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Review

Generation of *in vivo*-like multicellular liver organoids by mimicking developmental processes: A review

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ABSTRACT

Liver is involved in metabolic reactions, ammonia detoxification, and immunity. Multicellular liver tissue cultures are more desirable for drug screening, disease modeling, and researching transplantation therapy, than hepatocytes monocultures. Hepatocytes monocultures are not stable for long. Further, hepatocyte-like cells induced from pluripotent stem cells and *in vivo* hepatocytes are functionally dissimilar. Organoid technology circumvents these issues by generating functional *ex vivo* liver tissue from intrinsic liver progenitor cells and extrinsic stem cells, including pluripotent stem cells. To function as *in vivo* liver tissue, the liver organoid cells must be arranged precisely in the 3-dimensional space, closely mimicking *in vivo* liver tissue. Moreover, for long term functioning, liver organoids must be appropriately vascularized and in contact with neighboring epithelial tissues (e.g., bile canaliculi and intrahepatic bile duct, or intrahepatic and extrahepatic bile ducts). Recent discoveries in liver developmental biology allows one to successfully induce liver component cells and generate organoids. Thus, here, in this review, we summarize the current state of knowledge on liver development with a focus on its application in generating different liver organoids. We also cover the future prospects in creating (functionally and structurally) *in vivo*-like liver organoids using the current knowledge on liver development.
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Contents

Abbreviations: hiPSC, Human induced pluripotent stem cell; BD, Bile Duct; PV, Portal Vein; JAG1, Jagged1; SM22, smooth muscle protein 22; aSMA, a-smooth muscle actin; IHBD, intrahepatic bile duct; OPN, Osteopontin; CK19, cytokeratin 19; qPCR, quantitative PCR; RNA-seq, RNA sequencing; ECM, extracellular matrix; 3D, three-dimensional; HE, hepatic endoderm cells; CFTR, Cystic Fibrosis Transmembrane Conductance Regulator; GO, Gene ontology; EHBD, extrahepatic bile duct.

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1. Introduction

An organoid is a miniature cellular structure that partly mimics organ structure and function [1]. Therefore, an organoid culture recapitulates a developmental process *in vitro*. Earlier, organoids were generated by culturing tissue-specific stem/progenitor/pluripotent stem cells in optimized growth conditions comprising extracellular matrices and growth factors [2,3]. Later, organoids were also generated by coculturing epithelial progenitors with mesenchymal cells and endothelial cells [4]. More recently, organoids are generated by simultaneously inducing differentiation of epithelial and mesenchymal cells in cocultures; the mesenchymal-epithelial developmental interactions promote *in vitro* organogenesis [5]. This third strategy seems reasonable for *in vitro* organogenesis, given that both epithelial and mesenchymal cells emerge simultaneously, and their interaction is crucial for organogenesis *in vivo*. However, in this method, regulating the ratio of epithelial, mesenchymal, and other types of cells (such as hematopoietic cells) is challenging. Therefore, the second method—in which component cells are induced from pluripotent stem cells independently and then co-cultured—still has some advantages, e.g. adjusting the ratio among different types of cells, to generate multicellular organoids.

In establishing an organoid culture system, the culture medium, growth factors, and cytokines are optimized by referring to *in vivo* essential factors governing the development of the tissue of interest. Therefore, understanding *in vivo* organ/tissue developmental processes is crucial. Mostly, *in vivo* organ/tissue developmental processes are studied using mice. Therefore, one must consider interspecies differences when inducing human induced pluripotent stem cells (hiPSCs) differentiation using organogenesis knowledge from mice studies [6]. Recent progresses in scRNA-seq analysis of human fetal cells may fill the knowledge gaps between mouse and human organogenesis.

In this review, we first summarize our current knowledge on liver development and epithelial morphogenesis that are essential in generating liver organoids. We then review the current liver organoid culture systems that aim to mimic *in vivo* liver tissue structure and function with a view of modeling liver diseases.

2. Liver organogenesis

Recapitulation of the developmental process *in vitro* is a widely used strategy for generating functional *ex vivo* tissue. Therefore, knowing how multipotent stem/progenitor cells differentiate and establish three-dimensional (3D) tissue structures could help establish organoids. In the following sections, unless otherwise mentioned, the liver development findings pertain to that in mice.

2.1. Early development of hepatobiliary organs

2.1.1. Liver bud formation and growth

The liver, gallbladder (GB), extrahepatic bile duct (EHBD), and ventral pancreas (VP) arise from the ventral side of the posterior region of the foregut endoderm. Signals from the surrounding mesoderm induce foregut endoderm differentiation. The BMP4 signal from septum transversum mesenchyme (STM) and the FGF2 signal from cardiac mesoderm induce foregut endoderm to differentiate into liver primordium rather than VP [7–9] (Fig. 1A). While the FGF10 signal from STM induces pancreato-biliary characteristics via FGF10/FGFRIIb/Sox9 signal axis [10,11]. The analyses of *zebrafish* and *medaka* mutants which show abnormal liver organogenesis demonstrates that retinaldehyde dehydrogenase type2 (RALDH2; the enzyme responsible for retinoic acid (RA) biosynthesis) modulates hepatic fate by regulating WNT2bb expression in the lateral plate mesoderm [12,13]. Hepatic diverticulum tissue undergoes morphological conversion from columnar monolayered epithelium to pseudostratified epithelium around embryonic day 9.0 (E9.0), and hepatoblasts migrate into STM, mixing with mesenchymal and endothelial cells around E9.5 [14]. Later around E13.5, $WT1^{+}$ mesothelial cells promote liver growth by secreting pleiotrophin and midkine [15], whereas fibroblastic cells including hepatic stellate cells inside the liver tissue contribute to the growth by secreting FGF10 [16]. These cellular interactions support liver specification and growth.

In addition to cellular/tissue interactions, blood flow through liver vasculatures generates mechanical forces and thereby contributes to substantial liver growth. Lorenz et al. showed that the liver endothelial cells sense the blood flow via Integrin β1 and VEGFR3 and secret angiocrine growth factors (e.g., HGF) that contribute to hepatocyte proliferation around E12.5-E13.5 [17].

2.1.2. Extrahepatic biliary development

Until recently, human extrahepatic biliary tissue (GB and EHBD) was considered to arise from AFP^{+} hepatoblasts in the liver bud, based on a descriptive study of the human fetal growth [18]. However, the organ developmental trajectory analyses using gene-editing and lineage tracing experiments in mice demonstrated that extrahepatic biliary tissue and VP arises from a common progenitor, and not from hepatoblasts. *Pdx1* (*pancreatic and duodenal homeobox-1*) lineage tracing experiments showed that extrahepatic biliary tissue, including pancreas, is lineage labeled in *Pdx1*-Cre: ROSA26-LacZ mice [19]. The liver primordium is Hnf4a(+)Pdx1(−)Sox9(−), whereas EHBD, including cystic, hepatic, and common bile ducts, GB, and VP are differentiated from Hnf4a(−)Pdx1(+)Sox9(+) region [11] (Fig. 1B). SOX17 loss-of-function in posterior foregut hinders extrahepatic biliary development, whereas SOX17 gain-of-function

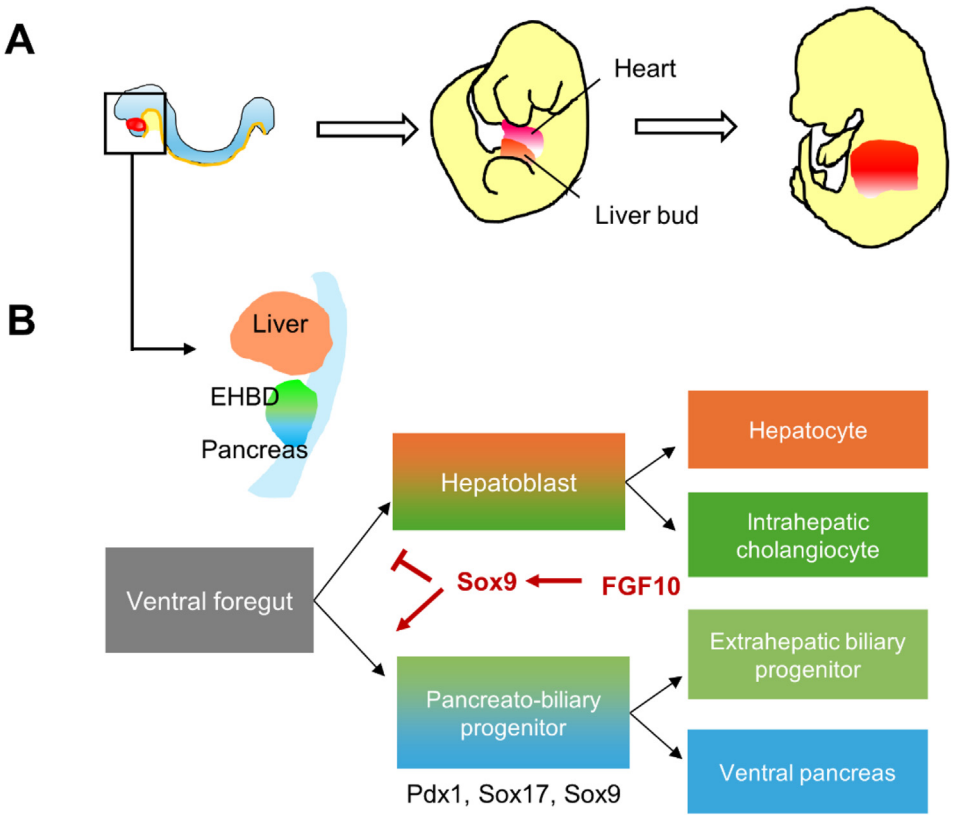

Fig. 1. Schematic representation of fetal liver development. (A) Liver develops from the foregut endoderm next to cardiac mesoderm; (B) Early segregation of intra and extrahepatic tissue.

suppresses pancreas development and is associated with ectopic biliary differentiation [19]. Sox17$^{-/+}$ mice showed perinatal biliary atresia and hepatitis, and nearly 95% of mice died perinatally [20–22]. Hes1$^{-/-}$ mice showed ectopic pancreatic differentiation in the EHBDs and GB regions, indicating that Hes1 contributes to the pancreas/biliary lineage segregation [19,23,24]. Hnf6 (Onecut1) and Hnf1b are essential for normal development of EHBD and IHBD [25,26]. In zebrafish, EphrinB ligands and EphB receptors family coordinate the hepatopancreatic duct tubulogenesis [27]. However, further studies are needed to confirm if mammals share a similar mechanism. EHBDs have unique glandular structures called peribiliary gland (PBG), which produce mucus and act as "stem cell compartments" [28–32]. Histological analyses indicate EHBDs start as simple tubular structures in the fetus (Okumura A unpublished data), and transitions from "mural" to "extramural" gland associated with the cellular hierarchy [31] probably during postnatal development. Depending on studies using knockout mice, Hhex [33], Pdx1 [34,35], Lgr4 [36], and Robo1/2 [37] have been correlated with extrahepatic biliary development. In addition to mouse studies, recent single-cell transcriptome analysis strongly supports the hypothesis that extrahepatic biliary tissue arises from pancreato-biliary common progenitor (not from hepatoblast) also in humans [38]. Thus, indicating that mouse developmental studies can translate well in understanding human extrahepatic biliary development and diseases. However, extrahepatic biliary tissue morphogenesis and its underlying mechanisms remain largely unknown.

2.2. Hepatic tissue structure formation

Fetal liver functions as the hematopoietic organ in midgestation; while hematopoietic stem cells migrate from aorta-gonad-mesonephros, and erythroid-myeloid progenitors migrate from the yolk sac [39,40]. Even though the vascular network is evident, epithelial tissue structures are not established at this stage. Around E15, mice hepatoblasts adjacent to the portal vein (PV) start to differentiate into cholangiocytes [41]. Beyond this point, bile canaliculus (BC) between hepatocytes and bile duct consisting of cholangiocytes gradually form and become established in the neonatal stage after hematopoietic cells migrate from the liver to the spleen and bone marrow [42] (Fig. 2A).

2.2.1. Bile canaliculi (BC) network formation

Hepatocytes possess unique apicobasal polarity; the tiny apical lumen (namely BC) forms between neighboring hepatocytes whose basal domain is not supported by the basement membrane, but rather faces the sinusoidal endothelial cells via the space of Disse.

The results from *in vitro* experiments unraveling the mechanisms of BC network development are contradictory. The cell polarity protein Partitioning defect 1b (Par1b) (or microtubule affinity-regulating kinase 2 (MARK2)) and the bile acid taurocholate activated intracellular signal pathway control hepatocyte-type polarity and BC network formation, respectively [43,44]. Moreover, cultured primary adult hepatocytes form BC networks during cytokinesis [45], suggesting that cell proliferation is the major driving force for BC formation. However, *in vivo*, BC networks develop significantly around E17 in mice [46], when hepatocytes are proliferating at a reduced rate after the midembryonic period, with only 5.6% hepatocytes proliferating at E17.5 [47]. Consistently, using live cell imaging of the BC formation in E13.5 mouse hepatoblasts cultures), Belicova et al. demonstrated that BC network can form without cell division. They found that luminal spaces between hepatocytes elongate, branch, and fuse to form BC

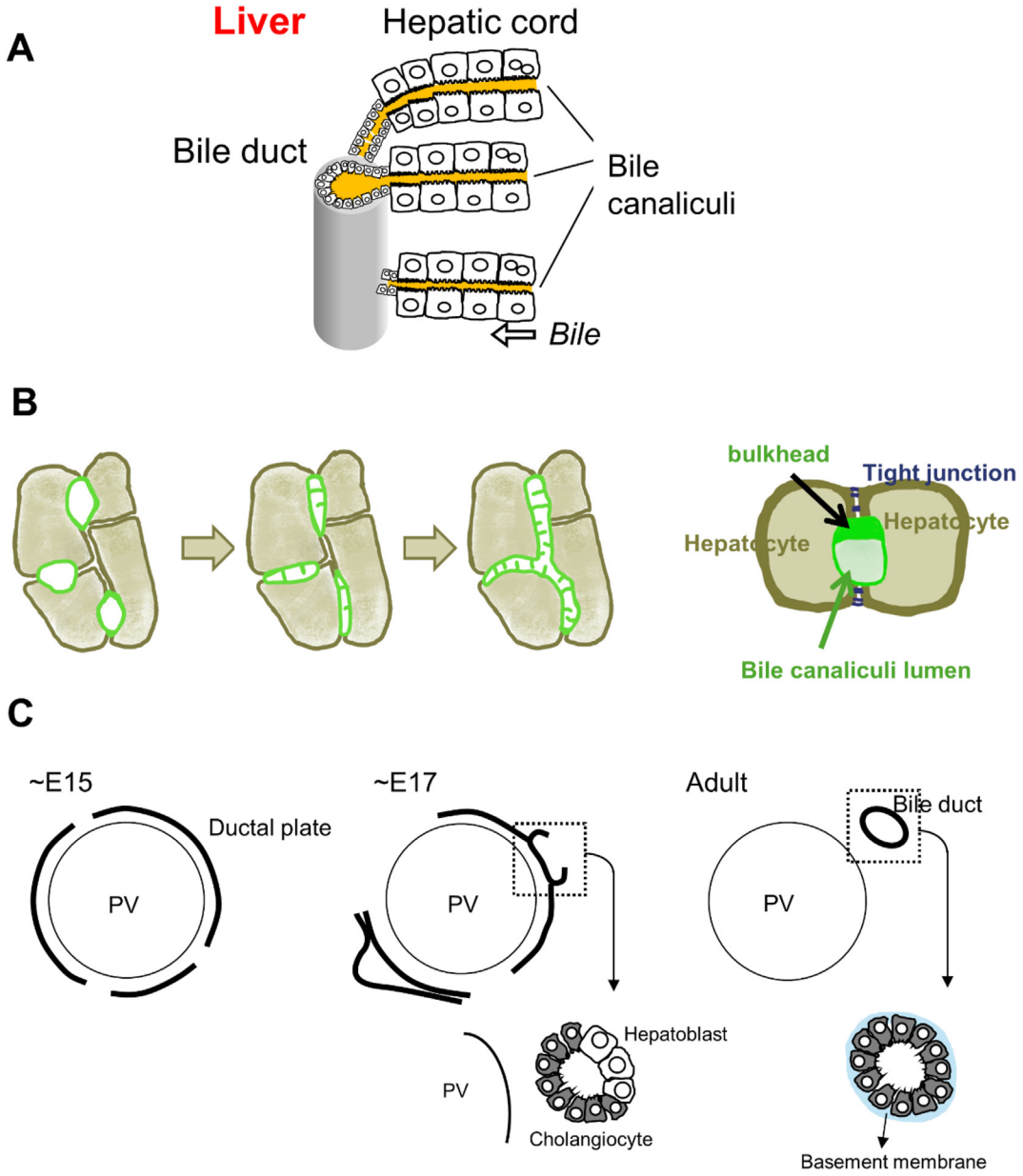

Fig. 2. Liver epithelial tissue structure. (A) Hepatocytes form bile canalicular network connected to bile ducts which drain the bile secreted by hepatocytes. (B) Hepatocytes first form tiny lumens along cell–cell boundary, which fuse and branch to become the bile canalicular network. The F-actin rich structure called "bulkhead" protrudes in the bile canalicular lumen, conferring mechanical strength to the lumen. (C) Cholangiocytes differentiate from hepatoblasts around the portal vein (PV); they first form "asymmetric duct" with hepatoblasts and then become mature "symmetric" ducts.

networks. Notably, they revealed with super-resolution microscopy that a F-actin-rich structure called "bulkhead" protrudes into the BC lumen. The bulkhead protrusion is named based on its visual similarities with the ship bulkheads (vertical wall-like partition between compartments) (Fig. 2B) [48]. They also demonstrated that Rab35 (endosome recycling and membrane trafficking protein) is essential for bulkhead formation; with Rab35 knockdown, hepatocytes in hepatoblast cultures and in fetal liver achieve hepatocyte-type polarity but eventually form bile duct like lumens. Bulkheads in BC lumen function like bulkhead in a small ship; they mechanically support the BC lumen and help it withstand the intraluminal pressure due to bile excretion [49].

2.2.2. Bile duct development

2.2.2.1. Formation of the biliary structure. Cholangiocytes exhibit the typical epithelial apicobasal polarity; the basal domain is supported by the basement membrane, whereas the apical domain faces to the central lumen surrounded by cholangiocytes. Cholangiocytes differentiate from hepatoblasts, around midgestation and then form intrahepatic bile ducts (IHBDs). In the classic model of IHBD development, ductal plates comprised a single cholangiocyte layer form around the PV, which later attains a double-cell layered structure. Lumens emerge between the two cell layers, which are reorganized into tubular BDs [41] (Fig. 2C). All processes start in the hilum region and proceed along the hilum-to-

periphery axis [50]. Classical BD formation model is based on 2D imaging results. Recently, 3D imaging analyses have revealed models contrasting with the classical BD formation model. Retrograde resin injection into IHBDs from the common bile duct revealed branching structures in the entire liver tissue [51]. Kaneko et al. demonstrated that the IHBD network around the PVs in the adult liver is formed by large ducts and small ductules, establishing a novel 3D imaging methodology with carbon ink injection [52]. We followed IHBD morphogenesis from E17 to adulthood, using carbon ink injection method (Fig. 3) [53]. We found that between E17 and E18, the immature homogeneous luminal network is rearranged into a mature hierarchical network (Fig. 4). This is evidenced by the color of the intestine turning yellow from white at E18, suggesting the bile starts to flow through biliary transport system around E18 after bile canaliculi network is established. Therefore, the bile flow enhances the transition of IHBDs network from its homogeneous to hierarchical luminal network state. At E17, by when most of the luminal structures are interconnected, BD lumens are in “asymmetric ducts” stage in the peripheral tissue, surrounded by cholangiocytes and hepatoblasts; they later differentiate into “symmetric ducts” surrounded uniformly by cholangiocytes [54]. We have not examined whether hepatoblasts surrounding the luminal structure are incorporated into the continuous luminal network. However, luminal connectivity formation may precede the completion of the hepatoblast-to-cholangiocyte fate decision.

2.2.2.2. Regulation of cholangiocyte differentiation and IHBD morphogenesis. Our understanding of molecular mechanisms governing hepatoblast-to-cholangiocyte differentiation and bile duct morphogenesis has progressed due to studies on abnormal bile duct development seen in human congenital biliary diseases and mutant mice (Fig. 3). In the following sections we will discuss the various signaling pathways and factors involved in hepatoblast-to-cholangiocyte differentiation and IHBD morphogenesis.

2.2.2.2.1. Jagged1-Notch pathway in liver epithelial cell fate decision and IHBD morphogenesis. Alagille syndrome, a congenital disease showing multiple developmental defects, including a paucity of IHBDs, has been intensively analyzed. *Jagged-1* (*JAG1*) and *Notch2* mutations are responsible for Alagille syndrome [55]. *Notch2* knockout mice (Alfp-Cre:*Notch2* $^{flox/flox}$ mice)—with Alfp-Cre (hepatoblast specific Cre-recombinase system expressing Cre under control of the Albumin regulatory element and α-fetoprotein enhancer)—showed a complete deficiency of the cholangiocyte

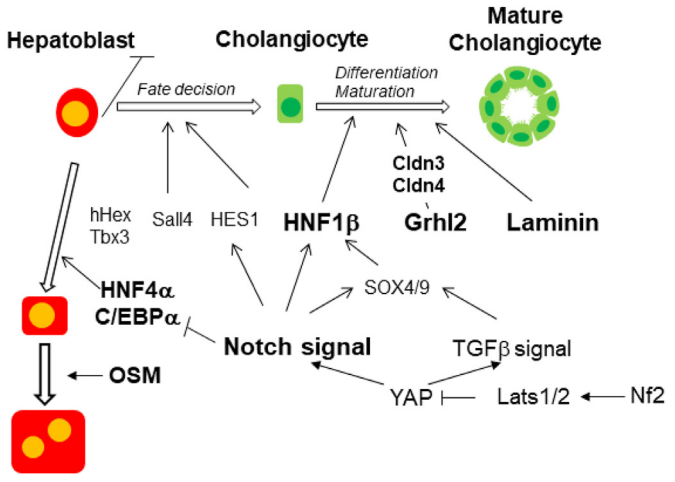

Fig. 3. Molecular mechanism of hepatoblast fate decision.

differentiation [56]. While, in SM22-Cre: *JAG1*$^{flox/flox}$ mice—in which JAG1 was depleted in vascular smooth muscle cells (VSMCs)—cholangiocyte number was diminished, and tubular morphogenesis was abnormal [57]. These results suggest that the cholangiocyte differentiation is mediated by interactions between JAG1^{+} VSMCs or periportal fibroblasts and Notch2^{+} hepatoblasts. Contrastingly, mice harboring *JAG1* missense mutation (H268Q; *JAG1*$^{Ndr/Ndr}$) phenocopied human Alagille syndrome, including eye, heart, and liver defects [58]. H268Q JAG1 has reduced Notch1 binding capacity, while its Notch2 binding is unaffected. Given that Notch2 is the critical receptor mediating the signal in hepatoblast-to-cholangiocyte fate decision, H268Q JAG1 phenocopying Alagille syndrome seems counter-intuitive. Thus, further studies are necessary to understand how Notch1 and Notch2 cooperatively regulate biliary development.

Hippo-YAP pathway is upstream of the Notch signaling pathway [59]. Overexpression of activated YAP or loss of plasma membrane localization (and subsequent activation) of the YAP-negative regulator Lats1/2 via knockout of neurofibromatosis type 2 protein (NF2; also known as merlin or neurofibromin 2) results in upregulated Notch 2 and aberrant Notch pathway activation. These changes lead to abnormal expansion of biliary structures, and subsequently to cholangiocarcinoma [60]. However, YAP depletion in Alb-Cre:*YAP*$^{flox/flox}$ and FoxA3-Cre:*YAP*$^{flox/flox}$ mice results in irregular duct structures and the complete loss of IHBD, respectively, in neonatal liver. YAP nuclear localization is detected in cholangiocytes in adult liver [61]. These results further indicate that Notch signaling pathway is crucial in liver epithelial cell fate decision and IHBD morphogenesis.

2.2.2.2.2. Extracellular matrix proteins in IHBD formation and maintenance. The extracellular matrix (ECM) has a pivotal role in morphogenesis and maintenance of epithelial structures including tubular structures such as IHBD, pancreatic duct, kidney tubules, and respiratory airway [62]. The basement membrane, comprised ECM proteins (such as laminins, collagens, and proteoglycans), supports epithelial tissue. Among ECM proteins, laminins crucially establish epithelial polarity and morphogenesis [63]. IHBDs, PVs, CVs, and hepatic arteries are surrounded by a laminin layer. In E14 liver tissue, the laminin 111 (supplied from p75NTR^{+} fibroblasts) induces establishment of apicobasal polarity in liver progenitor cells and formation of cyst structure *in vitro* [64]. Thus, laminin 111 regulates initiation of bile duct tubule formation, whereas laminin 511/521 regulates bile duct tubule maturation. Mature cholangiocytes express laminin 511/521; and bile duct maturation is suppressed in laminin-α5 knockout mice [64].

2.2.2.2.3. Transcription factors involved in bile duct development. Several transcription factors implicated in IHBD development have been identified by studying mutant mice with abnormal IHBD structures. Depletion of hepatocyte nuclear factor 6 (HNF6) and onecut 2 (OC2) disturbs the PV-CV gradient of the TGFβ signal, via increasing TGFβRII and decreasing β2-macroglobulin and follistatin (negative regulators for the TGFβ signal) [65]. These changes induce hepatoblasts with “hybrid phenotypes” (double positive for HNF4α and biliary cytokeratin) which form cystic structures. Cystic structures surrounded by “hybrid cells” are also abundantly formed in Foxa3-Cre: *Hhex*$^{flox/flox}$, *C/EBPα*$^{-/-}$, and Foxa3-Cre: *Prox1*$^{flox/flox}$ mice [33,66,67]. Among these three transcription factors, C/EBPα suppresses HNF6 and HNF1β expression. Therefore, the appropriate HNF6 expression in hepatoblasts may essentially control cholangiocyte differentiation around the PV.

Alfp-Cre: *HNF1β*$^{flox/flox}$ mice showed severe defects in bile duct morphogenesis [26]. HNF1β regulates primary cilia-related gene expression, including *polycystic kidney and hepatic disease 1* (*Pkhd1*) gene. Studying HNF1β−/− kidney revealed that HNF1β could be involved in cell divisions through regulating “planar cell polarity”

(see section 2.2.2.2.4); in the absence of HNF1β, elongation perpendicular to the axis dominated over that along the axis, resulting in lumen expansion and polycystic disease [68]. Alfp-Cre: *Sox9* $^{flox/flox}$ mice showed delayed IHBD morphogenesis, which is enhanced in Sox4$^{-/-}$Sox9$^{-/-}$ mice [54,56]. Sox4 and Sox9 cooperatively regulate laminin α5 and components of the Notch, TGFβ, and Hippo signaling pathways.

Grainyhead like-2 (Grhl2) expressed in tubular BDs induces expansion of the central lumen of cholangiocyte cysts *in vitro*. Grhl2 regulates claudin 3 (CLDN3), CLDN4, and Rab25, which are involved in tight junction formation crucial for lumen expansion [69]. Notably, HNF1β, SOX9, and GRHL2 are widely expressed in epithelial tubular structures. Mechanistic analysis of these factors in bile duct development may lead to understanding common regulatory mechanisms that govern epithelial tubulogenesis.

2.2.2.2.4. Planar cell polarity pathway. The planar cell polarity (PCP) pathway is involved in the zebrafish bile duct, mammalian lung, kidney, and pancreatic duct formation [70]. Raab et al. analyzed the single cell RNA-seq (scRNA-seq) data of mouse fetal liver cells and found that the PCP-related gene *Vangl2* whose protein interacts with desmosomal proteins and patterns cellular contact is highly expressed in EpCAM^{+} cholangiocytes isolated from mice fetus aged ≥ E14.5 [71]. Based on their observation that discontinuous bile duct fragments were evident in the *Vangl2* hypomorphic mutant mice, they concluded that the Vangl2/ROCK/RHO or JNK pathway activation is essential for the formation of continuous tubular network.

2.2.2.2.5. Epigenetic regulation of hepatoblast-to-cholangiocyte differentiation. We previously found that the methylation status of promoter regions of key hepatocyte transcription factors is different between hepatocytes and cholangiocytes [72]. Epigenetic regulation also occurs during hepatocyte de-differentiation [73]. Further, Wang et al., through their scRNA-seq analysis of fetal mice and human liver genomes, demonstrated the epigenetic regulation of liver development and proposed the “default vs. directed” model of hepatoblast differentiation [74]. According to this model, the hepatocyte differentiation is the “default” lineage program, whereas cholangiocyte differentiation is highly “directed” to deviate from the default hepatocyte program. Hepatoblasts express hepatocytic functional genes before cholangiocytes emerge. Whereas in cholangiocytes these genes are turned off immediately after differentiation. Furthermore, using integrated multi-omics analysis (RNA-seq, ATAC-seq, and ChiP-seq) they revealed that E12.5 hepatoblasts express high levels of EZH2 (histone methyltransferase) and JMJD3 (histone demethylase) than E15.5 or E17.5 hepatoblasts [75]. EZH2 binds to the promotor of genes related to cholangiocyte differentiation and maturation, and it suppresses their expressions. EZH2 and/or JMJD3 inhibition and knockout experiments suggested that these H3K27me3 modifiers antagonistically regulate cholangiocyte differentiation. Gene ontology analysis revealed several genes (including *WNT*, *TGFβ*, and Notch signaling components) that selectively lose H3K27me3 modification in cholangiocytes. Meanwhile, only a few genes in hepatocytes lose H3K27me3 modification. Thus, elimination of H3K27me3 modification from the bivalent promoters likely requires hepatoblast-to-cholangiocyte differentiation.

2.2.2.2.6. Connection between intrahepatic and extrahepatic bile ducts. The EHBD-IHBD luminal connection is crucial for bile drainage. During early embryogenesis, the lineages of cells forming EHBD and liver segregate around E9.0 (Fig. 1B), and after that, epithelial cells of IHBD and EHBD seem to develop independently. Although the mechanisms generating the luminal continuity remain largely unknown, histological analyses suggests that IHBD and EHBD lumens are discontinuous in the boundary region at gestational week 19 (GW19) and they connect by GW25 [76]. EHBD maintains its luminal structure during its developmental

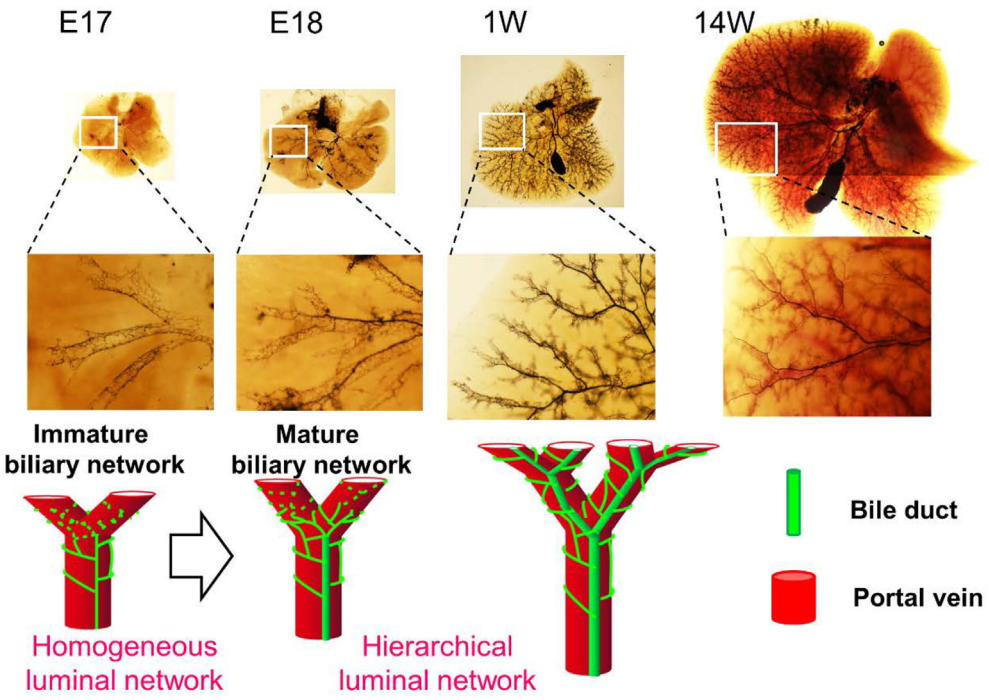

Fig. 4. Bile duct morphogenesis. The continuous luminal network of intrahepatic bile duct (IHBD) is mostly established by E17.5; the IHBD network is still immature homogeneously consisting of fine tubules. Between E17.5 and E18.5, the homogeneous fine luminal network is rearranged into the hierarchical network that contains large ducts and mesh-like fine ductules. [To help visualize, carbon ink was injected from the junction between EHBD and the duodenum, and the liver tissue was cleared in BABB after dehydration in ethanol].

morphogenesis, whereas IHBD gradually generates luminal structure along the hilum-to-periphery. During the process, the nascent lumen of IHBD may eventually connect to the EHBD lumen. Even though EHBD tissue lies apposing the liver epithelial tissue (from which cholangiocytes emerge) in the liver hilum throughout embryogenesis [76], it has not been ascertained if the final connecting step is led by IHBD outgrowth or EHBD outgrowth. Genetic lineage tracing is unable to segregate the origin of the connecting tissue due to the lack of early tissue specification markers of EHBD and IHBD. Further imaging analysis and lineage tracing studies are necessary to understand the development of the whole biliary system.

2.2.3. Liver vasculature formation

Liver is a highly vascularized organ. Blood enters liver tissue through PV and hepatic artery, flows through liver tissue via sinusoids, and flows out through CV. Liver sinusoidal endothelial cells (LSEC) forming the sinusoid have fenestrations, through which various substances and metabolites are exchanged in the space of Disse. Some of the substances and metabolites in the space of Disse flow into lymphatic vessels around the PV [77]. LSEC differentiation and sinusoid formation have been studied in mouse embryos. Endothelial cells (ECs) which contribute to hepatoblast proliferation are observed on the surface of the liver bud at E9.0. Beyond this stage, liver bud expands, invades into STM, while ECs inside the liver tissue form luminal structures at E10.5 [78]. Macroscopic observation and scRNA-seq data reveal that erythroblast exists in E10.5 liver [79,80], indicating that blood flow from the placenta via umbilical vein or the heart into the liver starts by this stage. The lineage tracing experiments demonstrate that LSECs originate from endocardial cells of sinus venosus (SV). Liver vasculature endothelial cells are negative for nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) protein throughout the developmental process. NFATc1 is a unique lineage marker for endocardial cells. When endocardial cells of SV were labeled in the Nfatc1-IRES-creER:ROSA-LSL-RFP mice (at E8.0 but not at E9.5), most hepatic endothelial cells were labeled with RFP. This demonstrated that endocardial cells of SV migrate to the liver around E8.0-9.5 and contribute to the formation of liver vasculature. Furthermore, dual lineage tracing experiments using NFATc1-IRES-Dre:Npr3-CreER:ROSA-RSR-LSL-RFP identified endocardial cells in SV (but not in atrial and ventricular endocardium) as the origin of the hepatic endothelial cells [81]. In addition to SV-derived cells, liver vasculature is partly formed by ECs surrounding the E8.5 liver bud which invade into the liver tissue alongside angiogenesis from vitelline or umbilical veins (Fig. 5).

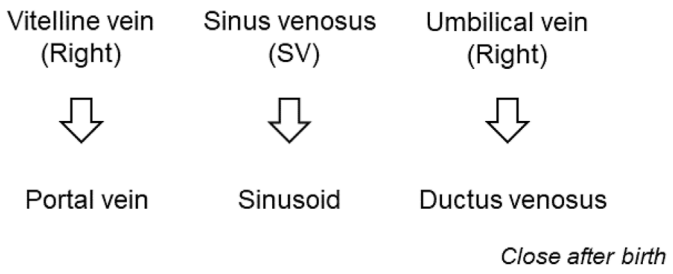

Fig. 5. Origin of liver vasculatures. The liver bud migrates into septum transversum mesenchyme (STM) and includes part of vitelline and umbilical veins. Right vitelline vein is incorporated into the liver tissue as the portal vein (PV) that carries nutrient-rich blood from the intestine. Umbilical veins carry the placental blood to the liver, and later the left umbilical vein persists to form ductus venosus, which bypasses the placental blood to the heart in the upper part of fetus; and eventually close after birth (DeSesso JM 2016). The lineage tracing experiments demonstrated that endothelial cells of sinusoid (consisting of sinusoidal endothelial cells, LSEC) are derived from endocardial cells of SV (Zhang H et al., 2016). In addition, endocardial cells of the cardiac vein juxtaposed to the liver bud become the hepatic vein that drains blood from the liver to the inferior vena cava (DeSesso JM 2016).

LSECs are distinct from other ECs, forming microvasculature in their permeable and discontinuous structure owing to fenestration. *GATA4* deletion in LSEC in Stab2-Cre:*Gata4*$^{fl/fl}$ mice results in the loss of LSEC-specific characters including fenestration, and induces embryonic lethality between E15.5 and E17.5 [82]. Activin receptor-like kinase 1 (ALK1) mutation causes hereditary hepatic vessel malformation disease. Indeed, BMP9 (stellate cell derived ALK1-ligand) knockout mice showed loss of the LSEC character in the liver microvasculature, accompanied by the downregulation of GATA4 and other LSEC markers such as Lyve1 and Stab2 [83]. SMAD6—which is downstream of BMP9 is a negative regulator of the ALK1-signal pathway—is necessary for forming endothelial cell junctions, indicating that tuning the ALK1 signal is important for liver vasculogenesis [84]. Gomez-Salinero et al. performed temporal scRNA-seq analysis for LSEC from E12 to P30 and found that transcription factor c-Maf is continuously upregulated over time. Deletion of *c-Maf* in endothelial cells of VE-cadherin (Cdh5/CD144)-Cre^{Ert2}: c-Maf$^{flox/flox}$ mice resulted in the loss of LSEC features [85]. Their scRNA-seq data showed that Gata4 is expressed in all types of endothelial cells in the liver, which is in sharp contrast to c-Maf, which is expressed only in LSECs. Moreover, *in vitro* overexpression studies showed that GATA4 overexpression induces LSEC marker genes in HUVECs but not the formation of LSEC-specific structures (e.g., fenestrae). Whereas c-Maf overexpression in HUVEC not only induces LSEC marker expression but also fenestration. Thus, c-MAF could be a master regulator of LSEC differentiation. However, the functional correlation between c-Maf and GATA4 in LSEC differentiation should be further clarified.

Xia et al. investigated the role of sialylation in LSEC using endothelial/hematopoietic cell-specific Slc15a1 (CMP—sialic acid transporter) knockout (Tie2-Cre: Slc15a1$^{flox/flox}$) mice [86]. These mice die postnatally due to severe hepatic injury, accompanied by hepatic lipid deposition. They found that endothelial Slc15a1 knockout leads to loss of LSEC characteristics (e.g., reduced fenestrae, decreased expression of LYVE1, increased deposition of Laminin ECM, and increased expression of CD34). Protein sialylation modulates the affinity of ligands for their receptor. Therefore the phenotypes of Slc15a1 mice were at least partly due to the desialylation of VEGFR2, resulting in overactivation of the VEGF signaling. Although it is still controversial whether VEGF signaling has positive or negative effects on LSEC differentiation and identity maintenance [87], VEGF signaling inhibitor partly rescued the Tie2-Cre: *Slc15a1*$^{flox/flox}$ phenotype. Notably, previous studies have focused mainly on the transcriptome data, but less on post-translational modifications. This work highlights that in addition to translational regulation, posttranslational modification (such as sialylation) can affect the endothelial function.

In addition to their specialized functions as hepatic endothelial cells, LSECs have a crucial role in determining hepatocyte characteristics. Hepatocytes are characterized by region-specific metabolism type, and the liver tissue consists of Zone-1; periportal, 2; interlobular, 3; pericentral hepatocytes lined along the PV-CV axis [87], which is established in the postnatal period [88–90]. The region specific-LSEC-derived growth factors (Wnt2, Wnt9b, R-spo3, and BMP2) contribute to the zonation [85,91–95]. Indeed, developmental defects of LSEC differentiation disrupt hepatic parenchymal zonation; Slc15a knockout resulted in poor expression of the pericentral metabolic enzyme (e.g., GS: glutaminase synthetase, Cyp2e1). LSECs themselves develop distinctive cellular characteristics along the PV-CV axis and then regulate hepatocyte zonation. However, further studies are needed to reveal whether disrupted hepatocyte zonation, in turn, affects LSEC zonation and

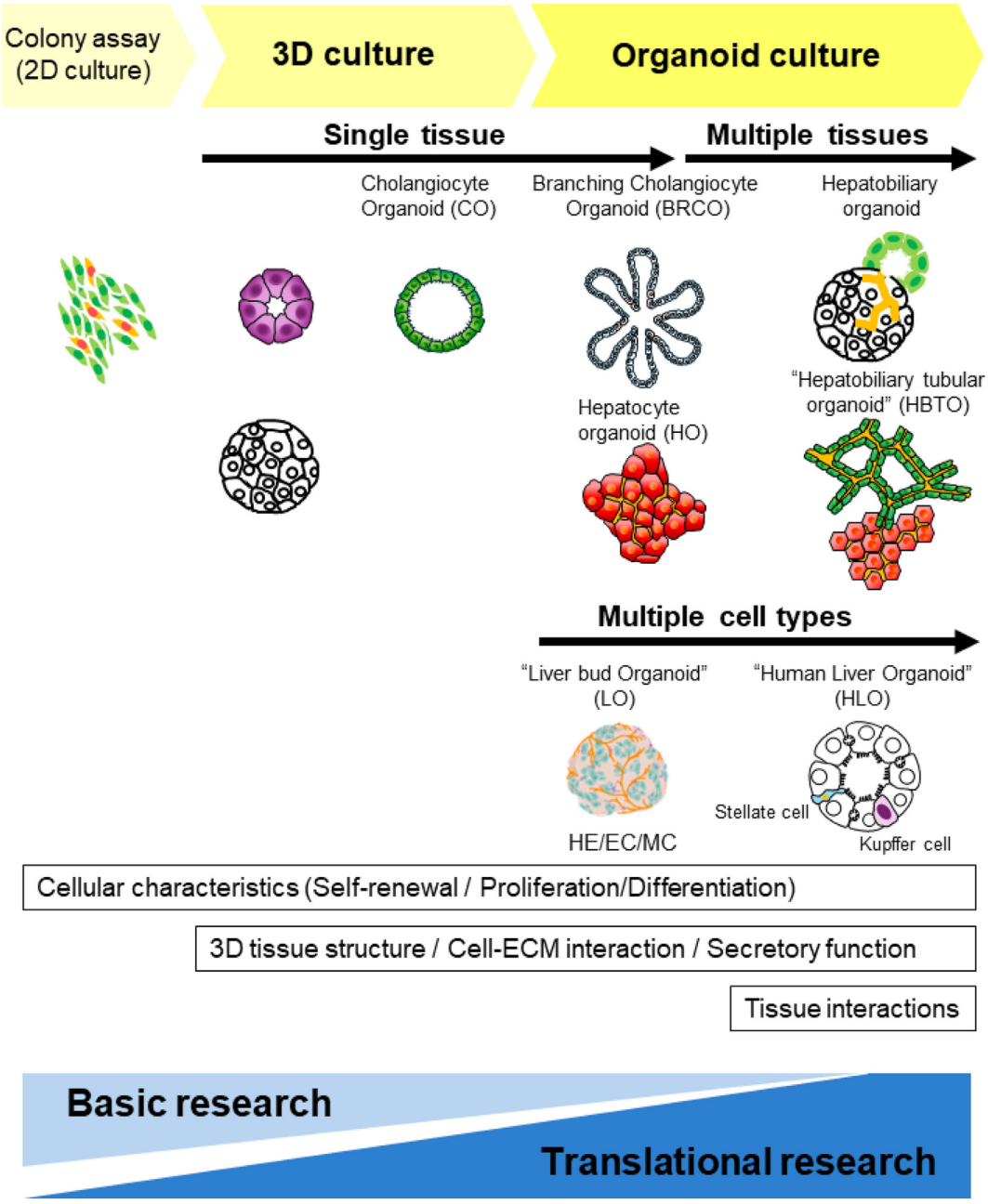

Fig. 6. Progressive improvement of liver buds to mimic *in vivo* liver tissue structures.

how LSEC zonation is regulated during embryonic and neonatal liver development.

3. Liver tissue reconstitution *in vitro*

A culture system containing tissue structures rather than a planar cell culture is complex but better mimics the *in vivo* functions of tissue/organ (Fig. 6). Organotypic or organoid cultures using tissue explants, or a mixture of primary cells have been reported since the late 1960s. Among various liver culture methods, spheroid culture has been widely used to maintain hepatocyte functions *in vitro*, whereas sandwich cultures of primary mature hepatocytes are a major culture method for inducing bile canalicular structure. Furthermore, Mitaka et al. generated a liver organoid using primary rat cells, in which hepatocyte progenitors cultured with "nonparenchymal cells" form the bile canalicular network [96]. These prototype culture techniques are still used to induce or maintain the function of hepatic cells *ex vivo*.

Since intestinal organoids derived from mouse intestinal columnar stem cells [2] or human pluripotent stem cells (hPSCs) [97] and an optic-cup organoid derived from human embryonic stem cell (hES) [3] were reported, organoid culture systems are being explosively established for many tissues and organ types. Although we reported cholangiocyte organotypic culture in 2007 [98], liver organoids have been widely recognized since the publication of two innovative works: 1) a cystic liver organoid created from a facultative liver stem cell derived from chronically injured mouse liver [99]; and 2) a liver bud organoid derived from hiPSCs [4]. Until now, many types of liver organoids have been reported. The simplest organoid (which includes hepatocyte or cholangiocyte

organoids) is comprised of a single cell type and has a uniform structure. However, since cellular or tissue interactions are essential for physiological functioning, the recent approach to generating liver organoids is to integrate multiple types of cells and tissues. There are two approaches in generating liver organoids containing various cell types: 1) in the first method, multiple lineage cells, such as endoderm and mesoderm cells, are simultaneously induced from multipotential progenitors, including ES and hiPSC, and then directed to form liver tissue; and 2) in the second method several lineage cells are independently differentiated and then mixed to induce tissue morphogenesis. In this section, we summarize liver organoids consisting of single or multiple types of cells and then touch upon disease modeling with liver organoids.

3.1. Liver organoid containing a single type of cell (hepatocyte organoid, cholangiocyte organoid)

3.1.1. Hepatocyte organoid

Hepatocytes are the major functional liver cells and are crucial for pharmacological assays. However, isolated hepatocytes stop exhibiting physiological functions in a planar culture. Spheroid and sandwich culture have been used for maintaining hepatocyte functions *in vitro*. In spheroid culture, hepatocytes aggregate without attaching to the plate. Whereas in sandwich culture, hepatocytes are plated between two layers of collagen gel. Hepatocytes form tissue structures, especially the bile canalicular network, in the sandwich method, which is further enhanced by coculturing with non-parenchymal cells [96].

Although primary hepatocytes lose their proliferative capacity, there exists a subpopulation called “small hepatocytes” (located in ZONE1 and 2), which can clonally proliferate and have redifferentiation potential. These small hepatocytes can be used for organoid culture. In addition, with recent discovery that hepatocytes exhibit lineage plasticity, Katsuda et al. successfully generated progenitor cells from primary mature hepatocytes [100]. Thus, organoid cultures are feasible using primary hepatocytes. Indeed, Hu et al. generated “hepatocyte organoid” containing bile canaliculi using human fetal liver cells [101], whereas Peng et al. expanded hepatocytes using TNFα stimulation and formed a hepatocyte organoid [102]. Spheroid culture usually helps hepatocyte-like cells induced from ESC and iPSC to achieve normal metabolic functioning [103]. Although, bile canaliculi have been successfully induced in hepatocytes derived from hiPSCs [104,105], the robust generation of bile canalicular network has not been reported in hepatocyte organoids created from pluripotent stem cells.

3.1.2. Cholangiocyte organoid

Epithelial cells (such as cholangiocytes) cultured in ECM gel form a “cyst,” which is a spheroid with a central lumen. Further, within the cyst the epithelial cells establish apicobasal polarity and acquire secretory functions. In contrast to mature cholangiocytes, bipotential liver progenitors require exogenous laminin to form cysts [98]. Cholangiocytes lie on the basement membrane from the onset of bile duct development. Using this knowledge, cholangiocyte organoids have been generated by mimicking cell-ECM interactions in culture. When the cell-ECM interaction is reproduced by culturing liver progenitor cells in EHS-gel that contains the basement membrane components, apicobasal epithelial polarity and secretory function are induced in the 3D culture [98].

A liver organoid with a cyst-like morphology was generated from “liver progenitor cells” isolated from damaged livers [99]. However, differentiation of the liver progenitor cells in the cystic liver organoid was biased toward developing into cholangiocytes. Therefore, these organoids are called “Cholangiocyte organoids” in subsequent papers [101]. A similar protocol was followed with cholangiocytes derived from human adult liver, and a cyst-like “Cholangiocyte organoid” structure was induced *in vitro* [106]. Further, tube-shaped bile ducts networks form in collagen gel sandwich cultures of liver progenitor cells or cholangiocytes isolated from adult liver [107,108]. Roos FJM et al. recently generated branching cholangiocyte organoid from human primary cholangiocytes by controlling the WNT-canonical and noncanonical signal pathways. They first generated cholangiocyte cysts by culturing the primary cholangiocytes in WNT-canonical signal activating conditioned medium containing EGF, HGF, FGF, A8301, and Forskolin with WNT and R-spondin (RSPO). Subsequently, they induced branching morphogenesis by culturing the cholangiocyte cysts in WNT-noncanonical signal activating conditioned medium containing EGF, dexamethasone, Dkk1 (WNT-canonical signal inhibitor), and RSPO [109].

Cholangiocyte-like cells have been induced from hiPSCs. Generally, the culture protocols inducing cholangiocytes from hiPSCs contain a hepatoblast differentiation step. As mentioned in Sections 2.2.2.2.1 and 2.2.2.2.3, intrahepatic cholangiocyte differentiation and IHBD formation are driven by Jagged1-Notch and TGFβ signaling. TGFβ ligands are soluble and active in the medium, whereas Notch ligands show limited activity in soluble forms [110,111]. Previously, IHBD organoids were generated using mainly two approaches: 1) coculture with JAG1-expressing or JAG1-overexpressing cells [112,113], or 2) activate an alternative signal pathway (e.g., RA) to induce the expression of cholangiocyte genes (e.g., *Sox9*, *CFTR*, *Onecut1*) [114,115]. Instead of using JAG1-expressing cells, Rizwan et al. devised a JAG1-immobilized-hyaluronan (HA) hydrogel in which JAG1 activity could be induced by photochemical stimuli [116] Using this system, they not only successfully induced the JAG1 driven cholangiocytes development (without using exogenous cells) but also created the tubular IHBD structure from human ES cells *in vitro*.

3.2. Hepato-biliary organoid

Bile canaliculi are connected to bile ducts via a junctional structure called the canal of Hering (CoH) near the PV. Because of their bidirectional differentiation potential, the bipotential liver progenitor cells can differentiate into hepatocytes and cholangiocytes and, in appropriate culture condition, they might be able to eventually generate hepatobiliary organoids containing bile canaliculi connected to bile ducts via CoH. We attempted to create organoids using bipotential neonatal EpCAM(+) cells [117] in collagen-containing EHS-gel. We found that EpCAM(+) cells form a cellular structure associated with a lumen containing both hepatocytes and cholangiocytes. However, lumen surrounded by a cell layer consisting of hepatocytes and cholangiocytes is different from the *in vivo* hepatobiliary structure. Hepatobiliary organoids have also been generated from human fetal liver cells and hiPSCs [118,119]. These organoids contain both hepatocytes and cholangiocytes, and absorb the bile acid, which accumulates in cystic structures consisting of cholangiocyte-like cells. Ramli et al. demonstrated that fluorescent-labeled bile acid taken up by hepatocytes is secreted into bile canalicular structures, and then transported to cholangiocyte cysts [120]. Although these organoids achieve a hepatobiliary tissue structure, organoids with *in vivo*-like liver tissue structure in which bile canaliculi are connected to tubular bile ducts via CoH remain to be established.

Histochemical analyses of mouse fetal liver demonstrate that the connection between the bile canaliculi and bile ducts is already established at E17. Even though the bile canaliculi is discontinuous, an immature fine homogeneous luminal network of IHBDs is already established [53,121]. These data suggest that the generation of hepatobiliary connection is an early event in liver epithelial

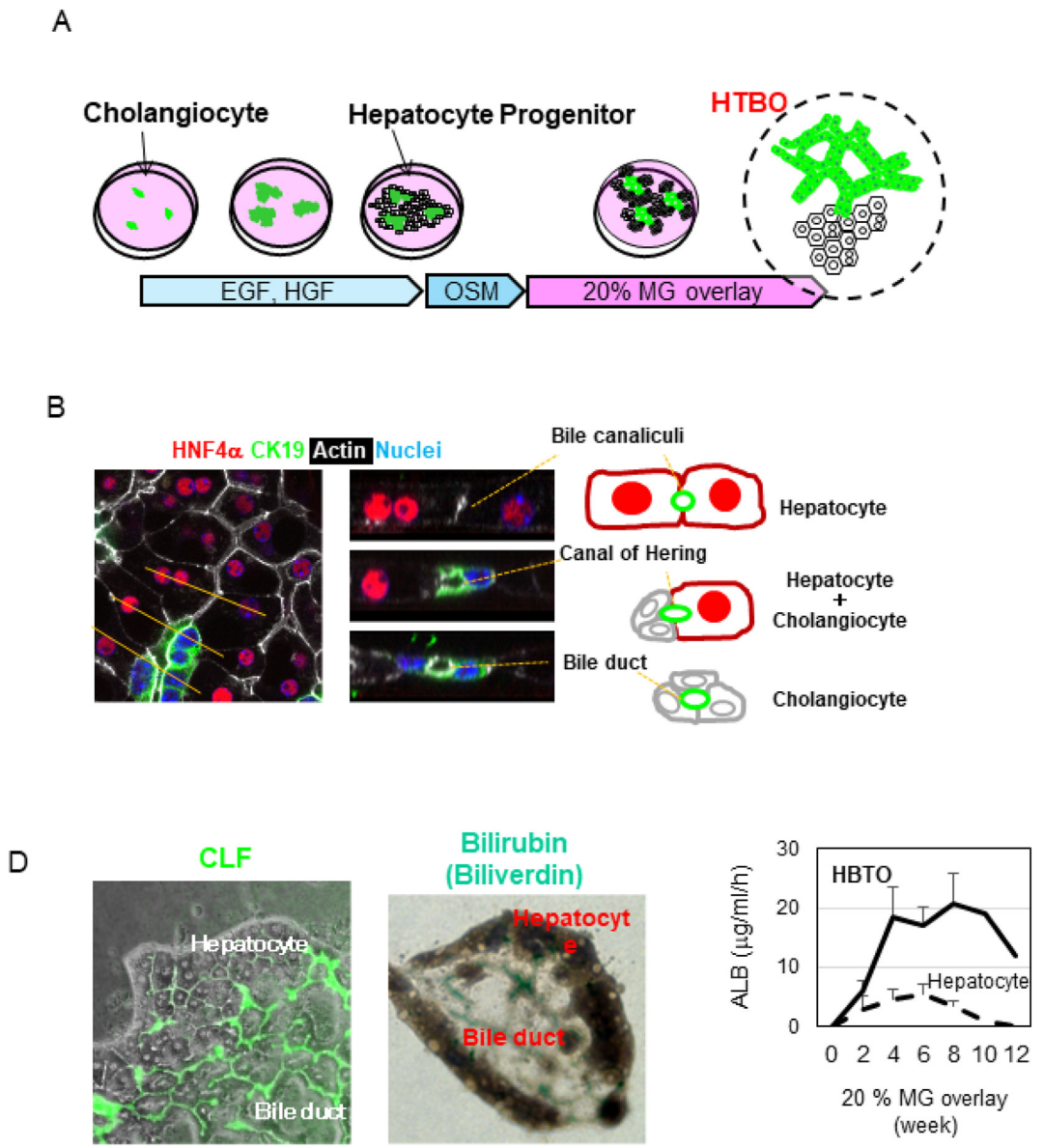

Fig. 7. Generation of hepatobiliary tubular organoid (HBTO). Mouse cholangiocytes are cultured on collagen. After they form colonies, hepatocyte progenitors are plated. Overlaying collagen gel containing 20% EHS-gel induces simultaneous formation of bile canaliculi and bile ducts comprised hepatocytes. At the boundary, bile canaliculi are connected to bile duct lumens through "the canal of Hering (CoH)."

morphogenesis. Thus, we hypothesized that the early contact between hepatocytes and cholangiocytes is crucial to induce hepatobiliary connection. Therefore, to generate hepatobiliary organoids, we used primary cholangiocytes and hepatocyte progenitors freshly isolated from adult mice. We maintained cholangiocytes on collagen gel until they formed colonies. Subsequently, to allow an interface between hepatocytes and cholangiocytes to form, we seeded hepatocyte progenitors at the beginning of coculture (Fig. 7A). Next, we overlayed collagen gel containing 20% EHS-gel on the coculture to induce formation of bile ducts and bile canaliculi, which are connected through CoH (Fig. 7B). We named this organoid as hepatobiliary tubular organoid (HBTO). Our data indicated that hepatocyte progenitors that are positive for E-cadherin and located in ZONE1 and 2 of the liver tissues, dominantly interact with $ECAD^{+}$ cholangiocytes, as compared to the rest of hepatocytes. The liver organoids containing intrahepatic biliary system has several advantages, including mimicking bile transport within liver tissue and maintaining hepatocyte function over a long period (around 1 month). Further, HBTO is expected to be useful in recapitulating chronic liver injury caused by hepatocyte damage, such as cholestasis. Currently, we are constructing a hepatocyte damage induced chronic liver injury model, by incorporating Kupffer and stellate cells, which play key roles in liver disease, in HBTO.

3.3. *Vascularization of liver organoids*

Vascularization of the liver organoid is crucial for increasing organoid size and for maintaining their functionality over long periods of time. When organoids made from a mixture of hepatoblast-like cells, mesenchymal cells, and vascular endothelial cells made from hiPSCs are transplanted into the mouse brain, a vascular network with blood perfusion is generated. However, *ex vivo* organoids with perfusable vasculature remain to be

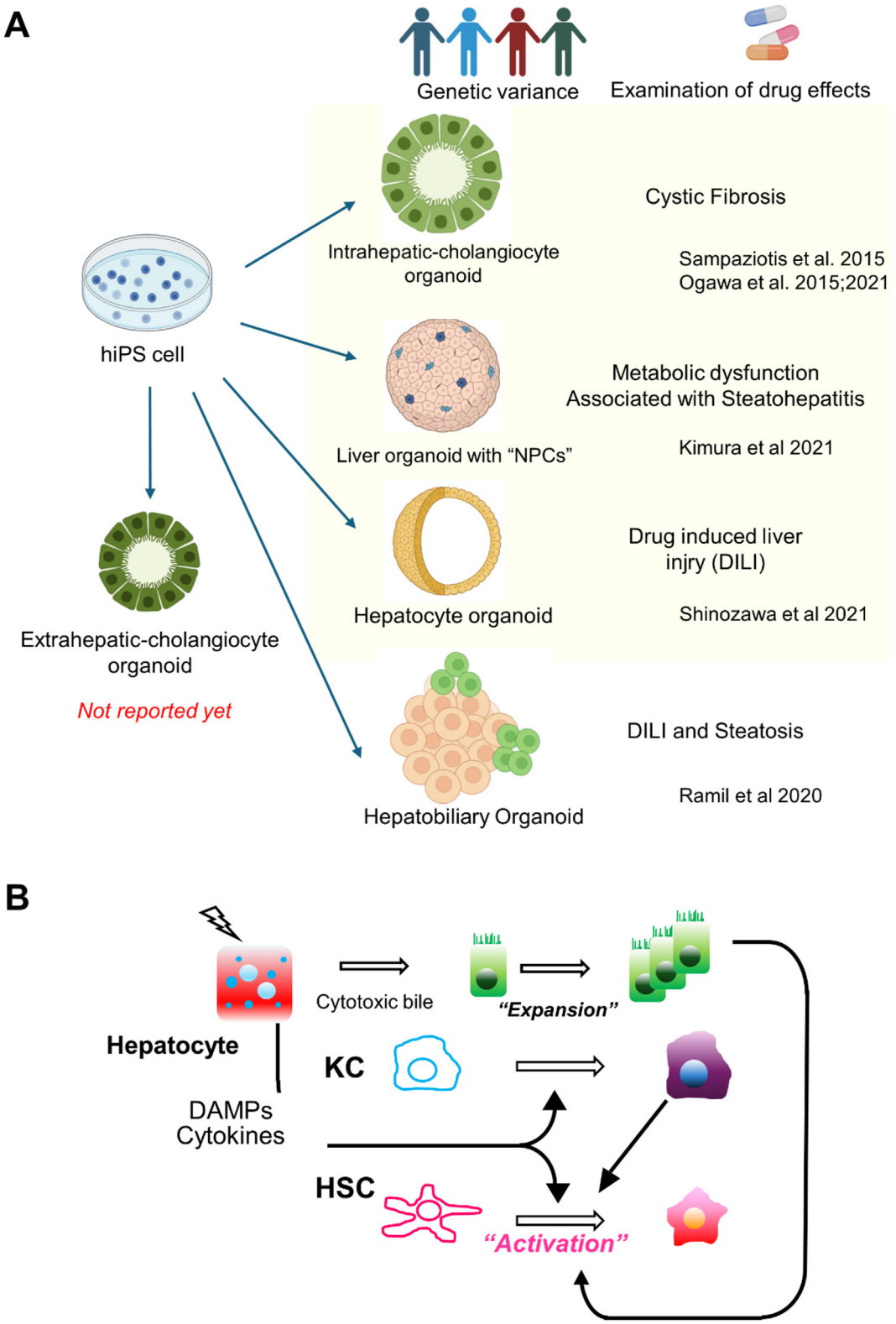

Fig. 8. Creating liver organoid models of various hepatic diseases. (A) Human induced pluripotent stem cells (hiPSCs) have been used to generate intrahepatic cholangiocyte and hepatocyte organoids (both are cysts) and multicellular liver organoids containing hepatocytes, Kupffer cells, HSCs, and LSECs. Cholangiocyte cysts and multicellular liver organoids generated from patient-derived hiPSCs can be used as liver organoid disease models reflecting patients' genetic background. hiPSC-hepatocyte organoids can be used in drug screening, even though they lack bile canalicular network. hiPSC-hepatobiliary organoids have bile canaliculi formed by hepatocytes (which connect to the lumen of cholangiocyte cysts). However, extrahepatic cholangiocyte organoids have not been generated from hiPSCs. Created with BioRender.com. (B) Hepatobiliary organoid recapitulating *in vivo*-like hepatobiliary connection and including Kupffer cells and HSCs are yet to be established from hiPSCs. Cholestasis caused by hepatocyte injuries may affect proliferative capacity of cholangiocytes in multicellular hepatobiliary organoids. Kupffer cells may sense hepatocyte damage and produce secretory factors including inflammatory cytokines, which activates HSCs.

established. Thereby, even if they have potential to connect via vasculature with the host system, it is difficult to control vascularization within the organoid after transplantation.

Organoids associated with perfusion systems have been developed using various types of culture devices. Ya et al. developed a hexagonal shaped lifelike bionic liver lobule chip (LLC). In the LLC, culture medium enters via the "PV" and "HA (hepatic artery)" channels, which then flows through the vascular network—called "a perfusable liver sinusoidal network" by the authors—and leaves via the "CV (central vein)" channel. Hepatocytes are lined along vasculature-like structures [122]. Further, the "liver-on-chip" system—in which hepatocyte spheroid is placed in a v-bottom microwell and medium flows above the spheroid—improves hepatic function [123]. Furthermore, a microfluid device with a large vessel-like structure with hepatocyte spheroids placed on both sides and medium perfused over them also exists. In this system, medium perfusion can increase hepatic functions [124]. However, in the above microfluid device systems, the culture medium flows around spheroids and not inside them. To circumvent this limitation, HepG2 was plated on a permeable membrane and the medium could flow through the cell layer. Such systems enhance and maintain HepG2 hepatic functions [125]. Further, a micropattern plate was used to generate a "vascular bed" (between two main vessels by sprouting) on which a human hepatocyte spheroid was cultured. Endothelial luminal structures were observed inside the spheroid, and electron-microscopy data showed the "space of Disse-like" structure between hepatocytes and endothelial cells. In addition, FITC-Dextran flowed between two main channels via vasculatures incorporated in the spheroid. However, it was not clear whether vasculature inside the hepatocyte organoid is perfusable [126]. Clément Quintard C et al. generated a large vasculature system using HUVEC connected to mesenchymal organoids. Vasculature hierarchy comprising large blood vessels to small blood vessels was observed. Further, they clearly showed that microbeads flow through this vascular network. As demonstrated, in vascularized hiPSC-pancreatic islets, this approach may be applicable in generating perfusable liver organoids [127].

3.4. Disease modeling with liver organoids

The most prominent feature of liver organoid is its ability to better mimic the structure and function of the liver tissue, compared to that by conventional 2D and spheroid cultures. Thus, hepatic diseases induced by morphological abnormalities can be modeled using liver organoids. In addition, given that absorption, secretion, and transport of substances metabolized in the liver are directional processes *in vivo*, the *in vivo*-like tissue structure of organoids is an appropriate feature applicable in drug screening and disease modeling.

Several groups have established cholangiocyte organoids using primary cholangiocytes and pluripotent stem cells, which have a "cystic" structure [128]These organoids are useful in understanding biliary diseases that induce abnormal expansion of biliary tubules. The *Cystic Fibrosis Transmembrane Conductance Regulator* (*CFTR*) gene encodes an ion channel responsible for chloride and water secretion. *CFTR* mutations cause cystic fibrosis (CF), affecting the airway and pancreas commonly, while the bile ducts are affected in about 10% of patients [129]. Functions of CFTR, coupled with anion exchanger 2 (AE2) and aquaporin, eventually affect bicarbonate ion and water transport in the liver. The CF phenotype manifests as the expansion of the apical lumen of tubular structures followed by extensive fibrosis. Verstegen et al. generated extrahepatic cholangiocyte organoids from cells derived from CF patients' biopsy samples [130] to model CF. However, Sampaziotis et al. and Ogawa et al. demonstrated that intrahepatic cholangiocyte organoids derived from CF patients' iPSC (*ΔF508*) could be applicable to evaluating the therapeutic potential of candidate drugs [112–114]; CF-iPSC-derived intrahepatic cholangiocyte organoids do not swell in response to forskolin (cAMP activator). The CFTR corrector VX809 corrects the misfolding of CFTR by acting as a chaperone. VX809 restores CFTR function in CF-iPSC-derived intrahepatic cholangiocyte organoids to levels similar to that in organoids derived from healthy hiPSCs [114]. Furthermore, the improvement of CFTR functionality in CF-iPSC-derived intrahepatic cholangiocyte organoids was achieved by combining CFTR correctors (VX809 or Corr-4a) with the CFTR potentiator VX770 (which enhances CFTR function) [113]. Thus, they suggested that these iPSC-derived intrahepatic cholangiocyte organoids could be a potential tool in drug development targeting intrahepatic cholangiopathy.

Structural and functional abnormalities in the biliary system (which comprises bile canaliculi, IHBD, EHBD, and GB) (Fig. 2A) lead to obstructed bile flow, and results in "cholestasis." Among many types of cholestatic liver injury, drug-induced liver injury (DILI)—which is an adverse reaction to pharmaceutics intake—is a big issue for clinics and the pharmaceutical industry [131]. Most of the oral pharmaceutics are taken up by hepatocytes (via transporter proteins in sinusoids, including the organic cation transporter family) and then metabolized by cytochromes P450 (CYP) family (phase 1) enzymes. Subsequently, to increase metabolite solubility, these metabolites are conjugated with glucuronic or sulfonic acid by phase 2 enzymes. Eventually, these soluble drug metabolites are pumped out into the sinusoid or bile canaliculi via ATP-binding cassette family proteins. Therefore, drugs and/or their metabolites with high cytotoxicity or ability to inhibit bile acid transporters damage hepatocytes.

During drug development, DILI injury is assessed in hepatocyte culture. Considering interspecies differences and highly variable susceptibility among individuals, a high throughput assay system for DILI is desirable for evaluating drug toxicity. Further, organoids with appropriately expressed hepatocyte polarity and bile canaliculi structure, are necessary to predict DILI. Shinozawa et al. developed the liver organoid-based toxicity screen (LoT) [132]. The high throughput LoT assay platform (consisting of 384 plates) uses live-imaging techniques to monitor cell viability, bile acid uptake, and bile excretion into a central lumen surrounded by hepatocytes. They confirmed that drugs known to induce DILI cause cholestasis in liver organoids by inhibiting bile salt export pump (BSEP, ABCB11) activity. Genotype-specific susceptibility to drugs can be studied by generating liver organoids generated from hiPSCs derived from multiple donors and using LoT assay system. Thus, this type of liver organoid enables creation of high throughput drug screening systems, even though hepatocyte polarity is different from that in liver tissue.

The LoT assay may be useful in large scale screening of drugs for their cholestasis inducing ability. Moreover, given that hepatocytes secrete bile into bile canaliculi, which is then transported to the bile duct, a liver organoid comprising *in vivo* tissue structures is suitable to assess cholestatic injury *in vitro*. Ramli et al. generated hepatobiliary organoids—comprised hepatocyte spheroids connected to cholangiocyte cystic structures—from hiPSC and treated them with troglitazone (a BSEP inhibitor) [120]. Bile accumulated in the hepatobiliary organoid. Genetic cholestatic diseases such as progressive familial intrahepatic cholestasis may be studied using *in vitro* pathological liver organoid models generated from patient iPSC or genome-edited hiPSC.

Hepatocytes are the main cell type affected in liver injury. However, various "non-parenchymal cells (NPCs)" are involved in the pathogenesis of acute and chronic liver injury. Therefore, when generating liver organoid models of chronic liver injury, NPCs (such as hepatic stellate cells (HSCs), Kupffer cells (KCs) and LSECs) must

be incorporated into the organoid. HSCs are fibroblasts that show myofibroblast features in injured liver and produce fibrous collagen while KCs are liver resident macrophages. Non-alcoholic fatty liver disease (NAFLD) is a major widespread chronic liver disease [133] Continuous progression of NAFLD leads to non-alcoholic steatohepatitis (NASH), cirrhosis, culminating in hepatic carcinoma. Recently, new terms for steatosis liver disease—metabolic dysfunction-associated steatotic liver disease (MASLD) and metabolic dysfunction-associated steatohepatitis (MASH)—have been proposed [134]. Currently, the pathogenic mechanisms causing MAFLD are unclarified and treatment options are absent. There is an urgent need to understand MAFLD pathogenesis to develop new therapies. Several groups have reported hiPSCs derived multicellular organoids which recapitulates MAFLD progression. To recapitulate disease progression *in vitro*, Ouchi et al. generated multicellular liver organoids containing KCs and HSCs [135]. With fatty acid treatment, these organoids showed steatohepatitis phenotypes, including lipid accumulation, inflammation, and fibrosis [136]. They developed a genetically diverse population organoid panel (PoP) for screening MASH genetic factors by generating liver organoids from 24 donor iPSC lines in a single well. They performed en masse liver organoid genotyping of these 24 organoids. They found that presence of a glucokinase regulatory protein gene variant—(*GCKR*)-rs1260326:C > T single nucleotide polymorphism (SNP) variant—implicated in MASH, increases de novo lipogenesis in the organoids. Thus, they highlighted that PoP panels facilitate genome-wide association study of MASH/MASLD. The PoP method is riddled with difficulties in obtaining reliable liver organoids using multiple donor iPSCs in a well. However, it is expected that this high throughput screening system will unravel mechanisms of human MASH and help in the discovery of novel MASH treatment targets.

In summary, organoids are emerging as powerful tools for studying congenital diseases and in drug screening. Currently, liver organoids and intrahepatic cholangiocyte organoids can be derived from hiPSCs in addition to primary human cells, enabling their application in disease modeling and drug screening. Such organoids also reflect the individual patient differences enabling personalized treatment. However, hiPSC-extrahepatic bile duct organoids have not been reported yet (Fig. 8). Generally, the hiPSC-organoid is generated by mimicking the developmental process. Given that biliary atresia (BA) is a neonatal cholangiopathy characterized by the obstruction of EHBD in the neonatal period, extrahepatic biliary organoids derived from a patient-derived hiPSC will enable understanding the pathogenesis of BA development and progression and help develop new diagnostic and treatment methods.

4. Conclusion

Liver organoid technologies enable us to generate *ex vivo* miniature physiologically functional liver tissues. In particular, human liver organoids can be generated using hiPSCs by recapitulating the *in vivo* developmental processes. However, creating *in vivo*-like multicellular human hepatic organoids with bile canalicular network, tubular bile ducts, and sinusoids in exact special orientation remains challenging. There are two approaches in generating (functionally and structurally) *in vivo*-like multicellular liver organoids: 1) The first approach is recapitulating organogenesis. This approach induces endodermal and mesodermal cells simultaneously and creates liver organoids comprising hepatocytes, HSC-like, and KC-like cells; and 2) The second approach involves coculture of spheroids derived from different origin. For instance, an organoid containing the liver, extrahepatic biliary bile duct, and pancreas was generated by coculturing hiPSC-anterior and posterior-endoderm spheroids [137,138]. However, these approaches have not been able to control functional connections of neighboring epithelial tissues.

In this review we aim to bridge the gap in the current knowledge on hepatic development and the methods used for establishing liver organoid systems. At present, information from liver development studies allows us to directedly induce various hepatic cells, including hepatocytes, cholangiocytes, HSCs, LSECs, and KCs, *in vitro*. In contrast, we still rely on “self-organization” of cells and tissues when creating *in vivo*-like tissue structures, *in vitro*. In depth knowledge of molecules involved in liver organogenesis will identify factors essential for tissue morphogenesis. This knowledge will help us directedly induce (functionally and structurally) *in vivo*-like multicellular liver organoids, without solely depending on *in vitro* tissue self-organization.

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Author contribution

A.O., A.K. and NT: wrote the manuscript; A.O. and N.T.: reviewed the manuscript.

Declaration of Generative AI and AI-assisted technologies in the writing process

Statement: During the preparation of this work, the authors used Grammarly to improve readability and language. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the publication's content.

Declaration of competing interest

The authors declare no conflicts of interest in association with this study.

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