distal PBG from the luminal epithelium showed a tendency to be TROP2 negative. These results suggested that TROP2 is a useful cell surface marker to isolate PBECs in humans as well as mice for their characterization.

Discussion

Since the discovery of PBG association with the biliary tree in humans, the anatomic and histochemical studies have mainly been performed using human samples. It has been reported that PBGs are tubuloalveolar glands with mucinous and serous glandular acini composed of heterogeneous cell compartments. The expression of a variety of secretory factors, including mucins, neuroendocrine protein (eg, Chga), and pancreatic enzymes (eg, α -amylase, trypsin, and lipase), has been reported in adult PBGs in healthy and diseased states.^{12,13} The structure of PBG in EHBD is reminiscent of the crypt structures of the intestine and stomach. In contrast to numerous studies on the localization and differentiation status of crypt-forming component cells in the gastrointestinal system, the nature of PBG in EHBD remains uninvestigated and poorly understood. In addition, the similarity and difference of PBECs between human and mouse also remain unclear. In the present study, we have demonstrated that Trop2 is differentially expressed between the luminal epithelium and PBG of EHBD, which is applicable to FACS-based separation of each cell component. The gene expression analysis of LBECs and PBECs

derived from mouse EHBD revealed that the expression of neuroendocrine protein (eg, Chga), pancreatic markers (eg, Amy2a5, Sst, and Pnlip), and mucin (Muc6) was exclusively detected in the PBEC fraction, consistent with the previous reports about human PBG.²⁻⁴ Several human BTSC markers (eg, EpCAM, sex-determining region Y-box 9, and Pdx1) were expressed in both LBECs and PBECs in mice (Figure 1), and Lgr5 was differentially expressed in PBEC fraction (Figure 3B), suggesting that Lgr5 might be an advantageous marker for characterization of BTSC in mice. In addition, further gene expression analysis of PBECs may provide useful clues as to how the extrahepatic biliary system is constructed by various types of component cells.

Since Cardinale and colleagues³ reported that multipotent stem/progenitor cells are capable of differentiating into hepatocytes and BECs and pancreatic islets are present in the human extrahepatic biliary tree, the role of PBG has been highlighted as a reservoir of BTSCs. Considering the location of multipotent stem/progenitor cells in PBGs, the physiological role of BTSC may be involved in the maintenance or repair of biliary epithelium in the vicinity of PBG. In fact, it has been reported that the hyperplasia and the proliferation of PBG occur in patients with hepatolithiasis and cholangitis, as well as in rodent models of bile duct injury,^{13,23,27-29} although there is no direct evidence that the PBG contributes to regeneration of EHBD. In this article, we showed that PBECs isolated from normal EHBD contain highly proliferative progenitor-like cells with the colony formation capacity *in vitro*, which can also form cystic organoid with luminal epithelial polarity and Mdr1-dependent secretory function in the 3D organoid culture system. More important, the PBEC-derived organoids comprised epithelial cells with gene expression profiles similar to LBEC (ie, up-regulation of Trop2 and down-regulation of Chga, Sst, and Muc6), suggesting that PBECs possess a potential of supplying LBEC. Consistently, the PBECs proliferated *in vivo* on biliary damage by BDL, reexpressing Trop2 dramatically. Concomitantly, the Trop2⁺ BECs isolated from EHBD 2 days after BDL showed the colony formation capacity comparable to the Trop2⁻ PBECs before biliary injury. From these *in vivo* and *in vitro* data, it is plausible that PBGs play a role as a source of regenerating biliary epithelial cells by producing transit amplifying cells (Figure 8).

Trop2 was initially discovered as a marker of invasive trophoblasts and a molecule structurally related to paralogous EpCAM. The expression of these two related molecules has been described in various organs during development as well as tumorigenesis.³⁰⁻³³ Trop2 has been reported to be expressed in adult or fetal-type stem/progenitor cells in various organs.^{11,22,32,33} Goldstein and colleagues²² reported that Trop2 identifies a subpopulation of murine and human prostate basal cells with stem cell characteristics. We have reported previously that Trop2 is expressed in proliferating LPCs induced by 3,5-diethoxycarbonyl-1,4-dihydro-collidine diet injury, whereas it is hardly detected in the liver at steady

state.¹¹ A similar observation has been reported in two recent articles that Trop2 is reexpressed or induced in adult regenerating gastric glands or remodeling airway epithelium after epithelial damage, whereas it is absent in normal tissues.^{33,34} In this article, we also showed that Trop2 is up-regulated in PBGs after BDL, whereas it seems to be absent in normal PBGs. All of these reports imply the role of Trop2 in the process of stem/progenitor cell-mediated organogenesis or regeneration. In fact, a variety of functions of Trop2 have been reported in cell proliferation, differentiation, adhesion, and migration.^{30,35,36} However, the present study is slightly different from the above mentioned cases, because the luminal epithelium of normal EHBD expresses Trop2 constitutively. In the corneal epithelia, Trop2 has been reported to enhance the expression and localization of tight junctional proteins, including claudin-1 and claudin-7.³⁷ Therefore, there is a possibility that Trop2 may play a role in reinforcing the barrier of EHBD to avoid the leakage of toxic bile. Thus, it remains unknown whether the up-regulation of Trop2 in PBG is a step in stem/progenitor cell activation or a process of differentiation into LBECs. Nonetheless, the drastic change of Trop2 expression as well as the colony and organoid formation capacity of PBECs showed the possibility that PBGs contribute to EHBD regeneration. Given that PBGs are a niche for BTSCs, multipotent hepatobiliary-pancreatic stem/progenitor cells in EHBD must be Trop2 negative. Considering that Trop2 is induced in LPCs, bipotential hepatobiliary progenitors in 3,5-diethoxycarbonyl-1,4-dihydro-collidine-damaged liver, a mouse model of cholangiopathy, the expression of Trop2 may account for the commitment of hepatic lineage. Although it remains unclear whether Trop2⁺ cells in injured PBGs are derived from BTSCs, they may originate from PBECs by dedifferentiation, similar to injured intestine and stomach. Further studies using genetic ablation or lineage tracing experiments will reveal functional role(s) for Trop2 and PBG in tissue repair of biliary tree as well as other organs.

In conclusion, the present study demonstrates the differential Trop2 expression between the luminal epithelium and PBGs of normal EHBD and the drastic phenotypic change in PBGs on biliary injury. FACS-based isolation and characterization of PBECs will provide useful information to further characterize mouse and human BTSCs and reveal the role of PBGs in the homeostasis and regeneration of EHBD.

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Supplemental Data

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