Suppression of Hepatic Bile Acid Synthesis by a non-tumorigenic FGF19 analogue Protects Mice from Fibrosis and Hepatocarcinogenesis

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Critical regulation of bile acid (BA) pool size and composition occurs via an intensive molecular crosstalk between the liver and gut, orchestrated by the combined actions of the nuclear Farnesoid X receptor (FXR) and the enterokine fibroblast growth factor 19 (FGF19) with the final aim of reducing hepatic BA synthesis in a negative feedback fashion. Disruption of BA homeostasis with increased hepatic BA toxic levels leads to higher incidence of hepatocellular carcinoma (HCC). While native FGF19 has anti-cholestatic and anti-fibrotic activity in the liver, it retains peculiar pro-tumorigenic actions. Thus, novel analogues have been generated to avoid tumorigenic capacity and maintain BA metabolic action.

Here, using BA related Abcb4−/− and Fxr−/− mouse models of spontaneous hepatic fibrosis and HCC, we explored the role of a novel engineered variant of FGF19 protein, called FGF19-M52, which fully retains BA regulatory activity but is devoid of the pro-tumoral activity. Expression of the BA synthesis rate-limiting enzyme Cyp7a1 is reduced in FGF19-M52-treated mice compared to the GFP-treated control group with consequent reduction of BA pool and hepatic concentration. Treatment with the non-tumorigenic FGF19-M52 strongly protects Abcb4−/− and Fxr−/− mice from spontaneous hepatic fibrosis, cellular proliferation and HCC formation in terms of tumor number and size, with significant reduction of biochemical parameters of liver damage and reduced expression of several genes driving the proliferative and inflammatory hepatic scenario. Our data bona fide suggest the therapeutic potential of targeting the FXR-FGF19 axis to reduce hepatic BA synthesis in the control of BA-associated risk of fibrosis and hepatocarcinoma development.

Hepatocellular carcinoma (HCC) is the sixth most common malignancy and the third most frequent cause of cancer-related death1. The lack of effective therapeutic options makes the quest for novel putative treatment strategies of paramount importance. The gut-liver axis homeostasis relies on a tight control of bile acid (BA) levels in order to avoid BA overload that is critical in the pathogenesis of hepatic diseases.

BAs are the end products of cholesterol catabolism, synthesized in the liver and released into the small intestine after meal ingestion. BA production and circulation are tightly regulated via the nuclear receptor, farnesoid X receptor (FXR). In the liver, FXR reduces conversion of cholesterol to BAs by downregulating the rate limiting enzyme of BA synthesis cytochrome P450 A1 (CYP7A1), via the small heterodimer partner (SHP). Moreover, FXR promotes hepatic bile secretion by increasing the expression of crucial BA transporters. In the enterocytes, BA-bound FXR induces the transcription of the fibroblast growth factor FGF15/19 (mice and human, respectively), an enterokine secreted into the portal circulation, able to reach the liver and bind to the fibroblast growth factor receptor 4 (FGFR4)/β-Klotho complex. This initiates a phosphorylation cascade in the c-jun N-terminal kinase-dependent pathway, ultimately inhibiting CYP7A1 expression hence BA synthesis2, a mechanism working in synergy with the hepatic FXR-SHP-dependent one3.

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Altered BA signaling in the liver and intestine is associated with severe diseases including the development of cholestasis and HCC. Hepatic diseases causing intrahepatic cholestasis, such as the progressive familial intrahepatic cholestasis type 2 and 3 (PFIC2-3) caused by the multidrug resistance protein 3 (MDR3) deficiency, represent a specific risk of HCC development, especially in children. ATP-binding cassette transporter (Abcb4)−/− and Fxr−/− mice are commonly used as elective models of HCC development.10–15 Abcb4−/− mice lack the liver-specific permeability-glycoprotein responsible for phosphatidylcholine flipase on the outer leaflet of the hepatocyte canalicular membrane and therefore for secretion of phosphatidylcholine in bile. The absence of phospholipids from bile causes bile regurgitation into the portal tracts, inducing accumulation of toxic BA levels, and consequent fibrosis that leads to hepatocyte dysplasia first and HCC at 12–15 months of age, mimicking human progressive familial intrahepatic cholestasis.17 Fxr−/− mice exhibited increased BA pool size and display cell hyperproliferation leading to the development of spontaneous HCC at 12 months of age.18

Strategies aimed at limiting BA overload are anticipated to provide hepatoprotection, as earlier reported in Fxr−/− mice treated with BA-sequestering agents.9 We have recently shown that specific intestinal Fxr activation is sufficient to restore BA homeostasis in Fxr−/− mice, thus protecting them from age-related hepatic inflammation, fibrosis, and cancer.18 Also, we have recently shown that long-term administration of a Fxr agonist enriched diet prevents spontaneous hepatocarcinogenesis in Abcb4−/− mice via Fxr-Fgf15-dependent suppression of hepatic Cyp7a119.

The discovery of the role of FXR target gene, FGF15/19, in the feedback regulation of hepatic BA synthesis shed light on the physiological relevance of the crosstalk between the liver and intestine in the context of BA homeostasis.20,21 FGF19 has also been implicated in HCC development. In fact, it is amplified in HCC and its expression is induced in liver of patients with extrahepatic cholestasis.22–24 Interestingly, induction of Fgf15 expression in mice by intestinal Fxr overexpression and Bile, along with a reduction of the BA pool size25 suggesting that modulation of FGF19 levels could offer benefits in a plethora of BA-related metabolic disorders. However, despite its protective action, FGF19 has been shown to be protumorigenic and accelerate hepatic tumour formation in Abcb4−/− mice in an FGR4-dependent fashion,26–27 raising doubts on the safety of a chronic administration of this hormone.28 Recently, the generation of a FGF19 variant (M70) that is equally effective as endogenous FGF15/19 in terms of bile acid metabolic regulatory actions but does not show any pro-tumorigenic activity in 8 months old Abcb4−/− mice29 was described. Moreover, administration of FGFR-FGF19-M70 in healthy human volunteers potently reduces BA synthesis30. This data provided us with the impetus to investigate the potential protective role of a novel non-tumorigenic FGF19 variant in spontaneous HCC development during BA dysregulation.

In the present work, we show for the first time that the non-tumorigenic variant of FGF19, namely FGF19-M52, retaining its intrinsic metabolic effects on Cyp7a1 repression and consequent reduction of hepatic BA synthesis, protects Abcb4−/− and Fxr−/− mice against spontaneous hepatocarcinogenesis thus electing hepatic BA suppression as a metabolic strategy to prevent fibrosis and HCC in susceptible models.

**Results**

**M52 is a non-tumorigenic variant of FGF19 that retains activity in regulating BA synthesis.**

The novel engineered variant of the FGF19 protein M52 has been recently generated. M52 differs from wild-type FGF19 by five amino acid substitutions (A30S, G31S, H33L, V34L, H35Q) and five–amino acid deletion at the N terminus (Fig. 1a). In order to characterize the M52 variant and compare it to the full length FGF19, we tested CYP7A1 repression in primary human hepatocytes. qRT-PCR analysis shows that relative mRNA levels of CYP7A1 do not change in both FGF19 and M52 cell treatment, indicating that the M52 variant retains his biological activity of repression of de novo BA synthesis (Fig. 1b). Further in vivo analysis in db/db mice revealed that M52 is present in plasma as well as FGF19 (Fig. 1c). Moreover, while FGF19 administration causes an increased number of tumors per liver as well as a raised liver weight and liver/body weight ratio compared to controls, the M52 variant does not show any tumorigenic activity (Fig. 1d–f, respectively).

**FGF19-M52 metabolically protects Abcb4−/− mice from age-related liver damage.**

The non-tumorigenicity of the novel FGF19-M52 variant and its concomitant ability to maintain CYP7A1 repression, prompt us to explore its metabolic and antitumoral ability in aged Abcb4−/− mice, a murine model of impaired BA homeostasis-induced HCC. Aged Abcb4−/− mice have been elected as a unique animal model for studying HCC pathogenesis because they resemble many features of human HCC progression. 100% of aging Abcb4−/− mice display metabolic derangement due to liver inflammation and toxicity induced by a progressive increase and accumulation of BAS. ELISA measurement shows a strikingly higher amount of FGF19 in adenovirus (AV)-FGF19-M52-treated mice compared to AV-GFP-control (AV-GFP 8.38 ± 6.28 vs AV-FGF19-M52 434.5 ± 83.65 pg/ml). Compared to the control group, AAV-FGF19-M52-injected mice displayed a significant lower hepatic mRNA expression of the key limiting enzyme of BA synthesis Cyp7a1 (Fig. 2a). Also, the cytochrome P450 family 8 subfamily B member 1 (Cyp8b1) — another critical enzyme controlling the ration between Cholic Acid (CA) and Chenodeoxycholic acid (CDCA) — by regulating the synthesis of CA - results inhibited in AAV-FGF19-M52–treated mice compared to control (Fig. 2b).

These changes were translated in an extremely powerful reduction of the plasmatic total BA pool size (AV-GFP 74.65 ± 12.54 vs AAV-FGF19-M52 0.63 ± 0.11 μM) and a shift in both plasma and liver BA composition to a more hydrophilic BA pool profile due to the enrichment in muricholic acid (MCA) (Fig. 2c, Tables 1 and 2).

In order to investigate whether the metabolic changes induced by the FGF19-M52 analogue would translate in protection from HCC occurrence, macroscopic analysis of 16 months old Abcb4−/− mice liver was performed at the day of sacrifice. Earlier studies suggested that attenuation of systemic BA overload reduce number and size of liver tumors.8 As expected, 100% of Abcb4−/− mice injected with the control adenovirus AV-GFP showed macroscopically identifiable tumours. Remarkably, almost no tumour could be identified in Abcb4−/− mice injected with the control adenovirus AV-GFP showed macroscopically identifiable tumours. Remarkably, almost no tumours could be identified in Abcb4−/− mice
FGF19-M52 protects Abcb4−/− mice from hepatic collagen deposition and fibrosis. Liver fibrosis results from chronic damage to the liver in conjunction with the accumulation of extracellular matrix (ECM) proteins, which is a characteristic of most types of chronic liver diseases. Lack of the Abcb4 gene elicits a plethora of detrimental cell responses, including hepatic fibrosis, thus laying the foundation to hepatocarcinogenesis. In order to explore the effect of our FGF19 analogue on fibrogenesis we examine the extent of hepatic collagen deposition in liver isolated from aged Abcb4−/− mice (Fig. 3f,g). This finding was paralleled by mRNA inhibition of Collagen type 1 alpha 1 (Col1a1) in M52 Abcb4−/− mice compared to the control group (Fig. 3h).

FGF19-M52 protects Abcb4−/− mice from overexpression of HCC oncogenes. 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. Abcb4−/− mice also present with cell hyperproliferation. CyclinD1 (Cnd1) is a key regulator of cell cycle progression, and its overexpression has been reported to be sufficient to initiate hepatocarcinogenesis. Accordingly, mouse models of disrupted BA homeostasis, such as Fxr−/− and Shp−/− mice, display enhanced Cnd1 expression. AAV-FGF19-M52 lowered Cnd1 protein, as shown by immunohistochemical analysis, and transcript in aged Abcb4−/− mice (Fig. 3e). Furthermore, dysregulated cyclinE1 (Ccne1) expression, as well as c-myc gene, have been shown to act as potent oncogenes, and amplification of both genes promotes HCC formation. mRNA analysis also revealed a marked inhibition of Ccne1 and c-myc expression in AAV-FGF19-M52-injected Abcb4−/− mice compared to controls (Fig. 3e).

FGF19-M52 protects Fxr−/− mice from HCC. We have previously shown that intestinal-specific Fxr reactivation, and in particular the entero-hepatic Fxr-Fgf15 axis activation, is able to prevent liver damage and its spontaneous hepatocarcinogenesis progression even in the absence of hepatic Fxr. In order to bypass the Fxr involvement and corroborate the importance of the intestinal hormone FGF19, we performed the same
experiment and analysis conducted in Abcb4−/− mice in aged Fxr−/− mice. ELISA measurement shows a striking amount of FGF19 in AAV-FGF19-M52-treated mice compared to AAV-GFP controls (AAV-GFP 0.33 ± 0.06 vs AAV-FGF19-M52 1021 ± 178.3 μM). AAV-FGF19-M52-injected mice displayed a significant lower hepatic mRNA expression of the key limiting enzymes of BA synthesis Cyp7a1 and Cyp8b1 compared to the control GFP-injected group (Fig. 4a, b). These changes were translated in a reduction of the plasmatic total BA pool size (AAV-GFP 11.48 ± 13.88 vs AAV-FGF19-M52 8.20 ± 3.59 μM) and a shift in both plasma and liver BA composition to a more hydrophilic BA pool profile due to the enrichment in MCA (Fig. 4c, Tables 1 and 2). 14 months old AAV-FGF19-M52 Fxr−/− mice presented with a striking lower number of macroscopically visible liver tumors and smaller in size compared to controls (Fig. 4d). This was accompanied by a significantly decreased level of plasma ALP level and a lowered trend of ALT and AST compared to control mice (Fig. 4e). Liver morphology and HE staining shows liver injury and disrupted hepatic parenchymal structure in GFP-injected Fxr−/− mice, in contrast with a more preserved hepatic parenchyma and less inflammatory infiltrates observed in FGF19-M52-treated mice (Fig. 5a). In parallel, a decrease in Clec7a and MCP-1 was observed in FGF19-M52-injected mice compared to the control group (Fig. 5b). Previous studies have shown that, during aging, Fxr−/− mice are characterized by enhanced fibrogenesis and cell proliferation, therefore we performed Sirius Red and Ccnd1 stainings along with q-RTPCR analysis. M52-treatment revealed a protection in terms of fibrosis and collagen deposition at

Figure 2. FGF19-M52 analogue protects Abcb4−/− mice from age-related liver damage and HCC progression. qPCR of hepatic (a) Cyp7a1 (b) Cyp8b1. Expression was normalized to Cyclophilin. (c) BA composition (CA/MCA Ratio) in murine plasma and liver. (d) Macroscopic appearance of livers, normalized tumor number and maximum tumor size of 16-months-old Abcb4−/− mice (n = 14–24). (e) Biochemical parameters of liver damage in 16 months old Abcb4−/− mice. Statistical significance (*p < 0.05, **p < 0.01, ***p < 0.001) was assessed by Mann-Whitney’s U test.
Table 1. Serum Bile Acid Composition. CA, cholic acid; UDCA, ursodeoxycholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; MCA, muricholic acids; TCA, taurocholic acid; TUDCA, tauroursodeoxycholic acid; TCDCA, taurinechenodeoxycholic acid; TDCA, taurinedeoxycholic acid; TLCA, taurinelithocholic acid; TMCA, taurinemuricholic acids.

| Genotype | CA% UDCA% CDCA% DCA% LCA% MCA% TCA% TUDCA% TCDCA% TDCA% TLCA% TMCA% |
|----------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Abcb4−/−-GFP | 0.45 ± 0.04 | 0.23 ± 0.02 | 0.12 ± 0.01 | 0.12 ± 0.03 | 0.03 ± 0.01 | 5.42 ± 1.15 | 24.46 ± 1.58 | 2.08 ± 0.73 | 3.22 ± 0.13 | 0.17 ± 0.03 | 0 ± 0 | 63.69 ± 1.24 |
| Abcb4−/−-M52 | 4.98 ± 1.2 | 0.02 ± 0.01 | 5.8 ± 1.65 | 0.03 ± 0.02 | 2.6 ± 0.6 | 18.32 ± 3.81 | 4.92 ± 3.32 | 0.12 ± 0.1 | 0.69 ± 0.61 | 0.03 ± 0.02 | 0 ± 0 | 62.63 ± 2.73 |
| Fxr−/−-GFP | 12.5 ± 3.17 | 0.83 ± 0.25 | 1.25 ± 0.38 | 8.2 ± 1.64 | 0.2 ± 0.06 | 8.1 ± 1.24 | 47.81 ± 3.69 | 0.68 ± 0.166 | 0.68 ± 0.166 | 3.78 ± 0.67 | 0 ± 0 | 15.93 ± 2.29 |
| Fxr−/−-M52 | 11.76 ± 3.03 | 1.22 ± 0.27 | 0.56 ± 0.14 | 3.22 ± 0.94 | 0.15 ± 0.05 | 28.88 ± 8.29 | 23.9 ± 6.86 | 0.53 ± 0.12 | 0.08 ± 0.04 | 0.36 ± 0.13 | 0 ± 0 | 29.37 ± 9.5 |

Table 2. Liver Bile Acid Composition. CA, cholic acid; UDCA, ursodeoxycholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; MCA, muricholic acids; TCA, taurocholic acid; TUDCA, tauroursodeoxycholic acid; TCDCA, taurinechenodeoxycholic acid; TDCA, taurinedeoxycholic acid; TLCA, taurinelithocholic acid; TMCA, taurinemuricholic acids.

Figure 3. FGF19-M52 analogue protects Abcb4−/− mice from fibrosis and cellular proliferation. (a) Liver histology assessed by hematoxylin and eosin (H&E) staining and observed by light microscopy (magnification, 10–20X). (b) Mcp-1 and Clec7a gene expression levels assessed by real time qPCR in tumor-free liver extracts. (c) Hepatic immunohistochemical staining of the oncogene Ccnd1. (d) Quantification of Ccnd1 staining assessed with ImageJ and expressed as % of the area occupied by positive cells. (e) Ccnd1, Ccne1 and c-myc gene expression levels assessed by real time qPCR in tumor-free liver extracts. Expression was normalized to Cyclophilin. (f) Hepatic collagen deposition assessed by Sirius Red staining (magnification, 10–20X). (g) Quantification of collagen deposition assessed with ImageJ and expressed as % of collagen staining/field. (h) Col1a1 gene expression levels assessed by real time qPCR in tumor-free liver extracts. Expression was normalized to Cyclophilin. All values represent mean ± SEM. Statistical significance (**p < 0.01, ***p < 0.001) was assessed by Mann-Whitney's U test.
mRNA and protein level (Fig. 5f–h) as well as significantly lower hyperproliferative molecular status compared to GFP-treated controls as indicated by a decrease in protein and mRNA levels of Ccne1, p21 and c-myc (Fig. 5c–e).

Discussion

FGF19 is a post-prandial enterokine and a cornerstone of BA synthesis control, also regulating carbohydrate, lipid and energy homeostasis. The landmark discovery of the FXR-FGF19 axis in the regulation of the BA homeostasis core opened new avenues for intestinal-specific therapeutic management of chronic diseases of the gut-liver axis. The therapeutic exploitation of the intestinal FXR/FGF19 axis activation in cholestasis and its translation into protection from HCC development in Abcb4−/− mice prompt us to further examine the feasibility of a FGF19-based therapy in chronic liver and intestinal disease. However, literature data are debatable whether the enterokine FGF19 is per se implicated or not in HCC development. The in-depth characterization of FGF19 molecular structure and function allowed us to design a novel FGF19-based pre-clinical therapeutic agent uncoupling its metabolic activities from the proliferative ones. In fact, another novel engineered...

Figure 4. FGF19-M52 analogue protects Fxr−/− mice from age-related liver damage and HCC progression. qPCR of hepatic (a) Cyp7a1 (b) Cyp8b1. Expression was normalized to Cyclophilin. (c) BA composition (CA/MCA Ratio) in murine plasma and liver. (d) Macroscopic appearance of livers, normalized tumor number and maximum tumor size of 14-months-old Fxr−/− mice (n = 8–16). (e) Biochemical parameters of liver damage in 14 months old Fxr−/− mice. Statistical significance (*p < 0.05, **p < 0.01, ***p < 0.001) was assessed by Mann-Whitney's U test.
variant of FGF19 protein (M70) which fully retains BA regulatory activity but is devoid of murine pro-tumoral activity has been recently identified. M70 differs from wild-type FGF19 by three amino acid substitutions (A30S, G31S, H33L) and a five amino acid deletion at the N terminus (P24-S28). Zouh et al. demonstrated that M70 interacts with the FGFR4 receptor and exhibits the pharmacologic characteristics of a biased ligand that selectively activates certain signalling pathways (e.g., cytochrome P450 7A1, phosphorylated extracellular signal–regulated kinase) and exclusion of others (e.g., tumorigenesis, phosphorylated signal transducer and activator of transcription 3). The identification of these types of FGF19 variants, including our M52, has greatly helped in overcoming the severe side effect of therapies targeting FGF19, due to derangement in the gut-liver axis regulation of BA homeostasis and consequent development of liver toxicity and diarrhoea. The therapeutic potential of FGF19-M70 analogue has been well characterized and, unlike the natural form of FGF19, it does not show any pro-tumorigenic activity in 8 months old Abcb4−/− mice, age at which these mice have not developed hepatic tumours yet.

Here, we study the novel non-tumorigenic analogue FGF19-M52 as putative drug to inhibit Cyp7a1, reduce BA synthesis and eventually protect against BA-induced cancer in the liver. To this end, we show for the first time that the FGF19-M52 analogue protects against spontaneous hepatic tumorigenesis in 16 months old mice Abcb4−/−, through the reduction of BA concentrations and modification of the BA pool. Moreover, our results demonstrate that M52 retains control of BA negative feedback of the gut-liver loop and prevents BA-induced spontaneous hepatocarcinogenesis in 14 months old Fxr−/− mice. Moreover, FGF19-M52 is also able to reduce Cyp8b1 expression, while no changes in the expression of BA transporters (Bsep, Ntcp, Oatp1 and Oatp2) and FGF19 receptors (Fgfr4 and β-klotho) were found (data not shown), indicating that FGF19-M52 globally reduces BA levels without a direct transcriptional impact on their secretory or transport genes.

It is well known that both Fxr and Abcb4 genes ablation in mice leads to liver damage, fibrosis, cholestasis and spontaneous HCC induced by high level of hydrophobic cytotoxic BAs. Indeed, the absence of Abcb4 leads to accumulation of intraductal and biliary BAs that in absence of phosphatidylcholine are cytotoxic and exert their detergent activity that represent the primum movens for the sequela of events that lead to HCC. On a different
angle, the absence of Fxr leads to de-repression of hepatic Cyp7a1 with consequent potent increased BA synthesis and concentration systemically and within the liver. These events represent the leading step for liver damage, inflammation and fibrosis that bring Fxr−/− mice to spontaneous HCC formation.

FGF19-M52-dependent inhibition of Cyp7a1 and the consequent decrease of intrinsically harmful BA pool size and composition protect from hepatic tumour formation, even in the absence of Fxr. Mechanistically, the decrease of BA chronically high level and the shift of their composition into a more favourable hydrophilic one result in an outstanding inhibition of hepatic fibrosis that promptly translates into a blockage of overexpression of the typical HCC oncogenes Ccnd1, c-myc and others. Thus, in both models, FGF19-M52-dependent modulation of BA concentration with reduction of their levels and toxicity protect from liver damage and HCC.

Our findings support the concept that the control of BAs synthesis is definitely of great importance and could effectively reverse Abcb4- and Fxr-deficiency-associated hepatocarcinogenesis, suggesting that multiple metabolic players are involved in the hepatocarcinogenesis-preventing scenario. Our finding leverages the fine effort of testing novel FGF19 engineered variants that could display anti-fibrotic and anti-inflammatory effects but also antitumoral actions, thus opening bona fide a novel pharmacological strategy for example in PFIC patients who are susceptible to HCC formation even in young age.

Methods

AAV Production. AAV293 cells (Agilent Technologies) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Mediatech) supplemented with 10% fetal bovine serum and 1X antibiotic-antimycotic solution (Mediatech). Cells were transfected with three different plasmids (20 μg/plate, each) including AAV transgene plasmids, pHHelper plasmid (Agilent Technologies) and AAV2/9 plasmid. Cells were harvested forty-eight hours after transfection. Viral particles in cell lysates were purified using a discontinued iodixanol gradient (Sigma-Aldrich). To determine the viral titer or genome copy number, viral stock was incubated in a solution containing 50 units/mL Benzonase, 50 mM Tris-HCl pH 7.5, 10 mM MgCl2, and 10 mM CaCl2 at 37 °C for 30 minutes. Viral DNA was cleaned with mini DNeasy Kit (Qiagen) and eluted with 40 μL of water. Viral genome copy was determined by quantitative PCR.

Animal studies. For the characterization of the FGF19 analogue M52 obtained from NGM Biopharmaceuticals, Inc (se WO 2013/006486), 10–12 week old male db/db mice were purchased from Jackson Laboratory, and housed in a pathogen-free animal facility at 22°C under controlled 12 hour light/12 hour dark cycle. All mice were kept on standard chow diet (Harlan Laboratories, Teklad 2918) and autoclaved water ad libitum. For in vivo tumorigenicity studies, cohorts db/db mice (n = 5) were randomized into treatment groups based on body weight. All animals received a single 200 μl intravenous injection of 3 × 1011 genome copies of either AAV-FGF19, AAV-M52, or a control virus encoding green fluorescent protein via tail vein. Viral vectors for histology and gene expression analysis. These animal studies were approved by the Institutional Animal Care and Use Committee at NGM. For the HCC murine models, Abcb4−/− mice on an FVB/N background were obtained from Charles River Laboratory (Charles River, Lecco, Italy) and whole-body Fxr−/− mice were originally obtained from Dr. Frank J. Gonzalez (National Institutes of Health, Bethesda, MD). Male 8-weeks-old Abcb4−/− and Fxr−/− mice (n = 14–24 and n = 8–16, respectively) received a single intravenous dose of 1 × 1011 vector genome of AAV containing genes encoding either the FGF19-M52 form or control GFP. All mice were housed under a standard 12-hour light/dark cycle and fed standard rodent chow diet and autoclaved tap water ad libitum. Mice were aged until 14 and 16-months-old (Fxr−/− and Abcb4−/−, respectively), sacrificed and analyzed for tumor development. All experiments were approved by the Italian Ministry of Health in accord with internationally accepted guidelines for animal care.

Primary human hepatocytes. Primary hepatocytes from human livers (Life Technologies) were plated on collagen I-coated 96-well plates (Becton Dickinson) and incubated overnight in Williams’ E media supplemented with autoclaved tap water ad libitum. Mice were aged until 14 and 16-months-old (Fxr−/− and Abcb4−/−, respectively), sacrificed and analyzed for tumor development. All experiments were approved by the Italian Ministry of Health in accord with internationally accepted guidelines for animal care.

Blood Parameters. Levels of ALT, AST and ALP were measured with a colorimetric kit (BioQuant Heidelberg, Germany) according to manufacturer’s instructions.

Chemicals. CA and other endogenous BAs were purchased from Sigma-Aldrich (St. Louis, MO). All solvents were of high purity and used without further purification. Acetonitrile for HPLC was from Merck (Darmstadt, Germany); methyl alcohol RPE, ammonia solution 30% RPE, glacial acetic acid RPE were from Carlo Erba Reagent (Milan, Italy); activated charcoal was from Sigma-Aldrich; and ISOLUTE C18 cartridges (500 mg, 6 ml) for the plasma sample pretreatment were purchased from SteBio (Bologna, Italy). Plasma BA free rat plasma was treated with activated 50 mg/ml charcoal and stirred at 4°C overnight. After centrifugation at 3000 g for 5 minutes the plasma was filtered through Millipore A10 Milli-Q Synthesis (0.45 μm) and stored at −20°C.

Bile Acid Measurements. Plasma and hepatic BAs were identified and quantified by high-pressure liquid chromatography-electrospray-mass spectrometry/mass spectrometry (HPLC-ES-MS/MS) by optimized methods suitable for use in pure standard solution, plasma and liver samples after appropriate clean-up preanalytical procedures. Liquid chromatography analysis was performed using an Alliance HPLC system model 2695 from Waters combined with a triple quadruple mass spectrometer QUATTRO-LC (Micromass; Waters) using an electrospray interface. The analytical column was a Waters XSelect CSH Phenyl-hexyl column, 5 μm, 150 × 2.1 mm, protected by a self-guard
column Waters XSelect CSH Phenyl- hexyl 5 μm, 10 × 2.1 mm. BAs were separated by elution gradient mode with a mobile phase composed of a mixture ammonium acetate buffer 15 mM, pH 8.0 (Solvent A) and acetonitrile:methanol = 75:25 v/v (Solvent B). Chromatograms were acquired using the mass spectrometer in multiple reaction monitoring mode. Plasma and hepatic bile acids were extracted using a standard, previously validated protocol19.

**mRNA extraction and quantitative real time qRT-PCR analysis.** Total RNA was isolated from tumor-free livers using RNaseasy Micro kit (Qiagen, Milano, Italy). cDNA was generated from 4 μg total RNA using High Capacity DNA Archive Kit (Applied Biosystem, Foster City, CA) and following the manufacturer’s instructions. mRNA expression levels were quantified by qRTPCR using Power Syber Green chemistry and normalized to cyclophilin mRNA levels. Relative quantification was performed using the ΔΔCT method. Validated primers for real time PCR are available upon request.

**Histology and Immunohistochemistry.** Macroscopically tumor-free liver samples were fixed in 10% buffered formalin for 24 hours, dehydrated, and embedded in paraffin. Five-micrometer- thick sections were stained with hematoxylin-eosin (H&E) following standard protocols. Liver fibrosis was analyzed with Sirius Red by using Direct Red 80 and Fast Green PFC (Sigma Aldrich, Milan, Italy). Hepatocyte proliferation was assessed by immuno-histochemical detection of cyclin D1 (CCND1). All stained sections were analyzed through a light microscope. Histological features of hepatice disease have been assessed according to Histological Scoring System by Kleiner DE et al.20 and Brunt EM et al.20.

**Statistical Analysis.** All measurements were performed in technical triplicates. All results are expressed as means ± standard error of the mean (SEM). Significant differences between three groups were determined by one-way ANOVA followed by Dunnett’s post hoc test, while between two groups were determined by Mann-Whitney’s U 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.

**Ethics Statement.** The Ethical Committee of the University of Bari approved this experimental set-up, which also was certified by the Italian Ministry of Health in accordance with internationally accepted guidelines for animal care.

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Author Contributions

R.G. contributed to study design, performed experiments and contributed to paper writing; N.S. performed animal studies; C.P. performed histology; M.C. performed gene expression analysis; B.K. and J.L. generated AAVFGF19M52 and performed experiments in db/db mice; E.P. performed BA measurements; A.R. supervised the BA profiling; C.S. provided expertise in hepatic physiology and contributed to the translational relevance of the data analysis; A.M. designed the study, supervised the project and wrote the paper.

Additional Information

Competing Interests: Brian Ko and Jian Luo are employers of NGM Biopharmaceuticals, San Francisco CA and provided the AAV with FGF19-M52 analogue and GFP controls. The other authors declare no conflicts of interest.

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