Epithelial Morphogenesis during Liver Development

Naoki Tanimizu and Toshihiro Mitaka

Department of Tissue Development and Regeneration, Research Institute for Frontier Medicine, Sapporo Medical University School of Medicine, Chuo-ku, Sapporo 060-8556, Japan

Correspondence: tanimizu@sapmed.ac.jp

Tissue stem/progenitor cells supply multiple types of epithelial cells that eventually acquire specialized functions during organ development. In addition, three-dimensional (3D) tissue structures need to be established for organs to perform their physiological functions. The liver contains two types of epithelial cells, namely, hepatocytes and cholangiocytes, which are derived from hepatoblasts, fetal liver stem/progenitor cells (LPCs), in mid-gestation. Hepatocytes performing many metabolic reactions form cord-like structures, whereas cholangiocytes, biliary epithelial cells, form tubular structures called intrahepatic bile ducts. Analyses for human genetic diseases and mutant mice have identified crucial molecules for liver organogenesis. Functions of those molecules can be examined in in vitro culture systems where LPCs are induced to differentiate into hepatocytes or cholangiocytes. Recent technical advances have revealed 3D epithelial morphogenesis during liver organogenesis. Therefore, the liver is a good model to understand how tissue stem/progenitor cells differentiate and establish 3D tissue architectures during organ development.

Functional differentiation/maturation of epithelial cells and establishment of three-dimensional (3D) epithelial tissue structures are indispensable for each organ performing physiological functions. The mammalian liver performs various types of metabolism, produces serum proteins, detoxifies bilirubin and ammonia, and protects the body from infection. Those physiological functions are achieved with 3D tissue architecture of liver epithelial cells, namely, hepatocytes and cholangiocytes (biliary epithelial cells) (Fig. 1). Hepatocytes and cholangiocytes are derived from hepatoblasts, fetal liver stem/progenitor cells (LPCs), in mid-gestation

and then differentiate functionally and structurally. Hepatocytes acquire many physiological functions and are polarized to form hepatic cord structure that is typically one-cell thick in an adult liver. The tiny apical lumen is called bile canaliculus (BC) to which hepatocytes secrete bile juice. Their basal domains face to sinusoidal endothelial cells that have fenestrations, through which hepatocytes efficiently exchange nutrients and metabolites with the bloodstream. Cholangiocytes establish a typical apicobasal epithelial polarity and form intrahepatic bile duct (IHBD) tubules that connect the BCs to the duodenum via hepatic and common bile

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Figure 1. Tissue structures of the liver. Hepatocytes are polarized and form hepatic cords. The apical domains form the thin tubular structure called bile canaliculi (BCs). Hepatocytes secrete bile into the BC. Their basal domains face to sinusoidal endothelial cells via the space of Disse. The space is not abundant in the extracellular matrix (ECM). Cholangiocytes develop the typical epithelial apicobasal polarity and form bile duct tubules adjacent to the portal vein (PV).

ducts to drain bile secreted from hepatocytes. In addition, cholangiocytes modulate the composition of bile by secreting chloride and carbonate ions and by secreting and absorbing water while bile flows through IHBDs.

Analyses for human congenital hepatic diseases and for mutant mice showing abnormal liver development have identified crucial molecules and signals for liver organogenesis. Since immunological definition of liver cells has progressed, LPCs and differentiated epithelial cells can be prospectively isolated from fetal, neonatal, and adult livers and can be used for genome-wide gene expression analyses to identify genes that regulate differentiation and morphogenesis of LPCs and liver epithelial cells (Miyajima et al. 2014). In addition, purified LPCs can be induced to differentiate into hepatocytes or cholangiocytes by using in vitro culture systems, in which we can address molecular mechanisms regulating differentiation and morphogenesis

of hepatocytes and cholangiocytes (Kamiya et al. 1999; Tanimizu et al. 2003, 2007; Senga et al. 2012).

Liver epithelial morphogenesis in vivo had been mostly studied depending on two-dimensional (2D) tissue sections prepared from developing livers (Lemaigre 2003). As compared with other epithelial organs such as gastrointestinal tracts, kidney, and lung, wherein epithelial morphogenesis proceeds in optically transparent tissue, it is hard to recapitulate and visually follow epithelial morphogenesis of the liver ex vivo because liver tissue structures develop from the mid-fetal to neonatal period when the liver becomes large and opaque. Recent advances in 3D imaging combined with tissue clearing techniques (Hama et al. 2011; Yang et al. 2014) enable us to visualize the processes of liver epithelial morphogenesis in 3D (Kaneko et al. 2015; Takashima et al. 2015; Tanimizu et al. 2016).

In this section, we summarize molecular mechanisms regulating cellular differentiation of LPCs and epithelial morphogenesis during liver development and the recent progress in our knowledge about in vivo 3D morphogenesis of liver epithelial cells.

OVERVIEW OF LIVER ORGANOGENESIS

Liver organogenesis starts as the foregut endoderm is stimulated by the fibroblast growth factors (FGFs) and bone-morphogenetic proteins (BMPs) secreted from the adjacent cardiac and the septum transversum mesenchyme, respectively, in mice (Zaret 2002). WNT2bb and retinaldehyde dehydrogenase type 2 (RALDH2), which is the enzyme responsible for retinoic acid (RA) biosynthesis and regulates WNT2bb expression in the lateral plate mesoderm, are involved in specification of the liver in zebrafish and medaka (Ober et al. 2006; Negishi et al. 2010). Around embryonic day 8.0 (E8.0), the foregut endoderm is segregated to $Hhex^{+}Pdx1^{-}$ and $Hhex^{-}Pdx1^{+}$ portions (Spence et al. 2009). $Hhex^{+}Pdx1^{-}$ tissue develops into the liver bud, whereas $Hhex^{-}Pdx1^{+}$ tissue generates the extrahepatic biliary structure consisting of extrahepatic bile ducts and gallbladder as well as the ventral pancreas (Bort et al. 2006; Spence et al. 2009). Hepatoblasts in the liver bud expand and migrate into surrounding mesenchymal tissue depending on intrinsic $Hhex$ and $Prox1$ (Sosa-Pineda et al. 2000; Bort et al. 2006) and on growth factors, including human growth factor (HGF) secreted from primitive endothelial cells, FGF10 from fibroblasts, and pleiotrophin/midkine from mesothelial cells (Matsumoto et al. 2001; Berg et al. 2007; Onitsuka et al. 2010). During migration into mesenchymal tissue, hepatoblasts lose epithelial characteristics, which they have established in the foregut (e.g., degradation of the basement membrane and reduction of E-cadherin expression; Sosa-Pineda et al. 2000). Subsequently, hepatoblasts near the portal veins (PVs) are committed to cholangiocytes. In the parenchymal region, hepatoblasts receive oncostatin M (OSM) from hematopoietic cells via gp130 and differentiate to hepatocytes (Kamiya et al. 1999).

IDENTIFICATION OF LIVER STEM/PROGENITOR CELLS AND THEIR CELLULAR CHARACTERISTICS

Efforts have been performed to identify specific surface antigens for various types of cells in developing, adult normal, and injured livers, which enable us to isolate LPCs at any developmental stages. $Dlk-1$ (Tanimizu et al. 2003) and $Liv2$ (Watanabe et al. 2002) are markers for hepatoblasts, whereas epithelial cell-adhesion molecule (EpCAM) (Okabe et al. 2009), CD13, and CD133 (Kamiya et al. 2009) have been used to identify neonate and adult LPCs (Fig. 2A). In addition to clonal culture systems assessing bidirectional differentiation potential of LPCs, two culture systems that induce hepatocytic or cholangiocytic differentiation have been used to examine molecular mechanism regulating differentiation and morphogenesis of LPCs. Hepatocytic differentiation can be induced by sequential treatment of OSM and Matrigel overlay (Kamiya et al. 1999, 2002). Bipotential LPCs not only acquire gene expression similar to mature hepatocytes but establish some metabolic functions and bile canaliculi-like structures (Fig. 2B, top). On the other hand, cholangiocyte differentiation is examined in 3D culture in which LPCs differentiate along the cholangiocyte lineage, establish cholangiocyte-type epithelial polarity, and form the cyst structure with a single lumen (Fig. 2B, bottom) (Tanimizu et al. 2007). Studies using these two culture systems revealed that neonatal $EpCAM^{+}$ LPCs differentiate hepatocytes and cholangiocytes, whereas adult $EpCAM^{+}$ cells differentiate cholangiocytes but only partly to hepatocytes, indicating that LPCs alter their differentiation potential during development (Tanimizu et al. 2014). It should be pointed out that most of markers used for isolation of adult LPCs are also those for cholangiocytes (Miyajima et al. 2014). Although LPCs can be enriched in $EpCAM^{+}$ or $CD133^{+}$ fraction, identification of novel surface antigens is necessary to distinguish LPCs and cholangiocytes. Recently, we prospectively isolated liver progenitor cells distinctive to LPCs; $CD45^{-}TER119^{-}CD31^{-}ICAM-1^{+}EpCAM^{-}$ cells efficiently differentiate to mature

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A Isolation of liver stem/progenitors

Stage	Surface markers
Fetal	Dlk1, Liv2, CD13
Neonate	EpCAM
Adult	EpCAM, CD133

B In vitro culture

Hepatocyte differentiation

Culture conditions

- Sequential treatment with oncostatin M and Matrigel overlay

Differentiation assay

- Increased expression of metabolic enzymes
- Acquisition of metabolic functions
- Formation of bile canaliculus-like structures

Cholangiocyte differentiation

Culture conditions

- 3D culture in ECM gel containing laminin 111

Differentiation assay

- Formation of cystic structures with a single lumen
- Expression and appropriate cellular localization of cholangiocyte markers
- Directional transport of a substrate of Mdr2

Figure 2. Identification of liver stem/progenitor cells and their in vitro culture systems. (A) Surface markers for fetal, neonatal, and adult liver stem/progenitor cells (LPCs). (B) In vitro culture systems for inducing differentiation of hepatocytes and cholangiocytes. Hepatocyte differentiation is induced by sequential treatment of oncostatin M and Matrigel overlay. In this culture system, round nuclei and dense cytoplasm become evident. In addition to increased expression of a number of metabolic enzymes, metabolic functions, including albumin secretion, ammonia detoxification, and accumulation of cytoplasmic glycogen as well as cytochrome P450 activities, are induced in this culture condition. Cholangiocyte differentiation is induced in 3D culture in which LPCs are embedded in the extracellular matrix gel containing laminin 111. Cells develop the cyst structure and properly localize cholangiocyte markers, including CK19, EpCAM, aquaporin-1, and integrin $\beta 4$. Cells in the cyst can directionally transport a substrate of multidrug resistance protein 2 (MDR2, ABCB4) from the basal to the apical.

hepatocytes in vitro and in vivo, whereas they have less potential to become cholangiocytes (Tanimizu et al. 2016c). Comparison among these LPC populations and mature liver epithelial cells may help us to understand molecular mechanisms determining a type of epithelial polarity and regulating the liver stem-cell system.

DEVELOPMENT OF INTRAHEPATIC BILE DUCTS

Tubular Morphogenesis of Cholangiocytes

Cholangiocytes, differentiated from hepatoblasts, develop IHBD tubules in late fetal and

perinatal periods. In a conventional model for mouse IHBD morphogenesis, a single cell layer of cholangiocytes called the ductal plate is first generated around the portal veins (PVs) in E14.5 ~ 15.5 livers, which becomes a double cell layer and is progressively reorganized to bile duct tubules from the liver hilum to the periphery (Fig. 3A) (Lemaigre 2003; Antoniou et al. 2009). At an early stage of morphogenesis, cholangiocytes in the ductal plate surround luminal space with neighboring hepatoblasts (Fig. 3B) (Antoniou et al. 2009). Since those structures consist of heterogeneous types of cells, they are called “asymmetric ducts.” After hepa-

Figure 3. Process of the development of intrahepatic bile ducts (IHBDs). (A) A conventional model of bile duct morphogenesis. Cholangiocytes first form a cell layer, ductal plate, near the portal vein (PV) around E15 and then progressively reorganize it to tubules. (B) The transition from asymmetric to symmetric ducts. Cholangiocytes first generate the luminal space with hepatoblasts ("asymmetric duct" structure). Either by cholangiocyte differentiation of hepatoblasts in "asymmetric duct" or by reshaping the layer of cholangiocytes, the "symmetric duct structure" is generated. (C) The structures of IHBDs in 8W liver. The hierarchical luminal network containing large ducts and small mesh-like ductules covers the whole liver tissue in the adult (panel 2). 3D confocal imaging of the liver stained with anti-osteopontin (OPN) antibody also visualizes this hierarchical network (panel 3). Boxes in panels 1 and 2 are enlarged in panels 2 and 3, respectively. (D) The structures of IHBDs in E17 liver. The continuous homogeneous luminal network is established in E17 liver (panels 2 and 3). In the periphery, where the continuous luminal network has not been established, OPN⁺ cholangiocytes are discontinuously differentiated around the PV (panel 4). Boxes in panels 1 and 2 are enlarged in panels 2 and 3 ~ 4, respectively. (E) The 3D model of IHBD morphogenesis. Cholangiocytes are discontinuously differentiated from hepatoblasts (panel 1), which are eventually interconnected to form the homogeneous luminal network in a Notch-signal-dependent manner (panel 2). The homogeneous luminal network is rearranged to the hierarchical one (panel 3). This transition is highly correlated with formation of the bile canalicular network.

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toblasts in “asymmetric ducts” differentiate into cholangiocytes, “symmetric” mature ducts are generated. Alternatively, a layer of cholangiocytes may be rearranged to surround a lumen (Fig. 3B). This type of morphogenesis was observed in a sandwich culture of a liver progenitor cell line in which a monolayer of progenitors folded up and formed tubular structures (Tanimizu et al. 2009). The model that includes the transition from “asymmetric” to “symmetric” ducts well explains tubulogenesis through the formation of intermediate structures that are observed in embryonic liver tissue sections.

Recently, several 3D-imaging techniques have been developed and applied on analyses for liver tissue structures. Retrograde injection of resin into IHBDs from the common bile duct revealed branching structures in the entire liver tissue (Walter et al. 2014). However, only large ducts might be visualized with this method. On the other hand, 3D confocal imaging and retrograde carbon ink injection could visualize not only large ducts but also fine ductular networks and showed that large ducts run along the PVs and small ductules, forming a mesh-like network around the PVs in developing livers as well as in the adult one (Kaneko et al. 2015; Takashima et al. 2015).

We combined confocal imaging and retrograde carbon ink injection into the luminal network to examine tubular morphogenesis of IHBDs (Fig. 3C,D) (Tanimizu et al. 2016). Since small ductules are branched out from large ducts, the mature IHBD structure is referred to as the “hierarchical network” (Fig. 3C). In contrast to the adult liver, the size of the lumen is fine and homogeneous in E17 liver, although the continuous luminal network is established (Fig. 3D2). This luminal network is also evident as the network of OPN⁺ cholangiocytes in 3D confocal imaging (Fig. 3D3). On the other hand, OPN⁺ cholangiocytes discontinuously exist in the liver periphery in which the luminal network has not yet been established (Fig. 3D4). Extensive analyses of IHBD structures in fetal and neonatal livers have revealed that IHBDs showing the “hierarchical network” thoroughly cover the whole liver tissue by 1 week after birth. Prior to this stage, the “homogeneous network”

of IHBDs, consisting of only small ductules, is observed in a substantial region of the periphery of the liver tissue. Based on these results, IHBD morphogenesis can be segregated into three steps. First, cholangiocytes are discontinuously differentiated from hepatoblasts around the PVs (Fig. 3E1), and then they form the continuous homogeneous luminal network consisting of only small ductules (Fig. 3E2). Finally, the homogeneous luminal network is dramatically rearranged into the hierarchical mature network between E17 and 18 (Fig. 3E3).

Molecular Mechanisms Governing IHBD Development

The Notch and transforming growth factor β (TGF- β) pathways are two major signals regulating the development of IHBDs. In addition, several transcription factors have been implicated in IHBD development (Table 1; Fig. 4) (Tanimizu and Mitaka 2016).

Jagged1-Notch Pathway

Biliary atresia, IHBD paucity, and autoimmune diseases, including primary biliary cirrhosis and primary sclerosing cholangitis, are caused by genetic abnormality affecting development and maintenance of the biliary system. Alagille syndrome, a congenital disease showing multiple developmental defects including paucity of IHBDs, is caused by haploinsufficiency of the Notch signaling. In most cases, point mutations in the allele of Jagged-1 lead to generation of truncated protein and reduce the signal (Piccoli and Spinner 2001). Notch2 mutation is also implicated in Alagille syndrome (Kamath et al. 2012). Although Jagged-1 heterozygous mice did not show any major developmental defects (Xue et al. 1999), Notch2^{+/-}Jagged1^{+/-} mice showed multiple developmental abnormalities similar to human Alagille syndrome, including growth retardation, jaundice, paucity of IHBDs, and abnormal development of kidney (McCright et al. 2002). In addition, components of the Notch signaling have been depleted in liver at different developmental stages. Although the recombination using Cre-loxP sys-

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Table 1. Mutant mice showing developmental defects in intrahepatic bile ducts (IHBDs) and extrahepatic bile ducts (EHBDs)

Gene	Model	Phenotype of mutant mice	Gene functions	References
Notch signal				
Jagged-1	SM22-Cre:Jag1 ^{flox/flox}	Abnormal IHBD morphogenesis	Regulate BEC differentiation and IHBD morphogenesis	Hofmann et al. 2010
	Notch2 ^{del/+} Jag1 ^{-/+} (a model for Alagille syndrome)	Abnormal IHBD morphogenesis		McCright et al. 2002
Notch2	Alb-Cre: Notch2 ^{flox/flox}	Abnormal IHBD morphogenesis	Regulate BEC differentiation and IHBD morphogenesis	Falix et al. 2014
	Alfp-Cre: Notch2 ^{flox/flox}	No cholangiocyte differentiation, Notch-independent ductular differentiation in postnatal liver		
RBPJ κ	Alfp-Cre:RBPJ κ ^{flox/flox}	Reduced cholangiocyte differentiation, abnormal IHBD morphogenesis	Regulate BEC differentiation and IHBD morphogenesis	Zong et al. 2009
Hes1	Hes1 ^{-/-}	Conversion of EHBDs to pancreatic tissue, abnormal IHBD morphogenesis	Regulate BEC differentiation and IHBD morphogenesis	Kodama et al. 2004; Sumazaki et al. 2004
	Alfp-Cre:HES1 ^{flox/flox}	Abnormal IHBD morphogenesis		Zong et al. 2009
Transcription factor				
β -catenin	Foxa3-Cre: β -catenin ^{flox/flox}	Reduced cholangiocyte differentiation	Regulate BEC differentiation from hepatoblasts	Tan et al. 2008
C/EBP α	C/EBP α ^{-/-}	Ectopic cystic structures in the parenchyma	Inhibit BEC program in hepatoblasts	Yamasaki et al. 2006
Foxa1/a2	Alfp-Cre:Foxa1 ^{flox/flox} , Foxa2 ^{flox/flox}	Cholangiocyte hyperplasia	Inhibit IL6-mediated cholangiocyte proliferation	Li et al. 2009
Foxm1b	Foxm1b ^{-/-} , Alfp-Cre:Foxm1b ^{flox/flox}	No cholangiocyte differentiation	Promote hepatoblast proliferation Regulate expression of HNF1 β	Krupczak-Hollis et al. 2004
HNF6	HNF6 ^{-/-}	No GB formation, abnormal BD morphogenesis (cystic structures near the PVs and ectopic cystic structures in the parenchyma)	Inhibit BEC program in hepatoblasts in the parenchymal region by downregulating the TGF- β signal	Clotman et al. 2002
	HNF6 ^{-/-} OC2 ^{-/-}	Abnormal BD morphogenesis (ectopic cystic structures in the parenchyma)		
HNF1 β	Alfp-Cre-HNF1 β ^{flox/flox}	Abnormal IHBD morphogenesis (some cystic structures near the PVs)	Regulate IHBD morphogenesis	Coffinier et al. 2002

Continued

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Table 1. *Continued*

Gene	Model	Phenotype of mutant mice	Gene functions	References
Hhex	FoxA3-Cre:Hhex ^{flox/flox}	No GB formation, abnormal IHBD morphogenesis (ectopic cystic structures in the parenchyma)	Regulate expression of HNF1 β , HNF4 α , and HNF6	Hunter et al. 2007
	Alfp-Cre:Hhex ^{flox/flox}	Abnormal IHBD morphogenesis (progressive polycystic phenotype in postnatal livers)		Senga et al. 2012
Prox1	Foxa3Cre:Prox1 ^{flox/flox}	Ectopic cystic structures in parenchyma	Inhibit cholangiocyte differentiation	Seth et al. 2014
Sall4	Not available	Enhanced cholangiocyte differentiation	Promote cholangiocyte differentiation	Oikawa et al. 2009
Sox4	Alfp-Cre:Sox4 ^{flox/flox} Sox9 ^{flox/flox}	Abnormal cholangiocyte differentiation and IHBD morphogenesis	Promote cholangiocyte differentiation and morphogenesis	Poncy et al. 2015
Sox9	Alfp-Cre:Sox9 ^{flox/flox}	Delay of IHBD morphogenesis	Promote IHBD morphogenesis	Antoniou et al. 2009
Tbx3	Tbx3 ^{-/-}	Reduced proliferation of hepatoblasts	Inhibit cholangiocyte differentiation	Suzuki et al. 2008
Molecules related to protein sorting and epithelial polarity				
Pkhd1	Pkhd1 ^{del2/del2} (a model for ARPKH)	Progressive IHBD dilatation	Regulate primary cilia formation	Woollard et al. 2007
Mks1	Mks1 ^{-/-} (a model for Meckel–Gruber syndrome)	Abnormal IHBD morphogenesis (ductal plate is not reorganized into tubules)	Regulate primary cilia formation	Weatherbee et al. 2009
Wnt5a	Wnt5a ^{-/-}	Increased number of cholangiocytes	Inhibit cholangiocyte differentiation	Kiyohashi et al. 2013
Vps33b	Zebrafish Vps33b mutant (a model for ARC syndrome)	Paucity of IHBDs	Regulate tight junction formation and apicobasal polarity	Matthews et al. 2005
Cldn15b	Zebrafish Cld15b mutant	Disorganized IHBD network	Promote IHBD morphogenesis	Cheung et al. 2011

ARPKH, Autosomal recessive polycystic kidney and hepatic disease; ARC syndrome, arthrogryposis, renal dysfunction, and cholestasis syndrome; TGF- β , transforming growth factor β .

Table is modified from Tanimizu and Mitaka (2016).

tem occurs in a period of time, by using Foxa3-Cre, Alfp-Cre, and Alb-Cre mice, depletion of the targeted gene is expected in liver bud, fetal liver, and neonatal liver, respectively. Additionally, Jagged-1 was depleted in mesenchymal cells including periportal fibroblasts, which express Jagged-1 at the onset of IHBD development, by using SM22-Cre mice. Cholangiocyte

differentiation from hepatoblasts were partly and almost completely abolished in Foxa3-Cre:RBPJ κ ^{flox/flox} (Zong et al. 2009) and Alfp-Cre:Notch2^{flox/flox} mice (Falix et al. 2014), respectively, whereas a reduced but substantial number of cholangiocytes emerged in SM22-Cre:Jagged1^{flox/flox} (Hofmann et al. 2010), Alfp-Cre:RBPJ κ ^{flox/flox} (Zong et al. 2009), and

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Figure 4. Genes and signaling pathways regulating the development of intrahepatic bile ducts (IHBDs). TGF- β , Transforming growth factor β .

Alb-Cre:Notch2^{flox/flox} mice (Geisler et al. 2008; Tchorz et al. 2009). The number of mature duct structures was decreased in the latter three mutant mice, indicating that tubular morphogenesis depends on the Notch signal. These results indicate that the Notch signal mediated by interactions between Jagged-1⁺ periportal fibroblasts and Notch2⁺ hepatoblasts induces fate decision of hepatoblasts along the cholangiocyte lineage beyond mid-gestation and then later controls tubular morphogenesis.

Extensive analyses of mice lacking an intact Notch signal have further shown that this signal governs the development of IHBDs. However, it remains unknown how this signal regulates tubular morphogenesis of IHBDs. When the signal was inhibited by intraperitoneal injection of DAPT, a γ -secretase inhibitor, into newborn mice between P2 and 6, the tubular network of IHBDs was not established in the periphery of the liver where cholangiocytes are newly differentiated and undergo tubular morphogenesis in postnatal development (Tanimizu et al. 2016). Even in the presence of DAPT, cholangiocytes normally established the apicobasal polarity in most parts of liver tissue. However, the number of cholangiocytes was significantly reduced in the periphery of the liver and thereby cholangiocytes could not be interconnected to generate the continuous network of IHBD in the presence of DAPT. These results suggest that the appro-

priate activation of the Notch signal is crucial to supply enough cholangiocytes to generate the continuous luminal network of IHBDs.

Although hepatoblast-to-cholangiocyte differentiation at the fetal and perinatal stages was totally blocked in Alb-Cre:Notch2^{flox/flox} mice and Alb-Cre:HNF6^{flox/flox}RBPJ^{flox/flox} mice, duct cells emerged around 3W after birth independent of the Notch signal (Falix et al. 2014; Walter et al. 2014). The duct structures that emerged in these mutant mice were similar to those in chronically injured livers associated with expansion of ductular structures, which is called the ductular reaction. Recent works have suggested that part of expanded ductular cells are derived from hepatocytes (Sekiya and Suzuki 2014). Therefore, hepatocytes-to-cholangiocytes conversion may compensate for the defective formation of IHBDs. However, the Notch pathway is the major signal implicated both in ductular expansion and “transdifferentiation” from hepatocytes to cholangiocytes in injured livers (Boulter et al. 2012; Yanger et al. 2013). Therefore, other unknown mechanisms may regulate expansion of duct cells in postnatal livers lacking the Notch signal.

Transcription Factors

The TGF- β signaling pathway has been implicated in biliary differentiation and the expres-

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sion pattern of its components are regulated by HNF6/Onecut-1 (OC-1). The fate determination of hepatoblasts into cholangiocytes was perturbed in mice lacking HNF6 alone or both HNF6 and OC-2. In HNF6^{-/-} and HNF6^{-/-}OC-2^{-/-} mice, hepatoblasts showed “hybrid phenotypes” (positive both for hepatocyte and cholangiocyte markers) and formed cystic structures (Clotman et al. 2002, 2005). Since TGF-βRII and negative regulators for the TGF-β signal, including α2-macroglobulin and follistatin, increased and decreased, respectively, in HNF6^{-/-}OC-2^{-/-} livers, the TGF-β signal was ectopically activated in the parenchymal region, which may induce cholangiocytic characteristics in hepatoblasts in that region.

HNF1β, a homeobox transcription factor, is expressed in various tubular structures including IHBDs (Coffinier et al. 1999). Its expression is regulated by HNF6 and the Notch signal in the liver (Clotman et al. 2002; Tanimizu and Miyajima 2004; Zong et al. 2009). In Alfp-Cre:HNF1β^{flox/flox} mice, CK⁺ cholangiocytes emerged near PVs but tubular morphogenesis was severely disturbed (Coffinier et al. 2002). HNF1β regulates proteins localizing in the primary cilia, including polycystic kidney and hepatic disease 1 (Pkh1). Although the precise mechanisms of how HNF1β regulates IHBD morphogenesis remains unknown, the planar cell polarity might be affected as shown in the kidney lacking HNF1β, in which cell divisions along the perpendicular axis dominated those along the axis of elongation, resulting in the lumen expansion and eventually polycystic disease (Verdeguer et al. 2010). Sox9 is regulated by the Notch and TGF-β signals and regulates expression of extracellular matrix proteins. IHBD morphogenesis was delayed in Alfp-Cre:Sox9^{flox/flox} mice (Antoniou et al. 2009), and severer defects in IHBD development were observed in Sox4^{-/-}Sox9^{-/-} mice (Poncy et al. 2015). These results indicate that redundancy exists between Sox4 and Sox9.

Grainyhead like-2 (Grhl2) is one of mammalian homologs of *Drosophila melanogaster* grainyhead. GRHL2 is expressed in many types of epithelial cells (Auden et al. 2006). In the liver, GRHL2 is specifically and thoroughly expressed

in cholangiocytes in the adult, whereas cholangiocytes forming tubules but not those of peripheral ductules express Grhl2 in the neonatal liver, suggesting that Grhl2 is involved in epithelial maturation and morphogenesis (Fig. 4) (Senga et al. 2012; Tanimizu et al. 2014). Overexpression of Grhl2 in liver progenitor cells augmented the epithelial integrity of cholangiocytes by upregulating claudin 3 (CLDN3) and CLDN4. Grhl2 also upregulated Rab25, a member of the Rab11 family that have been implicated in apical protein sorting and formation of the apical lumen (Casanova et al. 1999; Bryant et al. 2010), and thereby increased CLDN4 localized at tight junctions (TJs) (Senga et al. 2012). By coordinating this molecular network, Grhl2 induced expansion of the apical luminal space in vitro. Furthermore, Grhl2 limited hepatocyte differentiation of LPCs and the conversion of cholangiocyte-to-hepatocyte by inhibiting the expression of HNF4α, C/EBPα, and miR122 (Tanimizu et al. 2013, 2014). On the other hand, it has been reported that Grhl2 regulates airway regeneration by controlling differentiation of basal stem cells (Gao et al. 2015) and that Grhl2 suppresses epithelial–mesenchymal transition during neural tube closure (Ray and Niswander 2016). Taken together, Grhl2 may widely regulate not only epithelial morphogenesis but also the lineage plasticity of epithelial cells.

C/EBPα, Hhex, and Prox1 regulate hepatocyte differentiation and maturation (Kimura et al. 1998; Sosa-Pineda et al. 2000; Bort et al. 2006; Yoshida et al. 2006). However, development of IHBDs was abnormal and cystic structures consisting of hybrid cells emerged in Foxa3-Cre:Hhex^{flox/flox}, C/EBPα^{-/-}, and Foxa3-Cre:Prox1^{flox/flox} mice (Yamasaki et al. 2006; Hunter et al. 2007; Seth et al. 2014). Cystic structure formation is similar to that observed in HNF6^{-/-} mice. Abnormal development of IHBDs in mice lacking any one of them indicates that they not only promote hepatocyte characteristics but also suppress cholangiocyte ones in hepatoblasts.

In contrast to mice lacking HNF6, C/EBPα, Hhex, or Prox1, cystic structures did not emerge in the parenchymal region of Alfp-

Cre:HNF4 $\alpha^{flox/flox}$ nor Alfp-Cre:HNF1 $\beta^{flox/flox}$ livers. Hepatic cords and sinusoids were not properly formed in Alfp-Cre:HNF4 $\alpha^{flox/flox}$ mice (Parviz et al. 2003). HNF4 α depletion resulted in the loss of junctional proteins such as ZO1 and E-cadherin, which may be a reason why hepatoblasts do not form cystic structures. Although loss of HNF1 β induced cystic structures near PVs, we found that overexpression of HNF1 β in liver progenitor cells induced lumen expansion in a sandwich culture in which progenitor cells develop tubular structures (Tanimizu et al. 2009) and in the 3D culture, suggesting that HNF1 β is a crucial factor for lumen formation. This result is consistent in that HNF1 $\beta^{-/-}$ hepatoblasts do not form cystic structures in Alfp-Cre:HNF1 $\beta^{flox/flox}$ mice.

Extracellular Matrix Proteins

Laminin, a heterotrimer of α , β , and γ chains, has been implicated in establishment of epithelial polarity and morphogenesis (Timpl et al. 2000; Sasaki et al. 2004; Yu et al. 2005; Durbeej 2010). The layer of laminin is observed underneath cholangiocytes from the early stage of IHBD development as well as PVs, central veins (CVs), and hepatic arteries (Shiojiri and Sugiyama 2004; Kikkawa et al. 2005). In addition, the extracellular matrix (ECM) layer containing laminin surrounded cystic structures of “hybrid cells” appeared in livers lacking HNF6, Prox1, or C/EBP α (Clotman et al. 2005; Yamasaki et al. 2006; Seth et al. 2014). These results indicate that liver epithelial cells are associated with laminins just after hepatoblasts are committed, even if partly, to the cholangiocyte lineage.

We examined expression and localization of laminin α 1 and α 5 during IHBD development. Laminin α 1 and α 5 were detected at the basal side of cholangiocytes in E15.5 liver, whereas only laminin α 5 was detected in the adult. In embryonic livers, p75NTR $^{+}$ fibroblasts including periportal mesenchymal cells and EpCAM $^{+}$ cholangiocytes expressed laminin α 1 and α 5, respectively. These results suggest that laminin 111 (α 1 β 1 γ 1) supplied from mesenchymal cells support the beginning of biliary morphogenesis in a paracrine manner, and then laminin

511(α 5 β 1 γ 1)/521(α 5 β 2 γ 1) produced by cholangiocytes themselves promote tubular morphogenesis in an autocrine manner (Tanimizu et al. 2012).

This sequential contribution of laminin isoforms to IHBD morphogenesis was shown in the 3D culture system of liver progenitor cells (Tanimizu et al. 2012). HPPL, a bipotential liver progenitor cell line, developed apicobasal polarity and formed cysts, spheroids with the single central lumen depending on exogenous laminin 111 (Tanimizu et al. 2007). On the other hand, HPPL produced α 5-containing laminin (probably laminin 521) and deposited it on the basal domain. In laminin α 5 KO mice, the delay of IHBD morphogenesis was evident: immature and mature ducts increased and decreased, respectively, in E17.5 laminin α 5 KO livers. These results suggest that cholangiocytes start tubular morphogenesis depending on laminin 111, whereas they complete it and maintain the tubular structure of IHBDs on laminin 511/521 (Tanimizu et al. 2012).

Physical Stress

A recent work suggests that blood flow affects lumen formation and morphogenesis of blood vessels (Gebala et al. 2016). Given that IHBDs are the route for the bile juice, their morphogenesis could be affected by the flow of the bile. Notably, fragmented BCs become the continuous network between E17 and 18. We noticed that the color of the intestine changed from white to yellow and expression of *Ugt1a1*, a crucial enzyme for direct bilirubin formation, was significantly upregulated between E17 and 18. These results suggest that direct bilirubin production and the bile flow increase between E17 and 18, when the “homogenous fine luminal network” is reorganized to the hierarchical one (Fig. 3E). When a multidrug resistance protein 2 (MRP2, ABCC2) inhibitor was intraperitoneally injected into a pregnant mouse, extension of the BC network was suppressed in the fetus and the structural transition of IHBDs from a “homogenous” to “hierarchical” network was attenuated in the periphery of the liver (Tanimizu et al. 2016). Moreover, it was shown

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that rapid increase of bile volume in the luminal network of IHBDs after bile duct ligation altered the round lumen to the corrugated lumen to increase the surface area (Vartak et al. 2016). These results suggest that physical stress, such as fluid flow is an important factor promoting epithelial tubular morphogenesis.

HEPATOCTE MORPHOGENESIS

The apicobasal polarity of hepatocytes is different from that of typical epithelial cells including cholangiocytes (Treyer and Musch 2013). The basal domain without the basal lamina faces the space of Disse, which is generated between hepatocytes and sinusoidal endothelial cells (SECs). In developing livers, BC structures are progressively generated in the fetal liver, and the continuous network of BC is evident in neonatal livers. Abnormal BC formation caused by genetic mutations eventually lead to cholestasis in the neonatal period. Claudin 1 (CLDN 1) is a gene mutated in neonatal ichthyosis and sclerosing cholangitis (NISCH) syndrome. CLDN1^{-/-} mice showed abnormal paracellular permeability and defects in BC formation (Hadj-Rabia et al. 2004; Grosse et al. 2012). Mice lacking Radixin, a member of ERM (ezrin–radixin–moesin), lacked microvilli and MRP2 on the BC membrane, resulting in defective bile secretion and hyperbilirubinemia (Kikuchi et al. 2002). Mice lacking liver kinase B1 (LKB1, also known as Par4) in hepatocytes (Alb-Cre:LKB1^{flox/flox} mice) lost the BC localization of the bile salt export pump (BSEP) and did not generate the luminal space of IHBD (Woods et al. 2011). Furthermore, the luminal space of IHBD was not generated in Alb-Cre:LKB1^{flox/flox} mice, suggesting that BC formation affects tubular morphogenesis of IHBDs.

The process of BC formation and the underlying molecular mechanisms have been investigated using in vitro culture systems. By sequential treatment with OSM and Matrigel, E14.5 hepatoblasts show hepatocyte morphology and acquire metabolic functions (Fig. 2) (Kamiya et al. 1999, 2002; Kojima et al. 2000). Hepatoblasts first develop typical intercellular junctions, including adherence and tight junctions (TJs) in

the presence of OSM depending on K-Ras (Matsui et al. 2002) and then establish BC-like structures with Matrigel overlay. Overexpression of Par1b induced hepatocyte-type polarity in MDCK cells by orienting microtubules via activation of myosin V (Cohen et al. 2004a,b). The PKA → cAMP → LKB1 cascade activates Par1b during BC formation (Fu et al. 2011). In addition to MDCK cells, we showed that taurocholic acid, which activates the PKA → cAMP → LKB1 cascade (Fu et al. 2011), promotes formation of BC-like structures in hepatocytes derived from neonatal LPCs (Tanimizu et al. 2013). However, it remains unclear whether the hepatocyte-type polarity is directly generated or rearranged from a typical intercellular junction during hepatocyte differentiation and maturation.

BCs are initially generated as discontinuous luminal spaces in hepatocyte clusters, where it turns to the continuous network (Fig. 5A). Given that fetal hepatocytes actively proliferate, it is important to coordinate cell division and extension of the BC network. Recently, it was shown that Par1b contributes to maintaining the hepatocyte-type polarity during cell division by controlling the orientation of microtubules (Fig. 5B1) (Slim et al. 2013). In this model, the spindle orientation tends to be perpendicular to an existing BC and thereby abscission does not perturb the BC. On the other hand, asymmetric cytokinesis occurred to share the existing BC to two daughter cells in a culture of a rat hepatocyte cell line (Fig. 5B2) (Wang et al. 2014). In this model, the BC tubule is eventually generated between two rows of hepatocytes. To examine the effect of hepatocyte–ECM interactions in BC formation, a doublet of rat mature hepatocytes was plated into wells of a culture plate in which the surfaces are separately coated with fibronectin and pluronic acid to make adhesive and nonadhesive surfaces, respectively (Li et al. 2016). When the doublet was inoculated into a well in which the bottom and the side are nonadhesive and adhesive, respectively, the BC space was extended between two cells. It would be interesting to examine whether these mechanisms also operate in vivo to generate the continuous BC network in developing and regenerating livers.

Epithelial Morphogenesis during Liver Development

Figure 5. Hepatocyte morphogenesis. (A) Schematic view of the extension of the bile canaliculus (BC) network. BCs are initially generated within hepatocyte clusters. These BCs turn to the continuous network during the following development of the liver. These illustrations depend on observation for E17 and 18 liver sections stained with anti-ZO1 and HNF4 α antibodies. (B) Models for directional extension of the BC. The BC is maintained during cell division, and then a new one is generated between daughter cells. In this model, BC spaces generated independently are eventually interconnected to form the network (panel 1). The spindle is generated parallel to the existing BC. During the oriented cell division, cytokinesis occurs asymmetrically to maintain the BC. In this model, the BC tubule is extended between two rows of hepatocytes (panel 2). When a doublet of rat hepatocytes is inoculated into a well of a culture plate in which the bottom and the side walls are coated with extracellular matrix and nonadhesive substrate, respectively, a round luminal space is expanded between two cells (the *upper* image in panel 3). On the other hand, when a doublet is plated into a well where the sides are adhesive and the bottom is nonadhesive, a luminal space is elongated between two cells (the *lower* image in panel 3).

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CONCLUDING REMARKS

Intensive studies using 3D culture systems indicate that establishment of apicobasal polarity, de novo lumen formation, and generation of tubular structures could be proceeded within a homogenous cell cluster if environmental factors including ECM components and growth factors are properly supplied to the culture system (Martin-Belmonte et al. 2008; Bryant et al. 2010; Matsumoto et al. 2014). When an organ becomes functional, connections are correctly established among tissue structures consisting of a one-cell population. In the case of liver, the BC network and the luminal network of IHBDs should be properly connected to drain cytotoxic bile into the duodenum. Interestingly, our recent data suggest that such “inter-tissue” interactions accelerate tubular morphogenesis. Thus, in addition to the precise studies about epithelial morphogenesis of homogeneous cell populations, the contribution of inter-tissue interactions to epithelial morphogenesis and organ development should be investigated. Further technical advances for visualizing 3D tissue architectures may help us visualize the process of connection between the hepatic cord and IHBDs in fixed and, hopefully, in living mice. A culture system recapitulating both hepatic cord structures and IHBDs in the same culture condition may also become a powerful tool to understand formation of “liver lobule,” a functional unit of the liver. Such studies are expected to provide important information to understand tissue morphogenesis proceeding in other epithelial organs.

It is well known that the liver has strong regenerative capability. Hepatocytes and cholangiocytes proliferate and replace damaged tissues on acute injuries, whereas they rearrange tissue architectures to adapt the liver to chronically injured situations. In particular, dynamic rearrangements of IHBDs known as ductular reactions are observed in various types of chronic liver injuries. Notably, IHBDs are differentially rearranged depending on types of injuries (Kaneko et al. 2015). However, we do not know the molecular mechanisms determining how IHBDs alter their luminal network. It is also

unknown how cellular properties, including apicobasal polarity are regulated during rearrangement of IHBDs. Comparative studies between tubular morphogenesis of IHBDs and their adaptive rearrangement are expected to reveal novel molecular mechanisms governing formation, maintenance, and regeneration of epithelial tissue structures.

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