Notch signaling controls liver development by regulating biliary differentiation

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In the mammalian liver, bile is transported to the intestine through an intricate network of bile ducts. Notch signaling is required for normal duct formation, but its mode of action has been unclear. Here, we show in mice that bile ducts arise through a novel mechanism of tubulogenesis involving sequential radial differentiation. Notch signaling is activated in a subset of liver progenitor cells fated to become ductal cells, and pathway activation is necessary for biliary fate. Notch signals are also required for bile duct morphogenesis, and activation of Notch signaling in the hepatic lobule promotes ectopic biliary differentiation and tubule formation in a dose-dependent manner. Remarkably, activation of Notch signaling in postnatal hepatocytes causes them to adopt a biliary fate through a process of reprogramming that recapitulates normal bile duct development. These results reconcile previous conflicting reports about the role of Notch during liver development and suggest that Notch acts by coordinating biliary differentiation and morphogenesis.

KEY WORDS: Notch, Bile ducts, Liver, Mouse

INTRODUCTION

Bile plays an important role in metazoan biology by emulsifying fats and transporting the products of liver detoxification. After its synthesis by hepatocytes, bile is carried from the liver to the intestine by the bile ducts. Dysfunction of the biliary system, either through obstruction, destruction, congenital malformation or cancer, is a significant cause of morbidity and mortality. The large proximal ducts of the liver [extrahepatic bile ducts (EHBDs)] arise by branching of a primitive gut-derived diverticulum, whereas the smaller intrahepatic bile ducts (IHBDs), which constitute the largest component of the biliary tree, form in situ. During IHBD development, hepatic progenitor cells [hepatoblasts] adjacent to portal veins undergo ductal commitment, forming a structure known as the ductal plate, while progenitors located in the parenchyma, away from the portal veins, become hepatocytes (Lemaigre and Zaret, 2004). Prior to birth, tubular structures arise at discrete sites within the ductal plate, ultimately giving rise to IHBDs, while the remaining progenitor cells regress during the first few weeks of life (see Fig. 1A). It is not known how communication is established between the extra- and intrahepatic biliary systems.

Notch signaling is necessary for normal bile duct development. In humans, mutations in the Notch ligand JAG1 or in the NOTCH2 receptor are responsible for Alagille syndrome (AGS), an autosomal-dominant disorder, the features of which include IHBD paucity and several studies have implicated Notch in the regulation of differentiation and morphogenesis. We describe a novel mechanism for duct morphogenesis that relies upon sequential differentiation of adjacent layers of precursor cells. In addition, we report that Notch functions earlier than previously described in the embryonic liver, where it plays important roles in differentiation and tubule formation at distinct stages of development. Taken together, these results indicate that Notch acts in a temporal- and dose-dependent manner to coordinate biliary fate and morphogenesis.

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Accepted 17 March 2009
MATERIALS AND METHODS

Mouse studies
Mice were maintained in a pathogen-free environment. All strains have been described: Albumin-CreER; Foxa3-Cre (Lee et al., 2005), RbpjloxP/loxP (Han et al., 2002) mice were kindly provided by G. Schutz, K. Kaestner, P. Chambon, D. Melton and T. Honjo (RIKEN BioResources), respectively. A null allele of Rbpj (Rbpj<sup>−/−</sup>) was made by crossing Rbpj<sup>loxP/loxP</sup> and Sox2-Cre mice (Hayashi et al., 2002). For activating Notch signaling in differentiated hepatocytes, 6 mg tamoxifen (TM) was administered to Albumin-CreER; Rbpj<sup>loxP/loxP</sup> mice on alternating days for a total of 3-5 doses, and sections were examined 5-21 days later. Serum chemistries were measured by Analytics (Gaithersburg, MD, USA). All studies were performed in accordance with policies for the humane use of animals established by the University of Pennsylvania and the NIH.

Immunostaining and immunoblotting
Tissues were fixed in zinc-buffered formalin (Polysciences), embedded in paraffin and cut at 5 μm. Immunofluorescence. For Hes1 and Jag1 immunostaining, tyramide signal amplification was performed using the TSA Fluorescence System (PerkinElmer). Anti-Ck19 and anti-Hes1 antibodies were made by synthesizing peptides as described (Ito et al., 2000; Tanimizu et al., 2003), conjugating to Keyhole limpet hemocyanin (KLH), and immunizing rabbits with each peptide (Covance). For immunohistochemistry, sections were sequentially incubated with 1:250 donkey serum or CAS Block (Invitrogen). Primary antibodies are listed in Table 1. Rabbit anti-Ck19 and anti-Hes1 antibodies were made by synthesizing peptides as described (Ito et al., 2000; Tanimizu et al., 2003), conjugating to Keyhole limpet hemocyanin (KLH), and immunizing rabbits with each peptide (Covance). For immunohistochemistry, sections were sequentially incubated with biotinylated secondary antibodies (Jackson ImmunoResearch), peroxidase-conjugated streptavidin (ABC Staining Kit; Vector Labs), DAB substrate and Hematoxylin. Alexa 488- or Alexa 594-conjugated secondary antibodies (Invitrogen) were used with DAPI counterstaining for immunofluorescence. For Hes1 and Jag1 immunostaining, tyramide signal amplification was performed using the TSA Fluorescence System (PerkinElmer). All reported results were observed in at least three animals. For western blotting, total protein was extracted from whole liver, separated by SDS-PAGE and transferred to nitrocellulose. Membranes were blocked For western blotting, total protein was extracted from whole liver, separated by SDS-PAGE and transferred to nitrocellulose. Membranes were blocked for 1 hour in 5% skimmed milk in Tris-buffered saline (TBS) at 4°C. Membranes were then incubated with primary antibodies overnight in blocking buffer, washed with TBS containing 0.1% Tween-20, and incubated with secondary antibodies for 1 hour at room temperature. Quantitative PCR
Total RNA was extracted from whole liver using the RNaseq Mini Kit (Qiagen) and 1 μg used to synthesize cDNA using the SuperScript Kit (Invitrogen, 11752). Quantitative PCR was performed with SYBR Green Master Mix Reagent (Applied Biosystems) using an ABI 7900 sequence detector. Transcript quantities were determined using the difference of Ct method; standard curves were constructed for each primer pair and values were normalized to Hprt. Primer sequences are listed in Table 2.

Chromatin immunoprecipitation (ChIP) analysis
ChIP was performed using the ChIP Assay Kit (Upstate). Liver tissue (100 mg) was mixed in PBS and cross-linked using 1% formaldehyde for 10 minutes. Cross-linking was quenched by the addition of glycine to a final concentration of 0.125 M. Cells were lysed with 1 ml lysis buffer supplemented with protease inhibitor (Roche). DNA was sheared into fragments of 100-500 bp by BioRuptor sonication (Diagenode), and cross-linked proteins were immunoprecipitated using Notch1 antisera (Fang et al., 2007). After protein-A bead pull-down, cross-links were reversed and the DNA was purified using the QIAquick PCR Purification Kit (Qiagen). DNA copy number was measured by quantitative PCR, normalized to 28S ribosomal DNA sequences (Rubins et al., 2005). Enrichment of DNA was analyzed by comparing DNA copy number in ChIP samples with that of input. Primer sequences are listed in Table 2.

RESULTS

Notch signaling during IHBD development
We sought to confirm the normal sequence of events during IHBD development by examining the expression of the duct-specific cytokeratin Ck19 (Krt19 – Mouse Genome Informatics) at various stages (Fig. 1A-D). As described previously (Lemaigre, 2003), IHBD development is characterized by the appearance of ductal plate precursor cells adjacent to branches of the portal vein (~E14-16), the appearance of dilations at discrete points along the ductal plates (~E16-P2), and the postnatal disappearance of unincorporated biliary precursor cells (~P2-15). In addition, we observed that nascent ducts pass through a previously undescribed intermediate stage characterized by the asymmetric expression of biliary and hepatoblast markers. Specifically, Ck19 was expressed by cells on the portal side, but not the parenchymal side, of these asymmetric tubules, whereas Hnf4α was expressed by cells on the parenchymal side, but not the portal side (Fig. 1B,E; see Fig. S1 in the supplementary material). Other markers of BECs, including Epcam, acetylated tubulin (AcT), Sox9, Hnf1, and osteopontin (Opn; Spp1 – Mouse Genome Informatics) (Antoniou et al., 2009; Coffinier et al., 2002; Zhang et al., 2008), were also expressed by cells on the portal side, but not the parenchymal side, of these primitive ductal structures (Fig. 1A-D). As described previously (Lemaigre, 2003), IHBD development is characterized by the appearance of ductal plate precursor cells adjacent to branches of the portal vein (~E14-16), the appearance of dilations at discrete points along the ductal plates (~E16-P2), and the postnatal disappearance of unincorporated biliary precursor cells (~P2-15). In addition, we observed that nascent ducts pass through a previously undescribed intermediate stage characterized by the asymmetric expression of biliary and hepatoblast markers. Specifically, Ck19 was expressed by cells on the portal side, but not the parenchymal side, of these asymmetric tubules, whereas Hnf4α was expressed by cells on the parenchymal side, but not the portal side (Fig. 1B,E; see Fig. S1 in the supplementary material). Other markers of BECs, including Epcam, acetylated tubulin (AcT), Sox9, Hnf1, and osteopontin (Opn; Spp1 – Mouse Genome Informatics) (Antoniou et al., 2009; Coffinier et al., 2002; Zhang et al., 2008), were also expressed exclusively by cells on the portal side of these primitive ductal structures (Fig. 1B,F,H; see Movies 1, 2 in the supplementary material; data not shown). The asymmetry resolved after birth (P2), by which point the ducts had adopted a mature configuration and were symmetrically encircled by BECs (Fig. 1C,G,I, J). These

Table 1. Primary antibodies used for immunostaining experiments

<table>
<thead>
<tr>
<th>Species</th>
<th>Source</th>
<th>Catalog #</th>
<th>Dilution</th>
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<tr>
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<td>D. Melton</td>
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<td>DSHB</td>
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<td>Goat</td>
<td>Abcam</td>
<td>6673</td>
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<tr>
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<td>Covance</td>
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<td>Goat</td>
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<td>R&amp;D</td>
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<tr>
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<td>AF808</td>
<td>1:1000</td>
</tr>
<tr>
<td>Sox9</td>
<td>Goat</td>
<td>Chemicon</td>
<td>AB5535</td>
<td>1:500</td>
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DSHB, Developmental Studies Hybridoma Bank.
changes in biliary tubule composition were also apparent ultrastructurally (Fig. 1I; see Fig. S2 in the supplementary material). Thus, during IHBD development, lumen formation precedes the terminal differentiation of cells that will ultimately line the outer layer of the ducts.

To directly examine the expression of Notch signaling components during liver development, we measured stage-specific transcript levels for all four Notch receptors, Jagged and Delta ligands, and targets from the Hes and Hey families by real-time PCR. At all stages examined, multiple signaling pathway components were expressed (see Fig. S3 in the supplementary material), indicating that functional redundancy might mitigate the loss of any single pathway component. We further characterized the possible functional redundancy, we employed mice with a conditional mutation in the gene (Han et al., 2002). Rbpj constitutes the DNA-binding portion of the Notch transcription complex and is a necessary effector of canonical Notch signaling (Bolos et al., 2007; Oka et al., 1995). To examine the consequences of Rbpj loss on liver development, we obtained Foxa3-Cre; RbpjloxP/loxP mutants (Fig. 3B,C; see Fig. S6 in the supplementary material) and a

### Notch regulates embryonic biliary fate

The role of Notch signaling in liver development has previously been assessed in mice bearing deletions in Jag1, Notch1, Notch2 or Hes1 (Geisler et al., 2008; Kodama et al., 2004; Loomes et al., 2007; Lozier et al., 2008; McCright et al., 2002). To circumvent possible functional redundancy, we employed mice with a conditional mutation in the Rbpj gene (Han et al., 2002). Rbpj constitutes the DNA-binding portion of the Notch transcription complex and is a necessary effector of canonical Notch signaling (Bolos et al., 2007; Oka et al., 1995). To examine the consequences of Rbpj loss on liver development, we obtained Foxa3-Cre; RbpjloxP/loxP (Foxa3-RBP) embryos, in which the DNA-binding domain of Rbpj was deleted on one allele and flanked by loxP recombination sequences on the other allele. Rbpj deletion was confirmed by genomic PCR, and loss of Notch signaling was documented by a reduction of Hes1 staining in the ductal plate region (Fig. 3A). Compared with controls, Foxa3-RBP mutants exhibited a reduced number of ductal plate cells at E16.5 and P0 (Fig. 3B,C; see Fig. S6 in the supplementary material) and a
significant decrease in the number of bile ducts at P0 (Fig. 3C). These results indicate that Rbpj is necessary for normal ductal plate development in the embryonic liver.

Previous in vitro studies have suggested that Notch signaling induces biliary differentiation in hepatic cells (Kodama et al., 2004; Tanimizu et al., 2003; Tanimizu et al., 2004). To determine whether Notch plays an instructive role in biliary differentiation in vivo, we employed Rosa26Notch1ICD mice (Murtaugh et al., 2003) (henceforth referred to as RosaNICD), which harbor a constitutively active form of Notch1 downstream of loxP-flanked transcriptional stop sequences (Fig. 3D) (Murtaugh et al., 2003). This strain has been used to activate the Notch signaling cascade in a variety of tissues (Cheng et al., 2007; Jadhav et al., 2006; Niranjan et al., 2008; Stanger et al., 2005). We obtained bigenic Foxa3-Cre; RosaNICD/+(Foxa3-NICD) embryos at the expected Mendelian ratio. Widespread Hes1 staining was observed in Foxa3-NICD livers prior to E16.5, confirming that Notch signaling was activated in hepatic precursor cells (Fig. 3D). Ectopic BECs expressing a full repertoire of ductal markers were observed in the parenchyma of Foxa3-NICD livers as early as E16.5 (Fig. 3D; see Fig. S6B in the supplementary material; data not shown). Remarkably, some of these cells had assembled into bile ducts of mature appearance (Fig. 3D, inset; see Fig. S6B in the supplementary material). Taken together, these results suggest that Notch signaling (acting via Rbpj) regulates the differentiation of embryonic biliary precursors.

Notch regulates formation of the second biliary layer

We next sought to determine whether Notch plays a role in the development of primitive ductal structures. We employed AFP-Cre mice, in which Cre recombinase is expressed under the regulatory
control of the α-fetoprotein (Afp) enhancer and albumin promoter (Kellendonk et al., 2000), and confirmed by Rosa<sup>TFF</sup> reporter analysis that recombination occurs later with AFP-Cre mice than with Foxa3-Cre mice. At E15.5, 36% of Hnf4α<sup>+</sup> cells were labeled in AFP-Cre; Rosa<sup>TFF</sup> mice, significantly less than the 81% of Hnf4α<sup>+</sup> cells labeled in Foxa3-Cre; Rosa<sup>TFF</sup> mice at this stage. By contrast, 88% of Hnf4α<sup>+</sup> cells were labeled in AFP-Cre; Rosa<sup>TFF</sup> mice at E16.5 (see Fig. S5 in the supplementary material). By P2, 95% of Hnf4α<sup>+</sup> cells (hepatocytes) and 98% of Ck19<sup>+</sup> cells (BECs) were labeled in AFP-Cre; Rosa<sup>TFF</sup> mice. Thus, AFP-Cre exhibits peak activity (as measured by this assay) during the formation of the second ductal layer (~E16.5). AFP-Cre; Rbpj<sup>loxP/loxP</sup> (AFP-RBP) livers exhibited a less severe reduction in peri-portal Hes1<sup>+</sup> cells at E16.5 than that observed with Foxa3-Cre (e.g. compare Fig. 3A with Fig. 4A). Consistent with less efficient deletion at this stage, mutant animals had ductal plates of normal appearance at E16.5 (Fig. 4B, top panels). At P1 and P2, however, AFP-RBP livers exhibited a significant reduction in the number of bile ducts (Fig. 4B,C). This defect was also apparent at P6 (Fig. 4B, bottom panels), indicating that the phenotype was not due to delayed bile duct maturation. These results suggest that following induction of the first ductal plate layer, Rbpj is required for the subsequent formation of mature ducts.

To determine whether Notch directly regulates tubulogenesis, we crossed Rosa<sup>NICD</sup> mice to AFP-Cre mice, yielding bigenic AFP-Cre; Rosa<sup>NICD</sup> (AFP-NICD) embryos. AFP-NICD livers exhibited an increase in Hes1 transcript levels and protein, confirming that Notch signaling was activated throughout the hepatic lobule (Fig. 5A). Whereas the ductal plates appeared normal at E16.5, AFP-NICD mice exhibited an increase in portal vein-associated BECs at P0 and P2 (Fig. 5B). This change was
associated with an increase in the size and number of bile ducts at P2 from a mean of 2.3 (control) to 3.5 ducts per portal vein (AFP-NICD) (n=3 for each genotype, P<0.001). Although most BECs were confined to the portal region, ectopic Ck19+ cells were also detected in the lobules starting at P2 (Fig. 5B, Fig. 6). These Ck19+ cells failed to undergo regression, leading to the persistence of BECs at P15 in a portal-to-lobular gradient (Fig. 5C). Ck19+ cells showed higher proliferation in AFP-NICD livers compared with control (1.44% versus 1.06%, respectively; P=0.042), indicating that enhanced proliferation might contribute to the phenotype.

**Notch regulates tubulogenesis in a dose-dependent manner**

At P2, AFP-NICD mice exhibited ectopic tube formation in the lobules, an area normally occupied by hepatocyte-lined sinusoids (Fig. 6). The tubes were lined by Ck19+ cells and Hnf4α cells in a manner reminiscent of the asymmetric tubules present during normal biliary tubulogenesis (Fig. 6A, top right inset). Acetylated tubulin (AeT), a cilia marker that is confined to ductal plate BECs in control livers, was expressed in these ectopic structures (Fig. 6A, bottom panels). The tubes disappeared over the first 2 weeks of life and were replaced by duct-like structures (Fig. 5C; see Fig. S7E in the supplementary material), indicating that Notch-induced tubulogenesis is transient in nature.

Human bile duct development is sensitive to changes in JAG1 and NOTCH2 gene dosage. Therefore, we hypothesized that biliary tubulogenesis might be influenced by increasing the dose of Notch signaling. To test this possibility, we bred two copies of the RosaNICD allele into the AFP-Cre background (AFP-N/N), resulting in graded levels of Notch signaling in relation to RosaNICD copy number (Fig. 6B). AFP-N/N mice exhibited a profound tubulogenesis phenotype with dilated tubules in the ductal plate region as early as E16.5 (see Fig. S7C in the supplementary material). At P2, AFP-N/N livers exhibited dilated bile ducts and ectopic tubules throughout the lobule that completely disrupted normal hepatic architecture (Fig. 6C). Cells lining the tubules resembled their counterparts in AFP-NICD mice (i.e. cells expressed either Hnf4α or Ck19). A majority of the cells lining the tubules also expressed Hes1 (Fig. 6C, inset), again evoking the primitive ductal structures normally present at E16-17. In contrast to AFP-NICD mice, AFP-N/N mice exhibited bile ducts of mature appearance in the lobules at P15 (see Fig. S7F in the supplementary material). Surprisingly, these animals
exhibited preserved liver chemistries (see Fig. S8 in the supplementary material). Notably, serum bilirubin was undetectable in AFP-N/N animals, raising the possibility that the ectopic ducts in these animals were functional. Notch signaling can therefore exert a direct effect on morphogenesis during liver development, promoting tubule formation and bile duct maturation in a dose-dependent manner.

**Sox9 is a Notch target**

To better understand the mechanism underlying Notch-induced biliary differentiation and tubulogenesis, we examined the expression of known regulators of biliary development — *Oc1* (Onecut1 – Mouse Genome Informatics), *Oc2*, *Hnf1b*, *Hhex* and *Sox9* — by real-time PCR. Transcripts for *Hnf1b* and *Sox9*, but not *Oc1*, *Oc2* or *Hhex*, were significantly increased in AFP-NICD livers compared with controls at P0 (Fig. 7A). Immunostaining confirmed that *Sox9* and *Hnf1β* were ectopically expressed throughout the lobules of AFP-NICD livers at E16.5 and P0 (Fig. 7B,C). Conversely, no increase in *Oc1* or *Hhex* staining was detected (data not shown). To determine whether *Sox9* is direct target of Notch, we scanned upstream sequences of the *Sox9* gene and identified ten consensus Rbpj binding sites. We chose four elements – three sites close to the *Sox9* promoter region and one conserved element 14 kb upstream – for chromatin immunoprecipitation (ChIP) studies in AFP-NICD livers. When compared with a control sequence, the two sequences closest to the *Sox9* transcriptional start site were significantly enriched following ChIP with an anti-Notch1 antibody, whereas the other sequences showed no enrichment (Fig. 7D). This result suggests that Notch1 is capable of binding directly to the *Sox9* promoter in vivo. Because these two sites (#9 and #10) are within ~400 bp of each other, this result could represent binding of NICD to both or a single site.

**Notch signaling reprograms postnatal albumin+ cells**

Because BECs first appear in the lobules of AFP-NICD mice postnatally, several days after the onset of ectopic Hes1, Sox9 and Hnf1β expression, we hypothesized that terminally differentiated hepatocytes might retain competence to respond to Notch signals. To test this possibility, we employed the Albumin-CreER strain (Schuler et al., 2004), which mediates loxP recombination in hepatocytes following tamoxifen (TM) administration (see Fig. S5 in the supplementary material). We induced recombination by giving TM to 6-day-old Albumin-CreER; *RosaNICD/+* (AlbuminCreER-NICD) mice and examined liver sections 5, 11 or 21 days following the first dose (Fig. 8).

Within 5 days after receiving TM, Hes1 expression was observed in a pan-lobular distribution, indicating broad activation of Notch signaling in hepatocytes. Widespread expression of *Sox9*, and to a lesser extent *Hnf1β* and AcT, was also observed at this stage. Eleven days after TM administration, lobular expression of Hes1, Sox9, Hnf1β and AcT remained high, and expression of Opn (and, to a lesser extent, Ck19) was also detected in the lobules. All markers exhibited robust staining 21 days after TM injection, resulting in an extensive lobular Ck19+ ductal network. Notably, the morphology of the BEC-like cells also changed between 5 and 21 days after TM treatment, transitioning from a scattered distribution and assembling into duct-like structures (e.g. compare Sox9 staining in Fig. 8F and 8H). To determine whether these neo-biliary cells resulted from cell-autonomous or non-cell-autonomous effects of Notch, we gave a low dose of TM to AlbuminCreER-NICD mice to induce clones with activated Notch signaling. In these clones, identified as small clusters of ectopic Opn+ cells within the hepatic lobule, Hes1 staining co-localized completely.
with the biliary marker Opn (Fig. 8D, inset). These results suggest that Notch acts in a cell-autonomous manner to induce a biliary program in hepatocytes.

**DISCUSSION**

**Notch regulates biliary fate in vivo**

Among the factors that could regulate biliary differentiation, the Notch pathway has long stood out as a compelling candidate. Our results provide strong in vivo evidence in support of this hypothesis: (1) spatial and temporal expression of Jag1 and Hes1 in the developing liver is consistent with a role in biliary induction; (2) early deletion of Rbpj, an essential mediator of Notch signaling, results in a reduced number of BEC precursors and bile ducts; and (3) activation of the pathway with an NICD transgene results in ectopic biliary differentiation. Remarkably, activation of Notch signaling in the postnatal liver resulted in widespread biliary differentiation. Although we cannot exclude the possibility that Notch mediates this effect by acting in a rare subpopulation of albumin+ progenitor cells, our data are most consistent with the conclusion that Notch signaling converts differentiated hepatocytes into BECs.

Transdifferentiation of hepatocytes to BEC-like cells has been observed following biliary injury (Michalopoulos et al., 2005) and in hepatocytic spheroids in vitro, where the fate change was accompanied by an increase in the expression of Notch pathway components (Nishikawa et al., 2005). The molecular mechanisms underlying biliary reprogramming by Notch are unclear. We found that the process recapitulated features of normal biliary development, including induction of Sox9 and Hnf1β. Since components of the Notch signaling pathway are upregulated in a number of adult liver diseases (Flynn et al., 2004; Nijjar et al., 2001; Nijjar et al., 2002), our finding that hepatocytes retain biliary competence in response to Notch signals raises the possibility that Notch regulates hepatobiliary remodeling following injury.

How does Notch regulate the biliary program? During development, progenitor cells near the portal vein appear to be more sensitive to the effects of ectopic Notch signaling than those within the lobules, indicating that Notch might act in concert with other factors located near the portal vein (e.g. see Fig. 5 and Fig. S7 in the supplementary material). One candidate for such a cooperating signal is the TGFβ/activin pathway, an important regulator of embryonic biliary differentiation (Clopton et al., 2005). TGFβ/Notch cross-talk occurs in several settings, including myogenesis, where Hes1 is synergistically regulated by both pathways (Blokhzil et al., 2003; Dahlqvist et al., 2003). Since TGFβ/activin is active in a portal-to-central gradient during liver...
development (Clotman et al., 2005), cooperation between the Notch and TGFβ/activin pathways could confer additional spatial cues during bile duct development, as has been proposed (Ader et al., 2006; Clotman and Lemaigre, 2006). Our results also suggest a role for Sox9, a transcription factor that modulates TGFβ signaling during biliary development (Antoniou et al., 2009) and the expression of which was increased in AFP-NICD mutants and decreased in Foxa3-RBP mutants. Cross-talk between Notch and Sox9 has been reported in the pancreas (Seymour et al., 2007) and central nervous system (Taylor et al., 2007), and our ChIP results suggest that Sox9 is a direct target of Notch signaling. A connection between Notch signaling and Sox9 is also consistent with recent observations that Sox9 controls the timing of maturation of primitive ductal structures (Antoniou et al., 2009).

Although our findings are consistent with the in vitro observation that Notch can induce a biliary fate, they are at odds with in vivo studies suggesting that Notch is dispensable for biliary specification (Geisler et al., 2008; Kodama et al., 2004; Lozier et al., 2008; Tanimizu et al., 2003; Tanimizu et al., 2004). This discrepancy might in part be due to functional redundancy. We and others have observed the expression of multiple Notch ligands, receptors and Hes/Hey family members in embryonic liver (Crosnier et al., 2000;
Therefore, our studies relied on deletion of $Rbpj$, an essential mediator of canonical Notch signaling, to achieve complete pathway inactivation. It is also possible that differences in timing might account for the earlier phenotypes we observed. Ductal plate phenotypes appeared only when the early acting Foxa3-Cre strain was used to delete $Rbpj$ (Figs 3-5; see Fig. 6A in the supplementary material). Although deletion of $Rbpj$ with AFP-Cre had no effect on ductal plate development, it did result in a reduced number of bile ducts at birth, similar to the phenotypes resulting from Albumin-Cre-mediated deletion of $Notch2$ (Geisler et al., 2008; Lozier et al., 2008). In our hands, the Albumin-Cre strain mediates recombination late in embryonic development, exhibiting kinetics similar to those of AFP-Cre (data not shown). Therefore, the absence of an embryonic phenotype in previous studies might have resulted from $Notch2$ loss after ductal plate specification. As discussed below, we propose that duct morphogenesis, which is a late event in liver development, occurs through Notch-dependent regulation of differentiation in the second biliary layer.

**Biliary morphogenesis and Notch**

We have shown that bile ducts form through a process of sequential differentiation of two adjacent cellular layers, a mechanism that is distinct from other types of tube formation in the body (Hogan and Kolodziej, 2002; Lubarsky and Krasnow, 2003). During biliary tubulogenesis, lumens form at discrete sites along the first layer of the ductal plate, giving rise to asymmetric, primitive ductal structures at E16-17. Cells lining the two sides of the lumen are similar ultrastructurally but cells comprising the inner (first) layer express a set of distinctive BEC markers: Ck19, Sox9, and $Hnf1\beta$. This asymmetric intermediate can be independently observed (Antoniou et al., 2009), and our results extend their findings. We do not know why bile ducts form through this process, as opposed to the ‘budding’ or ‘wrapping’ mechanisms used in many other tissues (Lubarsky and Krasnow, 2003). One possibility stems from the fact that, unlike many other tubes, bile ducts must retain connectivity in two planes – hepatocyte canaliculi ($x$ axis) and the ductal tree ($z$ axis) – and thus lack a ‘terminal’ branch. Sequential differentiation might facilitate the development of this interconnected arrangement by ensuring contact between hepatocytes and BECs throughout development.
Several lines of evidence suggest that Notch functions during the formation and/or maturation of primitive ductal structures. First, the expression of Notch signaling components is consistent with a role in the differentiation of the second layer. Within asymmetric tubules, Jag1 expression is detected in BECs of the first layer, whereas Hes1 expression is detected in second-layer cells that still express Hnf4α. This suggests that Hes1 expression precedes biliary differentiation in the second layer of the ductal plate. Second, late deletion of Rbpj (with AFP-Cre) permits differentiation of the first layer but results in a significant reduction in the number of bile ducts, consistent with a role in the formation of the second layer and associated tubulogenesis. Third, Notch activation results in an increase in the number of bile ducts at birth. Finally, ectopic Notch activation promotes the formation of tubules that resemble primitive ductal structures; these ectopic tubules arise in a dose-dependent manner and gradually acquire a ductal morphology (Fig. 6 and see Fig. S7 in the supplementary material).

Taken together, these findings are consistent with a model in which Notch controls bile duct development by regulating biliary fate at successive stages of development (Fig. 9). Initially, endothelial Jag1 activates Notch signaling in peri-portal hepatoblasts, resulting in biliary differentiation and the appearance of the first (portal) layer of the ductal plate (E14.5-16.5). The nascent BECs also express Jag1, promoting activation of Notch signaling in the adjacent second layer, lumen formation, and the emergence of primitive ductal structures (E16.5-17.5). Subsequently, cells in the second layer complete the biliary program, giving rise to mature symmetrical ducts (P2). This model accommodates our results and reconciles conflicting reports from the literature regarding whether Notch acts as a regulator of

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**Fig. 8. Notch activation in differentiated hepatocytes promotes biliary differentiation.** (A-T) Albumin-CreER; RosaNICD mice were given five doses of tamoxifen (TM) (6 mg) starting at P6 and examined by immunofluorescence for the indicated markers 5, 11 or 21 days after the first dose. Expression of Hes1, Sox9, Opn, Hnf1β, Act and Ck19 was initially restricted to the portal tract (arrowheads, left column; insets show high magnification). TM treatment leads to rapid induction of Hes1, Sox9 and Hnf1β, but a more gradual induction of Act, Opn and Ck19. Note the rearrangement of ectopic biliary cells from a diffuse lobular distribution to a more organized ductal configuration. Clones of ectopic biliary cells generated by administering a low dose of TM are found in the lobule and show a tight correspondence between Hes1 and Opn expression (inset, upper right panel). All sections are counterstained with DAPI (blue). Scale bars: 100 μm.
Fig. 9. Model of bile duct development. Early in liver development (E12.5-14.5), endothelial-derived Jag1 (yellow) activates Notch signaling and Hes1 expression in adjacent hepatoblasts (blue nuclei), resulting in the formation of the first ductal plate layer at E14.6-16.5 (cells outlined in green). Between E16.5 and E17.5, tubulogenesis occurs at discrete sites of active Notch signaling in adjacent hepatocytes (pink nuclei), giving rise to a primitive ductal structure (asterisk). Cells comprising the second (outer) layer of this asymmetric tubule undergo biliary differentiation between E17.5 and P2. Subsequent growth of the portal mesenchyme and loss of unincorporated BECs leads to the formation of a mature portal tract by P15. Note that BECs in both the first and second layers of the ductal plate express Jag1. See text for details.

This model leaves several questions unanswered. First, how is biliary development controlled spatially? Although Notch signaling is activated throughout the first layer of the ductal plate, lumens arise at discrete locations, and it is unclear what governs the focal formation of these primitive structures. A related question concerns how expression of the ligand (Jag1) spreads to BECs. One possibility is ‘lateral induction’, a process by which Notch signaling in a cell results in the activation, rather than repression, of ligand expression in that cell (Eddison et al., 2000; Timmerman et al., 2004). In addition, other mechanisms are likely to restrict Notch signaling. For example, Jag1 is highly expressed in newborn BECs, where it results in the induction of Hes1, but other cells adjacent to these ligand-producing cells do not express Hes1 (see Fig. 2D,H). Furthermore, although our study demonstrates that biliary tubulogenesis is responsive to increasing Notch dosage, consistent with the known sensitivity of human bile duct development to Jag1 or Notch2 haploinsufficiency (Li et al., 1997; McDaniell et al., 2006; Oda et al., 1997), the mechanism underlying this dose responsiveness remains unclear.

It is worth pointing out two caveats. First, despite the fact that Notch2 is the major Notch receptor involved in bile duct development (Geisler et al., 2008; McDaniell et al., 2006), our experiments used the intracellular domain of Notch1 for gain-of-function. Despite this mismatch, we believe that the Notch1 ICD serves as a reasonable surrogate for Notch activity in the liver. Domain-swapping experiments have shown that the C-terminal portion of the Notch1 and Notch2 ICDs are functionally interchangeable in vivo (Kraman and McCright, 2005). In addition, the Rosa<sup>ICD</sup> strain we used is capable of rescuing a renal fate specification phenotype caused by Notch2 deficiency (Cheng et al., 2007). This indicates that Notch2 targets are appropriately activated by this transgene. Furthermore, our observations with the Notch1 ICD are in agreement with the loss-of-function phenotype resulting from Rbpj deletion. Nevertheless, confirmation that Notch2 promotes biliary differentiation will ultimately be needed. Second, our model proposes a role for Jag1 in the induction of the second layer in primitive ducts. However, conditional deletion of Jag1 in the hepatic epithelium is not associated with bile duct abnormalities during development (Loomes et al., 2007). Timing of deletion or functional redundancy with other hepatic ligands (Jag2, Dll1 or Dll4) could account for the lack of an embryonic Jag1 mutant phenotype, issues that can be addressed by earlier Jag1 deletion and with compound mutants.

We thank G. Schutz, K. Kaestner, P. Chambon, T. Honjo and D. Melton for sharing mice; T. Sudo, A. Miyajima and C. Bogue for sharing aliquots of Hes1, Ck19 and Hhex antisera, respectively; J. Leley and Y. Ohtani for help with ChIP; K. Kaestner, W. Pear, K. Loomes, M. Ryan and M. Pack for helpful discussions; J. Friedman for reading the manuscript; D. Ludwig and the AFCRI Histology Core for assistance with sample preparation; and Y. Sofer and A. Stout for help with confocal imaging. Monoclonal antibody G8.8 was provided by the Developmental Studies Hybridoma Bank. B.Z.S. was supported by grant DK076583 from NIDDK and support from the Penn Center for Molecular Studies in Digestive and Liver Disease. F.L. was supported by the Intramural Attraction Poles Program (Belgian Science Policy), the DG Higher Education and Scientific Research of the French Community of Belgium, the Alphonse and Jean Forton Fund, and the Fund for Scientific Medical Research. A.A. and P.R. hold fellowships from the Université Catholique de Louvain. Deposited in PMC for release after 12 months.

Supplementary material
Supplementary material available online at http://dev.biologists.org/cgi/content/full/136/10/1727/DC1

References


