Phosphorylation of hepatic farnesoid X receptor by FGF19 signaling-activated Src maintains cholesterol levels and protects from atherosclerosis

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ABSTRACT

The bile acid (BA) nuclear receptor, farnesoid X receptor (FXR/NR1H4), maintains metabolic homeostasis by transcriptional control of numerous genes, including an intestinal hormone fibroblast growth factor-19 (FGF19, FGF15 in mouse). Besides, activation by BAs, the gene-regulatory function of FXR is also modulated by hormone or nutrient signaling-induced posttranslational modifications. Recently, phosphorylation at Tyr-67 by the FGF15/19 signaling-activated nonreceptor tyrosine kinase Src was shown to be important for FXR function in BA homeostasis. Here, we examined the role of this FXR phosphorylation in cholesterol regulation. In both hepatic FXR-knockout and FXR-knockdown mice, reconstitution of FXR expression up-regulated cholesterol transport genes for its biliary excretion, including scavenger receptor class B member 1 (Scarb1) and ATP-binding cassette subfamily G member 8 (Abcg5/8), decreased hepatic and plasma cholesterol levels, and increased biliary and fecal cholesterol levels. Of note, these sterol-lowering effects were blunted by substitution of Phe for Tyr-67 in FXR. Moreover, consistent with Src’s role in phosphorylating FXR, Src-knockdown impaired cholesterol regulation in mice. In hypercholesterolemic apolipoprotein E-deficient mice, expression of FXR, but not Y67F-FXR, ameliorated atherosclerosis, whereas Src downregulation exacerbated it. Feeding or treatment with an FXR agonist induced Abcg5/8 and Scarb1 expression in WT, but not FGF15-knockout, mice. Furthermore, FGF19 treatment increased occupancy of FXR at Abcg5/8 and Scarb1, expression of these genes, and cholesterol efflux from hepatocytes. These FGF19-mediated effects were blunted by the Y67F-FXR substitution or Src downregulation or inhibition. We conclude that phosphorylation of hepatic FXR by FGF15/19-induced Src maintains cholesterol homeostasis and protects against atherosclerosis.
INTRODUCTION

The bile acid (BA)-sensing nuclear receptor, Farnesoid X Receptor (FXR, NR1H4), plays an important role in the maintenance of metabolic homeostasis by transcriptional control of numerous genes, including a gut hormone, Fibroblast Growth Factor-19 (human FGF19, mouse FGF15), and an orphan nuclear receptor, Small Heterodimer Partner (SHP, NR0B2) (1-3). After a meal, FXR is activated by transiently elevated BAs, inhibits hepatic BA synthesis, and promotes biliary excretion to maintain BA homeostasis and protect the liver from bile toxicity. FXR also has many other beneficial functions, including cholesterol-lowering effects. FXR-knockout (KO) mice have increased plasma cholesterol levels and pro-atherogenic lipoprotein profiles (4-7). Conversely, treatment with FXR agonists in mice was shown to decrease sterol levels, in part, by increasing expression of hepatic genes involved in reverse cholesterol transport (RCT), including Scarb1, Abcg5 and Abcg8 (8-11). RCT is an anti-atherogenic pathway, in which high density lipoprotein (HDL) cholesterol is transported to the liver, where it can be secreted into bile as free cholesterol or converted into BAs for biliary excretion (2,11-13). Although a role for FXR in the sterol regulation has been shown from genetic mouse and pharmacological studies, the molecular signaling mechanisms, particularly in response to nutrient and hormonal cues, have not been demonstrated.

An intestinal hormone, FGF15/19, is induced by FXR and acts at the liver to maintain metabolic homeostasis during the transition from the fed to fasted states independent of insulin action (3,14). FGF15/19 mediates postprandial hepatic responses, which include the inhibition of BA biosynthesis, activation of glycogen and protein synthesis, and repression of hepatic autophagy and one carbon metabolism (3,14-16). In addition to these functions, FGF15/19 has been implicated in the regulation of cholesterol metabolism. FGF15-KO mice had elevated cholesterol levels and treatment of mice with FGF19 led to decreased plasma sterol levels (17-19). The mechanisms by which FGF15/19 decreases cholesterol levels are unclear, although recent studies have shown that SHP mediates FGF15/19 function to inhibit hepatic cholesterol biosynthesis and intestinal cholesterol absorption (20,21).

The transcriptional function of FXR is primarily regulated by its physiological ligands, BAs, but can also be profoundly modulated by signaling-induced post-translational modifications (PTMs), sometimes in a gene-selective manner, and dysregulated PTMs are associated with metabolic disease (22-24). For example, acetylation of FXR is normally dynamically regulated during feeding/fasting cycles via p300 acetyltransferase and SIRT1 deacetylase, but constitutively elevated in obese mice (22). The elevated FXR acetylation in obesity blocks SUMOylation of FXR, which contributes to activation of proinflammatory transcriptional responses (22,24). Further, FXR is O-GlcNAcylated in response to high glucose levels in the fed-state, leading to increased FXR stability and transcriptional function (25). Recently, phosphorylation of FXR at Tyr-67 by a non-receptor tyrosine kinase, c-Src, in response to postprandial FGF15/19 signaling was shown to be important for FXR function in BA homeostasis (26).

In this study, we examined a role of Src-mediated phosphorylation of FXR in sterol regulation in mice. Utilizing Y67-FXR mutations and Src downregulation, we show that phosphorylation of hepatic FXR at a single residue, Y67, is important for the maintenance of cholesterol homeostasis and protection from atherosclerosis in mice. In mechanistic studies in mice and primary mouse hepatocytes, we further show that both the FXR phosphorylation at Y67 and Src are important for FGF19-induced FXR binding and transcriptional induction of Scarb1 and Abcg5/8, direct FXR target genes involved in hepatic cholesterol transport for biliary secretion.

RESULTS

FXR-mediated hepatic expression of cholesterol transport genes is blunted by substitution of Phe for Tyr-67 in FXR. To examine the effect of the FXR phosphorylation on expression of FXR target genes, we examined the changes in gene expression after rescue of FXR expression with hepatic-specific expression of FXR-WT or p-defective Y67F-FXR in mice in which FXR was specifically downregulated in the liver by expression of hepatocyte-targeting AAV-TBG-Cre (27) in FXR-floxed mice (26) (Fig. 1A,B). Analysis of previously published RNA-seq data (16) from these
mice revealed that expression of hepatic genes involved in the uptake and transport of cholesterol for hepatobiliary secretion, including a HDL uptake transporter, Scarb1 (8-11); key transporters for biliary cholesterol excretion, including Abcg5 and Abcg8 (8,9); and BA transporters, Abcb11 (Bsep) and Atp8b1, are upregulated by hepatic expression of WT-FXR compared to Y67F-FXR (Fig. 1A). To verify these RNA-seq data, FXR-WT or Y67F-FXR was expressed in the hepatic FXR-downregulated mice, and the mice were briefly fed cholic acid (CA) chow to activate FXR which induces FGF15/19 and subsequently phosphorylation of FXR (26) (Fig. 1B, top). FXR-WT or Y67F-FXR was expressed at levels similar to FXR in control FXR-floxed mice and WT, but not Y67F-FXR, was phosphorylated at Y67 as previously reported (26) (Fig. 1B, bottom). The mRNA levels of nearly all of the genes involved in cholesterol transport were increased by expression of WT-FXR compared to expression by Y67F-FXR (Fig. 1C). In contrast, mRNA levels of Pltp, a HDL cholesterol metabolism gene, were not increased. These results, together with the role of Y67-FXR phosphorylation in upregulating Abcb11 (Bsep) (26), a key BA excretion transporter, suggest a potential role for FXR phosphorylation in induction of genes involved in hepatic cholesterol and BA transport into the bile duct for biliary excretion as illustrated in Fig. 1D.

To further determine the role of the Y67-FXR phosphorylation in the regulation of expression of hepatic sterol transport genes, we utilized FXR liver-specific knockout (FXR-LKO) mice and examined the effects of in vivo rescue of FXR expression with WT-FXR or the p-defective Y67F-FXR mutant (Fig. 1E). Compared to control GFP, expression of WT-FXR increased hepatic expression of sterol transport genes, including Scarb1, Ldlr, Abcg5, and Abcg8, and lipoprotein-related genes, Pltp, Apoa1, Apoa2, and Apoe, in the liver (Fig. 1F, left), but did not affect expression of Scarb1, Abcg5, and Abcg8 in the intestine (Fig. 1F, right). Expression of Y67F-FXR, in contrast to FXR-WT, did not result in increased expression of hepatic transport genes, but expression of either FXR-WT or Y67F-FXR resulted in similar increases in expression of lipoprotein-related genes (Fig. 1F, left). Notably, in FXR-LKO mice, reconstitution of WT-FXR expression restored mRNA levels of Scarb1, Abcg5, and Abcg8 to levels similar to those in control C57BL6 mice and in FXR-floxed mice (Fig. 1G). These results demonstrate that phosphorylation of FXR at Y67 is important for expression of hepatic genes involved in cholesterol transport.

**Cholesterol-lowering effects by hepatic FXR are blocked by the p-defective Y67F mutation.** We next tested whether expression of cholesterol transport genes mediated by FXR phosphorylation leads to changes in cholesterol levels in mice. FXR expression was reconstituted with WT-FXR or Y67F-FXR in the liver of FXR-LKO mice and plasma and tissue cholesterol levels were measured. In vivo rescue of WT-FXR expression mice decreased plasma/liver cholesterol levels and increased biliary/fecal cholesterol levels to levels similar to those in control FXR-floxed mice and each of these effects was blunted by the p-defective FXR-Y67F mutation (Fig. 2A). Consistent with the decreased plasma cholesterol levels, levels of plasma lipoproteins, particularly VLDL and HDL, were decreased by expression of WT-FXR in FXR-downregulated mice, but these decreases were attenuated by the Y67F mutation (Fig. 2B). Similar effects on the plasma, liver, biliary, and fecal cholesterol levels and plasma lipoprotein levels were also observed after in vivo rescue of hepatic FXR expression with WT-FXR or Y67F-FXR in the hepatic FXR-downregulated mice (Fig. 2C, D). Similar results in both the FXR-LKO mice and the viral-mediated hepatic FXR-downregulated mice strongly suggest that phosphorylation at a single residue in FXR can impact cholesterol homeostasis in mice.

**Downregulation of Src increases systemic cholesterol levels in mice.** Phosphorylation of FXR at Y67 is mediated by FGF15/19-activated Src kinase (26), which suggests that Src may have a role in sterol regulation. To test this possibility, Src was downregulated in mice by lentiviral expression of Src shRNA (Fig. 3A, top). Downregulation of Src did not result in changes in protein levels of FXR but p-Y67-FXR levels were decreased (Fig. 3A, bottom), consistent with previous results (26). Downregulation of Src decreased hepatic expression of all cholesterol transport and metabolism genes tested, except for Pltp (Fig. 3B), increased plasma and liver cholesterol levels, and decreased biliary and fecal cholesterol levels (Fig. 3C). Further, plasma lipoprotein cholesterol levels were also increased by downregulation of Src (Fig. 3D).
These results demonstrate that Src has a previously unknown function in sterol regulation, in part by increasing hepatic expression of cholesterol transport genes. 3D). Y67-FXR phosphorylation is protective against atherosclerosis in ApoE-KO mice. Our findings that both Y67-FXR phosphorylation and Src are important for the regulation of cholesterol levels in mice (Fig. 2, 3) suggested that Y67-FXR phosphorylation might be important for the known protective effects of FXR against atherosclerosis in atherosclerosis-prone mice (4,5). To test this idea, the effects on atherosclerosis of liver-specific expression of WT-FXR or Y67F-FXR in atherosclerosis-prone ApoE-KO mice chronically fed a western diet were examined (Fig. 4A). WT-FXR and Y67-FXR were expressed at similar levels in the mice, and levels of p-Y67-FXR increased for WT-FXR, but not Y67F-FXR as expected (Fig. 4B). In these mice, mRNA levels of most hepatic sterol transport genes, including Scarb1 and Abcg1/5/8, were increased by expression of WT-FXR, and this increase was blocked by the Y67F mutation (Fig. 4C). Expression of WT-FXR resulted in decreased plasma/liver cholesterol levels and increased biliary/fecal cholesterol levels and each of these changes was blocked by the Y67F mutation (Fig. 4D). These results indicate that phosphorylation of hepatic FXR at Y67 is important for its beneficial effects in atherosclerotic-prone mice.

We further examined the effects of FXR on atherosclerosis by examining the histology of the aortic sinus and brachiocephalic artery. The brachiocephalic artery is the first branch of the aortic arch that supplies blood to the head and neck, and atherosclerosis in this artery is the major risk factor for stroke (28). Since cholesterol accumulation in macrophages in the artery wall promotes inflammatory responses (12,29), infiltration of macrophages was also examined. Expression of WT-FXR resulted in significant decreases in atherosclerotic plaques (Oil Red-O staining), necrotic area (H&E staining), collagen content (Picrosirius red staining), and macrophages (F4/80 antibody) in the atherosclerotic lesions of both the aortic sinus (Fig. 4E) and the brachiocephalic artery (Fig. 4F). Strikingly, each of these FXR-mediated beneficial atheroprotective effects was lost when p-defective Y67F-FXR was expressed, and collagen content and necrotic area in brachiocephalic artery were substantially increased over the control. These findings indicate that phosphorylation of FXR at a single residue can have atheroprotective effects.

Endogenous FGF15 signaling is important for induction of Scarb1 and Abcg5/8, after feeding or FXR activation. Our findings indicated that both Y67-FXR phosphorylation and Src are important for lowering cholesterol levels in mice in part through transcriptional induction of hepatic cholesterol transport genes (Fig. 1-3). Since Y67-FXR phosphorylation is mediated by FGF15/19 (26), an intestinal hormone that is induced by the bile acid-activated FXR in the late fed-state (3), we further examined the role of endogenous FGF15 signaling in the regulation of hepatic genes involved in sterol metabolism. FGF15-KO mice and control C57BL/6 mice were treated with an FXR agonist, GW4064 or refeed after fasting and expression of sterol transport genes was measured. Hepatic expression of Scarb1 and Abcg5/8 was increased after GW4064 treatment (Fig. 6A) or feeding (Fig. 6B) in control C57BL/6 mice, but these effects were severely blunted in FGF15-KO mice. These results suggest that increased expression of Scarb1 and Abcg5/8 after feeding or
GW4064 treatment is dependent on physiologically induced endogenous FGF15 signaling in mice.

To understand the mechanisms by which the FGF19-induced phosphorylation of FXR upregulates genes involved in sterol transport and metabolism, we first tested whether these genes are direct targets of FXR. In published hepatic FXR ChIP-seq data from mice treated with GW4064 (30,31), FXR binding peaks were detected at numerous cholesterol transport genes (Fig. 6C). Consistent with this observation, in mouse liver ChIP assays, occupancy of FXR at Abcg5, Abcg8, and Scarb1 (Fig. 6D) and the mRNA levels of these genes were increased by FGF19 treatment (Fig. 6E).

To determine whether occupancy of FXR at sterol transport genes was dependent on Y67-FXR phosphorylation, WT-FXR or Y67F-FXR was expressed in FXR-KO mice and liver ChIP assays were performed. FXR occupancy at Abcg5/8 and Scarb1 was detected with antibodies to either FXR or specifically p-Y67-FXR in mice expressing WT-FXR treated with FGF19 (Fig. 6F). Occupancy of FXR at these genes was not detected in mice expressing Y67F-FXR (Fig. 6F), suggesting Y67-FXR phosphorylation is required for binding to these genes. In contrast, at Pltp, similar occupancy of FXR in both groups of mice expressing WT or Y67F-FXR was detected (Fig. 6F, center), but occupancy of p-Y67-FXR was not detected (Fig. 6F, right), indicating that phosphorylation is not required for FXR binding to Pltp. This results is consistent with the increases in mRNA levels of Pltp, a direct FXR target (32), by expression of either Y67F-FXR or WT-FXR (Fig. 1C, F). These results suggest that FXR binding at Scarb1 and Abcg5/8, but not at Pltp, requires the Y67 phosphorylation and further suggest that the FXR phosphorylation contributes to gene-selective regulation of hepatic genes involved in cholesterol transport and metabolism.

FGF19-induced sterol transport gene expression and cholesterol efflux from hepatocytes are dependent on Y67-FXR phosphorylation. To further understand the mechanism by which the FXR phosphorylation at Y67 regulates hepatic sterol transport genes, we examined the effects of expression of p-mimic and p-defective Y67 mutants on expression of Abcg5/8 and Scarb1 in primary mouse hepatocytes derived from FXR-KO mice to avoid confounding effects of endogenous FXR (Fig. 7A).

Treatments with FGF19 increased mRNA levels of Abcg5/8 and Scarb1 in hepatocytes expressing WT-FXR, but these effects were blunted by the Y67F mutation (Fig. 7B). In contrast, the mRNA levels were increased even in untreated cells by expression of the p-mimic Y67E mutant, and FGF19 treatment did not further increase the levels (Fig. 7B). Consistent with the changes in mRNA levels, in luciferase reporter assays, expression of WT-FXR increased transactivation of Abcg8-Luc and Scarb1-Luc, which was further increased by FGF19 treatment, while expression of Y67F-FXR did not increase transactivation and FGF19 had no effect (Fig. 7C). Notably, expression of the p-mimic mutant, Y67E-FXR, increased transactivation independently of FGF19 treatment, similarly to that of WT-FXR in FGF19-treated cells and FGF19 did not further increase transactivation (Fig. 7C). These results indicate that the phosphorylation of FXR at Y67 is required for induction of Abcg5/8 and Scarb1 in hepatocytes.

Since Y67-FXR phosphorylation is important for hepatic expression of Abcg5 and Abcg8, cholesterol transporters for biliary excretion (10,11), we further examined the effect of the FXR phosphorylation on cholesterol efflux from primary hepatocytes. Consistent with the changes in expression of Abcg5 and Abcg8, expression of WT-FXR increased cholesterol efflux, and FGF19 treatment further increased the efflux (Fig. 7D). Expression of the p-defective Y67F-FXR did not increase cholesterol efflux with or without FGF19 treatment while the p-mimic Y67E-FXR increased efflux independent of FGF19 treatment (Fig. 7D). These findings suggest that Y67-FXR phosphorylation is important for FGF19-mediated induction of Abcg5/8 and Scarb1 and increased cholesterol excretion from hepatocytes.

Src is important for FGF19-induced sterol transport gene expression and cholesterol efflux from hepatocytes. We further examined the effects of downregulation or inhibition of Src on FXR occupancy at Abcg5, Abcg8 and Scarb1 genes and gene expression. In mice (Fig. 8A) or primary mouse hepatocytes (Fig. 8B) treated with FGF19, shRNA-mediated downregulation of Src reduced occupancy of FXR at Abcg5/8 and Scarb1 genes and decreased expression of these genes. Similarly, in primary mouse hepatocytes, FGF19 treatment...
increased mRNA levels of these genes and the FGF19-mediated increases were blocked by treatment with a Src inhibitor, dasatinib, which as expected, decreased levels of p-Y67-FXR (Fig. 8C, D). Consistent with these transcriptional changes, the FGF19-mediated increase in cholesterol efflux from hepatocytes was largely blocked by either downregulation or inhibition of Src (Fig. 8E). These results from hepatocytes and mice, together, strongly suggest that Src is required for FGF19-mediated induction of direct FXR targets, Abcg5/8 and Scarb1, and increased cholesterol efflux transport for hepatobiliary excretion.

**DISCUSSION**

In this study, utilizing in vivo rescue of FXR expression with FXR-WT and a p-defective Y67F-FXR mutant in FXR-LKO and hepatic FXR-downregulated mice and downregulation of Src, we demonstrate that phosphorylation of hepatic FXR at a single residue by Src can impact cholesterol homeostasis in mice. FXR, phosphorylated at Y67 by FGF15/19 signaling-activated Src, mediates the transcriptional induction of genes important for hepatic uptake and transport of cholesterol for hepatobiliary excretion, which contributes to decreases in plasma cholesterol levels and protection against atherosclerosis (Model, Fig. 9).

In vivo rescue of FXR-WT in the liver of FXR-LKO or FXR-downregulated mice resulted in decreased levels of plasma and liver cholesterol and increased cholesterol excretion in the feces, but these FXR-mediated sterol-lowering effects were not observed after rescue with of p-defective Y67F-FXR. The similar results in both the chronic FXR-LKO mice, in which developmental compensation could occur, and the acute AAV-TBG-Cre viral-mediated downregulation of hepatic FXR in FXR-floxed mice, in which unwanted viral effects could occur, strongly support the conclusion that phosphorylation of hepatic FXR at a single residue Y67 plays an important role in the regulation of cholesterol levels in mice. Consistent with these effects on regulating sterol levels, expression of FXR decreased atherosclerosis in atherosclerosis-prone ApoE-KO mice, while expression of the p-defective Y67F-FXR had little beneficial effect.

Our studies provide an explanation for previous studies that treatment with an FXR agonist, GW4064, increased hepatic expression of Abcg5, Abcg8, and Scarb1 in mice in vivo, but not in cultured hepatic cells (9). Our present studies show that phosphorylation of FXR by intestinal FGF15/19 is required for expression of these hepatic cholesterol transport genes. Thus, activation of FXR in mice, which induces intestinal FGF15/19, would be expected to induce the genes in vivo, but not in the cultured cells due to the absence of the intestinal FGF15/19.

FXR has been shown to regulate cholesterol levels via multiple pathways. FXR promotes hepatic cholesterol transport for biliary excretion, intestinal cholesterol absorption and transintestinal cholesterol excretion via modulation of BA composition (6,8,33). In addition, FXR-induced SHP decreases cholesterol levels via inhibition of de novo sterol biosynthesis and intestinal absorption (20,26). FXR also regulates plasma HDL levels through induction of microRNA-144 (34). In the present study, we identify a molecular signaling pathway for regulation of cholesterol levels via a hepatic FGF15/19-Src-FXR phosphorylation signaling cascade. Utilizing FGF15-KO mice, we further demonstrated that physiologically induced endogenous FGF15 signaling by feeding has a role in the regulation of expression of hepatic sterol transport genes. BA levels quickly increase after a meal, resulting in FXR induction of intestinal FGF19, and plasma FGF15/19 levels peak about 2-3 h after feeding, so that FGF15/19 is considered a late-fed state hormone (35,36). Thus, FXR-induced intestinal FGF15/19 feeds back to activate hepatic FXR via Src-mediated phosphorylation, which contributes to sustained FXR function in the transcriptional regulation of cholesterol metabolism in the late-fed state when the hepatic BA levels have declined.

Recently, we demonstrated that Y67-FXR phosphorylation is important for function of FXR in the regulation of hepatic BA levels, including transcriptional repression of BA synthetic genes, Cyp7a1 and Cyp8b1 (26). Inhibition of the conversion of cholesterol into BAs could potentially result in a detrimental increase in hepatic cholesterol levels. The present study, however, reveals that the FXR phosphorylation by FGF15/19-activated Src decreases hepatic cholesterol levels in part by induction of Scarb1 and Abcg5/8, genes involved in reverse cholesterol transport (RCT). Although we did not directly
examine the effect of Src-mediated FXR phosphorylation on RCT in vivo, our studies are consistent with a role for this phosphorylation in RCT. Both FXR and Src mediated increased biliary and fecal cholesterol levels, decreased plasma and liver cholesterol levels, and increased expression of cholesterol transport genes in mice, and increased cholesterol excretion from primary mouse hepatocytes. These results support the conclusion that the FXR phosphorylation mediated by Src regulates sterol levels in part via promoting hepatic cholesterol transport for biliary excretion.

An intriguing finding in this study is the demonstration of a previously unknown function for c-Src in cholesterol regulation and protection against atherosclerosis. Src is a well-known proto-oncogene and a target for cancer treatment (37). During cancer therapy, treatment with tyrosine kinase inhibitors, including dasatinib, an inhibitor of Src, increases the risk for cardiovascular disease and heart failure (38). Intriguingly, we observed that atherosclerosis in ApoE-KO mice was exacerbated in Src-downregulated mice, which could underlie the increased risk in cancer patients treated with Src inhibitors. Thus, the decreased FXR functions as a result of Src inhibition would lead to increased sterol levels as shown in the current study, which could also contribute to the cardiovascular risk of Src inhibitor therapy.

In conclusion, we demonstrate that phosphorylation of hepatic FXR at a single residue can impact whole-body cholesterol homeostasis and protect against atherosclerosis in mice and further show that Src has a previously unknown function in regulating cholesterol levels. FXR is the molecular target of obeticholic acid, an FDA-approved drug for liver fibrosis, and other FXR agonists and FGF19 analogues are under clinical trials for the treatment of metabolic disorders (1,39,40). Since PTMs of nuclear receptors like FXR have gene-selective effects (22,24,26), as is also suggested by the present studies, targeting the phosphorylation of FXR by FGF15/19 signaling-activated Src may provide more specific therapeutic options for hypercholesterolemia-related cardiovascular disease.

**EXPERIMENTAL PROCEDURES**

**Materials and reagents**- Antibodies for FXR (1:5,000, sc-13063) were purchased from Santa Cruz Biotechnology and for β-actin (1:10,000, sc-4970) and Src (1:3,000, sc-2108) from Cell Signaling. M2 antibody (1:3,000, F3165) was purchased from Sigma, Inc. F4/80 (AP10243PU-M) was obtained from Acris Antibodies. Antibodies for p-Y67-specific FXR were purchased commercially (1:10,000, Abmart, Inc.) and validated in FXR-KO mice (26). ON-TARGETplus mouse siRNAs for Src (L-040877) were purchased from GE Healthcare Dharmacon, Inc. GW4064 was obtained from Tocris bioscience. A Src inhibitor (dasatinib monohydrate) was obtained from Selleckchem. Purified FGF19 was provided by Dr. H. Eric Xu at Van Andel Research Institute, Grand Rapids, USA. In the present study, human FGF19 was used since mouse FGF15 is less stable and both FGF15 and FGF19 have the similar metabolic effects and FGF19 have been utilized in previous studies (20,26,41).

**Animal experiments**- FXR-floxed mice were provided by Drs. Kristina Schoonjans and Johan Auwerx (Ecole Polytechnique Fédérale de Lausanne, Switzerland). For viral-mediated liver-specific downregulation of FXR, AAV serotype 8 (AAV8) vectors containing a liver hepatocyte-specific TBG promoter were used. Eight weeks-old FXR-floxed male mice were injected via the tail vein with 100 µl of a mixture of AAV-TBG-Cre and either AAV8-TBG-GFP, AAV8-TBG-FXR WT or AAV8-TBG-Y67F-FXR at 5 x10^10 virus particles per mouse. Four weeks later, mice were briefly fed 0.5% cholic acid (CA)-supplemented chow for 6 h to activate FXR signaling. FXR-LKO mice were generated by breeding of homozygous FXR-floxed mice with Albumin-Cre mice (Jackson lab). Eight weeks-old FXR-LKO male mice were injected via the tail vein with either AAV8-TBG-GFP, AAV8-TBG-FXR WT or AAV8-TBG-Y67F-FXR viruses, and four weeks later, mice were fasted for 4-6 h before sacrifice to avoid metabolic fluctuation. For Src downregulation in mice, lentivirus expressing control shRNA or shRNA for Src (VectorBuilder, 0.5-1.0 x 10^6 IU per ml in 100 µl saline) was injected into C57BL6 mice via the tail vein, and after one month, plasma and tissues were collected. ApoE-KO male mice (Jackson lab) that had been fed a western diet (40 to 45% fat and 0.2 % cholesterol, Harlan Teklad, TD88137) for 6 months were injected with viruses, feeding with the...
western diet was continued, and mice were sacrificed 1-3 months later. FGF15-KO mice have been described in previous studies (42). FGF15-KO mice and C57BL/6 mice were fasted for 12 h or refed for 6 h after fasting or treated daily with vehicle or GW4064 (100 mg/kg) for 2 days, and livers were collected. **Evaluation of atherosclerosis**- Mice were euthanized by CO2 inhalation, and their hearts and brachiocephalic artery were perfused with PBS. After fixation with 10% formaldehyde overnight, the hearts and brachiocephalic artery were embedded in OCT compound for frozen section preparation. The aortic sinus and brachiocephalic artery were sectioned at a thickness of 10 μm. For IHC studies, frozen sections were stained with either Oil Red O (Abcam, ab150678), H&E (Vector Labs, H-3502), or Picrosirius red (Abcam, ab150681) or were incubated with the F4/80 antibody overnight at 4°C, which was detected with the rabbit-specific HRP/DAB Detection IHC Kit (Abcam). The sections were imaged with a NanoZoomer Scanner (Hamamatsu). **Cholesterol measurement**- Liver, biliary, fecal and plasma cholesterol levels were determined using a cholesterol quantitation kit (MAK043, Sigma, Inc). For measuring plasma cholesterol levels, plasma was prepared by spinning freshly collected blood at 1,500 x g for 20 min at 4°C. The supernatant was collected and stored at -80°C until use. For fractionation of plasma lipoproteins, plasma (100 μl) was subjected to FPLC in the Proteomics Center at University of Illinois at Urbana-Champaign. **Primary mouse hepatocyte (PMH)**- Primary mouse hepatocytes (PMH) were isolated from FXR-KO mice by collagenase (0.8 mg/ml, Sigma, Inc) perfusion through the portal vein of mice anesthetized with isoflurane. Hepatocytes were filtered through a cell strainer (100 μm nylon, BD), washed with M199 medium, centrifuged through 45% Percoll (Sigma, Inc.), and cultured in M199 medium containing 10% fetal bovine serum. PMHS from FXR-KO mice were transfected with plasmids, and 72 h later, the cells were treated with vehicle or 50 ng/ml FGF19 for 12 h. Luciferase activities were normalized to β-galactosidase activities. **RT-qPCR**- Total RNA was isolated using Trizol (Invitrogen), RT-qPCR was performed, and the amount of mRNA for each gene was normalized to that of 36B4 mRNA. Sequences of primers are in the Supporting Information Table S2. **Cholesterol efflux assays**- Cellular cholesterol excretion/efflux from isolated PMHs was measured using fluorescently-labelled cholesterol according to the manufacturer’s instruction (Abcam, ab196985). Fluorescence was measured in a fluorescent microplate reader was equipped with a filter for Ex/Em = 482/515 nm 4 h after addition of the fluorescently-labelled cholesterol for the calculation of the efflux. **IB analysis**- Liver tissues or cells were washed with ice-cold PBS and homogenized in RIPA buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1% NP40, 1% sodium deoxycholate, 1 mM DTT, and 0.1% SDS). Proteins in the cell lysates were separated and transferred to PVDF membranes, which were blocked and incubated with primary antibodies,
followed by incubation with secondary HRP-linked antibody.

**Statistics** - Statistical significance was determined by Student’s two-tailed t test, Mann-Whitney test, or one- or two-way ANOVA with the Tukey post-test for single or multiple comparisons as appropriate. Whenever relevant, the assumptions of normality were verified using the Shapiro–Wilk test, Kolmogorov-Smirnov test or the D’agostino-Pearson omnibus test. P-values < 0.05 were considered as statistically significant.

**Study Approval** - All animal use and biosafety protocols were approved by the Institutional Animal Use and Care and Biosafety Committees of the University of Illinois at Urbana-Champaign and were in accordance with NIH Guidelines.

**CONFLICT OF INTEREST:** The authors declare no conflict of interest.

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**AUTHOR CONTRIBUTIONS:** SB, HJ and JK designed research; SB, HJ, JC, YK, and DK performed experiments; SB, HJ, JC, YK, DK, and BKe analyzed data; YK performed genomic analyses; BKo and GG provided key materials for the study; and SB, HJ, BKe, and JK wrote the paper.

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FOOTNOTE

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The abbreviations used are: Abcg5/8, ATP binding cassette subfamily G member 5/8; ApoE, Apolipoprotein E; Bsep, Bile salt excretion pump; CoIP, coimmunoprecipitation; FGF15/19, Fibroblast Growth Factor 15/19; FXR, Farnesoid X Receptor; H&E, hematoxylin and eosin; HDL, High-density lipoproteins; IB, immunoblotting; IF, immunofluorescence; IHC, immunohistochemistry; LKO, liver-specific knockout; Pltp, Phospholipid transfer protein; PMH, Primary mouse hepatocytes; RCT, Reverse cholesterol transport; Scarb1, Scavenger receptor class B member 1; SHP, Small Heterodimer Partner; WT, wild type
FIGURE LEGENDS

Fig. 1. FXR-mediated increased expression of hepatic cholesterol transport genes is blocked by mutation of FXR at Tyr-67. (A) Heat maps of changes in hepatic gene expression in mice expressing Y67F-FXR compared to mice expressing WT-FXR from published RNA-seq data (26) (n= 3 mice). (B,C) FXR-floxed mice were co-infected with AAV-TBG-Cre and either AAV-TBG-GFP or AAV-TBG expressing WT-FXR or Y67F-FXR for 4 weeks (10 mice/group) and mice were fed a 0.5% CA chow for 6 h. (B) Levels of the indicated proteins were determined by IB. (C) The mRNA levels of the indicated genes were measured by RT-qPCR (n= 10). (D) Diagram of hepatic genes involved in cholesterol transport and efflux for biliary excretion. (E-G) FXR-LKO mouse studies: (E) FXR-LKO mice were infected with AAV-TBG-GFP or AAV-TBG expressing WT-FXR or Y67F-FXR for 4 weeks (6-7 mice/group). (E) Protein levels of hepatic FXR and p-Y67-FXR determined by IB are shown. (F) The mRNA levels of the indicated genes were measured by RT-qPCR in the liver (left), duodenum (Duod), jejunum (Jejun) or ileum, right. (G) The hepatic mRNA levels of Scarb1 and Abcg5/8 compared to their mRNA levels in control C57BL6 mice (set to 1) and FXR-floxed mice. All values are presented as mean ± SD. Statistical significance was measured using the (C) Mann-Whitney test or (F, G) one-way ANOVA with the Tukey post-test. *P<0.05, **P<0.01, and NS, statistically not significant.

Fig. 2. Hepatic FXR-mediated cholesterol-lowering effects are blocked by the p-defective Y67F mutation in mice. (A, B) FXR-LKO mice were infected with AAV-TBG-GFP or AAV-TBG expressing WT-FXR or Y67F-FXR for 4 weeks (6-7 mice/group). (A) Cholesterol levels in the plasma, liver, gallbladder, and feces compared to cholesterol levels of control FXR-floxed mice. A horizontal line indicates the mean. (B) Plasma was subjected to FPLC analysis and plasma lipoprotein levels were measured in different fractions. (C, D) FXR-floxed mice were co-infected with AAV-TBG-Cre and either AAV-TBG-GFP or AAV-TBG expressing WT-FXR or Y67F-FXR for 4 weeks (10 mice/group), and mice were fed a 0.5% CA chow for 6 h, and then, (C) plasma and tissue cholesterol levels and (D) plasma lipoprotein levels were measured. Statistical significance was measured using the one-way ANOVA with the Tukey post-test. *P<0.05, **P<0.01.

Fig. 3. Downregulation of Src increases systemic cholesterol levels and decreases hepatic expression of cholesterol transport genes in mice. C57BL/6 mice were infected with lentivirus expressing shRNA for Src for 4 weeks (8 mice/group). (A) Experimental outline (top). Src, p-Y67 FXR and FXR levels in the liver determined by IB (bottom, n= 3). (B) The mRNA levels of the indicated hepatic genes measured by RT-qPCR (n= 8). (C) Cholesterol levels in the plasma, liver, gallbladder, and feces (n= 8). A horizontal line indicates the mean. (D) Plasma pooled from 8 mice was subjected to FPLC analysis for lipoproteins as described in Methods. (B) All values are presented as mean ± SD. Statistical significance was measured using the (B, C) Mann-Whitney test. *P<0.05, **P<0.01, and NS, statistically not significant.

Fig. 4. Atheroprotective effects by hepatic FXR in ApoE-KO mice are diminished by the p-defective Y67F mutation. ApoE-KO mice that had been fed a western diet for 24 weeks were infected with AAV-TBG-GFP or AAV-TBG expressing WT-FXR or Y67F-FXR (7 mice/group) and feeding of the western diet continued until sacrifice 12 weeks later. (A) Experimental outline. (B) Protein levels of FXR, p-Y67-FXR and actin in liver extracts were determined by IB. (C) The mRNA levels of the indicated genes were measured by RT-qPCR (n= 7). (D) Cholesterol levels in the plasma, liver, gallbladder, and feces (n= 7). (E, F) From the left image panel to the right image panel, representative cross-sections of Oil Red O staining for atherosclerotic lesions, hematoxylin and eosin (H&E) staining for necrotic core detection, Picrosirius red staining of collagen (red), and immunohistochemical staining with anti-macrophage (F4/80) antibody to detect macrophages (brown) for the aortic sinus (E) or the brachiocephalic artery (F). At the right, quantification of plaques, necrotic core, collagen content and macrophages for the aortic sinus (E) or brachiocephalic artery (F). (C) All values are presented as mean ± SD. (D-F) A horizontal line indicates the
mean in the graphs. (C-F) Statistical significance was measured using the one-way ANOVA with the Tukey post-test. *P<0.05, **P<0.01, and NS, statistically not significant.

Fig. 5. Atherosclerosis in ApoE-KO mice is exacerbated by downregulation of Src. ApoE-KO mice that had been fed a western diet for 24 weeks were infected with lentivirus expressing shRNA for Src and feeding of the western diet continued until sacrifice 4 weeks later (8 mice/group). (A) Experimental outline and (B) levels of p-Y67 FXR, FXR and Src detected by IBD in the liver (right, n=3 mice). (C) The mRNA levels of the indicated genes were measured by RT-qPCR (n=8). (D) Cholesterol levels in the plasma, liver, gallbladder, and feces (n=8). (E, F) From the left image panel to the right image panel, representative cross-sections of Oil Red O staining for atherosclerotic lesions, hematoxylin and eosin (H&E) staining for necrotic core detection, Picrosirius red staining of collagen (in red) and immunohistochemical staining with anti-macrophages (F4/80) antibody to detect macrophages (brown) for the aortic sinus (E) or the brachiocephalic artery (F). Quantification of plaques, necrotic core, collagen content and macrophage for the aortic sinus (E, right) or the brachiocephalic artery (E, right). (C) All values are presented as mean ± SD. (D-F) A horizontal line indicates the mean in the graphs. (C-F) Statistical significance was measured using the Mann-Whitney test. *P<0.05, **P<0.01, and NS, statistically not significant.

Fig. 6. Endogenous FGF15 signaling is required for induction of direct FXR targets, Scarb1 and Abcg5/8, after FXR activation or feeding in mice. (A, B) The hepatic mRNA levels of the indicated genes measured by RT-qPCR of C57BL/6 or FGF15-KO mice that were either (A) treated daily with vehicle or GW4064 (100 mg/kg) for 2 days (n=4-5) or (B) fasted for 12 h (Fs) or fed for 6 h (Fd) after fasting (n=5). (C) UCSC genome browser displays of binding of FXR to representative genes involved in sterol transport and metabolism in mice treated with GW4064 from published FXR liver ChIP-seq data (30,31). The Y-axis shows the numbers of mapped sequence tags. Gene positions are indicated at the bottom of each display with the arrows indicating the direction of transcription and the start site. (D, E) C57BL/6 mice (n=5) were fasted for 12 h and injected via the tail vein with FGF19 (1 mg/kg) or vehicle for 2 h. (D) FXR occupancy in liver was detected by ChIP at the indicated genes. (E) The mRNA levels were detected by RT-qPCR. (F) FXR-KO mice were infected with adenovirus expressing WT-FXR or Y67F-FXR for 4 weeks and injected via the tail vein with FGF19 (1 mg/kg) for 2 h. Occupancy of FXR (left) and p-Y67 FXR (right) in the liver was detected by ChIP analysis at the indicated genes (n=5). (A-B, D-F) All values are presented as mean ± SD. Statistical significance was measured using the (E) Mann-Whitney test or (A, B, D, F) two-way ANOVA with the Tukey post-test. **P<0.01, and NS, statistically not significant.

Fig. 7. Y67-FXR phosphorylation is important for FGF19-induced cholesterol transport gene expression and cholesterol excretion/efflux from hepatocytes. Hepatocytes were isolated from FXR-KO mice and transfected with expression plasmids for flag-WT-FXR, flag-Y67F-FXR, flag-Y67E-FXR, or GFP. After 48h, cells were treated with vehicle or 50 ng/ml FGF19 for 2 h (A, B), 12 h (C), or 6 h (D). (A) FXR and p-Y67 FXR levels in the cells detected by IBD and (B) mRNA levels of indicated genes measured by RT-qPCR (n=5). (C) Luciferase reporter assay: Luciferase activities were normalized to β-galactosidase activity (n=4). (D) Cholesterol excretion/efflux assay: Cholesterol efflux was measured as described in Methods. (A, B, D) All values are presented as mean ± SD. (D) A horizontal line indicates the mean. (A, B, D) Statistical significance was measured using the (B-D) two-way ANOVA with the Tukey post-test. **P<0.01, and NS, statistically not significant.

Fig. 8. Src important for FGF19-induced cholesterol transport gene expression and cholesterol excretion/efflux from hepatocytes. (A) C57BL/6 mice were infected with lentivirus expressing shRNA for Src for 4 weeks and injected with FGF19 (1 mg/kg) for 2 h. FXR occupancy in liver detected by ChIP at the indicated genes (left), and mRNA levels detected by RT-qPCR (right) (n=5). (B) Primary mouse hepatocytes (PMH) were infected with lentivirus expressing shRNA for Src for 72 h and treated with 50 ng/ml FGF19 for 2 h. FXR occupancy detected by ChIP at the indicated genes (left) and mRNA levels detected by RT-qPCR (right) (n=5). (C, D) Primary mouse hepatocytes (PMHs) were pre-treated with
vehicle or 1 nM of dasatinib for 1 h and then treated with vehicle or 50 ng/ml FGF19 for 2 h. (C) The p-Y67 FXR and FXR levels determined by IB. (D) The mRNA levels of the indicated genes measured by RT-qPCR (n= 8). (E) PMH from C57BL/6 mice were infected with lentivirus expressing shRNA for Src for 72 h and treated with 50 ng/ml FGF19 for 6 h (left) or were pre-treated with vehicle or 1 nM dasatinib for 2 h and then, treated with vehicle or 50 ng/ml FGF19 for 6 h (right). Cholesterol efflux from hepatocytes was measured as described in Methods. (A-D) All values are presented as mean ± SD. (E) A horizontal line indicates the mean. (A-E) Statistical significance was measured using the two-way ANOVA with the Tukey post-test. *P<0.05, **P<0.01.

**Fig. 9. Model:** Phosphorylation of hepatic FXR at a single residue, Y67, mediated by FGF15/19 signaling-activated c-Src is important for the transcriptional induction of hepatic sterol transport genes, including *Scarb1, Abcg5,* and *Abcg8,* as shown in this study, and the resultant increased cholesterol efflux from hepatocytes for biliary excretion. This hepatic FGF19-Src-FXR phosphorylation signaling cascade contributes to decreased cholesterol levels and protects against atherosclerosis in mice.
Figure 1

(A) (FXR-floxed) AAV-TBG-Cre
AAV-TBG: FXR-WT Y67F

(B) AAV-TBG-GFP
AAV-TBG-FXR WT, or Y67F
(4 weeks) CA (6h)

(C) Rel mRNA level

(D) Hepatic Cholesterol and Bile Acid Transport for Biliary Excretion

(E) (FXR-LKO) AAV-TBG-GFP
AAV-TBG-FXR WT, or Y67F
(4 weeks) Sac (E-G)

(F) AAV-GFP AAV-FXR WT AAV-FXR Y67F

(G) C57BL/6 FXR-LKO (AAV-GFP) FXR-LKO (AAV-FXR WT) FXR-LKO (AAV-Y67F)
Figure 2

A (FXR-LKO) Injection w/ AAV-TBG-GFP, FXR WT or Y67F

(FXR floxed) Sac

Co-injection w/ AAV-TBG-Cre + AAV-TBG-GFP, FXR WT or Y67F

(B) Plasma lipoprotein profile

Cholesterol (μg/fraction)

Fraction number

AAV-GFP  AAV-FXR WT  AAV-FXR Y67F

(B) Plasma lipoprotein profile

Cholesterol (μg/fraction)

Fraction number

AAV-GFP  AAV-FXR WT  AAV-FXR Y67F

Liver cholesterol (µg/mg protein)

Fecal cholesterol (µg/mg)

Plasma total cholesterol (μg/µl)

Biliary cholesterol (mg/dL)

(FXR floxed) CA (6h)

(FXR-LKO) Sac

Liver cholesterol (µg/mg protein)

Biliary cholesterol (µg/mg)

Fecal cholesterol (µg/mg)

Plasma total cholesterol (μg/µl)

Biliary cholesterol (mg/dL)
Figure 3

A) Lenti-shGFP and Lenti-shSrc in (C57BL/6) mice 4 weeks after injection. Sac:

B) Relative mRNA level of Rel, Abcg1, Abcg5, Abcg8, Abca1, Abca9, Abcb1, and Ppar in Lenti-shGFP and Lenti-shSrc mice.

C) Plasma total cholesterol (µg/µl) in Lenti-shGFP and Lenti-shSrc mice.

D) Plasma lipoprotein profile in Lenti-shGFP and Lenti-shSrc mice.
Figure 4

A

AAV-TBG-GFP
AAV-TBG-FXR WT, or Y67F
24 weeks
Western diet
Sac
(ApoE-KO)

B

AAV:

FXR
p-Y67
FXR
Actin

C

Rel mRNA level

GFP
FXR WT
FXR Y67F

D

Plasma total cholesterol

Liver cholesterol (µg/mg protein)

Biliary cholesterol (mg/dL)

Fecal cholesterol (µg/mg)

E

Aortic sinus; ApoE-KO

ORO
H&E
Picrosirius red
F4/80

F

Brachiocephalic artery; ApoE-KO
Figure 5

A

24 weeks 4 weeks
(ApoE-KO) Western diet
Lenti-shGFP or Lenti-shSrc

B

shGFP shSrc
Src FXR p-Y67 FXR Actin

C

Rel mRNA level
Scarb Abag Abcd9 Abcd8 Abcd9 Abcd11 AlpG1 Ptg

D

Plasma total cholesterol (µg/µl)
Liver cholesterol (µg/mg protein)
Biliary cholesterol (µg/mg)
Fecal cholesterol (µg/mg)

E

Aortic sinus; ApoE-KO

Oro H&E Picrosirius red F4/80

F

Brachiocephalic artery; ApoE-KO

ORO H&E Picrosirius red F4/80

Downloaded from http://www.jbc.org/ on April 27, 2019
Figure 6

A

![Bar chart for Rel mRNA level comparison between Veh and GW4064 treatments for C57BL/6 and FGF15-KO conditions.](chartA)

B

![Bar chart for Rel mRNA level comparison between Fs and Fd treatments for C57BL/6 and FGF15-KO conditions.](chartB)

C

![FXR ChIP-seq results for (+) GW4064 treatment.](chartC)

D

![Graph showing % of input for ChIP and q-RTPCR experiments.](chartD)

E

![Graph showing % of input for liver ChIP experiments.](chartE)

F

![Graph showing % of input for liver ChIP experiments.](chartF)
Figure 7

A

PMH (FXR-KO)

| Ad: GFP | WT | Y67F | Y67E |
|---------|----|------|------|
| FGF19   | +  | +    | +    |
| FXR     | +  | +    | +    |
| p-Y67FXR| +  | +    | +    |
| Actin   | -  | -    | -    |

B

PMH (FXR-KO)  

![Bar charts showing Rel mRNA level for different treatments](chart1.png)

C

PMH (FXR-KO)

![Bar charts showing luciferase activity for different treatments](chart2.png)

D

PMH (FXR-KO)

![Scatter plot showing cholesterol efflux](chart3.png)
Scarb1
HDL Cholesterol
Abcg5
Abcg8
Gallbladder
Cholesterol Transport for Biliary Excretion
FGF15/19 Signaling
Src Activation
Y67-FXR Phosphorylation
Cholesterol
Abcg5
Abcg8
Gallbladder
Cholesterol Transport for Biliary Excretion
Plasma Cholesterol
Atherosclerosis
Src Activation
Y67-FXR Phosphorylation
FGF15/19 Signaling
Scarb1
HDL Cholesterol
Phosphorylation of hepatic farnesoid X receptor by FGF19 signaling-activated Src maintains cholesterol levels and protects from atherosclerosis
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