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Organoids in biliary atresia

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ABSTRACT

Organoids are three-dimensional and self-organizing cell cultures of various lineages that resemble structures and functions of an organ in many ways, and they are versatile tools in disease modeling and patho-mechanistic study of human diseases affecting their tissues of origin. Biliary atresia (BA), a cholangiopathy affecting the bile ducts of the liver, is a heterogeneous and multifaceted liver disease of complex pathogenesis. Cholangiopathies refer to a category of liver diseases that affect the cholangiocytes, the epithelial cells lining the lumen of the biliary trees. Biliary organoids consist of cholangiocytes in a spherical monolayer epithelium, which favorably resembles the structures and functional properties of the bile duct cholangiocytes. Biliary tissue-derived cells, pluripotent stem cells or embryonic stem cells, and hepatic progenitor cells are capable of generating biliary organoids. In the last decade, a considerable advancement has been made in the generation of biliary organoids for modeling liver physiology and pathophysiology. Using biliary organoids, scientists have advanced our knowledge underlying the pathogenic roles of genetic susceptibility, dysregulated hepatobiliary development/structure, environmental factors, and dysregulated immune–inflammatory responses to an injury in BA. This review will summarize and discuss the derivation and the use of biliary organoids in the disease modeling and patho-mechanistic study of BA.

ORGANOIDS IN DISEASE MODELING AND PATHO-MECHANISTIC STUDY

An organoid is a self-assembled three-dimensional structure which leverages the self-renewal and differentiation properties of stem/progenitor cells and the intrinsic self-organization ability to form organized structures, that resemble structures and functions of an organ in many ways.[1] Normal and patients' tissues/cells-derived organoids are versatile tools in disease modeling and patho-mechanistic study of human diseases affecting their tissues of origin.

Biliary atresia (BA) is a devastating inflammatory obliterative disease of the bile ducts, a cholangiopathy affecting the cholangiocytes, the epithelial cells lining the lumen of the biliary trees of the liver. This review will discuss the generation and the application of biliary organoids in the disease modeling and patho-mechanistic study of BA.

Development of biliary tree

Hepatocytes produce and secrete bile into their canaliculi (thin tubules between adjacent hepatocytes), then the canaliculi empty bile into bile ductules (known as canals of Hering), which connect with interlobular bile ducts. Bile travels from interlobular bile ducts down to intrahepatic bile ducts, left and right hepatic ducts, common hepatic duct, cystic duct, and the gall bladder, where bile is stored. When food is eaten, the gallbladder contracts pushing bile through the cystic duct and into the common bile duct and then into the duodenum to help to break down the dietary fats.[2]

The biliary tree refers to the network consisting of hepatocytes, intrahepatic bile ducts (IHBDs), and extrahepatic bile ducts (EHBDs). Cholangiocytes, the epithelial lining of the bile duct lumen, are joined together by intercellular tight junctions at the upper part of the intercellular membrane and polarize the plasma membrane with the apical side facing the bile duct lumen.[3] Cholangiocytes in the EHBDs and IHBDs are of different developmental origins. During human embryogenesis, a portion of the endoderm-lined yolk sac is incorporated into the embryo to form the primitive gut, from which the liver primordium is developed. The anterior portion of the primitive foregut (the hepatic diverticulum) develops and gives rise to the liver and IHBDs, while the posterior portion forms the EHBDs. The hepatic diverticulum endoderm gives rise to hepatoblasts, which are bipotential and differentiate into IHBD cholangiocytes and hepatocytes. Periportal hepatoblasts differentiate into ring structures of cholangiocyte progenitors, namely ductal plates around the portal vein branches. Then, primitive ductal structures (PDS) form at discrete locations of the ductal plate, which finally develop into IHBDs. PDS that fail to be incorporated into the bile ducts regress, leaving mature ducts in place.[4] The architecture organization of the hepatic parenchyma and vasculature, and the EHBDs are substantively developed by gestation week 16. In contrast, IHBD development continues throughout gestation and after birth.

Derivation of biliary organoids

Biliary organoids consist of cholangiocytes in a spherical monolayer epithelium.[5 6] Biliary organoids could be derived from both intrahepatic and extrahepatic biliary tissues.[6–9] Pluripotent stem cells (PSCs) or embryonic stem cells, hepatic progenitor cells, and tissue-derived cells are capable of generating biliary organoids.[10] Huch *et al.* reported the derivation and clonal expansion of organoids from single Lgr5+ (leucine-rich repeat-containing G-protein coupled receptor 5 expressing) liver cells in R-spondin 1-based culture medium.[7] Later, the same group reported the establishment of intrahepatic biliary organoids (IBOs) from bipotent EPCAM-expressing IHBD cells of human liver, and these biliary organoids contained bipotent hepatoblast-like stem cells that could be induced to form functional hepatocytes.[6] The epithelial cell adhesion molecule (EPCAM, also known as CD326) is a surface epithelial marker selectively expressed in ductal cells.[11] EPCAM-expressing ductal cells of human livers developed into organoids with a high efficiency, while EPCAM non-expressing hepatocytes failed to generate organoids.[6] Single cell transcriptomics studies have identified the EPCAM-expressing population within the canals of Hering as bipotent progenitors capable of differentiating into cholangiocytes and hepatocytes.[12 13] Derivation of biliary organoids from cells isolated from mouse and human extrahepatic biliary tissues including the gall bladders and the common bile ducts has also been reported.[8 9] The EHBD-derived biliary organoids could repair the biliary epithelium following transplantation into a mouse model of injury.[9] Sampaziotis *et al.* reported a stepwise differentiation of human PSCs into endoderm and subsequently into foregut progenitor cells, followed by the generation of hepatoblasts, cholangiocyte progenitors, and cholangiocyte-like cells in the form of biliary organoids,[14] which resembles the embryonic development of cholangiocytes in many ways.

Biliary organoids in biliary atresia study

BA is a heterogeneous and multifaceted liver disease of complex pathogenesis.[15] BA is largely a non-syndromic disease. It is likely that the injury to the liver in the fetus or around birth, and that the injury progression and pathological repair in the postnatal period, lead to BA.[16] A number of pathogenic pathways have been proposed for BA, including genetic susceptibility, dysregulated hepatobiliary development/structure, environmental factors (toxin or virus infection induced injury), and dysregulated immune–inflammatory responses to an injury.[16] Biliary organoids have been used to investigate the patho-mechanisms underlying some of these pathogenic pathways for BA (figure 1).

Genetic susceptibility

Genetic factors are known to play a role in BA.[17 18] Large-scale genome-wide association studies identified a group of genes involved in hepatobiliary development/structure, including *Adducin 3* (*ADD3*),[19 20] *Glypican 1* (*GPC1*),[21] *ARF GTPase 6* (*ARF6*),[22] *Mannosidase-1-Alpha-2* (*MAN1A2*),[23] *EGF containing fibulin extracellular matrix protein 1* (*EFEMP1*),[24] *ArfGAP with dual PH domains 1*, (AFAP1) and *Tumor Suppressor Candidate 3* (*TUSC3*).[25] Clustered regularly interspaced palindromic repeats/Cas9-(CRISPR/Cas9)-mediated knockout of BA susceptibility genes *ADD3* and *GPC1* led to reduced ductal structure formation in biliary organoids.[26] It is likely that mutations/variants of BA susceptibility genes involved in hepatobiliary development/structure perturbed embryogenesis of the biliary tree, although insufficient alone to cause BA, increases susceptibility to biliary injury and BA.

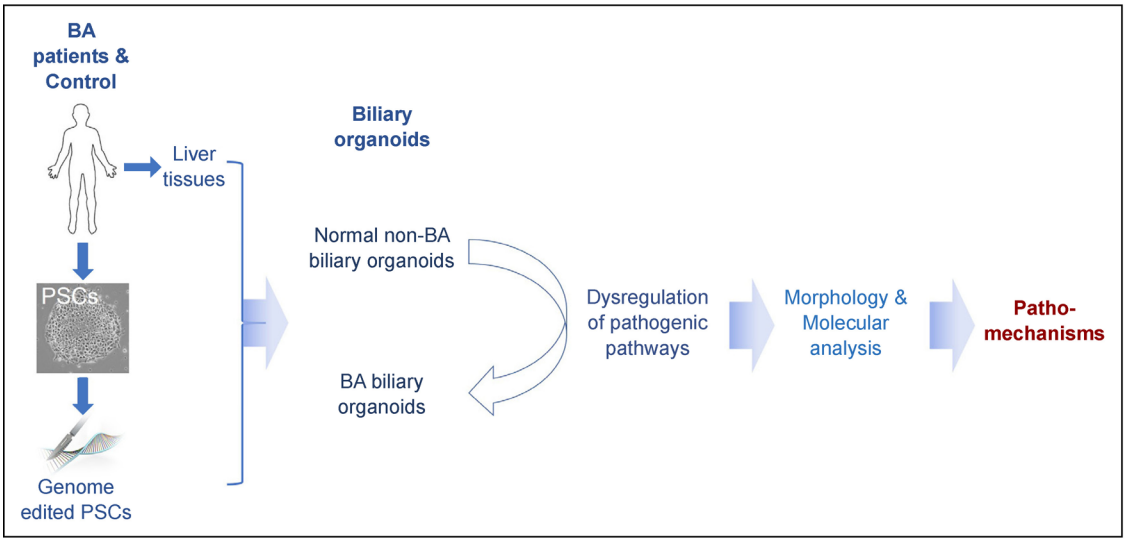


Figure 1 Generation and application of biliary organoids for biliary atresia (BA) disease modeling and patho-mechanistic study. The scheme represents an overview of the generation and the use of biliary organoids from BA/normal liver tissues, BA/normal pluripotent stem cells (PSCs), and genome-edited PSCs for disease modeling and patho-mechanistic study for BA.

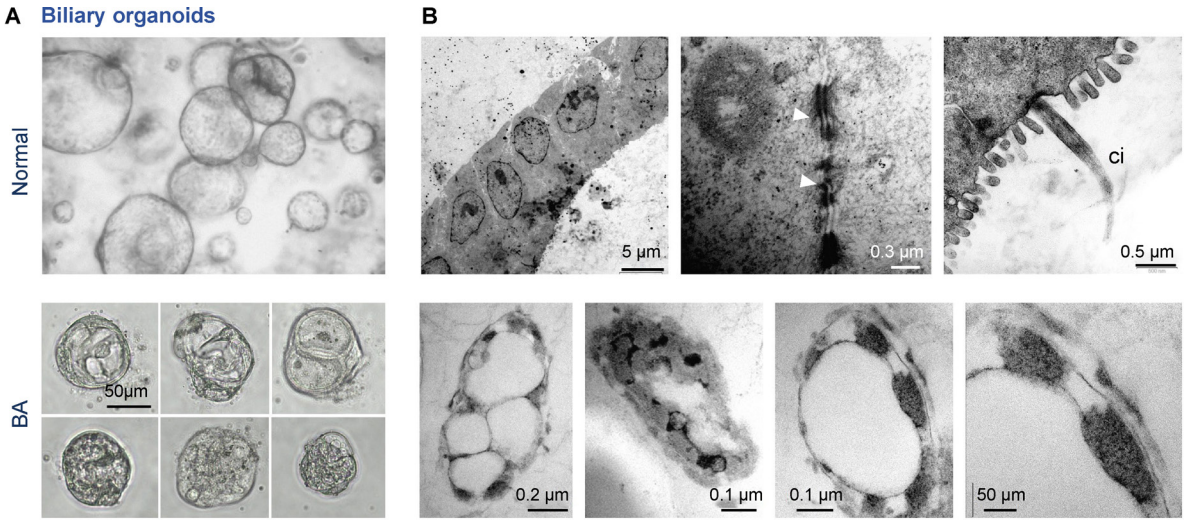


Figure 2 Morphology and electron microscopy (EM) study of biliary atresia (BA) and non-BA biliary organoids. (A) Non-BA organoids are large, well-expanded cystic structures with a single outer layer. BA organoids are tiny, frequently appearing as poorly expanded structures with multiple vacuoles and a thick cell layer or as unexpanded cell clusters. (B) EM study showed a single layer of tightly packed columnar bile duct cells, with tight junctions (arrowheads) and primary cilia (ci) in BA organoids. In contrast, non-BA organoid cells are elongated and non-columnar; loosely packed with no tight junctions and primary cilia.

Dysregulated hepatobiliary development/structure

The hepatobiliary system is not fully developed and functionally mature at birth;[27] hence, injury to the abnormally developed/immature hepatobiliary system could initiate a cascade of events, manifested as BA, suggesting dysregulated hepatobiliary development/structure could contribute to BA development.[28]

Dysregulated cholangiocyte development

EPCAM-expressing cells of BA and non-BA livers were cultured to generate organoids and revealed aberrant morphology and apical-basal organization in BA organoids (figure 2), an aberrant cholangiocyte development (a partial shift from a cholangiocyte towards a hepatocyte transcriptional profile) and changes in expression of genes related to amyloid-beta (Aβ) biology.[29] The apical-basal disruption and aberrant cholangiocyte development in BA organoids have also been corroborated in another study.[30] Importantly, the addition of exogenous Aβ to normal control biliary organoids was sufficient to cause BA-like morphological changes (figure 3).[29 31] Aβ depositing in the livers of infants with BA was shown to suppress mitochondrial respiration and mammalian target of rapamycin (mTOR) signaling, affecting liver regeneration after injury.[31] Plasma Aβ levels correlated with impaired hepatic functions and were suggested as an adjuvant biomarker for the diagnosis of BA.[32 33]

Biliatresone treatment of control biliary organoids caused a similar apical-basal disruption, accompanied by reduced glutathione and Sox17 levels.[34–36] rotavirus (RRV) infection of human biliary organoids led to a disrupted organoid morphology combined with the induction of an immune response, which resembled the viral infection elicited bile duct damage and immune reaction in BA development.[37] CRISPR-based loss-of-function screen followed by in vivo validation and single-cell RNA sequencing in biliary epithelial cell-like organoids has identified that yes-associated protein 1 (YAP) and mammalian target of rapamycin complex 1 (mTORC1) signaling are required for the process of ductular reaction in response to liver injury.[38] Dysregulated activation of Hippo-yes associated protein 1 (Hippo-YAP1) signaling has also recently been shown to induce Pancreatic And

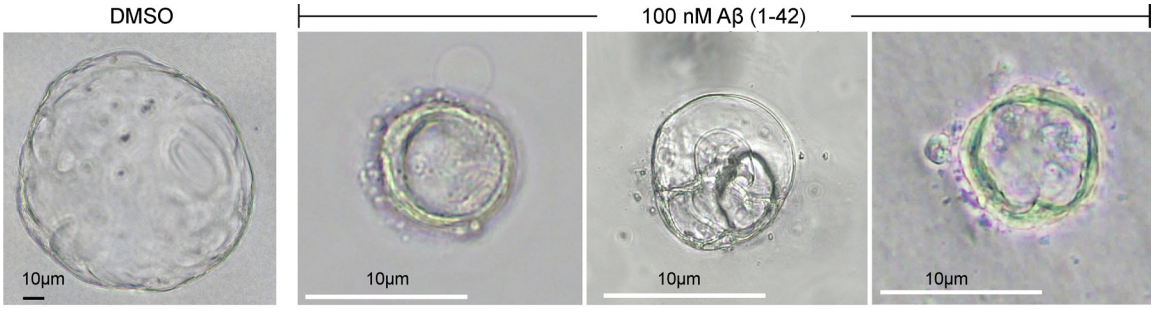
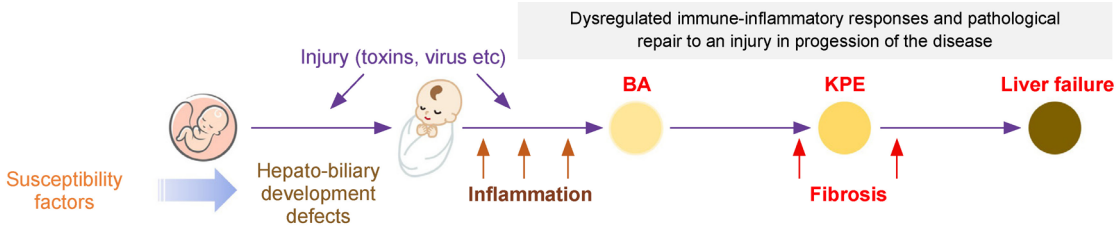


Figure 3 Amyloid-beta (Aβ) induced aberrant biliary organoid development. Untreated organoids are large, well-expanded cystic structures with a single outer layer. Aβ-treated organoids are tiny, and some are with multiple vacuoles. DMSO, Dimethyl sulfoxide.



Studies using tissue - or human PSC-derived biliary organoids	Major findings	Implications in BA pathogenesis
Comparative study of BA and non-BA biliary organoids	Bile duct cell progenitors in BA livers displayed aberrant cholangiocyte development	Defective bile duct regeneration and repair after injury
Environmental factors (biliatresone, CMV, Aβ) induced injury on biliary organoids	Environmental factors induced bile duct damages (oxidative stress; breakdown of apical-basal polarity and barrier functions; ciliogenesis defect; EMT)	Bile duct leakage and bile backflow into the liver parenchyma causing damages to liver cells
Transcriptomics analysis of biliary organoids from different regions of the bile ducts	Regional differences in human biliary tissues and corresponding in vitro-derived biliary organoids	Tissue specificity of cholangiopathies
Co-culture of injured biliary organoids with immune cells	CMV infected macrophages induced aberrant development of cholangiocytes	CMV infected macrophages caused bile duct injury
Developmental study of biliary organoids derived from genome edited hPSCs	Mutations/variants of BA susceptibility genes involved in hepato-biliary development/ structure caused aberrant cholangiocyte development	Perturbed embryogenesis of the biliary tree, although insufficient to cause BA, increases susceptibility to biliary injury and BA
Dysregulation of signalling pathways on biliary organoids	Identification of signalling pathways involved in ductular reaction in response to injury	Molecular patho-mechanisms underlying pathological biliary repair in BA

Figure 4 A diagram showing the current understanding of the multifactorial nature of biliary atresia (BA), and a table summarizing the insights from the studies using tissue-derived and/or human pluripotent stem cell (PSC)-derived biliary organoids on the interplay of different factors in the pathogenesis of BA. Aβ, beta-amyloid; CMV, cytomegalovirus; EMT, epithelial mesenchymal transition; hPSCs, human PSCs; KPE, Kasai portoenterostomy.

Duodenal Homeobox 1 (PDX1) downregulation and oxidative stress and impair the development of IBOs.[39]

Disruption of apical-basal polarity of cholangiocytes

Cholangiocytes, the epithelial lining of the bile duct lumen, are joined together by intercellular tight junctions at the upper part of the intercellular membrane and polarize the plasma membrane into the apical and basal sides. The apical side of the cholangiocyte faces the bile duct lumen, and the establishment of apical-basal polarity is fundamental for cholangiocyte development and functions.[3] Cholangiocytes modify bile acids via secretion and absorption through transporters on their apical and basolateral plasma membranes.[40 41] In the liver, defective cystic fibrosis transmembrane conductance regulator (CFTR) function causes dysregulation of chloride and bicarbonate secretion into bile duct,[42] impaired biliary secretion, and ductal cholestasis.[43] The observations of disruption of apical-basal polarity and lumen obstruction in BA livers and organoids,[29 30] in bile ducts of *Cdc42* knockout mouse cholangiocytes,[44] in biliatresone-treated biliary organoids,[34–36] corroborate the importance of apical-basal polarity in the development and functions of cholangiocytes. Disruption of apical-basal polarity and tight junction formation of cholangiocytes could result in bile leakage of bile ducts, which can lead to inflammation, scarring, or fibrosis of the liver.

Primary cilia dysfunction of cholangiocytes

Each cholangiocyte presents a primary cilium at its apical plasma membrane, and these non-motile primary cilia detect flow, composition, osmolality, and pH of the bile.[45] Shorter, misoriented, or less abundant cholangiocyte primary cilia were commonly observed in patients with BA.[46–49] A number of genes important for ciliogenesis, including *Polycystic Kidney Disease 1 Like 1* (*PDK1L1*), were mutated in patients with syndromic BA with laterality abnormalities.[46 48] Rare, deleterious de novo or biallelic variants of liver-expressed ciliary genes, including *Pericentrin* (*PCNT*), *Kinesin-like protein KIF3B,* (*KIF3B*) and *Tetratricopeptide Repeat Protein 17* (*TTC17*), were reported in around 30% patients with non-syndromic BA,[50] which further underpins the pathological roles of primary cilia dysfunction in BA. Biliatresone treatment reduced the number of primary cilia formation in control human biliary organoids.[36] Primary cilia are involved in a number of signaling pathways that are crucial for bile duct development including Hedgehog, Neurogenic locus notch homolog protein 1 (NOTCH), wingless-type MMTV integration site family, member 1 (WNT), and transforming growth factor-β/bone morphogenetic protein pathways.[51–53] Defects in these signaling pathways could cause abnormal biliary development and contribute to the pathogenesis of BA.[3 54]

Table 1 Landmark publications on biliary organoids in biliary atresia study

Year	Main findings	Refs.
2015	Establishment of biliary organoids from bipotent EPCAM-expressing IHBD cells of human liver, and these biliary organoids contained bipotent hepatoblast-like stem cells that could be induced to form functional hepatocytes	7
2016	Biliatresone-treated normal mouse biliary organoids displayed aberrant morphology with apical-basal disruption and reduced GSH and Sox17 levels	35
2017	Reconstruction of the mouse extrahepatic biliary tree using primary human extrahepatic biliary organoids	9
2019	Deletion of BA susceptibility genes *ADD3* and *GPC1* resulted in reduced biliary development in human PSC-derived biliary organoids	26
2019	CRISPR-based loss-of-function screen in BEC-like organoids and identification of YAP and mTORC1 signaling for the process of ductular reaction in response to liver injury	38
2020	Establishment of BA liver tissue-derived organoids. BA biliary organoids exhibited aberrant morphology, disruption of apical-basal organization, and aberrant cholangiocyte development	29
2020	RRV infection of human biliary organoids exhibited similar bile duct damage and immune reaction in BA development	37
2021	Demonstrate regional differences in human biliary tissues and corresponding in vitro-derived biliary organoids	77
2022	Biliary organoids uncover delayed epithelial development and barrier function in BA	30
2022	Poly I:C treated normal human liver tissue-derived biliary organoids resembled the organoids developed from BA liver samples	71
2023	HCMV infected human biliary organoids exhibited low-level productive or persistent infection and promoted the EMT process of organoids	67
2023	HCMV infection of macrophages induced abnormal development of cholangiocytes in a macrophage and biliary organoid co-culture	68
2024	Dysregulated activation of Hippo-YAP1 signaling induced PDX1 down-regulation and oxidative stress and impaired IBOs development	39
2024	Abnormal development, disruption of apical-basal polarity, defective primary cilia formation, and increased permeability in biliatresone-treated human biliary organoids	36
2024	Organoid-based transcriptomic profiling could be used to inform Kasai portoenterostomy (KPE) success and guide BA management	75

ADD3, Adducin 3; BA, biliary atresia; BEC, biliary epitehlial cells; CRISPR, Clustered regularly interspaced palindromic repeats; EMT, epithelial mesenchymal transition; EPCAM, epithelial cell adhesion molecule; GPC1, Glypican 1; HCMV, human cytomegalovirus; Hippo-YAP1, Hippo-yes associated protein 1; IBOs, intrahepatic biliary organoids; IHBD, intrahepatic bile duct; mTORC1, mammalian target of rapamycin complex 1); PDX1, Pancreatic And Duodenal Homeobox 1; Poly I:C, polyinosinic:polycytidylic acid; PSC, pluripotent stem cell; RRV, rotavirus; YAP, yes-associated protein 1.

Environmental factors

Viral infection

A viral infection induced biliary injury has long been suggested as a possible etiological factor for BA, and viruses including cytomegalovirus (CMV), Epstein-Barr virus, and human papillomavirus have been implicated.[55 56] Viral exposure may cause defective bile duct development[57 58] or bile duct injury and/or infection triggered secondary autoimmune reaction.[59–61] Among these viruses, CMV is the most studied virus with associations to BA, but reports are inconclusive.[62–64] Infants with BA with higher anti-CMV IgM antibody levels were shown to have the worst prognosis following Kasai surgery.[63 65 66] However, the cellular/molecular mechanisms underlying CMV-BA have not been elucidated. Ye *et al*. recently reported that human CMV (HCMV)-infected human biliary organoids exhibited low-level productive or persistent infection and promoted the epithelial mesenchymal transition (EMT) process of organoids, which could induce inflammation and EMT and contribute to the development of BA.[67] Furthermore, HCMV infection of macrophages induced injury and abnormal development of cholangiocytes in a macrophages and biliary organoid co-culture model for BA.[68]

Group A rotaviruses produced extrahepatic biliary obstruction in orally inoculated newborn mice.[69] Polyinosinic:polycytidylic acid (poly I:C) is a synthetic immunostimulant that is structurally similar to a double-stranded RNA found in the reovirus and rotavirus, which induces immune-mediated inflammation in rats.[70] Poly I:C treated normal human liver tissue-derived biliary organoids exhibited an aberrant morphology and a distinct pattern of expression of immune-mediated signaling pathways, resembling the organoids developed

from BA liver samples, which indicated that biliary organoids are potential research materials to study immune-mediated inflammation in BA.[71]

Environmental toxins

Outbreaks of a BA-like disease in livestock in Australia have led to the identification of a plant biliary toxin called biliatresone.[72] BA-like pathology was observed in biliatresone-treated larval zebrafish and in the offspring of biliatresone-fed pregnant mice. Disruption of apical-basal polarity, loss of monolayer integrity, increased permeability, and obstruction were observed in murine extrahepatic bile ducts and biliary organoids treated with biliatresone.[35] Abnormal development, disruption of apical-basal polarity, defective primary cilia formation, and increased permeability have also been observed in biliatresone-treated control biliary organoids.[34–36] Mice injected with biliatresone displayed biliary obstruction with associated inflammation and fibrosis.[73] The limited distribution of biliatresone suggests that exposure to biliatresone is not relevant for human BA, but it provides evidence that prenatal exposure to a toxin could lead to BA in neonates, and the characterization of biliary toxic structural motifs may lead to identification of toxins with human relevance.

Immune–inflammatory dysregulation to an injury

Liver injury induced exaggerated autoimmune and auto-inflammatory responses targeting cholangiocytes has been postulated to cause progressive bile duct damage, fibrosis, and obliteration in BA.[74] Cumulative evidence indicated that innate immune cells (natural killer cells, macrophages, dendritic cells, and neutrophils) and adaptive immune cells (T cells and B cells) contribute to BA pathogenesis (introduction of an article by Tam *et al*.[16]). Multicellular co-cultures which comprise of immune cells and biliary organoids are needed to delineate the mechanisms underlying dysregulated immune-inflammatory responses in BA pathogenesis. A biliary organoids and macrophages co-culture was recently established for the investigation of HCMV infection of macrophages in the induction of biliary injury for BA.[68] More advanced multicellular organoid platforms which comprise of bile duct cells and various types of immune cells are needed to delineate the roles of interactions between injured bile duct cells, innate and adaptive immune cells in BA.

In sum, studies using tissue-derived and/or human PSC-derived organoids have provided important insights into the mechanisms underlying susceptibility factors in the perturbation of biliary tree development in embryos, environmental factors (biliatresone, CMV, Aβ) induced bile duct damages, CMV-infected macrophages in bile duct injury, bile duct regeneration, and ductular reaction in response to injury. BA is a multifactorial disease, and biliary organoids have enabled us to start to understand the interplay of genetic susceptibility, bile duct injury, inflammatory responses, and pathological repair in the disease causation and/or progression of BA (figure 4).

Clinical translation of findings from biliary organoids

Taken all the above indicated that biliary organoids could be used as a disease model for patho-mechanism study for BA, and findings from biliary organoids could be translated into clinical application for the prognosis, therapeutic development, and personalized patient management for BA. We have recently shown that organoid-based transcriptomic profiling could be used to inform Kasai portoenterostomy (KPE) success and guide BA management.[75] Furthermore, dysregulated signaling pathways in BA biliary organoids are potential therapeutic targets for drug development to mitigate the disease progression and improve the outcomes of KPE by promoting biliary repair and regeneration in patients with BA.

Limitations and challenges in biliary organoid research

The liver is a complex organ which mainly comprises of hepatocytes, and other cell types including the parenchymal cholangiocytes and the non-parenchymal liver cells (liver sinusoidal endothelial cells, hepatic stellate cells, Kupffer cells), and other immune cell types.[76] Together, these cells do not only maintain the structural integrity and functionality of the liver during homeostasis, but also contribute to repair after injury and pathophysiology for BA.[16] Although we have seen the advancement in the generation and the application of biliary organoids in BA study (table 1), there are clearly limitations as these biliary organoids only consist of cholangiocytes, in that the interactions between bile duct cells, hepatocytes, and other non-parenchymal liver cells in the disease causation/progression of BA cannot be studied in these biliary organoids. Furthermore, Rimland and co-workers have demonstrated that differences exist not only between extrahepatic biliary organoids and their tissue of origin, but also between IHBD and EHBD organoids.[77] Therefore, one has to understand the differences and the limitations in biliary organoids derived from different parts of the bile duct and to interpret the findings with caution to understand the tissue specificity of cholangiopathies.

SUMMARY AND FUTURE PROSPECT

In the last decade, we have witnessed a considerable advancement in the generation of biliary organoids for modeling liver physiology and pathophysiology, which has yielded important insights in the pathogenesis for BA. In the future, advanced multicellular liver organoid platforms which comprise of parenchyma cells and non-parenchymal cells are needed to delineate the roles of interactions between different cell types, virus-elicited innate and adaptive immune responses, extracellular matrix (ECM), toxins, and genetic susceptibility in BA disease initiation and progression. Custom-designed scaffolds, decellularized liver ECM scaffolds, and improvements to culture reagents to maintain the structural organization while supporting the function and survival of multiple cell components of multicellular organoids will likely bring significant breakthroughs in elucidating

disease mechanisms for BA. Furthermore, with the advancements in microfluidic chip technology, in the future, biliary organoids-on-chips will enable a high-throughput and cost-effective screening of thousands of small molecules targeting the dysregulated signaling pathways identified in BA biliary organoids for therapeutic development for BA.

Findings on the biliary biology, disease mechanisms, therapeutic targets, biliary repair, and regeneration for BA will have broader implications for other cholangiopathies including primary biliary cirrhosis, primary sclerosing cholangitis, cystic fibrosis involving the liver, polycystic liver disease, and cholangiocarcinoma that, like BA, affect a central target: the cholangiocytes.

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