

Original Paper

# Inhibition of the Notch Signaling Pathway Reduces the Differentiation of Hepatic Progenitor Cells into Cholangiocytes in Biliary Atresia

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## Key Words

Biliary atresia • Notch pathway • Cholangiocyte differentiation • Hepatic progenitor cells

## Abstract

**Background/Aims:** Viral infections, especially with rotavirus, are often considered an initiator of the pathogenesis of biliary atresia (BA). However, the mechanism by which rotavirus induces BA is still unclear. **Methods:** A BA mouse model was induced in newborn mice by i.p. inoculation with rhesus rotavirus within 6 h of birth. The expression of Notch pathway-associated molecules (JAG1, JAG2, Notch1, Notch2, Notch3, Notch4, DII1, DII3, and DII4) was measured by quantitative PCR and western blot analysis. Bile duct obstruction was detected by hematoxylin and eosin staining and CK-19 immunohistochemical staining. DAPT was used to inhibit the Notch pathway *in vivo* and *in vitro*. **Results:** In the livers of patients with BA and rotavirus-induced BA mice, the expression of JAG1 and Notch2 was significantly increased. Inhibition of the Notch pathway by DAPT *in vivo* ameliorated bile duct obstruction and delayed BA-induced mortality. The serum levels of inflammation cytokines (TNF- $\alpha$ , IL-2, IL-8, and IL-18) were reduced by inhibiting the Notch pathway. The expression of CK19, Sox9, and EpCAM was significantly increased in BA liver, while DAPT treatment decreased the expression of CK19, Sox9, and EpCAM. **Conclusion:** Notch activation is involved in the pathogenesis of BA by promoting the differentiation of hepatic progenitor cells into cholangiocytes.

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## Introduction

Biliary atresia (BA) is a life-threatening liver and bile duct disease that occurs in infants and is characterized by progressive fibroinflammatory obliteration of the extrahepatic biliary tree and rapid progression of intrahepatic biliary fibrosis [1]. BA is the leading cause of end-stage liver disease and the main indication for pediatric liver transplantation; in the absence

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of treatment, it is inevitably fatal within the first 2 years of life [2]. Although there have been reports that viral infections, immune-mediated injury, and other factors are related to BA, the pathogenesis of BA is still not understood completely.

Notch signaling is a developmental pathway that regulates several fundamental cellular processes including cell fate and differentiation [3]. Four transmembrane Notch receptors (Notch1, 2, 3, and 4) and two types of ligands, Serrate/Jagged (Jag1 and 2) or Delta-like (Dll1, 3, and 4) constitute the Notch system, although there are several other components that can transduce and adjust the signal [4]. Notch signaling is essential for biliary development and repair by inducing the differentiation of hepatic progenitor cells (HPCs) into biliary epithelium [5-7]. Mutations in the genes encoding the Notch ligand Jag1 and the receptor Notch2 cause Alagille syndrome due to a paucity of bile ducts [6, 8]. Jag1 expression has been observed in the biliary epithelium and adjacent small vessel endothelium; biliary-specific expression of Dll4 has been found in human liver. Of the four Notch receptor isoforms, Notch1 and Notch2 are expressed in the biliary epithelium [9]. The persistence of Hairy/enhancer split-1, a downstream target of Notch pathway activation, in the liver of patients with BA at the time of Kasai portoenterostomy suggests that Notch signaling may play a role in the pathogenesis of BA [10].

Viruses, especially rotavirus, are generally considered to be initiators of the pathogenesis of BA [11]. According to reports, in addition to viral load, the incidence of BA caused by rotavirus infection is also related to the time of infection. The highest incidence of BA occurs after infection within the first 12 h postpartum, resulting in a BA mortality rate of 100%. However, the later a newborn mouse is infected, the less likelihood that BA is triggered, and the infection of pregnant mice fails to induce BA [12]. Previous studies on the mechanism of BA induced by rotavirus infection have focused on immune system activation. In murine BA, rotavirus infection is followed by lymphocyte activation and the secretion of inflammatory cytokines targeting extrahepatic bile ducts [13, 14]. However, it is still unknown if the Notch signaling pathway is involved in the pathogenesis of rotavirus-induced BA.

DAPT is a small molecule inhibitor of  $\gamma$ -secretase that prevents proteolytic cleavage of the Notch intracellular domain, which is necessary for the Notch signaling response because it translocates to the nucleus to induce transcription [15, 16]. DAPT can inhibit the mRNA expression of Notch pathway-related genes (Notch1, Notch2, and Dll1) [17]. Although DAPT is not a specific inhibitor of the Notch pathway, and has a broad impact on other transduction pathways such as the Wnt pathway [18] and PI3K/Akt pathway [19], many studies have used DAPT to elucidate the functions of the Notch pathway.

In this study, we report a novel role for the Notch signaling pathway in the pathogenesis of rotavirus-induced BA and the beneficial effect of DAPT on BA-induced liver injury. For the mechanism, activation of the Notch pathway was found to promote HPC-cholangiocyte phenotypic differentiation. Our study provides evidence that the Notch pathway may be a new therapeutic target for patients with BA.

## Materials and Methods

### Human subjects

A total of 20 liver specimens were retrieved from patients with BA undergoing surgery. Ten normal adjacent non-tumor tissues from patients with hepatoblastoma were used as controls. This study was approved by our institution's ethics committee, and written informed consent was obtained from each participant. The study protocol complied with the ethical guidelines of the 1975 Declaration of Helsinki and was reflected by the *a priori* approval of our institution's human research committee. Table 1 lists the clinical characteristics of the patients.

## Mouse BA model and blocking studies

C57BL/6J mice were purchased from Charles River Laboratories and maintained in pathogen-free vivarium rooms under a 12-h dark-light cycle. BA was induced in newborn mice by i.p. inoculation of  $1.5 \times 10^6$  ffu of rhesus rotavirus within 6 h of birth, and an equivalent amount of 0.9% NaCl (saline) was used as a control. For Notch pathway blocking studies, DAPT (GSI-IX, a prototypical gamma-secretase inhibitor; inhibiting gamma-secretase can prevent Notch receptor cleavage and thereby block Notch signal transduction) was injected (i.p., 50 mg/kg) prior to challenge with rhesus rotavirus. Serum and liver samples were collected at 2 weeks post-inoculation after euthanasia.

## Cell culture and treatment

WB-F344 cells were maintained in Ham's F12 medium supplemented with 10% fetal bovine serum at 37°C and 5% CO<sub>2</sub> humidity. For the treatment of cells, the final concentration of sodium butyrate (SB, B5887; Sigma) was 3.75 mM, and DAPT (D5942; Sigma) was used at 50 μM.

**Table 1.** Characteristics of patients with biliary atresia and controls

No.	Gender	Age	TBA (μM)	ALT (U/L)	AST (U/L)	GGT (U/L)	TBIL (μM)	DBIL (μM)
BA1	Male	3 months	75	99	155	402	195	144
BA2	Male	5 months	93	115	157	249	224	151
BA3	Male	9 months	164	135	137	989	68	135
BA4	Female	2 months	78	206	435	1092	197	116
BA5	Female	2 years	65	103	147	238	143	104
BA6	Male	15 days	243	410	406	689	240	190
BA7	Female	8 months	58	104	118	419	28	21
BA8	Female	2 months	79	66	119	832	108	91
BA9	Male	2 months	124	203	297	160	261	182
BA10	Male	3 months	136	157	148	534	150	80
BA11	Female	5 months	87	147	384	447	197	121
BA12	Male	4 months	72	95	128	148	129	103
BA13	Female	50 days	147	160	428	288	199	153
BA14	Female	2 months	93	109	126	1156	136	103
BA15	Male	12 days	79	64	93	219	144	119
BA16	Male	3 months	48	35	91	216	177	130
BA17	Male	10 months	53	77	68	671	109	90
BA18	Female	5 months	183	258	236	649	217	168
BA19	Female	9 months	92	270	262	443	163	75
BA20	Male	73 days	71	130	141	224	268	192
Control 1	Male	2 months	12	26	44	76	4.7	1.7
Control 2	Male	5 months	8.3	76	170	400	21.3	9.8
Control 3	Male	3 years	11	53	82	178	16.3	11.2
Control 4	Female	2 years	9.2	35	51	132	7.8	4.3
Control 5	Male	8 months	3.6	62	83	213	18.4	11.2
Control 6	Female	6 months	7.8	14	18	51	3.9	1.2
Control 7	male	9 months	16	32	70	115	18	5.4
Control 8	Female	12 months	10	28	47	110	4.6	1.9
Control 9	Female	2 years	5.2	26	26	21	5.8	2.3
Control 10	Male	3 years	2.4	14	22	15	3.2	1.7

## Quantitative PCR (qPCR) and western blotting

qPCR and western blot analyses were performed as described previously [20]. All PCR primers used are shown in Table 2. The antibodies used in this study for western blot analysis were as follows: anti-Notch2 (1:1000, #5732; Cell Signaling Technology, Beverly, USA), anti-JAG1 (1:1000, #70109; Cell Signaling Technology, Beverly, USA), anti-CK19 (1:10000, ab52625; Abcam, Cambridge, USA), anti-SOX9 (1:500, sc-166505; Santa Cruz Biotechnology, CA, USA), anti-EpCAM (1:1000, ab32392; Abcam, Cambridge, USA), and anti-β-actin (1:1000, #4970; Cell Signaling Technology, Beverly, USA).

## Statistical analysis

All data are reported as the mean ± standard error of the mean (SEM). For comparisons of different groups, statistical significance was determined based on Student's t test or analysis of variance using SPSS19 software. The relationship between two factors was tested with two-tailed Pearson's correlations. P-values < 0.05 were considered statistically significant.

## Results

### *Notch signaling pathway is activated in patients with BA and experimental BA mice*

We first examined the levels of molecules associated with the Notch signaling pathway in liver tissue from patients with BA and control subjects. The results demonstrated that the mRNA levels of JAG1, JAG2, and Notch2 were increased, while other Notch receptors (Notch1, 3, and 4) and ligands (DII1, 3, and 4) were increased only slightly or did not change significantly in the liver of patients with BA compared with controls (Fig. 1A). Consistently, western blot analysis confirmed that the protein levels of JAG1 and Notch2 were also increased (Fig. 1B).

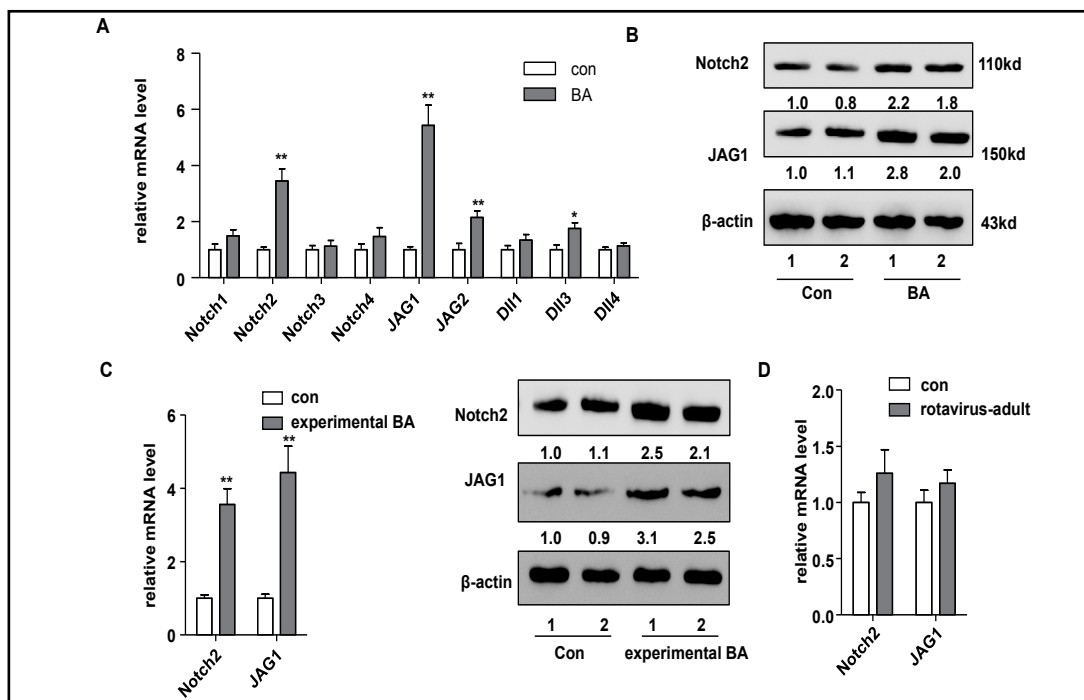
We also generated a BA mouse model by rotavirus injection within 6 h of birth. Compared with control mice, the mRNA and protein levels of JAG1 and Notch2 were increased in experimental BA mice (Fig. 1C). As a negative control, we also injected adult mice with rotavirus, which could not induce BA, and the expression of JAG1 and Notch2 was not altered significantly between the experimental and control groups (Fig. 1D).

### *Inhibition of Notch signaling reduces mortality in experimental BA mice*

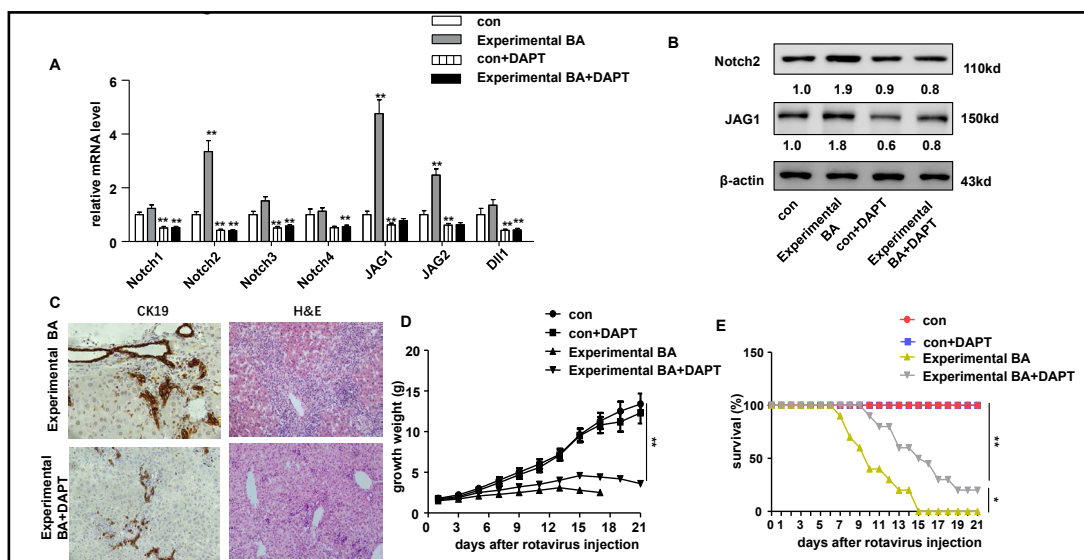
To elucidate further the role of the Notch pathway in the pathogenesis of BA, we used DAPT injection to inhibit the Notch pathway before rotavirus injection. The data indicated that the mRNA and protein levels of Notch1, Notch2, Notch3, Notch4, JAG1, JAG2, and DII1 were appreciably lower in the experimental BA+DAPT group compared to the experimental BA+saline group (Fig. 2A&B). The administration of DAPT to experimental BA mice reduced bile duct obstruction (Fig. 2C). Although poor weight gain was observed at 2–3 weeks after infection (Fig. 2D), mortality was delayed in the experimental BA+DAPT group (Fig. 2E).

**Table 2.** PCR primers used in this study

Name	Forward	Reverse
Human Notch1	GAGGCGTGGCAGACTATGC	CTTGACTCCGTCAGCGTGA
Human Notch2	CAACCGCAATGGAGGCTATG	GCGAAGGCACAATCATCAATGTT
Human Notch3	TGGCGACCTCACTTACGACT	CACTGGCAGTTATAGGTGTTGAC
Human Notch4	GATGGGCTGGACACCTACAC	CACACGCAGTGAAAGCTACCA
Human JAG1	GTCCATGCAGAACGTGAACG	GCGGGACTGATACTCCTTGA
Human JAG2	TGGGCGGCAACTCCTTCTA	GCCTCCACGATGAGGGTAAA
Human DII1	GATTCTCTGATGACCTCGCA	TCCGTAGTAGTGTTCGTCACA
Human DII3	CACTCCGGATGCACTCAAC	GATTCCAATCTACGGACGAGC
Human DII4	GTCTCCACGCCGTATTGG	CAGGTGAAATTGAAGGGCAGT
Human $\beta$ -actin	CACCAACTGGGACGACAT	ACAGCCTGGATAGCAACG
Mouse Notch1	GATGGCCTCAATGGGTACAAG	TCGTTGTTGTTGATGTCACAGT
Mouse Notch2	GAGAAAAACCGCTGTCAGAATGG	GGTGGAGTATTGGCAGTCCTC
Mouse Notch3	AGTGCCGATCTGGTACAACCT	CACTACGGGGTTCTCACACA
Mouse Notch4	GAACGCGACATCAACGAGTG	GGAACCCAAGGTGTTATGGCA
Mouse JAG1	ATGCAGAACGTGAATGGAGAG	GCGGGACTGATACTCCTTGAG
Mouse JAG2	GCGACCACTACGGCAACAA	CCGTGGAGCAAATTACATCCTT
Mouse DII1	GCAGGACCTTCTTTCGCGTAT	AAGGGGAATCGGATGGGGTT
Mouse Ck19	CTCCCGAGATTACAACCACTAC	GTTCTGTCTCAAACCTTGTTCTG
Mouse SOX9	AGTACCCGATCTGCACAAC	ACGAAGGGTCTCTTCTCGCT
Mouse EpCAM	TGGTGTGATTAGCAGTCATCG	TCAGTTCAGCACTCAGCAC
Mouse $\beta$ -actin	CTGTCCCTGTATGCCTCTG	ATGTCACGCACGATTTC

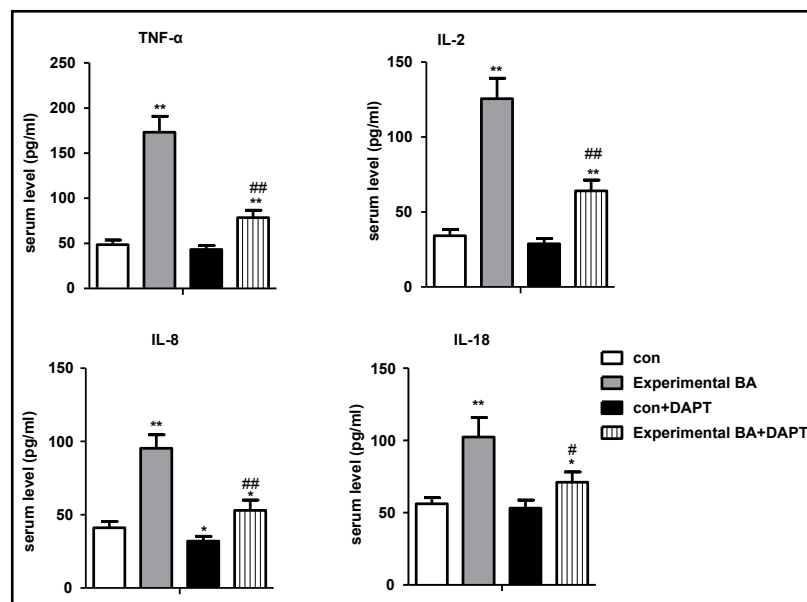


**Fig. 1.** The Notch signaling pathway is activated in patients with BA and experimental BA mice. (A) qPCR detection of Notch receptors (Notch1, Notch2, Notch3, and Notch4) and Notch ligands (JAG1, JAG2, Dll1, Dll3, and Dll4) in the liver of patients with BA (n = 20) and controls (n = 10). (B) Western blot analysis of Notch2 and JAG1 in the liver of patients with BA. (C) qPCR and western blot detection of Notch2 and JAG1 in the liver of rotavirus-induced experimental BA mice (2 weeks old, n = 6–8/group for triplicate assays). (D) qPCR detection of Notch2 and JAG1 in the liver of adult mice injected with rotavirus, which are the negative control for the experimental BA mice (8 weeks old, n = 5/group for triplicate assays). Data are shown as the mean ± SEM. \*P<0.05, \*\*P<0.01 vs. control.



**Fig. 2.** Inhibition of Notch signaling reduces mortality in experimental BA mice. (A) qPCR detection of Notch receptors (Notch1, Notch2, Notch3, and Notch4) and Notch ligands (JAG1, JAG2, and Dll1) in the liver of rotavirus-induced experimental BA mice pretreated with DAPT, a Notch pathway inhibitor (2 weeks old, n = 6–8/group for triplicate assays). (B) Protein levels of Notch2 and JAG1, (C) hematoxylin and eosin staining, (D) growth weight, and (E) survival curve of the mice in A. Data are shown as the mean ± SEM. \*P<0.05, \*\*P<0.01 vs. control.

**Fig. 3.** Inhibition of Notch signaling reduces the expression of inflammatory cytokines in experimental BA mice. The serum levels of inflammatory cytokines (TNF- $\alpha$ , IL-2, IL-8, and IL-18) in rotavirus-induced experimental BA mice that were pretreated with DAPT (2 weeks old, n = 6–8/group for triplicate assays). Data are shown as the mean  $\pm$  SEM. \*P<0.05, \*\*P<0.01 vs. control; #P<0.05, ##P<0.01 vs. experimental BA.



#### *Inhibition of Notch signaling reduces inflammatory cytokines in experimental BA mice*

The immune system plays a major role in the progressive fibrosis and obliteration of bile ducts in BA [21]. Release of proinflammatory cytokines from activated immune cells permanently damages bile ducts and causes biliary destruction, followed by fibrosis, producing the atresia phenotype [22]. As shown in Fig. 3, the expression of several inflammation cytokines (TNF- $\alpha$ , IL-2, IL-8, and IL-18) was significantly higher in the serum of experimental BA mice than in the control group. With DAPT treatment, the increased expression of inflammatory cytokines was partially blocked.

#### *Inhibition of Notch signaling reduces cholangiocyte differentiation in vitro and in vivo*

HPCs are the major source of proliferating cholangiocytes [23]. The WB-F344 cell line has morphological and functional characteristics similar to those of freshly isolated HPCs [24]. SB is a commonly used reagent for inducing cholangiocyte differentiation [25]. CK19 is a marker of bile epithelial cells [26], Sox9 is an endodermal transcription factor [27], and EpCAM is a stem/progenitor cell surface marker [28]. When WB-F344 cells were treated with SB, the mRNA and protein levels of CK19, Sox9, and EpCAM were dramatically increased in the SB group compared with the control group, which was reduced by DAPT treatment (Fig. 4A&B). In addition, the mRNA expression of Notch signaling components including Notch1, Notch2, JAG1, and JAG2 was notably increased in the SB group compared with the control group, and their expression was significantly decreased after DAPT treatment (Fig. 4C).

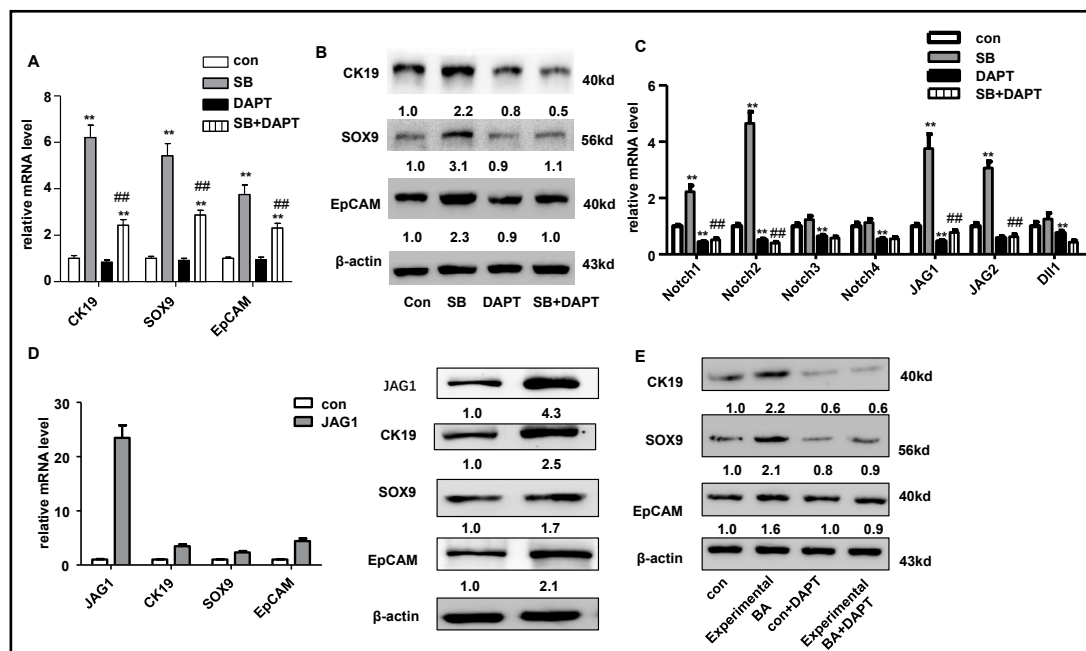
We also transfected WB-F344 cells with a JAG1-overexpression plasmid to activate the Notch pathway. As shown in Fig. 4D, the overexpression of JAG1 increased the mRNA and protein levels of CK19, Sox9, and EpCAM.

*In vivo*, we also found that the expression of CK19, SOX9, and EpCAM was induced in BA+saline mice, which was reduced in BA+DAPT mice (Fig. 4E). Therefore, inhibition of Notch signaling reduces the differentiation of cholangiocytes from HPCs *in vitro* and *in vivo*.

## Discussion

In humans, abnormal bile duct proliferation and growth are hallmarks of cholestatic disorders and are associated with many biliary disorders, including BA [29, 30]. In this study, we discovered a novel role for the Notch signaling pathway in the pathogenesis of BA by promoting the differentiation of HPCs into cholangiocytes.





**Fig. 4.** Inhibition of Notch signaling reduces cholangiocyte differentiation in vitro and in vivo. (A) qPCR detection and (B) western blot analysis of CK19, SOX9, and EpCAM in WB-F344 cells treated with SB and DAPT. (C) qPCR detection of Notch receptors (Notch1, Notch2, Notch3, and Notch4) and Notch ligands (JAG1, JAG2, and Dll1) in WB-F344 cells treated with SB and DAPT. (D) mRNA and protein levels of JAG1, CK19, SOX9, and EpCAM in WB-F344 cells transfected with a JAG1-overexpression plasmid. (E) Western blot analysis of CK19, SOX9, and EpCAM in the liver of rotavirus-induced experimental BA mice pretreated with DAPT. Data are shown as the mean  $\pm$  SEM (triplicate assays). \*\* $P < 0.01$  vs. control; # $P < 0.05$ , ## $P < 0.01$  vs. SB.

To demonstrate the potential role of the Notch signaling pathway in the pathogenesis of BA, we first selected patients with BA and normal control subjects and found that the mRNA and protein levels of JAG1 and Notch2 was increased in the liver of patients with BA compared with controls, which indicated that the Notch signaling pathway is activated in patients with BA. It should be noted that we did not consider the heterogeneity of the patients in this study, which may lead to misinterpretation. A more uniform group of patients may be needed in a future study, e.g., patients before Kasai portoenterostomy.

Notch signaling is an evolutionarily conserved intercellular signaling pathway required during development for cell specification, lineage commitment, and the maintenance of stem/progenitor cells in adults [31]. Besides, the Notch signaling pathway is essential for the specification of the biliary tree [32], whereas ablation of the Notch pathway results in the failure of hepatoblast differentiation into cholangiocytes, resulting in bile duct paucity [33]. On the basis of our results, the expression of Notch2 and the Notch ligand JAG1 was significantly increased in patients with BA and experimental BA mice. To confirm whether Notch signaling plays a key role in BA, we used DAPT to inhibit the activation of Notch signaling, resulting in the significant reduction of bile duct obstruction. SB treatment of WB-F344 cell lines promoted their differentiation into cholangiocytes, and DAPT administration significantly inhibited this process. Therefore, Notch signaling is required for the pathogenesis of BA, which may occur through the differentiation of enhanced HPCs into cholangiocytes.

Stem cells self-renew and differentiate into a range of cell populations. It has been confirmed that a bipotential cell type with stem cell properties exists in the liver of adults and can differentiate into hepatocytes and cholangiocytes. The Notch pathway is activated in the HPC subset that is identified as ductal cells by coordinating biliary differentiation and morphogenesis during development. Disruption of this pathway, as characterized by Alagille syndrome, results in disorganized or absent biliary morphogenesis, [34, 35]. In our study, we

found that inhibition of the Notch pathway led to a decrease in the differentiation of HPCs into bile duct epithelium in experimental BA mice, which is consistent with the results of previous studies in cholestatic mice induced by bile duct ligation [20].

In this study, we found that DAPT suppressed the mRNA expression of Notch pathway-related genes, namely, Notch1, Notch2, and Dll1, which is consistent with a previous report [17]. In addition, we found that DAPT pretreatment can block the activation of the Notch pathway and ameliorate liver injury in a BA animal model. As mentioned above, DAPT is not a specific inhibitor of the Notch pathway, and a more specific inhibitor or gene-editing method, such as Jag1 knockdown or liver-specific knockout, needs to be used to confirm the role of Notch in the pathogenesis of BA.

In summary, we have discovered a novel role for the Notch pathway in the pathogenesis of BA by targeting cholangiocyte differentiation as well as inflammation. This may be a therapeutic pathway for human BA.

## Disclosure Statement

The authors declare to have no conflict of interests.

## Acknowledgements

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