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A Novel Fibroblast Growth Factor 15 Dependent- and Bile Acid Independent-Promotion of Liver Regeneration in Mice

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Abstract

The role of intestine-derived factors in promoting liver regeneration after partial hepatectomy (PHx) are not entirely known, but bile acids (BAs) and fibroblast growth factor 15 (Fgf15) that is highly expressed in the mouse ileum could promote hepatocyte proliferation. Fgf15 strongly suppresses the synthesis of BAs and emerging evidence indicates that Fgf15 is important for liver regeneration. The mechanisms by which Fgf15 promotes liver regeneration are unclear, but Fgf15 may do so indirectly by reducing BA levels and/or directly by promoting cell proliferation. However, it's undetermined whether these two mechanisms are independent or integrated. In this study, we aimed to clarify these relationships by generating novel *Fgf15* tet-off, transgenic mice (*Fgf15* Tg) that had very low BA levels resulted from overexpressed Fgf15-mediated suppression of BA synthesis. Compared to wild-type mice, the *Fgf15* Tg mice showed increased hepatocyte proliferation even without surgery, and a further induction of the genes in cell-cycle progression

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after PHx. Moreover, overexpression of *Fgf15* by AAV-*Fgf15* transduction or treatment with the recombinant Fgf15 protein led to increased cell proliferation *in vivo*. Furthermore, *Fgf15*Tg mice exhibited an earlier and greater activation of MAPK, Stat3, and NF- κ B signaling pathways in the priming stage, and a disruption of the hippo signaling pathway in the termination stage of liver regeneration.

Conclusion—Direct *in-vivo* evidence demonstrates that Fgf15 is critical in stimulating the phases of priming and termination of liver regeneration that are critical for cell survival and liver-size determination, independent of BA levels.

Keywords

intestine-liver crosstalk; transgenic mice; cell proliferation; Fgf15; FGF19

Introduction

The liver is capable of a full regeneration following partial resection or chemical-induced injury through a highly orchestrated process. Two-thirds partial hepatectomy (2/3 PHx) in rodents has been widely used as a model to study liver regeneration since its introduction (1), with the complex regenerative processes and a cohort of factors being well-characterized (2).

Bile acids (BAs) have emerged as a group of critical factors in liver regeneration. BAs are synthesized in the liver from cholesterol, undergone enterohepatic circulation, and indispensable to emulsify lipids and lipid-soluble vitamins for intestinal absorption. Moreover, BAs function as important physiological signaling molecules to activate nuclear receptors, including farnesoid X receptor (FXR), pregnane X receptor and vitamin D receptor. Through their effects on these nuclear receptors, BAs regulate hepatic lipid, glucose, and energy homeostasis, and maintain metabolic balances (3, 4). BAs can also activate G protein-coupled receptors (GPCR), including sphingosine 1-phosphate receptor (S1PR) 2 and G protein-coupled bile acid receptor 1 (also called TGR5), to regulate lipid and glucose homeostasis (5–7). Disorders in BA homeostasis have been implicated in cholestatic liver diseases, fatty liver disease, cardiovascular disease, obesity and diabetes (4, 8–10).

Fgf15 (human ortholog FGF19) is expressed in the ileum, and highly induced upon the activation of FXR (11, 12). It is well known that Fgf15 functions as a gut-derived hormone to suppress BA synthesis via the Fgf15/Fgfr4 axis (13). Briefly, secreted Fgf15 interacts with Fgfr4/ β -Klotho receptor complexes at the cell membrane of hepatocytes, which leads to activation of JNK and ERK pathways and ultimately the suppression of the expression of genes in BA synthesis, including *Cyp7a1/CYP7A1* and *Cyp8b1/CYP8B1* (11, 13, 14).

BAs have been shown to promote liver regeneration. Specifically, depletion of BAs by BA-sequestering resin (cholestyramine) resulted in a decrease in liver regeneration (15, 16), which is consistent with a report showing that external biliary drainage led to decreased liver regenerative capacity (17). Mechanistically, BAs promote liver regeneration by activating FXR either in the liver to directly induce cell cycle progression or in the intestine to induce

Fgf15/FGF19 that activates hepatocyte proliferation and suppresses toxic BA levels (16, 18, 19). Indeed, the role of BA receptors in liver regeneration have been shown and mice deficient in FXR or TGR5 displayed significantly higher mortality, severe liver necrosis, and delayed liver regeneration after PHx (16, 20). (21, 22)

The intestine derived Fgf15/FGF19 is important to liver growth. We and others have shown that Fgf15 and FGF19 are critical in promoting liver regeneration and defining hepatic size, and mice deficient in Fgf15 showed impaired liver regeneration, marked liver injury and higher mortality (19, 21, 22). The underlying mechanisms by which Fgf15/19 promotes liver regeneration could be by reducing the BA levels to prevent BA-induced liver injury or via direct promotion of cell proliferation (21, 22). Therefore, we need to determine the direct effects of Fgf15 on liver regeneration versus the indirect mechanism regulated through suppressing BA synthesis. To determine the direct, BA-independent effects of Fgf15 on liver regeneration *in vivo*, we generated a new transgenic (Tg) mouse model, with Fgf15 overexpression in both the liver and intestine using the tetracycline inducible Tet-Off system. This model allows us to determine the role of Fgf15 in liver regeneration *in vivo* independent of BA levels.

Materials and Methods

Animals and treatments

Tissue-specific, inducible *Fgf15* Tg mice were generated on the C57BL/6J background (Supplementary Methods). Whole-body *Fgf15* knockout (KO) mice were previously described (22, 23). Male mice, n=5–12/group, were used in this study, with age-matched C57BL/6J mice as wild-type (WT) controls. Mice were maintained in a pathogen-free animal facility at 22°C under the 12-hr light/dark cycle. The animal studies were approved by the Rutgers University Institutional Animal Care and Use Committee (IACUC) and complies with all relevant federal guidelines and institutional policies. *Fgf15* Tg and WT mice were administered by oral gavage with water (10 ml/kg BW) containing 2 mg/ml doxycycline (Dox, Sigma, D9891) daily for 5 days to stop the *Fgf15* transgene expression. Two days after the treatment with Dox, 2/3 PHx was performed as described previously (22), with details in Supplementary Methods as well. AAV-mediated overexpression of *Fgf15* was achieved in *Fgf15* KO and WT mice via a single tail-vein injection of 1×10^{13} PFU/kg of AAV or AAV-*Fgf15*. Mice were kept in quarantine for 7 days before undergoing 2/3 PHx. Tissues were collected at 48 hrs post-surgery. For cholic acid (CA) feeding, WT and *Fgf15* Tg mice were fed chow diet containing 0% or 0.1% CA for 7 days. Recombinant Fgf15 protein (rFgf15) produced in *E. coli* and purified as described previously (24, 25) was injected into mice through tail vein at a dosage of 50 µg/kg. Tissues were collected at 3 hrs after injection.

Serum biochemical parameter analysis

Serum activities/levels of alanine transaminase (ALT), aspartate transaminase (AST), Alkaline phosphatase (ALP), total triglycerides (TGs), total cholesterol (TC), and total bilirubin were determined by kits from Pointe Scientific (Canton, MI) and the total BAs assay kit was from Diazyme Laboratories (Poway, CA).

Determination of BA pool size

Total BA pool size was determined and expressed as micromoles of BAs per 100 g of body weight ($\mu\text{mol}/100\text{g BW}$). BA concentration in the liver was expressed as $\mu\text{mol}/\text{g}$ liver weight ($\mu\text{mol}/\text{g LW}$), as described previously (13) and with details in Supplementary Methods as well.

Histological and immunohistochemical analysis

Hematoxylin and eosin (H&E) staining and immunohistochemistry staining of Fgf15 and BrdU are described in detail in the supplemental Methods.

Isolation of RNA, quantitative RT-PCR and western blot analysis

Samples were prepared and analyzed according to previous publications (13, 22). Gene expression at mRNA levels was normalized to the housekeeping gene, β -Actin, with primers listed in the Supplementary Table. β -actin or α -tubulin protein levels were used as the loading control for Western blot analysis.

Statistical analysis

All experimental data are expressed as the mean $\pm$ SD. Statistical differences among multiple groups were tested using two-way ANOVA followed by the Student–Newman–Keuls test. A value of $p < 0.05$ was considered statistically significant.

Results

Generation and characterization of a novel *Fgf15* Tg mice

To determine the direct effect of Fgf15 on liver regeneration *in vivo*, a novel line of *Fgf15* Tg mice were generated by using the Tet-Off expression system to create a tissue-specific expression of *Fgf15* that can be turned off by Dox treatment (Fig. 1A). Specifically, two lines of mice were cross-bred to make the *Fgf15*Tg mice, with one expressing tetracycline transactivator fusion protein (tTA) driven by the promoter of fatty acid binding protein (FABP) 1 gene for tissue-specific overexpression of tTA protein in both the liver and intestine (26, 27), and with the other line carrying a tTA-responsive element (TRE) composed of repeated tetracycline operator (tetO) linked to the target gene of interest, herein *Fgf15* (described in detail in Supplementary Methods). Therefore, *Fgf15*Tg mice have *Fgf15* overexpression in both enterocytes and hepatocytes where FABP1 promoter functions. Oral administration of Dox by gavage can efficiently prevent the binding of tTA protein to the TRE sequence, which prevents *Fgf15* transgene overexpression. Time-course of changes in Fgf15 mRNA levels following Dox treatment was shown in Supplementary Fig. 1. *Fgf15* Tg mice have developed and reproduced normally. When the Tg mice reached 2–3 months of age, serum activities/levels of ALT, AST, ALP, TGs, TC, BAs and total bilirubin were measured. Despite elevated serum BA levels in Tg mice compared to WT mice, these levels were still within the normal range. All other serum parameters were similar between Tg and WT mice (Fig. 1B). *Fgf15* Tg mice had lower body weight (BW) and liver weight (LW)/BW ratio compared to WT mice (Fig. 1C). No significant difference in liver histology/pathology was observed between WT and Tg mice (Fig. 1D). In WT mice, Fgf15 protein was

expressed in differentiated epithelial cells of the intestine and not detected in the livers, but in *Fgf15*Tg mice, strong Fgf15 protein expression was detected in both periportal hepatocytes and intestinal epithelial cells (Fig. 1E).

Levels of Fgf15 mRNA in Tg mice were 300–600 folds higher than those in WT mice (Fig. 1F). Treatment with Dox for 5 days terminated the *Fgf15* overexpression in both liver and intestine, and interestingly, 5 days after Dox withdrawal, the expression of *Fgf15* transgene was only recovered in the intestine but not the liver (+/–Dox, Fig. 1F), suggesting this strategy could be used as an intestine specific *Fgf15* overexpression model. With overexpression either in both the liver and intestine (Veh, Fig. 1F) or only in intestine (+/–Dox, Fig. 1F), *Fgf15*Tg mice showed a markedly reduced mRNA levels of *Cyp7a1*, *Cyp8b1* and *Cyp7b1*, but not *Cyp27a1* (Fig. 1F). The total BA pool size in Tg mice was 20–30% of that in WT mice (Fig. 1G). Specifically, *Fgf15* overexpression did not change BA levels in the liver, but reduced BA concentration in the intestines (Fig. 1G), where most of the BAs are stored in the body. To specifically determine the role of *Fgf15* overexpression in the intestine, the Tg mice treated with Dox for 5 days were used for all of the subsequent experiments.

Overexpression of *Fgf15* regulates genes involved in BA homeostasis and cell proliferation with or without BA supplement

To restore their BA pool size, *Fgf15*Tg mice were fed a 0.1% CA-containing diet (Fig. 2). With CA feeding, Tg mice showed a similar increase in liver BA levels compared to WT mice, but markedly increased BA levels in the intestine, and as a result, the total BA pool size increased in Tg mice (Fig. 2A). Hepatic Fgf15 mRNA levels were induced with CA feeding, with a significantly higher induction in Tg than in WT mice. Suppression of the gene expression of *Cyp7a1*, *Cyp8b1*, *Cyp7b1*, and *Cyp27a1* was achieved with CA feeding, and further suppression of *Cyp7a1* and *Cyp8b1* was noted in *Fgf15*Tg mice (Fig. 2B). Without CA feeding, *Fgf15*Tg mice had lower mRNA levels of *Shp* compared to WT mice. The CA feeding reduced mRNA levels of *Ntcp*, but increased mRNA levels of *Shp*, *Bsep* and *Ostβ* in WT mice (with the latter two not significant). However, CA feeding did not alter *Ntcp* and *Shp* mRNA levels in *Fgf15*Tg mice, and markedly increased those of *Bsep* and *Ostβ* (Fig. 2B). In the ileum, the higher *Fgf15* expression in Tg mice was associated with lower expression of *Ibabp*, which was highly induced by CA in both WT and Tg mice. Even though on chow diet, *Ostβ* expression was reduced in *Fgf15*Tg mice compared to WT, CA feeding significantly increased *Ostβ* expression only in *Fgf15*Tg mice (Fig. 2C).

A previous study showed that BAs stimulate hepatocyte proliferation (16). Therefore, to determine to what extent *Fgf15* overexpression enhances cell cycle progression directly without liver surgery, levels of proteins involved in cell cycle progression were determined in Tg mice without (basal level) and with CA feeding. Without CA feeding, though BA pool size was very low in the Tg mice, the basal mRNA levels of *Ccnd1* (Cyclin D1), *Pcna* (Pcna), and *Cdkn1a* (p21), but not *Ccne1* (Cyclin E1), were significantly higher than those in WT mice (Fig. 2D). With CA feeding, WT mice didn't show an increase in the mRNA levels of *Ccnd1*, *Ccne1* and *Pcna* except for *Cdkn1a*, which were highly induced. In Tg mice, CA further induced the expression of all these mediators (Fig. 2D). The protein

expression of cell signaling mediators related to cell proliferation and *Fgfr4* was further determined. JNK and ERK signaling pathways were activated in the Tg mice, as evidenced by increased p-JNK and p-ERK levels, as well as their downstream targets including c-Jun and c-Fos, but not c-Myc. CA increased protein levels of Cyclin D1 and PcnA, but reduced those of Cyclin E1 and c-Myc, in WT and Tg mice (Fig. 2E). Overall, these results suggest that *Fgf15* may directly promote cell proliferation in addition to modulating BA levels at basal levels.

Overexpression of *Fgf15* promotes liver regeneration upon 2/3 PHx

The induction of pro-proliferative genes in the liver by *Fgf15* transgene prompted us to determine the effects of *Fgf15* overexpression on liver regeneration at low BA levels. PHx was performed in Tg mice with overexpression of *Fgf15* in both liver and intestine (to mimic human expression pattern)-Veh/PHx, and in Tg mice with *Fgf15* overexpression only in the intestine by treatment with Dox for 5 days followed by Dox-free for 2 days (mouse expression pattern)-Dox/PHx (Fig. 3A). Twenty-four hrs following PHx, the total BA pool size in Tg mice remained lower (Fig. 3B). *Ccnd1* and *Ccne1* gene expression started to increase at 24 hrs post PHx (22), and the current study showed that overexpression of *Fgf15* in both liver and intestine induced *Ccnd1*, *Ccne1* and *Cdkn1a* following PHx, while overexpression of *Fgf15* only in the intestine induced *Ccnd1* and *Ccne1* to a lesser extent, and significantly increased levels of *Cdkn1a* (Fig. 3C), suggesting that *Fgf15* in both liver and intestine contributes to cell proliferation after PHx. The ERK, but not JNK, pathway showed higher activation following PHx in Tg mice (Fig. 3D). The protein levels of Cyclin D1, Cyclin E1, Cdk4, PcnA, and c-Myc, but not c-Jun, were markedly increased in *Fgf15* Tg mice (Fig. 3D).

Thirty-six hrs post PHx, overexpression of *Fgf15* in the liver remained to be suppressed by Dox, but it was fully recovered in the intestine (Fig. 4A). At this time point, *Ccnd1* gene expression was still much higher in mice with *Fgf15* overexpression in both the liver and intestine (Veh/PHx), but not in Tg mice with overexpression of *Fgf15* in intestine only (Dox/PHx). *Ccne1* expression tended to be lower in *Fgf15* Tg than WT mice, which may reflect a compensation. DNA proliferation, measured by BrdU incorporation in hepatocytes during liver regeneration, was higher in Tg mice with *Fgf15* overexpression in both liver and intestine (Veh/PHx) compared to WT (Fig. 4B). Interestingly, Dox treatment markedly increased BrdU incorporation in WT mice with PHx (Dox/PHx), and to a similar degree when Tg mice was under the same treatment (Fig. 4B). At 36 hrs post PHx, the BA pool size remained lower in Tg mice, but the hepatic BA levels were similar between WT and Tg mice, and it is noted that Tg mice tended to have higher BA levels in the liver (Fig. 4C). *Fgf15* overexpression, regardless in both liver and intestine (Veh/PHx) or in intestine only (Dox/PHx), led to higher expression of cell cycle related proteins, including Cyclin D1, Cdk4, and c-Myc (Fig. 4D). Intestine-overexpression of *Fgf15* reduced c-Jun protein levels at this time point after PHx (Fig. 4D).

In another independent gain-of-function model, *Fgf15* was overexpressed in the liver by AAV-mediated transduction in *Fgf15* KO mice. Transduction by AAV-*Fgf15* led to reduced BW, and decreased mortality following PHx (from 80 to 30 %) (Fig. 5A). This liver-specific

AAV-mediated Fgf15 overexpression reduced mRNA levels of hepatic *Shp* and intestinal *Ibabp*, genes induced by BAs (Fig 5B). The *Fgf15* KO mice normally had higher BA levels (Fig. 5C), and following PHx, they presented with 8–10 folds higher hepatic BA concentration than WT mice (Fig. 5C). AAV-*Fgf15* overexpression inhibited BA synthesis, resulting in a significant reduction in liver BA concentration, with the total BA pool size returning to levels in WT mice (Fig. 5C, AAV-*Fgf15*/PHx). Furthermore, the protein levels of Cyclin D1, Cyclin E1 and PcnA were induced in the AAV-*Fgf15* mice (Fig. 5D).

Fgf15 enhances hepatocyte priming in liver regeneration upon 2/3 PHx

The effects of Fgf15 on the priming of liver regeneration were examined at 30 and 60 min following PHx in WT and *Fgf15* Tg mice, by determining the mRNA and protein levels of genes involved in the initial stage of cell proliferation (Fig. 6). At 30 min, PHx increased the mRNA levels of *c-Jun* and *c-Fos* by 5- and 100-fold, respectively, in WT mice, and 13 and 250 folds, respectively, in Tg mice (Fig. 6A). This marked induction returned to baseline levels at 60 min (Fig. 6A). A marked induction of *c-Myc* and *Igf1p1* was also observed, which reached their maximal expression at 60 min, and with significant difference in *c-Myc* expression between WT and *Fgf15* Tg mice (Fig. 6A).

Activation of Stat3 with PHx, measured by increased p-Stat3 (S727) and p-Stat3 (Y705), is well-known as one of the major priming events during liver regeneration. Shown in Fig. 6B, the basal levels of p-Stat3 (S727) and p-Stat3 (Y705) seemed higher in *Fgf15* Tg mice (0 min). Both WT and *Fgf15* Tg mice demonstrated increased activation of Stat3 at 30 and 60 min after PHx, but Tg mice had a higher induction of p-Stat3 (S727) at 30 min (Fig. 6B). NF- κ B signaling pathway is activated by IKK-mediated phosphorylation of the inhibitory subunit I κ B α at Ser32 and Ser36, which leads to proteasomal degradation of inhibitor I κ B, thus releasing NF- κ B subunits and activating gene transcription in the nucleus (28). The phosphorylation and activation of NF- κ B (p65) at Ser536 and p-I κ B α at Ser32 in *Fgf15* Tg mice was significantly higher than in WT mice at 30 min after PHx, and returned to basal levels in both strains at 60 min after PHx. There were no significant changes in protein level for NF- κ B (p65) and I κ B α . The basal levels of c-Jun, c-Fos and c-Myc tended to be higher in Tg mice (0 min), but were not further induced by PHx in both strains (Supplemental Fig. 2).

To further determine the signaling cascades activated by Fgf15, *Fgf15* KO mice were injected with the recombinant Fgf15 protein (rFgf15) that was developed in our lab. The rFgf15 was functional as it significantly suppressed *Cyp7a1* gene expression (Fig. 6C). Despite having little effect on the gene expression of *c-Jun* and *c-Fos* (Supplemental Fig. 3), injection of rFgf15 increased p-JNK, p-ERK, and p-Stat3, indicating that Fgf15 could activate both MAPK and Stat3 signaling cascades *in vivo* (Fig. 6D).

Fgf15 may regulate the termination of liver regeneration after 2/3 PHx

In mice, the liver regenerates and grows back to its original mass within 14 days after 2/3 PHx. Hepatocytes reenter the cell cycle and replicate after incision and return to quiescence state after liver recovers its mass (2). However, the mechanisms for the termination of regeneration are complex and not fully understood. Extensive studies have elucidated that

the hippo pathway plays critical roles in organ size determination and is involved in the termination stage of liver regeneration. Activation of the Hippo pathway leads to phosphorylation of Yes-associated protein (Yap) and transcriptional co-activator with PDZ binding motif (Taz), which promotes Yap/Taz subsequent cytoplasmic sequestration and inactivation (29, 30).

The effects of *Fgf15* on liver regeneration termination is unknown. To study this effect, activation of the hippo pathway was assessed in *Fgf15* Tg mice at day-7 and -14 post PHx (Fig. 7). Consistent with the previous result, *Fgf15* Tg mice showed lower LW/BW ratio compared to WT mice (Fig. 7A, Sham). Livers in WT mice were still regenerating at day-7 post PHx and recovered their original weight 14 days after, whereas livers in *Fgf15* Tg mice stopped growing at day-7 post PHx and kept a similar weight at 14 days post PHx (Fig. 7A). Consistent with the liver weight changes, Cyclin D1 level was still much higher in WT mice 7 days post PHx, but similar in *Fgf15* Tg mouse livers between Sham and PHx, indicating that hepatocyte replication remained active in WT but not Tg mice (Fig. 7B). There were no significant differences for p-Yap, Yap and Taz in WT mice between sham and PHx, but in *Fgf15* Tg mice, a slight decrease in p-Yap and Taz levels following PHx, indicating an activation of the hippo pathway in *Fgf15* Tg mice 7 days post PHx (Fig. 7B). At day-14 post PHx, hepatocytes in both WT and *Fgf15* Tg mice returned to a more quiescence state, revealed by similar Cyclin D1 levels between Sham and PHx groups (Fig. 7C). Comparing to WT/Sham group, the protein levels of p-Yap, Yap and Taz were much higher in *Fgf15* Tg mice (Fig. 7C). PHx decreased p-Yap (inactive form) but increased Yap (active form) and Taz expression in WT. However, comparing to Sham, PHx significantly decreased both p-Yap and Yap in *Fgf15* Tg mice (Fig. 7C). These data indicate that after PHx, hepatocytes from WT and *Fgf15* Tg mice may have entered termination stage differently and *Fgf15* may have promoted the Hippo pathway activity after hepatectomy in mice.

In order to determine the effects of *Fgf15* on changes in biliary mass after PHx, biliary mass in WT and *Fgf15* Tg mice was measured at 7 and 14 days post PHx (Supplemental Fig. 4). However, there was no significant difference in the biliary mass between WT and *Fgf15* Tg mice.

Discussion

BAs are synthesized in the liver from cholesterol and mainly function to facilitate the absorption of lipids and fat-soluble vitamins in the intestine (3). Moreover, BAs are a component of bile, undergone enterohepatic circulation, and act as signaling molecules (3, 4, 10). An emerging function of BAs is to promote liver regeneration. However, accumulation of BAs are toxic and can lead to cellular damage and death, cholestasis, inflammation, and tumor genesis (3, 8, 9, 31). BA receptors, including FXR and TGR5, are critical for maintaining BA homeostasis, and deficiency of FXR and TGR5 in mice results in impaired liver regeneration after 2/3 PHx (16, 20). It has previously been suggested that BAs are involved in liver cell proliferation and physiological concentrations of BAs may activate FXR to promote liver regeneration (16). Novel findings from our laboratory and other groups have identified that a FXR target gene in the intestine, *Fgf15* (human orthologue, *FGF19*), is critical in promoting liver regeneration and maintaining liver size following PHx,

but not after chemical-induced liver injury (19, 21, 22, 32). It's a consensus that FGF15/19 suppresses BA synthesis and reduces total BA pool size (13, 14), but it is unclear to what degree Fgf15 directly stimulates cell proliferation *in vivo*. Therefore, through generation of the *Fgf15*Tg mice overexpressing *Fgf15* in liver and/or intestine by supplementing these mice with or without CA, we are able to use this model to distinguish the direct role of Fgf15 in promoting hepatocyte proliferation and liver regeneration *in vivo* versus the indirect effects mediated through BA suppression.

Human FGF19 is abundantly expressed in the gallbladder, intestine and common bile duct, as well as in hepatocytes during cholestasis (33). Contrastingly, mouse Fgf15 is only expressed in the intestines. The *Fgf15*Tg mice generated in the current study expressed Fgf15 in both the liver and intestine, with the transgene driven by the promoter of FABP1. There are two major benefits using this model system. First mouse *Fgf15* but not human *FGF19* gene is overexpressed in these mice, as a previous publication has shown that FGF19 may not activate Fgfr4 in mice (19), therefore, this model is suitable to study Fgf15 function *in vivo*. Second, by using the Tet-off system, we overexpressed *Fgf15* transgene expression in both the liver and intestine without Dox treatment, and with Dox treatment, rebound overexpression is only achieved in the intestine. This generates a more human relevant mouse model. Indeed, the results from current study showed that overexpression of Fgf15 in either liver plus intestine or in intestine only promotes hepatocyte proliferation and liver regeneration, suggesting that Fgf15 is mitogenic regardless of tissue origin.

FGF15/19 is secreted from the enterocytes under physiological conditions, circulates to the liver, and activates Fgfr4 in the presence of β -Klotho. Activation of Fgfr4 leads to activation of several signaling pathways, including MAPK and Stat3, which are believed to be the major mechanism to suppress BA synthesis and promote cell cycle progression (13, 22, 34). Activation of these pathways could contribute to the priming of liver regeneration. The early stage of liver regeneration (0 ~ 4 hr after PHx in mice) is known as the "priming" phase, which requires an orchestration of various molecular signaling pathways and complex tissue interactions. In this stage, a wide variety of genes are differentially expressed and cytokine signaling pathways are activated to prepare for the regeneration process (2, 35). Cytokines of tumor necrosis factor α (Tnfa) and interleukin-6 (Il-6) (36, 37), and activation of signaling cascades of the transcription factors Nf- κ B and Stat3 play critical roles in the priming phase and triggering of the G0/G1 transition of the cell cycle (28, 38, 39). Our current results suggest that activating Nf- κ B is also an important mechanism for Fgf15-mediated priming in liver regeneration. Specifically, the Tg mice showed greater activation of NF- κ B than Stat3 or ERK minutes following PHx. The mechanism of NF- κ B activation needs to be further studied in the future, as contrasting result has been reported that Fgfr4 activation inhibits Nf- κ B signaling (40).

The hippo pathway has been demonstrated as a key regulator of organ size determination and tumor suppression in mammals (29, 30). Activation of the hippo pathway leads to Yap phosphorylation and thus decreased Yap nuclear localization (30), so Yap/Taz activation in hepatocytes induces cell senescence and suppresses transcription activation. Total Yap and Taz levels were higher in *Fgf15*Tg mice, suggesting that the hippo pathway was more active

in *Fgf15*Tg mice (Fig. 7) and Fgf15 might be involved in the suppression of cell proliferation in the termination stage of liver regeneration.

Mst1/2 and Sav1 are upstream core components of hippo signaling and activation of Mst1/2 and Sav1 leads to phosphorylation of Yap/Taz. However, Mst1 and Sav1 protein levels in *Fgf15*Tg mice didn't show any significant differences (data not shown), indicating the phosphorylation of Yap/Taz might be independent of the activation of Mst1/2 and Sav1 pathways. Previous studies have shown that Yap/Taz can be phosphorylated by many other kinases, including JNK, EGFR-Ras-MAPK, and Src family tyrosine kinases (41). It has been well known that Fgf15/FGF19 activates JNK and ERK, however, it is still unclear how this Yap/Taz suppression signaling cooperates with cell proliferation signaling to precisely regulate cell division and hepatocyte proliferation during liver regeneration.

The most important discovery of this study is that we demonstrate that Fgf15 directly stimulates cell proliferation under low BA levels, suggesting that Fgf15 could directly promote cell proliferation independent of BA suppression during liver regeneration. Our study is in agreement with previous reports showing that Fgf15 is responsible for the effects of bile salts on liver growth and the direct mitogenic activity of FGF19 on primary hepatocytes and cultured cholangiocytes *in vitro* (21, 33). To our knowledge, the current study provides the first line of evidence to show that Fgf15 directly promotes liver growth *in vivo* independent of BA levels. In summary, Fgf15 effectively suppresses BA synthesis regardless of tissue origin, and following PHx, Fgf15-mediated suppression of BA synthesis aids in prevention of BA toxicity (Fig. 8). Furthermore, Fgf15 directly promotes cell proliferation and liver regeneration by activating signaling pathways that drive proliferation and cell survival (Fig. 8). In conclusion, we provide evidence showing that Fgf15 mediates an important crosstalk by which the intestines directly stimulate liver regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations

BAs	bile acids
BW	body weight
CA	cholic acid
CAR	constitutive androstane receptor
FABP	fatty acid binding protein
FGF15/19	Fibroblast growth factor 15/19

FXR	farnesoid X receptor
GPCR	G protein-coupled receptors
H&E	hematoxylin and eosin
KO	knockout
LW	liver weight
PHx	partial hepatectomy
rFgf15	recombinant Fgf15 protein
S1PR2	sphingosine 1-phosphate receptor 2
Taz	transcriptional co-activator with PDZ binding motif
TC	total cholesterol
tetO	tetracycline operator
Tg	transgenic
TG	triglycerides
TGR5	G protein-coupled bile acid receptor 1
TRE	tTA-responsive element
tTA	tetracycline transactivator
WT	wild type
Yap	Yes-associated protein

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Figure 1. Generation and characterization of inducible, tissue-specific *Fgf15* Tg mice

(A) Schematic diagram showing the Tet-Off system and constructs used for generating the tTA transactivator and tetracycline-responsive promoter element (TRE) controlled *Fgf15* Tg mice. The fatty acid binding protein 1 (FABP1) promoter drives the expression of tTA in both the liver and intestine, which binds to the TRE and activates transcription of the target gene *Fgf15* without treatment with doxycycline (Dox). Dox treatment resulted in transcriptional repression of the *Fgf15* transgene in both liver and intestine, respectively; (B) Serum biochemical parameters in WT and *Fgf15* Tg mice. ALT, AST and ALP: IU/L; TG and TC: mg/dL; BA: μmol/L; (C) Body weight (BW) and ratio of liver weight (LW) to BW in WT and *Fgf15* Tg mice; (D) Representative photomicrographs of H&E-stained liver sections (×200), and (E) immunohistochemistry staining of Fgf15 in both liver and intestine sections from WT and *Fgf15* Tg mice; (F), mRNA levels of *Fgf15* in liver (L) and intestine (I), and expression of genes in BA synthesis after Dox treatment (Dox) for 5 days or Dox withdrawal for another 5 days (+/-Dox). n=5/group, * indicates statistical significance (p<0.05) between WT and Tg mice with the same treatment. # indicates statistical significance (p<0.05) within the same strain between vehicle (Veh) and Dox treatment.

Figure 2. Both BAs and overexpressed *Fgf15* promoted cell proliferation

Decreased total BA pool in *Fgf15* Tg mice was rescued by 0.1% cholic acid (CA) feeding for 7 days. (A) Liver and intestine BA concentrations and total pool size in WT and *Fgf15* Tg mice; (B) Liver and (C) intestinal expression of genes in BA homeostasis and transport; (D) Expression of genes in cell proliferation and liver regeneration; (E) Protein levels of MAPK pathway and cell cycle markers from WT and *Fgf15* Tg mice. $n=5$ /group, * indicates statistical significance ($p<0.05$) within the same treatment compared to WT group. # indicates statistical significance ($p<0.05$) within the same strain between CA-fed and chow-fed group.

Figure 3. Tissue specific overexpression of *Fgf15* in both liver and intestine (Veh/PHx) or intestine only (Dox/PHx) by Dox treatment promoted liver regeneration at 24 hrs following 2/3 PHx

(A) Hepatic mRNA levels of *Fgf15* and *Cyp7a1* and intestinal *Fgf15* levels in WT and *Fgf15* Tg mice; (B) BA levels in liver and intestine and total BA pool size; (C) Relative gene expression levels of cell-cycle genes in WT and *Fgf15* Tg mice; (D) Protein levels of MAPK pathway and cell cycle markers from WT and *Fgf15* Tg mice. n=5/group, * indicates statistical significance (p<0.05) between WT and Tg mice with the same treatment. # indicates statistical significance (p<0.05) within the same strain compared to Veh/Sham treatment.

Figure 4. Tissue specific overexpression of *Fgf15* in both liver and intestine (Veh/PHx) or intestine only (Dox/PHx) promoted liver proliferation at 36 hrs following 2/3 PHx (A) Hepatic mRNA levels of *Fgf15* and cell cycle genes, and intestinal *Fgf15* in WT and *Fgf15*Tg mice; (B) Representative photomicrographs of BrdU staining (×200) and BrdU-positive cell counts per field of BrdU-stained liver sections from WT and *Fgf15*Tg mice; (C) BA levels in liver and total BA pool size at 36 hrs in WT and *Fgf15*Tg mice; (D) Hepatic protein levels of MAPK pathway and cell cycle markers from WT and *Fgf15*Tg mice. n=5/group, * indicates statistical significance (p<0.05) between WT and Tg mice with the same treatment. # indicates statistical significance (p<0.05) within the same strain compared to Veh/Sham treatment.

Figure 5. AAV-*Fgf15* promoted liver proliferation

WT and *Fgf15* KO mice underwent 2/3 PHx 7 days after AAV-*Fgf15* or AAV-GFP injection, and were analyzed 48 hrs following PHx. (A) BW changes and survival rate; (B) Liver and intestinal gene expression of *Fgf15* and FXR target genes in BA homeostasis; (C) Total BA pool size; (D) Protein levels of Cyclin D1, Cyclin E1, and PcnA in the liver. n=7 (Veh/sham), 9 (WT Veh/PHx) and 9 (AAV-*Fgf15*/PHx) in WT mice; n= 8 (Veh/sham), 8 (WT Veh/PHx) and 12 (AAV-*Fgf15*/PHx) in *Fgf15* KO mice. * indicates statistical significance ($p < 0.05$) between WT and Tg mice with the same treatment. # indicates statistical significance ($p < 0.05$) within the same strain compared to Veh/Sham treatment.

Figure 6. Fgf15 increased expression of immediate-early response genes and accelerated the activation of Stat3 and NF-κB signaling 30 and 60 mins following PHx
 (A) mRNA levels of hepatic early response genes in WT and *Fgf15* Tg mice; (B) Activation of Stat3 and NF-κB signaling; (C) Expression levels of liver genes in WT and *Fgf15* KO mice 2 hrs after rFgf15 protein treatment; (D) Activation of JNK, ERK and Stat3 signaling in the liver in WT and *Fgf15* KO mice 3 hrs after rFgf15 protein treatment. n=5/group, * indicates statistical significance (p<0.05) within the same time point (panel A) or the same treatment (panel C) compared to WT group, # indicates statistical significance (p<0.05) within the same strain compared to 0 time point (panel A) or saline treatment (panel C).

Figure 7. Activation of hippo signaling in WT and *Fgf15* Tg mice after PHx
 Ratio of LW to BW (A) and western blot analysis showed protein level of Yap/Taz at day 7 (B) and day 14 (C) post PHx in WT and Tg mice (n= 5–8). # indicated statistical significance ($p < 0.05$) between the PHx and Sham group within the same strain.

Figure 8. Schematic representation of the effects of BA and Fgf15 on BA homeostasis and liver regeneration

Intestinal Fgf15 is induced by BA-activated FXR, released into portal circulation and transported to the liver where it binds to its receptor complex of Fgfr4 and β Klotho on the hepatocyte membrane, and activates downstream MAPK (JNK/ERK), Stat3, and NF- κ B signaling pathways. Activation of MAPK may lead to suppression of BA synthesis and activation of STAT3 and NF- κ B signaling pathways may be crucial for cell survival and “priming” of liver regeneration. BAs under physiological conditions could also promote liver regeneration. In summary, Fgf15 promotes liver regeneration via BA-dependent and -independent mechanisms.