Notch Signaling Regulates Bile Duct Morphogenesis in Mice

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Abstract

Background: Alagille syndrome is a developmental disorder caused predominantly by mutations in the Jagged1 (JAG1) gene, which encodes a ligand for Notch family receptors. A characteristic feature of Alagille syndrome is intrahepatic bile duct paucity. We described previously that mice doubly heterozygous for Jag1 and Notch2 mutations are an excellent model for Alagille syndrome. However, our previous study did not establish whether bile duct paucity in Jag1/Notch2 double heterozygous mice resulted from impaired differentiation of bile duct precursor cells, or from defects in bile duct morphogenesis.

Methodology/Principal Findings: Here we characterize embryonic biliary tract formation in our previously described Jag1/Notch2 double heterozygous Alagille syndrome model, and describe another mouse model of bile duct paucity resulting from liver-specific deletion of the Notch2 gene.

Conclusions/Significance: Our data support a model in which bile duct paucity in Notch pathway loss of function mutant mice results from defects in bile duct morphogenesis rather than cell fate specification.

Introduction

The primary functional cells of the mammalian liver are the hepatocytes and the epithelial bile duct cells, or cholangiocytes (for recent reviews, see references [1–3]). During liver development, both hepatocytes and cholangiocytes differentiate from bipotential progenitor cells termed hepatoblasts [4,5]. Hepatoblasts located in the liver parenchyma differentiate into hepatocytes, while hepatoblasts located at the interface of the portal mesenchyme (which surrounds the portal vein) and the liver parenchyma differentiate into the biliary epithelial cells. Initially, biliary epithelial cells form a continuous single cell layer termed the ductal plate [reviewed in [5]]. The ductal plate subsequently undergoes morphogenesis and remodeling to generate the epithelial bile ducts. Defects in bile duct formation can lead to an impairment of bile duct flow (cholestasis), and result in a diverse group of both genetic and acquired biliary tract disorders termed cholangiopathies [reviewed in [6,7]].

The Notch signaling pathway is an evolutionarily conserved intercellular signaling mechanism [reviewed in [8,9]], and mutations in its components disrupt embryonic development in diverse organisms and cause inherited disease syndromes in humans. Mutations in the JAG1 gene, which encodes a ligand for Notch family receptors, cause Alagille syndrome [10,11]. Alagille syndrome (OMIM #118450) is a pleiotropic developmental disorder characterized by cholestasis and jaundice caused by intrahepatic bile duct paucity, congenital heart defects, vertebral defects, eye abnormalities, facial dysmorphism, and kidney abnormalities [12–14]. Alagille syndrome exhibits autosomal dominant inheritance, and analysis of the types of JAG1 mutations in Alagille syndrome patients suggest JAG1 haploinsufficiency as the primary cause of Alagille syndrome.

We have described previously a mouse model for Alagille syndrome [15]. Mice heterozygous for a Jag1 null allele, which have the same genotype as Alagille syndrome patients, exhibited haploinsufficient eye defects but did not exhibit other phenotypic abnormalities characteristic for Alagille syndrome [16]. However, mice doubly heterozygous for a Jag1 null allele and a Notch2 hypomorphic allele exhibited most of the clinically relevant features of Alagille syndrome, including bile duct paucity [15]. Our previous studies of these mice concentrated on analysis of late embryonic and postnatal livers, and did not establish whether bile duct paucity in Jag1/Notch2 double heterozygous mice was due to defects in differentiation of bile duct precursors from the bipotential hepatoblast, or defects in morphogenesis of the ductal plate.

A recent study of Hairy and enhancer of split 1 (Hes1)-null mice suggested that the role of Notch signaling during biliary development was in the control of biliary tract morphogenesis, rather than in a hepatocyte-cholangiocyte cell fate specification.
decision [17]. However, other genes encoding Hes-related bHLH proteins are also Notch targets, raising the possibility that Hes1-null mice may not reflect the full extent of the role played by the Notch signaling pathway during biliary development. In addition, since Hes1-null mice die perinatally from defects unrelated to the liver defects [18], morphogenesis and maturation of the intrahepatic biliary system cannot be followed during the early postnatal period when major biliary tract remodeling and maturation events take place [5].

In this paper, we characterize embryonic biliary tract formation in the previously described Jag1/Notch2 double heterozygote mouse model of Alagille syndrome. We also describe another mouse model of bile duct paucity resulting from liver-specific deletion of the Notch2 gene. Our data demonstrate a requirement for Jag1/Notch2-mediated signaling in bile duct formation in mice, and support a model in which bile duct paucity in Notch pathway mutations in mice results from defects in bile duct morphogenesis rather than cell fate specification.

Results

Analysis of bile duct morphogenesis during embryogenesis in Jag1dDSL/+ Notch2del1/+ double heterozygous mice

Our previous study [15] analyzed late embryonic and postnatal livers, and did not establish whether bile duct paucity in mice doubly heterozygous for a Jag1 null allele [Jag1−/−] [16] and a Notch2 hypomorphic allele (Notch2+/-) [19] was due to defects in differentiation of bile duct precursors from the bipotential hepatoblast, or whether it was due to defects in morphogenesis of the ductal plate. Therefore, we analyzed livers of Jag1dDSL/+ Notch2del1/+ double heterozygous mice by cytokeratin immunostaining from embryonic day (E) 16.5 through postnatal day (P) 0. At E16.5 in control littermate embryos, cytokeratin immunostaining revealed the presence of a partly bilayered ductal plate at the interface of the portal mesenchyme and the liver parenchyma (Fig. 1A). Over the next several days, the ductal plate remodels by a process in which focal dilations appear between the two cell layers of the plate (Fig. 1C,E). By P7, some of these focal dilations give rise to patent epithelial bile ducts incorporated into the portal mesenchyme (Fig. 1G), while the remainder of the ductal plate involutes. Cytokeratin immunostaining of liver sections from Jag1dDSL/+ Notch2del1/+ double heterozygous mice revealed that they were very similar to control littermate sections through at least P0. In the Jag1dDSL/+ Notch2del1/+ mice, a ductal plate formed (Fig. 1B) and focal dilations appeared (Fig. 1D,F). However, postnatal remodeling to form a patent epithelial bile duct did not occur. Instead, as we reported in our initial study [15], by P7 only ductal plate remnants remained in most portal tracts (Fig. 1H). These results indicate that in the Jag1/Notch2 double heterozygote mouse, bile duct paucity results from defects in bile duct morphogenesis, not from defects in differentiation of bile duct precursors from the bipotential hepatoblast.

Liver-specific Notch2 deletion results in defects in bile duct morphogenesis, but not ductal plate formation

Our previous study was the first to implicate a critical role for the Notch2 gene in bile duct formation [15]. The Notch2 protein is expressed in periporal hepatoblasts near or adjacent to Jag1-expressing cells surrounding the portal veins in mice [15,17,20]. Further support for a critical role for the Notch2 gene in bile duct formation and/or maintenance comes from recent studies on Alagille syndrome patients. While improved mutation detection protocols can now identify JAG1 mutations in approximately 94% of patients diagnosed with Alagille syndrome [21], there are still some Alagille syndrome patients in whom no JAG1 mutations can be identified. Recently, heterozygous NOTCH2 mutations were identified in a subset of Alagille syndrome patients who lack JAG1 mutations [22].

To specifically assess the role of the Notch2 gene in bile duct formation in mice, we disrupted Notch2 function in the liver utilizing mice expressing Cre recombinase under the control of the Albumin 1 promoter (Alb1-Cre) [23,24]. We crossed Notch2flox/Notch2flox mice with mice doubly heterozygous for the Alb1-Cre transgene and either the Notch2del1 or Notch2del2 alleles. Both of these Notch2 mutant alleles behave genetically as null alleles [25]. Offspring with the genotypes Alb1-Cre/+; Notch2del1/Notch2del1 or Alb1-Cre/+; Notch2del2/Notch2del2 were analyzed. Since no differenc-
es were detected in the phenotypes of the Alb1-Cre+/+; Notch2\textsuperscript{del2}/Notch2\textsuperscript{del2} and Alb1-Cre/++; Notch2\textsuperscript{del2}/Notch2\textsuperscript{del2} mice, of both genotypes were designated Notch2-cko (for Notch2 conditional knockout) in this report. Excision of the Notch2\textsuperscript{del2} allele was observed in liver DNA of Notch2-cko mice, but not in kidney DNA of Notch2-cko mice (lanes 5,6). Genotype of Notch2-cko mice is Notch2\textsuperscript{flox}/Notch2\textsuperscript{flox}; Alb1-Cre/+.

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At 4–5 weeks of age, clinical chemistry analysis of serum revealed that, as a group, Notch2-cko mice had elevated levels of alkaline phosphatase, alanine aminotransferase, and total bilirubin (Table 1). Elevated levels of these parameters are indicative of liver and biliary dysfunction. However, some Notch2-cko mice had alkaline phosphatase, alanine aminotransferase, and total bilirubin levels within the normal range. We also tested blood urea nitrogen levels, which when elevated is indicative of kidney dysfunction. As expected, blood urea nitrogen levels in Notch2-cko mice were not elevated (Table 1), in contrast to Jag1\textsuperscript{del}/+ Notch2\textsuperscript{del}/+ mice [15].

Notch signaling regulates bile duct morphogenesis independently of HNF6 and HNF1\(\beta\) expression

Previous studies have shown that biliary tract morphogenesis is dependent on the transcription factors Hepatocyte Nuclear Factor-6 (Hnf6; Onecut1 – Mouse Genome Informatics) and HNF1\(\beta\) (Tcf2 – Mouse Genome Informatics). Mice homozygous for a targeted null mutation of the Hnf6 gene [27], or with liver-specific deletion of the Hnf1\(\beta\) gene [23], fail to properly remodel the ductal plate to form patent bile ducts and exhibit persistence of ductal plate remnants. HNF1\(\beta\) expression was strongly downregulated in livers of Hnf6-null mice, indicating that the Hnf6 gene functioned upstream of the Hnf1\(\beta\) gene [27].

We tested by immunohistochemistry whether the HNF6 and HNF1\(\beta\) proteins were expressed in the perportal region of Jag1\textsuperscript{del}/+ Notch2\textsuperscript{del}/+ and Notch2-cko mice. HNF1\(\beta\) protein expression was observed in the perportal region of Notch2-cko mice at P0 and P7 (Fig. 5B,D). Similarly, HNF6 protein expression was observed in the perportal region of Jag1\textsuperscript{del}/+ Notch2\textsuperscript{del}/+ mice at P0 (Fig. 5F). These data suggest that the etiology of the biliary duct morphogenesis defects in the Notch pathway mutants is independent of the function of the HNF6 and HNF1\(\beta\) proteins. Independent functioning of the Notch pathway and the HNF6/HNF1\(\beta\) pathway is supported by the finding that Jag1 and Hes1 expression is unaffected in fetal livers of Hnf6-null mice [27].

Discussion

The Notch signaling pathway is frequently utilized to specify cell fate during bipotential cell fate decisions [8,9], so an attractive
model to explain the defects in bile duct formation in Jag1\textsuperscript{DSL}+/+ Notch2\textsuperscript{del1}/+ mice was reduced differentiation of cholangiocytes from the bipotential hepatoblast. The first indication that this model was likely incorrect came from analysis of mice homozygous for a null mutation of the \textit{Hes1} gene, which encodes a basic helix-loop-helix protein that is a downstream effector of the Notch pathway. The \textit{Hes1}-null mice formed a relatively-normal ductal plate consisting of cytokeratin- and DBA-positive cholangiocyte precursors, suggesting that the primary defect in these mice was not in the initial bipotential cell fate decision of the hepatoblast [17]. However, by P0 in wildtype littermates, patent bile ducts were beginning to form, while none were evident in the \textit{Hes1}-null mice. Unfortunately, \textit{Hes1}-null mice die at birth from severe central nervous system defects [18], precluding the analysis of later stages of ductal plate remodeling and bile duct morphogenesis in these mice.

Our analysis of Jag1\textsuperscript{DSL}/+ Notch2\textsuperscript{del1}/+ mice supports the model that Notch signaling regulates ductal plate remodeling and bile morphogenesis independently of HNF6 and HNF1\beta. Figure 5. Notch signaling regulates bile duct morphogenesis. A–D. HNF1\beta expression in Notch2-cko mice at P7 and P0. E,F. HNF6 expression in Jag1\textsuperscript{DSL}/+ Notch2\textsuperscript{del1}/+ mice at P0. The HNF1\beta and HNF6 proteins were expressed similarly in the perportal region (arrowheads) of both control littermate and mutant livers. doi:10.1371/journal.pone.0001851.g005

### Table 1. Blood Chemistry Analysis of Notch2-cko Mice.

| Genotype            | n  | Alkaline Phosphatase | Alanine Aminotransferase | Blood Urea Nitrogen | Total Bilirubin |
|---------------------|----|----------------------|--------------------------|---------------------|-----------------|
| Notch2-cko          | 23 | 366 ± 34             | 135 ± 27                 | 24 ± 0.7            | 0.71 ± 0.14     |
| Controls            | 29 | 246 ± 9              | 52 ± 9                   | 22 ± 0.8            | 0.49 ± 0.02     |

Serum from 4–5 week old Notch2-cko mice and their littermates were analyzed for the indicated parameters. Values shown are the mean (in International Units/Liter) ± standard error of the mean. All genotypes other than Notch2-cko were combined for controls.

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duct morphogenesis rather than cholangiocyte differentiation, and suggests that the Jag1/Notch2-mediated signal responsible for bile duct morphogenesis acts, at least in part, by modulating Hes1 expression. Our analysis of bile duct formation in Notch2-cko mice is consistent with this model. While the Alb1-Cre transgene does not delete early enough during embryogenesis to study the role of Notch2 gene function during ductal plate formation [29], the essentially identical biliary tract defects exhibited by the Jag1<sup>dlst</sup>/Notch2<sup>del1/+</sup> and Notch2-cko mice at late embryonic and postnatal stages strongly suggest that these defects arise by the same mechanism in both mouse models. However, it remains possible that Notch signaling may play some role in cholangiocyte differentiation, since none of the three mouse models analyzed (Hes1-null mice, Jag1<sup>dlst</sup>/+, Notch2<sup>del1/+</sup> mice, and Notch2-cko mice) are likely to be entirely deficient in Notch signaling when the cholangiocyte-hepatocyte cell fate decision is made.

In contrast to Notch2 deletion, deletion of the Jag1 gene in liver hepatoblasts did not lead to defects in bile duct development [30], suggesting that Jag1 expression in endothelial cells and/or vascular smooth muscle cells was sufficient for signaling to Notch2-expressing hepatoblasts during ductal plate remodeling and bile duct morphogenesis. Interestingly, this study also demonstrated that in mice that were compound heterozygotes for a Jag1 null allele and the Jag1 conditional allele deleted in hepatoblasts, a subset of animals exhibited bile duct proliferation [30]. Other recent studies support a model in which cholangiocyte differentiation is controlled by a gradient of Activin/TGFβ factors, such as HNF6 and Onecut2 (OC2) [31,32]. Our results suggest that Notch signaling regulates bile duct morphogenesis independently of the Activin/TGFβ/Onecut pathway.

In summary, we demonstrate here that similar defects in bile duct formation were observed in both Jag1<sup>dlst</sup>/+, Notch2<sup>del1/+</sup> and Notch2-cko mice. However, Jag1<sup>dlst</sup>/Notch2<sup>del1/+</sup> mice exhibit defects in many organ systems other than the biliary tract, such as the heart and the kidney [15]. We suggest that liver-specific deletion of the Notch2 gene in Notch2-cko mice represents an improved and more specific model than Jag1<sup>dlst</sup>/Notch2<sup>del1/+</sup> mice for studying the role of Notch signaling during bile duct morphogenesis and remodeling.

**Materials and Methods**

**Mice**

Jag1<sup>dlst</sup>, Notch2<sup>del1/+</sup>, Notch2<sup>del2</sup>, Notch2<sup>del3</sup>, and Notch2<sup>cko</sup> mice were described previously [15,16,19,25]. Albumin-Cre (Alb1-Cre) mice [23,24] were obtained from the Jackson Laboratory. To produce Alb1-Cre/+; Notch2<sup>del1</sup> mice (referred to as Notch2-cko, for Notch conditional knockout), Notch2<sup>del1</sup> mice were mated to mice heterozygous for both the Alb1-Cre transgene and either the Notch2<sup>del2</sup> or Notch2<sup>del3</sup> alleles. Animal maintenance and experimental procedures were in accordance with the NIH Guidelines for Animal Care and Use and the principles of the Helsinki Declaration, and were approved by the Institutional Animal Care and Use Committee of the Jackson Laboratory.

**Immunohistochemistry and lectin binding**

The antibiotics and lectins used in these studies were rabbit polyclonal anti-human cytokeratin (Dako, Cat. A0575); rabbit polyclonal anti-HNF6 (Santa Cruz, Cat. sc-22840); rabbit polyclonal anti-HNF6 (Santa Cruz, Cat. sc-13050); and biotinylated *Dolichos biflorus* agglutinin (DBA) lectin (Vector Laboratories, Cat. B-1035). Mutant sections were either stained with antibodies against HNF6 (rabbit polyclonal, Santa Cruz, Cat. sc-13050) and Onecut2 (OC2) [31,32].

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**Author Contributions**

Conceived and designed the experiments: TG, JL, BM. Performed the experiments: JL. Analyzed the data: TG, JL. Wrote the paper: TG.
24. Postic C, Magnuson MA (2000) DNA excision in liver by an albumin-Cre transgene occurs progressively with age. Genesis 26: 149–150.
25. McCright B, Lozier J, Gridley T (2006) Generation of new Notch2 mutant alleles. Genesis 44: 29–33.
26. Shiojiri N, Nagai Y (1992) Preferential differentiation of the bile ducts along the portal vein in the development of mouse liver. Anat Embryol (Berl) 185: 17–24.
27. Clotman F, Lannoy VJ, Reber M, Cereghini S, Cassiman D, et al. (2002) The onecut transcription factor HNF6 is required for normal development of the biliary tract. Development 129: 1819–1826.
28. Coffinier C, Gresh L, Fiette L, Tronche F, Schutz G, et al. (2002) Bile system morphogenesis defects and liver dysfunction upon targeted deletion of HNF1beta. Development 129: 1829–1838.
29. Sund NJ, Ang SL, Sackett SD, Shen W, Daigle N, et al. (2000) Hepatocyte nuclear factor 3beta (Foxa2) is dispensable for maintaining the differentiated state of the adult hepatocyte. Mol Cell Biol 20: 5175–5183.
30. Loomes KM, Russo P, Ryan M, Nelson A, Underkoffler L, et al. (2007) Bile duct proliferation in liver-specific Jag1 conditional knockout mice: effects of gene dosage. Hepatology 45: 323–330.
31. Clotman F, Jacquemin P, Plumb-Rudewicz N, Pierreux CE, Van der Smissen P, et al. (2005) Control of liver cell fate decision by a gradient of TGF beta signaling modulated by Onecut transcription factors. Genes Dev 19: 1049–1054.
32. Clotman F, Lemaigre FP (2006) Control of hepatic differentiation by activin/TGFbeta signaling. Cell Cycle 5: 168–171.