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Cell Lineages in Hepatic Development and Molecular Mechanisms of Cell-Cell Interactions Underlying Hepatoblast Differentiation into Mature Hepatocytes and Biliary Epithelial Cells

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ABSTRACT

The endodermal epithelium in the mammalian liver consists of hepatocytes and biliary epithelial cells. Both cell types originate from hepatoblasts, which may be hepatic stem cells in fetal stages. When hepatoblasts are located around the portal veins, they give rise to biliary epithelial cells under an inductive action by portal connective tissues. Nonperiportal hepatoblasts differentiate into mature hepatocytes. Recent studies on naturally mutated or genetically engineered animals have highlighted several new aspects of the hepatoblast differentiation. Mosaic analysis using random inactivation of either paternal or maternal X-chromosome carrying a *spf*^{ash} mutation in the heterozygous females has demonstrated that hepatoblasts may be bipotential for their differentiation potency into hepatocytes and biliary epithelial cells. This idea is strongly supported by the observation that hepatoblasts in *C/EBP*α-knockout mouse livers abundantly develop pseudoglandular structures, which resemble precursor structures for intrahepatic bile ducts (pearl-like structures or ductal plates). The data have also shown that the suppression of C/EBPα expression in periportal hepatoblasts is a key for biliary cell differentiation, which leads to elevation of *HNF6* and *HNF1β* expression. In this review article, molecular mechanisms for bile duct formation are discussed, including roles of the suppression of C/EBPα expression and Jagged1/Notch2 or activin/TGFβ signaling. For hepatocyte maturation and growth, cell-cell interactions such as hepatoblast-stellate or endothelial cell interactions are also required. Our present understanding of their molecular mechanisms is summarized with cell lineages and roles of nonparenchymal cells in liver development.

Keywords: biliary cell differentiation, C/EBPα, hepatic stem cells, hepatocyte maturation, Notch signaling, zonation

CONTENTS

INTRODUCTION

The liver is the largest gland of the body, which has a large number of important functions. It synthesizes bile, which drains the gall bladder via intra- and extrahepatic bile ducts before being released into the duodenum. It is also an important site of metabolism of carbohydrates, proteins and fats. The liver synthesizes and secretes plasma proteins such as fibrinogen and prothrombin (essential blood-clotting substances) into the blood. The functional and structural unit of the liver is known as "liver lobules". Various kinds of cell-cell interactions among several cell types constituting the liver, including hepatocytes, stellate cells, sinusoidal endothelial cells and Kupffer cells, play decisive roles in establishment of the liver lobule. Classical tissue recombination experiments and morphological analysis of liver development have indicated that successive interactions of the hepatic endoderm or hepatoblasts with mesodermal tissues such as the hepatocardiac mesoderm, septum transversum mesenchyme, stellate cells and endothelial cells are essential for liver development (Houssaint 1980; Gualdi *et al*. 1996; Matsumoto *et al*. 2001). Periportal biliary cell differentiation from hepatoblasts can also be induced by connective tissue (Doljanski and Roulet 1934; Shiojiri 1997). Although molecular mechanisms for liver development or hepatic cell-cell interactions have been veiled for a long time, recent approaches using molecular biological techniques, including molecular marking of hepatic cells and gain-of-functions or loss-of-functions experiments both *in vitro* and *in vivo*, have revealed several new aspects of liver development (Kinoshita and Miyajima 2002; Lemaigre and Zaret 2004; Zhao and Duncan 2005). Intriguing animal models such as knockout or transgenic mice, which show impaired liver development, are also leading our study of liver developmental biology (Keng *et al*. 2000; Martinez *et al*. 2000; Sosa-Pineda *et al*. 2000; Lee *et al*. 2005; Zhao and Duncan 2005; Battle *et al*. 2006). New molecular reagents (monoclonal antibodies or cDNAs) are now dramatically in-

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Invited Review

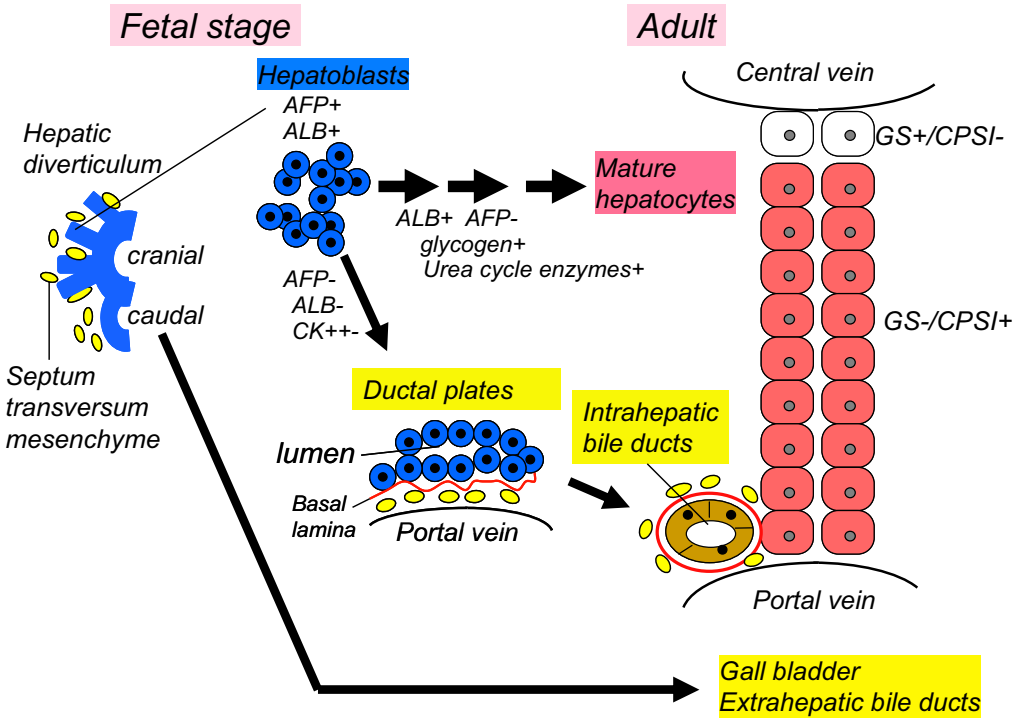

Fig. 1 Cell lineages for the hepatic endodermal cells during development. The cranial portion of the liver primordium gives rise to mature hepatocytes and epithelial cells of intrahepatic bile ducts. The caudal portion gives rise to epithelial cells of the gall bladder and extrahepatic bile ducts. The hepatic ducts originate from the cranial portion, which is not depicted. AFP, α-fetoprotein; ALB, albumin; CK, bile-duct-specific cytokeratin; CPSI, carbamoylphosphate synthase I; GS, glutamine synthase.

creasing from molecular biological analysis such as serial analysis of gene expression (SAGE), signal sequence trap analysis and microarray DNA data (Tanimizu *et al*. 2003; Nonaka *et al*. 2004; Kojima *et al*. 2005). These can specifically characterize liver development (Tanimizu and Miyajima 2004; Kojima *et al*. 2005). This review focuses on our understanding of cell lineages and histogenesis in liver development, and of molecular mechanisms of cell-cell interactions in hepatocyte maturation and biliary cell differentiation, including intra- and extrahepatic bile duct and gall bladder development.

CELL LINEAGES AND HISTOGENESIS OF LIVER DEVELOPMENT

The endodermal epithelium in the mammalian liver consists of hepatocytes and intrahepatic biliary epithelial cells. Both cell types originate from the cranial part of the hepatic diverticulum at E9.5 in the mouse embryo (**Fig. 1**), from which hepatoblasts are extended as cell cords into the subjacent mesenchyme (the septum transversum mesenchyme) or the blood vessels, including the omphalomesenteric veins and posterior cardinal veins (Shiojiri 1997; Shiojiri *et al*. 2006). The caudal part of the hepatic diverticulum gives rise to the epithelium of the gall bladder and the extrahepatic bile ducts (**Fig. 1**). Hepatoblasts, which may be hepatic stem cells in fetal stages, are scattered among hemopoietic cell islands culminating in the fetal mouse liver at midgestational stages.

Immunohistochemical analysis using monoclonal antibody against PECAM-1, which specifically reacts with endothelial cells, has demonstrated that even the fetal liver, in which hemopoiesis culminates, is also well vascularized like adult liver (Matsumoto *et al*. 2001; Shiojiri and Sugiyama 2004). The primitive sinusoidal endothelial cells are already well developed in early stages of liver development, which are supported by stellate cells and extracellular matrix components (Shiojiri and Sugiyama 2004). However, their origin has not been elucidated yet; the septum transversum mesenchyme or the endothelium of the omphalomesenteric veins and the posterior cardinal veins can give rise to endothelial cells of the primitive sinusoid structures (**Fig. 2**). Portal and hepatic veins may develop from the omphalomesenteric veins and posterior cardinal veins, respectively (**Fig. 2**). The expression of the gap junctional protein, connexins, is different among the hepatic blood vessels; connexin37 and 40 are expressed only in the endothelial cells of the portal veins and hepatic arteries, but are never expressed in other blood vessels throughout liver development (Shiojiri *et al*. 2006). Although the hepatic artery is always associated with a branch of the portal vein and also with a branch of the intrahepatic bile duct to form the portal triad in the adult liver, its development has not been examined in detail. The hepatic artery appears to be observed only around large portal veins in fetal mouse livers (Clotman *et al*. 2003). The injection experiments of fluorescent gelatin into the mouse fetus proper through umbilical veins demonstrated that the hepatic blood vessels (the portal veins, primitive sinusoidal structures and hepatic veins) are well connected with one another at early stages, including E11.5 and E13.5 (Sugiyama *et al*. unpublished data). Functional maturation of sinusoidal endothelial cells, which express SE-1 antigen, starts in midgestational stages (Morita *et al*. 1998). Although molecular markers for hepatic sinusoidal endothelial cells had been limited, their possible markers have recently been identified by SAGE, which consist of more than 20 genes (Nonaka *et al*. 2004). Their application in liver developmental studies may be very productive.

The origin of stellate cells has been controversial, but recent cell marking analysis using a transgenic mouse line that express fluorescent protein in all neural crest cells and their derivatives has demonstrated that they do not descend from neural crest cells (Cassiman *et al*. 2006). The stellate cells may derive from the septum transversum mesenchyme or from the mesothelial liver capsule (**Fig. 2**).

When hepatoblasts are located around the portal veins, they commence to construct pearl-like structures (ductal plates) expressing bile-duct-type cytokeratin and several lectin-binding sites in midgestational stages (from E13.5 on in the mouse fetus) (**Fig. 1**). They also have the basal lamina that is positive for laminin and nidogen while hepatoblasts giving rise to mature hepatocytes lack that structure. Our immunohistochemical study using antibodies against carbamoylphosphate synthase I (CPSI) and ornithine transcarbamylase (OTC), both of which are urea cycle enzymes, has demonstrated that cells of pearl-like structures transiently express these enzymes but their expression is suppressed in this cell population within a few days (Shiojiri *et al*. 2001). This result suggests that the final default phenotype of hepatoblasts may be hepatocytes, and the periportal microenvironment may restrict the fate of hepatoblasts to biliary epithelial cells. The pearl-like structures are excessively and

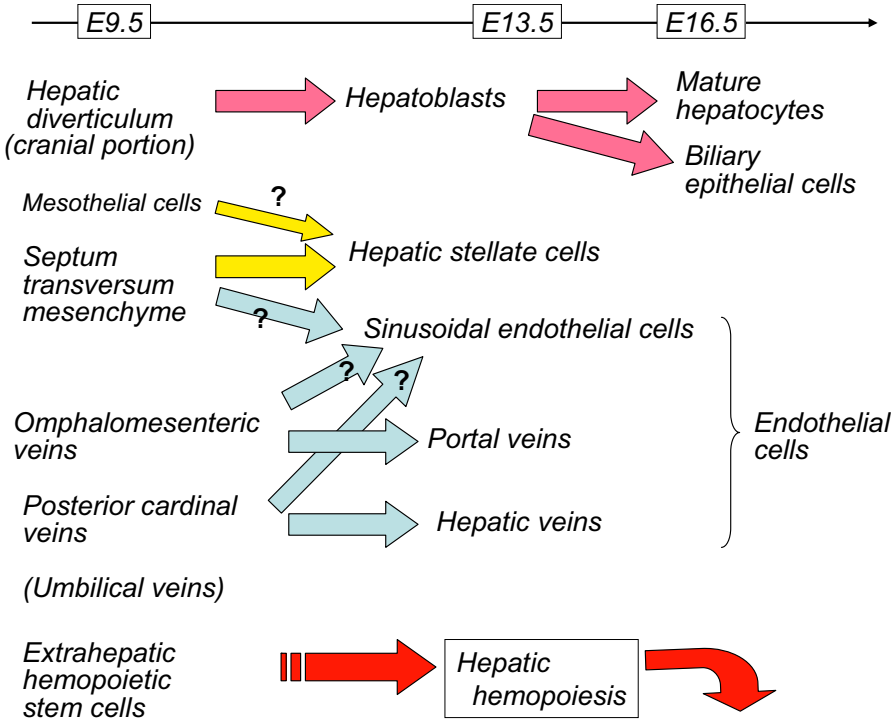

Fig. 2 Cell lineages for nonparenchymal cells during hepatic organogenesis. The origin of the sinusoidal endothelial cells is not clarified.

discontinuously formed around portal veins in the beginning of their formation, but they gradually connect with one another and decrease in number (Shiojiri 1997). These processes could be a stochastic event. Around E16.5, cuboidal or squamous biliary epithelial cells differentiate in the periportal area. Nonperiportal hepatoblasts differentiate into mature hepatocytes.

Hepatocyte maturation starts at E12.5-13.5, including the expression of bile canalicular enzymes, and progressively proceeds. Although urea cycle enzyme expression, including CPSI, starts in hepatocytes around E15.5 with that of other liver-specific markers, it become negative in pericentral hepatocytes within 10 days after birth (Shiojiri *et al*. 1998). Glutamine synthase (GS) expression takes place in pericentral hepatocytes in a few days after birth. In 2-3 weeks old liver, the liver lobule having the adult-type complementary expression of GS and CPSI is established (**Fig. 1**).

The extrahepatic bile ducts (the cystic duct and common bile duct) and the gall bladder develop with the liver primordium formation, and their primordium is located caudally to the latter at E9.5 as described (**Fig. 1**). Although the extrahepatic bile ducts develop independently of intrahepatic bile ducts, the former connects with the latter via the hepatic ducts at midgestational stages, leading to the formation of a confluent bile drainage system.

MOSAIC ANALYSIS OF MURINE LIVER DEVELOPMENT

Because the *spf*^{ash} (sparse fur, abnormal skin and hair) mutation is located on the X chromosome and causes OTC deficiency, hepatoblasts or hepatocytes in the heterozygous females (X/X^{spfash}) are mosaic in the expression of this enzyme due to the random inactivation of either X-chromosome at early stages of development (Shiojiri *et al*. 1997, 2000, 2001). This mosaic animal may be useful for analysis of the cell lineages and growth pattern of hepatoblasts/hepatocytes in liver development. During fetal and postnatal liver development of the heterozygous females, complicated mosaic patterns of OTC-positive and -negative hepatocytes are observed. Sizes of patches, which are aggregates of OTC-positive or -negative hepatocytes, increase during development, suggesting that migration and mixing of hepatocytes appear to be more extensive at fetal stages than in the adult liver. Patches are slender and comparatively simple in fetal and neonatal livers (**Fig. 3**). Quantitative analysis of patch shapes has demonstrated that undulation of patches is maximal at 7 days after birth (Shiojiri *et al*. 2000). Patches with nodular shapes also start to increase in number at this stage. Isolated patches in sections of fetal livers and post-natal livers three-dimensionally connect with one another. However, especially in fetal livers, in which OTC-positive patches are minor due to the presence of abundant hemopoietic cells, isolated three-dimensional patches consisting of 5 to 70 cells are often observed. They are shaped like slender branching or zigzag-shaped cords, but no definite orientation such as portal-central is observed in them at any stage. These results suggest that hepatocytes contiguously but randomly allocate their daughter cells as zigzag-shaped or branching cords at younger stages (**Fig. 3**).

Immunohistochemical analyses of intercellular junction proteins (E-cadherin, occludin, ZO-1, etc.) have shown that their expression and distribution change in hepatocytes during development, which may be correlated with the OTC mosaic pattern (Stamatoglou *et al*. 1992; Shiojiri *et al*. 2000). Adult-type distribution of extracellular matrix components in the liver starts to be established at postnatal stages (at 2-3 weeks after birth) (Shiojiri and Sugiyama 2004).

Mosaic analysis for periportal hepatoblasts of the heterozygous fetal livers has also suggested that hepatoblasts are bipotential for their differentiation into hepatocytes and biliary epithelial cells (Shiojiri *et al*. 2001). There is no special orientation of OTC-positive or -negative patches for periportal hepatoblasts such as ductal plates running along portal veins.

HEPATOCYTE MATURATION AND CELL-CELL INTERACTIONS

Molecular mechanisms of the hepatic induction by the hepatocardiac mesoderm and septum transversum mesenchyme have extensively been studied by using a primary culture system of the foregut endoderm and the gene inactivation technology, or their combinations (Zaret 2002; Lemaigre and Zaret 2004; Zhao and Duncan 2005). The extracellular inductive signals from the hepatocardiac mesoderm and septum transversum mesenchyme are mediated by FGF1, 2 and 8, and BMPs, respectively (Gualdi *et al*. 1996; Rossi *et al*. 2001). Although endothelial cells, which are closely ad-

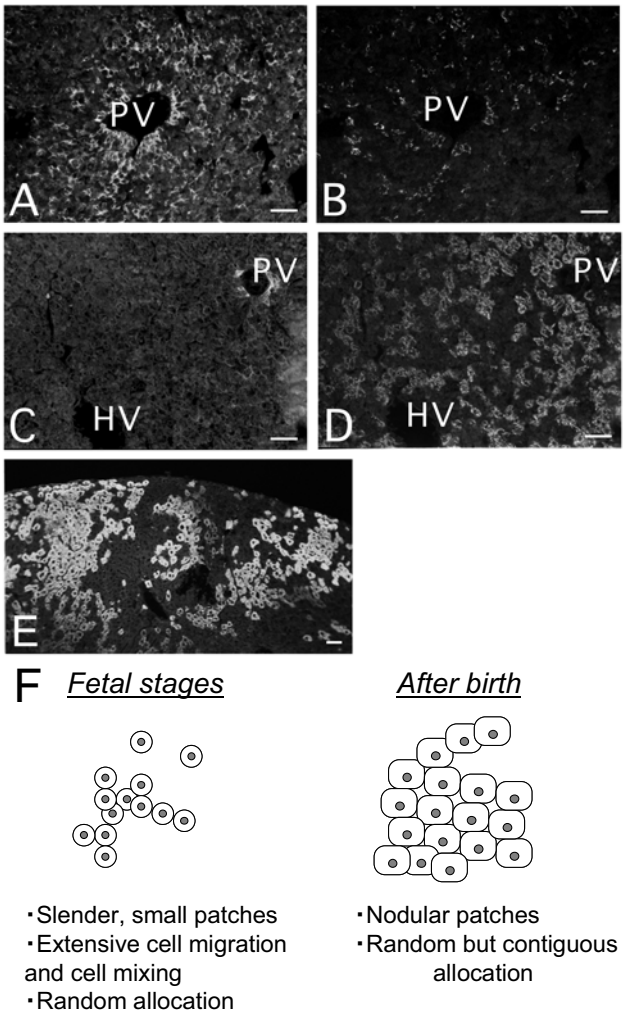

Fig. 3 OTC mosaicism during liver development of the *spf*^{ash}-heterozygous female mouse (B, D, E). (**A, B**) E15.5 liver. (**C, D**) Neonatal liver. (**E**) Adult liver. (**A, C**) Bile-duct-specific cytokeratin immunostaining of **B** and **D**, respectively. Hepatoblasts and periportal biliary epithelial cells in fetal and neonatal livers are positively immunostained for this cytokeratin. Although OTC-positive patches are small and simple in fetal and neonatal livers (**B, D**), those of adult liver are nodular and take complex patterns (**E**). HV, hepatic vein; PV, portal vein. Bar indicates 50 μm. (**F**) Schematic line drawing of developmental changes of the OTC mosaicism.

joining with the hepatic diverticulum, also play an important role in hepatoblast growth and maturation from the hepatic endoderm, the exact molecular players are still unknown at present (Matsumoto *et al*. 2001). The transcriptional factors expressed in the hepatic endoderm or young hepatoblasts include GATA4/6, Hex, Prox1, HNF3α (Foxa1), HNF3β (Foxa2), HNF4α, HNF6 and C/EBPα (Zaret 2002; Lee *et al*. 2005; Zhao and Duncan 2005). Most of their respective knockouts have impaired liver primordium development. Their link to the extracellular inductive ligands has been discussed elsewhere (Zaret 2002; Lemaigre and Zaret 2004; Zhao and Duncan 2005). Calmont *et al*. (2006) have recently revealed that hepatic gene induction is elicited by an FGF/MAPK pathway, and that the inhibition of the PI3K pathway does not block hepatic gene induction, but does block tissue growth.

After invading into the septum transversum mesenchyme, hepatoblasts are still immature, and require various cell-cell or cell-matrix interactions to give rise to mature hepatocytes expressing urea cycle enzymes (**Fig. 4**). Highly purified hepatoblasts exhibit lower viability, growth and maturation *in vitro* (Nitou *et al*. 2002). Although it has been well known that glucocorticoids play a pivotal role in hepatocyte maturation, recent studies have demonstrated that oncostatin M (OSM), an interleukin 6-related cytokine, which is emanated from hemopoietic cells, accelerates hepatocyte maturation (Kamiya *et al*. 1999; Kinoshita and Miyajima 2002) (**Fig. 4**). That stimulation depends on STAT3. Hepatocyte growth factor (HGF) also in the presence of dexamethasone induces expression of liver-specific enzymes, although to a lesser extent than OSM (Kamiya *et al*. 2001). HGF does not activate STAT3 and HGF-induced differentiation is independent of STAT3. While OSM expression in the liver starts in midgestational stages and decreases in postnatal stages, HGF is mainly expressed in the liver in the first few days after birth. TGFβ might also be a possible soluble regulator of liver development. Matrigel, a gel containing basal laminar components, also stimulates hepatocyte maturation in fetal hepatocyte culture, suggesting that extracellular matrices can control hepatocyte maturation in liver development (Kamiya *et al*. 1999) (**Fig. 4**).

The Miyajima group recently highlighted the specific role for *K-Ras* in regulation of homophilic adhesion during OSM-induced hepatocyte maturation (Matsui *et al*. 2002). By retroviral expression of dominant-negative forms of signaling molecules, Ras was shown to be required for the OSM-induced adherens junction formation. Fetal hepatocytes derived from *K-Ras* knockout mice fail to form adherens junctions in response to OSM, whereas adherens junction formation is induced normally by OSM in mutant hepatocytes lacking both *H-Ras* and *N-Ras*. Moreover, the defective phenotype of *K-Ras*-/- hepatocytes is restored by expression of *K-Ras*, but not by *H-Ras* and *N-Ras*. From DNA microarray analysis using mRNA from fetal hepatocytes, neuritin, a GPI-anchored protein, is found as a membrane protein expressed in hepatocytes (Kojima *et al*. 2005). Its expression increases along liver development and the maximum expression is established from the neonatal and adult stage, and may be a useful tool to define the differentiation stage of hepatocytes.

By contrast, TNFα may negatively regulate fetal hepatic maturation stimulated by OSM and Matrigel (Kamiya and Gonzalez 2004) (**Fig. 4**). TNFα is expressed in the prenatal and postnatal liver but not in adult liver, whereas TNFR1, a TNFα receptor, is expressed in both fetal and adult livers. TNFα suppresses expression of mature liver-specific genes such as tyrosine aminotransferase and apolipoproteins. In addition, the expression of hemopoietic cytokines and cyclin A2, repressed by OSM and Matrigel, is induced by TNFα in the fetal hepatic cultures coincident with cell division. Downregulation of HNF4α expression may be involved in the mechanism of suppression of hepatic maturation by TNFα. It remains to be clarified which hepatic cell type produces TNFα in fetal livers.

Although some cytokines or growth factors, or extracellular matrices have already been elucidated to be involved in liver development as described, our knowledge of additional regulators underlying hepatic cell-cell interactions and the exact cell types producing such maturation regulators is still limited (**Fig. 4**).

Several studies using liver tumors have shed light on molecular mechanisms on the complementary GS and CPSI expression in the liver lobule (Loeppen *et al*. 2002; Ovejero *et al*. 2004; Hailfinger *et al*. 2006). Based on observations of differential expression of genes in liver tumors from mice that harbor activating mutations in either *Catnb* (which codes for β-catenin) or *Ha-Ras*, a model for zonal heterogeneity in normal liver is proposed (Hailfinger *et al*. 2006). In this model, the regulatory control consists of two opposing signals, one delivered by endothelial cells of the central veins activating a β-catenin-dependent pathway (retrograde signal), the other by blood-borne molecules activating Ras-dependent downstream cascades (anterograde signal).

Sekine *et al*. (2006) have indicated that, in conditional knockouts of β-catenin gene in hepatocytes, genes for required for glutamine synthesis, including those of GS, ornithine aminotransferase and glutamate transporter 1, are downregulated as well as cytochrome P450 enzymes. Benhamouche *et al*. (2006) have also provided compelling evidence that the Wnt/β-catenin pathway plays a key role in the

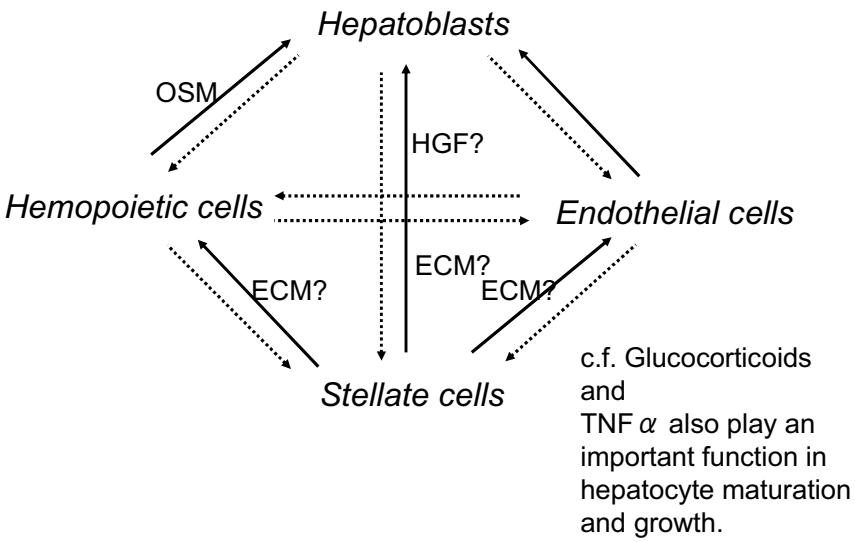

Fig. 4 Cellular interactions and extracellular signaling molecules in hepatocyte maturation and growth during development. Although each hepatic cell type intimately interacts with one another during liver development, molecular players such as growth factors, cytokines and extracellular matrices are still poorly known. Solid lines indicate proved cell-cell interactions, whereas dotted lines indicate a hypothetical action.

hepatic zonation. The complementary localization of activated β-catenin in the pericentral area and the negative regulator Apc in periportal hepatocytes is found, which is well consistent with that of the ammonia-metabolizing enzymes. The authors also demonstrated that Wnt/β-catenin signaling inversely controls the pericentral and periportal genetic programs by analyzing the immediate consequences of either a liver-inducible Apc disruption or a blockade of Wnt signaling after infection with an adenovirus encoding the Wnt antagonist, Dkk1. Genes involved in the periportal urea cycle and the pericentral glutamine synthesis systems are critical targets of β-catenin signaling (Benhamouche *et al*. 2006; Werth *et al*. 2006). It should be demonstrated in the next step whether endothelial cells of the central veins really emanate Wnt signals or not, or whether the Wnt signals are enough for that restricted expression of the glutamine synthesis pathway in pericentral hepatocytes.

MECHANISMS OF BILIARY CELL DIFFERENTIATION AND BILE DUCT MORPHOGENESIS

Periportal hepatoblasts have been demonstrated to give rise to biliary epithelial cells under an inductive action from the subjacent connective tissue, but its molecular mechanisms have not been fully understood. Mutations in the *Jagged1* gene, which encodes a ligand for Notch family receptors, are associated with Alagille syndrome, a human autosomal dominant developmental disorder characterized by liver, heart, eye, skeletal, craniofacial and kidney abnormalities. In this syndrome, the liver displays a paucity of intrahepatic bile ducts. Recent studies using mice of doubly heterozygous for the *Jagged1* null allele and a *Notch2* hypomorphic allele have demonstrated that they exhibit developmental abnormalities characteristic of Alagille syndrome, and also show absence of intrahepatic bile ducts (McCright *et al*. 2002). Jagged1 signals emanated in endothelial cells of the portal veins may induce periportal hepatoblasts to differentiate into biliary epithelial cells through Notch2-expressing connective tissue cells (**Fig. 5**). However, other roles or mechanisms in Jagged1-Notch2 signaling should be considered in biliary cell differentiation because biliary epithelial cells and their progenitors also express both Jagged1 and Notch2. Loomes *et al*. (2007) have indicated a role for the Notch pathway in postnatal growth and remodeling of the bile ducts by analyzing mice heterozygous at the *Jagged1* locus for both the null and conditional alleles (the liver-specific *Jagged1* conditional knockout made under the control of the *albumin* promotor and *α-fetoprotein* enhancer). An *in vitro* study with fetal hepatoblasts has demonstrated that active Notch signaling could inhibit hepatic differentiation and significantly reduce the expression of albumin (Tanimizu and Miyajima 2004). By contrast, downregulation of the Notch signaling can promote the hepatic differentiation. Activation of the Notch signaling also upregulates HNF1β expression, which is consistent with the differentiation state of biliary epithelial cells, whereas it downregulates the expression of liver-enriched transcription factors, HNF1α, HNF4α and C/EBPα (Tanimizu and Miyajima 2004). On the other hand, Clotman *et al*. (2005) have elegantly demonstrated that a gradient of activin/TGFβ signaling modulated by onecut transcription factors (HNF6) is required to segregate the hepatocytic and biliary lineages (**Fig. 5**). How activin/TGFβ and Notch2 signalings synergistically act on biliary cell differentiation is one of important questions to be resolved in the future.

It has been shown that *HNF6* knockout fetuses and fetuses with a liver-specific inactivation of the *HNF1β* gene fail to form intrahepatic bile ducts. Their knockouts exhibit transient cystic structure development, in which biliary epithelial cells remain irregularly arranged around the branch of the portal veins (Clotman *et al*. 2002; Coffinier *et al*. 2002). Because HNF6 upregulates the transcription of the *HNF1β* gene (Clotman *et al*. 2002; Coffinier *et al*. 2002), it is reasonable that respective knockouts exhibit similar abnormalities in intrahepatic bile duct development. In normal intrahepatic bile duct development, their expression is confined to periportal pearl-like structures in midgestational stages, and biliary epithelial cells in later stages. Several genes such as *PKHD1*, which cause polycystic kidney diseases and work for ciliary functions, are also known to be involved in biliary cyst formation (Lemaigre and Zaret 2004). Furthermore, it is noteworthy that *inv* mice at neonatal stages have still the immature state for intrahepatic bile duct development as ductal plates or pearl-like structures in addition to abnormal extrahepatic bile duct formation, which results in severe jaundice (Mazziotti *et al*. 1999). The *inv* gene codes for inversin, a protein involving ciliary functions, and inactivation of this gene induces the situs inversus with polycystic kidney diseases as other abnormalities. Thus, in intrahepatic bile duct morphogenesis, ciliary genes may be important and might be downstream of HNF6 and HNF1β signaling (**Fig. 5**). It may be curious that intrahepatic bile duct development in *inv* mice remains as ductal plates instead of cystic formation as seen in the extrahepatic bile duct and kidneys.

We have shown that C/EBPα, which is a basic-leucine zipper transcription factor and upregulates the transcription of liver-specific genes, is downregulated in biliary epithelial cells during liver development, suggesting that the suppression of C/EBPα expression may be a prerequisite to biliary cell differentiation (Shiojiri *et al*. 2004). This idea is also supported by the observation that hepatoblasts in *C/EBPα*-knockout mouse livers abundantly develop pseudoglandular structures, which resemble precursor structures for intrahepatic bile ducts (pearl-like structures or ductal plates) (Tomi-

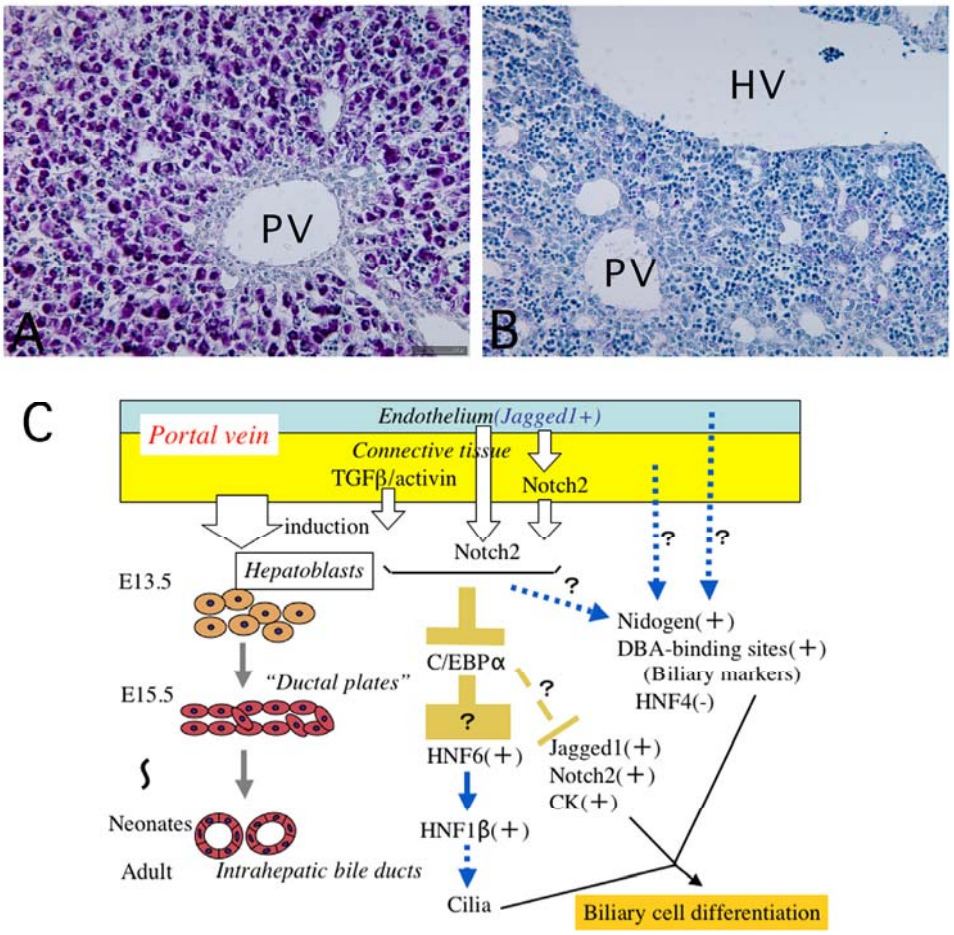

Fig. 5 The inactivation of *C/EBPα* gene induces pseudoglandular structures in the liver parenchyma at E17.5. (**A**) Wild-type liver. (**B**) Null liver. Periodic acid-Schiff (PAS)-hematoxylin staining. HV, hepatic vein; PV, portal vein. Bar indicates 50 μm. (**C**) Molecular mechanisms for bile duct formation. Portal tissues induce adjacent hepatoblasts to differentiate into biliary epithelial cells through suppression of their C/EBPα expression.

zawa *et al*. 1998; Yamasaki *et al*. 2006) (**Fig. 5**). In these pseudoglandular structures, *HNF6* and *HNF1β* mRNA are upregulated. The data also showed that the suppression of C/EBPα expression in periportal hepatoblasts is a key for biliary cell differentiation, which may result in elevation of *HNF6* and *HNF1β* expression (**Fig. 5**). C/EBPα can bind to regulatory sequences of the *HNF6* gene and block its transcription (Rastegar *et al*. 2000), though it is still possible that C/EBPα can indirectly suppress transcription of the *HNF6* gene.

Conditional deletion of the mouse *Forkhead Box* (*Fox*) *m1b* targeted allele in adult hepatocytes has demonstrated that the *Foxm1b* transcription factor is essential not only for hepatocyte mitosis during liver regeneration, but also for intrahepatic bile duct development (Krupczak-Hollis *et al*. 2004). Fetal *Foxm1b* knockout livers display a 75% reduction in the number of hepatoblasts, and have reduced hepatoblast mitosis, which contributes to abnormal liver development with significant reduction in the number of large hepatic veins compared to wild-type livers. Fetal *Foxm1b* knockout livers do not develop intrahepatic bile ducts, and these presumptive biliary hepatoblasts fail to express either biliary cytokeratins or nuclear levels of HNF1β. *Foxm1b* may be critical for hepatoblast precursor cells to differentiate toward biliary epithelial cell lineage. It is intriguing to examine the development of portal veins and hepatic veins in this animal model, which can unveil a novel mechanism of cell-cell interactions in bile duct development.

Although the hepatic artery is always developed around the portal veins with the intrahepatic bile ducts in normal adult livers, agenesis or dilation of the hepatic artery is induced with the abnormal intrahepatic bile duct development in *HNF6* and *HNF1β* knockout mouse livers (Clotman *et al*. 2002; Coffinier *et al*. 2002). These results suggest that bile duct formation may be tightly coupled with the portal triad development (Clotman *et al*. 2003). The molecular mechanism underlying this coupling is unknown.

Though the extrahepatic bile ducts and gall bladder develop independently of intrahepatic bile ducts, expression of the pancreatic transcription factor Pdx1 marks both extra- and intrahepatic biliary tracts. The epithelial cells of the extrahepatic biliary tract, including those of the gall bladder, weakly express Pdx1, whereas those of intrahepatic biliary tract do not express Pdx1 (Sumazaki *et al*. 2004), suggesting that the former is close to the pancreas in its differentiation state. Inactivation of *Hes1* (bHLH transcription factor working downstream of Notch signaling) gene induces pancreatic conversion of extrahepatic bile duct cells in addition to gall bladder agenesis and severe hypoplasia of extrahepatic bile ducts (Sumazaki *et al*. 2004). The absence of *Hes1* may not affect intrahepatic bile duct development according to the work by Sumazaki *et al*. (2004). Biliary epithelium in *Hes1* knockout mice ectopically expresses the proendocrine gene *Neurog3*, differentiates into endocrine and exocrine cells and forms acini and islet-like structures in the mutant bile ducts. *Hes1* may determine extrahepatic biliary organogenesis by preventing the pancreatic differentiation program, probably by directly repressing transcription of *Neurog3*.

Knockout mice for genes of *HNF6* and *HNF1β* also display deficient development of the extrahepatic bile ducts and gall bladder (Clotman *et al*. 2002; Coffinier *et al*. 2002). These data also agree well with the fact that HNF6 upregulates the transcription of *HNF1β* gene (Clotman *et al*. 2002; Coffinier *et al*. 2002). The crosstalk between Notch signaling and the HNF6-HNF1β cascade should be clarified in the future, including that in intrahepatic bile duct development.

inv newborn mice have abnormalities in extrahepatic bile duct development; anatomic variation of the extrahepatic biliary system and extrahepatic biliary obstruction leading to jaundice (Mazziotti *et al*. 1999). The anomaly is often accompanied by cystic dilation of the extrahepatic bile duct. As inversin is important in ciliary functions and is involved in polycystic kidney diseases, the absence of this molecule may induce cystic formation of the extrahepatic

bile duct like in kidneys. HNF6 and HNF1β might be upstream of inversin action also in extrahepatic bile duct development.

Haploinsufficiency of the *Forkhead Box f1* (*Foxf1*) transcription factor gene is known to cause abnormal gall bladder formation with perinatal lethality from lung hemorrhage in a subset of *Foxf1*+/- newborn mice (Kalinichenko *et al*. 2002). *Foxf1* is expressed in embryonic septum transversum mesenchyme and gall bladder mesenchyme. Its disruption (null) results in embryonic lethality at midgestational stages. *Foxf1*+/- gall bladders are significantly smaller than those of wild-type mice and have severe structural abnormalities characterized by a deficient external smooth muscle cell layer, reduction in mesenchymal cell number, and in some cases, lack of a discernible biliary epithelial cell layer. *Foxf1* appears to regulate the transcription of genes that are critical for cell adhesion, migration, and mesenchymal cell differentiation. The integrin β3 subunit gene is a direct transcriptional target of *Foxf1* protein (Malin *et al*. 2007). *Foxf1*+/- newborn mice have normal liver and intrahepatic bile ducts.

CONCLUDING REMARKS

This review summarized our understanding of cell lineages in liver development and mechanisms of cell-cell interactions during hepatoblast differentiation into mature hepatocytes and biliary epithelial cells at present. A large number of knockout or transgenic mice that are targeted to induce impaired liver development has been developed, and is being made. Their use is one of the right ways to elucidate molecular mechanisms of liver development because the liver is a complex organ, and it is not easy to construct the three-dimensional hepatic architecture *in vitro*. Although not all knockout mice may be useful among them for our analysis, their mating may generate interesting animal models to examine interactions among genes that may be important for liver development. Further gene inactivation studies such as conditional or site-directed mutagenesis in mice can unveil molecular mechanisms of liver development as a molecular scalpel. Development of new molecular reagents that characterize molecular machineries of each hepatic cell type, including its cell surface antigens, is also still required in analyzing abnormalities of the hepatic architecture as well as the normal architecture. Especially, we have shortage of molecular markers, the expression of which is restricted only for liver nonparenchymal cells. Such reagents can also allow us to purify or exclude special sets of hepatic cell types in liver development. Furthermore, coupling of a primary culture technique with genetic studies of mutant animals can link extracellular signals to intracellular signaling pathways that trigger hepatic cell differentiation and morphogenesis. Gene transfer technology utilizing viral vectors will expand the molecular analysis of liver development. The developmental studies focusing on the cellular interactions and molecular mechanisms of liver development can also lead to construct three-dimensional liver tissues from embryonic stem cells, and may be invaluable for the regenerative medicine.

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