HHS Public Access

Author manuscript

Curr Mol Med. Author manuscript; available in PMC 2016 February 26.

Published in final edited form as:
Curr Mol Med. 2014 ; 14(6): 703–711.

Hepatocyte FRS2α is Essential for the Endocrine Fibroblast Growth Factor to Limit the Amplitude of Bile Acid Production Induced by Prandial Activity

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Abstract

In addition to being positively regulated by prandial activity, bile acid production is also negatively controlled by the endocrine fibroblast growth factor 19 (FGF19) or the mouse ortholog FGF15 from the ileum that represses hepatic cholesterol 7 α-hydroxylase (Cyp7a1) expression through activating FGF receptor four (FGFR4). However, how these two regulatory mechanisms interplay to control bile acid homeostasis in the body and the downstream pathways by which FGFR4 regulates Cyp7a1 expression are not fully understood. Here we report that hepatocyte FGFR substrate 2α (FRS2α), a scaffold protein essential for canonical FGFRs to activate the ERK and AKT pathways, was required for the regulation of bile acid production by the FGF15/19-FGFR4 signaling axis. This occurred through limiting the extent of increases in *Cyp7a1* expression induced by prandial activity. Excess FGFR4 kinase activity reduced the amplitude of the increase whereas a lack of FGFR4 augmented the increase of *Cyp7a1* expression in the liver. Ablation of *Frs2α* alleles in hepatocytes abrogated the regulation of *Cyp7a1* expression by FGFR4. Together, the results demonstrate that FRS2α-mediated pathways are essential for the FGF15/FGF19-FGFR4 signaling axis to control bile acid homeostasis.

Keywords

bile acid; FRS2α; FGF15/FGF19; FGFR4; CYP7A1

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Conflict of Interests:
None.

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Introduction

Bile acids are hydrophilic derivatives of cholesterol, which are a major ingredient in bile and function as emulsifiers to help absorption of lipids and lipid-soluble vitamins. The deficiency of bile acids leads to malnutrition. In addition, since bile acids are the exit strategy for extra cholesterol in the body, excretion of bile acids into feces helps preventing high cholesterol levels in the body, which is associated with elevation of cardiovascular diseases. Recently, bile acids have been increasingly appreciated as hormonal molecules that regulate energy metabolism together with their nuclear receptor FXR [1] and cell membrane receptor TGR5 [2].

Excessive bile acids in hepatocytes are toxic to the cells and can lead to liver damage. Therefore, the bile acid levels need to be tightly regulated by multiple mechanisms [3]. The enterohepatic circulation of bile acids after a meal occurs with secretion of bile acids into the intestine and reabsorption of up to 95% of them back into the circulation. About 5% of bile acids are excreted constantly into feces. Bile acids are synthesized from cholesterol in the liver through two pathways. The major bile acid synthesis pathway is catalyzed by CYP7A1, a key cytochrome P450 enzyme in converting cholesterol into bile acids. The alternative pathway is controlled by sterol 27-hydroxylase (CYP27A1), which is account for approximate 30% of bile acid pool size. The enzyme activity of CYP7A1 is regulated mainly at the transcription level, which is induced by cholesterol and inhibited by bile acid feedback controls [4]. Several mechanisms responsible for the negative feedback controls have been uncovered [5-7]. Among them, the fibroblast growth factor (FGF) pathway has been recognized as a key mechanism to negatively regulate the enterohepatic circulation [8]. Bile acids bind and activate intestinal FXRs, resulting in increased FGF15 (human ortholog FGF19) expression in the ileum [9]. FGF15/19 then activates the hepatocyte FGF receptor 4 (FGFR4)-betaKlotho (KLB) signaling complex to repress *Cyp7a1* expression, resulting in reduced conversion of cholesterol to bile acids in the liver [10, 11]. However, the molecular mechanism downstream of the FGF15/19-FGFR4 complex signals inhibition of *Cyp7a1* in hepatocytes is not fully understood.

The FGF family comprises of 18 receptor binding members and four transmembrane tyrosine kinase receptors [12]. Among the FGF ligands, FGF19, FGF21, and FGF23 belong to the endocrine FGF (eFGF) subfamily [13], which are different from the rest of other canonical FGFs in two key aspects. First, the eFGF functions as a circulating hormone that activates targets distal from its origin and is involved in metabolic regulation. This is in contrast to classic FGFs that function as autocrine or paracrine factors that target the cells producing them or the cells near the site of their origins. Second, many classic FGFs have a high affinity for heparan sulfate and require it as a cofactor to bind and activate their receptors. The eFGFs, however, have weak affinities for heparan sulfates, but require Klothos as a coreceptor [12]. Klothos are not only required for eFGFs binding to FGFRs with high affinity, but also function as a determinant of ligand and signaling specificities of FGFRs [14-20]. The ERK, AKT, PLCγ, and several other signaling cascades have been implicated to relay FGF signals intracellularly downstream of the membrane. FGF receptor substrate 2α (FRS2α) functions as a scaffold protein recruiting two downstream signaling

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molecules, GRB2 and SHP2, to the FGFR kinases, which are required for canonical FGFRs to activate the ERK and AKT pathways, respectively [12, 21]. The activation of PLCγ and several other pathways, however, are not FRS2α dependent. Furthermore, FRS2α has been implicated in other growth factor signaling pathways. Germ line disruption of *Frs2α* causes early embryonic lethality [22]. Depleting FRS2α in many organ sites does not always phenocopy the loss of FGFs or FGFRs, although sometimes results in similar defects [23-25]. This is in line with the findings that FGFR elicits signals both via FRS2α-dependent and FRS2α-independent pathways.

Here we reported that hepatocyte-specific depletion of *Frs2α* abrogated the activity of the FGF15-FGFR4 signaling axis on limiting the amplitude of bile acid production increases induced by prandial activities. The finding that ablation of *Frs2α* phenocopied *Fgfr4* deficiency with respect to bile acid regulation indicated that FGF15/FGF19-FGFR4 signaling elicits such regulatory activities through FRS2α-mediated pathways. The results that the FRS2α-mediated FGFR4 signals restrict the amplitude of bile acid production induced by prandial activity unravel a mechanism by which the food intake induced bile acid production is restrained by eFGFs.

Materials and Methods

Animals and Diets

All animals were housed in the Program of Animal Resources at the Institute of Biosciences and Technology, Texas A&M Health Science Center, and were handled in accordance with the principles and procedures of the *Guide for the Care and Use of Laboratory Animals*. All experimental procedures were approved by the Institutional Animal Care and Use Committee. C57BL/6 mice carrying the *Fgfr4* null [26], Alb-*caFgfr4* transgenic [27], *Frs2α* floxed [28], Alb^{Cre} transgenic allele [29] were maintained and genotyped as previously described. Only 8 to 12 week-old adult male mice were used in this study. Mice were maintained in 12-hour light/12-hour dark cycles and were given free access to food and water. Standard rodent chow and the standard chow supplemented with 1% (w/w) cholic acid were purchased from Alief Purina Feed Store, Inc. (Alief, TX). FGF19 in PBS was administrated by intraperitoneal (I.P.) injection at a dosage of 1 mg/kg in the morning, four hours after fasting. PBS was used for vehicle control. The liver was harvested at the indicated times after the injection for Western blot analyses or gene expression analyses.

Histological Procedures

Liver tissues were fixed overnight in Histochoice Tissue Fixative MB (no. H120–4L, Amresco), dehydrated through a series of ethanol treatments, and embedded in paraffin according to standard procedure. Sections of 5 μm thickness were prepared and stained with hematoxylin and eosin [10].

Bile Acid Analysis

Bile acids were measured using the Bile Acids kit (no. 450-A, Sigma) as described [10]. To determine fecal bile acid excretion, the feces from individually housed mice were collected, weighed, and dried over a 72-hour period. Then 0.5 g of dried feces was minced and

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extracted in 10 ml of 75% ethanol at 50 °C for 2 hours. The extract was centrifuged, and 1 ml samples of supernatant were diluted to 4 ml with a 25% PBS solution for assay according to manufacturer's suggestions. The total bile acid pool size was determined as bile acid contents in the small intestine, gallbladder, liver, and feces. Fresh organs were collected, minced, and extracted with 15 ml of 75% ethanol at 50 °C for 2 hours as aforementioned.

Analysis of mRNA

Total RNA was extracted with the Ribopure RNA isolation reagent (Ambion, TX) as described [30]. Reverse transcription was carried out with SuperScript III (Life Technologies, Grand Island, NY) and random primers. Real-time PCR was performed on Mx3000 (Strategene), using the SYBR Green JumpStart Taq ReadyMix (Sigma, St. Louis, MO) with pairs of primers specific for each transcript according to manufacturer's protocols. The primer sequences are: Cyp7a1-F: GCATCTCAAGCAAACACCATTCC, Cyp7a1-R: AGTCAAAGGGTCTGGGTAGATTTC; Klb-F: GGGATCATGGCGCCCGTCTT, Klb-R: TGGCATGGGTTTGGCACAGGT; Fgfr4-F: GCTGCTGGCCGGGGTGTATC, Fgfr4-R: CCGAGCACCAACCTGTCCCG; Bsep-F: GCCCTCATACGGAAACCCAA, Bsep-R: TCATGGGTGCCTCTTTCCAC; Cyp8b1-F: GGTACGCTTCCTCTATCGCC, Cyp8b1-R: GAGGGATGGCGTCTTATGGG; Ibabp-F: CAGGAGACGTGATTGAAAGGG, Ibabp-R: GCCCCCAGAGTAAGACTGGG; Asbt-F: TGGGCTTCCTCTGTCAGTTTGGAA, Asbt-R: AGT GTGGAGCAAGTGGTCATGCTA; Ostα-F: AGCAATTTCCTTGCTGTGTCCACC, Ostα-R: AGGATGACAAGCACCTGGAACAGA; Ostβ-F: TCCGTTCAGAGGATGCAACTCC TT, Ostβ-R: CATTCCGTTGTCTTGTGGCTGCTT; Fgf15-F: ACAATTGCCATCAAGGACGTCAGC, Fgf15-R: TGAAGATGATGTGGAGGTGGTGCT. The ratio between expression levels in the two samples was calculated by relative quantification, using β-actin as a reference transcript for normalization.

In Situ hybridization

Paraffin-embedded tissue sections were rehydrated followed by digestion with 20 μg/ml protease K for 7 minutes at room temperature. After prehybridization at 65°C for 2 hours, the hybridization was carried out by overnight incubation at 65°C with 0.5 μg/ml digoxigenin labeled RNA probes for the indicated genes. Non-specifically bound probes were removed by washing four times with 0.1 × DIG washing buffer at 60°C for 30 minutes. Specifically bound probes were later detected using alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche, Indianapolis, IN). The *in situ* probe for FRS2α mRNA and its sense probe were generated as described [24].

Western Blotting Analysis

Liver tissues were homogenized in the cell lysis buffer (0.5% Triton-X-100-PBS). The lysates were harvested by centrifugation, separated SDS-PAGE, electric blotted on PVDF membrane. Mouse anti-ERK (1:3,000), anti-β-actin (1:3,000) and Rabbit anti-pJNK were purchased from Santa Cruz (Santa Cruz, CA). Rabbit anti-pERK (1:2,000), Rabbit anti-pFRS2 (1:1,000), anti-AKT (1:1,000), anti-pAKT (1:1,000), and anti-pSTAT3 were from Cell Signaling (Beverly, MA). Mouse anti-CYP7A1 (1:1,000) was from Millipore (Billerica,

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MA). The specific bands recognized by the aforementioned antibodies were visualized with ECL Substrate Kit (BIO-RAD) as previously described [31]. The intensity of the bands was quantitated using the NIH Image J software (http://rsb.info.inh.gov/ij).

Statistical Analyses

Values are expressed as the mean ± standard deviation (S.D.) with the number of replicates described in the legends to figures. The statistical significance of differences between mean values ($p < 0.05$) was evaluated using the two-tailed Student's *t* test.

Results

Ablation of *Frs2α* in hepatocytes does not affect liver histology and body weight

Emerging evidence demonstrates that FGF signaling plays important roles in liver homeostasis and function. Although being an essential adaptor protein for FGF signaling to activate the ERK and PI3K/AKT pathways, the function of FRS2α in the liver has not been fully characterized. To determine whether *Frs2α* was expressed in hepatocytes, *in situ* hybridization was carried out to assess *Frs2α* expression in the liver. The results showed that *Frs2α* was strongly expressed in the liver, evidenced by universal but specific purple staining in the cytosol of hepatocytes (Fig. 1A). The sense probe failed to generate any detectable signal demonstrating the hybridization was sequence-specific. To investigate the role of FRS2α in the liver, *Frs2α* alleles were tissue-specifically ablated in hepatocytes by crossing mice bearing *Frs2α* floxed ($Frs2\alpha^{Flox}$) alleles and mice bearing the Alb^{Cre} transgenic allele [29], in which the albumin promoter driven Cre recombinase was highly expressed in hepatocytes. Both $Frs2\alpha^{Flox}$ and Alb^{Cre} mice were back crossed to C57/BL6 for more than 5 generations. The mice harboring homozygous $Frs2\alpha^{Flox}$ alleles and one Alb^{Cre} were designed as $Frs2\alpha^{CN}$. *In situ* hybridization showed that expression of *Frs2α* was diminished in the $Frs2\alpha^{CN}$ liver (Fig. 1A).

The body weight and liver/body weight ratios of mutant mice were compatible to those of $Frs2\alpha^{Flox}$ mice at young adulthood (Fig. 1B). Although FGF signaling has been shown to be required for liver organogenesis [32], $Frs2\alpha^{CN}$ mice did not exhibit apparent defects in gross organ morphology (Fig. 1C), and tissue histology of the liver (Fig. 1D), as well as did not have abnormal proliferation (Fig. 1E) or apoptotic activities (data not shown). Furthermore, the mutant mice at the age of up to 12 months also appeared to be normal (data not shown), which were consistent with the mice with ablation of *Fgfr4,* the major FGFR isoform in the liver. No unexpected death related to liver failure was observed in $Frs2\alpha^{CN}$ mice. The results indicate that ablation of *Frs2α* in hepatocytes with the Alb^{Cre} driver did not result in vital function defects in the liver.

FRS2α deficiency causes elevation of bile acid productions in the liver

Our previous study shows that FGFR4 regulates bile acid production in the liver and that activation of FGFR4 induces FRS2α phosphorylation [10, 33]. To test whether the FRS2α-mediated pathways were required for regulation of bile acids, both fecal pools of single-housed $Frs2\alpha^{Flox}$ and $Frs2\alpha^{CN}$ mice were measured. The results showed that the daily fecal bile acid excretion of $Frs2\alpha^{CN}$ mice was two folds of the control value (Fig. 2A). To

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determine whether the increase in bile acids excretion was due to overproduction in the liver or due to decreased re-absorption in the intestine, the bile acid pool including bile acids from the liver, gallbladder, intestine, and feces was assessed, which represented the total amount of bile acid circulation of the animal. The results showed that the bile acid pool of $Frs2\alpha^{CN}$ mice was 2.4 folds higher compared to that of $Frs2\alpha^{Flox}$ mice (Fig. 2B). This data suggests that $Frs2\alpha^{CN}$ mice had an increase in bile acid production. CYP7A1 is the rate limiting enzyme for the classic pathway of bile acid production, which catalyzes the cholesterol oxidation in the liver. The expression of this key enzyme in the liver was measured to further determine the production of bile acids was increased. Since expression of *Cyp7a1* varies between day and night due to feeding patterns, unless otherwise specified, the samples for *Cyp7a1* and other gene expressions were collected only in mid mornings. The results showed that *Cyp7a1* expression in the $Frs2\alpha^{CN}$ liver was upregulated to 3.5 folds of that in the $Frs2\alpha^{Flox}$ liver (Fig. 2C). In contrast, CYP8B1, a key enzyme for the chenodeoxycholic acid production, was not changed significantly in the $Frs2\alpha^{CN}$ liver. In addition, expression of *Fgfr4* which is the major FGFR isoform in hepatocytes and KLB which is the co-receptor for FGF15/FGF19 in hepatocytes was not changed. Furthermore, the bile salt export pump (BSEP/ABCB11), a transporter for bile acid secretion, in the $Frs2\alpha^{CN}$ liver remained at a similar level as in the $Frs2\alpha^{Flox}$ liver (Fig. 2C). Similarly, the key transporters mediating absorption of bile acids in the intestine were not changed (Fig. 2D). Immunostaining further demonstrated that CYP7A1 abundance in $Frs2\alpha^{CN}$ liver was higher than that in control mice (Fig. 2E). Although most experiments were carried out with 12 to 16-week-old mice, we also assessed bile acid production and *Cyp7a1* expression in 10 to 12-month-old mice and found no difference between the two groups. Together, the data here indicate that the deficiency of FRS2α in liver increases bile acid production by upregulating *Cyp7a1* expression. Consistent with increasing bile acid production, expression of *Fgf15* was upregulated two fold in the ileum of $Frs2\alpha^{CN}$ mice (Fig. 2E). Since ileum FGF15/FGF19 downregulates bile acid production in the liver, the results suggest a failure of feedback control of enterohepatic bile acid circulation in $Frs2\alpha^{CN}$ mice.

Ablation of *Frs2α* in hepatocytes abrogates the feedback control of bile acid production mediated by the ileum-liver FGF15-FGFR4 signaling axis

FGFR4 is the cognate receptor for FGF15/FGF19 in hepatocytes, which plays a key role in negative feedback control of *Cyp7a1* gene expression [10]. It has been shown that FRS2α can be phosphorylated by FGFR4 kinases, and is essential for FGFR kinases to activate the ERK and PI3K/AKT pathways [14, 18, 19]. Since $Frs2\alpha^{CN}$ and *Fgfr4* null mice showed a similar phenotype with respect to increased bile acids, we then tested whether FRS2α mediated FGFR4 signals to repress *Cyp7a1* expression. Both $Frs2\alpha^{Flox}$ and $Frs2\alpha^{CN}$ mice at the age of 8-10 weeks were fed with normal chow food or the diet with 1% cholic acids (CA) for 1 week. As expected, feeding with CA diets repressed *Cyp7a1* expression in $Frs2\alpha^{Flox}$ mice to a level of 5% of those fed with normal chow (Fig. 3A). In contrast, the reduction in $Frs2\alpha^{CN}$ mice was modest comparing with that in $Frs2\alpha^{Flox}$ mice. Expression of *Cyp7a1* expression in CA diet fed $Frs2\alpha^{CN}$ mice remained at 38% of those fed with regular chow, which was still about 2 folds higher than that in $Frs2\alpha^{Flox}$ mice fed with normal chow and 40 folds higher than that in CA diet fed $Frs2\alpha^{Flox}$ mice (Fig. 3A). These

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results indicate a critical role of FRS2α-mediated signaling pathways in negative feedback control of bile acid production.

To further test whether FRS2α was required for FGF15/FGF19, the ligand for the FGFR4-KLB complex, to downregulate *Cyp7a*1 expression, recombinant FGF19, the human ortholog of mouse FGF15, was administered to mice bearing either $Frs2\alpha^{Flox}$ or $Frs2\alpha^{CN}$ alleles by I.P. injection. In $Frs2\alpha^{Flox}$ mice, liver expression of *Cyp7a1* in the FGF19-injected group was reduced to 30% of control animals that were injected with PBS at 2 hours after the injection. However, injection of FGF19 failed to reduce *Cyp7a1* expression in $Frs2\alpha^{CN}$ mice, which remained to be 2.5 to 3.0 folds higher than that in $Frs2\alpha^{Flox}$ animals regardless administration of FGF19 (Fig. 3B). Western blot also showed that CYP7A1 abundance was significantly increased in $Frs2\alpha^{CN}$ liver and that FGF19 failed to reduce CYP7A1 expression in $Frs2\alpha^{CN}$ liver (Fig. 3C). This indicated that FRS2α was required for FGF19 to regulate CYP7A1 expression and bile acid production. Interestingly, expression of squalene epoxidase (SE), one of the rate-limiting enzymes in sterol biosynthesis, was not affected by *Frs2α* ablation, although it was enhanced by FGF19 treatment (Fig. 3B), suggesting that FRS2α was not essential for FGF19 to activate SE expression in the liver.

The ERK and PI3K/AKT pathways are major downstream mediators of canonical FGF signaling cascade [12]. We then tested whether FRS2α was required for FGF19 to activate ERK or PI3K/AKT in the liver. Administration of recombinant FGF19 via I.P. injection induced phosphorylation of FRS2α and ERK, but not AKT, within 30 minutes after the injection (Fig. 3C). Ablation of *Frs2α* led to failure of FGF19 to induce ERK phosphorylation, without affecting baseline phosphorylation of AKT. The results indicated that FRS2α was essential for FGF19 to activate the ERK pathway as has demonstrated generally for canonical FGFs. Consistently, loss of *Fgfr4* abolished the activity of FGF19 to induce phosphorylation of FRS2α and ERK. The mice carrying wildtype *Fgfr4* or one *Fgfr4* null alleles did not exhibit detectable differences and hereafter designated as *Fgfr4* controls. The data here further demonstrate that FGFR4 was the only FGF receptor in the liver mediating FGF19 signals (Fig. 3D). However, FGF19 failed to induce phosphorylation of AKT on Thr308. Ablation of *Fgfr4* or *Frs2α*, also did not affect AKT phosphorylation at the baseline level. The results suggest that the AKT pathway is not involved in the FGF19-FGFR4 signaling axis in the liver and that other signaling pathways are the upstream regulators for the AKT pathways. Interestingly, ablation of *Fgfr4*, but not *Frs2α*, increased phosphorylation of AMPK in the liver; ablation of *Frs2α*, but not *Fgfr4*, affected STAT3 phosphorylation. This is in line with the reports that FGFR4 elicits both FRS2α dependent and independent pathways and that FRS2α does not just serve as a downstream mediator for FGFR4.

FRS2α is required for FGFR4 to control the amplitude of *Cyp7a1* expression in response to prandial stimulation

In addition to being negatively regulated by the FGF19/FGFR4 signaling axis, bile acid production is also regulated by other mechanisms, including prandial activities [34, 35]. However, how FGF signaling intertwines with other bile acid regulatory mechanisms has

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not been characterized. To investigate whether ablation of *Fgfr4* compromised prandial control of bile acid production, both *Fgfr4^{null}* and wildtype littermates were fasted overnight and re-fed with regular chow. Both *Cyp7a1* expression and bile acid pool were measured one hour after re-feeding (Fig. 4A&B). The results showed that although re-feeding increased *Cyp7a1* expression, the effects were significantly augmented in *Fgfr4^{null}* mice, indicating that FGFR4 signaling limited the amplitude of the response to feeding.

We previously reported that forced overexpression of a constitutively active FGFR4 (caFGFR4) mutant in the liver represses *Cyp7a1* expression and bile acid production [14]. To determine whether constitutively exposure to FGFR4 kinase further attenuated prandial influences on bile acid production and whether FRS2α was required for FGFR4 to elicit such activities, transgenic mice overexpressing caFGFR4 in the liver were crossed with both *Frs2α^{Flox}* and *Frs2α^{CN}* mice. As described above, the mice were fasted overnight then re-fed with regular chow or continued fasting for an hour. Consistently, expression of caFGFR4 significantly reduced *Cyp7a1* expression both in fasted or re-fed conditions. Ablation of *Frs2α* abolished the effects of caFGFR4 on *Cyp7a1* expression in both conditions since the *Frs2α^{CN}* mice essentially had a similar level of *Cyp7a1* expression regardless of forced expression of caFGFR4 mutants in both groups (Fig. 4C). The data indicate that FRS2α is required for FGFR4 to suppress *Cyp7a1* expression. It was noteworthy that regardless of overexpression of caFGFR4, the extent of prandial pattern-induced *Cyp7a1* expression change was higher in *Frs2α^{CN}* mice (11-fold increase) than that in *Frs2α^{Flox}* mice (2.5-fold increase). This demonstrated that FRS2α is essential for FGFR4 to control the amplitude of *Cyp7a1* expression changes in response to prandial activity, and therefore, regulating bile acid homeostasis as illustrated in Fig. 5.

Discussion

FRS2α has been well established as a membrane proximal mediator of canonical FGF signaling. The endocrine FGF15/19-FGFR4 signaling axis is a key mechanism regulating cholesterol/bile acid homeostasis. Whether FRS2α-mediated pathways are required for FGFR4 to control hepatic bile acid production has not been reported. Herein we showed that ablation of *Frs2α* in the hepatocytes abrogated the FGF15/FGF19-FGFR4 feedback control of bile acid production without effect on overall liver morphology. Both ablation of *Fgfr4* or tissue-specific ablation of *Frs2α* in hepatocytes increased augmented effects of the prandial induction on the *Cyp7a1* expression. Since CYP7A1 is the rate-limiting enzyme for bile acid production, the results indicate that FRS2α is essential for FGFR4 to control the amplitude of prandially-induced bile acid production via inhibiting *Cyp7a1* expression. As conversion to bile acid is one exit strategy for extra cholesterol in the body, manipulation of FRS2α-mediated signaling pathways may provide a new avenue for controlling the cholesterol/bile acid homeostasis.

Frs2α null embryos die in early development [22]; cell type-specific disruption of *Frs2α* causes significant defects in many organs and frequently phenocopies the deficiency of FGF or FGFR [24, 36, 37]. Although the *Alb^{Cre}* driver is expressed in embryonic stages, no significant developmental defects in the liver were observed. This is consistent with the data that *Fgfr4* null mice have no obvious developmental defects [26], which is the only FGFR

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isoform in mature hepatocytes. *Alb*^{Cre} is only expressed in the liver after E15.5, and the expression is mosaic at the stage. It is possible that late and low penetration of the *Frs2α* disruption was not sufficient to disrupt liver development. It is also possible that other adaptor protein, such as FRS2β, may compensate the loss of FRS2α to support liver development. However, it is also possible that FRS2α-mediated pathways are not essential for liver development. Although beyond the scope of this study, further investigations will be taken to address this issue. No significant difference in longevity was observed between *Frs2α*^{CN} and *Frs2α*^{Flox} mice. This indicated that the loss of hepatocyte FRS2α did not impact the maintenance and vital functions of the liver and is in line with the finding that FGFR4-deficient mice are relatively normal [26]. However, whether FRS2α plays a role in other cell types of the liver remains to be characterized.

There is no change in liver and intestine bile acid transporters that are responsible for bile acid secretion and reabsorption, respectively. Thus, the increase in fecal bile acids and total bile acid pool size represents the upregulated bile acid production. The expression of *Cyp8b1* that encodes a key enzyme for the chenodeoxycholic acid synthesis was not changed in the FRS2α deficient mice. Therefore, increase in *Cyp7a1* expression is solely responsible for elevation of bile acid production in *Frs2α*^{CN} liver, which is consistent with *Fgfr4* ablation [10]. Since ileum FGF15/19 expression is regulated by bile acids through the FXR receptor [38], increase in bile acids in the intestine will enhance FGF15/19 expression. This explains why *Frs2α*^{CN} mice had increased *Fgf15* expression in the ileum.

Increased bile acid production in the FRS2α-deficient mice indicates that FRS2α-mediated pathways negatively regulate bile acid production. The significance of this finding was further demonstrated in the two studies with bile acid diet challenge and the fasting-feeding condition. In both cases, the differences between *Frs2α*^{Flox} and *Frs2α*^{CN} mice were intensified with respect to *Cyp7a1* expression. It is worthwhile to notice that *Cyp7a1* expression was increased upon re-feeding in *Frs2α*^{CN} mice, suggesting prandial upregulation of *Cyp7a1* expression is mediated by FRS2α-independent mechanisms. However, FRS2α-mediated pathways controlled the amplitude of the surge since ablation of *Frs2α* augmented the escalation of *Cyp7a1* expression. The detailed molecular mechanism underlying this augmentation needs to be studied.

FRS2α-deficiency in hepatocytes blocked FGFR4 repression of *Cyp7a1* expression (Fig. 4C), as well as FGF15/FGF19-regulated ERK activation and gene expression in the liver (Fig. 3), indicating that FRS2α is an essential component in the FGF15/FGF19-FGFR4 signaling axis. In addition, this suggests that eFGFs share similar downstream signaling pathways with other classical FGFs and that FRS2α is needed for FGF19 to regulate expression of these genes. The molecular mechanism of how shared signals between eFGFs and canonical FGFs requiring klotho co-receptors are limited to metabolic regulation in the case of eFGFs is of great interest.

In conclusion, the FRS2α-mediated signaling pathway is essential for the ileum-liver directionally specific FGF15/FGF19-FGFR4 signaling axis to regulate bile acid homeostasis through controlling expression of *Cyp7a1* that encodes the rate-limiting enzyme in bile acid

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synthesis. The finding suggests a new avenue for pharmaceutical control of bile acid/ cholesterol metabolism.

Conclusion

FGF15-FGFR4 signaling from the ileum to the liver limits the amplitude of bile acid production induced by prandial activities via the *Frs2α*-mediated pathway in hepatocytes. The results unravel a feedback control mechanism by which the food intake induced bile acid production is restrained by eFGFs.

Acknowledgements

We thank Dr. Chuxia Deng for mice bearing the original *Fgfr4* null allele and Samantha Del Castillo for critical reading of the manuscript. This work was supported by the National Institutes of Health CA96824, CA140388, DE023106, and The Cancer Prevention and Research Institution of Texas CPRIT110555 to FW, the J.S. Dunn Research Foundation to WLM, the Natural Science Foundation of Zhejiang Province of China (Y2110492) to CW, and the National Natural Science Foundation of China (81101712, 81270761) to XL.

List of abbreviation

FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FRS2α	fibroblast growth factor receptor substrate 2 alpha
CN	conditional null
eFGF	endocrine fibroblast growth factor
SE	squalene epoxidase
Cyp7a1	cholesterol 7 α-hydroxylase
KLB	beta Klotho

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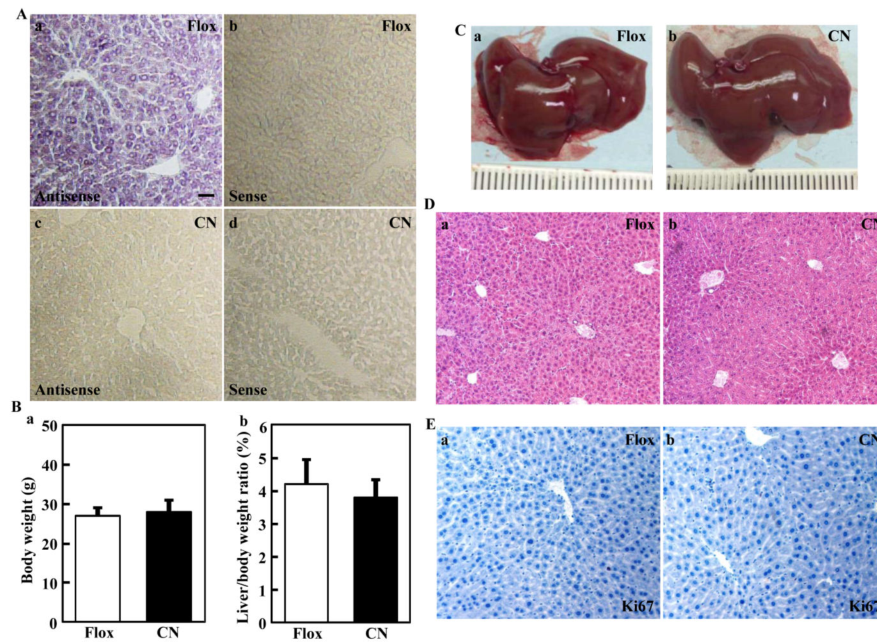

Fig.1. Ablation of *Frs2α* in hepatocytes does not cause apparent liver defects

(A) *In situ* hybridization showing *Frs2α* expression (purple staining) in the liver of $Frs2\alpha^{Flox}$ but not in $Frs2\alpha^{CN}$ liver. Sense probe was used as a negative control. (B) Average body weight and the liver/body weight ratios of $Frs2\alpha^{Flox}$ and $Frs2\alpha^{CN}$ mice at the age of 2 months. (C) Gross morphology of $Frs2\alpha^{Flox}$ and $Frs2\alpha^{CN}$ liver of 2-month-old mice. (D) H&E staining showing no obvious histological difference between $Frs2\alpha^{Flox}$ and $Frs2\alpha^{CN}$ livers. (E) Ki67 staining showing no proliferative differences between two groups. Flox, $Frs2\alpha^{Flox}$; CN, $Frs2\alpha^{CN}$; Solid bars represent 50 μm.

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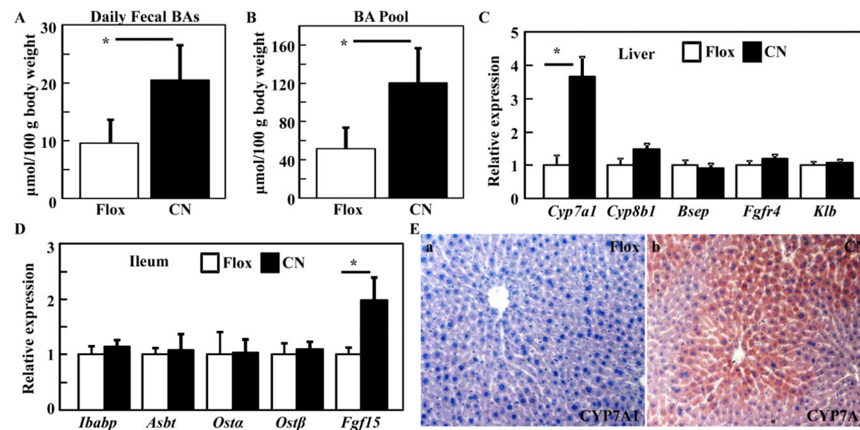

Fig.2. Ablation of *Frs2α* in hepatocytes increases bile acid production

(A) Fecal bile acid secretion. Fecal pellets were collected daily from single housed 12-week-old *Frs2α*^{Flox} and *Frs2α*^{CN} mice for bile acid extraction. Data are mean±sd from 6 mice. (B) Total bile acid pool was measured from liver, gallbladder, intestine and their contents. (C&D) Real-time RT-PCR analyses showing key genes in the bile acid homeostasis in the liver (C) or ileum (D). (E) Immunostaining of CYP7A1 in 3-month-old mouse livers. Data are mean±sd from 5 mice. *, $p<0.05$; BA, bile acid; Flox, *Frs2α*^{Flox}; CN, *Frs2α*^{CN}.

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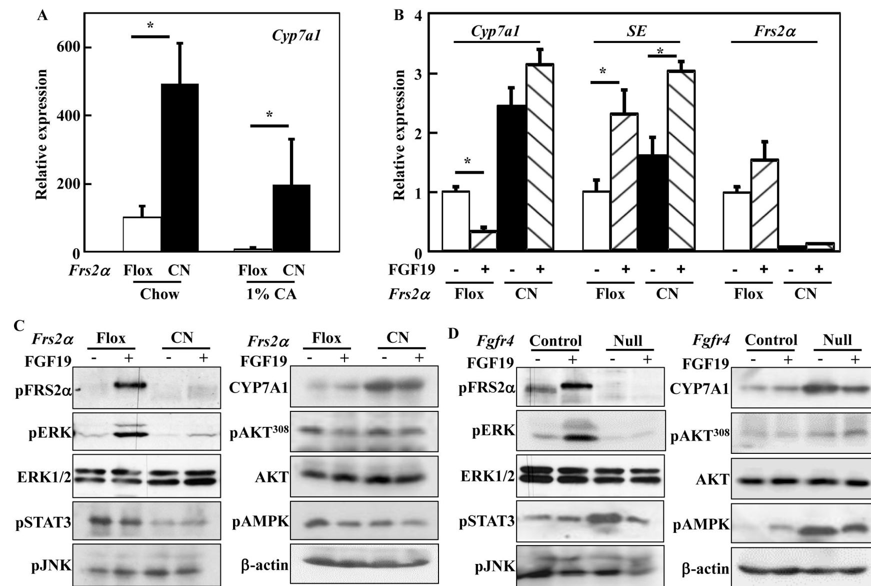

Fig. 3. Ablation of *Frs2α* in hepatocytes compromises the bile acid-FGF15 feedback control of *Cyp7a1* expression

(A) $Frs2\alpha^{Flox}$ and $Frs2\alpha^{CN}$ mice were fed with normal chow or 1% cholic acid-containing diet for 1 week. The liver was then harvested for real-time RT-PCR analyses of *Cyp7a1* expression. (B) $Frs2\alpha^{Flox}$ and $Frs2\alpha^{CN}$ mice were I.P. injected with FGF19 (1 mg/kg body weight). Two hours after the injection, the liver was harvested for real-time RT-PCR analyses of the indicated genes. (C&D) The indicated mice were fasted for 4 hours in the morning. FGF19 in PBS was I.P. injected at a dosage of 1 mg/kg. The liver was harvested 30 minutes later for Western blot analyses of indicated proteins. RT-PCR data derived from 5 mice were normalized to actin loading control and presented as means±sd. *, $p<0.05$; SE, Squalene epoxidase; Flox, $Frs2\alpha^{Flox}$; CN, $Frs2\alpha^{CN}$.

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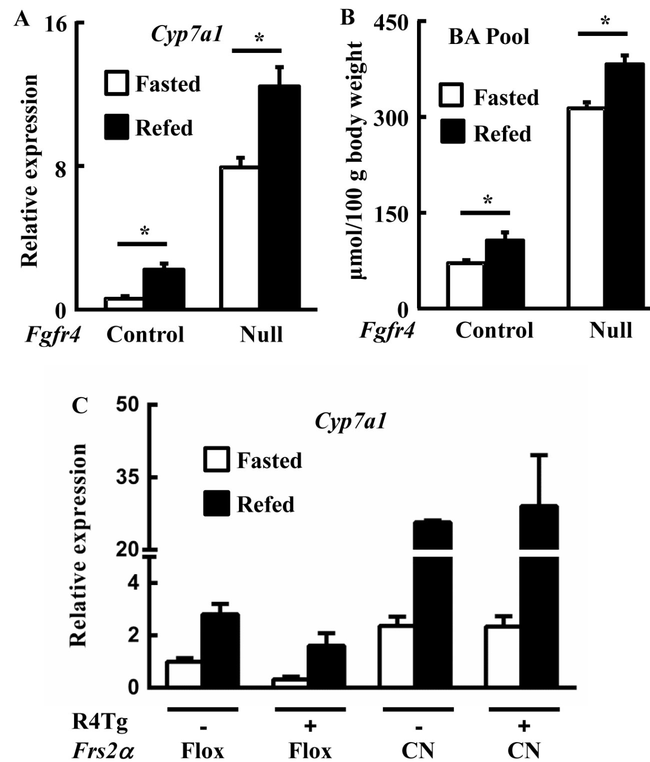

Fig. 4. Ablation of *Frs2* in hepatocytes attenuates FGFR4 control of the amplitude of post-prandial activation of *Cyp7a1* expression

(A) The mice bearing the indicated *Fgfr4* alleles were fasted overnight followed by feeding (Refed) with normal chow or continuing to fast (Fasted) for 1 hour. Expression of *Cyp7a1* was analyzed by real-time RT-PCR. (B) The bile acid pools of mice with similar treatments as in (A) were analyzed as described. (C) Mice with the indicated genotype were treated as in (A) and the expression of *Cyp7a1* was analyzed by real-time RT-PCR. Data derived from 5 mice were normalized to actin loading controls and presented as means±sd. BA, bile acid; R4Tg, transgenic mice expressing constitutively active FGFR4 in hepatocytes; *, $p<0.05$; Flox, $Frs2\alpha^{Flox}$; CN, $Frs2\alpha^{CN}$.

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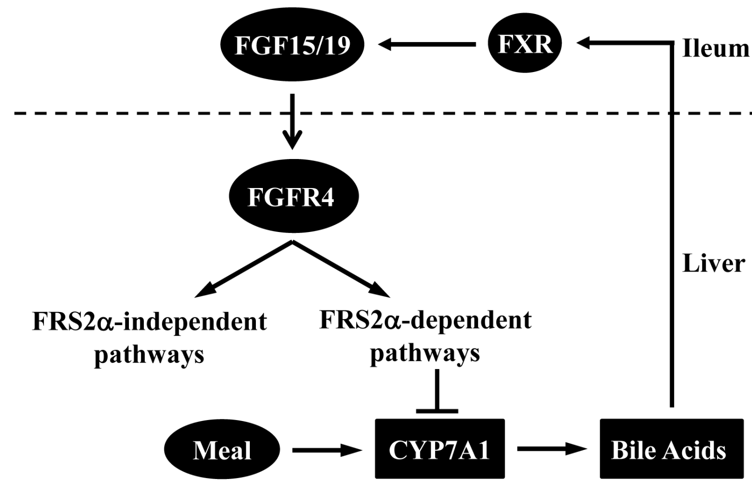

Fig. 5. FRS2α mediates FGFR4 signals to control the amplitude of *Cyp7a1* expression induced by meal intake

Meal intakes induce bile acid production via promoting *Cyp7a1* expression in the liver. Elevated bile acids in the ileum induce *Fgf15/Fgf19* expression in the ileum, which then activates the FRS2α-dependent pathways of FGFR4 and suppresses expression of *Cyp7a1* in the liver.