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Cholangiocyte organoids for disease, cancer, and regenerative medicine

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ABSTRACT

The biliary tract is a ductal network comprising the intrahepatic (IHBDs) and extrahepatic bile duct (EHBDs). Biliary duct disorders include cholangitis, neoplasms, and injury. However, the underlying mechanisms are not fully understood. With advancements in 3D culture technology, cholangiocyte organoids (COs) derived from primary tissues or induced pluripotent stem cells (iPSCs) can accurately replicate the structural and functional properties of biliary tissues. These organoids have become powerful tools for studying the pathogenesis of biliary diseases, such as cystic fibrosis and primary sclerosing cholangitis, and for developing new therapeutic strategies for cholangiocarcinoma. Additionally, COs have the potential to repair bile duct injuries and facilitate transplantation therapies. This review also discusses the use of organoids in genetically engineered mouse models to provide mechanistic insights into tumorigenesis and cancer progression. Continued innovation and standardization of organoid technology are crucial for advancing precision medicine for biliary diseases and cancer.

1. Introduction

The biliary system is a network of branched tubular forms, such as a tree, consisting of epithelial cells called cholangiocytes. The biliary tree contains intrahepatic (IHBDs) and extrahepatic bile ducts (EHBDs) (Roskams and Desmet, 2008; Cardinale et al., 2012). EHBDs include the common bile duct (CBD), common hepatic duct, cystic duct, and gallbladder (GB) (Fig. 1).

During development, the biliary system arises from a region of the ventral foregut endoderm proximate to the liver and ventral pancreas (Zong and Stanger, 2011; Spence et al., 2009). EHBDs and the ventral pancreas originate from the pancreatobiliary domain (Hex1-/Sox17 +/Pdx1 +) and hepatocytes, and IHBDs are derived from the hepatic domain (Hex1 +/Sox17-/Pdx1-) (Fig. 2A). The EHBD/ventral pancreas and IHBD/liver are derived from distinct progenitors.

The primary role of the biliary system is to drain bile produced by hepatocytes to the duodenum through IHBDs and EHBDs. Cholangiopathies and biliary duct disorders include cystic fibrosis (CF), fibropolycystic diseases (i.e., Caroli syndrome and congenital hepatic fibrosis), primary biliary cholangitis (PBC), primary sclerosing cholangitis (PSC), immune-related adverse events (irAE), cholangitis, and neoplasms. To resolve these disorders of the biliary system, an understanding of the function of the biliary system and the underlying mechanisms is required (Lazaridis et al., 2004).

Sato et al. (2009) found that leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5)-positive intestinal stem cells can clonally generate intestinal organoids with a crypt-villus architecture in 3D culture systems using matrigel. Intestinal organoids can survive by providing in vivo intestinal stem cell niche components, including epidermal growth factor (EGF), Noggin, and R-spondin-1. Matrigel contains an extracellular matrix (ECM) that provides structural and biochemical support to the epithelial cells. Recent advances in 3D culture technology have allowed for deriving organoids from primary tissues, embryonic stem cells (ESCs), or induced pluripotent stem cells (iPSCs). Organoids have the capacity for self-renewal and self-organization and reflect key structural and functional properties of the original organs, such as the lungs, gut, liver, and bile ducts (Clevers, 2016; Fatehullah et al., 2016; Marsee et al., 2021).

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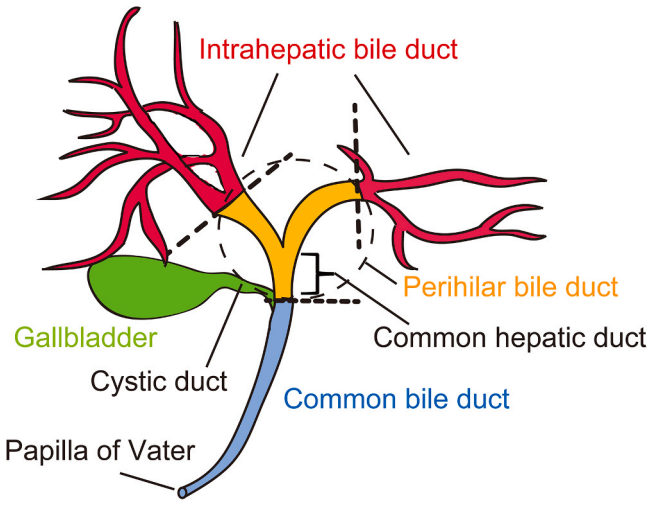

Fig. 1. Anatomy of the biliary network system. Shema of the bile ducts.

Organoids are powerful tools for studying the mechanisms of bile duct development, disease modeling, and drug screening. In this review, we describe various types of cholangiocyte organoids: (1) Cholangiocyte organoids (COs) derived from primary tissue (IHBD, EHBD, and GB), (2) COs derived from iPSCs, (3) hepatobiliary organoids derived from iPSC, and (4) multiorgan organoids derived from iPSCs (Fig. 3).

2. Cholangiocyte organoids

Huch et al. (2013) established murine intrahepatic cholangiocyte organoids (ICOs) from Lgr5-positive progenitor cells in the damaged liver tissues of adult mice for the first time. Lgr5-positive cells in the mouse liver differentiate into hepatocytes and cholangiocytes.

Broutier et al. (2016) demonstrated that long-term culture of intrahepatic cholangiocyte organoids and hepatocyte organoids derived from both mouse and human primary hepatocytes was possible in a similar way. Broutier and colleague also showed that both murine and human organoids have the similar cholangiocyte properties. Hereafter, human COs reports have been gradually increasing in the last 10 years.

2.1. Human COs derived from primary tissue

2.1.1. Intrahepatic cholangiocyte organoids (ICOs)

Huch et al. (2015) generated human ICOs from Lgr5-positive liver stem cells. These stem cells differentiated into organoids with biliary epithelial characteristics in the presence of specific growth factors and culture conditions. Hu et al. (2018) demonstrated the establishment of long-term ICOs and hepatocyte organoids for human primary hepatocytes using cholangiocyte medium and hepatocyte medium respectively. Hepatocyte organoids expressed the hepatocyte marker albumin (Alb) and hepatocyte nuclear factor 4α (Hnf4a). ICOs expressed the cholangiocytes progenitor/cholangiocyte markers like EPCAM, SRY-box transcription factor 9 (SOX9), and Cytokeratin7/19 (KRT7/19). In terms of organoid function, low-density lipoprotein (LDL) uptake and glycogen accumulation were detected in hepatocyte organoids.

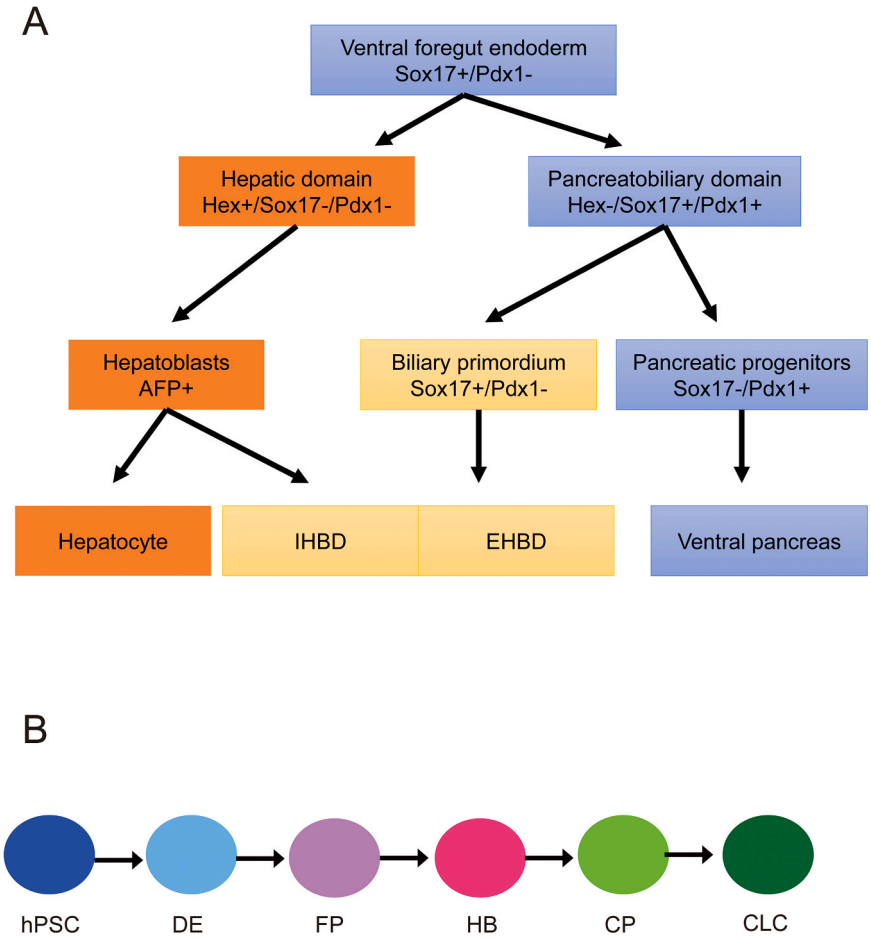

Fig. 2. Development and differentiation of Cholangiocyte. (A) Development of the bile duct, hepatocyte, and pancreas. (B) Protocol for iPSC differentiation towards cholangiocytes. DE: Definitive Endoderm, FP: Foregut progenitors, HB: Hepatoblasts, CP: Cholangiocyte Progenitors, CLC: Cholangiocyte-like cell.

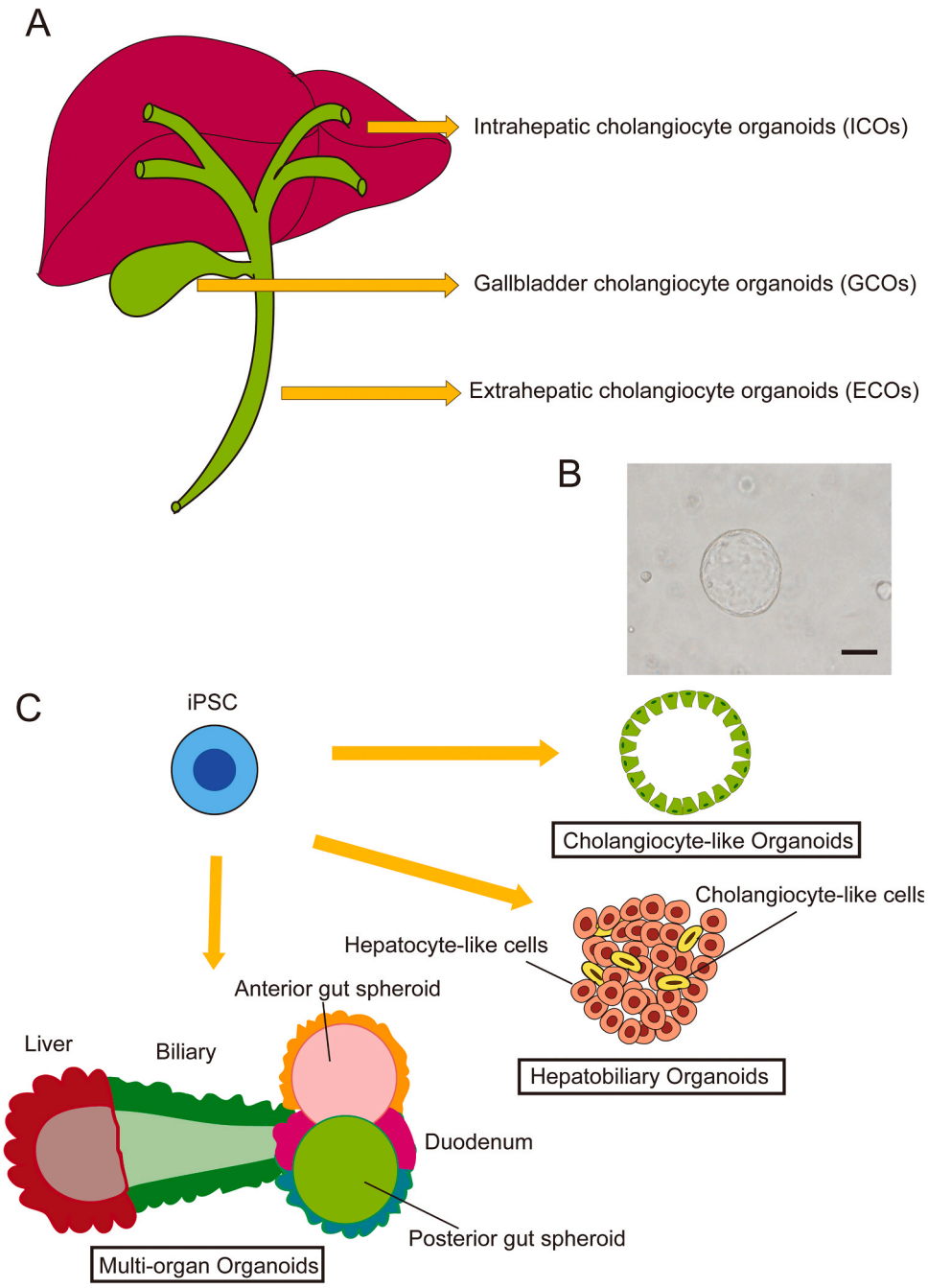

Fig. 3. Cholangiocyte organoid (CO) models. (A) CO derived from the primary tissue. (B) Human COs. Scale bar, 100μm. (C) CO models derived from induced pluripotent stem cells (iPSC).

Meanwhile, Rhodamine123, a fluorescent substrate for the cholangiocyte surface glycoprotein multidrug resistance protein-1 (MDR1), was actively transported into the lumen of ICOs. Notably, ICOs not only express biliary markers but also have a biliary duct-like function.

2.1.2. Gallbladder cholangiocyte organoids (GCOs)

Lugli et al. (2016) found that the growth and cholangiocyte properties of gallbladder cholangiocyte organoids (GCOs) depended on R-spondin 1 and noggin, whereas the organoids differentiated partially toward the hepatocyte fate without these growth factors. Human GCOs were propagated in tissue culture for at least 16 weeks and expressed biliary cell markers in these conditions.

2.1.3. Extrahepatic cholangiocyte organoids (ECOs)

Human extrahepatic cholangiocyte organoids (ECOs) were first established from extrahepatic biliary trees in 2017 (Sampaziotis et al., 2017). The ECOs generated from the obtained cholangiocytes expressed duct markers, including KRT7, KRT19, HNF-1β, GGT, cystic fibrosis transmembrane conductance regulator (CFTR), and SOX9, and maintained their functional properties in culture (alkaline phosphatase (ALP) and γ-glutamyltransferase (GGT) activities) even after long-term culturing.

2.1.4. Regional diversity of cholangiocyte organoids (COs)

Verstegen et al. (2020) clarified the differences in characteristics and functions between human ECOs derived from the common bile duct and ICOs derived from the liver with canonical Wnt-stimulated culture

medium. No significant differences in gene expression were observed between the ECO and ICO groups. Both ECOs and ICOs differentiated into cholangiocytes. However, only ICOs differentiated into a hepatocyte-like fate.

Rimland et al. (2021) generated COs derived from the human GB, CBD, pancreatic duct (PD), and IHBD. Regardless of their tissue of origin, COs display similar morphology, proliferation, and capacity for single-cell clonality. RNA-seq showed that the region-specific gene expression in both tissues and organoids differed according to their location of origin. Remarkably, the IHBD and EHBD organoids differed in their responses to canonical Wnt signaling. Continuous activation of canonical Wnt signaling is detrimental to the proliferation and self-renewal of IHBD but not EHBD organoids.

Sampaziotis et al. (2021) performed single-cell RNA sequencing (scRNA-seq) on cholangiocytes isolated from the human IHBD, CBD, and GB. All cholangiocytes expressed KRT7, KRT19, SOX9, and GGT. However, a deep analysis revealed non-overlapping expression modules in the three populations. Gene expression in environmentally similar regions (e.g., IHD and CBD versus GB) displayed higher transcriptional similarity in different individuals. scRNA-seq of COs from these cholangiocytes revealed that COs assume a similar transcriptional signature independent of their region of origin. In other words, these COs lost expression of markers specific to their regions of origin. However, upon exposure to bile they could regain the lost region-specific identity.

2.2. COs derived from iPSCs

Since Takahashi and Yamanaka (2006) reported the reprogramming of mature somatic cells into iPSCs by the introduction of the Yamanaka factors, Oct4 (octamer-binding transcription factor 4), Sox2 (SRY-box transcription factor 2), Klf4 (Kruppel-like factor 4), and c-Myc (myelocytomatosis oncogene), numerous protocols have been reported to direct the differentiation of iPSCs into specific cells.

Dianat et al. (2014) induced differentiation of hESCs and hiPSCs into endoderm and mesoderm hepatoblasts expressing HNF3b, GATA4, HNF6 HNF4a, α-fetoprotein (AFP), and CK19. Hepatoblasts differentiate into cholangiocyte-like cells (CLCs) and hepatocytes upon exposure to specific signaling pathways (Fig. 2B) (Sampaziotis et al., 2015; Sampaziotis et al., 2015; Ogawa et al., 2015). CLCs express cholangiocyte markers, such as CK7, CK19, CFTR, Transmembrane G protein-coupled receptor 5 (TGR5), and SOX9, as well as the functional characteristics of cholangiocytes, including bile acid transfer, alkaline phosphatase activity, GGT activity, and physiological responses to secretin, somatostatin, and vascular endothelial growth factor.

2.3. Hepatobiliary organoids derived from iPSC

Recently, hepatobiliary structures containing differentiated hepatocytes and cholangiocytes derived from human iPSCs were generated simultaneously (Vyas et al., 2018; Wu et al., 2019). The induced hepatocyte-like cells could take up indocyanine green, accumulate lipids and glycogen, and display appropriate secretion ability (albumin and urea) and drug metabolic ability (CYP3A4 activity and inducibility). The biliary structures in the system showed GGT activity and the ability to efflux rhodamine and store bile acids. Furthermore, after transplantation into immunodeficient mice, the organoids survived for more than 8 weeks (Sampaziotis et al., 2015). Bile duct formation was interrupted by the inhibition of Notch signaling (DAPT), a phenotype similar to that of Alagille syndrome (Vyas et al., 2018).

2.4. Multiorgan organoids derived from iPSC

Koike et al. (2019) demonstrated that the dynamic morphogenesis of hepatic, biliary, and pancreatic structures could be generated from iPSCs. The fusion of anterior and posterior gut spheroids derived from iPSCs leads to the formation of HBP domains containing the liver, biliary, ventral pancreas, and duodenum. Retinoic acid and HES1 function are essential for organogenesis of the HPB domain. Because HPB multiorgan organoids can be maintained in culture for at least 90 days, they are promising for studying organogenesis and tissue interactions.

2.5. Limitations of iPSC-derived organoids

A limitation of iPSC-derived organoids in clinical use is their genomic instability due to exposure to reprogramming factors (Prior et al., 2019). The properties of iPSC-derived organoids can be easily altered by the surrounding environment, such as medium components.

3. Repair and transplantation therapy

The proliferative and differentiation potential of cholangiocyte organoids holds promise for the repair of bile duct injuries prior to transplantation. Sampaziotis et al. (2017) produced biodegradable polyglycolic acid (PGA) scaffolds populated with primary human ECOs. These PGA scaffolds rescued immunodeficient mice with injured GB walls. Engrafted COs maintained cholangiocyte gene expression. Researchers also generated an ECO-populated collagen tube and successfully replaced the murine bile duct with the ECO-populated collagen tube (Fig. 4). Tysoe et al. (2019) described a protocol for establishing ICOs and ECOs from human bile ducts, gallbladders, and EPCAM-positive sorted duct cells from liver biopsies. The harvested COs were maintained and seeded on both synthetic and biological scaffolds. Finally, the bioengineered biliary tissue was generated from COs as densified collagen sheets and tubes expressing key biliary markers and retaining the function of cholangiocytes.

Recently, Sampaziotis et al. (2021) established COs from cholangiocytes isolated from human IHBD, CBD, and GB. Injection of COs into IHBD-rescued immunodeficient mice with drug-induced cholangiopathy repaired the damaged intrahepatic bile ducts. In human liver experiments, organoids engrafted into the biliary ducts restored bile duct function and improved bile pH and volume, demonstrating their potential for human bile duct repair (Fig. 4).

4. Disease modeling with organoids

COs are strong tools for researching biliary disease mechanisms and drug screening. Herein, we provide examples of organoid research on two common biliary diseases, cystic fibrosis (CF) and primary sclerosing

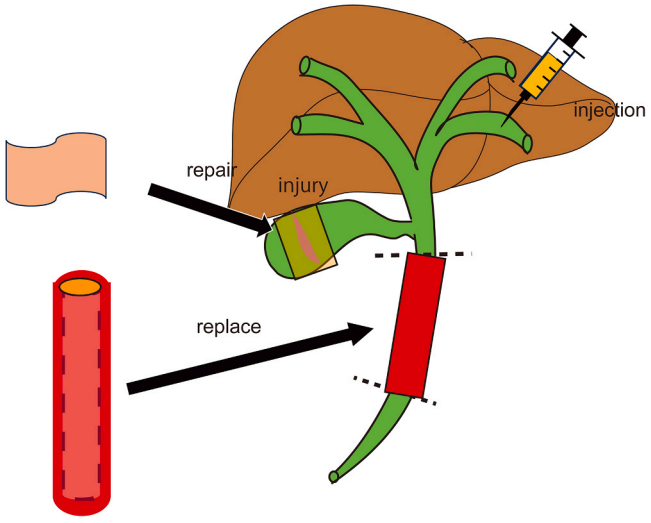

Fig. 4. Repair and transplantation therapy using human cholangiocyte organoids (COs) in a mouse model.

cholangitis (PSC), using COs.

4.1. Cystic fibrosis (CF)

CF is caused by mutations in the CFTR gene, which encodes a chloride channel normally expressed in the epithelial cells of many organs, including the lung, intestine, pancreatic duct, and biliary tract. Abnormal CFTR protein levels in the biliary system impair cholangiocyte chloride transport, leading to a lack of alkalinization and subsequent blockage of biliary ducts in the liver (Kobelska-Dubiel et al., 2014).

Two studies have generated COs from iPSCs of patients with CF carrying the most common mutation in CFTR (ΔF508). Functional CFTR was absent in CF-CLCs, and administration of the experimental CF drug improved CFTR function. CF-CLCs do not form cysts in response to forskolin (Sampaziotis et al., 2015; Ogawa et al., 2015; Verstegen et al., 2020). These CF-COs encourage further research on CF.

4.2. Primary sclerosing cholangitis (PSC)

PSC is a heterogeneous chronic cholestatic liver disease characterized by fibroinflammatory biliary tract strictures in IHBD and EHBD. PSC is rare, progressive, and often fatal. However, PSC has an unknown etiology and there is no effective treatment other than liver transplantation.

Soroka et al. (2019) established that COs could be derived from the bile of individuals with and without PSC. Compared to individuals without PSC, PSC-COs from patients with PSC expressed markers of an immune phenotype consisting of CCL20, HLA-DMA, and CD74. Furthermore, IL−17A treatment significantly increased the secretion of CCL20.

Garcia Moreno et al. (2024) performed transcriptomic profiling of PSC and non-PSC ECOs using scRNA-seq. The scRNA-seq revealed that PSC-ECOs do not have a unique cell population of cholangiocytes compared to non-PSC ECOs. However, differences in gene expression exist throughout the cholangiocyte populations between PSC and

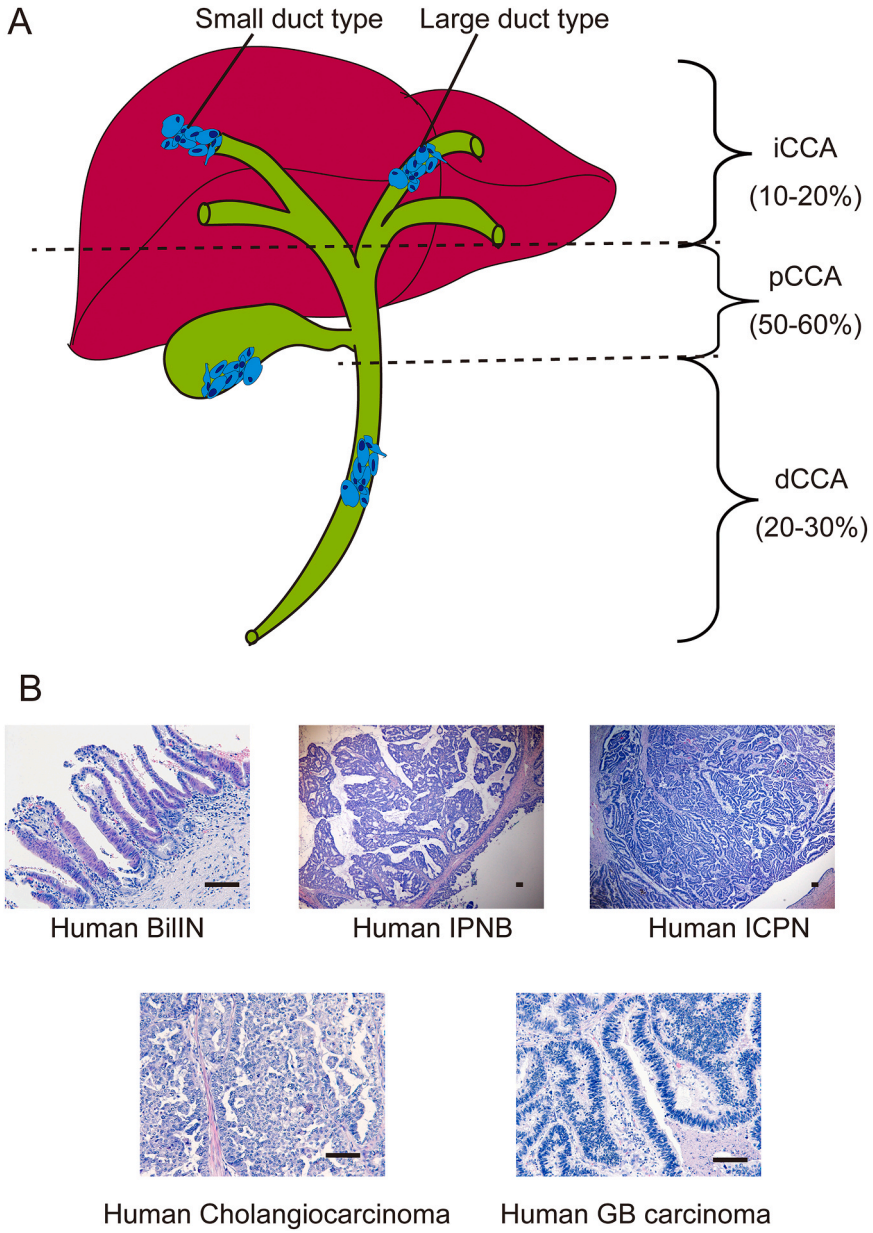

Fig. 5. Subclassification of biliary tract carcinoma (BTC). (A) Anatomical classification of cholangiocarcinoma. (B) Histology of human BTC and precanceraous lesion. Scale bar, 100 μm.

non-PSC ECOs. The abundance of secreted pro-inflammatory proteins was greater in PSC ECOs. In response to IL-17A stimulation, both PSC and non-PSC ECO exhibited changes in the expression of genes such as CCL20, CCL28, and CXCL1. However, PSC ECO showed a greater number of differentially expressed genes.

5. Cancer

Biliary tract cancer (BTC) is classified into cholangiocarcinoma (CCA) or gallbladder carcinoma (GBC). Based on their anatomical origin, CCAs include intrahepatic CCAs (iCCA or ICC), perihilar CCAs (pCCA), and distal CCAs (dCCA) (Rizvi and Gores, 2013). pCCA and dCCA can also be collectively referred to as 'extrahepatic' CCA (eCCA or ECC). Recently, the world health organization (WHO) classified iCCA into two subtypes, the small duct type (SD-type) and large duct type (LD-type), based on their different etiologies and clinical behaviors (WHO Classification of Tumours Editorial Board, 2019) (Fig. 5A). CCA accounts for approximately 3 % of all adult malignancies. Mortality from CCA has increased worldwide, according to the WHO and Pan American Health Organization databases for 32 selected locations in Europe, America, Asia, and Oceania (Bertuccio et al., 2019). The incidence of CCA in East Asia, including Thailand, Korea, Japan, and China, is particularly high, with $>$ 4 deaths per 100,000 inhabitants (Banales et al., 2020). The risk factors for both eCCA and iCCA include choledochal cysts, choledocholithiasis, cirrhosis, cholelithiasis, primary sclerosing cholangitis, inflammatory bowel disease, primary biliary cholangitis, type 2 diabetes mellitus, and viral hepatitis (Clements et al., 2020). Liver fluke is a risk factor for cholangiocarcinoma, particularly in Thailand, Indochina, China, and Korea (Sithithaworn et al., 2014; Khan et al., 2019). Surgical resection is the most effective treatment for CCA. Because biliary cancer is initially asymptomatic, it is often diagnosed in the late stages, such as the locally advanced or metastatic phases. Therefore, fewer than 50 % of patients with GBC and 35–68 % of patients with eCCA are eligible for surgical resection (Wang et al., 2021). Currently, durvalumab (anti-PD-L1), in combination with gemcitabine and cisplatin, is the most efficient treatment for advanced biliary tract cancer (Oh et al., 2022). Nevertheless, the 5-year survival rate of biliary cancer is only 5 %–15 % (Hundal and Shaffer, 2014; WHO Classification of Tumours Editorial Board, 2019; Banales et al., 2020). In Japan, CCA has the second worst prognosis (Cancer Statistics. Cancer Information Service, National Cancer Center, Japan). To improve the poor prognosis, the development of novel diagnostic methods and therapeutic strategies is urgently required. Therefore, it is important to elucidate the molecular mechanisms underlying the development of biliary cancer and its precursor lesions.

5.1. Precursor lesions of BTC

BTC progresses through several precursor lesions. The WHO classification defines the precursors of biliary tract carcinoma as biliary intraepithelial neoplasm (BilIN), intraductal papillary neoplasm of the bile duct (IPNB), intracholecytic neoplasm of the gallbladder (ICPN), pyloric gland adenoma (PGA) of the gallbladder, and mucinous cystic neoplasm (MCN). (Fig. 5B)

BilIN is a microscopically identifiable, noninvasive, flat, or (micro) papillary lesion and IPNB is a grossly visible premalignant neoplasm with intraductal papillary or villous growth of the biliary-type epithelium. ICPN is a counterpart of IPNB in the GB. PGA of the GB is a grossly visible noninvasive neoplasm of the gallbladder composed of uniform back-to-back mucinous glands arranged in a tubular configuration. MCN are cystic epithelial neoplasms associated with ovarian-type subepithelial stroma (WHO Classification of Tumours Editorial Board, 2019). The epithelium of IPNBs and ICPN show 4 types (intestinal, biliary, oncocystic, and gastric), based on the histological appearance and immunophenotype (Zen et al., 2006; Adsay et al., 2012). However, tumorigenesis of these premalignant lesions remains unclear.

5.2. Genetic alternation of BTC

Notably, whole-genome and whole-exome sequencing analyses of BTCs have been reported (Table 1) (Nakamura et al., 2015; Jusakul et al., 2017; Wardell et al., 2018; Pandey et al., 2020). CCAs are a heterogeneous group of malignancies. Moreover, the frequency of alternation differs according to the anatomical location (intrahepatic, perihilar, and distal) (Banales et al., 2020) (Fig. 6). Notably, the most common gene alterations are *TP53* and *KRAS*. However, genetically targeted therapies for these mutations are challenging (Bekaii-Saab et al., 2021).

6. Tumor organoids

6.1. PDOs derived from BTC

Recently, reports on BTC organoid analyses have increased (Table 2) (Yáñez-Bartolomé et al., 2023).

Broutier et al. (2017) demonstrated that tumor organoids could be established from neoplastic epithelial cells in hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma (ICC), and combined HCC/CC (CHC) tumors. Intrahepatic cholangiocarcinoma organoids (iCCOs) were similar to the original cancer tissue in terms of gene and protein expression (positive for KRT19 and EpCAM and negative for AFP and HepPar1). iCCOs retained the mutational landscape of the original tumor. When subcutaneously transplanted into mice, the xenografted iCCOs adopted structures similar to those of primary iCCAs. Finally, drug screening of 29 compounds revealed that tumor organoids were sensitive to specific drug therapy compounds depending on the

Table 1
Gene alternation of BTC according to the most representative reports.

	publish year	**2015**	**2017**	**2018**	**2020**
	Author	**Nakamura et al.**	**Jusakul et al.**	**Wardell, Fujita et al.**	**Pandey et al.**
	Type of Sequence	**WES**	**WGS, WES, Targeted-seq**	**WGS, WES, Targeted-seq**	**WES**
The number of patients	**Total**	**239**	**489**	**412**	**160**
	iCCA	**137**	**310**	**136**	**-**
	pCCA	**-**	**128**	**109**	**-**
	eCCA	**74**	**45**	**121**	**-**
	GBC	**28**	**-**	**46**	**160**
	N/A	**6**	**6**		
category	***gene***				
TP53	*Tp53*	25.9 %	32.0 %	25.7 %	53.1 %
	ATM	4.2 %	5.0 %	3.2 %	5.0 %
Kinase-RAS	*KRAS*	18.0 %	16.6 %	17.0 %	3.8 %
	EGFR	5.0 %	0.4 %	0.5 %	0.6 %
	ERBB2	4.6 %	3.7 %	1.5 %	16.3 %
	FGFR2	4.6 %	2.8 %	0.2 %	1.3 %
	PIK3CA	6.7 %	4.6 %	5.3 %	5.0 %
	STK11	3.3 %	5.0 %	1.5 %	6.3 %
RB–cell cycle	*CDKN2A*	4.6 %	2.8 %	2.4 %	7.5 %
TGF-β	*SMAD4*	8.8 %	13.1 %	8.3 %	8.1 %
WNT	*RNF43*	2.1 %	4.8 %	1.5 %	2.5 %
	CTNNB1	2.1 %	1.3 %	2.9 %	11.3 %
	APC	4.6 %	7.2 %	4.4 %	7.5 %
SWI/SNF	*ARID1A*	11.3 %	17.4 %	6.1 %	9.4 %
	ARID2	6.3 %	5.5 %	3.6 %	14.4 %
	ARID1B	3.8 %	3.9 %	0.7 %	3.1 %
	PBRM1	5.4 %	6.5 %	5.8 %	3.8 %
Epigenetic	*BAP1*	7.5 %	8.5 %	4.1 %	1.3 %
	IDH1	3.3 %	3.5 %	3.4 %	1.3 %
Other	*ELF3*	5.4 %	6.3 %	2.4 %	21.3 %
	GNAS	6.3 %	7.0 %	1.2 %	4.4 %
	BRCA2	4.6 %	3.7 %	2.2 %	6.9 %

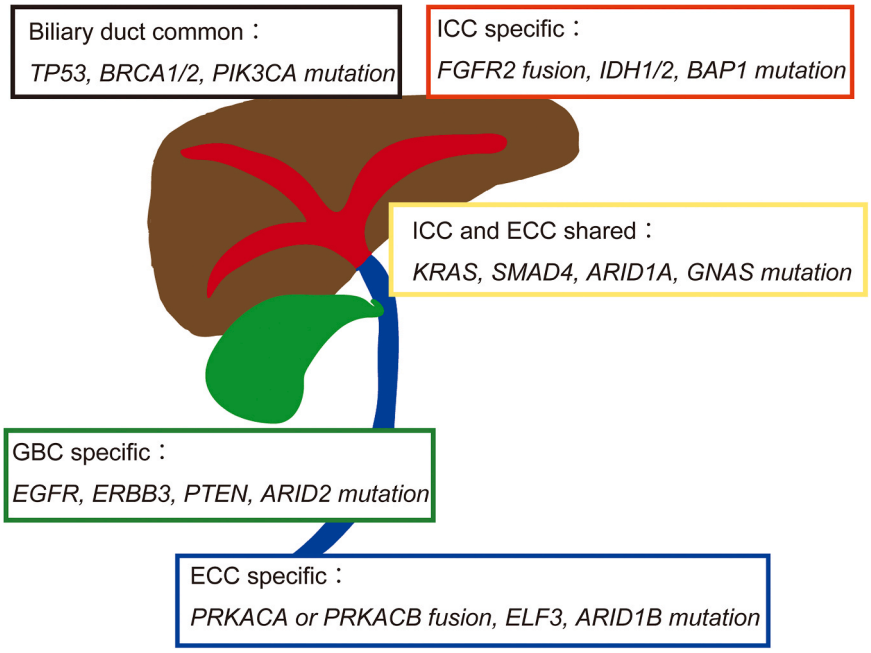

Fig. 6. Mutational profile of biliary tract carcinoma (BTC). Characteristic BTC gene mutation by anatomical region.

Table 2
Reports of recent PDO from BTC patients.

published year	Author	PDO model	Tumor types				Sampling method	Characterizations			Drug screening
		Total	iCCA	eCCA	GBK	Others		Histopathology	Genome	Transcriptome	
2017	Broutier et al.	8	3	–	–	5	Liver biopsy	H&E, AFP, HepPar−1, EpCAM, KRT19	WES	RNA-seq	29 compounds
2023	Lee et al.	16	16(SD-type, n = 5; LD-type, n = 8)	–	–	–	Surgical resection or biopsy	H&E, CK19, SOX9, Alb, vimentin, S100P, N-cadherin, and CD56 PD-L1	WES	RNA-seq	gemcitabine and cisplatin
2019	Saito et al.	6	3	–	1	2	Surgical resection	H&E, CK7, MUC1	WES	microarray	339 compounds
2023	Kinoshita et al.	60	5	49 (hCCA, n = 19; eCCA, n = 30)	1	5	Bile	H&E, CK7, HepPar−1	Targeted sequencing (TP53, KRAS)	–	–
2023	Ren et al.	61	44	13	4	–	Surgical resection, ascities, and biopsy	H&E, CK19, CK7	WES	RNA-seq	gemcitabine, 5-FU, cisplatin, SN−38, oxaliplatin, mitomycin C, paclitaxel

underlying mutation. iCCAs are subclassified into small-duct (SD) and large-duct (LD) subtypes according to histological characteristics. Lee et al. (2023) reported that iCCOs retain two ICCA subtypes in primary cancer cells. Transcriptome analysis of iCCOs revealed that the gene expression of tumor organoids from SD-type iCCA and LD-type iCCA is significantly distinct.

Saito et al. (2019) analyzed tumor organoids from gallbladder carcinoma organoids (GBCOs), iCCOs, and other carcinoma. Drug screening with 339 drugs revealed that 18 anticancer drugs can suppress the growth of iCCOs. Surprisingly, two antifungal drugs, a hyperlipidemia drug and a Parkinson's disease drug, also had anti-tumor efficiency against the growth of iCCOs.

Kinoshita et al. (2023) reported a method for generating bile-derived organoids from patients with biliary cancer. One problem with bile-derived organoids is the contamination with non-cancer cells. However, three methods: (1) repeated passage, (2) xenograft model, (3) use of Nutlin-3a (for cancer cell with *P53* mutation) enriched cancer cell-derived organoids.

Li et al. (2023) found that only iCCO is sensitive to bortezomib (BTZ), a proteasome inhibitor, but the co-culture of iCCO with cancer-associated fibroblasts (CAFs) leads to BTZ resistance. Evaluation of fibroblast gene expression revealed that CXCR4 was upregulated in CAFs. Inhibition of CXCR4 reduced CAFs, and combination therapy with bortezomib, a CXCR4 inhibitor, and a checkpoint inhibitor was effective against iCCA in vivo.

Recently, Ren et al. (2023) generated 61 BTC patient-derived organoids (PDOs) and screened conventional chemotherapeutics, including gemcitabine, cisplatin, capecitabine, 5-fluorouracil (5-FU), SN-38 (an active metabolite of irinotecan), oxaliplatin, mitomycin C, and paclitaxel. The response to chemotherapeutic drugs varied among patients

with PDOs. In a patient-derived organoid xenograft (PDOX) mice model, the response to each chemotherapeutic agent was similar to that observed in the in vitro assay. Moreover, the gene expression signatures of BTC PDOs with different drug responses can potentially predict chemotherapy responses in patients with BTC.

The PDO and PDOX models show promise to support tumorigenesis analysis, drug discovery, and precision medicine for BTC.

6.2. Tumor organoids derived from genetic engineered COs

Alternative approaches to establish tumor organoids for cancer modeling have also been explored. Using the CRISPR/Cas9 technology, normal COs were transformed by introduction of relatively high-frequent cancer driver gene mutations such as *KRAS, Tp53, phosphatase and tensin homolog (Pten), APC, C-MYC, BAP1, Fibroblast growth factor receptor 2 (FGFR2) fusion,* to generate tumor organoids. Moreover, transplantation of mutant organoids into immunodeficient mice presents malignant potential and carcinoma features with characteristics of cancer by the inserted oncogenic driver.

6.2.1. Murine genetic engineered cancer organoids

Saborowski et al. (2019) cultured murine liver organoid and performed subcutaneous injection of recombinased $Kras^{G12D/wt}$; $p53^{\Delta/\Delta}$ organoids. CCAs were developed with a 100 % success rate. This model is suitable for preclinical drug testing in an immunocompetent *in vivo* situation. Moreover, by using Cre-recombinase, CRISPR/Cas9, they transfected a plasmid co-encoding for a Cre-recombinase, Cas9 protein, and sgRNAs directed against the tumor suppressors *Pten* and *p53* into $Kras^{LSL\text{-}G12D/wt}$ organoids. As a result, *Kras-mutant/sgp53/sgPten* organoids gave rise to CK19-positive, moderately differentiated murine CCAs. Meanwhile, normal murine liver organoids were transfected with retroviruses encoding stably overexpressing-Myc, shRNA directed against p53, and transiently transfected with *sgAPC*. These mutant liver organoids were differentiated into CK19-negative tumors resembling hepatocellular carcinoma (HCC).

Ochiai et al. (2019) showed that oncogenic *Kras* and silencing of *Cdkn2a*, *Pten, Apc,* or *Trp53* in liver-derived organoids cooperate to induce iCCA while oncogenic *Kras* alone did not result in tumorigenesis. On the other hand, activation of the PI3K pathway ($Pik3ca^{H1047R}$ knocked-in) in liver-derived organoids did not induce formation of tumor even if either *Cdkn2a* or *Pten* was additionally silenced. FGFR2 fusion proteins (FFs) were reported to occur in 12–15 % of patients with iCCA (Sirica et al., 2019). *FGFR2-AHCYL1* fusion in liver-derived organoids did not induce formation of tumor in spite of additional knocked-in $Pik3ca^{H1047R}$ or silencing of *Cdkn2a*, *Pten,* or *Trp53*.

Cristinziano et al. (2021) generated mouse liver organoids expressing FGFR2 fusion Proteins. FGFR2 fusion proteins (FFs) were reported to occur in 12–15 % of patients with iCCA (Sirica et al., 2019). *Tp53-/-* organoids expressing ectopic FFs were transplanted in immuno-deficient mice or in the liver sub-capsular region or subcutaneously to assess tumorigenesis. FFs-driven organoids transformed moderately to poorly differentiated Ck19 + /HepPar1- adenocarcinomas at both orthotopic and subcutaneous transplantation sites. They showed that the FGFR inhibitor + MEK inhibitor combination therapy was efficient as therapeutic targeting of FFs-driven iCCA.

6.2.2. Human genetic engineered cancer organoids

Artegiani et al. (2019) found that BAP1 loss-of-function by using CRISPR/Cas9 in normal human COs lead to disruptions in epithelial organization, cell polarity, and increased cell motility. *TP53, SMAD4, NF1,* and *PTEN* were frequently observed among the top BAP1-co-mutated tumor suppressor genes. By subcutaneous and intrahepatic injections with alternation of *TP53/SMAD4/NF1/PTEN/BAP1* COs, adenocarcinoma resembling human cholangiocarcinoma developed.

Huang et al. (2014) have previously generated human induced hepatocytes (hiHeps) cells transdifferentiated from fibroblasts have shown prominent liver functions. Sun et al. (2019) generated liver organoids were generated from hiHep cells by transfection with SV40 large T antigen (SV40LT), which inactivation of p53 and RB. Liver organoids possessed liver architecture and function. The hiHep organoids overexpression of *c-Myc* resulted in to model human HCC. Meanwhile, RAS^{G12V}, YAP^{5SA}, *NICD,* $IDH2^{R172K}$, *FGFR2, MCL1,* and $PTPN3^{A90P}$ transformed organoids develop histopathological changes similar to human ICCs. Notably, all RAS^{G12V}-transformed hiHep organoids formed human ICC-like cancers in vivo after orthotopic transplantation.

7. Genetically engineered mouse models (GEMM) and organoid research

GEMMs are powerful tools for several reasons. Lineage tracing experiments and observations over time using GEMMs can reveal the tumor origin, initiation, cancerization, progression, and metastasis mechanisms. GEMMs are useful for investigating gene abnormalities, inflammation, and the surrounding environment in vivo because these factors can be manipulated. Therefore, studies on GEMMs and organoids could be useful for elucidating tumorigenic mechanisms and identifying the cellular origin of tumors and cancer. Here, we reviewed the GEMMs of biliary cancer and its precursor lesions.

7.1. $Hnf1b^{CreER}$; KrasG12D; $Ctnnb1^{lox(ex3)/t}$ (HKβ) mice

The role of the KRAS and canonical Wnt pathways in the tumorigenesis of the extrahepatic biliary system were examined. Analysis of $Hnf1b^{CreER}$; KrasG12D; $Ctnnb1^{lox(ex3)/+}$ (HKβ) mice revealed that concurrent activation of KRAS and Wnt pathway induced biliary tumors that resembled BilIN in the EHBD and ICPN in the GB (Fig. 7). Xenograft experiments using murine mutant biliary organoids have demonstrated that ICPN and BilIN are precancerous lesions that progress to biliary cancer. Global gene expression analysis of mutant biliary organoids revealed that c-Myc and TGF-β signaling were upregulated in *HKβ* biliary organoids. Furthermore, silencing of *Smad4 or Tgfbr2* increased the growth ratio of *HKβ* organoids and the formation rate of allografted cancer. Murine biliary organoid analyses demonstrated that c-Myc contributes to tumorigenesis, whereas the Tgfb pathway inhibited it in *HKβ* mice (Nagao et al., 2022).

7.2. $Hnf1b^{CreER}$; KrasG12D; Rosa-NICD (HKN) mice

Next, we reviewed the functional role of the KRAS and Notch pathways in tumorigenesis of the biliary tract. Analysis of $Hnf1b^{CreER}$; *KrasG12D; Rosa-NICD (HKN)* mice revealed that simultaneous activation of the KRAS and Notch pathways induced BilIN expression in the EHBD and GB (Fig. 8). mTORC1 signaling was upregulated in *HKN* biliary organoids, and an mTOR inhibitor suppressed cell growth in *HKN* organoids and Notch-activated human biliary cancer cells. Therefore, the mTORC1 pathway contributes to the growth of murine *HKN* organoids and Notch-activated human biliary cancer cells (Namikawa et al., 2023).

7.3. $Pdx1\text{-}Cre$; $Kras^{G12D}$; $Trp53^{R172H}$; $Elf3^{f/f}$ (KPCE) mice

Loss-of-function mutations in *the E74-like ETS transcription factor 3* (*ELF3*) are frequently observed in human GB cancer (Pandey et al., 2020). Analysis of $Pdx1\text{-}Cre;Kras^{G12D};Trp53^{R172H};Elf3^{f/f}$ (KPCE) mice revealed the formation of massive papillary lesions with a higher grade of cellular atypia in the GB. ERBB and EGFR/mTORC1 signaling were significantly enriched in *KPCE* GBOs. RT-PCR and ChIP assays on *KPCE* GBOs and *Elf3*-overexpressed *KPCE* GBOs revealed that ELF3 directly downregulates epiregulin (Ereg). CRISPR/Cas9-mediated *Ereg* KO in *KPCE* organoids showed that the formation of the mesenchymal

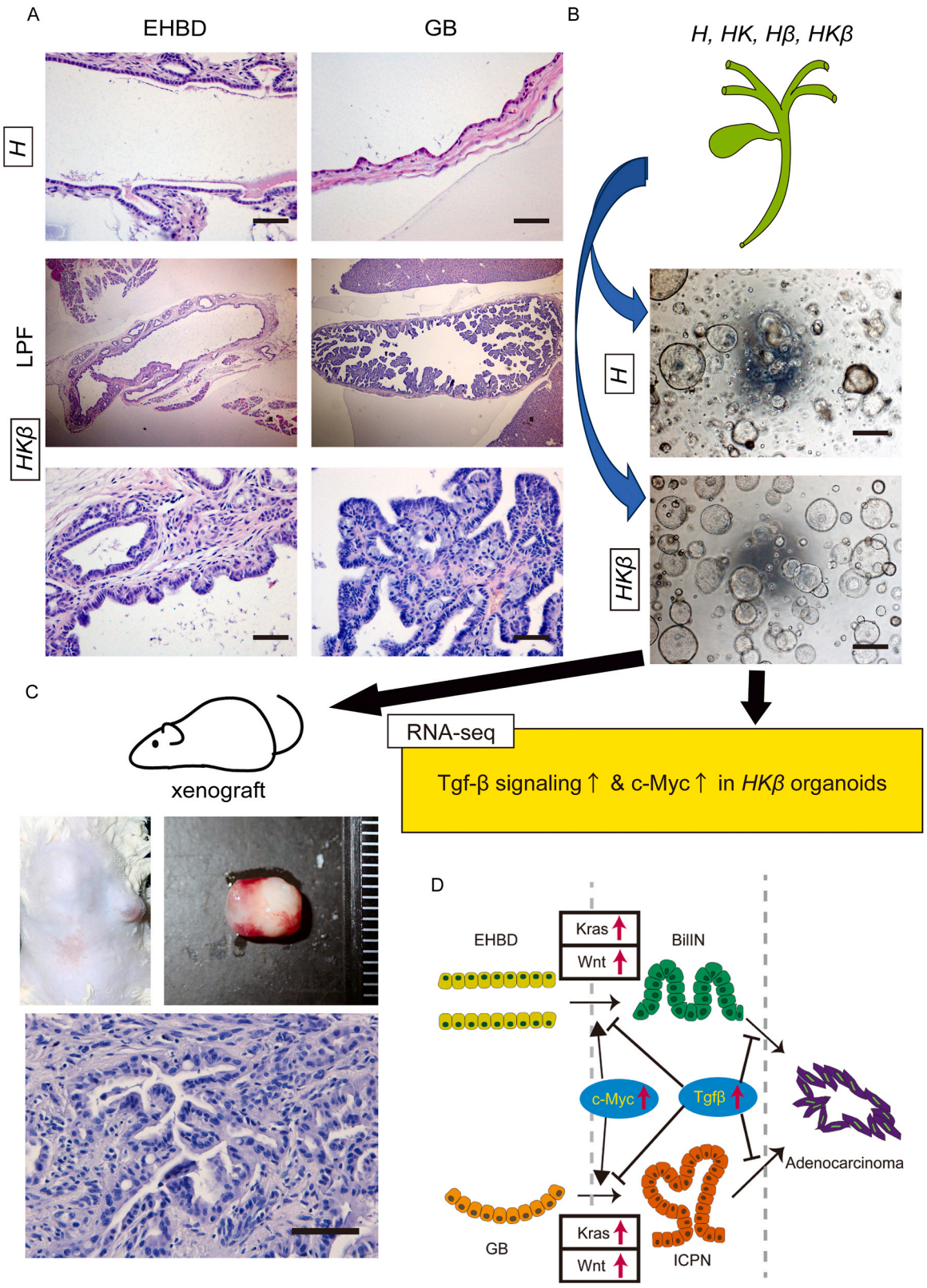

Fig. 7. Concurrent activation of KRAS and Wnt signaling mouse model (*HKβ*). (A) H&E staining of extrahepatic bile duct and gallbladdar of *HKβ* and control mice *(H)*. Scale bar, 50μm (B) Cholangiocyte organoids (COs) derived from a mutant biliary duct. Scale bar, 500μm (C) Allografted tumor from *HKβ* COs and global gene expression analysis. Scale bar, 50μm (D) Schema of *HKβ* mouse and mutant COs in vitro and in vivo analysis.

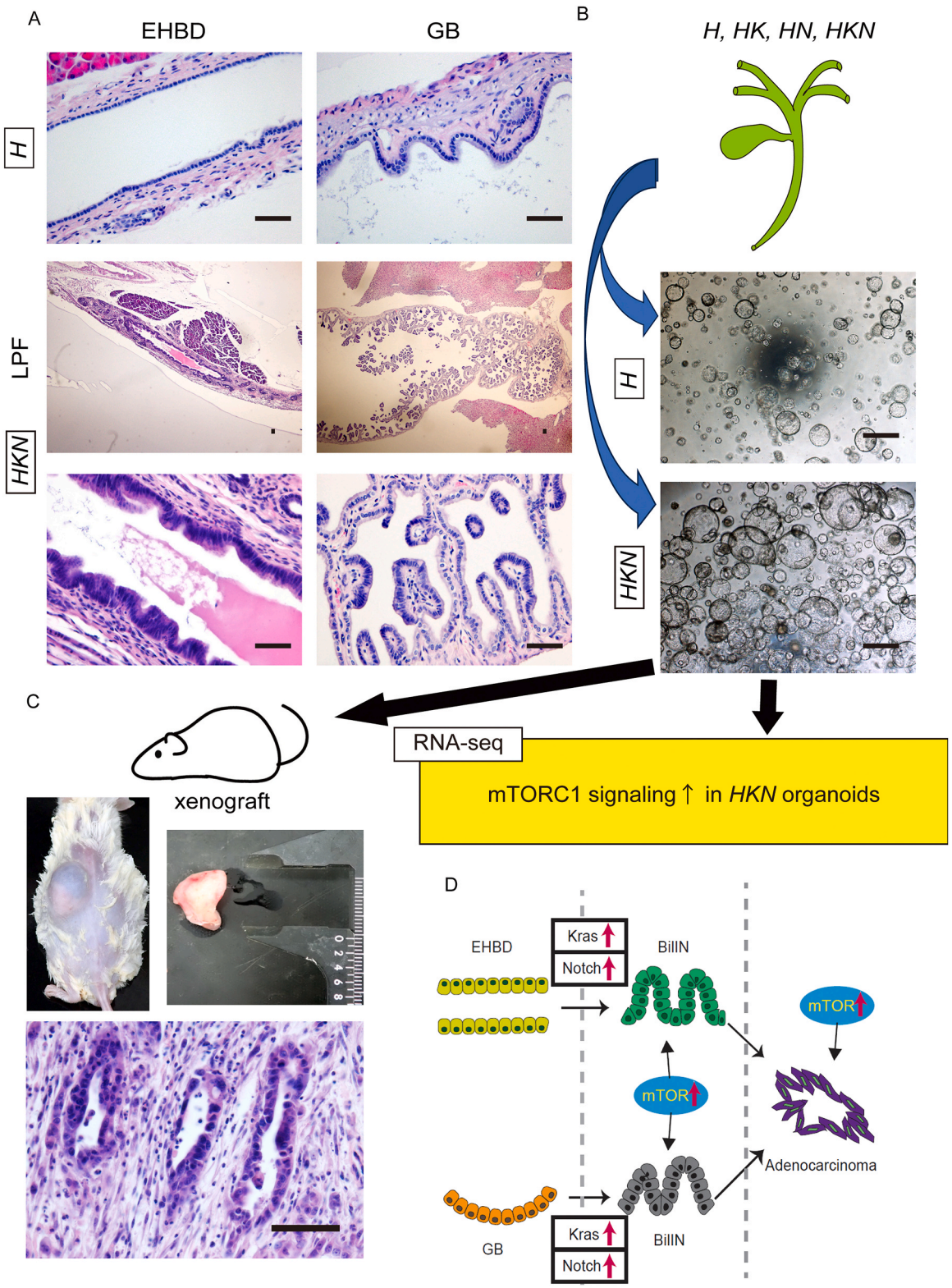

Fig. 8. Simultaneous activation of KRAS and Notch signaling mouse model (*HKN*). (A) H&E staining of extrahepatic bile duct and gallbladdar of *HKN* and control mice *(H)*. Scale bar, 50μm (B) Cholangiocyte organoids (COs) derived from a mutant biliary duct. (C) Allografted tumor from *HKN* COs and global gene expression analysis. Scale bar, 500μm (D) Schema of *HKN* mouse and mutant COs in vitro and in vivo analysis. Scale bar, 50μm.

structure of *KPCE* allograft tumors was regulated by EGFR/mTORC1 signaling via Ereg. Therefore, loss of *Elf3* induces the formation of massive papillary lesions in the *KPCE* gallbladder and the mesenchymal structure of *KPCE* allograft tumors via the EREG/EGFR/mTORC1 pathway (Nakamura et al., 2023).

Although GEMMs are useful for deciphering the biology of biliary tumorigenesis, they have limitations. First, the GEMMs are mice, not humans. Second, engineering multiple alleles increases the time and cost required for the breeding and maintenance of GEMMs. Organoids can be used for biological investigations. For example, genetic manipulation of key driver mutations in normal organoids could mimic cancer

development in vitro. The co-culture of organoids with fibroblasts could provide mechanistic insights into biliary cancer. Furthermore, organoids are a good platform for drug testing prior to patient treatment, as their treatment response correlates well with that of the patients.

8. Future perspective

Organoid technology has been rapidly developing over a decade. COs can provide us some benefits. On the other hand, we should know limitation of organoid research.

Advantages:

- **Cryopreservation and Repeatability:** Both COs and iPSCs can be cryopreserved, allowing for repeated experiments and validation in vitro.
- **Safe Sampling:** Sampling of primary cultures is safe and non-invasive for patients.
- **3D Culture Versatility:** The 3D culture system supports the growth of both cancerous and non-cancerous cells, making it easier to investigate gene function, drug efficacy, and responses to external factors.

Limitation

- **Dependency on Culture Conditions:** The outcomes of organoid research are significantly influenced by specific culture conditions.
- **Challenges in Replicating the Tumor Microenvironment (TME):** It is difficult to fully replicate the TME, as essential components like cancer-associated fibroblasts (CAFs) and immune cells are challenging to model accurately in organoid cultures.
- **Safety in human medical applications:** Organoid technology is currently limited to research purposes in regenerative medicine. To ensure its safety for therapeutic use in humans, further validation is needed.

However, these limitations are expected to be resolved with future advancements in organoid technology. Precision medicine for CCA and regenerative therapies for humans hold the potential to bring significant breakthroughs.

9. Conclusions

Cholangiocyte organoids derived from primary tissues or iPSCs have emerged as potent tools in disease modeling, cancer research, and regenerative medicine. These 3D structures recapitulate the key functional and structural properties of the tissue of origin, allowing the detailed study of biliary diseases, including cholangiopathies and biliary tract cancers. Organoids demonstrate a significant potential for understanding disease mechanisms, screening drugs, and repairing bile duct injuries through transplantation. Advances in organoid technology and iPSC differentiation protocols have enabled the generation of complex organoids encompassing various biliary regions and functions, thereby furthering their applicability in research and therapy.

Key insights include the establishment of cholangiocyte organoids (COs) from the intrahepatic and extrahepatic bile duct, gallbladder, and pancreatic duct tissues. These organoids have facilitated significant discoveries of biliary disease mechanisms such as cystic fibrosis and primary sclerosing cholangitis and have been pivotal in developing therapeutic strategies for cholangiocarcinoma. Additionally, the use of genetically engineered mouse models (GEMMs) in conjunction with organoids has provided deeper insights into tumorigenesis and cancer progression, thereby offering promising avenues for precision medicine.

Although organoid technology holds tremendous promise, limitations such as genomic instability of iPSC-derived organoids need to be addressed to ensure their safe clinical application. Moreover, standardization of organoid nomenclature and classification systems is crucial for consistent scientific communication and reproducibility.

In conclusion, cholangiocyte organoids represent a transformative advancement in biomedical research with broad implications for understanding biliary biology, modeling diseases, discovering new drugs, and developing regenerative therapies. Continued innovation and standardization in this field will likely yield significant breakthroughs in treating biliary diseases and cancers.

CRediT authorship contribution statement

Tsuruyama Tatsuaki: Investigation. **Shinnosuke Nakayama:** Investigation. **Yuki Nakanishi:** Methodology, Funding acquisition. **Kei Iimori:** Investigation. **Go Yamakawa:** Investigation. **Hiroshi Seno:** Supervision, Project administration, Funding acquisition. **Kenta Mizukoshi:** Investigation. **Yukiko Hiramatsu:** Investigation, Funding acquisition. **Akihisa Fukuda:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Tomonori Masuda:** Investigation. **Munemasa Nagao:** Writing – original draft, Investigation, Data curation, Conceptualization. **Takahisa Maruno:** Investigation. **Sho Matsuyama:** Investigation. **Yu Muta:** Investigation, Funding acquisition. **Hirotaka Kashima:** Investigation. **Mayuki Omatsu:** Investigation. **Mio Namikawa:** Resources, Investigation, Data curation. **Munenori Kawai:** Investigation.

Declaration of Competing Interest

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

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Data availability

No data was used for the research described in the article.

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