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Biliary epithelial cell differentiation of bipotent human liver-derived organoids by 2D and 3D culture

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ABSTRACT

Organoid culture is a technology for creating three-dimensional (3D) tissue-like structures *in vitro*, and is expected to be used in various fields. It was reported that human adult bile duct cells derived from human biopsy can be expanded as organoids *in vitro* that exhibit stem cell-like properties including high proliferative ability and differentiation ability toward both hepatocytes and biliary epithelial cells (BECs). Although many studies have achieved the efficient differentiation of bipotent human liver-derived organoids (hLOs) toward mature hepatocytes, the differentiation potency toward mature BECs remains unclear. In this study, we attempted to evaluate the differentiation potency of bipotent hLOs, which were generated from primary (cryopreserved) human hepatocytes (PHHs), toward BECs by sequential treatment with epidermal growth factor (EGF), Interleukin-6 (IL-6), and sodium taurocholate hydrate. Along with the differentiation toward bipotent hLOs-derived BECs (Org-BECs), increases in the gene expression levels of BEC markers and formation of the lumen-like structures typical of BECs were observed. In addition, Org-BECs exhibited P-glycoprotein-mediated drug transport capacity. Finally, in order to expand the applicability of Org-BECs, we succeeded in the differentiation of bipotent hLOs toward BECs in a two-dimensional (2D) culture system. Our findings demonstrated that bipotent hLOs can indeed differentiate into mature BECs, meaning that they possess a capacity for differentiation toward both hepatocytes and BECs.

1. Introduction

Liver progenitor cells are the main components of liver parenchyma in the late embryonic stage. They have the ability to differentiate into both hepatocytes and biliary epithelial cells (BECs) [1]. BECs include cells of the intrahepatic bile ducts, extrahepatic bile ducts, cystic ducts and gallbladder. They are responsible for regulating the flow rate, solubility, ionic concentration, pH and micelle-forming capacity of bile secreted by hepatocytes [2]. Dysfunction of BECs leads to various diseases, such as cystic fibrosis-related cholangitis, Alagille syndrome, autoimmune cholangitis, primary biliary cirrhosis and numerous diseases caused by drugs and toxins [3]. These cholangiopathies, which occur in approximately 33% of adult liver transplants and more than 70% of pediatric liver transplants, have high morbidity and mortality. Therefore, BECs are physiologically and pathologically important, though *in vitro* cell models for understanding their functions at the molecular level are limited. BECs derived from mice or rats are widely used for research today, since human primary BECs are difficult to obtain. However, some functions of murine-derived BECs differ from those of human BECs— e.g., the former do not express cystic fibrosis transmembrane conductance regulator (CFTR) [4], an important determinant of biliary secretion. A new human BECs model is thus urgently needed for research purposes.

Human induced pluripotent stem (iPS) cells can undergo self-renewal and differentiate into various cell types [5]. It is expected that functional BECs differentiated from human iPS cells would provide a new platform for studying the pathogenesis of bile duct diseases and for developing therapeutic agents. In 2014, Dianat et al. [6] reported that human iPS cell-derived hepatoblast-like cells can differentiate into BECs when treated with growth factors, EGF, IL-6, and sodium

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taurocholate. These cells formed functional cysts when cultured in a 3D matrix. Sampaziotis et al. [7] reported that the differentiation efficiency toward BECs was improved when human iPS cell-derived hepatoblast-like cells and OP9 stromal cells were co-cultured in the presence of hepatocyte growth factor (HGF), EGF, and transforming growth factor-β (TGF-β). Furthermore, stimulation of human iPS cell-derived hepatoblast-like cells with fibroblast growth factor 10 (FGF10), Activin A, and retinoic acid also improved the differentiation efficiency into BECs [8]. Our group previously reported that extracellular matrix laminin411 or laminin511 promoted differentiation of BECs from human iPS cells and increased the percentage of cells positive for a BEC marker, Aquaporin1 (AQPR1) [9]. BECs differentiated from human iPS cells were reported to reproduce various cholangiopathies *in vitro*. For example, it was reported that an experimental compound (VX809) for the treatment of polycystic liver disease (CF) rescued the disease phenotype of CF cholangiopathy in an *in vitro* model using human iPS cell-derived BECs generated from CF patients [8].

Organoid culture is a technology for creating three-dimensional (3D) structure derived from pluripotent stem cells, somatic stem cells or differentiated cells that self-organize through cell-cell and cell-matrix interactions, and recapitulate characteristics of the biological tissues or organs *in vitro* [10,11]. Organoids, unlike conventional cultured cells, are anatomically and functionally similar to biological organs [10,11], making it possible to study the functions of human organs in greater detail than was previously possible. Considering that the efficiency for generating iPS cell-derived cholangiocytes is 75% due to contamination by hepatic-lineage cells [7], and that the differentiation procedure is time-consuming, organoid-derived BECs generated by a comparatively easy procedure are expected to be used more efficiently for drug discovery and in fields such as developmental biology. Huch et al. [12,13] showed that human adult bile duct cells derived from human biopsy could be expanded as organoids *in vitro*. They showed that, compared to PHHs, the organoids had lower expression levels of hepatocyte markers and higher expression levels of BEC and stem cell markers. They also showed that the organoids could be differentiated into hepatocytes by the maturation medium [13–15]. However, the efficient differentiation toward mature BECs have not been fully evaluated.

In this study, we established organoids from commercially available PHHs. Since the organoids were at liver progenitor cell-like state, we attempted to differentiate the organoids, described as bipotent human liver derived organoids (hLOs) in this paper, into mature BECs. Bipotent hLOs embedded in Matrigel or collagen-gel were sequentially treated with BECs differentiation medium containing EGF, IL6, and sodium taurocholate hydrate to generate Org-BECs. Finally, we evaluated whether the differentiation could be performed in a 2D culture system yielding an Org-BECs derived monolayer.

2. Materials and methods

2.1. Organoid culture

Three lots of PHHs (lots DOO; Celsis, HC10-10; XENOTECH, HC4-24; XENOTECH) were used to generate organoids (Table S1). PHHs were washed with cold Advanced DMEM/F12 (Thermo Fisher Scientific) and spun at 400 g for 5 min. The cell pellet was mixed with Matrigel (growth factor reduced, Corning) and 1×10^4 cells were seeded per well in a 24-well plate. After the Matrigel had solidified, 500 μl of organoid expansion medium was added to each well. The organoid expansion medium was prepared as described in a previous report [13]. Briefly, advanced DMEM/F12 was supplemented with 1% Antibiotic Antimycotic Solution and 1 × GlutaMAX (GIBCO), 10 mM HEPES (Nacalai Tesque), 2% B27 supplement (GIBCO), 1.25 mM N-Acetylcysteine (Sigma), 10 mM Nicotinamide (Sigma), 10 nM recombinant gastrin (Merk), 50 ng/ml EGF (R&D), 10% R-Spondin1 conditioned medium (homemade), 100 ng/ml recombinant human FGF10 (peprotech), 25 ng/ml recombinant human HGF (R&D), 5 μM A83-01 (Wako), and 10 μM Forskolin (Wako). During cultivation, the medium was refreshed every 3 days. For the establishment of the organoids, the medium was supplemented with 25 ng/ml Recombinant Noggin (R&D), 7.5 ng/mL Recombinant Wnt3a (R&D) and 10 μM Y27632 (Wako) for the first 3–4 days. Passage was performed in a 1:3 split ratio once every 10–14 days. Organoids passaged 5–10 times were used in all experiments.

2.2. BEC differentiation protocols

To induce the differentiation of BECs, bipotent hLOs were cultured in BECs differentiation medium for 14 days. BECs differentiation medium was prepared according to our previous report [9]. Briefly, bipotent hLOs were cultured for 5 days in Advanced DMEM/F12 supplemented with 2% FBS (GIBCO), 10 mM nicotinamide, 5.0 mM sodium pyruvate, 20 mM HEPES, 1 × GlutaMAX and 50 nM EGF. For the next 5 days, cells were cultured in Advanced DMEM/F12 supplemented with 2% FBS (GIBCO), 10 mM nicotinamide, 5.0 mM sodium pyruvate, 20 mM HEPES, 1 × GlutaMAX and 20 nM IL-6 (Peprotech). For the last 4 days, cells were cultured in Advanced DMEM/F12 supplemented with 2% FBS (GIBCO), 10 mM nicotinamide, 5.0 mM sodium pyruvate, 20 mM HEPES, 1 × GlutaMAX and 1 mM sodium taurocholate hydrate (Sigma). For the control experiment without inducer-treatment, cells were cultured without EGF, IL-6 and sodium taurocholate hydrate. For the BECs differentiation with Matrigel, bipotent hLOs were cultured with the organoid expansion medium described above for 7–10 days. Then the medium was changed to the BECs differentiation medium. For the BECs differentiation with collagen gel, we first established collagen gel plates by adding 500 μl collagen gel solution (consisting of 400 μl type I-A Collagen (Nitta Gelatin), 50 μl Advanced DMEM/F12 and 50 μl of reconstitution buffer containing 260 mM $NaHCO_3$, 50 mM NaOH and 200 mM HEPES) to each well of a 12-well plate SUMILON), and the plates were incubated at 37 °C for 30 min. Bipotent hLOs (4×10^5 cells) resuspended in 500 μl of the BECs differentiation medium were mixed with 500 μl collagen gel solution, and plated onto the basal layer of collagen gel plates. After incubating for 30 min at 37 °C, 1 ml of the BECs differentiation medium was added to each well. For the BECs differentiation in a 2D culture system, bipotent hLOs (2×10^5 cells) were seeded on 0.03% collagen-coated 48 well plates and cultured with 300 μl of the BECs differentiation medium.

2.3. Real-time RT-PCR

Total RNA was isolated using Sepasol-RNA I Super G (Nacalai Tesque). Using 500 ng of the total RNA, cDNA was synthesized with a SuperScript VILO cDNA synthesis kit (Thermo Fisher Scientific). qRT-PCR was performed with Fast SYBR Green master mix (Thermo Fisher Scientific) using a StepOnePlus real-time PCR system (Applied Biosystems). The relative quantitation of target mRNA levels was performed by using the $2^{-\Delta\Delta CT}$ method. The values were normalized by those of the housekeeping gene, *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*). PCR primer sequences (described in Table S2) were obtained from PrimerBank (https://pga.mgh.harvard.edu/primerbank/).

2.4. Immunohistochemistry

To perform the immunohistochemistry, the cells washed with PBS were fixed with 4% paraformaldehyde for 10 min. After blocking the cells with PBS containing 2% bovine serum albumin (Nacalai Tesque) and 2% Triton X-100 (Merck) for 30 min at room temperature, the cells were incubated with the blocking buffer containing a primary antibody (described in Table S3) overnight at 4 °C, and finally with the blocking buffer containing a secondary antibody (described in Table S3) for 1 h at room temperature. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (Nacalai Tesque). Images were acquired with fluorescence microscope (Biozero BZ-9000; KEYENCE).

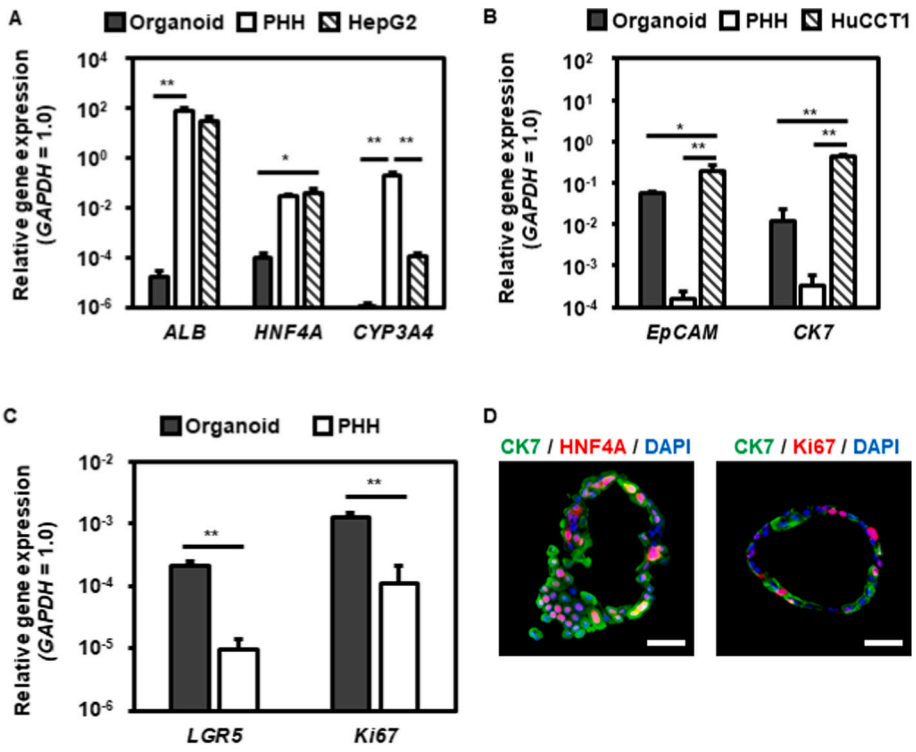

Fig. 1. Gene expression profile of bipotent hLOs (**A**) The gene expression levels of hepatocyte markers (*ALB*, *HNF4A*, *CYP3A4*) in bipotent hLOs (Organoid), PHHs (PHH) and HepG2 cells (HepG2). (**B**) The gene expression levels of BEC markers (*EpCAM*, *CK7*) in bipotent hLOs (Organoid), PHHs (PHH) and HuCCT1 cells (HuCCT1). (**C**) The gene expression levels of a Wnt-target gene (*LGR5*) and a cell proliferation marker (*Ki67*) in bipotent hLOs (Organoid) and PHHs (PHH). Parental PHHs, which were used for the generation of organoids and cultured for 48 h, were used as a positive control. The gene expression level of *GAPDH* was taken as 1.0. All data are represented as the means ± SD ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$. (**D**) The expressions of marker proteins for BECs (CK7; green), hepatocytes (HNF4A; red) and cell proliferations (Ki67; red) in bipotent hLOs were examined by immunostaining. Nuclei were counterstained with DAPI (blue). Images were acquired with fluorescence microscope (Biozero BZ-9000; KEYENCE). Scale bars represent 50 μm. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.5. Rhodamine transport assay

Org-BECs were incubated with Hanks' balanced salt solution (HBSS) containing 10 μM rhodamine 123 (Sigma) for 15 min at 37 °C, then washed 3 times with cold PBS. To demonstrate that rhodamine 123 transfer indeed reflected the activity of the membrane channel multidrug resistance protein 1 (MDR1), Org-BECs were incubated with 20 μM verapamil (Sigma), a widely used inhibitor of MDR1, for 30 min at 37 °C before rhodamine 123 treatment. Following completion of each experiment, images were acquired with fluorescence microscope (Biozero BZ-9000; KEYENCE).

2.6. Transepithelial electrical resistance (TEER) measurement

Cell culture inserts (24-well, 0.4-μm pore size, Corning) was coated with 250 μL of 2% (v/v) Matrigel suspended in advanced DMEM/F12 for 1 h at 37 °C. Org-BECs (4×10^5 cells/well) were dissociated into single cells and applied to the Matrigel-coated cell culture inserts. The BECs differentiation medium supplemented with 1 mM sodium taurocholate hydrate was added to the apical side for 5 days until the assay. For the control experiment without inducer-treatment, cells were cultured on Matrigel-coated cell culture insert with BECs differentiation medium without sodium taurocholate hydrate. TEER values of Org-BECs were measured using a Millicell ERS-2 voltohmmeter with an STX01 electrode (Merck). The raw data were converted to $\Omega \times cm^2$ based on the culture insert area. The blank resistance was then subtracted from the measured resistance to obtain the effective TEER.

2.7. Statistical analysis

Statistical analyses were performed using the unpaired two-tailed Student's *t*-test. All data are represented as means ± SD.

3. Results

3.1. Characterization of bipotent hLOs established from cryopreserved primary human hepatocytes

First, we examined the characteristics of the organoids which were established from PHHs. The gene expression levels of hepatocyte markers (*albumin (ALB), hepatocyte nuclear factor 4 alpha (HNF4A), cytochrome P450 family 3 subfamily A member 4 (CYP3A4)*) in the organoids were significantly lower than those in PHHs and HepG2 cells, a human hepatoma cell line (Fig. 1A). Although HepG2 cells are known to express little CYP3A4, the gene expression levels of *CYP3A4* in the organoids were much lower than those in HepG2 cells. In contrast, the gene expression levels of BEC markers (*epithelial cell adhesion molecule (EpCAM), cytokeratin 7 (CK7)*) in the organoids were much higher than those in PHHs, while they were lower than those in HuCCT-1 cells, a human bile duct carcinoma cell line (Fig. 1B). Similar results to Fig. 1A and B were obtained by western blotting (Fig. S1). These results suggested that the organoids we established from PHHs lost the characteristics of hepatocytes, but gained some of the characteristics of BECs, albeit not to a sufficient degree. To further characterize the organoids, we examined the gene expression level of a Wnt-target gene (*leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5)*) generally used as a marker for adult stem cells. The results showed that the gene expression level of *LGR5* in the organoids was higher than that in PHHs (Fig. 1C). In addition, the organoids showed a higher expression level of *Ki67,* a cell proliferation marker, compared to PHHs. We also analyzed the organoids immunohistochemically and confirmed that they were CK7-, HNF4A- and Ki67-positive (Fig. 1D). Collectively, these results suggested that the organoids lost the characteristics of hepatocytes and gained the characteristics of both BECs and liver progenitor cells. Furthermore, we confirmed that the generated organoids could be partially differentiated into hepatocytes by using the previously reported differentiation method (Fig. S2) [15]. In consideration of these results, we hypothesized that the organoids we established from PHHs were at liver progenitor cell-like state and decided to describe the organoids as bipotent hLOs in this paper.

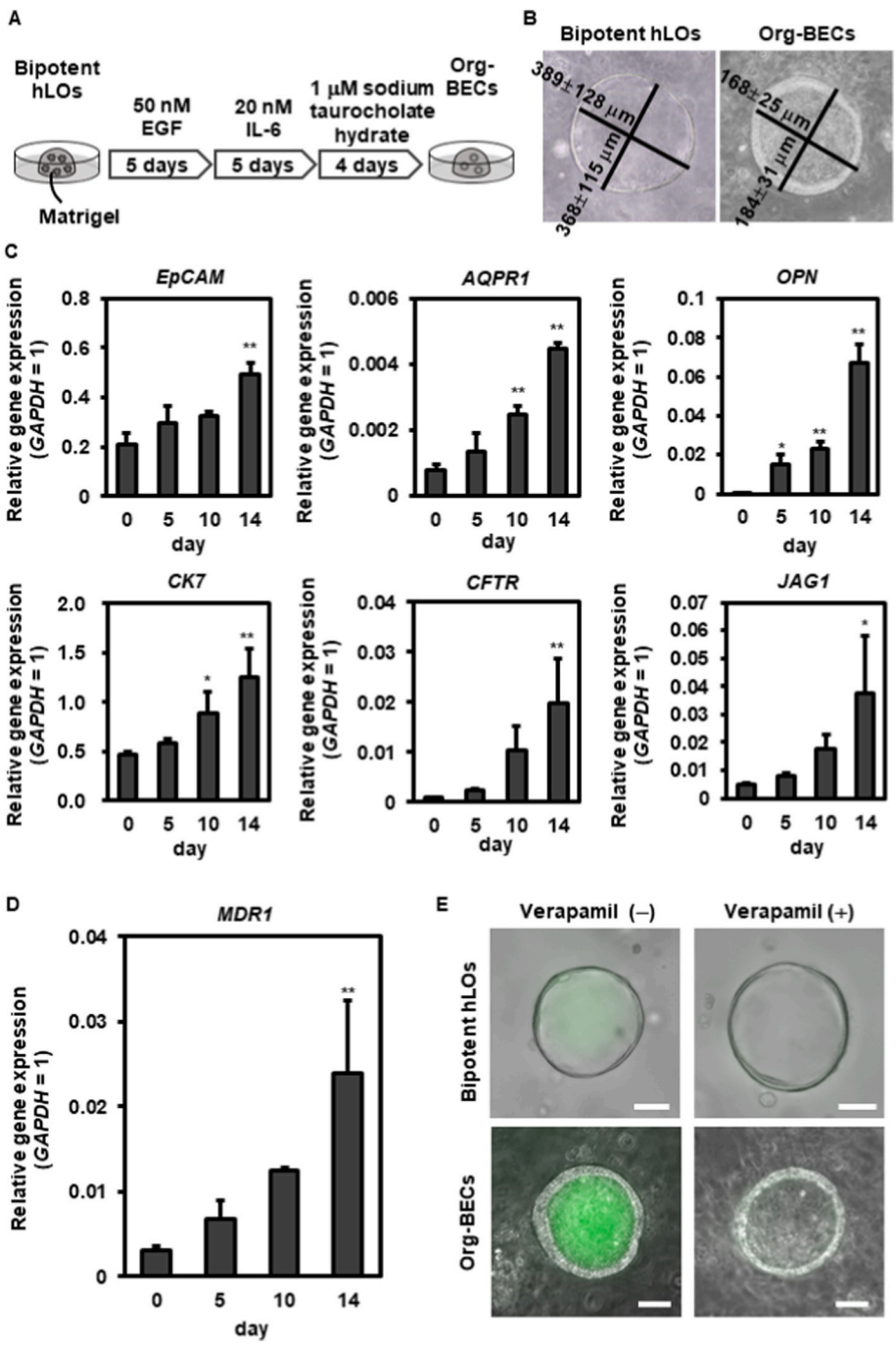

Fig. 2. Characterization of Org-BECs differentiated with Matrigel (**A**) The procedure for differentiation of bipotent hLOs toward BECs with Matrigel is presented schematically. Details of the differentiation procedure are described in the Materials and Methods. (**B**) Phase contrast images of bipotent hLOs and Org-BECs are shown. (**C, D**) The temporal gene expression levels of (**C**) BEC markers (*EpCAM*, *CK7, AQPR1*, *CFTR*, *OPN*, *JAG1*) and (**D**) *MDR1* in the cells at days 0, 5, 10 and 14 of the BECs differentiation were examined. The gene expression level of *GAPDH* was taken as 1.0. All data are represented as the means ± SD ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$. (**E**) Rhodamine 123 transport assay for bipotent hLOs (upper panels) and Org-BECs (lower panels) without (left panels) or with an MDR1 inhibitor, verapamil, treatment (right panels). Images were acquired with fluorescence microscope (Biozero BZ-9000; KEYENCE). Scale bars represent 50 μm.

3.2. Bipotent hLOs embedded in Matrigel differentiate into BECs

As described above, we hypothesized that bipotent hLOs at liver progenitor cell-like state have the differentiation potency toward both BECs and hepatocytes. Thus, we attempted to differentiate bipotent hLOs into mature BECs using previously developed protocol in our earlier report which differentiate human iPS cells toward BECs [9]. The bipotent hLOs were differentiated toward BECs, as described in Fig. 2A. Along with the differentiation, Org-BECs formed lumen-like structures that are typical to BECs (Fig. 2B). The diameters of the lumen-like structures were about 168–184 μm on day 14 of the differentiation, which was about half the diameter of the spheroids formed in bipotent hLOs. The difference in the size may be due to forskolin in the organoid expansion medium that stimulates cAMP and thus CFTR mediated transport to the inside of the organoids was promoted. The gene expression levels of BEC markers (*EpCAM, CK7, AQPR1, CFTR, Osteopontin (OPN), Jagged 1(JAG1)*) in Org-BECs increased temporally along with the differentiation (Fig. 2C). Similar results were obtained with the bipotent hLOs generated from two different PHH lines, HC10-10 and HC4-24 (Fig. S3A and S**3B**). To confirm the qRT-PCR results, we did western blotting of bipotent hLOs and Org-BECs using anti-SOX9 (SRY-box transcription factor 9) antibody which is known to be a cholangiocyte marker. We showed that protein expression level as well as gene expression level of SOX9 in Org-BECs were higher than those in bipotent-hLOs. (Fig. S4). Furthermore, to evaluate the gene expression levels when bipotent hLOs were not properly differentiated towards Org-BECs, we differentiated bipotent hLOs with the BEC differentiation medium without EGF, IL6 and sodium taurocholate hydrate. The

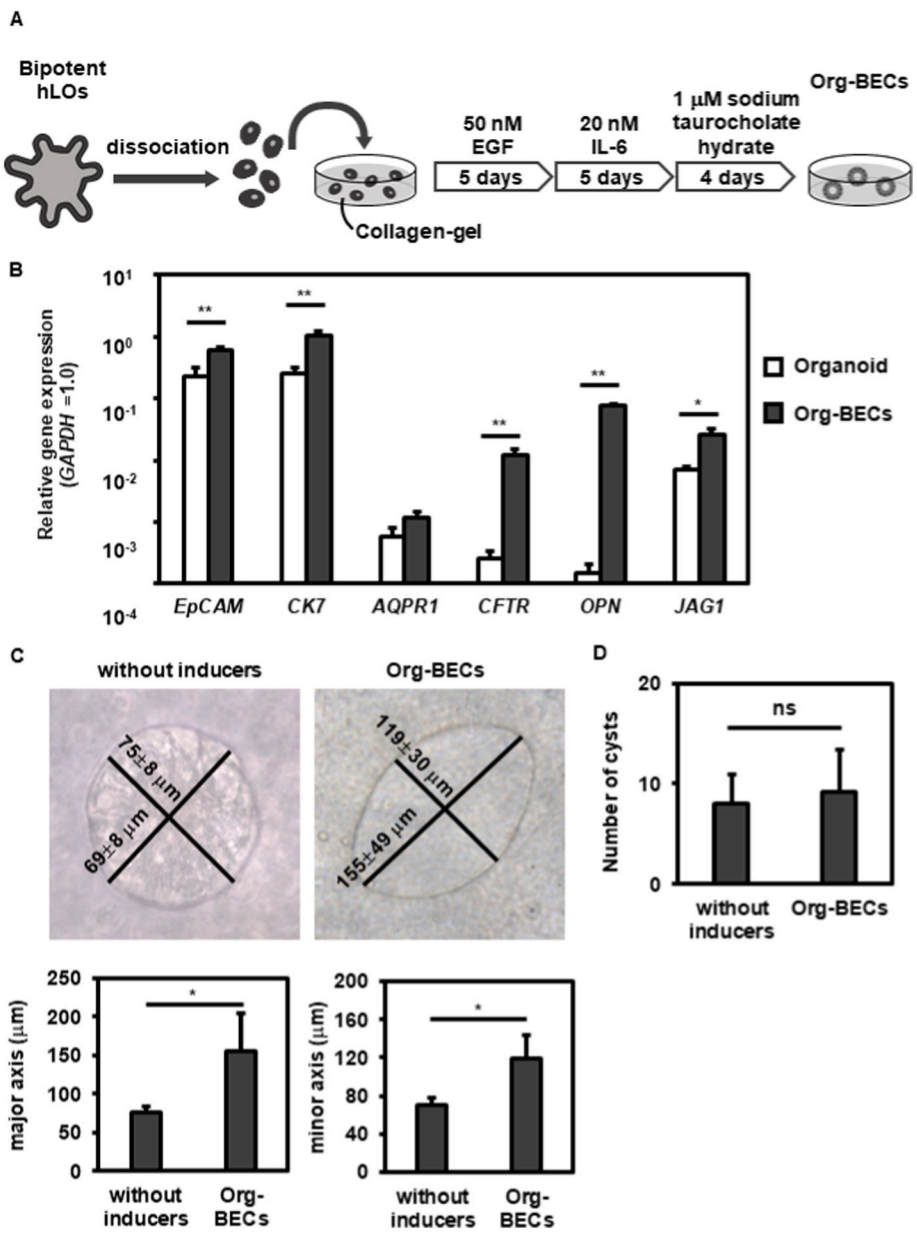

Fig. 3. Characterization of Org-BECs differentiated in a 3D culture system with collagen gel (**A**) The procedure for differentiation of bipotent hLOs toward BECs with a 3D culture system in collagen gel is presented schematically. Details of the differentiation procedure are described in the Materials and Methods. (**B**) The gene expression levels of BEC markers (*EpCAM*, *CK7*, *AQPR1*, *CFTR*, *OPN*, *JAG1*) in bipotent hLOs (Organoid) and Org-BECs were examined. The gene expression level of *GAPDH* was taken as 1.0. (**C**) The phase contrast images, and the diameters of lumen-like structures generated in the cells not properly differentiated (without inducers) and properly differentiated (Org-BECs) are shown. (**D**) The number of lumen-like structures formed in each well of a 24-well plate when bipotent hLOs were not properly differentiated (without inducers) or properly differentiated (Org-BECs) into BECs. All data are represented as the means ± SD ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$.

expression levels of BEC markers (*CK7, AQPR1, CFTR, OPN, JAG1*) in the cells not properly differentiated (without inducers) were lower than those properly differentiated (Org-BECs) (Fig. S5), suggesting that sequential treatment with EGF, IL6 and sodium taurocholate hydrate are necessary to efficiently differentiate bipotent hLOs toward BECs.

Rhodamine 123 is a substrate for P-glycoprotein encoded by the *multi drug resistance (MDR1)* gene. The ability to transport rhodamine 123 is one of the major functions of BECs. Since we confirmed that the gene expression level of *MDR1* increased with the differentiation toward Org-BECs (Fig. 2D), we next attempted to examine whether the lumen-like structures of Org-BECs gained the ability to transport rhodamine 123. At day 14 of the differentiation, Org-BECs transported more rhodamine 123 into the lumen compared to bipotent hLOs (Fig. 2E, left panel) apparently. When the Org-BECs were treated with an MDR1 inhibitor, verapamil, the transport of rhodamine123 was inhibited (Fig. 2E, right panel). These results indicated that Org-BECs gained the MDR1 activity along with the differentiation. We also performed the BECs differentiation from bipotent hLOs which were dissociated into single cells and embedded in Matrigel. Lumen-like structures were formed (Fig. S6A), and the gene expression levels of BEC markers were increased with the differentiation (Figs. S6B and S6C). These results suggested that bipotent hLOs-derived single cells could differentiate into BECs in the same manner as bipotent hLOs-derived spheroids.

3.3. Bipotent hLOs embedded in collagen gel differentiate into BECs

Bile ducts are surrounded by dense periportal collagenous matrices *in vivo*. Therefore, BECs are generally cultured in a 3D culture system with collagen-gel *in vitro* [9,16,17]. Thus, to confirm the differentiation potency of bipotent hLOs toward BECs in a 3D culture system with collagen-gel, we performed the differentiation as described in Fig. 3A. At day 14 of the differentiation, we confirmed that the gene expression levels of most BEC markers (*EpCAM, CK7, CFTR, OPN, JAG1*) in Org-BECs were higher than those in bipotent hLOs (Fig. 3B), suggesting that we succeeded in the differentiation with a 3D culture system in collagen-gel. Similar results were obtained with bipotent hLOs generated from two different PHH lines, HC10-10 and HC4-24 (Fig. S7A and **S7B**). Fig. 3C showed that Org-BECs formed lumen-like structures, and

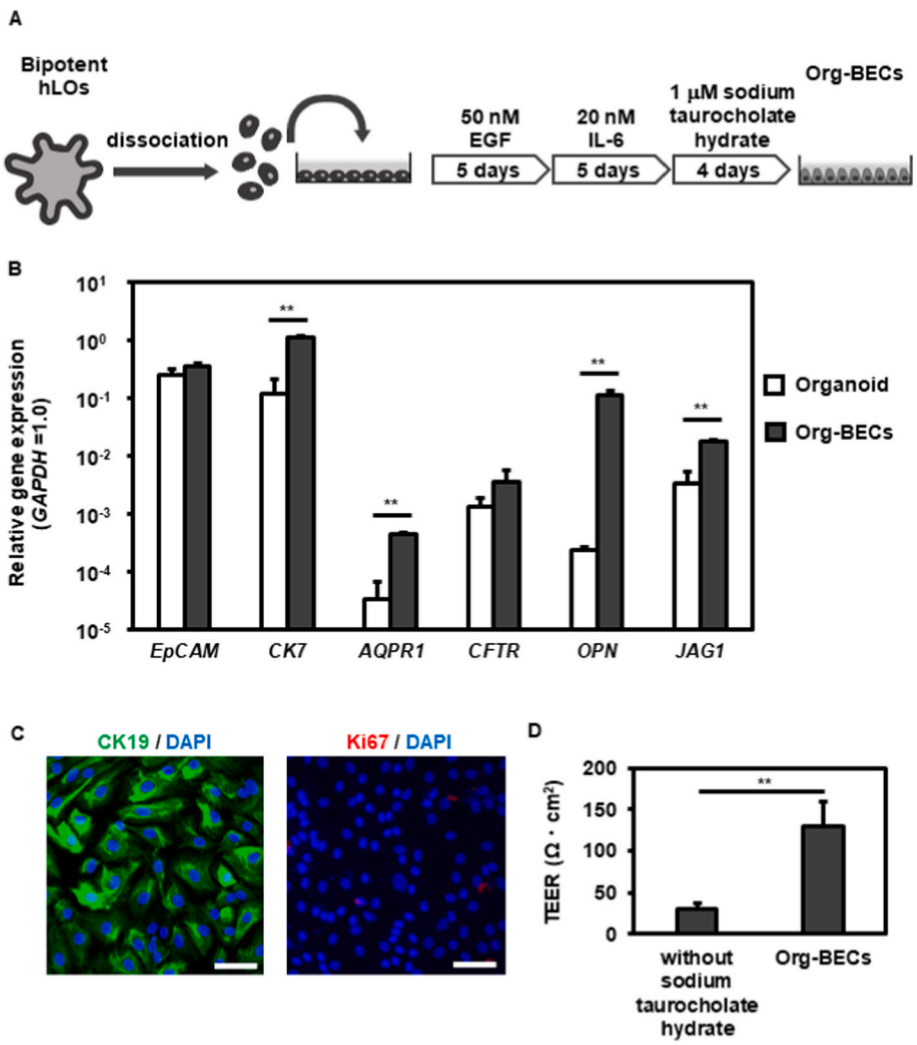

Fig. 4. Establishment of Org-BECs differentiation in a 2D culture system (**A**)The procedure for differentiation of bipotent hLOs toward BECs in a 2D culture system is presented schematically. Details of the differentiation procedure are described in the Materials and Methods. (**B**) The gene expression levels of BEC markers (*EpCAM*, *CK7*, *AQPR1*, *CFTR*, *OPN*, *JAG1*) in bipotent hLOs (Organoid) and Org-BECs were examined. The gene expression level of *GAPDH* was taken as 1.0. (**C**) The expression of marker proteins for BECs (CK19; green) and cell proliferations (Ki67; red) in Org-BECs were examined by immunostaining. Nuclei were counterstained with DAPI (blue). Images were acquired with fluorescence microscope (Biozero BZ-9000; KEYENCE). The scale bars represent 100 μm. (**D**) The barrier function in the cells not properly differentiated (without sodium taurocholate hydrate) and properly differentiated (Org-BECs) applied to cell culture inserts for 5 days were examined by TEER measurement. All data are represented as the means ± SD ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the diameters of the lumen-like structures in Org-BECs were about 119–155 μm. On the other hand, the diameters of the lumen-like structures in the cells not properly differentiated (without inducers) were about 75–69 μm, which was smaller than those in Org-BECs (Fig. 3C). There was no significant difference in the number of cysts formed in each well of the 24-well plates (Fig. 3D). Collectively, these results suggested that EGF, IL6 and sodium taurocholate hydrate are necessary to effectively differentiate bipotent hLOs toward BECs.

3.4. Bipotent hLOs differentiate into BECs in a 2D culture system

In order to apply Org-BECs to pharmaceutical research, it is necessary to develop a 2D culture system of Org-BECs. Thus, we performed differentiation of bipotent hLOs toward BECs in a 2D culture system as described in Fig. 4A. At day 14 of the differentiation, the gene expression levels of BEC markers (*CK7, AQPR1, CFTR, OPN*) in Org-BECs were higher than those in bipotent hLOs, suggesting that we succeeded in the differentiation in a 2D culture system (Fig. 4B). Similar results were obtained with bipotent hLOs generated from two different PHH lines, HC10-10 and HC4-24 (Figs. S8A and S8B). In order to examine their proliferation abilities, we next performed immunohistochemistry of Org-BECs. Most of the cells were positive for a BEC marker, CK19, in Org-BECs (Fig. 4C). Although bipotent hLOs were positive for a cell proliferation marker, Ki67 (Fig. 1D), the proportion of Ki67 positive cells decreased in Org-BECs (Fig. 4C), suggesting a decrease in the proliferation ability of bipotent hLOs. Finally, to determine whether Org-BECs could form a tight monolayer, the TEER value of Org-BECs applied to cell culture inserts was evaluated. The TEER value of Org-BECs was 130.5 Ω • cm^2, while that of the cells not properly differentiated (without sodium taurocholate hydrate) was 30.9 Ω • cm^2 (Fig. 4D). Thus, sodium taurocholate in addition to EGF and IL6 are essential for Org-BECs to form a tight monolayer.

4. Discussion

The purpose of this study was to examine whether bipotent hLOs established from PHHs were able to differentiate into mature BECs. The data suggested that they could differentiate into mature BECs under various conditions, such as when embedded in Matrigel or collagen-gel and even in single cell-dissociated 2D culture. We also succeeded in producing a tight monolayer from Org-BECs. Similar studies have reported the generation of mature BECs from hLOs [18,19]. However, the bipotent hLOs used in our study were generated from commercially available primary cryopreserved human hepatocytes (PHHs), while those in other studies were mostly generated from human biopsy [18,19]. Since the use of commercially available PHHs is not hampered by ethical issues, and since these cells are easier to obtain than human biopsy tissue, we believe that this study could provide a more versatile method to generate functional Org-BECs. Thus, Org-BECs generated from PHHs are expected to be widely used as a new BEC model for drug discovery and in fields such as developmental biology. Here, we showed that bipotent hLOs established from PHHs (more than 99% of the cells

were ALB-positive (data not shown)) lost the characteristics of hepatocytes, but partially gained the characteristics of BECs during the process of establishment and/or cultivation of organoids (Fig. 1A–D). When bipotent hLOs were cultured with a stepwise protocol using BECs differentiation medium, they could differentiate into BECs (Fig. 2A–E, Figs. S3–6). Moreover, our results showed that bipotent hLOs could gain hepatocyte-like functions under the optimal condition (Fig. S2). Further study is necessary to identify the cell origin of bipotent hLOs. Since bipotent human liver organoids showed cystic like morphology that is typical to intrahepatic cholangiocyte organoids [11,13], while hepatocyte organoids have a dense grape-like morphology [14], one possibility for the cell origin of the organoid generation may be the cholangiocytes which were contaminated in PHHs. Mature hepatocytes have been demonstrated to show plasticity *in vivo* by hepatocyte-to-BECs conversion [20–22]. Therefore, another possibility may be the cholangiocytes or bipotent liver progenitors transdifferentiated from hepatocytes, since the culture medium we used in this study was initially established to culture intrahepatic cholangiocytes organoids [13].

In this study, we showed that Org-BECs could be differentiated from bipotent hLOs under three different culture conditions, i.e., embedded in Matrigel or collagen-gel, and single cell-dissociated 2D culture. The optimal culture condition could thus be selected according to the purpose of the studies. Matrigel is effective for liver organoid culture and differentiation [23], while collagen-gel is generally used to culture BECs, since bile ducts are surrounded by dense periportal collagenous matrices *in vivo* [9,16,17]. The 2D culture and monolayer platform of Org-BECs are suitable for the application of Org-BECs to pharmaceutical research.

We used a differentiation method by which we previously reported the differentiation of human iPS cell-derived hepatoblast-like cells into BECs [9]. Several previous studies have reported methods for culturing primary BECs. Yang et al. [24] succeeded in establishing a BEC line from rat bile duct fragments. The BEC line could form lumen-like structures, though they soon disappeared during the culture. Hashimoto et al. [17] reported that rat BECs formed bile ductular networks when the culture conditions were sequentially controlled. Under their culture condition, the cells obtained polarity and expressed transporter activity. Auth et al. [25–27] showed that human BECs cultured in collagen-gel could form lumen-like structures more efficiently when they were cocultured with hepatocytes than when cultured alone. Wang et al. proposed a bioengineered 3D platform to reproduce functional BECs from organoids derived from human intrahepatic bile ducts with Matrigel/collagen type I mixture [18]. The differentiation method we used in this study [9] was different from that in other studies which generated mature BECs from hLOs [18]. We showed that EGF, IL6 and sodium taurocholate hydrate were effective for the differentiation of hLOs towards functional BECs. Since an effective protocol for differentiation of hLOs into BECs have not been fully developed, we believe that our findings would offer helpful perspectives for the future optimization of the differentiation methods of bipotent hLOs into BECs.

In conclusion, we showed that bipotent hLOs established from PHHs were able to effectively differentiate into BECs by sequential treatment with EGF, IL6 and sodium taurocholate hydrate. Org-BECs could be a new *in vitro* cell model for understanding bile duct functions at the molecular level.

Author contributions

Y.T., Y.U. and H.M. designed the experiments. Y.T. performed the experiments. Y.T., analyzed the data. Y.T., Y.U. and H.M. wrote the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2023.101432.

References

[1] A. Miyajima, M. Tanaka, T. Itoh, Stem/progenitor cells in liver development, homeostasis, regeneration, and reprogramming, Cell Stem Cell 14 (2014) 561–574, https://doi.org/10.1016/J.STEM.2014.04.010.
[2] M. Strazzabosco, L. Fabris, Functional anatomy of normal bile ducts, Anat. Rec. 291 (2008) 653–660, https://doi.org/10.1002/AR.20664.
[3] J.M. Banales, R.C. Huebert, T. Karlsen, M. Strazzabosco, N.F. LaRusso, G.J. Gores, Cholangiocyte pathobiology, Nat. Rev. Gastroenterol. Hepatol. 16 (2019) 269–281, https://doi.org/10.1038/S41575-019-0125-Y.
[4] G. Alpini, C. Ulrich, S. Roberts, J.O. Phillips, Y. Ueno, P.V. Podila, O. Colegio, G. D. LeSage, L.J. Miller, N.F. LaRusso, Molecular and functional heterogeneity of cholangiocytes from rat liver after bile duct ligation, Am. J. Physiol. 272 (1997), https://doi.org/10.1152/AJPGI.1997.272.2.G289.
[5] K. Takahashi, K. Tanabe, M. Ohnuki, M. Narita, T. Ichisaka, K. Tomoda, S. Yamanaka, Induction of pluripotent stem cells from adult human fibroblasts by defined factors, Cell 131 (2007) 861–872, https://doi.org/10.1016/J.CELL.2007.11.019.
[6] N. Dianat, H. Dubois-Pot-Schneider, C. Steichen, C. Desterke, P. Leclerc, A. Raveux, L. Combettes, A. Weber, A. Corlu, A. Dubart-Kupperschmitt, Generation of functional cholangiocyte-like cells from human pluripotent stem cells and HepaRG cells, Hepatology 60 (2014) 700–714, https://doi.org/10.1002/HEP.27165.
[7] F. Sampaziotis, M.C. De Brito, I. Geti, A. Bertero, N.R.F. Hannan, L. Vallier, Directed differentiation of human induced pluripotent stem cells into functional cholangiocyte-like cells, Nat. Protoc. 12 (2017) 814–827, https://doi.org/10.1038/NPROT.2017.011.
[8] F. Sampaziotis, M.C. De Brito, P. Madrigal, A. Bertero, K. Saeb-Parsy, F.A.C. Soares, E. Schrumpf, E. Melum, T.H. Karlsen, J.A. Bradley, W.T.H. Gelson, S. Davies, A. Baker, A. Kaser, G.J. Alexander, N.R.F. Hannan, L. Vallier, Cholangiocytes derived from human induced pluripotent stem cells for disease modeling and drug validation, Nat. Biotechnol. 33 (2015) 845–852, https://doi.org/10.1038/NBT.3275.
[9] K. Takayama, S. Mitani, Y. Nagamoto, F. Sakurai, M. Tachibana, Y. Taniguchi, K. Sekiguchi, H. Mizuguchi, Laminin 411 and 511 promote the cholangiocyte differentiation of human induced pluripotent stem cells, Biochem. Biophys. Res. Commun. 474 (2016) 91–96, https://doi.org/10.1016/j.bbrc.2016.04.075.
[10] M.A. Lancaster, J.A. Knoblich, Organogenesisin a dish: modeling development and disease using organoid technologies, Science (80–.) (2014) 345, https://doi.org/10.1126/SCIENCE.1247125/ASSET/D8794BAA-40A0-43FF-901B-011021094BBA/ASSETS/GRAPHIC/345_1247125_F4. JPEG.
[11] A. Marsee, F.J.M. Roos, M.M.A. Verstegen, F. Roos, M. Verstegen, H. Clevers, L. Vallier, T. Takebe, M. Huch, W.C. Peng, S. Forbes, F. Lemaigre, E. de Koning, H. Gehart, L. van der Laan, B. Spee, S. Boj, P. Baptista, K. Schneeberger, C. Soroka, M. Heim, S. Nuciforo, K. Zaret, Y. Saito, M. Lutolf, V. Cardinale, B. Simons, S. van Ijzendoorn, A. Kamiya, H. Chikada, S. Wang, S.J. Mun, M.J. Son, T.T. Onder, J. Boyer, T. Sato, N. Georgakopoulos, A. Meneses, L. Broutier, L. Boulter, D. Grün, J. Ijzermans, B. Artegiani, R. van Boxtel, E. Kuijk, G. Carpino, G. Peltz, J. Banales, N. Man, L. Aloia, N. LaRusso, G. George, C. Rimland, G. Yeoh, A. Grappin-Botton, D. Stange, N. Prior, J.E.E. Tirnitz-Parker, E. Andersson, C. Braconi, N. Hannan, W. Y. Lu, S. Strom, P. Sancho-Bru, S. Ogawa, V. Corbo, M. Lancaster, H. Hu, S. Fuchs, D. Hendriks, S.J. Forbes, L.J.W. van der Laan, Building consensus on definition and nomenclature of hepatic, pancreatic, and biliary organoids, Cell Stem Cell 28 (2021) 816–832, https://doi.org/10.1016/J.STEM.2021.04.005.
[12] M. Huch, C. Dorrell, S.F. Boj, J.H. Van Es, V.S.W. Li, M. Van De Wetering, T. Sato, K. Hamer, N. Sasaki, M.J. Finegold, A. Haft, R.G. Vries, M. Grompe, H. Clevers, In vitro expansion of single Lgr5+ liver stem cells induced by Wnt-driven regeneration, Nat 494 (2013) 247–250, https://doi.org/10.1038/nature11826, 2013 4947436.
[13] M. Huch, H. Gehart, R. Van Boxtel, K. Hamer, F. Blokzijl, M.M.A. Verstegen, E. Ellis, M. Van Wenum, S.A. Fuchs, J. De Ligt, M. Van De Wetering, N. Sasaki, S. J. Boers, H. Kemperman, J. De Jonge, J.N.M. Ijzermans, E.E.S. Nieuwenhuis, R. Hoekstra, S. Strom, R.R.G. Vries, L.J.W. Van Der Laan, E. Cuppen, H. Clevers, Long-term culture of genome-stable bipotent stem cells from adult human liver, Cell 160 (2015) 299–312, https://doi.org/10.1016/j.cell.2014.11.050.
[14] H. Hu, H. Gehart, B. Artegiani, C. Löpez-Iglesias, F. Dekkers, O. Basak, J. van Es, S. M. Chuva de Sousa Lopes, H. Begthel, J. Korving, M. van den Born, C. Zou, C. Quirk, L. Chiriboga, C.M. Rice, S. Ma, A. Rios, P.J. Peters, Y.P. de Jong,

H. Clevers, Long-Term expansion of functional mouse and human hepatocytes as 3D organoids, Cell 175 (2018) 1591–1606.e19, https://doi.org/10.1016/j.cell.2018.11.013.
[15] L. Broutier, A. Andersson-Rolf, C.J. Hindley, S.F. Boj, H. Clevers, B.K. Koo, M. Huch, Culture and establishment of self-renewing human and mouse adult liver and pancreas 3D organoids and their genetic manipulation, Nat. Protoc. 11 (2016) 1724–1743, https://doi.org/10.1038/nprot.2016.097.
[16] Y. Nishikawa, Transdifferentiation of mature hepatocytes into bile duct/ductule cells within a collagen gel matrix, Methods Mol. Biol. 826 (2012) 153–160, https://doi.org/10.1007/978-1-61779-468-1_13.
[17] W. Hashimoto, R. Sudo, K. Fukasawa, M. Ikeda, T. Mitaka, K. Tanishita, Ductular network formation by rat biliary epithelial cells in the dynamical culture with collagen gel and dimethylsulfoxide stimulation, Am. J. Pathol. 173 (2008) 494–506, https://doi.org/10.2353/AJPATH.2008.071024.
[18] Z. Wang, J. Faria, L.J.W. van der Laan, L.C. Penning, R. Masereeuw, B. Spee, Human cholangiocytes form a polarized and functional bile duct on hollow fiber membranes, Front. Bioeng. Biotechnol. 10 (2022) 922, https://doi.org/10.3389/FBIOE.2022.868857/BIBTEX.
[19] O.C. Tysoe, A.W. Justin, T. Brevini, S.E. Chen, K.T. Mahbubani, A.K. Frank, H. Zedira, E. Melum, K. Saeb-Parsy, A.E. Markaki, L. Vallier, F. Sampaziotis, Isolation and propagation of primary human cholangiocyte organoids for the generation of bioengineered biliary tissue, Nat. Protoc. 146 (14) (2019) 1884–1925, https://doi.org/10.1038/s41596-019-0168-0, 2019.
[20] N. Tanimizu, Y. Nishikawa, N. Ichinohe, H. Akiyama, T. Mitaka, Sry HMG box protein 9-positive (Sox9+) epithelial cell adhesion molecule-negative (EpCAM-) biphenotypic cells derived from hepatocytes are involved in mouse liver regeneration, J. Biol. Chem. 289 (2014) 7589–7598, https://doi.org/10.1074/JBC.M113.517243.
[21] S. Sekiya, A. Suzuki, Direct conversion of mouse fibroblasts to hepatocyte-like cells by defined factors, Nat 475 (2011) 390–393, https://doi.org/10.1038/nature10263, 2011 4757356.
[22] S. Sekiya, A. Suzuki, Hepatocytes, rather than cholangiocytes, can Be the major source of primitive ductules in the chronically injured mouse liver, Am. J. Pathol. 184 (2014) 1468–1478, https://doi.org/10.1016/J.AJPATH.2014.01.005.
[23] S. Nuciforo, M.H. Heim, Organoids to model liver disease, JHEP Reports 3 (2021), https://doi.org/10.1016/j.jhepr.2020.100198.
[24] L. Yang, R.A. Faris, D.C. Hixson, Long-term culture and characteristics of normal rat liver bile duct epithelial cells, Gastroenterology 104 (1993) 840–852, https://doi.org/10.1016/0016-5085(93)91021-9.
[25] M.K.H. Auth, M. Okamoto, Y. Ishida, A. Keogh, S.H.G. Auth, J. Gerlach, A. Encke, P. McMaster, A.J. Strain, Maintained function of primary human hepatocytes by cellular interactions in coculture: implications for liver support systems, Transpl. Int. 11 (Suppl 1) (1998) S439–S443, https://doi.org/10.1007/S001470050516.
[26] M.K.H. Auth, R.E. Joplin, M. Okamoto, Y. Ishida, P. McMaster, J.M. Neuberger, R. A. Blaheta, T. Voit, A.J. Strain, Morphogenesis of primary human biliary epithelial cells: induction in high-density culture or by coculture with autologous human hepatocytes, Hepatology 33 (2001) 519–529, https://doi.org/10.1053/JHEP.2001.22703.
[27] M.K.H. Auth, D. Woitaschek, M. Beste, T. Schreiter, H.S. Kim, E. Oppermann, R. E. Joplin, U. Baumann, P. Hilgard, S. Nadalin, B.H. Markus, R.A. Blaheta, Preservation of the synthetic and metabolic capacity of isolated human hepatocytes by coculture with human biliary epithelial cells, Liver Transplant. 11 (2005) 410–419, https://doi.org/10.1002/LT.20367.