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The onecut transcription factor HNF6 is required for normal development of the biliary tract

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SUMMARY

During liver development, hepatoblasts differentiate into hepatocytes or biliary epithelial cells (BEC). The BEC delineate the intrahepatic and extrahepatic bile ducts, and the gallbladder. The transcription factors that control the development of the biliary tract are unknown. Previous work has shown that the onecut transcription factor HNF6 is expressed in hepatoblasts and in the gallbladder primordium. We now show that HNF6 is also expressed in the BEC of the developing intrahepatic bile ducts, and investigate its involvement in biliary tract development by analyzing the phenotype of *Hnf6*$^{-/-}$ mice. In these mice, the gallbladder was absent, the extrahepatic bile ducts were abnormal and the development of the intrahepatic bile ducts was perturbed in the prenatal period. The morphology of the intrahepatic bile ducts was identical to that seen in mice whose *Hnf1β* gene has been conditionally inactivated in the liver. HNF1β expression was downregulated in the intrahepatic bile ducts of *Hnf6*$^{-/-}$ mice during development. Furthermore, we found that HNF6 can stimulate the *Hnf1β* promoter. We conclude that HNF6 is essential for differentiation and morphogenesis of the biliary tract and that intrahepatic bile duct development is controlled by a HNF6→HNF1β cascade.

Key words: HNF6, Biliary tract, Liver, Mouse, HNF1β

INTRODUCTION

The bile is made in the liver by the hepatocytes and it transits to the gut via the biliary tract, which consists of intrahepatic and extrahepatic bile ducts and of the gallbladder. The bile is secreted into canaliculi delineated by the apical surface of the hepatocytes. These canaliculi connect to the network of intrahepatic bile ducts (IHBD), which transport the bile towards the hilum of the liver. The bile produced in each liver lobe is collected in individual hepatic ducts, transported to and from the gallbladder via the cystic duct, and excreted into the gut via the common bile duct. The hepatic, cystic and common bile ducts are collectively termed extrahepatic bile ducts (EHBD). The lumen of the IHBD, of the EHBD and of the gallbladder is delineated by an epithelium consisting of biliary epithelial cells (BEC), also called cholangiocytes.

The formation of the biliary tract during embryogenesis is intimately linked to liver development (reviewed by Shiojiri, 1984; Shiojiri, 1997). In mammalian embryos, the liver starts developing as a diverticulum on the ventral side of the anterior intestinal portal (reviewed by Zaret, 1998; Darlington, 1999; Zaret, 2000). The diverticulum consists of a cranial part and a caudal part, although in mice the boundary between these two parts is not histologically clear. The cranial part gives rise to the hepatocytes and to the BEC that delineate the IHBD and part of the EHBD. The caudal part develops into the common bile duct and the gallbladder.

The BEC arise from the hepatoblasts which are precursor cells that have the potential to differentiate into either hepatocytes or BEC (Shiojiri, 1984; Germain et al., 1988; Shiojiri et al., 1991; Spagnoli et al., 1998). The morphogenesis of the IHBD has been described in humans and rats by a combination of histological and immunohistochemical methods (Van Eyken et al., 1988a; Van Eyken, 1988b). The hepatoblasts located at the interface between the portal mesenchyme and the liver parenchyma differentiate into BEC that form a single cell layer called the ductal plate. Segments of the ductal plate become bilayered, and focal dilations appear between the two BEC layers. The ductal plate is then remodeled around these dilations to form ducts, while the BEC located between the ducts eventually disappear. Subsequently, the ducts become incorporated within the portal mesenchyme. The differentiation of IHBD progresses from the hilum of the liver towards its periphery, and the ducts progressively connect with one another to form a network that will drain the bile towards the EHBD (Shiojiri and Katayama, 1987). In humans,

lack of remodeling of the ductal plate is thought to be a basic anomaly in several congenital diseases of the IHBD (Desmet, 1992).

Much attention has been paid to the initial steps of hepatic differentiation from the endoderm and to their control by extracellular signals and transcription factors (reviewed by Zaret, 1998; Darlington, 1999; Duncan, 2000; Zaret, 2000). However, no transcription factor that controls biliary tract development has been identified. A good candidate is the onecut transcription factor HNF6 (Onecut1 – Mouse Genome Informatics) (Lemaigre et al., 1996; Lannoy et al., 1998). Its mRNA is expressed in mouse liver starting at the onset of its development, and is detected not only in hepatoblasts, but also in the gallbladder primordium and in the EHBD (Landry et al., 1997; Rausa et al., 1997). To study the involvement of HNF6 in biliary tract development, we have now investigated in detail the expression of HNF6 in fetal liver and have analyzed the consequences of inactivating the *Hnf6* gene for biliary tract development. Our results show that, in wild-type fetuses, HNF6 protein is present in the BEC of the gallbladder, of the EHBD and of the IHBD, starting at the onset of their differentiation. We have found earlier that the *Hnf6*$^{-/-}$ mice display an abnormal pancreatic phenotype characterized by perturbed differentiation of the endocrine cells (Jacquemin et al., 2000). We now analyze the liver phenotype and demonstrate that HNF6 is required for gallbladder formation, for normal differentiation of the BEC and for proper morphogenesis of the IHBD and EHBD.

MATERIALS AND METHODS

Fouchet's staining

Livers from newborn mice were dissected in phosphate buffered saline (PBS) and fixed in Bouin's fixative. Paraffin sections (5 μm) were rehydrated, stained for 5 minutes in Fouchet's reagent (trichloroacetic acid 25%, ferric chloride 10% in water) and counterstained for 5 minutes with Van Gieson.

Immunohistochemistry

Dissected fetuses or livers were directly frozen in liquid nitrogen-cooled isopentane or fixed in formaldehyde [1% in phosphate-buffered saline (PBS) for 2 hours], embedded in gelatin/sucrose and stored at –80°C. Cryosections (5 or 10 μm) were made. Sections of fresh-frozen tissue were post-fixed in acetone. Primary antibodies were affinity-purified polyclonal rabbit anti-mouse HNF6 (a gift from R. Costa) (Rausa et al., 2000), polyclonal rabbit anti-PDX-1 (a gift from O. Madsen), polyclonal rabbit anti-human keratin (DAKO, A0575), mouse monoclonal anti-human Ki-67 (PharMingen International 36521A) and rabbit polyclonal anti-laminin (Eurodiagnostics). The rabbit polyclonal anti-HNF1β antibody was raised against residues 32 to 89 of the mouse HNF1β protein fused to gluthathione S-transferase, as described (Cereghini et al., 1992). The serum used in this study, obtained after three boosts, recognizes the HNF1βA and B splicing isoforms, but it does not crossreact with the closely related protein HNF1α. Primary antibodies were detected by immunoperoxidase using EnVision Peroxidase secondary reagents (DAKO), except for the anti-PDX-1 antibody which was detected using biotinylated sheep anti-rabbit (Roche) followed by streptavidine-peroxidase conjugate (Roche). The peroxidase was detected using 3-amino-9-ethylcarbazole (ICN Biochemicals) for cytokeratin and laminin staining on acetone-fixed sections, or DAB+ (DAKO) for HNF6, PDX-1 and HNF1β on formaldehyde-fixed sections. For immunofluorescence, primary antibodies were detected with secondary antibodies coupled to FITC or Texas Red (Immunoresearch Laboratories).

Semi-quantitative RT-PCR analysis

Total RNA was diluted to 0.2 μg/μl, denatured at 85°C for 3 minutes, and quickly chilled on ice. Total RNA (1 μg) was incubated in a final volume of 25 μl containing 50 mM TrisCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 200 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies), 40 units of RNAsin (Promega), 3 μg of random hexamers (Life Technologies) and 1 mM dNTPs (Amersham Pharmacia Biotech), and left for 10 minutes at room temperature and subsequently for 1 hour at 37°C. After cDNA synthesis, the incubation was diluted with 50 μl of H_2O. For the PCR, 3 μl of the diluted cDNA incubation were incubated in a volume of 50 μl containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% (vol/vol) Triton X-100, 1.5 mM $MgCl_2$, 40 μM dATP, dTTP and dGTP, 20 μM dCTP, 10 pmol of each primer, 2.5 units of Taq polymerase (Promega) and 2.5 μCi of 3000 Ci/mmol [^{32}P]dCTP (Amersham Pharmacia Biotech). Mineral oil (50 μl) was added to each tube. Standard thermal cycle profile was as follows. A single denaturing step at 94°C for 300 seconds was followed by the chosen number of cycles: 94°C for 30 seconds; 55°C for 30 seconds; 72°C for 30 seconds. The number of cycles was determined for each amplicon as required to reach mid-logarithmic phase of amplification. Primer sequences were 5′-GGCATCGTGATGGACTCCG-3′ and 5′-GCTGGAAGGTGGACAGCGA-3′ (β-actin), 5′-TTCTAAGCTGAGCCAGCTGCAGACG-3′ and 5′-GCTGAGGTTCTCCGGCTCTTTCAGA-3′ (HNF1α), 5′-GAAAGCAACGGGAGATCCTC-3′ and 5′-CCTCCACTAAGGCCTCCCTC-3′ (HNF1β), 5′-ACCCCACGAATCTCAGCTGCA-3′ and 5′-CAAGGTAGCGCCATAAGGAGA-3′ (HNF3α), 5′-CCATCCAGCAGAGCCCCAACA-3′ and 5′-GTCTGGGTGCAGGGTCCAGAA-3′ (HNF3β), 5′-TCGTCCACACCTTATTTCAGCG-3′ and 5′-AGGATGCATTAAGCAGAGAGC-3′ (HNF3γ), 5′-ACACGTCCCCATCTGAAG-3′ and 5′-CTTCCTTCTTCATGCCAG-3′ (HNF4α), 5′-CAGCACCTCACGCCCACCTC-3′ and 5′-CAGCCACTTCCACATCCTCCG-3′ (HNF6), 5′-ACCCTTCACCAATGACTCCTATG-3′ and 5′-ATGATGACTGCAGCAAATCGC-3′ (Tbp).

Electrophoretic mobility shift assay (EMSA)

EMSA was performed as described (Jacquemin et al., 2000). The sense strand sequence of the double-stranded oligonucleotide probes, derived from the mouse *Hnf1β* gene (*Tcf2* – Mouse Genome Informatics), was 5′-AACTATAAAACAATTGATAAATAAGTTAGT-3′ (site I) and 5′-CCTGGCCAGCCTATGGATTTTAATGATAAT-3′ (site II) (the HNF6-binding consensus is underlined).

Cell culture and transient transfection

Human embryonic kidney (HEK293) cells (3.5×10^5 cells) were transfected with 2 μg of the indicated *Hnf1β* promoter-CAT reporter construct and the indicated amount of pCB6-HNF6 expression vector or pCB6 empty vector, and 1 μg of pRSV-β-gal internal control vector, using the standard calcium phosphate co-precipitation procedure. Cells were harvested 36 hours later and CAT and β-galactosidase activities were measured as described (Power and Cereghini, 1996). All transfections were performed in duplicate in at least three independent experiments.

RESULTS

HNF6 protein is detected in hepatocytes and in biliary epithelial cells

In situ hybridization studies showed that *Hnf6* is expressed in the liver and in the gallbladder, starting at the onset of their formation (Landry et al., 1997; Rausa et al., 1997). In these

experiments, HNF6 mRNA was not detectable in liver between embryonic day (E) 12.5 and E15. We first reassessed these observations with the more sensitive technique of RT-PCR. Data in Fig. 1A show that the *Hnf6* gene is expressed in the liver throughout this period, but that HNF6 mRNA concentration decreases between E12.5 and E15.5. To identify which cell types express HNF6 during development, we performed immunostaining with an antibody directed against a recombinant HNF6 protein (Fig. 1B-D). At E15.5, the strongest expression within liver was seen in the BEC which, at that stage, form a ductal plate at the interface between the portal mesenchyme and the liver parenchyma. Lower expression was observed in hepatocytes (Fig. 1B). Strong HNF6 immunostaining was also detected in the BEC of the gallbladder (Fig. 1C) and of the common bile duct (Fig. 1D).

HNF6 is essential for development of the gallbladder and morphogenesis of the extrahepatic bile ducts

To determine whether HNF6 controls the development of the biliary tract, we analyzed the phenotype of mice in which the *Hnf6* gene had been inactivated by homologous recombination (Jacquemin et al., 2000). The biliary tract of $Hnf6^{+/-}$ mice was indistinguishable from that of wild-type mice. By contrast, several biliary anomalies were observed in $Hnf6^{-/-}$ mice. First, the gallbladder was missing (Fig. 2A,B) and this resulted from the lack of gallbladder primordium. At E10.5, the earliest stage at which this primordium can be detected (Kaufman, 1992), it was absent in $Hnf6^{-/-}$ embryos, whereas it was clearly identified in the septum transversum of wild-type embryos (Fig. 2C,D). Because of the lack of gallbladder-specific marker, we could not investigate further the mechanism of the absence of gallbladder in $Hnf6^{-/-}$ mice. Second, the morphology of the EHBD in $Hnf6^{-/-}$ mice was abnormal (Fig. 2A,B). In control animals, each hepatic duct drains the bile from an individual liver lobe. The hepatic ducts merge with the cystic duct and form the common bile duct, which ends in the duodenum (Fig. 2A). In $Hnf6^{-/-}$ mice, the hepatic ducts were not individualized but were replaced by an enlarged structure that connects the liver to the duodenum (Fig. 2B).

We conclude from these observations that HNF6 is required to initiate the development of the gallbladder and that it controls the morphogenesis of the EHBD.

HNF6 controls development of the intrahepatic bile ducts

Newborn $Hnf6^{-/-}$ mice were icteric and had high levels of bilirubin in the urine (not shown), suggesting that they suffer from a cholestatic syndrome. This hypothesis was confirmed by analysis of liver sections with Fouchet's staining. This method allows detection of bilirubin only when it accumulates under pathological conditions. Accumulation of bile was detected in the canaliculi of $Hnf6^{-/-}$ mice (Fig. 2E). Large bile lakes surrounded by necrotic parenchyma were observed (Fig. 2F). Such observations are diagnostic for liver necrosis resulting from cholestasis. They may explain in part the high mortality rate (75%) of $Hnf6^{-/-}$ mice between postnatal day (P) 1 and P10 (see Discussion). We hypothesized that this cholestatic syndrome results from abnormal differentiation of the BEC or from perturbed morphogenesis of the IHBD.

Little information on intrahepatic biliary development is available in the mouse. Thus, we first had to establish the time-course of IHBD formation in wild-type fetuses. The morphogenesis of IHBD progresses from the hilum towards the periphery of the lobes. We therefore characterized the pattern of IHBD development at each stage according to the most differentiated biliary structure found around branches of the portal vein located close to the hilum. To identify the developing BEC we immunostained liver sections with an anti-

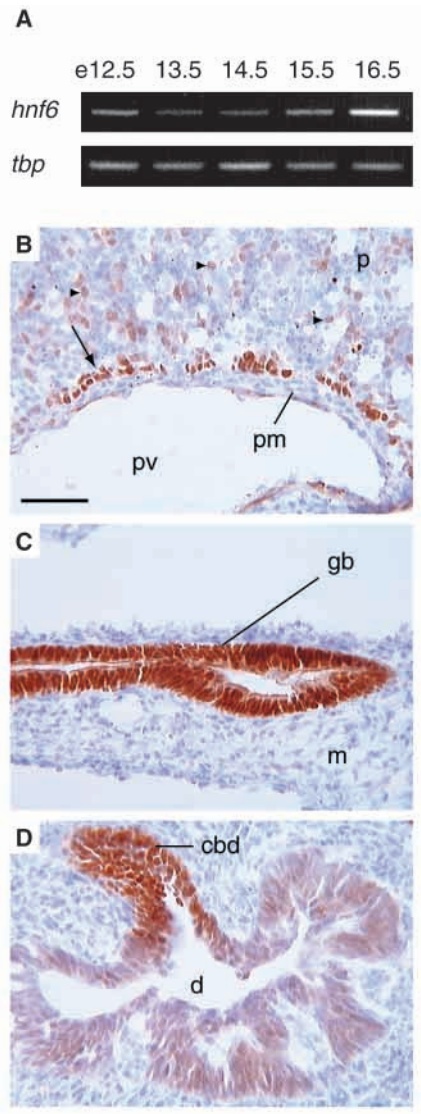


Fig. 1. HNF6 is expressed in mouse fetal liver and biliary tract. HNF6 expression was assessed by RT-PCR with TATA box-binding protein (*Tbp*) as a reference (A) or by immunohistochemistry at E15.5 (B-D). (A) In the liver, *Hnf6* mRNA is detected throughout the fetal period, although its concentration is lower between E12.5 and E15.5. (B) At E15.5, a strong nuclear staining is observed in the ductal plate, in cells located at the interface between the portal mesenchyme and the liver parenchyma (arrow), and a weaker nuclear signal is detected in hepatocytes (arrowheads). (C) HNF6 is also detected in the epithelium of the gallbladder primordium, but not in the surrounding mesenchyme. (D) The epithelium of the common bile duct shows a strong HNF6 signal, while a weak signal is present in the epithelium of the duodenum. cbd, common bile duct; d, duodenum; gb, gallbladder primordium; m, mesenchyme; p, liver parenchyma; pm, portal mesenchyme; pv, portal vein. Scale bar: 50 μm.

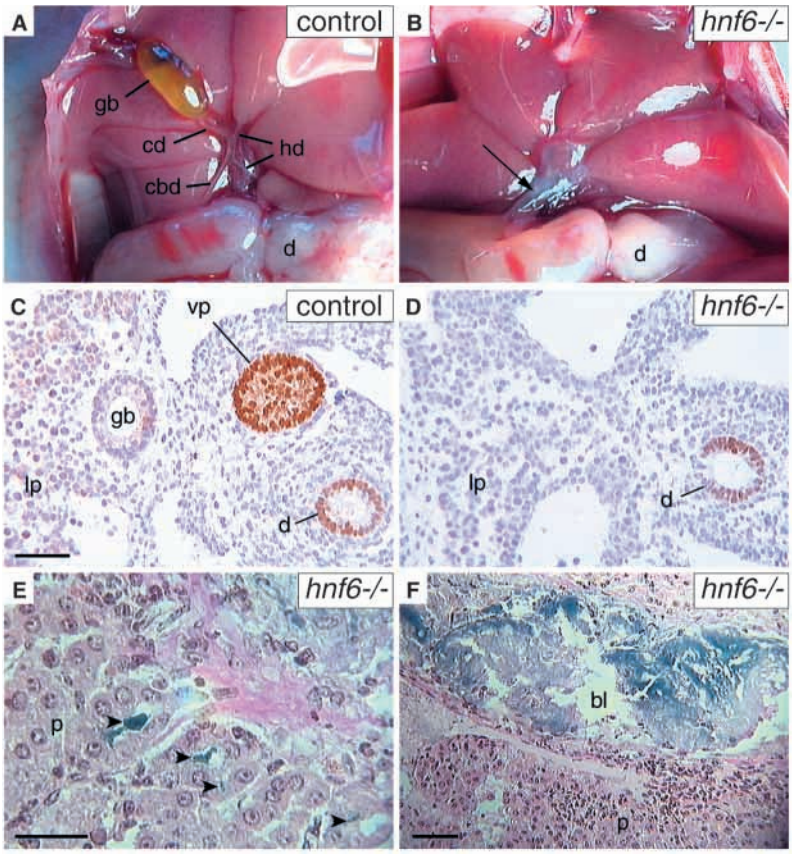


Fig. 2. Lack of HNF6 results in absence of gallbladder, abnormal morphogenesis of the extrahepatic bile ducts and cholestasis. (A) In wild-type adult mice, the gallbladder is seen on the ventral side of the liver and the hepatic ducts merge with the cystic duct to form the common bile duct, which ends up in the duodenum. (B) In *Hnf6*$^{-/-}$ adult animals, the gallbladder is absent. An enlarged structure (arrow) connects the liver to the duodenum. (C,D) Transverse sections in E10.5 embryos. The sections were stained for Pdx-1 to identify the ventral pancreatic bud (strong staining) and the duodenum (weak staining). Rostral is towards the left, caudal towards the right. (C) In wild-type embryos, the gallbladder primordium, devoid of Pdx-1 staining, is observed between the liver primordium and the ventral pancreatic bud. (D) In *Hnf6*$^{-/-}$ embryos, the gallbladder primordium is absent. Note that the ventral pancreatic bud is also missing at E10.5, as formation of the pancreatic buds is delayed in *Hnf6*$^{-/-}$ mice (P. J., G. G. R. and F. P. L., unpublished). (E,F) Fouchet's staining on liver sections from *Hnf6*$^{-/-}$ mice at P6. (E) Abnormal bile accumulation between the hepatocytes (arrowheads) is observed. (F) Large areas of bile accumulation surrounded by necrotic parenchyma are also detected. bl, bile lake; cbd, common bile duct; cd, cystic duct; d, duodenum; gb, gallbladder; hd, hepatic duct; lp, liver primordium; p, liver parenchyma; vp, ventral pancreatic bud. Scale bars: 50 μm in C,E (bar in C also applies to D); 100 μm in F.

cytokeratin (CK) antibody known to label the BEC specifically (Van Eyken et al., 1987). At E13.5 in wild-type fetuses, a few CK-positive cells were found dispersed throughout the liver parenchyma (Fig. 3A). At E14.5, CK-positive cells formed a discontinuous layer at the interface between the portal mesenchyme and the liver parenchyma (Fig. 3C). This layer was continuous at E15.5 and formed the ductal plate (Fig. 3E). At E16.5, regions of the ductal plate became bilayered and focal dilations appeared between the two cell layers, thereby forming tubular structures that are the precursors of the bile ducts (Fig. 3G). At E17.5, the remodeling of the ductal plate progressively gave rise to individualized bile ducts, which started to be incorporated within the portal mesenchyme (Fig. 3I). The regions of ductal plate not involved in bile duct formation were regressing. At P10, the bile ducts were completely surrounded by mesenchyme. Remnants of the ductal plate were still visible at that stage (Fig. 3K).

We then analyzed IHBD development in *Hnf6*$^{-/-}$ fetuses and compared it with that of control fetuses. In *Hnf6*$^{-/-}$ livers at E13.5, an abnormally high number of CK-positive cells accumulated around the branches of the portal vein and formed cord-like extensions within the liver parenchyma. In addition, a few lumina were visible between the CK-positive cells (Fig. 3B). At E14.5, these cells delineated irregular duct-like structures (Fig. 3D), which had increased in number and in size at E15.5. These structures were then characterized by a regular and continuous epithelium of CK-positive cuboidal cells. These cells expressed laminin as do differentiated BEC (Fig. 4A-D). However, such structures were never seen incorporated within the portal mesenchyme. At that stage, no ductal plate was seen (Fig. 3F). At E16.5, large cysts were found in some animals, in addition to the duct-like structures already seen at E15.5. The cysts were delineated by an epithelium of CK-expressing cells (Fig. 3H) and therefore most probably derived from the duct-like structures observed at earlier stages. At E17.5, the cysts and duct-like structures were disrupted and the anti-CK immunoreactivity was no longer observed on all the cells of their epithelium (Fig. 3J). Moreover, a single layer of CK-positive cells was now observed around the portal mesenchyme (Fig. 3J), suggesting that a new wave of differentiation was initiated to form a ductal plate. At P10, the duct-like structures had almost completely regressed, and discontinuous ductal plates were observed (Fig. 3L).

The numerous CK-positive cells observed at E13.5 in the *Hnf6*$^{-/-}$ livers could arise either from an excessive proliferation of early BEC or from an increased number of hepatoblasts that have differentiated towards the biliary lineage. To discriminate between these two hypotheses, we determined by double-immunofluorescence experiments whether CK and the proliferation marker Ki-67 were co-expressed in the BEC (Fig. 4E,F). As the number of CK-positive cells is too small at E13.5, control fetuses were analyzed at E14.5. At that stage, the CK-positive cells were not expressing Ki-67 (Fig. 4E), consistent with the earlier proposal that these cells are postmitotic (Fabris et al., 2000). In *Hnf6*$^{-/-}$ fetuses, the numerous CK-positive cells detected at E13.5 did not express Ki-67 either (Fig. 4F). This suggests that the excess of BEC found at E13.5 in the *Hnf6*$^{-/-}$ fetuses does not result from increased proliferation, but results from an increased number of hepatoblasts that have differentiated towards the biliary lineage.

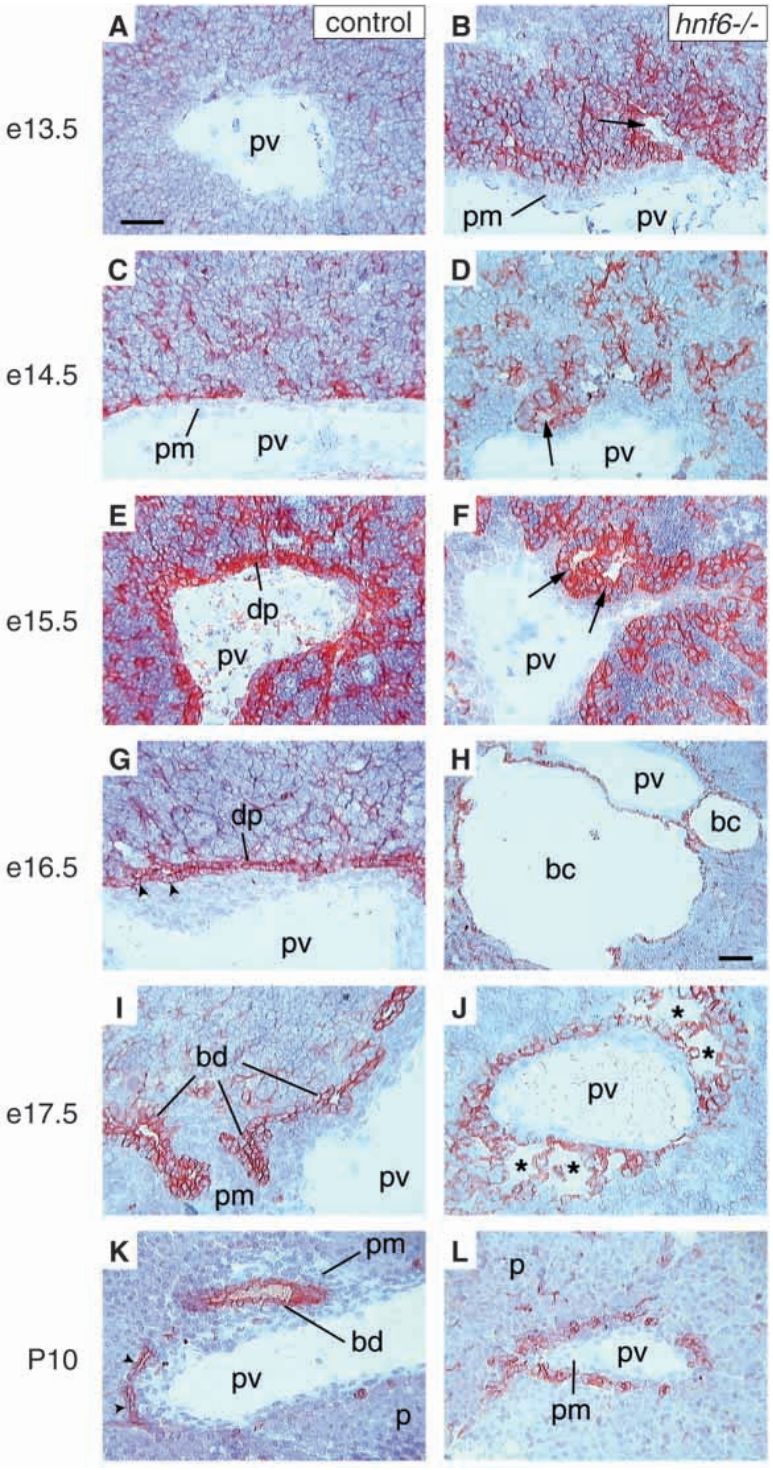


Fig. 3. Time-course of intrahepatic bile ducts development in control and in *Hnf6*$^{-/-}$ animals. The biliary tract of *Hnf6*$^{+/-}$ mice was indistinguishable from that of wild-type mice. Heterozygotes were therefore used as controls together with wild-type fetuses. The biliary epithelial cells (BEC) were visualized on liver sections by cytokeratin (CK) immunostaining. (A,B) At E13.5 in the controls, a few CK-positive cells are found dispersed throughout the liver parenchyma. In *Hnf6*$^{-/-}$ fetuses, numerous CK-positive cells are observed in the vicinity of the portal vein. These cells extend as cords within the liver parenchyma and a few lumina (arrow) are visible between the cells. (C,D) At E14.5 in the controls, CK-positive cells form a discontinuous layer at the interface between the portal mesenchyme and the liver parenchyma. In *Hnf6*$^{-/-}$ fetuses, CK-positive cells form irregular duct-like structures (arrow). (E,F) At E15.5 in the controls, BEC form a single continuous layer of cells called the ductal plate around the portal vein. In the *Hnf6*$^{-/-}$ fetuses, disorganized biliary structures, including duct-like structures (arrows), are observed. These duct-like structures are delineated by a continuous cuboidal epithelium of CK-positive cells. (G,H) At E16.5 in the controls, the ductal plate is partly duplicated and focal dilations (arrowheads) are visible between the two layers of BEC. In some *Hnf6*$^{-/-}$ livers, large biliary cysts delineated by CK-positive cells and located in the vicinity of the portal vein are observed (note that magnification is lower in H). (I,J) At E17.5 in the controls, the first bile ducts are identified as CK-positive cells surrounding a lumen and incorporated into the portal mesenchyme. Between the bile ducts, CK-positive cells have disappeared from the interface between the portal mesenchyme and the liver parenchyma. In *Hnf6*$^{-/-}$ fetuses, lumina (asterisks) are still present around the portal vein but the surrounding epithelium is disorganized and partly consists of CK-negative cells. A single layer of CK-positive cells is observed around the portal mesenchyme. (K,L) At P10 in the controls, mature bile ducts are seen within the portal mesenchyme and remnants of the ductal plate ongoing regression (arrowheads) are still visible between the liver parenchyma and the portal mesenchyme. In *Hnf6*$^{-/-}$ animals, CK-positive cells are located discontinuously around the portal mesenchyme. bc, biliary cyst; bd, bile duct; dp, ductal plate; p, liver parenchyma; pm, portal mesenchyme; pv, portal vein. Scale bar: in A, 50 μm in A-G,I-L; in H, 100 μm.

We conclude from these experiments that HNF6 controls the differentiation of hepatoblasts into BEC, as well as the morphogenesis of the IHBD.

HNF6 is essential for the expression of *Hnf1β* in biliary epithelial cells

HNF6 participates to the network of liver-enriched transcription factors (Landry et al., 1997; Lahuna et al., 2000; Rastegar et al., 2000). We therefore investigated by semiquantitative reverse transcription-PCR (RT-PCR) whether the lack of HNF6 induced perturbations in the liver expression of those factors (Fig. 5). The expression of *Hnf3β* (*Foxa2* – Mouse Genome Informatics), *Hnf3γ* (*Foxa3* – Mouse Genome Informatics) and *Hnf4α* (*Hnf4* – Mouse Genome Informatics) was similar in control and *Hnf6*$^{-/-}$ livers at all stages tested. A slight but reproducible increase in the expression of *Hnf1α* and *Hnf3α* (*Foxa1* – Mouse Genome Informatics) was observed at E14.5 and P3, suggesting that HNF6 controls these genes negatively in liver. The most strongly affected factor was HNF1β. Expression of its two splicing isoforms was almost abolished at E12.5 (Fig. 5B) and at E14.5 (Fig. 5A), but it had returned to near normal levels at P3 (Fig. 5A).

Liver-specific inactivation of the *Hnf1β* gene induces, before birth, the same morphological defects in IHBD development as those seen in *Hnf6*$^{-/-}$ fetuses [see accompanying paper (Coffinier et al., 2002)]. The *Hnf1β* defective livers at E17.5 show disrupted duct-like structures and a single layer of CK-positive cells around the portal mesenchyme. This suggests that

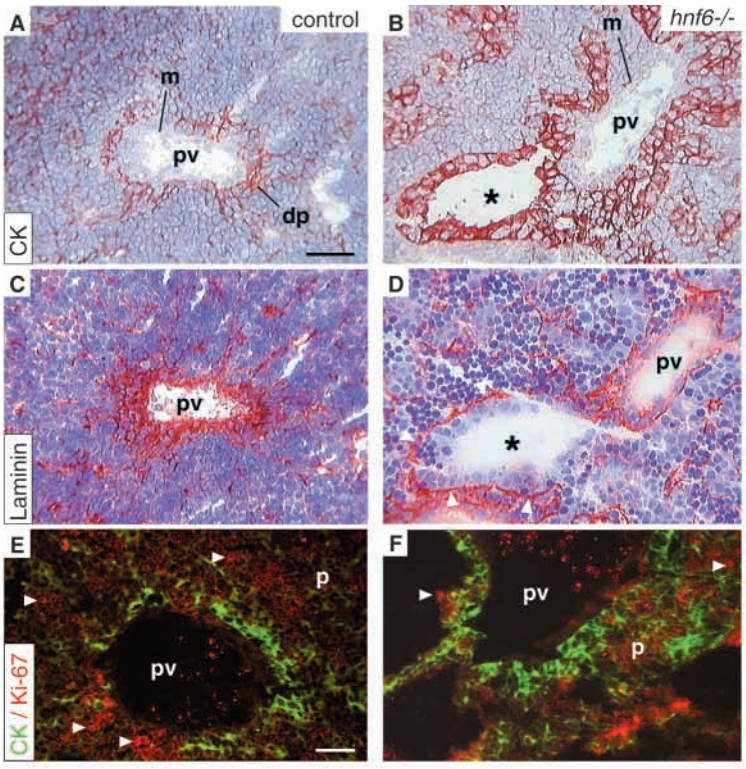
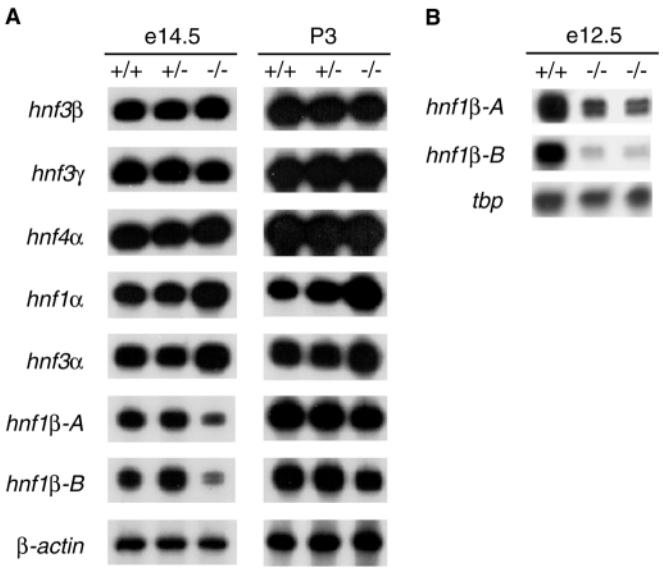


Fig. 4. Differentiation of biliary epithelial cells (BEC) in control and *Hnf6*$^{-/-}$ fetuses. (A-D) CK-positive cells and portal mesenchyme express laminin as detected by immunohistochemistry in control and in *Hnf6*$^{-/-}$ fetuses at E15.5. In *Hnf6*$^{-/-}$ fetuses, laminin (arrowheads in D) is found at the basal pole of the BEC surrounding the lumen (asterisks in B,D) of duct-like structures. (A,C) and (B,D) show adjacent sections. (E,F) Double immunofluorescence for CK (green) and for the proliferation marker Ki-67 (red) on liver sections. These were analyzed when the number of CK-positive cells was high enough, i.e. at E14.5 in control fetuses and at E13.5 in *Hnf6*$^{-/-}$ fetuses. Both in control (E) and in *Hnf6*$^{-/-}$ (F) livers, the CK-positive cells do not express Ki-67, which is detected in CK-negative cells (arrowheads). dp, ductal plate; m, portal mesenchyme; p, liver parenchyma; pv, portal vein. Scale bars: 50 μm in A,E.



HNF6 controls IHBD development via HNF1β. To buttress this hypothesis, we looked in detail at the expression of HNF1β in wild-type and *Hnf6*$^{-/-}$ livers. Using *lacZ* knock-in experiments, it has been shown that *Hnf1β* is expressed in the liver diverticulum (Barbacci et al., 1999; Coffinier et al., 1999a), in the gallbladder primordium and in late gestation bile ducts (Coffinier et al., 1999b). We therefore examined by immunohistochemistry if HNF1β was expressed in the ductal plate (Fig. 6A,B). In control fetuses at E15.5, strong staining for HNF1β was observed in the BEC-forming ductal plates, whereas a diffuse and weak background staining was seen in the liver parenchyma (Fig. 6A). The staining for HNF1β was nuclear (see high magnification inset in Fig. 6A). This shows that the ductal plates express both HNF1β and HNF6 (compare Fig. 1B with Fig. 6A, which are adjacent sections). The absence of clear HNF1β staining of hepatocyte nuclei is consistent with the low expression level of *Hnf1β* seen in *lacZ* knock-in experiments (Coffinier et al., 1999b). In the *Hnf6*$^{-/-}$ fetuses at E15.5, expression of HNF1β in the BEC was undetectable (Fig. 6B). Lack of HNF1β expression was transient, as HNF1β staining in BEC was again detectable starting around E17.5 (inset in Fig. 6B). All these experiments lead to the conclusion that HNF6 is required for *Hnf1β* expression in the BEC until the end of gestation.

To investigate how HNF6 controls HNF1β, we tested if HNF6 can stimulate the *Hnf1β* gene promoter. We sequenced a 3.65 kb fragment of the mouse *Hnf1β* gene located immediately upstream of the transcription start site. Two potential binding sites matching the HNF6 consensus (Lannoy et al., 1998) were found at positions –3228/–3221 and –3150/–3143. These sites bound in vitro synthesized HNF6 in electrophoretic mobility shift assays (Fig. 6C). Under our conditions the binding to the –3228/–3221 site was weaker than that to the –3150/–3143 site. The response of the *Hnf1β* promoter to HNF6 was tested by co-transfecting HEK293 cells with an expression vector for HNF6 and with reporter constructs containing *Hnf1β* promoter fragments cloned upstream of the chloramphenicol acetyltransferase (CAT) gene (Fig. 6D). The activity of the *Hnf1β* promoter region that spans nucleotides –3650 to +25, which contain the two HNF6 binding sites, was increased

Fig. 5. Expression of liver-enriched transcription factors in control (+/+ and +/–) and in *Hnf6*$^{-/-}$ mice. The expression levels were assessed by semi-quantitative RT-PCR on liver RNA. The data are representative of three independent experiments. (A) At E14.5, the expression levels of *Hnf3β*, *Hnf3γ* and *Hnf4α* are not affected by inactivation of the *Hnf6* gene. The expression levels of *Hnf1α* and *Hnf3α* are slightly increased in *Hnf6*$^{-/-}$ fetuses, whereas the two splicing isoforms of *Hnf1β* (Hnf1β-A and Hnf1β-B) are strongly downregulated. At P3, the expression levels of *Hnf3β*, *Hnf3γ* and *Hnf4α* are not affected, the expression of *Hnf1β* has normalized and that of *Hnf1α* and *Hnf3α* is still upregulated. (B) At E12.5, the expression of the two splicing isoforms of *Hnf1β* is strongly downregulated in the *Hnf6*$^{-/-}$ livers, as assessed with *Tbp* as a reference.

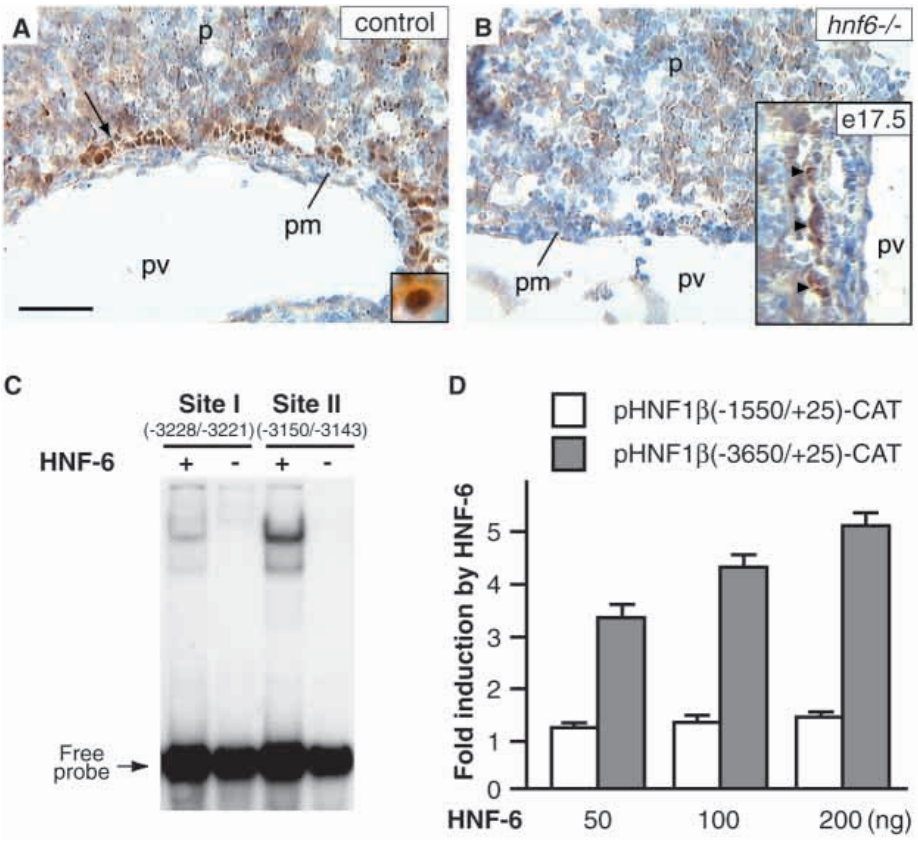


Fig. 6. HNF6 controls the expression of HNF1β. (A,B) Transient downregulation of HNF1β in the BEC of *Hnf6*$^{-/-}$ fetuses. (A) In control livers at E15.5, a strong signal for HNF1β is detected in the ductal plate, namely in cells located at the interface between the portal mesenchyme and the liver parenchyma (arrow). The inset at higher magnification demonstrates that staining is nuclear. The weak and diffuse staining of the parenchyma corresponds to nonspecific background. Note that the section shown in A is directly adjacent to the section stained for HNF6 shown in Fig. 1B. (B) In *Hnf6*$^{-/-}$ livers at E15.5, no HNF1β nuclear staining is observed. The inset in B shows restoration of HNF1β expression (arrowheads) in BEC of E17.5 *Hnf6*$^{-/-}$ fetuses. (C) Electrophoretic mobility shift assays show that HNF6 can bind two sites in the 5′-flanking sequence of the *Hnf1β* gene. Oligonucleotide probes corresponding to each of these sites give a retarded band with wheatgerm extracts programmed for HNF6 (+), but not with unprogrammed extracts (–). (D) Co-transfection experiments demonstrate that HNF6 can stimulate transcription from the *Hnf1β* promoter. HEK293 cells were transfected with the indicated amount of HNF6 expression plasmid and with a CAT reporter construct controlled by the indicated fragments of 5′-flanking sequence of the *Hnf1β* gene containing (gray bars) or not (white bars) the HNF6-binding sites. To calculate the fold increase in induction by HNF6, the activity of the reporter plasmids in the presence of HNF6 expression vector was normalized for the reporter activity in the presence of empty expression vector (mean values±s.e.m. of at least three experiments). p, liver parenchyma; pm, portal mesenchyme; pv, portal vein. Scale bar: 50 μm.

fivefold by HNF6. By contrast, the activity of the –1550/+25 region, which is devoid of HNF6-binding sites, was not stimulated.

We conclude from these experiments that HNF6 is required for the expression of HNF1β in BEC during formation of the ductal plate and that HNF6 can exert this control by stimulating the *Hnf1β* promoter.

DISCUSSION

Transcription factors that control liver development from the endoderm and hepatocyte differentiation have been extensively studied (reviewed by Cereghini, 1996; Zaret, 1998; Darlington, 1999; Duncan, 2000; Zaret, 2000). By contrast, factors that control development of the gallbladder, the EHBD and the IHBD have not yet been identified. To our knowledge, the present work is the first to identify transcription factors involved in biliary tract differentiation and morphogenesis.

HNF6 regulates *Hnf1b* expression

We demonstrate here that HNF6 controls expression of the *Hnf1β* gene in liver during development. RT-PCR data show that *Hnf6*$^{-/-}$ hepatoblasts express reduced levels of *Hnf1β* at E12.5, i.e. before the onset of BEC differentiation. Furthermore, our immunohistochemical data show that, at later stages, expression of *Hnf1β* is undetectable in BEC of *Hnf6*$^{-/-}$ fetuses. HNF6 is therefore crucial for the expression of *Hnf1β* in hepatoblasts and BEC of the ductal plate. However, despite the absence of HNF6, expression of HNF1β was detected in BEC in late gestation and RT-PCR on liver RNA from *Hnf6*$^{-/-}$ mice demonstrate that HNF1β expression levels return to near normal levels after birth. The mechanism that restores *Hnf1β* expression in *Hnf6*$^{-/-}$ mice is unknown. We are currently investigating whether HNF6 homologs of the onecut class (Jacquemin et al., 1999) are involved in this process.

HNF6 regulates the differentiation of hepatoblasts into biliary epithelial cells

There is strong evidence that hepatocytes and BEC derive from the same hepatoblast precursors (Shiojiri, 1984; Germain et al., 1988). Our data show that HNF6 controls the initiation of BEC differentiation. HNF6 is expressed in the liver when BEC differentiation is initiated. Moreover, in the absence of HNF6, differentiation of BEC starts prematurely and more hepatoblasts differentiate into BEC. These BEC form cord-like extensions within the liver parenchyma. At E13.5, HNF6 is therefore required not only to restrict BEC differentiation but also to confine BEC differentiation to the vicinity of the portal vein. In wild-type fetuses, BEC differentiate from hepatoblasts at the interface between the portal mesenchyme and the liver parenchyma. Because of this specific location, it has been hypothesized that a signal arising from the portal mesenchyme or from the endothelium of the branches of the portal vein induces BEC differentiation (Shiojiri, 1984; Van Eyken et al.,

1988a; Shah and Gerber, 1990). We speculate that HNF6 controls the response to this signal. The existence of an inducing activity produced by the mesenchyme is further suggested by experiments showing that differentiation of hepatoblasts into BEC is stimulated when co-cultured with hepatic or lung mesenchyme (Shiojiri and Koike, 1997). The signaling mechanisms are unknown. Candidates are hepatocyte growth factor (HGF) and its receptor Met, as HGF induces the formation of biliary-like cells or structures in various in vitro models (Johnson et al., 1993; Block et al., 1996). Although we found a potential binding site for HNF6 in the promoter of the *Met* gene, *Met* expression was not modified in *Hnf6*$^{-/-}$ fetal livers (data not shown).

In the pancreas, HNF6 regulates expression of *Ngn3* (*Atoh5* – Mouse Genome Informatics) a target of the Notch signaling pathway that regulates endocrine differentiation via a lateral inhibition process (Jacquemin et al., 2000). In the liver, the Notch receptors and their ligand jagged 1 are expressed during development (Weinmaster et al., 1992; Louis et al., 1999; Crosnier et al., 2000). This led us to hypothesize that an HNF6-regulated lateral inhibition mechanism controls the differentiation of hepatoblasts into BEC. However, we found no evidence for such a mechanism, as control and *Hnf6*$^{-/-}$ fetal livers expressed identical levels of *Ngn3*, *Jag1* and *Hes1* (data not shown).

As shown here, HNF1β is controlled by HNF6 at the onset of BEC differentiation. This, together with the role of HNF1β in BEC differentiation [see accompanying paper (Coffinier et al., 2002)], prompts us to propose that initiation of BEC differentiation is regulated by the HNF6→HNF1β cascade.

HNF6 regulates morphogenesis of the intrahepatic bile ducts

The morphogenesis of the IHBD was severely altered in *Hnf6*$^{-/-}$ livers. BEC never organized as a proper ductal plate. Still, NCAM (data not shown) and laminin, which are markers of mature BEC (Van Eyken et al., 1987; Terada and Nakanuma, 1994; Fabris et al., 2000; Libbrecht et al., 2001), were detected in *Hnf6*$^{-/-}$ BEC. Duct-like structures did form but were not found incorporated within the portal mesenchyme, as normally happens for bile ducts around birth. This suggests a lack of maturation of these structures and possible perturbations in their relationship with the portal mesenchyme. These duct-like structures regressed and disappeared. Using the TUNEL assay, we detected no increase in the rate of apoptosis in the regressing structures of *Hnf6*$^{-/-}$ fetuses (data not shown). It should be noted that the involvement of apoptosis in IHBD morphogenesis in rodents remains controversial (Terada and Nakanuma, 1995; Sanzen et al., 1995).

Liver-specific inactivation of the *Hnf1β* gene induces, at E17.5, morphological defects identical to those seen at the same stage in *Hnf6*$^{-/-}$ fetuses [see accompanying paper (Coffinier et al., 2002)]. As in *Hnf6*$^{-/-}$ mice, the *Hnf1β*$^{-/-}$ livers do not show a proper ductal plate. Instead, the *Hnf6*$^{-/-}$ and *Hnf1β*$^{-/-}$ livers show ductal plate-like structures consisting of a single layer of CK-positive cells around the portal mesenchyme. This indicates that HNF6 and HNF1β are required for proper ductal plate formation. The absence of detectable HNF1β in the BEC of *Hnf6*$^{-/-}$ fetuses at E15.5 and our transfection data showing that HNF6 stimulates the Hnf1β promoter suggest that a HNF6→HNF1β cascade controls ductal plate formation and intrahepatic bile duct development.

At E16.5 in wild-type fetuses, the single-layered ductal plate has become partly bilayered without proliferation of BEC (our data) (Fabris et al., 2000). This suggests that the second layer of BEC arises from differentiation of as yet unidentified precursors and not from proliferation of the existing single-layered ductal plate. These precursor cells may still be present in *Hnf6*$^{-/-}$ livers and could be at the origin of the wave of differentiation that gives rise to ductal plate-like structures starting around E17.5. According to this hypothesis, the BEC precursors in *Hnf6*$^{-/-}$ livers must acquire the capacity to form ductal plate-like structures by the end of the fetal period. This capacity does not require the presence of HNF6 or of HNF1β, as a ductal plate-like structure is observed at E17.5 both in *Hnf6*$^{-/-}$ and *Hnf1β*$^{-/-}$ livers [this work and accompanying paper (Coffinier et al., 2002)].

Approximately 75% of the *Hnf6*$^{-/-}$ mice die between P1 and P10. These mice are strongly icteric, have high bilirubin levels in the urine (not shown) and show foci of liver necrosis. As described earlier (Jacquemin et al., 2000), the newborn mice also suffer from a pancreatic defect characterized by a major reduction in the number of pancreatic endocrine cells, which is associated with low insulinemia, low glucagonemia and hypoglycemia. The combination of defects in glucose homeostasis with liver necrosis is therefore the likely cause of death. The liver phenotype is not fully penetrant. Our RT-PCR data (not shown) indicate that approximately 25% of the embryos at E12.5 show no significant reduction in Hnf1b gene expression in the liver. The histology of the liver appeared to be normal in the *Hnf6*$^{-/-}$ mice that reached adulthood. These mice showed no sign of overt hepatic defects, suggesting that they correspond to mice which had no deficient *Hnf1β* expression during development.

HNF6 is required for gallbladder formation and proper extrahepatic bile duct morphogenesis

No gallbladder primordium was seen in *Hnf6*$^{-/-}$ embryos around E10, the earliest stage at which the gallbladder can be identified histologically (Kaufman, 1992). HNF6 is detected in the gut endoderm before the onset of the hepatic bud formation (P. J., G. G. R. and F. P. L., unpublished), and it is still present in the gallbladder epithelium at E15.5. Therefore, any step of gallbladder development earlier than E10 is potentially affected by the absence of HNF6. The absence of a gallbladder-specific marker precluded analysis of gallbladder development at a stage earlier than E10.

Potential targets for HNF6 expressed in the gallbladder and EHBD are the homeodomain transcription factor genes *Hex* (Bogue et al., 2000) and *Hnf1β* (Coffinier et al., 1999b). The expression level of *Hex* was normal in the *Hnf6*$^{-/-}$ fetal livers (data not shown). By contrast, HNF1β was absent in EHBD of E15.5 *Hnf6*$^{-/-}$ fetuses (data not shown). Liver-specific inactivation of the *Hnf1β* gene does not result in the absence of gallbladder as seen in *Hnf6*$^{-/-}$ mice, but it is associated with abnormal gallbladder morphology [see accompanying paper (Coffinier et al., 2002)]. One explanation is that Hnf1β was not inactivated in all BEC of the gallbladder in these experiments. Thus, one cannot exclude the possibility that the HNF6→HNF1β cascade is also required for initiation of gallbladder development.

An absence of gallbladder is compatible with normal bile flow to the gut as rats have no gallbladder. In *Hnf6*$^{-/-}$ mice, the EHBD were morphologically abnormal but allowed the bile to flow to the gut. The cholestatic syndrome in these mice most probably results from defective IHBD morphology. Cholestasis could have resulted from deficient expression of bile transporters in hepatocytes or in BEC (reviewed by Trauner et al., 1998). This is unlikely, as expression at P3 of the transporters NTCP, MRP2, MDR1, MDR2, SPGP, AE2 and CFTR was unaffected in the *Hnf6*$^{-/-}$ mice (data not shown).

In humans, several congenital diseases of the IHBD, collectively termed 'ductal plate malformations' (DPM), are histologically characterized by the presence of biliary structures in ductal plate conformation (reviewed by Desmet, 1992; Birnbaum and Suchy, 1998). Caroli's disease, an autosomal-recessive syndrome, shows DPM and ectasias of the intrahepatic bile ducts similar to the cysts found in the *Hnf6*$^{-/-}$ embryos. To our knowledge, none of the human DPM is associated with the lack of gallbladder and with the pancreatic defects seen in *Hnf6*$^{-/-}$ mice. These observations do not rule out the involvement of HNF6 in the pathogeny of DPM. Indeed, mutations in the *Hnf6* promoter that would affect expression of HNF6 specifically in the liver could cause DPM without inducing deficiencies in other organs.

In summary, our data demonstrate that HNF6 is crucial for differentiation and morphogenesis of the biliary tract. Our earlier work has shown that in the mouse HNF6 controls differentiation of pancreatic precursors into endocrine cells (Jacquemin et al., 2000). In *Drosophila* (Nguyen et al., 2000) and in the ascidian *Halocynthia roretzi* (Sasakura and Makabe, 2001), HNF6 controls the differentiation of neural precursors. The fact that HNF6 controls the differentiation of hepatoblasts into BEC further demonstrates that HNF6 plays a crucial role for decision-making in pluripotent precursor cells.

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