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In vitro generation of cholangiocyte-like cells from human liver organoids

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Reality is for people that lack imagination.
(Hayao Miyasaki)

ABSTRACT

The fact that biliary diseases carry high morbidity and mortality has motivated researchers to understand the mechanisms behind it by trying to design an *in vitro* model to study the pathophysiology of these diseases.

To achieve this goal, we propose an *in vitro* system using human LGR5^{+} liver stem cells (liver organoids) to generate cholangiocyte-like cells (CLCs). This protocol consists of a 9-day differentiation using a combination of growth factors and extracellular matrix components, followed by gene expression and immunofluorescence analysis, and functionality assays.

CLCs generated in our protocol acquired differentiated cholangiocyte characteristics, including expression of key biliary markers, cilia, and the ability to transport small molecules and respond to farnesoid X receptor agonist.

Consequently, our novel *in vitro* model provides a new tool to study the biological mechanisms controlling cholangiopathies, a potential use in regenerative medicine therapies, as well as drug screening.

Keywords: Biliary diseases, cholangiocytes, organoids, regenerative medicine

RESUMO

As elevadas taxas de morbidade e mortalidade causadas pelas doenças biliares têm motivado os esforços levados a cabo pelos investigadores em entender os mecanismos que lhes estão inerentes, tentando, para tal, conceber um modelo *in vitro* que permita o estudo da fisiopatologia destas doenças.

Com esse intuito, este estudo propõe um sistema *in vitro* com células estaminais hepáticas humanas LGR5 + (organóides de fígado) a fim de gerar *cholangiocyte-like cells* (CLCs). Este protocolo implica 9 dias de diferenciação utilizando, para tal, uma combinação de fatores de crescimento e componentes da matriz extracelular, prosseguido pela análise de expressão genética e de imunofluorescência, e testes de funcionalidade.

As células geradas através do protocolo supracitado incorporaram características de colangiócitos diferenciados, incluindo a expressão dos principais marcadores biliares, cílios e a capacidade de transportar moléculas pequenas e responder ao agonista do receptor X farnesóide.

Consequentemente, nosso novo modelo *in vitro* fornece uma nova ferramenta para estudar os mecanismos biológicos que controlam as colangiopatias, o seu uso em medicina regenerativa, bem como *drug screening*.

Palavras-chave: Doenças biliares, colangiócitos, organóides, medicina regenerativa

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LIST OF ABBREVIATIONS

3D Three-dimensional

A

AGS Alagille Syndrome

ALP Alkaline Phosphatase

ALT Alanine Aminotransferase

AMA Antimitochondrial Antibody

B

BEC Biliary Endothelial Cell

BMP Bone Morphogenic Protein

C

cAMP Cyclic adenosine monophosphate

CCA Cholangiocarcinoma

CDM Cholangiocyte Differentiation Medium

CEA Carcinoembryonic Antigen
CA Cancer Antigen

CF Cystic Fibrosis

CLC Cholangiocyte-like Cell

D

DILI Drug-induced Liver Injury

DP Ductal plate

E

ECM Extracellular matrix

EGF Endothelial Growth Factor

EHBD Extrahepatic Bile Duct

F

FGF Fibroblast Growth Factor

FXR Farnesoid X Receptor

G

GABA Gamma-aminobutyric

GF Growth Factor

H

HGF Hepatocyte Growth Factor

HLA Human Leucocyte Antigen

HSC Hepatic Stem Cell

I

IBD Inflammatory Bowel Disease

IHBD Intrahepatic Bile Duct

iPSC induced Pluripotent Stem Cell

M

MDR Multidrug Resistance Protein

O

OEM Organoid Expansion Medium

OSM Oncostatin M

OST Organic Solute Transporter

P

PBC Primary Biliary Cirrhosis

PBG Peribiliary Glands

PGA Poly(glycolic acid)

PSC Primary Sclerosing Cholangitis

S

STM Septum Transversum Mesenchyme

T

TB Total Bilirubin

TGF-β Transforming Growth Factor β

TLR Toll-like Receptor

TNF Tumor Necrosis Factor

U

UDCA Ursodeoxycholic acid

INTRODUCTION

Biliary diseases are progressive disorders that can cause acute and chronic liver illnesses and have accounted for approximately 15% of all liver transplants performed in Europe between 1989 and 2009 [1]. Consequently, a liver transplant is required for survival of the patients.

The fact that these diseases not only carry high morbidity and mortality but also have limited treatments available has prompted researchers to try to understand the exact mechanisms leading to these diseases and ways to treat them [2].

The biliary tree is a complex three-dimensional (3D) tubular network that drains the bile secreted from the liver to the digestive tract [3]. The bile is first secreted by hepatocytes (i.e. primary bile) into the bile canaliculi and then flows through the bile ducts, which are lined by biliary endothelial cells (BECs), termed cholangiocytes, that will be responsible for its final composition [4].

Cholangiocytes are involved in both bile secretion and in the inflammatory cascade as they are able to secrete different pro-inflammatory mediators, cytokines, and chemokines. The pathophysiology of these cells has been the target of many studies that have the goal of providing clues for future therapeutic strategies once there is an absence of *in vitro* systems and animal models to model and to study biliary diseases [5].

Table 1. Summary of Cholangiopathies [2]

Disease	Clinical Manifestations	Diagnosis	Treatments	Animal Models
Primary Biliary Cirrhosis	Progressive destruction of intrahepatic bile ducts	• Autoantibodies • Liver biochemistry changes • Liver histology	• UDCA • Liver transplantation • Symptoms therapy	• dnTGFβRII Mice • IL-2Rα−/− Mice • NOD.c3c4 Mice • Ae2a,b−/− Mice • Scurfy Mice • MRL/lpr Mice
Primary Sclerosing Cholangitis	Inflammation and fibrosis of the intra- and extrahepatic bile ducts	• Cholangiography • Liver histology • Liver biochemistry changes	• Liver transplantation • Symptoms Therapy	• Mdr2 (Abcb4) Gene Knockout Mice • Cftr−/− Mice • Mice with a Point Mutation in the Ferrochelatase Gene (fch/fch) • Mice Fed the Porphyrinogenic Substance DDC
Drug-Induced Cholestasis	Variable	• Liver biochemistry changes	• Withdrawal of the suspected drug • Avoiding drug rechallenge • Treatment of symptoms	N/A
Cholangiocarcinoma	Features of primary sclerosing cholangitis; Inflammation and cholestasis	• Magnetic resonance cholangiopancreatography • Serum carbohydrate antigen	• Surgery • Chemo-radiotherapy	Xenograft and Orthotopic Models

1.1. Biliary Diseases

Biliary diseases represent a complex group of liver diseases that affect the extra- and intrahepatic bile ducts (**TABLE 1**), which includes primary biliary cholangitis, primary sclerosing cholangitis, cholangiocarcinoma and drug-induced liver cholangitis.

To better understand the pathophysiology of biliary diseases, a brief description of each disorder is followed:

1.1.1. Primary Biliary Cholangitis

Primary biliary cholangitis (PBC) is an autoimmune liver disease characterized by progressive destruction of intrahepatic bile ducts, leading to cholestasis, cirrhosis, and liver failure [6]. Typically, PBC affects more women than men.

A high sensitivity and specificity of antimitochondrial antibodies (AMA) for PBC, along with high levels of serum alkaline phosphatase and liver histology allow the correct diagnosis of this disorder. Additionally, serum alkaline phosphatase (ALP) can be used as a measure of disease activity and response to treatment [7].

PBC is characterized by an enrichment of autoreactive $CD4^+$ and $CD8^+$ T cells, natural killer (NK) cell activity and monocyte responses and accumulation of Th17 cells around damaged bile ducts [6].

As for treatments, ursodeoxycholic acid (UDCA) [8] remains the only FDA-approved drug to treat patients with PBC. While liver transplantation remains the only effective option for those patients who do not respond to UDCA treatment, a shortage of liver donors, lifelong medication and follow-up are aspects to be taken into consideration. Novel therapeutic approaches for PBC patients include bile acid-based therapies [9], immune-based therapies [10], and cell-based therapies [11].

1.1.2. Primary Sclerosing Cholangitis

Primary sclerosing cholangitis (PSC) is a chronic liver disease, predominantly manifested in men, characterized by inflammation and scarring of the bile ducts that ultimately leads to cirrhosis [12].

Not much is known about the causes that lead to PSC, however, it seems to be immune-mediated and strongly associated with inflammatory bowel disease (IBD). PSC is characterized by some important features that include portal tract inflammation with lymphocytes, progressive fibrosis, and bile duct destruction [13].

For its diagnosis, high levels of ALP and γ-glutamyltranspeptidase, combined with a supportive cholangiography can help secure the diagnosis [12].

PSC carries high morbidity and mortality once not only it has an indeterminate pathogenesis but also lacks effective medical therapy. UDCA used to treat patients with PBC was thought useful as an alternative treatment, unfortunately, its effect is lost upon drug cessation. Appropriate treatment of symptoms and infective complications are available for patients; however, liver transplantation remains the only effective treatment [2].

1.1.3. Cholangiocarcinoma

Cholangiocarcinoma (CCA) is an epithelial biliary malignancy that may arise anywhere along the biliary tree. It has been classified into three different subtypes: intrahepatic (iCCA), perihilar (pCCA), and distal (dCCA) [2]. The cellular origin of CCA is still controversial, while the majority of studies show that transformed cholangiocytes [14] are involved, others have evidence that it may also originate from hepatic progenitor cells [15] and peribiliary glands (PBGs) [16].

The pathophysiology of CCA is not entirely clear; however, inflammation and cholestasis are key factors present in all different subtypes. Other risk factors include the presence of PSC, liver fluke infections, hepatolithiasis, and cirrhosis [17].

Having such a dismal prognosis, a correct diagnostic including biochemical tests and radiological data is necessary. High levels of ALP and bilirubin alone are not satisfactory, moreover, expression of tumor markers, such as carcinoembryonic antigen (CEA) [18] and cancer antigen (CA) 19-9 [19] are elevated in cholangiocarcinoma.

The different types of CCA display different clinical behavior and molecular patterns, consequently, their management and clinical trials must be treated considering distinct malignancies. Several rodent models developed for the study of CCA aim to provide suitable tools for future therapeutics, nevertheless, they harbor important limitations and difficulties, such as species-specific microenvironment and bile duct size, which is far more time-consuming and technically difficult [2].

1.1.4. Drug-induced Liver Injury (Cholestasis)

Drug-induced liver injury (DILI) is a cause of acute and chronic liver disease that is induced by a wide range of prescription or non-prescription drugs, including alternative medicines such as Chinese medicine, natural medicine, health products, and dietary supplements. The clinical picture of DILI resembles every other liver disease, and while different drugs can lead to the same phenotype, DILI due to a specific drug often manifests characteristic clinical features of that same drug [20].

Levels of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and total bilirubin (TB) are assessed for the diagnosis of DILI, that even though not specific for DILI, remain the hallmark for detecting and classifying liver damage [21]. For the clinical characterization, the RUCAM scale is widely used when DILI is suspected. An R value based on the pattern of serum enzymes is calculated, and it consists on the ratio of ALT to ALP $\frac{ALT\ value \div ALT\ upper\ normal\ limit}{ALP\ value \div ALP\ upper\ normal\ limit}$. Depending on the R value, DILI can be classified as hepatocellular DILI ($R \geq 5$), cholestatic ($R \leq 2$), and mixed ($2 < R < 5$) [22].

An objective and specific diagnostic is still not possible once, for patients with polypharmacy, it is difficult to find out which drug used was a potential hepatotoxin. With this in mind, a test system called MetaHeps was developed to help identify the DILI-causing drug out of a panel of comedications. This test uses monocyte-derived hepatocyte-like cells (MH cells) from the patient, which can be exploited for investigation of drug hepatotoxicity *in vitro* once they carry donor-specific hepatocyte characteristics [23].

1.2. HEALTHY BILIARY TREE: Cellular and Immune biology

The biliary tree is composed of intra- and extrahepatic ducts, both anatomically and functionally different, that connect the liver to the intestine. Lining the biliary ducts, a subset of cells, known as cholangiocytes, are responsible to modify the hepatocyte-derived bile. Even though bile modification is the main physiological function of these cells, several other functions have been highlighted, including their capacity to proliferate, secrete proinflammatory mediators and antimicrobial peptides, in response to liver injury [24].

1.2.1. Bile Duct Development and Biliary Differentiation

During liver development, both hepatocytes and cholangiocytes arise from common progenitor cells named hepatoblasts [27]. In addition, cholangiocytes lining the intrahepatic and extrabiliary ducts have different embryonic origins, while the intrahepatic cholangiocytes originate from hepatoblasts, the extrahepatic cholangiocytes derive directly from the endoderm [28].

Gastrulation is a key process in embryonic development once it is responsible for the formation of the three germ layers: ectoderm, mesoderm, and endoderm. From the endoderm, a primitive gut tube is formed that is divided into foregut, midgut and hindgut regions. Studies in mice have shown that at embryonic day 8 (E8.0) of gestation the embryonic liver originates from the ventral portion of the foregut endoderm [27].

Looking more closely at the development of the liver, at E9.0, a structure known as hepatic/liver diverticulum (**FIGURE 1**), bearing morphological signs of the embryonic liver starts forming. The anterior part of this structure will give rise to the liver and intrahepatic bile ducts (IHBD), whereas from its posterior portion, the gallbladder and extrahepatic bile ducts (EHBD) will be formed [28]. From this stage, hepatic endoderm cells identified as hepatoblasts start proliferating and invading the adjacent septum transversum mesenchyme (STM), ending in the formation of the liver bud. Between E10–15 the liver bud undergoes intense growth as a result of vascularization and colonization by hematopoietic cells, ultimately giving rise to the fetal hematopoietic organ [31].

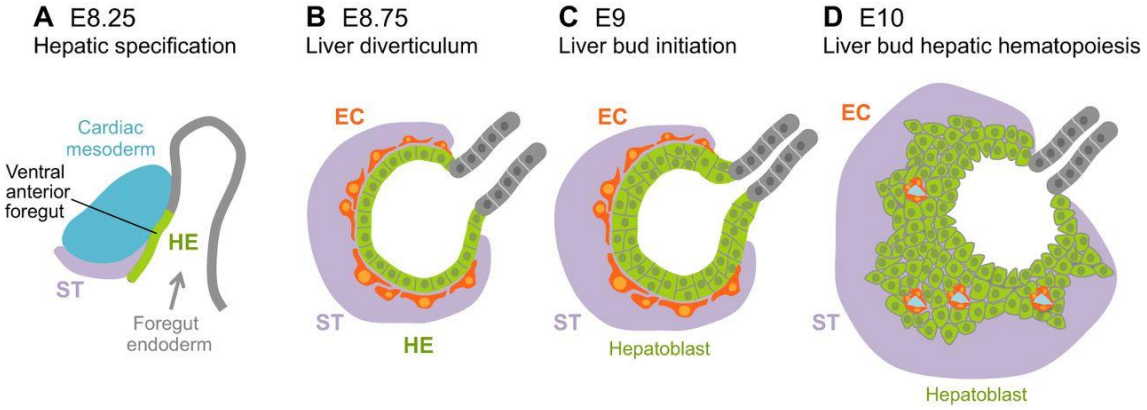

Figure 1. Liver diverticulum and bud formation in mice. At E8.75, endothelial cells (ECs, orange) are found surrounding the thickened hepatic endoderm, which initiates a budding process into the septum transversum. Endothelial cells contribute to hepatic specification. (C) At E9, the hepatic endoderm transitions from a columnar to a pseudostratified epithelium. (D) At E10, hepatic endoderm cells, identified as hepatoblasts, proliferate and migrate into the septum transversum to form the liver bud [29].

Hepatoblasts in the liver bud that reside adjacent to the portal veins will become biliary endothelial cells lining the lumen of the IHBD, while the hepatoblasts residing in the parenchyma will differentiate into hepatocytes [28].

As mentioned previously, intrahepatic and extrahepatic bile ducts do not share the same embryological origin. Extrahepatic bile ducts derive from the caudal part of the ventral foregut, from a $Hex^{-}/Pdx\text{-}1^{+}/Sox17^{+}$ primordial cell population, while the intrahepatic bile ducts arise from hepatoblasts, bipotent parenchymal cells, characterized by a $Hex^{+}/Pdx\text{-}1^{-}/ Sox17^{-}/AFP^{+}$ phenotype [30].

Hepatoblasts start differentiating toward a biliary phenotype when the ductal plate (DP) first emerges. The ductal plate, set between the portal mesenchyme and hepatoblasts, progressively undergoes remodeling, leading to the migration of part of its cells while the others disappear. The migrated cells will then become immature bile ducts and peribiliary glands. It is important to reiterate that the DP is characterized by expression of both hepatocellular (Keratin 8 (KRT8) and KRT18) and biliary markers (KRT19). The hepatocellular markers will gradually be downregulated while other biliary markers will start being expressed in the development of the bile duct [31].

For the development of the bile duct, hepatoblasts furthermore need stimuli from Notch, TGFβ and Wnt/b-catenin signals, growth factors, and the surrounding extracellular matrix (ECM) [32].

1.2.2. Signals Regulating Biliary Differentiation

Notch signaling

Notch signaling is essential during both development and in adults [33]. In mammals, four Notch receptors (Notch1-4) and five Notch ligands (Jagged1-2, Delta-like 1,3,4) have been found; However, particularly Notch2 and Jagged1 seem to play an essential role in biliary fate.

The interaction between Jagged1 (JAG1) and Notch2 begins when JAG1 starts being expressed by the nascent portal mesenchyme, prompting the transdifferentiation of Notch2-expressing hepatoblasts, located in the bile mesenchyme, into a biliary phenotype. Along with these two mediators, the hairy and enhancer of split-1 (Hes1) is also activated and will stimulate the activation of the cholangiocyte-specific transcription factors, HNF1β and Sox9, resulting in the development of the ductal plate [2].

This evidence was supported by patients diagnosed with Alagille syndrome (AGS), an autosomal-dominant disease that is caused by a mutation in either JAG1 or Notch2. Patients with AGS exhibit features that comprise IHBD paucity, cholestasis, and are also accompanied by heart and skeletal anomalies [34]. Moreover, when trying to *in vitro* differentiate cholangiocytes from human pluripotent stem cells (hPSCs), it was shown that Notch signaling is fundamental to generate structurally and functionally mature cholangiocytes [35].

Wnt/b-catenin pathway

Evidence that Wnt signaling plays an important role during hepatic development is supported by experiments in various species [36].

While repression of Wnt/b-catenin is needed in an early phase of development to allow the expression of transcription factors implicated in liver development, its stimulation is required for liver specification and growth in a later stage [37]. With respect to differentiation of hepatoblasts into cholangiocytes, while activation of Wnt signaling after the development of the liver bud results in a loss of hepatocytes, whereas biliary differentiation is conserved, its loss contributes to biliary hypoplasia [38]. In accordance with this, another study demonstrated that β-catenin is dispensable for this differentiation to take place, but that overexpression of β-catenin is detrimental to biliary differentiation and morphogenesis [39].

TGFβ signaling

The transforming growth factor beta (TGFβ) signaling family includes various ligands including TGF-βs, bone morphogenetic proteins (BMPs) and activins. A gradient of Activin/TGFβ signaling is essential for hepatocyte and cholangiocyte differentiation. Around the portal vein, where cholangiocytes start differentiating, the signaling is high, whereas in the surroundings is low, where hepatocytes differentiate [40].

For TGFβ signaling to be transmitted, TGFβ-receptor 2 (TGFBR2) must be stimulated by TGFβ1, TGFβ2 or TGFβ3. The interaction between TGFβ and TGFBR2 causes a conformational shift that results in SMAD complexes (main signal transducers for receptors of the TGFβ family) moving into the nucleus and function as transcriptional factors that regulate the expression of differentiation-related genes. Overexpression of TGFBR2 has been demonstrated to inhibit hepatocyte differentiation from hepatoblast-like cells (HBCs) while stimulating cholangiocyte differentiation [41].

Another study has shown that mice deficient in either HNF-6 or OC-2, Onecut transcription factors required for normal biliary development, manifested abnormal biliary structures after birth and cholangiocytes remained in ductal plate configuration [40].

FGF signaling

Fibroblast growth factors (FGFs) are needed to regulate hepatic fate within the definitive endoderm through activation of FGF receptors (FGFRs) [42].

Experiments using chick transplants have demonstrated that FGF is the hepatic inducing signal from the cardiac mesoderm. Low expression of FGF in endodermal cells adjacent to the cardiac mesoderm induced hepatoblast formation while a high concentration of FGF gave rise to lung progenitor cells [43].

Among FGF receptors, FGF2 (also known as bFGF) has shown to play an important role in the hepatic specification. To support this evidence, Yanai et al. used a mesenchyme-free culture of liver bud to demonstrate that bFGF and FGF7 induced hepatoblast differentiation into BECs and that this differentiation was enhanced by BMP

[44]. Another study using human iPSCs was performed by Twaroski et al. where it was reported that FGF2 inhibited canonical Wnt signaling during hepatic specification of the endoderm which is required for further differentiation along the hepatic lineage [45].

Extracellular matrix (ECM) molecules

During liver development, growth factors (GFs) and components of the extracellular matrix (ECMs) contribute to the differentiation of liver parenchymal cells. Suzuki et al. using freshly generated ALB-positive stem cells from mouse fetal liver were able to describe that the hepatocyte growth factor (HGF) in an early stage is important to achieve this phenotype and that the combination of HGF and oncostatin M (OSM) suppressed the development of bile ducts. Other ECM components such as laminin, type IV collagen, and type I collagen contributed, in a similar manner to HGF and OSM, to the differentiation of hepatic stem cells. As for biliary differentiation, HNF6 proved to be fundamental for differentiation and maturation of cholangiocytes rather than hepatocytes, since in its absence intrahepatic and extrahepatic bile ducts displayed abnormalities [46].

Another study that highlights the role of ECM components in the differentiation of HPCs is the work of Vyas et al. who used decellularized liver tissue [47]. Moreover, the seeding of PSCs on Matrigel-coated surfaces improves hepatic differentiation, and Matrigel is required to culture liver organoids over long time periods [48]. The interaction cell-matrix was again shown for laminin 411 and 511 which contributed to the differentiation of hiPSCs towards cholangiocytes expressing major biliary markers such as aquaporin-1 (AQP1), cystic fibrosis transmembrane conductance regulator (CFTR), secretin receptor (SCTR) and gamma-glutamyltransferase 1 (GGT1) [49].

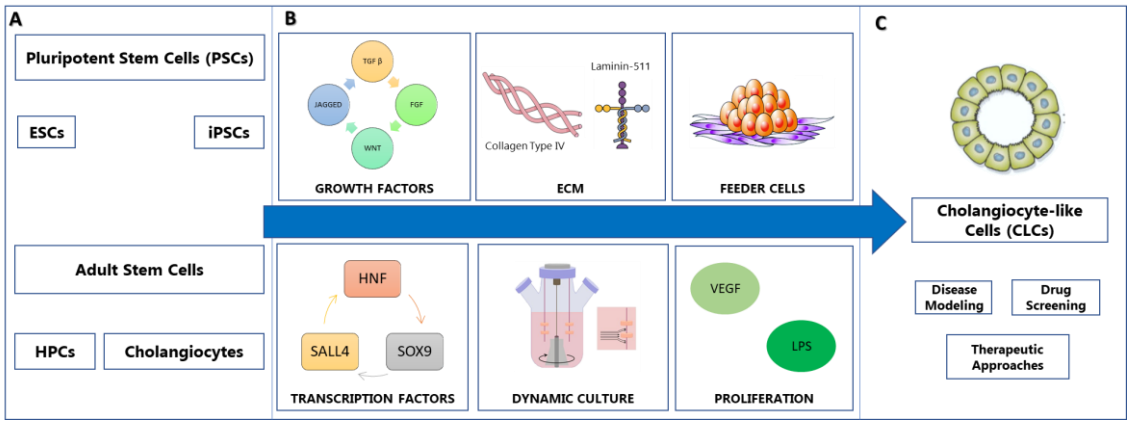

Figure 2. Factors influencing the differentiation of pluripotent stem cells and adult (stem cells) towards cholangiocytes. Within the approaches to differentiate cholangiocytes, **(A)** different cell sources are used, which are subsequently cultured in specific culture systems that use **(B)** growth factors, transcription factors, extracellular matrix molecules, feeder cells or dynamic culture elements or a combination of these options. Alternatively, the proliferation of obtained cells is stimulated to increase the pool of cholangiocyte-like cells. Next, **(C)** a heterogeneous cell population is produced that, after classification, can be used in a range of applications.

1.3. Biological Properties of Cholangiocytes and Response to Liver Damage

As pointed out before, cholangiocytes display several significant biological activities, from proliferation, to regulation of both immune and adaptive immunity, to their main physiological role in bile secretion.

1.3.1. Cholangiocyte Heterogenicity

It is known that intrahepatic and extrahepatic bile ducts do not share the same embryological origin. Cholangiocytes lining the IHBD derive from hepatoblasts, and therefore have a close embryological link to hepatocytes. In comparison, cholangiocytes lining the EHBD arise from endoderm and share a common development origin with the pancreas and duodenum [24].

Between cholangiocytes that line the biliary tree, individual and morphological features have been used to identify and classify these cells into small and large cholangiocytes. In humans, IHBD can be divided by size and proximity, small bile ductules, interlobular ducts, septal ducts, area ducts, segmental ducts and hepatic ducts (**TABLE 2**). The hepatic ducts mark the starting point of the extrahepatic biliary tree. In the rat, the classification is based on the function of small and large ducts [24], [50].

Table 2. Morphological characteristics of bile ducts in human and rat [50]

Human Bile Ducts (diameter in µm)	Rat Bile Ducts (diameter in µm)
(Large Bile Ducts)	
Interlobular Ducts (15-100)	Large Bile Ducts (>15)
Septal Ducts (100-300)	
Area Ducts (300-400)	
Segmental Ducts (400-800)	
Hepatic Ducts (>800)	
(Small Bile Ducts)	
Small Bile Ductules (<15)	Small Bile Ducts (<15)
Interlobular Ducts (~15)	

Regarding their cellular structure, small cholangiocytes are cuboidal and have a high nucleus/cytoplasm ratio, while large cholangiocytes are column-shaped and have more organelles and a small nucleus/cytoplasm ratio (**TABLE 3**) [51]. In large cholangiocytes, primary cilia can be found on their apical membrane which is important for the role of mature cells in secretion and proliferation. Both small and large cholangiocytes express the biliary marker CK19; However, only large cholangiocytes are involved in bile secretion once these cells express SR, CFTR and AE2 which are crucial for biliary fluid secretion [52].

On the other hand, administration of gamma-aminobutyric acid (GABA) in mice has shown that large cholangiocytes undergo apoptosis whereas small cholangiocytes resist and are able to differentiate into large cholangiocytes, illustrating the relevance of small cholangiocytes in restoring the biliary epithelium when large ducts are damaged [53].

Table 3. Known differences between small and large cholangiocytes [51]

Phenotype	Small Cholangiocytes	Large Cholangiocytes
Location	Smaller bile ducts	Larger bile ducts
Shape	Cuboidal	Columnar
Intracellular Structures	High nucleus to cytoplasm ratio Multilobulated nucleus, few mitochondria, abundant Golgi apparatus, minimal rough endoplasmic reticulum	Low nucleus to cytoplasm ratio Multilobulated nucleus, few mitochondria, abundant Golgi apparatus, more rough endoplasmic reticulum
Apical Surface	Lysosomes and vesicles localized to the apical membrane, tight junctions between cells, coated pits, microvilli	Lysosomes and vesicles localized to the apical membrane, tight junctions between cells, coated pits, microvilli
Protein Expression	• Express lipase, α-amylase and trypsin • AE1 absent, AE2 present • CFTR absent • CYP4502E1 absent • Blood group antigens present (e.g., A, B, O, Lewis) • Bcl-2 present	• Express lipase, α-amylase and trypsin • AE1 absent, AE2 present • CFTR present • CYP4502E1 present • Blood group antigens absent • Bcl-2 absent
Response to Injury	Resistant to liver injury Can proliferate (e.g., behave as liver progenitor cells)	Susceptible to liver injury

1.3.2. Role in Bile Secretion

The bile produced by hepatocytes (i.e. primary bile) is then modified by cholangiocytes (**FIGURE 3**). Hepatocytes first start secreting conjugated bilirubin, bile salts, cholesterol phospholipids, proteins, ions, and water into the canaliculi. As bile flows along the biliary tree, it is subjected to cholangiocyte hormone-regulated secretory and absorptive processes that will dilute and alkalinize the bile [54].

This process is modulated by the balance between pro-secretory signals: acetylcholine [55], bombesin [56], glucagon [57], secretin [57], vasointestinal peptide (VIP) [58], and antisecretory factors: dopamine [59], endothelin-1 (ET-1) [60], gastrin [61], insulin [62], and somatostatin [63].

Cholangiocytes contribute to bile modification by secreting Cl^-, $HCO3^-$, and water and reabsorbing bile acids, amino acids, and glucose. At the molecular level, bile secretion initiates when Cl^- channels open in the apical membrane, resulting in Cl^- flowing out of the cell. Activation of the $Cl^-/HCO3^-$ anion exchanger (AE2) follows the movement of Cl^- out of the cell allowing $HCO3^-$ to be secreted into the bile duct lumen while driving at the same time the efflux of water into the ductal lumen. The $HCO3^-$ secreted by cholangiocytes is also important for the formation of the bicarbonate umbrella, which establishes a protective apical layer against bile acid-induced injuries. Moreover, other ion channels are present on mature large cholangiocytes, including

cystic fibrosis transmembrane conductance regulator (CFTR), aquaporin 1 (AQP1) and apical Na+-dependent bile acid transporter (ASBT) [24].

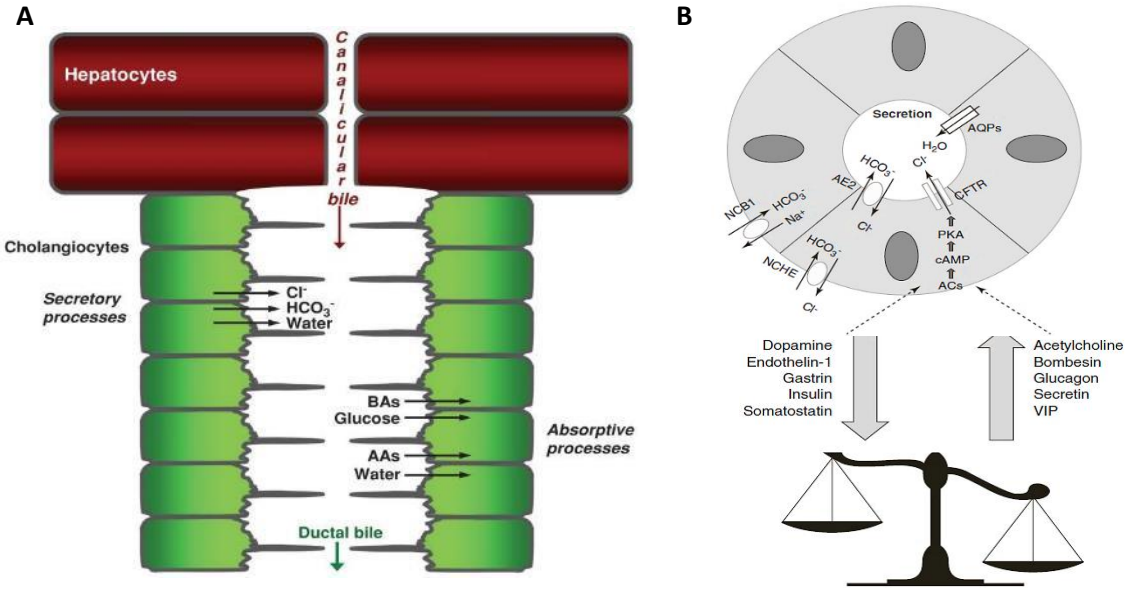

Figure 3. (A) Schematic representation of the role of cholangiocytes in bile secretion. Primary bile secreted by hepatocytes flows along the bile ducts where it is subjected to cholangiocyte secretory and absorptive processes [24]. **(B)** The bile secreted by cholangiocytes is under the control of pro- (acetylcholine, bombesin, glucagon, secretin, VIP) and antisecretory (dopamine, endothelin-1, gastrin, insulin, somatostatin) signals of hormonal and cellular origin. Activation of PKA by ACs expressed in cholangiocytes leads to the secretion of Cl^- into the lumen of bile ducts through the cAMP-dependent channel CFTR. This will then lead to the extrusion of $HCO3^-$ and the reabsorption of Cl^- by the AE2 ion channel, providing the alkalinization of the bile and its fluidification by AQPs which extrude water into the lumen [2].

In vivo cholangiocyte proliferation can be induced through biliary injury or exposure to alcohol, drugs, and toxins. The cAMP pathway is involved in the proliferation of these cells. This regulation has been shown in rats when cAMP levels were increased by administration of forskolin (an adenylate cyclase activator) which not only activated cholangiocyte proliferation but also secretion [64].

Administration of bile acids is also known to trigger cholangiocyte proliferation both *in vivo* and *in vitro* [65]. Additionally, gastrointestinal hormones such as secretin [66], somatostatin [67], and gastrin [61] have also been shown to play a role in cholangiocyte proliferation. Some other important mediators include the vascular endothelial growth factor (VEGF) [68], hepatocyte growth factor (HGF) [69], epidermal growth factor (EGF) [70], acetylcholine [71], and estrogen [72].

It is well known that during cholestasis cholangiocytes start proliferating, and even though some treatments can decrease their proliferation, it can also affect the role of these cells in liver injury. For this reason, further investigation is still necessary to better understand how to target irregular proliferation that is characteristic of cholangiopathies.

1.3.4. Immunobiology of Cholangiocytes

The human bile is sterile under normal conditions; however, the biliary tract is connected to the duodenum which is colonized by numerous microorganisms that can lead to microbial infections [73].

Cholangiocytes that are quiescent in non-pathological conditions become reactive when exposed to toxins, drugs, or when in the process of a liver injury. These cells are involved in both innate and immune responses once they express multiple pattern recognition receptors, such as toll-like receptors (TLRs), that when activated allow cholangiocytes to start expressing several adhesion molecules, pro-inflammatory mediators and antimicrobial peptides that will result in a cascade of responses against the pathogen or injury [73].

BECs have the ability to interact and cross-talk with other cells, including hepatocytes, hepatic stellate cells (HSC), stem cells, myofibroblasts, endothelial cells, and inflammatory cells. This interaction is mediated via pro-inflammatory and pro-fibrotic mediators, such as tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6) [74]. Additionally, cholangiocytes are also involved in the maintenance of tissue homeostasis via modulators of apoptosis (e.g. AkT1), senescence (e.g. N-RAS transforming protein), and proliferation (e.g. platelet-derived growth factor); However, during pathologic conditions the same modulators that contribute to tissue homeostasis will result in ductopenia, dysplasia, or malignant transformation of the bile ducts [75].

Another important feature of cholangiocytes is their ability to act as antigen-presenting cells. Under healthy conditions, cholangiocytes express low levels of human leucocyte antigen (HLA) class I, while class II which is required for the activation of CD4+ T cells is not detectable. The expression of these cell-surface proteins is increased under pathological conditions; However, a close interaction with other inflammatory cells is necessary for T cell activation [2].

1.4. Recent Advances in Biliary Research

Studies using PSCs were used in order to differentiate these cells into hepatoblast, allowing researchers to acquire knowledge about development processes [76]. As researchers were able to induce hepatoblast differentiation for the sole purpose of generating hepatocyte-like cells for cell therapy, they further discover that during the process some cells with cholangiocyte characteristics were also identified [77], [78]. With this in mind, new studies started with the goal of generating cholangiocytes from stem/progenitor cells for disease modeling, drug screening, and therapeutic approaches. A thorough examination of study details is listed in **TABLE 4A** and **TABLE 4B**, summarizing nine differentiation protocols by stage-specific differentiation steps, culture conditions, characterization elements and application purposes.

Table 4A. Differentiation of stem/progenitor cells towards hepatocytes/cholangiocytes.

Author	Stage-specific differentiation	Culture Conditions	Characterization	Aplication Purposes
Zhao (2009) Ref: [76]	1. ESC ->DE	Activin A	**Morphology** Round cysts with luminal center, surrounded by monolayer cells **Immunostaining** CK7, CK19, apicobasal polarity (β catenin, F-actin, E-cadherin, integrin α6) and AFP	**Modeling** 3D cholangiocyte model
	2. DE -> HE	a.FGF4 + BMP2 b.HGF		
	3. HE -> CLC	a.Feeder Cells b.Matrigel		
Cardinale (2011) Ref: [77]	HPC -> CLC	a.FGF + VEGF + dexamethasone b.Hyaluronan gel + collagen type I	**Immunostaining** CK7, CK19, SCTR, CFTR **Gene expression** In 3D model: increase GGT1, CFTR, AE2	**Modeling** 3D cholangiocyte model **Transplantation** SCID mice: lining bile ducts with human CK7+ cells
Nakamura (2012) Ref: [80]	1, PSC -> DE	a. Activin A + Wnt3a b. Activin A	**Morphology** Bile duct-specific structures with microvilli	**Modeling** 2D cholangiocyte model
	2. DE -> HB	FGF2 + BMP4 + Shh		
	3. HB -> CLC	a. FGF2 + BMP4 + HGF b. OSM + dexamethasone		
Yanagida (2013) Ref: [81]	1. PSC -> HPC	a.Activin A + 10% O_2 b.FGF + BMP4 +5% O_2 c.HGF + 5% O_2	**Morphology** Epithelial cysts **Immunostaining** CK7, apicobasal polarity (F-actin, PKC, β catenin, integrin α6) and AFP	**Modeling** 3D cholangiocyte model
	2. HPC -> CLC	a.Feeder cells + HGF + EGF + A8301 + Y27632 b.Matrigel + EGF + Wnt3a + R-spondin 1 + A8301 + Y27632		

A8301, ALK inhibitor; **AA**, ascorbic acid; **AChR M3**, M3 muscarinic cholinergic receptor; **AE2**, $Cl^-/HCO3^-$ anion exchanger; **AFP**, alpha-fetoprotein; **ALB**, albumin; **ASBT**, apical Na^+-dependent bile acid transporter; **ATP**, adenosine triphosphate; **BMP**, bone morphogenic protein; **CFTR**, cystic fibrosis transmembrane conductance regulator; **CK**, cytokeratin; **CLC**, cholangiocyte-like cell; **CLF**, cholyl-lysyl-fluorescein; **DE**, definitive endoderm; **EGF**, epidermal growth factor; **EpCAM**, epithelial cell adhesion molecules; **ESC**, embryonic stem cell; **FGF**, fibroblast growth factor; **FOXM1B**, forkhead (fox) factor M1B; **FP**, foregut progenitor; **GGT**, gamma glutamyl transpeptidase; **GH**, growth hormone; **HB**, hepatoblast; **HE**, hepatic endoderm; **HES**, hairy and enhancer of split; **HEY**, hairy/enhancer of split related with YRPW motif protein; **HGF**, hepatic growth factor; **HNF**, hepatic nuclear factor; **HPC**, hepatic progenitor cell; **IL**, interleukin; **InsP3R III**, inositol 1,4,5-triphosphate receptor type 3; **iPSC**, induced pluripotent stem cell; **JAG1**, JAGGED 1; **KDR**, kinase insert domain receptor; **LY294008**, phosphoinositide 3-kinase inhibitor; **MDR**, multidrug resistance protein; **NCAM**, neural cell adhesion molecule; **OPN**, osteopontin; **OSM**, oncostatin M; **P2RY1**, purinergic receptor P2Y G-protein coupled 1; **PKC**, protein kinase C; **PKD2**, polycystin-2; **PROX1**, prospero homeobox protein-1; **PSC**, pluripotent stem cell; **RA**, retinoic acid; **SALL4**, sal-like protein 4; **SB-431542**, transforming growth factor-β signaling inhibitor; **SCID**, severe combined immunodeficiency; **SCTR**, secretin receptor; **Shh**, sonic hedgehog; **SOX9**, sex determining region Y (SRY)-box 9; **STH**, sodium taurocholate hydrate; **Tbx3**, T-box transcription factor 3; **TD02**, tryptophan 2,3-dioxygenase; **TGFB**, transforming growth factor-β; **TGR5**, G protein-coupled bile acid receptor 1; **VEGF**, vascular endothelial growth factor; **Y27632**, ROCK-inhibitor; **ZO-1**, zonula occludens-1

Table 4B. Differentiation of stem/progenitor cells towards cholangiocytes.

Author	Stage-specific differentiation	Culture Conditions	Characterization	Aplication Purposes
Dianat (2014) Ref: [82]	1. PSC ->DE	Activin A + LY294002 + Wnt3a (only iPSCs)	**Immunostaining** Both ESC and iPSC-derived: CK7, CK19, SOX9, acetylated α-tubulin and HNF4α ESC-derived only: OPN, HNF1β, CFTR, SCTR, ASBT, TGR5, KDR **Gene expression** Both ESC and iPSC: CFTR, TRG5, AQP1 iPSC only: SSTR2, P2RY1, InsP3R III, AchR M3 ESC only: SOX9, SCTR, JAG1, CK7, AE2, NCAM, SALL4, NOTCH2, FOXM1B **Functional analysis** Both ESC and iPSC: transport of rhodamine 123 and CLF ESC only: responsive to ATP, acetylcholine and somatostatin	**Modeling** 3D cholangiocyte model to investigate cell functionality
	2. DE -> HE	Activin A + FGF2 + BMP4		
	3. HE -> HB	FGF4 + HGF + EGF + retinoic acid		
	4, HB -> CLC	Collagen type I + GH + EGF + IL-6 + STH		
De Assuncao (2015) Ref: [83]	1, PSC -> DE	Matrigel + Activin A, Wnt3a	**Gene expression** CK7, CK19, CFTR, PDK2, AE2 and negative for SOX2, NANOG, EpCAM, AFP, PROX1, Tbx3, MDR3, ALB, AAT, TDO2 **Western blottinng** CK7, CK19, acetylated α-tubulin, AQP1, SSTR	**Modeling** 3D cholangiocyte model to investigate cell functionality **Transplantation** In mouse liver, CK7-expressing cells were observed within existing bile ducts and in developing clusters
	2. DE -> HE	Matrigel + FGF2, BMP4, Shh		
	3. HE-> HB	Matrigel + Shh, JAG1		
	4. HB -> CLC	Collagen type I + TGFβ (+ Matrigel)		
Ogawa (2015) Ref: [35]	1. PSC ->DE	Activin A + FGF2 + AA + CHIR99021	**Immunostaining** CK19, SOX9, CFTR, SCTR, ASBT, acetylated α-tubulin, E-cadherin, ZO-1 and ALB- **Gene expression** CK19, SOX9, CFTR, SCTR, SSTR, AQP1, AE2, GGT, TGR5, HNF1β, HNF6, NOTCH targets (HES1, HES5, HEY1) and ALB- **Functional analysis** Transport of rhodamine 123, inhibited transport of rhodamine 123 using verapamil, forskolin-induced swelling via the CFTR and inhibition of the CFTR using CFTRinh-172	**Transplantation** In a Matrigel plug in NSG mice, duct-like structures were observed after 6-8 weeks, expressing CK19, CFTR and primary cillia **Disease Modeling** 3D CF patient-derived cholangiocyte model to determine patient-specific responses to CFTR medication (VX-809 and Corr-4a)
	2. DE -> HE	FGF2 + BMP4 + AA		
	3. HE -> HB	HGF + OSM + AA + dexamethasone		
	4, HB -> CLC	Feeder cells + Matrigel + HGF + EGF + TGFβ + AA		
Sampaziotis (2015) Ref: [84]	1, PSC -> DE	Activin A + FGF2 + BMP4 + LY294002	**Immunostaining** CK7, CK18, CK19, HNF6, SOX9, CFTR, HNF1β, acetylated α-tubulin and AFP- **Gene expression** CK7, CK19, CFTR, SCTR, SSTR2, AE2, AQP1, SOX9, HNF1β, GGT, JAG1, NOTCH2, HES1 and AFP-, TBX3- and HNF4α-	**Disease Modeling** 3D polycystic liver disease patient-derived cholangiocyte model response to medication (Octreotide) 3D CF patient-derived cholangiocyte model to CFTR medication (VX-809)
	2. DE -> FP	Activin A		
	3. FP-> HB	SB-431542 + BMP4		
	4. HB -> CP	Activin A + FGF10 + RA		
	5. CP -> CLC	Matrigel + EGF + dexamethasone + AA		
Takayama (2016) Ref: [85]	1. iPSC -> DE	Activin A + Wnt3a-conditioned medium	**Gene expression** AQP1, CFTR, SCTR, GGT1, JAG1, GPBAR-1, SOX9 **Functional analysis** Cyst-forming capacity of CLCs	**Biomaterial design** Laminin incorporation in the biomaterial to promote cholangiocyte differentiation
	2. DE -> HB	FGF4 + BMP4		
	3. HB -> CLC	Collagen type I + laminin + EGF + IL-6 + STH + AA		

Among research groups, Sampaziotis et al. made a lot of progresses during the last few years as they were able to successfully differentiate hiPCS into cholangiocytes, and using these cells they were able to demonstrate that VX809, a therapeutic drug in phase 2 of clinical trials for the treatment of cystic fibrosis (CF) lung disease, could potentially be used for CF liver disease [84]. Moving a step forward, they then decided to investigate the possibility of repairing the biliary epithelium and replacing the native common bile duct using healthy extrahepatic cholangiocytes. The extrahepatic cholangiocytes were scrapped from the inner part of bile ducts and cultured it in Matrigel as well as in the presence of three growth factors (Dkk-1, EGF and R-spondin), allowing the formation of extrahepatic cholangiocyte organoids (ECOs). To repair the biliary epithelium ECOs were seeded in a poly(glycolic acid) (PGA) bioengineered scaffold and transplanted into damaged gallbladders whereas to replace the native common bile duct ECOs were harbored in a bioengineered collagen duct. Both experiments were successful as ECOs adopted the shape and structure of the native tissue, and animals survived without any complications [86].

Raven et al. (2017) decided to investigate if other liver cells were able to induce hepatocyte regeneration, bearing in mind that upon severe liver injury hepatocytes lose their capacity to proliferate. To impair hepatocyte regeneration, they used two mouse models: β1-integrin knockdown and viral p21 overexpression. Their findings revealed that when hepatocyte regeneration is impaired, cholangiocytes are able to regenerate hepatocytes.

Animal models have been widely used to study the pathogenesis of biliary diseases as well as to test drugs; Nevertheless, differences between rodents and humans are still a major limitation. Taken together the studies mentioned above provide *in vitro* alternatives to currently used animal models. *In vitro* alternatives are available as cell lines or primary cells; However, primary cholangiocytes are heterogeneous and can only be kept in culture for a limited time. Moreover, long-term *in vitro* models that resemble biliary epithelial structures, which are necessary to investigate transepithelial transport, are lacking.

The use of organoids has already been proven valuable for fundamental research, disease modeling, drug testing, and transplantation purposes [87]. A protocol to culture bipotent human liver progenitor cells derived from human bile ducts showed that the organoids these cells form *in vitro* remain long-term proliferative and are genetically stable [88]. Even though it was possible to differentiate human liver organoids into hepatocytes, to our knowledge, differentiation of these organoids into functional cholangiocytes has not been reported.

For all the reasons stated above and bearing in mind that generating iPSCs remains low efficient, time-consuming, and the fact that the cells are often genetically instable, this study proposes a method to overcome the limitations of *in vitro* culturing of primary cholangiocytes and cholangiocytes derived from iPSCs.

This project will investigate a novel method to generate cholangiocyte-like cells (CLCs) from human liver organoids and apply them to bioengineering approaches. In a first step, human biliary fragments will be isolated to generate organoids. These

organoids will then be cultured using an optimized protocol to allow their efficient differentiation into CLCs.

After differentiation, gene-expression profiling will be performed, and its results compared to freshly isolated cholangiocytes. Immunofluorescence staining will provide information on the availability of various cholangiocyte markers, and functionality assays such as MDR1 transport and FXR signaling.

With this technology, CLCs derived from human liver organoids could provide a valuable tool to develop new therapeutic agents and may be used in cell-based tissue regeneration therapies.

MATERIALS AND METHODS

Samples

Samples of human liver (n=4 donors) and gallbladder were obtained from surplus material of liver donors used for liver transplantations performed at the Erasmus Medical Center, Rotterdam (approved by the Medical Ethical Council of the Erasmus MC).

Isolation of bile duct fragments for organoid formation

Human liver organoids were isolated and cultured as previously described [48]. Briefly, the liver sample was first minced using a scalpel and washed with isolation medium (IM), consisting of DMEM/F12 with 1% v/v GlutaMAX (Gibco) supplemented with 1% v/v fetal calf serum (FCS, Gibco) and 1% v/v penicillin/streptomycin (Gibco). After removal of the supernatant, an enzyme buffer containing IM supplemented with 125 µg/ml collagenase II (Gibco), 125 µg/ml dispase (Gibco), and 100 µg/ml DNase I (Roche) was used to digest the sample.

After checking for the presence of biliary duct fragments under the microscope, ducts were collected in a new tube and more enzyme buffer was added to continue the digestion. Collected samples were centrifuged (1,500 rpm, 5 min., 4°C) to discard the supernatant, washed once with 8-10 ml cold IM, and centrifuged again after which the pellet was made as dry as possible. The organoid fragments were mixed with Matrigel (BD Biosciences) and 50 µl droplets were plated out in the center of each well of a 24-wells plate. The Matrigel droplets solidified at 37 °C (~15 min.), after which 500 µl of human organoid expansion medium (OEM, composition is described in the section below). Medium was refreshed every 2-3 days.

Culture of human liver organoids

Organoids from 4 liver donors were kept in culture using human organoid expansion medium that contained Advanced DMEM/F12 supplemented with 1% v/v penicillin/streptomycin, 1% v/v HEPES (Gibco), 1% v/v GlutaMAX, 10% v/v R-spondin-1 conditioned medium (RCM, Rspo1-Fc-expressing cell line was kindly provided by Calvin J. Kuo), 1% v/v B27 minus vitamin A (Invitrogen), 1% v/v N2 (Invitrogen), 10 mM nicotinamide (Sigma-Aldrich), 1.25 mM N-acetylcysteine (Sigma-Aldrich), 100 ng/ml FGF10 (Peprotech), 10 nM gastrin (Sigma-Aldrich), 10 µM forskolin (Sigma-Aldrich), 100 µg/ml primocin (Invitrogen), 50 ng/ml EGF (Invitrogen), 25 ng/ml HGF (Peprotech), and 5 µM A83-01 (TGFβ inhibitor, Tocris Bioscience).

The Matrigel and organoids were mechanically dissociated into smaller fragments using fresh cold Advanced DMEM/F12. Following a centrifuge step (1,500 rpm, 5 min., 4°C) the organoid fragments were mixed with fresh Matrigel and a 50 µl droplet was seeded in the center of each well of a 24-wells plate. Passage was performed weekly at a 1:4 split ratio. The medium was changed every other day.

Differentiation of human liver organoids to CLCs

Organoids were split by removal from Matrigel using cold DMEM/F12, mechanical dissociated into smaller fragments, and transferred into a mixture of Matrigel and 1.2mg/ml rat-tail type I collagen (Merck Millipore) at the ratio of 2:3. After two hours of incubation at 37°C and, when the gel mixture was solidified, cholangiocyte differentiation medium (cholangiocyte differentiation medium, CDM) was added. CDM was based on Advanced DMEM/F12 supplemented with 1% v/v penicillin/streptomycin, 1% v/v HEPES (Gibco), 1% v/v GlutaMAX, 500 ng/ml R-spondin (R&D Systems), 100 ng/ml Dkk-1 (R&D Systems), ITS+ Premix (BD Biosciences), 1% v/v B27 minus vitamin A (Invitrogen), 1.25 mM N-acetylcysteine (Sigma-Aldrich), 100 ng/ml FGF10 (Peprotech), 10 nM gastrin (Sigma-Aldrich), 100 µg/ml primocin (Invitrogen), 50 ng/ml EGF (Invitrogen), 25 ng/ml HGF (Peprotech), and 5 µM A83-01 (TGFβ inhibitor, Tocris Bioscience). Differentiation medium was replaced every other day until the end of differentiation (day 9).

RNA isolation, cDNA synthesis and RT-qPCR

RNA was isolated from three culture replicates of both liver organoids and CLC organoids at different time points during the differentiation experiment using the RNeasy Micro Kit (Qiagen). Briefly, 350 µl of Buffer RLT was added into the wells containing organoids, followed by RNA extraction according to the manufacturer's instructions.

cDNA was obtained using the iScript™ cDNA synthesis kit as described by the manufacturer (Bio-Rad). A mix of random hexamers and oligo-dT primers were used. Relative gene expression of the selected genes was measured using RT-qPCR. Primer design, validation, RT-qPCR conditions, and data analysis was performed as previously described [89]. Gene expression was normalized against two housekeeping genes, HPRT (hypoxanthine phosphoribosyltransferase 1) and RPL19 (ribosomal protein L19). Primers are listed in **SUPPLEMENTARY TABLE 1**.

Cholangiocyte functionality studies

Rhodamine 123 transport assay

The Rhodamine123 (Rh123) transport test was performed at day 4, in which cold DMEM/F12 was used to remove Matrigel and collagen from CLCs. CLCs were pretreated with DMSO or 10 µM Verapamil (Sigma-Aldrich) for 30 minutes, followed by 5 minutes of incubation with 100 µM Rh123 (Sigma-Aldrich). Then CLCs were washed 3 times with CDM. Following completion of each experiment, images were taken using a Nikon A1R/STORM confocal microscope.

FXR activity

For the FXR activity test, CLCs (day 7) were incubated in CDM supplemented with 10 µM GW4064 (Sigma-Aldrich) for 48 hours and RNA was isolated to determine the expression of downstream signaling of FXR target genes.

Bright field microscopy

Imaging of the organoids was performed using an Olympus CKX41 microscope in combination with a Leica DFC425C camera.

Immunofluorescence analysis

At day 9, liver organoids and CLC organoids were taken out of the Matrigel and fixed in 4% PFA for 30 minutes on ice. After fixation, the organoids were permeabilized and blocked with PBS, 0.5% Triton X, 1% DMSO and 1% BSA for 1h at room temperature. Primary antibodies were incubated overnight at 4°C. The following day, samples were washed 3 times in PBS for 20 minutes per wash, incubated with secondary antibody and washed again 3 times in PBS. Nuclei were stained with DAPI (Sigma-Aldrich). Organoids were mounted on slides with Secure-Seal™ adhesive spacer (Invitrogen) and ProLong® Diamond Antifade Mounting Medium (Invitrogen). Images were acquired using a Nikon A1R/STORM confocal microscope. Antibody details for each protein are shown in **SUPPLEMENTARY TABLE 2**.

Timing of experiments on CLC organoids

All the experiments and characterization with regards to CLC organoids were performed after 9 days of 3D culture unless stated otherwise.

Statistical analysis

Statistical analysis and graphs were performed using GraphPad Prism 6.0 (GraphPad Software Inc., USA).

RESULTS

1. *In vitro* generation of cholangiocyte-like cells (CLCs) from human liver organoids

We based our strategy on established protocols [86], [90], with some modifications (See Supporting Materials for details), to promote the differentiation of human liver organoids into CLCs (**FIG. 4A**). Human liver organoids were first isolated from the liver ductal compartment as previously described [88]. Subsequently, the organoids were mechanically dissociated into smaller fragments and mixed in a matrix, consisting of Matrigel and collagen type I. Organoids were then maintained in cholangiocyte differentiation medium (CDM) and started to form ring-like structures within 24h. After 4 days of culture, CLC organoids grew in size and formed cystic and tubular-like structures (**FIG. 4B**).

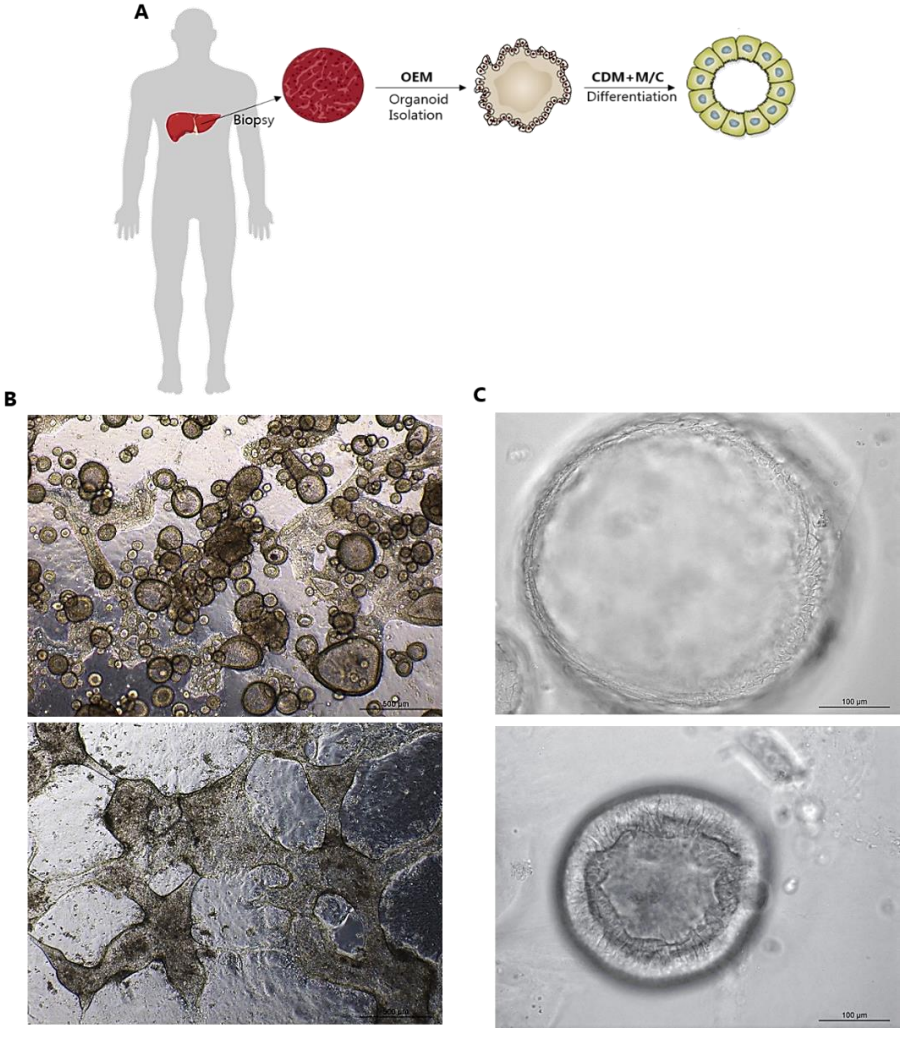

Figure 4. Differentiation of human liver organoids towards cholangiocyte-like cells (CLCs).
(A) Schematic overview of the protocol for differentiation of human liver organoids towards CLCs. OEM, organoid expansion medium; CDM, cholangiocyte differentiation medium; M/C, Matrigel/collagen type I mixture. **(B)** Brightfield microscopy images of tubular and cystic structures formed by CLCs in CDM condition. Scale bar=500 µm. **(C-D)** Brightfield microscopy images of organoids in OEM and CDM conditions, respectively. Scale bar=100 µm.

As organoids start undergoing differentiation some differences can be observed when comparing organoids growing in OEM and CDM conditions (**FIG. 4C-D**). The size of the cells that comprise the organoids increases, which is translated into the thickening of the organoid's wall (**FIG. 4C**). A "network" in the central lumen also becomes more visible due to the fact that cells get more tight junctions, therefore, the cell membrane is clearly more visible (**FIG. 4D**).

By the end of our differentiation protocol, RNA was isolated and qPCR analysis was performed to evaluate the expression of stem cell, hepatocyte and cholangiocyte markers. A decrease in expression of the stem cell marker leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5) was the first indication that our conditions were promoting differentiation (**FIG. 5**). Gene expression profiling showed that CLCs displayed expression of the early biliary markers keratin 19 (KRT19), SRY-box 9 (SOX9), and HNF1 homeobox B (HNF1b) (**FIG. 5**). The combined expression of the two cytoskeleton markers keratin 19 (KRT19) and keratin 7 (KRT7), characteristic of cholangiocytes was also observed in our cells (**FIG. 5**). Important transporters involved in several mature cholangiocyte functions were present in our cells at higher levels when compared with OEM conditions, that included the solute carrier family 10, member 2 (SLC10A2, also known as ASBT), ATP-binding cassette, sub-family B, member 1B (ABCB1b, also known as MDR1b), aquaporin 1 (AQP1), solute carrier family 4, member 2 (SLC4A2, also known as AE2), and the cystic fibrosis transmembrane conductance regulator (CFTR) which was significantly increased in CDM conditions (**FIG. 5**). The G protein-coupled receptors secretin receptor (SCTR), somatostatin receptor 2 (SSTR2), and G protein-coupled receptor 1 (GPBAR1, also known as TGR5), known to be present in mature cholangiocytes and for their role in bile secretion were also expressed at higher levels in CDM conditions (**FIG. 5**). Another important biliary marker, gamma-glutamyltransferase 1 (GGT1), involved in the transfer of amino acids across the cell membrane and glutathione metabolism, was present in our cells at levels that were significantly higher than OEM conditions (**FIG. 5**).

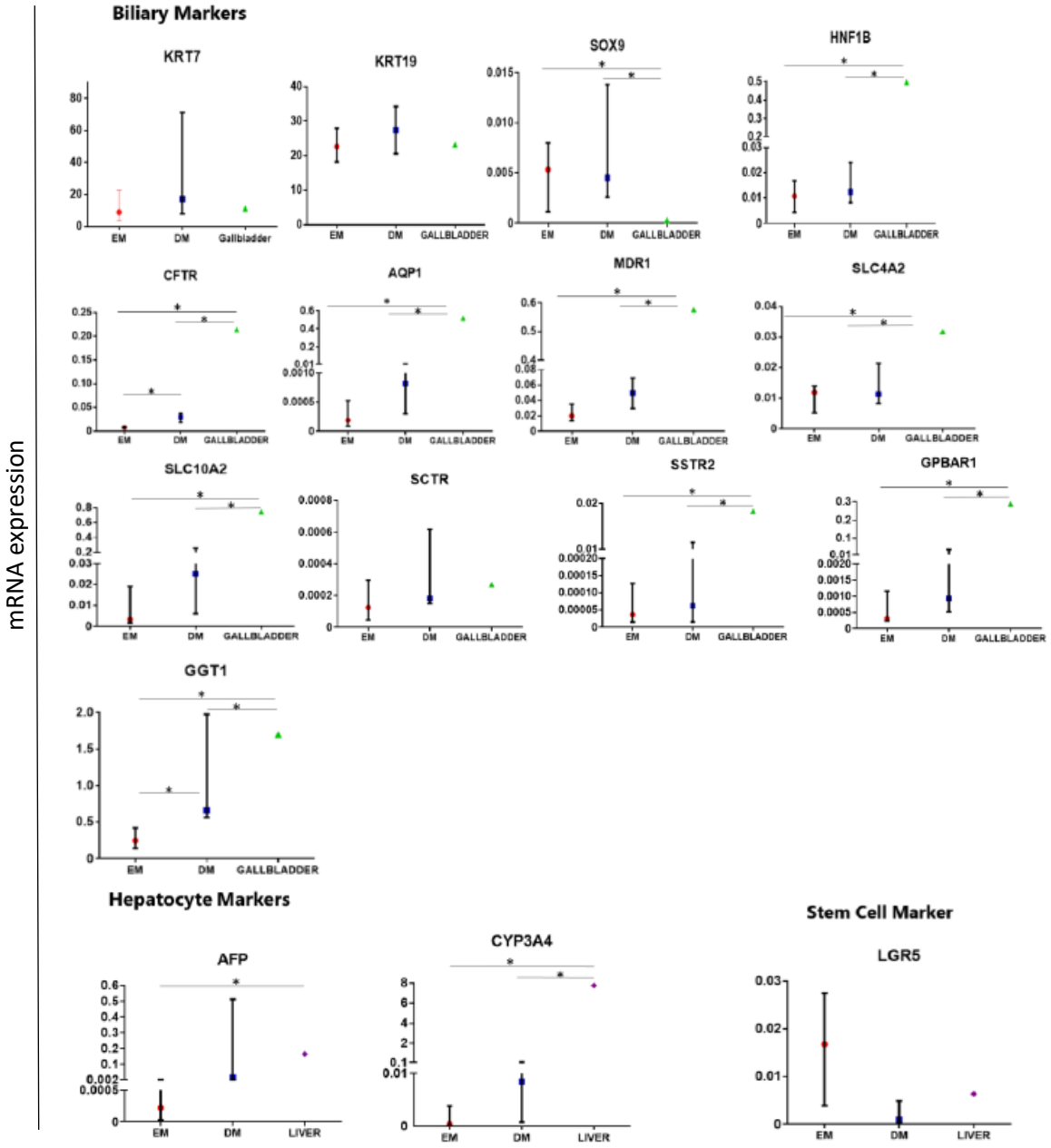

Figure 5. Gene expression analysis for cells under OEM and CDM conditions. n=4 independent samples for OEM and CDM. Results are shown as dot plots (median of four independent experiments for each group) for cholangiocyte, hepatocyte and stem cell markers, and their correspondent controls (Gallbladder or Liver). Asterisks represent statistically significant differences *p<0.05 (two-tailed Mann–Whitney U test).

To validate the results obtained from the gene expression profiling, immunofluorescence stainings were performed to provided additional information on the generated cells. The presence of cells at different stages of differentiation were observed when we stained for CK19 and TGR5 (**FIG.6A**), as some cells were stained for the mature marker TGR5 and not the early biliary marker CK19. The presence of the secretin receptor, vital for bile secretion, was also observed in our cells (**FIG.6B**). The epithelial phenotype of our cells was confirmed by CDH1 expression (FIG.6C), however, when trying to acquire information regarding the proliferation rate of our cells, using the proliferation marker ki67, an unspecific signal of this marker precluded to conclude about it (**FIG.6C**). More importantly, the presence of cilia, which plays an important role in fully differentiated cholangiocytes, was detected using an antibody against a ciliary marker, acetylated α-tubulin (**FIG.6D**).

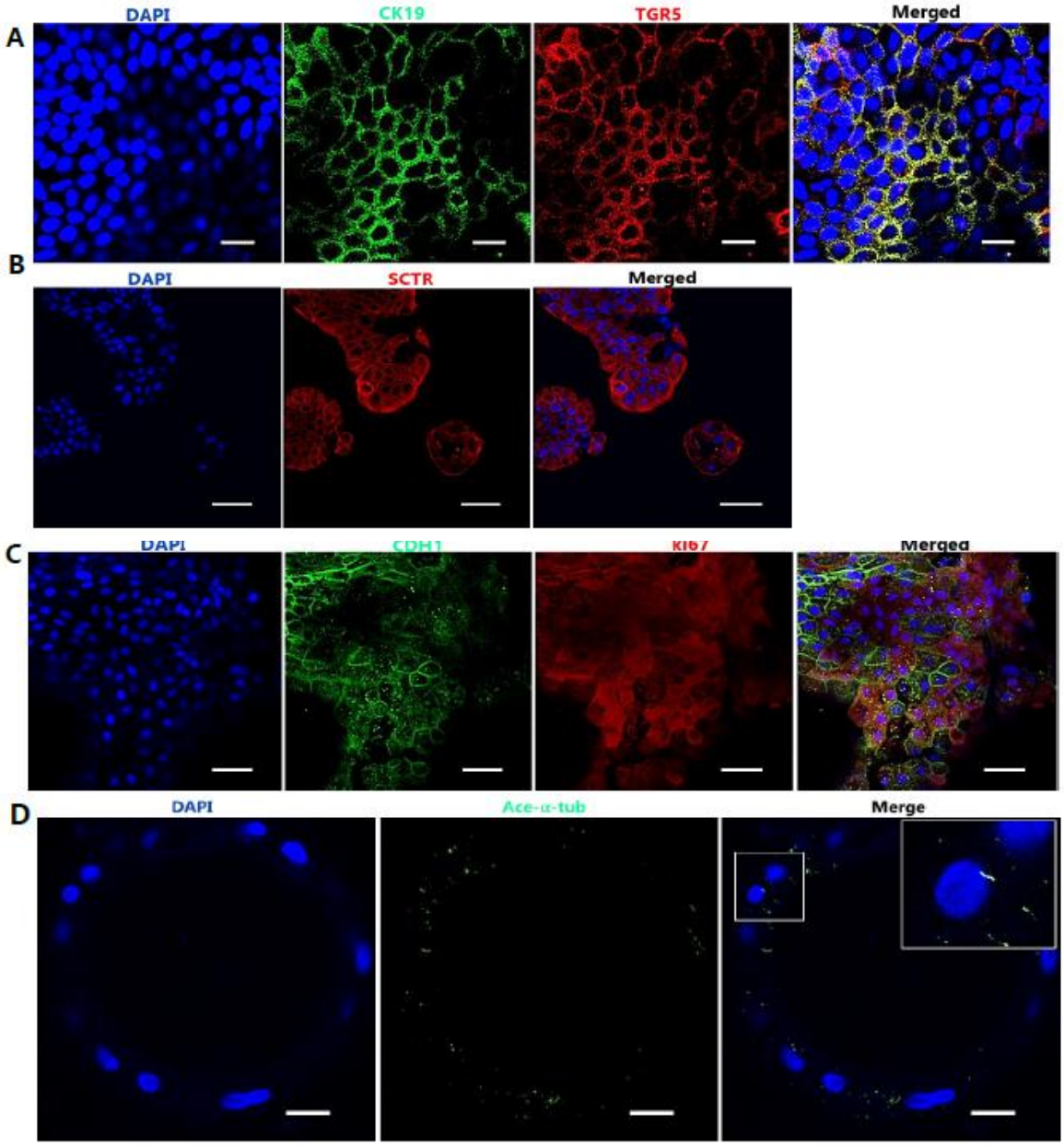

Figure 232. (A-C) IF analysis demonstrating the expression of biliary markers by CLC organoids (Keratin (KRT) 19, G-protein-coupled bile acid receptor 1 (GPBAR1/TGR5), secretin receptor (SCTR), epithelial marker (cadherin1 (CDH1), and the proliferation marker, ki67). Scale bar=50μm **(D)** CLCs acquired cilia as indicated by the staining for acetyl-α-tubulin (Ace-α-tub). Scale bar=20μm. Nuclear staining with DAPI, 4',6-diamidino-2-phenylindole dihydrochloride for all conditions.

2. Functional characterization of CLCs

Next, we characterized the functionality of the CLC organoids that were generated. *In vivo*, cholangiocytes are responsible to modify bile that flows along the biliary tree through a series of secretory and absorptive processes [54]. The presence of transmembrane channel proteins such as multidrug resistance protein 1 (MDR1) is important for the secretory activity of cholangiocytes as these cells are able to secrete xenobiotics through these efflux pumps at the apical membrane [91]. The efficacy of MDR1 transport was demonstrated after incubating our CLC organoids with rhodamine 123, a MDR1 fluorescent substrate. After incubation, fluorescence accumulation of rhodamine 123 was detected inside the central lumen of our CLC organoids (**FIG. 7A**) whereas after incubation with verapamil an MDR1 inhibitor, rhodamine 123 transport was abolished (**FIG. 7B**).

Bile acids are potent ligands for the farnesoid X receptor (FXR), which is implicated in the excretion of bile acids. When activated, FXR leads to bile excretion whereas its inhibition causes bile accumulation which can lead to injury. A mechanism by which bile acids are excreted involves two transporters, one located on the apical surface of cholangiocytes and the other on the basolateral membrane. SLC10A2 (also known as ASBT) located on the apical membrane, responsible for the uptake of bile acids, is downregulated by FXR. On the other hand, the organic solute transporter α/β (OST α/β) (also known as SLC51A and SLC51B, respectively) is upregulated and contributes to the excretion of bile acids [92], [93]. This physiological balance was demonstrated in our CLC organoids upon incubation with GW4064, a potent FXR agonist. Incubation with GW4064 resulted in the downregulation of SLC10A2 (**FIG. 7C**), and in the upregulation of SLC51A and SLC51B (**FIG. 7C**).

Together, these results confirm that the CLC organoids possess key functionalities of primary cholangiocytes and response to external stimuli.

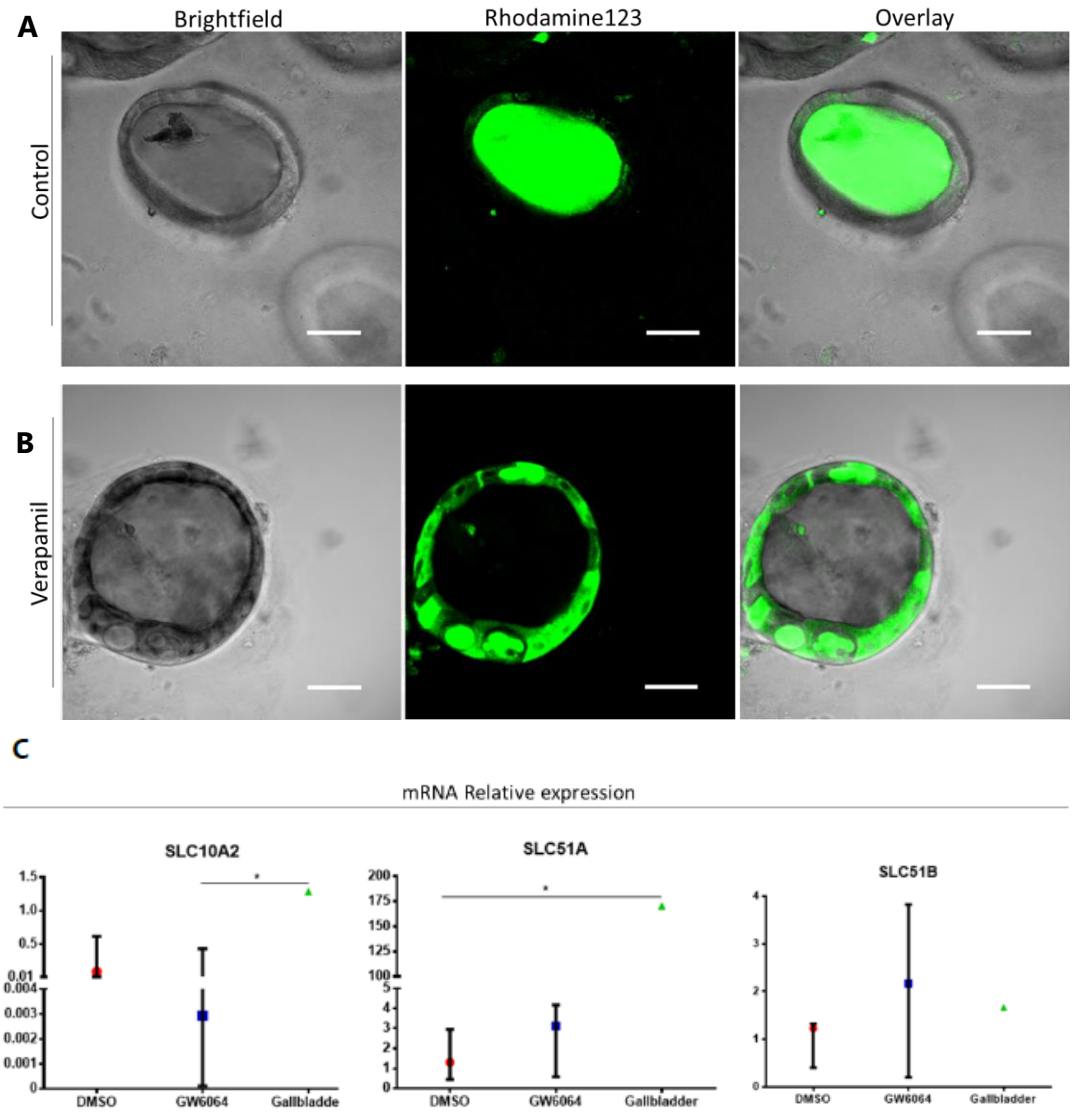

Figure 6. Functional characterization of CLC organoids. **(A-B)** Brightfield and confocal microscopic images demonstrating the MDR1-dependent transport of the fluorescent substrate Rhodamine123 into the lumen of a cystic-like structure. CLC organoids were pretreated with DMSO **(A)** and Verapamil **(B)** for 30 minutes. Scale bar = 50µm. **(C)** Gene expression analysis of the FXR signaling downstream target genes, Slc10a2, Slc51a and Slc51b upon incubation with a FXR agonist (GW4064) versus control (DMSO). n=3 independent samples for each group. Data are shown as median of three independent experiments for each group (two-tailed Mann-Whitney U test). Asterisks represent statistically significant differences (*p<0.05).

DISCUSSION

This study describes, for the first time, the potential of differentiating human liver organoids into mature cholangiocytes. Our serum-free protocol describes the generation of cholangiocyte-like cells over a period of 9 days. The CLCs generated express key biliary markers, show cilia and remain proliferative by the end of our protocol.

The use of liver organoids provides an alternative approach that overcomes limitations associated with the use of iPSC which still remain low efficient, time-consuming, and are genetically compromised [88]. For this protocol, four different liver donors were used, and some variability was observed throughout the process as some donors were able to grow and differentiate better than others. In our protocol, collagen type I was combined with Matrigel to better mimic the environment around developing bile ducts. This combination required a high cell density as cells do not grow at the same rate as in just Matrigel, however, they were still able to grow, proliferate and express key biliary markers. With this in mind, some minor optimization steps may be required to understand what the best clump size and plating density for each donor is, since the use of different liver donors already provides a high variability in our differentiation protocol.

Our *in vitro* system provides a faster alternative to generate CLCs when compared with previous protocols that used hiPSC, which needed 26 days to achieve the same result [94]. However, both our protocol and the ones relying on hiPSC possess two major limitations, one that involves the use of Matrigel in the culture system and the other about the maturity of the generated cells. The use of Matrigel raises issues such as batch-to-batch variability which could affect our differentiation protocol [95]. Another important aspect is the fact that CLC organoids express both early (SOX9, CK19, HNF1β) and mature (CK7, SCTR, CFTR, ASBT) biliary markers which should be taken into consideration when trying to model adult biliary disorders as some mature markers may not be present.

Our data showed that CLCs express SCTR, CFTR, TRG5/GPBAR1 and Slc4a2/AE2 which are expressed in large, but not small cholangiocytes. The expression of these markers and the fact that cilia were also present lead us to speculate that our population of cells resemble large cholangiocytes, however, it is feasible that a mixed population is present. Further investigation is necessary to conclude the existence of small cholangiocytes. For better characterization of biliary markers, the use of fresh bile duct tissue or frozen isolated common bile duct cholangiocytes (Celprogen) instead of gallbladder should be taken into consideration since intrahepatic cholangiocytes are not commercially available.

In vitro it was possible to demonstrate that CLCs generated with our protocol possess key functionalities of primary cholangiocytes, however, our culture system cannot be used to test bile salt transport activity because it is not possible to access the apical membrane. Introducing polyethersulfone hollow fiber membranes (HFM) into our culture system will overcome this obstacle [96]. Bioengineered bile ducts can be built by culturing CLC organoids on the outside of HFM and then apply these structures to perfusion chambers compatible with microscopy, making it possible to observe them by

confocal imaging, and therefore, allowing functional assessment [97]. The use of these structures would represent a useful model for studying features of intrahepatic bile ducts as they are the ones responsible to transport taurocholic acids.

Our method describes a novel *in vitro* model that can be used for studying cholangiopathies but also for drug screening in the pharmaceutical industry. A relevant field of application is the use of our CLCs in regenerative therapies of patients suffering from cholangiopathies by implanting them into a human patient, as an allogeneic graft or, after genetic correction, as an autologous graft. Infusing these cells with other hepatic cells into decellularized livers can too present a useful tool to construct bioengineered livers. On the other hand, CLCs can be used to search for new drugs with clinical applications or for toxicology tests.

CONCLUSION

Biliary diseases have a heterogeneous pathogenesis and respond differently to therapy which is the reason until now there is no effective therapy that can replace liver transplantation.

The main goal of this research is to develop a system that will facilitate the growth of human cholangiocytes outside the body and to use these cells to understand and treat cholangiopathies. By modeling cholangiopathies *in vitro,* it is possible to evaluate, test, and identify new drugs that ultimately will lead to new and effective therapies.

Our findings have shown the possibility to direct the differentiation of human liver organoids into cholangiocyte-like cells through a combination of growth factors and extracellular matrix components. The differentiated cells expressed key biliary markers, such as KRT7, CFTR, GGT1, SCTR, SSTR2, and ASBT, formed a lumen with epithelial polarity, and exhibited *in vitro* tubulogenesis. To further determine the identity of the differentiated cells, two functionalities assays were performed to prove these cells could transport a fluorescent dye, an indicator of functional MDR1 expression, and to respond to farnesoid X receptor agonist.

Altogether, these results indicate that the differentiated cells are similar to functional cholangiocytes. This opens up exciting prospects for major advances in the understanding of the complex immunobiological functions played by cholangiocytes, and the treatment of biliary diseases.

FUTURE WORK

Following the present study, some recommendations for future work are pointed out in order to complement the obtained results, emphasizing:

- Addition of more liver donors, including samples from cholangiopathy and ciliopathy patients to verify the efficacy of our differentiation protocol;
- Acquiring additional information regarding the morphology of the generated cells, namely, SEM or TEM pictures, could elucidate if a heterogenous population of cholangiocytes is present;
- Testing other drugs, besides Verapamil, to have an insight of how the cells react to the components and if they are able to secrete them or internalize them in case of therapies;
- Performing other functionality assays, such as the chloride transfer through cystic fibrosis transmembrane regulator (CFTR), and responses to hormonal stimuli (secretin and somatostatin);
- Building bioengineered bile ducts with the use of hollow fiber membranes and the generated cells to test bile salt transports, and the possibility to transplant them into NSG mice before transplanting them in humans.

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SUPPLEMENTARY INFORMATION

Supplementary Table 1. List of primers used.

Gene	Direction	Primer sequence (5′ à 3′)
CK7	**Forward**	GGACATCGAGATCGCCACCT
	Reverse	ACCGCCACTGCTACTGCCA
CK19	**Forward**	CTTCCGAACCAAGTTTGAGAC
	Reverse	AGCGTACTGATTTCCTCCTC
SCTR	**Forward**	TGCTCACCAGCAGAAATGGT
	Reverse	AGGTAGGAGTGCCGCTTCTC
CFTR	**Forward**	TTCTGGGAGGAGGGATTTGG
	Reverse	GTGAGAAATTACTGAAGAAGAGGC
GGT1	**Forward**	CCTCAAAGGGTACAACTTCTC
	Reverse	TTGTAGTAGGAGATCGGGTG
SOX9	**Forward**	CCCAACGCCATCTTCAAGG
	Reverse	CTGCTCAGCTCGCCGATGT
SLC4A2	**Forward**	ACTACCTGAGTGACTTCCGA
	Reverse	TGCTACAGAACGAGAAGAAGG
ABCB1	**Forward**	AATGATGCTGCTCAAGTTAAAGGG
	Reverse	TCAGTAGCGATCTTCCCAGAACC
SLC10A2	**Forward**	CTGTGCCTCCTTATCTATACCA
	Reverse	AGAGAAACCAGAGATGTACCT
SLC51A	**Forward**	TTGTTCGCCTCCCTATTCC
	Reverse	TTGTGGTCTTTCCTTCGGT
SLC51B	**Forward**	TGTGGTGGTCATTATAAGCATGG
	Reverse	TCTTAGGTTGTTTAGGCTGTTGTG
AQP1	**Forward**	CACCTCCTGGCTATTGACTACAC
	Reverse	ATCCAGTGGTTGCTGAAGTTGTG
GPBAR1	**Forward**	CATTGCCCACATTGCCAG
	Reverse	GAGCCAAGTAGACGAGGAG
LGR5	**Forward**	GCAGTGTTCACCTTCC
	Reverse	GGTCCACACTCCAATTCTG
HNF1B	**Forward**	ATGATCAAGGGTTACATGCAG
	Reverse	GTCTGGTTGAATTGTCGGAG
AFP	**Forward**	GATGAAACATATGTCCCTCCTG
	Reverse	ATGAGAAACTCTTGCTTCATCG
CYP3A4	**Forward**	CACAGGCTGTTGACCATCAT
	Reverse	TTTTGTCCTATAAGGGCTTT

Supplementary Table 49. List of antibodies used.

Target Protein	Dilution	Company	Catalog Number
Cytokeratin 19	IF 1:100	Novacastra	NCL-CK19
Secretin Receptor	IF 1:100	Atlas Antibodies	HPA007269
Alpha-Acetyl Tubulin	IF 1:100	Thermo Scientific	32-2700
Ki67	IF 1:100	Thermo Scientific	RM-9106-S
TGR5	IF 1:100	Atlas Antibodies	HPA062890
E-Cadherin	IF 1:100	BD Bioscience	610181
Alexa Fluor Goat Anti-Mouse 488	IF 1:100	Life technologies	A11029
Alexa Fluor Goat Anti-Rabbit 568	IF 1:100	Life technologies	A11036