Mice with hepatocyte-specific FXR deficiency are resistant to spontaneous but susceptible to cholic acid-induced hepatocarcinogenesis

Bo Kong, Yan Zhu, Guodong Li, Jessica A. Williams, Kyle Buckley, Ossama Tawfik, James P. Luyendyk, and Grace L. Guo

1Department of Pharmacology and Toxicology, School of Pharmacy, Rutgers University, Piscataway, New Jersey; 2Department of General Surgery, Xuanwu Hospital, Capital Medical University, Beijing, China; 3Department of General Surgery, the Fourth Hospital of Harbin Medical University, Harbin; 4Division of Biobank Research, Department of General Surgery, the Fourth Hospital of Harbin Medical University, Harbin; 5Department of Pharmacology, Toxicology, and Therapeutics, University of Kansas Medical Center, Kansas City, Kansas; 6Department of Pathology and Laboratory Medicine, University of Kansas Medical Center, Kansas City, Kansas; and 7Pathobiology and Diagnostic Investigation, Michigan State University, East Lansing, Michigan

Submitted 4 May 2015; accepted in final form 12 December 2015

Kong B, Zhu Y, Li G, Williams JA, Buckley K, Tawfik O, Luyendyk JP, Guo GL. Mice with hepatocyte-specific FXR deficiency are resistant to spontaneous but susceptible to cholic acid-induced hepatocarcinogenesis. Am J Physiol Gastrointest Liver Physiol 310: G295–G302, 2016. First published January 7, 2016; doi:10.1152/ajpgi.00134.2015.—Farnesoid X receptor (FXR) belongs to the nuclear receptor superfamily with its endogenous ligands bile acids. Mice with whole body FXR deficiency develop liver tumors spontaneously, but the underlying mechanism is unclear. Moreover, it is unknown whether FXR deficiency in liver alone serves as a tumor initiator or promoter during liver carcinogenesis. This study aims to evaluate the effects of hepatocyte-specific FXR deficiency (FXRhep−/−) in liver tumor formation. The results showed that FXRhep−/− mice did not show spontaneous liver tumorigenesis with aging (up to 24 mo of age). Therefore FXRhep−/− mice were fed a bile acid (cholic acid)-containing diet alone or along with a liver tumor initiator, diethylnitrosamine (DEN). Thirty weeks later, no tumors were found in wild-type or FXRhep−/− mice without any treatment or with DEN only. However, with cholic acid, while only some wild-type mice developed tumors, all FXRhep−/− mice presented with severe liver injury and tumors. Interestingly, FXRhep−/− mouse livers increased basal expression of tumor suppressor p53 protein, apoptosis, and decreased basal cyclin D1 expression, which may prevent tumor development in FXRhep−/− mice. However, cholic acid feeding reversed these effects in FXRhep−/− mice, which is associated with an increased cyclin D1 and decreased cell cycle inhibitors. More in-depth analysis indicates that the increased in cell growth might result from disturbance of the MAPK and JAK/Stat3 signaling pathways. In conclusion, this study shows that hepatic FXR deficiency may only serve as a tumor initiator, and increased bile acids is required for tumor formation likely by promoting cell proliferation.

HCC; tissue specific FXR; bile acids

FARNESOID X RECEPTOR (FXR) is a ligand-activated nuclear receptor and is highly expressed in the liver, intestine, kidney, and adrenals. The endogenous ligands of FXR are bile acids, including primary and secondary bile acids (15, 18, 24). FXR is essential in maintaining bile acid homeostasis, and FXR deficiency has been involved in the pathogenesis of a variety of liver and intestinal diseases. Particularly, FXR serves as a tumor suppressor in the liver and colon, and this emerging concept has been strongly supported by a few studies conducted in whole body FXR knockout mice demonstrating that FXR deficiency leads to spontaneous liver tumor development and increased colon cancer susceptibility; furthermore, the expression and function of FXR was found to be reduced in human liver tumors and colon cancers, indicating a strong link between FXR deficiency and human hepatic and colon cancer development (7, 25, 26, 28).

However, the mechanisms by which FXR deficiency results in tumor development are not clear. Whole body FXR deficiency elevates bile acid levels, and increased bile acids are known to promote tumor development in several carcinogenesis models, including liver and colon cancers (2, 8, 23). However, it is unknown whether liver-specific FXR deficiency serves as a tumor initiator as well, in addition to promoting liver tumor development via increasing bile acid levels. This is because, for the last few years, we and others have identified that FXR likely suppresses tumor development via direct and indirect pathways. In detail, FXR directly serves as a transcription factor to induce the gene expression of several tumor suppressors, including small heterodimer partner (Shp), E-cadherin, and SOCS3, which are mediators critical in suppressing cell cycle proliferation, β-catenin activation, and Stat3 activation, respectively (13, 14, 27, 32). Indirectly, FXR activation prevents overt bile acid levels, and increased bile acids have been shown to lead to increased inflammation, IL-6 production, Stat3 activation, and β-catenin activation (13, 14, 27). Therefore, there is an urgent need to develop an experimental model to test to what degree that specific FXR deficiency in the liver causes liver tumor via direct tumor initiation and/or promotion. This knowledge will help to better design strategies in the future to prevent and/or treat liver tumors associated with FXR malfunction/deficiency.

Thereby, in the present study, we used hepatocyte-specific FXR knockout (KO) mice (FXRhep−/−) to determine to what degree hepatocyte-specific FXR deficiency is a tumor initiator and/or promoter in vivo. In detail, the role of FXR deficiency in hepatocytes as either tumor initiator and/or promoter was tested in four groups of mice: role as both initiator and promoter (mice without chemical treatment: spontaneous tumor development), role as tumor initiator [mice with bile acid treatment as promoter: dietary supplement of low-dose cholic acid (CA); 0.2%], role as tumor promoter [mice with prior treatment as promoter: dietary supplement of low-dose cholic acid (CA); 0.2%], role as tumor promoter [mice with prior treatment as promoter: dietary supplement of low-dose cholic acid (CA); 0.2%].
tumor initiation with \( N, N \)-diethylnitrosamine (DEN), and no effects on liver tumor development (mice treated with both DEN and CA) (20).

MATERIALS AND METHODS

Animals and treatments. FXR hepatocyte-specific knockout mice (FXR\textsuperscript{floxed/floxed}) have been described previously (9). In brief, mice with "floxed" FXR (FXR\textsuperscript{floxed/floxed}) were crossed with FXR\textsuperscript{floxed/floxed} mice carrying albumin promoter-driven Cre (Cre\textsuperscript{cre/cre}) to generate FXR\textsuperscript{floxed/floxed}Cre\textsuperscript{cre/cre} (wild-type mice, herein referred to as WT) and FXR\textsuperscript{floxed/floxed}Cre\textsuperscript{cre/+} (hepatocyte-specific FXR KO mice, herein referred to as FXR\textsuperscript{hko/+} mice). Both WT and FXR\textsuperscript{hko/+} were on a C57BL/6J genetic background and were maintained in pathogen-free animal facilities in the Laboratory of Animal Research under a standard 12-h light-dark cycle with food and water provided ad libitum. All the protocols and procedures were approved by the Institutional Animal Care and Use Committee at the University of Kansas Medical Center.

Male WT and FXR\textsuperscript{hko/+} mice \( (n = 4–15) \) were treated with a single intraperitoneal injection of DEN at 90 mg/kg or PBS vehicle at 0.2% (wt/wt) at 4 wk of age, followed by feeding with 0.2% (wt/wt) CA-containing diet for 30 wk. The gross tumor prevalence is shown in the table. WT, wild-type mice; FXR\textsuperscript{hko/+}, farnesoid X receptor-deficient mice.

Four-weeks-old mice were treated with a single dose (90 mg/kg ip) of \( N, N \)-diethylnitrosamine (DEN), followed by feeding with 0.2% (wt/wt) cholic acid (CA)-containing diet for 30 wk. The gross tumor prevalence is shown in the table. WT, wild-type mice; FXR\textsuperscript{hko/+}, farnesoid X receptor-deficient mice.

**Table 1. Tumor morphological prevalence of CA- and DEN-treated mice**

<table>
<thead>
<tr>
<th>Group</th>
<th>Mouse number</th>
<th>Tumor prevalence, %</th>
<th>Mouse number</th>
<th>Tumor prevalence, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chow + Vehicle</td>
<td>5</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>CA + Vehicle</td>
<td>7</td>
<td>0</td>
<td>15</td>
<td>100</td>
</tr>
<tr>
<td>Chow + DEN</td>
<td>9</td>
<td>0</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>CA + DEN</td>
<td>9</td>
<td>44</td>
<td>7</td>
<td>100</td>
</tr>
</tbody>
</table>

 Four-wk old mice were treated with a single dose (90 mg/kg ip) of \( N, N \)-diethylnitrosamine (DEN), followed by feeding with 0.2% (wt/wt) cholic acid (CA)-containing diet for 30 wk. The gross tumor prevalence is shown in the table. WT, wild-type mice; FXR\textsuperscript{hko/+}, farnesoid X receptor-deficient mice.

**Table 2. Summary of neoplastic prevalence in CA- and DEN-treated mice**

<table>
<thead>
<tr>
<th>Ctrl/Veh</th>
<th>CA/Veh</th>
<th>Ctrl/DEN</th>
<th>CA/DEN</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT ( (n = 5) )</td>
<td>CA/Veh ( (n = 4) )</td>
<td>Ctrl/DEN ( (n = 9) )</td>
<td>CA/DEN ( (n = 7) )</td>
</tr>
<tr>
<td>High-grade dysplasia</td>
<td>0.0%</td>
<td>0.0%</td>
<td>28.6%</td>
</tr>
<tr>
<td>Low-grade dysplasia</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
</tr>
<tr>
<td>Focal portal inflammation</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
</tr>
</tbody>
</table>
low-grade and 60% high-grade dysplasia and 27% focal portal inflammation. With DEN treatment only, none of the WT mice developed dysplasia or portal inflammation, whereas 29% of FXRhep^-/-^ mice developed low-grade dysplasia and 57% showed focal portal inflammation. For DEN/CA treatment, all WT mice presented with high-grade dysplasia, none with low-grade dysplasia, and 33% with focal portal inflammation; and for FXRhep^-/-^ mice, 57% showed high-grade dysplasia, 43% low-grade dysplasia, and 14% focal portal inflammation.

The body weight was increased in WT mice with CA feeding (Fig. 1C) but decreased in FXRhep^-/-^ KO mice. DEN treatment prevented CA-associated weight gain in WT mice but resulted in more weight loss in FXRhep^-/-^ mice. CA reduced the liver-to-body weight (LW/BW) ratio in WT mice but increased that in FXRhep^-/-^ mice. DEN slightly reduced the LW/BW ratio in not only WT and but also FXRhep^-/-^ mice. DEN/CA cotreatment did not change the LW/BW ratio in WT mice but markedly increased that in the FXRhep^-/-^ mice.

Serum lipid biochemistry and markers of liver injury. Since bile acid metabolism is closely related to lipid homeostasis, we measured triglyceride levels in these mice. The FXRhep^-/-^ mice had higher serum triglyceride levels than the WT mice. Treatment with CA reduced lipid levels in WT mice but further increased those in FXRhep^-/-^ mice, indicating that hepatic FXR is mainly responsible for the lipid-lowering effect following FXR activation. DEN/CA cotreatment increased triglyceride levels in both WT and FXRhep^-/-^ mice (Fig. 2). With vehicle or DEN treatment, both WT and FXRhep^-/-^ mice had normal ALT levels, indicating lack of obvious liver injury. The CA treatment increased ALT levels fourfold in FXRhep^-/-^ mice, but not in the WT mice, but the combined DEN/CA treatment increased ALT levels in FXRhep^-/-^ as well as WT (fourfold). ALP levels were increased markedly in FXRhep^-/-^ mice with CA or DEN/CA treatment and moderately in WT mice with DEN/CA treatment, indicating degree of bile duct epithelial cell injury. Bile acids levels were increased in WT mice under CA or DEN/CA treatment, but the levels were within the
normal range. However, FXR$^{−/−}$ mice showed much higher bile acid levels with CA treatment (100–150 μM), suggesting that hepatic FXR is required for the defense against bile acid toxicity. The increases in ALP and bile acids shared the same trend in FXR$^{−/−}$ mice, indicating that increases in bile acid levels are associated with bile duct epithelial cell injury. The increased liver damage in FXR$^{−/−}$ mice was also reflected by a marked increase in bilirubin levels (Fig. 2).

Expression of genes involved in bile acid homeostasis in the liver. Figure 3 shows that basal mRNA expression of Fxr, Shp, and Bsep in the liver was lower in FXR$^{−/−}$ mice than that in WT mice, and CA treatment increased the mRNA levels of these genes in WT mice, but not in FXR$^{−/−}$ mice, suggesting that Fxr activation is required to maintain and induce the expression of Shp and Bsep. Moreover, CA treatment further reduced Bsep expression in the FXR$^{−/−}$ mice, likely reflecting a suppression of Bsep expression via injury/inflammation. In agreement with our previous publication (9), Cyp7a1 mRNA levels were moderately higher in FXR$^{−/−}$ mice than those in WT mice. Treatment with CA markedly suppressed Cyp7a1 mRNA levels, but the degree of suppression was smaller in FXR$^{−/−}$ mice than in WT mice.

Cell proliferation and death. Figure 4 shows the protein levels of genes critical for cell proliferation and cell apoptosis, including cyclin D1, cdk4, cyclin E1, cdk2, PCNA, PARP1, and caspase-3. The basal levels of cyclin D1 were lower in WT mice and almost undetectable in FXR$^{−/−}$ mice, consistent with our previous finding that cyclin D1 is a FXR target gene in the liver (1, 22). The cyclin D1 levels were slightly higher in WT mice after CA treatment, but markedly higher in the FXR$^{−/−}$ mice, suggesting that cyclin D1 is induced via-FXR independent mechanism(s). Cyclin D1 levels were slightly reduced in WT mice by DEN treatment, but markedly induced in both strains by the combined DEN/CA treatment. These data suggest that cyclin D1 induction may serve as a marker for tumor development in this study. Interestingly, the trend of change for cyclin E1 was opposite of that of cyclin D1. Furthermore, despite reduced basal cyclin D1 in FXR$^{−/−}$ mice, the levels of its partner, cdk4, were higher (perhaps to compensate for the loss of cyclin D1). The levels of cdk4 were further induced by CA and DEN/CA in WT and FXR$^{−/−}$ mice. Cyclin E1’s partner, cdk2, was elevated strongly in FXR$^{−/−}$ mice and moderately in WT mice with DEN/CA treatment, but its expression was very low in other groups. PCNA, a marker for DNA replication, was markedly induced by DEN/CA in both strains. Cleaved caspase-3 was present only in FXR$^{−/−}$ mice after DEN/CA treatment, indicating that hepatic FXR may prevent apoptosis. The levels of cell cycle inhibitors were also determined (Fig. 5). The level of p15 showed no change with CA or DEN treatment, whereas p21 and p27 levels followed a similar trend of changes in FXR$^{−/−}$ mice. In detail, WT mice had reduced expression of p21 following CA treatment with and without DEN, but p21 level was almost undetectable by treatment with CA regardless of DEN treatment in FXR$^{−/−}$ mice. These data suggest that one of the mechanisms by which FXR deficiency and bile acids cause liver tumor formation is by reducing cell cycle inhibitors. The basal levels of p53 were undetectable in the WT mouse livers but were induced by CA or DEN and also synergistically induced by DEN/CA cotreatment. Basal p53 expression was very strong in FXR$^{−/−}$ mice compared with that of WT mice, but treatment with CA or DEN reduced its expression. These changes in FXR$^{−/−}$ mice in response to CA or DEN treatment were opposite to that of WT mice, suggesting that FXR is critical in regulating p53 expression in the liver. Levels of Gadd45a expression followed the same trend as p53 with the treatment of vehicle, CA, and DEN in both WT and FXR$^{−/−}$ mice. But under DEN/CA treatment, despite increased p53 levels, the levels of Gadd45a were very low. Survivin levels were slightly higher in FXR$^{−/−}$ mice than those in WT mice, but treatment with DEN/CA markedly diminished its levels.

Activation of MAPK pathway. Increased bile acids are known to activate MAPK signaling pathways, especially via activation of JNK and ERK (5, 19). However, in our study, we found that the total JNK1/2 levels were not changed in these groups, but the p-JNK1/2 levels were markedly reduced by CA and/or DEN treatments (Fig. 6). Even though the total bile acid levels were not increased by hepatocyte-specific deletion of FXR, the FXR$^{−/−}$ mice had increased total and phosphor-
ylated c-Jun, which was, surprisingly reduced by CA and/or DEN treatment (Fig. 6). CA treatment increased p-Erk1 in FXRhep/H11002/H11002 mice and DEN/CA increased p-Erk1 in both strains. The results suggest that long-term bile acid treatment may desensitize MAPK activation to some degree, and levels of T- or p-c-Jun were negatively, whereas the levels of p-Erk1 were positively, correlated with liver tumor formation in this study.

Activation of Stat3 pathway. Stat3 activation has been demonstrated to promote cell cycle progression and tumorigenesis by increasing the gene transcription of genes involved in prosurvival and proinflammatory pathways (3, 6, 30). Our previous study also indicates that constitutive activation of Stat3 may be involved in tumorigenesis in liver of FXR whole body knockout mice (14). Here we further examined activation of the Stat3 pathway in FXRhep/H11002/H11002 mice (Fig. 7). The FXRhep/H11002/H11002 mice appeared to have higher Stat3 activation, suggested by increased GP130, JAK1, JAK2, and p- and total-Stat3, and decreased Socs3. This result is consistent with our previous report that there was a FXR/Socs3/Stat3 regulatory loop by which FXR positively induces Socs3 to suppress Stat3 activation (14). In the FXR whole body KO mice, p-Stat3 was increased at both Y705 and S727; however, in the present study, both CA and DEN treatment resulted in downregulation of total and p-Stat3 (Y705) protein levels, whereas p-Stat3 (S727) protein levels were increased. This differential phosphorylation of Stat3 protein in FXR whole body KO and FXRhep/H11002/H11002 mice suggests a more complicated regulation of Stat3 activation by FXR deficiency and increased bile acid concentration.

Altered liver bile acids profiles in FXR knockout mice. The gathered data shown in this study indicate that hepatic FXR serves a cytoprotective role during the initiation stage of carcinogenesis. As the master regulator of bile acid homeostasis, FXR regulates the synthesis and transport of bile acids. Moreover, the alteration in bile acid pool and composition could play a role in the tumorigenesis, especially tumor promotion. The total bile acid pool size has also been published by us previously, showing that bile acid pool size was only slightly increased in FXRhep/H11002/H11002 mice compared with WT mice (9). The liver bile acid composition and bile acid pool were measured in the present study by the UPLC/MS method (Fig. 8). Levels of four bile acids (GDCA, LCA, GUDCA, and T-H9275-MCA) were below the UPLC-MS detection limits among 23 bile acids measured. For bile acids that have relatively higher concentration in the liver (Fig. 8A, left) and compared with WT mice, the relative percentage of β-MCA and ω-MCA in the liver was increased significantly in FXR KO and FXRhep/H11002/H11002 mice. Among the bile acids that have relatively lower concentration in the liver, CDCA, α-MCA, and HDCA were also significantly higher in FXRhep/H11002/H11002 mice (Fig. 8A, right). In the total bile acid pool, the individual bile acid composition was similar to that in the liver (Fig. 8B). Overall, the levels of ω-MCA, DCA, CDCA, and α-MCA were significantly increased in FXRhep/H11002/H11002 mice, whereas HDCA and β-MCA also showed a slight increase in these mice compared with WT mice.

DISCUSSION

The bile acid-activated nuclear receptor, FXR, is known to suppress hepatocellular carcinoma (HCC) and colon cancer development, and particularly, whole body FXR deficiency in mice leads to spontaneous liver tumor development (7, 16, 27, 28). However, the mechanism by which FXR suppresses carcinogenesis is not entirely clear. FXR is essential in maintaining bile acid homeostasis, and deletion of FXR in mice results in increased bile acids level in the body because of the
increasing bile acid biosynthesis and altered transport. Bile acid is well known for its tumor promotion effects and has been found to promote carcinogenesis in several tumor models, including HCC, cholangiocarcinoma, colon cancer, and breast cancer. Consequently, the spontaneous liver tumorigenesis in FXR KO mice can result from the direct effects of loss of FXR-dependent protection and/or the increased bile acid toxicity. A recent study (4) shows that whole body FXR knockout mice with intestine-specific FXR overexpression could prevent the spontaneous hepatocarcinogenesis, likely due to the reestablishment of suppression of Cyp7a1 expression with the intact intestinal FXR/FGF15 signal to reduce bile acid levels. Another study had been conducted on an intestinal tumorigenesis model of FXR KO/ApcMin/H11001 mice, which showed that reducing bile acid levels with bile acid binding resin, cholestyramine, did not modify the intestinal tumor susceptibility of FXR/H11002/H11002 mice (17). All these studies indicate that not only elevated bile acid concentrations increase susceptibility to tumorigenesis, but loss of FXR is also involved; however, the exact role of FXR as tumor initiator or promoter is not clear.

In this study, we used the hepatocyte-specific FXR knockout mice, which have serum bile acid levels comparable to those of WT mice, and determined the role of hepatic-specific deficiency of FXR as initiator and/or promoter in liver tumor development by using the two-stage tumorigenesis model (DEN initiation/bile acid promotion) in hepatocyte-specific FXR KO (FXRhep/H11002/H11002) mice. This study highly suggests that FXR deficiency in hepatocytes likely serves as a tumor initiator. The present study clearly showed that FXR deficiency in hepatocytes did not result in spontaneous liver tumor development in mice, indicating that, in contrast to whole body FXR deficiency, hepatocyte-specific FXR deficiency is not sufficient for tumor development, i.e., FXR deficiency in hepatocytes is not a complete tumor initiator and promoter. The basal bile acid levels in the FXRhep/-/- mice were comparable to those in WT mice, suggesting that additional injury due to elevated bile...
acids is likely required for tumorigenesis in the liver. We have measured the relative bile acid composition in the livers of WT, FXR KO, and FXR\textsuperscript{hep\textsuperscript{-/-}} mice and found overall the bile acid composition among these three strains was similar, with the exception for a few bile acid species that were altered with whole body FXR deficiency or hepatic FXR deficiency. Furthermore, the liver-specific FXR deficiency only slightly altered the bile acid composition. For example, ω-MCA was higher in the FXR\textsuperscript{hep\textsuperscript{-/-}} mice. The MCAs are found to be FXR antagonists than agonists, and this differential FXR modulating function may alter the homeostasis by which FXR maintains in vivo in mice (12, 21). Indeed, the FXR\textsuperscript{hep\textsuperscript{-/-}} mice showed much higher tumor prevalence under chronic bile acid feeding, confirming that additional injury is required for liver tumorigenesis under hepatic FXR-deficient conditions. Moreover, FXR deficiency is likely a tumor initiator rather than a promoter because treatment with the tumor initiator, DEN, did not render the FXR\textsuperscript{hep\textsuperscript{-/-}} mice more susceptible to tumor development. These results are in agreement with the earlier findings of others (4, 17), indicating that liver tumorigenesis depends on both the initiation effects from loss of hepatic FXR function and tumor promotion from increased bile acids.

FXR deficiency increased liver injury in the DEN/CA model, as reflected by body weight loss and increased levels of liver enzymatic activities, bile acids, and bilirubin. It is surprising that FXR\textsuperscript{hep\textsuperscript{-/-}} mice showed marked injury following bile acid feeding. Our previous studies suggest that the intestine serves is critical for regulating bile acid synthesis under physiological conditions (9), but this study clearly indicates an important role of hepatic FXR in bile acid detoxification. We found that expression of Bsep was low in the FXR\textsuperscript{hep\textsuperscript{-/-}} mice and was further downregulated after CA treatment, indicating a critical role of Bsep in removing bile acids from the liver and thus reducing toxicity. The importance of Bsep in cholestasis development has been well documented in human patients with Bsep gene (ABCB11) mutation. In mice, deletion of the Bsep gene led to an upregulation of hepatic sinusoidal transporters transporting bile acids out of hepatocytes (10), but this upregulation of sinusoidal efflux transporters may not be sufficient to replace the central role of Bsep in removing bile acids out of hepatocytes.

Our laboratory and others have provided emerging evidence suggesting that FXR may directly suppress cell proliferation by inducing a few tumor suppressors (Shp, Socs3, and E-cadherin) and/or indirectly suppress cell proliferation by reducing bile acid levels as increased bile acids activate Stat3, MAPK, and β-catenin (13, 14, 27), which are briefly summarized in Fig. 9.

Interestingly, one of the central tumor suppressors, p53, was markedly induced in the FXR\textsuperscript{hep\textsuperscript{-/-}} mouse livers. The mechanism, for which we have hypothesized, is likely due to the reduction of Shp in FXR\textsuperscript{hep\textsuperscript{-/-}} mice. Shp is a classical FXR target gene in the liver and the reduction of p53 by Shp has been reported with the mechanism showing that Shp mediates the stabilization of mdm2, the protein that promotes p53 degradation (29, 32). Furthermore, Shp has also been shown to inhibit p53 occupancy in its target genes (11); therefore, the lack of Shp in FXR\textsuperscript{hep\textsuperscript{-/-}} mice could additionally enhance p53 transcriptional activity (Fig. 9). But, apparently, p53 is also regulated by mechanism(s) other than Shp, as bile acid feeding induced Shp in WT mice, which did not lead to a reduction of p53.

Hepatocarcinogenesis is a complicated and multistep process through differential mechanisms, and dysregulation of multiple signaling pathways is implicated in HCC carcinogenesis and progression, including the WNT/β-catenin, mitogen-activated protein kinase (MAPK), IL-6/Stat3 pathways, etc. This study also shows the paradoxical effects of FXR modulation on cell proliferation and regulation of the signaling pathways. We have reported that FXR directly induces several tumor suppressors, including Shp, E-cadherin, and Socs3, to suppress cell proliferation. In addition, FXR is well known to suppress bile acid levels and thus to inhibit bile acid-induced cell proliferation. In contrast, the present study showed reduced expression of Cyclin D1 in FXR\textsuperscript{hep\textsuperscript{-/-}} mice, which is in agreement with our previous report that FXR positively regulates Cyclin D1 gene expression (27). The mechanism of increased p21 and p27 levels needs to be further determined but likely could be due to indirect regulation with FXR deficiency.

In summary, this study clearly suggests that male mice with hepatocyte-specific FXR deficiency are resistant to spontaneous liver tumor formation. However, hepatocyte-specific FXR deficiency may initiate liver carcinogenesis. Furthermore, increased bile acid level, commonly observed in a variety of liver diseases, strongly leads to liver injury and promotes liver tumorigenesis under conditions of FXR deficiency.

GRANTS
This study was funded by National Institute of General Medical Sciences Grant R01GM104037 (principal investigator G. Guo).

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES
BILE ACID-INDUCED BUT NO SPONTANEOUS HCC IN FXRHEP \(^{-/-}\) MICE


