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Organogenesis and Development of the Liver

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Embryonic development of the liver has been studied intensely, yielding insights that impact diverse areas of developmental and cell biology. Understanding the fundamental mechanisms that control hepatogenesis has also laid the basis for the rational differentiation of stem cells into cells that display many hepatic functions. Here, we review the basic molecular mechanisms that control the formation of the liver as an organ.

The liver is the largest gland in the body exhibiting both endocrine and exocrine properties. Endocrine functions include the secretion of several hormones such as Insulin-like growth factors, Angiotensinogen, and Thrombopoietin, while the major exocrine secretion is in the form of bile. The liver is also essential for glycogen storage, drug detoxification, control of metabolism, regulation of cholesterol synthesis and transport, urea metabolism, and secretion of an extensive array of plasma proteins including Albumin and Apolipoproteins. Since the liver is such an important regulator of normal physiological processes, liver disease, such as hepatic fibrosis, cirrhosis and hepatitis, and hepatocellular carcinoma, results in high rates of morbidity and mortality, so much so that liver disease is the fourth leading cause of death among middle-aged adults in the United States. The high economical and health burden resulting from liver disease has prompted a call to increase understanding of the basic developmental mechanisms that control liver cell differentiation and function (Action Plan for Liver Disease Research: http://www2.niddk.nih.gov/).

The advances made over the last two decades of hepatic research have been substantial. The fact that liver mass accounts for between 2%–5% of body weight and because the majority of cells within the liver are hepatocytes made the liver highly accessible for the purification of proteins using traditional biochemical procedures. This included the isolation of transcription factors, growth factors, signaling molecules, and hormones that were challenging to identify in other organs and tissues. During the latter half of the 20th century, facilitated by the explosion of molecular biology, our depth of understanding of control of gene expression within the context of the hepatocyte was arguably better understood than within in any other cell type. Many technologies that became key to the investigation of cell function in general, such as the production of cDNA libraries, electromobility shift assays to identify DNA binding proteins, and in vivo DNA footprinting to identify the occupancy of transcription factors within promoters, were first established in the liver. In addition to facilitating rapid advances in the study of gene expression, the ability of the liver to regenerate in response to insult also allowed identification of the mechanisms that regulate mammalian cell proliferation, cell cycle, and DNA repair in vivo. Most of these advances, whether it be in understanding control of gene expression or cell proliferation, describe fundamental mechanisms that are applicable to all aspects of biology.

Architecture of the Liver

To understand the molecular basis of hepatogenesis it is first necessary to first consider the structure of the adult liver. In contrast to most complex organs, histological sections through the liver reveal a rather homogeneous landscape of hepatocytes periodically infiltrated with vascular tissue and bile ducts (Figure 1A). This somewhat bland histological appearance masks an extremely complex and under appreciated tissue architecture that is crucial for normal hepatic function (Figures 1B and C). The basic architectural unit of the liver is the liver lobule. The lobule consists of plates of hepatocytes lined by sinusoidal capillaries that radiate toward a central efferent vein. Liver lobules are roughly hexagonal with each of six corners demarcated by the presence of a portal triad of vessels consisting of a portal vein, bile duct, and hepatic artery (Figure 1B). Both the portal vein and hepatic artery supply blood to the lobule, which flows through a network of sinusoidal capillaries before leaving the lobule through the central vein. Although hepatocytes are the major parenchymal cell type of the liver and account for 78% of liver volume (Blouin et al., 1977), they function in concert with cholangiocytes (biliary epithelial cells), endothelial cells, sinusoidal endothelial cells, Kupffer cells (resident liver macrophages), pit cells (natural killer cells), and hepatic stellate cells (see Table 1).

The hepatocytes, which are polarized epithelial cells, are arranged as cords that are one cell thick in mammals. The basolateral surfaces of the hepatocyte face fenestrated sinusoidal endothelial cells, which facilitates the transfer of endocrine secretions from the hepatocytes into the blood stream (Figure 1C). Tight junctions formed between neighboring hepatocytes generate a canaliculus that surrounds each hepatocyte and is responsible for collection of bile acids and bile salts that are transported across the hepatocyte's apical surface. Bile collected by the canaliculi is carried to the bile ducts within the portal triad and subsequently transported for storage in the gall bladder. As shown in Figure 1C, the complex arrangement between the polarized hepatocytes with the capillaries and cholangiocytes underlies both endocrine and exocrine functions of the liver. The challenge facing developmental biologists is to understand the molecular events that lead to the generation of each cell type within the liver and to determine how the cells arrange to form the three-dimensional architecture that is so crucial for hepatic function.

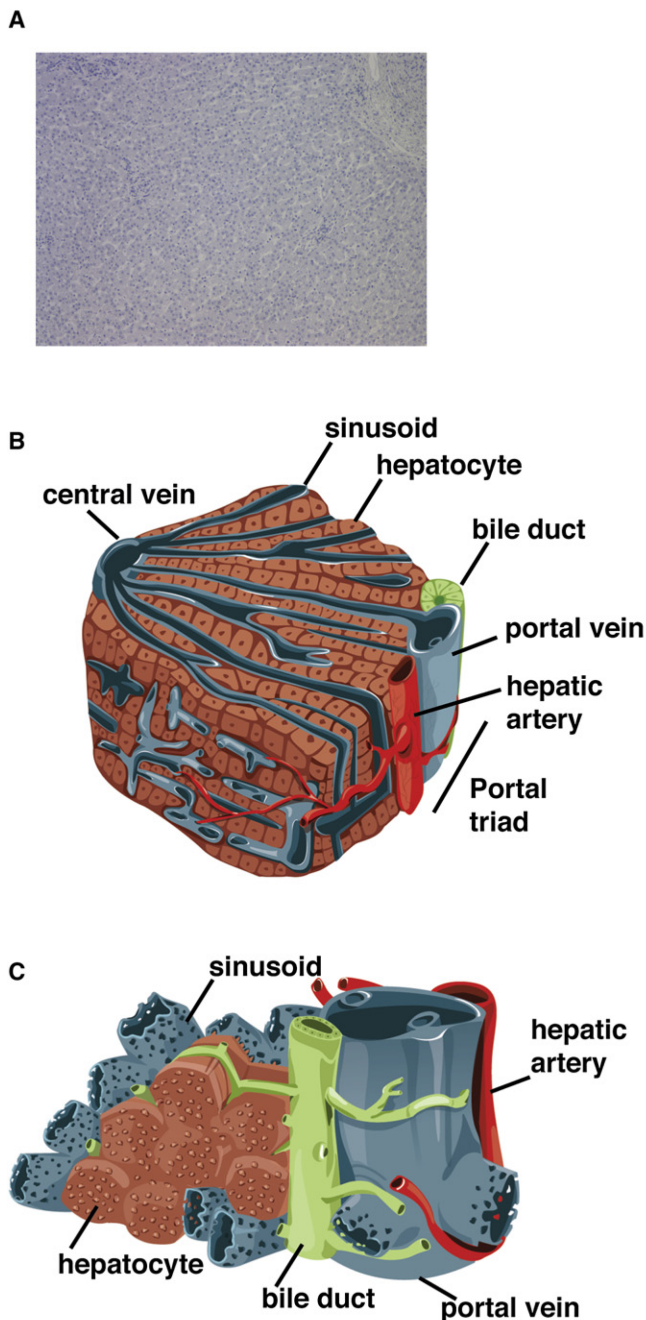

Figure 1. Architecture of the Liver
(A) Hematoxylin-stained section through a human liver showing homogenous distribution of cells.
(B) Illustration showing overall structure of a portion of a liver lobule.
(C) Higher resolution of the relationship between key cellular compartments of the liver.

Onset of Hepatic Development

Signaling Pathways Controlling Onset of Liver Parenchymal Cell Differentiation

The parenchymal cells of the liver derive from the anterior portion of the definitive endoderm (Figure 2), which itself is established in the embryo during gastrulation (Le Douarin, 1975). Labeling of anterior endoderm in mouse embryos with vital dyes has revealed the presence of three distinct domains of hepatic progenitor cells that are located in the medial and bilateral regions of the foregut (Tremblay and Zaret, 2005). As the foregut closes, the progenitor cells within these regions converge to lie adjacent to the developing heart and in close apposition to regions of lateral plate mesoderm that will ultimately generate the mesothelial cells of the proepicardium and septum transversum. Coculture studies using either chick or mouse embryos demonstrated that the developing cardiac mesoderm plays a crucial instructive role during the induction of hepatic cell fate (when the embryo has generated roughly 7–8 pairs of somites; LeDouarin, 1964; Houssaint, 1980; Gualdi et al., 1996). Using modern molecular techniques these inductive signals were found to be members of the fibroblast growth factor family, since FGF1 and FGF2 could substitute for cardiac tissue in inducing the onset of Albumin expression, a characteristic marker of hepatic cell fate, in explants of mouse anterior endoderm (Gualdi et al., 1996; Jung et al., 1999). Moreover, FGF-mediated specification of hepatic cell fate is concentration dependent and this appears to be controlled by the position of the endoderm relative to the heart, which is the major source of hepatogenic FGF (Deutsch et al., 2001; Serls et al., 2005).

The induction of hepatic gene expression by FGF is controlled specifically through activation of the MAPK pathway and is independent of PI3K signaling (Calmont et al., 2006). Several FGFs including *Fgf1*, *Fgf2*, *Fgf8*, and *Fgf10* are expressed in the cardiac mesoderm during the onset of hepatogenesis, and studies of knockout mice suggest that functional redundancy exists between these factors in controlling liver development (Miller et al., 2000). Nevertheless, the requirement for FGF signaling in controlling the onset of liver development is evolutionarily conserved, with FGFs displaying hepatogenic properties in *Xenopus*, chick, and *Zebrafish* embryos (Chen et al., 2003; Shin et al., 2007; Zhang et al., 2004).

Coculture studies using either chick-quail grafts or mouse embryos uncovered a mesenchymal cue necessary for early hepatic development (LeDouarin, 1968; Houssaint, 1980). A cluster of mesenchymal cells that give rise to the mesothelium of the peritoneal cavity, the cardiac epicardium, as well as aspects of the septum transversum, arises from lateral plate mesoderm and is closely associated with the pre-hepatic endoderm. Several lines of evidence support the contention that the septum transversum mesenchyme is required for early stages of hepatic development, when the embryo has only generated about 2–4 somites. GATA4, a zinc finger transcription factor, is robustly expressed in septum transversum mesenchymal cells that surround the liver bud. Examination of *Gata4*$^{-/-}$ embryos revealed that the septum transversum and proepicardial mesenchyme is absent in these embryos (Watt et al., 2004) and that the liver bud fails to expand (Watt et al., 2007). GATA4 regulates expression of the secreted bone morphogenetic protein, BMP4 (Nemer and Nemer, 2003), which, like GATA4, is highly expressed in the septum transversum mesenchymal cells at the eight somite stage of mouse development (Rossi et al., 2001). Analyses of *Bmp4*$^{-/-}$ mouse embryos also revealed a delay in expansion of the liver bud and addition of BMP inhibitors were found to block hepatic specification in vitro (Rossi et al., 2001). Although these data support a model whereby GATA4 controls

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Table 1. Predominant Cell Types and Their Functions within the Adult Liver

Cell Type	Position in Liver	Function
Hepatocyte	Parenchyma	~70% of liver cell population Protein secretion Bile secretion Cholesterol metabolism Detoxification Urea metabolism Glucose/glycogen metabolism Acute phase response Blood clotting
Cholangiocyte/bile duct cell	Duct epithelium	~3% of liver cell population Form bile ducts to transport bile Control rate of bile flow Secrete water and bicarbonate Control pH of bile
Endothelial cell	Vasculature	Form veins, arteries, venuoles, and arterioles Control blood flow Contribute toward parenchymal zonation
Liver sinusoidal endothelial cell	Sinusoids	~2.5% of lobular parenchyma Form sinusoidal plexus to facilitate blood circulation Highly specialized Allow transfer of molecules and proteins between serum and hepatocytes Scavenger of macromolecular waste Cytokine secretion Antigen presentation Blood clotting
Pit cell	Liver natural killer cells	Rare Cytotoxic activity
Kupffer cell	Sinusoids	~2% of liver Scavengers of foreign material Secrete cytokines and proteases etc.
Hepatic stellate cell	Perisinusoidal	~1.4% of liver cells Maintenance of extracellular matrix, Vitamin A, and retinoid storage Controls microvascular tone Activated to become myofibroblast Contributes toward regenerative response to injury Secretion of cytokines

BMP4 expression in the septum transversum to regulate early hepatic development, it is worth noting that both factors are expressed in other cell types in the immediate vicinity and, as is the case for FGFs, several members of the BMP family are also present, including BMP2. Nevertheless, the requirement for both GATA4 and BMP4 in controlling early liver development is conserved, since both are essential for hepatogenesis in zebrafish (Holtzinger and Evans, 2005; Shin et al., 2007).

Recent studies in mouse embryos have also highlighted the highly dynamic nature of the signaling events that control hepatic specification (Wandzioch and Zaret, 2009). Using a combination of mouse embryo culture, conditional genetic ablation, and pharmacological intervention, Zaret and colleagues provided evidence to support a model whereby TGFβ signaling acts to restrict endoderm specification as cell movements position progenitors within the appropriate inductive environments. Moreover, the authors found that there is a measure of flexibility on the relative timing of BMP and FGF signaling that induces distinct populations of hepatic progenitors to differentiate. In the proposed model, TGFβ acts as a developmental timer to ensure that the endoderm retains hepatic competency and is prevented from inappropriately differentiating.

The WNT signaling pathway has also been implicated during the onset of hepatic development, although in contrast to a clear inductive role for FGF and BMP, the contribution of WNT signaling appears to be complex. Studies in *Xenopus* have shown that canonical WNT signaling makes different contributions depending on the developmental stage (McLin et al., 2007). At early somite stages WNT signaling acts in the posterior endoderm to repress expression of *Hhex*, an essential transcriptional regulator of hepatic development. If canonical WNT signaling is blocked in the posterior endoderm it results in ectopic liver development. Repression of WNT signaling by expression of WNT antagonists in the anterior endoderm is, therefore, required to relieve repression of *Hhex* in the anterior endoderm and so facilitate commitment of the endoderm to a hepatic fate. In contrast to the repressive effects of WNTs at early somite stages, following specification, WNT signaling appears to promote hepatogenesis in multiple systems including *Xenopus* (McLin et al., 2007) and zebrafish (Ober et al., 2006;

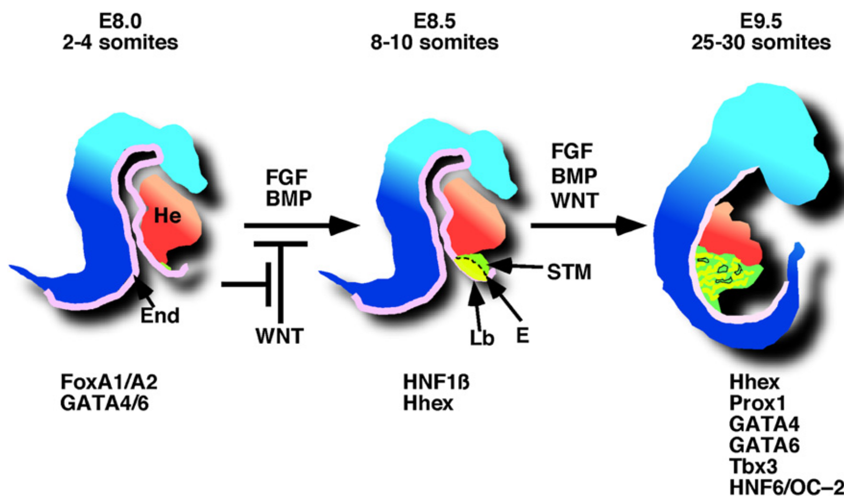

Figure 2. Mechanisms Controlling Early Development of the Hepatic Parenchymal Cells

Illustration showing the onset of differentiation of liver parenchymal cells indicating signaling molecules and transcription factors with proven regulatory roles. Early functions of Wnt signaling promote posterior endodermal identity at the expense of anterior (e.g., prospective hepatic) identity, and must be inhibited anteriorly by local Wnt antagonist expression before liver development can proceed further. Later functions of Wnt signaling act in parallel with BMP and FGF signaling to drive hepatic specification, expansion, and differentiation. Foregut endoderm (End; pink), heart (He; red), liver bud (Lb; yellow), septum transversum mesenchyme/lateral plate mesoderm (STM; green), and vascular endothelial cells (E; black).

Goessling et al., 2008). In the case of zebrafish, Wnt2b was identified as a being essential for the onset of differentiation of the hepatic progenitor cells. This factor is expressed in the lateral plate mesoderm that is positioned adjacent to the endoderm that is destined to become the fish liver. Interestingly, although Wnt2bb mutants fail to initially specify hepatic progenitors, most embryos recover to form normal livers and develop into adult fish (Ober et al., 2006). This implies that additional mechanisms exist that can compensate for Wnt2bb signaling in zebrafish. Whether the compensatory growth of the liver occurs through a response of the original liver field or whether other regions of the gut adopt a liver cell fate in response to loss of Wnt2bb is an outstanding question.

Transcriptional Control of Hepatic Progenitor Cell Differentiation

In addition to identifying signaling molecules, a substantial effort has been devoted to describing the transcription factors that control the initial stages of hepatic development (for reviews see Zaret, 2008; Lemaigre, 2009; Hannenhalli and Kaestner, 2009). Although expressed at low levels, Albumin is one of the best characterized markers of nascent hepatic cells (Cascio and Zaret, 1991). DNA footprinting analyses of the transcriptional regulatory elements controlling the onset of *Albumin* expression during hepatic development revealed that FoxA and GATA4 both of which are expressed in the anterior endoderm, were capable of binding to the *Albumin* enhancer before the onset of *Albumin* expression (Gualdi et al., 1996; Bossard and Zaret, 1998). Both FoxA and GATA4 have the capacity to interact with their respective binding sites in the context of compacted chromatin (Cirillo et al., 1998, 2002; Cirillo and Zaret, 1999) and the binding of these factors results in displacement of linker histone H1 and repositioning of nucleosomes. This has led to the model that such transcription factors can act as ‘‘pioneer’’ factors to mark domains of chromatin as competent to be expressed in response to appropriate developmental cues (reviewed in Kaestner, 2005). Recent studies have shown that FoxA has an unusually slow nuclear mobility which is consistent with high non-specific nucleosome binding (Sekiya et al., 2009). It has been suggested that the slow nuclear mobility of FoxA may facilitate its ability to probe sites in nuclear DNA that are inaccessible to conventional transcriptional activators due to the presence of chromatin, which would be consistent with FoxA acting as a pioneer factor. Further support for this model also comes from the finding that mice lacking both FoxA1 and FoxA2, which are functionally redundant, fail to initiate development of the hepatic lineage both in mice and in response to FGF treatment of cultured endoderm (Lee et al., 2005). Interestingly, hepatocyte differentiation and control of gene expression, following specification of the liver progenitors, appears to be independent of FoxA1/A2 (Li et al., 2009).

Studies in mouse embryos have found that the homeodomain transcription factor HNF1β is also essential for hepatic specification (Lokmane et al., 2008). In embryos lacking HNF1β, the mesenchymal portion of the liver forms relatively normally, however, the liver bud fails to express any markers of hepatic parenchymal cell progenitors and the level of mRNAs encoding the FoxA factors was severely reduced. In addition, when cultured in the presence of FGF, in contrast to control endoderm, *Hnf1*$\beta^{-/-}$ ventral endoderm failed to express albumin, which is consistent with a crucial contribution of HNF1β in controlling hepatic specification.

Between the 7 and 11 somite stages of development in the mouse, around embryonic day (E)8.5, in response to the inductive cues from the heart and mesenchyme, the cells forming the hepatic endoderm that lie proximal to the *sinus venosus* transition to a columnar morphology (Bort et al., 2006) and express several hepatic genes including *Albumin*, *Afp*, *Ttr* (transthyretin), *Rbp* (retinol binding protein), and the transcription factor *Hnf4a*, all of which are reliable indicators of early hepatic cell fate. The transition in cellular morphology results in a thickening of the epithelium, which bulges into the surrounding stroma. The basal face of the diverticulum is surrounded by a matrix which contains laminin, nidogen, type IV collagen, fibronectin, and heparin sulfate proteoglycans (Shiojiri and Sugiyama, 2004). At around 21 somites in the mouse, nuclear migration within the epithelial cells results in a pseudostratified epithelial morphology. The matrix surrounding the basal surface of the epithelium is then degraded and E-cadherin expression is downregulated in the hepatic cells as they delaminate and invade the surrounding stroma as migrating cords of hepatoblasts (Medlock and Haar, 1983; Bort et al., 2006). The migration of the hepatic progenitor cells into the stroma requires the action of matrix

metalloproteinases (MMPs) (Margagliotti et al., 2008). Several MMPs have been identified in the vicinity of the liver bud including MMP-14 in the hepatic progenitors and MMP-2 in the surrounding mesenchyme.

Analyses of mutant mouse embryos have identified several transcription factors that control the formation and expansion of the primary liver bud. The homeobox transcription factor Hhex regulates proliferation and positioning of the ventral endoderm within the cardiogenic field that controls induction of hepatic cell fate (Bort et al., 2004) and is required to ensure pseudostratification. In the absence of Hhex, mutant mouse embryos initiate hepatic specification (Bort et al., 2004) but fail to complete liver bud morphogenesis, resulting in hepatic structures that lack a parenchymal cell component (Keng et al., 2000; Martinez Barbera et al., 2000). Conditional ablation of the Hhex gene in the early hepatoblasts also disrupts their differentiation into hepatocytes (Hunter et al., 2007) suggesting that Hhex has multiple roles in controlling the onset of hepatogenesis. In addition to the mesodermal functions discussed above, GATA4 and/or GATA6 may also contribute more directly to hepatoblast development by transactivating the Hhex promoter (Denson et al., 2000; Watt et al., 2007; Zhao et al., 2005). However, further study is required to establish a cell autonomous requirement.

A number of other transcriptional regulators have been characterized as playing a role in later events. For example, the homeodomain factors HNF6 (also called Onecut-1) and Onecut-2 are redundantly required for hepatoblast migration (Margagliotti et al., 2007). The prospero-related homeobox transcription factor Prox1 also promotes hepatoblast proliferation and migration from the primary liver bud (Sosa-Pineda et al., 2000). Although the mechanism through which Prox1 controls hepatoblast migration is unclear, the mutant hepatoblasts were found to maintain high levels of E-cadherin and failed to degrade the basal matrix surrounding the liver bud. Recent work has shown that the T box transcriptional repressor Tbx3 may act upstream of Prox1 (Lüdtke et al., 2009). Interestingly, the block in expansion of the *Tbx3*$^{-/-}$ liver bud is accompanied, not only by persistence of epithelial adhesion and matrix characteristics as discussed above in the context of *Prox1*$^{-/-}$, but also by an apparent change in the fate of the hepatoblasts. In control embryos the expression of key regulators of hepatocyte differentiation including Hnf4a and c/EBPa are strongly expressed in the migrating hepatoblasts, whereas expression of transcription factors that primarily control cholangiocyte fate, such as HNF6 and HNF1β, are found to be at very low levels. In Tbx3 mutant mice expression of Hnf4a and c/EBPα is lost while levels of HNF6 and HNF1β are increased suggesting that Tbx3 normally promotes a hepatocyte fate and represses a cholangiocyte fate. Moreover, the authors of this work promote the interesting possibility that the inability of the cells to delaminate from the hepatogenic endoderm is a secondary consequence of failure of the endoderm to initiate differentiation toward the hepatocyte lineage (Lüdtke et al., 2009). If this is true then the interpretation of other mutations affecting liver bud expansion and hepatoblast migration may have to be revisited. In summary, analyses of mouse embryos harboring mutations in genes encoding several transcription factors suggest that establishment of a network of transcription factors during liver bud morphogenesis is essential for both commitment of the ventral endoderm to a hepatic fate as well as for subsequent morphogenesis of the early liver bud and that these processes are intricately intertwined.

Transcriptional Transitions during Differentiation

The hepatoblasts that migrate into the septum transversum appear to have the potential to differentiate into either cholangiocytes or hepatocytes. Cells that follow a hepatocyte cell fate progressively mature and, during the remainder of both embryonic and postnatal development, accumulate the gene expression and physiological profile of mature hepatic parenchymal cells (Ge et al., 2005; Jochheim et al., 2003). The maturation of hepatocytes is facilitated through an expanding and complex network of transcription factors that regulate hepatocyte gene expression. Detailed expression and chromatin immunoprecipitation studies of 12 hepatic transcriptional regulators during hepatogenesis have revealed a dynamic and complex set of interactions that are required to establish mature hepatocyte identity (Kyrmizi et al., 2006). By comparing developmental time points, the complexity of cross-regulation among factors was found to gradually increase as development progressed and the number of transcription factors binding a given promoter also became greater. The increase in cross-regulation between liver transcription factors during hepatogenesis is likely to stabilize the regulatory circuitry to ensure terminal differentiation of the hepatocytes as development progresses. Six transcription factors, (HNF1a, HNF1β, FoxA2, HNF4a1, HNF6, and LRH-1 [Nr5a2]), were found to form the core of this regulatory circuitry by occupying each others promoters as well as the promoters of peripheral hepatic transcription factors.

As discussed above, gene deletion studies in mouse embryos have found that HNF1β, FoxA2, and HNF6 all have roles in controlling the onset of hepatic gene expression during specification and liver bud formation, which is consistent with these factors having important roles in establishing the transcription factor network within the liver progenitor cells. Mice lacking HNF1a complete embryogenesis with little impact upon liver development (Pontoglio et al., 1996), which likely reflects the observation that HNF1β occupies most HNF1-binding sites during development. As development progresses, however, promoter sequences that are occupied by HNF1β in fetal hepatocytes are found to be bound by HNF1a in the adult. Loss of HNF4a does not have an impact on hepatic specification; however, subsequent differentiation of the hepatic progenitors is blocked (Li et al., 2000; Parviz et al., 2003). When HNF4a is specifically removed from fetal hepatoblasts, hepatic architecture is also severely affected, with livers exhibiting loss of endothelial cells and disrupted hepatocellular polarity. The loss of hepatocyte polarity in *Hnf4a*$^{-/-}$ livers appears to reflect a requirement for HNF4a in controlling expression of several proteins involved in cell junction assembly (Battle et al., 2006). Moreover, in the absence of HNF4a the core regulatory network is severely disrupted in fetal hepatic progenitors (Kyrmizi et al., 2006); however, in adult hepatocytes, maintenance of the transcription factor network appears to be less dependent on HNF4a, although HNF4a does continue to have an important role in maintaining adult hepatocyte function (Hayhurst et al., 2001). The importance of HNF4a in maintaining mature hepatocyte character in adult livers is also supported

by genome-wide ChIP studies in which HNF4a was found to occupy 12% of genes in human hepatocytes (Odom et al., 2004).

Maturation of Hepatocytes within the Liver Parenchyma

Several reviews have covered the role of transcription factors in regulating gene expression in mature hepatocytes (Friedman and Kaestner, 2006; Spear et al., 2006; Lemaigre, 2009). The networks of transcriptional activators and cofactors that control the liver's metabolic and cellular functions are extremely diverse relying on members of all known transcription factor families. In addition to the expression of hepatic genes throughout the parenchyma, expression of some genes is restricted to zonal regions that are often related to the position of the portal triad (periportal) or central veins (pericentral/perivenous; Jungermann and Katz, 1989). Heterogeneous expression can be described as either forming a gradient, where expression is gradually diminished across hepatocytes within a zone, or compartmentalized, where strict boundaries of expression within hepatocytes is observed (Spear et al., 2006). From a functional perspective the zonation of the liver lobules has been studied intensely (reviewed by Kaestner, 2009). However until recently, surprisingly little was known about the molecular mechanisms controlling zonal gene expression (Burke and Tosh, 2006).

Recent studies have highlighted an important contribution of the WNT/β-catenin signaling pathway in controlling the positional identity of hepatocytes within the liver lobule (Kaestner, 2009). Work in mice revealed that β-catenin is important for zonal gene expression in perivenous hepatocytes and this activity is antagonized by adenomatous polyposis coli (APC) in the periportal regions (Benhamouche et al., 2006; Burke et al., 2009). Analyses using mice in which a transgene is expressed from a regulatory element from within the alphafetoprotein enhancer have suggested that compartmentalized zonal expression may be due to the action of a transcriptional repressor (Peyton et al., 2000). Moreover, examination of mice that lack HNF4a specifically in hepatocytes found an increase in periportal expression of a subset of perivenous expressed genes including glutamine synthetase (Stanulović et al., 2007). Although oligonucleotide array analyses are consistent with HNF4a acting predominantly as a transcriptional activator (Battle et al., 2006), HNF4a was found to directly interact with the glutamine synthetase enhancer suggesting that HNF4a inhibits expression of pericentral mRNAs in periportal hepatocytes possibly by recruiting the histone deacetylase protein HDAC1 (Stanulović et al., 2007). More recently a direct link between the repressive activity of HNF4a and β-catenin signaling has been established (Colletti et al., 2009). Activation of the Wnt pathway converted hepatocytes that exhibited a periportal character to those that expressed perivenous markers. In response to activation of Wnt signaling, a transcription factor activated by β-catenin called LEF1 was found to physically interact with HNF4a. In perivenous gene promoters, including that of glutamine synthetase, binding of both HNF4a and LEF1 was required for gene activation and when HNF4a alone was bound expression was repressed. In contrast, periportal gene promoters could be activated by binding of HNF4a alone and ectopic activation of LEF1 inhibited gene expression. Why HNF4a fails to activate expression of the perivenous promoters and instead act as a repressor remains to be determined as does the nature of the Wnt pathway agonists whose activity presumably defines the perivenous expression profile (Kaestner, 2009).

In addition to the heterogeneous expression of subsets of genes throughout different parenchymal zones, expression of several genes, including alphafetoprotein, H19, and Glypican-3 (Spear et al., 2006), is robust in fetal hepatocytes and sharply reduced in fully differentiated cells. This switch in expression from a fetal to adult expression program is of biomedical interest because it is often reversed as hepatic cells become cancerous. How such differential regulation is controlled has been studied for over three decades. Recent studies of the alphafetoprotein promoter have revealed an important role of transcriptional repressors in controlling the transition of the gene expression profile within hepatocytes from fetal to an adult. Alphafetoprotein regulator 1 (Afr) was genetically defined as a locus that conferred a high level of AFP expression in the livers of adult Balb/cJ mice. Recently, genetic mapping studies revealed that the increase in AFP expression in adult livers was a consequence of a retrovirus insertion into the *Zhx2* gene (Perincheri et al., 2005). Although all data support an important role for Zhx2 in mediating repression of Afp expression, a direct interaction with the *Afp* promoter has not been identified, raising the possibility that Zhx2 regulates Afp indirectly. Further studies have shown that the negative regulation mediated by Zhx2 is not restricted to the Afp gene, since Zhx2 also represses expression of H19 and glypican-3 (Morford et al., 2007; Perincheri et al., 2005). In addition to Zhx2, hepatocyte specific deletion of the zinc finger protein ZBTB20 resulted in a dramatic increase in expression of AFP in adult hepatocytes (Xie et al., 2008). Molecular analyses found that ZBTB20 could directly bind the *Afp* promoter and was able to repress expression mediated by this promoter. While both of these factors clearly have important roles in defining the gene expression profile of fully differentiated hepatocytes any relationship between the two factors is yet to be determined.

The Hepatic Vasculature and Stromal Compartment

Development of the Large Blood Vessels

Following specification, hepatic progenitors interact with stromal cells and several studies have shown that the stromal compartment of the liver has important roles in controlling diverse aspects of hepatic development. In the adult liver, the stromal cell population primarily includes hepatic sinusoidal endothelial cells, hepatic stellate cells, and Kupffer cells (KC). At E9.5 in the mouse, before formation of functional blood vessels, endothelial cells have been found to promote the outgrowth of the hepatic progenitors from the liver bud (Matsumoto et al., 2001).

The acquisition of the hepatic vasculature advances throughout embryogenesis, relying on both angiogenesis and vasculogenesis to generate the complex hepatic vascular network that underlies liver function (Gouysse et al., 2002). The fetal liver is in contact with two major venous systems, the umbilical veins and the vitelline veins. The vitelline veins participate in the formation of the efferent venous system of the liver. The umbilical vein is the major afferent vessel in the fetal liver, but its presence is transient and it disappears after birth. When the umbilical vein collapses, the portal vein replaces it as the major afferent vein (for review, see Collardeau-Frachon and Scoazec, 2008). Hepatic artery development occurs later than venous development. It starts to form along the intrahepatic portal vein within

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the parenchyma and gradually extends toward the periphery (Gouysse et al., 2002). The current model suggests that intrahepatic arterial development in humans is driven by the ductal plate that forms at the same stage and is a source of VEGF. In mice, the same model is likely to apply with the exception that arteries form near well-developed ducts instead of in the vicinity of the less mature ductal plate (Clotman et al., 2003; Coffinier et al., 2002).

Development of the Hepatic Sinusoidal Capillaries

Sinusoids, the smallest blood vessels of the liver, form a complex three-dimensional plexus through which blood is transported throughout the liver lobules. The sinusoids consist of hepatic sinusoidal endothelial cells and stellate cells. Sinusoidal endothelial cells are highly specialized, having important roles in the transfer of solutes between serum and hepatocytes as well as the clearance of soluble macromolecules (Elvevold et al., 2008). They have a number of specialized features including the presence of multiple fenestrations that are arranged as clusters called sieve-plates that may facilitate transfer of factors between the sinusoidal lumen and the surface of the hepatocytes (Figure 1C; Wisse, 1972). Sinusoidal endothelial cells share some similarities to lymphatic endothelial cells including the expression of the lymphatic vascular endothelial hyaluronan receptor-1 (LYVE-1) (Mouta Carreira et al., 2001). Furthermore, sinusoidal endothelial cells were shown to express VAP1, Stabilin 1 and 2, L-SIGN, and Reelin, have a low expression of PECAM (CD31) and von Willebrand factor, and do not express the type 1 transmembrane sialomucin (CD34), which is typically found in classical endothelial cells (Lalor et al., 2006; Nonaka et al., 2007).

Sinusoids are the first blood vessels to form during hepatogenesis, where they develop by angiogenesis from existing vessels in the septum transversum mesenchyme (Collardeau-Frachon and Scoazec, 2008; Couvelard et al., 1996; Enzan et al., 1983). As development progresses, the sinusoidal endothelial cells gradually adopt the functional and structural characteristics of mature sinusoids and this correlates with changes in expression of extracellular matrix components that may influence the maturation process (Couvelard et al., 1996, 1998; Nonaka et al., 2007). Although angiogenesis appears to be the primary mechanism through which the sinusoids are formed, some studies have suggested that the growth of the sinusoids during embryogenesis, at least in the case of avian embryos, may be partially facilitated by the introduction of endothelial cells that originate from mesothelial precursors, (Pérez-Pomares et al., 2004). Although the molecular mechanisms that control growth and maturation of sinusoidal endothelial cells are not well defined, several studies support a role for Wnt signaling in their proliferation and differentiation (Klein et al., 2008; Matsumoto et al., 2008; Zeng et al., 2007). In particular, Wnt2 was shown to be expressed in rat hepatic sinusoidal endothelial cells and could increase their proliferation through activation of canonical β-catenin signaling (Klein et al., 2008). Moreover, when Wnt2 levels were depleted it resulted in a decrease in expression of VEGF receptor 2 in rat sinusoidal endothelial cells. This implies that the autocrine activity of Wnt2 cooperates with VEGF signaling to control sinusoidal endothelial cell growth in the liver. The sinusoidal endothelial cells can also impact proliferation of the hepatocytes (LeCouter et al., 2003). Treatment of mice with VEGF-A results in an increase in liver parenchymal cell proliferation and liver mass. Studies using primary hepatocyte-sinusoidal cell cocultures revealed that this increase in hepatocyte proliferation was due to the secretion of a number of mitogenic factors, including HGF and IL6, from the sinusoidal endothelial cells in response to activation of the VEGF receptor.

Developmental Interactions of the Liver with the Fetal Hematopoietic Environment

In mammals soon after the liver progenitors invade the surrounding mesenchyme, the fetal liver is colonized by hematopoietic progenitors and transiently becomes the principal hematopoietic organ. Coculture studies have suggested that immature hepatic progenitor cells can generate an environment that supports hematopoiesis (Hata et al., 1993); however, when hepatic progenitor cells are induced to differentiate to a mature form, the resulting cells can no longer support blood cell development (Kinoshita et al., 1999), consistent with the movement of hematopoietic stem cells from the fetal liver to the adult bone marrow, during this general timeframe. In addition to the parenchymal cells, fetal liver-derived stromal cells have been shown to enhance hematopoietic progenitor cell proliferation possibly through Wnt signaling pathways (Martin and Bhatia, 2005). Conversely, hematopoietic cells within the fetal liver express the cytokine Oncostatin M (OSM) (Yoshimura et al., 1996). Addition of Oncostatin M to liver progenitor cells in culture was found to enhance their differentiation into hepatocytes and loss of the gp130 subunit of the Oncostatin M receptor had a negative impact on hepatocyte differentiation in mice (Kamiya et al., 1999). Together, these studies suggest that there exists a dynamic interplay between the blood and parenchymal compartments within the fetal liver that controls the timing of both hepatogenesis and hematopoiesis.

Stellate and Kupffer Cells during Hepatogenesis

Hepatic stellate cells reside in the perisinusoidal space (space of Disse) between the basolateral surface of the hepatocytes and abluminal surface of the sinusoidal endothelial cells. Recent studies have revealed that the cells have many important activities that impact liver function and development (Friedman, 2008). The better characterized roles include the ability of stellate cells to store vitamin A and to modulate hepatic microcirculation in response to endothelin signaling (Housset et al., 1993; Watanabe et al., 2007). After hepatic lesion or under pathological conditions, hepatic stellate cells can also become activated to adopt a myofibroblast character and chronic activation of stellate cells leads to liver fibrosis (for review see Friedman, 2008). The origin of hepatic stellate cells has been debated, with various lines of evidence suggesting that the cells are of endodermal, neural crest, or mesenchymal origin. Most of the conclusions drawn from such studies were based on expression of shared sets of marker genes; however, shared gene expression does not necessarily correlate with cell lineage. Direct lineage tracing experiments have been performed in avian embryos (Pérez-Pomares et al., 2004), which led to the conclusion that mesothelial cells derived from the proepicardium and septum transversum mesenchyme could give rise to both endothelial and stellate cells within the hepatic sinusoids. Recent studies in human (Loo and Wu, 2008) and in mouse (Asahina et al., 2009) support a mesothelial origin of hepatic stellate cells. Submesothelial cells express activated leukocyte cell adhesion molecule (ALCAM) and after FACS sorting, ALCAM-positive cells were found to acquire

a myofibroblastic phenotype in culture and were capable of forming lipid droplets when cultured in a three-dimension collagen gel in the presence of retinol, a feature that is characteristic of quiescent hepatic stellate cells (Asahina et al., 2009). Whether mesothelial cells are the sole source of hepatic stellate cells or whether they can differentiate from other fetal lineages will require further lineage tracing experiments.

Gene knockout studies in mice have suggested that hepatic stellate cells and derivatives of the septum transversum mesenchyme may contribute toward the fate of other hepatic cell lineages. The homeobox protein Hlx is expressed in the septum transversum and visceral mesenchyme but is not detected in the endoderm or its derivatives. Examination of *Hlx*$^{-/-}$ mouse embryos found that at E12.5 the livers were severely hypoplastic containing only 3% of the cells found in control livers (Hentsch et al., 1996). Although at E9.5 the mutant livers appeared to form hepatic cords and parenchymal cell lineages were specified and had initiated a differentiation program, the parenchymal progenitors failed to proliferate. Whether this is due to regulation of expression of mitogens or growth factors by Hlx remains to be determined. Wilm's Tumor (Wt1) and retinoic acid signaling are involved in hepatic stellate cell development and liver morphogenesis (Ijpenberg et al., 2007). Analyses of Wt1 null fetal livers revealed that that the absence Wt1 led to a decrease in expression of the retinoic acid synthesizing enzyme RALDH2 in hepatic stellate cell progenitors. This in turn affected retinoic acid-mediated liver growth resulting in liver hypoplasia and abnormal liver lobe formation (Ijpenberg et al., 2007; Sucov et al., 1994). The LIM homeobox gene Lhx2 is also expressed in the septum transversum mesenchyme and stellate cells throughout liver development (Kolterud et al., 2004). Loss of Lhx2 in mice resulted in abnormal stellate activation and mice developed hepatic fibrosis, which is consistent with the proposal that Lhx2 is required to maintain stellate cells in a quiescent state. Moreover, examination of fetal *Lhx2*$^{-/-}$ livers found an increase in deposition of extracellular matrix proteins, which appeared to result in disorganization of the parenchyma, including increased expression of hepatocyte genes and disrupted architecture of the sinusoidal trabeculae.

Kupffer cells are resident macrophages on the surface of hepatic sinusoidal endothelial cells. They represent 15% of the liver cell population and 50% of resident macrophages in the body. There is no clear report on the role of Kupffer cells in liver organogenesis. However, some data suggest that Kupffer cells or their progenitors may be involved in maturation of erythrocytes during fetal liver hematopoiesis (for review, see Naito et al., 2004). In addition to a possible contribution to erythyropoiesis, selective depletion of Kupffer cells using liposome-encapsuled dichloromethylene diphosphonate (Cl2MDP) in adult mice or rats after partial hepatectomy (PH) lead to a delay in hepatic regeneration (Meijer et al., 2000). In the absence of Kupffer cells, there is a decrease in the levels of secreted TNF-α and IL-6 compared to normal liver and the delay in liver regeneration was attributed to a lack of NF-κB activation (Abshagen et al., 2007).

Development of the Biliary Tree

A major function of the liver is to generate bile that is transported to the intestine where it is required for the emulsification of fat. These secretions are released from the apical surface of the hepatocytes and are transported through a network of intrahepatic ducts into the extrahepatic biliary tract which consists of the hepatic and cystic ducts, the gallbladder, and the common bile duct.

Development of the Extrahepatic Biliary Tract

The extrahepatic biliary tract originates from a portion of the ventral endoderm that is positioned immediately rostral to the ventral pancreatic bud. A recent report demonstrated that the extrahepatic biliary tract derives from pancreatobiliary precursors coexpressing PDX1 and SOX17 (Spence et al., 2009). This precursor population gives rise to SOX17^{+}/PDX1^{-} extrahepatic biliary cells and SOX17^{-}/PDX1^{+} pancreatic cells. The segregation of the pancreatobiliary precursor population depends on SOX17. This factor is required for extrahepatic biliary tract development and overexpression inhibits pancreas development. The expression of SOX17 is controlled by homolog of hairy/enhancer-of-split (Hes-1): in the absence of Hes-1 the mice not only display accelerated differentiation of pancreatic endocrine cells from pancreatic progenitors (Jensen et al., 2000), the bile duct cells also differentiate to a pancreatic phenotype (Fukuda et al., 2006; Sumazaki et al., 2004). Other transcription factors involved in extrahepatic biliary development include Hhex: in Hhex null embryos the common bile duct is replaced by duodenal-like tissue suggesting that the decision between a dudodenal or biliary fate appears to depend, at least in part, on the function of this transcription factor (Hunter et al., 2007). Mice deficient in HNF6, Hes-1, HNF1β, or FoxF1 show lack or abnormal shape of the gallbladder (Clotman et al., 2002; Sumazaki et al., 2004; Coffinier et al., 2002; Kalinichenko et al., 2002).

Cell Signals Controlling Development of Intrahepatic Bile Ducts

While the extrahepatic cholangiocytes derive directly from the endoderm, the cholangiocytes that line the intrahepatic bile ducts arise from hepatoblasts. The earliest sign of biliary differentiation is expression of Sox9, a transcription factor that controls the timing of bile duct development (Antoniou et al., 2009). Sox9-positive cells are first found close to the branches of the portal vein where they form the ductal plate (Figures 3B and 3C). The ductal plate is a continuous ring of cells arranged as a monolayer that surrounds the periportal mesenchyme. Two signaling mechanisms have emerged as key determinants of localized biliary differentiation. Transforming growth factor-beta (TGF-β) ligands generate a gradient of TGF-β signaling with high activity near the vein and lower activity in the parenchyma (Antoniou et al., 2009; Clotman et al., 2005). The appropriate concentration of TGF-β is required to induce differentiation of biliary cells from periportal hepatoblasts. When TGF-β signaling in the parenchyma is excessive, a biliary differentiation program is superimposed upon the hepatocytes leading to the development of hybrid hepato-biliary cells (Clotman et al., 2005). The Notch signaling pathway was also suspected of contributing to biliary development based on the finding that patients affected with Alagille syndrome, a polymalformative disease with bile duct paucity, had mutations in the *JAGGED1* and *NOTCH2* genes (Li et al., 1997; Oda et al., 1997; McDaniell et al., 2006). The analysis of this pathway in the liver has been challenging due to the presence of multiple ligands and

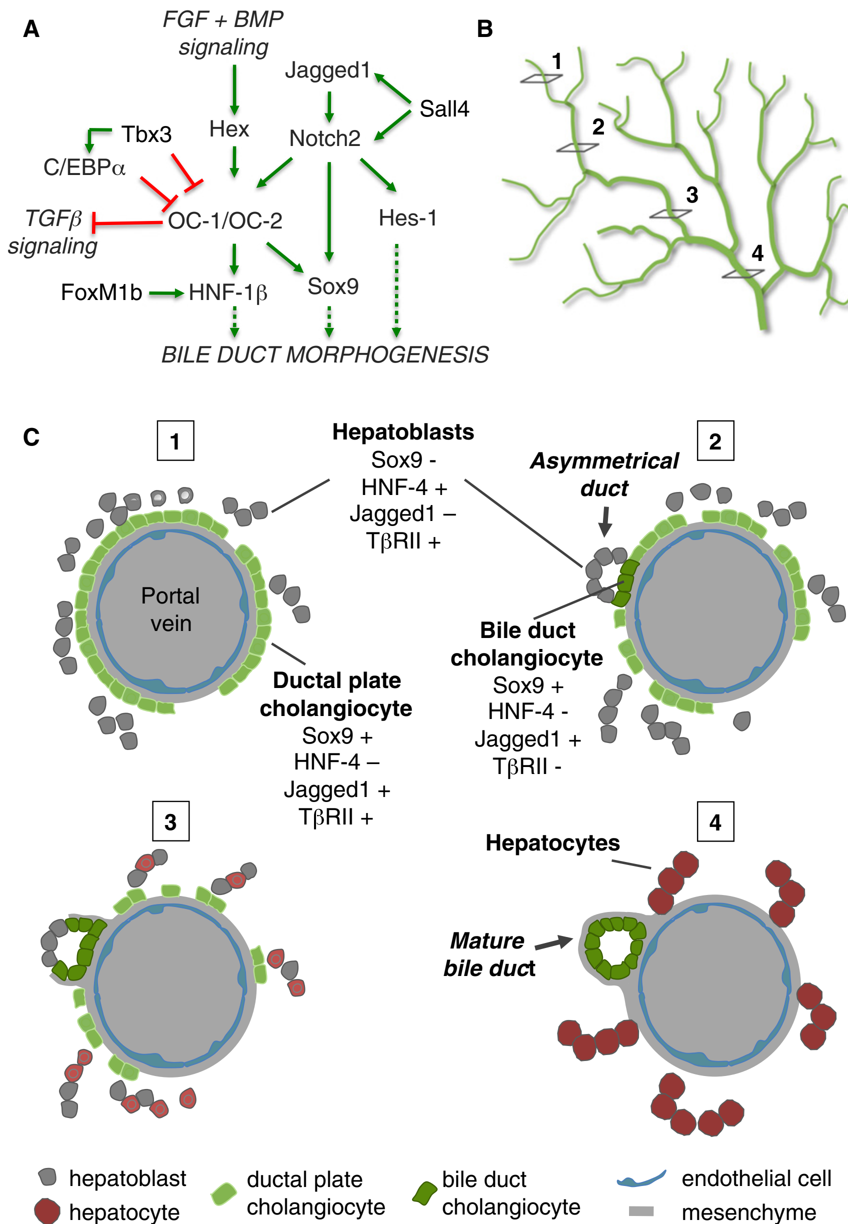

Figure 3. Development of the Bile Ducts
(A) Gene network regulating bile duct development.
(B and C) Bile duct morphogenesis progresses from the hilum of the liver to the periphery of the lobes. The biliary tree is schematically represented at a stage when ducts have reached maturity near the hilum (section 4), while peripheral structures still show cholangiocytes forming a single-layered ring of cells (ductal plate; section 1). Two intermediate maturation stages illustrate the progression from asymmetrical to radially symmetrical ducts, as well as markers that characterize the hepatoblasts and cholangiocytes that line the developing ducts are mentioned (sections 2 and 3). When morphogenesis progresses, the ductal plate areas are not involved in duct formation regress.

receptors with overlapping functions (reviewed in Lemaigre, 2008). However, recent data favor a model in which Notch signaling controls multiple steps in biliary development, including the initial differentiation of cholangiocytes. Analysis of mice that have a liver-specific inactivation of RBP-Jκ, a common transcriptional mediator of Notch signaling, revealed a reduced number of biliary cells differentiating from hepatoblasts (Zong et al., 2009). Since expression of Jagged1 occurs in the periportal mesenchyme and biliary cells while Notch2 is present in the biliary cells(Zong et al., 2009; Geisler et al., 2008), it appears that Notch signaling contributes not only to differentiation of biliary cells but also restricts differentiation to a periportal location. Other signals, including Wnt (Hussain et al., 2004; Monga et al., 2003; Tan et al., 2008; Decaens et al., 2008), FGF, and BMP (Yanai et al., 2008), also regulate the differentiation of hepatoblasts to cholangiocytes; however, in contrast to TGF-β and Notch their role in restricting biliary differentiation to the periportal area is less clear.

Transcriptional Control of Intrahepatic Bile Duct Development and Duct Remodeling

Transcriptional regulation of biliary differentiation has also been a focus of intense research (Figure 3A), with most data collected from the analyses of transcription factor-deficient mice. Such animals commonly retained hepatic cells that exhibited characteristics of both hepatocytes and cholangiocytes, as was the case in animals lacking Hhex, HNF6, Onecut-2, or C/EBPα (Hunter et al., 2007; Clotman et al., 2005; Yamasaki et al., 2006). In the case of mice lacking both HNF6 (OC-1) and Onecut-2 (OC-2), which act redundantly, the phenotype was explained by repression exerted by the factors on TGF-β signaling. When both HNF6 and Onecut-2 were absent, TGF-β signaling was enhanced resulting in an expansion of the signaling gradient (Clotman et al., 2005). Other transcription factors act to control cholangiocyte differentiation in a cell-autonomous manner by controlling biliary cell gene expression. For example, FoxM1B and Sall4 drive biliary differentiation, while Tbx3 represses the process (Oikawa et al., 2009; Krupczak-Hollis et al., 2004; Lüdtke et al., 2009; Suzuki et al., 2008). In addition, recent studies revealed that when both FoxA1 and FoxA2 were deleted in the developing hepatoblasts, the mutant mice developed hyperplasia of the biliary tree as a consequence of excessive cholangiocyte proliferation (Li et al., 2009). This appeared to result from perturbed expression of IL-6, which can induce cholangiocyte proliferation: in the absence of FoxA1/12 the glucocorticoid receptor, an inhibitor of IL6 gene transcription, no longer binds to the IL-6 promoter leading to increased and prolonged expression of IL6. This implies that FoxA1/A2 have an important role in terminating bile duct expansion during development.

The ductal plate must also undergo a complex process of remodeling that leads to the formation of bile ducts (Figures 3A–3C). The ductal plate initially consists of a primary layer of polarized cholangiocytes, then at specific locations a second layer forms that is separated from the primary layer by a luminal space. This second layer was recently shown to consist of hepatoblasts, thereby creating asymmetrical ductal structures, with the portal side of the lumen delineated by cholangiocytes and the parenchymal side by hepatoblasts (Antoniou et al., 2009). This asymmetric cellular arrangement is transient as the hepatoblasts on the parenchymal side of the primitive ducts differentiate to form cholangiocytes, thereby producing ducts entirely lined by cholangiocytes. The Notch pathway is instrumental in biliary tubulogenesis. In the absence of the Notch effector Hes-1, ductal structures failed to form (Kodama et al., 2004) and stage-specific inactivation of the Notch pathway impaired duct formation beyond the formation of a monolayered ductal plate (Zong et al., 2009). The TGF-β receptor type II (TβRII) is expressed in the ductal plate monolayer and then becomes repressed when the cholangiocytes mature during duct formation suggesting that TGF-β signaling may also contribute to duct formation (Antoniou et al., 2009).

Several of the hepatic transcription factors (HNF6, Onecut-2, HNF1β, C/EBPα, Hhex) required for differentiation of cholangiocytes (Clotman et al., 2002, 2005; Coffinier et al., 2002; Yamasaki et al., 2006; Hunter et al., 2007) are also required for tubulogenesis of the ducts. However, whether the abnormal duct formation in mouse embryos lacking these transcription factors result from deficient differentiation or from abnormal morphogenesis is unclear. Nevertheless, a tentative gene regulatory cascade can be proposed, based on the expression of the factors in the various mouse mutants (Figure 3A). Target genes directly regulated by cholangiocyte transcription factors have in most cases not yet been described. Candidates are genes that modulate Activin/TGF-β signaling, such as Follistatin, TβRII, and α2-macroglobulin, as well as the vesicular membrane fusion protein vps33b, known to be required for duct development in both humans and zebrafish (Matthews et al., 2005; Gissen et al., 2004). These factors all require HNF6 and HNF1β for normal expression (Matthews et al., 2005; Clotman et al., 2005).

Proliferation of cholangiocytes facilitates growth of the ducts and starts at the end of gestation, when differentiation is terminated and symmetrical ducts are formed. Polycystic diseases affecting the liver are characterized by abnormal cholangiocyte proliferation and often result from mutations in genes regulating primary cilia function (Masyuk et al., 2009; Adams et al., 2008). Primary cilia in cholangiocytes function as osmo-, mechano-, and chemosensors and exert a tight control on cholangiocyte proliferation (Masyuk et al., 2008). In the pancreas and kidneys, ciliopathic genes are controlled by HNF6 and HNF1β (HNF6: *Cys1 and Pkhd1*; HNF1β: *Ift88/Tg737/Polaris*, *Pkd2*, and *Pkhd1*), indicating that they may be similarly targeted by HNF6 and HNF1β during duct development (Pierreux et al., 2006; Gresh et al., 2004).

Finally, while most efforts have been devoted to the identification of signaling pathways and transcription factors regulating biliary development, recent evidence points to important posttranscriptional control exerted by a host of microRNAs. miR-30a and miR-30c are expressed in developing ducts and their inhibition in zebrafish results in abnormal duct development (Hand et al., 2009a). Interestingly, mRNAs targeted by the miR-30a and miR-30c miRNAs include those encoding ActivinA and epidermal growth factor receptor. Along the same lines, miR-15a was found to repress proliferation of cholangiocytes by inhibiting the expression of cdc25a (Lee et al., 2008). Moreover, the levels of miR15a were decreased in livers isolated from the PCK rat, a model of autosomal recessive polycystic kidney disease, as well as in patients with polycystic liver disease. Although these data imply that repression of miR15a may contribute to hepatic cystogenesis, studies in which *Dicer*, which is essential for production of all miRNAs, was specifically deleted in hepatocytes revealed that liver function was surprisingly unaffected, although liver mass and hepatocyte proliferation were modestly increased (Hand et al., 2009b). Further, studies in which specific miRNAs are depleted in the liver will be neccessary before the role of miRNAs in controlling liver development and hepatic function can be deciphered.

Conclusion

Through the rapid evolution of molecular genetic technologies and the growth in the study of several new animal models our understanding of the molecular mechanisms controlling liver development has also become advanced compared to many other organ systems. Several findings first found in the context of liver development are generally applicable to the development of other tissues and organs. For example, communication between the vasculature and the endoderm as an essential signaling event that governs hepatic cell fate has been found to be reproduced in other organ systems including the pancreas (Zaret, 2008). While such advances are exciting, it is clear that we do not have a complete picture. We still do not understand the mechanisms that regulate organ size, and our knowledge of how individual tissue compartments interact to control cell maturation, although improving, remains vague. Moreover, we still have only a rudimentary understanding of why adult primary hepatocytes rapidly dedifferentiate when placed in culture. As more pathways and factors that regulate liver development are revealed, it is likely that such questions will be answered.

The information gleaned from developmental studies, even at its current level, has now been successfully applied to control the differentiation of hepatocytes from stem cells. This has opened up the possibility of using stem cell approaches in both the study and possible treatment of liver disease. The feasibility of using stem cells as a source of hepatocytes is also supported by studies in both animal models (Grompe, 2006) and humans using primary hepatocytes isolated from cadavers (Fisher and Strom, 2006). Recent work has shown that up to 90% of the mouse hepatic parenchyma can be replaced with human hepatocytes when such cells are transferred into immunocompromised fumarylacetoacetate hydrolase (Fah)-deficient animals (Azuma et al., 2007; Bissig et al., 2007). When combined with the ability to generate hepatocytes from either adult or embryonic stem cells, this opens the possibility of using humanized mouse livers to study and treat inborn errors of hepatic metabolism. Several candidate diseases that could benefit from such approaches have been described (Fisher and Strom, 2006; Grompe, 2006) including urea cycle disorders, Wilson disease, Crigler-Najjar syndrome type I, hyperlipidaemia, glycogen storage diseases,

alpha-1 anti-trypsin deficiency, Factor VII deficiency, familial hypercholesterolemia, and tyrosinaemia type I.

Not only could the availability of an indefatigueable source of primary hepatocytes facilitate the study of liver pathologies, it could potentially allow the generation of bioartificial liver devices (Strain and Neuberger, 2002). At the moment liver transplantation is successful in the treatment of liver disease; however, the availability of donor organs is extremely limiting. If a device could be established that could temporarily maintain basic liver functions, it could act as a bridge until the patient's own liver completed a regenerative response or until a transplant was available. Although the use of xenogeneic cells or cultured hepatic cell lines in bioartifical liver devices have been described, success has been limited by the need for large numbers of highly differentiated human hepatocytes. However, building on basic information gleaned from developmental studies, microscale culture of highly differentiated primary human hepatocytes has been described (Khetani and Bhatia, 2008). Moreover, three-dimensional culture techniques have now been established that support hepatocytes in a highly differentiated state that could potentially be used as disease models and to study drug toxicity and function (Sivaraman et al., 2005).

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