Separating Tumorigenicity from Bile Acid Regulatory Activity for Endocrine Hormone FGF19

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All authors are employees and stockholders of NGM Biopharmaceuticals, Inc.

Running Title: Separating Tumorigenicity from Bile Acid Activity for FGF19
ABSTRACT

Hepatocellular carcinoma (HCC), one of the leading causes of cancer-related death, develops from premalignant lesions in chronically damaged livers. While it is well-established that FGF19 acts through receptor complex FGFR4-β-Klotho (KLB) to regulate bile acid metabolism, FGF19 is also implicated in the development of HCC. In humans, FGF19 is amplified in HCC and its expression is induced in the liver under cholestatic and cirrhotic conditions. In mice, ectopic overexpression of FGF19 drives HCC development in a process that requires FGFR4. In this study, we describe an engineered FGF19 (M70) which fully retains bile acid regulatory activity but does not promote HCC formation, demonstrating that regulating bile acid metabolism is distinct and separable from tumor-promoting activity. Mechanistically, we show that FGF19 stimulates tumor progression by activating the STAT3 pathway, an activity eliminated by M70. Furthermore, M70 inhibits FGF19-dependent tumor growth in a rodent model. Our results suggest that selectively targeting the FGF19-FGFR4 pathway may offer a tractable approach to improve the treatment of chronic liver disease and cancer.

INTRODUCTION

FGF19 (also called FGF15 in rodents) is an endocrine hormone of the FGF family that regulates bile acid, carbohydrate, lipid and energy metabolism (1). FGF19 selectively binds to FGFR4, which can be further enhanced by co-receptor KLB (2-5). The interaction between FGF19 and FGFR4 is crucial in repressing hepatic expression of cholesterol 7α-hydroxylase (CYP7A1), the first and rate-limiting enzyme in the conversion of cholesterol into bile acids (6-9). Interestingly, FGF19-FGFR4 signaling is also implicated in hepatocellular tumorigenesis. In humans, FGF19 is co-amplified with Cyclin D1 (CCND1) at ~15% frequency in HCC on 11q13.3 (10). Clonal growth and tumorigenicity of HCC cells harboring the 11q13.3 amplicon can be inhibited by RNAi-mediated knockdown of FGF19, as well as an anti-FGF19 antibody (10). Transgenic mice with ectopic expression of FGF19 in skeletal muscle develop...
HCC at the age of 10-12 months old (11). This tumorigenic activity is thought to be mediated by the liver-enriched FGFR4, because inactivation of FGFR4 via gene knockout or by a neutralizing antibody reduces tumor burden in FGF19-transgenic mice (12, 13). Therefore, targeting the FGF19-FGFR4 pathway has the potential for treating HCC. However, early efforts have encountered setbacks. Severe toxicity was observed in non-human primates by an anti-FGF19 neutralizing antibody due to on-target inhibition of endogenous FGF19, leading to dysregulation of bile acid metabolism (14). Hence, alternative approaches are needed to overcome these barriers to developing successful therapy.

In an effort to eliminate the tumorigenic activity of FGF19 without compromising its beneficial role in bile acid homeostasis, we established an in vivo liver tumorigenicity model in mice to evaluate FGF19-induced hepatocarcinogenicity. Using an adeno-associated virus (AAV)-mediated gene delivery approach (15), we introduced FGF19 transgene in mice and evaluated a panel of FGF19 variants in vivo to identify tumor-free variants. In this paper, we show that one such variant, M70, fully retains the biological activity of FGF19 in regulating bile acid homeostasis while devoid of tumorigenicity. Notably, we found that M70 binds to and activates FGFR4, which is assumed to mediate FGF19-associated tumorigenicity (13, 16). Mechanistically, we show that FGF19 stimulates tumor progression by activating the STAT3 pathway, an activity completely eliminated by M70. Furthermore, M70 inhibits FGF19-dependent tumor growth in a rodent model.

MATERIALS AND METHODS

DNA Constructs. Human FGF19 (NM_005117), human FGFR4 (NM_022963), mouse FGFR4 (NM_008011), human KLB (NM_175737) and mouse KLB (NM_031180) cDNAs were purchased from Genecopoeia. Mutations were introduced in the FGF19 constructs using the QuickChange Site-Directed Mutagenesis kit (Stratagene).
**AAV Production.** AAV293 cells (Agilent Technologies) were cultured in Dulbecco’s Modification of Eagle’s Medium (DMEM; Mediatech) supplemented with 10% fetal bovine serum and 1x antibiotic-antimycotic solution (Mediatech). The cells were transfected with 3 plasmids (AAV transgene, pHelper (Agilent Technologies) and AAV2/9) for viral production. Viral particles were purified using a discontinued iodixanal (Sigma-Aldrich) gradient and re-suspended in phosphate-buffered saline (PBS) with 10% glycerol and stored at −80°C. Viral titer or genome copy number was determined by quantitative PCR.

**Animal Experiments.** All animal studies were approved by the Institutional Animal Care and Use Committee at NGM. Mice were 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. Male mice were used unless otherwise specified. C57BL/6J, FVB/NJ, BDF, ob/ob and db/db mice were purchased from Jackson Laboratory. Heterozygous rasH2 transgenic mice were obtained from Taconic. All animals received a single 200µL intravenous injection of 3x10¹¹ genome copies of AAV via tail vein on Day 1. Animals were euthanized and livers were collected 24 or 52 weeks after dosing with AAV. Liver weight and liver tumor nodule numbers were recorded upon necropsy.

**Histological and Immunohistochemical analysis.** Formalin-fixed paraffin-embedded tissue sections were stained with hematoxylin and eosin (H&E) for histological assessment at Nova Pathology (Bellingham, WA). For immunohistology, anti-PCNA (Dako), anti-Ki67 (Dako), anti-glutamine synthetase (ThermoFisher) or anti-β-catenin antibodies (Cell Signaling) were used. Biotinylated secondary antibody, ABC-HRP reagent and DAB colorimetric peroxidase substrate (Vector Laboratories) were used for detection.

**Measurement of Serum FGF19 Protein.** Whole blood was collected from mouse tail snips into capillary tubes (Becton Dickenson). Levels of human FGF19 and variants were measured in serum using an
enzyme-linked immunosorbence (ELISA) assay (Biovendor). The assay recognizes both FGF19 and M70 in an indistinguishable manner.

**Clinical Chemistry.** Serum levels of liver enzymes (ALT, AST, and ALKP), triglycerides, total cholesterol, HDL-C and LDL-C were measured using enzymatic reactions on COBAS Integra 400 clinical analyzer (Roche Diagnostics). Concentrations of total bile acids in serum were determined using a 3α-hydroxysteroid dehydrogenase method (Diazyme).

**Gene Expression Analysis.** Total RNA was extracted from tissues or cells using the RNeasy kit (Qiagen). qRT-PCR analysis was performed using QuantiTect multiplex qRT-PCR master mix (Qiagen) and premade primers and probes (Life Technologies). Reactions were performed in triplicates on Applied Biosystems 7900HT Sequence Detection System. Relative mRNA levels were calculated by the comparative threshold cycle method using GAPDH as the internal standard.

**Expression of Recombinant Proteins.** FGF19 and M70 were produced in E. coli and purified as described with modification (17). Methionine was added to the mature peptides to facilitate expression in bacteria. Proteins were then purified to homogeneity via successive rounds of ion-exchange and hydrophobic-interaction chromatography. Protein sequence was confirmed via LC/MS and monodispersity via SEC-HPLC (TOSOH TSKgelG3000).

**Surface Plasmon Resonance (SPR) Assays.** SPR experiments were performed on a Biacore T200 instrument at 25°C. For direct binding between FGFR4 and ligands, anti-Fc antibody was immobilized on a CM5 sensor chip using standard amine coupling procedure and mouse FGFR4(ECD)-Fc (R&D Systems) was captured subsequently. K\textsubscript{D} values were determined using the Biacore T200 evaluation software V.1 using a 1:1 binding model.

**Solid-Phase Binding Assay.** 96-well ELISA plates (Thermo Fisher) were coated with goat-anti-Fc, blocked with 3% bovine serum albumin (BSA), and then incubated with 1 μg/mL mouse FGFR4(ECD)-Fc (R&D Systems) in PBS containing 3% BSA. The plates were incubated with various concentrations of
FGF19 or M70 (in the presence of 20μg/mL heparin (Sigma) and 1μg/mL mouse KLB (R&D Systems)) in PBS/3% BSA. The bound proteins were detected using biotinylated FGF19-specific polyclonal antibody (R&D Systems) followed by streptavidin-HRP and the TMB peroxidase colorigenic substrate (KPL).

**Luciferase Assays.** Rat L6 myoblasts (ATCC) were cultured in DMEM supplemented with 10% FBS at 37°C under 5% CO2. Cells were transiently transfected with expression vectors encoding mouse KLB, mouse FGFR4, GAL4-Elk-1 transcriptional activator (pFA2-Elk1, Stratagene), and firefly luciferase reporter driven GAL4 binding sites (pFR-luc, Stratagene) using Fugene 6 transfection reagent (Roche Applied Science).

**CYP7A1 Expression in Primary Hepatocytes and in mice.** Primary hepatocytes from mouse or rat livers (Life Technologies) were plated on collagen I-coated 96-well plates (Becton Dickinson) and incubated overnight in Williams’ E media supplemented with 100 nM dexamethasone and 0.25 mg/mL matrigel. Cells were treated with recombinant FGF19 or M70 proteins for 24 hours. CYP7A1 expression in cell lysates was determined by qRT-PCR analysis. For assessing CYP7A1 regulation *in vivo*, 12-week old *db/db* mice were injected intraperitoneally with FGF19 or M70 proteins. Mice were euthanized 4 hours after dosing and livers were harvested for qRT-PCR analysis.

**In vivo Signaling Analysis.** *db/db* mice (9-11 weeks old) (Jackson Laboratories) were given intraperitoneal injections (1mg/kg) of FGF19 or M70 recombinant proteins. Livers were collected after injection and snap frozen in liquid nitrogen. Signaling proteins were detected in liver lysates with antibodies to pSTAT3 (Cell Signaling#9145), STAT3 (Cell Signaling#8768) or antibody cocktail I (Cell Signaling#5301).

**Statistical Analysis.** All results are expressed as the mean±standard error of the mean (SEM). One-way ANOVA followed by Dunnett’s post-test was used to compare data from multiple groups (GraphPad


Prism). When indicated, unpaired Student’s t-test was used to compare two treatment groups. A p-value of 0.05 or smaller was considered statistically significant.

RESULTS

An AAV-mediated transgene system for evaluation of hepatocellular tumorigenesis in vivo

AAV-mediated gene delivery provides a means to achieve continuous transgene expression without inflammatory responses that are commonly associated with other viral vectors (18). Sustained expression of up to one year has been observed with the AAV gene delivery method when introduced into adult mice (19). The first AAV vector was recently approved as a treatment for a genetic disorder in humans (20).

In the previously-reported FGF19 transgenic models, FGF19 was ectopically expressed in the skeletal muscle, a non-physiological site of FGF19 expression (8, 11). Under pathological conditions, such as cirrhosis or cholestasis, FGF19 expression is induced in the liver (21-24). As an alternative to the conventional approach of generating transgenic mice, we introduced FGF19 via AAV in 6-12 week old mice (Fig. 1A). The primary tissue of transgene expression using this method is liver (25).

Multiple mouse strains were evaluated for latency and robustness of FGF19-mediated liver tumor formation (Table 1). A control AAV virus encoding green fluorescent protein (AAV-GFP), did not promote liver tumor formation the mouse strains tested (Table 1). In general, mice injected with AAV-GFP exhibited similar phenotype as saline-injected animals (data not shown). For simplicity, only results from AAV-GFP-injected animals were shown as controls in following studies. Interestingly, the tumor latency varied depending upon the mouse genetic background. In particular, among several mouse strains tested, db/db mice exhibited the shortest latency and high tumor penetrance, with the appearance of multiple, large, raised tumor nodules protruding from the liver surface 24 weeks following AAV-FGF19
delivery (Fig. 1B). This finding is consistent with the observation that mutations in the leptin receptor are frequently found in cirrhotic livers and are linked to HCC in human (26, 27). *db/db* mice, which carry a genetic defect in the leptin receptor (28), provide a clinically relevant genetic context for evaluating candidate HCC-promoting genes.

AAV-mediated delivery of wild type FGF19 transgene into *db/db* mice resulted in high circulating levels of FGF19, reaching ~1μg/ml 1 week after a single tail vein injection and persisting throughout the 24-week study period (Fig. 1C). 24 weeks after gene delivery, the mice were euthanized and subjected to necropsy. Visible tumor nodules on the entire surface of the liver were counted (Fig. 1D). The maximum diameter of the liver tumor nodules was recorded (Fig. 1D). Occasionally a few liver tumor nodules were observed in *db/db* mice injected with control virus or saline, probably reflecting an increased background level of hepatic tumorigenesis in this genetic model (Fig. 1D and data not shown).

Microscopic examination classified the AAV-FGF19-induced *in situ* liver tumors as solid HCC, which resembled those reported in FGF19-transgenic animals (Fig. 1E). Cellular proliferative status, examined by immunohistochemical staining for Ki-67 and PCNA, indicated that the tumors were highly proliferative. Similar to the tumors observed in FGF19-transgenic mice, liver tumors in AAV-FGF19 mice were glutamine synthetase-positive, suggestive of a pericentral origin (11) (Fig. 1E). Liver tumors from AAV-FGF19 mice also showed increased nuclear staining for β-catenin (Fig. 1E). Taken together, these data suggest that AAV-mediated transgene expression in mice provides a robust system to evaluate FGF19-induced hepatocarcinogenesis *in vivo*.

*M70 is an engineered, non-tumorigenic FGF19*

Using AAV-mediated gene delivery method, we evaluated a panel of FGF19 variants in *db/db* mice for their tumorigenicity. A FGF19 variant carrying 3 amino acid substitutions (A30S, G31S, H33L) and a 5-amino acid deletion, referred as M70, was selected for further studies (Fig. 2A).
Whereas ectopic expression of FGF19 promoted significant liver tumor formation in db/db mice (15.6±2.8 tumor nodules per liver), livers from mice with high systemic exposure to M70 for 24 weeks were completely free of hepatic tumor nodules (Fig. 2B). FGF19-expressing mice exhibited a significant increase in liver weight (Fig. 2C), which closely correlates with liver tumor burden. In contrast, mice expressing M70 did not show any increase in liver weight (Fig. 2C). Similar results were obtained when the liver-to-body weight ratio was calculated (Fig. 2D and Fig. 2E). Average serum concentration of M70 was 2-3μg/ml in these mice, about 10,000-fold higher than circulating FGF19 levels in human (Fig. 2F). Histological analysis of livers from M70-expressing mice showed no evidence of neoplastic lesions associated with FGF19 overexpression in mice, including hepatocellular dysplasia, hepatocellular adenomas, or hepatocellular carcinomas (Fig. 2G). Moreover, as indicated by Ki-67 staining, overexpression of M70 did not promote hepatocellular proliferation observed in mice expressing FGF19 (Fig. 2G). Furthermore, while liver tumor lesions in FGF19 expressing mice became prominently stained for glutamine synthetase (a marker for pericentral hepatocytes), no increased expression of glutamine synthetase was observed in the liver of M70-expressing mice (Fig. 2G). Finally, no liver toxicity, as reflected by serum levels of liver enzymes, was observed following 24 weeks of exposure to M70 (Fig. 2H). Additional serum parameters (e.g. glucose, triglycerides, and cholesterol) are included in Supplementary Table S1. Taken together, these results demonstrate that M70 lacks the ability to promote hepatocellular tumorigenesis in db/db mice.

We also evaluated the liver tumorigenic potential of M70 in a rasH2 transgenic mouse model. CB6F1-RasH2 mice hemizygous for a human H-RAS transgene have been extensively used as an accelerated alternative to the conventional two-year carcinogenicity assessment in rodents (29). Sensitive to genotoxic and nongenotoxic carcinogens, rasH2 mice develop both spontaneous and induced neoplasms earlier than wild type mice. Since activation of RAS signaling pathway is frequently observed in human HCC (30), this strain provides a relevant genetic background for studying hepatocarcinogenicity.
During the course of a 52-week study, rasH2 mice expressing FGF19 or M70 had a significant reduction of body weight gain compared with control mice (Fig 3A). After 52 weeks of continuous exposure, clear differences in liver morphology were observed in mice expressing FGF19 compared to those expressing M70. Gross morphological changes, including the appearance of multiple tumor nodules, were observed in the livers of mice expressing FGF19 (Fig. 3B). In contrast, the livers from mice expressing M70 showed normal gross morphology and were completely free of tumor nodules (Fig. 3B). A low level of spontaneous liver tumor formation was observed in control rasH2 mice (Fig. 3B). Moreover, M70-expressing animals showed a dramatic decrease in liver weight compared with mice expressing FGF19 (Fig. 3C). M70 also normalized the ratio of liver to body weight in rasH2 mice (Fig. 3D). The serum levels of FGF19 and M70 in these mice are comparable, 155±28ng/ml and 209 ± 22 ng/ml, respectively (Fig. 3E).

H&E stained liver sections from these mice were evaluated for the presence of neoplastic lesions (Fig. 3F). In addition, anti-glutamine synthetase staining was carried out as a marker of FGF19-induced liver tumor (Fig. 3F). rasH2 mice expressing FGF19 displayed a variety of cellular abnormalities, including hepatocellular adenoma and hepatocellular carcinomas. Remarkably, none of the livers from mice expressing M70 exhibited histological evidence of neoplastic lesions (Fig. 3F). Consistent with results of histological analysis, increased hepatic expression of Ki-67 and AFP, an embryonic hepatic protein often induced in HCC (31), was observed in FGF19-expressing rasH2 mice, but not in mice expressing M70 (Fig. 3G).

In summary, unlike FGF19, prolonged exposure to high levels of M70 did not promote liver tumor formation, in either db/db or rasH2 mice.
M70 binds and activates FGFR4 in vitro and in vivo

To elucidate the molecular mechanism that underlies the inability of M70 to induce liver tumors, we assessed the interaction of M70 to the known receptor complex for FGF19. Surface plasmon resonance (SPR) analysis was used to measure direct binding of either M70 or FGF19 to FGFR4. As shown in Fig 4A and 4B, the affinity of M70 for FGFR4 was comparable to that of FGF19 (dissociation constant \(K_D = 134 \pm 47\) nM and \(167 \pm 5\) nM, respectively). Using a solid phase assay, M70 interacted with the FGFR4-KLB receptor complex (Fig. 4C), with the presence of KLB dramatically increased ligand-receptor affinity. The dissociation constant of M70 binding to the FGFR4-KLB receptor complex indicated a high-affinity interaction that was virtually identical to that of FGF19 (\(K_D = 2.14\) nM and \(K_D = 2.49\) nM; respectively).

We also evaluated the ability of M70 to activate its receptors in a cell-based assay using rat L6 cells transfected with an FGF-responsive GAL-Elk1 luciferase reporter (9, 32). In this assay, effective binding of a ligand to FGFR results in the activation of the endogenous ERK kinase pathway, leading to subsequent activation of a chimeric transcriptional activator comprising of an Elk-1 activation domain and a GAL4 DNA-binding domain. L6 cells lack functional FGFR or KLB and are only responsive to FGF19 when co-transfected with cognate receptors (data not shown). M70 activated intracellular signaling pathways in L6 cells co-expressing FGFR4 and KLB as effectively as FGF19 (EC50 = 38pM and 52pM for M70 and FGF19, respectively; Fig. 4D). In contrast, signaling in cells transfected with FGFR4 alone was much less responsive to either ligand, showing a > 500-fold reduction in potency upon addition of either FGF19 or M70 (Fig. 4D). These results suggest that the formation of a ternary complex between FGFR4-KLB co-receptors and the cognate ligands is important for potent activation of intracellular signaling.

In addition, we analyzed FGFR4 pathway activation in Hep3B, a human HCC cell line that expresses KLB and, among the FGFR isoforms, predominantly FGFR4. Recombinant M70 protein
induced phosphorylation and activation of ERK with a similar potency and efficacy as wild type FGF19 (half maximum effective concentration EC$_{50}$=0.38 nM and 0.37nM for M70 and FGF19, respectively; Fig. 4E).

Finally, we assessed a physiologically relevant activity of M70. FGF19 have been implicated in the regulation of hepatic bile acid metabolism in humans and in rodents (7) (8). FGF19 potently represses hepatic expression of CYP7A1, in a process that requires FGFR4 (8, 9). We evaluated the ability of M70 to regulate CYP7A1 in primary hepatocytes. Upon addition to the culture media, M70 effectively repressed CYP7A1 expression in primary hepatocytes derived from mouse or rat liver, showing an activity comparable to that of wild-type FGF19 (Fig. 4F).

To evaluate the acute effects of M70 administration on hepatic expression of CYP7A1 in vivo, mice were injected intraperitoneally (i.p.) with recombinant M70 or FGF19 protein at doses ranging from 0.001 to 10mg/kg (Fig. 4G). A single i.p. injection of M70 potently suppressed the expression of CYP7A1 mRNA with an ED$_{50}$ of 1.29µg/kg (Fig. 4G). Consistent with the repression of bile acid synthesis, serum levels of total bile acids were lower in mice with long-term exposure to M70 (Fig. 4H). These data demonstrate that systemic administration of M70 can potently and rapidly trigger FGFR4-mediated response in vivo.

In summary, M70 and wild type FGF19 exhibit a comparable profile of biological activity that leads to the activation of ERK and suppression of CYP7A1.

*M70 Exhibits Differential Signaling Pathway Activation Compared with FGF19*

M70 binds to the FGFR4 receptor complex and activates the intracellular signaling pathway leading to CYP7A1 repression, but does not promote liver tumor formation in either *db/db* or rasH2 mouse models. The identification and characterization of M70 allows us to define two distinct and
separable biological processes regulated by the FGF19-FGFR4 pathway, bile acid homeostasis and tumorigenesis.

In order to elucidate the molecular basis for this lack of tumorigenic potential, we analyzed the activation of key signaling proteins involved in tumorigenesis, including ERK, PI3K/AKT, STATs and WNT/β-catenin pathways. M70 and FGF19 proteins (1mg/kg) were injected intraperitoneally into db/db mice. Livers were collected and phosphorylation of signaling proteins was measured by immunoblotting. Consistent with the ability of both molecules to signal in cultured primary hepatocytes, FGF19 and M70 stimulated ERK phosphorylation to a similar extent in liver tissues in vivo. In line with previous reports on the role of FGF19 in modulating hepatic protein synthesis (33), both wild type FGF19 and M70 induced phosphorylation of ribosomal S6 protein (Fig. 5). These data are consistent with results described in the previous sections that M70 retains activity on the FGFR4-KLB receptor complex. Neither M70 or FGF19 had any effect on hepatic levels of phosphorylated AKT, nor did they significantly activate GSK3β and β-catenin at any of the time points tested (Fig. 5).

Remarkably, FGF19 induced STAT3 phosphorylation 2 hours after dosing (Fig. 5A). This effect lasted to 4 hours post dosing (data not shown). In contrast, M70 completely lacked the ability to induce STAT3 phosphorylation (Fig. 5A). The pSTAT3 activation by FGF19 is likely due to non-cell autonomous mechanisms on the liver, since no induction of pSTAT3 was observed 15 minutes after protein injection or in primary mouse hepatocyte culture (data not shown). Consistent with these observations of differential STAT3 phosphorylation and activation, including Survivin, Cyclin D1 and Bcl-XL, were dramatically upregulated in db/db and rasH2 livers expressing FGF19, but not M70 (Fig. 5B and 5C). Since STAT3 is an oncogene frequently activated in HCC (34), its activation by FGF19 provides a plausible mechanism for FGF19-induced hepatocarcinogenicity. Conversely, the inability of M70 to activate the STAT3 pathway could contribute to its lack of tumorigenicity in vivo.
Thus, M70 only activates a subset of signaling pathways downstream of its receptors. This property, a hallmark of selective modulator or “biased agonist” (35), suggests that M70 may act as a selective modulator of FGFR4 to regulate metabolism without causing tumorigenicity.

*M70 inhibits FGF19-mediated tumor formation*

Our observations suggest that M70 behaves as a selective modulator (or “biased ligand”) to activate the metabolic signaling of, but not the tumorigenic pathway from, FGFR4. Next, we examined whether the biased agonism of M70 could inhibit FGF19-dependent tumor formation.

*db/db* mice were injected with AAV-FGF19, with or without 10-fold molar excess of AAV-M70. Mice were necropsied 24 weeks after transgene expression and the livers were excised for analysis. Although ectopic expression of FGF19 in *db/db* mice promoted the formation of tumor nodules on the hepatic surface, livers from mice expressing both FGF19 and M70 were completely free of tumor nodules (Fig. 6A). Liver weights mice co-expressing FGF19 and M70 were significantly lower relative to mice expressing FGF19 only (Fig. 6B). The ratios of liver to body weight in M70 and FGF19 co-treated mice were not significantly different from those of control mice (Fig. 6C). The serum levels of FGF19 were 94±12ng/ml when dosed alone, and the combined serum level of FGF19 and M70 was 453±169ng/ml (Fig. 6D). Histological analysis of the livers confirmed that, unlike FGF19-expressing mice, mice co-expressing M70 and FGF19 did not exhibit any histological evidence of liver tumors (Fig. 6E). These data clearly demonstrate that M70 can effectively blocks tumor formation induced by wild type FGF19.

These results are consistent with the notion that M70 acts as a biased ligand that is capable of antagonizing wild type FGF19 in tumorigenic signaling, and demonstrates the potential of using a selective modulator, such as M70, to suppress FGF19-dependent tumor growth.
DISCUSSION

FGF19 is an endocrine hormone of the FGF family that regulates bile acid, carbohydrate, lipid and energy metabolism (33, 36, 37). Among FGFRs, FGF19 binds selectively to FGFR4 (2). A direct link between FGF19 and FGFR4 in hepatocellular oncogenesis is established in a mouse study where FGF19-mediated liver tumorigenesis was abrogated in FGFR4 KO mice (13). Although predominantly expressed in the ileum, FGF19 is induced in liver under cholestatic conditions caused by either destruction or obstruction of bile duct, cirrhosis or bile acid accumulation. Since chronic liver cirrhosis and cholestasis often trigger compensatory regeneration and HCC, FGF19 might present a missing link between liver injury, regeneration and cancer.

HCC represents an important unmet medical need (38). Given the potential role of FGF19 in HCC development, efforts have been devoted to developing therapies by targeting FGF19. Indeed, a neutralizing anti-FGF19 monoclonal antibody was developed and demonstrated anti-tumor activity in xenograft models (22). However, such strategy has suffered setback due to serious adverse effects. Administration of this antibody to cynomolgus monkeys led to dose-related liver toxicity accompanied by severe diarrhea (14). This adverse effect is apparently due to on-target inhibition of endogenous FGF19, resulting in increased hepatic bile acid synthesis, elevated serum bile acid, perturbation of enterohepatic circulation, and the development of diarrhea and liver toxicity (14). Thus, alternative approaches must be developed.

Through a high throughput in vivo screen, we identified an engineered FGF19 variant M70, that does not cause any liver tumors even after prolonged exposure at supra-physiological levels in mice. Although there were previous reports of generating tumor-free FGF19 variants (9, 32), those variants were specifically designed to eliminate FGFR4 binding, and therefore impaired in regulating bile acid metabolism. In contrast, M70 retains the ability to maintain bile acid homeostasis. Importantly, we show
that not only does M70 lack tumorigenic potential, but that it can effectively block the tumorigenic effects associated with wild type FGF19.

The major difference between M70 and FGF19 resides in the N-terminus of the protein. Each FGF family protein consists of the structurally conserved central globular domain, and the flanking N-terminal and C-terminal segments that are structurally flexible and are divergent in primary sequences (1). In X-ray crystal structures of multiple FGF/FGFR complexes, the N-terminal segment of the FGF molecule makes specific contact with the FGFR and is believed to play an important role determining the specificity of the FGF-FGFR interaction (1). Through systematic efforts utilizing an in vivo screen, we found that changing 3 amino acids at the N-terminus coupled with a 5-aa deletion eliminated tumorigenicity without impairing its ability to activate FGFR4-dependent process such as bile acid regulation.

In order to elucidate the molecular basis for M70’s lack of tumorigenic potential, we carried out in vivo analyses to assess the activation of key signaling proteins involved in tumorigenesis, including ERK, PI3K/AKT, STATs, and WNT/β-catenin pathways. We found that FGF19, but not M70, activates STAT3 in mouse liver. STAT3 is a major player in hepatocellular oncogenesis (34). Phosphorylated (i.e., activated) STAT3 is found in approximately 60% of HCC in humans (39), and correlates with poor prognosis in HCC patients (30). Constitutively-active STAT3 acts as an oncogene in cellular transformation (40). Hepatocyte-specific ablation of STAT3 prevented HCC development in mice (39). Inhibitors of STAT3 activation block the growth of human cancer cells and are being tested in the clinic for treating various cancers, including HCC (41-43). However, the events that lead to STAT3 activation in human HCC are not known. IL-6, among other inflammatory cytokines, is postulated to be the major STAT3 activator in the liver (39). Here we show that FGF19 also activates STAT3 signaling in vivo, an effect that could be directly mediated by the FGFR4 receptor complex, or indirectly through induction of cytokines or growth factors. The precise mechanism for FGF19-induced STAT3 activation remains to be investigated. Although previous studies reported that FGF19 appears to have a strong effect on β-catenin
activation in cultured cell lines (10, 12), we did not observe an impact of FGF19 on levels of dephosphorylated (i.e., activated) β-catenin or P-GSK3β in an acute in vivo setting in mice. At present, the basis for these disparate observations is unclear.

Our data demonstrate that M70 exhibits the pharmacologic characteristics of a “biased ligand” or a selective modulator. M70 exhibits bias toward certain FGFR4 signaling pathways (e.g., CYP7A1, pERK) to the relative exclusion of others (e.g., tumor, pSTAT3). Studies of GPCRs and ion channels have demonstrated that selective receptor modulators offer opportunities for fine-tuning biologic responses in a manner that is not attainable via classic orthosteric mechanisms (44). As a selective FGFR4 modulator, M70 antagonizes the oncogenic activity of FGF19 in HCC.

FGF19 demonstrates an array of biological effects. The therapeutic potential for FGF19 includes the treatment of chronic liver diseases, as well as obesity and diabetes (45). However, the carcinogenicity of FGF19 challenges the development of an FGF19 therapeutic for chronic use. With the identification of M70, an engineered FGF19 devoid of tumorigenicity with potent metabolic properties, therapeutic benefits could be achieved without unwanted side effects. Our study opens new avenues for modulating FGF19 pathway to treat cancer, diseases with bile acid dysregulation, type 2 diabetes, and other metabolic disorders.

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23. Hasegawa Y KH, Kawai M, Ueno T, Miyahara Y,. FGF19 is aberrently expressed in cholestatic hepatocytes and its signaling pathways were downregulated in biliary atresia children. Hepatology. 2013;58:802A.
Table 1. FGF19 Promotes Hepatocarcinogenesis in Multiple Mouse Models. Various strains of mice (6-12 week old) were injected with $3 \times 10^{11}$ genome copies of AAV vectors encoding FGF19 or a control gene (green fluorescent protein). Tumor incidence was determined at 24 or 52 weeks after AAV administration, and expressed as number of animals with liver tumor over the total number of animals in the group. *n.d.*, not determined.

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<td>db/db</td>
<td>5/5 (100%)</td>
<td><em>n.d.</em></td>
<td>0/5</td>
<td><em>n.d.</em></td>
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FIGURE LEGENDS

Figure 1. An AAV-mediated Transgene System for Studying Hepatocellular Tumorigenesis.

(A) A diagram of experimental protocol. Mice were given a single injection of 3x10^{11} genome copies of AAV-FGF19 via tail vein when 6-12 week old. Mice were sacrificed 24 or 52 week later for liver tumor analysis. ITR, inverted terminal repeat; EF1a, elongation factor 1α promoter. (B) Representative livers of db/db mice 24 weeks after administration of AAV-FGF19. A control virus encoding green fluorescent protein (Control) is included. (C) Serum levels of FGF19 were measured by ELISA at 1, 4, 12 and 24 weeks after AAV administration in db/db mice (n = 5). (D) Liver tumor multiplicity and size in db/db mice (n=15) expressing FGF19 transgene. Tumors per liver were counted and maximal tumor sizes were measured. (E) Histological and immunohistochemical characterization of FGF19-induced liver tumors in db/db mice. FGF19-induced neoplastic cells are strongly glutamine synthetase-positive. Tumors (T) are outlined by dotted lines. All values represent mean±SEM. ***p<0.001, *p<0.05 denote significant differences vs. control group by two-tailed t test.

Figure 2. M70 is a Tumor-Free FGF19 Variant After Continuous Exposure in db/db Mice for 24 Weeks.

(A) Alignment of protein sequences of M70 and FGF19 in the N-terminal region. Mutations introduced into M70 are underlined. Number of tumors per liver (B), liver weight (C) and ratio of liver to body weight (D) of db/db mice (n=5) expressing FGF19 or M70 for 24 weeks. Growth curve (E) and serum levels of transgene expression (F) were also determined. (G) Representative liver sections from db/db mice after 24 weeks of transgene expression. H&E staining and immunohistochemical detection of Ki-67 and glutamine synthetase of liver tissue sections were included. Tumors (T) are outlined by dotted lines. (H) Serum levels of liver enzymes (ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP: alkaline phosphatase; n=5) were measured prior to termination of the study. All
values represent mean±SEM. *p<0.05, **p<0.01, ***p<0.001 denotes significant differences vs. control group by one-way ANOVA followed by Dunnett’s post test.

**Figure 3. No Liver Tumor Formation in rasH2 Mice Treated with M70 for 52 Weeks.**

Growth curve (A), number of tumors per liver (B), liver weight (C), liver-to-body weight ratios (D) and serum levels of M70 or FGF19 (E) of rasH2 mice (n=9) expressing FGF19 or M70 transgenes for 52 weeks. (F) Representative images of livers stained with H&E or anti-glutamine synthetase. Portal (p) and central (c) veins are indicated. Tumors (T) are outlined by dotted lines. (G) qRT-PCR analysis of Ki-67 and AFP expression in the liver. All values represent mean±SEM. *p<0.05, **p<0.01, ***p<0.001 denotes significant differences vs. control group by one-way ANOVA followed by Dunnett’s post test.

**Figure 4. M70 Binds and Activates FGFR4 in vitro and in vivo.**

(A) Biacore SPR assay of the interaction between FGF19 and FGFR4. Left column shows binding curves obtained over a range of FGF19 concentrations, while right column shows the steady state fits of the data for obtaining K_D values. (B) Binding of M70 to FGFR4 by Biacore. (C) Solid phase binding of M70 or FGF19 to FGFR4-KLB receptor complex. (D) Receptor activation by M70 or FGF19. L6 cells were transiently transfected with FGFR4 and ELK-luciferase reporter in the presence or absence of KLB. (E) M70 induces ERK phosphorylation in Hep3B cells. (F) M70 represses CYP7A1 expression in primary hepatocytes of mouse or rat origin. (G) Repression of hepatic CYP7A1 expression by M70 in mice. 12-week-old db/db mice were injected intraperitoneally with recombinant M70 or FGF19 protein. Hepatic CYP7A1 expression was evaluated by qRT-PCR. Dose response curves of CYP7A1 repression in mice were shown. (H) M70 reduces serum levels of total bile acids. Serum samples were taken from db/db mice (n=5) 24 weeks following administration of AAV vectors expressing FGF19, M70, or control virus. All values represent mean±SEM. *p<0.05 denotes significant differences vs. control group by one-way ANOVA followed by Dunnett’s post test.

**Figure 5. Differential Activation of Cell Signaling Pathways by M70 and FGF19 in vivo.**
(A) Livers were harvested from *db/db* mice (n=6) injected intraperitoneally with saline, FGF19, or M70 proteins 2 hours post injection. Liver lysates were examined by western blot for expression and phosphorylation of the indicated proteins. Each lane represents an individual mouse. Rab11 serves as a loading control. (B) Livers were harvested from *db/db* mice (n=5) 24 weeks following administration of AAV vectors expressing FGF19 or M70 transgenes. STAT3 target genes (*Survivin* and *Cyclin D1*) mRNA levels in livers were measured by qRT-PCR. (C) qPCR showing expression of mRNAs for STAT3 target genes (*Survivin, Cyclin D, and Bcl-X<sub>L</sub>*) in rasH2 mice 52 weeks following administration of AAV vectors expressing FGF19 or M70 transgenes. Results are represented as fold expression relative to control animals. All values represent mean±SEM. **p<0.01, ***p<0.001 denotes significant differences vs. control group by one-way ANOVA followed by Dunnett’s post test.

**Figure 6. M70 Inhibits FGF19-induced Tumor Growth in *db/db* mice.**

(A)-(D) 11 week old *db/db* mice (n=5) were injected with AAV-FGF19 (3x10<sup>10</sup> genome copies) in the absence or presence of M70 (3x10<sup>11</sup> genome copies). Liver tumor score (A), liver weight (B), ratio of liver to body weight (C) and serum levels of transgene expression (D) were determined 24 weeks later. (E) Histology of livers of mice expressing FGF19 or co-treated with M70. Liver sections were stained with H&E or anti-glutamine synthetase, a marker for FGF19-induced liver tumors. Portal (p) and central (c) veins are indicated. Tumors (T) are outlined by dotted lines. All values represent mean±SEM. *p<0.05 denotes significant differences vs. control group by one-way ANOVA followed by Dunnett’s post test; ##p<0.01 denotes significant differences by two-tailed t test.
One-time injection of AAV via tail vein

Assess tumor burden

One-time injection of AAV via tail vein

Assess tumor burden

FGF19

Focus on FGF19

One-time injection of AAV via tail vein

Assess tumor burden

FGF19
Figure 2: (A) Schematic representation of FGF19-M70 and RPLAFSDAGPHVHYGWGDPI-M70. (B) Tumors per liver. (C) Liver weight. (D) Liver percent body weight. (E) Body weight over weeks post injection. (F) FGF19 levels. (G) H&E staining. (H) Liver function tests: ALT, AST, ALP.
CAN-14-0208 (Zhou M. et al. Figure 3).

**A**

Weeks Post Injection vs Body Weight (g)

- Control (black line)
- FGF19 (green dots)
- M70 (red dots)

**B**

Tumors/Liver

- Control (white bar)
- FGF19 (green bar)
- M70 (red bar)

**C**

Liver (g)

- Control (white bar)
- FGF19 (green bar)
- M70 (red bar)

**D**

Liver % BW

- Control (white bar)
- FGF19 (green bar)
- M70 (red bar)

**E**

FGF19 (ng/mL)

- Control (white bar)
- FGF19 (green bar)
- M70 (red bar)

**F**

H&E

- Control (left)
- FGF19 (middle)
- M70 (right)

Glutamine Synthetase

- Control (top left)
- FGF19 (top middle)
- M70 (top right)

**G**

Ki-67

- Control (left)
- FGF19 (middle)
- M70 (right)

AFP

- Control (left)
- FGF19 (middle)
- M70 (right)
CAN-14-0208 (Zhou M. et al. Figure 4).
CAN-14-0208 (Zhou M. et al. Figure 5).

A  

<table>
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<th>M70</th>
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B  

- **Survivin**
  - mRNA (Fold)  
  - Control: 1.0 ± 0.2  
  - FGF19: 4.5 ± 0.5  
  - M70: 4.0 ± 0.3  

- **Cyclin D1**
  - mRNA (Fold)  
  - Control: 1.0 ± 0.1  
  - FGF19: 4.0 ± 0.3  
  - M70: 3.5 ± 0.2

C  

- **Survivin**
  - mRNA (Fold)  
  - Control: 1.0 ± 0.1  
  - FGF19: 2.0 ± 0.2  
  - M70: 1.5 ± 0.1

- **Cyclin D1**
  - mRNA (Fold)  
  - Control: 1.0 ± 0.1  
  - FGF19: 5.0 ± 0.5  
  - M70: 4.5 ± 0.4

- **Bcl-XL**
  - mRNA (Fold)  
  - Control: 1.0 ± 0.1  
  - FGF19: 2.5 ± 0.3  
  - M70: 1.8 ± 0.2
A) Number of Tumors per Liver

B) Liver Weight (g)

C) Liver % BW

D) FGF19 (ng/ml)

E) H&E and Glutamine Synthetase images for Control and FGF19 + M70 conditions.
Separating Tumorigenicity from Bile Acid Regulatory Activity for Endocrine Hormone FGF19

Lei Ling, Mei Zhou, Xueyan Wang, et al.

Cancer Res  Published OnlineFirst April 11, 2014.

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