Genetic Lineage Tracing Analysis of the Cell of Origin of Hepatotoxin-Induced Liver Tumors in Mice

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The expression of biliary/progenitor markers by hepatocellular carcinoma (HCC) is often associated with poor prognosis and stem cell-like behaviors of tumor cells. Hepatocellular adenomas (HCAs) also often express biliary/progenitor markers and frequently act as precursor lesions for HCC. However, the cell of origin of HCA and HCC that expresses these markers remains unclear. Therefore, to evaluate if mature hepatocytes give rise to HCA and HCC tumors and to understand the molecular pathways involved in tumorigenesis, we lineage-labeled hepatocytes by injecting adeno-associated virus containing thyroxine-binding globulin promoter-driven causes recombination (AAV-TBG-Cre) into RosaYFP mice. Yellow fluorescent protein (YFP) was present in >96% of hepatocytes before exposure to carcinogens. We treated AAV-TBG-Cre; RosaYFP mice with diethylnitrosamine (DEN), followed by multiple injections of carbon tetrachloride to induce carcinogenesis and fibrosis and found that HCA and HCC nodules were YFP+ lineage-labeled; positive for osteopontin, SRY (sex determining region Y)-box 9, and epithelial cell adhesion molecule; and enriched for transcripts of biliary/progenitor markers such as prominin 1, Cdh44, and delta-like 1 homolog. Next, we performed the converse experiment and lineage-labeled Foxo1-positive hepatic progenitor cells simultaneously with exposure to carcinogens. None of the tumor nodules expressed YFP, indicating that Foxo1-expressing cells are not the origin for hepatotoxin-induced liver tumors. We confirmed that HCA and HCC cells are derived from mature hepatocytes and not from Foxo1-Cre-marked cells in a second model of toxin-induced hepatic neoplasia, using DEN and 3,3',5,5'-tetrachloro-1,4-bis(pyridyloxy)benzene (TCPPOBOP). Conclusion: Hepatocytes are the cell of origin of HCA and HCC in DEN/carbon tetrachloride and DEN/TCPPOBOP induced liver tumors. (HEPATOLOGY 2016;64:1163-1177)

Hepatocellular carcinoma (HCC) is the most common type of liver cancer. Risk factors associated with liver cancer include viral hepatitis type B and C, alcohol, nonalcoholic fatty liver disease, diabetes, environmental toxin exposure, and obesity.1,2 It has been estimated that more than 33,000 new cases of liver cancer are diagnosed in the United States yearly and that 80% of these are
Therefore, it is critical to understand the cellular mechanisms of HCC pathogenesis in order to develop effective anticancer therapy.

Human HCC cells frequently express markers of biliary/progenitor cells that are absent from normal hepatocytes, including osteopontin (OPN), CD133 (encoded by prominin 1), CD44, delta-like 1 homolog, SRY (sex determining region Y)-box 9 (SOX9), and epithelial cell adhesion molecule (EpCAM). Expression of progenitor markers by HCC has been linked to aggressive behavior of tumors and poor prognosis, highlighting the importance of understanding the cell of origin of these cancers. It also has been reported that biliary/progenitor markers are expressed in hepatocellular adenomas (HCAs), benign tumors with the risk for malignant transformation. Hepatic progenitor cells (HPCs), the biliary epithelial component of ductular reactions, have been proposed to be the cell of origin of HCC based on correlative data. Surprisingly, the cellular origin of HCA and HCC nodules (referred to as “tumor nodules” hereafter) that are positive for biliary/progenitor markers has not been fully addressed experimentally.

Mature hepatocytes acquire markers of biliary/progenitor cells in response to injury, which led us to hypothesize that mature hepatocytes give rise to tumor nodules positive for progenitor markers. Therefore, to test our hypothesis, we lineage-traced mature hepatocytes using adeno-associated virus (AAV)-based genetic labeling. We included HCA nodules as well as HCC nodules in our analyses to gain insight into cellular mechanisms of hepatocyte dedifferentiation at the early stages of tumor progression. It has been well documented that HCA is a neoplastic lesion that progresses to HCC in mouse models and that there is an increased risk of malignant transformation in a subset of HCA in humans.

We employed two different combinations of hepatotoxins that induce progenitor marker–expressing HCA and HCC nodules accompanied by ductular reactions and fibrosis. In addition, we lineage-traced cells that express the winged helix transcription factor forkhead box protein (Foxl1) using Foxl1-Cre;Rosa<sup>YFP</sup> mice because our previous studies indicated that Foxl1 is a marker for HPCs. We also investigated whether tumor nodules express c-myc and components of the Wnt, Notch, and Hippo signaling pathways, key regulators of hepatic cell specification and tumorigenesis. Our study clarifies the long-debated cellular origin of tumor cells that express progenitor markers by tracing hepatocytes to tumor nodules in two mouse models of toxin-induced HCA and HCC.

Materials and Methods

MICE

For lineage tracing of hepatocytes, 6-day-old Rosa<sup>YFP</sup> (Rosa26<sup>loxP-stop-loxP-YFP</sup>) reporter mice were injected with serotype 8 AAV-thyroxine-binding globulin (TBG)-Cre (4 × 10<sup>10</sup> gene copies per mouse, intraperitoneally) (University of Pennsylvania Vector Core). For lineage tracing of Foxl1-expressing cells, Foxl1-Cre mice were crossed to Rosa<sup>YFP</sup> reporter mice. Two different strategies were used to induce HCC, as described. First, 15-day-old mice were injected with diethylnitrosamine (DEN; 25 mg/kg body weight, intraperitoneally; Sigma-Aldrich, St. Louis, MO). Beginning at 29 days, mice were injected with carbon tetrachloride (CCl<sub>4</sub>; 0.5 mg/kg...
body weight, intraperitoneally; Sigma-Aldrich) weekly for 14–21 weeks. Tissues were harvested 1–8 weeks after the last injection. Second, 15-day-old mice were injected with DEN (20 mg/kg body weight, intraperitoneally). Beginning at 29 days, mice were injected with 3,3',5,5'-tetrachloro-1,4-bis(pyridyloxy)benzene (TCPOBOP; 3 mg/kg body weight, intraperitoneally; Sigma-Aldrich) biweekly for 16–26 weeks. Tissues were harvested 2–8 weeks after the last injection. All protocols were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

HISTOLOGY AND CELL COUNTING

HCA and HCC nodules were identified by a board-certified veterinary anatomic pathologist based on histomorphology of hematoxylin and eosin-stained sections according to published guidelines. Colocalization analysis for hepatocyte, biliary, and/or progenitor cell markers as well as yellow fluorescent protein (YFP) on stained sections was performed as described. Briefly, liver lobes were fixed in 4% paraformaldehyde for 24 hours at 4°C and embedded in paraffin. Slides (5-μm sections) were subjected to antigen retrieval using a 2100 Retriever (Electron Microscopy Sciences, Hatfield, PA). Slides were incubated with primary antibodies diluted in CAS-Block (Life Technologies, Grand Island, NY) overnight at 4°C and then incubated with appropriate secondary antibodies diluted in CAS-Block for 2 hours at room temperature. 4',6-Diamidino-2-phenylindole (DAPI) was used to stain nuclei. For analysis of the ductular response, 10 random pictures centered on the portal triad were taken for each section. For measurement of lineage labeling efficiency, approximately 1,300 cells were counted per mouse. For analysis and quantification of tumor nodules, serial sections were stained by immunofluorescence or immunohistochemistry as described. High-resolution slide scan images were obtained using a light microscope (Keyence BZ-X700, Japan). Image J Software was used for analyses. The following antibodies were used: hepatocyte nuclear factor 4 alpha (HNF4α; PP-H1415-00; R&D Systems, Minneapolis, MN); YFP (ab6673 from Abcam, Cambridge, MA, and GFP-1210 from Aves Labs, Tigard, OR); Opn (AF808; R&D Systems); Epcam (ab71916; Abcam), Sox9 (AB5535; Millipore, Norwood, OH), vimentin (5741; Cell Signaling Technologies, Danvers, MA), yes-associated protein 1 (Yap1; 4912; Cell Signaling Technologies), and alpha-fetoprotein (AFP; sc8108; Santa Cruz Biotech, Santa Cruz, CA). Cytokeratin-19 (CK19) antibody was a kind gift from Dr. Joshua R Friedman (University of Pennsylvania).

RNA EXTRACTION AND QUANTITATIVE REVERSE-TRANSCRIPTION POLYMERASE CHAIN REACTION

Total RNA was extracted from liver samples using the PerfectPure RNA Tissue Kit (5 PRIME, Gaithersburg, MD) based on the manufacturer’s protocol. Superscript II reverse transcriptase (Life Technologies) was used for generating complementary DNA. Polymerase chain reactions (PCRs) were performed using SYBRGreen QPCR Master Mix (Agilent Technologies, Santa Clara, CA) on the Mx3000 PCR cycler (Agilent Technologies). Reactions were performed in triplicate and normalized relative to the ROX reference dye, and median cycle threshold values were used for subsequent analyses. TATA-box binding protein was used to normalize messenger RNA (mRNA) expression. The following primers were used: collagen, type 1, alpha 1, encoded by Coll1a1 (F) GTGGAGCGGTGCACAGTCA, (R) GGCTGGGTGGGAGGAGG-GAACCA; matrix metalloproteinase 2, encoded by Mmp2 (F) GACCTGCAGGGTGGTGTCAT, (R) TATGGCCCCGTGGGAACAGGG; tissue inhibitor of metalloproteinase 1, encoded by Timp1 (F) ACCAGACGATACCATGATGCC, (R) TGGGGTGAGGCACAGCTAC; prominin 1, encoded by Prom1 (F) GAAAATGTGCTCTGCAGAAACC, (R) TCTCAAGCTGAAAAAGCAGCA; Cd44 (F) TGACCCGTTGTGCTGATCCT, (R) GCACAAAAGGACTGAAGCTGC; delta-like 1 homolog, encoded by Dlk1 (F) GTGGCCATCGTCTTTTCAC, (R) GATGATATTGACCGCCAGCC; Sox9 (F) GAGGTTCAGTACAGTGAGGA, (R) TGTCACAACACACGACACA; Myc (F) CTCCACTCACCAGCACA, (R) CTTGCAATTTGGCCTC; betacatenin (Ctnnb1) (F) TGACACCTCCCAAGCTCTGT, (R) CTATGATATTGCCAGCC; Notch1 (F) CCTCAGATTTGCTGCTCTCT, (R) TGGTCAAAACTTGCTGCC; Notch2 (F) CAGCAGATTTGGCTGCTCTCT, (R) GAAAATGTGCTCTGCAGAAACC; and hairy and enhancer of split-1 (Hes1) (F) AGAGGCTGCAAGTTTT, (R) TCCACACTTGGCTGTGCTGAGA. TATA box protein, encoded by Tbp, primers were as described.

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STATISTICAL ANALYSIS

The Student t test was used to determine the significance of differences between two groups. P < 0.05 was considered statistically significant.

Results

HEPATOCYTES GIVE RISE TO TUMOR CELLS IN THE DEN/ CCL4-INDUCED TUMOR MODEL

We asked whether established mature hepatocytes are the major source of hepatotoxin-induced tumor nodules. In RosaYFP mice, expression of YFP is blocked by transcriptional stop sequences flanked by loxP sites. Expression of Cre-recombinase allows for permanent labeling of the Cre-expressing cells and all its descendants with YFP. Injection of an AAV serotype with a high tropism for hepatocytes that expresses Cre recombinase under the control of the hepatocyte-specific TBG promoter (AAV-TBG-Cre) into RosaYFP mice resulted in efficient labeling of nearly all hepatocytes (96.9 ± 0.7%, n = 3) with YFP (Fig. 1A–C). Other hepatic cells, including CK19-expressing cholangiocytes and vimentin-expressing mesenchymal cells, remained YFP-negative, confirming the specificity of this labeling strategy (Fig. 1B,C).

For the induction of HCC, we employed the recently developed paradigm in which a one-time administration of the mutagen DEN is combined with multiple injections of the hepatotoxic CCl4 (Fig. 1D). This model of chemical carcinogenesis is thought to approximate human pathophysiology because it induces chronic inflammation, elevation of endotoxin levels, and fibrosis in the liver, creating an environment permissive for the development of cancer. As expected, treatment of mice led to the activation of ductular reactions positive for biliary markers Opn and Epcam (Fig. 1E), development of bridging fibrosis, as well as up-regulation of markers of fibrosis (Fig. 1F). A small number of Epcam-positive cells within ductular reactions were labeled with YFP (8.7 ± 6.1%, n = 4) (Fig. 1E, right panel), indicating a hepatocyte origin. Our finding is in line with two recent studies reporting that ductular reactions mostly, but not exclusively, originate from biliary cells in mouse models of liver cancer.

At the end of the treatment with DEN/CCl4, we detected multiple nodules with the morphology of HCA and HCC, all of which contained AFP and Opn-positive cells (Table 1 and Fig. 2; Supporting Fig. S1). OPN is a frequently used marker of human HCC, although it is also found in cholangiocytes and HPCs. All of the HCC tumor nodules were YFP-positive (Table 1 and Fig. 2), and 26 of 27 HCA nodules were YFP-positive (96.2%) (Table 1; Supporting Fig. S1), indicating that tumor cell positivity for YFP was in line with the observed efficiency of labeling of hepatocytes with AAV. In addition, we confirmed that most of the HCA and HCC nodules contained cells marked with Sox9 in tumor parenchymal cells or in tumor-associated duct structures, or both, and that cells expressing Sox9 in the tumors were also YFP-positive (Fig. 2). SOX9 is a progenitor cell marker and is associated with tumor progression of human HCC. Similarly, Epcam, a marker of an HCC subtype with progenitor features, was present in tumor-associated ductular structures that were YFP-positive in HCCs and in HCAs (Fig. 2; Supporting Fig. S1). Peritumoral ductular structures were positive for Opn, Sox9, Epcam, and CK19 but negative for YFP (Fig. 2). We also investigated whether tumor nodules express Yap1, a component of the Hippo signaling pathway that is involved in reprogramming of hepatocytes into cells with progenitor characteristics. Yap1 was present in nuclei in both tumor and nontumor tissues in HCAs and HCCs, with marked Yap1 cytoplasmic expression in HCCs (Fig. 2; Supporting Fig. S1). AFP, a tumor marker and marker of...
hepatoblasts, was expressed in tumor and nontumor cells, which is likely a reflection of the injury induced by CCl4, though many of the tumors had darker staining than the surrounding tissues (Fig. 2; Supporting Fig. S1). CK19, a marker of cholangiocytes and of hepatoblasts, was expressed in peritumoral bile ducts but absent from the tumors in the DEN/CCl4 model (Fig. 2; Supporting Fig. S1).

Quantitative PCR analysis indicated that tumor nodules express significantly higher mRNA levels of the markers of biliary/progenitor cells including prominin 1, Cd44, delta-like 1 homolog, and Sox9 than the normal liver (Fig. 3). While Sox9 mRNA levels were significantly higher in tumor nodules compared to tumor-adjacent areas, other markers were expressed in tumor-adjacent areas at levels slightly lower than or comparable to tumor nodules. The similar levels of expression in tumor and tumor-surrounding areas is consistent with hepatotoxin-induced ductular reactions in tumor surrounding areas and with dedifferentiation of mature hepatocytes. Both ductular reactions and tumors came to express progenitor cell and ductal cell markers. Our results demonstrate that mature hepatocytes give rise to tumor nodules positive for progenitor markers in the DEN/CCl4 model.

To further understand which signaling molecules are associated with dedifferentiation of hepatocytes and tumor formation, we investigated expression of Myc, Ctnnb1, Notch1, Notch2, and Hes1 on the transcript level (Fig. 3). It has been demonstrated that activation of c-myc is required to reprogram mature hepatocytes into cancer stem cells. Activation of Wnt signaling has been suggested as a marker of malignant transformation of HCA into HCC. Activation of Notch signaling has been associated with HCC formation in mice. Furthermore, the Wnt and Notch signaling pathways are key regulators of hepatic cell specification. Surprisingly, the levels of Myc mRNA were down-regulated in DEN/CCl4-induced tumors compared to normal liver, and the transcript levels of Ctnnb1, Notch1, Notch2, and Hes1 remained unchanged.

**FOXL1-CRE-MARKED CELLS DO NOT CONTRIBUTE TO TUMORS IN THE DEN/CCl4 MODEL**

We have previously demonstrated that Foxl1+ cells appear in the portal triad following certain types of liver injury, including bile duct ligation and with the choline-deficient, ethionine-supplemented diet. Foxl1-Cre-labeled cells express markers of biliary/progenitor cells and contribute to parenchymal regeneration. Therefore, we asked whether Foxl1+ HPCs contribute to DEN/CCl4-induced tumor nodules. To follow Foxl1+ HPCs, we employed Foxl1-Cre;Rosa26YFP mice in which Foxl1-expressing cells as well as their descendants are marked by YFP. To test whether the administration of DEN and CCl4 activates Foxl1-Cre expression in the liver prior to tumorigenesis, we treated Foxl1-Cre;Rosa26YFP mice with DEN on postnatal day 15 and once with CCl4 on postnatal day 29 and harvested the liver 1 week later (Fig. 4A).
We detected Ck19\(^+\)YFP\(^+\) cells in the portal triad (Fig. 4B), indicating that these hepatotoxins activate the Foxl1\(^+\) progenitor cell compartment prior to the induction of liver tumors. Next, to induce tumors in the mouse liver, Foxl1-Cre, Rosa\(^{YFP}\) mice received DEN followed by up to 21 weekly injections of CCl\(_4\) (Fig. 4C). At the end of the treatment with DEN/CCl\(_4\), Foxl1-Cre-labeled cells were confined to nontumor areas, and YFP expression was detected in 32% of all Ck19\(^+\) cells (which include both cholangiocytes and HPCs) but only in 0.2% of HNF4\(\alpha\) hepatocytes (Fig. 4D). We detected six HCA nodules that contained cells expressing Opn, AFP, Sox9, and Epcam, similar to what was seen in the hepatocyte lineage-tracing model, except that in this case the tumor cells were all YFP-negative (Fig. 4E). None of the tumors in this experimental group were classified as HCC by histopathological criteria, but the staining pattern of

**FIG. 2.** Lineage tracing demonstrates that HCC originates from hepatocytes when carcinogenesis is induced by DEN and CCl\(_4\) treatment. Serial sections of a representative HCC stained with hematoxylin and eosin, Opn, AFP, YFP/Sox9, YFP/Epcam, Yap1, and CK19. Dotted lines indicate tumor margins. White arrows point to tumor-associated ductular structures. Yellow arrows mark extratumoral bile ducts. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; H&E, hematoxylin and eosin.
tumor markers was similar between HCAs and HCCs (Fig. 4E). These results indicate that Foxl1+ cells are not tumor-initiating cells in this model of hepatic tumorigenesis.

HEPATOCYTES, BUT NOT FOXL1-CRE-MARKED HPCS, GIVE RISE TO TUMORS IN THE DEN/TCPOBOP MODEL OF LIVER TUMORIGENESIS

Having established that hepatocytes are the cells of origin of tumor cells in the DEN/CCl4 model, we tested another tumorigenesis paradigm, the combination of DEN with TCPOBOP.(33) TCPOBOP is an agonist for the constitutive androstane receptor and promotes hepatocarcinogenesis through multiple mechanisms including activation of the c-Myc-FoxM1 pathway and up-regulation of antiapoptotic proteins.(47-49) To lineage-trace hepatocytes, we injected AAV-TBG-Cre into RosaYFP mice before hepatotoxin treatment (Fig. 5A). Treatment of mice led to activation of the ductular reaction as indicated by Opn and Epcam expression (Fig. 5B), as well as up-regulation of markers of fibrosis (Fig. 5C), although the fibrosis was not extensive enough to cause bridging of fibrous septa (data not shown). Consistent with our results in the DEN/CCl4 model, only a small fraction (fewer than 2%) of Epcam positive cells within ductular reactions were labeled by YFP (1.6 ± 1.0%, n = 4) (Fig. 5B, right panel).

Next, to induce tumor formation, we treated AAV-TBG-Cre-injected RosaYFP mice with DEN, followed by biweekly injections of TCPOBOP for up to 26 weeks. Toxin treatment led to development of HCA and HCC nodules in both AAV-TBG-Cre-treated and Foxl1-Cre-marked groups (Table 1). In AAV-TBG-Cre-treated mice, HCAs and an HCC induced by DEN/TCPOBOP treatment were positive for AFP, Opn, Epcam, and Yap1 (Fig. 5D; Supporting Fig. S2). The HCAs and HCC were YFP-positive, demonstrating that the tumors had originated from mature hepatocytes in the DEN/TCPOBOP model (Fig. 5D and Table 1; Supporting Fig. S2). Our quantitative reverse-transcription PCR (RT-PCR) analyses...

FIG. 3. Tumors induced by DEN and CCl4 treatment express progenitor markers. Quantitative RT-PCR analyses for mRNA levels of progenitor markers in the tumor nodules compared to tumor-adjacent areas and the liver of normal chow-fed mice (n = 4 mice per group). Note that we pooled HCA and HCC tumor nodules isolated from the same mouse, six nodules per mouse. *P < 0.05, tumor-adjacent areas versus normal and tumors versus normal; A P < 0.05, tumors versus adjacent areas. Error bars represent the standard error of the mean. Abbreviations: AT, adjacent tumor; Dlk1, delta-like 1 homolog; Prom1, prominin 1.
FIG. 4. Foxl1-Cre-marked HPCs are not tumor-initiating cells in the DEN/CCl₄ hepatotoxin-induced tumor model. (A) Schema of the treatment paradigm to label HPCs prior to the induction of liver tumors. (B) Ck19⁺ HNF4α-YFP⁺ HPCs (yellow arrows) derived from Foxl1-Cre-expressing cells are clearly visible in the portal tract. (C) Schema of the treatment paradigm to induce liver tumors. (D) Ck19⁺ HNF4α-YFP⁺ cells were detected in nontumor areas at the end of the treatment period (left panel). Quantification of the percentage of YFP⁺ Ck19⁺ or YFP⁺ HNF4α⁺ cells in DEN/CCl₄-treated mice (n = 4 per each group; right panel). Error bars represent the standard error of the mean. (E) YFP-expressing cells are absent from HCA tumor nodules. Serial sections of a representative HCA stained with hematoxylin and eosin, Opn, AFP, YFP/Sox9, YFP/Epcam, Yap1, and CK19 are shown. Dotted lines indicate the margins of the tumor. White arrows point to tumor-associated ductular structures. Yellow arrows mark extratumoral bile ducts. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; H&E, hematoxylin and eosin.
FIG. 5. HCC induced by DEN and TCPOBOP treatment originates from hepatocytes. (A) Schema of the treatment paradigm to induce liver tumors. (B) Ductular reactions were detected in tumor-adjacent areas at the end of the treatment period by staining for Opn and Epcam. Yellow arrow indicates Epcam<sup>1</sup>YFP<sup>1</sup> cells. (C) Quantitative RT-PCR analyses revealed higher mRNA levels of fibrotic markers in the liver of mice treated with DEN/TCPOBOP compared to normal chow-fed mice (n = 3-4 per each group). *P < 0.05. Error bars represent the standard error of the mean. (D) Serial sections of an HCC stained with hematoxylin and eosin, Opn, AFP, YFP/Sox9, YFP/Epcam, Yap1, and CK19 are shown. Dotted lines indicate the tumor margin. Yellow arrows mark extratumoral bile ducts. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; H&E, hematoxylin and eosin; Mmp2, matrix metalloproteinase 2; Timp1, tissue inhibitor of metalloproteinase 1.
indicated that tumor nodules were enriched for biliary progenitor markers as well as for c-myc transcripts (Fig. 6). The levels of Ctnnb1 and Hes1 remained unchanged, while expression of Notch1 and Notch2 was down-regulated in tumor nodules.

We then asked whether Foxl1+ HPCs contribute to DEN/TCPOBOP-induced tumor formation. First, to test whether the administration of DEN and TCPOBOP activates Foxl1 expression in the liver prior to tumor formation, we treated Foxl1-Cre;Rosa26YFP mice with DEN 15 days after birth and once with TCPOBOP on postnatal day 29 and harvested the liver 1 week later (Fig. 7A). The presence of Ck19+YFP+ cells in the portal triad at this early time point (Fig. 7B) indicated that the DEN/TCPOBOP protocol activates the Foxl1+ progenitor cell compartment prior to the induction of HCA/HCC. Next, to induce tumor formation, we treated Foxl1-Cre;Rosa26YFP mice with DEN, followed by biweekly injections of TCPOBOP for up to 26 weeks (Fig. 7C). YFP expression was detected in Ck19+ cells in nontumor areas (Fig. 7D). HCA and HCC nodules expressed AFP, Opn, Sox9, Epcam, and Yap1 (Table 1 and Fig. 7E; Supporting Fig. S3). In contrast to the DEN/CCl4 model, the DEN/TCPOBOP condition led to CK19-positive tumor and nontumor parenchymal cells, albeit at lower levels than at the bile ducts (Fig. 7E and Table 1). Despite the presence of multiple YFP+ cells in tumor-adjacent areas, all tumor nodules remained YFP-negative, indicating that Foxl1+ cells are not a source of tumor nodules in this model (Table 1 and Fig. 7E,F).

**Discussion**

Identifying the cell of origin of HCC is crucial to understanding tumorigenesis in the liver. However, which types of liver cells give rise to HCC is still a subject of debate. HCCs frequently express biliary/progenitor markers,\(^{(7,50,51)}\) which are associated with poor prognosis, and stem cell-like features such as ability to self-renew and differentiate.\(^{(4,7)}\) These findings have led to the notion that the cell of origin of HCC might be a progenitor or biliary epithelial cell. Indeed, Holzbaeur and colleagues demonstrated that oncogenic H-Ras and SV40LT transform HPCs, hepatoblasts, and mature hepatocytes into cells that express biliary/
progenitor markers and give rise to tumors upon transplantation, indicating that a strong oncogenic stimulus can transform normal cells regardless of their lineage.\(^{(45)}\) However, two independent studies demonstrated that endogenous biliary lineages do not contribute to HCC in mouse models of liver cancer using lineage tracing\(^{(37,38)}\) and that wild-type HPCs do not give rise to HCC upon transplantation,\(^{(52)}\) putting the progenitor hypothesis of HCC origin into question.

On the other hand, increasing evidence suggests a hepatocyte origin of HCC. Multiple studies have demonstrated the remarkable plasticity of mature hepatocytes.\(^{(16,53,54)}\) Furthermore, He and colleagues identified...
HCC progenitor cells that express biliary/progenitor markers such as Sox9 and Cdh4 in foci of altered hepatocytes of DEN-treated mouse liver. Although the authors did not perform lineage-tracing experiments to identify the cell of origin of HCC progenitor cells, correlative evidence strongly suggested that mature hepatocytes are the cells of origin of DEN-induced HCC progenitor cells. Based on these reports, we hypothesized that mature hepatocytes give rise to HCC, at least in models that include exposure to hepatotoxins, even if they come to express biliary/progenitor markers. Our results clearly demonstrate that in two different mouse models of hepatotoxin-induced liver tumorigenesis, hepatocytes are the cellular source for HCA or HCC that is positive for biliary/progenitor markers.

Two recent studies identified novel cell populations that contribute to parenchymal regeneration—hybrid periportal hepatocytes and Axin2⁺ pericentral cells. While hybrid periportal hepatocytes did not give rise to HCC in three different mouse models of liver cancer, the tumorigenic potential of Axin2⁺ cells has not yet been evaluated. In the current study, AAV labeled both periportal and pericentral hepatocytes. Further studies using alternative labeling strategies will be required to identify the subpopulation of hepatocytes that contributes to tumorigenesis.

Using a complementary approach, we investigated whether Foxl1⁺ HPCs can give rise to tumor cells because a subset of biliary cells within ductular reactions marked by Foxl1-Cre become HPCs that possess the ability to self-renew and differentiate toward both cholangiocyte and hepatocyte lineages. Foxl1⁻⁻ marked HPCs express the progenitor/biliary markers Epcam, Sox9, prominin 1, and Cdh4. Our Foxl1-Cre lineage labeling is constitutive, marking cells with YFP whenever Foxl1 is expressed during development or injury. Thus, if we had found that liver tumors in Foxl1-Cre,Rosa²[YFP] mice express YFP, we would not have been able to state conclusively that these tumors arose from HPCs as the Foxl1 promoter might have been activated in hepatocytes, mesenchymal cells, or other potential tumor precursor cells at any time during toxin exposure and tumorigenesis. However, the fact that all HCA and HCC nodules remained YFP-negative in Foxl1-Cre,Rosa²[YFP] mice indicates that Foxl1-expressing cells do not contribute to HCA/HCC nodules. We conclude that Foxl1-Cre-marked HPCs are not tumor-initiating cells in two models of hepatotoxin-induced tumorigenesis.

Our findings do not rule out the possible contribution of different stem/progenitor populations within the liver to tumor cells, as the Foxl1-Cre transgene does not mark all Ck19-positive cells. However, the fact that AAV-TBG-Cre labels more than 96% of HCAs and all HCC tumor nodules suggests that the contribution of nonhepatocyte cells to DEN/CCl₄ or DEN/TCPOBOP-induced tumors is minimal. Our results are consistent with a recent report demonstrating that cells expressing Hnf1β, a transcription factor specific to biliary cells including ductular reactions, do not contribute to HCC. Furthermore, biliary cells marked with Opn-iCreERT2 also do not contribute to HCC, further supporting our results. Thus, the biological significance of the expansion of ductular reactions during tumor development in models of chemically induced liver cancer appears limited.

Finally, we note that our study does not rule out the potential contribution of Foxl1⁺ HPCs to tumorigenesis in other models of HCC. The hepatotoxin DEN is metabolically activated in hepatocytes, implying that Foxl1⁺ HPCs may not have been exposed to genotoxic stress in our mouse models. Nevertheless, our results indicate that mature hepatocytes can give rise to tumor cells positive for biliary/progenitor markers despite the presence of ductular reactions in tumor-adjacent areas, thus indicating that expression of biliary/progenitor markers by HCC on its own does not support the progenitor hypothesis of HCC origin. In conclusion, our study demonstrates that mature hepatocytes are the cell of origin in two mouse models of liver tumors induced by hepatotoxin treatment. An important question for the future is to determine why HCCs that express progenitor cell markers have a worse prognosis than those that do not.

Acknowledgment: We thank Tia Bernard-Banks (University of Pennsylvania) for managing the mouse colony and Dr. Joshua R. Friedman (University of Pennsylvania) for providing the anti-Ck19 antibody.

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