Intrahepatic cholangiocarcinomas (ICCs) are primary liver tumors with a poor prognosis. The development of effective therapies has been hampered by a limited understanding of the biology of ICCs. Although ICCs exhibit heterogeneity in location, histology, and marker expression, they are currently thought to derive invariably from the cells lining the bile ducts, biliary epithelial cells (BECs), or liver progenitor cells (LPCs). Despite lack of experimental evidence establishing BECs or LPCs as the origin of ICCs, other liver cell types have not been considered. Here we show that ICCs can originate from fully differentiated hepatocytes. Using a mouse model of hepatocyte fate tracing, we found that activated NOTCH and AKT signaling cooperate to convert normal hepatocytes into biliary cells that act as precursors of rapidly progressing, lethal ICCs. Our findings suggest a previously overlooked mechanism of human ICC formation that may be targetable for anti-ICC therapy.
Cholangiocarcinomas can originate from hepatocytes in mice

Biao Fan,1,2 Yann Malato,3,4 Diego F. Calvisi,5 Syed Naqvi,3 Nataliya Razumilava,6 Silvia Ribback,5 Gregory J. Gores,6 Frank Dombrowski,5 Matthias Evert,5 Xin Chen,1,7 and Holger Willenbring3,4,7

1Department of Bioengineering and Therapeutic Sciences, UCSF, San Francisco, California, USA. 2Department of Surgery, Beijing Cancer Hospital and Institute, Peking University School of Oncology and Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Beijing, China. 3Eli and Edythe Broad Center of Regeneration Medicine and Stem Cell Research and 4Department of Surgery, Division of Transplantation, UCSF, San Francisco, California, USA. 4Institut für Pathologie, Ernst-Moritz-Arndt-Universität, Greifswald, Germany. 5Division of Gastroenterology and Hepatology, Mayo Clinic, Rochester, Minnesota, USA. 6Division of Gastroenterology and Hepatology, Mayo Clinic, Rochester, Minnesota, USA. 7Liver Center, UCSF, San Francisco, California, USA.

Intrahepatic cholangiocarcinomas (ICCs) are primary liver tumors with a poor prognosis. The development of effective therapies has been hampered by a limited understanding of the biology of ICCs. Although ICCs exhibit heterogeneity in location, histology, and marker expression, they are currently thought to derive invariably from the cells lining the bile ducts, biliary epithelial cells (BECs), or liver progenitor cells (LPCs). Despite lack of experimental evidence establishing BECs or LPCs as the origin of ICCs, other liver cell types have not been considered. Here we show that ICCs can originate from fully differentiated hepatocytes. Using a mouse model of hepatocyte fate tracing, we found that activated NOTCH and AKT signaling cooperate to convert normal hepatocytes into biliary cells that act as precursors of rapidly progressing, lethal ICCs. Our findings suggest a previously overlooked mechanism of human ICC formation that may be targetable for anti-ICC therapy.

Introduction
Cholangiocarcinomas (CCs) are adenocarcinomas that can be distinguished by their anatomic location. Extrahepatic CCs may cause symptoms such as jaundice and are therefore occasionally detected at resectable stages (1). In contrast, intrahepatic CCs (ICCs) long remain asymptomatic and are therefore typically diagnosed at therapy-resistant advanced stages. The resulting poor prognosis and increasing incidence of ICCs necessitate a better understanding of their pathogenesis.

ICCs frequently grow alongside or into the lumen of bile ducts and are thought to originate from biliary epithelial cells (BECs). The heterogeneous histology of ICCs suggests that they can derive from both cylindrical BECs lining large bile ducts (ductal ICCs) or cuboidal BECs lining small bile ducts or ductules (peripheral ICCs) (2). Ductules also contain liver progenitor cells (LPCs), which can give rise to both BECs and hepatocytes and are also being considered as an origin of ICCs, particularly of rare tumors such as cholangiocellular carcinomas that exhibit both ICC and hepatocellular carcinoma (HCC) characteristics.

An alternative mechanism explaining the formation of ICC could be lineage conversion occurring during malignant transformation of hepatocytes. To our knowledge, this possibility has so far not been considered. However, the common developmental origin of BECs and hepatocytes, and the previous finding that forced activation of NOTCH signaling can convert hepatocytes into cells resembling BECs in mice, provide indirect support for this concept (3). The clinical observation that peripheral ICCs can form masses in the liver parenchyma, where mainly hepatocytes reside, is also compatible with a hepatocyte origin (4). Furthermore, ICCs can be caused not only by diseases affecting BECs, such as parasitic infections, primary sclerosing cholangitis, biliary duct cysts, and hepatolithiasis, but also by diseases that cause hepatocyte injury, such as hepatitis C or B infection, alcohol abuse, and nonalcoholic steatohepatitis (5).

The first direct evidence in support of a hepatocyte origin of ICCs was provided by our previous finding that hydrodynamic tail vein injection of a human AKT overexpression plasmid into mice produced mainly HCCs, but also a small number of cholangiocellular lesions (6). However, although this technique delivers plasmids mainly to hepatocytes in the liver (7), we could not rule out plasmid entry and AKT overexpression in BECs or LPCs, especially because AKT was under the control of a ubiquitous promoter. Thus, a hepatocyte origin of the cholangiocellular lesions could not be definitely established. In addition, our finding of only benign tumors, but not ICCs, suggested that overexpression of AKT alone is not sufficient for ICC formation.

Here, we hypothesized that activated NOTCH signaling can convert hepatocytes into ICC precursors because of its ability to induce biliary differentiation of hepatocytes in mice (3) and its emerging role as a driver and prognostic marker in human CCs (8, 9). Indeed, using our previously reported hepatocyte fate–tracing model (10), we found that activated NOTCH signaling, not alone, but in combination with AKT overexpression, causes rapid formation of ICCs from hepatocytes.

Results and Discussion
To activate NOTCH signaling in the liver, we stably overexpressed the intracellular domain of the NOTCH1 receptor (NICD; Myc-tagged). For this purpose, we delivered plasmids for sleeping beauty transposase–mediated genomic transgene integration to livers of wild-type FVB/N mice by hydrodynamic tail vein injection (6). We did not detect histological changes within 10 weeks after NICD plasmid injection (data not shown). By 20 weeks, we found cystic cholangiocellular tumors resembling human biliary cystadenomas (Supplemental Figure 1, A and B). Some of these tumors contained cytologically malignant cells that not only expanded intracytically, but also invaded the sur-
rounding liver tissue (Supplemental Figure 1, C–E), consistent with progression to invasive cystadenocarcinomas. All tumors were derived from cells that stably overexpressed NICD (Supplemental Figure 1F).

Considering the rapid onset of hepatocyte proliferation and formation of premalignant lesions in mice overexpressing AKT in the liver (6), we decided to inject the NICD plasmid together with an AKT overexpression plasmid (HA-tagged; combination referred to as NICD/AKT) into mice. Macroscopically, livers of these mice appeared normal 1.5 and 2.5 weeks after injection (Figure 1A). However, small, white, cyst-like lesions were present on the liver surface after 3.5 weeks. These lesions rapidly expanded and by 4.5 weeks occupied most of the liver surface. At 5 weeks after plasmid injection, the lesions had replaced most of the normal liver tissue, and the mice rapidly deteriorated and either died or needed to be euthanized (Supplemental Figure 2).

At the microscopic level, single or clusters of cytologically malignant cells could be identified 1.5 weeks after plasmid injection (Figure 1B and Supplemental Figure 3, A and B). These clusters progressed into small tumors of ductular phenotype by 2.5 weeks (Supplemental Figure 3, C and D). The tumors grew markedly by 3.5 weeks and exhibited either a ductular or cystic phenotype (Supplemental Figure 3, E and F). Although cytologically malignant, many tumors still had well-defined borders at this stage. By 4.5 weeks, however, most tumors showed additional signs of malignancy, including necrosis, high mitotic activity, and invasion of the surrounding liver parenchyma (Supplemental Figure 3, G and H), characteristics that correspond to human ICCs. Immunostaining and immunoblotting for the Myc and HA tags showed that tumors invariably derived from cells stably overexpressing NICD/AKT (Supplemental Figure 4, A and B). Additional immunostainings for the biliary marker cytokeratin 19 (Ck19) and major urinary protein (Mup), a marker specific for hepatocytes (10), confirmed that the tumors exhibited exclusively biliary differentiation (Supplemental Figure 5, A and B), providing further support for their classification as ICCs. Along these lines, expression of Afp, a gene overexpressed in HCCs, remained at low levels, whereas expression of Epcam, a gene specific for biliary cells in the liver, markedly increased with time after injection (Supplemental Figure 6, A and B). Thus, overexpression of NICD/AKT in the liver induces specifically ICCs.

Many ICCs formed in the central area of the liver lobule, where normally hepatocytes, but not BECs or LPCs, reside (Supplemental Figure 3B). Therefore, we hypothesized that the NICD/AKT-induced ICCs originated from hepatocytes. To rule out migrant BECs or LPCs as the origin, we used our previously reported hepatocyte fate–tracing model, in which all hepatocytes and their progeny, but no other liver cells, express enhanced yellow fluorescent protein (EYFP) (10). To generate the model, we intravenously injected $4 \times 10^{11}$ viral genomes of a double-stranded adenovector serotype 8 expressing Cre recombinase from the hepatocyte-specific transthyretin promoter (AAV8-Ttr-Cre) into mice that carry EYFP disrupted by a floxed stop codon in the ubiquitously expressed Rosa26 locus (R26R-EYFP mice) (Figure 2A). To replicate the experiments described above, we used FVB/N R26R-EYFP mice. We analyzed a subset of the mice 1 week after injection to ascertain that AAV8-Ttr-Cre looped out the stop codon and activated EYFP expression in hepatocytes with the
same efficiency and specificity as previously reported (Figure 2B and ref. 10). We hydrodynamically injected the remaining mice with the NICD/AKT plasmids.

As expected, all mice receiving the NICD/AKT plasmids harbored numerous large ICCs 4.5 weeks later. HA immunostaining showed that all ICCs originated from cells stably overexpressing NICD/AKT (Supplemental Figure 7A). Positive EYFP immunostaining revealed that the cells of origin of ICCs were hepatocytes (Figure 2, C–E). Confirming our results described above (Supplemental Figure 5, A and B), the ICCs expressed the biliary markers Sox9, Ck8, and Muc1, but were negative for the hepatocyte marker Mup (Figure 2, C–E, and Supplemental Figure 7B). Ck8 is expressed in mouse BECs from early developmental stages onward (Supplemental Figure 7C and ref. 11). The Muc1 protein is normally localized in the apical membrane of BECs (Supplemental Figure 7D and ref. 12). High or cytoplasmic expression of Muc1 is associated with progression and invasiveness of human ICCs (13, 14). Indeed, we observed intense, nonpolarized Muc1 labeling in many ICC cells (Supplemental Figure 7E). Because Sox9 is also expressed in bipotential LPCs (17) and Ck8 is expressed in bile duct development at earlier stages than Ck19 (18), this find-
ing suggests that NICD/AKT-expressing hepatocytes gradually acquired biliary differentiation. In accordance with this assessment, we found that EYFP and Sox9 double-positive cells gradually lost hepatocyte differentiation, as indicated by declining levels of Mup, which is only expressed in mature hepatocytes (Figure 3D). Furthermore, electron microscopy revealed loss of glycogen stores in hepatocytes undergoing lineage conversion, and, illustrating their hepatocyte origin and difference from normal BECs, these cells showed formation of cell junctions and bile canaliculi with adjacent hepatocytes, lack of a basement membrane, and cytological atypia (Supplemental Figure 10, A–C). These findings show that NICD/AKT-induced conversion of hepatocytes into biliary cells occurs already at the single-cell stage and suggest concurrent initiation of malignant transformation.

In conclusion, our results show that overexpression of two factors, NICD and AKT, is sufficient for rapid conversion of fully differentiated, normal hepatocytes into lethal ICCs. Our finding that ICC formation involves conversion of hepatocytes into atypical biliary cells at an early, single-cell stage suggests that lineage reprogramming gives way to or promotes initiation of malignant transformation, which is then manifested by cell proliferation. Although the mechanism by which NICD and AKT cooperate to induce hepatocyte-derived ICCs remains to be determined, our finding that overexpression of NICD alone induces invasive cystadenocarcinomas suggests that it is the driving oncogene. AKT is likely acting to accelerate ICC formation by providing metabolic and thus proliferation-promoting support (6). In support of this assessment, overexpression of AKT alone produces mainly HCCs and a few benign cholangiocellular lesions (6), whereas overexpression of both NICD and AKT induced only ICCs in our model. NOTCH and AKT signaling are frequently coactivated in human ICCs (Supplemental Figure 11, A–C), which suggests that their cooperation is also driving human ICC formation.
Whether ICCs originate from hepatocytes also in humans remains to be determined. However, this possibility is strongly suggested by recent findings of intracytoplasmic p62+ hyaline inclusions, which are thought to be specific for injured or malignantly transformed hepatocytes, in common peripheral ICCs (that is, not rare, potentially LPC-derived, cholangiokelocellular carcinomas) (19). Most ICCs with p62+ hyaline inclusions emerged in patients with liver disease due to hepatitis C or B infection or alcohol abuse, which suggests that chronic hepatocyte injury caused lineage conversion and ICC formation. In accordance with these observations, epidemiologic studies have identified viral hepatitis as a risk factor for ICC (5). By establishing that hepatocytes can give rise to ICCs, our study sheds light on the pathogenesis of this cancer and suggests molecular targets for much-needed new therapies.

Methods

Supplemental Methods are available online with this article.

**Mice.** Six- to 10-week-old wild-type (Charles River) or R26R-EYFP (20) mice on the FVB/N strain background were used.

**Plasmids.** HA-tagged AKT (human AKT1) and hyperactive sleeping beauty transposase plasmids were described previously (6). AKT was myristoylated for constitutively active kinase activity. Mouse NOTCH1 receptor NICD sequence with Myc tag was obtained from Addgene. NICD and AKT were expressed from the EF1α receptor NICD sequence with Myc tag was obtained from Addgene. NICD and AKT were expressed from the EF1α promoter. Transposase was expressed from the CMV promoter.

**Hydrodynamic tail vein injection.** Twenty micrograms of NICD, 4 μg AKT, and 1 μg transposase plasmids were diluted in 2 ml 0.9% NaCl, sterile filtered, and injected into a lateral tail vein within 5–7 seconds as previously described (6). AAV8-Ttr-Cre. Production, titering, and injection of AAV8-Ttr-Cre was performed as previously described (10).

**Immunostaining.** Liver samples were fixed overnight in zinc formalin (Anatech Ltd.), embedded in paraffin, cut into 5-μm-thick sections, and placed on Superfrost Plus slides (Fisher Scientific). Sections were deparaaffinized and boiled in Antigen Retrieval Citra Solution (BioGenex) for 10 minutes. After blocking in 10% serum for 1 hour, sections were incubated with primary antibodies overnight at 4°C and secondary antibodies for 1 hour at room temperature (Supplemental Tables 1 and 2). Nuclear DNA was stained with 300 nM DAPI (Millipore).

**Statistics.** Data are expressed as mean ± SEM. Statistical significance was determined by 2-way ANOVA followed by 2-tailed Student’s t test. A P value less than 0.05 was considered significant.

**Study approval.** All mouse procedures were approved by the Institutional Animal Care and Use Committee at UCSF.

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Address correspondence to: Xin Chen, UCSF, 513 Parnassus Avenue, Campus Box 0912, San Francisco, California 94143, USA. Phone: 415.502.6526; Fax: 415.502.4322; E-mail: chenx@pharma.ucsf.edu. Or to: Holger Willenbring, UCSF, 35 Medical Center Way, Campus Box 0665, San Francisco, California 94143, USA. Phone: 415.476.2417; Fax: 415.514.2346; E-mail: willenbringh@stemcell.ucsf.edu.


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