Development 133, 4233-4243 (2006) doi:10.1242/dev.02591

Suppression of C/EBPα expression in periportal hepatoblasts may stimulate biliary cell differentiation through increased *Hnf6* and *Hnf1b* expression

Harufumi Yamasaki[1], Aiko Sada[1], Takeyuki Iwata[1], Tohru Niwa[1], Minoru Tomizawa[2], Kleanthis G. Xanthopoulos[3], Toru Koike[1] and Nobuyoshi Shiojiri[1,*]

The expression of C/EBPα, which may govern transcription of mature hepatocyte marker genes, was suppressed in periportal hepatoblasts in mouse liver development, leading to biliary cell differentiation. This study was undertaken to analyze how inactivation of the *Cebpa* gene affects biliary cell differentiation and gene expression of the regulatory genes for that differentiation, including *Hnf1b* and *Hnf6*. In the knockout mouse liver at midgestation stages, pseudoglandular structures were abundantly induced in the parenchyma with elevated expression of *Hnf6* and *Hnf1b* mRNAs. The wild-type liver parenchyma expressed mRNAs of these transcription factors at low levels, though periportal biliary progenitors had strong expression of them. These results suggest that expression of *Hnf6* and *Hnf1b* is downstream of C/EBPα action in fetal liver development, and that the suppression of C/EBPα expression in periportal hepatoblasts may lead to expression of *Hnf6* and *Hnf1b* mRNAs. Immunohistochemical studies with biliary cell markers in knockout livers demonstrated that differentiated biliary epithelial cells were confined to around the portal veins. The suppression of C/EBPα expression may result in upregulation of *Hnf6* and *Hnf1b* gene expression, but be insufficient for biliary cell differentiation. When liver fragments of *Cebpa*-knockout fetuses, in which hepatoblasts were contained as an endodermal component, were transplanted in the testis of *Scid* (*Prkdc*) male mice, almost all hepatoblasts gave rise to biliary epithelial cells. Wild-type hepatoblasts constructed mature hepatic tissue accompanied by biliary cell differentiation. These results also demonstrate that the suppression of C/EBPα expression may stimulate biliary cell differentiation.

KEY WORDS: C/EBPα, Knockout, Hepatoblasts, Biliary epithelial cells, Bile ducts, Morphogenesis, Mouse

INTRODUCTION

Hepatoblasts are immature hepatocytes appearing in the early stages of liver development that have bipotent differentiation capacity into mature hepatocytes and intrahepatic biliary epithelial cells (Lemaigre, 2003; Lemaigre and Zaret, 2004; Shiojiri, 1997; Shiojiri et al., 1991; Zhao and Duncan, 2005; Zaret, 2002). Intrahepatic biliary epithelial cells differentiate from periportal hepatoblasts under the influence of the subjacent mesenchyme (Enzan et al., 1974; Van Eyken et al., 1988a; Van Eyken et al., 1988b; Wilson et al., 1963; Wood, 1965). During that differentiation, in the bipotent hepatoblasts, transient expression of hepatocyte-markers such as α-fetoprotein (AFP), albumin, urea cycle enzymes and CCAAT/enhancer binding protein α (C/EBPα), is suppressed and instead they begin to express basal laminar components such as laminin and peanut agglutinin (PNA)-binding sites, and bile duct-type cytokeratins (Shiojiri et al., 2004). Recent studies with targeted inactivation of *Hnf6* (*Onecut1* – Mouse Genome Informatics) and *Hnf1b* (*Tcf2* – Mouse Genome Informatics) genes demonstrated that both transcription factors play important roles in biliary cell differentiation, and that the HNF6 action is upstream of that of HNF1β (Clotman et al., 2002; Coffinier et al., 2002). Clotman et al. (Clotman et al., 2005) have also shown that a gradient of activin/TGFβ signaling modulated by onecut transcription factors is required to segregate the hepatocytic and the biliary lineages. Jagged 1 (*Jag1*), which encodes one of the ligands for Notch receptors, is a causal gene for Alagille syndrome, which is an autosomal-dominant disorder characterized by intrahepatic cholestasis and abnormalities of the heart, eye and vertebrae (Li et al., 1997; Lorent et al., 2004; Oda et al., 1997). Notch2 signaling has also been demonstrated to play a decisive role in biliary cell differentiation, with targeted gene inactivation or overexpression (McCright et al., 2002; Tanimizu and Miyajima, 2004).

The extrahepatic bile duct and the gall bladder have a different origin and develop independently of intrahepatic bile ducts (Van Eyken et al., 1988b; Shiojiri, 1997). Their epithelial cells do not express hepatocyte markers such as albumin, urea cycle enzymes and C/EBPα during development, whereas intrahepatic bile duct cells transiently express these markers (Shiojiri et al., 2004). *Hes1* gene inactivation induces hypoformation of extrahepatic bile duct with its conversion to pancreatic tissue (Sumazaki et al., 2004).

Targeted disruption of the *Cebpa* gene in mice, which governs the transcription of hepatocyte-specific genes, leads to development of pseudoglandular structures in the liver parenchyma co-expressing antigens specific for hepatocyte and biliary cell lineages, implying its involvement in hepatocyte or biliary cell differentiation (Flodby et al., 1996; Tomizawa et al., 1998; Wang et al., 1995). However, it has not yet been analyzed in detail how biliary cell differentiation takes place in the knockout liver in terms of the expression of *Hnf6*, *Hnf1b*, *Jag1* and *Notch2*, which may reveal

[1]Department of Biology, Faculty of Science, Shizuoka University, 836 Oya, Suruga-ku, Shizuoka City, Shizuoka 422-8529, Japan. [2]Department of Medicine and Clinical Oncology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba City, Chiba 260-8670, Japan. [3]Anadys Pharmaceuticals, 3115 Merryfield Row, San Diego, CA 92121, USA.

*Author for correspondence (e-mail: sbnshio@ipc.shizuoka.ac.jp)

Accepted 23 August 2006

their up- or downstream relationships with the action of C/EBPα in biliary cell differentiation. The knockout mice are neonatal lethal because of hypoglycemia accompanied by hyperammonemia (Flodby et al., 1996; Kimura et al., 1998; Wang et al., 1995). Thus, it is intriguing to study what type of histology the knockout liver exhibits and how biliary epithelial tissue is induced when the knockouts survive. In vitro studies have demonstrated that knockout hepatocytes can immortalize at a higher frequency (Soriano et al., 1998).

In the present study, we demonstrate the expression of biliary cell markers in pseudoglandular cells of *Cebpa*-knockout livers. Inactivation of the *Cebpa* gene not only suppresses hepatocyte maturation, but also upregulates regulatory genes for biliary cell differentiation such as *Hnf6* and *Hnf1b* genes in the liver parenchyma, suggesting that the absence of C/EBPα in normal biliary cells induces *Hnf6* and *Hnf1b* expression, leading to biliary cell differentiation. *Jag1* and *Notch2* mRNAs were also upregulated in knockout livers, but not to the same extent as *Hnf6* and *Hnf1b* mRNAs. Testicular transplants of the knockout livers developed abundant biliary epithelial tissues.

MATERIALS AND METHODS

Animals

Knockout mice for the *Cebpa* gene were used (Flodby et al., 1996; Tomizawa et al., 1998). The heterozygotes were mated during the night, and noon of the day the vaginal plug was found was considered to be 0.5 days of gestation. Fetuses at 9.5, 10.5, 11.5, 12.5, 13.5, 14.5, 15.5 and 17.5 days of gestation, newborns (1 day old), and adult animals (8 weeks old) were used for immunohistochemistry and in situ hybridization. Male C.B-17/Icr-*scid*Jcl mice (CLEA Japan, Tokyo, Japan) were also used as hosts for transplantation of fetal liver fragments.

Histochemistry

Tissues for laminin, nidogen, AFP, albumin, ornithine transcarbamylase (OTC), carbamoylphosphate synthase I (CPSI), cytokeratin, HNF4 and proliferating cell nuclear antigen (PCNA) immunohistochemistry, and fluorescent lectin or periodic acid-Schiff (PAS) staining were fixed in a cold mixture of 95% ethanol and glacial acetic acid (99:1 v/v) and embedded in paraffin. Frozen sections (cold acetone-fixed for 10 minutes) were also used for immunohistochemistry of C/EBPα, integrin subunits, E-cadherin and N-cadherin.

Hydrated sections were incubated with a rabbit anti-C/EBPα antibody (Santa Cruz Biotechnology, Santa Cruz, CA) [1 μg IgG/ml in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA)], rabbit anti-mouse HNF4 antibody (Santa Cruz Biotechnology) (1/100 dilution), rabbit anti-mouse AFP antiserum (Organon Teknika, Durham, NC) (1/200), rabbit anti-mouse albumin antiserum (1/100) (Organon Teknika), a rat anti-mouse nidogen antibody (Chemicon International, Temecula, CA) (1/200), rabbit anti-mouse laminin antiserum (E-Y Laboratory, San Mateo, CA) (1/200), guinea pig anti-cytokeratin 8 and 18 antiserum (Progen Biotechnik Gmbh, Heidelberg, Germany) (1/200), rabbit anti-calf keratin antiserum (Dako, Carpinteria, CA) (1/300), rabbit anti-human OTC antiserum (Shiojiri et al., 2001) (1/1000), rabbit anti-rat CPSI antiserum (1/1000), rat anti-E-cadherin antibody (Takara Biomedicals, Otsu, Japan) (1/100) and rabbit anti-N-cadherin antiserum (Dako) (1/100) for 1 hour at room temperature. Rabbit anti-calf keratin antiserum recognizes 39 (cytokeratin 19) and 53 kDa cytokeratin polypeptides in adult mouse liver (Shiojiri, 1994). Rat anti-β4 integrin subunit (BD Biosciences, Tokyo, Japan) (1/100) or rabbit anti-α3 integrin subunit antiserum (Chemicon International) (1/100) was also used as the primary antibody. After thorough washing with PBS, sections were incubated with a fluorescein-labeled goat anti-rabbit IgG antibody, anti-rat IgG antibody or anti-guinea pig IgG antibody (Organon Teknika) (1/100) for 1 hour at room temperature, washed again and mounted in buffered glycerol containing *p*-phenylenediamine (Johnson and de C. Nogueira Araujo, 1981). In some immunofluorescence experiments, nuclei were stained with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI). In double immunofluorescent analysis, the following species-specific secondary antibodies were used: a Cy3 or a fluorescein-labeled donkey anti-rabbit, rat or guinea pig IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) (1/500 dilution for Cy3-labeled antibodies and 1/50 dilution for fluorescein-labeled antibodies). Control incubations were carried out in PBS containing 1% BSA in place of the primary antibodies.

PCNA immunohistochemistry was carried out by using mouse anti-PCNA antibody (Dako) (1/100) and ABC kit (Vector Laboratories, Burlingame, CA), according to the manufacturers' instructions.

For histochemistry of *Dolichos biflorus* agglutinin (DBA)-, soybean agglutinin (SBA)- or PNA-binding sites, sections were incubated with fluorescein-labeled DBA, SBA or PNA (Vector Laboratories) (50 μg/ml) for 30 minutes in the dark. Control sections were incubated with the lectins and their haptenic sugars (0.1 M N-acetylgalctosamine or lactose). Hematoxylin-Eosin or PAS-H staining was carried out to indicate histology and glycogen, respectively.

In situ hybridization

cDNAs coding for partial sequences of mouse *Hnf6*, *Hnf1b*, *Jag1* and *Notch2* mRNAs were cloned by reverse transcription-polymerase chain reaction (RT-PCR). The primers used were as follows: forward 5′-AAA-GAGGTGGCGCAGCGTAT-3′ and reverse 5′-GTTGGAGCCGCCCT-CGTC-3′ for *Hnf6*; forward 5′-CGGCGACGACTATGACATC-3′ and reverse 5′-GCTTCTGCCTGAACGCCTCT-3′ for *Hnf1b*; forward 5′-CGAACACATTTGCAGCGAAT-3′ and reverse 5′-GCGGTGCCCTCA-AACTCT-3′ for *Jag1*; and forward 5′-GGAAGCTTGGGCAGGTTAC-3′ and reverse 5′-TTGGGCTGGTGGTCACATCT-3′ for *Notch2*. These were designed based on the sequences of mouse genes [DDBJ/EMBL/GenBank Accession Numbers: U95945 (*Hnf6*), AB052659 (*Hnf1b*), AF171092 (*Jag1*) and D32210 (*Notch2*)]. Both sense and antisense digoxigenin-labeled riboprobes were synthesized from plasmids containing their cDNAs, and the cDNAs of AFP and albumin (gifts from Drs T. Mitaka and S. Nishi, respectively) (Sargent et al., 1981) by using a DIG RNA labeling kit (Roche Diagnostics, Mannheim, Germany). Sense and antisense digoxigenin-labeled riboprobes for *Cebpa* mRNA were directly prepared using T7 and T3 RNA polymerases from PCR fragments (corresponding sequence from 127 to 350 bp of the mouse *Cebpa* cDNA; NM007678) that are franked by T7 and T3 promoters on each side. The primers used for amplifying *Cebpa* fragments were as follows: forward 5′-CCGACTTCTACGAGGTGGAG-3′; reverse 5′-CAGGAACTCGTCGTTGAAGG-3′.

In situ hybridization on paraffin sections was carried out according to Ishii et al. (Ishii et al., 1997) with some modifications, which included changing the hybridization temperature from 70 to 42°C. The proteinase K concentration was 4 μg/ml, and the length of the proteinase K treatment was modified according to the size of the tissue.

Semi-quantification of positive signals in in situ hybridization

Photographs of sections for in situ hybridization of *Hnf6*, *Hnf1b*, *Jag1* and *Notch2* mRNAs were taken on an Olympus BX60 equipped upright microscope by using a PDMCle/OL camera (Olympus, Tokyo, Japan) with input into a PC. Positive signals in digitized photographs were semi-quantitatively analyzed by using NIH image 1.61/ppc. The use of 'weak', 'moderate', or 'strong' expression of *Hnf6*, *Hnf1b*, *Jag1* and *Notch2* mRNAs in the text is based on this analysis.

Organ culture

Distal fragments of the 12.5-day liver, in which intrahepatic bile duct structures have not developed yet, were cultured in DM-160 (Kyokuto Pharmaceuticals, Tokyo, Japan) supplemented with 10% fetal bovine serum, dexamethasone (100 nM) and antibiotics on RA-type Millipore filter on a stainless grid for 5 days (Shiojiri, 1984; Shiojiri and Mizuno, 1993). Recombinant TGFβ1 (R & D Systems, Minneapolis, MN) were also added in the culture medium (50 or 200 pg/ml) (Clotman et al., 2005).

Testicular transplantation

The liver fragments at 12.5 days of gestation were transplanted into the testis of *Scid* (*Prkdc* – Mouse Genome Informatics) mice for 2 months (Shiojiri, 1984).

RESULTS

Expression of C/EBPα and its mRNA during liver development

To determine whether expression of C/EBPα and its mRNA are suppressed during biliary cell development, immunohistochemical and in situ hybridization analyses were carried out. During liver development, biliary cell differentiation occurred in periportal areas at 13.5 to 15.5 days of gestation, when hepatoblasts were induced to form pearl-like structures, as described by Van Eyken et al. (Van Eyken et al., 1988b). With that formation, C/EBPα expression was suppressed in periportal hepatoblasts and differentiated biliary epithelial cells (Fig. 1C-F), which exhibited cuboidal or squamous morphology and appeared after 16.5-17.5 days of gestation. Nuclei of hepatoblasts and hepatocytes were positive for C/EBPα immunohistochemistry (Fig. 1A,B). Epithelial cells of the gall bladder and extrahepatic bile duct, including the cystic duct and common bile duct, did not express this transcription factor during development. The presence of a competitive peptide abolished the positive signals in immunohistochemistry and immunoblotting (data not shown). In situ hybridization of C/EBPα mRNA also demonstrated that the expression of this mRNA was downregulated in biliary epithelial cells in 16.5- and 17.5-day livers (Fig. 1G-I), which agreed well with the distribution pattern of C/EBPα protein.

Bile duct marker expression in pseudoglandular structures in the *Cebpa* knockout liver

Targeted inactivation of the C/EBPα gene results in abundant generation of pseudoglandular structures during perinatal liver development (Tomizawa et al., 1998; Wang et al., 1995). To determine how the pseudoglandular structures develop, and what kind of differentiation states exist, we histologically and immunohistochemically examined knockout liver development with special attention given to the expression of mature hepatocyte and biliary cell markers. Histologically, pseudoglandular or pearl-like structural development in the liver parenchyma commenced earlier and more markedly in knockouts than in wild-type fetuses at around 12.5-13.5 days of gestation, although that in the wild-type liver occurred only around the portal vein, which led to periportal biliary duct development in later stages (Fig. 2A,E). The pseudoglandular structural development became more conspicuous in the whole liver parenchyma of the 17.5-day and neonatal knockout livers (Fig. 2B-D,F-H). Glycogen accumulation was completely suppressed in the 17.5-day knockout liver (Fig. 3A,G). Almost all cells of the knockout pseudoglandular structures were strongly reactive with anti-calf keratin antiserum (Fig. 3I), which reacted only with biliary epithelial cells in the adult wild-type liver. However, not all pseudoglandular cells were positively stained for DBA- and SBA-binding sites and basal laminar proteins, including nidogen, laminin and PNA-binding sites; only pseudoglandular cells around the portal vein or under the hepatic capsule expressed these bile duct markers (Fig. 3J-L; Fig. 4). In the wild-type liver, only periportal biliary epithelial cells and their progenitors expressed these biliary markers, although all hepatoblasts transiently reacted with anti-calf keratin antiserum, and their reactivity was confined to biliary epithelial cells with fetal development (Fig. 3C-F). Immunohistochemistry of HNF4 showed that this transcription factor was expressed in nuclei of wild-type hepatocytes and knockout nonperiportal pseudoglandular cells, but not in wild-type biliary epithelial cells and their counterparts in the knockout liver (Fig. 3B,H). Periportal epithelial cells of the pseudoglandular structures in the knockouts, especially near the hilus, were columnar or low columnar and expressed markers for extrahepatic biliary epithelial cells. These

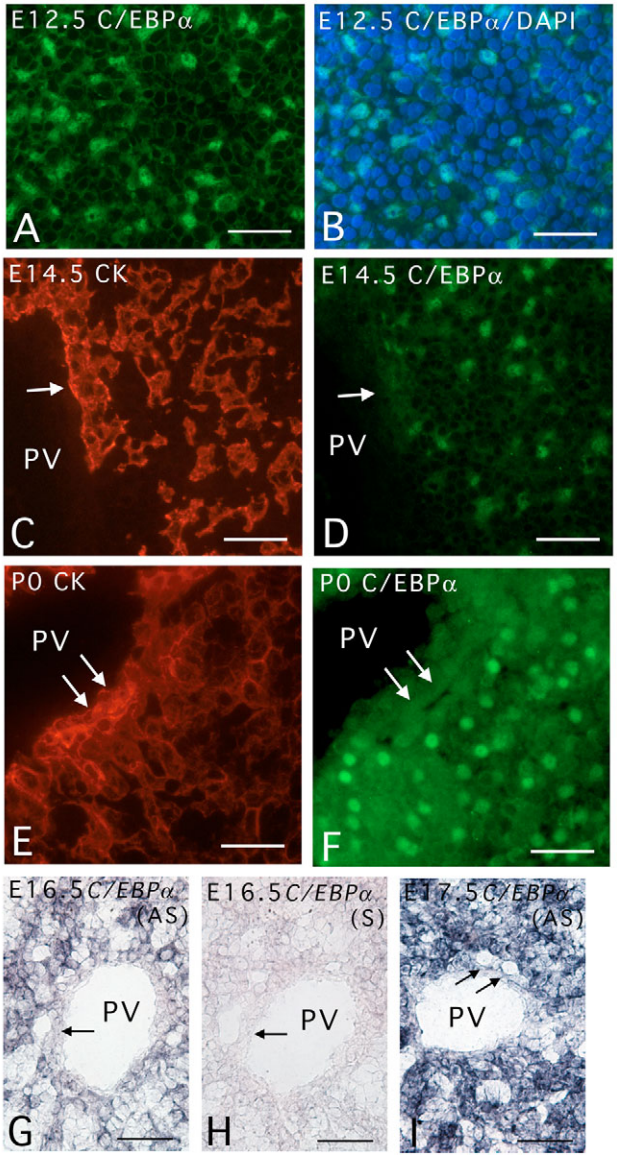

Fig. 1. C/EBPα and its mRNA expression in mouse liver development. Immunohistochemistry reveals that hepatoblasts and hepatocytes express C/EBPα, localized in their nuclei, in 12.5- (**A**,**B**), 14.5-day (**D**) and neonatal (**F**) liver sections. (B) Double staining of C/EBPα and DAPI. (**C**,**E**) Cytokeratin immunostaining of D and F, respectively. Pearl-like structures and biliary epithelial cells are negative for C/EBPα (arrows in C-F). In situ hybridization using *Cebpa* antisense probes shows the absence of *Cebpa* mRNA in biliary epithelial cells (arrows) of 16.5-day (**G**) and 17.5-day (**I**) mouse liver sections. Hepatocytes are positive for *Cebpa* mRNA. Sense probes give no significant signal on a 16.5-day liver section (**H**). PV, portal vein. Scale bars: 50 μm.

results showed that they resembled extrahepatic bile duct cells in terms of their strong expression of DBA-binding sites and β4 or α3 integrin subunits, as well as their morphology (Fig. 5A,B,E,F). By contrast, progenitors of biliary cells of the wild-type liver were squamous or cuboidal, and faced small lumina. Their weak expression of DBA-binding sites and β4 or α3 integrin subunits also occurred after 17.5 days of gestation (Fig. 5A,B). These progenitor cells for periportal biliary cells of both wild-type and knockout livers were negative for AFP and albumin, and their mRNAs.

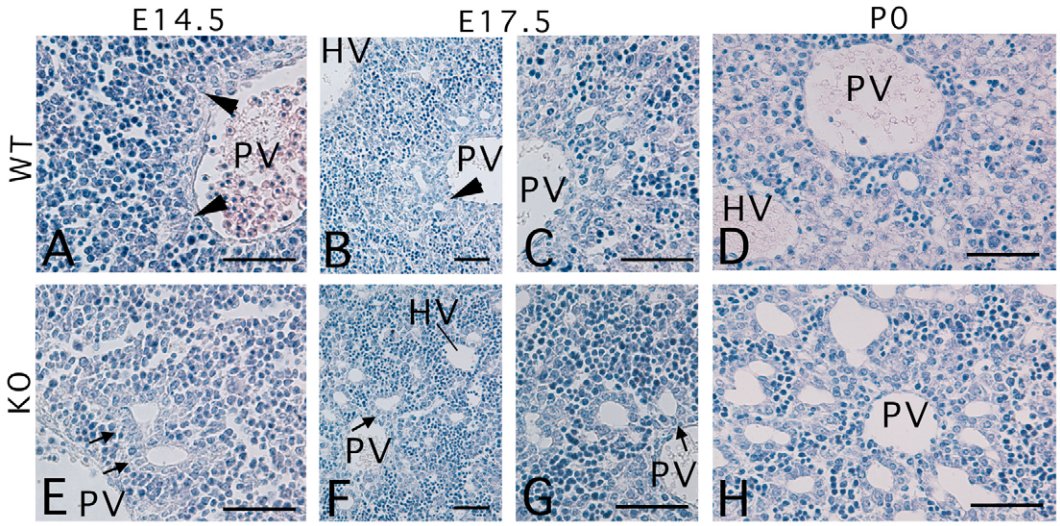

Fig. 2. Pseudoglandular development in *Cebpa* knockout mouse livers. Hematoxylin and Eosin staining indicates that periportal hepatoblasts form pearl-like structures in 14.5-day and 17.5-day wild-type livers (arrowheads) (**A-C**), while pseudoglandular or pearl-like structures are abundant in the whole liver parenchyma of knockout livers (small arrows) (**E-G**). Biliary epithelial cells differentiate only around the portal vein and a typical intrahepatic bile duct is formed in a neonatal wild-type liver (**D**). In a neonatal knockout liver, pseudoglandular structures with large lumina still abundantly develop and no normal bile duct is seen even around the portal vein (**H**). HV, hepatic vein; PV, portal vein. Scale bars: 50 μm.

Expression of E-cadherin and N-cadherin was also conspicuously different between wild-type and knockout livers at 17.5 days (Fig. 5C,G). E-cadherin was expressed in hepatoblasts, hepatocytes and biliary epithelial cells in liver development of wild-type fetuses, but its immunostaining in biliary epithelial cells was stronger than that in hepatoblasts and hepatocytes. By contrast, in the knockout liver, almost all epithelial cells of the pseudoglandular structures strongly expressed this adhesion molecule. N-cadherin was also expressed in cells of the wild-type fetal liver, mainly in hepatoblasts and hepatocytes, but its immunostaining was weak in biliary epithelial cells at this stage. N-cadherin expression declined in biliary epithelial cells during postnatal development. A similar situation was seen in the knockout liver; periportal pseudoglandular cells were negative, and nonperiportal cells were positive for N-cadherin (Fig. 5D,H).

During the neonatal stage, periportal cells and nonperiportal cells of pseudoglandular structures were morphologically distinguished in the knockout liver; periportal cells were comparatively small, and many nonperiportal cells appeared to be hepatocytes. Although periportal cells expressed biliary cell markers, they never formed normal bile ducts lined with connective tissue. They connected with hepatocyte-like cells, and formed large lumina. In the wild-type liver, bile ducts, which were lined with connective tissue cells and isolated from the liver parenchyma, were often observed around the portal vein; at least one bile duct always existed there. Nonperiportal hepatocytes, which expressed no CPSI and OTC throughout development, faced large lumina and accumulated little glycogen in the knockout liver.

Increased proliferation of pseudoglandular cells

Lack of C/EBPα gene expression results in increased DNA synthesis of freshly isolated mouse hepatocytes (Soriano et al., 1998). To test whether pseudoglandular cells had high proliferative activity in 17.5-day and neonatal knockout livers, we counted cells marked by the expression of PCNA. PCNA-positive cells were found in knockout pseudoglandular cells, wild-type hepatocytes and biliary epithelial cells, with the highest levels found in wild-type biliary epithelial cells (Fig. 6A-D). Measurement of the proliferation indexes, defined as the number of PCNA-positive cells in each cell population, revealed increased proliferation in pseudoglandular cells in knockout mice compared with lower proliferation in hepatocytes of wild-type animals (Fig. 6E). Knockout biliary epithelial cells showed higher and lower proliferation than in nonperiportal pseudoglandular cells and wild-type biliary epithelial cells, respectively (Fig. 6E). However, the area of pseudoglandular cells in the knockout liver was smaller than that of wild-type hepatocytes, and the knockout liver had a normal size (data not shown).

Pseudoglandular cells highly express *Hnf6* and *Hnf1b* mRNAs

C/EBPα can bind to regulatory sequences of the *Hnf6* gene and block its transcription (Rastegar et al., 2000), and HNF6 is upstream of HNF1β for bile duct development (Clotman et al., 2002; Coffinier et al., 2002). To test whether C/EBPα was upstream of *Hnf6* and *Hnf1b*, we compared their expression in wild-type and knockout livers by in situ hybridization.

Both *Hnf6* and *Hnf1b* mRNAs were expressed weakly in liver parenchymal cells but moderately in extrahepatic bile duct cells in the 12.5-day wild-type liver (Fig. 7A,D). In the 15.5-day liver, their strong expression was confined to cells of periportal pearl-like structures and the expression in extrahepatic bile duct cells became stronger (Fig. 7B,E,F). In 17.5-day and neonatal livers, epithelial cells of intrahepatic bile ducts were also moderately positive for both mRNAs (Fig. 7C,G). Hepatocytes were not reactive for either mRNA except for very weak staining in the 17.5-day liver.

In the knockout liver, expression of *Hnf6* and *Hnf1b* mRNA was upregulated in cells of pseudoglandular structures at 12.5 days, appearing especially around the portal vein (Fig. 7H,M). At 15.5 and 17.5 days of gestation, pseudoglandular structures of the liver parenchyma were moderately positive for both mRNAs but the staining intensity in the structures around the portal vein and under the hepatic capsule was much stronger, and was comparable with that in extrahepatic bile ducts (Fig. 7I-K,N-P,S). In the neonatal liver of the knockout, periportal cells were moderately positive for both

DEVELOPMENT

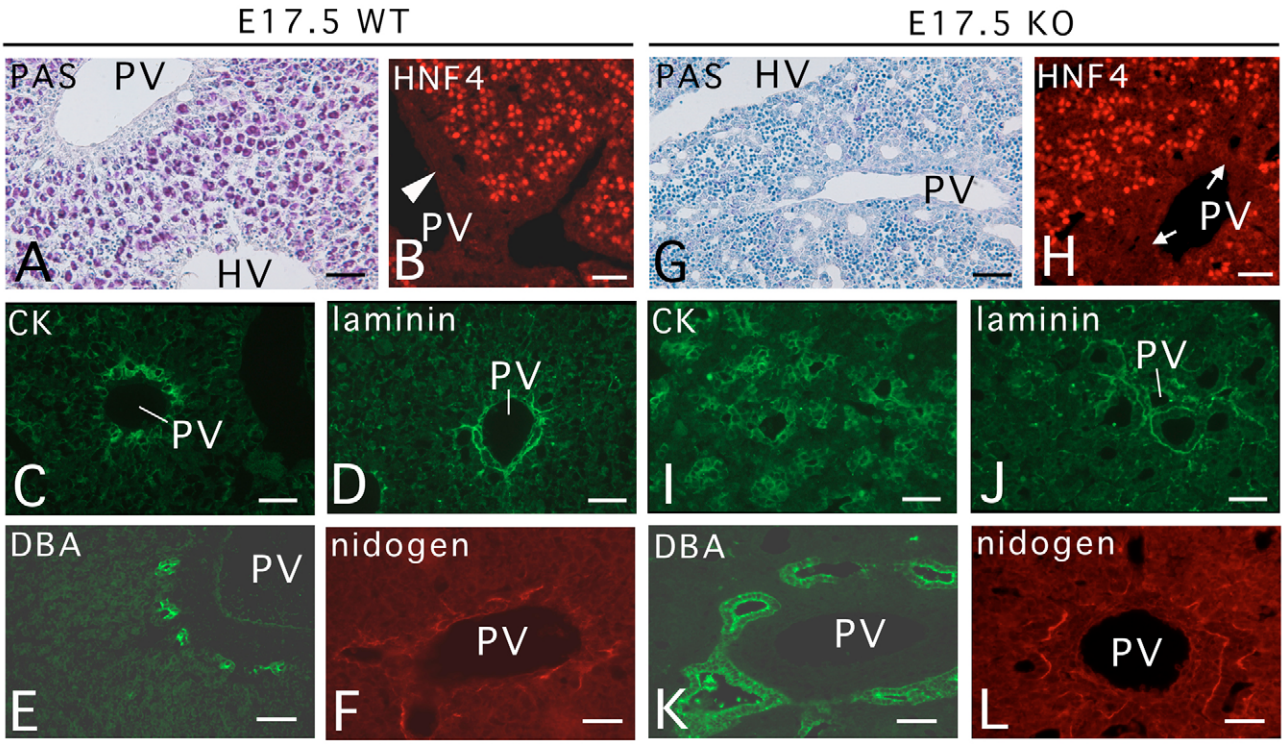

Fig. 3. Periportal cells of pseudoglandular structures in the 17.5-day knockout liver express biliary cell markers. PAS staining indicates that wild-type hepatocytes store abundant glycogen (**A**). Immunohistochemistry for cell type-specific markers and lectin histochemistry also show that wild-type hepatocytes express HNF4 in their nuclei (**B**), and that periportal biliary epithelial cells are negative for HNF4 (arrowhead; B), but are positive for bile duct-specific cytokeratin, laminin, DBA-binding sites and nidogen (**C-F**). The knockout liver develops many pseudoglandular structures, which are composed of hepatocyte-like cells poorly accumulating glycogen (**G**). Although most pseudoglandular structures are positive for bile duct-type cytokeratin (**I**), other bile duct markers are reactive only with periportal pseudoglandular structures (**J-L**). Staining pattern of HNF4 in the knockout liver resembles that of the wild-type liver; periportal biliary cells are negatively immunostained (arrows; **H**). HV, hepatic vein; PV, portal vein. Scale bars: 50 μm.

mRNAs, and nonperiportal parenchymal cells were weakly or moderately (heterogeneously) positive (Fig. 7L,Q,R). Their expression levels for both mRNAs were upregulated compared with those of the wild-type liver.

Pseudoglandular cells express *Jag1* and *Notch2* mRNAs

Recent work has established that defects in bile duct cell differentiation and morphogenesis are caused by mutations in components of the Notch signaling pathway, including the *Jag1* and *Notch2* genes (McCright et al., 2002). To examine the relationship between the Notch signaling pathway and C/EBPα, we compared their expression in wild-type and knockout livers by in situ hybridization.

In the wild-type liver at 12.5 days of gestation, moderate *Jag1* mRNA expression was observed in some endothelial cells and connective tissue cells of the large portal vein and the hepatic artery, and liver parenchymal cells were also weakly positive (Fig. 8A,B). At this stage, *Notch2* mRNA was also weakly positive in liver parenchymal cells and in cells of the gall bladder (Fig. 8G,H).

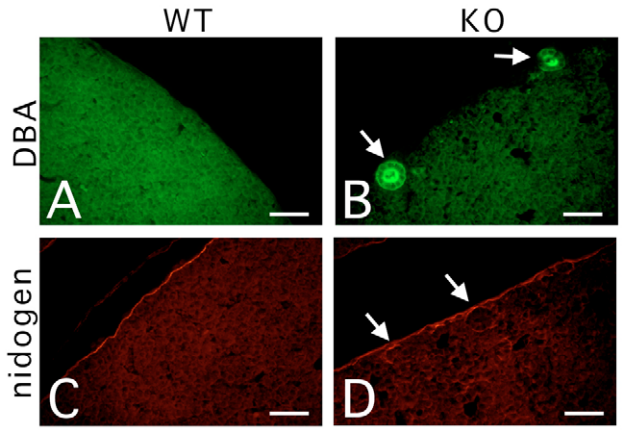

Fig. 4. Pseudoglandular structures under the hepatic capsule express bile duct markers in the 17.5-day knockout liver. Pseudoglandular structures under the hepatic capsule (arrows) are reactive with fluorescent DBA (**B**) and an anti-nidogen antibody (**D**) in the knockout liver. Hepatocytes under the capsule react with neither DBA (**A**) nor the anti-nidogen antibody (**C**) in the wild-type liver. Scale bars: 50 μm.

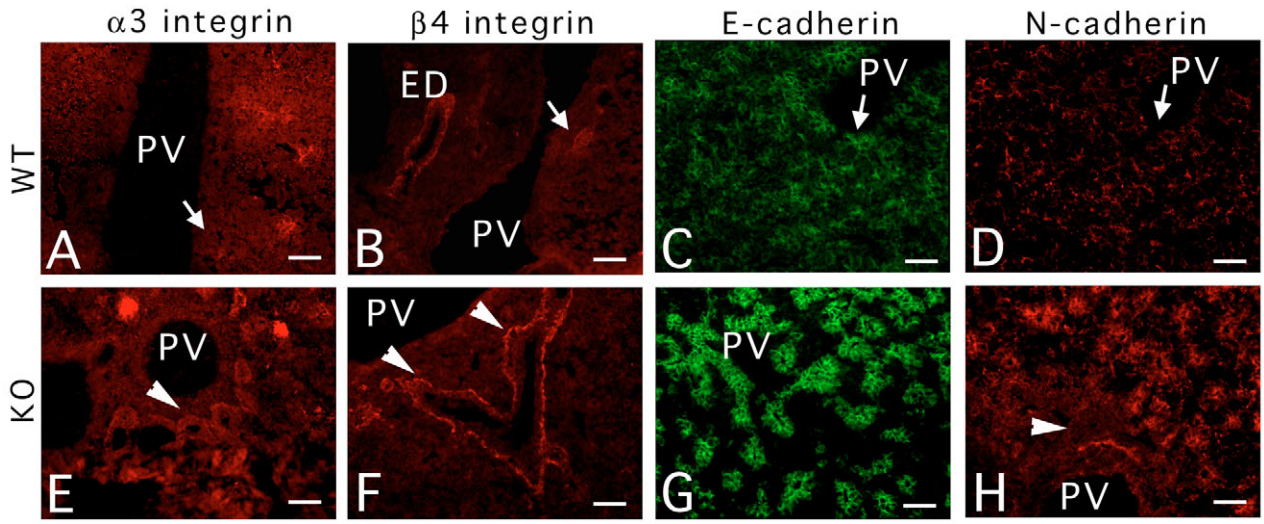

Fig. 5. Expression of cell-adhesion molecules in pseudoglandular structures of the 17.5-day knockout liver. Intrahepatic biliary epithelial cells (arrows) are negatively or weakly immunostained for α3 and β4 integrin subunits in the wild-type liver (**A**,**B**) although extrahepatic bile duct cells are moderately immunostained for β4 integrin subunits (B). E-cadherin expression is strong in biliary epithelial cells, but they have weak N-cadherin expression in the wild-type liver (arrows) (**C**,**D**). In the knockout liver, cells of periportal pseudoglandular structures moderately express both integrin subunits (arrowheads) (**E**,**F**). In addition, almost all cells of pseudoglandular structures strongly or moderately express E-cadherin and N-cadherin (**G**,**H**) but those around the portal vein are negative for N-cadherin (arrowhead, H). PV, portal vein. Scale bars: 50 μm.

Extrahepatic bile duct cells were moderately positive for *Jag1* and *Notch2* mRNAs (Fig. 8A,G). At 15.5 days, *Jag1* mRNA was strongly expressed in endothelial cells of the portal vein and the hepatic artery, and some periportal connective tissue cells, which persisted in 17.5-day and neonatal livers (Fig. 8C-F). Some cells of pearl-like structures, especially cells located close to periportal connective tissue, were also positive (Fig. 8C). *Notch2* mRNA was strongly expressed in cells of pearl-like structures, but moderately in nonperiportal hepatoblasts or hepatocytes (Fig. 8I). Extrahepatic bile duct cells were heterogeneously moderately or weakly positive for *Jag1* and *Notch2* mRNAs (Fig. 8J). At 17.5 days, strong *Jag1* mRNA expression was still observed in some biliary epithelial cells

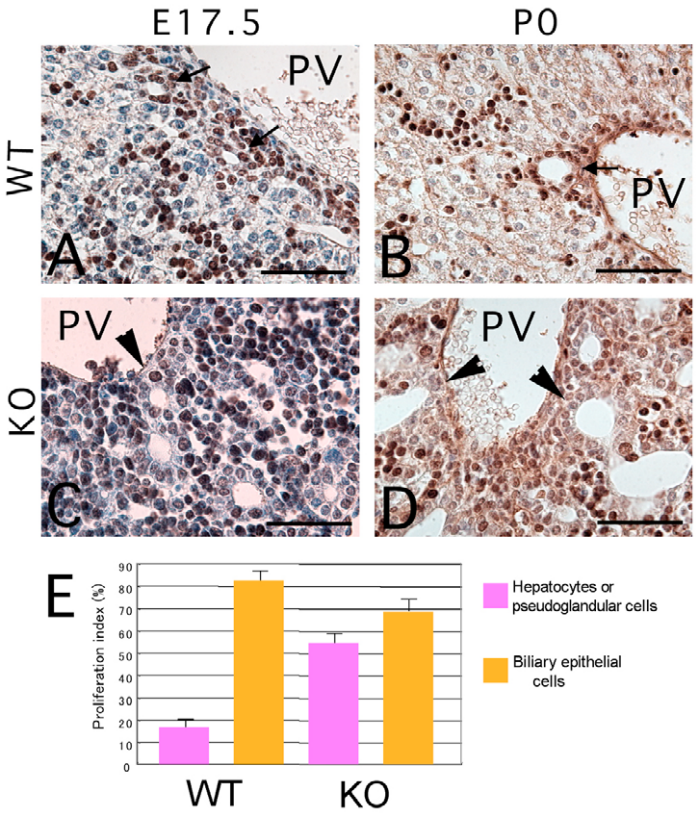

Fig. 6. Higher proliferation of pseudoglandular cells in the knockout mouse liver. (**A-D**) PCNA immunostaining shows an increase of proliferation in pseudoglandular cells in 17.5-day (C) and neonatal (D) knockout mouse livers (arrowheads), compared with wild-type mice (arrows, A,B). PV, portal vein. Scale bars: 50 μm. Proliferation indexes of hepatic cells, given as number of PCNA-positive cells per portal biliary epithelial cell (light brown) or nonperiportal hepatocyte/pseudoglandular cell (purple), show a decrease and increase of proliferation in periportal biliary epithelial cells and nonperiportal pseudoglandular cells of the knockout mouse liver, respectively, compared with wild-type mice (**E**; wild type, *n*=3; knockout, *n*=3). Bars indicate the standard deviations of the means.

and periportal connective tissue cells, although hemopoietic cells also became weakly positive. In the neonatal liver, hemopoietic cells and free leukocytes were moderately positive (Fig. 8F) and hepatocytes were faintly positive for *Jag1* mRNA. Some biliary epithelial cells of extrahepatic bile ducts and intrahepatic bile ducts were moderately positive. *Notch2* mRNA was present in biliary epithelial cells around the portal vein (Fig. 8K,L). Some hemopoietic cells and free leucocytes were weakly positive for *Notch2* mRNA. Hepatocytes were mostly negative.

In the knockout liver, the localization of *Jag1* mRNA was similar to that in the wild-type liver, but its expression level was slightly lower at 12.5 days (Fig. 8M). *Notch2* mRNA was

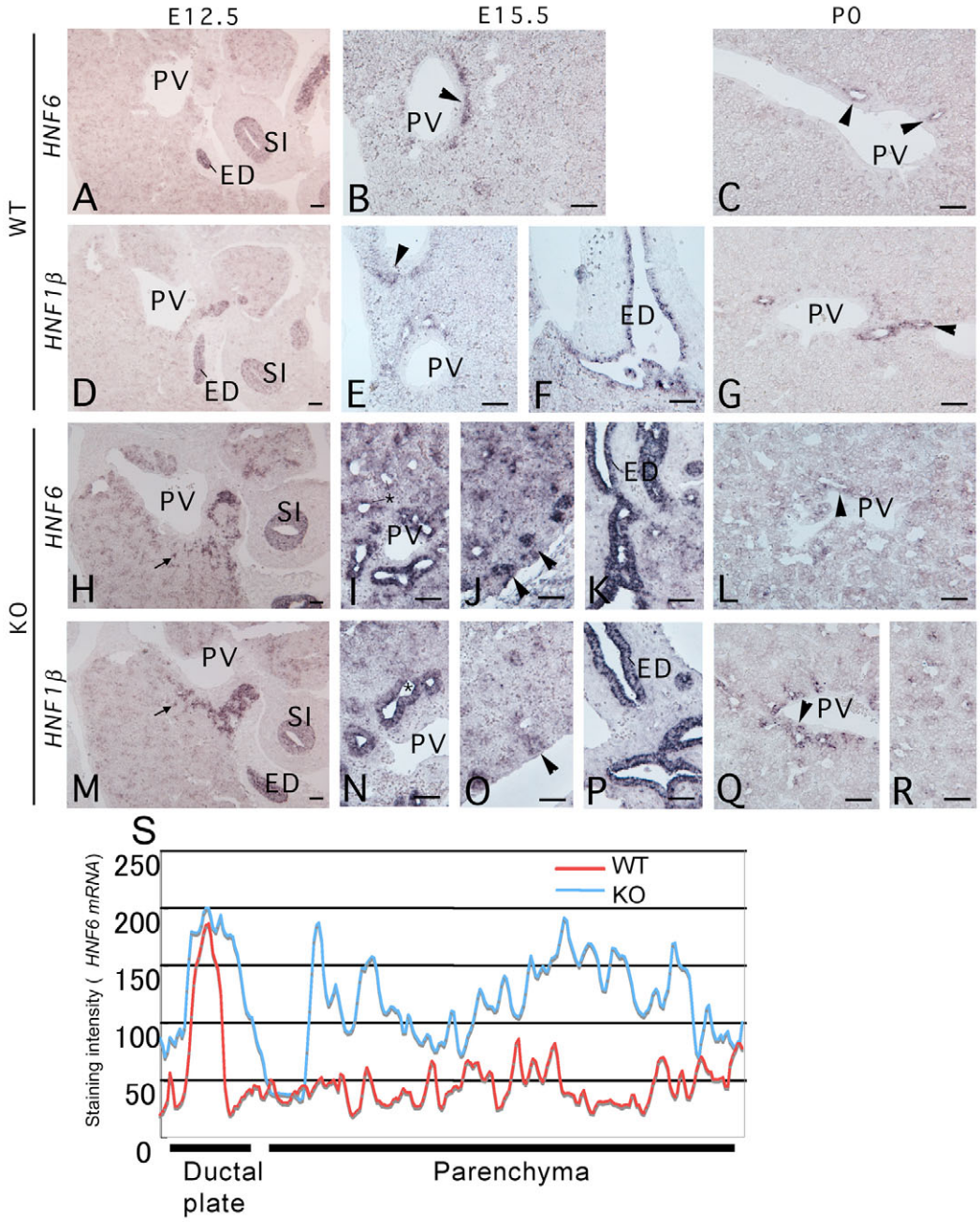

Fig. 7. In situ hybridization analysis of *Hnf6* and *Hnf1b* mRNAs in fetal and neonatal livers. In the wild-type liver, hepatoblasts weakly or moderately express *Hnf6* and *Hnf1b* mRNAs at 12.5 days (**A**,**D**), but strong signals of both mRNAs are confined to periportal biliary structures in later development (arrowheads; **B**,**C**,**E**,**G**). Epithelial cells of the extrahepatic bile duct are strongly positive for both mRNAs (A,D,**F**). In the knockout liver, *Hnf6* and *Hnf1b* mRNAs are upregulated in hepatoblasts at 12.5 days (arrows; **H**,**M**), and in pseudoglandular structures at 15.5 days (star; **I**,**N**). Cells of pseudoglandular structures under the hepatic capsule (arrowheads; **J**,**O**) and extrahepatic bile duct cells (**K**,**P**) strongly express both mRNAs. Also in the neonatal the knockout liver, *Hnf6* and *Hnf1b* mRNAs are upregulated in hepatocytes (**L**,**Q**,**R**) compared with those in the wild-type liver. (R) The nonperiportal region. Periportal epithelial cells and hepatocytes are strongly positive (arrowheads; L,Q). ED, extrahepatic bile duct; PV, portal vein; SI, small intestine. Scale bars: 50 μm. (**S**) Positive signal profile of *Hnf6* mRNA localization from the portal vein to the parenchyma in 15.5-day wild-type (red tracing) and knockout (blue tracing) livers. The level of *Hnf6* mRNA expression is upregulated in the parenchymal region of the knockout liver. The use of 'weak', 'moderate' or 'strong' expression of *Hnf6* mRNA in the text is based on the profile analyses of positive signals.

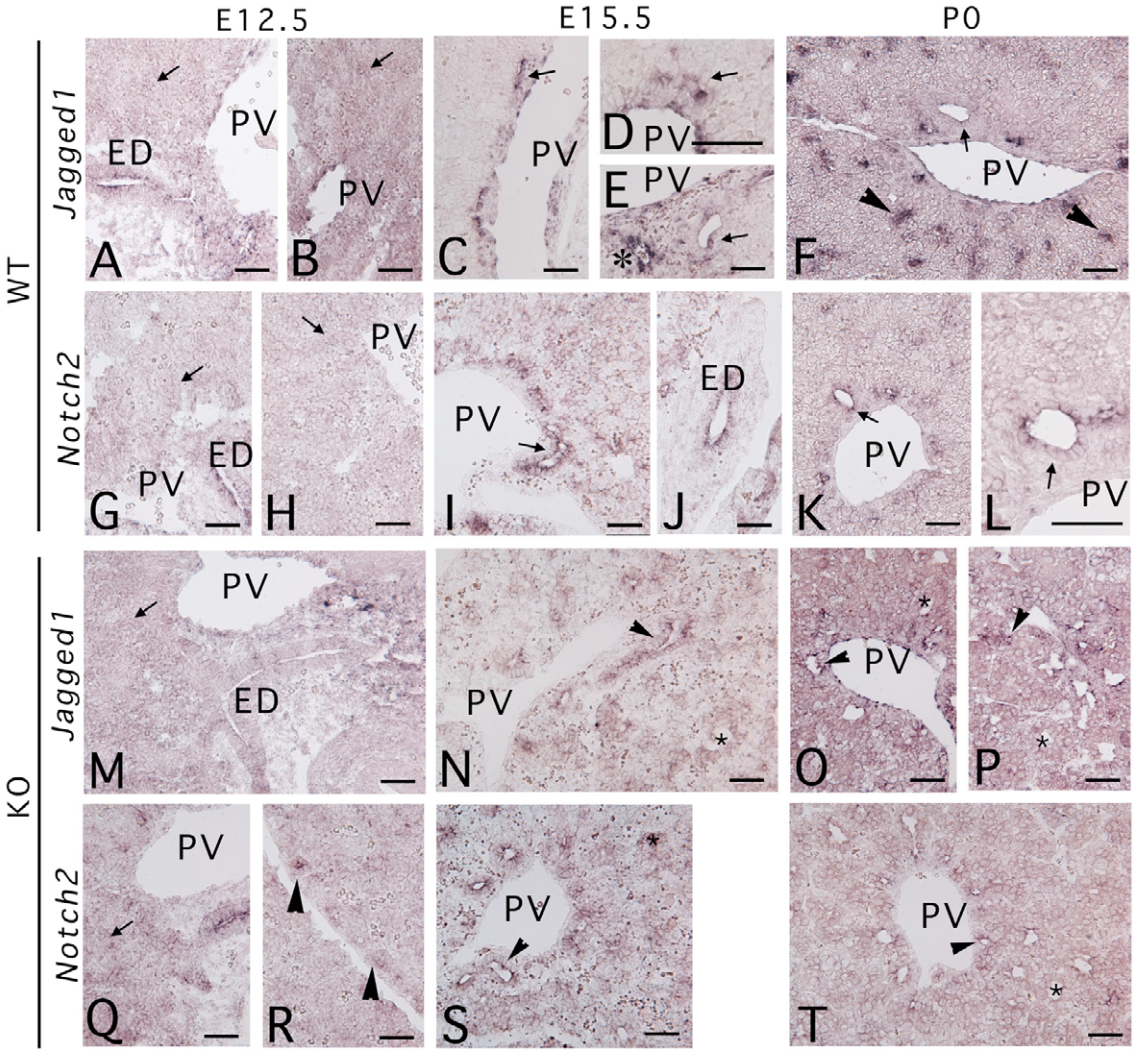

Fig. 8. In situ hybridization analysis of *Jag1* and *Notch2* mRNAs in fetal and neonatal livers. In the wild-type liver, hepatoblasts very weakly express *Jag1* and *Notch2* mRNAs at 12.5 days (arrows; **A**,**B**,**G**,**H**), but epithelial cells of periportal biliary structures become positive for both mRNAs in later development (arrows; **C-F**,**I**,**K**,**L**). Epithelial cells of the extrahepatic bile duct are moderately positive for both mRNAs (A,G,**J**). Endothelial cells of the portal vein and hepatic artery (*; E), and their connective tissue cells express *Jag1* mRNA (A-F). Arrowheads indicate *Jag1* mRNA-positive hemopoietic cells in the neonatal wild-type liver (F). In the 12.5-day knockout liver, although *Jag1* mRNA expression in hepatoblasts is at a normal level (arrow; **M**), *Notch2* mRNA is upregulated in hepatoblasts (arrow; **Q**). Hepatoblasts under the capsule (large arrowheads) are positive for *Notch2* mRNA (**R**). In 15.5-day and neonatal knockout livers, *Jag1* and *Notch2* mRNAs are upregulated in pseudoglandular structures (star), but periportal epithelial cells are more strongly stained (small arrowheads; **N-P**,**S**,**T**). *Jag1* mRNA expression is downregulated in endothelial cells of the portal vein (N), compared with that in the 17.5-day wild-type liver (C,D,E). ED, extrahepatic bile duct; PV, portal vein. Scale bars: 50 μm.

upregluated in the hepatoblast population and extrahepatic bile duct cells (Fig. 8Q,R). In the 15.5-day liver, pseudoglandular cells were weakly or moderately positive for *Jag1* mRNA (Fig. 8N). However, the expression was downregulated in endothelial cells of the portal vein. *Notch2* mRNA was positive but heterogeneous in pseudoglandular cells at 15.5 days (Fig. 8S). In the neonatal liver, endothelial cells of the portal vein were positive for *Jag1* mRNA (Fig. 8O,P). Almost all hepatocytes were weakly positive, but periportal epithelial cells and some nonperiportal hepatocytes were moderately positive. *Notch2* mRNA was upregulated in hepatocytes of the liver parenchyma (Fig. 8T). The staining intensity of periportal epithelial cells was comparatively strong, but weaker than that of biliary epithelial cells of the wild-type liver.

Effect of TGFβ1 on biliary cell differentiation in the knockout liver

TGFβ signaling may stimulate biliary differentiation from hepatoblasts (Clotman et al., 2004). To examine whether the knockout hepatoblasts were more susceptible to TGFβ signaling for their biliary differentiation, we cultured distal fragments of the 12.5-day liver in the presence of TGFβ1 in vitro. Wild-type hepatoblasts in the explants mostly gave rise to large hepatocytes facing large lumina and expressing bile duct-type cytokeratin, but no biliary cell differentiation was observed even in the presence of TGFβ1 in terms of the expression of bile duct markers (DBA- and PNA-binding sites) (see Fig. S1 in the supplementary material). Knockout liver explants also showed histology similar to that of wild-type liver explants (see Fig. S1 in the

DEVELOPMENT

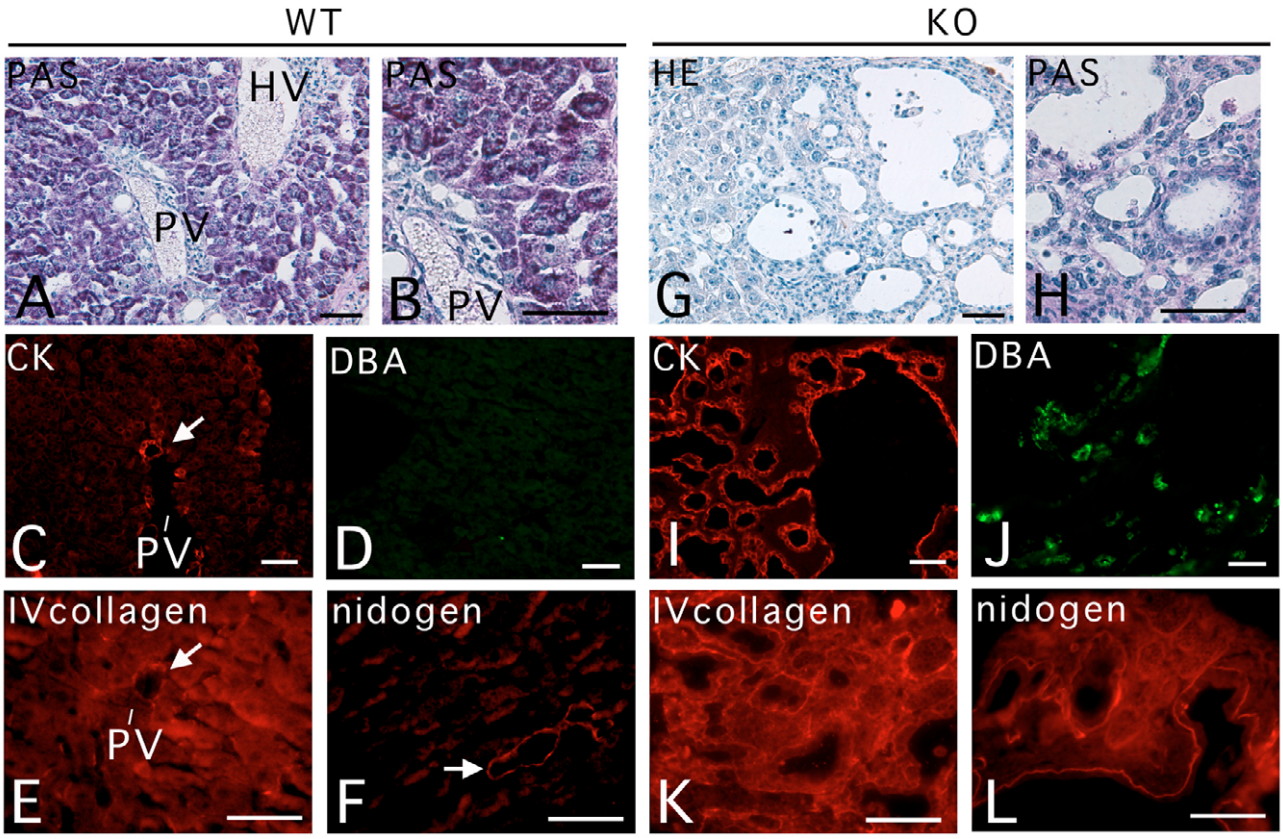

Fig. 9. Liver fragments of knockout fetuses develop cystic structures in the testes of *Scid* mice. Liver fragments of 12.5-day wild-type and knockout fetuses were transplanted and maintained for 2 months in the testes of *Scid* mice, and histologically and histochemically analyzed for expression of cell type-specific markers. The wild-type liver developed normal hepatic tissues, in which the portal vein and hepatic vein differentiated (**A**,**B**). Hepatocytes store PAS-positive glycogen. Periportal biliary cells (arrows) are immunostained for bile duct-specific cytokeratin (**C**), type IV collagen (**E**) and nidogen (**F**) but are negative for DBA-binding sites (**D**). By contrast, the knockout liver developed abundant cystic structures strongly expressing bile duct markers in the testis (**G-L**). Normal hepatic tissues are also observed with cystic structures in a few knockout transplants (G; Hematoxylin and Eosin staining). HV, hepatic vein; PV, portal vein. Scale bars: 50 μm.

supplementary material). Knockout hepatocytes treated with TGFβ1 had a tendency to express bile duct-type cytokeratin more strongly.

Knockout liver fragments generate cystic structures

To investigate whether knockout pseudoglandular cells gave rise to hepatocytes or biliary epithelial cells, we transplanted 12.5-day liver fragments into testes of *Scid* mice. Testes are a good place for hepatic histogenesis from fetal liver fragments (Shiojiri, 1984). In testicular transplants of liver fragments of 12.5-day wild-type fetuses, mature liver tissues developed with periportal bile ducts after 2 months (Fig. 9A,B). The biliary epithelial cells strongly expressed bile duct-specific cytokeratin, but only weakly expressed nidogen and type IV collagen (Fig. 9C,E,F). They hardly expressed laminin or DBA-, SBA- and PNA-binding sites (Fig. 9D). By contrast, the knockout liver fragments formed abundant cystic structures lined with dense connective tissue that expressed bile duct-markers such as DBA- and SBA-binding sites and basal laminar components (laminin, nidogen and PNA-binding sites) (Fig. 9G-L). Not all epithelial cells of the structures expressed these markers. In some transplants of the knockout liver, hepatic tissues containing hepatocytes were obtained with cystic or ductal structures (Fig. 9G).

DISCUSSION

The present study demonstrated that inactivation of the *Cebpa* gene induced abundant pseudoglandular structures or pearl-like structures that resembled precursors for intrahepatic bile ducts in the wild-type fetal liver but their lumina were very large, especially in later development. These results were consistent with those of previous studies (Flodby et al., 1996; Tomizawa et al., 1998; Wang et al., 1995), and may support the idea that downregulation of *Cebpa* gene expression is a necessary step for bile duct formation (Shiojiri et al., 2004) (Fig. 10). In normal intrahepatic bile duct development, C/EBPα is downregulated, which results in downregulation of liver-specific genes such as those of urea cycle enzymes, and may also lead to biliary cell differentiation (Shiojiri et al., 2004). In the present study, testicular transplants of knockout fetal liver fragments generated abundant cystic structures composed of biliary epithelial cells that expressed DBA-binding sites and basal laminar components, whereas those of wild-type liver fragments developed normal hepatic tissues. However, our immunohistochemical analyses showed that, in the knockout liver, only pseudoglandular structures around the portal vein and under the hepatic capsule expressed DBA-binding sites and basal laminar components and were negative for albumin, AFP and N-cadherin. These results suggest that although the downregulation of C/EBPα is an important step, it is not sufficient for bile duct development. It is also of note

that transplanted knockout liver fragments formed cystic structures, but not morphologically normal bile ducts, in the testis. Hepatocytes or hepatocyte-like cells of the knockout liver may tend to generate cystic structures. Soriano et al. (Soriano et al., 1998) have demonstrated that the lack of *Cebpa* gene expression increases the immortalization frequency of freshly isolated mouse hepatocytes.

The preferential periportal development of HNF4-negative pseudoglandular structures with basal lamina in the knockout liver suggested the presence of some bile duct-inducing factors in the periportal environment (Fig. 10). Clotman et al. (Clotman et al., 2005) have demonstrated that a gradient of activin/TGFβ signaling modulated by onecut transcription factors is required to segregate the hepatocytic and biliary lineages. The data of the present study did not support their conclusion, suggesting that TGFβ signaling was not sufficient by itself and required other factors to stimulate hepatoblast differentiation toward the biliary cell lineage. Although further studies will be required to resolve what types of molecules in the periportal environment induce biliary cell differentiation, the induction might also include signaling from Jag1 of portal cells to Notch2 receptors of periportal hepatoblasts (McCright et al., 2002; Tanimizu and Miyajima, 2004). However, there were not as many *Jag1*-positive periportal connective tissue cells, and periportal hepatoblasts and biliary epithelial cells expressed both *Jag1* and *Notch2* mRNAs as shown in the present study, which implied their signaling in hepatoblast and biliary cell populations. Furthermore, the cells of the hepatic capsule never expressed *Jag1* mRNA, which can induce pseudoglandular structures expressing *Jag1* and *Notch2* mRNAs, resembling biliary cells in terms of basal laminar deposition and lectin-binding sites. The deposition of basal laminar components in pseudoglandular structures under the hepatic capsule might be controlled by the presence of extracellular matrix components or its bile-duct-inducing property similar to that of the portal connective tissue.

In addition to *Jag1* and *Notch2*, *Hnf6* and *Hnf1b* have already been demonstrated to be involved in bile duct development, and their knockout mice are deficient in biliary cell development, including extrahepatic and intrahepatic bile ducts (Clotman et al., 2002; Clotman et al., 2003; Coffinier et al., 2002). The present study examined their down- and upstream relationships to the action of C/EBPα in biliary cell differentiation, the absence of which produced pseudoglandular structures. We found that *Hnf6* and *Hnf1b* mRNAs were upregulated in almost all pseudoglandular structures of the knockout liver, suggesting that these transcription factors were downstream of the action of C/EBPα. C/EBPα can bind to regulatory sequences of the *Hnf6* gene and block its transcription (Rastegar et al., 2000), though it is still possible that C/EBPα can indirectly suppress transcription of the *Hnf6* gene. Because HNF6 is upstream of HNF1β for bile duct development (Clotman et al., 2002; Coffinier et al., 2002), suppression of C/EBPα expression in periportal hepatoblasts may lead to the elevation of HNF6 expression and then HNF1β expression (Fig. 10). Our data agreed well with this proposed signaling. Because *Notch2* and *Jag1* were also upregulated in cells of pseudoglandular structures in nonperiportal areas, their actions might also be downstream of C/EBPα (Fig. 10). The direct control of *Jag1* and *Notch2* genes by C/EBPα has not been demonstrated. An in vitro study with fetal hepatoblasts demonstrated that active Notch signaling could downregulate the expression of C/EBPα, HNF1α and HNF4 (Tanimizu and Miyajima, 2004). Downregulation of *Jag1* in endothelial cells of portal veins was seen in *Cebpa* knockout fetal mice, suggesting an interaction between the liver parenchymal region and portal endothelial cells.

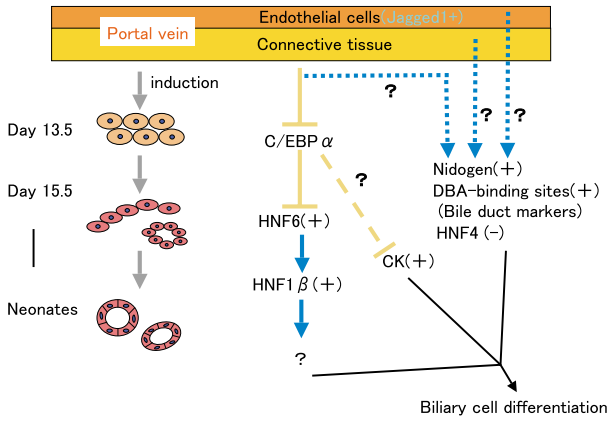

Fig. 10. Bile duct development and C/EBPα. The portal environment induces suppression of C/EBPα expression, which leads to downregulation of hepatocyte-specific genes and upregulation of *Hnf6* and *Hnf1b* genes. The deposition of basal laminar components, expression of DBA- and SBA-binding sites, and suppression of HNF4 expression are not downstream of the action of C/EBPα. *Jag1* and *Notch2* mRNA expression might be downstream of C/EBPα. CK, bile duct-specific cytokeratin.

Cells of pseudoglandular structures appearing around portal veins near the hilus expressed α3 and β4 integrin subunits, both of which are expressed by extrahepatic bile duct cells from early stages of their development, but intrahepatic bile duct cells express them weakly only after 17.5 days (Shiojiri and Sugiyama, 2004). Thus, they might be comparable with extrahepatic bile duct cells. They also morphologically resembled extrahepatic bile duct cells. These results suggested that inactivation of the *Cebpa* gene might induce extrahepatic bile duct development in the liver. Extrahepatic bile ducts did not express this transcription factor at any time throughout normal development, which might be related to the appearance of extrahepatic bile duct cell-like cells in the *Cebpa* knockout liver.

The present study also demonstrated that, in the neonatal knockout liver, typical bile ducts developed poorly irrespective of the stimulation of periportal pseudoglandular structural formations containing biliary marker-positive cells. Because C/EBPα expression was suppressed in periportal hepatoblasts during intrahepatic bile duct development of the wild-type liver (this factor is probably not required for bile duct formation), normal bile duct morphogenesis theoretically could take place in the knockout liver. Our data on abnormal bile duct development in the knockout liver suggest that periportal bile duct development may require maturation of liver parenchymal cells, including active C/EBPα in them, in addition to the portal environment inducing bile duct differentiation. Graded expression of *Jag1* and *Notch2* mRNAs in cells of periportal pseudoglandular cells may block segregation of bile duct marker-positive cells from marker-negative cells. Notch signaling can act on such segregation phenomena (Rhee et al., 2003; Rida et al., 2004). The lack of C/EBPα expression in the hepatoblast and hepatocyte populations induced strong E-cadherin expression in all pseudoglandular structures, which might have resulted in poor segregation of the bile duct marker-positive cells. Although knockout hepatocyte-like cells did have increased proliferative activity compared with wild-type hepatocytes, knockout biliary epithelial cells showed lower proliferative activity than did their wild-type counterparts, which might have been related to poor formation of bile ducts in the knockout. The opposite results of the effects of C/EBPα gene inactivation on cell proliferation might be

explained by the level of phosphorylation of the C/EBPα protein; in hepatocytes, C/EBPα might be hyperphosphorylated and inhibit proliferation, whereas in biliary epithelial cells, in addition to a reduction of the protein level, it is likely to be dephosphorylated and might accelerate proliferation by sequestering retinoblastoma protein (Wang and Timchenko, 2005; Wang et al., 2006). Although testicular transplants of knockout liver fragments produced many cystic structures, this might also have been related to the poor development of typical bile ducts in the neonatal knockout liver.

The weak expression of *Hnf6*, *Hnf1b*, *Jag1* and *Notch2* mRNAs in hepatoblasts in the early stages, observed in the present study, might be related to their bipotentiality for differentiation. These can be used as markers for the immature state of hepatocytes.

In conclusion, the absence of *Cebpa* gene expression may stimulate biliary cell differentiation. The portal environment plays a decisive role in periportal bile duct development. The morphogenesis of intrahepatic bile ducts, including their segregation from the liver parenchyma along portal veins, can be coupled with maturation of the liver parenchyma. HNF6 and HNF1β may be downstream of C/EBPα in bile duct development (Fig. 10).

We thank Professor Emeritus Takeo Mizuno of the University of Tokyo and Prof. Nelson Fausto of the University of Washington for their interest in our study and encouragement, and Mr Kim Barrymore for his help in preparing our manuscript. This work was performed through Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science and Technology, the Japanese Government, and by a Grant for Child Health and Development (17C-4) from the Ministry of Health, Labor and Welfare, the Japanese Government.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/21/4233/DC1

References

Clotman, F., Lannoy, V. J., Reber, M., Cereghini, S., Cassiman, D., Jacquemin, P., Roskams, T., Rousseau, G. G. and Lemaigre, F. P. (2002). The onecut transcription factor HNF6 is required for normal development of the biliary tract. *Development* **129**, 1819-1828.

Clotman, F., Libbrecht, L., Gresh, L., Yaniv, M., Roskams, T., Rousseau, G. G. and Lemaigre, F. P. (2003). Hepatic artery malformations associated with a primary defect in intrahepatic bile duct development. *J. Hepatol.* **39**, 686-692.

Clotman, F., Jacquemin, P., Plumb-Rudewiez, N., Pierreux, C. E., Van der Smissen, P., Dietz, H. C., Courtoy, P. J., Rousseau, G. G. and Lemaigre, F. P. (2005). Control of liver cell fate decision by a gradient of TGFb signaling modulated by Onecut transcription factors. *Genes Dev.* **19**, 1849-1854.

Coffinier, C., Gresh, L., Fiette, L., Tronche, F., Schutz, G., Babinet, C., Pontoglio, M., Yaniv, M. and Barra, J. (2002). Bile system morphogenesis defects and liver dysfunction upon targeted deletion of HNF1β. *Development* **129**, 1829-1838.

Enzan, H., Ohkita, T., Fujita, H. and Iijima, S. (1974). Light and electron microscopic studies on the development of periportal bile ducts of the human embryo. *Acta Pathol. Jap.* **24**, 427-447.

Flodby, P., Barlow, C., Kylefjord, H., Ährlund-Richter, L. and Xanthopoulos, K. G. (1996). Increased hepatic cell proliferation and lung abnormalities in mice deficient in CCAAT/enhancer binding protein α. *J. Biol. Chem.* **271**, 24753-24760.

Ishii, Y., Fukuda, K., Saiga, H., Matsushita, S. and Yasugi, S. (1997). Early specification of intestinal epithelium in the chicken embryo: a study on the localization and regulation of CdxA expression. *Dev. Growth Differ.* **39**, 643-653.

Johnson, G. D. and de C. Nogueira Araujo, G. M. (1981). A simple method of reducing the fading of immunofluorescence during microscopy. *J. Immunol. Methods* **43**, 349-350.

Kimura, T., Christoffels, V. M., Chowdhury, S., Iwase, K., Matsuzaki, H., Mori, M., Lamers, W. H., Darlington, G. J. and Takiguchi, M. (1998). Hypoglycemia-associated hyperammonemia caused by impaired expression of ornithine cycle enzyme gene in C/EBP α knockout mice. *J. Biol. Chem.* **273**, 27505-27510.

Lemaigre, F. P. (2003). Development of the biliary tract. *Mech. Dev.* **120**, 81-87.

Lemaigre, F. and Zaret, K. S. (2004). Liver development update: new embryo models, cell lineage control, and morphogenesis. *Curr. Opin. Genet. Dev.* **14**, 582-590.

Li, L., Krantz, I. D., Deng, Y., Genin, A., Banta, A. B., Collins, C. C., Qi, M., Trask, B. J., Kuo, W. L., Cochran, J. et al. (1997). Alagille syndrome is caused by mutations in human *Jagged1*, which encodes a ligand for Notch1. *Nat. Genet.* **16**, 243-251.

Lorent, K., Yeo, S. Y., Oda, T., Chandrasekharappa, S., Chitnis, A., Matthews, R. P. and Pack, M. (2004). Inhibition of Jagged-mediated Notch signaling disrupts zebrafish biliary development and generates multi-organ defects compatible with an Alagille syndrome phenocopy. *Development* **131**, 5753-5766.

McCright, B., Lozier, J. and Gridley, T. (2002). A mouse model of Alagille syndrome: *Notch2* as a genetic modifier of *Jag1* haploinsufficiency. *Development* **129**, 1075-1082.

Oda, T., Elkahloun, A. G., Pike, B. L., Okajima, K., Krantz, I. D., Genin, A., Piccoli, D. A., Meltzer, P. S., Spinner, N. B., Collins, F. S. et al. (1997). Mutations in the human *Jagged1* gene are responsible for Alagille syndrome. *Nat. Genet.* **16**, 235-242.

Rastegar, M., Rousseau, G. G. and Lemaigre, F. P. (2000). CCAAT/enhancer-binding protein-α is a component of the growth hormone-regulated network of liver transcription factors. *Endocrinology* **141**, 1686-1692.

Rhee, J., Takahashi, Y., Saga, Y., Wilson-Rawls, J. and Rawls, A. (2003). The protocadherin papc is involved in the organization of the epithelium along the segmental border during mouse somitogenesis. *Dev. Biol.* **254**, 248-261.

Rida, P. C., Le Minh, N. and Jiang, Y.-J. (2004). A Notch feeling of somite segmentation and beyond. *Dev. Biol.* **265**, 2-22.

Sargent, T. D., Yang, M. and Bonner, J. (1981). Nucleotide sequence of cloned rat serum albumin messenger RNA. *Proc. Natl. Acad. Sci. USA* **78**, 243-246.

Shiojiri, N. (1984). The origin of intrahepatic bile duct cells in the mouse. *J. Embryol. Exp. Morphol.* **79**, 25-39.

Shiojiri, N. (1994). Transient expression of bile-duct-specific cytokeratin in fetal mouse hepatocytes. *Cell Tissue Res.* **278**, 117-123.

Shiojiri, N. (1997). Development and differentiation of bile ducts in the mammalian liver. *Microsc. Res. Tech.* **39**, 328-335.

Shiojiri, N. and Mizuno, T. (1993). Differentiation of functional hepatocytes and biliay epithelial cells from immature hepatocytes of the fetal mouse in vitro. *Anat. Embryol.* **187**, 221-229.

Shiojiri, N. and Sugiyama, Y. (2004). Immunolocalization of extracellular matrix components and integrins during mouse liver development. *Hepatology* **40**, 346-355.

Shiojiri, N., Lemire, J. M. and Fausto, N. (1991). Cell lineages and oval cell progenitors in rat liver development. *Cancer Res.* **51**, 2611-2620.

Shiojiri, N., Inujima, S., Ishikawa, K., Terada, K. and Mori, M. (2001). Cell lineage analysis during liver development using the *spf^{ash}*-heterozygous mouse. *Lab. Invest.* **81**, 17-25.

Shiojiri, N., Takeshita, K., Yamasaki, H. and Iwata, T. (2004). Suppression of C/EBP α expression in biliary epithelial cells during mouse liver development. *J. Hepatol.* **41**, 790-798.

Soriano, H. E., Kang, D. C., Finegold, M. J., Hicks, M. J., Wang, N. D., Harrison, W. and Darlington, G. J. (1998). Lack of C/EBP α gene expression results in increased DNA synthesis and an increased frequency of immortalization of freshly isolated mice hepatocytes. *Hepatology* **27**, 392-401.

Sumazaki, R., Shiojiri, N., Isoyama, S., Masu, M., Keino-Masu, K., Osawa, M., Nakauchi, H., Kageyama, R. and Matsui, A. (2004). Conversion of biliary system to pancreatic tissue in *Hes1*-deficient mice. *Nat. Genet.* **36**, 83-87.

Tanimizu, N. and Miyajima, A. (2004). Notch signaling controls hepatoblast differentiation by altering the expression of liver-enriched transcription factors. *J. Cell Sci.* **117**, 3165-3174.

Tomizawa, M., Garfield, S., Factor, V. and Xanthopoulos, K. G. (1998). Hepatocytes deficient in CCAAT/enhancer binding protein α (C/EBP α) exhibit both hepatocyte and biliary epithelial cell character. *Biochem. Biophys. Res. Commun.* **249**, 1-5.

Van Eyken, P., Sciot, R., Callea, F., Van der Steen, K., Moerman, P. and Desmet, V. J. (1988a). The development of the intrahepatic bile ducts in man: a keratin-immunohistochemical study. *Hepatology* **8**, 1586-1595.

Van Eyken, P., Sciot, R. and Desmet, V. (1988b). Intrahepatic bile duct development in the rat: a cytokeratin-immunohistochemical study. *Lab. Invest.* **59**, 52-59.

Wang, G.-L. and Timchenko, N. A. (2005). Dephosphorylated C/EBP α accelerates cell proliferation through sequestering retinoblastoma protein. *Mol. Cell. Biol.* **25**, 1325-1338.

Wang, G.-L., Shi, X., Salisbury, E., Sun, Y., Albrecht, J. H., Smith, R. G. and Timchenko, N. A. (2006). Cyclin D3 maintains growth-inhibitory activity of C/EBP α by stabilizing C/EBP α-cdk2 and C/EBP α-Brm complexes. *Mol. Cell. Biol.* **26**, 2570-2582.

Wang, N.-D., Finegold, M. J., Bradley, A., Ou, C. N., Abdelsayed, S. V., Wilde, M. D., Taylor, L. R., Wilson, D. R. and Darlington, G. J. (1995). Impaired energy homeostasis in C/EBP α knockout mice. *Science* **269**, 1108-1112.

Wilson, J. W., Groat, C. S. and Leduc, E. H. (1963). Histogenesis of the liver. *Ann. N. Y. Acad. Sci.* **111**, 8-24.

Wood, R. L. (1965). An electron microscope study of developing bile canaliculi in the rat. *Anat. Rec.* **151**, 507-530.

Zaret, K. S. (2002). Regulatory phases of early liver development: paradigms of organogenesis. *Nat. Rev. Genet.* **3**, 499-512.

Zhao, R. and Duncan, S. A. (2005). Embryonic development of the liver. *Hepatology* **41**, 956-967.