Generation of human hepatobiliary organoids with a functional bile duct from chemically induced liver progenitor cells

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Abstract

Background Liver disease imposes a significant medical burden that persists due to a shortage of liver donors and an incomplete understanding of liver disease progression. Hepatobiliary organoids (HBOs) could provide an in vitro mini-organ model to increase the understanding of the liver and may benefit the development of regenerative medicine.

Methods In this study, we aimed to establish HBOs with bile duct (BD) structures and mature hepatocytes (MHs) using human chemically induced liver progenitor cells (hCLiPs). hCLiPs were induced in mature cryo-hepatocytes using a small-molecule cocktail of TGF-β inhibitor (A-83-01, A), GSK3 inhibitor (CHIR99021, C), and 10% FBS (FAC). HBOs were then formed by seeding hCLiPs into ultralow attachment plates and culturing them with a combination of small molecules of Rock-inhibitor (Y-27632) and AC (YAC).

Results These HBOs exhibited bile canaliculi of MHs connected to BD structures, mimicking bile secretion and transportation functions of the liver. The organoids showed gene expression patterns consistent with both MHs and BD structures, and functional assays confirmed their ability to transport the bile analogs of rhodamine-123 and CLF. Functional patient-specific HBOs were also successfully created from hCLiPs sourced from cirrhotic liver tissues.

Conclusions This study demonstrated the potential of human HBOs as an efficient model for studying hepatobiliary diseases, drug discovery, and personalized medicine.

Keywords Chemically induced progenitor cells, Biliary function, Hepatobiliary organoid, Bile canaliculi

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Introduction

Liver diseases impose significant healthcare burdens that persist due to a shortage of liver donors and an incomplete understanding of the progression of liver diseases [1]. The liver is a remarkably intricate multicellular organ that houses complex metabolic functions executed by both parenchymal and nonparenchymal cell populations [2]. Hepatocytes, which comprise 78% of the liver volume, are the predominant parenchymal cell type responsible for the primary functions of the liver. However, hepatocytes should work collaboratively with other cell types, including cholangiocytes (biliary epithelial cells), endothelial cells, sinusoidal endothelial cells, Kupffer cells, pit cells, and hepatic stellate cells, in a coordinated effort to ensure overall well-being and optimal functioning of the liver [3]. Hepatocytes and cholangiocytes are important contributors to liver metabolism, with the integrity of their cellular structures forming the foundation of the liver function [4, 5]. This important secretion originates from the bile canaliculus of hepatocytes and enters the extrahepatic biliary tract through a complex network of intrahepatic ducts derived from cholangiocytes, encompassing the hepatic duct, cystic duct, gallbladder, and common bile duct (BD) [4, 6]. The BDs contribute to approximately 70% of pediatric liver transplantations and 30% of adult liver transplantations [7, 8]. To maintain the viability and optimal functionality of hepatocytes both in vitro and in vivo, it is imperative that the liver tissue possesses an intact and efficient bile excretory system [4, 5, 9]. Hepatobiliary diseases, such as progressive familial intrahepatic cholestasis, are caused by bile canaliculi disruption, which requires in vitro pathophysiological research, drug development, and screening [10–12]. Establishing in vitro disease models that faithfully replicate the intricacies of such conditions requires a holistic approach, with a particular focus on the overall functionality of the liver, notably the integrity of bile transport. Multilineage hepatobiliary organoids (HBOs), also called multi-tissue organoids, are excellent in vitro models for exploring hepatobiliary diseases and provide in vitro research models for the establishment of hepatobiliary diseases and drug screening, which remains a challenge for the generation of HBOs in vitro [13].

Multiple HBO-containing cell types have been reported in the relevant literature [14, 15]. Hepatic organoids can be derived from induced pluripotent stem cells (iPSCs) or adult organ-specific stem/progenitor cells [9, 15, 16]. HBOs generated from iPSCs go through a long period of time and complex combinations and steps of growth factors, inhibitors, and small-molecule compounds. Although iPSCs can expand indefinitely and differentiate into multiple cell lineage, their omnipotence leads to more complex differentiation processes and a higher likelihood of heterogeneity when generating HBOs in vitro [17]. Recently, chemically induced liver progenitor cells (CLiPs) from liver tissue have the potential for bidirectional differentiation of cholangiocytes and hepatocytes and the capacity of expansion indefinitely, which raise a satisfy cell source for liver organoid generation and liver regenerative medicine [18–20]. Liver progenitor cells (LPCs) can self-assemble into HBOs [21]. As for the functional integrity and structure of hepatic organoids generated from LPC or CLiPs (hCdHOs), Salas-Silva, S., et al. generated hepatic organoids from human iPSCs (hiPSOs) and compared the differences between the two [22]. They found that hCdHOs and hiPSOs displayed comparable generation efficiency (95%) and biochemical characteristics, but hiPSOs took doubling time and expressed higher LPC markers (AFP, EpCAM) and comparable levels of KRT-19, CFTR but lower cholangiocytic markers [22]. As for isolated LPCs from tissue, the low isolation efficiency and limited cell expansion capacity have limited their use in generating HBOs or regenerative medicine compared to CLiPs [23]. Wu et al. generated HBOs from human iPSCs with the functions closed to the fetal liver but not adult liver [16]. Also, CLiPs can be induced from mature hepatocytes (MHs) isolated from healthy or diseased liver tissue and has the characteristics of LPCs [24, 25]. In addition, human CLiPs (hCLiPs) can assemble functional three-dimensional BD structures and MHs in vitro [26]. These CLiPs retain unique signatures associated with specific diseases, making them invaluable for personalized medical approaches and facilitating precise drug screening procedures.

In this study, we developed a novel method to create HBOs using hCLiPs. These organoids feature BD structures and MHs derived from cryo-hepatocytes and patient-isolated hepatocytes, respectively. By adding specific small molecules directly to a matrix-free culture medium in an ultralow attachment dish, we established HBOs with functional bile canaliculi and BD connections that mimicked hepatobiliary physiology. This is a promising model for the study of hepatobiliary diseases.

Methods

Preparation of human CLiPs

Human CLiPs were converted by small molecules, as previously reported [26]. Human Cryo-Hepatocytes (CHHs) (Lot.416, Corning) were seeded into collagen type I-coated dishes (Asahi Techno Glass, Tokyo, Japan) at a density of 2×10^4 cells/cm^2 in STIM medium to promote attachment to the plate surface [26]. The STIM medium used was a hepatocyte culture media kit with 10 ng/μL epidermal growth factor (EGF) containing 1x penicillin–streptomycin-glutamine (100X) (Gibco™, Tokyo, Japan) and 10% fetal bovine serum (FBS, Gibco™, Tokyo, Japan). Four hours later, the culture medium was changed to small chemically reprogrammed culture medium, which

was described as the reported that small hepatocyte medium (SHM) was DMEM/F12 containing 2.4 g/L NaHCO$_3$ and L-glutamine (Life Technologies, Tokyo, Japan) and supplemented with 5 mM HEPES, 30 mg/L L-proline, 0.05% BSA, 10 ng/mL EGF (all from Sigma−Aldrich Japan, Tokyo, Japan), insulin-transferrin-serine (ITS)-X (Life Technologies, Tokyo, Japan), 10−7 M dexamethasone (Dex) (Fuji Pharma Co. Ltd., Tokyo, Japan), 10 mM nicotinamide (Sigma−Aldrich, Tokyo, Japan), 1 mM ascorbic acid-2 phosphate (Wako Pure Chemical, Osaka, Japan), 100 U/mL penicillin, and 100 mg/mL streptomycin (Life Technologies, Tokyo, Japan) supplied with two small chemical molecules of 0.5 µM A-83-01 (TGF-b type I receptor inhibitor, Wako Pure Chemical, Tokyo, Japan), 3 µM CHIR99021 (GSK-3 inhibitor, AdooQ Bio-Science, CA, USA) and 10% FBS, which would be called FAC medium [26]. The culture medium was changed one day after seeding and every two or three days thereafter. It takes 14–16 days to generate up to 90% hCLiPs from CHHs.

Hepatobiliary organoid formation from hCLiPs

hCLiPs at 90–100% confluence were treated with TrypLE Express (Life Technologies) for 15–20 min. hCLiPs were then seeded into ultralow attachment plates (Lot. 3471, Life Science, Corning, NY, USA) at 0.5-1×10^5 cells/cm^2 and incubated in small molecule induction medium. For comparison, three types of small molecule induction media were prepared: FAC medium was the hCLiP induction medium mentioned above; biliary epithelial cell induction medium (BIM) was the mTeSR™1 Complete Kit (Catalog #85850, STEMCELL Technologies, Tokyo, Japan), including mTeSR™1 basal medium supplemented with the addition of three small chemical molecules of 10 µM Y-27,632, 0.5 µM A-83-01, and 3 µM CHIR99021; YAC medium was similar to the FAC medium except that FBS was replaced by 10 µM Y-27,632. On days 1–3 after seeding, the cells were gently mixed with 5 mL pipettes without changing the culture medium. From day 4, HBOs were collected into 15 mL tubes and centrifuged at 7×*g* for 2 min. The HBO pellets were then resuspended in fresh culture medium and gently transferred to the original culture dishes. The culture medium was changed every 3–5 days.

Gene expression analysis by real-time quantitative reverse transcription polymerase chain reaction (qRT–PCR)

Samples were cultured in dishes under various conditions and mRNA was extracted using a spin column (NucleoSpin RNA II; Macherey-Nagel, Düren, Germany). cDNA was synthesized using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Tokyo, Japan). Samples were then stored at −20 °C until their analysis by polymerase chain reaction (PCR), which was performed using an Applied Biosystems StepOne Plus Real-time PCR System with TaqMan Gene Expression Assay Kits (Applied Biosystems, Tokyo, Japan) according to the manufacturer's instructions. Briefly, the PCR mixtures contained 1 µL of cDNA, 1 µL of TaqMan Gene Expression Assay probe, 5 µL of TaqMan Fast Advanced Master Mix (both from Applied Biosystems), and 13 µL of nuclease-free water. All the TaqMan gene primers used are listed in Supplementary Table 1. The thermocycling conditions were 95 °C for 20 s, followed by 40 cycles of 95 °C for 1 s and 60 °C for 20 s. Expression levels were quantified using the comparative cycle time method. Cycle threshold (Ct) values were automatically determined using the Applied Biosystems StepOne Plus Real-Time PCR System, and fold changes in gene expression were calculated using the 2^(−ΔΔCT) method. The expression levels were normalized to those of the housekeeping gene and the internal control glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Immunofluorescence staining

The cells and HBOs were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) (Wako Pure Chemical, Osaka, Japan) for 10 min. The fixed samples were then incubated in 0.1% Triton X-100 (Sigma−Aldrich, Tokyo, Japan) in PBS for 10 min and blocked in PBS containing 1% BSA for 1 h at room temperature. The cells were then incubated with primary antibodies diluted in PBS+1% BSA at 4 °C overnight. After washing with PBS three times, they were incubated with the appropriate secondary antibodies diluted in PBS+1% BSA for 2 h. All primary and secondary antibodies used are listed in Supplementary Table 2. Nuclei were stained with 4′,6-diamidiono-2-phenylindole (DAPI) (DOJINDO, Kumamoto, Japan) for 30 min. The samples were washed three times with PBS for 30-−60 min. Fluorescence and bright-field images were captured using a microscope (Ti-U and C-HGFI; Nikon, Tokyo, Japan).

Rhodamine 123 assay

A rhodamine 123 assay was performed. We incubated the HBOs with Hanks' balanced salt solution (HBSS) containing 100 µM rhodamine 123 (both from Sigma-Aldrich, Tokyo, Japan) for 30 min at 37 °C and washed twice with HBSS. To inhibit the transporter activity of multidrug-resistant protein 1 (Mdr1), we incubated the samples with 20 µM verapamil (Tokyo Chemical Industry Co., Ltd. Tokyo, Japan) at 37 °C for 2 h before adding rhodamine 123. Fluorescence and bright-field images were captured using a microscope (Ti-U and C-HGFI; Nikon, Tokyo, Japan). Fluorescence intensity along the line was evaluated by imageJ (imageJ 1.53 K, National Institutes of Health, USA).

Cholyl-lysyl-fluorescein (CLF) dye assay

We loaded the HBOs with 1 µM CLF (Corning Life Sciences, Bedford, USA) for 30 min at 37 °C and washed them twice with HBSS. The cells were observed, and images were captured using a confocal microscope. Fluorescence and bright-field images were captured using a microscope (Ti-U and C-HGFI; Nikon, Tokyo, Japan). The organoids were cultured in the original medium for another two days to observe CLF metabolism.

Formation of hepatobiliary organoids from patient derived hCLiPs

This study was approved by the local ethics committee of Nagasaki University Hospital (ID 09022449-6) and informed consent was obtained from all patients. The clinical characteristics of the liver donors with liver cirrhosis are described in Supplementary Table 3. The specimens and samples were obtained after obtaining written informed consent from each individual. Liver tissues were procured from surgically resected specimens and promptly cleaned after extraction. After the cleansing process, the liver specimen was immersed in cold UW solution and expeditiously transported to the research laboratory. Hepatocytes (MHs) were isolated from acquired tissues using a modified two-step collagenase perfusion technique [24]. The isolated cells were filtered through a cotton mesh membrane and a 63-µm stainless mesh. This was followed by triple purification by centrifugation at 50 ×*g* for 2 min each at 4℃. The resulting cell suspension was blended with a 25% Percoll Plus solution (GE Healthcare, Tokyo, Japan) and then subjected to centrifugation at 70 ×*g* for 7 min at 4℃. The isolated MHs were cultured in FAC medium for several weeks to induce CLiP conversion. The induced human CLiPs from patients were detached with Tryple solution, conditioned in YAC medium, and seeded onto ultralow-adhesion plates (Lot. 3471, Life Science, Corning, NY, USA).

Statistical analysis

Data are provided as the mean±standard deviation (SD) unless otherwise stated. Statistical analyses were performed, and graphs were generated using GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA). Data were analyzed using a one-way analysis of variance, Student's t test, or an analysis of variance with repeated measures when appropriate. A probability (p) value<0.05 was considered statistically significant. The details of the statistical analyses and associated values are described in the respective figure legends.

Results

The induction of HBO formation in ultralow attachment dishes by different induction media

hCLiPs were induced by a small chemical molecule culture medium and exhibited distinct characteristics of liver progenitor cells, such as expressing the LPC markers of SOX-9, EpCAM, KRT-19, and CD133 and having the morphology of small cell size, large nucleus and proliferation capacity, which were endowed with the capability for biodifferentiation (Fig. S1A, C, D) [19, 26]. The hepatocytes culturing without the FAC macules kept the characteristic of mature hepatocytes including the multi-faceted shapes, single or two round nuclei and lipid droplets, and the cell density on day14 was lower, which indicated that cell proliferation was slower than the hCLiPs (Fig. S1B). HBOs were generated from human CLiPs in an ultralow attachment dish prompted by a specific combination of small molecules, as described previously in Methods (Fig. 1A). After approximately 20 days, hCLiPs exhibited the ability to develop various types of organoids in ultralow attachment plates induced by different small-molecule media. These organoids, influenced by different induction media, displayed diverse shapes and sizes, and their growth continued to expand with prolonged culture times (Fig. 1B, Fig. S2A). On the 20th day, the average diameter of the organoids was approximately 200 µm. (Fig. 1C). On the 34th day, YAC-HBOs were subjected to immunofluorescence staining, and the results showed that YAC-HBOs displayed positive staining for Ki-67 in some cells inside HBOs, indicating ongoing cell proliferation within YAC-HBOs (Fig. S2B). Cross-sections of these organoids revealed their internal architecture, characterized by the presence of multiple orifices and luminal structures (Fig. 1D). In summary, our findings demonstrate that varying the induction medium could efficiently generate hepatobiliary-like organoids from hCLiPs on ultralow attachment culture dishes in a single-step process.

HBOs contain BD structures integrated with MHs

HBOs derived from different conditioned media exhibit diverse morphologies. In FAC-HBOs and YAC-HBOs, CK-7-positive ducts were identified both on the surface and within the organoids, along with the observation of sac-like bulges on the organoid surface, which serves as a key indicator of the existence of BD structures (Fig. 2A). Additionally, the positive expression of ALB further confirmed the presence of MHs within the organoids (Fig. 2A). Moreover, the qRT-PCR results revealed a notable upregulation of biliary markers, including MRP-2, BSEP, and KRT-19, along with mature hepatocyte markers, such as CYP1A2, HNF4A, and ALB, in the organoids induced by YAC medium on day 20. Conversely, the organoids exhibited the upregulation
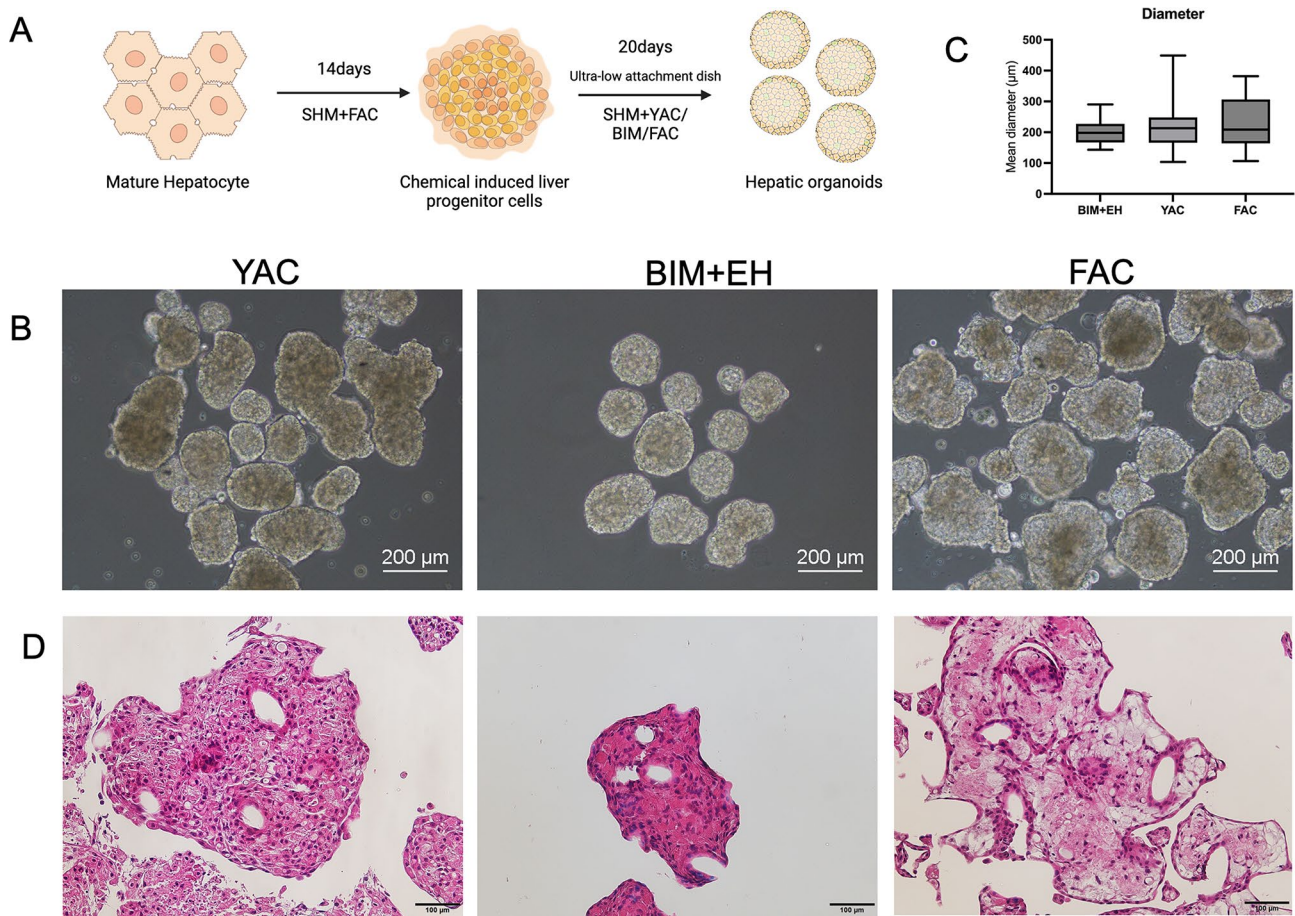
Fig. 1 The induction of HBO formation in an ultralow attachment dish by different induction media. **A**. Schematic representation of HBO induction. SHM?small hepatocyte medium, was described in the Methods section; FAC, SHM supplied with 10% FBS with A-83-01 and CHIR99021; BIM, BD induction medium; YAC, SHM medium with 10μM Y-27632, 0.5μM A-83-01, 3μM CHIR99021. **B**. The morphology of HBOs in YAC, BIM and FAC media. Scale bar = 200 μm. **C**. The diameters of the HBOs in different induction media were heterogeneous. The diameters of YAC-HBOs showed a high degree of variation. **D**. H&E staining of a cross-slide of an HBO, the HBOs showed a biliary lumen. Scale bar = 100 μm

of selected biliary and mature hepatocyte markers when cultured in FAC and BIM media (Fig. 2B). The data revealed that the internal architecture of the organoids encompasses three-dimensional BD structures and MHs, forming a fundamental structure for bile transportation and hepatic metabolism in YAC-HBOs. Furthermore, CK-19 and MRP-2 served as shared markers for BD and bile canaliculi. The immunofluorescence results underscored the distinctive characteristics of bile canaliculi and BD structures within YAC-HBOs (Fig. 2C). YAC-HBOs were subjected to paraffin fixation and subsequent sectioning for immunofluorescence and immunohistochemistry staining. The results indicated that cells surrounding the lumen were positive for CK-7, CK-19 and MRP2, indicative of biliary epithelial cells, whereas the adjacent stromal cells displayed ALB positivity, signifying the presence of MHs (Fig. 2D, and Fig. S3 A-C). Furthermore, immunofluorescence staining results showed that the lumen structure inside YAC-HBOs was CK-7 positive, indicating that it was a bile duct structure (Fig. S3D).

As for the function and structure of YAC-HBOs after prolonged the culture time, immunofluorescence staining of YAC-HBOs was performed on the 34th day, and the results showed that YAC-HBOs still showed CK-7 and ALB positivity (Fig. S2C). These findings strongly indicated that YAC-HBOs represent multicellular, multitissue hepatobiliary organoids that include both BD structures and MHs. Notably, the application of YAC medium demonstrated its capability to efficiently transform hCLiPs into HBOs on ultralow attachment plates.

HBOs have bile metabolism and transport functions
To examine the bile transport functionality of YAC-HBOs, rhodamine-123, which serves as a bile acid analog capable of being transported via bile acid transporters MDR-1/2 and MRP-2, was utilized to assess their bile transport capacity. Rhodamine-123 was found to accumulate within the lumen (Fig. 3A). This transportation process was impeded by verapamil, a known inhibitor of MDR-1 (Fig. 3A). Fluorescence intensity along
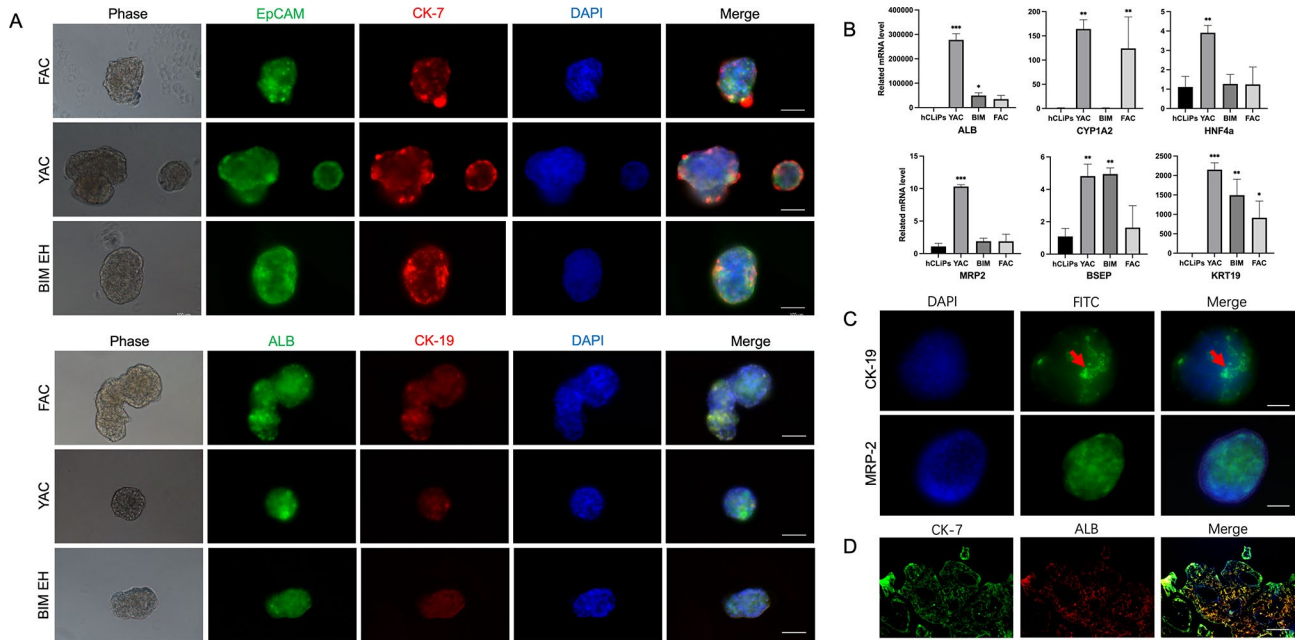
Fig. 2 HBOs contain BD structures integrated with MHs. **A**. Immunofluorescent staining of HBOs showed that HBOs were positive for CK-7 and CK-19 (biliary markers), ALB (a mature hepatocyte marker) and EpCAM (progenitor cell marker). Scale bar = 50 µm. **B**. RT-qPCR of the HBOs showed that the expression of MRP-2, BSEP and KRT19 (biliary markers), and ALB, CYP1A2 and HNF4a (mature hepatocyte markers) was significantly upregulated in YAC-HBOs in comparison to hCLiPs, while only some of these factors were upregulated in FAC-HBOs and BIM-HBOs (n=3-6). GAPDH was used as internal control. Data were mean ± SD. At least 3 independent experiments with triplicate measurements were performed and analyzed by Student's t test, *p<0.05, **p<0.01 ***p<0.001. **C**. The results of immunofluorescent staining of YAC-HBOs showed that the CK-19-positive line structure showed the structure of bile canaliculi. Immunofluorescent staining of MRP-2 showed that YAC-HBOs expressed the capillary BD structure. Scale bar = 50 µm. **D**. The immunofluorescence results of sections showed that the cavities within YAC-HBOs were composed of CK-7-positive epithelial cells, while the surrounding cells were ALB-positive. Scale bar = 50 µm
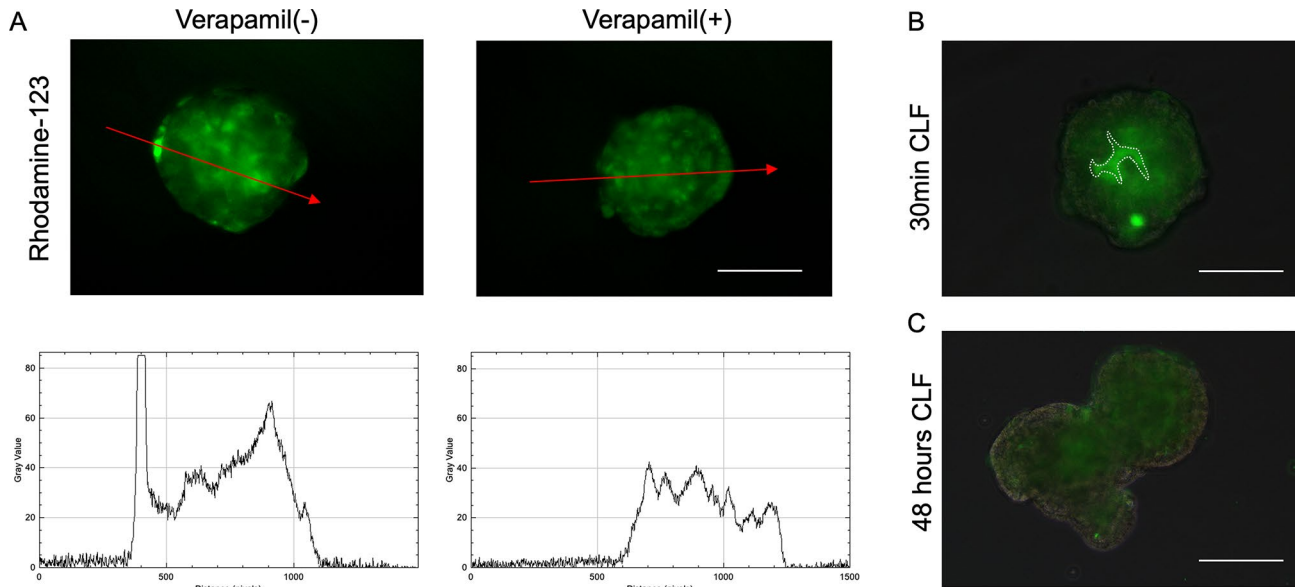

Fig. 3 HBOs have bile metabolism and transport functions. **A**. YAC-HBOs could accumulate Rhodamine-123 in their BDs, which could be inhibited by the verapamil. Fluorescence intensity along the line was evaluated by image J. Scale bar = 100 µm. **B**. YAC-HBOs could accumulate CLF in their BDs through MHs, with a high concentration observed in the BDs at 30 mins, which showed the tubular shape. Scale bar = 100 µm. **C**. After 48 h, the CLF fluorescence intensity observed in YAC-HBOs was weaker than that at 30 min. Scale bar = 100 µm. At least three independent experiments were conducted, and the representative images were shown in this figure

the line evaluated by image J showed that the verapamil could significantly reduce the accumulation of rhodamine-123 (Fig. 3A). CLF is an analog of bile and can be directly absorbed by hepatocytes, but not bile epithelial cells, which could be transported into the into the biliary network and lumen. The CLF dye assay shows that YAC-HBOs accumulated CLF into the the bile duct lumen from the culture medium, which showed the tubular shape (Fig. 3B). After 48 h, the CLF fluorescence intensity observed in YAC-HBOs was weaker than that at 30 min (Fig. 3C). These data combinate with the date of the structures inside the YAC-HBOs indicated that YAC-HBOs induced from hCLiPs exhibits both structural and functional attributes associated with bile transport, which there was a functional connection between the bile duct and mature hepatocytes.

The formation of HBOs from human CLiPs induced from cirrhosis patients

Following the successful formation of HBOs from hCLiPs induced from mature cryo-hepatocytes, we explored the potential of utilizing hCLiPs derived from MHs sourced from diseased livers to establish HBOs. The cell morphology, RT-qPCR, and immunofluorescence staining results demonstrated that hCLiPs showed the characteristics of liver progenitor cells (Fig. S4). Cells and structures positive for BD-specific markers CK-7, MRP-2, and CK-19 were observed in both the interior and surface cystic structures of HBOs, while ALB (a mature hepatocyte marker) was predominantly distributed within the interior of the organoids (Fig. 4A). These HBOs demonstrated the ability to take up CLF, a bile analog of hepatic bile metabolism (Fig. 4B). In addition, rhodamine 123, which represents the bile transport function of the BD,

can also be transported to specific regions of D-HBOs, and these transporters could be inhibited by verapamil (Fig. 4C). These data suggest that human CLiPs derived from diseased livers can form HBOs, demonstrating hepatobiliary organoid functions similar to those derived from healthy sources.

Discussion

To preserve the functionality of hepatocytes and the liver, whether in vitro or in vivo, liver tissue requires the presence of an effective bile excretion system [27]. In this study, we constructed a hepatobiliary organoid in vitro from hCLiPs with bile canaliculi of MH-connected BDs, which exhibited complete mature liver and bile excretion functions.

Cholestasis due to BD injury and biliary dysfunction related to bile transport ultimately lead to liver damage [11]. A requirement arises for an in vitro HBO that truly reflects the complex reactions within BD structures, including hepatocytes and interconnected bile canaliculi that connect the various components of BD structures [5, 9, 28]. In this study, we used human CLiPs to generate hepatobiliary organoids. These hCLiPs with bidirectional differentiation potential spontaneously generated HBOs in matrix-free culture medium supplied with small molecule compounds on 3D culture in an ultra-low adhesion dish. Although the principle of their generation and the process of cell connection are not clear, HBOs generated after approximately two weeks have the ability to form BD structures and hepatocyte-connected bile canaliculi structures. We previously reported the use of rat CLiPs to establish cystic organoids containing MHs and cholangiocytes in gelatin-coated culture dishes using YAC medium with the ability to transport rhodamine 123 from
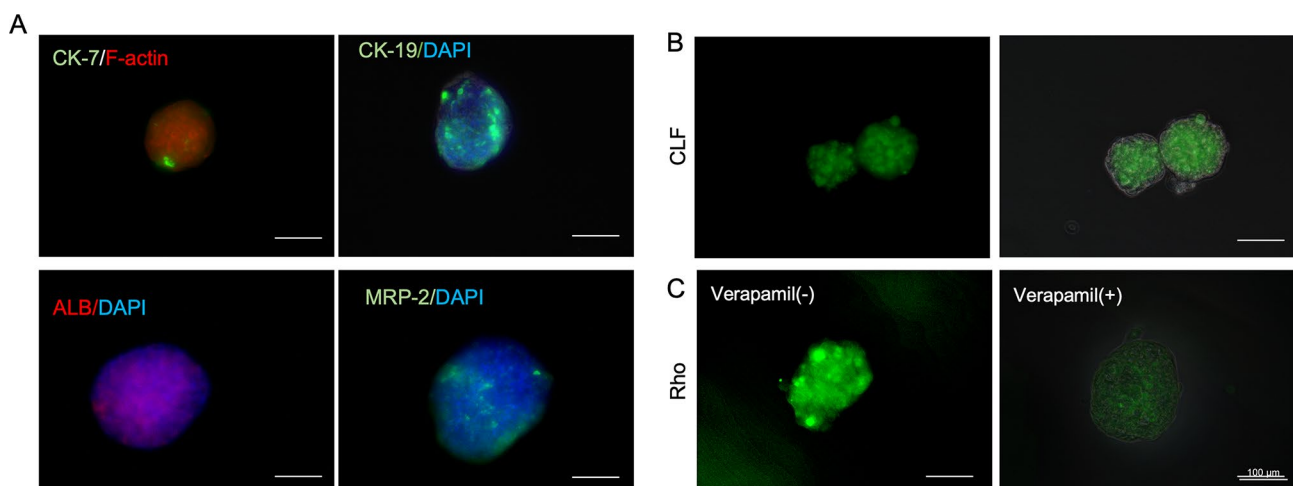

Fig. 4 HBO formation from human CLiPs induced from cirrhosis patients. **A**. The immunofluorescence results showed that the HBOs expressed CK-7, MRP-2 and CK-19 (biliary markers), and ALB (a mature hepatocyte marker). Scale bar = 100 µm. **B**. The HBOs took up CLF from MHs in the culture medium and accumulated in the biliary lumen. Scale bar = 100 µm. **C**. The uptake of rhodamine 123 was observed in the biliary lumen of HBOs. This could be inhibited by verapamil. Scale bar = 100 µm

the culture medium into the cyst cavity [29]. One reason for this difference may be differences in the use of extracellular matrix (ECM) [30]. In research on BD organoids or liver organoids, gels are typically used as an extracellular matrix to support cell differentiation and maturation [31]. Laminin, collagen IV, collagen I, and fibronectin are related to the development and maturation of BDs and hepatocytes [21]. Also, our previous study, we first generated three-dimensional bile ducts from FAC-hCLiPs embedding in an ECM system with Matrigel, and then co-cultured isolated MHs with the previously induced three-dimensional bile ducts, thus constructing a hepatobiliary tissue with functional connection between MHs and BD [26, 32]. However, the encapsulation and non-homogeneity of gels also negatively impact the generation of organoids [33]. In addition, comparing with the previous step-by-step co-culture Matrigel system, the ECM-free culture method is cheap, rapid, sustainable and avoids the limitations of clinical applications caused by the introduction of xenogeneic Matrigel and the further analysis caused by adhesion and compositional complexity of Matrigel [34]. A previous study found that fetal liver progenitor cells may provide ECM during the self-assembly process [21]. FAC-induced hCLiPs are also prone to produce fibroblast cells during induction, and fibroblast cells with collagen production that cannot be completely removed may also provide support for the development and maturation of 3D HBOs [26]. Functionally and structures, in YAC-HBOs, CLF can be taken up by hepatocytes and accumulated in the BDs. The results also showed the structural details of the transporter-related genes BSEP and MRP-2 in YAC-HBOs, coupled with the marker CK-19, indicating bile canaliculi, collectively unveiling the presence of the bile canaliculi connecting to the BD in YAC-HBOs. Tanimizu et al. generated a functional liver organoid on combining hepatocytes and cholangiocytes with hepatobiliary connections ex vivo [9]. They demonstrated that hepatobiliary connection successfully created based on the functional assay of CLF transportation and the connection between the lumen consisting of CK19+cholangiocytes and HNF4α+hepatocytes [9]. In our study, the CK-19 positive duct and lumen were confirmed in Fig. 2C, D and figure S3. The presence of CK-19, MRP2, and CK-7 positive luminal structures in YAC-HBOs indicated that these lumen structures were composed of CK-7, CK19 and MRP2 positive cholangiocyes. The successful metabolism and accumulation of CLF in the bile duct lumen requires a structural basis for the functional connection between the bile duct and mature hepatocytes that can actively take up CLF. Our results also showed that YAC-HBOs have both internal tubular and cystic BD structures. The lecture reported that tubular BD structures also maintained better BD organization in comparison to cysts

[35]. These results shed light on the intricate interplay and functional dynamics between hepatocytes and cholangiocytes within this context. Additionally, the presence of ALB-positive MHs within YAC-HBOs confirmed their hepatobiliary organoid nature. Remarkably, the persistence of EpCAM-positive cells within the organoids indicated sustained growth potential over time. YAC-HBOs can survive and expressed Ki-67 when cultured from 20 days to 34 days, indicating that the volume can be stably increased by extending the culture time. At the same time, immunohistochemical staining was performed on day 34, and YAC-HBOs can still stably express CK-7 and ALB. In an ultra-low adhesion matrix-free culture medium, hCLiPs could self-assemble into approximately 200 µm-sized functional HBOs. In essence, the demonstrated ability to modulate the characteristics of YAC-HBOs through alterations in the culture medium composition highlights the versatility and capacity of the method for inducing specific organoids.

Recently, HBOs have been studied using iPSC-derived liver progenitor cells [9, 15, 16]. Tanimizu et al. created functional liver organoids with hepatobiliary connections by co-culturing small hepatocytes and cholangiocytes to enhance MHs ex vivo [9]. Ramli et al. developed iPSC-derived HBOs with a functional BD and bile canaliculi system, disrupted by cholestasis-inducing drugs, and exhibited gene expression signatures resembling NASH liver tissues upon incubation with free fatty acids [15]. The above studies describe the methods of multi-cell co-culture and stepwise induction from iPSCs to generate HBOs. iPSCs undergo stepwise transformation with complex medium adjustments into hepatocytes or cholangiocytes, mimicking embryonic liver development [36]. This involves committing cells to the endodermal lineage, followed by specialization into hepatocytes or cholangiocytes, resulting in structures resembling liver tissue and facilitating the creation of diverse multilineage organoid models [37]. Although the induction of iPSCs into HBOs has many advantages, induced organoids are often immature and difficult to culture for long periods of time, which requires further research [38]. The iPSCs were differentiated into HBOs using a multi-step approach in 3D suspension culture with Matrigel and the pathways targeted by growth factors, cytokines, and small molecules in each step of differentiation. The combination of BMP4, BMP7, and FGF7 signaling factors, HGF, EGF, FGF, R-spondin-1 (Rspo1), TGFβ inhibitor A8301, and cAMP inducer FSK [15, 39, 40] were included in the differentiation process. In comparison to HBOs established from iPSCs or multi-cell co-culture [9, 15, 16, 41], the generation of HBOs from hCLiPs induced from isolated MHs through a small-molecule cocktail has the advantages of wide sources, simple induction steps, and special sources of patient disease backgrounds. hCLiPs solve the

problem of insufficient somatic cell sources or genetic changes in iPSCs. hCLiPs closely resemble LPCs, demonstrating sustained culture, proliferation, and differentiation akin to the intrahepatic BDs and MHs development processes [19]. Although the precise organizational process underlying the formation YAC-HBOs from hCLiPs remains unclear, CHIR-99,021 as an agonist of the WNT pathway may maintain the ability of LPCs and MHs to maintain the proliferation capacity, while A83-01, as an inhibitor of TGF-β, is beneficial for the development of hCLiPs into cholangiocytes [42, 43]. BIM+EH has been proven to be more beneficial for BD development [26, 44]. Further investigations are required to understand the complex organization processes of HBOs with hepatocytes and functional BDs.

In addition, HBOs generated from hCLiPs induced from patient-specific isolated hepatocytes have the potential for application in personalized medicine in specific diseases, such as those caused by gene defects. To ensure a standardized procedure, this study first employed stable cryopreserved MHs, from which non-parenchymal cells would be removed. We then established HBOs sourced from patients with cirrhosis. These HBOs have internal structures and functions similar to those of normal hCLiPs. This provides a methodology that can establish individual patient-specific as well as disease-specific HBOs, which may also provide the possibility for personalized medicine. Verstegen et al. reported the use of patient-derived extrahepatic cholangiocyte organoids in a model cystic fibrosis biliary disease [45]. It is feasible for HBOs derived from similar disease backgrounds to be used for disease model research and personalized treatment of specific disease backgrounds. In future investigations, efforts will be directed towards the establishment of HBOs derived from specific diseases, including primary biliary sclerosis and biliary cystic fibrosis. This will further expand our understanding of disease-related variations in HBOs and be useful in exploring effective drugs or treatments.

However, regarding to the limitations in this study, due to the lack of patients with bile metabolism-related diseases, such as primary cholangitis, primary sclerosing cholangitis, or cystic fibrosis, there are deficiencies but potentials in the establishment of disease models and the analysis of related disease mechanisms based on this YAC-HBOs. The further practical application research of this YAC-HBOs would be carried out, such as creating the hepatobiliary disease model based on the diseased tissue and use this model to search the bile, drug and other toxic substances metabolism in the liver, such as hepatic steatosis, cholangitis induced by troglitazone. Additionally, there is a lack of research on the in vivo application of YAC-HBOs. In terms of methodology, the potential and specific cell types and developmental stages within YAC-HBOs have not been explored at the single-cell level. However, since no new genes are introduced and the stemness of mature cells is closer, the potential for carcinogenesis in future in vivo studies would be lower than with iPSCs, as verified by in vivo experiments on CLiPs [19, 20].

Conclusions

Our study presents a novel and expedited approach to establish HBOs from hCLiPs derived from cryo-hepatocytes or patient-isolated hepatocytes in ultralow attachment plate culture dishes utilizing a specialized small-molecule culture medium. Furthermore, if we can combine co-culture based on YAC-HBOs in the future to generate mini-liver organs containing non-parenchymal cells and vascular structures, it will be beneficial to in vitro research on liver diseases and liver development.

Abbreviations

iPSCs	Induced Pluripotent Stem Cells
HBOs	Hepatobiliary Organoids
CLiPs	Chemically Induced Liver Progenitor Cells
LPCs	Liver Progenitor Cells
MHs	Mature Hepatocytes
BD	Bile Duct
SHM	Small Hepatocyte Medium
EGF	Epidermal Growth Factor
HGF	Hepatocyte Growth Factor
TGF-b	Transforming Growth Factor Beta
GSK-3	Glycogen Synthase Kinase-3
FBS	Fetal Bovine Serum
ALB	Albumin
MRP-2	Multidrug Resistance-Associated Protein 2
BSEP	Bile Salt Export Pump
KRT-19	Keratin 19
CYP1A2	Cytochrome P450 1A2
HNF4A	Hepatocyte Nuclear Factor 4 Alpha
CK-7	Keratin 7
CLF	Cholyl-L-Lysyl-Fluorescein
MDR-1	Multidrug Resistance Mutation-1
ECM	Extracellular Matrix
EpCAM	Epithelial Cell Adhesion Molecule

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13287-024-03877-z.

Supplementary Material 1

Supplementary Material 2

Data availability
All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate
 (1) This study was approved under the project of "Basic research related to isolation, culture, preservation and transplantation of surgically resected liver tissue" by the local ethics committee of Nagasaki University Hospital. (2) The approval date for these experiments is 2020-09-15. (3) And the Approval Number is 09022449-6. The informed consent was obtained from all patients and their guardians.

Consent for publication
Not applicable.

Conflict of interest
The authors declare no financial conflicts of interest in association with the present study.

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