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Functional and structural features of cholangiocytes in health and disease

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Abstract

Cholangiocytes are the epithelial cells that line the bile ducts. Along the biliary tree, two different kinds of cholangiocytes exist; small and large cholangiocytes. Each type has important differences in their biological role in physiological and pathological conditions. In response to injury, cholangiocytes become reactive and acquire a neuroendocrine-like phenotype with the secretion of a number of peptides. These molecules act in an autocrine/paracrine fashion to modulate cholangiocyte biology and determine the evolution of biliary damage. The failure of such mechanisms is believed to influence the progression of cholangiopathies, a group of diseases that selectively target biliary cells. Therefore, the understanding of mechanisms regulating cholangiocyte response to injury is expected to foster the development of new therapeutic options to treat biliary diseases. In the present review, we will discuss the most recent findings in the mechanisms driving cholangiocyte adaptation to damage, with particular emphasis on molecular pathways that are susceptible of therapeutic intervention. Morphogenic pathways (Hippo, Notch, Hedgehog), which have been recently shown to regulate biliary ontogenesis and response to injury, will also be reviewed. In addition, the results of ongoing clinical trials evaluating new drugs for the treatment of cholangiopathies will be discussed.

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INTRODUCTION

The liver is the largest gland in the body and is endowed with critical metabolic functions that involve digestion of food and clearance of toxic substances. At the level of the bile canaliculus, hepatocytes secrete bile, which is then carried to the duodenum through a complex network of bile ducts lined by cholangiocytes [1–3].

Under physiological conditions, cholangiocytes actively contribute to the final composition and volume of bile secretion by basal and hormone regulated events [4]. In normal conditions, one of the most important and well-studied functions of cholangiocytes is secretin-induced release of bicarbonate into bile. The binding of secretin to the secretin receptor (SR) on the basolateral membrane of cholangiocytes leads to the formation of cAMP, PKA-dependent phosphorylation of CFTR, and the subsequent extrusion of Cl^- in the lumen of bile ducts. Driven by the Cl^- gradient across the plasma membrane, the activation of the apical Cl^-/HCO_3^- anion exchanger 2 (AE2) culminates in the net excretion of bicarbonate in bile [5], with passive influx of water (Figure 1). As a result, cholangiocytes participate to up to 40% of the so-called bile salt-independent bile flow [6].

Cholangiocytes are the specific target of a heterogeneous group of human diseases, termed cholangiopathies, which have deep consequences on the biology of these cells [7]. In the present review, we will discuss the differences in the structure and function of cholangiocytes and underline the main findings in biliary pathophysiology of the last 10 years. The clinical implications of ongoing research will also be specifically addressed.

MORPHOLOGY, HEPATIC VASCULATURE AND FUNCTION OF CHOLANGIOCYTES

The biliary epithelium is composed of intra and extra-hepatic bile ducts lined by cholangiocytes [8]. The human intrahepatic biliary epithelium is classified by size: hepatic ducts (>800 μm), segmental ducts (400–800 μm), area ducts (300–400 μm), septal bile ducts (100 μm), interlobular ducts (15–100 μm), and bile ductules (<15 μm) [9–11]. The intrahepatic biliary epithelium of rodents is formed by ducts of different sizes, small (<15 μm in diameter) and large (>15 μm) [12, 13]. Cholangiocytes lining small and large bile ducts have been morphologically and functionally categorized into small and large cholangiocytes, respectively [12–15]. With regards to cellular structure, while small cholangiocytes are cuboidal, larger cholangiocytes in larger bile ducts are more columnar in shape [9–11]. Moreover, small cholangiocytes are poorly specialized and have a high nucleus/cytoplasm ratio, whereas large cholangiocytes are supplied with plenty of organelles and a small nucleus/cytoplasm ratio [16]. Large, but nor small cholangiocytes have cilia, which act as chemo and mechanosensors within bile duct lumen [17]. Expression of molecules involved in secretin-stimulated biliary secretion also differs along the biliary epithelium. SR, CFTR and

AE2 are only expressed by large cholangiocytes and are responsible for the majority of biliary fluid secretion, through the activation of a cAMP-dependent pathway [12, 13]. In small cholangiocytes, on the other hand, Ca^{2+}-activated signaling pathways seem predominant. Indeed, the activation of purinergic receptors in small and large cholangiocytes induces Ca^{2+}-dependent Cl^- secretion via TMEM16A, providing an alternative route to the secretin-stimulated cAMP-dependent ductal fluid secretion [18, 19]. Functionally, large cAMP-dependent cholangiocytes are more susceptible to damage, whereas small cholangiocytes are more resistant to liver injury [12, 20–22]. During damage of large cholangiocytes, however, small cholangiocytes replenish the biliary epithelium. Again, the amplification of Ca^{2+}-dependent signaling pathways in small cholangiocytes are essential in driving the *de novo* acquisition of large cholangiocyte phenotypes (Figure 1) [20, 21].

Cholangiocytes are normally quiescent in liver but respond to injury or stress by enhanced proliferation [3, 23, 24]. Compensatory responses to liver injury include biliary hyperplasia, ductular reaction and ductopenia. Biliary hyperplasia (characterized by proliferation/loss of cholangiocytes as observed in cholestatic liver diseases such as primary sclerosing cholangitis) is associated with enhanced biliary secretion of HCO_3^- in bile, which may be a compensatory protective mechanism for the injured biliary epithelium [25]. On the other hand, ductopenia is evidenced by the damage of bile ducts in response to toxins or in certain diseases such as biliary atresia [15, 20, 21, 26, 27]. The hepatic artery is the main blood supplier of the biliary epithelium within the peribiliary vascular plexus (PBP). The PBP secretes a number of angiogenic factors such as VEGF that have been shown to regulate biliary proliferation in experimental models of cholestasis [28–31].

CHANGES OF THE BILIARY EPITHELIUM IN PATHOLOGIC CONDITIONS

Pathophysiology of biliary response to injury

Cholestatic liver diseases represent a heterogeneous group of diseases characterized by an impairment of bile formation or bile flow that can arise at the hepatocellular or cholangiocellular level [32]. Emblematic diseases in this group are primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) [33]. The current various animal models allow a better insight into the signaling pathways involved in the development of cholestasis. Such studies may provide potential treatment strategies to restore impaired secretory functions of hepatocytes and cholangiocytes, or to modulate the response of these cells to liver injury. Specific types of liver injury activate the proliferation of particular cholangiocyte subpopulations (i.e., large/small) [15, 20, 21]. In most instances, biliary proliferation contributes to the major part of the ductular reaction. However, new ductules may also originate from activated progenitor cells or from cells that have entered from the circulation and differentiate into liver cells [23, 34, 35].

Cholangiocyte response to injury is an articulated event, which retains a “double face” in pathophysiologic terms. After an initial insult, cholangiocytes become activated and start to proliferate. This modification is functional to compensate for the anatomical loss of biliary cells and also to sustain their secretory activities [36]. In most instances, however, biliary proliferation eventually subsides and apoptotic mechanisms become prevalent with the development of ductopenia [7]. Along with proliferation, cholangiocyte response to injury is

characterized by the so-called neuroendocrine-like trans-differentiation, which plays an essential role not only in sustaining biliary proliferation itself, but also in immune responses, hepatic inflammation and development of liver fibrosis [4, 23]. To this extent, a number of neuroendocrine factors are synthetized *de novo* by reactive cholangiocyte and have been shown to modulate biliary damage by autocrine/paracrine mechanisms (Table 1) [23]. An elegant morphological study has provided strong evidence for the autocrine/paracrine role of VEGF in the regulation of biliary damage. This study has shown that following BDL-induced cholestasis, the PBP undergoes extensive proliferation supporting the increased nutritional and functional needs of the proliferating biliary epithelium. However, the proliferation of the PBP only occurs after cholangiocytes, supporting the autocrine role of the biliary system (secreting angiogenic factors) in the regulation of biliary function [31]. To support the important role of angiogenic factors in sustaining biliary growth, we have shown that secretin stimulates biliary proliferation by microRNA 125b and let7a–dependent upregulation of VEGF-A and NGF, respectively [37]. Knockout of the secretin receptor (that is only expressed by large cholangiocytes) decreases biliary hyperplasia in cholestatic mice by downregulation of cAMP signaling [38]. Other studies have demonstrated the pro-proliferative effect of VEGF-A and VEGF-C, which increased biliary growth of normal rats by interaction with VEGFR2 and VEGFR3, respectively. The same study also showed that the *in vivo* administration of neutralizing antibodies for VEGF-A/C decreased BDL-induced biliary hyperplasia [29]. The paracrine effect of VEGFs on biliary functions was also demonstrated in experiments where the ligation of the hepatic artery resulted in disappearance of the PBP (source of angiogenic factors such as VEGFs), significant reduction of biliary growth (accompanied by enhanced apoptosis), and reduced secretion of VEGF and bicarbonate by cholangiocytes [28]. Another study has shown that inhibition of VEGF expression in cholangiocytes by overexpression of miR-125b and knockdown of histidine decarboxylase (HDC, the enzyme regulating histamine synthesis) decreased BDL-induced biliary hyperplasia [39]. Consistent with the role of VEGF on biliary functions, a recent study has shown that VEGF plays an important role on infection-induced increase of biliary cystogenesis in cholangiocytes of the polycystic kidney (PCK) rat model [40]. Prolonged feeding of taurocholic acid to BDL rats prevents biliary damage induced by hepatic artery ligation or caffeic acid by overexpression of VEGF-A [41, 42]. Also, cholangiocyte neuroendocrine-like trans-differentiation, driven by the *de novo* expression of pancreatic duodenal homeobox-1, has been associated with enhanced biliary VEGF expression [43]. Another study has shown that cholangiocytes generate a VEGF gradient that is key during arterial vasculogenesis, whereas angiopoietin-1 signaling from hepatoblasts participates in the remodeling of the hepatic artery to sustain the nutritional demands of the proliferating biliary epithelium [44].

Biliary hyperplasia is also promoted by a number of growth factors such as nerve growth factor (NGF), follicle stimulating hormone (FSH), gonadotropin-releasing hormone (GnRH), estrogens, and the biogenic amine histamine, by the interaction with their specific receptors [22, 45–47]. For example, we have shown that: (i) intrahepatic bile ducts secrete NGF and express NGF receptors; and (ii) NGF stimulates (in combination with estrogens) biliary proliferation by activating the ERK pathway as well as the phosphatidylinositol 3-kinase pathway [48]. The decrease in intrahepatic bile duct mass (concomitant with reduced

expression of estrogen receptor-alpha and beta and enhanced biliary apoptosis) support the role of endogenous estrogens in sustaining the enhanced proliferative and secretory activities of cholangiocytes during cholestasis, which may be important during ductopenic states [49]. Supporting this concept, another study has shown that estrogens maintain biliary mass and reduce apoptosis after biliodigestive anastomosis in cholestatic BDL rats [50]. A recent study has also shown that cholangiocytes express FSH and its receptor (FSHR) and also secrete FSH. *In vivo*, FSH increases biliary mass, whereas administration of antide (a gonadotropin-releasing hormone antagonist blocking FSH secretion) and anti-FSH antibody to BDL rats decreased cAMP-dependent cholangiocyte proliferation and biliary mass. Modulation of biliary FSH expression may be a target for the management of cholestatic liver diseases [45]. FSH as well as other growth factors including estrogens either directly or by synergizing NGF, IGF1, FSH and VEGF have been shown to regulate the proliferative and secretive activities of cystic epithelium of polycystic liver diseases (PCLDs) in rodent models and human cell lines. Also, GnRH (secreted by the hypothalamus as well as cholangiocytes) has been shown to stimulate biliary proliferation by both paracrine/autocrine pathways [51]. Disruption of GnRH/GnRHR cascade may be an important target for the management of cholangiopathies.

Conversely, a number of other molecules have been shown to reduce cholangiocyte proliferation. For example, the activation of serotonin 1A and 1B receptors inhibits biliary hyperplasia in cholestatic rats by enhanced IP_3/Ca^{2+}/protein kinase C signaling and subsequent inhibition of the cAMP/protein kinase A/Src/extracellular signal-regulated kinase 1/2 pathway. Cholangiocytes also secrete serotonin that reduces biliary proliferation during the course of cholestasis in an autocrine fashion [52]. In addition, they express the neuronal isoform of neuronal tryptophan hydroxylase (TPH) and synthesize serotonin and use serotonin as an autocrine/paracrine signal to regulate biliary remodeling [53]. Other studies provide evidence for the growth limiting function of the hormone melatonin. In cholestatic BDL rats, melatonin both *in vivo* and *in vitro* decreased biliary hyperplasia by cAMP-dependent downregulation of clock gene expression through the interaction with MT1 receptor subtype [24]. Furthermore, when BDL rats were housed in prolonged darkness there was reduced biliary hyperplasia and fibrosis, which was accompanied by a significant increase in the serum levels of melatonin likely originating from the pineal gland [54]. Hepatic inhibition of arylalkylamine N-acetyltransferase (AANAT, the rate limiting enzyme regulating melatonin synthesis) by Vivo-Morpholino sequence of AANAT (that decreases melatonin hepatic secretion) increases biliary growth and the expression of angiogenic factors in cholestatic rats [55, 56]. A number of elegant studies have also been performed to evaluate the role of histamine on cholangiocyte proliferation. It has been found in rodent models of cholestasis that histamine increases or inhibits biliary proliferation by interacting with either H1-H2HR histamine receptors (stimulatory) or H3-H4HR histamine receptors (inhibitory) [22, 47, 57, 58]. Stimulation of H3HR by H3HR agonist decreases BDL-induced cholangiocyte hyperplasia via inhibition of cAMP signaling, thus suggesting a possible beneficial effect of histamine in cholangiopathies. Histamine also interacts with the H1HR receptor and increases the proliferation of small cholangiocytes by activation of IP_3/Ca^{2+}/CaMKI/CREB-dependent signaling [58, 59]. This differential response induced by histamine may be employed in variable conditions of liver diseases where either reduction in biliary

hyperplasia or regeneration of liver would be desirable depending on the injury. It is evident from these studies that modulation of the different receptors could be of prime importance while managing the balance between biliary growth/loss in cholangiopathies or post transplantation. *In vivo*, GABA induces the damage of cAMP-dependent large cholangiocytes concomitant with *de novo* proliferation of small cholangiocytes which amplify their Ca^{2+}-dependent signaling and acquire phenotypes of large cholangiocytes to repair the damaged biliary epithelium [20, 21].

Emerging morphogenic pathways regulating biliary pathophysiology

Hippo signaling pathway—The Hippo signaling pathway is an evolutionarily conserved pathway that regulates bile duct differentiation and homeostasis in the liver [60]. The core Hippo signaling pathway is a kinase cascade [61]. The apical membrane-associated FERM domain protein neurofibromin 2 (NF2) directly binds and recruits the Nuclear Dbf2-related family kinase, large tumor suppressor homolog 1/2 (LATS1/2), to the plasma membrane. Membrane recruitment, in turn, promotes LATS1/2 phosphorylation by the Ste-20 family protein kinase, Mammalian STE20-Like Protein Kinase 1/2 (MST1/2), together with the adaptor protein salvador homolog 1 (SAV1). In turn, LATS1/2, in a complex with small regulator protein Mps One Binder Homolog A (MOB1), phosphorylates Yes-associated protein (YAP), a transcription coactivator. Phosphorylation of YAP deactivates its transcription coactivator activity through sequestering YAP in cytoplasm. YAP is highly expressed in cholangiocytes of both mouse and human liver, suggesting a role of YAP in cholangiocyte biology [62, 63]. Using genetically modified mouse models, transcriptional regulation activity of YAP was found to be required for bile duct development [64]. Liver specific *Yap* deletion leads to postnatal bile duct paucity due to failure formation of primitive ductal structures around E18.5. In agreement, increasing YAP activity through ablating upstream negative regulator, *Nf2*, significantly increases the numbers of primitive ductal structures and results in bile duct hyperplasia [64]. However, the YAP downstream targets involved in regulating bile duct development remain to be elucidated. YAP is also important for determining biliary cell fate [62, 65]. Comparing to cholangiocytes, hepatocytes keep a lower YAP activity. Increasing hepatocyte YAP activity through ectopic YAP expression or ablating upstream negative regulator Mst1/2 dedifferentiate periportal hepatocytes into ductal cells. Furthermore, Carmago et al. demonstrated that YAP regulates hepatic cell fate determination directly through the Notch signaling, another critical signaling pathway for bile duct development[62].

Notch signaling pathway—The Notch signaling pathway contains four transmembrane Notch receptors (Notch-1, −2, −3, −4), and two types of cell surface ligands, Serrate/Jagged (Jag-1, −2) or Delta-like (Dll-1, −3, −4)[66]. The activation of the Notch signaling requires a cell-cell interaction between the “transmitting” cell expressing Notch ligands and the “receiving” cell expressing Notch receptors. Upon ligand engagement, the Notch receptor is cleaved by the γ-secretase complex, leading to the cytoplasmic release of the Notch intracellular domain (NICD). NICD will then translocate into the nucleus where it binds to the recombination signal binding protein immunoglobulin kappa J (RBP-Jκ) to displace the RBP-Jκ associated co-repressors, thereby allowing the transcription of the Notch-target genes. Among them, the Hairy enhancer of split homologs transcription factors (Hes and

Hey), the family of the hepatocyte nuclear factors (HNF) and the Sex determining region Y-box 9 (Sox-9) are involved in biliary cell differentiation. Mouse models deficient in Notch receptor Notch-2 [67–69], Notch ligands Jag-1 [70,71], Notch nuclear effector RBP-Jκ [72,73], Notch transcription target Hes-1 [74], Sox-9 and HNF1β (Ref 13) [75,76], all show defects in intrahepatic bile duct tubulogenesis during fetal development and early post-natal life. Consistently, constitutive activation of the Notch-2 intracellular domain (NICD) in hepatoblasts during development leads to ectopic formation of tubular and cystic structures, resembling early malignant biliary lesions [77,78]. In agreement with their physiological role in the commitment of the biliary lineage, Notch2, Jagged1, Hes1, Sox-9 and HNF1β are highly expressed in biliary cells [73, 74].

Hedgehog Signaling

Both immature and mature cholangiocytes produce and respond to the Hedgehog (Hh) signaling ligands, Sonic hedgehog (Shh) and Indian hedgehog (Ihh) [79–81]. Shh and Ihh ligands then bind to their transmembrane Hedgehog receptor Patched (Ptc), which relieve the suppression of Smoothened (Smo), leading to activation of the Glioblastoma (Gli) family of transcription factors (Gli1, Gli2, Gli3) [79]. The important role of the Hedgehog pathway in cholangiocyte pathogenesis was demonstrated with a cholestatic injury model [80, 82]. Dramatic increases in hepatic expression of Hh ligands and up-regulation of Hh pathway activity occur post bile duct ligation (BDL) in rodents. Moreover, mice with a genetic ablation of *Ptc* exhibited exacerbated ductular and fibrogenic responses. However, the physiological role and the molecular mechanism of the Hedgehog signaling during maintenance of bile duct homeostasis are not fully understood and remains to be further investigated.

Role of cholangiocytes in the development of human chronic cholestatic conditions

Primary Sclerosing Cholangitis—Signaling mechanisms fueling PSC development are being studied in several different animal models. Among the different rodent models, the MDR2$^{-/-}$ mice have been particularly helpful in studying the development of fibrosis in PSC [83]. MDR2$^{-/-}$ mice have decreased concentration of phosphatidylcholine in bile, which is known to potentiate the toxicity of bile acids. Additionally, leakage of bile into portal tracts, caused by disrupted tight junctions of the biliary epithelium, has been demonstrated in MDR2$^{-/-}$ mice and is responsible for causing inflammation and fibrosis [84]. Fibrosis is regulated by the expression of several pro/anti fibrotic genes. For example, TIMP-1 mRNA expression is increased, whereas MMP-13 is suppressed in this model. Additionally, a number of pro-inflammatory molecules such as TNF-α, IL-1β, IL-6, TGF-β1 and interferon-γ are overexpressed in MDR2$^{-/-}$ mice compared to controls. Mainly based on experiments on MDR2$^{-/-}$ mice [85], new possible treatment options for PSC are currently being evaluated in clinical trials (as discussed below).

In a recent study, cholangiocytes where isolated from livers of PSC patients [86]. The authors were able to culture intrahepatic cholangiocytes and further confirm their purity by immunofluorescence studies for cholangiocyte specific markers such as CK-19. The authors also showed that PSC cholangiocytes expressed less tight junction proteins, ZO1 (indicating impaired epithelial junctions) and were enlarged in size with robust filamentous structures

throughout the cell body. Further, these cholangiocytes exhibited characteristics of cellular senescence when compared with normal human cholangiocytes and H69. Next Generation Sequencing confirmed the elevated expression of pro-inflammatory cytokines and chemokines compared to controls. Thus, this study has provided targets that could potentially be used for devising treatment protocols for the management of PSC [86].

As for many other diseases, genome-wide association studies (GWASs) represent a promising approach not only for dissecting the pathophysiology of PSC but also for the identification of possible therapeutic targets. To date, a total of 16 genes have been associated with increased risk of PSC [87]. Among others, the single nucleotide polymorphism (SNP) located at chromosomal region *2q35* has attracted the interest of researchers. This SNP is in close proximity to the TGR5 gene and has been associated both with PSC and ulcerative colitis [88]. TGR5 is the first G-protein coupled receptor for bile acids with important roles in energy expenditure and basal metabolism [89]. Interestingly, five mutations in the TGR5 gene have been shown to reduce or abolish the function of the protein [90]. The activation of TGR5 in cholangiocytes is thought to stimulate bicarbonate secretion [91], possibly contributing to the protection of the biliary epithelium via the biliary bicarbonate umbrella [25].

Primary Biliary Cirrhosis—Primary Biliary Cirrhosis (PBC) is an immune-mediated pathology of the biliary tree characterized by the generation of anti-mitochondrial antibodies (AMAs) directed against the pyruvate dehydrogenase complex (PDC-E2) [92]. Recent studies have shown that TLR9 and CD86 expression is enhanced in B cells of PBC patients [93, 94]. Profiling studies for cytokines and chemokines have shown that these molecules are important in the pathogenesis of PBC [95]. Further, there is often involvement of autoreactive CD4+ and CD8+ T cells in PBC. A number of animal models for PBC have been proposed. Despite the fact that none of them is able to perfectly recapitulate the complex interactions of the human disease, they have proved to be valuable tools in order to study PBC alterations and explore possible therapeutic targets. Briefly, the NOD.c3c4 mouse was the first animal model to develop PBC like characteristics [96]. The second mouse model, which is most frequently used for studying PBC, owing to the similarity of human PBC, is the one expressing the dominant negative form of TGFβ receptor II (dnTGF βRII). This particular mouse model is characterized by higher serum level of TNF-α, IFNY and IL-6 when compared to control animals [97]. Similarly, elevated serum cytokines, lymphocyte infiltration around portal tracts and cholangiocyte injury are noted in a third rodent model of PBC. This is called the IL-2Ra knockout mice model[98]. In genetically susceptible individuals, environmental factors may trigger an immune-mediated injury of cholangiocytes. The immunological events then occur in a step-by-step manner, starting from antigen presentation, T-cell differentiation, proliferation and recruitment, finally resulting in an effector cell response and production of autoantibodies[99] . In this context, a number of different signaling pathways have been implicated in this disease development or progression, and as such, any of these steps could theoretically be targeted for treatment of PBC. Since many pathophysiological events of the human disease remain obscure and may differ from animal models, caution should be implemented while evaluating the experimental effects of the manipulation of signaling pathways. Nonetheless, antibody

mediated therapy, targeted inhibition of cellular pathways relevant to immune regulation and cell therapy methods directed towards reprogramming the immunomodulatory axis remain an intriguing opportunity to treat PBC patients [100–102].

Biliary Atresia—Biliary atresia (BA) is a disease caused by obstructive cholangiopathy resulting from inflammation and fibrosis of extra-hepatic bile ducts. Inflammatory reactions triggered by viral infection have been proposed as the possible cause of BA by several population studies as well as in murine models [103]. Population studies have proved the presence of human papillomavirus, cytomegalovirus and reovirus in livers of BA patients [104–106]. Evidences from studies in rhesus-rotavirus infected murine model of BA as well as from fixed liver tissues from BA patients have shown that there are structural as well as pathological changes in the extrahepatic cholangiocytes only. It was observed that primary cilia were selectively lost from the extrahepatic and not intrahepatic cholangiocytes post rotavirus infection in experimental mice [107, 108]. Chemokine expression levels were also increased in cholangiocytes isolated from rotavirus-infected mice as well as in virus-infected cholangiocytes in culture [109]. In this study, quantitative and qualitative assessments of several chemokines were performed. MIP-2 and monocyte chemotactic protein 1 were up-regulated after rotavirus infection when compared to normal both *in vivo* and *in vitro*.

Cholangiocyte proliferation and subsequent enlargement of extrahepatic bile ducts in BA has been linked to over expression of IL-33 and activation of Th-2 cells. Serum levels of IL-33 are elevated in biliary atresia patients, and in livers and bile ducts of experimental mice.. Moreover, treatment of normal wild-type mice with IL-33 promoted cholangiocyte proliferation and cell growth culminating in significantly enlarged extra-hepatic bile ducts. In the same study it was shown that bile ducts genetically primed to cholangiocarcinoma (by constitutive activation of Akt-Yap pathway) responded to administration of IL-33 via development of advanced tumors with intrahepatic metastases compared to controls. Such data suggests that activation of IL-33 pathway may help biliary repair, and disruption of the same may halt carcinogenesis [110]. Other studies indicate the involvement of factors such as granzymes, which are secreted by hepatic NK and CD8 T cells, and injure cholangiocytes in short-term culture. Consistent with *in vitro* data, it has been noted that in infants with BA there are increased hepatic mRNA expression of granzymes A and B [111]. Thus these studies offer multiple targets that could be manipulated to manage cholangiocyte proliferation accompanying liver conditions such as BA. Recently, the role of microRNAs in liver diseases is being increasingly recognized [112]. For example, miR-21 was found up regulated during early stages of liver regeneration by targeting pellino 1 antibody [113]. Let-7 family members, miR-127, miR-26a, miR-34a and miR-23b were all found dysregulated during liver regeneration. Similarly, microRNA expression profiles have been found altered during treatment of mice with rhesus rotavirus, in a time dependent fashion, in the extrahepatic bile ducts from the experimental animals. For instance, changes in expression pattern of miR-30b/c, −133a/b, −195, −200a, −365 have been proposed in the development of BA [114]. Hence, these reports could provide alternative treatment protocols for life threatening conditions such as biliary atresia in small children.

CLINICAL IMPLICATIONS OF RECENT ADVANCES IN BILIARY RESEARCH

Despite the enormous progresses of recent years, the pathophysiology of cholangiopathies is far from being completely understood, with severe consequences on the development of effective new treatments.

Important signals come from the clinical practice. To date, orthotopic liver transplantation (OLT) remains the only curative treatment of cholestatic liver diseases (these represent as much as 20% of OLT indications in adults) [115]. Moreover, symptoms such as fatigue and pruritus are often scarcely alleviated by standard medical approaches [116,117].

Ursodeoxycholic acid (UDCA) remains the only approved drug for the treatment of fibrosing cholangiopathies. UDCA exert its effects on multiple levels, from the protection of cholangiocytes against toxic bile acid, to the stimulation of choleresis through post-transcriptional effects on hepatocellular and cholangiocyte transporters [118, 119].

The administration of UDCA in a dose of 13–15 mg/kg/day has well-established favorable effects on long-term survival of PBC patients [120, 121]. Transplant-free survival in early-stage PBC patients treated with UDCA has been shown to be similar to healthy controls, matched for age and gender [122, 123]. However, not all PBC patients respond to UDCA administration. A good biochemical response was achieved only in 61% of PBC patients treated with UDCA, as defined by the “Paris criteria” that strongly correlates with transplant-free survival at 10 years [124].

If the administration of UDCA is universally recognized as the standard treatment for PBC, definitive evidence to recommend its use in PSC is still lacking. Moreover, high doses of 28–30 mg/kg/day of UDCA in PSC patients have been associated with increased risk of liver transplantation and development of esophageal varices [125]. As a matter of fact, the latest available European guidelines do not propose any specific recommendation for UDCA use in PSC [126].

In this scenario, the development of alternative therapies for cholestatic liver diseases is utterly needed and intense research is ongoing. Promising results have recently emerged from the study of two bile acids derivatives, obeticholic acid and norursodeoxycholic acid.

Obeticholic acid (OCA), also known as INT-747, is a semisynthetic analogue of chenodeoxycholic acid (CDCA) that possesses a strong farnesoid X receptor (FXR) affinity [127]. The role of FXR in bile acid homeostasis has clearly emerged in recent years. Endogenous bile acids bind to FXR which, in turn, is able to repress or induce the expression of various genes involved in their synthesis and secretion, such as CYP7A1, BSEP and NTCP [115]. CDCA is the most potent endogenous FXR ligand (with a 100-fold less affinity than OCA), while UDCA has no affinity. Interestingly, $Fxr^{-/-}$ mice have elevated serum bile acids levels and the infusion of OCA in rats is able to stimulate bile flow and protect against lithocholic acid-induced liver damage [127, 128]. Given these premises, the efficacy and safety of OCA has been recently tested in 165 PBC patients who failed to achieve a good biochemical response to UDCA alone. The results of the study demonstrated that the administration of 10, 25 or 50 mg of OCA was able to significantly reduced levels of

ALP, gamma-GT, and alanine aminotransferase, compared to placebo. However, a significant increase in pruritus was also registered. The severity of itch was worse than placebo with all 3 OCA dosages used, while the incidence of pruritus was higher only in the 2 higher-dosing groups [129]. Phase 2 and phase 3 studies involving OCA are currently underway, with extremely promising preliminary results. Indeed, the administration of 5 or 10 mg of OCA has been shown to be superior to placebo in determining the improvement of biochemical parameters correlated with clinical outcome in patients with inadequate response to UDCA [130].

Norursodeoxycholic acid (*nor*UDCA) is a C23 homologue of UDCA, with one fewer methylene group in the side chain of the molecule. The biology of *nor*UDCA has peculiar characteristics; in fact, this bile acid derivative is usually not conjugated with taurine or glycine. It is secreted into the bile canaliculi and reabsorbed by cholangiocytes, from where it returns to the liver. The resulting cholehepatic shunting leads to a bicarbonate rich-choleresis, which is thought to protect cholangiocytes against the toxicity of bile acids [131, 132]. Fickert et al. have tested the possible therapeutic effect of *nor*UDCA in Mdr2$^{-/-}$ mice, a model for sclerosing cholangitis [84]. The authors demonstrated that the administration of *nor*UDCA was able to ameliorate liver tests and liver histology in Mdr2$^{-/-}$ mice, in contrast to UDCA that had detrimental effects [85]. A recent study of the same group confirmed that *nor*UDCA was able to improve liver injury in the selective bile duct ligation model in mice, where UDCA administration resulted again significantly more toxic than *nor*UDCA [133]. Based on these results, a phase 2 trial is currently recruiting patients to test the safety and efficacy of *nor*UDCA in PSC patients.

Monoclonal antibodies have also attracted the interest of researches as a possible therapeutic tool to treat cholangiopathies. Given the encouraging results obtained with anti-CD20 antibodies in the dnTGF βRII mice [134], the monoclonal antibody rituximab has been tested in a phase 1 trial in 6 PBC patients with incomplete response to UDCA. Rituximab treatment proved to be safe in PBC patient and was able to transiently reduce serum levels of total IgG, IgM, and IgA and AMAs [135]. Based on the results of recent GWAS showing a genetic association between variants of the IL-2 and IL-23 pathways and PBC [136, 137], a phase 2 clinical trial is currently underway evaluating the safety and efficacy of ustekinumab, an anti-p40 monoclonal antibody.

The safety and efficacy of two different monoclonal antibodies (BTT1023 and simtuzumab) are being investigated in PSC patients. This study is currently in phase 2 clinical trials. BTT1023 is a human monoclonal antibody targeting the vascular adhesion protein-1 (VAP-1), a molecule that has been shown to stimulate the recruitment of effector lymphocytes to the liver through the upregulation of the endothelial cell adhesion molecule MadCAM-1 [138, 139]. Simtuzumab is directed against the lysyl oxidase-like protein 2 (LOXL2), an enzyme that favors the cross-linking of collagen and elastin fibers [140]. The results of these studies will hopefully lay the basis for possible new and effective treatments for biliary diseases.

CONCLUSION/FUTURE PERSPECTIVE

The knowledge of the mechanisms regulating biliary cell response to injury has enormously grown in the last few decades [141]. The studies of recent years have clarified that cholangiocytes are not the passive targets of biliary diseases. Indeed, reactive cholangiocytes undergo a series of profound modifications and acquire a neuroendocrine-like phenotype that allows cells to regulate the complex molecular interactions that occur in the diseased liver [4, 23]. As discussed in the review, a number of molecular pathways have been shown to deeply influence cholangiocyte response to injury. Moreover, animal models have proved an invaluable tool in order to dissect the pathophysiological changes occurring in the biliary tree in response to injury, providing important clues on the complex interactions occurring *in vivo*. As a result of these continuous efforts, new potential treatments for PBC and PSC have been developed and are currently investigated in clinical trial with promising results. However, the etiology of many cholangiopathies is still obscure, and much work remains to be done to translate the large amount of data collected on disease pathogenesis into effective medical treatments capable of influencing the natural history of biliary diseases.

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Abbreviations

AE2	anion exchanger 2
BA	biliary atresia
BDL	bile duct ligation
SR	secretin receptor
cAMP	3',5'-cyclic monophosphate
CFTR	cystic fibrosis transmembrane conductance regulator
FSH	follicle stimulating hormone
GnRH	gonadotropin-releasing hormone
IP_3	d-myo-inositol 1,4,5-triphosphate
MT1	melatonin Receptor 1
PBC	primary biliary cirrhosis
PBP	peribiliary plexus

PKA	protein kinase A
PSC	primary sclerosing cholangitis
TMEM16A	transmembrane member 16A
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
UDCA	ursodeoxycholic acid

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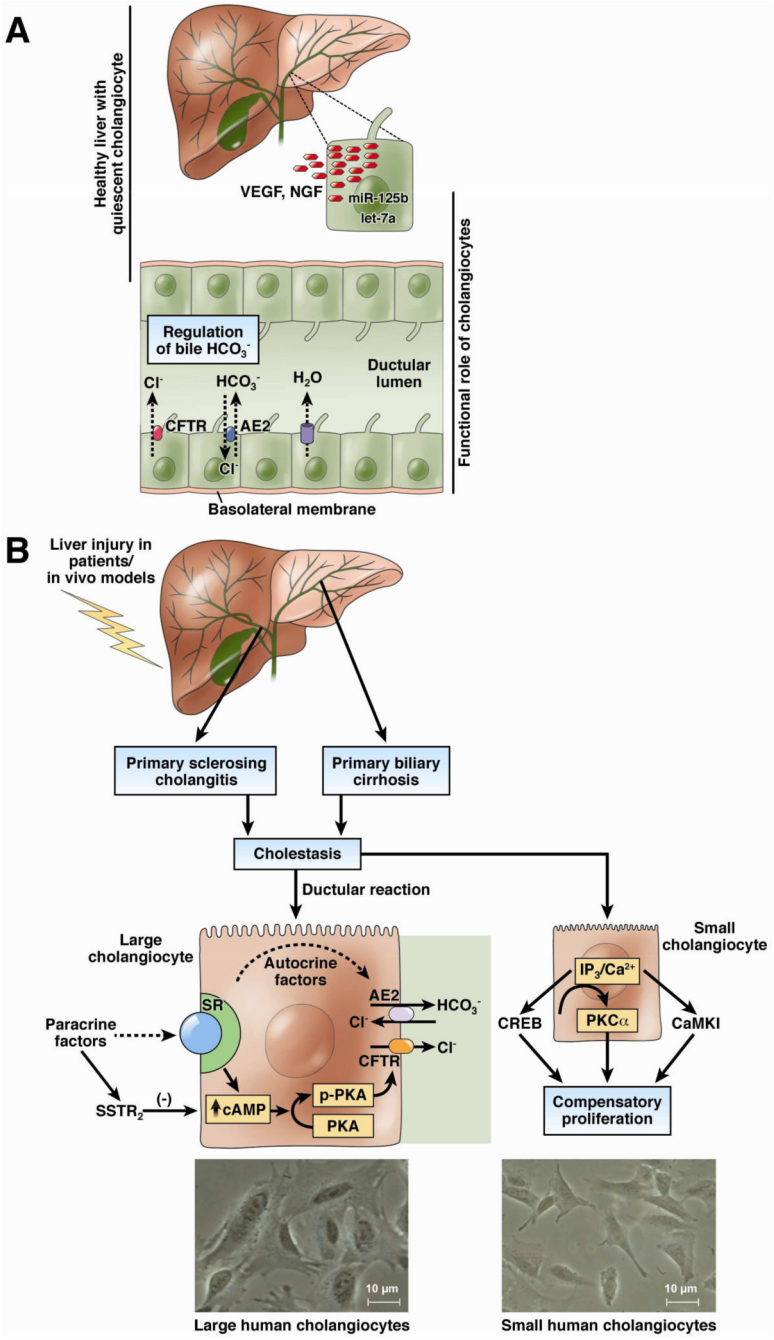

Figure 1.

Overview of cholangiocyte role in biliary functions. [A] Intrahepatic bile ducts are lined by both large and small cholangiocytes. Under physiological conditions, cholangiocytes (large cholangiocytes preferentially) modify ductal bile by a sequence of secretory and absorptive processes mediated by membrane exchangers. This modification mainly leads to the formation of bicarbonate rich bile. Cholangiocytes also secrete VEGF and NGF that are regulated by microRNA 125b and let-7a respectively. [B] The formation of bicarbonate rich bile is enhanced by stimulation with secretin and cAMP, which increase in response to liver

insult. Liver behaves as a neuroendocrine compartment in response to injury and starts to respond to hormones and peptides in an autocrine as well as paracrine manner. Liver injury is subsequently followed by large cholangiocyte proliferation under the influence of these factors (neurotransmitters, gastrointestinal peptides, steroids). Large but not small bile ducts express SR and somatostatin receptor 2 ($SSTR_2$) and respond to secretin and somatostatin. Biliary hyperplasia results in cholestasis, which further results in human biliary disorders such as PSC and PBC. Occasionally, in response to specific events injury or drug administration, small cholangiocytes proliferate by an IP_3 mediated signaling pathway, often to compensate for the lack of large cholangiocyte proliferation and thus maintain the biliary mass. [Bottom] Isolation of small (right), approximately 9 μm diameter and large (left), approximately 13 μm diameter cholangiocytes from human SV-40 transformed cholangiocytes (H69 cells). Small and large human cholangiocytes were purified by counterflow elutriation followed by immunoaffinity purification. Original magn., × 800.

Table 1

Summarizing the main neuroendocrine factors involved in cholangiocyte response to injury

Molecule	Functions	Ref.
Secretin	• Stimulates the proliferation of both normal and BDL large cholangiocytes	38
	• Produced by cholangiocytes and S cell, induces the upregulation of VEGF and NGF via downregulation of microRNA 125b and let7a	37, 39
VEGF	• As a component of PDX-1-induced neuroendocrine-like trans-differentiation of cholangiocytes, stimulates biliary proliferation via an autocrine mechanism	29, 43
	• Sustains cholangiocyte proliferation and PBP reactive expansion after BDL	28
	• Stimulates biliary cystogenesis in cholangiocytes of the polycystic kidney rat model	40
	• Participates to arterial vasculogenesis during human liver embryogenesis	44
FSH	• Stimulates cholangiocyte proliferation	45
Histamine	• Stimulates small cholangiocyte proliferation via the activation of the histamine receptor H1	22, 47
	• Stimulates large cholangiocyte proliferation via the activation of the histamine receptor H2	47
	• Reduces cholangiocyte proliferation via the activation of the histamine receptor H3	57
	• Increases the growth of cholangiocarcinoma cells and the synthesis of VEGF	59
Estrogens	• Stimulate cholangiocyte proliferation and prevent apoptosis in BDL rats	49, 50
NGF	• Stimulates cholangiocyte proliferation (additive effect in combination with estrogens)	48
Serotonin	• Inhibits cholangiocyte proliferation and secretory activities	52, 53
Melatonin	• Produced by both pineal gland and cholangiocytes, inhibits biliary proliferation and secretory functions of BDL rats	24, 54, 55
	• Down regulates VEGF synthesis by cholangiocytes	56