

# Hepatobiliary Organoids and Their Applications for Studies of Liver Health and Disease: Are We There Yet?

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Organoid culture systems have emerged as a frontier technology in liver and biliary research. These three-dimensional (3D) cell cultures derived from pluripotent and adult hepatobiliary cells model organ structure and function. Building on gastrointestinal organoid establishment, hepatobiliary organoid cultures were generated from mouse leucine-rich repeat-containing G-protein-coupled receptor 5-positive liver progenitor cells. Subsequently, 3D hepatobiliary organoid cultures were developed from hepatocytes and cholangiocytes to model human and animal hepatobiliary health and disease. Hepatocyte organoids have been used to study Alagille syndrome, fatty liver disease, Wilson disease, hepatitis B viral infection, and cystic fibrosis. Cholangiocyte organoids have been established to study normal cholangiocyte biology and primary sclerosing cholangitis and to test organoid potential to form bile ducts and gallbladder tissue *in vitro*. Hepatobiliary cancer organoids, termed tumoroids, have been established from frozen and fresh human tissues and used as a drug-testing platform and for biobanking of cancer samples. CRISPR-based gene modifications and organoid exposure to infectious agents have permitted the generation of organoid models of carcinogenesis. This review summarizes currently available adult cell-derived hepatobiliary organoid models and their applications. Challenges faced by this young technology will be discussed, including the cellular immaturity of organoid-derived hepatocytes, co-culture development to better model complex tissue structure, the imperfection of extracellular matrices, and the absence of standardized protocols and model validation. (HEPATOLOGY 2021;74:2251-2263).

Organoids are defined as three-dimensional (3D) structures grown *in vitro* from stem/progenitor cells that consist of organ-specific cell types that self-organize.<sup>(1)</sup> These “organ-in-a-dish” systems model tissue-specific composition, architecture, and function and allow more physiological spatial cell organization and polarization than two-dimensional cell lines, many of which are cancer-derived. The organoid technology has evolved in parallel with the definition of mechanisms of tissue development. To mimic the progenitor cell niche, organoids are cued with cytokines

and growth factors and are cultured in an extracellular matrix (ECM).<sup>(2)</sup>

The liver is a vital organ with numerous functions, including detoxication, digestion, and metabolism,<sup>(3)</sup> provided by parenchymal and nonparenchymal liver cells organized into functional units, termed lobules. Parenchymal cells are derived from the endodermal hepatic diverticulum and are represented by hepatocytes and biliary epithelial cells. Biliary cells line intrahepatic bile ducts (IHBDs) and extrahepatic bile ducts (EHBDs), cystic ducts, and the gallbladder (Fig. 1).

*Abbreviations:* 3D, three-dimensional; A1AT, alpha-1 antitrypsin; CCA, cholangiocarcinoma; CFTR, cystic fibrosis transmembrane conductance regulator; COMMD1, copper metabolism domain-containing 1 gene; ECM, extracellular matrix; EHBD, extrahepatic bile duct; EpCAM, epithelial cell adhesion molecule; FFA, free fatty acid; GGT, gamma-glutamyl transferase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; Hnf, hepatocyte nuclear factor; IHBD, intrahepatic bile duct; KRT, keratin; LGR5, leucine rich repeat containing G-protein-coupled receptor 5; SOX, sex-determining region Y-box transcription factor.

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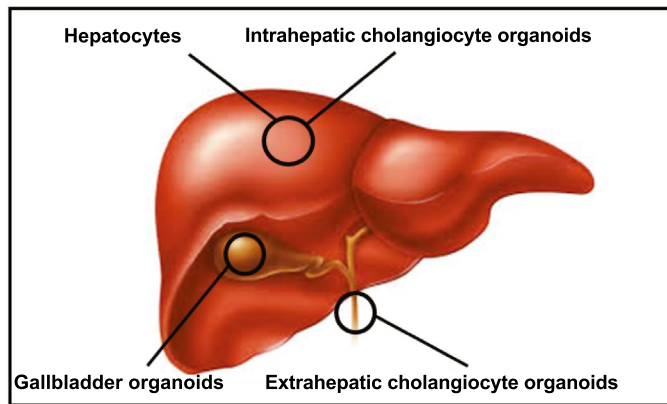
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**Applications:**

- Hepatobiliary biology
- Disease modeling: Alpha-1 antitrypsin deficiency, Wilson's disease, hepatic steatosis, primary sclerosing cholangitis, hepatobiliary cancers, viral hepatitis B, cystic fibrosis
- Regenerative medicine: cell transplantation, gene therapy (Wilson's disease)
- Personalized medicine:
  - Drug testing platforms
  - Animal cancer avatars (xenografts)
  - Biobanking

**Pluripotent stem cell-derived organoids:**

- Induced pluripotent stem cell-derived
- Embryonic stem cell-derived

**Adult tissue-specific stem cell-derived organoids:**

- Bipotential ductal cell-derived
- Hepatocyte-derived
- Cholangiocyte-derived
  - Intrahepatic
  - Extrahepatic
- Gallbladder-derived

**Challenges:**

- Hepatocyte maturation and large-scale production
- Human issue access
- Suboptimal extracellular matrices
- Co-culture with other cell types
- Standardization of generation and validation
- Undetermined safety for liver regeneration

**FIG. 1.** Human hepatobiliary organoid classification, tested applications, and challenges. The hepatocyte organoid image is reprinted with permission from Elsevier.<sup>(24)</sup>

Hepatobiliary tissues have varying developmental processes. Embryonic hepatoblasts are the primary progenitor cells for the developing liver, yielding both hepatocytes and IHBD cholangiocytes. In contrast, EHBD cells and large IHBD cells originate from the ventral pancreatic region.<sup>(3,4)</sup> The gallbladder is derived from a hepatic diverticulum close to the ventral pancreatic bud and the developing EHBDs. Hepatobiliary organs also include nonparenchymal cells, such as resident macrophages, stromal fibroblasts (e.g., stellate cells and portal fibroblasts in the liver), and endothelial cells.

Regenerative capacity in the adult liver is astonishing. At homeostasis, less than 2% of hepatocytes

and biliary cells are proliferating.<sup>(3,5-7)</sup> However, after injury, a robust proliferative response is induced to regenerate both hepatocytes and cholangiocytes. After partial hepatectomy in mice and rats, the liver regains its original size by 3-7 days.<sup>(8)</sup> In humans, after 60% liver resection, the organ achieves 75% of its original size in 10 days, which is sufficient to normalize liver biochemistry results.<sup>(9)</sup> This response is especially intriguing because no resident progenitor cells have been identified in the adult liver. Recent studies using unbiased lineage-tracing approaches in genetic mouse models have shown that hepatocytes in all liver zones participate in slow cell replacement during

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homeostasis and rapid replacement after injury.<sup>(5-7)</sup> This contrasts with earlier *Axin2*-Cre lineage-tracing studies that suggested only pericentral vein region hepatocytes support liver regeneration after injury.<sup>(10)</sup>

Biliary tree progenitor cells, termed oval cells in rodents,<sup>(11)</sup> located in the canal of Hering are thought to be an additional pool of multipotent progenitors capable of giving rise to both hepatocytes and cholangiocytes to maintain adult tissues.<sup>(12)</sup> Oval cells are WNT-responsive and express the R-spondin receptor leucine rich repeat containing G-protein-coupled receptor 5 (LGR5), which amplifies wingless-type MMTV integration site family (WNT) signaling.<sup>(13,14)</sup> Regeneration of large IHBDs and EHBDs during normal tissue turnover and after injury is mainly attributed to progenitor-like cells residing in peribiliary glands.<sup>(15)</sup> Importantly, it has been shown that mature adult hepatocytes and cholangiocytes have plasticity to transdifferentiate upon injury to regenerate liver tissue.<sup>(16-18)</sup> Identification of cellular mechanisms driving liver regeneration has relied on genetically engineered mouse models, with some variable findings depending on the extent of injury. Defining mechanisms of human hepatobiliary regeneration will likely require complex human organoid models. It is possible that specific regenerative mechanisms vary depending on the type and extent of injury.

When liver function cannot be restored, the single option for patient survival is transplantation. There is a vigorous search for cell-based technologies that will provide alternatives for liver transplantation. Furthermore, there is a need for better cell-culture models of human hepatobiliary tissues to study normal tissue function and the mechanisms of disease. The organoid technology, which is based on principles of organ development and repair, has become an exciting *in vitro* model system to answer questions about human liver disease and repair (Fig. 1).

## Classification of Hepatobiliary Organoids

Organoids can be derived from pluripotent stem cells<sup>(1)</sup> or from adult organ-specific stem/progenitor cells<sup>(19)</sup> (Fig. 1). Pluripotent stem cells include human embryonic stem cells and induced pluripotent stem cells.<sup>(1)</sup> Although no professional stem cells have been identified in the liver, hepatobiliary organoids

have been successfully generated from the adult liver (Table 1), bile ducts, and gallbladder (Table 2) using biopsy specimens, surgical samples, and bile and biliary brushings. These organoids have also been generated from numerous mammalian species, including humans, mice, rats, cats, and dogs. Their sources are likely facultative progenitor cells.

Differentiation of pluripotent stem cells into liver cells is a step-wise process recapitulating stages of embryonic liver development, involving commitment into the endodermal lineage, differentiation into hepatoblasts, and eventual hepatocyte-like cell development.<sup>(20)</sup> It permits generation of multilineage human organoid models simultaneously containing hepatocyte, stellate, and Kupffer-like cells<sup>(21)</sup> or hepatic cells with mesenchymal stem cells and umbilical vein endothelial cells.<sup>(22)</sup> There are multiple sources for human pluripotent stem cells,<sup>(23)</sup> including embryonic cells and skin fibroblasts, as well as commercial sources, making them more accessible than adult hepatobiliary tissues. Despite several advantages, pluripotent stem cell-derived liver organoids are generally immature and difficult to culture over the long term, which can make drug and functional studies problematic.<sup>(24)</sup>

This review focuses on adult tissue-derived hepatobiliary organoid models and their applications (Fig. 2). It also discusses the challenges of this young and rapidly evolving technology and its future directions.

## Hepatobiliary Organoid Models and Their Application

### HEPATOCTYTE ORGANOID MODELS

The discovery that WNT is a crucial signal for stem cell maintenance in epithelial tissues became a scientific rationale for contemporary organ-in-a-dish technology.<sup>(25)</sup> Hans Clevers' group reported in 2009 the long-term culture of mouse intestinal LGR5<sup>+</sup> stem cells in ECMs that assemble into 3D structures of progenitor and differentiated intestinal cells, organized into crypt- and villus-like compartments.<sup>(26)</sup> The key was defining the niche factors required to maintain stem-cell function. For the intestine, this included WNT3a, R-spondin-1, EGF, and Noggin. Subsequently, human organoids have been generated

**TABLE 1. Selected Hepatocyte Organoid Models, Their Validation, and Applications**

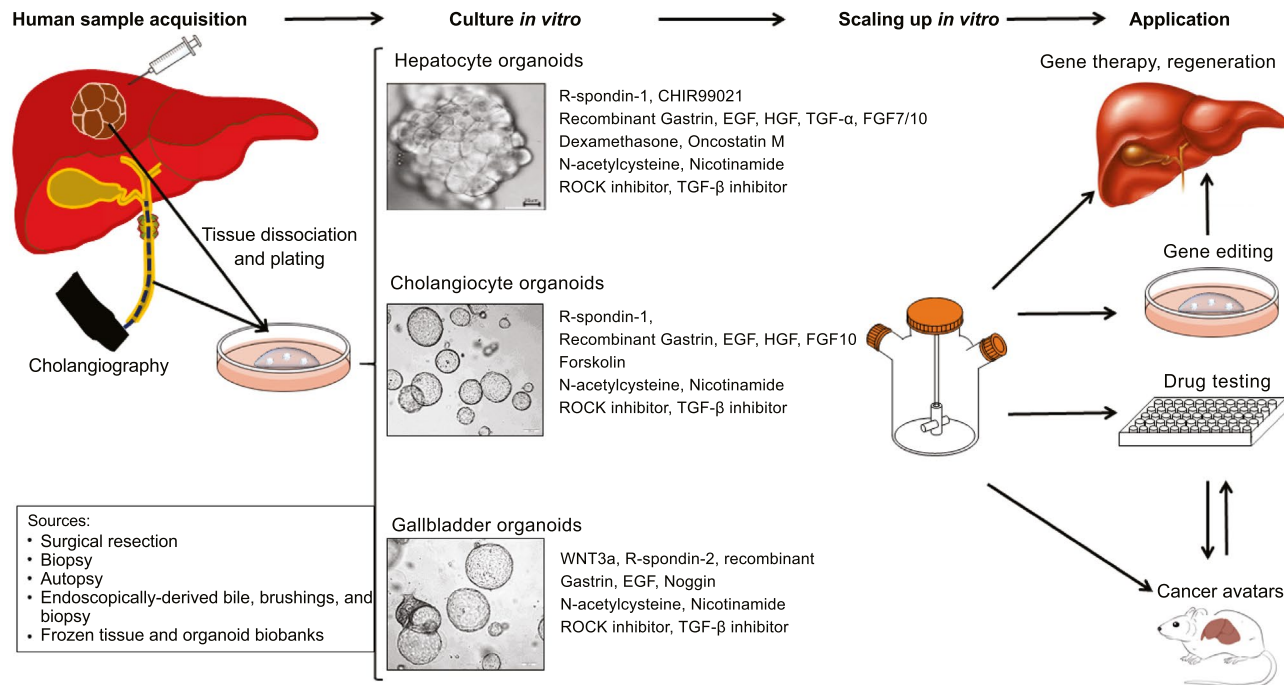
Cellular Source	Validation	Applications	References
Human and mouse LGR5 <sup>+</sup> /EpCAM <sup>+</sup> bipotential liver cells	<ul style="list-style-type: none"> <li>IHC and mRNA abundance of progenitor, epithelial, hepatocyte, biliary, and proliferation markers</li> <li>Functional studies for albumin secretion, LDL uptake, cytochrome P450 activity, glycogen storage, rhodamine-123 transport, bile acid production, and ammonia elimination</li> </ul>	Disease modeling (A1AT), Alagille syndrome, hepatitis B viral infection, tumorigenesis, regenerative medicine, gene therapy, large-scale hepatocyte-like organoid production	Huch et al, <sup>(28,29)</sup> Schneeberger et al, <sup>(33)</sup> De Crignis et al <sup>(53)</sup>
Primary human hepatocytes and mouse AXIN2 <sup>+</sup> hepatocytes	<ul style="list-style-type: none"> <li>IHC and mRNA abundance of progenitor, epithelial, hepatocyte, biliary, and proliferation markers</li> <li>Functional studies for albumin secretion, LDL uptake, cytochrome P450 activity, glycogen storage, and rhodamine-123 transport</li> </ul>	Liver regeneration potential	Hu et al, <sup>(24)</sup> Peng et al <sup>(31)</sup>
Commercially purchased primary mature human hepatocytes	<ul style="list-style-type: none"> <li>IHC and mRNA abundance of progenitor, epithelial, hepatocyte, biliary, and proliferation markers</li> </ul>	Large-scale hepatocyte-like organoid production	Garnier et al. <sup>(34)</sup>
Primary human liver cancer	<ul style="list-style-type: none"> <li>Histological and genetic correlation with primary tumors</li> </ul>	Xenograft studies, drug screening, biobanking	Broufrier et al, <sup>(56)</sup> Nucifora et al, <sup>(57)</sup> Saito et al, <sup>(58)</sup> Li et al <sup>(63)</sup>
Cat and dog bipotential liver cells	<ul style="list-style-type: none"> <li>IHC and mRNA abundance of progenitor, epithelial, hepatocyte, biliary, and proliferation markers</li> <li>Functional studies for albumin secretion, AST and ALT levels, cytochrome P450 activity, and glycogen storage</li> </ul>	Disease modeling (hepatic steatosis, Wilson disease), gene therapy	Nantasaniti et al, <sup>(49)</sup> Kruitwagen et al. <sup>(50,51)</sup>

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; IHC, immunohistochemistry; LDL, low-density lipoprotein.

TABLE 2. Selected Bile Duct and Gallbladder Organoid Models, Their Validation, and Tested Applications

Cellular Source	Validation	Application	References
Primary human liver cells, EHBD cholangiocytes and gallbladder	<ul style="list-style-type: none"> <li>IHC and mRNA abundance of progenitor, epithelial, hepatocyte, and biliary markers</li> <li>Functional studies for rhodamine-123 and choly-L-lysyl-fluorescein transport and GGT and ALP activity</li> <li>Secretory response to forskolin</li> </ul>	Bile duct bioengineering, regenerative medicine, cystic fibrosis	Sampaziotis et al, <sup>(36)</sup> Tysoe et al, <sup>(37)</sup> Versteegen et al <sup>(54)</sup>
Human bile-derived cells	<ul style="list-style-type: none"> <li>IHC and mRNA abundance of progenitor, epithelial, hepatocyte, biliary, and immune markers</li> <li>Functional studies for rhodamine-123 transport and GGT and ALP activity</li> </ul>	Disease modeling (primary sclerosing cholangitis)	Soraka et al <sup>(38)</sup>
Human fetal gallbladder	<ul style="list-style-type: none"> <li>IHC and mRNA abundance of progenitor, epithelial, hepatocyte, biliary, and proliferation markers</li> </ul>	Regenerative medicine	Manohar et al <sup>(46)</sup>
Mouse bile ducts	<ul style="list-style-type: none"> <li>IHC and mRNA abundance of progenitor, epithelial, hepatocyte and biliary markers</li> <li>Functional studies for Rhodamine 123 transport and response to FXR agonists</li> </ul>	Bile duct bioengineering	Chen et al <sup>(40)</sup>
Mouse EHBD cholangiocytes	<ul style="list-style-type: none"> <li>IHC and mRNA abundance of progenitor, epithelial, hepatocyte, biliary, and proliferation markers</li> </ul>	Genetic models of biliary cancer and disease modeling	Nakagawa et al, <sup>(43)</sup> Shiota et al <sup>(44)</sup>
Mouse gallbladder	<ul style="list-style-type: none"> <li>IHC and mRNA abundance of progenitor, epithelial, hepatocyte, and biliary markers</li> </ul>	CRISPR-Cas9-assisted mutagenesis, carcinogenesis, and regenerative potential	Lugli et al, <sup>(47)</sup> Scanu et al, <sup>(64)</sup> Erfangga et al <sup>(65)</sup>

Abbreviations: ALP, alkaline phosphatase; FXR, Farnesoid X-activated receptor; IHC, immunohistochemistry.



**FIG. 2.** Human hepatobiliary organoid translational workflow. Abbreviation: ROCK, Rho associated coiled-coil containing protein kinase.

from normal and diseased tissues, including hepatobiliary, gallbladder (Tables 1 and 2), stomach, esophagus, pancreas, brain, lung, heart, urinary bladder, mammary gland, and fallopian tube tissues.<sup>(27)</sup>

## Taking a Step From Mouse to Human Hepatocyte Organoids

Hepatobiliary organoids were established in 2013<sup>(28)</sup> from mouse LGR5<sup>+</sup> progenitor-like oval cells that appear in the portal triad area after liver injury and were cultured in the presence of WNT3a, R-spondin-1, EGF, HGF, FGF10, and Noggin.<sup>(28,29)</sup> The resulting organoids were bipotential, forming both hepatocyte and cholangiocyte lineages. Hepatocyte differentiation of these mouse bipotential organoid cells could be induced by removal of R-spondin-1 and HGF, treatment with Notch and TGF- $\beta$  pathway inhibitors to lessen stemness, and the addition of FGF, bone morphogenetic protein 7 (BMP7), EGF, and dexamethasone to promote differentiation.<sup>(28,29)</sup> Hepatocytes formed in this mouse organoid model were binucleated and expressed mature hepatocyte markers and function, evidenced by low-density

lipoprotein uptake, albumin secretion, glycogen accumulation, and induction of the cytochrome P450 system.<sup>(28-30)</sup> However, these cultures still included cells expressing the ductal cell marker keratin 19 (KRT19). Interestingly, transplantation of these organoids into the injured livers of mice with fumarylacetoacetate hydrolase gene double knockout promoted further hepatocyte maturation, evidenced by induction of the hepatocyte nuclear factor 4 alpha gene (*Hnf4a*) and loss of the cholangiocyte marker *Krt19*.<sup>(29)</sup>

Human liver organoids were reported in 2015<sup>(29)</sup> and were similar to mouse organoids<sup>(28)</sup> in that they were derived from bipotential epithelial cell adhesion molecule-positive (EpCAM<sup>+</sup>) cells sorted from dissociated human liver and in that they contained both hepatocyte- and cholangiocyte-like cells.<sup>(29)</sup> Inhibition of TGF- $\beta$ , and stimulation of cAMP and WNT signaling were requisite to maintain the organoid cultures over the long term. They could be differentiated toward hepatocyte-containing organoids using culture conditions defined for mouse organoids.<sup>(28,29)</sup> Engraftment of human hepatocyte-like organoids into carbon tetrachloride-injured mouse livers was confirmed by detection of cells marked by human KRT19

and albumin and human albumin and alpha-1 anti-trypsin (A1AT) in mouse blood, signifying the presence of functional transplanted human hepatocytes.<sup>(29)</sup>

More recently, organoids have been established from purified AXIN2<sup>+</sup> mouse hepatocytes, which produced more mature, longer-lasting hepatocyte organoids.<sup>(24,31)</sup> These liver organoids, reported independently by the Nusse<sup>(31)</sup> and Clevers groups<sup>(24)</sup> in 2018, were cultured in media containing CHIR99021<sup>(24,31)</sup> and TNF- $\alpha$ ,<sup>(31)</sup> in addition to R-spondin-1, HGF, EGF, gastrin, and FGF10.<sup>(24)</sup> CHIR99021 is an aminopyrimidine derivative, which potently inhibits glycogen synthase kinase, thus functioning as a WNT activator.<sup>(32)</sup> The inclusion of TNF- $\alpha$  was based on the observation that liver repair involves recruitment of immune cells secreting TNF- $\alpha$ .<sup>(31)</sup> Human fetal and adult primary hepatocyte-derived organoids can also be cultured long-term in this medium, producing grape-like clusters composed of large cells with a low nuclear-cytoplasm ratio and hepatocyte appearance and function.<sup>(24)</sup> However, these human and mouse hepatocyte-like cells differ from mature hepatocytes because they are cycling,<sup>(31)</sup> express the fetal hepatocyte marker alpha fetoprotein and can be transdifferentiated into cholangiocytes upon WNT pathway withdrawal,<sup>(24)</sup> suggesting cell immaturity and plasticity. Exposure to the cytokine oncostatin M<sup>(24)</sup> and dexamethasone<sup>(24,31)</sup> promoted maturation of human<sup>(24)</sup> and mouse<sup>(31)</sup> hepatocyte-like cells, which could be successfully transplanted into injured livers of mice.<sup>(24,31)</sup>

Cell production on a large scale is a challenging prerequisite for regenerative medicine. Bipotential liver organoids produced from biobanked human fresh-frozen hepatocytes<sup>(33,34)</sup> and hepatocytes freshly isolated from patient donors<sup>(33)</sup> have been expanded in suspension in spinner flasks containing Matrigel ECM (Corning).<sup>(33,34)</sup> This increased hepatocyte cell number over a 2-week period by sevenfold compared with static cultures,<sup>(33)</sup> which was attributed to improved oxygenation and more efficient access to growth factors in spinner flasks. Importantly, spinner culture-derived liver organoids could be matured into hepatocytes in the presence of FGF19, BMP7, and dexamethasone.<sup>(33,34)</sup> These differentiated human hepatocytes failed to propagate upon subcutaneous transplantation into immunodeficient mice<sup>(33)</sup> supporting their nontumorigenic properties and potential safety for liver regeneration therapy.

Collectively, these studies in both mice and humans suggest that activation of WNT signaling is crucial for hepatocyte organoid generation.<sup>(24)</sup> Additionally, HGF, a known hepatocyte mitogen, and TNF- $\alpha$  are factors promoting hepatocyte culture success.<sup>(29,31)</sup> When liver organoids are derived from hepatocytes and not from bipotential ductal or oval cells, they contain more functionally mature hepatocytes. In turn, both human and mouse hepatocyte maturation determines cell-engraftment success.<sup>(28,29,35)</sup> Organoid-derived hepatocyte maturity has been assessed by measuring key hepatocyte features, such as bile acid production and transport, maintenance of metabolic homeostasis, and detoxication.<sup>(30)</sup>

## CAN WE BIOENGINEER BILE DUCTS?

The human biliary system includes EHBDs, IHBDs, cystic ducts, and the gallbladder, which have distinct progenitor cell populations. Biliary organoids have been generated from a variety of human tissue sources, including liver biopsy specimens, liver and EHBD surgical samples from live and deceased donors, and cholangiogram-obtained samples<sup>(36-38)</sup> (Table 2). Cholangiocyte organoids are commonly cystic with a large lumen.<sup>(39)</sup> Interestingly, less mature mouse cholangiocyte organoids have been observed to form more branching structures.<sup>(40)</sup>

Human bile ducts were bioengineered in 2017<sup>(36)</sup> from cholangiocyte cell organoids cultured long-term with R-spondin-1, EGF, and Dickkopf WNT signaling pathway inhibitor 1, a WNT signaling antagonist thought to promote biliary maturation.<sup>(36)</sup> The cells demonstrated typical cholangiocyte features, including cilia, organelles crucial for cholangiocyte sensing and signaling; tight junctions; biliary cell markers such as KRT7, KRT19, gamma-glutamyl transferase (GGT), and sex-determining region Y-box transcription factor 9 (SOX9); and alkaline phosphatase and GGT activities. Functionality characteristic for cholangiocytes<sup>(41)</sup> was demonstrated by rhodamine-123 accumulation, confirming the properly functioning multidrug resistance-associated protein 1, bile acid extrusion, and response to secretin with luminal fluid accumulation. These human cholangiocyte organoids formed bile duct-like tubes upon transplantation under the mouse kidney capsule.<sup>(36)</sup> After seeding on polyglycolic acid scaffolds or densified collagen, they

were also shown to repair EHBD and gallbladder defects in immunodeficient mice.<sup>(37)</sup> This provides an important step forward demonstrating that human cholangiocyte organoids can serve as a platform for regenerative needs.

In 2018, bile duct organoids were generated from mouse LGR5<sup>+</sup> bipotential liver cells cultured in media containing gastrin and acetylcysteine.<sup>(40)</sup> These cultures contained cells possessing several features of cholangiocytes, including cilia; *Krt7*, *Krt19*, *Hnf1b*, and aquaporin 1 gene expression; and GGT activity. They exhibited functional transport of small molecules and responded to an Farnesoid X receptor agonist. The cholangiocyte-like organoids could be integrated with hollow, collagen-coated polyethersulfone fiber membranes to bioengineer autologous bile ducts that exhibited polarized bile acid transport activity.<sup>(40)</sup> Mouse studies also identified a clonogenic subpopulation of suppression of tumorigenicity 14–positive cholangiocytes that can proliferate and produce long-term biliary organoids.<sup>(42)</sup> Cholangiocyte-like cell organoids could also be generated from dissociated mouse EHBDs,<sup>(43,44)</sup> expressing markers of mature cholangiocytes (*Krt19*, aquaporin 1) and progenitor cells (*Lgr5*), which have been used to study normal cholangiocyte biology<sup>(44)</sup> and EHBD cancer.<sup>(43)</sup>

A major advance was reported in 2018 when Soroka et al.<sup>(38)</sup> showed that human organoid cultures could be generated from cholangiogram-obtained bile, thereby overcoming the limitation of invasive access to human biliary tissue. These bile-derived organoids were cultured long-term with R-spondin-1, EGF, HGF, gastrin, and FGF10 and exhibited cyst-like morphologic characteristics. They expressed typical cholangiocyte markers, including EpCAM, SOX9, and KRT19, and demonstrated rhodamine-123 transport and GGT activity. This approach takes advantage of a common procedure for patients with cholangiopathies and indicates the presence of cells with progenitor properties in bile.

It was recently shown that human IHBD and EHBD organoids generated from dissociated liver or EHBD tissue and cultured long-term in medium<sup>(45)</sup> containing R-spondin-1, EGF, HGF, and gastrin had a similar gene-expression profile. However, only IHBD, and not EHBD, organoids showed potential to differentiate into hepatocyte-like cells when exposed to hepatocyte-differentiation medium,<sup>(39)</sup> as evidenced by induction of albumin, cytochrome P450 family 3 subfamily A member 4, and HNF4A.<sup>(45)</sup>

This suggests an intrinsic IHBD-specific cell plasticity to regenerate liver tissue that is not contained in EHBD-derived cells.

## GALLBLADDER ORGANOID

Human fetal gallbladder has been shown to contain clonogenic EpCAM<sup>+</sup>/cluster of differentiation 44–positive (CD44<sup>+</sup>)/CD133<sup>+</sup> cells, which, when embedded in Matrigel and stimulated with R-spondin-2, WNT3a, EGF, and Noggin, form organoids that can engraft subcutaneously in mice.<sup>(46)</sup> It was also shown that cultured human fetal EpCAM<sup>+</sup> gallbladder cells and EpCAM<sup>+</sup> IHBD cells express distinctive transporters<sup>(46)</sup> (Table 2), suggesting that organoids from different parts of the biliary tree cannot substitute for each other in pharmacotoxicology and biology studies.

To take advantage of tissue accessibility, the gallbladder has also been studied as a source for hepatocyte generation.<sup>(47)</sup> Upon removal of growth factors and nicotinamide, mouse gallbladder organoids differentiate toward a hepatocyte fate and can be used for liver transplantation when injected under the liver capsule or through the mesenteric vein in mice<sup>(47)</sup> (Table 2). The transdifferentiation potential underscores the exceptional cellular plasticity of hepatobiliary cells and identifies another more readily available source of cells for regenerative purposes.

## CAN ORGANOID MODEL LIVER DISEASES?

### A1AT Deficiency

An organoid model of human liver disease was reported in 2015 using liver biopsies from patients with A1AT deficiency.<sup>(29)</sup> These hepatocyte-like, A1AT-deficient organoids accumulate intracellular A1AT aggregates, and their supernatants exhibited a decreased ability to block neutrophil elastase activity, the functional defect in patients with A1AT deficiency. A1AT-deficient organoids also demonstrated endoplasmic reticulum stress and increased apoptosis, suggesting mechanisms of the patient liver pathologic condition.

### Alagille Syndrome

Mutations in the genes encoding JAGGED-1 and NOTCH2 underlie the pathologic mechanism in Alagille syndrome, which is characterized by the



inability to form functional bile ducts, leading to biliary atresia. Huch et al.<sup>(29)</sup> reported in 2015 that undifferentiated bipotential organoids generated from liver biopsy specimens from patients with Alagille syndrome failed to up-regulate biliary markers when cued with cholangiocyte-differentiation media, suggesting that these organoids are a valid model of human liver morphogenesis. Similar findings were observed with bile duct-derived organoids from a genetic mouse model of Alagille syndrome.<sup>(48)</sup>

## Wilson Disease

The Bedlington Terrier dog breed has a defect in the copper metabolism domain-containing 1 gene (*COMMD1*), leading to hepatitis with liver fibrosis due to copper accumulation, modeling human Wilson disease. Hepatocyte-like organoids from these *COMMD1*-deficient dogs, reported in 2015, demonstrated increased intracellular copper accumulation, which could be reversed by infection with a lentiviral construct encoding *COMMD1*.<sup>(49)</sup> Autologous organoids with the corrected gene defect transplanted through the portal vein into the dog liver engraft and survive for up to 2 years.<sup>(50)</sup> These studies show the potential of gene therapy by correcting underlying genetic defects in autologous organoids to treat genetic liver diseases.

## Primary Sclerosing Cholangitis

Studies of bile-derived organoids from patients with primary sclerosing cholangitis<sup>(38)</sup> showed suitability to model this multifactorial human disease *in vitro*. These organoids altered expression of genes associated with immune regulation, a heightened inflammatory response to IL-17, and increased expression of the major histocompatibility complex, class II, DM alpha gene and the C-C motif chemokine ligand 20 gene, genes reported to be associated with primary sclerosing cholangitis.<sup>(45)</sup> Future co-culture studies involving primary sclerosing cholangitis organoids, stromal cells, and immune cells would be needed to tease out the mechanisms of cellular crosstalk leading to biliary fibrosis, the underlying pathologic mechanism of this devastating disease.

## Steatosis

Although there are currently no human organoid models of steatohepatitis, IHBD-derived cat liver

organoids have been used to model nonalcoholic fatty liver disease,<sup>(51)</sup> as cats are prone to a severe form of steatohepatitis, feline hepatic lipidosis. Cat hepatocyte-like organoids showed up-regulation of beta-oxidation genes and decreased cell viability upon treatment with free fatty acids (FFAs). This phenotype could be rescued by co-treatment with L-carnitine required for transfer of FFAs across the mitochondrial membrane. Conversely, inhibition of carnitine palmitoyl transferase-1, which blocks transmembrane FFA transfer, exacerbated the phenotype.

## Alcohol-Associated Liver Disease

Wang et al.<sup>(52)</sup> used human hepatocyte organoids co-cultured with mesenchymal cells, both derived from fetal liver tissue, to study nonmonogenic alcohol-associated liver disease. Upon exposure to alcohol, hepatocyte-like cells in these organoid co-cultures developed oxidative stress with increased reactive oxygen species, steatosis with intracellular lipid droplet accumulation, and release of inflammatory mediators. The mesenchymal cells promoted hepatocyte maturation and showed fibrogenic responses to alcohol, as evidenced by up-regulation of the lysyl oxidase-like 2, collagen type I alpha 1 chain, collagen type III alpha 1 chain, actin alpha 2, and *TGF-β1* genes, recapitulating the pathophysiology of alcohol-associated liver disease.

## Hepatitis B Viral Infection

Hepatocyte organoids from livers of healthy donors and patients infected with hepatitis B virus (HBV) were recently shown to model HBV infection and tumorigenesis.<sup>(53)</sup> Exposure of healthy liver organoids to recombinant virus or serum from patients with HBV led to organoid infection and active virus replication. HBV-infected organoids could be used to test anti-HBV drugs and drug toxicity. Transcriptomic analysis of liver organoids from the liver tissue of patients with HBV and nontumor cirrhosis showed the presence of an early cancer-gene signature and therefore showed a potential use of these organoids in cancer biomarker discovery.<sup>(53)</sup>

## Cystic Fibrosis

EHBD organoids have been generated from a patient with a known compound cystic fibrosis

transmembrane conductance regulator (CFTR) gene mutation. These organoids were shown to have a defective secretory response to forskolin because of loss of CFTR-mediated chloride-ion transport.<sup>(54)</sup> This model system could be useful to characterize the molecular defects observed with different *CFTR* mutations.

## ORGANOIDS IN LIVER CANCER RESEARCH

Hepatocellular carcinoma (HCC), cholangiocarcinoma (CCA), and mixed HCC–CCA are the most common primary hepatobiliary cancers, with increasing incidence and poor outcomes worldwide.<sup>(55)</sup> Until recently, *in vitro* models of hepatobiliary cancers were limited to immortalized cancer cell lines established decades ago. Hepatobiliary cancer organoids can provide advanced *in vivo* and *in vitro* models to test drugs and study cancer biology in the era of personalized medicine. Several groups have shown that tumoroids, organoids derived from human hepatobiliary cancer tissue, can be successfully established from all three major hepatobiliary cancer subtypes<sup>(56,57)</sup> and gallbladder cancer.<sup>(58)</sup> The rate of organoid establishment from primary tumor tissue is directly proportional to the proliferative index.<sup>(56,57)</sup> Notably, organoids from healthy tissue contaminants can outgrow tumoroids in standard liver medium.<sup>(29,56)</sup> Thus, tumoroid culture requires distinct media, including withdrawal of R-spondin-1, WNT3a, and Noggin and supplementation with dexamethasone and a rho-kinase inhibitor to decrease apoptosis.<sup>(56)</sup> Culture conditions for HCC, intrahepatic CCA, and mixed HCC–CCA<sup>(29)</sup> are quite similar, which might indicate a similar mechanism of tumorigenesis. Notably, tumoroids from extrahepatic CCA are more difficult to establish,<sup>(58)</sup> suggesting differences between intra- and extrahepatic cancers.

Human tumoroid phenotypes *in vitro* and in mouse xenografts *in vivo* closely resemble the histologic presentation of primary tumors. HCC and mixed HCC–CCA tumoroids exhibit a solid structure without a lumen, and CCA tumoroids form gland-like structures invading the lumen.<sup>(57-59)</sup> The cancer-related somatic genetic variants present in the primary tumors are retained in 84% to 88% of corresponding tumoroids and are preserved in late passages.<sup>(56,57)</sup> Mutations in the catenin beta 1, KRAS proto-oncogene, GTPase (*KRAS*), AT-rich interaction domain 1A (*ARID1A*),

and *ARID2* genes, which are common in hepatobiliary cancers, are observed in tumoroids.<sup>(60,61)</sup> Some genetic aberrations can predict patient outcomes. For example, tumoroids enriched in SOX2 expression were associated with poor cancer prognosis.<sup>(58)</sup>

Cultured human tumoroids and their xenografts can be used to test sensitivity to anticancer compounds to predict clinical outcomes. Accordingly, the EGFR inhibitor erlotinib reduced the viability of a subset of intrahepatic CCA tumoroids independent of *KRAS* or other commonly observed mutations.<sup>(58)</sup> Tumoroid treatment with sorafenib, a Food and Drug Administration–approved multikinase inhibitor for HCC,<sup>(62)</sup> decreased viability of HCC tumoroid lines.<sup>(57)</sup> Screening of drugs for treatment of HCC and intrahepatic CCA tumoroids generated from different parts of the same tumor demonstrated high intratumor drug-response heterogeneity, indicating that a tumor biopsy specimen might not represent whole-tumor responses.<sup>(63)</sup> Tumoroid production from several tumor sites, including metastatic foci, and co-culture with mesenchymal cells and immune cells might help to account for tumor heterogeneity and microenvironment influence.

Organoids from genetically engineered mouse models provide an additional instrument for hepatobiliary cancer research. Exposure of tumor protein p53 gene (*Tp53*) mutant mouse gallbladder organoids to *Salmonella* induced epithelial-cell transformation to carcinoma, even in the absence of immune cells.<sup>(64)</sup> CCA tumoroids generated from mice with epithelial KRAS activation and deletion of the cadherin 1 and TGF- $\beta$  receptor 2 genes<sup>(43)</sup> unveiled peribiliary glands as a source of CCA and demonstrated a role for the alarmin IL-33 in carcinogenesis.<sup>(43)</sup> Mouse gallbladder organoids with loss-of-function mutations in the *Tp53* and phosphatase and tensin homolog genes and activating mutations in *Kras* or erb-b2 receptor tyrosine kinase 2 (*ErbB2*), were studied to show that tumoroid histologic characteristics *in vitro* are dependent on driver oncogenes and to test the antitumor activity of liposomal irinotecan delivery in xenografts in mice *in vivo*.<sup>(65)</sup> Studies of mouse liver tumors identified the tumor-initiating properties of LGR5<sup>+</sup> cells.<sup>(66)</sup>

Collectively, the data demonstrate a high translational potential of cancer-derived organoid models that recapitulate patient tumor histologic characteristics and transcriptional and genetic landscapes and could be used to predict clinical outcomes.

## Challenges and Future Directions

We have witnessed remarkable progress with hepatobiliary organoid technology over the last few years. Disease modeling shows great promise for organoid use for cancer research and autologous transplantation therapy, especially after gene-defect correction.<sup>(56,58,67)</sup> EHBD and gallbladder repair using human cholangiocyte organoids incorporated into scaffolds suggests the potential of using patient-derived cells in regenerative structures to avoid problems with immune rejection. However, hepatocyte maturation, culture longevity, and large-scale production of pure cultures remain challenges. In addition, access to freshly isolated human hepatocytes is very limited, and maintenance of cultures in spinner flasks<sup>(33,34)</sup> could be cost prohibitive.

A general challenge for the organoid field is inherent to epithelial-cell monocultures, which do not reflect the tissue complexity and heterogeneous cell-cell interactions in the native tissue. For hepatobiliary organoid co-cultures, it would be preferable to include mesenchymal cells, including fibroblasts, derived from liver or biliary tissue sourced from the same patient. Mesenchymal cells can potentially promote hepatocyte maturation, as demonstrated in human fetal cell-derived co-cultures.<sup>(52)</sup> Furthermore, the addition of umbilical vein endothelial cells together with mesenchymal stem cells has been shown to provide paracrine signals to induce hepatocyte differentiation and spatial 3D organoid organization in induced pluripotent cell-derived organoids.<sup>(68)</sup> Co-culture of tumoroids with immune cells would be an invaluable immune-oncology research tool, as would co-culture with cancer-associated fibroblasts, as reported in pancreatic cancer models.<sup>(69,70)</sup>

Another general challenge for the organoid field is the need to identify an appropriate ECM to support growth. Matrigel, the most commonly used matrix, is a mixture of ECM proteins extracted from mouse Engelbreth-Holm-Swarm sarcoma cells.<sup>(71)</sup> It is complex,<sup>(72)</sup> not well defined, and rich in growth factors, including insulin-like growth factor 1 and EGF,<sup>(73)</sup> and does not allow stiffness manipulation. Furthermore, as a mouse-derived ECM, Matrigel can introduce zoonosis as well as xenogenic contaminants, which might induce immune responses.<sup>(74)</sup> It is also prone to batch-to-batch variability.

ECM effects on hepatobiliary progenitor cells are not yet determined and may be problematic in cancer research. By definition, cancer cells possess anchorage-independent growth, which is difficult to model with organoids requiring an ECM. Ideally, matrices would mimic native ECM biological and mechanical properties and support a complex cell mixture. Furthermore, matrix stiffness could be manipulated to recreate homeostatic and fibrotic microenvironments to study cirrhosis and fibroproliferative cholangiopathies. If used for preclinical models, drug testing, and hepatobiliary regeneration in humans, matrices need to be more clinically relevant and meet requirements of the Federal Drug Administration's Good Manufacturing Practices. Type 1 collagen,<sup>(40)</sup> polyglycolic acid scaffolds,<sup>(36)</sup> and decellularized ECM hydrogels<sup>(75)</sup> have been tested among other alternative synthetic matrices.<sup>(74)</sup> In spheroid organoid models, the apical surface is directed toward the lumen and is challenging to access. A recently reported "bile duct-on-a-chip" collagen device showed proper cholangiocyte polarization and permitted separate access to apical and basolateral surfaces.<sup>(76)</sup>

Lastly, journal requirements for publishing organoid work have not been standardized. To interpret human organoid studies, one should consider the interpersonal variability affecting disease phenotypes, and the passage number, as organoid growth slows down with time,<sup>(24)</sup> indicating changes in organoid biology in culture. Preservation of earlier passages and primary tissue biobanking for organoid recovery can help standardize experimental conditions and increase the sample number. Finally, protocols for organoid establishment from the same tissue vary by cell-isolation techniques and growth-factor use, which likely results in cultures with different levels of cell maturity. Thus, it is important to report functional and cellular fidelity. The most optimal and standardized organoid culture conditions are yet to be discovered.

## Conclusions

The hepatobiliary organoid technology is evolving as culture conditions and validation techniques are being refined. Biobanking of primary human tissues and organoids with simultaneous collection of clinical data could be the basis for future, more advanced translational studies (Fig. 2). At the current stage, hepatocyte, biliary, and gallbladder organoids already

provide an unparalleled tool to study homeostasis, disease processes, and alternatives to organ transplantation in relation to one of the most complex organs in the human body, the liver.

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