Engineered Fibroblast Growth Factor 19 Reduces Liver Injury and Resolves Sclerosing Cholangitis in Mdr2-Deficient Mice

Mei Zhou, R. Marc Learned, Stephen J. Rossi, Alex M. DePaoli, Hui Tian, and Lei Ling

Defects in multidrug resistance 3 gene (MDR3), which encodes the canalicular phospholipid flippase, cause a wide spectrum of cholangiopathy phenotypes in humans. Mice deficient in Mdr2 (murine ortholog of MDR3) develop liver diseases that closely reproduce the biochemical, histological, and clinical features of human cholangiopathies such as progressive familial intrahepatic cholestasis and primary sclerosing cholangitis. We hypothesized that modulating bile acid metabolism by the gut hormone fibroblast growth factor 19 (FGF19) may represent a novel approach for treating cholangiopathy and comorbidities. We introduced adeno-associated virus carrying the gene for either the endocrine hormone FGF19 or engineered FGF19 variant M70 to 12-week old Mdr2-deficient mice with fully established disease. Effects on serum levels of liver enzymes, liver histology, and bile acid homeostasis were evaluated. FGF19 and M70 rapidly and effectively reversed liver injury, decreased hepatic inflammation, attenuated biliary fibrosis, and reduced cholelithiasis in Mdr2-deficient mice. Mechanistically, FGF19 and M70 significantly inhibited hepatic expression of Cyp7a1 and Cyp27a1, which encode enzymes responsible for the rate-limiting steps in the classic and alternate bile acid synthetic pathways, thereby reducing the hepatic bile acid pool and blood levels of bile acids. Importantly, prolonged exposure to FGF19, but not M70, led to the formation of hepatocellular carcinomas in the Mdr2-deficient mice. Furthermore, M70 ameliorated the hepatosplenomegaly and ductular proliferation that are associated with cholangiopathy. Conclusion: These results demonstrate the potential for treating cholangiopathy by safely harnessing FGF19 biology to suppress bile acid synthesis. (HEPATOLOGY 2016;63:914-929)
in abnormal excretion of biliary phosphatidylcholine and the onset of intrahepatic cholestasis.\(^4,5\) To date, more than 30 mutations in MDR3 have been reported that are causally associated with a variety of biliary diseases, including PFIC type 3 (PFIC3),\(^6,7\) intrahepatic cholestasis of pregnancy,\(^8,9\) low phospholipid-associated cholelithiasis,\(^10,11\) anicteric cholestasis,\(^12\) oral contraceptive-induced cholestasis,\(^11\) and cirrhosis.\(^13\)

Under physiological conditions, biliary phospholipids are transported into the bile through the canalicular phospholipid flippase MDR3 and subsequently form mixed phospholipid-bile acid micelles that protect cholangiocytes from bile acid–induced cell injury. In patients with MDR3 deficiency (e.g., PFIC3), biliary accumulation of nonmicellar, free bile salts lead to bile duct injury, fibrosis, and cirrhosis, requiring liver transplant in the first decade of life.\(^6\) These histological and biochemical characteristics are reproduced in mice with targeted disruption of the orthologous multidrug resistance 2 gene (\(Mdr2^{−/−}\) mice).\(^14\) In addition, \(Mdr2^{−/−}\) deficient mice develop multifocal strictures and segmental dilatations, “onion skin”-type periductal fibrosis, and focal fibro-obliteration of bile ducts, closely resembling biliary abnormalities occurring in primary and secondary sclerosing cholangitis in humans.\(^15,16\) Ursodeoxycholic acid (UDCA), a hydrophilic bile acid thought to function by diluting and displacing the “toxic bile,” improves outcomes for some PFIC3 patients; but even UDCA responders still progress to cirrhosis at age 15–20.\(^17\) Studies of UDCA in PSC patients have produced controversial results, including one report of worsened overall survival.\(^18–20\)

Physiological regulation of bile acid synthesis is maintained through a feedback mechanism mediated by the fibroblast growth factor 19 (FGF19) signaling pathway.\(^21–24\) In addition to its role in regulating bile acid homeostasis, FGF19 plays a role in regulating cell proliferation in the liver and has been implicated in the formation of hepatocellular carcinomas (HCCs).\(^25–30\) As a means of harnessing the therapeutic potential of the FGF19 pathway for treating bile acid–related diseases, we have engineered and characterized a nontumorigenic variant of FGF19, M70, that retains activities in regulating bile acid metabolism but does not promote hepatic tumorigenesis.\(^31,32\) M70 differs from wild-type FGF19 by three amino acid substitutions (A30S, G31S, and H33L) and a five–amino acid deletion at the N terminus. Unlike FGF19, M70 does not cause liver tumors even after prolonged exposure at supraphysiologic levels in \(db/db\) or rasH2 mice.\(^33\) M70 interacts with the FGFR4 receptor but exhibits the pharmacologic characteristics of a biased ligand that selectively activates certain signaling pathways (e.g., cytochrome P450 7A1, phosphorylated extracellular signal–regulated kinase) to the relative exclusion of others (e.g., tumorigenesis, phosphorylated signal transducer and activator of transcription 3).\(^31\) In the current study, we evaluated the effects of ectopic expression of FGF19 and M70 in the \(Mdr2^{−/−}\) mouse model and demonstrate that a one-time viral delivery of genes encoding these hormones efficiently produces long-lasting hepatoprotective, anti-inflammatory, and antifibrotic effects. Importantly, we show that, unlike FGF19, M70 not only fails to trigger hepatic tumor formation even after prolonged exposure but also exhibits antiproliferative effects in the context of sclerosing cholangitis.

Materials and Methods

ANIMALS AND ANIMAL CARE

Mice were handled and experiments were performed according to the protocols approved by the Institutional Animal Care and Use Committee at NGM based on the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals. \(Mdr2^{−/−}\) mice on an FVB/N background (stock number 002539) and sex-matched and age-matched wild-type FVB/N mice were obtained from Jackson Laboratory; 12-week-old \(Mdr2^{−/−}\) mice (males and females) received a single
intravenous dose of $1 \times 10^{11}$ vector genome of adenovirus (AAV) containing genes encoding either FGF19, M70, or green fluorescent protein.

**BLOOD PARAMETERS**

Blood was collected from the tail vein or after death using microvette tubes (Sarstedt). Alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate transaminase (AST), triglyceride, and cholesterol levels were measured on a Cobas Integra 400 Clinical Analyzer (Roche Diagnostics). Plasma FGF19 level was determined by FGF19 enzyme-linked immunosorbent assays (Biovendor). All assays were performed according to the manufacturers’ instructions.

**HEPATIC BILE ACID POOL SIZE AND SERUM BILE ACID CONCENTRATIONS**

Livers from female Mdr2$^{-/-}$ mice were homogenized in 75% ethanol and incubated at 50°C for 2 hours. After centrifugation at 6000g for 10 minutes, the pellets were extracted again with 50% ethanol. Supernatants from the two extraction steps were pooled, evaporated, and reconstituted in 50% ethanol. Concentrations of total bile acids in liver extracts or serum were determined using a 3z-hydroxysteroid dehydrogenase method (Diazyme).

**HEPATIC HYDROXYPROLINE CONTENT**

Livers from female Mdr2$^{-/-}$ mice or wild-type mice were homogenized in water and hydrolyzed in concentrated hydrochloric acid at 120°C for 3 hours. Hydroxyproline concentrations were determined by the reaction of oxidized hydroxyproline with 4-(dimethylamino)benzaldehyde using a colorimetric kit from Sigma.

For detailed information, please refer to the Supporting Information.

**Results**

**FGF19 AND M70 DIMINISH LIVER INJURY IN MDR2$^{-/-}$ MICE**

Bile duct injury in Mdr2$^{-/-}$ mice is a progressive process resulting from the detergent-like properties of nonmicellar-bound, free biliary bile acids. In this study, Mdr2$^{-/-}$ mice were treated at 12 weeks of age, at which point segmental biliary strictures, dilatations, “onion skin”-type fibrosis, and severe cholangiocyte injury were already fully developed. To assess potential therapeutic efficacy in the context of Mdr2$^{-/-}$ mice, we introduced genes encoding FGF19 or M70 into Mdr2$^{-/-}$ mice by AAV-mediated gene delivery (Fig. 1A,B), which allows stable transgene expression for up to 1 year without inflammatory responses.

Four weeks following gene delivery, significant reductions of serum levels of ALP, a marker of biliary damage, were observed in mice expressing FGF19 (69% reduction from baseline of 349 ± 26 U/L to 107 ± 16 U/L and 83% reduction from baseline of 565 ± 49 U/L to 98 ± 7 U/L in male and female mice, respectively; n = 6, P < 0.001; Fig. 1C; Supporting Fig. S1A). A similarly profound reduction in ALP levels was observed in Mdr2$^{-/-}$ mice in response to M70 transgene expression (59% reduction from baseline of 343 ± 18 U/L to 142 ± 12 U/L and 78% reduction from baseline of 598 ± 29 U/L to 130 ± 7 U/L in males and female mice, respectively; n = 5, P < 0.001). Notably, these reduced serum levels of ALP were maintained throughout the course of the study period, 24 weeks after gene delivery. The improvement in ALP levels associated with the ectopic expression of FGF19 and M70 in Mdr2$^{-/-}$ mice was accompanied by marked reductions in the serum levels of ALT and AST (Fig. 1D,E; Supporting Fig. S1B,C). Overall, hepatobiliary damage in female Mdr2$^{-/-}$ mice appears to be more severe than that in their male counterparts, as manifested by higher serum levels of ALP, ALT and AST. Nonetheless, expression of FGF19 and M70 transgenes restores liver enzymes to normal levels and reverses liver injury in both male and female mice. Mean plasma FGF19 levels were 41 ± 9 ng/mL and 22 ± 9 ng/mL in males and females, respectively, and mean plasma M70 levels were 18 ± 10 ng/mL and 9 ± 3 ng/mL in males and females, respectively, at the end of the study (Fig. 1F). Both FGF19 and M70 reduced body weights in these animals (Supporting Fig. S1D), consistent with a previous report on the regulation of energy metabolism by FGF19. Thus, we conclude that ectopic expression of FGF19 or M70 in Mdr2$^{-/-}$ mice results in rapid, robust, and sustained reduction in serum levels of ALP, ALT, and AST, markers of biliary and hepatocellular damage.

**FGF19 AND M70 AMELIORATE HEPATIC INFLAMMATION AND FIBROSIS IN MDR2$^{-/-}$ MICE**

Most cholangiopathies are associated with portal inflammation in proximity to the biliary epithelium, and...
FIG. 1. FGF19 and M70 diminish liver injury in Mdr2⁻/⁻ mice. (A) Schematics of the experiment. Twelve-week-old Mdr2⁻/⁻ mice were injected with AAV carrying FGF19, M70, or a control gene (the gene for green fluorescent protein; n = 5 per sex per group). Liver enzymes were determined before and 4 and 24 weeks after AAV administration. (B) Sequence alignment of FGF19 and M70. Only N termini of the proteins are shown. (C) Serum levels of ALP over time (n = 5 mice per group). (D) Serum ALT (n = 5). (E) Serum AST (n = 5). (F) Circulating levels of FGF19 and M70 measured by enzyme-linked immunosorbent assay (n = 5). Values are mean ± standard error of the mean. **P < 0.01, ***P < 0.001 versus control group by two-way analysis of variance.
the initiation of inflammation plays a critical role in the pathogenesis of sclerosing cholangitis. We determined the expression profiles for a panel of genes encoding cytokines and chemokines in livers isolated from \textit{Mdr2}^{-/-} mice (Fig. 2A-C). Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis showed marked reductions in the expression of \textit{Tnf-\alpha}, \textit{Il-6}, \textit{Ccl2}, \textit{Cxcl2}, and \textit{Icam-1} in mice expressing either FGF19 or M70. Histologically, \textit{Mdr2}^{-/-} mice develop pronounced hepatic inflammation as the disease progresses, characterized by extensive mixed inflammatory infiltrates in the portal tracts and the parenchyma (Fig. 2D). In contrast, FGF19 and M70 clearly decreased the numbers of infiltrating leukocytes. Both FGF19 and M70 reduced
hepatic Cdi1b (Ifgam)-positive myeloid cells, F4/80 (Emr1)-positive macrophages, and Cd4-positive T cells as shown by qRT-PCR analysis (Fig. 2E).

Whereas pronounced “onion skin”-like fibrotic rings were evident in Mdr2\(^{2-/-}\) mice injected with the control virus, as revealed by staining with hematoxylin and eosin, sirius red, and trichrome. Livers from Mdr2\(^{2-/-}\) mice treated with control virus show characteristic "onion skin" morphology with a pronounced periductal fibrotic ring. Periductal collagen fibers appear red in sirius red staining and blue in trichrome staining. Scale bars = 100 μm. (B) mRNA levels of collagens (n = 5). (C) mRNA levels of profibrotic genes (n = 5). (D) mRNA levels of genes in tissue remodeling (n = 5). (E) Hepatic hydroxyproline content (n = 5). Values are mean ± standard error of the mean. *P < 0.05, **P < 0.01, ***P < 0.001 versus control group by one-way analysis of variance. Abbreviations: bd, bile duct; C, control; H&E, hematoxylin and eosin; KO, knockout; WT, wild type.

Taken together, these results indicate that the expression of FGF19 and M70 in Mdr2\(^{2-/-}\) mice...
markedly reduces the hepatic infiltration of inflammatory cells, the induction of proinflammatory cytokines, and the activation of periductal myofibroblasts, leading to the effective resolution of hepatic fibrosis and sclerosing cholangitis observed in this model.

**FGF19 AND M70 INHIBIT BILE ACID SYNTHESIS AND REGULATE THE EXPRESSION OF KEY GENES IMPLICATED IN BILE ACID HOMEOSTASIS IN MDR2<sup>−/−</sup> MICE**

To gain further insights into mechanisms leading to the reversal of chronic liver injury and cholangiopathy by FGF19 and M70 in Mdr2<sup>−/−</sup> mice, we examined the effects of these hormones on the hepatic expression of genes encoding key enzymes in the bile acid synthetic pathways. The expression of Cyp7a1, which catalyzes the first and rate-limiting step in the classic bile acid synthetic pathway,(24) is highly regulated through a negative feedback mechanism mediated by FGF19.(37,38) The mRNA levels of Cyp7a1 were markedly suppressed by FGF19 and M70 in Mdr2<sup>−/−</sup> mice (87% and 70% reduction by FGF19 and M70, respectively; n = 5, P < 0.001; Fig. 4A). In contrast, mRNA levels of Cyp8b1, a key enzyme controlling the synthesis of cholic acid, were not significantly different in Mdr2<sup>−/−</sup> mice expressing the FGF19, M70, or control transgene. Hepatic expression of Cyp27a1, which catalyzes the initial step of the alternative bile acid biosynthetic pathway, was also significantly

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**FIG. 4.** FGF19 and M70 inhibit bile acid synthesis and regulate key genes in bile acid homeostasis in Mdr2<sup>−/−</sup> mice. Twelve-week-old Mdr2<sup>−/−</sup> mice were injected with AAV carrying FGF19, M70, or a control gene (n = 5 per group). Hepatic gene expression was determined 24 weeks after AAV administration. Mdr2<sup>+/+</sup> mice (wild type) were included as comparators for Mdr2<sup>−/−</sup> mice (knockout) in gene expression analysis. (A) mRNA levels of bile acid synthetic enzymes in the classic pathway (n = 5). (B) mRNA levels of bile acid synthetic enzymes in the alternate pathway (n = 5). (C) Hepatic bile acid pool (n = 5). (D) Serum levels of total bile acids. (E) Expression profiles of bile acid uptake, canalicular efflux, and basolateral efflux transporters as measured by qRT-PCR (n = 5). Values are mean ± standard error of the mean. ***P < 0.001 versus control group by one-way analysis of variance. Abbreviations: BA, bile acid; C, control; KO, knockout; WT, wild type.
suppressed by FGF19 and M70 (41% and 40% reduction by FGF19 and M70, respectively; n = 5, P < 0.001, Fig. 4B), whereas mRNA levels of Cyp7b1 were not affected.

Importantly, expression of either FGF19 or M70 dramatically reduced the hepatic pool of bile acids in these mice compared with the severely elevated levels measured in livers isolated from Mdr2−/− mice expressing a control transgene (Fig. 4C). The dysregulated bile acid metabolism resulting from Mdr2 deficiency leads to elevated levels of bile acids in the serum of these mice, which continue to rise as the animals age and the disease progresses (Fig. 4D). Consistent with the observations regarding the increased serum levels of liver enzymes, female Mdr2−/− mice appear to manifest more severe disease symptoms, as evidenced by higher baseline serum levels of total bile acids and more pronounced progression over time compared with their male counterparts in the study. Strikingly, the elevated serum levels of total bile acids associated with the Mdr2 deficiency in these mice were completely normalized in both male and female mice expressing FGF19 or M70 (Fig. 4D).

In addition to the regulated synthesis of bile acids, a variety of hepatic membrane transporters play

FIG. 5. FGF19 and M70 restore lipid homeostasis in Mdr2−/− mice. Twelve-week-old Mdr2−/− mice were injected with AAV carrying FGF19, M70, or a control gene (n = 5 per sex per group). Serum concentrations of triglycerides and cholesterol and hepatic gene expression were determined 24 weeks after AAV administration. Mdr2+/+ mice (wild type) were included as comparators for Mdr2−/− mice (knockout) in gene expression analysis. (A) Serum levels of total cholesterol (n = 5). (B) Expression of key genes involved in cholesterol synthesis and clearance (n = 5). (C) Expression of cholesterol efflux pumps (n = 5). (D) Serum levels of triglycerides (n = 5). (E) Hepatic mRNA levels of genes involved in lipogenesis (n = 5). Values are mean ± standard error of the mean. *P < 0.05, **P < 0.01, ***P < 0.001 versus control group by one-way analysis of variance. Unpaired, two-tailed t test was used to compare two groups. Abbreviations: C, control; KO, knockout; WT, wild type.
critical roles in preserving bile acid homeostasis.\(^{(39)}\) Disruption of the Mdr2 transporter in Mdr2\(^{-/-}\) mice markedly disturbs bile acid homeostasis in these animals, leading to severe conditions of cholestasis and exacerbated bile acid–related liver damage.\(^{(40)}\) To determine whether changes in bile acid transport contribute to the beneficial effects of ectopic FGF19 and M70 expression in Mdr2\(^{-/-}\) mice, we examined the mRNA levels of key bile acid transporters. Neither FGF19 nor M70 inhibited expression of the hepatocellular uptake transporters (Ntcp, Oatp1, and Oatp2) or induced expression of the canalicular (Bsep, Mrp2, and Mdr2; note the lack of Mdr2 expression in these mice) or basolateral (Ost-β, Mrp3, and Mrp4) efflux pumps (Fig. 4E). Based on these observations, adaptive changes in the expression of hepatic bile acid transporters do not account for the restoration of bile acid homeostasis and the normalization of hepatic and serum bile acid levels in response to FGF19 or M70.

Collectively, these data strongly suggest that inhibition of de novo bile acid synthesis by FGF19 and M70, rather than induction of adaptive responses, limits hepatic accumulation of toxic bile and leads to improvement in liver health in Mdr2\(^{-/-}\) mice.

**FGF19 AND M70 RESTORE LIPID HOMEOSTASIS IN MDR2\(^{-/-}\) MICE**

Mdr2\(^{-/-}\) mice display multiple derangements of lipid homeostasis,\(^{(41)}\) including alterations in cholesterol and triglyceride metabolism, that closely resemble clinical observations in PFIC3 patients.\(^{(42,43)}\) For example, Mdr2\(^{-/-}\) mice exhibit lower serum levels of total cholesterol compared with wild-type mice. Strikingly, treatment with AAV-FGF19 and AAV-M70 restored serum cholesterol to normal levels 24 weeks after injection (Fig. 5A). Furthermore, gene expression analysis by qRT-PCR revealed that mRNA levels of Sreb2, the master regulator of cholesterol synthesis, and Hmgcr, the rate-limiting cholesterol biosynthetic enzyme, were significantly reduced following FGF19 or M70 treatment (Fig. 5B). On the other hand, hepatic expression of Ldlr was modestly reduced in mice treated with FGF19 or M70, which may at least partially contribute to the increase in serum cholesterol in these study groups. Interestingly, the mRNAs for Abcg5 and Abcg8, efflux pumps responsible for transporting cholesterol out of the liver, were increased in animals expressing the FGF19 or M70 transgene (Fig. 5C). No significant differences in Starb1 mRNA levels were observed in livers isolated from mice in the various treatment groups. Collectively, these data indicate that multiple genes involved in cholesterol metabolism and transport are regulated in response to FGF19 and M70 to reduce hepatic cholesterol synthesis and enhance cholesterol efflux.

Contrary to the effects on circulating cholesterol levels, serum triglycerides were reduced by administration of AAV-FGF19 or AAV-M70 in Mdr2\(^{-/-}\) mice (Fig. 5D). Consistent with reductions in hepatic triglyceride synthesis, we observed a dramatic decrease in hepatic expression of key regulators of lipogenesis, including Scd1, Fasn, Elovl6, Elovl7, Ady, and Aacd, in Mdr2\(^{-/-}\) mice expressing either the FGF19 or M70 transgenes (Fig. 5E).

These data suggest that the endocrine hormones FGF19 and M70 act to restore lipid homeostasis in Mdr2\(^{-/-}\) mice by mediating the transcriptional regulation of genes implicated in the efflux of cholesterol from the liver, as well as genes involved in the hepatic synthesis of cholesterol and triglycerides.

**FGF19 AND M70 REDUCE CHOLECYSTOLITHIASIS IN MDR2\(^{-/-}\) MICE**

To determine whether FGF19 and M70 affect bile composition, we performed a cholecystectomy on Mdr2\(^{-/-}\) mice 12 weeks after injection of AAV-encoded transgenes and examined gallbladder bile (Fig. 6A,B). Both FGF19 and M70 markedly reduced gallbladder bile volume in Mdr2\(^{-/-}\) mice (Fig. 6C). Gallbladder bile in Mdr2\(^{-/-}\) mice contains only trace amounts of phospholipids due to disruption of the Mdr2 phospholipid flippase, and this low level of phospholipids was not affected by FGF19 or M70 expression (Fig. 6D). In contrast, concentrations of total bile acids in gallbladder bile were significantly reduced by either FGF19 or M70 treatment (Fig. 6D).

Similar to human low phospholipid–associated cholestasis patients with MDR3 defects, Mdr2\(^{-/-}\) mice develop gallbladder stones over time, with female mice displaying a markedly higher susceptibility to gallstone formation.\(^{(44)}\) Multiple gallstones were observed in the gallbladders from 24-week-old female Mdr2\(^{-/-}\) mice (Fig. 6E), whereas treatment with FGF19 or M70 reduced both the number and the size of gallstones in these mice (Fig. 6E).

These data suggest that expression of FGF19 and M70 decreases biliary bile acids without altering levels of phospholipids in the bile and reduces the formation of gallstones associated with Mdr2 deficiency.
FGF19, BUT NOT M70, INDUCES HEPATOCELLULAR CARCINOMGENESIS IN MDR2−/− MICE

Despite impressive hepatoprotective effects in the weeks immediately following gene delivery, prolonged ectopic overexpression of FGF19 also drives the development of liver tumors in Mdr2−/− mice (Fig. 7A,B). In contrast, mice injected with AAV-M70 remained tumor-free for the duration of the experiment. Importantly, whereas all liver tumors in FGF19-expressing mice stained positive for glutamine synthetase, a marker of pericentral hepatocytes (Fig. 7C), increased staining of glutamine synthetase was not observed in Mdr2−/− mice expressing M70. The FGF19-induced liver tumors displayed macroscopic and microscopic characteristics typical of HCC. HCC tumor area...
occupied 69%, 0%, and 0% of the entire liver area in Mdr2<sup>−/−</sup> mice injected with recombinant AAV encoding FGF19, M70, or the control gene (n = 5 per group). Liver histology and gene expression were determined 32 weeks after AAV administration. (B) Macroscopic appearance of livers. (C) Representative hematoxylin and eosin and anti-glutamine synthetase staining of livers. White boxes denote area showing at higher magnifications below. 3,3′-Diaminobenzidine substrates were used for anti-glutamine synthetase immunostaining (brown color). The necrotic area in liver tumors induced by AAV-FGF19 is indicated. Scale bars = 5 mm (top panels) and 100 µm (bottom panels). (D) Morphometric quantification of glutamine synthetase-positive tumor areas (n = 5). (E) mRNA levels of key genes implicated in hepatocarcinogenesis (n = 5). (F) mRNA levels of Cyclins (n = 5). Values are mean ± standard error of the mean. **P < 0.01, ***P < 0.001 versus control group by one-way analysis of variance. Abbreviations: C, control; cv, central vein; H&E, hematoxylin and eosin; Nec, necrotic area; Tu, tumor.

FIG. 7. FGF19, but not M70, induces hepatocellular carcinoma in Mdr2<sup>−/−</sup> mice. (A) Study design. Twelve-week-old Mdr2<sup>−/−</sup> mice were injected with AAV carrying FGF19, M70, or a control gene (n = 5 per group). Liver histology and gene expression were determined 32 weeks after AAV administration. (B) Macroscopic appearance of livers. (C) Representative hematoxylin and eosin and anti-glutamine synthetase staining of livers. White boxes denote area showing at higher magnifications below. 3,3′-Diaminobenzidine substrates were used for anti-glutamine synthetase immunostaining (brown color). The necrotic area in liver tumors induced by AAV-FGF19 is indicated. Scale bars = 5 mm (top panels) and 100 µm (bottom panels). (D) Morphometric quantification of glutamine synthetase-positive tumor areas (n = 5). (E) mRNA levels of key genes implicated in hepatocarcinogenesis (n = 5). (F) mRNA levels of Cyclins (n = 5). Values are mean ± standard error of the mean. **P < 0.01, ***P < 0.001 versus control group by one-way analysis of variance. Abbreviations: C, control; cv, central vein; H&E, hematoxylin and eosin; Nec, necrotic area; Tu, tumor.

occupied 69%, 0%, and 0% of the entire liver area in Mdr2<sup>−/−</sup> mice injected with recombinant AAV encoding FGF19, M70, or the control gene, respectively (Fig. 7D). Increased hepatic expression of α-fetoprotein (Afp) and glypican-3 (Gpc3), markers often increased in human HCC, was induced by AAV-FGF19 but not AAV-M70 (Fig. 7E). Moreover, mRNA levels of cyclins involved in mitosis and cell cycle progression, including cyclin a2 (Ccna2), Ccnb1, and Ccnb2, were significantly elevated in the livers expressing FGF19 but not M70 (Fig. 7F).

In summary, we used intravenous delivery of AAV-FGF19 in Mdr2<sup>−/−</sup> mice to generate a mouse model of HCC in the context of severe cholangiopathy. The resulting tumors display macroscopic, histopathologic, and molecular features characteristic of HCC. Notably, unlike FGF19, expression of M70 does not promote the formation of hepatic tumors in this model.

**M70 EXHIBITS ANTIPROLIFERATIVE EFFECTS IN MDR2<sup>−/−</sup> MICE**

Closely resembling the histopathological aspects of the disease observed in patients with PSC, PFIC3,
and other cholangiopathies, ductular proliferation in \( Mdr2^{-/-} \) mice resulted in extensive portal expansion as the disease progressed. We evaluated cholangiocyte proliferation by Ki-67 staining. Strikingly, \( Mdr2^{-/-} \) mice expressing M70 displayed significantly decreased numbers of proliferating cholangiocytes compared with mice receiving a control virus (Fig. 8A). Morphometric analysis of the bile duct area, as revealed by Dolichos biflorus agglutinin-fluorescein and hematoxylin and eosin staining, showed a marked reduction in bile duct mass in M70-treated mice (Fig. 8B; Supporting Fig. S4A). Furthermore, mRNA levels of a ductal marker, cytokeratin-19, were also reduced in mice expressing M70.

Moreover, \( Mdr2^{-/-} \) mice treated with AAV-M70 showed a clear reduction of proliferating (Ki-67-positive) hepatocytes compared with those receiving a control virus (Fig. 8C). Evidence of advanced liver disease commonly associated with \( Mdr2 \) deficiency, including cytological atypia and dysplasia, architectural disorganization, and aberrant mitotic figures, were markedly improved in the livers of mice expressing M70 (Supporting Fig. S4B). Consistent with reductions in both proliferating cholangiocytes and hepatocytes, M70-treated \( Mdr2^{-/-} \) mice exhibited normal liver weight, as well as liver-to-body weight ratios (Fig. 8D), suggesting the reversal of the hepatomegaly commonly observed in \( Mdr2^{-/-} \) mice as well as in patients with cholangiopathy. In conjunction with reductions in liver weight, \( Mdr2^{-/-} \) mice treated with M70 also showed improvement in splenomegaly (Fig. 8E), another feature clinically associated with portal hypertension and cirrhosis.

In summary, M70 exerts antiproliferative effects in the context of sclerosing cholangitis and appears to reverse hepatosplenomegaly in \( Mdr2^{-/-} \) mice. These results indicate that M70 treatment may confer additional protective benefits, in addition to anti-inflammatory and antifibrotic effects.

**Discussion**

We report here that, in a mouse model of chronic cholangiopathy AAV-mediated delivery of FGF19, or engineered analogue M70, provides effective, long-term improvement of liver injury, hepatic inflammation, and fibrosis. Both FGF19 and M70 profoundly inhibit de novo bile acid synthesis, resulting in significant reduction in liver damage without induction of adaptive responses in membrane transporters. Furthermore, we show that FGF19 and M70 restore hepatic and systemic lipid homeostasis and reduce cholecystolithiasis. Importantly, in striking contrast to FGF19, M70 does not induce HCC formation, even after prolonged exposure, and further corrects ductular proliferation and hepatosplenomegaly in these mice. Thus, we have established an in vivo system for evaluating the tumorigenic effect of FGF19 in the context of chronic cholangiopathy and demonstrate that the engineered, nontumorigenic FGF19 variant M70 reverses sclerosing cholangitis in this mouse model with genetic, clinical, and phenotypic features that closely resemble human diseases.

As an endocrine hormone, FGF19 plays a key role in the regulation of hepatic bile acid metabolism under physiological conditions. Predominantly expressed in the ileum, FGF19 is released into portal circulation as part of the feedback mechanism to suppress bile acid synthesis in the liver. Hepatocytes are the only cell type in the liver that expresses the FGF19 receptor complex FGFR4-βKlotho. Therefore, the anti-inflammatory and antifibrosis effects of FGF19 and M70 likely result from direct targeting of hepatocytes. Concentrations of FGF19 increase under cholestatic and cirrhotic conditions, suggesting that FGF19 itself represents a component of the adaptive hepatic response to these pathophysiological circumstances. Seemingly paradoxically, we show here that, while providing important hepatoprotective benefits, prolonged exposure to FGF19 at circulating levels as low as 20 ng/mL strongly promotes HCC formation in the \( Mdr2^{-/-} \) model. Thus, FGF19 acts as a “double-edged sword” that, on the one hand, serves as part of an adaptive response to limit liver injury and, on the other hand, may play a causal role in tumor promotion and contribute to the increased HCC risk in patients with chronic liver diseases. Nontumorigenic FGF19 variants, such as M70, provide a novel approach to capitalize on our knowledge of this pathway for potential therapeutic use.

Enhanced transcription of MDR3 by experimental drugs such as farnesoid X receptor and peroxisome proliferator-activated receptor α agonists contributes, at least in part, to the proposed mechanism of action of such compounds. An understanding of the allelic variation and polymorphism of MDR3, as well as how MDR3 interacts with other genes to affect individual susceptibility to adult-onset liver disease, is only just beginning to emerge. Despite tremendous progress in understanding the molecular basis of cholangiopathy
in MDR3-related genetic disorders, effective treatments are still lacking. To date, there are no drugs approved by the US Food and Drug Administration, or other regulators, for treating cholangiopathies, including PSC and PFIC3. UDCA, a hydrophilic bile acid, is thought to reduce liver injury by promoting choleresis, diluting and displacing toxic bile acids, and inducing the bile acid detoxification and export.

**FIG. 8.** M70 reduces ductular and hepatocellular proliferation in Mdr2<sup>−/−</sup> mice. Twelve-week-old Mdr2<sup>−/−</sup> mice received a single dose of AAV carrying M70 or a control gene (n = 5 per group). Liver histology and gene expression were determined 24 weeks after AAV administration. (A) Representative Ki67 immunostaining of bile ducts, quantification of bile duct mass (n = 5), and qRT-PCR analysis of ductal marker cytokeratin-19 (n = 5). 3,3′-Diaminobenzidine substrates were used with anti-Ki67 staining (brown color). Scale bars = 100 μm. (B) Representative images of the bile ducts. Bile ducts were stained with fluorescein-labeled *Dolichos biflorus* agglutinin. Green (fluorescein) and blue (4',6-diamidino-2-phenylindole for nuclear counterstain) channel images are shown. The pronounced bile duct proliferation and biliary tract expansion in Mdr2<sup>−/−</sup> mice were reversed by M70 treatment. Scale bars = 100 μm. (C) M70 inhibits proliferation of hepatocytes as shown by reduced Ki-67 staining (brown color) and Ki-67 mRNA levels (n = 5). Scale bars = 100 μm. (D) Liver weight and ratios of liver-to-body weight (n = 5). (E) Spleen photo, spleen weight, and ratios of spleen-to-body weight (n = 5). Values are mean ± standard error of the mean. ***P < 0.001 versus control group by unpaired, two-tailed t test. Abbreviations: C, control; DAPI, 4',6-diamidino-2-phenylindole; DBA, *Dolichos biflorus* agglutinin.
systems. Previous studies have shown that when administered in the diet for 4 weeks, UDCA only slightly decreases periductal fibrosis but does not repair liver injury in Mdr2<sup>−/−</sup> mice. Administration of farnesoid X receptor agonist INT-747 aggravated liver damage in this model, presumably resulting from the induction of bile acid efflux transporters in the context of biliary strictures. None of these studies examined the effects on cholecystolithiasis in Mdr2<sup>−/−</sup> mice. In contrast, M70, acting through a novel mechanism by suppression of bile acid synthesis without promoting bile acid efflux, effectively resolves periductal fibrosis and sclerosing cholangitis in Mdr2<sup>−/−</sup> mice and appears significantly more effective than UDCA or farnesoid X receptor agonists in this setting. Previous studies describing therapeutic agents in the Mdr2<sup>−/−</sup> mouse model often start treatment in 8-week-old male mice, and with studies using female mice (which develop much more severe liver disease) mostly nonexistent, the efficacy of M70 in this setting is particularly notable. Additionally, the therapeutic approach described in this report requires only a single intravenous injection of AAV carrying M70 to achieve the sustained efficacy, a property especially attractive for treating genetic disorders where lifelong intervention is required.

The increase in serum cholesterol by FGF19 and M70 in the Mdr2<sup>−/−</sup> mouse model is of particular interest because low serum levels of cholesterol have been observed in patients with MDR3 mutations and in Mdr2<sup>−/−</sup> mice. Both FGF19 and M70 act to reduce hepatic synthesis of cholesterol and triglyceride and promote cholesterol efflux from the liver. These lipid regulatory mechanisms may further contribute to the beneficial effects ascribed to FGF19 and M70 expression.

In humans, high concentrations of FGF19 are detected in the gallbladder bile (>20 ng/mL, comparing with ~0.2 ng/mL in plasma). High levels of FGF19 mRNA are found in the gallbladders of mice expressing an FGF19 transgene, including regulatory sequences, from a human genomic BAC clone. Notably, the induction of FGF19 transcripts in human liver during cholestasis was shown to originate in the nonparenchymal cells rather than hepatocytes. Our current study does not exclude the intriguing possibility that FGF19 in the bile could have a direct, cytochrome P450 7A1-independent, protective effect on cholangiocytes. Previous studies also suggested a direct action of FGF19 on gallbladder motility. The mechanism underlying improvement in cholecystolithiasis by FGF19 and M70 in Mdr2<sup>−/−</sup> mice, particularly in the context of reducing biliary bile acids without altering biliary phospholipids, should be an area of future investigation.

In summary, we find compelling evidence that M70, an engineered analogue of the gut hormone FGF19, possesses profound anti-inflammatory, anti-biobotic, and hepatoprotective activities in mice exhibiting chronic cholangiopathy. Together with previous work, our studies support a unifying model that the suppression of de novo bile acid synthesis by FGF19 and analogues protects not only hepatocytes but also cholangiocytes from toxic bile-induced injury. These findings have broadened concepts regarding the role of bile acids in the initiation and progression of cholangiopathy and expanded our ability to potentially devise precision medicines for patients with MDR3 and/or phospholipid deficiency.

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Author names in bold denote shared co-first authorship.

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