

Liver ductal organoids reconstruct intrahepatic biliary trees in decellularized liver grafts

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Abstract

Three-dimensional scaffolds decellularized from native organs are a promising technique to establish engineered liver grafts and overcome the current shortage of donor organs. However, limited sources of bile duct cells and inappropriate cell distribution in bioengineered liver grafts have hindered their practical application. Organoid technology is anticipated to be an excellent tool for the advancement of regenerative medicine. In the present study, we reconstructed intrahepatic bile ducts in a rat decellularized liver graft by recellularization with liver ductal organoids. Using an *ex vivo* perfusion culture system, we demonstrated the biliary characteristics of repopulated mouse liver organoids, which maintained bile duct markers and reconstructed biliary tree-like networks with luminal structures. We also established a method for the co-recellularization with engineered bile ducts and primary hepatocytes, revealing the appropriate cell distribution to mimic the native liver. We then utilized this model in human organoids to demonstrate the reconstructed bile ducts. Our results show that liver ductal organoids are a potential cell source for bile ducts from bioengineered liver grafts using three-dimensional scaffolds.

Keywords:

Decellularization

Recellularization

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Extracellular matrix

Organoids

1. Introduction

Liver transplantation is currently the only curative option for patients with end-stage liver disease. However, the demand for liver organs greatly exceeds the supply of donor livers. To address this challenge, approaches such as cell transplantation, bioartificial organs, and liver support devices have been explored [1, 2]; however, none have yet been established as therapeutic alternatives.

Decellularization and recellularization, in which an extracellular matrix (ECM) is prepared from its native organs, retaining the inherent structure and biological properties, followed by recellularization with new cells to create transplantable functional organs, are promising techniques for tissue engineering [3]. Since the first report of a decellularized liver scaffold [4], recellularized liver graft models have been investigated from some liver cell sources. In addition to hepatocytes, a major functional cell type to be recellularized, the source cells include cholangiocytes and endothelial cells, as well as other stromal cells. Hepatocyte cell sources have been widely reported, and include primary hepatocytes, fetal hepatocytes, induced pluripotent stem cells (iPSC)-derived hepatocyte-like cells, and direct reprogramming of fibroblasts [5-9]. In contrast, the study of cholangiocyte cell sources has been rare owing to the difficulty of culturing primary cholangiocytes. Mouse immortalized cholangiocytes [10], normal rat cholangiocytes [11], and iPSC-derived cholangiocytes [9] have been studied as sources to recellularize the decellularized liver tissue ECM. Although there is a proof of concept to show that external cells can repopulate decellularized liver tissue, further physiological

choanocyte candidates are needed for cellular sources.

Recent advances have enabled the culture of tissue stem cells as three-dimensional (3D) organoids, which self-organize into 3D structures mimicking the original organs [12]. This organoid culture technique has also been applied to liver stem cells. A bile duct fragment embedded in Matrigel self-organized into liver ductal organoids with bipotential differentiation capacity into both hepatocyte and cholangiocyte lineages [13, 14]. Liver ductal organoids have been reported as a potential cell source for hepatocyte regeneration [15]. Therefore, we expect that the liver ductal organoids may also be a cell source for the regeneration of cholangiocytes, and the proliferating and self-organizing ability of the organoids provide a means to obtain cell networks for tissue engineering.

Here, we have described liver ductal organoids as a potential bile duct cell source for a bioengineered liver graft. We characterized the biliary properties of liver ductal organoids *in vitro* and those of repopulated bile ducts in a bioengineering liver graft *ex vivo*. The morphology and gene expression of the organoid-derived cells in recellularized livers indicate that liver ductal organoids are a useful cell source for the reconstruction of intrahepatic bile ducts.

2. Results

2.1. Mouse liver ductal organoids exhibit characteristics of functional cholangiocytes

Although liver ductal organoids are derived from intrahepatic bile ducts, these cells have mainly been investigated as a resource for differentiated

hepatocytes [13, 14, 16-18] rather than for cholangiocytes [19, 20]. We investigated to what extent the ductal organoids are characteristic of cholangiocytes. Liver ductal organoids showed rapid proliferation when cultured in Matrigel (Fig. 1A–C). Liver ductal organoids under maintenance culture were analyzed by RT-qPCR and immunofluorescence to determine the expression of markers specific to cholangiocytes and hepatocytes. In the RT-qPCR analyses (Fig. 1D and Supplementary Fig. S1), the expression of the cholangiocyte specific markers *Krt19*, *Sox9*, and *Cftr* was 0.68-fold (*p* value = 0.0053) lower, 2.0-fold (*p* value <0.0001) higher and 4.3-fold (*p* value <0.0001) higher in ductal organoids than extrahepatic bile ducts, respectively (Fig. 1D). In contrast, the expression of hepatocyte specific markers, *Alb*, *Hnf4a*, and *Cyp3a11* was quite low (1.66×10^7 -, 5.27-, and 1.49×10^5 -fold lower [*p* value <0.0001, 0.0002, and <0.0001], respectively) compared with that in primary hepatocytes (Fig. 1D). Immunofluorescence analyses revealed that ductal organoids exhibit cystic structures expressing cholangiocyte lineage markers, such as KRT19, SOX9, and CFTR, whereas ALB was absent (Fig. 1E, Supplementary Fig. S2). These findings indicate that liver ductal organoids under maintenance culture conditions, which reportedly possess bipotent stemness, exhibit characteristics of cholangiocytes but not of hepatocytes.

We subsequently characterized liver ductal organoids by focusing on their cholangiocyte function. The multidrug resistance protein 1 (MDR1) transporter is expressed in normal biliary epithelia and is involved in the efflux of a broad range of substrates into the lumen [21]. We thus evaluated the ability of organoids to efflux rhodamine 123, which is mainly transported by MDR1 transporters. Liver

ductal organoids were incubated with the dye rhodamine 123 (Fig. 2A), and the dye was transported into the lumen of the cystic organoids. In contrast, in the presence of 20 μ M verapamil, which inhibits MDR1 function, rhodamine 123 did not accumulate in the lumen (Fig. 2A). Quantification of the fluorescence intensity showed that verapamil significantly blocked the transportation of rhodamine 123 (p value < 0.0001) (Fig. 2B), indicating that this fluorescent dye was actively transported by MDR1.

In addition, to assess the function of another transporter expressed in normal biliary epithelia, the function of cystic fibrosis transmembrane conductance regulator (CFTR) was evaluated. CFTR expression was confirmed by both immunofluorescence (Fig. 1E) and immunoblotting (Supplementary Fig. S3), and we conducted a forskolin-induced swelling assay. This assay evaluates the increase in CFTR function induced by the activation of the cAMP pathway, which is stimulated by forskolin, and leads to organoid swelling [22, 23]. Liver ductal organoids were incubated with forskolin in the absence or presence of CFTR_{inh}-172, a specific CFTR inhibitor (Fig. 2C). Organoids increased the size by 2.66-fold after forskolin treatment (Fig. 2D). This forskolin-induced swelling ratio was significantly reduced to 1.55 by CFTR_{inh}-172 (p value < 0.0001), indicating that liver ductal organoids have CFTR functional activity. The inhibition of CFTR was not associated with cell death (p value = 0.42) (Fig. 2E, Supplementary Fig. S4) nor proliferation (p value = 0.28) (Fig. 2F, G). These findings suggest that liver ductal organoids have the properties of functional cholangiocytes.

2.2. Mouse liver ductal organoid cells repopulate the decellularized rat liver ECM to reconstruct biliary trees

As the ductal organoids exhibit the characteristics of cholangiocytes to a certain extent, we then investigated if the ductal organoids were a potential cell source for bioengineered livers. In this study, rat liver was used to generate decellularized tissue ECM because the mouse extrahepatic bile duct is too small to cannulate. The rat liver, which is also large enough for surgical implantation, is widely used for rodent decellularized liver experiments, while the mouse liver is widely used as a source for ductal organoids because of its technical robustness for exponential expansion. We have previously described a decellularized liver graft technique that offers a bioengineered scaffold with a physiological ECM [6, 8] (Supplementary Fig. S5). In addition to the native ECM, the decellularized liver also retains the vascular and biliary network frame structure (Supplementary Fig. S6). HE staining (Supplementary Fig. S7A) as well as DAPI staining (Supplementary Fig. S7B), and scanning electric microscopy (Supplementary Fig. S7C) confirmed the absence of both cells and nuclei in the decellularized liver scaffold. Quantification of DNA amount in the decellularized tissue also supported the complete decellularization (Supplementary Fig. S7D). After confirming that the biliary structure was preserved in the decellularized liver, liver ductal organoid cells were then injected into the biliary network of a decellularized rat whole liver scaffold via the common bile duct (Fig. 3A). The recellularized scaffold was cultured in an *ex vivo* perfusion culture system for 3–5 days (Fig. 3B); then, the tissue was formalin-fixed and paraffin-embedded for histological analysis. The ductal organoid-derived cells engrafted along the bile

duct walls, forming a monolayered structure lining the lumens (Fig. 3C). RT-qPCR analyses revealed that recellularized bile ducts expressed cholangiocyte marker genes, including *Krt19*, *Sox9*, *Cftr*, and *Hnf1b* at comparable levels with those in the extrahepatic bile duct *in vivo* (Fig. 3D), whereas hepatocyte markers (*Alb*, *Cyp3a11*, *Hnf4a*) remained at a low level. Moreover, the repopulated organoid cells sustained the expression of stemness markers (*Lgr5*, *Prom1*). In the immunofluorescence analyses, the repopulated organoid cells also exhibited key biliary markers (KRT19, SOX9, CFTR) (Fig. 4A). PCNA, a proliferation marker originally positive in ductal organoids, was maintained in some areas even after recellularization (Fig. 4B), indicating that the engrafted cells retained proliferative ability in the bile duct structure. These findings determined that repopulated liver ductal organoids were engrafted along the bile duct ECM, maintaining cholangiocyte properties.

Next, to demonstrate the 3D structure of recellularized bile ducts, liver ductal organoid cells expressing GFP were inoculated into a decellularized liver. The cells spread to the periphery of the liver, displaying a branched tree-like network (Fig. 5A). In addition, the confocal microscopy images showed engrafted cells on the decellularized bile duct ECM that formed luminal structures (Fig. 5B and Supplementary video S1). To evaluate the recellularization efficiency, we calculated the retention of the injected cells in the recellularized liver. The retention rate of the injected cells was as high as $98.3 \pm 1.46\%$ (average $\pm$ SD, N = 5 [*p* value = 0.0625, Wilcoxon's signed-rank test]) (Fig. 5C). Also, we have quantitatively evaluated the bile duct structure formation in the recellularized liver because the engraftment was not to the entire recellularized liver

(Supplementary Fig. S8A). The engraftment of ductal lumens was evaluated through the maximal section of the decellularized liver (Supplementary Fig. S8B). As shown in Fig. 5D, approximately 40% to 60% of the bile duct ECM were engrafted. The caudate and right lobes were likely to be engrafted more efficiently; the engraftment efficiency was high in some areas and low in others within a lobe, which may be due in part to the efficiency of the influx of injected cells. Note that only rarely was organoid-derived cell engraftment on the portal vein ECM observed (Supplementary Fig. S8B). As shown by these results, the intrahepatic bile ducts were recellularized efficiently by the injection of ductal organoid-derived cells via the common bile duct.

2.3. Liver ductal organoid cells retain cholangiocyte characteristics during simultaneous recellularization with primary hepatocytes

Hepatocytes are the main functional cellular unit in the liver. We evaluated whether the co-recellularization of repopulated liver ductal organoids and primary hepatocytes affected their engraftment or differentiation properties. As the optimal culture medium for hepatocytes and cholangiocytes differs, we first injected 5×10^6 liver ductal organoid-derived cells via the common bile duct, and the recellularized liver was perfused with expansion medium (EM) containing 10 μ M forskolin (Supplementary Table S1), a bile duct organoid EM, via the portal vein for 5 days. Then, freshly isolated mouse primary hepatocytes (5×10^7 cells) were injected via the common bile duct, followed by perfusion culture with HCM™ (Lonza Sales Ltd, Basel, Switzerland), a hepatocyte culture medium, via the portal vein for 2 days (Fig. 6A). Histological analyses of this co-recellularized

liver revealed the appropriate cell distribution of hepatocytes into the parenchymal space and of liver ductal organoid cells into the bile duct (Fig. 6B, C). Through the immunofluorescence analyses, repopulated primary hepatocytes were shown to express ALB and HNF4a. Repopulated liver ductal organoid cells expressed KRT19 and SOX9 but not ALB or HNF4a (Fig. 6D), showing that the repopulated cells maintained biliary lineage and did not differentiate to hepatocyte lineage when co-cultured with primary hepatocytes.

2.4. Human liver ductal organoid cells are capable of repopulating decellularized rat liver ECM

These findings must be extended to human cells to establish a transplantable human liver graft in the future. Human liver ductal organoids have bipotential capacity to differentiate into both hepatocytes and cholangiocytes, similar to mouse liver ductal organoids [14]. We generated three liver ductal organoid lines from residual liver specimens from patients undergoing hepatectomy in response to liver tumors (Table 1). Human liver ductal organoids cultured in Matrigel (Fig. 7A, B) expressed key biliary markers (KRT19, SOX9) but not ALB (Fig. 7C). Next, we injected the human organoid cells into a rat decellularized liver and achieved successful recellularization (Fig. 7D), similar to the mouse organoids, in a rat decellularized liver. The RT-qPCR analysis demonstrated that *KRT19* and *PROM1* were upregulated in all cases evaluated and *SOX9* was upregulated in two of three cases (Fig. 7E, Supplementary Fig. S9) exhibiting the cholangiocyte characteristics. In contrast, the gene expression of hepatocyte markers, *ALB*, and *CYP3A4*, a human orthologue of murine

Cyp3a11, remained low (Fig. 7E). In the immunofluorescence analyses, the recellularized bile ducts were shown to maintain the expression of KRT19 and SOX9 (Fig. 7F). Therefore, human liver ductal organoids maintained cholangiocyte properties after recellularization.

3. Discussion

Here, we have reported that liver ductal organoids can be a useful cholangiocyte cell source for bioengineered bile ducts. Luminal structures with a single cell layer lining were successfully reconstituted in the 3D scaffold decellularized from the liver which expressed appropriate bile duct markers. In addition, the co-recellularization with primary hepatocytes was possible via the common bile duct after recellularization with ductal organoids, suggesting the intact passage of the bile duct lumen after recellularization. Also, specific markers for each cell type were maintained.

Since the liver ductal organoid was first introduced as a bipotential stem cell culture [13], hepatocyte lineage differentiation from the ductal organoids has been well studied [17, 18]. Very recently, several papers investigating the cholangiocyte characteristics of this bipotent organoid were published [19, 20, 24]. The induction of biliary lineage differentiation in liver ductal organoids upregulated cholangiocyte-related genes and downregulated stem cell markers such as *Lgr5* and *Ki67* [19]. However, in this study, we found that the liver ductal organoids already possess cholangiocyte-like properties prior to differentiation, including representative cholangiocyte markers and functions. In addition, the repopulated liver ductal organoid-derived cells remained proliferative and

retained the expression of stem cell markers *Lgr5* and *Prom1* as well as key biliary markers (*Krt19* and *Sox9*). This characteristic appears to be a favorable feature as a source for tissue engineering because maintaining the proliferating potential is important to enable engrafted cells to expand and self-organize in a decellularized liver graft *ex vivo*, or even after transplantation. Liver ductal organoids morphologically consisted of flat cells forming a cystic structure *in vitro*, meanwhile, the recellularized bile ducts showed luminal structures in the form of a simple columnar epithelium. We also confirmed that repopulated liver ductal organoids were not differentiated into hepatocyte lineage, despite their bipotential capacity and perfusion with hepatocyte medium in the co-recellularized liver.

With regard to cholangiocyte cell sources for recellularization, a similar approach was recently reported [20], in which engineered extrahepatic bile duct models *in vitro* were established using human decellularized extrahepatic bile ducts repopulated with bile duct-derived organoids. They compared the recellularizing efficiency among three types of biliary cell-derived organoids; extrahepatic bile duct-derived organoids (EDO), bile-derived organoids (BDO), and intrahepatic bile duct-derived organoids (IDO, i.e. liver ductal organoids). In their study, EDO and BDO, but not IDO repopulated decellularized extrahepatic bile ducts efficiently *in vitro*. Although, as such, the liver ductal organoid was not a promising cell source for recellularizing extrahepatic bile duct, our study demonstrated that liver ductal organoids from both mice and humans successfully repopulated intrahepatic bile ducts in decellularized rat livers. Given the regional differences in the cell characteristics and gene profiles between

extra and intrahepatic bile duct organoids [24, 25], it is reasonable to use region-specific organoids for each target of intra and extrahepatic bile ducts to establish an engineered liver graft.

Previous studies have shown that iPS-derived cholangiocytes [9] and primary cholangiocytes [11] can be the cell source to recellularize bile ducts in the decellularized liver. Although we have not compared side-by-side, recellularized liver with ductal organoids showed morphological advantages to these previous reports. Based on the images presented in these papers, iPS-derived cholangiocytes did not show a clear microscopic luminal structure and primary cholangiocytes did not show a macroscopic branched tree-like structure. In the present study, we showed that ductal organoids could construct branched tree-like structures with luminal morphology, at least in a portion of the recellularized liver. There are pros and cons to every approach: ductal organoids have the potential of exponential proliferation, while it requires sampling from the live liver; iPSC does not require invasive tissue sampling, while it requires gene manipulation; primary cholangiocyte is the most physiological cell source, while it has poor proliferative potential. Thus, studying multiple alternative cell sources would give suggestions to each other.

For clinical applications rather than an experimental preparation of a small amount of regenerative tissue, several problems must be overcome. The immune response and the number of the cells are examples of potential issues. To avoid the immune response, an autologous cell source that does not require immunosuppression therapy is a potential solution. As liver ductal organoids can be generated from a small piece of a patient's liver without gene editing,

engineered bile ducts can be reconstructed using autologous cells. Moreover, the liver ductal organoids are genetically stable during long-term expansion [14], which allows the supply of a large number of cells. Because liver ductal organoids are also envisioned as a hepatocyte cell source owing to their bipotential capacity, large numbers of the organoids will be needed for transplantation as the hepatocyte cell source. To supply large numbers of cells, a highly efficient method of culturing liver ductal organoids has been reported [16]. Given these advantages, liver ductal organoids have the potential to be a cell source for bioengineered liver grafts. By overcoming these problems, in the future, it is expected that recellularized liver would support or even substitute liver grafts from donors, which would help solve the issues related to donor shortage.

We demonstrated the cholangiocyte properties of the repopulated liver ductal organoids, although functional interactions between recellularized bile ducts and hepatocytes remained ambiguous. The integrated function of the bile efflux and transportation are important in recellularization. Primary hepatocytes quickly lose their functions *in vitro* [2, 26]. Likewise, it is difficult to maintain recellularized primary hepatocytes in perfusion culture for a long time, whereas repopulated liver ductal organoids were viable for more than a week. Such limited viability restricts the ability of recellularized hepatocytes to construct the functional structures. Thus, in future studies, it is important to improve hepatocytes as a resource for recellularization, as well as to evaluate the cellular integration between recellularized bile ducts and hepatocytes upon experimental transplantation.

In conclusion, we demonstrated that liver ductal organoids are a useful cell source for the reconstruction of intrahepatic bile ducts in a 3D scaffold of decellularized liver and to enable the appropriate cell distribution of recellularized hepatocytes and bile ducts in bioengineered liver grafts. It would be of great interest to develop liver ductal organoids as a hepatocyte source as well as to generate functional recellularized liver grafts *in vivo* for the clinical application of these cells in the future.

4. Materials and Methods

4.1. Animals and human liver samples

All the animal experiments were performed in accordance with the Animal Protection Guidelines of Kyoto University and with approval from the Animal Research Committee of Kyoto University.

Liver specimens were obtained from resected liver tissues of patients who underwent hepatectomy at Kyoto University Hospital and provided written informed consent, following the approval given by Kyoto University Graduate School and Faculty of Medicine, Ethics Committee (R1671).

4.2. Preparation and culture of mouse liver ductal organoids

Mouse liver ductal organoids were prepared and cultured as previously described [13, 14], but with slight modifications. Briefly, the liver was harvested from C57BL/6J mice (8–12 weeks old, female, CLEA Japan, Shizuoka, Japan), mechanically minced with scissors into ~2-3 mm cubes, and serially passed through syringes with 18-, 20-, 22-, and 23-gauge needles for further mechanical

fragmentation by hydrodynamic shear force. Then, the tissue fragments were enzymatically digested with 2.6 μ U/mL Liberase DH (Roche, Basel, Switzerland) in DMEM (Wako, Pure Chemical Industries, Osaka, Japan) containing 0.1 mg/mL DNase I (Sigma-Aldrich, St. Louis, MO) for 10 min at 37°C. The digested fragments were serially passed through mesh filters of 100, and 40 μ m (BD Falcon, Franklin Lakes, NJ, USA). The cell clusters trapped on 100 and 40 μ m filters were collected and suspended in DMEM (Wako) and centrifuged at 440 $\times g$ for 3 min, and the pelleted cell clusters were used for culture. Cell clusters were suspended in Matrigel-GFR (Corning, NY) and placed on non-treated culture plates (IWAKI, AGC Techno Glass, Shizuoka, Japan) at approximately 20 to 30 clusters per 8 μ l Matrigel drop. After gelation of the Matrigel for 10 min at 37°C, tissue fragments in Matrigel drops were cultured with mouse EM (Supplementary Table S1) [13] containing 10 μ M forskolin (Wako) for 7-10 days until the emergence of ductal organoids. Once established, organoids were passaged every 4–6 days by dispersion with TrypLE Express (Invitrogen, Carlsbad, CA, USA) for 15 min at 37°C. The dispersed cells were re-embedded in Matrigel-GFR for further culture or used for recellularization.

4.3. Preparation and maintenance of human liver ductal organoids

Human liver specimens (0.5–1.0 cm³) were obtained from the non-tumorous part of the resected liver from patients who underwent hepatectomy. The specimens were subjected to the same procedure as the mouse organoid culture described above. For passaging, the application time of TrypLE Express was 10 min. The organoids were cultured in human EM (Supplementary Table

S1) [14].

4.4. Isolation of mouse primary hepatocytes

Primary hepatocyte isolation was performed using a two-step collagenase perfusion technique, as previously described [27]. Briefly, a C57BL6/J mouse was anesthetized with isoflurane (Wako) and the inferior vena cava was exposed and cannulated with a 25-gauge needle. After clamping the superior vena cava and cutting the hepatic portal vein, the liver was perfused with 50 mL of Ca^{2+} -free HBSS containing 0.5 mM EGTA (Wako) and 2 U/mL heparin (Novo-Heparin; Mochida, Tokyo, Japan) for 7 min, followed by 100 mL of collagenase solution containing 0.2% dispase II (Sanko Junyaku, Tokyo, Japan), 0.2% collagenase type II (Gibco, Palo Alto, CA, USA), 0.1 mg/mL DNase I (from bovine pancreas, Sigma), heparin 2 U/mL, 150 mmol/L NaCl, 5.4 mmol/L KCl, 0.34 mmol/L NaHPO_4 , 0.1 mmol/L MgSO_4 , 5.0 mmol/L CaCl_2 , 4.2 mmol/L, NaHCO_3 , 5.6 mmol/L glucose, and 10 mmol/L HEPES (all of the chemical reagents other than those indicated were purchased from Wako) for 7 min at 37°C. The liver was then resected out and gently shaken to retrieve the cells. After filtering the isolated cells through a 100 μm strainer (BD Falcon, Franklin Lakes, NJ, USA), the cell suspension was washed three times by centrifugation at $50 \times g$ for 3 min at 4°C. The isolated primary hepatocytes were immediately used for RNA extraction or the recellularization procedure.

4.5. ATP assay (Cell viability assay)

After 3,000 single cells dispersed from liver ductal organoids were embedded in Matrigel, the organoids were cultured in EM for 5 days. ATP activity in each culture was measured every day to evaluate organoid viability from an aliquot of the organoid lysate using CellTiter-Glo® (Promega, Madison, WI) and GloMax Discover System Microplate Reader and Software (Promega).

4.6. Histological analysis

Organoids were retrieved from Matrigel, re-embedded in Cellmatrix Type I-A (Nitta Gelatin), and fixed in 10% formalin (Wako) overnight at room temperature. Recellularized liver grafts were fixed in 4% paraformaldehyde (Wako) for 24 h at 4°C or in 10% formalin overnight at room temperature, and then embedded in paraffin.

Paraffin-embedded sections (4 µm) were dewaxed, rehydrated, and subjected to either hematoxylin and eosin (HE) staining or immunohistochemical staining. For immunostaining, antigen retrieval was performed by autoclave for 15 min at 121°C, and the sections were by incubation in PBS containing 10% donkey serum and 0.1% Triton X-100 (Nacalai Tesque Inc., Kyoto, Japan). All antibodies were diluted with PBS with 5% donkey serum and 0.1% Triton X-100. The sections were incubated with primary antibody overnight at 4°C, followed by incubation with secondary antibody for 1 h at room temperature, and then mounted using ProLong™ Gold Antifade Mountant with DAPI (Invitrogen). The stained sections were visualized using an Olympus BX50F4 microscope (Olympus Optical, Tokyo, Japan). The antibodies used for immunostaining are listed in Supplementary Table S2.

4.7. Quantitative real-time PCR

Liver ductal organoids in maintenance culture were dissociated with TrypLE Express (Gibco), counted, and seeded in 3,000 cells per 5 μ l Matrigel drops. For each condition, approximately 10,000 organoids from 30 Matrigel drops, which account for $\sim 1 \times 10^6$ cells, were prepared. Before RNA extraction, liver ductal organoids in Matrigel were collected and washed twice with PBS. To extract RNA from the recellularized liver, the recellularized liver after perfusion culture was incubated with RPMI (Wako) containing 10 mg/mL collagenase type II (Gibco) for 15 min at 37°C. The cell suspension from the digested liver was centrifuged, the supernatant was removed, and the pellet was processed for RNA extraction.

Total RNA was prepared from organoids and engineered liver grafts using the RNeasy Mini kit (QIAGEN, Hilden, Germany) and reverse-transcribed into cDNA using QuantiTect Rev. Transcription Kit (QIAGEN). qPCR was performed on the StepOne™ system (Applied Biosystems, Foster City, CA) using Fast SYBR® Green Master Mix (Applied Biosystems). Gene expression levels were normalized to the reference gene *TATA box binding protein (Tbp)* for mouse cells and *ACTB* for human samples in the main figures. The results normalized by alternative reference genes were presented in Supplementary Fig. S1 and S9 to confirm the trend of expression pattern. A list of primers is presented in Supplementary Table S3.

4.8. Immunoblotting

Liver ductal organoids cultured in Matrigel-GFR for 4 days after passage

were used for protein extraction. The recellularized liver was incubated with RPMI containing 10 mg/mL collagenase type II for 15 min at 37°C to lyse ECM, and the cell suspension was centrifuged to obtain a cell pellet for protein extraction.

Protein was extracted with cell lysis buffer (10mM Tris pH 7.4, 0.15M NaCl, 1% NP40, 0.25% sodium deoxycholate, 2mM NaVO₄, 0.05M NaF, 2mM EDTA, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 0.1% SDS and 1mM PMSF). Protein concentration was determined with TAKARA BCA Protein Assay Kit (TAKARA Bio Inc., Japan), separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a PVDF membrane. The membranes were incubated with primary antibodies in TBS containing 0.1% tween-20 and 5 % nonfat skim milk at 4°C overnight. After washing, the membranes were incubated with secondary antibodies conjugated with horses radish peroxidase (HRP) at room temperature for 1 h. ECL™ Prime Western Blotting System (Cytiva, MA, USA) was used to detect HRP and visualized with the ChemiDoc Touch MP system (Bio-Rad, USA). Antibodies were listed in Supplementary Table S2.

4.9. Rhodamine 123 efflux assay

The rhodamine 123 efflux assay was performed as described elsewhere [23]. Briefly, organoids in Matrigel on day 3 or 4 after passage were incubated with 100 µM rhodamine 123 (Sigma-Aldrich) in HBSS (Wako) for 10 min. Then, the gel was washed with HBSS three times and incubated with EM for 30 min before imaging. To inhibit MDR1 transporter activity, the Matrigel culture was

preincubated with EM containing 20 μ M verapamil (Wako) for 30 min before the addition of rhodamine 123. Then, the medium was changed to HBSS containing 100 μ M rhodamine 123 and 20 μ M verapamil. After incubation for 5 min, the gel was washed and incubated with EM containing 20 μ M verapamil. Fluorescence images of the organoids were obtained using a Leica TCS SPE confocal microscope (Leica Microsystems, Wetzlar, Germany). The fluorescence intensity of images was measured and calculated using ImageJ (National Institutes of Health, Bethesda, Maryland, USA). Fluorescence intensity across an organoid was measured and shown graphically by the Plot Profile function. Normalized fluorescence intensity was determined by subtracting background intensity from the center-of-organoid intensity for each organoid.

4.10. CFTR functional assay

The CFTR functional assay was performed based on a previous report [23]. Briefly, 1000 individual cells were embedded in a 5 μ L Matrigel drop and incubated in EM for 4 days. Then, the gels were preincubated with EM containing DMSO or 60 μ M Inh-172 (Cayman, Michigan, USA), a CFTR-specific inhibitor, for 3 h. The gels were subsequently incubated with EM containing 10 μ M forskolin (Wako) with DMSO or Inh-172 for 24 h before imaging with a Leica DMI8 (Leica Microsystems) using the Leica Application Suite X (LAS-X) software. The swelling rate of the organoids was calculated by comparing the total area of each organoid before and after the stimulation, using ImageJ.

PCNA positivity rate was calculated by counting the number of positive cells per that of all cells in each organoid treated with DMSO or Inh-172.

For cell viability imaging after Inh-172 treatment, organoids were incubated with 1 µg/ml propidium iodide (PI) (Invitrogen) and 10 µg/ml Hoechst (Invitrogen) for 15 min in the culture medium. After washing three times by HBSS, the organoids were imaged with a Leica DMI8 using LAS-X software.

4.11. Caspase activation assay

Caspase activation was quantified after incubation for 24 h with DMSO or Inh-172, as described in *Section 4.9. CFTR functional assay*, by Caspase-Glo® 3/7 Assay (Promega) using GloMax Discover Microplate Reader (Promega).

4.12. Preparation of GFP-expressing organoids

pLX304-GFP, a GFP-expressing lentiviral vector, was generated by transferring a GFP coding sequence from pALB-GFP (Addgene #55759) into pENTR4 (Addgene #17424) followed by gateway cloning into pLX304 (Addgene #25890). To generate lentiviral particles, HEK293FT cells were co-transfected with pLX304-GFP, psPAX2 (Addgene #12260), and pMD2.G (Addgene #12259) using X-tremeGENE™ HD (Roche) following the manufacturer's instructions. Viral supernatant was harvested at 48 and 72 h post-transfection and filtered through a 0.45 µm PVDF membrane (Merck Millipore). For lentiviral infection, the liver ductal organoids were dissociated into single cells and mixed with the filtered viral supernatant. The cell-lentivirus mixture was supplemented with 10 µM Y27632 (LC Laboratories, Woburn, MA), 1 mM N-acetyl-L-cysteine (Wako), and 10 µg/mL polybrene (Sigma), transferred to an untreated plate and centrifuged at 2000 rpm for 1 h at room temperature. After centrifugation, the

supernatant was discarded, and the cells were embedded in Matrigel drops (5–8 μ L each) and cultured with EM containing forskolin. After expansion, the organoids were passaged and selected by blasticidin (Wako).

4.13. Harvest, decellularization, and recellularization of rat whole liver

Harvest and decellularization. Harvest and decellularization of the liver were performed as previously reported [8]. Under general anesthesia with isoflurane (Wako), Lewis rats (8–24 weeks old, female, SLC, Hamamatsu, Japan) underwent laparotomy. Heparin (1 U/g of body weight) was administered intravenously. The abdominal aorta was cannulated with a 20-gauge cannula after clamping the descending aorta in the thoracic cavity. The liver was perfused with 50 mL of PBS after the transection of the superior and inferior vena cava. The portal vein and the common bile duct were cannulated with 20-gauge and 24-gauge cannula, respectively. The liver was harvested and cryopreserved at -80°C . For decellularization, the liver frozen at -80°C was thawed overnight at 4°C and then perfused with 0.25 w/v% Trypsin-1 mmol/L EDTA-4Na solution with phenol red (Wako) at 37°C for 1 h, followed by 1% polyoxyethylene(10) octylphenyl ether (Wako)/0.05% EDTA (Sigma) solution for 48 h at room temperature. The decellularized liver was sterilized by perfusion with 0.1% peracetic acid (Sigma) for 2 h and washed with sterilized PBS.

Recellularization. Liver ductal organoids were dissociated into single cells and $3\text{--}5 \times 10^6$ cells suspended in 5 mL of EM were administered into the scaffold through the bile duct at a flow rate of 1 mL/min. Before perfusion, the recellularized liver was incubated at 37°C in EM supplemented with 50 $\mu\text{g/mL}$

gentamicin (Gibco), 2.5 µg/mL of amphotericin B (Gibco), and 10 µM forskolin for 3 h. We applied our previously described recellularization protocol for primary hepatocytes [8]. In total, 5×10^7 hepatocytes were suspended in 30 mL of HCM™ (Lonza, Sales Ltd, Basel, Switzerland) and injected via the bile duct at a flow rate of 1 mL/min. The recellularized liver was incubated at 37°C for 3 h before starting the perfusion culture.

Analysis of cell retention rate: $5\text{--}6 \times 10^6$ dissociated single cells from mouse liver ductal organoids were injected into a decellularized scaffold following the recellularization protocol. After the cell injection, the medium leaked from the scaffold was collected. Then, the cells leaked in the collected medium were counted.

Analysis of engraftment ratio: HE staining was performed with paraffin sections of the caudate, left, middle, and right lobes in a recellularized liver in which $5\text{--}6 \times 10^6$ ductal organoids were injected and cultured with EM in the perfusion culture system for five days. Whole lobe Images of HE staining were created by the image-joint function of BZ-X710 (Keyence, Japan). The number of portal veins with/without engrafted bile ducts was counted by the Cell Counter function of ImageJ.

4.14. Perfusion culture

Perfusion culture was performed as previously reported [6, 8]. After incubation in EM for 3 h following recellularization, the recellularized liver was connected to the circulation culture system by the cannula inserted into the portal vein and placed in a customized chamber. The recellularized liver was

perfused with EM using a peristaltic pump (Perista pump, ATTA, Japan) with the continuous flow at a rate of 0.7–1.0 mL/min at 37°C. The perfusate was drained from the recellularized liver mainly from the hepatic vein and re-entered into the circulation system from the outlet of the chamber.

4.15. Scanning electron microscopy (SEM)

Electron microscopy images were obtained as previously described [6]. Normal fresh liver and decellularized liver of Lewis rats were fixed with 2% glutaraldehyde (Nacalai Tesque) and 4% PFA in phosphate buffer at 4°C overnight. The fixed samples were micro-dissected and dehydrated with ethanol (50, 60, 70, 80, 90, 99, and 100%). Then, the samples were immersed in t-butanol, frozen at –20°C, and the t-butanol sublimated off. Samples were then sputter-coated with platinum-palladium alloy using an ion coater (JEC-3000FC, JEOL, Japan) and observed by SEM (JSM-7900F, JEOL).

4. 16. DNA quantification

Small pieces (20mg) of native fresh liver and decellularized liver of Lewis rats were applied for DNA extraction. DNA was extracted using DNeasy Blood & Tissue Kit (QIAGEN) following the manufacturer's instructions. Then, DNA content was measured using Qubit dsDNA HS Assay Kit (Invitrogen) and Qubit™ 3 Fluorometer (Invitrogen).

4.17. 3D bile duct imaging

The liver with repopulated bile ducts of GFP-expressing cells was cultured in

the circulation culture system for 5 days and imaged using confocal microscopy (Leica Microsystems). A reconstructed 3D image was prepared from Z-stack images using ImageJ.

4.18. Statistical analyses

A *p* value of < 0.05 was considered to indicate statistical significance; analyzes were performed by one-way ANOVA with post-hoc Dunnett's multiple comparisons test (Fig 1D, 3D, 7E), unpaired *t*-test (Fig. 2B, D, E, G, S7) and Wilcoxon's signed-rank test (Fig. 5C) using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA).

Table 1. Patient data for organoids

No.	I.D.	Age	Sex	Background disease
1	KUH2N	59	M	Colorectal liver metastases
2	KUH6N	71	M	Intrahepatic cholangiocarcinoma
3	KUH7N	16	F	Focal nodular hyperplasia

Figure legends

Fig. 1. Characterization of mouse liver ductal organoids.

(A) Bright-field image of mouse liver ductal organoids cultured in Matrigel. Scale bars: 100 μ m. (B) Images of hematoxylin and eosin (HE) staining. A higher magnification image of the outlined box is shown in the right panel. Scale bars: 50 μ m. (C) Time course of the viability of the cultured ductal organoids evaluated

by ATP assay. Relative fluorescence correlates to ATP activity in each condition. The values are the average $\pm$ SD. N = 3 for each condition. (D) Relative gene expression analyzed by RT-qPCR and normalized by *Tbp* in the liver ductal organoids (ORG), primary hepatocytes (PH), and extrahepatic bile duct tissue (EBD). The values are the average $\pm$ SD. N = 3 for each condition. *: $p < 0.01$, **: $p < 0.001$, ***: $p < 0.0001$, ns: not significant, one-way ANOVA, Dunnett's multiple comparisons test. (E) Immunofluorescence staining of ductal organoids labeled with the indicated cholangiocyte and hepatocyte markers. Higher magnification images of the white outlined boxed areas are shown in the right panels. Scale bars: 50 μ m. See also Supplementary Fig. S2 for negative controls.

Fig. 2. Mouse liver ductal organoids exhibit functional characteristics of cholangiocytes.

(A) Representative confocal microscopy images showing uptake of rhodamine 123 dye in liver ductal organoids in the absence (left) or presence (right) of verapamil, an MDR1 inhibitor. Higher magnification images of the outlined boxed areas are shown in the middle panels. Graphs below the fluorescence images depict the fluorescence intensity along the white line in each image. Scale bars: 50 μ m. (B) Quantification of mean intraluminal fluorescence intensity of the organoids normalized to background levels cultured in the absence or presence of verapamil for 30 minutes. * $p < 0.0001$, unpaired *t*-test. (C) Representative bright-field images in the absence or presence of CFTR_{inh}-172, a CFTR inhibitor, before treatment (Pre) and after incubation for 24 h (Post). Scale bars: 250 μ m. (D) Quantification of the organoid swelling rate after forskolin (FSK) stimulation

in the absence or presence of CFTR_{inh}-172, a CFTR inhibitor. N = 31 and N = 51 for control and CFTR_{inh}-172-treated groups, respectively. * $p < 0.0001$, unpaired t -test. (E) Comparison of caspase activity after treatment with DMSO or CFTR_{inh}-172. The values are the average $\pm$ SD. N = 3 for each condition. ns: not significant, unpaired t -test. (F) Representative fluorescence microscopy images for organoids treated with DMSO (upper panels) or CFTR_{inh}-172 (lower panels) and immunostained for PCNA (green). Scale bars: 50 μ m. (G) Quantification of PCNA positivity in organoids treated with DMSO or CFTR_{inh}-172. N = 29 and N = 31 for each condition. ns: not significant, unpaired t -test.

Fig. 3. Characterization of recellularized bile ducts.

(A) Macroscopic images of a recellularized liver graft before (left) and after (right) recellularization in the bile ducts. (B) Photograph of the perfusion culture set-up placed in a CO₂ incubator. (C) HE staining of the recellularized liver with engrafted bile ducts (yellow arrows). Scale bars: 200 μ m. (D) Relative gene expression, as analyzed by RT-qPCR and normalized for *Tbp*, in the recellularized bile ducts (RBD), primary hepatocytes (PH), and extrahepatic bile duct tissue (EBD). The values are the average $\pm$ SD. N = 3 for each condition. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, ns: not significant, one-way ANOVA, Dunnett's multiple comparisons test.

Fig. 4. The recellularized bile ducts maintain biliary properties and proliferation ability.

(A) Immunofluorescence staining images of the recellularized bile ducts labeled

with the indicated cholangiocyte and hepatocyte markers. Scale bars: 100 μm . Higher magnification images of the white outlined boxed areas are shown in the right panels. Scale bars in high-magnification images: 50 μm . (B) Immunofluorescence images of the recellularized bile ducts (upper panels) and liver ductal organoids (lower panels) stained with PCNA. Higher magnification images of the white outlined boxed areas are shown in the right panels. Scale bars: 50 μm .

Fig. 5. Recellularized bile ducts reconstruct biliary tree-like structures.

(A) Fluorescent microscopic images of the bile ducts recellularized with liver ductal organoids expressing GFP on day 5 of perfusion culture. Scale bars: 250 μm . (B) Upper panel: 3D reconstruction of laser confocal microscopy images of the bile ducts recellularized with liver ductal organoids expressing GFP. Lower panel: cross-sectional view (xz) of the white dashed plane in the upper panel. (C) Retention of the injected organoid-derived cells in the recellularized liver. The number of the cells both injected and incorporated in the decellularized liver was plotted. N=5, ns: not significant, Wilcoxon signed-rank test. (D) Engraftment of bile duct ECM by organoid-derived cells. Engraftment percentage was evaluated by histological quantification for the caudate (Cd), left (Lt), middle (Md), and right (Rt) lobes. Plots are for each sample (N = 3). The values are the average $\pm$ SD.

Fig. 6. Co-recellularization of engineered bile ducts and primary hepatocytes results in distinguished localization and maintenance of each

specific cell marker.

(A) The scheme for co-recellularization by mouse liver ductal organoid cells and mouse primary hepatocytes. BD-seeding: Cells were injected via the common bile duct. (B) Macroscopic images of the co-recellularized liver with primary hepatocytes and ductal organoids. (C) HE staining of the co-recellularized liver on day 7. Scale bar: 100 μ m. (D) Immunofluorescence staining of the indicated cholangiocyte and hepatocyte markers. White arrows indicate recellularized bile ducts. Scale bars: 100 μ m.

Fig. 7. Human liver ductal organoid cells can repopulate decellularized bile ducts.

(A) Bright-field image of human liver ductal organoids cultured in Matrigel. Scale bars: 100 μ m. (B) HE staining of human liver ductal organoids cultured in Matrigel. A higher magnification image of the outlined boxed area is shown in the right panel. Scale bars: 50 μ m (left), 25 μ m (right). (C) Immunofluorescence staining of ductal organoids cultured in Matrigel labeled with the indicated cholangiocyte and hepatocyte markers. Scale bars: 50 μ m. (D) An HE staining image of bile ducts recellularized with human liver ductal organoid cells. A higher magnification image of the outlined boxed area is shown in the lower panel. Scale bars: 100 μ m (upper), 50 μ m (lower). (E) Relative gene expression of gallbladder cholangiocytes (GB), recellularized bile ducts of each human organoid line (hRBD1, KUH2N; hRBD2, KUH6N; hRBD3, KUH7N), and liver tissue (Liver) normalized by *ACTB*. The values are the average $\pm$ SD. N = 3 for each condition. *: $p < 0.001$, **: < 0.0001 , ns: not significant, one-way ANOVA,

Dunnett's multiple comparisons test. (F) Immunofluorescence staining of bile ducts recellularized with human liver ductal organoid cells and labeled with the indicated cholangiocyte and hepatocyte markers. Scale bars: 50 μ m.

Author contributions

Katsuhiro Tomofuji: Investigation, Data curation, Writing-original draft. Ken Fukumitsu: Conceptualization, Project administration, Methodology, Writing-review & editing, Funding acquisition. Jumpei Kondo: Methodology, Writing-original draft, Funding acquisition. Takamichi Ishii, Satoshi Ogiso, Yu Oshima: Methodology. Takashi Ito, Satoshi Wakama, Kenta Makino, Hiroshi Horie: Validation. Masahiro Inoue: Supervision, Funding acquisition. Etsuro Hatano: Supervision.

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Appendix A. Supplementary data

The following is the supplementary data related to this article:

Supplementary Figure and Table.docx

Supplementary Video S1.avi

Competing financial interests

M.I. belongs and J.K. belonged to the Department of Clinical Bio-resource Research and Development at Kyoto University, which is sponsored by KBBM, Inc. The other authors declare no competing interests.

Data availability

The raw data required to reproduce these findings are available from the corresponding authors upon reasonable request.

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