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REVIEW

Tissue-Engineered Bile Ducts for Disease Modeling and Therapy

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Recent biotechnical advances in the *in vitro* culture of cholangiocytes and generation of bioengineered biliary tissue have a high potential for creating biliary tissue to be used for disease modeling, drug screening, and transplantation. For the past few decades, scientists have searched for a source of cholangiocytes, focused on primary cholangiocytes or cholangiocytes derived from hepatocytes or stem cells. At the same time, the development of scaffolds for biliary tissue engineering for transplantation and modeling of cholangiopathies has been explored. In this review, we provide an overview on the current understanding of cholangiocytes sources, the effect of signaling molecules, and transcription factors on cell differentiation, along with the effects of extracellular matrix molecules and scaffolds on bioengineered biliary tissues, and their application in disease modeling and drug screening.

Keywords: cholangiocytes, differentiation, bile duct engineering, disease modeling

Impact Statement

Over the past few decades, biliary tissue engineering has acquired significant attention, but currently a number of factors hinder this field to eventually generate bioengineered bile ducts that mimic *in vivo* physiology and are suitable for transplantation. In this review, we present the latest advances with respect to cell source selection, influence of growth factors and scaffolds, and functional characterization, as well as applications in cholangiopathy modeling and drug screening. This review is suited for a broad spectrum of readers, including fundamental liver researchers and clinicians with interest in the current state and application of bile duct engineering and disease modeling.

Introduction

THE BILIARY TRACT is a highly complex architectural and functional system in the human body, comprised by the liver, bile ducts, and gallbladder. After production by hepatocytes, bile is secreted into the bile canaliculi and then flows through the bile ducts that are lined by biliary epithelial cells, termed cholangiocytes, responsible for the final bile composition.[1] Their function is previously reviewed in detail and includes bile modification[2] and immune tolerance.[3] In addition, the biliary tree harbors putative stem cell niches, crucial for tissue homeostasis.[4] Human cholangiocyte function is regulated by bile salts, cytokines, growth factors, peptide hormones, nucleotides, and neurotransmitters.[5,6]

Cholangiopathies are a group of diseases originating from cholangiocyte injury, often causing liver failure. The most common cholangiopathies include cystic fibrosis, polycystic liver disease, primary biliary cholangitis, primary sclerosing cholangitis, biliary atresia, and cholangiocarcinoma (CCA). These cholangiopathies are caused by various factors (e.g., genetic, immune-mediated, idiopathic, infectious) resulting in cholestasis (impaired bile formation), followed by inflammation, fibrosis of bile ducts, and liver dysfunction, eventually leading to liver failure or end-stage liver disease.[7,8] End-stage liver disease is a leading cause of death and liver transplantation is the only curative strategy. However, liver transplantation is hampered by a lack of suitable healthy donors and life-long immunosuppressive therapy with its adverse effects.[9,10] Although precise numbers are lacking, cholangiopathies account for a substantial number requiring a liver transplant. The lack of sufficient donor livers poses a significant health and economic burden.

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To study cholangiopathies, testing of new drugs and the (clinical) application of tissue engineering approaches, *in vitro* long-term maintenance of cholangiocytes and generation of biliary tissue is of utmost importance.[11,12] Thereto, the anatomical and functional features of the tissue should be replicated *in vitro*, which is currently hindered by a lack of adequate *in vitro* culture systems and suitable scaffolds.[12] The possibility to induce differentiation of cholangiocytes from pluripotent stem cells (PSCs) has resulted in novel opportunities to establish reliable *in vitro* culture systems of cholangiocytes.[13–15] Cholangiocytes and cholangiocyte-based organoids derived from induced pluripotent stem cells (iPSCs) are advancements in the field of liver cell culture and the emerging field of tissue engineering, specifically bile duct engineering, offering a variety of choices regarding cell source and desired differentiated cell type.[16–18] However, heterogeneity of morphological features, functional assays, and marker-expression of stem cell-derived cholangiocytes is still observed *in vitro*. In this review, we focus on strategies of human cholangiocyte differentiation and the heterogeneity of obtained cell types. Subsequently, the use of these cells in various *in vitro* and *in vivo* applications studying cholangiopathies such as cholestasis is described.

Cell and Tissue Sources for Bile Duct Engineering

Cholangiocytes are primarily responsible for bile modification and transport. Furthermore, the biliary tree contains stem cell niches harboring the bipotential precursors able to differentiate into cholangiocytes and hepatocytes. During embryogenesis, hepatocytes and cholangiocytes differentiate via distinctive pathways from a shared progenitor cell, referred to as hepatoblasts.[19] This bipotential progenitor cell source is the product of a series of differential steps, starting with specification of cells in the mesendoderm forming the definitive endoderm, which gives rise to the foregut, midgut, and hindgut. Subsequently, a part of the foregut generates progenitors that are capable of differentiating toward pancreatic progenitors and hepatoblasts (Fig. 1). Details on these intermediate cell types and signaling pathways involved in embryogenesis are reviewed elsewhere.[20]

For bile duct tissue engineering, the preferred cell source is primary cholangiocytes closely mimicking native cell physiology.[21–26] Shortage of primary cholangiocytes hampered bile duct engineering and encouraged researchers to seek for substitutes. Alternative cell sources include differentiated hepatic cells, hepatic progenitor cells (HPCs), iPSCs, embryonic stem cells (ESCs), and immortalized or
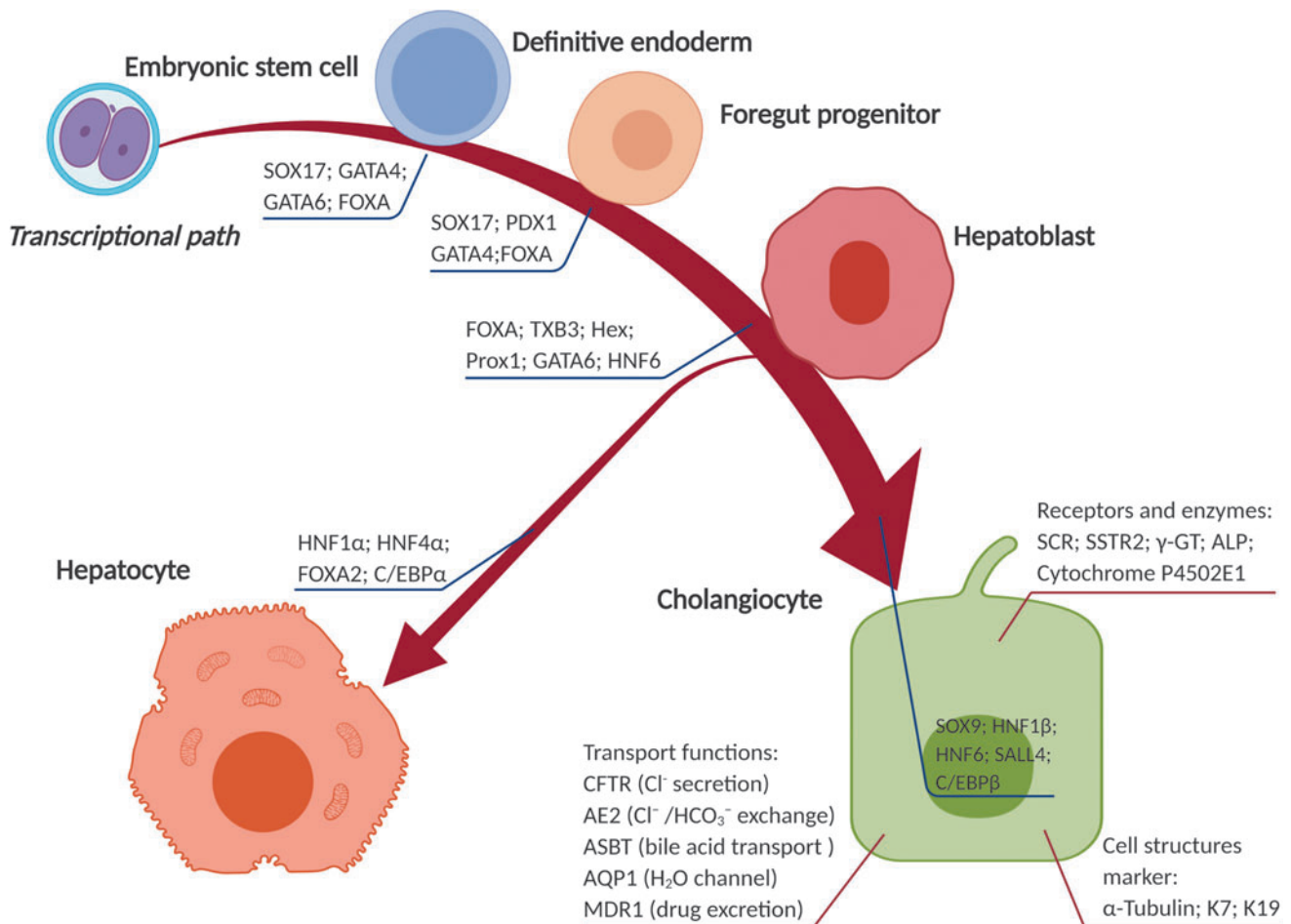

FIG. 1. Differentiation of embryonic stem cells (ESCs) into cholangiocytes. The key transcription factors that guide ESCs differentiate via a series of stages toward hepatoblasts, serving as a bipotential progenitor cell source that is able to differentiate into both cholangiocytes and hepatocytes. Several structural features, transport function, receptor- and enzyme-markers are shown that can be used to identify mature cholangiocytes, and to evaluate the differentiation process.

tumor-derived cholangiocyte cell lines. Table 1 presents an overview of cell sources used for bile duct tissue engineering, discussed in the next paragraphs.

Adult cell sources

Primary cholangiocytes. Primary cholangiocytes, isolated from biliary tract tissue, have been used widely in biliary tract regenerative medicine.[23,24,26] Purified primary cholan-

giocytes proliferate, but lose their differentiated/functional phenotype in culture. Especially in two-dimensional (2D) culture conditions, lack of proliferation after a few passages is observed and functionality is lost due to absence of the native microenvironment of the cholangiocytes. One well-known nonimmortalized human cholangiocyte line is the NCH-3 cells, which has been used for biliary disease modeling and to uncover that inhibition of the Wnt-ligand production should be considered as a therapy strategy

TABLE 1. CELL SOURCE FOR BILE DUCT TISSUE ENGINEERING

Cell source and microtissue	Species	Application	Pros	Cons	Ref.
Adult cell					
Primary cholangiocytes	Human	2D/3D cell modeling	Normal morphology and function. Phenotypic stability	Poor *in vitro* proliferation Not compatible with large scale experiments	28,29
	Mice	Bioengineering bile ducts	Easy to obtain compared with human hepatocytes	Incomplete morphology and functionality	24
Primary hepatocytes	Rat	3D cell modeling Cell transplantation Transdifferentiation	Easy to obtain compared with human hepatocytes	Incomplete morphology and functionality	30,31
	Mice	3D cell modeling Cell transplantation Transdifferentiation	Easy to obtain compared with human hepatocytes	Incomplete morphology and functionality	32,33,76
Immortalized cell line	Human	Study of anion exchanger activity and EGF receptor inhibition	Easy *in vitro* proliferation, maintenance and storage	Potential tumorigenicity Reduced functional performance	40–42
	Mice	3D printing 3D cell modeling	Easy *in vitro* proliferation, maintenance and storage	Potential tumorigenicity Incomplete morphology and functionality	114,164
Tumor-derived cell line	Human	Transdifferentiation 3D cell modeling Drug screening	Easy *in vitro* proliferation, maintenance and storage	Tumorigenicity Incomplete morphology and functionality	34,48,50,51
Stem cell					
ESCs	Human	3D cell modeling	High proliferation potential	Ethical issues Limited availability	48,54
iPSCs	Human	3D cell modeling Drug validation	Unlimited availability and indefinite proliferation Easily genetically modified	Possible teratoma formation	13,14,16,56
HPCs	Human	Disease modeling	Unlimited availability and indefinite proliferation Less safety concerns	Low isolation and purification rate	59,60
	Mice	Cell transplantation	Unlimited availability and indefinite proliferation Less safety concerns	Low isolation and purification rate	61
Organoid					
Organoids/ Cholangioids	Human	3D cell modeling Drug screening Diseases modeling Transplantation	Easy method for generation cholangiocytes within a limited time period	Functional stability	14,18,26
	Mice	Bioengineering bile ducts	Extensive *in vitro* proliferation	Incomplete functionality	128

2D, two-dimensional; 3D, three-dimensional; EGF, epidermal growth factor; ESC, embryonic stem cell; HPC, hepatic progenitor cell; iPSCs, induced pluripotent stem cells.

against scar formation.[27] Three-dimensional (3D) culture on collagen gels[28] or fibrous scaffolds[29] delayed dedifferentiation and allowed long-term proliferation compared with conventional cell cultures. In 3D cultures, primary human cholangiocytes formed spheroids with a central lumen phenotype (cholangioids), which were characterized as suitable 3D models of bioengineered biliary tissue.[26] However, utilization of isolated cholangiocytes remains suboptimal, encouraging alternative sources.

Hepatocytes. Hepatocyte-to-cholangiocyte transdifferentiation has been evidenced by an increase of cells expressing the cholangiocyte marker cytokeratin 19 in the spleen after hepatocyte transplantation in rats.[30] Furthermore, transdifferentiation was demonstrated upon severe injury of the biliary epithelium,[31] chronic liver injury,[32] or after activation of Notch signaling.[33] Although transdifferentiation of human HepaRG-derived hepatocyte-like cells into biliary-like cells was observed a decade ago,[34] no additional work has been done to investigate the use of human hepatocytes as a cell source for cholangiocytes. Primary human hepatocytes (PHHs) with high proliferation ability *in vivo* are hampered in *in vitro* utilization by their rapid dedifferentiation and loss of specific hepatic functions.[35] Some efforts have been made to the *in vitro* expansion of PHHs, which retain their hepatic features.[36–39] Although the PHHs were dedifferentiated to a biphenotypic state by defined media conditions, the biliary differentiation has not been studied in-depth for this cell source.

Cholangiocyte-derived/like cell lines. For long-term experiments investigating the function of cholangiocytes, immortalized or tumor-derived cell lines were used as cell source, such as the SV-40 immortalized human intrahepatic biliary epithelial cell line H69.[40] H69 cells presented key biliary markers and were used to study anion exchanger (AE) activity[41] and epidermal growth factor (EGF) receptor inhibition.[42] In addition, MMNK-1 cell line, a SV40T, and hTERT transformed human fetal liver cell clone, expressed key biliary markers and demonstrated specific cholangiocyte functions, including responses to lipopolysaccharide,[43] clarification of the transforming growth factor-β1 (TGF-β1) signaling pathway targets,[44] and effects of cholesterol-derivatives[45] and were used in genotoxicity studies.[46] Interestingly, MMNK-1 cells formed tubular structures upon seeding in Matrigel.[43,44]

The HepaRG cell line was isolated from a liver tumor of a female patient suffering from hepatocarcinoma and hepatitis C virus infection.[47] The HepaRG cell line is commonly used for its ability to differentiate toward hepatocytes[34] and cholangiocytes.[48] In monolayers, HepaRG-derived hepatoblasts were treated with human growth hormones, EGF, interleukin 6, and sodium taurocholate to differentiate them toward the cholangiocyte lineage. These cholangiocyte-like cells (CLCs) showed high levels of cholangiocyte markers and formed primary cilia. They also formed a lumen with apico-basal polarity and transporter functions.[48] Due to its stable phenotype, it is suitable for drug discovery/metabolism, as reviewed elsewhere.[49]

CCA is an epithelial biliary malignancy that may arise anywhere along the biliary tree.[50] The majority of research in CCA was reported with two CCA-derived cell lines, EGI-

1, and TFK and included drug discovery, such as sorafenib or bosutinib combined with gemcitabine or cisplatin to suppress proliferation.[51] Immortalized or tumor-derived cell lines could be very useful in the modeling cholangiopathies, however, due to safety concerns, they cannot be used for cell therapy purposes.[52]

Stem cells

Stem cells, including ESCs, iPSCs, and adult stem cells (ASCs), have been utilized for biliary tissue engineering. Proliferation, a hallmark of stem cells, allows scalable populations of cells to high quantities. In addition, stem cells can be directed to differentiate into cholangiocytes by mimicking embryonic development.

Embryonic stem cells. ESCs are used extensively in recent years for their self-renewal and differentiation into cells of all germ cell layers.[53] Zhao *et al.* directed the differentiation of human ESCs via a bipotent hepatic progenitor stage into CLCs. These CLCs lacked some cholangiocyte functions, but expressed the cholangiocyte markers *KRT7* and *KRT19*, formed cysts and polarized tubule-like structures under 3D *in vitro* culture, and demonstrated multidrug resistance gene-1 (MDR1; P-glycoprotein) transport function.[54] Noushin *et al.* directed ESCs-derived hepatoblasts into functional CLCs with cilia expression and bile acid transport. Under 3D *in vitro* culture conditions, these CLCs form polarized tubule-like structures.[48] However, ESCs research is restricted by ethical and immunological guidelines, issues that can be circumvented by iPSCs.

Induced pluripotent stem cells. iPSCs are derived from somatic cells upon reprogramming by, for example, Yamanaka factors (OCT3/4, SOX2, KLF4, and c-MYC),[55] can be cultured long-term, and differentiate into various cell types under controlled conditions. To differentiate iPSCs toward CLCs, a stepwise, specific growth factor dependent, protocol mimicking embryonic development was used, via definitive endoderm, to initiate hepatoblast specification.[13,14,16,56] *In vivo*, these hepatoblasts can give rise to both hepatocytes and cholangiocytes during the embryonic development (Fig. 1). Hepatoblasts eventually form cholangiocytic cysts with basolateral polarity and cholangiocytic marker expression.[56,57] Sampaziotis *et al.* used patient-derived iPSCs for drug screening and to model polycystic and cystic fibrosis-associated liver disease.[14]

Adult stem cells. After severe or chronic liver injury, ASCs replenish hepatocytes or cholangiocytes and/or support regeneration.[58] Cholangiocytes have been generated from HPCs *in vitro*.[59–61] Unfortunately, the low number of HPCs limits their isolation and purification and hampers research on progenitor cells-derived cholangiocytes. Isolation of HPCs from human tissue is based on the membranous expression of epithelial cell adhesion molecule (EpCAM),[62] a marker for ASCs.[63] EpCAM-positive cells are found in different parts of the human biliary tree, including the canals of Hering and the peribiliary glands.[59] Transplantation of these cells in the liver of immune-deficient mice demonstrated differentiation toward hepatocytes and cholangiocytes with *de novo* bile duct formation.[59,62]

The low percentage of human HPCs (0.5–2.5%) in a liver biopsy[62] appeared insufficient for a human application, thus restricting this research to mouse transplantations.[62] Therefore, isolation and expansion protocols require improvements to obtain adequate cell numbers for disease models, drug screening, or regenerative therapies. This is where the organoid culture system comes into focus.[60,64]

Organoids/cholangioids

Organoids are self-organized 3D tissue cultures that can be developed from various species, organs, and cell types[65] when seeded in Matrigel or other hydrogels. The cholangiocyte-specific *in vitro* organoid model, termed cholangioids, formed proliferating spheroid-like structures with epithelial polarity. In addition, K7 and K19 remained present on the membrane during culture, and the cholangioids respond to secretin and rhodamine 123 accumulation indicating active transport. Lgr5-positive stem cells isolated from damaged mice liver tissue were able to differentiate and expand *in vitro*, forming liver organoids and further differentiated into hepatocytes and cholangiocytes.[66] Of great importance, primary human bile duct-derived organoids remained genetically stable in long-term cultures.[64] In another study, primary extrahepatic cholangiocytes in Matrigel formed extrahepatic cholangiocyte-organoids (ECOs) with native biliary features and functions. Furthermore, the ECOs populated on scaffolds created biliary tract tissue suitable for transplantation. Loarca *et al.*, used patient-specific cholangioids to study the pathogenesis of primary sclerosing cholangitis, showing the cholangioids' potential for *in vitro* disease modeling.[18]

Enhancing Directed Differentiation Toward Cholangiocytes

Differentiation protocols for the generation of CLCs from PSCs have been reported.[13,14,48,67] A thorough examination of the signaling pathways used in directed differentiation toward cholangiocytes is listed in Tables 2 and 3, summarizing nine protocols by stage-specific differentiation steps, culture conditions, characterization, and application purposes. Early differentiation protocols (Table 2) aimed at the bipotential differentiation capacity of hepatoblasts or HPCs, protocols from 2014 onward (Table 3) primarily focused on the differentiation of PSCs toward CLCs. A consensus view was reached on the intermediate steps in the differentiation protocol.

It is beyond the scope of this review to discuss all stages of liver development. However, some information regarding signaling pathways and transcriptional regulation of these processes offer valuable suggestions for differentiation of

TABLE 2. DIFFERENTIATION OF STEM/PROGENITOR CELLS TOWARD HEPATOCYTES AND CHOLANGIOCYTES

Author	Stage specific differentiation	Culture conditions	Characterization of CLC	Application purposes
Zhao et al.[54]	1. ESC→DE 2. DE→HE 3. HE→HPC 4. HPC→Hep 5. HPC→CLC	Activin A FGF4+BMP2 STO feeder cells HGF+OSM+dexamethasone Matrigel	Morphology Round cysts with luminal center, surrounded by monolayer cells Immunostaining K7, K9, apicobasal polarity (β catenin, F-actin, E-cadherin, integrin α6) Functional analysis Transport rhodamine 123	Modeling 3D cholangiocyte modeling
Vincenzo et al., 2011[59]	HPC→Hep HPC→CLC	Hyaluronan gel+collagen type IV +laminin+T3+OSM+EGF +HGF+dexamethasone Hyaluronan gel+collagen type I+VEGF+HGF	Immunostaining K7, K19, SCTR, CFTR Gene expression In 3D model: increased GGT, CFTR, AE2	Modeling 3D cholangiocyte modeling Transplantation SCID mice: lining bile ducts with human K7+ cells
Nakamura et al.[15]	1. PSC→DE 2. DE→HB 3. HB→Hep/CLC	a. Activin A+Wnt3α b. Activin A FGF2+BMP4+Shh a. FGF2+BMP4+HGF b. OSM+dexamethasone	Morphology Bile duct-specific structures with microvilli	Modeling 2D cholangiocyte modeling
Ayaka et al.[56]	1. PSC→DE 2. DE→HB 3. HB→Hep/CLC	a. Activin A+Wnt3α b. Activin A FGF2+BMP4+Shh a. FGF2+BMP4+HGF b. OSM+dexamethasone	Morphology Bile duct-specific structures with microvilli	Modeling 2D cholangiocyte modeling

AE2, anion exchanger 2; BMP, bone morphogenetic protein; CFTR, cystic fibrosis transmembrane conductance regulator; CLC, cholangiocyte-like cell; DE, definitive endoderm; FGF, fibroblast growth factor; GGT, gamma glutamyl transpeptidase; HB, hepatoblast; HE, hepatic endoderm; Hep, hepatocyte; HGF, hepatocyte growth factor; PSC, pluripotent stem cell; SCID, severe combined immunodeficiency; SCTR, secretin receptor; Shh, sonic hedgehog; OSM, oncostatin M; VEGF, vascular endothelial growth factor.

TABLE 3. DIRECTED DIFFERENTIATION OF HUMAN STEM/PROGENITOR CELLS TOWARD CHOLANGIOCYTES

Author	Stage-specific differentiation	Culture conditions	Characterization	Application purposes
Noushin et al.[48]	1. PSC→DE 2. DE→HE 3. HE→HB 4. HB→CLC	Activin A+LY294002 +Wnt3α (only iPSC) Activin A+FGF2+BMP4 FGF4+HGF+EGF+RA Collagen type I +GH+EGH +IL-6+STH	Gene expression Both ESC and iPSC: *CFTR, TRG5, AQP1* iPSC only: *SSTR2, P2RY1, InsP3R III, AChR M3* ESC only: *SOX9, SCTR, JAG1, KRT7, AE2, NCAM, SALL4, NOTCH2, FOXM1β* Immunostaining Both ESC and iPSC-derived: K7, K19, HNF6, SOX9, acetylated α-tubulin and HNF4α⁻ ESC-derived only: OPN, HNF1β, CFTR, SCTR, ASBT, TGR5, KDR Functional analysis Both ESC and iPSC: transport of rhodamine 123 and CLF ESC only: responsive to ATP, acetylcholine and somatostatin	Modeling 3D cholangiocyte modeling to investigate cell functionality
De Assuncao et al.[67]	1. PSC→DE 2. DE→HE 3. HE→HPC 4. HPC→CLC	Matrigel+Activin A, Wnt3α Matrigel+FGF2, BMP4, Shh Matrigel+Shh, JAG1 Collagen type I+TGF-β	Gene expression *KRT7, KRT19, CFTR, PKD2, AE2,* and negative for *SOX2, NANOG, EpCAM, AFP, PROX1, TBX3, MDR3, ALB, A1AT, TDO2* Western blotting K7, K19, acetylated α-tubulin, AQP1, SSTR2 Immunofluorescence K7, K19, acetylated α-tubulin Flow cytometry K7, K19, CFTR and lack of EpCAM Functional analysis Active intracellular calcium signaling in response to ATP	Modeling 3D cholangiocyte modeling to investigate cell functionality Transplantation In mouse liver, K7 expressing cells were observed within existing bile ducts and in developing cluster
Ogawa et al.[13]	1. PSC→DE 2. DE→HE 3. HE→HB 4. HB→CLC	a. Activin A+FGF2 +AA+CHIR99021 FGF2+BMP4+AA HGF+OSM+AA +dexamethasone Feeder cells+Matrigel+ HGF+EGF+ TGF-β+AA	Gene expression *SOX9, HNF1β, HNF6, KRT19, SCTR, CFTR, AQP1, AE2, ASBT, GGT, TGR, SSTR2, HES1, HES5, HEY1* and *ALB* Immunostaining K19, SOX9, CFTR, SCTR, ASBT, acetylated α-tubulin, E-cadherin, ZO-1 Functional analysis MDR1 transport rhodamine 123 CFTR function	Transplantation In a Matrigel plug in NSG mice, duct-like structures were observed after 6–8 weeks, expressing K19, CFTR and primary cilia Modeling 3D CF patient-derived cholangiocyte modeling response to CFTR medication (VX-809 and Corr-4a)

(*continued*)

TABLE 3. (CONTINUED)

Author	Stage-specific differentiation	Culture conditions	Characterization	Application purposes
Sampaziotis et al.[14]	1. PSC→DE 2. DE→FP 3. FP→HB 4. HB→CP 5. CP→CLC	Activin A+FGF2 +BMP4+LY294002 Activin A SB-431542+BMP4 Activin A+FGF10+RA Matrigel+EGF	Gene expression *KRT7, KRT19, CFTR, SCTR, SSTR2, AE2, AQP1, SOX9, HNF1β, GGT, JAG1, NOTCH2, HES1, AFP⁻, TBX3⁻* and *HNF4α⁻* Immunostaining K7, K18, K19, HNF6, SOX9, CFTR, HNF1β, NICD, acetylated α-tubulin and AFP⁻ Functional analysis Transport of rhodamine 123 via MDR1, fluorescent bile acid CLF via ASBT. Responsive to acetylcholine, ATP and VEGF	Modeling 3D polycystic liver disease model response to medication (Octreotide) 3D CF patient-derived cholangiocyte model to CFTR medication (VX-809)
Takayama et al.[87]	1. PSC→DE 2. DE→HB 3. HB→CLC	Activin A+Wnt3α+FBS FGF4+BMP4 Collagen type I +laminin+EGF +IL-6+STH+AA	Gene expression *AQP1, CFTR, SCTR, GGT1, JAG1, GPBAR1, SOX9* Functional analysis Cyst-forming capacity of CLCs	Biomaterial Laminin incorporation in the biomaterial to promote cholangiocyte differentiation

AA, ascorbic acid; AChR M3, M3 muscarinic acetylcholine receptors; AFP, alpha-fetoprotein; ALB, albumin; AQP1, aquaporin 1; ASBT, apical Na⁺-dependent bile acid transporter; ATP, adenosine triphosphate; CLF, cholyl-lysyl-fluorescein; EpCAM, epithelial cell adhesion molecule; FBS, fetal bovine serum; FOXM1β, forkhead (fox) factor M1B; FP, foregut progenitor; GH, growth hormone; HES, hairy and enhancer of split; HEY, hairy/enhancer of split related with YRPW motif protein; HNF, hepatic nuclear factor; IL, interleukin; InsP3R III, inositol 1,4,5-trisphosphate receptor type 3; JAG1, Jagged1; K, keratin gene; KDR, kinase insert domain receptor; KRT, keratin protein; LY294002, phosphoinositide 3-kinase inhibitor; MDR, multidrug resistance protein; NCAM, neural cell adhesion molecule; NICD, notch intracellular domain; OPN, osteopontin; P2RY1, purinergic receptor P2Y G-protein coupled 1; PKD2, polycystin-2; PROX1, prospero homeobox protein-1; RA, retinoic acid; SALL4, sal-like protein 4; SB-431542, transforming growth factor-β signaling inhibitor; SOX9, sex determining region Y (SRY)-box 9; SSTR, somatostatin receptor; STH, sodium taurocholate hydrate; TBX3, T-box transcription factor 3; TDO2, tryptophan 2,3-dioxygenase; TGF-β, transforming growth factor-β; TGR5, G protein-coupled bile acid receptor 1; ZO-1, zonula occludens-1.

stem cells into cells of the hepatic lineage. First, the effect of different signaling molecules and transcription factors (TFs) will be described, followed by a brief examination of the effects of extracellular matrix (ECM) molecules. Finally, clues for scaffolds induced differentiation are reviewed. Although most of these processes is performed in animal models, the focus in this review is on human cell differentiation and the development of human tissue engineering applications, unless otherwise stated (Fig. 2).

Signaling pathways

Wnt/β-catenin signaling. Wnt/β-catenin signaling is involved during hepatic development.[68] To date, an excellent overview of stage-specific effects during *in vitro* ESC differentiation toward hepatoblasts is provided by Touboul *et al.*.[69] Inhibition of the Wnt/β-catenin signaling during early stages of differentiation (definitive endoderm into hepatic cells) and stimulation of the Wnt/β-catenin signaling during later stages of differentiation (hepatic cells into hepatoblasts) resulted in an optimal proliferative hepatoblast population. Inhibition of Wnt signaling reduced the differentiation of hESC-derived HPCs toward hepatocytes as

evidenced by decreased expression of albumin and HNF1A.[70] Conversely, enhanced Wnt signaling during this differentiation process improved hepatocyte differentiation and decreased expression of the cholangiocyte marker *KRT7*.[71] The importance of Wnt/β-catenin signaling was confirmed in organoids,[64] in which its inhibition induced growth arrest that could be reversed by exogenous addition of Wnt3a. Activation of Wnt/β-catenin signaling during differentiation protocols should be done with careful considerations, since Wnt signaling is also linked to cholangiocarcinogenesis.[72,73] Initial manipulation of Wnt/β-catenin signaling is required to induce differentiation of PSCs into definitive endoderm.[74] Together, the use of Wnt/β-catenin signaling is limited to the part until hepatoblast differentiation, and no beneficial effects of Wnt/β-catenin signaling on further cholangiocyte differentiation has been described.

TGF-β signaling. The TGF-β signaling family includes various ligands such as TGF-βs, bone morphogenetic proteins, and activins. The role of TGF-β ligands on hepatic tissue is stage-specific. Being important during liver development and liver regeneration, high levels of TGF-β in
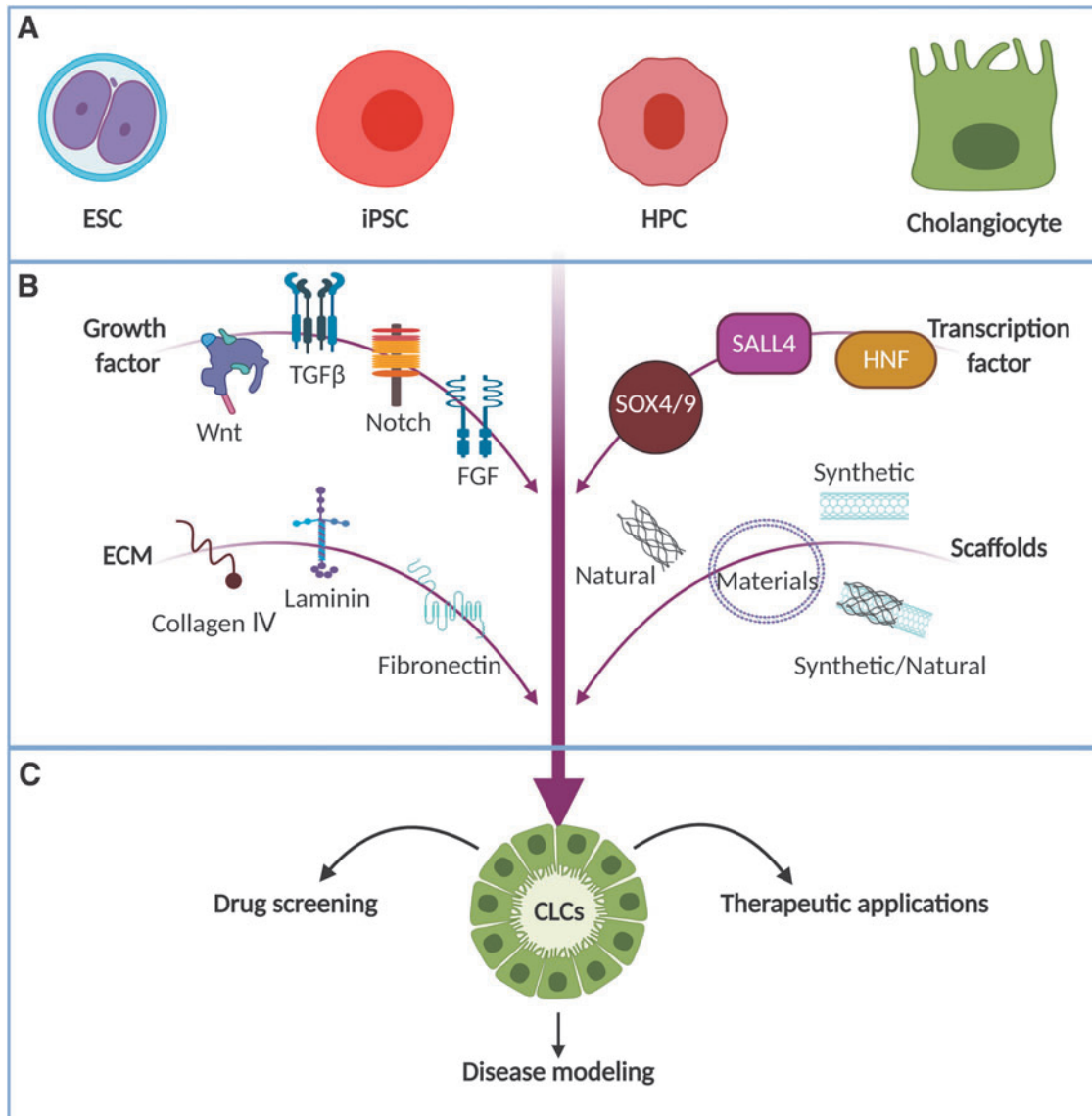
FIG. 2. Factors influencing the differentiation of PSCs and adult (stem) cells toward cholangiocytes. Within the approaches to differentiate cholangiocytes, **(A)** different cell sources (ESC; iPSC; HPC; primary cholangiocytes) are used, which are cultivated in specific culture systems that use **(B)** growth factors (Wnt, Wnt/β-catenin signaling; TGF-β; Notch, Notch signaling; fibroblast growth factor signaling), transcription factors (SOX9; SALL4; HNF), extracellular matrix molecules (Collagen, collagen type IV; laminin and fibronectin) and scaffold elements (synthetic material, natural materials, synthetic/natural materials) or a combination of these options. Next, a heterogeneous cell population is produced that, after classification, can be used **(C)** in a range of applications. HNF, hepatocyte nuclear factors; HPC, hepatic progenitor cells; iPSC, induced pluripotent stem cells; SALL4, sal-like protein 4; SOX9, sex-determining region Y (SRY)-box 9; TGF-β, transforming growth factor-β.

healthy hepatic tissue are linked to cell death and involved in the onset of various liver diseases (reviewed in detail by Fabregat *et al.*.[75]). Empirical evidence showed the necessity of TGF-β in cholangiocyte differentiation protocols.[13,67,76] After differentiation of cholangiocytes, however, the effect of TGF-β shifts toward decreased proliferation, as shown in H69-derived cholangiocytes.[77] Alternatively, inhibition of TGF-β can be used to differentiate hepatoblasts into hepatocytes[69] and TGF-β inhibition extends the culture of human liver organoids.[64] Kazuo *et al.* showed enhanced expression of cholangiocyte markers upon stimulation of human hepatoblast-like cells with TGF-β ligands, together

with reduced expression of hepatocyte markers.[78] TGF-β-induced senescence in hepatocellular carcinoma (HCC) cell lines has been reported, and this factor could be used as a therapeutic target for early HCCs.[79] These studies indicate a counter-acting role of TGF-β signaling in cholangiocyte and hepatocyte differentiation and provide a therapeutic strategy in cholangiopathies.

Notch signaling. Notch signaling is essential in development and regeneration.[80] In mammals, four Notch receptors (Notch1–4) and five membrane-bound Notch ligands (Jagged1–2, Delta-like 1, 3, 4) are described. Particularly

Notch2 and Jagged1 seem to play an essential role in biliary fate during embryonic development.[81,82] Jagged1 (JAG1), expressed on the nascent portal mesenchyme, stimulates the transdifferentiation of Notch2 expressing hepatoblasts into a biliary phenotype. Upon JAG1/Notch2 interaction, the hairy and enhancer of split-1 (HES1) genes are transcribed subsequently, activating the cholangiocyte-specific TFs HNF1β and SOX9 (sex determining region Y [SRY]-box 9), resulting in development of the ductal plate.[81–83] In Alagille syndrome patients, mutations in Notch ligand (JAG1) or receptor (NOTCH2) cause immaturely developed bile ducts despite normal hepatocyte differentiation.[77] Downregulation of Notch is linked to enhanced hepatocyte differentiation in cultured HPCs.[71,84]

Fibroblast growth factor signaling. Within the fibroblast growth factor (FGF) family, FGF2 (also known as bFGF) is the most important member involved in hepatic specification. FGF2 induced the expression of K19 during *in vitro* culture of chicken hepatoblasts,[85] while hepatic markers were not detected. A comprehensive evaluation of the working mechanism of FGF2 on human iPSCs was performed by Kirk *et al..*[86] Since FGF induced naked cuticle homolog 1 expression to transiently inhibit the canonical Wnt/β-catenin signaling during hepatic specification of the endoderm, FGF2 is used in early steps of the cholangiocyte differentiation protocol for PSCs.[13,14,48,67,87]

Transcriptional regulation

Among the plethora of TFs for embryonic biliary specification, hepatocyte nuclear factors (HNFs) are the most important TFs, further divided in four subfamilies (Table 4). Because the HNF4 subfamily is exclusively involved in hepatocyte differentiation,[88–90] it is not further discussed in this review.

Leading HNFs that are required for biliary specification are HNF1β and HNF6.[91,92] An insufficient presence of HNF1β in human cholangiocytes resulted in the lack of primary cilia on the apical membrane.[93] More recently, bile duct cysts were observed in extrahepatic bile ducts of patients with mutations in the *HNF1β* gene.[94] *Hnf6*-overexpressing mice showed increased hepatocyte proliferation due to induced transcription of genes involved in cell cycle regulation.[95] The role of HNF1β or HNF6 to control *in vitro* human cholangiocyte cultures has not been addressed.

Members of the HNF3 subfamily, also known as FOXA proteins, fulfil a species-specific role in cholangiocytes. Although their presence is mainly important during initial liver specification in mice,[96] some FOXA proteins remain specifically present in human adult cholangiocytes.[97]

TABLE 4. HEPATOCYTE NUCLEAR FACTOR

HNF1 subfamily	HNF4 subfamily
HNF1α	HNF4α
HNF1β	HNF4γ
HNF3 subfamily	*HNF6 subfamily*
HNF3α (FoxA1)	HNF6α (Onecut1)
HNF3β (FoxA2)	HNF6β (Onecut2)
HNF3γ (FoxA3)	

Inhibited expression of *Foxa1* and *Foxa2* in cholangiocytes resulted in enhanced bile duct proliferation in mice, however, this increased proliferation was also correlated to cholangiopathies *in vivo*.[98] Future work should investigate the potential to target these TFs for controlled human cholangiocyte behavior, as demonstrated for hepatocytes.[99]

Other interesting TFs that could possibly be used for TF delivery are sal-like protein 4 (SALL4) and SOX9. SALL4 is known to regulate cholangiocyte differentiation in early stages of liver development, initially shown in mouse livers.[100] Overexpression of SALL4 in embryonic hepatoblasts resulted in lineage commitment toward cholangiocytes, while inhibiting hepatocyte differentiation. This effect was partly mediated via increased gene expression of Notch signaling ligands and receptors. In human tissue, SALL4 suggested an oncofetal protein expressed in fetal hepatic tissue and in HCCs during the adult state.[101] However, SALL4 gene expression has been observed in adult cholangiocytes,[13] encouraging further investigations of this TF in differentiation of hepatoblasts.

SOX9 belongs to the SOX family of TFs that plays a pivotal role in liver organogenesis. During mouse liver development, intrahepatic bile duct development is controlled by SOX9. Inactivation of *Sox9* in mice cause a delay in the maturation of the asymmetric primary ductal structures into bile ducts.[102] SOX4 also controls the timing of cholangiocyte differentiation. SOX4 and SOX9 TFs cooperatively regulate the bile duct development through activating signaling pathways (e.g., TGF-β, Notch, and Hippo-Yap signaling). Both SOX factors also support apicobasal polarity in the developing cholangiocytes.[103] Moreover, the *Sox9* gene is a dose-sensitive modifier of bile duct paucity and liver phenotypes in *Jag1*^{+/−} mice.[104] Mouse studies implicated a function for SOX9 in biliary specification of hepatoblasts,[105,106] expression of SOX9 is observed in differentiated human CLCs.[13,14,48,87,107] This warrants further investigations onto SALL4 and SOX9 to guide biliary differentiation *in vitro*.

ECM molecules

Decellularized liver tissue is capable of initiating the differentiation of HPCs,[60] illustrating the importance of ECM components. Seeding of PSCs on Matrigel-coated surfaces improves hepatic differentiation[13,14,67] and supports long-term liver organoid cultures.[64] Matrigel is a gelatinous mouse-derived protein mixture composed of many ECM molecules, including collagen type IV, laminin, and entactin, and an array of growth factors.[108] Investigations into these individual ECM components, which are also present in the basement membrane in intrahepatic cholangiocytes, showed the importance of laminins on PSC differentiation into hepatocytes[109] and cholangiocytes.[87] The laminin-integrin interaction as shown for laminin 411 and 511 improves the differentiation of human iPSCs toward cholangiocytes.[87] Additional to ECM-components, ECM-stiffness properties influence cholangiocyte differentiation. Both collagen IV and fibronectin support cholangiocyte differentiation of liver progenitors on 30 kPa stiff substrates while only collagen IV does so on 4 kPa soft substrates.[110] Cell-matrix interactions should be investigated in-depth before being applied in therapeutic tissue engineering.

Scaffolds

Scaffolds are 3D biocompatible structures aiming to advance cell attachment and stimulate tissue regeneration *in vivo* or *in vitro*. Various sources of hydrogel or other scaffold materials in liver tissue engineering have been tested.[111,112] For instance, recent advances in 3D bioprinting technologies have been applied to biocompatible materials that create biliary tree constructs using decellularized ECM.[113] Compared to 2D, in 3D scaffolds, cholangiocytes showed a ductal phenotype in Matrigel,[114] whereas functional polymers accelerated HepaRG cell differentiated into mature hepatocytes and improved metabolic functions.[115] HepaRG in nanofibrillar cellulose and hyaluronan-gelatin hydrogels enhanced albumin expression along with CYP3A4 expression and efflux transporter functions,[116] resulting in cells closer to native tissue than 2D cultures.[117]

A large variety of synthetic and natural materials can be selected to manufacture (bio-)artificial scaffolds.[118] For tissue engineering purposes, different material properties need to be fine-tuned to optimize cell adherence and regeneration.[119,120] For example, 3D formation of small hepatocytes stacked morphology was influenced by the pore size of poly(D,L-lactide-*co*-glycolide).[121] Scaffold stiffness can also directly influence cholangiocyte behavior,[110,122,123] for example, cyst diameter and formation frequency of cholangioids are significantly induced by soft stiffness matrices.[124] In addition, biological properties of materials are equally important, as shown for proliferation and differentiation of a human hepatic cell line on a nanofibrillar cellulose hydrogel.[125]

Natural materials have superb biocompatibility to stimulate cell adhesion and growth, however, their use is limited by physical and mechanical stability.[126] Sampaziotis *et al.*

established a collagen tube scaffold populated with primary human ECOs that maintained expression of biliary markers such as K7, K9, HNF1β, SOX9, cystic fibrosis transmembrane conductance regulator (CFTR), alpha-fetoprotein, and gamma glutamyl transpeptidase enzymatic activity.[26] Synthetic material, on the contrary, with defined structures can be better controlled but often lack the necessary biocompatibility and absorbability.[127] Human biliary epithelial cells on polyglycolic acid fiber mesh scaffolds collapsed after 3 to 4 weeks. To improve the stability of the constructs, they developed a polycaprolactone-stabilized polyglycolic acid scaffold that allows cells to form spheroidal aggregates while supporting long-term proliferation and phenotypic stability.[29]

We recently combined synthetic and natural materials by coating polyethersulfone hollow fiber membranes (HFM) with L-dopa and collagen type I, and seeded CLCs that formed morphological and functional bile ducts.[128] As for the previous parts, this is an area of research that is making rapid progress.

Functional Characterization of Differentiated Cholangiocytes

The cholangiocytes are epithelial cells that line a complex system of interconnecting bile ducts extending from the canals of Hering to the hepatic ducts. The bile is first secreted by hepatocytes into the bile canaliculi and then flows through the bile ducts for the final bile composition (Fig. 3). To evaluate cholangiocyte differentiation, their heterogeneity *in vivo* must be addressed. Differences between small and large cholangiocytes are summarized in Table 5. These phenotypic differences
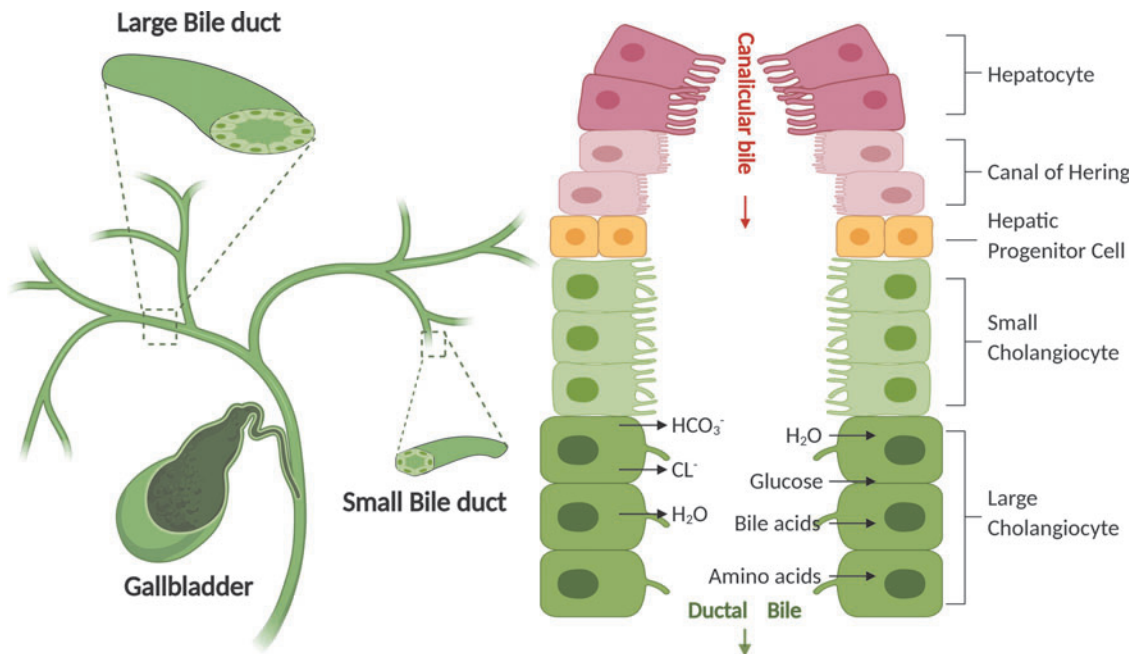

FIG. 3. Biliary tree, bile duct, cholangiocytes, and duct bile formation. Bile is secreted by hepatocytes (i.e., primary or hepatic bile) and subsequently delivered to the bile ducts. The canals of Hering provide the continuum between the hepatocyte canaliculus and the ductules or cholangioles. HPCs are bipotential cells that reside in the canals of Hering. Due to the heterogeneous of bile ducts, hepatic bile is modified by the large cholangiocytes which are lining the large bile ducts. Large cholangiocytes mainly modify the hepatic bile through secretory (e.g., HCO_3^-, Cl^-, H_2O) and absorptive (e.g., H_2O, glucose, bile acids, amino acids) processes, thus resulting in modified ductal bile.

TABLE 5. KNOWN DIFFERENCES BETWEEN SMALL AND LARGE CHOLANGIOCYTES

Phenotype	Small cholangiocytes	Large cholangiocytes	Ref.
Location	Small bile duct	Large bile duct	[7]
Shape	Cuboidal	Columnar	[7]
Intracellular structures	Greater nuclear/cytoplasmic ratio, more organelles	Lower nuclear/cytoplasmic ratio, more rough endoplasmic reticulum	[7,165]
	Multilobulated nucleus, few mitochondria, abundant Golgi apparatus		[7]
Apical surface	Microvilli	Primary cilia	[166]
	Lysosomes and vesicles localized to the apical membrane, tight junctions between cells, coated pits		[7]
Protein expression			
Lipase, α-amylase and trypsin	Human septal ducts	Large ducts, and peribiliary glands	[167]
BCL-2	Human small ducts	Not expressed	[168]
Blood group antigens (e.g., A, B, O, lewis)	Human small ducts	Not expressed	[169]
SCTR	Not expressed / Expressed *de novo* after large duct damage	Expressed in large rodent ducts	[170,171]
CFTR	Not expressed	Expressed in large ducts	[171]
Cl⁻/HCO₃⁻ AE2	Not expressed	Expressed in large ducts	[171]
SSTR2	Not expressed	Expressed in large ducts	[172,173]
γ-GT	Not expressed	Expressed in large ducts	[174]
ALP	Not expressed	Expressed in large ducts	[175,176]
Cytochrome P4502E1	Not expressed	Expressed in large ducts	[176]
Response to injury	Resistant to liver injury / Can proliferate (e.g., behave as liver progenitor cells)	More susceptible to liver injury	[172,177]

ALP, alkaline phosphatase; Cl⁻/HCO₃⁻ AE2, chloride bicarbonate anion exchanger 2; γ-GT, γ-glutamyl transpeptidase.

include for instance, villi or cilia presence, sensitivity to secretin, GABA-responsiveness, and cellular plasticity. An overview of relevant markers is given in the next paragraphs.

Cytoskeleton markers

Specific for cholangiocytes is the coexpression of K7 and K19 to provide cellular integrity and mechanical stability.[129] Staining of cytokines is used to distinguish between bile duct cells (K7/K19) and hepatocytes (K8/K18).[130] However, expression is not necessarily exclusive to these cells, as the expression of K7 and K19 is also shown in HPCs and intermediate CLCs;[131] that makes this marker combination not valid for discriminating between various stages of cholangiocyte maturation, let alone to distinguish large from small cholangiocytes. For this latter distinction, acetylated α-tubulins form highly stable microtubules,[132] which are required for the formation of a single, functional primary cilium on the apical membrane of large cholangiocytes.[21]

Transporters and channels

Various ion channels and transporters are present on mature large cholangiocytes, including Cl⁻/HCO₃⁻ anion exchanger 2 (AE2), CFTR, aquaporin 1 (AQP1), and apical Na⁺-dependent bile acid transporter (ASBT). Although not cholangiocyte-specific, the presence of these channels in combination with their polarized expression is commonly used to prove the cholangiocyte-like state of cells *in vitro*. For example, AE2, ASBT, and CFTR are present on the apical membrane, while AQP1 is detected on both the apical and basolateral membrane.[2]

Transport of fluorescent dyes provides information on transport activity. For example, rhodamine 123 is transported via the *MDR1* gene product, P-glycoprotein, into the cholangioid lumen[133] and cholyl-lysyl-fluorescein uptake reveals ASBT activity.[14] Furthermore, changes in intracellular calcium levels represent acetylcholine and secretin receptor (SCTR) activity on cholangiocytes.[134] Finally, forskolin addition leads to cyst swelling, indicative of CFTR function in cholangiocytes,[13] as initially proven in intestinal organoids.[135]

Ion channel activity can also be evaluated in relationship to diseases modeling *in vitro*. For example, cystic fibrosis (CF) patient-derived cells were used to create cholangiocytes to test for drug-restoring CFTR activity.[136] Further, overexpression of microRNA-506 in cholangiocytes inhibits AE2 and type III inositol 1,4,5-trisphosphate receptor (Ins3PR3), mimicking biliary cholangitis-like features.[137] In addition, a cholangiocyte responds to a high bile flow by activation of adenosine triphosphate-activated P2Y receptors on the apical membrane. This increases the intracellular Ca^{2+} concentration, which drives Ca^{2+}-activated Cl⁻ channels to secrete chloride ions, enabling the exchange of ions by AE2.[138,139] Within this process, all channels are required to facilitate the complete mechanosensitive secretory pathway, pointing to the complexity to acquire properly functioning cholangiocytes *in vitro*.

G-protein-coupled receptors

An important group of membrane receptors needed for cholangiocyte function are the G-protein-coupled receptors (GPCRs). G-protein-coupled bile acid receptor 1 (GPBAR1,

also known as TGR5) is one of these essential GPCRs, reviewed in detail elsewhere.[140] In addition, TGR5 is an important mediator of bile acid-induced cholangiocytes proliferation and it protects cholangiocytes from death-receptor-mediated apoptosis.[141]

Other GPCRs that can be used for cholangiocyte classification include secretin receptor (SCTR), somatostatin receptor (SSTR), and muscarinic acetylcholine receptor (AChR M3). The SCTR and SSTR counteract each other by increasing and decreasing intracellular cAMP levels after activation, respectively, thereby influencing cholangiocyte secretion function. Together with AChR M3, these receptors regulate cAMP and Ca^{2+} activated Cl^- channels. Besides the secretory function, SCTR is an important regulator of cholangiocyte proliferation[142] and involved in inflammatory cholangiocyte communication.[143] Together GPCR function is required for a definitive characterization of cholangiocytes.

Nuclear receptors

Nuclear receptors (NRs), shuttling between plasma membrane and nucleus, act like TFs.[144] Ligands for cholangiocyte NRs include bile acids, vitamins, and steroid hormones. For example, farnesoid X receptor, pregnane X receptor, and vitamin D receptor bind intracellular bile salts, leading to increased gene expression of genes involved in bile acid hydroxylation, detoxification, and secretion.[145] Because these NRs are not exclusively expressed in human cholangiocytes, they are not suitable for distinguishing between hepatocytes and cholangiocytes. However, their presence can be used to classify the functionality of cultured cholangiocytes and can be therapeutic targets in liver diseases.[146]

Polarity

The characterization of cholangiocytes can include cellular polarity. In native hepatic tissue, cholangiocytes are polarized with the apical side facing toward the luminal space of the bile duct. A frequently used marker for polarity is the tight junction marker zonula occludens-1 present in apical junctions. Recently, the apical orientation of the Golgi system was used to determine polarity in cholangioids, using an antibody against the Golgi protein GM130.[18] Together with the basolateral markers β-catenin and integrin α6, they are used to indicate apical-basal polarity in cholangiocytes.[58]

Taking into account the various characteristics described above, it is at present difficult to rationally design optimal cholangiocyte differentiation protocols. We believe that aiming for an open comparison and consensus on terminology/characterization is instrumental and will greatly accelerate the steps to optimize cholangiocyte culture systems and their application in future studies.

Modeling Cholangiopathy in Bioengineered Bile Ducts

Different biliary disease models have been established, both *in vivo* and *in vitro*, to improve the current therapeutic strategies.[147] For instance, *Mdr2* (*Abcb4*) knockout mice develop sclerosing cholangitis,[148] and rats present a human primary sclerosing cholangitis phenotype upon intrabiliary injection of 2,4,6-trinitrobenzenesulfonic acid.[149] Chronic feeding of DDC (3,5-diethoxycarbonyl-1,4-dihydrocollidine) in mice should serve as the small bile duct cholestatic model.[150] Primary sclerosing cholangitis is a chronic liver disease, characterized by inflammation and scarring of the bile ducts that ultimately lead to cirrhosis.[151] Interestingly, the frequently observed lymphocytic cholangitis in privately owned cats is similar to primary sclerosing cholangitis based on histology, the contribution of microbiological drivers, gender predisposition, and the involvement of Notch-signaling.[152–155] Preclinical (cats) and fundamental (rodents) models for cholangiopathies are taking the complexity of organ interactions into account. Other typical *in vivo* animal models to model cholangiopathy have been thoroughly reviewed elsewhere.[147,156] Although these models can significantly increase our basic mechanistic understanding of cholangiopathies, they are limited in use. *In vitro* models have a great advantage in maintaining cells in controlled experimental conditions. Thus, research groups developed cholangiocyte disease models to model *in vivo* cholangiopathies. Various *in vitro* models have been developed for cholangiopathies, including gene editing[14] and chemically induced[157] models. Here, we describe the current understanding of the disease modeling of cholangiopathies in 3D *in vitro* cultures.

Primary sclerosing cholangitis is a chronic liver disease, predominantly manifested in human, characterized by inflammation and scarring of the bile ducts that ultimately lead to cirrhosis. Loarca *et al.* reported a novel 3D model that mimics several features of primary sclerosing cholangitis and can be used to study pathogenesis and to identify potential therapeutic targets.[18]

Primary liver cancer is one of the most lethal malignancies worldwide, with the majority classified into HCC and CCA.[158] Laura *et al.* created organoids from HCC, CCA, and combined HCC/CCA with preserved histological architecture, gene expression, and genomic landscape of the original tumor[159] and, therefore, these organoids can be applied to better understand liver cancer biology even at a personalized level.

Cystic fibrosis (CF) is a systemic disease caused by mutations in the *CFTR* gene.[160] *CFTR* mutations in cholangiocytes influence chloride secretion, bile viscosity, and focal biliary cirrhosis secondary to bile plugs occluding the intrahepatic bile ducts. Sampaziotis *et al.* developed a biliary CF *in vitro* model by generating hiPSCs and hiPSCs-derived CLCs from patients with the common CF mutation ΔF508 to test the CF drug VX809's ability to rescue the disease phenotype *in vitro*.[14]

Future Outlook

The heterogeneous etiology of cholangiopathies explains the different responses to therapy and emphasizes the need to develop representative models and alternatives for liver transplantation. Tissue engineering as a new strategy provides a potential addition (or even alternative) for organ transplantations. Challenges not addressed include the use of Matrigel, feeder cells, or animal-derived supplements, each of these aspects render them impossible to implement in human clinical practice. Second, the effect of the *in vivo* transplantation site on the cells must be investigated.

An important need for future studies is to determine the specific effects of the *in vivo* environment. The delicate interplay between hepatocytes, cholangiocytes, and other cells present in hepatic tissue needs a deeper clarification. Tissue engineering allows studying the cellular interplay between different cell types. A 3D biomimetic patterning was demonstrated,[161] using rapid 3D bioprinting of human HPCs and complementary layered supporting cells, which had improved *in vitro* hepatocyte differentiation. Moreover, 3D hepatic lobule-like constructs were assembled by gear-like microstructures encapsulating multiple cell types. The 3D integrations constructs exhibited hepatic lobule-like morphology and a built-in lumen, replicating *in vivo* liver architecture and function. This construct showed a high proliferation rate and spreading during long-term coculture.[162]

Microfluidic devices provide precise control concentration gradient, spatial distribution of cells, and relatively easy to control flow systems to mimic the *in vivo* environment. Bile duct-on-chip is under development. Our group successfully bioengineered mouse CLCs on HFM, which acquired polarity and mimicked the native bile duct transport activity in a 3D-printed microfluidic device.[128] Others demonstrated that mouse cholangiocytes in a microfluidic device become monolayered, polarized, and formed tight junctions. This device allowed proof-of-concept toxicity studies with the biliary toxin, biliatresone, and specific bile acids.[24]

Although major improvement of tissue engineering strategies is still required, these bile duct-on-chip captured some essential features and functions of native bile duct, which makes them feasible for disease modeling and drug screening and allows studying interactions between biliary tract and other systems. Bioengineered bile ducts have been successfully transplanted into mice.[26] These bioengineered bile ducts could be considered as a possible therapy for cholangiopathies in human. Nevertheless, cell or tissue therapy for liver disease has a clear requirement for unlimited cell sources with native function, phenotypic stability, and without risk of tumorigenicity. HPCs have unlimited availability, indefinite proliferation, and less safety concerns, which are vital when considering cell-based therapies. Advanced therapy medical products (ATMPs) are innovative therapies that involve gene therapy, somatic cell therapy, and tissue-engineered products.[163] Legal and regulatory framework for ATMPs still needs to be established and applied to liver cell therapy.

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