Prevention of Spontaneous Hepatocarcinogenesis in Farnesoid X Receptor–Null Mice by Intestinal-Specific Farnesoid X Receptor Reactivation

Chiara Degirolamo, Salvatore Modica, Michele Vacca, Giuseppe Di Tullio, Annalisa Morgano, Andria D’Orazio, Kristina Kannisto, Paolo Parini, and Antonio Moschetta

Farnesoid X receptor (FXR) is the master regulator of bile acid (BA) homeostasis because it controls BA synthesis, influx, efflux, and detoxification in the gut/liver axis. Deregulation of BA homeostasis has been linked to hepatocellular carcinoma (HCC), and spontaneous hepatocarcinogenesis has been observed in FXR-null mice. This dreaded liver neoplasm has been associated with both FXR gene deletion and BA-mediated metabolic abnormalities after inactivation of FXR transcriptional activity. In the present study, we addressed the hypothesis that intestinal selective FXR reactivation would be sufficient to restore the fibroblast growth factor 15 (FGF15)/cholesterol-7alpha-hydroxylase (Cyp7a1) enterohepatic axis and eventually provide protection against HCC. To this end, we generated FXR-null mice with re-expression of constitutively active FXR in enterocytes (FXR<sup>+/−</sup>/iVP16FXR) and corresponding control mice (FXR<sup>+/−</sup>/iVP16). In FXR-null mice, intestinal selective FXR reactivation normalized BA enterohepatic circulation along with up-regulation of intestinal FXR transcriptome and reduction of hepatic BA synthesis. At 16 months of age, intestinal FXR reactivation protected FXR-null mice from spontaneous HCC development that occurred in otherwise FXR-null mice. Activation of intestinal FXR conferred hepatoprotection by restoring hepatic homeostasis, limiting cellular proliferation through reduced cyclinD1 expression, decreasing hepatic inflammation and fibrosis (decreased signal transducer and activator of transcription 3 activation and curtailed collagen deposition).

Conclusion: Intestinal FXR is sufficient to restore BA homeostasis through the FGF15 axis and prevent progression of liver damage to HCC even in the absence of hepatic FXR. Intestinal-selective FXR modulators could stand as potential therapeutic intervention to prevent this devastating hepatic malignancy, even if carrying a somatic FXR mutation. (Hepatology 2015;61:161-170)

Hepatocellular carcinoma (HCC) is the fifth-most prevalent type of cancer and the second-leading cause of cancer-related death, with over...
500,000 new cases and more than 695,000 deaths globally per year. This deadly malignancy mostly occurs within an already established chronic liver disease (CLD) and displays a highly heterogeneous etiology, with hepatitis B and C, alcoholic liver disease, and nonalcoholic steatohepatitis being the most prominent factors. Less common etiological factors are hereditary hemochromatosis, z1-antitrypsin deficiency, and some porphyrias.1

The most common and unifying condition associated with HCC is cirrhosis, which unfolds after long latencies of CLD; notably, HCC, is the leading cause of death in patients with compensated liver cirrhosis.2

An association between HCC development and altered bile acid (BA) metabolism has been documented in both animal and human studies.3-7 BAs are endproducts of cholesterol metabolism, synthesized and conjugated in the liver and secreted by bile into the small intestine, where they aid in the solubilization and absorption of lipids, nutrients, and vitamins. Gut/liver axis homeostasis relies on a tight control of BA levels to avoid BA overload. Elevated BA concentrations induce DNA oxidative damage, inflammation, nuclear factor kappa B (NF-kB) activation, resistance to apoptosis, and enhanced cell proliferation, thus promoting neoplastic transformation of hepatocytes and enterocytes.8 The nuclear receptor, farnesoid X receptor (FXR), is the master regulator of BA homeostasis by controlling BA synthesis, influx, efflux, and detoxification in the gut/liver axis and has been implicated in both liver and intestinal tumorigenesis.7,9-14

Earlier work from Drs. Moore, Huang, and Gonzalez’s laboratories first documented the link between FXR deficiency and hepatocarcinogenesis, thus electing aged FXR-null mice as a valuable animal model for studying HCC development in a context of diseased liver. Aged male and female FXR-null mice develop spontaneous HCC by the age of 12-16 months, and their livers exhibit increased inflammation, cell proliferation, activation of Wnt/β-catenin pathway, and c-myc signaling.7,13,15 Strategies aimed at limiting BA overload are anticipated to provide hepatoprotection, as earlier reported in FXR-null mice treated with BA-sequestering agents.7 However, it is still unclear whether FXR-mediated BA homeostasis restoration or the effective presence of a functional hepatic FXR transcriptional machinery would prevent hepatocarcinogenesis.

Tissue-specific knockout (KO) and selective transgenic (Tg) FXR constitutive activation studies revealed that some molecular pathways are primarily governed by hepatic FXR, whereas others are preferentially controlled by intestinal FXR.16-18 Moreover, the tissue-specific pattern of FXR regulation and its pathophysiological relevance to liver regeneration capacity and ability to cope with BA overload has been documented in rodents.17,19,20 Indeed, there is a compelling evidence that tissue-specific FXR modulators may hold promise in the prevention and/or treatment of CLD and, ultimately, HCC.

In the present work, we first show that intestinal FXR activation is sufficient to restore BA homeostasis in both young and aged FXR-null mice, thus protecting them from age-related hepatic inflammation, fibrosis, and cancer.

Materials and Methods

Animals. Intestinal-specific Tg, transgenic iVP16FXR and control iVP16 mice on a pure FVB/N background were previously generated.17 Whole-body FXR2−/− mice were originally obtained from Dr. Frank J. Gonzalez (National Institutes of Health, Bethesda, MD). FXR2−/−/iVP16 and FXR2−/−/iVP16FXR mice were created by cross-breeding, for more than eight generations, C57B6J pure strain FXR2−/− and FVBN pure strain iVP16 and iVP16FXR mice. FXR2−/− mice carrying the transgene, hFXR, under the control of the villin promoter were identified by polymerase chain reaction (PCR) of tail genomic DNA to confirm the presence of the hFXR coding sequence. Four- and sixteen-month-old male mice were used in the experiments. All mice were housed under a standard 12-hour light/dark cycle and fed standard rodent chow diet and autoclaved tap water ad libitum. All experiments were approved by the ethical committee of the Fondazione Mario Negri Sud (Chieti, Italy) and certified by the Italian Ministry of Health in accord with internationally accepted guidelines for animal care.

BA measurements, serum analysis, histology and immunohistochemistry (IHC), RNA isolation, quantitative real-time PCR, protein extract preparation and immunoblotting analysis, intestinal permeability assay, and microarray analysis are detailed in the Supporting Information.

Statistical Analysis. All results are expressed as means ± standard error of the mean (SEM). Significant
differences between two groups were determined by Mann-Whitney’s U test. Comparison between multiple groups was assessed using nonparametric analysis of variance (ANOVA; i.e., Kruskal-Wallis’ test). Posthoc, multiple, pair-wise comparisons were calculated with Nemenyi-Damico-Wolfe-Dunn’s test. Comparison between proportions was evaluated using the chi-square (χ²) test. All statistical analyses were performed with GraphPad Prism software (v5.0; GraphPad Software Inc., San Diego, CA) and conducted as a two-sided alpha level of 0.05.

**Results**

**Intestinal FXR Reactivation Restores BA Enterohepatic Circulation in Young FXR-Null Mice.** To investigate whether intestinal constitutive FXR activation would be sufficient to recover BA enterohepatic circulation in FXR-null mice, we cross-bred FXR-null mice with intestinal-specific Tg iVP16FXR and control iVP16 mice, thus generating FXR²⁻⁻ iVP16FXR and FXR²⁻⁻ iVP16 mice, respectively. Gene expression analysis of the ileum of FXR²⁻⁻ iVP16FXR mice revealed a significant up-regulation of intestinal FXR target genes, including fibroblast growth factor 15 (Fgf15), ileal BA-binding protein (Ibabp), and organic solute transporter alpha and beta (Ostα and β), thus confirming the transgene expression of FXR in intestinal epithelium, compared to control FXR²⁻⁻ iVP16 mice (Fig. 1A). Fgf15 functions as a hormone through activating FGF receptor (FGFR)4 in the liver, which triggers a signaling cascade involving the mitogen-activated protein kinase (extracellular signal-regulated kinase; ERK) and c-Jun N-terminal kinase (JNK). The expression of these downstream mediators was restored, as indicated by the increased expression of ERK1/2 and JNK1/3 in the liver of FXR²⁻⁻ iVP16FXR mice (Fig. 1B). The expression of fibrate-activated receptor (FXR) target genes was also restored, with increased expression of Cyp7a1, cholesterol-7β-hydroxylase; Cyp8b1, sterol-12α-hydroxylase; and Cyp7b1, cholesterol-7α-hydroxylase, compared to FXR²⁻⁻ iVP16 mice (Fig. 1D). The expression of BA-synthesizing enzymes, including Cyp7a1 and Cyp8b1, was also restored, with increased expression in the liver of FXR²⁻⁻ iVP16FXR mice (Fig. 1E). These results provide evidence that intestinal constitutive FXR activation is sufficient to restore BA enterohepatic circulation in FXR-null mice.
protein kinases 1 and 2 (ERK1/2)) pathway and synergizes with small heterodimer partner (SHP) to repress hepatic cholesterol-7-alpha-hydroxylase (Cyp7a1) expression through involvement of the c-Jun (NH2)-terminal kinase (JNK)-dependent pathway.16,18,21 Fgf15 requires β-klotho to stably bind to FGFR4,22,23 and both β-klotho and Fgfr4 transcript levels were found to be up-regulated upon intestinal selective FXR reactivation (Fig. 1B). FGFR4 activation was associated with JNK, but not ERK1/2, phosphorylation and with no changes in FGF receptor substrate 2 (FRS2) protein level (Fig. 1C), thus suggesting that FGF15-induced signaling cascades were partially activated. Cyp7a1 gene and protein (Fig. 1D) expression were down-regulated in FXR+/−/iVP16FXR, compared to control FXR+/−/iVP16, mice. In agreement with previous tissue-specific KO studies,16,18 intestinal FXR did not affect Cyp8b1 expression (Fig. 1E), thus confirming that FXR-mediated repression of Cyp8b1 was more dependent on the presence of FXR in the liver, rather than in the intestine. Interestingly, transcript levels of Cyp7b1, a major enzyme in the alternative BA synthetic pathway, were highly up-regulated in FXR+/−/iVP16FXR, compared to control FXR+/−/iVP16, mice (Fig. 1E). Down-regulation of Cyp7a1 and up-regulation of Cyp7b1 were associated with an increased percentage of beta-muricholic acid (β-MCA) at the expense of cholic acid (CA) in the biliary BA pool, as indicated in Table 1. Up-regulation of intestinal FXR transcriptope along with suppression of hepatic Cyp7a1 expression rescued young FXR-null mice from a massive BA overload, as indicated by the reduced BA content in serum, bile, liver, and feces of FXR+/−/iVP16FXR, compared to control FXR+/−/iVP16, mice (Fig. 2A). Finally, these metabolic changes were not accompanied by significant alterations in either basolateral, including multidrug resistance-associated protein (Mrp)4 or canalicular (Fig. 2B,C) BA transporters, but in Oatp1 and 2.

**Intestinal FXR Reactivation Protects From HCC Progression.** Aged FXR-null mice have been elected as a unique animal model for studying HCC pathogenesis because they resemble many features of human HCC progression: inflammation; steatohepatitis; fibrosis; and cancer.24 In the absence of FXR, chronically higher BA levels elicit a plethora of detrimental cell responses, including cell hyperproliferation, NF-κB activation, and DNA oxidative damage, thus laying the foundation to hepatocarcinogenesis. An earlier study suggested that attenuation of systemic BA overload by BA-sequestering agent treatment may reduce the number and size of liver tumors.7 Because FXR modulators are able to restore BA homeostasis, along with repression of NF-κB signaling, inhibition of collagen deposition, and apoptosis, one can hypothesize that FXR modulation may serve as a promising approach to prevent age-related HCC. To investigate whether the metabolic changes induced by intestinal FXR activation in young FXR-null mice (Figs. 1 and 2) would translate in hepatoprotection at older age, FXR+/−/iVP16 and FXR+/−/iVP16FXR mice were sacrificed at 16 months of age when HCC incidence was reported as occurring in almost 100% of aged FXR-null mice.7 Sixteen-month-old FXR+/−/iVP16 mice developed grossly identifiable liver tumors (tumor number ranging from 4 to 12), whereas none of them was found in Tg FXR−/−/iVP16FXR (P < 0.001, χ² test; Fig. 3A). In agreement with previous studies, FXR−/−/iVP16 mice exhibited a higher liver/body-weight ratio and a marked elevation of liver enzymes (alanine aminotransferase [ALT] and aspartate aminotransferase [AST] levels), compared to FXR+/−/iVP16FXR mice (Fig. 3B). Importantly, restoration of BA homeostasis in aged FXR-null mice (Fig. 3C,D) was paralleled by HCC prevention, even in the absence of hepatic FXR.

**At Young Age, Intestinal FXR Reactivation Induces Metabolic Changes That Set the Stage for HCC Prevention.** To elucidate the early events contributing to HCC formation, we first analyzed liver morphology and compared histological changes between FXR+/−/iVP16 and FXR+/−/iVP16FXR at both young (4 months) and older (16 months) age. At young age, FXR+/−/iVP16 livers clearly showed liver injury and a disrupted hepatic parenchymal structure, whereas at older age lobular inflammation (score 3) and a marked necrosis (Histological Activity Index score) worsened the observed phenotype (Fig. 4A). As early as 4 months of age, FXR+/−/iVP16FXR livers displayed a more preserved hepatic parenchyma, and by 16

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**Table 1. Biliary Bile Acid Composition**

| Genotype | Age | CA % | β-MCA % | 2-MCA % | CDCA % | DCA % | UDCA % |
|----------|-----|------|---------|---------|--------|-------|--------|
| FXR+/−/iVP16 | Y | 89.7 ± 2.3 | 7.2 ± 1.7 | 1.0 ± 0.25 | 0.2 ± 0.1 | 0.73 ± 0.5 | 0.27 ± 0.1 |
| FXR+/−/iVP16FXR | Y | 50.9 ± 6.6 | 39.8 ± 6.6 | 5.9 ± 0.1 | 0.4 ± 0.1 | 1.5 ± 0.1 | 0.63 ± 0.1 |
| FXR+/−/iVP16 | A | 70.6 ± 11.8 | 23.9 ± 13.9 | 1.5 ± 0.5 | 0.37 ± 0.2 | 1.87 ± 0.5 | 0.5 ± 0.25 |
| FXR+/−/iVP16FXR | A | 40.4 ± 6.0 | 49.8 ± 5.8 | 4.9 ± 0.2 | 0.43 ± 0.1 | 0.86 ± 0.1 | 1.2 ± 0.12 |

Abbreviations: Y, young (4 months old); A, aged (16 months old); CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; UDCA, ursodeoxycholic acid.
months of age, displayed less necrotic areas and inflammatory infiltrates. Finally, in contrast to aged FXR$^{-/-}$ iVP16 mice, no signs of lipid-containing vacuoles were found in any FXR$^{-/-}$ iVP16FXR mice at the same age. Previous studies have shown that, during aging, FXR-null mice are characterized by enhanced cell proliferation, fibrogenesis, and up-regulation of fibrolysis-related transcripts.7,13,24,25 To explore the mechanisms underlying intestinal FXR-mediated hepatoprotection, we examined the extent of collagen deposition in livers isolated from both young and aged mice, and we found less Sirius Red-positive cells in FXR$^{-/-}$ iVP16FXR livers (Fig. 4A).

Intestinal FXR Activation Prevents FXR-Deficiency-Associated Inflammatory Status in Both the Liver and the Gut. Liver histological analysis (Fig. 4A) revealed that intestinal FXR activation confers benefits to liver health status as early as 4 months of age, thus suggesting that FXR may regulate cellular responses in the initiation phases of liver tumorigenesis. HCC arises in a setting of a chronic inflammatory state and, accordingly, tumor-bearing FXR-null mice have higher serum and hepatic cytokine (interleukin [IL]-6, IL-1β, tumor necrosis factor alpha [TNF-α], and interferon gamma [IFN-γ]) levels,7,13,24 thus implicating a putative protective role for FXR in BA-induced hepatic inflammation. Several cytokines and their downstream mediators are able to divert inflammation to liver carcinogenesis, and among them, IFN-γ and signal transducer and activator of transcription 3 (STAT3) appear more prominent. IFN-γ has been found as the most up-regulated cytokine in FXR-null mouse livers,13 whereas STAT3 activation has been detected in aged FXR-null livers and considered a mechanism of hepatocarcinogenesis.26-28 Conversely, FXR agonists proved to be effective at suppressing NF-κB activation and countering STAT3 phosphorylation in hepatoma cell lines,29,30 thus suggesting that FXR modulation could stand as a strategy to suppress inflammation-driven hepatocarcinogenesis. To explore whether intestinal FXR activation could reverse or attenuate the chronic
inflammatory state observed in FXR-null mice, we first monitored cytokine gene expression levels, and we found that \( \text{Il6} \), \( \text{Tnf}a \), and \( \text{IFN}c \) transcripts were lower in FXR\(^{2/2}\)iVP16FXR livers, compared to FXR\(^{2/2}\)iVP16 (Fig. 4B). Moreover, upon intestinal FXR activation, STAT3 activation, as assessed by the amount of Tyr705-phosphorylated protein, was inhibited, whereas total STAT3 protein remained unchanged (Fig. 4C).

Given the importance of the intestinal mucosal barrier in systemic inflammatory inputs, we examined the gene-related changes induced by intestinal FXR reactivation in FXR-null enterocytes. Interestingly, genes involved in BA detoxification, including aldo-keto-reductase 1B7 (\( \text{Akr1b7} \)) and cytochrome P450 isoform 3A11 (\( \text{Cyp3a11} \)), and antibacterial defense, such as angiogenin 1 (\( \text{Ang1} \)), were found to be up-regulated (Fig. 5A), whereas intestinal epithelial integrity was preserved, as assessed by permeability test (Fig. 5B) and histological examination (Fig. 5C). Our findings support the concept that intestinal FXR activation could effectively reverse FXR deficiency-associated inflammatory status in both the liver and the gut, thus providing a contributing explanatory pathway to the hepatocarcinogenesis-preventing scenario.

**Down-Regulation of Cell-Cycle–Related Genes and Aryl-Hydrocarbon Receptor Signaling Contribute to Intestinal FXR-Mediated Hepatoprotection.**

In order to characterize the liver-specific transcriptional scenario activated by restoration of intestinal FXR transcriptome, we performed a microarray analysis (Supporting Tables 1-4). We identified 14 up-regulated and 39 down-regulated genes (\( P < 0.05; >1.5\)-fold) in both young and aged liver, and we clustered them in Ingenuity canonical pathways and discussed only those that were biologically significant. Data obtained from microarray (Fig. 6A), gene (Fig. 6B), and protein (Fig. 6C) analysis pointed to a down-regulation of genes involved in aryl hydrocarbon receptor signaling, glutathione-mediated detoxification, IL-1-mediated inhibition of RXR function, and nuclear factor erythroid 2-related factor 2 (NRF2)-mediated oxidative stress response in both young and aged livers (Supporting Table 4). One of the most significantly modulated pathways (\( P \) value 3.02E-07) is aryl-hydrocarbon receptor signaling, whose genes are ascribed to cell-cycle progression, cancer, and cell proliferation, with cyclins occupying a focal position in this pathway. Alterations of cell-cycle–related genes have been documented in hepatocarcinogenesis as well as a compensatory proliferative response to BA-induced hepatocellular damage, thus providing evidence for a prognostic role of G1-S modulators in HCC.\(^{33}\) CyclinD1 (\( \text{Ccdn1} \)) is a key regulator of cell-cycle progression, and its overexpression has been reported to be sufficient to initiate hepatocellular carcinogenesis.\(^{34}\) Accordingly, mouse models of disrupted BA homeostasis, such as FXR- and SHP-null mice, display enhanced \( \text{Ccdn1} \) expression.\(^{27,35}\) Intestinal FXR activation lowered cyclinD1 transcript and protein in both young and older mice (Fig. 6B,C). Dysregulated cyclinE1 (\( \text{Ccen1} \)) expression has been shown to act as a potent oncogene, and amplification of cyclinE1 promotes HCC formation.\(^{36}\) Interestingly, cyclinE1 transcript (Fig. 6B) and protein (Fig. 6C) levels were
lower in young FXR\(^{-/-}\)iVP16FXR mice, compared to FXR\(^{-/-}\)iVP16 while being unchanged at older age, thus underscoring a direct role in the early phases of hepatocarcinogenesis. Accordingly, IHC revealed more proliferating cell nuclear antigen–positive cells in FXR\(^{-/-}\)iVP16 livers than in FXR\(^{-/-}\)iVP16FXR at both young and older age (Fig. 6D). Of note, cyclinD1 is a negative target gene of Shp,\(^{35}\) and hepatic Shp overexpression has been associated with lower cyclinD1 transcript levels and reduced tumor progression in FXR-null mice.\(^{27}\) In agreement with previous data, hepatic Shp expression was found inversely associated with hepatic cyclinD1 gene and protein levels in both young and aged mice (Supporting Fig. 1). Thus, intestinal FXR reactivation modulated the hepatic pregnane X receptor (PXR) transcriptome by down-regulating, at both young and older age, PXR target genes involved in IL-1-mediated inhibition of RXR signaling, including glutathione S-transferase mu1 (Gstm1) and aldehyde dehydrogenase 1 family member B1 (Aldh1b1; Fig. 6E).

**Discussion**

A compelling evidence stemming from animal and human studies implicates nuclear receptors as regulators of liver metabolic homeostasis and candidate biomarkers in HCC development.\(^{5,7,13,24,37}\) The nuclear receptor, FXR, is the master transcriptional regulator of BA homeostasis by controlling BA synthesis, influx, efflux, and detoxification in the gut/liver axis and has been implicated in liver tumorigenesis.\(^{7,13}\) Accordingly,
abnormal BA levels resulting from disruption of metabolic homeostasis has been observed in both an animal model of liver tumors and patients of hepatitis B virus infection, cirrhosis, and HCC. To date, strategies aimed at restoring BA homeostasis have the potential to pave the way for future therapy of CLD and, ultimately, HCC. FXR is highly expressed in the gut/liver axis, and tissue-specific KO and Tg FXR activation studies revealed the relative contribution of hepatic versus intestinal FXR in regulating BA and lipid homeostasis, thus providing the rationale to exploit tissue-specific FXR modulation in the management of CLD. We previously reported on the generation of a Tg mouse model (iVP16FXR) able to protect mice from chemically and genetically induced cholestasis by down-regulation of BA synthesis and up-regulation of intestinal BA disposal.

The main aim of the present study was to investigate, in newly generated Tg tissue-specific mouse models, whether restoration of FXR-Fgf15-Cyp7a1 enterohepatic regulatory axis-driven BA homeostatic processes would provide protection against HCC, even in the absence of hepatic FXR. Herein, we provide evidence that intestinal FXR is sufficient to protect against hepatocarcinogenesis by limiting BA overload, restoring the Fgf15/FGF4 signaling axis and up-regulating BA detoxification and efflux pathways. Moreover, in agreement with previous results in vitro showing anti-inflammatory and fibrotic properties of FXR agonists, we show in vivo that intestinal FXR reactivation confers hepatoprotection through reduced inflammation and proliferation as well as limited collagen deposition, thus resulting in less liver injury. Our findings depict an integrated framework linking intestinal FXR-mediated transcriptional programs, endocrine Fgf15/FGFR4-signaling pathways, BA homeostasis, and cellular responses, such as inflammation and proliferation. Importantly, the pathophysiological scenario described in our work arises only upon aging and ascribes intestinal FXR as a crucial contributor to liver health status. Indeed, FXR tissue-specific gain- and loss-of-function studies provide further support to the idea that the Fgf15/FXR axis could serve as a promising target to promote liver regeneration and repair as well as protection against cholestasis. Finally, the therapeutic exploitation of intestinal targets in the clinical management of liver pathological conditions has received a great deal of attention. Bacterial translocation and dysbiosis are hallmarks of CLD and cirrhosis, and rifaximin, a nonabsorbable antibiotic, is currently employed in patients with advanced liver disease. Strategies aimed at promoting antimicrobial defense and preserving intestinal epithelial integrity and mucosa fitness are anticipated to offer protection against cirrhosis and its complications, including hepatic encephalopathy and, eventually, hepatocarcinogenesis. Previously, it has been shown that the synthetic FXR agonist, GW4064, protects mice against bacterial translocation by preservation of intestinal epithelium integrity. In our mice, intestinal FXR reactivation induces genes involved in BA detoxification and efflux (Akr1b7, Cyp3a11, and Ostab), antibacterial defense (Ang1), and anti-inflammatory response (Il-1β and Tnfα). These events, along with down-regulation of genes involved in lipid metabolism, hepatic system disease, and cell growth, as underscored by microarray analysis, may all stand as potential underlying mechanisms contributing to the HCC prevention.

In conclusion, we show that intestinal FXR activation is able to rescue FXR-null mice from BA overload and prevents hepatocarcinogenesis by controlling BA synthesis through restoration of the FGF15 axis, limiting hepatic inflammation and proliferation while preserving intestinal epithelium integrity. Besides the debated positive or negative association between hepatic FXR expression and HCC development, our...
findings may support the therapeutic exploitation of the intestinal FXR and FGF19 axis in the clinical management of HCC patients, even if carrying a somatic FXR mutation.

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Fig. 6. Network of genes modulated by intestinal FXR reactivation and identified by whole-genome microarray analysis. (A) Venn diagram of genes down-regulated in both young and aged livers of FXR<sup>−/−</sup> iVP16FXR versus FXR<sup>−/−</sup> iVP16 mice. (B-E) Gene and protein expression analysis of molecular contributors to intestinal Tg FXR activation-mediated hepatoprotection in livers from both young and aged mice. Immunoblotting with specific antibodies against Cyclin D1 and E1 has been performed prior stripping on the same nitrocellulose membrane shown in Figure 4C and, as consequence, the immunoblots share the same beta-actin loading control bands. Cyclophilin was used as a housekeeping gene to normalize data, and young FXR<sup>−/−</sup> iVP16 mice were used as calibrators. All values shown are mean ± SEM (n = 7-11 animals/genotype). Data from groups sharing the same lowercase letters were not significantly different, whereas data from groups with different case letters were significantly different (P < 0.05; ANOVA Kruskal-Wallis’ test followed by Dunn’s posthoc test). LPS, lipopolysaccharide; mRNA, messenger RNA. Scanned original blots are reported as Supporting Information.

References
1. Bruix J, Boix L, Sala M, Llovet JM. Focus on hepatocellular carcinoma. Cancer Cell 2004; 5(3):215-219.
2. Fattovich G, Stroffolini T, Zagni I, Donato F. Hepatocellular carcinoma in cirrhosis: incidence and risk factors. Gastroenterology 2004; 127(Suppl 1):S35-S50.
3. Cameron R, Imaida K, Ito N. Promotive effects of deoxycholic acid on hepatocarcinogenesis initiated by diethylnitrosamine in male rats. Gann 1981;72(4):635-636.
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4. Knisely AS, Strautnieks SS, Meier Y, Stieger B, Byrne JA, Portmann BC, et al. Hepatocellular carcinoma in ten children under five years of age with bile salt export pump deficiency. HEPATOLOGY 2006; 44(2): 478-486.

5. Wang X, Fu X, Van NC, Meng Z, Ma X, Huang W. Bile Acid Receptors and Liver Cancer. Curr Pathobiol Rep 2013;1(1):29-35.

6. Chen T, Xie G, Wang X, Fan J, Qiu Y, Zheng X, et al. Serum and urine metabolite profiling reveals potential biomarkers of human hepatocellular carcinoma. Mol Cell Proteomics 2011;10(7):M110.

7. Yang F, Huang X, Yi T, Yen Y, Moore DD, Huang W. Spontaneous development of liver tumors in the absence of the bile acid receptor farnesoid X receptor. Cancer Res 2007;67(3):863-867.

8. Perez MJ, Briz O. Bile-acid-induced cell injury and protection. World J Gastroenterol 2009;15(14):1677-1689.

9. Makishima M, Okamoto AY, Repa JJ, Tu H, Learned RM, Luk A, et al. Identification of a nuclear receptor for bile acids. Science 1999;284(5418):1362-1365.

10. Parks DJ, Blanchard SG, Bledsoe RK, Chandra G, Consler TG, Kliever SW, et al. Bile acids: natural ligands for an orphan nuclear receptor. Science 1999;284(5418):1365-1368.

11. Sinal CJ, Tohkin M, Miyata M, Ward JM, Lambert G, Gonzalez FJ. The nuclear receptor farnesoid X receptor-knockout mice: efficient intestinal bile salt absorption in the absence of bile acid-binding protein. J Biol Chem 2003;278(43):41930-41937.

12. Kok T, Huizhovs CV, Wolters H, Havinga R, Agellon LB, Stellaard F, et al. Identification of a nuclear receptor for bile acids. Science 1999;284(5418):1362-1365.

13. Parks DJ, Blanchard SG, Bledsoe RK, Chandra G, Consler TG, Kliever SW, et al. Bile acids: natural ligands for an orphan nuclear receptor. Science 1999;284(5418):1365-1368.

14. Borude P, Edwards G, Walesky C, Li F, Ma X, Kong B, et al. Hepatocellular carcinoma observed in farnesoid X receptor knockout mice. Hepatology 2011;53(1):289-298.

15. Wang X, Fu X, Van NC, Meng Z, Ma X, Huang W. Bile Acid Receptors and Liver Cancer. Curr Pathobiol Rep 2013;1(1):29-35.

16. Chen T, Xie G, Wang X, Fan J, Qiu Y, Zheng X, et al. Serum and urine metabolite profiling reveals potential biomarkers of human hepatocellular carcinoma. Mol Cell Proteomics 2011;10(7):M110.

17. Yang F, Huang X, Yi T, Yen Y, Moore DD, Huang W. Spontaneous development of liver tumors in the absence of the bile acid receptor farnesoid X receptor. Cancer Res 2007;67(3):863-867.

18. Perez MJ, Briz O. Bile-acid-induced cell injury and protection. World J Gastroenterol 2009;15(14):1677-1689.

19. Borude P, Edwards G, Walesky C, Li F, Ma X, Kong B, et al. Hepatocellular carcinoma observed in farnesoid X receptor knockout mice. Hepatology 2011;53(1):289-298.

20. Wang X, Fu X, Van NC, Meng Z, Ma X, Huang W. Bile Acid Receptors and Liver Cancer. Curr Pathobiol Rep 2013;1(1):29-35.

21. Chen T, Xie G, Wang X, Fan J, Qiu Y, Zheng X, et al. Serum and urine metabolite profiling reveals potential biomarkers of human hepatocellular carcinoma. Mol Cell Proteomics 2011;10(7):M110.

22. Yang F, Huang X, Yi T, Yen Y, Moore DD, Huang W. Spontaneous development of liver tumors in the absence of the bile acid receptor farnesoid X receptor. Cancer Res 2007;67(3):863-867.

23. Perez MJ, Briz O. Bile-acid-induced cell injury and protection. World J Gastroenterol 2009;15(14):1677-1689.

24. Borude P, Edwards G, Walesky C, Li F, Ma X, Kong B, et al. Hepatocellular carcinoma observed in farnesoid X receptor knockout mice. Hepatology 2011;53(1):289-298.

25. Kim I, Ahn SH, Inagaki T, Moschetta A, Lee YK, Peng L, Zhao G, Downes M, et al. Selective activation of nuclear bile acid receptor FXR in the intestine protects against intestinal tumorigenesis. Cancer Res 2009;69(18):5985-5994.

26. Parks DJ, Blanchard SG, Bledsoe RK, Chandra G, Consler TG, Kliever SW, et al. Bile acids: natural ligands for an orphan nuclear receptor. Science 1999;284(5418):1365-1368.

27. Borude P, Edwards G, Walesky C, Li F, Ma X, Kong B, et al. Hepatocellular carcinoma observed in farnesoid X receptor knockout mice. Hepatology 2011;53(1):289-298.

28. Perez MJ, Briz O. Bile-acid-induced cell injury and protection. World J Gastroenterol 2009;15(14):1677-1689.

29. Borude P, Edwards G, Walesky C, Li F, Ma X, Kong B, et al. Hepatocellular carcinoma observed in farnesoid X receptor knockout mice. Hepatology 2011;53(1):289-298.

30. Xu Z, Huang G, Gong W, Zhou P, Zhao Y, Zhang Y, et al. FXR ligands protect against hepatocellular inflammation via SOCS3 induction. Cell Signal 2012;24(8):1658-1664.

31. Sinal CJ, Tohkin M, Miyata M, Ward JM, Lambert G, Gonzalez FJ. The nuclear receptor farnesoid X receptor-knockout mice: efficient intestinal bile salt absorption in the absence of bile acid-binding protein. J Biol Chem 2003;278(43):41930-41937.

32. Nishida N, Fukuda Y, Komeda T, Kita R, Sando T, Furukawa M, et al. Amplification and overexpression of the cyclin D1 gene in aggressive human hepatocellular carcinoma. Cancer Res 1994;54(12):3107-3110.

33. Nishida N, Fukuda Y, Iwashita K, Nakao K. Alteration of cell cycle-related genes in hepatocarcinogenesis. Histol Histopathol 1997;12(4):1019-1025.

34. Ito Y, Matsuura S, Sakon M, Miyoshi E, Noda K, Takeda T, et al. Expression and prognostic roles of the G1-S modulators in hepatocellular carcinoma: p27 independently predicts the recurrence. HEPATOLOGY 1999;30(1):90-99.

35. Zhang Y, Xu P, Park K, Choi Y, Moore DD, Wang L. Orphan receptor small heterodimer partner suppresses tumorigenesis by modulating cyclin D1 expression and cellular proliferation. HEPATOLOGY 2008;48(1):289-298.

36. Ohashi R, Gao C, Miyazaki M, Hamazaki K, Tsuji T, Inoue Y, et al. Enhanced expression of cyclin E and cyclin A in human hepatocellular carcinomas. Anticancer Res 2001;21(8B):657-662.

37. Vacca M, Degirolamo C, Massuva V, Polimenio L, Mariani-Costantini R, Palasciano G, et al. Nuclear receptors in regenerating liver and hepatocellular carcinoma. Mol Cell Endocrinol 2013;368(1-2):108-119.

38. Li J, Zhang Y, Kurutba R, Gao X, Gandhi CR, Xie W, et al. Roles of microRNA-29a in the antifibrotic effect of farnesoid X receptor in hepatic stellate cells. Mol Pharmacol 2011;80(1):191-200.

39. Ridlon JM, Alves JM, Hylemon PB, Bajaj JS, Citr嗜咆, bile acids, and gut microbiota: Unraveling a complex relationship. Gut Microbes 2013;4(5).

40. Inagaki T, Moschetta A, Lee YK, Peng L, Zhao G, Downes M, et al. Regulation of antibacterial defense in the small intestine by the nuclear bile acid receptor FXR. Proc Natl Acad Sci U S A 2006;103(10):3920-3925.

41. Kurokawa K, Fujisawa Y, Ohtani H, Nishida N, Inagaki T, et al. Downregulation of hepatocellular carcinoma cells. Mol Cancer Res 2012;10(4):516-522.

42. Su H, Ma C, Liu J, Li N, Gao M, Huang A, et al. Downregulation of nuclear receptor FXR is associated with multiple malignant clinicopathological characteristics in human hepatocellular carcinoma. Am J Physiol Gastrointest Liver Physiol 2012;303(1):G1245-G1253.

43. Ohno T, Shirakami Y, Shimizu M, Kubota M, Saki H, Yasuda Y, et al. Synergistic growth inhibition of human hepatocellular carcinoma cells by acyclic retinoid and GW4064, a farnesoid X receptor ligand. Cancer Lett 2012;323(2):215-222.

44. Zhang Y, Gong W, Dai S, Huang G, Shen X, Gao M, et al. Downregulation of human farnesoid X receptor by miR-421 promotes proliferation and migration of hepatocellular carcinoma cells. Mol Cancer Res 2012;10(4):516-522.

Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.27274/suppinfo.