FGF21 ameliorates hepatic fibrosis by multiple mechanisms

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Abstract

Background Previous study reports that fibroblast growth factor 21 (FGF21) could ameliorate hepatic fibrosis, but its mechanisms have not been fully investigated.

Methods and results In this study, three models were used to investigate the mechanism by which FGF21 alleviates liver fibrosis. Hepatic fibrosis animal models were respectively induced by CCL4 and dimethylnitrosamine. Our results demonstrated that liver index and liver function were deteriorated in both models. Hematoxylin and eosin and Masson’s staining showed that the damaged tissue architectonics were observed in the mice of both models. Treatment with FGF21 significantly ameliorated these changes. ELISA analysis showed that the serum levels of IL-1β, IL-6 and TNF-α were significantly elevated in both models. However, administration of FGF21 significantly reduced these inflammatory cytokines. Real-time PCR and Western blot analysis showed that treatment with FGF21 significantly decreased mRNA and protein expressions of collagenI, α-SMA and TGF-β. Platelet-derived growth factor-BB (PDGF-BB) stimulant was used to establish the experimental cell model in hepatic stellate cells (HSCs). Real-time PCR and Western blot analysis demonstrated that the expression of collagenI and α-SMA were significantly upregulated by this stimulant in model group. Interestingly, our results showed that mRNA and protein expressions of leptin were also significantly induced in PDGF-BB treated HSCs. Administration of FGF21 significantly reduced leptin expression in a dose dependent manner and these effects were reversed in siRNA (against β-klotho) transfected HSCs. Furthermore, the leptin signaling pathways related protein p-ERK/t-ERK, p-STAT3/STAT3 and TGF-β were significantly downregulated by FGF21 treatment in a dose dependent manner. The expressions of SOCS3 and Nrf-2 were enhanced by treatment with FGF21. The underlying mechanism may be that FGF21 regulates leptin-STAT3 axis via Nrf-2 and SOCS3 pathway in activated HSCs.

Conclusions FGF21 ameliorates hepatic fibrosis by multiple mechanisms.

Keywords Liver fibrosis · FGF21 · TGF-β · SOCS3/leptin
Introduction

Fibroblast growth factor 21 (FGF21) is a novel metabolic regulator with diverse biological functions via FGF receptors and co-receptor β-klotho [1]. FGF21, as a hepatokine, adipokine and myokine, plays an important role in many diseases [2]. In the aspect of regulating blood glucose, insulin sensitivity and the expression of glucose transporter1 are improved by treatment with FGF21 in diabetic mice [3, 4]. Emerging evidence indicates that FGF21 can suppress hepatic sterol regulatory element-binding protein-2 to regulate lipid metabolism and related diseases [5]. Besides, FGF21 prevents Angiotensin II-induced hypertension and vascular dysfunction by activation of ACE2/angiotensin (1–7) axis in mice [6, 7]. Efforts have been made to elucidate the mechanism that FGF21 regulates hepatic metabolic pathways to improve steatosis and inflammation [8–10]. Though some studies demonstrate that FGF21 can attenuate hepatic fibrogenesis [11, 12], the therapeutic effects of FGF21 on liver fibrosis in different models and underlying mechanisms are not been fully investigated.

Hepatic fibrosis is a wound healing response to varying aetiologies, and as such affects the entire world population [13]. The major causes of hepatic fibrosis include non-alcoholic steatohepatitis (NASH), non-alcoholic fatty liver disease (NAFLD) and liver cirrhosis [13, 14]. The accumulation of extracellular matrix (ECM) proteins lead to fibrous scar formation and changes of liver architecture [15]. In the process of hepatic fibrogenesis, activation of hepatic stellate cells (HSCs), which mainly produce ECM, exhibit fibrogenic potential [16]. Among the activating agents, transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF) play critical roles in the regulation of activation of HSCs and the progression of fibrosis [17]. Besides, Leptin, a 16-kDa protein hormone, plays a key role in the development of liver fibrosis, which also regulates HSCs activation and ECM synthesis [18, 19]. Damaged hepatocytes release inflammatory, fibrogenic cytokines and reactive oxygen species (ROS), which could also induce the activation of HSCs [16, 20]. Liver fibrosis is a reversible process before progressing into liver cirrhosis or hepatic carcinoma [9, 14]. Therefore, developing therapeutic strategies that effectively reverse liver fibrosis are necessary for preventing the development of liver cirrhosis.

Researchers demonstrated that a lot of chemicals are known to induce liver fibrosis and hence are commonly used to establish experimental animal models to study this particular pattern of lesions [21, 22]. In order to further study the therapeutic effects of FGF21 on liver fibrosis, we simulated two animal hepatic fibrosis models and one cell model, namely Carbon tetrachloride (CCL₄)-induced model, dimethylnitrosamine (DMN)-induced model and PDGF-treated HSCs model. CCL₄ is the most widely used hepatotoxin in the study of liver fibrosis and cirrhosis in rodents [21, 22]. Previous studies have showed that lipid profile, liver enzymes and oxidative stress markers remarkably increase and high-density lipoprotein dramatically decrease in CCL₄-induced model [23]. CCL₄ is metabolized by cytochrome P450 (CYP450) enzymes to a trichloromethyl radical that can be further oxygenated to the trichloromethylperoxy radical. The radicals are highly reactive and induce complex cellular alterations that result in hepatotoxic damage, inflammation fibrosis and hepatocellular carcinoma [22]. The pathogenic mechanisms simulate human chronic disease associated with toxic damage in many aspects [21]. DMN, a carcinogenic and genotoxic compounds, is usually used to experimentally induce hepatic fibrosis in mice [21, 24]. DMN causes activation of lymphocytes and injury to sinusoidal endothelial cells, which produce potent fibrogenic factors like TGF-β1, CTGF and FGF1. Pro-inflammatory cytokines, including IL-1β, IL-6, IFN-γ and TNF-α, trigger hepatocytes to activate downstream signaling pathways such as nuclear factor-κB, which in turn induce activation of resting hepatic stellate cells [25, 26]. A number of papers have demonstrated that clusters of metabolic activation, immune response, oxidative stress metabolic disorders, ion homeostasis, HSCs activation and extracellular matrix deposition are significantly induced in DMN model [21, 25]. This model is mainly associated with potent fibrogenic factors and abundant production of inflammation [26]. Among the mechanisms of liver fibrogenesis, growth factor and hormone signaling play central roles in the activation of HSCs, mainly through PDGF-BB [27]. Therefore, the cell model of activated HSCs was induced by PDGF-BB. In this study, these three liver fibrosis models were successfully simulated. The therapeutic effects of FGF21 on liver fibrosis and underlying mechanisms were investigated in different models.

Materials and methods

Animal model and treatment

Male ICR mice (SPF) weighting 30–35 g and male C57BL/6 mice (SPF) weighting 18–22 g were purchased from Changchun YiSi Company. All mice were housed in the experimental animal center of Northeast Agriculture University at 22 ± 2 °C with 12:12 h light–dark cycles. Mice were randomly divided into 8 groups. ICR mice were used to establish DMN model and C57BL/6 mice were used to establish CCL₄ model [11, 22]. ICR
Mice were randomly divided into four groups (Control, DMN, DMN + FL, DMN + FH). C57BL/6 mice were randomly divided into 4 groups (Control, CCL4, CCL4 + FL, CCL4 + FH). Each group contains 15 mice. DMN-liver fibrosis model was induced by intraperitoneal injections (IP) of DMN (Sigma, USA) at a dose of 10 mg/kg body weight. The injections were given on the first three consecutive days of each week over a period of 4 weeks. From the 5th week onwards, FGF21 was subcutaneously treated to the mice once daily for 4 weeks. The low and high dose of FGF21 in DMN + FL and DMN + FH group were 0.75 mg/kg and 1.5 mg/kg. The mice in normal and model groups were intraperitoneally injected with same amount of saline for 4 weeks. Animals were sacrificed and sampled at the end of the experiment.

CCL4-liver fibrosis model was induced by gavage every other day with CCL4 solution (CCL4 was mixed with olive oil 1:1) 2 mL/kg for 7 weeks. The mice in normal control were given by gavage every other day with olive oil (2 mL/kg) for 7 weeks. From the 4th week onwards, the mice in FGF21 groups were intraperitoneally injected with FGF21 once daily (0.75 mg/kg, 1.5 mg/kg) for 4 weeks. The mice in normal and model groups were intraperitoneally injected with same amount of saline for 4 weeks. After 24 h of the last injection, animals were sacrificed and sampled.

Determination of the liver function and liver index

Serum of mice were collected at the end of experiment. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and Bilirubin (TBIL) activity were measured in Harbin Electricity Hospital. The body weight and liver weight in each group were measured by electronic scale.

Histological analysis

The liver tissues were fixed in 4% paraformaldehyde at room temperature for 48 h. After dehydration, the blocks were embedded in paraffin and sectioned. Sections were stained with hematoxylin and eosin (HE) and Masson’s trichrome staining. Photographs were taken in a blinded fashion at random fields.

Inflammatory cytokine levels analysis

The levels of IL-1β, IL-6 and TNF-α in serum were measured by ELISA detection kits (R&D, USA) according to the operation manual. The mRNA levels in the liver were detected by Real-time PCR.

Cell culture and siRNA-β-klotho transfection

Rat hepatic stellate cell line was purchased from American Type Culture Collection. The cells were grown in DMEM + 10% foetal bovine serum, 1% penicillin/streptomycin at 100% humidity atmosphere of 5% CO2 at 37 °C. PDGF-BB (Abcam, USA) was used to activate HSCs following previous study [11]. The cells were divided into four groups in the logarithmic phase of growth: the PBS group, the PDGF-BB group (20 ng/ml, the PDGF-BB + FGF21 (0.1 μM) and the PDGF-BB + FGF21 (1.0 μM), namely C, P, P + FL and P + FH. The β-klotho mRNA was specifically knocked down by using commercially available siRNA oligonucleotides. The sequences of the siRNA were designed by Sangon Biotech (Shanghai, China). The negative siRNA was used as control.

Cell proliferation, apoptosis and ROS analysis

HSCs were seeded at 1.5–5 × 104 cells/well and incubated for 12 h. Then the medium was replaced in FBS-free DMEM for cell synchronization. After 12 h incubation, the cells were treated with PDGF-BB and FGF21 for 12 h. Next, the Cell Counting Kit-8 (CCK-8) solution (Beyotime, China) was added into each well plates for 2 h. The absorbance was measured at 450 nm. The cells were managed by the foregoing method, then 10 μM 2,7-dichlorodihydro fluorescein diacetate (DCF-DA) (Beyotime, China) was added when cells were washed three times with PBS. After incubation, the HSCs were measured using a Flex Station 3 (Molecular Devices, USA). The cell apoptosis stained with hoechst33528 were photographed by fluorescence microscope (OLYMPUS, Japan) and analyzed by flow cytometry (BD, USA).

RNA isolation and Real-time PCR

Total RNA were isolated from the liver and HSCs with TRIzol (Invitrogen, USA), and quantitative gene expression was performed on a bio-rad CFX manager (ABI7500, Applied biosystems, USA) using SYBR green technology (TaKaRa, Japan). The primer sequences were show in Supplemental Table1.

Western blot

Lysates of liver tissues and HSCs were prepared using strong radioimmuno-precipitation assay buffer (RIPA) combination with a protease inhibitor PMSF (Sigma, USA) and phosphatase inhibitors (Beyotime, China). Protein concentrations were measured by the BCA quantitative kit. Then protein was separated by SDS-PAGE, electro-transferred to nitrocellulose filter membrane,
blocked with Quickblock™ Western block kit (Genscript, USA), and probed with the following antibodies overnight at 4 °C. (diluted with primary antibody diluent, Beyotime, China). Rabbit anti-collagenI and anti-leptin antibody (1:500, Abcam). Rabbit anti-α-SMA, anti-BCL-2 and anti-Nrf-2-antibody (1:1000, Abcam). Mouse anti-TGF-β-antibody (1:500, Abcam). Rabbit anti-β-actin, anti-Bax, anti-SOCS3, anti-caspase 3, anti-Lamin B, anti-ERK, anti-p-ERK (Thr202/Tyr204) and anti-p-STAT3 (Tyr705)-antibody (1:1000, Cell Signaling). Mouse anti-STAT3-antibody (1:1000, Cell Signaling). The membrane was subsequently incubated with HRP-conjugated Rabbit or Mouse secondary antibody (1:7500, Abcam) for 1 h at room temperature. Specific signals were detected using the enhanced ECL kit (Thermo Scientific, USA). The chemiDoc™ XRS + with Image Lab™ Software (BIO-RAD, USA) was used for development.

**Statistical analysis**

All data were expressed as mean±SD, followed by a two-way tails student’s t-test. Also, multiple comparisons were analyzed by one-way ANOVA on GraphPad Prism 6 software. P value <0.05 was regarded as statistically significant.

**Results**

**Administration of FGF21 ameliorates CCL₄-induced hepatic fibrosis in mice**

To evaluate the therapeutic effects of FGF21 against hepatic fibrosis, CCL₄ was used to induce hepatic fibrosis model in this study. The liver index in model group was significantly deteriorated compared with control. Treatment with FGF21 significantly ameliorated liver index compared with model group (Supplemental Table 2). Besides, CCL₄-treated mice developed elevated plasma

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**Fig. 1** FGF21 ameliorates CCL₄-induced hepatic fibrosis in mice. The livers were fixed in 4% paraformaldehyde, embedded in paraffin, sections were used for HE and Masson’s staining. The total protein were extracted from the liver tissues. Real-time PCR and western blotting were used to measure liver fibrosis related protein expression. CCL₄ means model group, FL means the low dose treatment of FGF21, FH means the high dose treatment of FGF21. A, B Representative Masson staining (×200) and HE staining (×200) of liver sections from CCL₄ induced model mice. Results of staining showed that the damaged tissue architectonics changes and extensive deposition of collagen were significantly ameliorated by treatment with FL or FH. C The mRNA expression of α-SMA and collagenI in the liver of each group. D The protein expression of α-SMA and collagenI in each group. E The relative mRNA expression of IL-1β, IL-6 and TNF-α in the liver of each group. F ELISA analysis of IL-1β, IL-6 and TNF-α in the serum of each group. G The mRNA and protein expression levels of TGF-β in the liver of CCL₄-induced model mice. The relative protein expression levels were expressed as the ratio of α-SMA/β-actin, collagenI/β-actin, TGF-β/β-actin. The bands were analyzed with Image J. These data represent mean±SD (n=6), *p<0.05, **p<0.01, ***p<0.001 compared to control, *p<0.05, **p<0.01, ***p<0.001 compared to model mice
concentrations of ALT AST, ALP and TBIL in the model mice compared with control. Treatment with FGF21 significantly ameliorated these parameters in a dose dependent manner (Supplemental Table 3). Results of Masson’s staining demonstrated that pathological changes were observed (Fig. 1A) and HE staining showed that normal tissue architectonics were damaged in the model mice compared to control (Fig. 1B). But, these changes were significantly ameliorated by treatment with FGF21 compared to model mice. These results indicate that administration of FGF21 ameliorates CCL4-induced liver lesion in CCL4-model mice.

The expressions of α-SMA and collagenI are regarded as markers of liver fibrosis. Real-time PCR and Western blot were used to detect the expression levels of mRNA and protein. Our results showed that the mRNA expression levels of α-SMA and collagenI were significantly upregulated in model group compared to control (Fig. 1C). Treatment with low and high dose of FGF21 significantly downregulated the mRNA levels of α-SMA and collagenI compared to model group. The protein expression levels of α-SMA and collagenI in model group were significantly increased compared to control and FGF21 significantly decreased these effects (Fig. 1D). These data suggest that administration of FGF21 significantly ameliorates CCL4-induced hepatic fibrosis of ECM production in mice.

To investigate the effect of FGF21 on inflammation cytokines in CCL4-induced model, the expression levels of IL-1β, IL-6 and TNF-α were measured by the methods of Real-time PCR and ELISA in the liver and the serum. Our results demonstrated that the mRNA levels of IL-1β, IL-6 and TNF-α were significantly elevated in the liver of model mice compared to control. Administration of FGF21 significantly reduced the mRNA expression levels of IL-1β, IL-6 and TNF-α compared to model group (Fig. 1E). Besides, the protein expression levels of IL-1β, IL-6 and TNF-α were elevated in the serum of model mice compared to control. Treatment with FGF21 significantly decreased these parameters compared to control group (Fig. 1F).

Although the mechanisms are multiple in different models, TGF-β plays a key liver fibrosis mediator [11, 14, 28]. To elucidate the potential mechanism by which FGF21 ameliorates CCL4-induced hepatic fibrosis, the expression levels of TGF-β were detected by Real-time PCR and Western blot in this study. Results of Real-time PCR and Western blot showed that the mRNA and protein expression levels of TGF-β were significantly increased in the liver of CCL4-induced model mice compared to control. FGF21 significantly downregulated TGF-β mRNA and protein expression levels compared to model group (Fig. 1G). All data indicate that FGF21 ameliorates CCL4-induced liver fibrosis and underlying mechanism is related to TGF-β signaling pathway.

**Administration of FGF21 ameliorates DMN-induced hepatic fibrosis in mice**

To evaluate the therapeutic effects of FGF21 on hepatic fibrosis in another model, DMN was also used to induce hepatic fibrosis model in this study. The liver index was significantly deteriorated and plasma concentrations of ALT AST, ALP and TBIL in model group were significantly elevated compared to control. FGF21 significantly ameliorated these parameters in a dose dependent manner compared to model group (Supplemental Tables 2, 3). Results of Masson’s staining demonstrated that pathological changes were observed (Fig. 2A) and HE staining showed that the normal tissue architectonics were damaged in the model group compared to control mice (Fig. 2B). However, these changes were significantly ameliorated by treatment with FGF21 compared to model mice. Real-time PCR and Western blot analysis showed that the mRNA and protein expression levels of α-SMA and collagenI in model group were significantly upregulated compared to control group (Fig. 2C, D). FGF21 significantly downregulated the expression levels of α-SMA and collagenI compared to model group. Besides, our results demonstrated that the mRNA and protein expression levels of IL-1β, IL-6 and TNF-α were significantly elevated in the liver and serum of model group compared with control. FGF21 significantly reduced the expression levels of IL-1β, IL-6 and TNF-α compared to model group (Fig. 2E, F).

To elucidate the potential mechanism of FGF21 in ameliorating DMN-induced hepatic fibrosis, the expression of TGF-β was detected by Real-time PCR and Western blot in this study. Our data demonstrated that the mRNA and protein expression levels of TGF-β in the liver of model group were significantly upregulated compared to control. These effects were attenuated by treatment with FGF21 compared to model group (Fig. 2G). Our results suggest that the therapeutic effects of FGF21 on DMN-induced liver fibrosis are realized and underlying mechanism is involved in TGF-β signaling pathway.

**FGF21 attenuates activation of HSCs and the expression of leptin in PDGF-BB-induced cell model**

Among the mechanisms of liver fibrosis, growth factor plays a significant role in the activation of HSCs [29]. To stimulate the fibrogenesis, HSCs were activated by PDGF-BB in this study for cell model. Our results showed that the expression levels of collagenI and α-SMA in model group were significantly increased compared with control. Treatment with FGF21 significantly decreased these effects compared to model group (Fig. 3A). Interestingly, we found that the mRNA and protein expression levels of leptin in model
group were significantly increased compared to control. Whereas, FGF21 significantly decreased the mRNA and protein expression levels of leptin compared to model group.

To further prove this result, we used small interfering RNA direct against β-klotho to silence the effect of FGF21. As anticipated, the expression of β-klotho was downregulated and the expression of leptin was recovered. HSCs were activated in transfected cells treated with PDGF-BB and FGF21 (Fig. 3B). Our data indicate that FGF21 attenuates PDGF-BB-induced activation of HSCs and one of the underlying mechanisms may be that FGF21 reduces the expression of leptin.

**FGF21 blocks the PDGF-BB-leptin axis in activated HSCs**

Emerging evidence demonstrates that leptin is a well-known profibrogenic cytokine [18]. PDGF-BB plays a key role in HSC proliferation and leptin expression [30]. Previous studies have showed that ERK, STAT3 and TGF-β exhibit central roles in PDGF-leptin-hepatic fibrosis axis [31–33]. To elucidate the relationship between FGF21 and PDGF-BB/Leptin downstream signaling pathway in HSCs, the expressions of ERK, STAT3 and TGF-β were detected by Real-time PCR and Western blot methods. Our results demonstrated that the mRNA and protein expressions of TGF-β were significantly attenuated by treatment with FGF21 in activated HSCs (Fig. 3C, D). The ratio of p-ERK1/2 to t-ERK1/2 and p-Stat3 to t-Stat3 in model group were significantly increased compared to control. These parameters were significantly decreased by administration of FGF21 in a dose dependent manner compared with model group (Fig. 3D). These results suggest that ERK, Stat3 and TGF-β are involved in the PDGF-leptin axis in the development and progression of HSCs activation. Administration of FGF21 could block this axis.

A line of evidences demonstrate that SOCS3 is an important inhibitor of the leptin signaling pathway [34]. Ga Young et al. reported anti-inflammatory mechanisms of suppressors of cytokine signaling target ROS via NRF-2/thioredoxin induction and inflammasome activation in macrophages [35]. Our previous studies have demonstrated that FGF21
attenuates pulmonary fibrogenesis via Nrf-2 signaling pathway [36]. Therefore, we measured the expression levels of SOCS3 and Nrf-2. Our results showed that the expression levels of Nrf-2 in model group were significantly increased compared to control. FGF21 further enhanced mRNA and protein expression levels of Nrf-2 compared with model group (Fig. 3E, F). The mRNA and protein levels of SOCS3 in model group were downregulated compared to control group. These parameters were significantly upregulated by treatment with FGF21 in a dose dependent manner compared to model group (Fig. 3E, F). These data suggest that Nrf-2 and SOCS3 are involved in the mechanism by which FGF21 ameliorates PDGF-BB-induced HSCs fibrogenesis.

FGF21 attenuates cell proliferation, renders HSCs sensitive to apoptosis and reduces the level of ROS

The expression of leptin is increased in PDGF-BB-treated HSCs, which exhibits the ability of mitogenesis and inhibition of apoptosis [31]. In this study, Hotecest33528 staining and flow cytometry analysis demonstrated that HSCs apoptosis in model group was reduced compared to control, but there was a significantly increased apoptosis rate of HSCs by treated with FGF21 compared to model group (Fig. 4A–C). Besides, the mRNA and protein expressions of Bcl-2 were significantly downregulated and the mRNA and protein expressions of Bax and caspase 3 were significantly

Fig. 3 FGF21 attenuates activation of HSCs and the expression level of leptin in PDGF-BB-induced cell model. C represents control, P means PDGF-BB-treated group, FL means low dose treatment of FGF21, FH means high dose treatment of FGF21. A The mRNA and protein expression levels of collagenI, α-SMA and leptin in PDGF-BB-treated cells. B The mRNA and protein expression levels of collagenI, α-SMA and leptin in PDGF-BB and siRNA-treated cells. C The mRNA expression level of TGF-β in each group. D The protein expression levels of p-ERK1/2, t-ERK1/2, p-STAT3, t-STAT3 and TGF-β in each group. E The mRNA expression levels of Nrf-2 and SOCS3 expression in each group. F The protein expression levels of SOCS3 and Nrf-2 in each group. The bands were analyzed with Image J. The relative protein expression levels were expressed as the ratio of Klotho/β-actin, α-SMA/β-actin, collagenIβ-actin and Leptin/β-actin. All data represent mean ± SD, *p < 0.05, **p < 0.01, ***p < 0.001 compared to model mice. *p < 0.05, **p < 0.01, ***p < 0.001 compared to control, ns mean no significant difference.
upregulated by administration of FGF21 compared with model group (Fig. 4F, G). The CCK-8 analysis showed that HSCs proliferation in model group was increased compared to control. It was attenuated by administration of FGF21 in a dose dependent manner compared to model group (Fig. 4D). Intracellular AKT signaling pathway regulates cell growth and apoptosis. Our results showed that the ratio of p-AKT to t-AKT was significantly decreased by administration of FGF21 compared with model group (Fig. 4G). Meanwhile, the analysis of ROS showed that the production of ROS in model group was increased compared to control and FGF21 significantly reduced the production of ROS compared to model group (Fig. 4E). These results indicate that FGF21 could attenuate cell proliferation, render HSCs sensitive to apoptosis and reduce the level of ROS. Our results suggest that one of the underlying mechanisms is associated with leptin-AKT pathway.

**Discussion**

Hepatic fibrosis leads to severe hepatic dysfunctions and even life-threatening [13, 14]. There is no specific therapeutic drugs. FGF21, as an endocrine hormone and important
regulator with pleiotropic effects, exerts beneficial effects in the liver diseases [1, 2]. Previous studies have demonstrated that treatment with FGF21 can attenuate pulmonary fibrogenesis and liver fibrosis [11, 36]. However, the therapeutic effects of FGF21 on liver fibrosis in different models and underlying mechanisms have not been fully investigated. In this study, we simulated three hepatic fibrosis models, which are similar to liver fibrosis in human. CCL4-induced model mimics human chronic disease and its mechanisms mainly involves retinol metabolism and PPARγ signaling pathway [21, 22]. There is a lot of discussions about administrated ways of CCL4 [21]. In principle, CCL4 can be administrated through three ways (IP injection, inhalation and gavage). Jang et al. report that liver fibrosis induced by gavage is the most effective to achieve sufficient fibrosis and greatest reproducibility with acceptable animal survival [37]. Therefore, the way of administration was adopted in this study. DMN-induced model, associated with hepatotoxic, genotoxic and immunotoxic, could cause liver damage. Its mechanism mainly involves fibrogenic factors, like CTGF and TGF-β, and pro-inflammatory cytokines [21, 25]. Our results showed that the liver index, the liver function and histopathology changes were significantly damaged to different degrees in the mice of both models and these parameters were significantly ameliorated by administration of FGF21 in a dose dependent manner. Interestingly, we found that the weight of mice in CCL4 groups were not gained. The reason may be that insufficient food intake affected by gavage can lead to starvation conditions. Our results also showed that FGF21 significantly reduced ECM in the liver of both models. The activation of TGF-β/Smad signaling pathway results in collagen production and fibrogenesis [13]. In this study, our results showed that FGF21 significantly downregulated the expression of TGF-β in the liver of both models. A body of evidences have demonstrated that inflammatory pathway exerts a key role in hepatic fibrogenesis [13, 20]. Our results demonstrated FGF21 could significantly decrease inflammatory cytokines expression levels. Yang et al. reported that targeted disruption of SMAD3 results in impaired mucosal immunity and diminished T cell responsiveness to TGF-β [38]. We speculated that administration of FGF21 downregulated the expression of TGF-β and then it regulated SMAD3 expression to reduce inflammatory cytokines. Through the main mechanisms of liver fibrosis induced by CCL4 and DMN are different, these results demonstrate that the therapeutic effects of FGF21 on liver fibrosis in both models are realized and underlying main mechanism is involved in TGF-β signaling pathway.

The activated HSCs have been shown to exhibit fibrogenic potential [14]. In this study, PDGF-BB was used to activate HSCs for imitating hepatic fibrosis cell model. Our results showed that the HSCs were activated by treated with PDGF-BB evaluated by the expression levels of collagenI and α-SMA. Administration of FGF21 significantly reduced the expression levels of collagenI and α-SMA. Interestingly, we also found that the expression level of leptin in model group was significantly increased compared with control, and FGF21 significantly decreased the expression of leptin compared with model group. These effects were reversed in si-RNA against β-klotho transfected HSCs. The results indicate that the HSCs activated by PDGF-BB promote the expression of leptin, and then leptin further promotes fibrogenesis by the way of autocrine. In mammals, leptin is primarily produced by adipocytes of white adipose tissue, thereby acting as a peripheral factor that signals nutritional status to the CNS [39]. Elevated serum leptin levels are detected in patients with liver cirrhosis. Normal liver tissue doesn’t express leptin, but its expression could be detected in the fibrotic liver correlated to the degree of fibrosis [40]. In this study, we preliminarily confirmed that leptin expression is increased in PDGF-BB-activated hepatic stellate cells and FGF21 could attenuate this effect in vitro. The effect of FGF21 on leptin in fibrotic livers of different degree will be further investigated in vivo. Previous studies have demonstrated that PDGF-BB induces activation of the downstream molecules ERK in activated HSCs and ERK signaling pathway plays a critical role in modulating major response of myofibroblast [41]. Meanwhile, the expression levels of phosphorylated Stat3 and TGF-β serve as central role in the leptin signaling pathway [33]. Therefore, we measured these protein expression levels in each group. Western blot analysis showed that the ratio of p-ERK to t-ERK and the ratio of p-Stat3 to t-Stat3 were significantly upregulated in PDGF-BB treated HSCs. Whereas, exposed HSCs to both FGF21 and PDGF-BB significantly decreased the ratios compared to exposed PDGF-BB alone. Besides, the expression level of TGF-β was also significantly downregulated by treatment with FGF21. These results suggest that ERK is involved in leptin expression in PDGF-BB activated HSCs and leptin further promotes HSCs fibrogenesis via Stat3/TGF-β pathway by autocrine. Jak2/Stat3 signals are regulated in negative feedback fashion by the supressors of cytokine signaling (SOCS3), which blocks Stat3 phosphorylation by either targeting the activated receptor complex for degradation or acting as a pseudosubstrate for Jak2 [34]. Accumulated data demonstrate that SOCS3 could serve as a major regulator of infection and inflammation and underlying mechanisms target ROS via Nrf-2/thioredoxin induction and inflammasome activation in macrophages [35, 42]. Zhang et al. reported that FGF21 could attenuate pulmonary fibrosis via activating Nrf-2 signaling pathway [36]. Therefore, We examined the expression levels of SOCS3 and Nrf-2 in each group. The mRNA and protein expression levels of SOCS3 were significantly decreased in model group. Treatment with FGF21 significantly upregulated the expression levels of SOCS3 and enhanced the expression of Nrf-2 in a dose dependent
manner. All data suggest that FGF21 provides a sustained production of SOCS-3 and Nrf-2 expression and subsequently inhibits leptin-induced STAT3 phosphorylation to prevent fibrogenesis and related inflammation. Meanwhile, FGF21 suppresses oxidative stress via enhancing Nrf-2 expression.

A large number of papers have demonstrated that the high expression level of leptin plays an important role in mitogenesis and inhibition of apoptosis in HSCs [18, 31]. In this study, our results demonstrated that FGF21 attenuated HSC proliferation and rendered HSCs sensitive to apoptosis in a dose dependent manner. Papers show that leptin mediates HSCs proliferation via AKT phosphorylation and inhibits HSCs apoptosis via caspase family [40, 43]. Results of our study showed that the ratio of p-Akt to t-Akt was significantly downregulated by administration of FGF21. The mRNA and protein expression levels of Bax and caspase 3 were significantly increased by treatment with FGF21. All data indicate that FGF21 attenuates HSCs proliferation and renders HSCs sensitive to apoptosis.

In conclusion, the therapeutic effects of FGF21 on liver fibrosis respectively induced by CCL4 and DMN are realized and underlying main mechanism is involved in TGF-β signaling pathway. Besides, FGF21 significantly attenuated PDGF-BB-induced HSCs proliferation, promoted apoptosis, reduced the level of ROS and then prevented ECM production in vitro. The FGF21-leptin-Stat3 axis plays an important anti-fibrogenic role in PDGF-BB activated HSCs. Therefore, our data suggest that FGF21 ameliorates hepatic fibrosis by multiple pathways.

Supplementary Information The online version contains supplementary material available at [https://doi.org/10.1007/s11033-021-06707-0].

Author contributions FRM performed the research, and analyzed the data. KMH, KK, QH, YKC and XHJ participated in data collection and analysis. WX and DSL contributed to the initial and consequent project discussion, manuscript discussion and revision. All the authors approved the final version of the manuscript.

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Data availability The data analyzed during the current study may be available upon reasonable request.

Declarations

Conflict of interest The authors declare that there are no conflict of interest.

Ethical approval This study was approved by the ethics committee of Northeast Agriculture University. All experimental protocols followed the guidelines issued by National Institute of Health and the Institutional Animal Care and Use Committee of Northeast Agriculture University. The mice were euthanized under anesthesia induced by intraperitoneal injection of 1.2% avertin (Sigma, USA) at a dose of 20 μL/g body weight.

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