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Development of the Intrahepatic and Extrahepatic Biliary Tract: A Framework for Understanding Congenital Diseases

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bile duct, liver development, biliary disease, differentiation, morphogenesis, gene network

Abstract

The involvement of the biliary tract in the pathophysiology of liver diseases and the increased attention paid to bile ducts in the bioconstruction of liver tissue for regenerative therapy have fueled intense research into the fundamental mechanisms of biliary development. Here, I review the molecular, cellular and tissular mechanisms driving differentiation and morphogenesis of the intrahepatic and extrahepatic bile ducts. This review focuses on the dynamics of the transcriptional and signaling modules that promote biliary development in human and mouse liver and discusses studies in which the use of zebrafish uncovered unexplored processes in mammalian biliary development. The review concludes by providing a framework for interpreting the mechanisms that may help us understand the origin of congenital biliary diseases.

INTRODUCTION

Reorganization of the intrahepatic biliary tract is observed in a number of liver diseases that are characterized by chronic cholestasis, inflammation, infection, or toxic injury (1), raising questions about the mechanisms of normal bile duct development and of biliary remodeling in disease. In parallel, in vitro production of biliary epithelial cells (cholangiocytes) and construction of bile ducts for regenerative therapy are often based on the implementation of developmental mechanisms (2–5), thereby prompting the need for understanding the fundamentals of intrahepatic and extrahepatic biliary development. Biliary development is intimately associated with the differentiation of other cell types in the liver and with the morphogenesis of the hepatocyte cords, vasculature, and nervous system. An extensive discussion of these issues can be found in other reviews and papers (6–10). Here, the focus is on biliary development in mammals, namely humans and mice. Despite zebrafish, *Danio rerio*, having a tubular liver architecture as opposed to the lobular architecture of mammals, the molecular actors and mechanisms are well conserved between mammals and fish (11, 12). Therefore, also described are the biliary tract developmental processes uncovered using zebrafish that have remained less explored in mammalian models.

The intrahepatic and extrahepatic biliary systems develop separately, and how they connect to each other is not yet clear. Consequently, the development of the intrahepatic and extrahepatic biliary trees is discussed separately, and for both systems, an attempt is made to summarize the transcriptional and signaling modules promoting differentiation and morphogenesis. These modules are highly dynamic, as they evolve in parallel with the maturation of the cholangiocytes and with the patterning and morphogenesis of the bile ducts. Acquisition of apicobasal polarity and, to a lesser extent, of planar cell polarity cannot be dissociated from morphogenesis. Therefore, also discussed is how the polarization of cholangiocytes impacts the formation of biliary lumina and duct morphogenesis. Further, the bile duct epithelium, both intrahepatic and extrahepatic, is directly connected to the peribiliary glands, which are considered to have regenerative potential and to harbor cells with progenitor properties (13–15). Consequently, the development of the peribiliary glands is briefly summarized. Finally, although a comprehensive review of congenital diseases of the bile ducts is beyond the scope of this article, the fundamental concepts of normal biliary development may influence how we understand the pathophysiology of such diseases. Therefore, a framework is provided for analyzing the potential causative mechanisms.

MECHANISMS OF NORMAL INTRAHEPATIC BILE DUCT DEVELOPMENT

Intrahepatic Cholangiocytes Originate from Bipotent Hepatoblasts

In a developing liver, the hepatocytes and cholangiocytes are derived from the bipotent hepatoblasts, which are hepatic precursor cells originating from the endoderm. This long-held view rests on a body of evidence obtained from gene expression studies, cell and liver explant cultures, and transgenic mouse phenotyping (6, 9, 16). Recent fate-tracing experiments in mouse embryos provided additional information that supports the lineage relationship from endoderm to hepatic epithelial cells. Individual cells in the endoderm were genetically labeled at embryonic day (E) 7.75–8.5, that is, prior to the onset of liver organogenesis, and their clonal progeny were analyzed in the liver at later stages. The results demonstrated that the endoderm contains multipotent cells, some of which evolve into hepatoblasts and subsequently differentiate into hepatocytes and cholangiocytes (17).

The concept of this simple lineage map is well established, but the exact stage at which the hepatoblasts become committed to a cholangiocyte fate was only recently deduced from single-cell

RNA sequencing experiments. Bile duct morphogenesis is initiated at around day 45 of gestation in humans or E14.5–15.5 in mice. It is initially characterized by the development of the ductal plate, which on two-dimensional (2D) sections of embryonic tissue resembles a discontinuous sleeve of cholangiocytes around the branches of the portal vein (18, 19). However, the earliest signs of biliary morphogenesis do not coincide well with the onset of biliary differentiation. Indeed, molecular signatures of early cholangiocyte specification are detectable within a subset of hepatoblasts prior to ductal plate formation. Transcriptomic analyses performed on single mouse hepatoblasts revealed that a few individual cells start expressing a set of biliary-specific genes already at E11.5–12.5 (20, 21). Further, biliary-specific protein expression detected by immunostaining on sections not only supported these transcriptomic data but also suggested that the earliest-developing cholangiocytes are located in the vicinity of the portal vein. Indeed, cells that express the transcription factor sex-determining region Y-box (Sox) 9, which is considered the earliest marker of cholangiocyte differentiation, are not evenly distributed throughout the parenchyma at E11.5, but instead are predominantly found near the veins (19). Later, at E14.5–15.5, when sufficient numbers of cholangiocytes have differentiated from hepatoblasts, a single-layered epithelium corresponding to the ductal plate is formed. Interestingly, mice that are knockout for the T-box transcription factor Tbx3 show premature upregulation at E9.5 of hepatocyte nuclear factor (Hnf) 6 (also called Onecut1) and Hnf1β (vHnf1 or Tcf2), two transcription factors that are known to drive cholangiocyte differentiation (22–24). This suggests not only that cell-intrinsic factors such as Tbx3 prevent cholangiocyte specification but also that early hepatoblasts are exposed to signals that may promote their premature differentiation into cholangiocytes under certain nonphysiological conditions.

Hepatoblasts give rise to hepatocytes or cholangiocytes and are considered bipotent. However, it is not clear whether single hepatoblasts are unipotent, giving rise to either hepatocytes or cholangiocytes, or whether instead hepatoblasts are truly bipotent, with each individual cell being capable of generating hepatocytes and cholangiocytes. This question remains unresolved, except for a population of leucine-rich repeat-containing G protein–coupled receptor (Lgr) 5–expressing hepatoblasts that was proven to be bipotent in lineage-tracing experiments (21). The issue is even more complicated by the observation that a subset of cholangiocytes belonging to the ductal plate may reorient toward a hepatocyte fate. Indeed, lineage-tracing experiments in which mouse cholangiocytes were genetically labeled at E15.5, taken together with morphological data, provided evidence that the ductal plate cells give rise to the cholangiocytes lining the bile ducts, as expected, but can also revert to a hepatocyte phenotype and generate a subset of periportal hepatocytes (25, 26). The latter would then be produced following successive cell fate transitions, namely from hepatoblast to ductal plate cholangiocyte, and from ductal plate cholangiocyte to periportal hepatocyte.

Interacting Signaling Modules Promote Cholangiocyte Specification

A number of cell-intrinsic and cell-extrinsic cues have been identified that promote differentiation of hepatoblasts toward either cholangiocytes or hepatocytes. There are several modes of representing such regulations, and recently we summarized the organization of gene regulatory network motifs at distinct stages of liver development (27). Here, hepatoblast fate decisions are discussed by identifying interacting signaling modules, each constituted by gene networks (**Figure 1**).

Given that cholangiocytes differentiate and organize near the branches of the portal vein, it is not surprising that several extrinsic signals emanating from the venous structures have been identified. The importance of transforming growth factor β (TGFβ) as a stimulator of cholangiocyte specification was uncovered in studies revealing that high expression of TGFβ ligands in

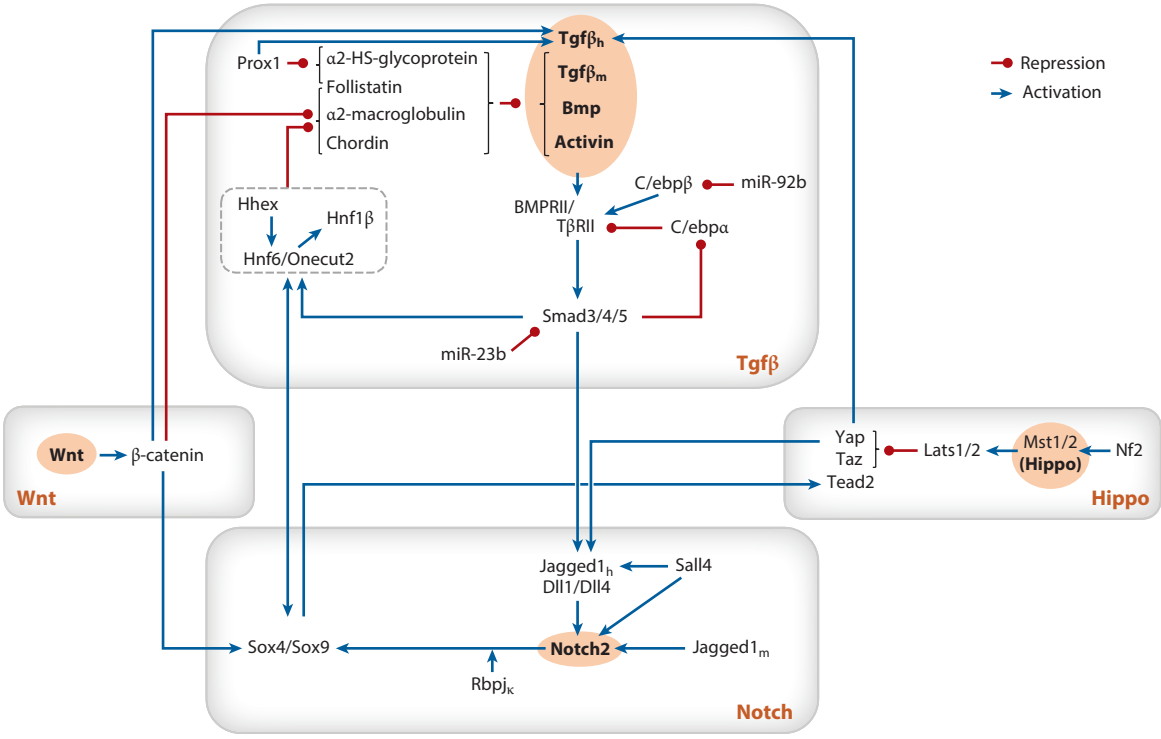

Figure 1

Interacting signaling modules control cholangiocyte specification in mammalian liver. The modules cooperate to promote the differentiation of hepatoblasts into the cholangiocyte lineage. Information about signaling by fibroblast growth factor and the extracellular matrix is not yet sufficiently consistent to be integrated into the scheme. The subscripts m and h refer to expression in the mesenchyme or differentiating hepatoblast. The dashed rectangle circumscribes a transcriptional network that collectively controls Follistatin, α2-macroglobulin, and Chordin.

the periportal mesenchyme creates a gradient response in the parenchyma. This response peaks in the hepatoblasts located along the periportal mesenchyme, and it induces their differentiation into ductal plate cells (19, 28). TGFβ was shown to bind to the ductal plate in immunostaining experiments, suggesting that the effects of the pathway on cholangiocyte differentiation are direct; yet the direct TGFβ target genes in the cholangiocytes remain unidentified. TGFβ also binds to the periportal mesenchyme cells, which may be stimulated to differentiate into portal myofibroblasts expressing Jagged1 (29). Within the liver parenchyma, TGFβ induces a signaling response of lower intensity than in the ductal plate, as shown by the activity of a TGFβ-responsive reporter transgene (28). Perturbation of the response intensity causes aberrant fate determination of the parenchymal hepatoblasts, which then fail to properly differentiate toward either the cholangiocyte or hepatocyte lineage and, instead, adopt a hybrid phenotype characterized by the coexpression of biliary and hepatocyte markers. This was illustrated in mice that were knockout for *Hnf6*, *Onecut2*, hematopoietically expressed homeobox (*Hhex*), or prospero homeobox 1 (*Prox1*) and revealed the existence of gene regulators that balance the TGFβ-dependent differentiation of hepatoblasts toward either cholangiocytes of hepatocytes (28, 30, 31). In that context, the expression ratio of two paralogs of the CCAAT/enhancer binding protein (C/ebp), namely C/ebpα and C/ebpβ, plays a critical role: C/ebpα stimulates expression of hepatocyte genes and simultaneously represses the TGFβ type II receptor (TβRII), thereby reducing the response to TGFβ

and adjusting it to the needs of hepatocyte differentiation. In contrast, C/ebpβ stimulates TβRII expression and so favors cholangiocyte differentiation (32–34). The C/ebpα:C/ebpβ ratio is kept within the correct limits by two additional inputs: First, the microRNA miR-92b represses C/ebpβ expression (35), and, second, TGFβ represses C/ebpα, thereby creating a double negative, that is, a positive feedback loop bolstering TGFβ signaling and cholangiocyte differentiation (**Figure 1**). Moreover, microRNAs of the miR-23 cluster contribute to the hepatoblast fate decision by favoring hepatocyte differentiation at the expense of cholangiocyte differentiation through repression of the bone morphogenetic protein (BMP) and TGFβ signaling mediators Smad3, 4, and 5 (36–38). Finally, when considering TGFβ ligands, it is noteworthy that TGFβ2 and TGFβ3 are most prominently expressed in the periportal mesenchyme, but they are also found in hepatoblasts, where their expression is stimulated by Prox1 and where they likely promote autocrine effects. In addition, expression of BMP and Activin contributes to the activation of TGFβ signaling, the effects of which are proposed to be further stimulated by Hnf6, Onecut2, and Prox1 via dampening of the BMP, Activin, and TGFβ antagonists Chordin, Follistatin, and α2-HS-glycoprotein (28, 30, 36) (**Figure 1**).

The involvement of Notch signaling in biliary development had long been suspected when it was found that Alagille syndrome, a genetic disease associated with biliary paucity, resulted from mutations in *JAG1* (JAGGED1) or *NOTCH2*. Several studies in mice concluded that *Notch2* expression in hepatoblasts is essential for their differentiation into cholangiocytes, and *Foxa3-Cre*-mediated inactivation of recombination signal binding protein for immunoglobulin kappa J region ($Rbpj_\kappa$), the DNA binding partner of the Notch intracellular domain, impaired ductal plate formation (39–47). Several arguments support Jagged1 as a key ligand of Notch2 in this process: First, Jagged1 is initially expressed in the periportal mesenchyme and, at later stages, also in the ductal plate cholangiocytes; second, double *Jag1–Notch2* heterozygotes develop a reduced number of cholangiocytes; third, coculture of human pluripotent stem cell–derived hepatoblasts with stromal cells expressing Jagged1 promotes biliary differentiation; and fourth, *Jag1* inactivation in the periportal mesenchyme prevents bile duct formation (39, 40, 48–50). It would have been overly simplistic if stimulation of cholangiocyte specification by Notch signaling was initiated only by Jagged1–Notch2 interactions. Not only is Jagged1 activity regulated by the glycosyltransferase Poglut1 (51), but in vitro studies using hepatoblast lines suggest that the ligands delta-like (Dll)-1 and -4 also induce Notch signaling and contribute to cholangiocyte specification (52). In the same set of experiments, TGFβ was shown to induce Jagged1 and delta-like ligands in cholangiocytes, which resulted in downregulation of hepatocyte genes. This not only suggested that autocrine Notch signaling takes part in the maintenance of the cholangiocyte phenotype but also revealed important cross talk between the Notch and TGFβ pathways. Such cross talk is not restricted to TGFβ-induced Notch ligand production, and it also occurs downstream, since it was shown that *Sox9*, a direct target of Notch (see the next paragraph), and Hnf6, a regulator of TGFβ signaling, reciprocally stimulate each other's expression (53, 54) (**Figure 1**).

Downstream targets of Notch signaling during cholangiocyte differentiation include Hairy and enhancer of split 1 (*Hes1*), yet there is some debate about its function in intrahepatic biliary development. Its expression is induced by Notch signaling, but it is not required for ductal plate formation. Indeed, constitutive $Hes1^{-/-}$ knockout mice develop a ductal plate, but it fails to remodel and to form tubular structures (48). Moreover, in a background of *Notch2* overexpression, *Hes1* is dispensable for Notch-induced biliary overgrowth (45). Strong evidence for *Sox9* being directly targeted by the Notch pathway was obtained by chromatin immunoprecipitation using anti-$Rbpj_\kappa$ antibodies (44). However, despite Sox9 being among the earliest markers of biliary differentiation, no data support the notion that Sox9 is required for cholangiocyte specification. Liver-specific knockout of Sox9 is indeed compatible with ductal plate formation, and the lack of

Sox9 only mildly impacts biliary development by delaying maturation of the ducts. However, the combined inactivation of *Sox4* and *Sox9*, which exert partly redundant functions, strongly affects differentiation of cholangiocytes, which then fail to repress the hepatoblast marker Hnf4 and to induce biliary markers such as Hnf6 and Hnf1β (54).

The most recently identified signaling cascade driving cholangiocyte specification is the Hippo/Yap pathway. Its role was initially uncovered in studies showing that Yes associated protein (Yap) is required for ductal plate formation and that Yap activation in neurofibromin 2 (*Nf2;* also called *Merlin*) knockout livers stimulates biliary development (55). The mode of action of the Hippo/Yap pathway was subsequently investigated and revealed upstream regulators and downstream effectors of Yap, as well as interactions with the other signaling pathways driving biliary development. The large tumor suppressor homologs (Lats)-1 and -2 kinases were shown to critically repress Yap activity, whose expression at the protein level in normal liver is detected in hepatoblasts and cholangiocytes, but becomes downregulated in maturing hepatocytes. Lee and coworkers (56) further found that activation of Yap and its paralog transcriptional coactivator with PDZ-binding motif (Taz; also called WWTR1) stimulates expression of biliary genes in parallel with activation of TGFβ target genes, including *Tgfβ2*, which then likely exerts autocrine effects (56). There are also several arguments supporting the idea that some of the effects of Yap depend on Notch signaling, yet how the Hippo and Notch pathways interact in this context is not yet clear (57, 58). However, Yap and Taz activate transcription only when bound to DNA binding proteins, such as the TEA domain (Tead) transcription factors, and it is noteworthy that Tead2 expression in the liver is cholangiocyte specific and requires functional *Sox4*. Therefore, we speculate that at least some of the Notch-dependent effects of Yap/Taz rely on the function of Sox factors (54) (**Figure 1**).

Wnt signaling pervades all stages of liver development (59). However, the study of this pathway is inherently complex due to redundant effects of the multiple Wnt ligands and receptors expressed in liver and the activation of downstream canonical and noncanonical cascades (60). Following initial work with cultured explants of embryonic livers, which showed that Wnt3a and β-catenin stimulate biliary specification (61, 62), it was further demonstrated using transgenic mice that β-catenin activation indeed promotes the hepatoblast-to-cholangiocyte transition (63). However, β-catenin depletion did not affect cholangiocyte specification (64), suggesting the existence of compensatory molecules, with γ-catenin being a good candidate (65). Genes directly targeted by β-catenin during cholangiocyte specification have not been identified. However, there is evidence that β-catenin stimulates TGFβ signaling: β-catenin activation by loss of adenomatous polyposis coli (Apc) is associated with increased levels of TGFβ2 expression (63), while β-catenin inactivation causes reduced expression of the TGFβ inhibitor α2-macroglobulin (66). Moreover, β-catenin-stimulated upregulation of Sox4 and of extracellular matrix components, which are inducers of cholangiocyte specification, further connects the Wnt/β-catenin pathway to other biliary-promoting pathways (63). Interestingly, in zebrafish, Wnt signaling functions as a non-cell autonomous driver of cholangiocyte development in which signaling in hepatocytes induces expression of Jagged ligands that promote Notch activation in cholangiocytes (67). Finally, the abovementioned complexity of Wnt signaling is well illustrated by the observation that Wnt5 represses cholangiocyte specification through activation of noncanonical calcium/calmodulin-dependent kinase II (68).

There is limited information about the role of fibroblast growth factor (FGF) and extracellular matrix–induced signaling in cholangiocyte specification. In vitro data using chicken hepatic progenitor cells indicate that basic FGF can induce the expression of biliary genes. However, in vivo, the expression of constitutively active FGF receptor 3 did not convincingly stimulate cholangiocyte specification, leaving open the question about the physiological role of FGF in

this process (69). The components of FGF and extracellular matrix were studied in parallel in chicken liver development, and the authors provided evidence that collagen type I, fibronectin, and laminins stimulate cholangiocyte gene expression (69). Whether this observation is relevant for mammalian liver is unknown, but Couvelard et al. (70) connect it with other findings showing that developing cholangiocytes in humans specifically express a set of laminin receptors, namely the α_2-, α_3-, α_6-, and β_4-integrin chains, and Tanimizu et al. connect it (71) with the observation that in vitro commitment of a human hepatoblast line to the cholangiocyte lineage is controlled by α_1-laminin/β_1-integrin signaling.

Morphogenesis of the Intrahepatic Bile Ducts in Mammalian Liver: A Tentative Three-Dimensional Model

In morphological terms, bile ducts are branched epithelial tubes, a priori much like similar structures found in other organs. Classical reviews categorized the main modes of tube and lumen formation as cord or cell hollowing, wrapping, budding, and cavitation, and cell intercalation (72, 73). Biliary tubulogenesis bears similarities to cord hollowing, yet its study has revealed unique features. Several specificities emerged from the analysis of mammalian bile duct development (18, 19, 25, 26, 74, 75) (**Figure 2**). First, bile ducts develop along the branches of the portal vein, starting at the hilum of the liver and progressing toward the periphery of the liver lobes. Second, the process is initiated by the formation of the ductal plate, which consists of a discontinuous and single-layered sheet of cholangiocytes lining the portal mesenchyme. The ductal plate becomes detectable in humans at approximately the fifth to seventh week of embryonic life and at approximately E13.5 in mice (19, 76). Third, the ductal plate gives rise to several lumina that initially are asymmetrically lined by cholangiocytes on the portal side and by hepatoblasts on the parenchymal side; these lumina delineate small cysts and tubes called primitive ductal structures. Fourth, when the cysts interconnect and the tubes elongate, the hepatoblasts lining the parenchymal side of the lumina differentiate into cholangiocytes. Fifth, maturation and finalization of duct morphogenesis involve remodeling of the tubes, with a decrease in the number of ducts and the differentiation of a subset of ductal plate cells into periportal hepatocytes. The model represented in **Figure 2** requires critical consideration as it represents an attempt to assemble disparate data obtained from

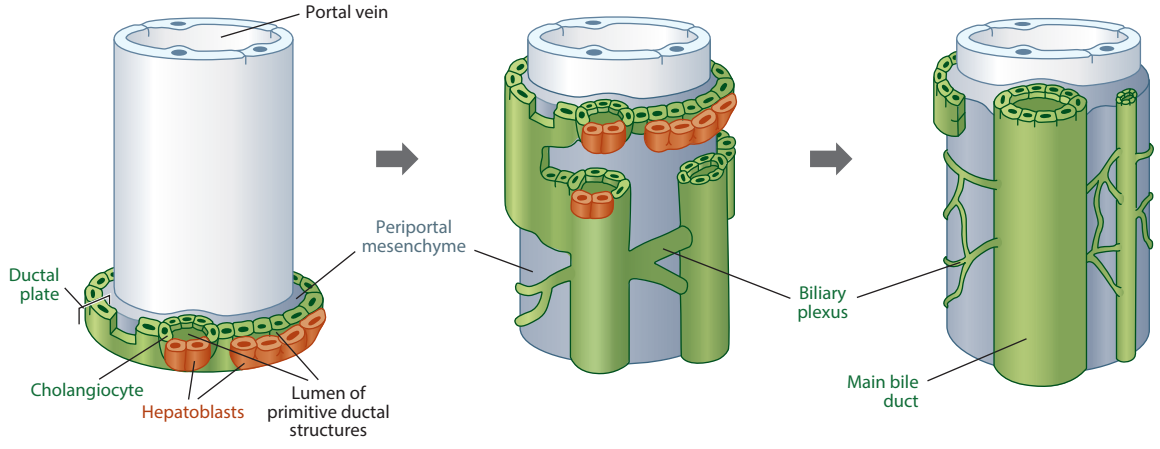

Figure 2

Morphogenesis of the intrahepatic bile ducts in mammalian liver. Adapted from Reference 7 with permission from Elsevier.

2D analyses of immunostained liver sections, computer-assisted 3D reconstructions from 2D sections, and 3D imaging of liver tissue that had been cleared after retrograde injection of ink into the common bile duct. Three-dimensional imaging, for example, using light-sheet microscopy, should provide more definitive insight into duct morphogenesis.

Dynamic Signaling Mechanisms Control Intrahepatic Biliary Morphogenesis

Little is known about the actual mode of biliary lumen formation. Data from in vitro cultures suggest that cells from the monolayered ductal plates depolarize, dedifferentiate, migrate out of the monolayer, and fold up to form a lumen (77). This process implies multiple cell state transitions: from hepatoblast to ductal plate cholangiocyte, then reversion of a subset of cholangiocytes to a hepatoblast state to form asymmetrical primitive ducts, and, again, redifferentiation into the cholangiocyte state in order to generate mature ducts entirely lined by cholangiocytes. In an alternative model, ductal plate cholangiocytes and hepatoblasts, which initially adhere to each other, become separated, thereby leaving a luminal space between the two cell types; later, the lumen expands and the ducts mature. This alternative model displays features of cord-hollowing morphogenesis and resembles lumen formation in the developing pancreas, where adjacent pancreatoblasts form apical poles that face each other and then separate to create lumina (78). It is also supported by data showing punctate expression of apical markers such as Na^+-H^+ exchanger regulatory factor 1 (Nherf1) and Mucin1 at the apical pole of cholangiocytes at the onset of lumen formation (26). The lumen then expands in parallel with recruitment of an increasing number of polarized cells to form the primitive ductal structures lined with cholangiocytes on the portal side and hepatoblasts on the parenchymal side. Importantly, the shaping of the ductal structures also requires apical constriction of the cells lining the lumen.

Several signaling mechanisms were identified as playing roles in biliary morphogenesis. TGFβ signaling, which is critical at the stage of cholangiocyte specification, is dynamically controlled at the morphogenic stage. Once hepatoblasts have differentiated into ductal plate cells, they need to shut off the expression of TβRII since prolonged expression of TβRII is associated with delayed maturation of cholangiocytes and abnormal duct morphogenesis. The mechanisms of this repression are not clearly understood, but there is evidence that it occurs via a TGFβ-induced negative feedback loop (19, 52).

Similar to the requirement for TGFβ signaling, Notch signaling is required at successive stages of bile duct development (79). Inactivation of *Jag1* in the mesenchyme or of Notch receptors and mediators in the hepatoblasts at the morphogenic stage has demonstrated that duct formation is controlled by Notch signaling (44, 49). Moreover, several studies pointed to dose–dependent effects of Notch signaling on tubulogenesis, revealing the existence of redundant Notch receptor functions and also of cooperation between Notch signaling and the transcription factor Hnf6/Hnf1β cascade (80, 81). Also, Notch and liver kinase B 1 (Lkb1; also called Stk11) signaling contribute to the maturation of biliary ducts, but how these pathways interact remains unclear (82). Which cells communicate via Notch signaling during duct formation is not fully clear either: *Jag1* is expressed both in the mesenchyme and cholangiocytes, raising the possibility that biliary differentiation of the hepatoblasts in the asymmetrical primitive duct structures depends on hepatoblast–mesenchyme or hepatoblast–cholangiocyte interactions, or both, mediated by Notch. The interactions between developing bile ducts and mesenchyme are intimate and bidirectional during duct formation as deficiencies originating in the biliary cells prevent normal development and expansion of the neighboring mesenchyme (54).

Unsurprisingly, the canonical Wnt pathway is active during biliary tubulogenesis. This conclusion results from studies in which β-catenin was either inactivated or stabilized in the ductal

plate cells. These studies showed that β-catenin was dispensable for morphogenesis, but that its activity must be contained within strict limits to avoid hyperplasia of the bile ducts (64). There is also evidence from in vitro experiments using a hepatoblast line that Wnt5a-induced noncanonical signaling reduces bile duct size (68), suggesting that harmonious biliary morphogenesis requires appropriately balanced canonical and noncanonical Wnt signaling.

The spatial distribution and function of extracellular matrix components during biliary morphogenesis have attracted much attention. An even distribution of α_1-laminin is detected at the basal side of all cholangiocytes at the onset of ductal plate development. This laminin chain is produced by the periportal mesenchyme, and its expression around the ducts becomes progressively undetectable as biliary development proceeds (71). Instead, α_5-laminin is produced by the developing cholangiocytes as laminin $\alpha_5\beta_2\gamma_1$. At the onset of ductal plate development, it is detected at the basal pole of the cholangiocytes, that is, facing the periportal mesenchyme; it is found at very low levels along the basal side of the cholangiocytes that belong to primitive ductal structures and at higher levels near the single-layered ductal plate cholangiocytes. Later, α_5-laminin is detected all around the ducts, indicating that the rise in its expression coincides with duct maturation. Further, gene inactivation studies in mice showed that α_5-laminin is dispensable for ductal plate formation, but that it is required for transformation of primitive ductal structures into mature ducts and for determination of luminal size (71). Together, these data combined with in vitro experiments addressing the functional role of α_1-laminin, indicate that α_1-laminin is necessary for cholangiocyte specification and that, subsequently, α_5-laminin controls tubulogenesis.

In line with the spatiotemporal expression of laminins, it is noteworthy that the laminin receptors, namely β_1- and β_4-integrins, also present distinct temporal expression patterns in the cholangiocytes, with β_1-integrin being expressed first and β_4-integrin afterward (71). In parallel, studies in human fetal liver revealed that Tenascin, a glycoprotein of the extracellular matrix, is abundant near cholangiocytes lining tubular structures but not near the other ductal plate cholangiocytes (83).

The spatial distribution of α_5-laminin and Tenascin reveals that there are distinct populations of cholangiocytes at E15.5 in mice, namely those involved in tubulogenesis that express low levels of α_5-laminin and are exposed to high levels of Tenascin, and those that belong to the single-layered portion of the ductal plate that, on the opposite end, produce more α_5-laminin and are not exposed to Tenascin (83). What drives the development of these distinct cholangiocyte populations remains elusive. We recently found that miR-337–3p has differential effects on the two types of cholangiocytes: Overexpression of miR-337–3p represses the expression of *Sox9*, *Hnf6*, and *Hnf1β* in the single-layered cholangiocytes but not in those forming primitive ductal structures (84).

The role of the extracellular matrix in biliary morphogenesis is likely linked with that of the Hippo/Yap pathway. Indeed activation of Yap and Taz induces the secretion of extracellular matrix proteins such as laminin and collagen IV, and these proteins are proposed to locally affect the stiffness of the matrix (56). In turn, we speculate that localized stiffness determinants contribute to the control of morphogenesis, similar to observations in the pancreas, where local stiffness cues determine the activity of Yap and the differentiation of β-cells (85).

The dynamic signaling that controls morphogenesis as described here does not provide much explanation about the mechanisms of biliary branching. Development of the portal vasculature constitutes a global template for the biliary architecture, but it is likely insufficient to determine branching because it occurs during the remodeling phase. Yet new insight has recently emerged in the field, with studies showing how the cyclin-dependent kinase 5/p21-activated kinase/LIM domain kinase/Cofilin cascade controls branching morphogenesis in zebrafish liver (86).

Polarity of Cholangiocytes as a Determinant of Intrahepatic Biliary Morphogenesis

The morphogenesis of ducts cannot be separated from the acquisition of apicobasal polarity by cholangiocytes. Initial work by Tanimizu and coworkers (87) pointed out the role of the extracellular matrix in the polarization of developing cholangiocytes in vitro. This was followed by descriptions of polarization and of formation of primary cilia in vivo (19, 53). Distinguishing the transcriptional control of differentiation from the control of polarization is difficult, given that the acquisition of polarity is an intrinsic feature of differentiation. Yet some studies have focused on the positioning of cellular components within developing cholangiocytes: Deficient expression of *Hnf6* and *Hnf1β* leads to the loss of apicobasal polarity and deficient ciliogenesis, and the transcription factor Grainyhead like 2 specifically controls the location of tight junction components in developing cholangiocytes (53, 88, 89). The importance of tight junctions in biliary development cannot be underestimated, in particular when considering the role of its constituent claudins. Not only are mutations in the human CLAUDIN1 (*CLDN1*) gene associated in humans with neonatal sclerosing cholangitis but also studies using zebrafish as a model revealed that the claudin 15-like b (*cldn15lb*) gene is essential for biliary remodeling and hepatocyte canaliculi formation (90). Tight junctions are also critical for the development of hepatocyte canaliculi, and this needs to be analyzed in light of the observation that in mice Lkb1 regulates both canaliculi formation and bile duct maturation, but blocking canaliculi formation using a multidrug resistance–associated protein 2 inhibitor leads to deficient biliary remodeling (74, 82, 91, 92). Likewise, the loss of the adherens junction components β-catenin and γ-catenin, ablated using *alb-cre*, which leads to deletion of floxed genes in both hepatocytes and cholangiocytes, led to progressive intrahepatic cholestasis (93). Whether the pathology is solely due to loss of the two catenins from hepatocytes or cholangiocytes, or both, will need to be addressed in future studies.

Apicobasal polarization also appears to be a driver of biliary lumen formation in the cord-hollowing process alluded to above. Lumen formation starts with single cholangiocytes expressing Moesin and Nherf1 at the apical pole, thereby marking the onset of lumen formation. The lumen then expands by recruiting an increasing number of Moesin- and Nherf1-expressing cholangiocytes, a process likely regulated by cell–cell communication and limited in space by the expression of Nf2. Ultrastructural studies revealed the presence of cytoplasmic vesicles near microlumens that were formed between adjacent cholangiocytes, suggesting active apical membrane formation. In addition, lumen expansion is coordinated with apical constriction to ensure correct shaping of the ducts (26). Interestingly, this mode of duct development, initiated by microlumen formation between adjacent cells, is similar to the development of duct lumina in the pancreas. This observation underscores one of the many developmental, tissular, and pathophysiological similarities between the liver and the pancreas. Indeed, the two organs share a common endodermal origin, display similar tissular organization, and have common dysregulatory mechanisms that lead to similar modes of tumorigenesis (78, 94, 95).

Most of our knowledge relates to apicobasal polarity, and there is still surprisingly little known regarding planar cell polarity in the morphogenesis of intrahepatic ducts. Cui and coworkers (96) used the zebrafish to demonstrate that the planar cell polarity gene *prickle*, acting via Rho and Jun kinases, controls biliary morphogenesis. The mode of action of prickle remains unclear: The knockdown affected both hepatocytes and cholangiocytes, and a combination of cell autonomous and non-cell autonomous mechanisms must be envisaged to explain how duct formation is controlled by planar polarity cues. Yet a link with the Hnf6/Hnf1β cascade has been proposed. Other genes typically associated with planar cell polarity, such as *Vangl2*, are expressed in cholangiocytes, suggesting that future work may link this process with morphogenesis (96, 97).

Development of the Extrahepatic Biliary Tree

The architecture of the extrahepatic biliary system is conceptually similar in humans, mice, and zebrafish (98). In humans, the common hepatic duct is formed by the merging of the right and left hepatic ducts. The common hepatic duct then combines with the cystic duct, which drains bile from the gallbladder, to form the common bile duct. The common bile duct, after collecting juice from the pancreatic ducts, namely from the Santorini and Wirsung ducts, becomes the short common hepatopancreatic duct and ends in the hepatopancreatic ampulla of Vater. Anatomical differences between humans and mice and zebrafish lie in the number of hepatic ducts emerging from the liver before forming the common hepatic duct and in the proportionate lengths of the common bile duct and hepatopancreatic duct.

During liver development in humans, a diverticulum forms at approximately day 26 after fertilization as an extrusion of the ventral endoderm, and the endodermal cells at the apex of the diverticulum generate a tissue bud. Within the bud one can identify a cranial part, which gives rise to the liver, and a caudal part, which develops into the gallbladder and common bile duct as well as developing into the ventral pancreas (**Figure 3**). The gallbladder anlage can be identified at approximately day 29 after fertilization, and the patent gallbladder, pancreas, and common

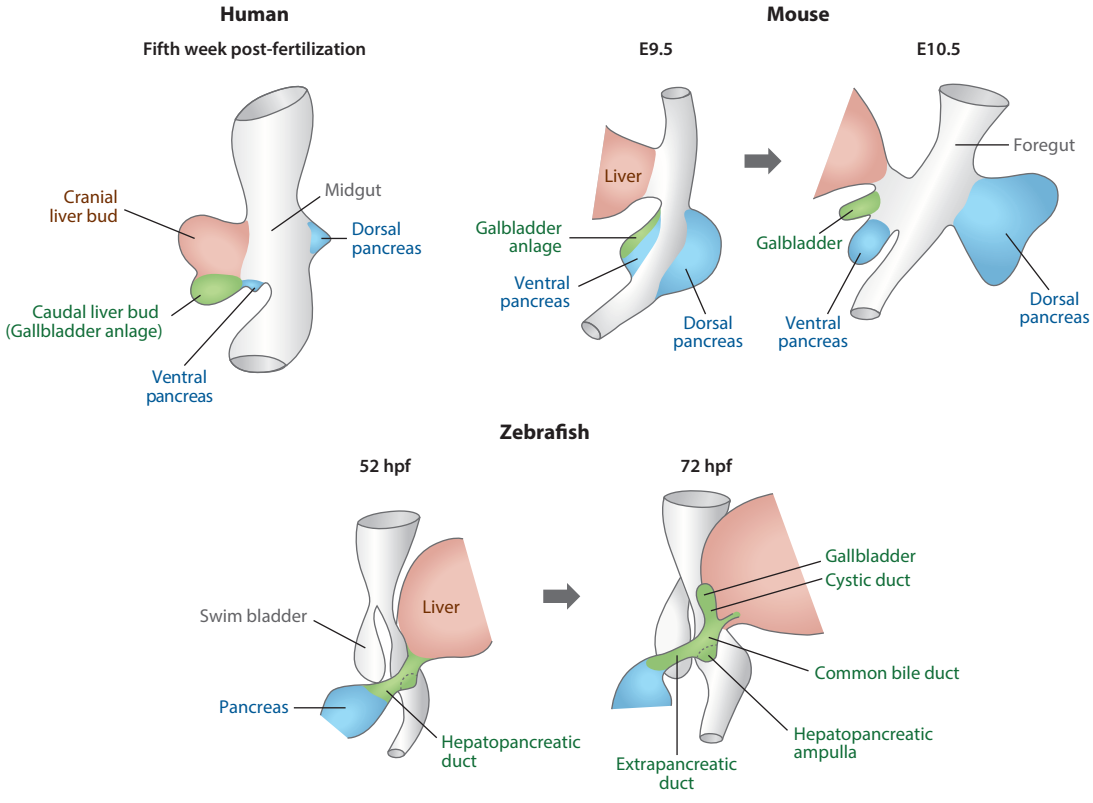

Figure 3

Development of the extrahepatic biliary tract. In humans, the extrahepatic tract derives from the caudal part of the liver bud, whereas in mice the gallbladder anlage is associated with the ventral pancreas. In zebrafish, the extrahepatic biliary tree develops from cells located between the ventral pancreatic bud and liver. At 52 hpf, the ventral and dorsal pancreas have fused. Abbreviations: E, embryonic day; hpf, hours post-fertilization. The panel showing development in humans is adapted from Reference 103 with permission from Elsevier.

bile duct are detectable at around the seventh week of gestation. The diverticulum remains connected to the liver lobes and evolves into the hepatic ducts (76, 99–103). In mice, the origin of the extrahepatic biliary tree differs slightly from that in humans. It is not derived from the liver bud but from an endodermal region that is located immediately caudal to the liver diverticulum (**Figure 3**). This region develops into ventral pancreatic tissue, the common bile duct, and the gallbladder, and it is characterized by the expression of the transcription factors Sox17 and pancreatic duodenal homeobox factor 1 (Pdx1) (104). In zebrafish, the extrahepatic biliary tract develops from cells located between the liver and ventral pancreas, and it becomes compartmentalized into an extrapancreatic duct, common bile duct, gallbladder, and cystic duct (105, 106) (**Figure 3**).

Key molecular players were identified in animal models. In particular, Sox17 is essential for specification of the biliary fate in mice since in its absence, the extrahepatic biliary tree fails to form and is replaced by pancreatic tissue (104, 107). In the endodermal progenitors, Sox17 forms a feedback loop with Hes1 during biliary specification (104). Hes1 is required for gallbladder development and is best known as a mediator of Notch signaling; however, strictly speaking, a direct role for Notch activation in gallbladder development has not been demonstrated (108, 109). Sox17 is epistatic to other factors that were shown to control development of the extrahepatic biliary tree, namely Hnf6, Hnf1β, and Hhex: In mice, inactivation of the genes encoding these proteins leads to, respectively, gallbladder agenesis and common bile duct enlargement, dysplasia of the gallbladder epithelia, or replacement of the extrahepatic epithelium by duodenal tissue (23, 24, 31). Recent studies in zebrafish further extended our understanding of the role of hhex and shed new light on the patterning of the extrahepatic biliary tree. Indeed, the analysis of *hhex* null mutants indicates that this gene is required for specification of pancreatobiliary progenitors and that the extrahepatic biliary tree develops from at least two cell populations (110). The gallbladder, common bile duct, and extrapancreatic duct are missing in *hhex* mutants, but these mutants still form a primitive extrahepatic duct, suggesting that the development of one biliary cell population depends on hhex, while another develops independently.

Beyond the stage of specification, the patterning of the extrahepatic biliary tree in different segments has been investigated in zebrafish. In *cloche* mutants, in which vascular development is impaired as a consequence of inactivation of a basic helix-loop-helix factor, a clear distinction cannot be made between the common bile duct and extrapancreatic duct. This provides strong evidence for patterning control being exerted by the adjacent vasculature (110). Further, mesenchymal signals contribute to the patterning of the extrahepatic biliary tree, as evidenced by *Fgf10* mutants, which show a shortened hepatopancreatic duct lacking clear delineation of its components (111). The *Fgf10* mutants also show ectopic hepatic and pancreatic cells and misdifferentiated cells in liver and pancreas, indicating that Fgf10 exerts wide control of the development of the hepatopancreatic endodermal domain. Fgf10 exerts part of its functions redundantly with *fgf24*, a zebrafish gene with no known mouse homolog, and stimulates expression of *sox9b*, a homolog of mammalian *Sox9*, which is required for establishing morphological boundaries between the cystic duct, extrahepatic duct, and common bile duct (112). Finally, in zebrafish, interactions with the surrounding mesenchyme that are mediated by Ephrin–Eph interactions dynamically control development and remodeling of the various segments of the extrahepatic biliary tree (M.I. Thestrup & E. Ober, personal communication).

When specifically considering gallbladder development, it is noteworthy that the level of *Sox17* expression is critical in mice since *Sox17* haploinsufficiency induces gallbladder hypoplasia and the development of short ectopic extrahepatic ducts, while intrahepatic ducts remain unaffected (113). In zebrafish gallbladder, *sox17* is controlled by sox9b (112, 114). Whereas the Sox factors mentioned so far are found in the epithelium, the transcription factor Forkhead box f1 (Foxf1) is detected both in the gallbladder epithelium and mesenchyme, and a subset of heterozygous *Foxf1*$^{+/-}$

mice develop an abnormal gallbladder with reduced mesenchyme, a deficient smooth muscle cell layer, and a lack of cholangiocytes (115). This phenotype reveals the existence of epithelium–mesenchyme interactions during gallbladder formation, a process whose importance is further substantiated by the phenotype of mice hypomorphic for Lgr4. Lgr4 is found in the epithelium, and deficient function impairs development of the gallbladder mesenchyme, resulting in the shortening, and eventually in the absence, of the gallbladder and cystic duct; however, the common bile duct remains intact (116). Along the same lines, glypican 1, a heparan sulfate proteoglycan that potentially binds Hedgehog signals and Fgf19, is necessary for determination of a normal-sized gallbladder (117).

Development of the Peribiliary Glands

The peribiliary glands are associated with the intrahepatic and extrahepatic biliary trees. They consist of mucinous and serous acini that form a plexus communicating with the bile duct lumen. Intrahepatic peribiliary glands are found at the level of large ducts and develop in the embryo from the ductal plate. In humans, the ductal plate is detectable at the seventh week of gestation, but the first morphological evidence for intrahepatic peribiliary gland development is found later, at the tenth week of gestation: Tubular extensions emerge from the ductal plate and progressively develop as acinar structures just before birth (118). The glands complete their development well after birth and constitute an extramural and intramural peribiliary plexus (119). Extrahepatic peribiliary glands develop along the common bile duct and cystic and hepatic ducts, but not along the gallbladder. They are detectable as acini-like structures at the 35th week of gestation and proliferate until the first year after birth, eventually forming an extensive network that connects the segments of the extrahepatic biliary tree (100, 120, 121). The cells forming the network coexpress mature markers, such as cytokeratin 19, and endodermal markers, such as Sox17 and Pdx1, and proliferate in response to injury, likely in order to restore mucosal integrity and function (121). There is accumulating evidence that the peribiliary glands harbor cells with multipotent stem cell properties (13–15), but whether these properties are instrumental in biliary morphogenesis during fetal development remains unknown. Moreover, the recent identification of trophoblast cell surface protein 2 (Trop2) as a marker that distinguishes Trop2-positive lumen-forming cholangiocytes from Trop2-negative peribiliary gland–constituting epithelial cells provides a tool to investigate the progenitor properties of the peribiliary gland cells (122).

A FRAMEWORK FOR UNDERSTANDING CONGENITAL BILIARY DISEASES

Diseases of the Intrahepatic Biliary Tract

Congenital diseases of the biliary tract affect the intrahepatic or the extrahepatic duct. A systematic analysis and description of those diseases is beyond the scope of this review, and the reader is referred to other comprehensive reviews of these diseases (123–126). Rather, this section discusses how the pathophysiology of these diseases may be better characterized in light of our current understanding of the mechanisms of biliary development.

The term ductal plate malformation applies to a set of congenital diseases of the intrahepatic ducts that is defined by the presence of embryonic biliary structures after birth. This typically occurs in ciliopathies such as polycystic liver diseases, congenital hepatic fibrosis, or Meckel–Gruber and related syndromes, as well as in congenital forms of biliary atresia (127). Our earlier work suggested that ductal plate malformations may arise as a consequence of abnormal differentiation and morphogenesis occurring at any stage of biliary development (53). Indeed, considering the

sequential steps in bile duct development, disease-initiating events may already be activated at the hepatoblast fate-decision stage. For instance, livers affected with Meckel–Gruber syndrome show aberrantly shaped ductal plates composed of cholangiocytes coexpressing biliary and hepatocyte markers; in parallel, hepatocytes in those livers express elevated levels of cytokeratin 19, suggesting that hepatoblasts fail to properly take the decision to adopt a biliary or hepatocyte fate and, instead, accumulate two differentiation programs to generate hepatobiliary hybrid cells (128). Along the same lines, using the congenital polycystic kidney mouse model, we proposed that ductal plate malformations in autosomal polycystic liver disease can be initiated by an excessive and accelerated differentiation of hepatoblasts toward the cholangiocyte lineage, leading to an accumulation of periportal cholangiocytes (129). The latter then start to overproliferate after birth, leading to the appearance of biliary cysts (129). The examples selected here are all ciliopathies, and, therefore, our analysis implies that primary cilia may play a role at the earliest stage of biliary differentiation, as already suggested above by the absence of primary cilia in patients or mice who have deficient expression of Hnf6 or Hnf1β. Primary cilia coordinate the activation of several signaling pathways (130). In cholangiocytes, cyclic adenosine monophosphate and protein kinase A are key effectors of primary cilia function, for instance, by controlling nuclear location of β-catenin via phosphorylation of Ser^{675} (131).

In contrast to ductal plate malformations, bile duct paucity is characterized by a reduced number of bile ducts. Alagille syndrome is among the most studied of the congenital biliary diseases; patients suffer from defects in multiple organs, with intrahepatic bile duct paucity in liver. The role of defective Notch signaling in this process has been discussed above and illustrates well how understanding the mechanisms of biliary development fuels our understanding of disease and vice versa. Importantly, compensatory regenerative biliary structures develop in some adult patients affected by Alagille syndrome, and there is evidence that this process depends on TGFβ-induced transdifferentiation of hepatocytes into cholangiocytes, thereby mimicking TGFβ-dependent differentiation of hepatoblasts into ductal plate cholangiocytes (132).

Following the hepatoblast-to-cholangiocyte transition, biliary lumen formation and expansion depend on proper apicobasal polarization of the cholangiocytes. Therefore, defective polarization should be considered as a mechanism with the potential to initiate biliary malformations. Supporting this assumption, perturbed polarization is a hallmark of cholangiocytes in a number of ductal plate malformations (126). Extended biliary lumina are also a frequent feature of congenital biliary diseases. Recent work by McClatchey and coworkers (26) revealed how the size of the lumen is restricted by Nf2 and apical constriction, and how pathological expansion of the lumen may secondarily affect the remodeling of developing bile ducts. Indeed, such remodeling implies differentiation of a subset of cholangiocytes into periportal hepatocytes, thereby limiting the total number of cholangiocytes. There is morphological evidence that such conversion to a hepatocyte fate fails to occur in Nf2-deficient liver, leading to expanded biliary structures (26). Of note, lineage tracing in the congenital polycystic kidney mouse model did not provide evidence that the cholangiocyte-to-hepatocyte conversion is impaired in autosomal polycystic liver disease (129).

Polarization of hepatocytes is necessary to allow formation of the biliary canaliculi at their apical pole (133). Canaliculi development is tightly linked with bile duct maturation and remodeling, suggesting that patients who suffer from arthrogryposis, renal dysfunction, and cholestasis syndrome have canalicular defects that may secondarily result in impaired bile duct development and bile duct paucity. This syndrome is caused by mutations in *VPS33B* or *VIPAS39* (*VIPAR*), which code for proteins controlling apical recycling and apicobasal polarity (134, 135). Interestingly, *vps33b* expression was shown in zebrafish to be stimulated by hnf1β and hnf6, two transcription factors belonging to the transcriptional network driving biliary development

and whose dysfunction leads to a biliary phenotype characterized by, among other dysfunction, deficient apicobasal polarity of the cholangiocytes (53, 136, 137).

Diseases of the Extrahepatic Biliary Tract

The most prevalent congenital disease of the extrahepatic biliary tract is biliary atresia. Patients are affected with progressive, inflammatory, and sclerosing obstruction of the extrahepatic biliary tract. The pathophysiology remains unclear, and several variants have been described, including prenatal-onset forms, which are characterized by the association of biliary atresia with non-hepatic malformations or by the presence of a cystic malformation near the site of common bile duct obstruction (138, 139). These prenatal forms suggest that aberrant development of the extrahepatic biliary tract may contribute to the pathophysiology. Susceptibility genes have been identified (reviewed in 139), including *GPC1*, which encodes glypican 1 and whose knockdown in zebrafish is associated with a reduced number of intrahepatic ducts and a smaller gallbladder (117). Along the same lines, a genome-wide association study in patients identified epidermal growth factor receptor (EGFR)/ADP ribosylation factor 6 (ARF6) signaling as a potential player in biliary atresia. Further investigation of the zebrafish homologs *arf6a* and *-b* and *egfra* using morpholino-mediated knockdown or treatment of larvae with an EGF inhibitor resulted in biliary dysgenesis, thereby uncovering a regulatory function for EGFR/ARF6 in intrahepatic bile duct development (140). While the discovery of *GPC1* and *ARF6* as biliary atresia susceptibility genes fueled research to try to understand normal development, developmental studies that uncovered the role of primary cilia formed the basis of research on biliary atresia. Mutations in ciliopathy genes were specifically searched for in patients with biliary atresia splenic malformation syndrome, leading to the identification of the candidate polycystin 1 like 1 (*PKD1L1*), a gene associated with ciliary calcium signaling (141).

Toxins were suspected to be at the origin of biliary atresia. In this context, the remarkable discovery of the flavonoid biliatresone as a causative agent for the disease in Australian livestock established a clear link between the agent and developmental regulators (142): Biliatresone-treated cholangiocyte spheroids not only show a loss of apical polarity but also have reduced expression of *Sox17*, a gene whose heterozygosity in mice was known to cause hypoplasia and shredding of the gallbladder epithelium, leading to bile duct stenosis and atresia (113, 143, 144).

Finally, choledochal cyst is a rare biliary malformation. Several variants have been described, according to the type and location of the cysts. Some variants are exclusively extrahepatic; others also affect intrahepatic ducts. The etiology is unknown, but the association of choledochal cyst with other malformations suggests a developmental defect (145). At the experimental level, it is noteworthy that zebrafish mutant in *nf2*, a gene discussed above in the context of mouse biliary development, display cysts affecting the common bile duct (146).

CONCLUSIONS

Much information has been collected about the mechanisms controlling intrahepatic cholangiocyte differentiation from hepatoblasts, and increasingly accurate descriptions of intrahepatic duct morphogenesis are now available. In contrast, many open questions remain with regard to the molecular mechanisms regulating intrahepatic duct morphogenesis, and our knowledge of extrahepatic biliary differentiation, morphogenesis, and patterning is still fragmentary. Given the impressive pace at which data have been collected in recent years, we may consider with confidence that in the next decade our knowledge of biliary development and pathophysiology will be pushed well beyond the current limits.

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