Constitutive Notch2 Signaling Induces Hepatic Tumors in Mice

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Hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCC) are the most common liver tumors and a leading cause for cancer-related death in men. Notch2 regulates cellular differentiation in the developing and adult liver. Although aberrant Notch signaling is implicated in various cancers, it is still unclear whether Notch2 regulates proliferation and differentiation in liver carcinogenesis and thereby contributes to HCC and CCC formation. Here, we investigated the oncogenic potential of constitutive Notch2 signaling in the liver. We show that liver-specific expression of the intracellular domain of Notch2 (N2ICD) in mice is sufficient to induce HCC formation and biliary hyperplasia. Specifically, constitutive N2ICD signaling in the liver leads to up-regulation of pro-proliferative genes and proliferation of hepatocytes and biliary epithelial cells (BECs). Using the diethylnitrosamine (DEN) HCC carcinogenesis model, we further show that constitutive Notch2 signaling accelerates DEN-induced HCC formation. DEN-induced HCCs with constitutive Notch2 signaling (DEN\textsuperscript{N2ICD} HCCs) exhibit a marked increase in size, proliferation, and expression of pro-proliferative genes when compared with HCCs from DEN-induced control mice (DEN\textsuperscript{ctrl} HCCs). Moreover, DEN\textsuperscript{N2ICD} HCCs exhibit increased Sox9 messenger RNA (mRNA) levels and reduced Albumin and Alpha-fetoprotein mRNA levels, indicating that they are less differentiated than DEN\textsuperscript{ctrl} HCCs. Additionally, DEN\textsuperscript{N2ICD} mice develop large hepatic cysts, dysplasia of the biliary epithelium, and eventually CCC. CCC formation in patients and DEN\textsuperscript{N2ICD} mice is accompanied by re-expression of hepatocyte nuclear factor 4α (HNF4α), possibly indicating dedifferentiation of BECs. Conclusion: Our data establish an oncogenic role for constitutive Notch2 signaling in liver cancer development. (HEPATOL 2013;57:1607-1619)

Hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCC) are the most common hepatic neoplasms of epithelial origin and are among the leading causes of cancer-related death.\textsuperscript{1,2} Deregulation of several signaling pathways has been described in both HCC and CCC, including the Wnt/β-catenin, AKT/mTOR, RAF/MEK/ERK, EGFR, and HGF/cMET pathways.\textsuperscript{3-8} Aberrant Notch signaling is implicated in the development of various solid and hematologic neoplasias.\textsuperscript{9,10} However, a role for Notch signaling in HCC and CCC formation is not established. Four transmembrane Notch receptors (Notch1-4) exist in mammals. Binding of ligands (e.g., Jagged1) triggers a proteolytic processing of Notch receptors and translocation of the Notch intracellular domain (NICD) to the nucleus. NICD binding to RBPjκ leads to transcriptional activation of effector genes such as Hairy and Enhancer of Split homologs (e.g., Hes1, Hes5, or Hey1),\textsuperscript{11-13} which are important regulators of differentiation, proliferation, and apoptosis in a variety of epithelial tissues and cancers.\textsuperscript{14}

Notch1 and Notch2 are key regulators of liver development.\textsuperscript{15-20} Jagged1 haploinsufficiency and Notch2 mutations have been shown to be associated with Alagille syndrome, a multisystem disorder that is characterized by severe developmental defects in the
liver, including intrahepatic bile duct paucity. Mouse models with liver-specific Notch2 deletion or Jagged1 mutations largely recapitulate this phenotype, while liver-specific Notch1 deletion showed no obvious phenotype. Transgenic Aalbmin-Cre (AlbCre)/intracellular domain of Notch2 (N2ICD) mice that constitutively express N2ICD in hepatoblasts revealed that Notch2 signaling regulates biliary epithelial cell (BEC) fate specification and tubulogenesis during bile duct development. Moreover, Notch2-mediated BEC differentiation in AlbCre/N2ICD mice results in the formation of ectopic bile ducts and an excess of BECs at the expense of hepatocytes during embryonic and early postnatal development. Interestingly, these mice restored their hepatocyte pool during the first postnatal weeks and survived into adulthood. Reappearance of hepatocytes expressing the transgenic N2ICD suggests that BECs are able to transdifferentiate into hepatocytes in the absence of constitutive N2ICD signaling. In support of this hypothesis, it was reported recently that Sox9+ BECs are able to dedifferentiate and function as facultative stem cells for BECs and hepatocytes. The BEC marker Sox9 is regulated by Notch and high levels of Sox9 messenger RNA (mRNA) in human HCC correlate with a less-differentiated tumor phenotype and poor prognosis. However, whether constitutive Notch2 signaling favors less-differentiated and more aggressive HCCs is not known.

To assess the oncogenic potential of constitutive Notch2 signaling in the liver, we analyzed hepatic tumor formation in aged AlbCre/N2ICD mice. Moreover, we studied AlbCre/N2ICD mice in a well-established diethylnitrosamine (DEN) carcinogenesis model to address whether constitutive Notch2 signaling can contribute to HCC and CCC formation. We show that constitutive Notch2 signaling not only resulted in spontaneous HCC formation but also accelerated HCC formation in a DEN carcinogenesis model by promoting proliferation and less differentiated HCC. In addition, DEN-treated AlbCre/N2ICD mice developed biliary cysts and eventually CCC, a process that we now show involves re-expression of Hepatocyte nuclear factor (HNF) 4x. Finally, we also observed expression of HNF4x in human CCC. Thus, our study reveals that constitutive Notch2 signaling in the liver is oncogenic and leads to HCC and CCC formation.

Materials and Methods

Mouse Breeding, Genotyping, and Experimentation. N2ICD mice express the Myc-tagged N2ICD from a modified Rosa26 locus upon Cre-mediated induction. Although the human Myc tag is functionally incompetent, it allows immunohistochemical detection of transgenic N2ICD protein expression using anti-Myc antibodies. N2ICD mice were crossed with AlbCre mice expressing Cre-recombinase under the liver-specific Albumin promoter. Single-transgenic AlbCre and N2ICD mice as well as wild-type mice were used as controls in our experiments. Genotyping of the mice was performed via TaqMan analysis as described. At postnatal day 16, a subgroup of mice was injected with 20 μg/g DEN intra-peritoneally (control mice, n = 44; AlbCre/N2ICD mice, n = 31). Mice were euthanized via CO2 inhalation 2 months (n = 6 each), 6 months (control mice, n = 9; AlbCre/N2ICD mice, n = 4), and 9-12 months (control mice, n = 36; AlbCre/N2ICD mice, n = 29) after DEN injection. A total of 7 DEN-treated AlbCre/N2ICD mice showed premature mortality, which we did not observe in DEN-treated control mice. Organs were excised and examined, then one part was shock frozen and one part was fixed in 10% formalin. Four-microgram-thick paraffin sections were stained with hematoxylin and eosin (H&E) and analyzed for tumor formation and other pathologies by two independent pathologists. Larger tumors were microdissected and divided into three parts. One part was fixed in 10% formalin, embedded in paraffin, stained with H&E, and classified by the pathologists. Another part was freshly frozen in optimal cutting temperature (OCT) compound and used for immunofluorescence studies. The last part was snap-frozen to isolate mRNA for real-time polymerase chain reaction (PCR) expression analysis. All animals were housed in
a 12-hour day/night cycle with food and drinking water ad libitum. The protocol was approved by the Veterinary Office of Basel.

**Patient Material.** Frozen CCC biopsies embedded in OCT compound (n = 13) and a tissue microarray (TMA; n = 59 CCCs) were fixed overnight with 4% paraformaldehyde in phosphate-buffered saline at 4°C for the preparation of cryosections or in 10% formalin at room temperature for subsequent paraffin-embedding. Human biopsies were freshly frozen in OCT compound, and 10-μm-thick cryosections were cut and fixed for 10 minutes in 4% paraformaldehyde. The CCC TMA was prepared as described, using primary antibodies or doli-

**Immunohistochemistry.** Samples for immunostain-

**RNA Extraction and Reverse-Transcription and Quantitative Real-Time PCR.** Total RNA was extracted from mouse liver tissue using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Promega Biosciences, Wallisellen, Switzerland) in the presence of random primers (Promega) and deoxynucleoside triphosphate. The samples were incubated for 5 minutes at 70°C and then for 1 hour at 37°C. The reaction was stopped by heating at 95°C for 5

**Results**

**Liver-Specific N2ICD Expression Induces HCC Formation in Mice.** In order to evaluate the oncogenic potential of constitutive Notch2 signaling in the liver, N2ICD mice were crossed with AlbCre mice to obtain AlbCre/N2ICD mice that allow liver-specific expression of N2ICD (Fig. 1A). Analysis at 12 months revealed hepatic tumor formation in 88% of all AlbCre/N2ICD mice (n = 16) (white arrows in Fig. 1B), while single transgenic N2ICD and AlbCre control mice (n = 32) did not show any hepatic tumors or other overt phenotypes (data not shown). Moreover, AlbCre/N2ICD mice showed a marked increase in liver weight (Fig. 1C). Histopathological examination of 12-month-old AlbCre/N2ICD mice identified these tumors as HCCs, cytologically characterized by polygonal cells with vesicular nuclei, prominent nucleoli, and higher nucleo-cytoplasmic ratio than the surrounding liver parenchyma (Fig. 1E). AlbCre/N2ICD mice showed small dysplastic foci (data not shown) but also large macroscopically visible HCCs (Fig. 1B,E), while control littermate mice had normal liver morphology (Fig. 1D). Immunostainings for the hepatocyte marker Alb and the biliary markers Sox9, CK19, and HNF1β showed that 95.7% of these HCCs (n = 23) expressed Alb and Sox9 but were devoid of CK19 and HNF1β (Fig. 1E and Supporting Fig. 1B). In addition, 13% of the Alb+/Sox9+ HCCs showed nondysplastic HNF1β+/CK19− BECs hap-hazardly distributed through the HCC (Supporting Fig. 2A). However, these BECs displayed neither cytological atypia nor architectural dysplastic changes. In addition, all AlbCre/N2ICD mice displayed Sox9+/− HNF1β+/CK19+ BEC hyperplasia (Fig. 1F and Supporting Fig. 1C), multiple disorganized ectopic BECs, and ectopic tubular structures in the liver parenchyma.
This is in line with previous findings showing that constitutive Notch2 signaling in the developing liver leads to the generation of ectopic lobular BECs and bile ducts. Moreover, we observed dilated bile ducts that developed into small benign cysts in 23% of all 12-month-old AlbCre/N2ICD mice (Fig. 1B and Supporting Fig. 3A,B). However, we did not observe BEC dysplasia or even CCC within the observation period of 1 year. Coinmunostaining of HCCs in AlbCre/N2ICD mice for the Myc-tagged transgenic N2ICD and the hepatocyte marker HNF4α indicated that the HCCs originated from cells with constitutive Notch2 signaling (Fig. 2A,C). Likewise, BEC hyperplasia costained for Myc-tagged transgenic N2ICD and HNF1β (Fig. 2B). In control mice, Sox9/CK19/HNF1β and HNF4α/Alb staining was restricted to bile duct BECs (Fig. 1D and Supporting Figs. 1A and 4A,B). Myc staining was absent in control livers, showing that the Myc antibody specifically detects transgenic N2ICD (Supporting Fig. 4A,B). Livers of AlbCre/N2ICD mice showed increased mRNA expression of the Notch2 target gene Hes1 when compared with control livers, demonstrating that constitutive
Notch2 expression induced canonical Notch2 signaling (Fig. 2D). Likewise, HCCs from AlbCre/N2ICD mice showed an eight-fold increase in Hes1 mRNA levels compared with control livers (Fig. 2D). Moreover, the Hes1 target gene and BEC marker Sox9 was increased in AlbCre/N2ICD livers and corresponding HCCs when compared with control mice (Fig. 2D), thus further confirming Sox9 expression in N2ICD-livers and tumors. (E) mRNA expression of the Hes1 target gene and BEC marker Sox9 is increased in AlbCre/N2ICD livers and HCCs when compared with control livers. Box plots show median ± quartiles measured via quantitative PCR and normalized to Rpl19 (n = 5 for each group). Scale bars, 50 μm (A-C). **P < 0.01; ***P < 0.001.

Notch2 Induces Cell Cycle Progression and Proliferation in AlbCre/N2ICD Mice. To gain mechanistic insight in the oncogenic potential of constitutive Notch2 signaling in AlbCre/N2ICD mice, we investigated whether Notch2 induces proliferation in hepatocytes and BECs. Immunostaining for the proliferation marker Ki67 was performed to assess proliferation in control livers, AlbCre/N2ICD livers, and HCCs from AlbCre/N2ICD mice (Fig. 3A). AlbCre/N2ICD livers showed a 3.5-fold increase in proliferating hepatocytes (Fig. 3B) and a 2.5-fold increase in proliferating BECs (Fig. 3C) when compared with control livers. A further 30-fold increase in proliferating tumor cells in AlbCre/N2ICD HCCs compared with nonmalignant AlbCre/N2ICD livers was observed (Fig. 3B). Likewise, mRNA expression of proteins regulating cell
cycle progression, such as CyclinD1 and CyclinA2 were highly up-regulated in Notch2-induced HCCs when compared with control livers (Fig. 3D). Intriguingly, CyclinD1 mRNA expression was already 10-fold increased in nonmalignant AlbCre/N2ICD livers compared with control livers (Fig. 3D). Dysregulation of the genes regulating cell cycle progression and the resultant increased proliferation of hepatocytes and BECs possibly explains why AlbCre/N2ICD mice develop HCC and biliary hyperplasia.

Constitutive N2ICD Expression in a DEN Carcinogenesis Model Results in Increased HCC Growth and Formation of Biliary Cysts and CCC. To further characterize the oncogenic role of constitutive Notch2 signaling in HCC and its potential to contribute to CCC formation, we studied AlbCre/N2ICD mice in the well-established DEN carcinogenesis model. Intrapерitoneal DEN injection was shown to efficiently cause HCC formation.28 AlbCre/N2ICD mice and control mice were injected with DEN at P16 and tumor formation was assessed 2, 6, and 9-12 months after the injection (Fig. 4A). Strikingly, diameters of HCCs in DEN-injected AlbCre/N2ICD (DENN2ICD) mice were increased five-fold compared with those in DEN-injected control (DENctrl) mice (Fig. 4B). Accelerated tumor formation in DENN2ICD mice was accompanied by a dramatic liver weight increase when compared with DENctrl mice (Fig. 4C). Fifty percent of the 6-month-old and 93% of the 9-to 12-month-old DENN2ICD mice developed HCC, compared with only 22% and 25% of the DENctrl littermates, respectively (Table 1). Eighty-eight percent of the analyzed DENN2ICD HCCs (n = 50) were Alb+/Sox9+/CK19+/HNF1β+ (Fig. 4E and Supporting Fig. 6A) and the remaining 12% were only Alb+, similar to DENctrl HCCs (Supporting Fig. 7 and data not shown). Moreover, 50% of the Alb+/Sox9+ HCCs contained nondysplastic Sox9+/HNF1β+/CK19+ BECs (data not shown) as also seen in AlbCre/N2ICD HCCs (Supporting Fig. 2A). Thirty-one percent of the 9- to 12-month-old and 25% of the 6-month-old DENN2ICD mice additionally developed Alb+/Sox9+/CK19+/HNF1β+ HCC-CCCs (Table 1, Fig. 4F, and Supporting Fig. 6B), whereas DENctrl mice did not develop HCC-CCCs. Like AlbCre/N2ICD mice without DEN treatment (Fig. 1C and Supporting Fig. 2C), all DENN2ICD mice
exhibited BEC hyperplasia (Table 1 and data not shown). In addition, all DEN\textsuperscript{N2ICD} mice had already developed multiple large Sox9\textsuperscript{+}/CK19\textsuperscript{+}/HNF1\textbeta\textsuperscript{+} biliary cysts 6 months after DEN injection and 33\% had developed them after 2 months, whereas DEN\textsuperscript{ctrl} mice did not develop any cysts (Table 1, Fig. 4D,G, and Supporting Fig. 6C). BEC dysplasia was observed in 75\% of the 6-month-old and 90\% of the 9- to 12-month-old DEN\textsuperscript{N2ICD} mice (Table 1). Finally, 50\% of the 6-month-old and 52\% of the 9- to 12-month-old DEN\textsuperscript{N2ICD} mice developed Alb\textsuperscript{−}/Sox9\textsuperscript{−}/HNF1\textbeta\textsuperscript{−}/CK19\textsuperscript{−} CCCs, which was not observed in DEN\textsuperscript{ctrl} mice (Table
1, Fig. 4F, and Supporting Fig. 6D). Biliary cysts and CCCs from DEN$^{N2ICD}$ mice (HNF1β+) expressed Myc-tagged N2ICD (Supporting Fig. 8), thus originating from BECs with constitutive Notch2 signaling. Together, our data indicate that constitutive Notch2 signaling accelerates HCC formation in the DEN tumor model, clearly establishing its oncogenic role in HCC formation. Moreover, DEN$^{N2ICD}$ mice also developed CCC in the DEN tumor model, further implicating Notch2 into CCC formation.

**Notch2 Promotes Proliferation and Less-Differentiated HCCs in the DEN Carcinogenesis Model.** Because constitutive Notch2 signaling was shown to increase proliferation in AlbCre/N2ICD mice (Fig. 3), we next analyzed whether DEN$^{N2ICD}$ HCCs showed increased proliferation compared with DEN$^{ctrl}$ HCCs. Immunostaining for the proliferation marker Ki67 was performed to assess proliferation in DEN$^{N2ICD}$ and DEN$^{ctrl}$ HCCs and respective nonmalignant liver tissues (Fig. 5A). Quantification showed that proliferation of hepatocytes increased five-fold in DEN$^{N2ICD}$ compared with DEN$^{ctrl}$ livers (Fig. 5B), whereas proliferation was increased 3.5-fold in DEN$^{N2ICD}$ compared with DEN$^{ctrl}$ HCCs, indicating that constitutive Notch2 signaling accelerates HCC formation in the DEN carcinogenesis model through increased proliferation (Fig. 5B). Interestingly, DEN$^{ctrl}$ HCCs showed increased Hes1 mRNA expression when compared with control livers, suggesting that increased Notch signaling is generally involved in DEN-induced HCC formation (Fig. 5C). Again, constitutive Notch2 signaling in DEN$^{N2ICD}$ HCCs further increased Hes1 mRNA expression (Fig. 5C). Both DEN$^{N2ICD}$ HCCs and DEN$^{ctrl}$ HCCs showed increased CyclinD1 mRNA expression when compared with control livers (Fig. 5D). Intriguingly, the mean CyclinA2 mRNA expression in DEN$^{N2ICD}$ HCCs was increased three-fold compared with DEN$^{ctrl}$ HCCs and even 4.2-fold more induced than in control liver (Fig. 5D), indicating that DEN-induced HCCs with constitutive Notch2 signaling have increased proliferative potential. Moreover, the antiapoptotic gene Bel2 was up-regulated in DEN$^{N2ICD}$ HCCs when compared with control livers, whereas DEN$^{ctrl}$ HCCs showed no increase (Fig. 5E), consistent with our recent finding that Notch2 up-regulates antiapoptotic mechanisms in glioblastoma.32 Similar to the observation in HCCs from AlbCre/N2ICD mice (Fig. 2D), DEN$^{N2ICD}$ HCCs showed high up-regulation of Sox9 mRNA levels compared with control livers, whereas DEN$^{ctrl}$ HCCs did not show any increase (Fig. 5F). In contrast, DEN$^{ctrl}$ HCCs showed highly increased expression of alpha-fetoprotein (AFP) mRNA when compared with control livers, while not increasing significantly in DEN$^{N2ICD}$ HCCs (data not shown). Moreover, Alb mRNA expression was increased in DEN$^{ctrl}$ HCCs but decreased in DEN$^{N2ICD}$ HCCs when compared with DEN$^{ctrl}$ HCCs and control livers (Fig. 5F). Likewise, mRNA expression of other hepatocyte-related genes (Gck, Trf, Cyp3a11, ApoE, HGFL1) was decreased in DEN$^{N2ICD}$ HCCs when compared with DEN$^{ctrl}$ HCCs and control livers (Supporting Fig. 9). These findings are consistent with high Sox9 and low AFP and Alb mRNA levels reported in less differentiated and more proliferative human HCCs.27,33

**CCC Development Is Accompanied by Increased Proliferation and Dedifferentiation of BECs.** Taking advantage of the CCC formation in DEN$^{N2ICD}$ mice, we next wanted to gain insight into the mechanisms involved in the carcinogenesis of these tumors. Through a systematic histopathological analysis of the livers of DEN$^{N2ICD}$ mice, we were able to follow the steps from cyst formation to CCC development, showing first dilated bile ducts that led to extended biliary cysts, then further progression to high-grade dysplastic biliary epithelium and eventually invasive CCC (Fig. 6A). Immunostaining for Ki67 was used to assess proliferation of BECs in DEN$^{ctrl}$ bile ducts, and DEN$^{N2ICD}$ bile ducts, cysts, dysplastic cysts, and CCCs (Fig. 6B). BECs in DEN$^{N2ICD}$ bile ducts showed increased BEC proliferation compared with DEN$^{ctrl}$ bile ducts (Fig. 6C). Whereas no significant increase in proliferation was observed in DEN$^{N2ICD}$ cysts compared with
DEN\(^{N2ICD}\) bile ducts, dysplastic cysts showed increased proliferation when compared with nondysplastic cysts (Fig. 6C). Proliferation in DEN\(^{N2ICD}\) CCCs showed no further significant increase in proliferation compared with dysplastic cysts (Fig. 6C). It is therefore possible that those cysts are the consequence of increased proliferation in bile duct BECs and that the transformation of BECs into CCC occurs during dysplastic progression of these cysts. Highly proliferating foci that were frequently observed within dysplastic cysts support this concept (Fig. 6Biii).

Under normal developmental conditions, immature BECs express both HNF4\(\alpha\) and the BEC marker HNF1\(\beta\), while further differentiation leads to loss of HNF4\(\alpha\) expression in mature BECs and loss of HNF1\(\beta\) in hepatocytes and thus can be used as a differentiation marker.\(^{20,34,35}\) We therefore analyzed whether CCC formation in DEN\(^{N2ICD}\) mice involved
dedifferentiation of mature HNF1β+/HNF4α− BECs into HNF1β+/HNF4α+ BECs. Coimmunostaining for the Myc-tagged Notch2 with HNF4α showed that BECs in cysts expressed HNF1β (Supporting Fig. 3A) but not HNF4α (Fig. 7A), indicating a mature phenotype. In contrast, 86% of the CCCs in DENN2ICD mice re-expressed HNF4α+ (Fig. 7B) while simultaneously expressing HNF1β (Supporting Fig. 3B), thus resembling immature BECs during liver development.19 To further investigate whether HNF4α is also re-expressed in human CCC, we analyzed human CCC biopsies (n = 13) and a human CCC tissue microarray (n = 59 CK19+ CCCs) after HNF1β and HNF4α immunostaining. Intriguingly, all cancer cells in human CCCs expressed HNF1β (Fig. 7C,E), and of these 81% also expressed HNF4α (Fig. 7D,F), suggesting that re-expression of HNF4α is a common feature during CCC formation.

Discussion

Aberrant Notch signaling is implicated in a large variety of developmental disorders and in many types of cancer.14,36 However, the role of Notch signaling in HCC and CCC formation remains controversial. N1ICD expression in HCC cell lines blocks proliferation and induces apoptosis in vitro.37 Moreover, γ-secretase inhibitors, which block Notch signaling, increase HCC formation in a mouse model with deletions of the three retinoblastoma family genes.37 This suggests a tumor-suppressive role for Notch signaling in HCC. However, liver-specific deletion of Notch2 or Notch1 did not result in hepatic tumor formation.15,25 We therefore investigated whether constitutive Notch2 signaling in AlbCre/N2ICD mice contributes to HCC and CCC formation. Strikingly, we found that constitutive Notch2 signaling by itself is sufficient to induce HCC formation. Expression of genes inducing cell cycle progression was already increased in livers from AlbCre/N2ICD mice prior to HCC formation, indicating that constitutive Notch2 signaling promotes proliferation. This is consistent with the pro-proliferative function of Notch2 signaling in the brain.32 Moreover, AlbCre/N2ICD mice displayed BEC hyperplasia but failed to develop spontaneous CCC during the observation period of 1 year. Given the role of Notch2 in specifying BEC fate, the induction of HCC rather than CCC is surprising.19 However, AlbCre/N2ICD HCCs expressed the biliary marker Sox9, showed decreased expression of hepatocyte-related genes, and contained infiltrating BECs, indicating that Notch2 biases BEC fate.
We next studied the consequence of constitutive Notch2 signaling in the DEN carcinogenesis model, which allowed us to directly compare HCCs derived from AlbCre/N2ICD and control mice. We show that constitutive Notch2 signaling accelerates HCC growth in this model. Likewise, mRNA levels of genes inducing cell cycle progression were increased in DEN\(^{N2ICD}\) HCCs when compared with DEN\(^{ctrl}\) HCCs. Low AFP and Alb mRNA levels have been associated with less-differentiated, advanced human HCCs.\(^{33}\) Moreover, high Sox9 mRNA levels correlate with less-differentiated (grade T3-T4) HCCs and poor prognosis.\(^{27}\) In our mouse model, Notch2 activation led to increased Sox9 and low AFP and Alb mRNA expression levels in DEN\(^{N2ICD}\) HCCs and HCCs from AlbCre/N2ICD mice, implicating Notch2 signaling in promoting less differentiated HCCs. Consistently, DEN-induced HCCs are much larger in DEN\(^{N2ICD}\) than DEN\(^{ctrl}\) mice and show increased proliferative potential, indicative of more aggressive tumors. Thus, our data clearly establish an oncogenic role for constitutive Notch2 signaling.

While constitutive Notch2 signaling is sufficient to induce HCC formation in AlbCre/N2ICD mice, CCC formation in AlbCre/N2ICD was only observed upon DEN injection. Therefore, additional mutations seem to be required to transform the BEC hyperplasia seen in AlbCre/N2ICD mice into dysplastic BECs and CCC. However, it cannot be excluded that AlbCre/N2ICD mice older than 12 months may develop spontaneous CCC.

The cellular origin for HCC and CCC is still controversial.\(^{38}\) We have established that differentiation of HNF4\(^{a}\)+ hepatoblasts into HNF1\(^{b}\)+ BECs occurs through a HNF4\(^{a}\)+/HNF1\(^{b}\)+ immature BEC precursors.\(^{19}\) Whether CCC formation includes...
dedifferentiation of HNF1β+/HNF4α- BECs into HNF1β+/HNF4α+ BECs is unknown. Recently, it was shown that Sox9+ BECs can dedifferentiate upon injury and act as facultative stem cells in the liver.\textsuperscript{26,39} It is conceivable that CCC formation includes dedifferentiation of BECs that thereby acquire the proliferative potential necessary to form tumors. In support of this hypothesis, we now found that CCC formation is accompanied by a re-expression of HNF4α in BECs, both in DEN-injected AlbCre/N2ICD mice and in human CCCs. Re-expression of HNF4α in human and mouse CCCs possibly indicates that malignant transformation of BECs during CCC formation includes a de-differentiation step that resets mature HNF1β+/HNF4α- BECs to immature HNF1β+/HNF4α+ BEC precursors. It is unclear whether the HCCs in our mouse model originate from hepatocytes or BECs. AlbCre-mediated constitutive Notch2 expression in AlbCre/N2ICD mice begins during embryonic development in hepatoblasts and leads to their differentiation into BECs.\textsuperscript{19} Almost all cells in the liver with constitutive Notch2 signaling are BECs around birth.\textsuperscript{19} Because no hepatomas or other early postnatal tumors were found in AlbCre/N2ICD mice,\textsuperscript{19} it is unlikely that HCCs in these mice derive from hepatoblasts. This is consistent with other transgenic experiments activating the Notch pathway in hepatoblasts.\textsuperscript{17,20} The reported appearance of hepatocytes with transgenic N2ICD expression in postnatal AlbCre/N2ICD mice suggests that BECs can transdifferentiate into hepatocytes despite persistent transgenic N2ICD expression.\textsuperscript{19} In the present study, we showed that HCCs in AlbCre/N2ICD mice derived from cells with transgenic N2ICD expression that expressed high levels of Sox9 mRNA and protein. In addition, DEN\textsuperscript{H1} HCCs showed increased mRNA expression of hepatocyte-specific genes (AFP, \textit{Alb}), whereas DEN\textsuperscript{N2ICD} HCCs showed an increase in the biliary marker Sox9 and decreased mRNA levels of various hepatocyte-specific genes. It is therefore possible that HCCs in AlbCre/N2ICD mice derive from BECs that transdifferentiated into hepatocytes and that persistent Notch2 signaling in these cells explains the high levels of Sox9 expression and reduced expression of hepatocyte markers. More research is required to clearly establish the possible origin of HCC and CCC by tracing of BECs and hepatocytes during tumor formation.

In conclusion, our data establish that constitutive Notch2 signaling can lead to spontaneous HCC formation. Moreover, we show that constitutive Notch2 signaling accelerates HCC formation in the DEN carcinogenesis model by promoting proliferation and less differentiated HCC. In addition, DEN-treated Alb-Cre/N2ICD mice develop biliary cysts and eventually CCC, a process that is characterized by re-expression of HNF4α in both mice and man. Our results support that inhibition of Notch2 is a promising strategy to treat HCC and CCC, for which effective therapies are direly needed.

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