SECTION I: PATHOPHYSIOLOGY OF PEDIATRIC LIVER DISEASE

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Liver Development: From Endoderm to Hepatocyte

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The liver is derived from the endoderm, one of the three germ layers formed during gastrulation. The initial endodermal epithelium consists of approximately 500 cells in the mouse [1], from which cells will be apportioned to the thyroid, lung, stomach, liver, pancreas, esophagus, and intestines. How is the endoderm patterned to generate such diverse tissues? Once the hepatic primordium is formed, how are the different hepatic cell types generated? How do they generate a proper liver architecture? And how do the principles of liver development apply to liver regeneration and the possibility of generating hepatocytes from stem cells? This chapter focuses on all of these questions.

A BRIEF OVERVIEW OF EMBRYONIC LIVER DEVELOPMENT

By late gastrulation in the mouse (embryonic day of gestation 7.5 [E7.5]) the anteroposterior pattern of the endoderm is already established [2], so that during E8.5–9.5 (mouse) the anterior-ventral domain develops the organ buds for the liver, lung, thyroid, and the ventral rudiment of the pancreas [3]. This corresponds to about 2–3 weeks' gestation in humans. The specification of liver progenitors occurs through a combination of positive inductive signals from the cardiogenic mesoderm and septum transversum mesenchyme and repressive signals from the trunk mesoderm [4–6]. This occurs at about 8.5 days gestation in the mouse, when the embryo contains six to seven pairs of somites, which are clusters of skeletal and muscle progenitors. The cells adopting the hepatic fate are characterized by the expression of two of the liver-specific markers, albumin and α-fetoprotein (AFP). Although albumin was initially considered to be an adult liver marker, it is now well established that it is among the earliest liver-specific markers to be expressed in development, along with AFP. The nascent hepatic epithelium, consisting of hepatoblasts, then invades a stromal cell field containing angioblasts, which are precursors to the blood vessels, and the septum transversum mesenchyme. Under the influence of the stromal cells, the hepatoblasts proliferate to form the liver bud, and then differentiate to form the fetal liver.

At E10–11 in the mouse, hematopoietic stem cells originating from the yolk sac and aorta-gonad-mesonephros regions colonize the fetal liver and expand their mass and lineage diversity. Therefore, the fetal liver in mammals is a primary site of hematopoiesis. At the same time, the resident hematopoietic cells secrete growth signals that promote maturation of the liver [7–10]. Around birth, hematopoietic cells migrate out of the liver and a functional switch from a hematopoietic microenvironment to a metabolic organ occurs.

DISTINCT HEPATIC PROGENITOR DOMAINS IN THE MAMMALIAN EMBRYO

A recent study investigated in detail which populations of undifferentiated endoderm cells generate the embryonic liver bud [11]. The authors isolated mouse embryos at E8.0, which is prior to hepatic specification, and used vital dyes to label different clusters of endoderm cells in different isolated embryos. They then cultured the embryos whole, into the organogenic phase, and then determined which tissues inherited the labeled cells. By comparing the descendant cell populations arising from different labeled endoderm cell domains in different embryos, they were able to develop a "fate map." The fate map (Figure 1.1) indicates the location of progenitor domains in the undifferentiated endoderm that will give rise to the embryonic liver bud. Interestingly, the authors found that two distinct types of endoderm-progenitor cells, lateral and medial, arising from three spatially separated embryonic domains, generate the epithelial cells of the liver bud (see Figure 1.1). The movement of these cells and the morphologic changes in the embryo during this period position the distinct progenitor domains close to the hepatic-inducing tissues. Although both lateral and medial liver bud descendants express early hepatoblast genes in common, it remains to be determined if the different progenitor domains give rise to functionally different cell populations in the adult liver.

LIVER-INDUCTIVE ROLE OF CARDIAC MESODERM

To be induced to a liver fate, the ventral endoderm has to interact with other tissues. An early finding was that the ventral

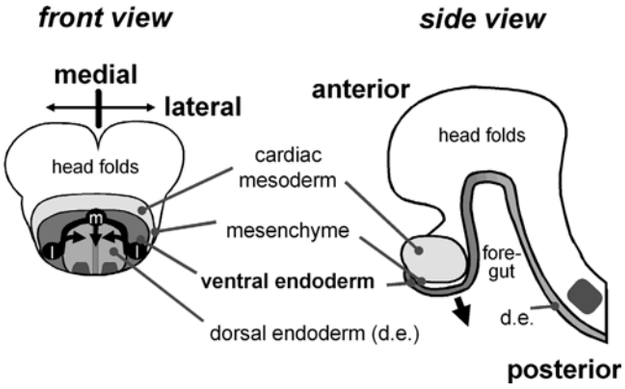

Figure 1.1. Fate map of liver progenitors in the ventral foregut endoderm. The ventral foregut endoderm is denoted by the dark-shaded area and surrounds the anterior intestinal portal of the foregut. Anterior halves of embryos are shown, corresponding to about day 8.0 of mouse gestation (~2.5 weeks of human gestation). The dark spots designated *m* (for medial) and *l* (for lateral) indicate progenitor domains of undifferentiated endoderm cells that will contribute to the liver bud [11]. The arrows indicate tissue movement. Also shown are the location of the cardiac mesoderm and prospective septum transversum mesenchyme cells ("mesenchyme"), both of which signal to the endoderm during this period to promote hepatic induction.

endoderm has to be in close contact with cardiac mesoderm (Figure 1.1), as first shown by transplant experiments with chick embryos [4,6,12]. This is consistent with the morphologic changes that occur during this time of embryo development. At the five- and seven-somite stages, the future hepatic part is brought in close proximity to the cardiac mesoderm through invagination of the foregut. Although the cardiac mesoderm is necessary for the induction of the hepatic fate, it is not sufficient. Further studies showed that the endoderm needs a second stimulus from the septum transversum mesenchyme [13–15] (Figure 1.1). Results in the chicken were confirmed in the mouse [5,16], suggesting a general mechanism of liver development in higher vertebrates. Although these pure morphologic studies showed clearly the importance of both cardiac mesoderm and septum transversum mesenchyme for liver development, they left open the question of what signals are produced by these tissues to facilitate hepatic lineage commitment.

Gualdi et al. [5] developed an in vitro assay to analyze hepatic specification, and showed that the signal from the cardiac mesoderm requires close proximity to the endoderm. Using this explant system, it was then found that fibroblast growth factor 1 (FGF1) and FGF2 could substitute for cardiac mesoderm to induce the hepatic fate (Figure 1.2) [17]. This is in agreement with the ability of FGFs to act locally, as secreted FGFs stay in close contact with the extracellular matrix. As the FGFs are secreted from the cardiac mesoderm, Jung et al. [17] found that the endodermal cells express FGF receptor 1 (FGFR1) and FGFR4, receptors specific for these FGF molecules. Although the group found weak liver gene inductive potential for FGF8, they showed that this factor has a positive effect on the outgrowth of hepatic cells, after the initial specification.

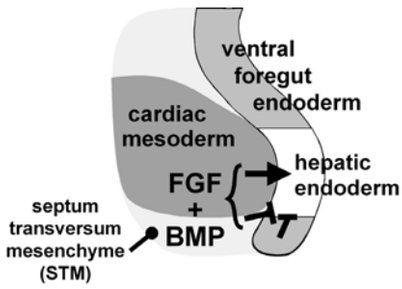

Figure 1.2. Signaling that induces hepatic genes in the endoderm in the mouse. See text for details.

Interestingly, in the absence of the FGF signaling from the cardiac mesoderm, the domain of the ventral foregut endoderm that normally becomes the liver rapidly defaults to a pancreatic fate [18]. Recent work by Serls et al. [19] showed that the FGF signals do not work as on/off switches for the liver program; rather, there are different thresholds of FGF concentrations that pattern the ventral foregut. This group cultured ventral endoderm explants in medium with different concentrations of FGF2. In the absence of FGF, the explants expressed pancreatic genes. At low concentrations of FGF, they expressed hepatic genes, and at high concentrations, lung marker genes were expressed.

Although it is clear that FGF1 and 2 can induce liver development and both factors are expressed by the cardiac mesoderm, it is important to keep in mind that no liver defects have been described in FGF1 and FGF2 double-knockout mice [20]. Because there are 22 known members of the FGF protein family, it is likely that other members can compensate for the loss of FGF1 and 2 or that other FGFs are involved in the induction of hepatic fate. For example, Cai et al. [21] showed in a study investigating heart development that FGF8 and 10 are expressed in the cardiac mesoderm. Therefore, there may be considerable redundancy of FGF signaling here, as in many other developmental contexts.

Further evidence for the importance of cardiac mesoderm as an inductive tissue for liver development comes from experiments with embryonic stem cells. Co-culture experiments of mouse embryonic stem cells with cardiac mesoderm found that under these conditions, the embryonic stem cells differentiate into hepatocyte-like cells [22]. The cells activate the crucial endodermal transcription factors, such as SOX17α, FOXA2, and GATA4, and express albumin and AFP. These results emphasize the predictive value of information from developmental biology on the productive differentiation of stem cells.

LIVER-INDUCTIVE ROLE OF SEPTUM TRANSVERSUM MESENCHYME

The septum transversum mesenchyme, the second most important tissue for liver fate decision, originates from the lateral plate mesoderm and gives rise to the epicardium of the heart and the diaphragm. Rossi et al. [23] used the in vitro hepatic induction

assay and included the bone morphogenetic protein (BMP) inhibitor Xnoggin. When they cultured cardiac mesoderm, septum transversum mesenchyme, and ventral endoderm together with the inhibitor, albumin gene induction was not observed. Addition of BMPs could rescue this phenotype, but not the addition of FGFs. This shows that in addition to FGFs, BMPs play a role in the hepatic fate decision (Figure 1.2). This group then used knock-in mouse embryos containing an LacZ reporter transgene controlled by BMP4 transcriptional regulatory elements. They found high expression of $BMP4^{LacZ}$ at the eight-somite stage within the septum transversum mesenchyme. In situ hybridization experiments for BMP2 and 4 also showed the expression of these genes in the septum transversum mesenchyme [24], supporting the role of the septum transversum mesenchyme as the source of BMP signaling for liver development (Figure 1.2). In addition to the cell fate decision, the secretion of BMPs seems to be important for the outgrowth of the budding hepatoblasts [23].

Summarizing our knowledge, the model suggests that FGF and BMP signals act cooperatively on the endoderm to induce hepatic cell fate and the outgrowth of the hepatoblasts. Interestingly, there is another example of cooperation of these two pathways in development in that the factors together induce cardiogenesis during chick development [25,26].

TRANSCRIPTION FACTORS IMPORTANT FOR THE HEPATIC FATE DECISION

Foxa transcription factors are important for liver-specific gene expression. Lee et al. [27] used in vitro transcription assays to show that Foxa1 can relieve the transcriptional repression of the *Afp* gene in chromatin-assembled DNA templates. Furthermore, FoxA1 and FoxA2 are expressed within the ventral endoderm prior to the induction of the hepatic fate [28–30]. Foxa1-null mice have no defect in early embryonic development [31,32] but this might result from compensation through Foxa2 (see below). In contrast, FoxaA2-null embryos show major developmental defects in the formation of the node, the notochord, the floor plate of the neural tube, and the morphogenesis of the foregut endoderm [33–35]. The early endoderm defect prevented an assessment of the role of Foxa2 on liver specification. To address this question, conditional gene inactivation approaches were used.

First, Sund et al. [36] made an AlbCre-$FoxA2^{loxp/loxp}$ mouse. These mice have loxp recombination sites flanking the *Foxa2* gene, so that in liver cells, where the AlbCre construct is expressed, the Cre recombinase will delete the *Foxa2* gene and the phenotype can be assessed. However, these mice developed a normal liver morphology. Because in this context the Cre recombinase is expressed late in development, the conclusion is that Foxa2 is not required for maintaining the hepatic fate. With FoxA3Cre-$FoxA2^{loxp/loxp}$ mice, in which the *Foxa2* gene is inactivated in the endoderm prior to liver induction, Lee et al. [37] also found normal hepatic induction and growth. However, this might be still through compensation by Foxa1. Lee et al. [37] pursued this further with an impressive set of genetic experiments to simultaneously knock out both the *Foxa1* and *Foxa2* genes in the undifferentiated endoderm. These embryos completely lacked the formation of the liver bud, and no liver-specific genes were activated. Thus, both *Foxa* genes cooperate in the establishment of the hepatic primordium and thus promote the hepatic competence of the foregut endoderm.

Narita et al. [38] found that the transcription factor Gata4 is intrinsically required for ventral foregut endoderm development. In addition, it was shown that this factor is necessary for early liver gene expression [39]. Gata4 and its family member Gata6 are expressed in the endoderm before the hepatic fate is induced [40,41]. The expression of Gata4 in the lateral mesoderm is downstream of BMP4 [42]. Corresponding with this, Gata4 mRNA could be detected within the hepatic endoderm in explant studies of ventral endoderm in co-culture with cardiac mesoderm and septum transversum mesenchyme. Adding the BMP inhibitor Xnoggin to the explants abolished Gata4 expression [39], and explants from $BMP4^{-/-}$ mice also exhibited a strong down-regulation of Gata4 [23]. Gata4 expression is activated by binding of forkhead and Gata transcription factors to a distal enhancer element [42]. The activity of this enhancer element is initially broad but eventually becomes restricted to the mesenchyme surrounding the liver. This activity of the enhancer is attenuated by the BMP antagonist Noggin, and the enhancer is not activated in BMP4-null embryos. This suggests a direct requirement of BMP signaling for the enhancer activity and that Gata4 is a downstream effector of BMP signaling in lateral mesoderm. $Gata4^{-/-}$ embryos show defects in foregut development similar to Foxa2 knockout embryos [40], again indicating that Foxa2 and Gata4 are acting on the same developmental process. Additional studies in *Drosophila* and *Caenorhabditis elegans* suggest that cooperation between Foxa2 and Gata transcription factors is crucial for the endoderm specification [43].

These factors also cooperated at a transcriptional enhancer sequence for the albumin gene, which binds both Foxa and Gata factors in the gut endoderm prior to albumin gene activation [5,39,44,45]. This suggests that these factors are mediators of competence in the foregut endoderm. Interestingly, Foxa and Gata4 can bind to their specific binding sites in compacted chromatin [46], which is usually inaccessible to transcription factors. Foxa and Gata4 were found to locally open the chromatin structure, allowing other transcription factors to enter and bind their specific nearby sites. From these results, it was proposed that Foxa and Gata4 represent "pioneer" transcription factors that could mark genes as competent, through opening of the local chromatin structure. These genes are finally expressed if they receive the correct inductive signals via the expression of other transcription factors. Supporting this idea is the fact that Foxa was found to relieve the p53-mediated transcriptional repression of the *Afp* gene in chromatin-assembled DNA templates [27,47,48].

Gata factors also exhibit genetic redundancy [36,49,50] and have roles in the early embryo, prior to liver induction [34,40,41,51–53]. To circumvent a requirement for Gata6 in yolk sac development [52,53], tetraploid embryo chimeras were

used to give Gata6$^{-/-}$ embryos a wild-type extra-embryonic endoderm [54]. Zhao et al. [54] could show that the Gata6 tetraploid chimeric embryos still induced the hepatic fate decision of the ventral endoderm, but exhibited a failure in the outgrowth of the liver bud beyond day 9.5 of gestation. As Gata4 is still expressed in the ventral endoderm of Gata6 knockout embryos, Gata4 may compensate for Gata6 loss. Gata4 is expressed only transiently in the prehepatic endoderm during hepatic specification and then the expression is normally lost during outgrowth of the liver bud.

Consistent with this finding in the mouse, zebrafish need Gata6 for liver bud growth. Experiments in the fish depleting both Gata4 and Gata6 found an earlier block in liver development and a complete lack of the liver bud [55]. Summarizing these results, it is now clear that both Gata factors have both redundant and specific functions during liver development.

Wandzioch et al. [56] showed another role for the septum transversum mesenchyme. The LIM homeobox gene *Lhx2* is expressed in hepatic stellate cells in the adult liver. In addition, liver development in Lhx2$^{-/-}$ mice is disrupted. In the embryo, *Lhx2* expression can be found in cells from the septum transversum mesenchyme, E9 onward. These cells build a subpopulation of mesenchymal cells in the liver and become hepatic stellate cells. The Lhx2 knockout mice show a disrupted cellular organization and altered gene expression pattern in intrahepatic endoderm cells. An increased deposition of extracellular matrix proteins precedes these abnormalities. Therefore, the septum transversum mesenchyme is not only an inductive tissue for the early liver; it also contributes to nonparenchymal cells.

STIMULATION OF HEPATOBLAST GROWTH

The second important step after the induction of the hepatic fate in endoderm cells and the differentiation into hepatoblasts is the proliferation of these cells. The mesenchymal component of the liver, derived from the septum transversum mesenchyme, is essential for proliferation of hepatoblasts [6,14,16].

There are other essential interactions for liver bud growth. Experiments with flk$^{-/-}$ mice [57], which lack endothelial cells [58], show a failure of hepatic endoderm morphogenesis and mesenchyme invasion, after the primary specification of hepatic endoderm. The requirement for endothelial cells for hepatic endoderm growth could be recapitulated with embryo tissue explants, showing that the effect is independent of oxygen and factors in the bloodstream. The important interactions between endothelial and liver cells appear to persist in the adult liver [59].

A signaling pathway that controls the proliferation of the fetal liver cells involves hepatocyte growth factor (HGF). Genetic studies in mouse embryos showed that the proliferation and outgrowth of the liver bud cells require the interaction of HGF with its receptor, c-met. Either knockout of HGF (expressed in the hepatic mesenchyme) or c-met (HGF-receptor expressed in hepatoblasts) showed similar phenotypes, a hypoplastic liver at E14.5 [60–62]. This again shows a clear interaction between the mesenchyme and the hepatoblasts. Interestingly, during regeneration of the adult liver, this pathway is important for the proliferation of the hepatocytes, since conditional c-met knockout mice show an inhibition in the proliferation after partial hepatectomy [63]. This is a good example in which pathways for the development of an organism function in a similar way in the adult.

The transcription factors Xbp1 and Foxm1b are also required for the liver bud cell proliferation [64]. Foxm1b knockout mice die in utero by E18.5. The fetal liver shows a 75% reduction in the number of hepatoblasts. This diminished proliferation of the hepatoblasts contributes to abnormal liver development. In addition, these animals do not develop intrahepatic bile ducts. Therefore, this factor seems to be critical for the hepatoblast precursor cells to differentiate toward the biliary epithelial cell lineage. The Xbp1 knockout mice also show hypoplastic livers [65]. These animals die from anemia caused by reduced hematopoiesis, with a reduced growth rate and increased apoptosis of hepatocytes. This again shows a link between hematopoiesis and liver development. Xbp1 is also highly expressed in hepatocellular carcinomas [65].

Using ex vivo cultured fetal mouse liver, Monga et al. [66] found a function of the Wnt pathway for fetal liver cell proliferation. Blocking the expression of β-catenin, a key component of the Wnt signaling pathway, leads to reduced cell proliferation. Consistent with these findings are studies in chicken embryos, in which an inhibition of Wnt signaling results in reduced liver size. In contrast, the overexpression of β-catenin increases the liver size [66,67].

Hlx is a homeodomain transcription factor whose expression is restricted to the hepatic mesenchyme. In studies by Hentsch et al. [68] and Lints et al. [69], deletion of this factor led to severe hepatic hypoplasia; the liver failed to expand and reached only 3% of its normal size. This was not associated with an increase in apoptotic cells. The animals also had severe anemia because the small liver provided insufficient support for fetal hematopoiesis.

The expression of the homeobox factor Hex is restricted to the ventral endoderm at the ten-somite stage. During further development, the expression is even more restricted to two areas, the future liver and thyroid [70,71]. Studies in Hex knockout mice established the importance of this factor for liver development [70,71]. More detailed investigations showed that at E9.0, the presumptive hepatic bud is formed but no albumin or Afp expression could be detected. Further analysis using reverse-transcriptase polymerase chain reaction of earlier stage embryos could detect the expression of Alb, Ttr, and Prox1 in the ventral endoderm [72]. This shows that Hex seems not to be necessary for the establishment of the hepatic fate but for the outgrowth of the hepatoblasts. It was further established that Hex promotes the hepatic endoderm to transition to a pseudostratified epithelium, which in turn allows hepatoblasts to emerge into the stromal environment and continue differentiating [73]. The function of Hex seems to be conserved in higher vertebrates, as in Hex$^{-/-}$ zebrafish no liver develops [74]. Zhang et al. [75] showed in the chicken that the expression of

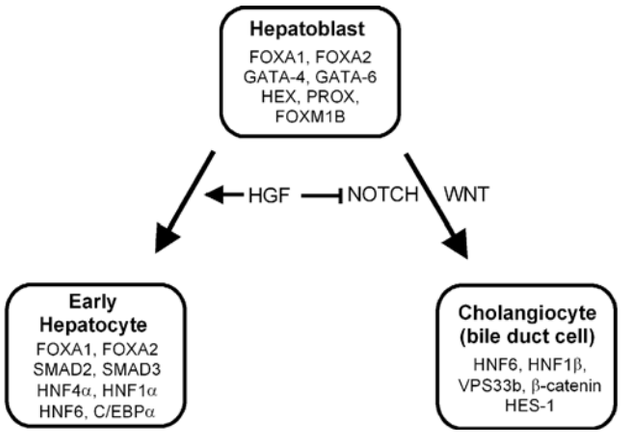

Figure 1.3. Transcription factors and signals promoting hepatoblast growth and differentiation into hepatocytes and cholangiocytes (bile duct cells). See text for details.

Hex in the ventral endoderm requires both FGF and BMP signaling. This is in agreement with a BMP-responsive element in the promoter of the *Hex* gene [76]. Other promoter studies show the binding of Foxa2 and Gata4 to the Hex promoter [77]. Both transcription factors are important for the promoter activity and contribute to liver-enriched expression of Hex (Figure 1.3).

Burke and Oliver [78] showed that the expression of homeobox transcription factor Prox1 is restricted to regions developing into mammalian pancreas and liver in the early endoderm. The liver of Prox1 knockout mice embryos at E14.5 is significantly smaller compared with control animals [79]. Still, these animals formed distinct liver lobes, but the hepatocytes were restricted to the central rudiment. At E10.0–E12.5, hepatoblasts were absent from the developing liver lobes. The proliferation of the hepatoblasts was strongly reduced in the knockout embryos. Further, it was shown that the $Prox^{-/-}$ cells failed to delaminate from the liver diverticulum. As these cells express Alb and Afp, Prox1 seems not to be necessary for the induction of the hepatic fate but for the expansion of the hepatoblasts (Figure 1.3). A newer study presented data that the early expression in hepatoblasts is evolutionarily conserved, as the same expression profile was found in chicken, mouse, rat, and human embryos [80–83]. Interestingly, this group showed that adult hepatocytes still express Prox1 and that the expression is strongly up-regulated in hepatoma cell lines. This might provide a link between a developmental factor and cancer.

FETAL HEMATOPOIESIS DURING LIVER DEVELOPMENT

As mentioned previously, after the liver bud emerges from the gut tube, hematopoietic cells migrate there and proliferate. The hematopoietic cells secrete oncostatin M (OSM), a growth factor belonging to the interleukin-6 (IL-6) family, and the surrounding liver cells express the gp130 receptor subunit OSMR. Supporting the importance of this interaction in fetal hepatocyte cultures, OSM stimulates the expression of hepatic differentiation markers and induces morphologic changes and multiple liver-specific functions as ammonia clearance, lipid synthesis, glycogen synthesis, detoxification, and cell adhesion [81–83].

Oncostatin M not only induces hepatic differentiation but also suppresses fetal liver hematopoiesis. For these experiments, Kinoshita et al. [8] used fetal hepatic cells from different developmental stages in co-culture with hematopoietic stem cells. Hepatic cells from E8.5 support the expansion of hematopoietic stem cells and give rise to myeloid, lymphoid, and erythroid lineages. The addition of OSM and glucocorticoid strongly suppresses this. In contrast, hepatic cells from E14.5 no longer support hematopoiesis in co-cultures. However, the hematopoietic cells induce further differentiation of hepatoblasts, and in consequence, the liver stops supporting local hematopoiesis and induces the hematopoietic stem cell switch to the bone marrow.

During liver development, the expression of the cyclins D1, D2, and D3 are down-regulated [83]. These cyclins are important for the initiation of the cell cycle and therefore for cell proliferation. In primary cultures of fetal hepatocytes, OSM can induce the down-regulation of the cyclins D1 and D2 [83]. This down-regulation is mediated by Stat3, which is activated through OSM and OSM receptor complex interaction.

SPECIFYING THE HEPATOCYTE AND BILIARY LINEAGE

Other studies showed that mice lacking either hepatocyte nuclear factor (HNF)6 or HNF1β in the liver show defects in the development of the biliary cell lineage, but little effects were found on the hepatocyte lineage [84,85]. Weinstein et al. [86] investigated the liver development in $Smad2^{+/-}$ and $Smad3^{+/-}$ mice; Smads are the downstream effectors of transforming growth factor-β (TGF-β). They found that the livers of these mice at E14.5 were dramatically hypoplastic, with a strong reduction of hepatocyte proliferation (Figure 1.3). In addition, there was more apoptotic cell death and the liver architecture was disrupted. These abnormalities were likely related to a defect in cell–cell adhesion, as β1-integrin was very strongly down-regulated. Interestingly, this phenotype could be rescued through the addition of HGF. Therefore, it seems that the TGF-β and HGF pathways cooperate on this aspect of liver development.

It is well known that hepatocytes and bile duct cells originate from a common precursor, the hepatoblast [87]. Notch signaling promotes hepatoblast differentiation into the biliary epithelial lineage, and HGF antagonizes this [88,89]. The expression of the Notch intracellular domain in hepatoblasts inhibits their differentiation into hepatocytes. In contrast, if Notch signaling was down-regulated by application of an siRNA against Notch2 mRNA, hepatic differentiation occurred. Therefore, HGF-based antagonism of Notch signaling would promote the commitment to the hepatocyte lineage (Figure 1.3).

Supporting the idea of HGF as a promoter for the hepatic fate decision is a study from Suzuki et al. [90]. They found that HGF induces the expression of C/EBPα in albumin-negative fetal liver cells. When C/EBPα activity is blocked through expression of a dominant negative form of C/EBPα, there is no transition of alb− to the alb+ stage (Figure 1.3).

The importance of the Notch signaling for biliary development is evolutionarily conserved. In humans, a haploinsufficiency of Jagged1 (a Notch ligand) leads to the Alagille syndrome. This disease is characterized by a reduction in intrahepatic bile ducts [91,92]. Notch signaling is also required for the biliary development in zebrafish [93], where disruption of signaling leads to a phenotype similar to humans with the Alagille syndrome. In mice, the Notch pathway controls the expression of the helix-loop-helix protein Hes1 [94,95]. Mice deficient in this factor show an absence of the gallbladder and severe hypoplasia of extrahepatic bile ducts, and the tubular formation of intrahepatic bile ducts is completely absent. In addition, the biliary epithelium of Hes1$^{-/-}$ mice ectopically expresses the proendocrine gene *Neuro3*, and pancreatic differentiation programs are activated. Thus, biliary epithelium has the potential for pancreatic differentiation, and Hes1 seems to determine biliary organogenesis by blocking the pancreatic cell fate (Figure 1.3).

Wnt signaling might be involved in regulating biliary epithelial cell fate. Ex vivo fetal liver culture experiments show that the addition of Wnt3A supports the biliary epithelial cell differentiation. In agreement with this finding, the inhibition of β-catenin prevents hepatoblasts from expressing biliary markers [66,96]. One important factor for activating this differentiation program is HNF6. HNF6 is expressed in hepatoblasts, in the gallbladder primordium, and in biliary epithelial cells of the developing intrahepatic bile ducts. HNF6 knockout mice developed no gallbladder, and the development of the intrahepatic and extrahepatic bile ducts was abnormal. The intrahepatic bile ducts had a similar phenotype in conditional HNF1β knockout mice. HNF1β was down-regulated in HNF6$^{-/-}$ mice, and HNF6 could activate the HNF1β promoter [84,85]. These results suggest that the effect of HNF1β is downstream of HNF6 (Figure 1.3). Supporting this relationship between HNF6 and HNF1β are results from zebrafish, which showed a biliary phenotype in HNF6-deficient animals and that this phenotype can be rescued by overexpressing HNF1β [97].

Another study in zebrafish found an additional gene involved in this cascade. The vps33b ortholog of a mammalian vacuolar sorting protein is expressed in the developing liver and intestine. A knockdown study showed similar biliary defects as in the HNF6 knockdown. The expression of vps33b is reduced in HNF6-deficient and vhnf1 mutated zebrafish embryos. This implies that vps33b is a downstream target gene of HNF6/vhnf1 [98]. It is important to realize that many of these pathways work together. HNF6 synergizes with Foxa2 to potentiate Foxa2 transcriptional activity by recruiting the p300/C/EBP coactivator proteins [99]. For a full understanding of liver development, it will be important to discern the feedback and regulatory loops between all pathways involved. For this, the use of bioinformatics will be crucial.

REGULATION OF LIVER-SPECIFIC GENE ACTIVITY AND DIFFERENTIATION

Odom et al. [100] have demonstrated the importance of HNF4α for gene regulation in hepatocytes. Microarray data suggest that HNF1α binds to 222 target genes in human hepatocytes corresponding to 1.6% of the genes assayed. HNF6 bound to 227 (1.7%), and HNF4α bound to 1575 (12%) of the genes, which means that HNF4α bound to nearly half of the active genes in the liver that were tested. In addition, most of the genes bound by HNF1α or HNF6 were also bound by HNF4α, but only a few genes were bound by both HNF1α and HNF6.

HNF4α knockout embryos have a severe defect in visceral endoderm formation, which prevents gastrulation and causes a stop in development at E6.5 [101]. This is consistent with the expression pattern of HNF4α, which is in the primary and extraembryonic visceral endoderm before gastrulation and in epithelial cells at the beginning of liver, pancreas, and intestine formation [102]. To circumvent this early mortality in HNF4α knockout mice, Duncan et al. [103] performed tetraploid rescue experiments. Applying this technology, the HNF4$\alpha^{-/-}$ embryos developed through gastrulation and developed a fetal liver. Analysis of the liver in rescued E12.5 embryos showed a critical function of HNF4α in the regulation of pregnane X receptor (PXR) and cross-regulation with HNF1α [104]. In addition, the expression of several liver genes, such as albumin, AFP, and transferrin was reduced. Using another strategy, Parviz et al. [105] made a conditional HNF4α knockout mouse specific for the fetal liver. At E18.5, the liver of these animals failed to store glycogen. Consistent with this, genes important for glucose homeostasis were down-regulated and the liver architecture was abnormal, including a decreased expression of cell adhesion and cell junction molecules in hepatocytes. Both these studies and others [106] together show clearly that HNF4α is broadly involved in diverse aspects of hepatocyte differentiation (Figure 1.3). As Gata4 is important for hepatic fate decision and HNF4α seems to be a downstream target, this again links the early steps of liver development with the later ones. To summarize, HNF4α is important for the regulation of many genes involved in the physiologic function of the liver, but this factor seems to be dispensable for the initial hepatic fate decision.

THE PHENOMENON OF TRANSDIFFERENTIATION

Transdifferentiation is the name used to describe the conversion of one differentiated cell type to another [107,108]. There is some evidence for a transdifferentiation of pancreas cells into hepatocyte-like cells. The first experiment to show this was performed by Scarpelli and Rao [109]. They used a methionine-deficient protocol to induce the regeneration of pancreas in the hamster. Analyzing the tissue, this group found hepatocytes in the regenerating pancreas. The authors suggested that the conversion of pancreas cells into hepatocytes was triggered by a single dose of the carcinogen N-nitrosobis (2-oxopropyl) amine,

given during the S-phase in the regenerating pancreatic cells. Since then, there have been different protocols in different animal systems that induce hepatocytes in the pancreas (e.g., rat [110–112], mice [113]). In addition to these experimental conditions, Paner et al. [114] found hepatocytes naturally in human pancreatic tumors.

In this context, the establishment of the pancreatic cell line AR42J was helpful. AR42J cells were originally isolated from a pancreatic carcinoma of an azaserine-treated rat [115,116]. These cells have an amphicrine character, meaning they possess both exocrine and neuroendocrine properties. A subclone of the parent cell line named AR42J-B13 was isolated later by Mashima et al. [117]. Shen et al. [118] showed that treatment with dexamethasone and OSM induces the formation of hepatocyte-like cells from AR42J-B13 or AR42J. These hepatocyte-like cells express liver markers such as albumin, glucose-6-phosphatase, transferrin, and transthyretin. The group found that the cells are induced to express the liver transcription factor C/EBPβ and that the ectopic expression of C/EBPβ alone induces the differentiation of AR42J cells into hepatocytes. Expression of liver inhibitory protein (LIP), a dominant negative form of C/EBPβ, blocks the transdifferentiation. In the same paper, the authors describe the use of isolated pancreatic buds from E11.5 mouse embryos. Cultures of the pancreatic bud exposed to dexamethasone and OSM leads to the transdifferentiation from an exocrine phenotype to a hepatic phenotype. These experiments suggest that C/EBPβ is an important switch for inducing transdifferentiation, and the experimental results support the idea that pancreatic exocrine cells have the potential to differentiate into hepatocyte-like cells. Still, it remains to be shown if these cells can completely function as hepatocytes in vivo.

Our knowledge of liver-to-pancreas cell transdifferentiation is more limited. Pancreatic-type exocrine tissue has been found in livers of rats treated with polychlorinated biphenyls [111], in fish liver tumors induced by chemical carcinogens [119], and in the liver of a human patient with hepatic cirrhosis [120]. But until now, there has been no clear evidence of transdifferentiation from hepatocytes to pancreatic cells. The pancreatic cells in the liver could come from oval cells or another kind of progenitor.

These reactions might be better understood in a developmental context. As mentioned before, the endoderm reacts to FGF signaling in a dose-dependent manner, and without FGF signaling, the endoderm takes the default pancreas fate. Therefore, both organs are strongly linked developmentally, and adult cells of these organs might retain the ability to activate the other determination program. For the future, it might be interesting to see if the ability for transdifferentiation is dependent on specific competence factors, such as Foxa and Gatas.

STEM CELLS AND THE LIVER

Recent studies suggest that bone marrow cells can differentiate into hepatocytes and cholangiocytes in the liver. Petersen et al. [121] performed bone marrow transplantation in mice, and then they induced liver damage and compensatory regeneration. Rare bone marrow donor cells migrated to the liver and differentiated into hepatocytes and bile duct cells. Theise et al. [122] showed later that this differentiation does not require liver damage or regeneration. Investigations in human patients who underwent bone marrow transplantation found hepatocytes derived from bone marrow cells [123,124]. Lagasse et al. [125] determined which bone marrow cell subpopulation possesses this ability. Only highly purified hematopoietic stem cells, but not other bone marrow cells, were able to restore liver function in mice. Interestingly, Ishikawa et al. [126] showed recently that bone marrow cells differentiate into hepatocytes via hepatoblast intermediates. They used green fluorescent protein–marked bone marrow cells for bone marrow transplantation experiments in carbon tetrachloride–treated mice (induces liver damage and consequently regeneration). In this model, FGF is an important growth factor for the differentiation process of bone marrow cells to hepatocytes. Treatment of these mice with recombinant FGF2 increased the repopulation by bone marrow cells and increased the expression of hepatoblast marker genes. In addition, these animals showed a higher survival rate. This links the phenomenon of bone marrow cell differentiation into hepatocytes to the events occurring during liver development.

A different perspective on these studies was gained from work on restoring liver function in fumarylacetoacetate hydrolase (FAH)-deficient mice, in which the application of myelomonocytic cells was sufficient [127,128]. Notably, it was shown that cell fusion is the source of apparent bone marrow–derived hepatocytes. That is, rare bone marrow–derived cells fuse with resident hepatocytes and activate the *Fah* gene on a bone marrow cell chromosome, thereby complementing the host cell Fah1 defect. A recent study using monkey embryonic stem cells also showed cell fusion events [129]. Monkey embryonic stem cells were cultured to form embryoid bodies. In these embryoid bodies, hepatocyte-like cells were found and transplanted into immunodeficient, urokinase-type plasminogen-activator transgenic mice. When these mice developed liver failure, hepatocytes expressing monkey albumin were identified. A more detailed analysis of the cells, however, showed that they originated from cell fusion. Interestingly, if undifferentiated embryonic stem cells were applied, cell fusion and liver repopulation did not occur. Therefore, cell fusion might occur more with partially differentiated cells and might be another mechanism distinct from stem cell differentiation.

Schwartz et al. [130] isolated multipotent adult progenitor cells (MAPCs) from postnatal bone marrow from human, mouse, and rat. Culturing these cells on Matrigel with FGF4 and HGF caused the cells to differentiate into hepatocyte-like cells. The cells expressed the typical liver genes *Foxa2*, *Gata4*, cytokeratin 19, transthyretin, *Afp*, and albumin. But more importantly, these cells showed functional characteristics of hepatocytes, such as the secretion of urea and albumin, expression of phenobarbital-inducible cytochrome P450, taking up of low-density lipoprotein, and storage of glycogen. These results are more supportive of the idea of a stem cell differentiating into

hepatocytes, as in the cell culture system, no cell fusion with hepatocytes should be possible.

Recent studies have shown that embryonic stem cells can efficiently be differentiated into definitive endoderm [131,132]. The differentiated cells could be purified to near homogeneity. Kubo et al. [133] described a protocol for the induction of differentiation into definitive endoderm from embryonic stem cells. After the embryonic stem cells differentiated into embryoid bodies, the cells were cultured in the presence of activin A under serum-free conditions. This induced the development of endoderm from a brachyury-positive population that also displays mesoderm potential. Activin belongs to the TGF-β family and, as discussed previously, members of this family are important for endoderm formation during normal development. Interestingly, it was previously shown that hepatic differentiation could be induced in embryonic stem cells by co-culturing them with cardiac mesoderm [22]. This, again, is a link to a process during development, in which the cardiac mesoderm is essential for hepatic fate decision. Additional studies have identified growth factors that allow direct hepatic fate specification from embryonic stem cells in cell culture [134]. A combination of FGF1, FGF4, and HGF can induce the hepatic fate, and the later addition of OSM to the cell culture induced an even more differentiated hepatocyte-like cell. Again, this in part is a recapitulation of the events during development. This study also showed that the transplantation of the differentiated cells into mice with cirrhosis had a significant therapeutic effect. This is a very important control to confirm that hepatocyte-like cells can function as hepatocytes in vivo. Using microarray analysis, the same group later showed that the gene expression profile of the appropriately differentiated embryonic stem cells is highly similar to that of adult mouse liver [135]. In addition, they used siRNA against Foxa2 and identified this transcription factor as having an essential role in hepatic differentiation from embryonic stem cells. This agrees with the findings during normal liver development.

For the future, studies of embryonic stem cells to endoderm or hepatocyte differentiation can increase our understanding of the molecular basis of liver development, as this technique overcomes the limitations of the small amount of tissue during normal development. The exact understanding of these developmental processes that lead to a specific cell fate might help us to recapitulate the events in vitro and engineer artificial liver cells and tissue to combat pediatric liver diseases.

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