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Fibroblast growth factor 7 inhibits cholesterol 7α-hydroxylase gene expression in hepatocytes

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ABSTRACT

Cholesterol 7α-hydroxylase (CYP7A1) is the initial and rate-limiting enzyme for bile acid synthesis. Transcription of the CYP7A1 gene is regulated by bile acids, nuclear receptors and cytokines. Fibroblast growth factor 7 (FGF7) secreted from activated hepatic stellate cells (HSC) during chronic liver fibrosis regulates hepatocyte survival and liver regeneration. In the carbon tetrachloride (CCl_4)-induced fibrotic mouse liver, we demonstrated that the expression of CYP7A1 was largely decreased while the expression of FGF7 was significantly increased. We further demonstrated that FGF7 inhibited CYP7A1 gene expression in hepatocytes. Knockdown study by short interfering RNA, kinase inhibition and phosphorylation assays revealed that the suppression of CYP7A1 expression by FGF7 was mediated by FGFR2 and its downstream JNK signaling cascade. The FGF7 neutralizing antibody restored CYP7A1 expression in Hep3B cells treated with conditioned medium from HSC. In summary, the data suggest that FGF7 is a novel regulator of CYP7A1 expression in hepatocytes and may prevent hepatocytes from accumulating toxic bile acids during liver injury and fibrosis.

1. Introduction

Bile acids play a key role in nutrient digestion, absorption and transportation, including resolution and excretion of cholesterol and sterols, absorption of lipid-soluble vitamins and management of toxic metabolites. However, bile acids have potential toxicity, noted of the cholestasis which results in accumulation of high concentration bile acids causing liver damage and ultimately leading to fibrosis and cirrhosis [1,2]. Hence, there must be precise and complicated regulations of bile acid homeostasis.

Cholesterol 7α-hydroxylase (CYP7A1) is an important enzyme in bile acid homeostasis, which catalyzes the initial and rate-limiting step of classical pathway in the bile acid biosynthesis [3]. It converses cholesterol into hydrophobic bile acids in the liver which then excreted into the bile and ileum. More than 90% bile acids are reabsorbed in the ileum, brought back to the liver via portal circulation and in reverse inhibit bile acid synthesis by suppressing the activity of CYP7A1. Less than 10% bile acids are excreted in feces which is replenished by de novo bile acid synthesis [4]. The bile acid-dependent feedback repression of CYP7A1 is essential to avoid bile acids going up to a toxic level. Several nuclear receptors activated by bile acids play vital roles in the regulation of CYP7A1, consisting of farnesoid X receptor (FXR), pregnane X receptor (PXR), vitamin D3 receptor (VDR) and liver X receptor (LXR) [5–8]. Besides, some growth factors and cytokines, for instance, hepatic growth factor (HGF) and transforming growth factor β1 (TGFβ1) which can be expressed by HSC inhibit CYP7A1 expression and bile acid synthesis [9,10].

Hepatic stellate cells (HSC) are recognized as the most important effector cells since they are highly activated and produce excessive extracellular matrix (ECM) and inflammation cytokines during the process of liver injury, fibrosis and cirrhosis [11,12]. It is reported that Fibroblast growth factor 7 (FGF7), a member of the fibroblast growth factor (FGF) family, was expressed by the activated HSC in chronic liver diseases associated with fibrosis [13]. FGF7, also known as keratinocyte growth factor (KGF), mainly produced in the stromal cells, promotes potent mitogen and differentiation for epithelia cells in which its selective receptor, a splice variant of FGF receptor 2 (FGFR2b), is predominately produced [14–16].

In the current study, we demonstrate that FGF7 suppresses transcription of CYP7A1 gene in hepatic cells through JNK pathway.

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This may provide a new insight of the regulation of bile acid biosynthesis during liver injury and fibrosis.

2. Materials and methods

2.1. Antibodies and reagents

Rabbit anti-JNK and anti-phospho-JNK were purchased from Cell Signaling Technology. Mouse anti human FGFR2 and anti-GAPDH antibodies were from Santa Cruz Biotechnology. Rabbit anti human FGF7 neutralizing antibody was from Abcam. Recombinant human FGF19 and FGF7 proteins were from Peprotech. SP600125 was from Beyotime. Leupeptin, aprotinin, phenylmethylsulfonylfluoride were purchased from Sigma–Aldrich Inc. Other reagents were commercially available in China.

2.2. Cell culture

Hep3B cells and LX2 cells were maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37 °C in 5% CO_2. Primary mouse hepatocytes were isolated from the male C57BL/C mice as previously described [17] and then cultured in the 6-well plate at a density of 5.0×10^5 cells per well in a 1:1 mixture of Dulbecco's modified Eagle's medium and F-12 (Gibco) supplemented with 5% FBS, 100U/ml penicillin, 0.1 mg/ml streptomycin, 0.005 mg/ml insulin, 0.005 mg/ml transferrin, 5 ng/ml selenium and 40 ng/ml dexamethasone at 37 °C with 5% CO_2.

2.3. Carbon tetrachloride (CCl4) model of liver fibrosis

All procedures involving animals were reviewed and approved by the Animal Care and Use Committee of Fudan University. Male BABL/c mice were injected subcutaneously with CCl_4 (2 μl/g/body weight, diluted 1:5 in olive oil) or olive oil alone three times per week, for 12 weeks (eight animals per group). Animals were killed at 24 h after the last subcutaneous injection. The livers were harvested for gene expression analysis and Sirius red staining.

2.4. Sirius red staining

Liver specimens were fixed and stained with Sirius red as previously described [18]. Briefly, tissue samples were fixed in 4% paraformaldehyde, embedded in paraffin, cut into five-micrometer thick sections and then stained with Sirius red.

2.5. RNA isolation and Real-time PCR

Total RNAs were prepared from liver samples and hepatocytes with Trizol reagent (Invitrogen). The cDNA syntheses were performed using RT Master Mix kit (Takara). Real-time PCR was performed using a SYBR-green Premix Real-time PCR kit (Takara). The relative mRNA expression was normalized by GAPDH. The Real-time PCR primers were showed in Supplementary Table S1.

2.6. Western blotting

Western blotting experiments were performed as previously described [19]. Briefly, cells were collected, washed with phosphate buffered saline (PBS, pH 7.4) and lysed with lysis buffer on ice for 15 min. Cell lysates then were separated by SDS–PAGE and transferred to a PVDF membrane. After blocking with 5% albumin from bovine serum (BSA), the membrane was incubated with indicated antibodies and detected by enhanced chemiluminescent (ECL) assay kit.

2.7. Small interfering RNA assay

The small interfering RNAs (siRNA) for human FGFR2 (Santa Cruz Biotechnology) were transfected into Hep3B cells using Lipofectamine 2000 reagent (Life Technologies). Cells were harvested 48 h after transfection. The mRNA and protein levels of FGFR2 were analyzed by Real-time PCR and western blotting.

2.8. Conditioned media from hepatic stellate cells (HSC-CM)

HSC-CM was prepared as previously described [20]. Human hepatic stellate cell line, LX2 cells (about 5×10^5 cells/ml) were seeded into 100-mm plastic dish with 10 ml DMEM containing 10% FBS for 3 days(covering about 70% area of dish). To obtain HSC-CM, LX2 cells were washed twice with sterile PBS and switched to 10 ml DMEM supplemented with 2% FBS for 2 days. Culture medium was collected from the dish and centrifuged at 1000*g* for 5 min. The supernatant was stored as HSC-CM at −80 °C until use.

2.9. Statistical analyses

All data were shown as the mean ± SD. All the experiments were carried out three times or as indicated. Difference between two groups was tested by Student's *t*-test. A P value of <0.05 was considered statistically significant.

3. Results

3.1. Expression of FGF7 and CYP7A1 in CCl_4 induced fibrotic mouse liver

We treated mice with CCl_4 to induce chronic hepatic fibrosis. Liver samples were examined for fibrosis using Sirius red staining. Fig. 1A shows a significant collagen deposition in the livers of CCl_4 treatment group. In addition, the mRNA expression levels of fibrosis markers α-SMA, collagen I and TGFβ1 were highly elevated (Fig. 1B), indicating the successful fibrotic liver induction. As expected, the mRNA level of FGF7 was largely increased in the fibrotic liver compared to the vehicle-treated normal liver, while its specific receptor FGFR2b showed no obvious change. Interestingly, the mRNA level of CYP7A1 was significantly decreased in the fibrotic liver compared to the vehicle-treated normal liver (Fig. 1C).

3.2. FGF7 inhibits expression of CYP7A1 in both primary mouse hepatocytes and Hep3B cells

To identify the possible regulation between FGF7 and CYP7A1, we measured the impact of FGF7 on CYP7A1 expression in hepatocytes. Treatment of primary mouse hepatocytes with FGF7 (40 ng/ml) caused a significant decrease of 80% CYP7A1 mRNA expression within 6 h (Fig. 2A). As a positive control, treatment of primary mouse hepatocytes with FGF19 (50 ng/ml) also caused a rapid decrease of CYP7A1 mRNA expression [21] (Fig. 2A). The FGF7 receptor, FGFR2b, remained unchanged during the treatment (Fig. 2A). Additionally, FGF7 treatment strongly inhibited CYP7A1 mRNA expression in a dose-dependent manner with an IC50 of 10 ng/ml for primary mouse hepatocytes (Fig. 2B) and 5 ng/ml for Hep3B cells (Fig. 2C). Fig. 2D shows that FGF7 (40 ng/ml) inhibited CYP7A1 expression in a time-dependent manner in primary mouse hepatocytes. FGF7 performed a rapid inhibition in the initial 6 h and a slow decrease in the later 18 h. These data demonstrate that FGF7 inhibits CYP7A1 gene expression in hepatocytes.

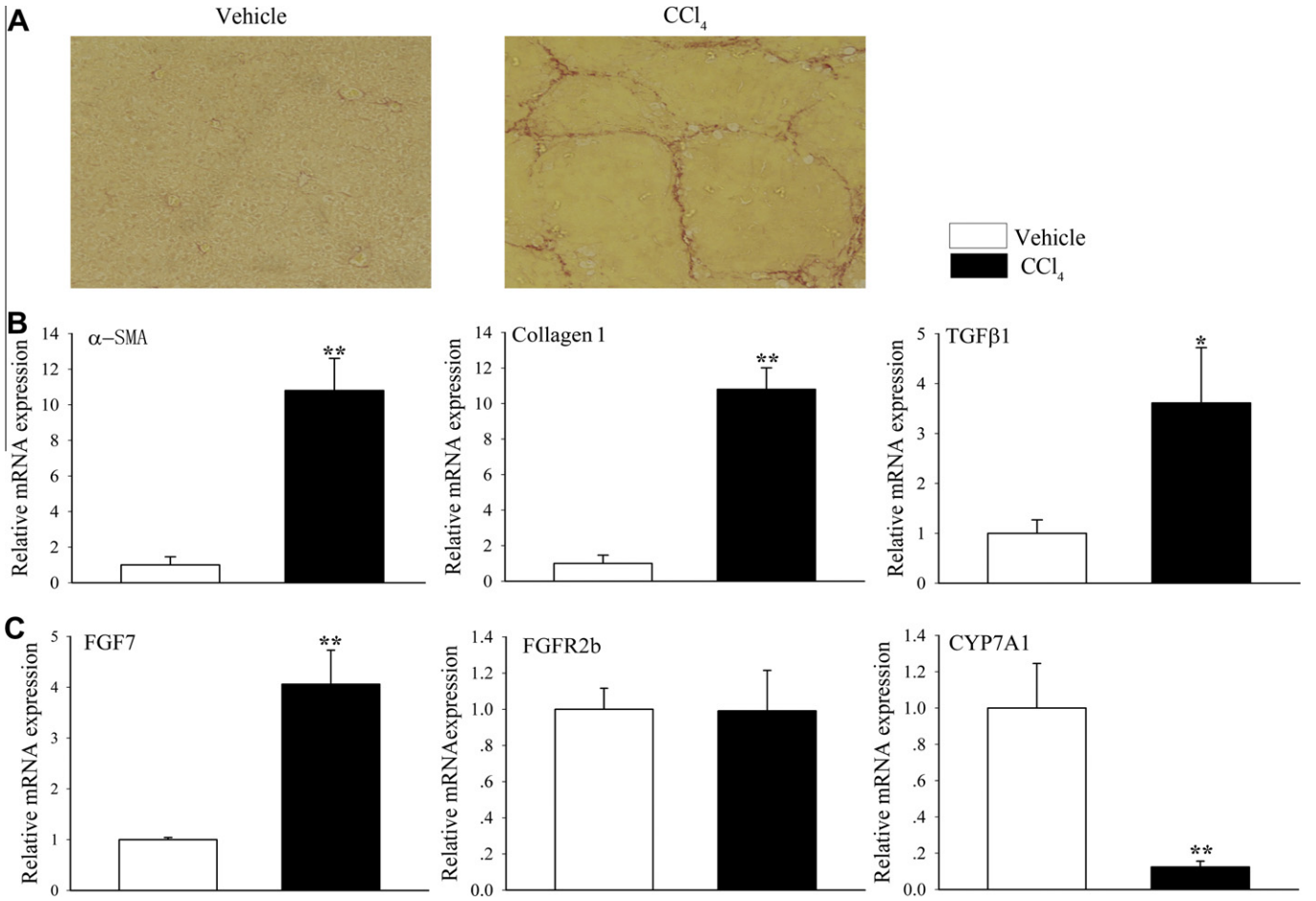

Fig. 1. Expression of FGF7 and CYP7A1 in CCl_4 induced fibrotic mouse liver. Male BALB/c mice were injected subcutaneously with CCl_4 (2 μl/g/body weight, diluted 1:5 in olive oil) (CCl_4) or olive oil alone (vehicle) three times per week, for 12 weeks. (A) Sirius red staining of liver tissue sections from mice treated with CCl_4 or vehicle. (B and C) Total RNA was isolated for Real-time PCR analysis of mRNA levels of α-SMA, collagen I, TGF-β1, FGF7, FGFR2b and CYP7A1 in liver samples. Data are expressed as the mean ± SD of eight C57BL/6 mice each group. $^{*}P < 0.05$, $^{**}P < 0.01$.

3.3. Suppression of CYP7A1 by FGF7 is mediated by FGFR2 and its downstream JNK signaling cascade

To further investigate the signaling pathway that mediates FGF7 inhibition of the CYP7A1 gene expression, we first studied the role of FGFR2 in the regulation by using FGFR2 siRNA. Hep3B cells were transfected with increasing amount of FGFR2 siRNA to knock down FGFR2 expression. The 80 pmol/ml of FGFR2 siRNA transfection resulted in the reduction of 90% FGFR2 protein level and the reduction of 70% FGFR2 mRNA level (Fig. 3A and B), which led to the abolishment of CYP7A1 regulation by FGF7 (Fig. 3B). The data indicate that the inhibition of CYP7A1 expression by FGF7 is mediated through FGFR2.

It has been well established that FGF/FGFR activates JNK signaling cascade [21–23]. Furthermore, several studies have indicated that JNK pathway is involved in the suppression of CYP7A1 expression [21,24,25]. To further investigate the role of JNK pathway in the regulation, we treated hepatocytes with the specific JNK inhibitor, SP600125. Fig. 3C shows that SP600125 (25 μM) totally blocked the inhibition of CYP7A1 expression by FGF7 (40 ng/ml). To determine whether JNK activity was elevated by FGF7 treatment in Hep3B cells, we measured the activation of JNK kinase by kinase phosphorylation assay. Hep3B cells were treated with FGF7 (40 ng/ml) for 10, 30 min. After treatment, lysates were analyzed via western blotting using phospho-JNK antibody. As shown in Fig. 3D, FGF7 treatment induced rapid JNK phosphorylation. To further confirm FGF7 activation of JNK pathway, Hep3B cells were pretreated with SP600125 (25 μM) for 1hour and then exposed to FGF7 (40 ng/ml) for additional 30 min. As shown in Fig. 3E, SP100 625 blocked JNK phosphorylation by FGF7 treatment. These data reveal that suppression of CYP7A1 expression by FGF7 is mediated by JNK signaling cascade.

3.4. Blocking FGF7 abrogates HSC-reduced inhibition of CYP7A1 expression in Hep3B cells

During the process of liver fibrosis, activated HSC secretes FGF7 [13]. In addition, several fibroblasts and stellate cell lines have been reported to secrete FGF7 [20,26,27]. LX2 is a widely utilized stellate cell line for the HSC research. To confirm the secretion of FGF7 from LX2 cells, we collected the conditioned media from LX2 cells (HSC-CM) as described in the Material and Methods. FGF7 expression in HSC-CM was measured by western blotting using FGF7 antibody with normal DMEM containing 2% FBS as the negative control and human recombinant FGF7 protein as the positive control. As shown in Fig. 4A, FG7 was highly expressed in the HSC-CM. To investigate the effect of FGF7 in HSC-CM on the regulation of CYP7A1 expression, we treated Hep3B cells with HSC-CM in the presence of the neutralizing antibody against FGF7 or control IgG. Fig. 4B shows that the FGF7 neutralizing antibody significantly increased the expression of CYP7A1. The data suggest

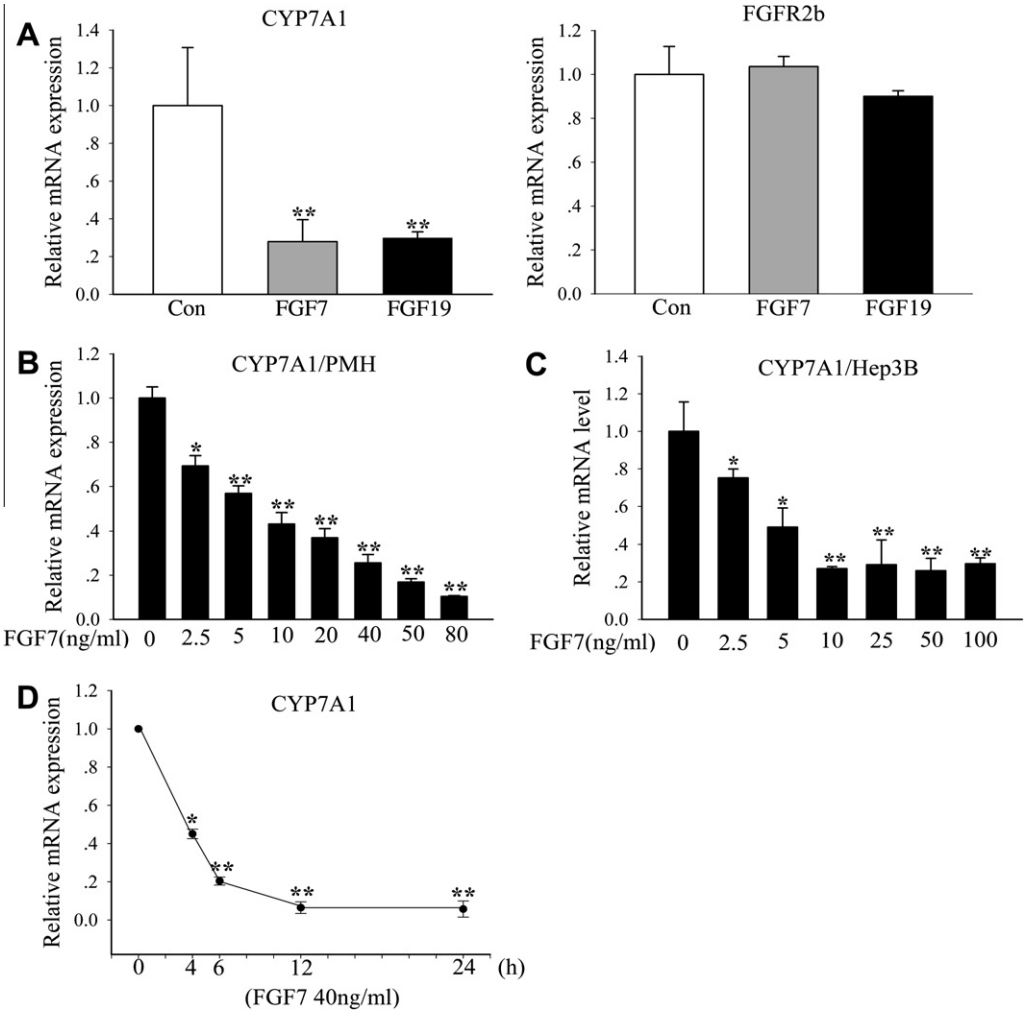

Fig. 2. FGF7 inhibits expression of CYP7A1 in hepatocytes. (A) Primary mouse hepatocytes (PMH) were treated with FGF7 (40 ng/ml) or FGF19 (50 ng/ml) for 6 h. PMH (B) and Hep3B cells (C) starved overnight were treated with increasing amount of FGF7 for 6 h. (D) PMH were treated with FGF7 (40 ng/ml) for a period of time. After treatment, total RNA was isolated for Real-time PCR analysis of mRNA level of CYP7A1. Data are expressed as the mean ± SD of at least three independent experiments. $^{*}P < 0.05$. $^{**}P < 0.01$.

that neutralizing FGF7 secreted by HSC abrogates its inhibition on CYP7A1 expression in hepatocytes.

4. Discussion

The fibroblast growth factor 7 (FGF7), produced by mesenchymal cells, acts as a key paracrine mediator for proliferation, migration, differentiation of epithelial cells. In this study, we demonstrated that FGF7 inhibited CYP7A1 expression in hepatocytes. FGF7 rapidly and strongly repressed CYP7A1 gene transcription in time- and dose-dependent manners in primary mouse hepatocytes and Hep3B cells. Further studies showed both siRNA knockdown of FGFR2 expression and SP600125 (a JNK inhibitor, blocking the JNK cascade) abolished the regulation of CYP7A1 by FGF7 in Hep3B cells. Moreover, treatment of Hep3B cells with the neutralizing antibody against FGF7 abrogated the paracrine inhibition of CYP7A1 by conditioned media from LX2 cells.

Carbon tetrachloride (CCl_4) induced murine liver fibrosis is a widely used animal model for liver fibrosis research. In this model, the liver showed normal bile acids excretion but lower ability of bile acid synthesis [28]. Consistent with the results, we found that the expression of CYP7A1 was largely decreased in the liver samples of CCl_4 induced murine model of chronic hepatic fibrosis (Fig. 1B). Bile acids develop a number of functions relying on their amphipathic structures. Because of their potential toxicity, accumulation of bile acids at a high level may cause harmful effects to the liver [29]. FGF7 secreted from HSC may play a significant role in CYP7A1 expression and keeping low rate of the bile acid synthesis to avoid the high toxic bile acid level in the fibrotic liver.

A recent study showed FGF7 was detected in the HSC after partial hepatectomy (PH). Overexpression of exogenous FGF7 notably promoted hepatic proliferation after PH [30]. In contrast, CYP7A1 is repressed after the 70% PH of normal liver [31]. The liver-specific overexpression of CYP7A1 impaired liver regeneration after 70% PH, which was accompanied by increased hepatocyte apoptosis and liver injury. Interestingly, there was a distinct JNK pathway in mediating CYP7A1 suppression and acting as a predominant role in the initial stage after 70% PH [32]. Our findings suggest FGF7 may be responsible for or involved in the JNK-dependent repression of CYP7A1 after PH.

It has been reported that JNK had two distinct pathways to regulate CYP7A1 expression: (1) JNK phosphorylated HNF-4α, released HNF-4α from the BARE site of CYP7A1 promoter and resulted in the decreased transcription of CYP7A1 [33]; (2) Activated c-Jun targeted SHP and promoted its expression to inhibit CYP7A1

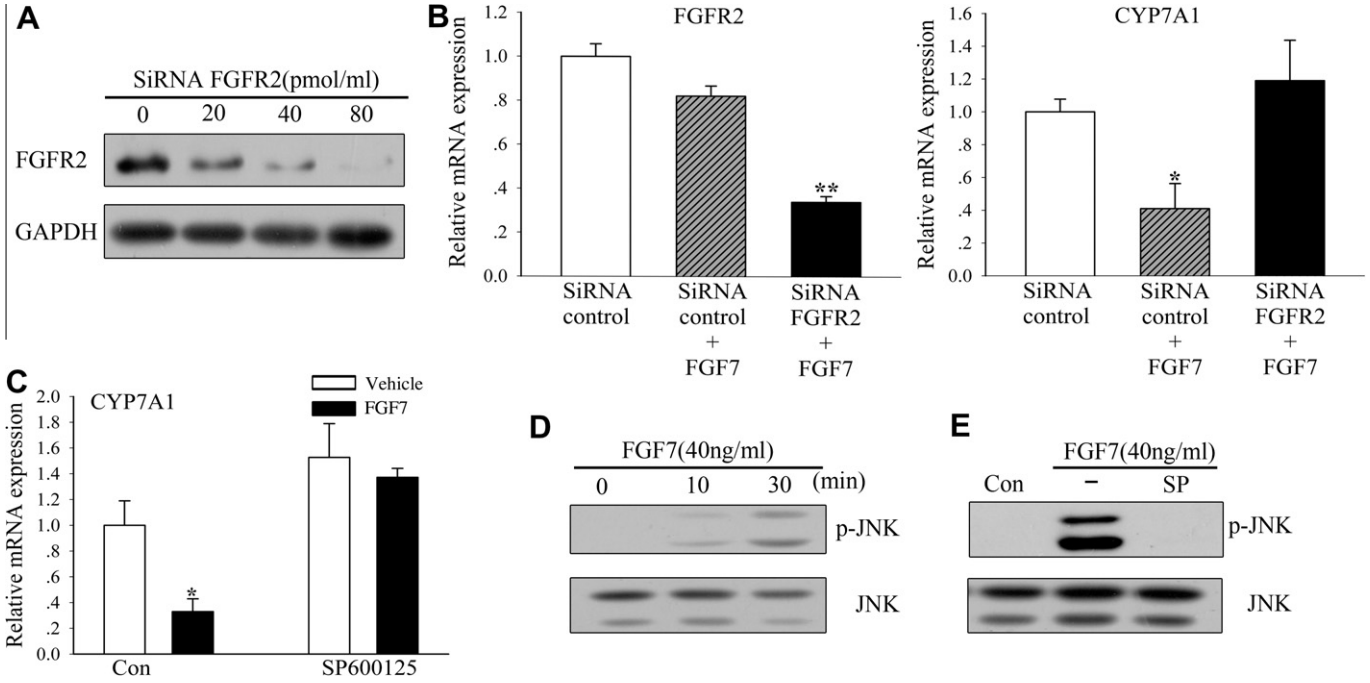

Fig. 3. Suppression of CYP7A1 by FGF7 is mediated by FGFR2 and its downstream JNK signaling cascade. (A) The control siRNA and increasing amount of siRNA-FGFR2 were transfected into Hep3B cells in six-well plate for 36 h. The knockdown effect was measured by western blotting using FGFR2 and GAPDH antibodies. (B) Hep3B cells were transfected with the control siRNA and siRNA-FGFR2 (80 pmol/ml) for 24 h, starved overnight and then treated with 40 ng/ml FGF7 for additional 6 h. Total RNA was isolated for Real-time PCR analysis of mRNA level of FGFR2b (left) and CYP7A1 (right). (C) Hep3B cells were pretreated with SP600125 (SP, 25 μM) for 1 h, starved overnight and then treated with FGF7 (40 ng/ml) for additional 6 h. Total RNA was isolated for Real-time PCR analysis of mRNA level of CYP7A1. (D) Hep3B cells were starved overnight and then treated with FGF7 (40 ng/ml) for the indicated time period or (E) starved overnight, pretreated with SP600125 (SP, 25 μM) for 1 h and then treated with FGF7 (40 ng/ml) for 30 min. Cell lysates were isolated for western blotting using antibodies against JNK or phospho-JNK. Data are expressed as the mean ± SD of at least three individual experiments. $^{*}P < 0.05$. $^{**}P < 0.01$.

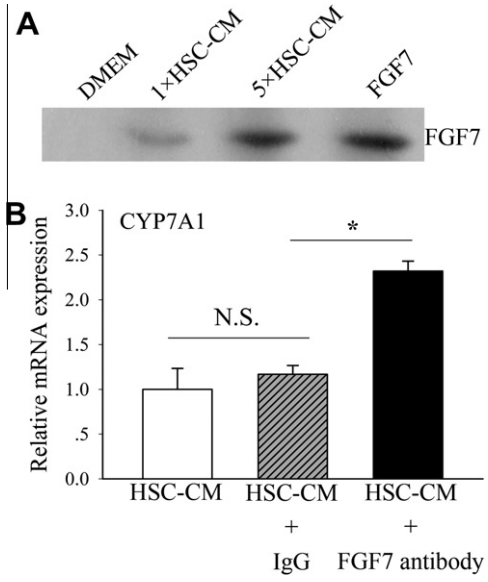

Fig. 4. Blocking FGF7 abrogates HSC-reduced inhibition of CYP7A1 expression in Hep3B cells. (A) The control DMEM containing 2% FBS, 5 μl HSC-CM (1×), 25 μl HSC-CM (5×) and 75 pg FGF7 protein were loaded for western blot using antibody against FGF7. Data represent one of three separate experiments. (B) Hep3B cells were starved overnight and then treated with HSC-CM in the presence of the neutralizing antibody against FGF7 (1 μg/ml) or control IgG for 12 h. Total RNA was isolated for Real-time PCR analysis of mRNA level of CYP7A1. Data are expressed as the mean ± SD of at least three individual experiments. $^{*}P < 0.05$.

transcription [24]. Further studies are needed to determine which pathway is responsible for CYP7A1 regulation.

In conclusion, this study reveals that FGF7 is a novel regulator of CYP7A1 expression in hepatocytes. The inhibition of CYP7A1 by FGF7 may play a significant role in maintaining bile acid homeostasis, protect hepatocytes from excessive bile acid toxicity and promote the liver regeneration during liver injury and fibrosis.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.06.035.

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