

Regenerative Medicine and the Biliary Tree

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Abstract

Despite decades of basic research, biliary diseases remain prevalent, highly morbid, and notoriously difficult to treat. We have, however, dramatically increased our understanding of biliary developmental biology, cholangiocyte pathophysiology, and the endogenous mechanisms of biliary regeneration and repair. All of this complex and rapidly evolving knowledge coincides with an explosion of new technological advances in the area of regenerative medicine. New breakthroughs such as induced pluripotent stem cells and organoid culture are increasingly being applied to the biliary system; it is only a matter of time until new regenerative therapeutics for the cholangiopathies are unveiled. In this review, the authors integrate what is known about biliary development, regeneration, and repair, and link these conceptual advances to the technological breakthroughs that are collectively driving the emergence of a new global field in biliary regenerative medicine.

Keywords

- cholangiocytes
- regenerative medicine
- biliary tree
- stem cells
- organoids

Because of the unique regenerative ability of the liver, many decades of research into liver regeneration have given us unique insights into various modes of organ regeneration¹; therefore, hepatology has historically been at the epicenter of the science of regeneration. Although the underpinnings of modern regenerative medicine have been developing for much of that time, the worldwide pivot toward regenerative medicine, which now pervades all of modern medicine, can be most clearly landmarked by the Nobel Prize-winning technology of induced pluripotent stem cells, reported a decade ago in 2006.² Although this technology was quickly adapted to the study of hepatocyte differentiation,^{3–12} hepatocellular disease modeling,^{13–16} and cell-based therapy,^{17–19} there has been a subsequent expansion of similar advances in the realm of biliary disorders.^{20–23} As we will see, the biliary tree has always been center stage in the quest to understand the regenerative capacities and limitations of the liver because it has a distinct developmental origin, is anatomically and functionally heterogeneous, harbors

several putative stem cell niches, and is the target for a vast array of liver pathologies. Furthermore, in many ways, the biliary tree may be an even more attractive target for regenerative therapeutics than parenchymal hepatocytes, given its broad distribution throughout the liver and its endoscopic accessibility by endoscopic retrograde cholangiopancreatography (ERCP). Recently, several excellent review articles have been published on individual aspects relevant to the topic of biliary regenerative medicine.^{24–27} However, a more comprehensive perspective has been lacking and may be useful to help further define this emerging field. In this review, we will revisit what is known about biliary development, regeneration, and repair; summarize current concepts related to biliary stem cells and cellular plasticity in the liver; review the current state of advances in biliary regenerative medicine; and provide a vision of where this field is most ideally poised for advances in basic discoveries and clinical applications in the future.

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Cholangiocytes and Cholangiopathies

The liver, which is responsible for bile acid production, serum detoxification, the synthesis of serum proteins, immune regulation, and metabolic activities, is composed of two types of epithelial cells: hepatocytes and cholangiocytes. Although a majority of the essential functions are performed by hepatocytes, which make up 95% of the liver parenchyma, specialized cholangiocytes form the biliary tree. The biliary tree is composed of intrahepatic and extrahepatic bile ducts, lined by the mature epithelial cholangiocytes.²⁸ They facilitate secretion and modification of biliary constituents and serve as a conduit for bile transport to the intestine. These cells are now known to be the target of a diverse group of biliary disorders, known as *cholangiopathies*, many of which can lead to progressive periportal fibrosis, portal hypertension, biliary cirrhosis, and cholangiocarcinoma.²⁹ The cholangiopathies can be proliferative (e.g., polycystic liver disease) or fibro-obliterative (e.g., primary sclerosing cholangitis) in character and have heterogeneous etiopathogenesis (e.g., genetic, toxic, immunomediated, vascular, etc.).³⁰ A brief outline of five major cholangiopathies will demonstrate the breadth of clinical issues facing these patients.

Primary sclerosing cholangitis (PSC) is an idiopathic, fibro-obliterative cholangiopathy characterized by the diffuse inflammation of intrahepatic and/or extrahepatic bile ducts.³¹ This chronic process can progress to end-stage biliary cirrhosis with portal hypertension and hepatic failure. Ursodeoxycholic acid (UDCA) has been used for the treatment of PSC and when administered in low doses, has shown improvement in serum liver biochemistries. Vancomycin has also been used, particularly in children with PSC.

Primary biliary cholangitis (PBC) is a complex, autoimmune-mediated cholangiopathy, characterized by the progressive destruction of the intrahepatic bile ducts, leading to cholestasis and portal inflammation, which when chronic, can progress to periportal fibrosis and cirrhosis.³² The prognosis for PBC has been improved with the use of UDCA.³³ Recently, the Food and Drug Administration also approved obeticholic acid as a treatment for PBC.

Autosomal-dominant polycystic kidney disease is the most common inherited kidney disease, caused by mutations in the PKD1 and PKD2 genes.³⁴ In many cases, autosomal-dominant polycystic kidney disease is also associated with polycystic liver disease, a proliferative cholangiopathy associated with ciliary dysfunction in which multiple cysts develop within the liver parenchyma as a result of alterations in calcium homeostasis and cyclic adenosine monophosphate activity and subsequent effects on protein kinase-mediated proliferation.

Biliary atresia is a rare childhood disease that affects the function of and the anatomy along the canalicular-bile duct continuum.³⁵ The obliteration or discontinuity of the extrahepatic biliary system results in the obstruction of bile flow, leading to cholestatic jaundice. Biliary atresia is treated with the Kasai hepatopertoenterostomy and/or liver transplantation. The condition remains the most common childhood indication for liver transplantation.

Cholangiocarcinoma is a group of rare, but devastating, hepatobiliary cancers that arise from the intrahepatic, perihilar, or distal biliary tree.³⁶ Chronic inflammation from liver fluke infestation, hepatitis B and C infections, and PSC are the main risk factors for cholangiocarcinoma. Other etiologic factors include inflammatory bowel disease, hepatolithiasis, cirrhosis, alcohol, smoking, and fatty liver disease. A highly selected subgroup of patients with hilar cholangiocarcinoma can benefit from neoadjuvant chemoradiation followed by liver transplantation.

Overall, treatment of the cholangiopathies consists of pharmacotherapies (e.g., UDCA, vancomycin, etc.) that are largely ineffective and surgical therapies (e.g., Kasai procedure, liver transplantation, etc.) that are limited in scope and availability.³⁷ As such, many times cholangiopathies remain essentially untreatable with high morbidity and mortality in both children and adults. Even after successful liver transplantation, patients can be stricken with devastating biliary complications such as ischemic cholangiopathy (in the case of donation after cardiac death or hepatic artery thrombosis). Living donor transplant (effectively a regenerative medicine therapy) has significantly expanded the pool of donor organs, but unfortunately, biliary complications such as strictures and leaks are notoriously prevalent following this procedure, effecting up to 20% of living donor recipients.

Biliary Regenerative Medicine and the R³ Paradigm

The unfortunate lack of effective therapies for biliary disease has prompted the aggressive evaluation of new therapeutic options in the realm of biliary regenerative medicine that have the potential to radically alter our management of these patients. An expanding understanding of biliary pathophysiology, the presence of endogenous stem cell niches within the biliary tree, and endoscopic access to the system make cholangiopathies attractive targets for regenerative medicine therapies. At the most simplistic level, biliary regenerative medicine is tasked with creating new cholangiocytes and building new bile ducts. Although there is no definitive blueprint for the development of regenerative medicine therapies, one useful concept is the R³ paradigm, which incorporates three distinct regenerative tactics: replacement, regeneration, and rejuvenation.³⁸ Replacement strategy refers to the transplantation of a cell-based product that reestablishes homeostasis (liver transplantation is an example of this approach).^{39,40} Regenerative strategy refers to the engraftment of progenitor cells that require in vivo growth and differentiation (stem cell transplant is an example).^{17,41,42} Rejuvenation strategy refers to the induction of self-renewal of tissues by the activation of endogenous stem cells.^{43–45} In the context of biliary disease, replacement would include therapies designed to directly replace the damaged biliary epithelium (e.g., cholangiocyte-based cell therapies, bioengineered tissue patches, etc.). Regeneration, in contrast, would encompass stem cell-based therapies (biodegradable stem cell-coated stents, for example). Lastly, rejuvenation therapies would be designed to activate a

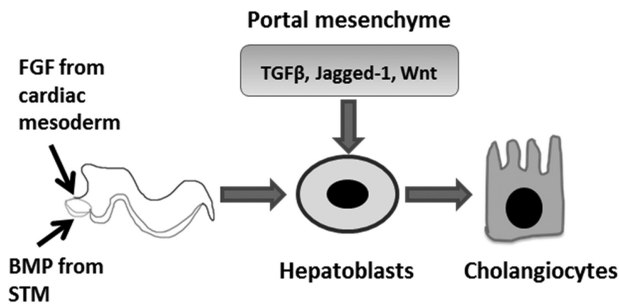


Fig. 1 Spatiotemporal regulation of cholangiocyte development. Schematic representation of key factors involved in biliary development from hepatoblasts. FGF, fibroblast growth factor; BMP, bone morphogenetic protein; STM, septum transversum mesenchyme; TGF β , transforming growth factor β .

therapeutic subset of the endogenous biliary stem/progenitor cell systems (gene therapy, therapeutic exosome delivery, etc.).

Biliary Development

To properly envisage new regenerative therapeutics for biliary disorders, it is useful, if not mandatory, to understand the normal embryological development of the biliary tree. The liver is formed from the ventral foregut endoderm, which also gives rise to the lung, the ventral pancreas, and the thyroid.⁴⁶ The transcription of liver specific genes, such as albumin, can be detected in the ventral foregut endoderm as early as embryonic day 8.5 (E8.5), specifying hepatic differentiation.⁴⁷ This hepatic induction is dependent on distinct, spatiotemporal regulation including signals of fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) from the cardiac mesoderm and the septum transversum mesenchyme, respectively (–Fig. 1).^{47–50} Subsequent to the FGF and BMP signaling cascades, Wnt signaling from the mesoderm is also required for liver specification.^{51,52}

Between E9.0 and E9.5, hepatic endoderm cells called *hepatoblasts* delaminate from the epithelium and expand into the adjacent septum transversum mesenchyme to form the liver bud, coordinated by signals from endothelial cells and a series of transcriptional events.^{53–55} Sonic hedgehog (SHH) is expressed in the ventral foregut endoderm during development, but at the onset of liver bud formation, its expression is downregulated. At E11.5, hepatoblasts show expression of SHH and its downstream transcription factor, Gli-1, which are then later attenuated. Thus, a temporally restricted activation of Hh signaling appears to be required to promote hepatoblast proliferation, a signal that is then shut off for normal hepatic differentiation of the hepatoblasts.⁵⁶

The hepatoblasts are bipotent and differentiate into both cholangiocytes and hepatocytes beginning around E13. Liver bud hepatoblasts residing adjacent to the portal tracts, upon the influence of the portal mesenchyme, adopt a cholangiocyte fate and form the lumen of the intrahepatic bile ducts, while the hepatoblasts in the parenchyma continue to differentiate toward hepatocytes. The maturation and specification of these cells are regulated by diverse growth factors,

cytokines, and transcription factors, which have been reviewed in detail elsewhere.^{24,57}

Parenchymal hepatocyte differentiation requires exposure to oncostatin M secreted from the hematopoietic cells in the liver in combination with hepatocyte growth factor and Wnt hormones.^{58,59} The activity of these factors is further balanced by tumor necrosis factor α , which maintains the proliferation of fetal hepatocytes for appropriate liver growth. These signals together regulate a network of liver-enriched transcription factors that control hepatocyte gene expression.

The biliary fate of the periportal hepatoblasts is orchestrated through temporally coordinated transforming growth factor (TGF) β , Notch, Wnt, and FGF signaling (–Fig. 1).^{60–65} Jagged-1 (Jag-1), a Notch ligand, is a key signaling molecule for biliary development, and is thought to be derived from the portal mesenchyme. Deletion of the Jag-1 gene in the portal mesenchyme results in profound defects in bile duct formation.⁶⁶ In humans, mutations in Jag-1 or Notch2 lead to bile duct paucity in patients with Alagille's syndrome.^{67–70} Furthermore, biliary differentiation is prevented by inhibiting Notch signaling, whereas ectopic Notch signaling promotes parenchymal hepatoblasts to adopt a biliary fate.^{62–64} Signaling through the Jag-1/Notch2 ligand-receptor pair, essential for biliary morphogenesis, is evolutionarily conserved in vertebrate liver development.^{66,71,72}

Another important signaling pathway essential for biliary development is the TGF β /activin pathway. A gradient of TGF β signaling exists in fetal liver, with high levels in the periportal region and low levels in the pericentral region, which controls the induction of the biliary fate. Wnt signals also regulate biliary differentiation from the hepatoblasts.⁷³ Taken together, a combination of spatially restricted signaling factors collectively allow for biliary differentiation in the periportal region.

Biliary Regeneration and Repair

Among the solid organs, liver is distinguished in its unique and remarkable capacity to regenerate upon injury (surgical resection or toxic insults). Humans can tolerate a 70% hepatectomy, rodents can tolerate a 90% hepatectomy, and zebrafish liver can regenerate after near total obliteration of parenchymal hepatocytes.^{74,75} Although the bulk of historical work on liver regeneration focuses on hepatocyte regeneration, restoration of the biliary tree is also essential for proper organ function. Additionally, the periportal location of putative liver stem cell niches and the expansion of biliary progenitors seen in the context of chronic liver diseases puts cholangiocytes at center stage in discussions of liver regeneration and repair mechanisms.²⁵ In contrast to biliary development, which occurs through the differentiation of hepatoblasts, it is useful to classify biliary regeneration and repair into three distinct domains: (1) homeostatic self-replication of cholangiocytes in normal liver, (2) accelerated biliary regeneration after liver resection, and (3) repair of the biliary tree upon injury (–Fig. 2).

One of the early proposed models for liver cell replacement was the so-called streaming liver hypothesis, where a

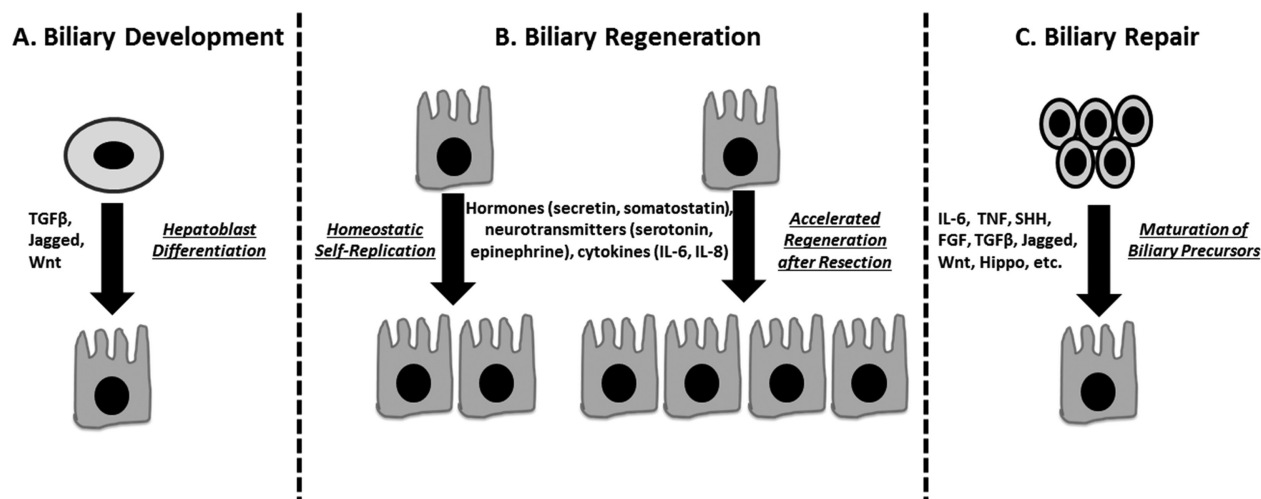


Fig. 2 Biliary development, regeneration, and repair. (A) Biliary development involving differentiation of hepatoblasts. (B) Biliary regeneration involving homeostatic self-replication of cholangiocytes in normal liver or accelerated self-replication following partial hepatectomy. (C) Biliary repair involving activation and maturation of biliary precursors.

continuous flow of new hepatocytes would emerge from the periportal stem cell niche and progress toward the central vein region for eventual apoptosis, analogous to the crypt-to-villi flow of intestinal epithelial cells.⁷⁶ Although elegant, lineage-tracing evidence eventually accumulated and argued against such a model^{77,78} and it is now fairly well-established that normal liver tissue maintenance is achieved via homeostatic self-replication of pre-existing hepatocytes and cholangiocytes. Occasional and apparently random apoptosis of adult cells is counterbalanced by occasional and apparently random mitotic events.

The partial hepatectomy (PHx) model in rat was the first classical model of liver regeneration, described by Higgins and Anderson in 1931.⁷⁹ They surgically excised two anterior lobes of rat liver, equating to 70% reduction in liver size. The cells of the remaining lobes proliferated to regain the lost mass over the course of 1 to 2 weeks. In this process, liver cells are able to overcome cell-cycle checkpoints and re-enter the cell cycle. This is followed by waves of DNA synthesis, cellular hypertrophy, and proliferation. Organ mass and function is eventually restored through compensatory hypertrophy of the remnant lobes as opposed to regrowth of resected lobes. There is apparently little activation of the periportal stem cell niche in this circumstance, arguing that normal liver regeneration can be achieved by simple proliferation of adult parenchymal cells without invoking any stem/progenitor cell population.

However, when the liver suffers from severe and/or chronic damage, cellular proliferation and regenerative capacity are thought to be attenuated. In this circumstance, historical paradigms have suggested that there is emergence of a facultative bipotent liver stem/progenitor cell compartment to contribute to the process of liver regeneration. These stem/progenitor cells are also referred to as oval cells in rodent models due to their stem cell-like morphology,^{80,81} and have been extensively evaluated as a potential source of new hepatocytes during liver regeneration.^{25,82} Even with sup-

pressed hepatocyte proliferation in rats by the administration of the chemical acetylaminofluorene, followed by PHx, oval cells can appear and expand in the periportal regions of the lobule within a few days and reach a peak at ~7 to 9 days after PHx. The administration of certain diets such as 3,5-diethoxycarbonyl-1,4-dihydro-collidine⁸³ feeding or the choline-deficient, ethanolamine supplemented⁸⁴ diet are well-known to produce robust oval cell responses in both mice and rats. Cells bearing a strong resemblance to rodent oval cells are also observed in human liver diseases. In humans, however, these cells are usually termed "hepatic progenitor cells" or "intermediate hepatobiliary cells."⁸⁵ The hematopoietic and epidermal systems as well as the small intestine have defined stem cell populations responsible for normal cell turnover that have been isolated and anatomically localized. These stem cells exhibit self-renewal properties and differentiate into mature cell types under normal physiological conditions to replace cell losses in blood, skin, and gut, thereby maintaining normal tissue homeostasis. In the liver, normal hepatocyte and cholangiocyte turnover is slow, and the concept of such a stem cell niche to support homeostatic self-renewal or tissue repair after injury has long been contemplated. The lack of a well-organized and universally accepted system of nomenclature for ductular cells and biliary precursors has led to a proliferation of poorly understood and loosely applied terminology (e.g., liver progenitor cells, biliary tree stem cells, atypical ductular cells, oval cells, biliary tree stem/progenitors, ductular hepatocytes, intermediate hepatocytes, reactive cholangiocytes). Although each term is meant to strictly refer to a specific subpopulation of a heterogeneous group of cells along the biliary lineage, the terms are often used synonymously and interchangeably, which has led to some confusion in the literature. Perhaps the simplest term to apply to the diverse expansion of the biliary compartment seen in chronic liver disease is the descriptive term, "ductular reactive cells" (DRCs), which describes the emergence of a histological lesion, known as the *ductular reaction*. The ductular

reaction consists of the activation and expansion of immature cholangiocytic cells that coalesce into primitive duct-like structures. The ductular reaction is, in fact, a collective signaling response to the surrounding hepatic stellate cells, macrophages, mature cholangiocytes, hepatocytes, portal myofibroblasts, and endothelial cells, which form a niche that regulates the formation, expansion, and differentiation of DRCs.⁸⁶ This reaction is apparently an attempt to activate endogenous repair mechanisms, but can also be viewed as an abnormal regenerative response because it is accompanied by excessive extracellular matrix deposition and promotes the progression of fibrosis.^{87–89}

The origin and fate of the ductular reactive cells is a subject of active debate in the literature and a topic on which concepts are rapidly evolving. What is clear is that a subpopulation of immature biliary-like cells is highly expanded in certain forms of chronic liver injury and that these cells have a bipotent differentiation capacity when isolated *in vitro*. There are varying theories on the origin of DRCs including a purely biliary origin, or alternatively, transdifferentiation of mature hepatocytes. Once established, however, the ductular reaction appears to be composed of cells of a biliary phenotype based on the following: (1) Many known oval cell markers are also markers for cholangiocytes^{90,91}; (2) the arrangement of these cells histologically is often in a ductular pattern⁹²; and (3) they typically emanate from and cluster near the portal tracts.⁹³ Historically, the canals of Hering, terminal structures where the hepatocyte canaliculi and the interlobular bile ducts interconnect, have been proposed as the site of origin for DRCs. Given the ideal anatomical location of this structure between the two epithelial cells, it was reasonable to postulate that it could serve as a niche to supply the putative stem cells for both the hepatocytes and cholangiocytes. Direct proof of this model is hampered due to a lack of specific marker proteins and the inexact nature of lineage-tracing studies. Additional stem cell niches are being described

including the peribiliary glands and a maturational lineage of stem and progenitor cells along the length of the biliary tree.⁹⁴ Recent studies also suggest that a self-renewing population of Axin2+ hepatocytes can regenerate damaged liver from a pericentral location through a reverse-streaming mechanism.⁹⁵

Stem Cells and Cellular Plasticity in Liver

In addition to the complexities surrounding the origin of DRCs, an even more relevant question is their ultimate fate (i.e., do ductular progenitors give rise to mature cholangiocytes to facilitate biliary repair and/or do they also contribute to parenchymal hepatocyte regeneration?). Pluripotent stem cell technology has revealed that adult cells, previously thought to be “terminally differentiated” retain remarkable cellular plasticity. This seems to be particularly true in the liver where complex transdifferentiation events are being documented, raising questions about whether cellular plasticity and reprogramming events may obviate the need for stem cell-based liver regeneration.^{96–98} The idea of biliary-derived DRCs being bipotent *in vivo* has been supported by lineage-tracing studies utilizing Cre/Lox technology with various Cre drivers and injury models; however, there are also several studies that do not support or show minimal contribution of biliary cells to regenerating hepatocytes (►Table 1).^{77,78,99–107} Studies done in zebrafish suggest that biliary cells contribute to hepatocyte restoration only in severe but not in moderate hepatocyte ablation.^{74,75} Another recent study in mice also showed that following deletion of 98% of hepatocytes, transplanted cells of biliary origin contributed significantly to the restoration of liver parenchyma, regenerating both hepatocytes and cholangiocytes.¹⁰⁶ The key limitations in definitively resolving the apparent contradictory findings lie in the imperfect and variable models of liver disease available (none of which

Table 1 Lineage tracing studies utilizing various Cre drivers and injury models to study the fate of ductular reactive cells

Reference	Genetic mice for lineage tracing	Injury model
Sackett et al, 2009 ⁹⁹	Foxl1-Cre	BDL, DDC, CDE
Furuyama et al, 2011 ¹⁰⁰	Sox9 Cre	CCI4, PHx, CDE, BDL, DDC, APAP
Malato et al, 2011 ⁷⁷	Ttr-Cre	CCI4, PHx, BDL, DDC
Espanol-Suner et al, 2012 ⁷⁸	OPN-Cre	PHx, CCI4, CDE, DDC
Huch et al, 2013 ¹⁰¹	LGR5-Cre	CCI4, MCDE, DDC
Rodrigo-Torres et al, 2014 ¹⁰²	HNF1β-Cre	CCI4, PHx, CDE, DDC, APAP
Schaub et al, 2014 ¹⁰³	Ttr-Cre, CK19-Cre, PDGFR-β-Cre	CDE
Tarlow et al, 2014 ¹⁰⁴	Sox9-Cre	PHx, CCI4, CDE, DDC
Yanger et al, 2014 ¹⁰⁵	KRT-19-Cre, TBG-Cre	CCI4, CDE, DDC, ANIT
Lu et al, 2015 ¹⁰⁶	KRT-19-Cre	CDE, ΔMdm2
Kamimoto et al, 2016 ¹⁰⁷	Prom1-Cre, AAV8-iCre	TAA, DDC

Abbreviations: AAV8, adeno-associated virus 8; ANIT, alpha naphthylisothiocyanate; APAP, acute acetaminophen; BDL, bile-duct ligation; CCI4, carbon tetrachloride; CDE, choline-deficient ethionine supplemented; DDC, 3,5-diethoxycarbonyl-1,4-dihydro-collidine; Foxl1, forkhead box 11; HNF1β, hepatocyte nuclear factor 1 homeobox B; KRT19/CK19, cytokeratin 19; LGR5, leucine-rich repeat-containing G-protein coupled receptor 5; OPN, osteopontin; PHx, partial hepatectomy; Prom-1, prominin-1; Sox9, SRY-related HMG-box transcription factor 9; TAA, thioacetamide; TBG, thyroid hormone-binding globulin; Ttr, transthyretin.

accurately represent human chronic liver disease) and some technical limitations of lineage-tracing studies. Genetic lineage tracing is a powerful strategy for in vivo fate-tracing experiments because it allows for cell-type specificity (using cell-specific Cre drivers) and Cre expression can be controlled temporally (with tamoxifen-inducible systems). Although lineage tracing remains the gold standard to trace the origin of new cells, several important caveats need to be kept in mind.^{108,109} Leaky or unexpected Cre expression in different cell types, even in small amounts, can bias results. Injury states may alter the cell-specificity of Cre. Furthermore, tamoxifen can persist within the animal so that temporal precision of labeling is not always possible. Tamoxifen is also excreted in feces and can lead to Cre activation in untreated animals if co-housed. Despite these known limitations, genetic lineage tracing is likely to continue to provide novel and important insights into liver cell plasticity in the future.

Stem Cell-Derived Cholangiocytes

Human embryonic stem cells and induced pluripotent stem cells (iPSCs) have self-renewal capacity, are pluripotent, and have the ability to differentiate into cells of all three primary germ layers. Soon after the development of iPSCs, several in vitro hepatocyte differentiation strategies quickly emerged. Although these protocols produced cells with many features of adult hepatocytes, it has been notoriously difficult to achieve a fully mature adult hepatocyte phenotype; because of this, the differentiated cells have typically been referred to as *hepatocyte-like* cells. Nonetheless, these protocols provide powerful tools for studying liver developmental biology, recapitulating specific disease phenotypes, and could potentially provide unlimited resources for drug-testing applications and cell-based therapies.

The natural and logical evolution of the application of iPSC technology to liver disease was to develop iPSC-derived cholangiocytes for modeling biliary disease. Indeed, several groups have now published unique protocols for the differentiation of iPSCs into cholangiocytes. The protocols available for the differentiation of stem cells to cholangiocytes were

mainly designed to mimic the patterns and stages observed during biliary development in utero. All were designed with a stepwise approach in which the cells were exposed to soluble factors as they proceed through various phases of endodermal differentiation. In principle, these phases should mimic normal embryologic development through phases including a definitive endoderm phase, a hepatic specification phase, a hepatoblast-like phase, and a differentiated cholangiocyte phase (► **Table 2**).

In 2014, Dianat et al showed that embryonic stem cells could be differentiated to cholangiocytes and subsequently applied the technique to iPSCs and HepaRG cells. They generated hepatoblasts using methods previously described in the generation of hepatocytes from pluripotent cells. In a monolayer, cells in the hepatoblast stage were matured to cholangiocytes with exposure to human growth hormone, epidermal growth factor (EGF), interleukin 6 (IL-6), and sodium taurocholate. Cells from the final stage of differentiation expressed high levels of cholangiocyte markers such as CK7, CFTR, TGR5, HNF6, SOX9, and AQP1. The cells also formed cilia and when cultured in a three-dimensional (3D) matrix, they developed epithelial/apical-basal polarity, and they formed functional cysts and biliary ducts.²⁰

Our group utilized defined media and feeder-free culture conditions along with temporal exposure to key biliary morphogens to achieve cholangiocyte differentiation from patient-derived iPSCs. We reported the use of temporally restricted Hh signaling during the differentiation of iPSC to cholangiocytes. Hepatic specification was achieved using a combination of SHH, BMP4, and FGF2. To induce hepatic progenitor cells, SHH was also used in combination with Jag-1 (to activate Notch signaling). For the cholangiocyte maturation, TGFβ was used in conjunction with a collagen-1 matrix. The resulting cholangiocytes showed expression of cholangiocyte markers (CK19, CK7, PKD2, CFTR, AE2), the presence of primary cilia, intact calcium signaling, and were able to form duct-like structures in 3D culture. This protocol also showed that iPSC-derived cholangiocytes were able to engraft within mouse liver in vivo, following retrograde intrabiliary infusion.²¹

Table 2 Growth factors employed by different groups for differentiation of stem cells to biliary cells

Reference	Definitive endoderm	Hepatic specification	Hepatic progenitor	Cholangiocyte
Dianat et al, 2014 ²⁰	Wnt3A, ActivinA, FGF2	ActivinA, FGF2, BMP4	FGF4, HGF, EGF, RA	HG, EGF, IL-6, ST
De Assuncao et al, 2015 ²¹	Wnt3A, ActivinA	SHH, BMP4, FGF2	Jagged1, SHH	TGFβ
Ogawa et al, 2015 ²²	ActivinA	FGF2, BMP4	HGF, Dex, OSM	HGF, EGF, TGFβ, OP9 co-culture
Sampaziotis et al, 2015 ²³	ActivinA,FGF2, BMP4	BMP4	FGF10, ActivinA, RA	EGF
Takayama et al, 2016 ¹¹⁰	ActivinA	FGF4, BMP4	Dex, HGF, EGF	EGF, IL-6, ST

Abbreviations: BMP, bone morphogenetic protein; Dex, dexamethasone; EGF, epidermal growth factor; FGF, fibroblast growth factor; HG, human growth hormone; HGF, hepatocyte growth factor; IL-6, interleukin-6; OSM, oncostatin M; RA, retinoic acid; SHH, sonic hedgehog; ST, sodium taurocholate; TGFβ, transforming growth factor β.

Ogawa et al used a combination of hepatocyte growth factor (HGF), dexamethasone, and oncostatin to obtain hepatic progenitors and a subsequent combination of HGF, EGF, and TGF β to obtain mature cholangiocytes. Notch signaling was activated using a co-culture with OP9 cells, a stromal cell line that expresses Notch ligands. The resulting cells, when grown in 3D culture, formed epithelialized cystic and/or ductular structures that expressed markers found in mature cholangiocytes. This study also showed that cholangiocytes generated from the iPSCs of patients with cystic fibrosis had impaired cyst swelling and that CFTR chemical correctors increased the levels of CFTR on the apical side of the lumen and augmented cyst swelling.²²

Sampaziotis et al showed that in their protocol FGF10, retinoic acid, and TGF β were key factors for biliary specification. Their iPSC-derived cholangiocytes were characterized using a range of assays that resembled primary common bile duct cholangiocytes at the transcriptional level. Patient-derived iPSCs in 3D culture conditions were used to model polycystic liver disease and cystic fibrosis.²³ 3D cultures of iPSC-derived cholangiocytes generated from polycystic patients were responsive to secretin and somatostatin; this response was blunted by the use of octreotide. They also showed that the cholangiocytes derived from cystic fibrosis patients had minimal CFTR protein expression and were unable to modify intracellular chloride. They corrected the disease phenotype with an experimental drug, VX809.

Takayama et al used an approach similar to the Dianat protocol to promote cholangiocyte differentiation. The maturation of their hepatoblast-like cells was done with human growth hormone, EGF, and IL-6 along with extracellular matrix molecules. The gene-expression levels of the cholangiocyte markers, AQP1, SOX9, CFTR, G protein-coupled bile acid receptor 1, Jag-1, secretin receptor, and GGT were all increased by using laminin 411 or laminin 511 as a matrix.¹¹⁰

Overall, the collective efforts at generating cholangiocytes derived from stem cells have been remarkably successful. These cells are already proving to be promising tools that can be used in many applications such as cell transplantation studies, biliary disease modeling, deciphering biliary development, and small molecule screening. Although each of the various differentiation protocols have some similarities and some unique aspects, it is fascinating that the disparate protocols all seem to result in a mature, adult cholangiocyte phenotype, insofar as these features have been evaluated. This may suggest that *in vitro* the default differentiation pathway of liver progenitors is biased toward cholangiocyte differentiation, especially given the difficulties noted in generating fully mature stem cell-derived hepatocytes. This is in contradistinction to the conceptualized sequence of events in liver development, where it is thought that intervening signals from the portal mesenchyme are required for cholangiocyte differentiation, whereas the “default” fate for most hepatoblasts is thought to be hepatocellular. Most likely, additional fine-tuning of the various cholangiocyte differentiation approaches will be needed to achieve the most robust and efficient cholangiocyte differentiation possible. It may also be that certain protocols will have advantages or disadvantages

in certain applications. Regardless, this powerful new technology has provided unique opportunities to study cholangiocyte development, pathogenesis, and treatment strategies in ways that were previously unthinkable. When combined with large-scale efforts that are underway to generate robust biorepositories of iPSCs, iPSC-derived cholangiocytes, and biliary organoids from patients with cholangiopathies, this technology will become an even more powerful tool for individualized medical applications.

Three-Dimensional Culture Systems and Biliary Organoids

Organoids are collections of organ-specific cell types that develop from stem cells or organ progenitors.¹¹¹ Organoid formation recapitulates the major processes of self-organization during development including cell sorting and spatially restricted lineage commitment in a manner similar to the *in vivo* environment.¹¹² Grouped together and spatially organized similar to an organ, these structures are capable of recapitulating specific functions of the adult organs (e.g., secretion / absorption, filtration, neural activity, contraction, etc.). An important distinction to bear in mind is the difference between 3D cultures of a single-cell type and true organoids, which consist of multiple self-organizing cell types. An example of this distinction from the intestine is the difference between an enteroid (which consists of a single-cell type in 3D culture) and a true intestinal organoid (a stem cell-derived and self-organizing multicellular cluster). Organoid research has tremendous potential across multiple organ systems to facilitate disease modeling, pharmacological testing, and therapeutic regenerative medicine applications (►Fig. 3). Indeed, organoids have been generated to model

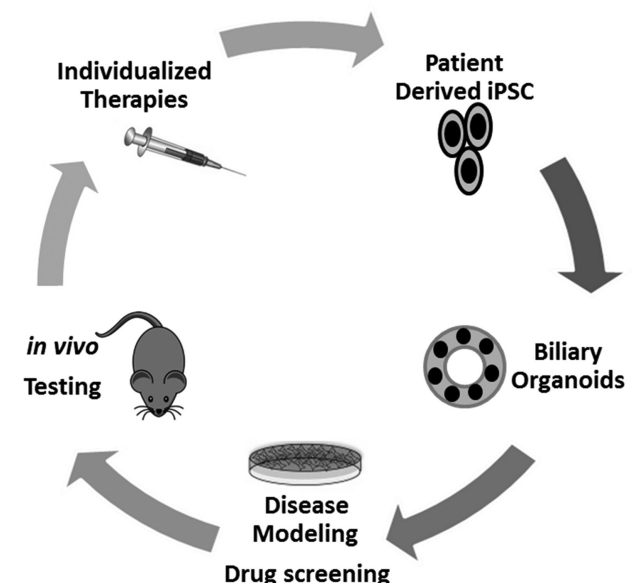


Fig. 3 Induced pluripotent stem cell- (iPSC-) derived cholangiocytes and applications. Schematic depicting patient-derived iPSC that could potentially be expanded as organoids, utilized for disease modeling and drug screening, tested *in vivo*, and eventually returned to the patient as an individualized therapy.

diverse organs including the kidney, the lung, the brain, the thyroid, and others.¹¹¹ Within gastroenterology, organoid systems have been generated to represent the esophagus, the stomach, the pancreas, the small intestine, the large intestine, and the pancreas.¹¹³ Liver organoids have been generated from both tissue-derived progenitor cells^{101,114} and from iPSCs.¹¹⁵ Organoid research is a logical extension of the existing iPSC technology that has been leveraged toward the biliary system. Furthermore, the infrastructure for overseeing biorepositories of biliary organoids likely already exists within existing iPSC biorepositories.

Several groups have utilized 3D culture systems to facilitate research in primary liver stem cells. As early as 2001, it was shown that primary liver cells cultured under 3D conditions could be maintained *in vitro* in the presence of EGF, HGF, and dexamethasone.¹¹⁶ Mouse Foxl1⁺ hepatic progenitor cells, when cultured in type 1 collagen gels, formed CK19 positive branches.¹¹⁷ In 2007, Tanimizu et al showed that HPPL, a mouse liver progenitor cell line, when cultured in 3D, formed cysts with a luminal space and apicobasal polarity.¹¹⁸ Kido et al showed that CPM⁺ cells, sorted and matured to cholangiocytes in 3D culture, formed cysts with a luminal structure and proper apicobasal polarity.¹¹⁹ Recently, it was also shown that rat liver stem cells can be isolated and grown as cystic structures when cultured in high levels of Wnt3a and noggin (a BMP signaling inhibitor). Gallbladder stem/progenitor cells from noninjured livers can form 3D structures that express stem cell markers in the presence of R-spondin 1, noggin, and nicotinamide.¹²⁰ Yu et al published a protocol for direct reprogramming of fibroblasts to induced hepatic stem cells. These cells expressed hepatocyte and cholangiocyte markers *in vitro* and were able to form cysts and branching structures that were positive for CK19 and CK7 under 3D culture.¹²¹

In 2013, an elegant study generated true liver organoids by combining human-iPSC-derived hepatic cells with mesenchymal stem cells and endothelial cells. Although the cells were cultured in two-dimensional conditions, the cells self-organized into 3D clusters, which resembled embryonic liver.¹¹⁵ When transplanted in mice, these organ buds developed into hepatic tissue with features of adult liver. Notably, however, these organoids lacked biliary elements. Huch et al have demonstrated that LGR5⁺ adult liver stem cells can be isolated and cultured as liver organoids in a 3D culture method involving matrigel with HGF, EGF, FGF, and R-spondin1.¹⁰¹ The expanded cells self-organized into 3D structures with a ductal (CK19⁺) single-layered epithelium compartment and a pseudo-stratified compartment expressing both ductal (CK7, CK19) and hepatocyte (E-cadherin, Hnf4 α) markers. When cultured in 3D, a single LGR5⁺ cell can be expanded as organoids and differentiated into hepatocytes and cholangiocytes. In 2015, the same group used a similar approach to expand liver biopsy tissue and single EPCAM⁺ cells as liver organoids.¹¹⁴ As powerful as these emerging systems are, organoid technology does have some notable limitations. Because they are generated *in vitro*, organoids in culture may not fully recapitulate all aspects of liver development that occur in the *in vivo* environment. In particular,

they lack several essential components of the intact liver such as a vascular system, interaction with other cell types, specific extracellular matrix interactions, and immune surveillance (although organoid transplantation studies may help to overcome some of these issues). Despite these known limitations, 3D culture systems and liver organoid technology are rapidly advancing and are likely to be increasingly utilized for individualized disease modeling and regenerative medicine applications for the biliary system.

Summary

In the past several decades, we have witnessed significant advances in our understanding of biliary development, the basic physiology of cholangiocytes, and the pathogenesis of the cholangiopathies. Despite these advances, effective treatment modalities remain elusive. For this reason, the recent explosion of work in biliary regenerative medicine is particularly encouraging and provides great hope for future regenerative therapeutics for biliary disease. In this review, we outlined some of the primary clinical challenges associated with the biliary system and we identified a standard regenerative medicine paradigm involving replacement, regeneration, and rejuvenation that may help to categorize the development of future regenerative therapies. We also highlighted both historical and more recent basic science advances in biliary development, regeneration, and repair, and we reviewed new regenerative technologies involving iPSC-derived cholangiocytes and biliary organoids. All of these conceptual and technical advances now set the stage for the future translation and application to ultimately develop new regenerative service lines for patients with biliary disease. When and how these new therapies will emerge is unknown, but their development and translation will likely require multidisciplinary transformational teams consisting of basic scientists, hepatologists, and biliary and transplant surgeons, as well as interventional radiologists and advanced endoscopists. It is likely that the field will take advantage of its existing endoscopic access to the biliary tree by building new platforms in regenerative endoscopy. The ERCP-based delivery of cell-based (e.g., stem cells or stem-cell derived cholangiocytes) or cell-free regenerative therapeutics (e.g., biodegradable stents or therapeutic exosome delivery) are particularly appealing because advanced endoscopy is available at academic centers throughout the world. Other technological applications being contemplated include 3D bioprinting of ductular tissue, biliary biostents incorporating cellular elements, and tissue-engineering approaches, such as recellularization of decellularized bile duct units. Our embrace of regenerative medicine also involves some challenges. We will need to face down the issues of inexact technologies, inefficient or incomplete differentiation from stem cells, epigenetic memory, and malignancy potential, as well as highly complex tissue engineering and regulatory challenges. In the end, however, we are left with great hope for clinical advances. Clearly, the future is bright in terms of regenerative medicine and the biliary tree. We are awakening to the dawn of a new golden age in regenerative hepatology that promises

the development and application of new regenerative therapeutics for previously untreatable liver diseases.

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Abbreviations

3D	three-dimensional
BMP	bone morphogenic protein
DRCs	ductular reactive cells
EGF	epidermal growth factor
ERCP	endoscopic retrograde cholangiopancreatography
FGF	fibroblast growth factor
HGF	hepatocyte growth factor
IL-6	interleukin 6
iPSCs	induced pluripotent stem cells
Jag-1	Jagged-1
PBC	primary biliary cholangitis
PHx	partial hepatectomy
PSC	primary sclerosing cholangitis
SHH	sonic hedgehog
TGF	transforming growth factor
UDCA	ursodeoxycholic acid

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