FGF21 Ameliorates Hepatic Fibrosis by Multiple Mechanisms

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Abstract

Previous study reports that FGF21 could ameliorate hepatic fibrosis, but its mechanisms have not been fully investigated. In this study, three models were used to investigate the mechanism by which FGF21 alleviates liver fibrosis. CCL4 and DMN were respectively used to induce hepatic fibrosis animal models. Our results demonstrated that liver index and liver function were deteriorated in both models. HE 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. RT-PCR and Western blot analysis showed that mRNA and protein expression of collagenI, α-SMA and TGF-β were significantly decreased by treatment with FGF21. PDGF-BB stimulant was used to establish the experimental cell model in HSCs. RT-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 expression of leptin were also significantly induced in PDGF-BB treated HSCs. Administration of FGF21 could significantly reduce 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 expression 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.

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 could suppress hepatic sterol regulatory element-binding protein-2 to regulate lipid metabolism and related diseases [5]. Besides, FGF21 prevents Angiotensin □-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, 9, 10]. Through previous studies demonstrate that FGF21 could attenuate hepatic fibrogenesis [11, 12], the therapeutic effects of FGF21 on liver fibrosis in different models and underlying mechanisms still need to be investigated.

Hepatic fibrosis is a wound healing response to varying aetiologies and as such affects the entire world population [13]. The main 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, exhibits

fibrogenic potential [16]. Among the activating agents, transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF) play the critical role 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 regulated HSC 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.

Accumulating researches demonstrate 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 show that lipid profile, liver enzymes and oxidative stress markers remarkably increased and high-density lipoprotein dramatically decreased 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 induced 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, an 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 induced 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 extracelluar matrix deposition were significantly induced in DMN model [21, 25] This model was mainly associated with potent fibrogenic factors and abundantly production of inflammation [26]. Among the mechanisms of liver fibrogenesis, growth factor signaling and hormone play central role in the activation of HSCs, mainly through PDGF-BB [27] Therefore, PDGF-BB is used to activate HSCs for cell model. In this study, these three liver fibrosis models were successfully simulated. The therapeutic effects of FGF21 on liver fibrosis and the underlying mechanisms were investigated in different models.

Materials And Methods

Animal model and treatment

Male ICR mice (SPF) weighting 30-35g were purchased from Changchun YiSi Company. All mice were housed in the experimental animal center of Northeast Agriculture University at 22 ± 2 ℃ with 12: 12h

light-dark cycles. Mice were randomly divided into 8 groups. DMN model contains 4 groups (Control, DMN, DMN + FL, DMN + FH). CCL4 model contains 4 groups (Control, CCL4, CCL4 + FL, CCL4 + FH). Each group contains 15 mice. DMN-liver fibrosis model were induced by intraperitoneal injections 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. After 12h administration of DMN, 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. Animals were sacrificed at the end of the experiment.

CCL4-liver fibrosis model were induced by gavage every other day with CCL4 solution 2ml/kg for 7 weeks. The mice in normal control were given by gavage with olive oil (2mg/kg) for 7 weeks. From the 4th week on, the mice in FGF21 group were injected with FGF21 intraperitoneal once daily (0.75 mg/kg, 1.5 mg/kg). Animals were sacrificed at the end of the experiment.

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 embeded 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 RT-PCR. Results were from triplicate experiments.

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 humidity atmosphere of 5% CO_2 at 37°C. PDGF-BB (Abcam, USA) was used to activate HSCs following previous study. The cells were divided into four groups in the logarithmic phase of growth: the PBS group; The PDGF-BB group (20ng/ml; The PDGF-BB + FGF21 (0.1 μM); 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 (Shang hai, China). The negative siRNA was used as control.

Cell proliferation, apoptosis and ROS analysis

HSCs were seeded at 1.5-5×10^4 cells/well and incubated for 12h. Then the medium were replaced in FBS-free DMEM for cell synchronization. After 12h incubation, the cells were treated with PDGF-BB and FGF21 for 12h. Next, the Cell Counting Kit-8 (CCK-8) solution (Beyotime, China) were added into each well plates for 2h. 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 quantitative PCR

Total RNA were isolated from the liver and HSCs with TRIzol (Invirogen, USA), and quantitative gene expression was performed on a bio-rad CFX manager (ABI7500, Applied biosystems, USA) using SYBR green technology (TaKaRa, Janpan). The primer sequences were show in Table 1.

Table 1
Sequence of RT-PCR primers were used in this study

Species	Gene	Sequence
mice	β-actin	F5'-ACATCTGCTGGAAGGTGGAC-3'
		R5'-GGTACCACCATGTACCCAGG-3'
	IL-1β	F5'-CCATGGCACATTCTGTTCAAA-3'
		R5'-GCCCATCAGAGGCAAGGA-3'
	IL-6	F5'-TAGTCCTTCCTACCCCAATTTCC-3'
		R5'-TTGGTCCTTAGCCACTCCTTC-3'
	TNF-α	F5'-CCCTCACmACTCAGATCATCTTCT-3'
		R5'-GCTACGACGTGGGCTACAG-3'
	α-SMA	F5'-CGATAGAACACGGCATCAT-3'
		R5'-GCATAGCCCTCATAGATAGGCA-3'
	Collagen I	F5'-CAGGCTGGTGTGATGGGATT-3'
		R5'-CCAAGGTCTCCAGGAACACC-3'
	TGF-β	F5'-CCGCAACAACGCAATCTA-3'
		R5'-TGAGGAGCAGGAAGGGTC-3'
	Bcl-2	F5'-GATTGTGGCCTTCTTTGAGT-3'
		R5'-ATAGTTCCACAAAGGCATCC-3'
	Bax	F5'-TGTTACAGG GTTTCATCCAG-3'
		R5'-ATCCTCTGCAGCTCCATATT-3'
	Caspase 3	F5'-CATGGCCCTGAAATACGAAGTC-3'
		R5'-GCAGGCCTGAATGATGAAGAGTTT-3'
	Nrf-2	F5'-CCATTTACGGAGACCCACCGC-3'
		R5'-GCCCAAGTCTTGCTCCAGCTC-3'
	Leptin	F5'-GGTTCCTGTGGCTTTGGTCCTATC-3'
		R5'-CCCTCTGCCTGGCGGATACC-3'
	SOCS3	F5'-AAGGCCGGAGACTTTGCTTCGG-3'
		R5'-GCGGGAAACTTGCTGCTCCCTGA-3'

Western blot

Protein were lysed from the the liver tissues and HSCs using radioimmuno-precipitation assay buffer 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 membrance, 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). The membrane was subsequently incubated with HRP-conjugated Rabbit or Mouse secondary antibody (1:7500, Abcam) for 1h at room temperature. Specific signals were detected using the enhanced ECL kit (Thermo Scientific, USA). The chemiDocTM XRS + with Image LabTM Software (BIO-RAD, USA) was used for development.

Statistical analysis

The results diversity conspicuousness between two groups were compared by a two-way tails student's t-test. All data were expressed as mean ± SD. Besides, multiple comparisons were analyzed by one-way ANOVA on GraphPad Prism 6 software.

Result

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 was significantly deteriorated compared to control mice. Treatment with FGF21 significantly ameliorated liver index compared to model group (**Table 2**). Besides, CCL4-treated mice developed elevated plasma concentrations of ALT AST, ALP and TBIL in the model mice compared with control mice. Treatment with FGF21 significantly ameliorated these parameters in a dose dependent manner (**Table 3**). Results of Masson's staining demonstrated that pathological changes were observed (Fig. 1A**)** and HE staining showed that the normal tissue architectonics were damaged in the model mice compared to control mice**(**Fig. 1B). But, these changes were significantly ameliorated by treatment with FGF21 compared to model mice. These studies indicate that administration of FGF21 ameliorates CCL4-induced liver lesion in CCL4-model mice.

Table 2

Impact of FGF21 on liver index in mice.

Groups	n	Body weight (g)	Liver weight (g)	Liver index (%)
Control	10	21.76±0.54	0.97±0.03	0.044±0.001
CCL_4	10	17.99±0.60**	1.09±0.04*	0.058±0.001**
CCL_4+FL	10	18.50±1.17	0.96±0.06	0.052±0.001##
CCL_4+FH	10	19.63±0.44	0.89±0.02#^	0.047±0.001##
Control	10	43.18±1.71	2.01±0.10	0.045±0.001
DMN	10	29.67±1.28**	2.13±0.10	0.062±0.005**
DMN+FL	10	35.21±1.61#	1.61±0.16#	0.048±0.001#
DMN+FH	10	33.76±0.44#	1.38±0.07##	0.045±0.001##
All data represent mean ± SEM, n=10 per group, #p<.0.05, ##p<0.01, ###p<0.001 compared to model mice. *p<.0.05, **p<0.01 compared to control.				

Table 3

Impact of FGF21 on ALT, AST, ALB and TBIL in mice.

Groups	n	ALT（IU/L）	AST（IU/L)	ALP（IU/L）	TBIL(μmol/L)
Control	10	83.67±13.86	132.5±10.50	114.0±8.02	3.345±0.24
CCL4	10	795.6±104.8**	527.1±91.70**	190.3±14.75*	8.060±0.63*
CCL4+FL	10	210.3±22.6##	189.7±22.28##	116.0±5.29##	4.705±0.05#
CCL4+FH	10	147.0±28.1##^	136.7±16.97##^	101.3±2.96##^	3.845±0.06#
Control	10	52.50±4.97	126.8±14.55	53.08±6.58	2.153±0.55
DMN	10	139.3±26.41*	180.2±10.57*	102.8±8.61**	6.606±0.70**
DMN+FL	10	96.80±17.41#	150.4±9.09#	82.3±11.00#	3.184±0.53##
DMN+FH	10	81.76±4.91#	135.0±16.34#	66.00±10.02#	3.073±0.29##
All data represent mean ± SEM, n=10 per group, #p<.0.05, ##p<0.01, ###p<0.001 compared to model mice. *p<.0.05, **p<0.01 compared to control.					

The expression of α-SMA and collagenI can be regarded as markers of liver fibrosis. RT-PCR and Western blot were used to detect the expression levels of mRNA and protein. Our results showed that the mRNA

levels of α-SMA and collagenI were significantly upregulated in the model mice compared to control mice (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 of α-SMA and collagenI were significantly increased in the model 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 of IL-1β, IL-6 and TNF-α were measured by the methods of RT-PCR and ELISA at the mRNA and protein levels 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 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 model group (Fig. 1F).

Although the mechanisms are different in different models, TGF-β play a key liver fibrosis mediator [11, 14, 28]. To elucidate the potential mechanisms that FGF21 ameliorates CCL4- induced hepatic fibrosis, we detected the mRNA and protein levels of TGF-β in the liver. Results of RT-PCR and Western blotting 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 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 effect of FGF21 against 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 were elevated compared to control group in the model group. FGF21 significantly ameliorated these parameters in a dose dependent manner compared to model group (**Table 2, Table 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 mice compared to control mice**(**Fig. 2B). However, these changes were significantly ameliorated by treatment with FGF21 compared to model mice. RT-PCR and Western blot analysis showed that the mRNA and protein expression levels of α-SMA and collagenI were significantly upregulated in the model group 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 levels of IL-1β, IL-6 and TNF-α were significantly elevated in the liver and serum of model mice compared to 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 mechanisms of the therapeutic of FGF21 on DMN-induced hepatic fibrosis, Our data demonstrated that the mRNA and protein expression levels of TGF-β were upregulated in the liver of DMN-induced model mice compared to control. These effects were attenuated by FGF21 treatment compared to model group (Fig. 2G). Our results suggest that the therapeutic effects of FGF21 on MDN-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 signaling play 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 mRNA and protein levels of collagenI and α-SMA were significantly increased in model group compared to normal control group. FGF21 significantly decreased these effects compared to model group (Fig. 3A). Interestingly, we found that the mRNA and protein expression levels of leptin were significantly increased in model group compared to control group. 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 (Klotho-siRNA) 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 the 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 [30]. PDGF-BB is known to play a key role in HSC proliferation and increase the expression level of leptin [31]. Previous studies show that ERK, STAT3 and TGF-β exhibit central roles in PDGF-leptin-hepatic fibrosis axis [32–34]. To elucidate the relationship between FGF21 and PDGF-BB/Leptin downstream signaling pathway in HSCs, the expression of ERK, STAT3 and TGF-β were detected by RT-PCR and Western blot methods. Our studies demonstrated that the mRNA and protein levels 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 were significantly increased in model group compared to control. These parameters were significantly decreased by administration of FGF21 in a dose dependent manner compared to model group (Fig. 3D). These results suggest that ERK, Stat3 and TGF-β are involved in the PDGF-leptin aixs in the development and progression of HSCs activation. FGF21 could block this axis.

A line of evidence demonstrate that SOCS3 are important inhibitors of the leptin signaling pathway [35]. Ga Young et al report anti-inflammatory mechanisms of suppressors of cytokine signaling target ROS via NRF-2/thioredoxin induction and inflammasome activation in macrophages [36]. Our previous studies demonstrate that FGF21 attenuates pulmonary fibrogenesis via Nrf-2 signaling pathway [37]. Therefore, we measured the expression levels of SOCS3 and Nrf-2. Our results showed that the expression levels of Nrf-2 were increased in model group compared to control group. 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 were downregulated in model group 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 mitogenesis and inhibition of apoptosis [32]. In this study, Hotecest33528 staining and flow cytometry analysis demonstrated that HSCs apoptosis was reduced in model group compared to control, but there was a significantly increased apoptosis rate of HSCs by treated with FGF21 compared to model group (Fig. 4A, B, C). Besides, the mRNA and protein expression of Bcl-2 were significantly downregulated and the expression of Bax and caspase3 were significantly upregulated by administration of FGF21 compared with model group (Fig. 4F, G). The CCK-8 analysis showed that HSCs proliferation was induced in model group 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 was increased in model group 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 and 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 conditions without effective treatment [13, 14]. FGF21, as an endocrine hormone and important regulator with pleiotropic effects, exists beneficial effects in the liver diseases [1, 2]. Previous studies demonstrate that treatment with FGF21 could attenuate pulmonary fibrogenesis and liver fibrosis [11, 37]. However, the therapeutic effects of FGF21 on liver fibrosis in different models and the underlying mechanisms have not been 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]. DMN -induced model, associated with hepatotoxic, genotoxic and immunotoxic, could cause liver damage and 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 damage in different degrees in the mice of both models and these parameters were significantly ameliorated by administration of FGF21 in

a dose dependent manner. Our results also showed that FGF21 significantly reduced ECM in both models. The activation of TGF-β/Smad signaling pathway results in collagen production and fibrogenesis. Besides, FGF21 significantly downregulated the expression of TGF-β. A body of evidences demonstrate that inflammatory pathway exhibits a significant role in hepatic fibrogenesis [13, 20]. Our results demonstrated FGF21 could significantly decreased inflammatory cytokines expression levels. Yang X et al report 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, our 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 activation of HSCs has 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 of collagenI and α-SMA. Administration of FGF21 significantly reduced the mRNA and protein expression levels of collagenI and α-SMA. Interestingly, we found that the expression of leptin was significantly increased in model group, and FGF21 significantly decreased the expression of leptin. These effects were reversed in si-RNA against β-klotho transfected HSCs. The results indicate that the HSCs activated by PDGF-BB promotes 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 leptin serum levels have been detected in patients with liver cirrhosis. Normal liver tissue doesn't express leptin, but its expression could be detected in the fibrotic livers 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 and serum will be further investigated *in vivo*. Previous studies have demonstrated that PDGF-BB induces activation of the downstream molecules ERK in activated HSC and ERK signaling pathway plays a critical role in modulating major response of myofibroblast [41]. Meanwhile, the expression of phosphorylated Stat3 and TGF-β serve as the central role in the leptin signaling pathway [34]. 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 mRNA and protein expression of TGF-β were also significantly downregulated by treatment with FGF21. These results suggest that ERK is involved in the expression of leptin in PDGF-BB activated HSCs and leptin further promotes HSCs fibrogenesis via Stat3/TGF-β pathway by autocrine. Howard and Flier report that 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 [35]. 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 [42, 43]. Zhang et al reports that FGF21 could attenuate pulmonary fibrosis via activating Nrf-2 signaling pathway [37]. We examined the expression levels of SOCS3 and Nrf-2 in each group. The mRNA and protein expression of SOCS3 were significantly decreased in model group. Treatment with FGF21 significantly upregulated the expression levels of SOCS3 and enhanced the expression 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 phosphroylation to prevent fibrogenesis and related inflammation. Meanwhile, FGF21 suppresses oxidative stress via enhancing Nrf-2 expresion.

Large number of papers demonstrate that the high expression level of leptin plays an important role in mitogenesis and inhibition of apoptosis in HSCs [30, 32]. In this study, our results demonstrated that FGF21 reduced HSC proliferation and rendered HSCs sensitive to apoptosis in a dose dependent manner. Papers show that leptin mediates HSC proliferation via AKT phosphorylation and inhibits apoptosis via caspase family [40, 44]. 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 of Bax and caspase 3 were significantly increased by treatment with FGF21. All data indicate that FGF21 attenuated HSCs proliferation and rendered 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.

Declarations

Conflicts interest

The authors declare that there are no conflicts of interest.

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Ethics statement

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 euthanatized under anesthesia induced by intraperitoneal injection of 1.2% avertin (Sigma, USA) at a dose of 20 μl/g body weight.

Availability of data and materials

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

Author' contributions statement

FR, M performed the research, and analyzed the data. Khoso, MH. K, K. Q, H, YK, C and XH, J participated in data collection and analysis. W, X and DS, Li contributed to the initial and consequent project discussion, manuscript discussion and revision. All the authors approved the final version of the manuscript.

References

1. Kharitonenkov A, Adams AC (2014) Inventing new medicines: The FGF21 story. Molecular Metabolism 3(3):221–229
2. Itoh N et al (2014) FGF21 as a Hepatokine, Adipokine, and Myokine in Metabolism and Diseases. Frontiers in Endocrinology 5(2):p.152–159
3. Dutchak PA, Katafuchi T, Bookout AL et al (2012) Fibroblast Growth Factor-21 Regulates PPARγ Activity and the Antidiabetic Actions of Thiazolidinediones. Cell 148:556–567
4. Berglund ED et al (2009) Fibroblast Growth Factor 21 Controls Glycemia via Regulation of Hepatic Glucose Flux and Insulin Sensitivity. Endocrinology 150(9):4084–4093
5. Lin Z et al (2015) Fibroblast Growth Factor 21 Prevents Atherosclerosis by Suppression of Hepatic Sterol Regulatory Element-Binding Protein-2 and Induction of Adiponectin in Mice. Circulation 131(21):1861–1871
6. Li S et al. Fibroblast growth factor 21 protects the heart from angiotensin II-induced cardiac hypertrophy and dysfunction via SIRT1. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease. 1865(6): p. 1241–1252. (2019)
7. Pan X et al (2018) FGF21 Prevents Angiotensin II-Induced Hypertension and Vascular Dysfunction by Activation of ACE2/Angiotensin-(1–7) Axis in Mice. Cell Metabolism 27(6):1323–1337
8. Lin X et al (2014) FGF21 inhibits apolipoprotein(a) expression in HepG2 cells via the FGFR1-ERK1/2-Elk-1 pathway. Molecular Cellular Biochemistry 393(1–2):33–42
9. Xu (2015) p et al. Long-Term Administration of Fibroblast Growth Factor 21 Prevents Chemically-Induced Hepatocarcinogenesis in Mice. DIGEST DIS SCI 60:3032–3043

10. Opoku YK et al (2020) Fibroblast growth factor-21 ameliorates hepatic encephalopathy by activating the stat3-socs3 pathway to inhibit activated hepatic stellates cells. Exclj Journal 19:567–581
11. Xu P et al (2016) Fibroblast growth factor 21 attenuates hepatic fibrogenesis through TGF-β/smad2/3 and NF-κB signaling pathways. Toxicology Applied Pharmacology 290:43–53
12. Lee KJ et al (2018) Expression of Fibroblast Growth Factor 21 and β-Klotho Regulates Hepatic Fibrosis through the Nuclear Factor-κB and c-Jun N-Terminal Kinase Pathways. Gut Liver 12(4):449–456
13. Aydin MM, Akcali KC (2018) Liver fibrosis. The Turkish Journal of Gastroenterology 29(1):14–21
14. Bataller R, Brenner DA (2005) Liver fibrosis. Journal of Clinical Investigation 115(2):209–218
15. Ramachandran P, Iredale JP (2012) Liver fibrosis: a bidirectional model of fibrogenesis and resolution. QJM 105(9):813–817
16. Tsuchida T, Friedman SL (2017) Mechanisms of hepatic stellate cell activation. Nature Reviews Gastroenterology Hepatology 14(7):397–411
17. Zhang C et al (2016) Liver fibrosis and hepatic stellate cells: Etiology, pathological hallmarks and therapeutic targets. World Journal of Gastroenterology 22(48):10512
18. Saxena NK et al (2004) Leptin as a novel profibrogenic cytokine in hepatic stellate cells: mitogenesis and inhibition of apoptosis mediated by extracellular regulated kinase (Erk) and Akt phosphorylation. The FASEB Journal 18(13):1612–1614
19. Košuta I et al (2020) Leptin as a Key Player in Insulin Resistance of Liver Cirrhosis? A Cross-Sectional Study in Liver Transplant Candidates. Journal of Clinical Medicine 9(2):560
20. Chen Z et al (2019) Targeted Drug Delivery to Hepatic Stellate Cells for the Treatment of Liver Fibrosis. J Pharmacol Exp Ther 370(3):695–702
21. Yanguas SC, Cogliati B, Eillebrods J (2016) Experimental models of liver fibrosis. Arch Toxicol 90(5):1025–1048
22. Scholten D et al (2015) The carbon tetrachloride model in mice. Laboratory Animals 49(1):4–11
23. Gary O, Korsrud, Harold C et al (1972) Sensitivity of several serum enzymes in detecting carbon tetrachloride-induced liver damage in rats. Toxicology appled pharmaclogy 22:474–483
24. Verna L, Gary JW, Williams M. N-Nitrosodiethylamine Mechanistic Data and Risk Assessment: Bioactivation, DNA-Adduct Formation, Mutagenicity, and Tumor Initiation. Pharmacol Ther.71:57–81. (1996)
25. Liu X et al (2017) Differential Proteomic Analysis of Dimethylnitrosamine (DMN)-Induced Liver Fibrosis. PROTEOMICS 17(22):1700267
26. George JM, Tsuchishima, Tsutsumi M (2019) Molecular mechanisms in the pathogenesis of N-nitrosodimethylamine induced hepatic fibrosis. Cell Death Dis 10(1):52–59
27. Van Dijk F et al (2015) Targeted Therapies in Liver Fibrosis: Combining the Best Parts of Platelet-Derived Growth Factor BB and Interferon Gamma. Frontiers in Medicine 2:257–260
28. Friedman SL et al (2008) Mechanisms of Hepatic Fibrogenesis Gastroenterology 134(6):1655–1669

29. Friedman SL, Yamasaki G, Wong L (1994) Modulation of transforming growth factor beta receptors of rat lipocytes during the hepatic wound healing response. J Biol Chem 269(14):10551–10558
30. Saxena NK et al (2004) Leptin as a novel profibrogenic cytokine in hepatic stellate cells: mitogenesis and inhibition of apoptosis mediated by extracellular regulated kinase (Erk) and Akt phosphorylation. The FASEB Journal 18(13):1612–1614
31. Borkham-Kamphorst E, Weiskirchen R (2016) The PDGF system and its antagonists in liver fibrosis. Cytokine Growth Factor Rev 28:53–61
32. Bethanis SK, Theocharis SE (2006) Leptin in the Field of Hepatic Fibrosis: A Pivotal or an Incidental Player? Digestive Diseases Sciences 51(10):1685–1696
33. Kwon OKW, Kim, Kim M (2016) Leptin signalling pathways in hypothalamic neurons. Cellular Molecular Life Sciences 73(7):1457–1477
34. Sahin GS, Dhar M (2019) Crystal Dillon. leptin stimulates synaptogenesis in hippocapal nerons via KLF4 and SOCS3 inhibition of STAT3 signaling. Molecular Cellular Neuroscience 20:123–128
35. Howard JK, Flier JS (2006) Attenuation of leptin and insulin signaling by SOCS proteins. TRENDS in Endocrinology Metabolism 17(9):365–370
36. Ga Y, Kim (2020) Hana and Yoon Hye-Min. Anti-inflammatory mechanisms of suppressors of cytokine signaling target ROS via NRF-2/thioredoxin induction and inflammasome activation in macrophages. BMB Rep 53(12):640–645
37. Zhang S et al (2018) FGF21 attenuates pulmonary fibrogenesis through ameliorating oxidative stress in vivo and in vitro. Biomed Pharmacother 103:1516–1525
38. Yang X, Lechleider RJ, Chen (1999) Targeted disruption of SMAD3 results in impaired mucosal immunity and diminished T cell responsiveness to TGF-beta. EMBO J 18(5):1280–1291 et al.
39. Trombley S, Maugars G, Kling P et al (2012) Effects of long-term restricted feeding on plasma leptin, hepatic leptin expression and leptin receptor expression in juvenile Atlantic salmon. 175(3):p.92–99
40. C.Otte and J, M.Otte. et al. Expression of Leptin and Leptin Receptor During the Development of Liver Fibrosis and Cirrhosis. Experimental and Clinical Endocrinology & Diabetes. 112(01): p. 10–17. (2004)
41. Foglia B et al (2019) ERK Pathway in Activated, Myofibroblast-Like, Hepatic Stellate Cells: A Critical Signaling Crossroad Sustaining Liver Fibrosis. International Journal of Molecular Sciences 20(11):2700
42. Kim GY (2020) Hana and Yoon Hye-Min. Anti-inflammatory mechanisms of suppressors of cytokine signaling target ROS via NRF-2/thioredoxin induction and inflammasome activation in macrophages. BMB Rep 53(12):640–645
43. Lu C et al (2017) Nrf2 induces lipocyte phenotype via a SOCS3-dependent negative feedback loop on JAK2/STAT3 signaling in hepatic stellate cells. International Immunopharmacology 49:203–211
44. Gong Z. Jiayu Lin. Jie Zheng et al Dahuang Zhechong pill attenuates CCl4-induced rat liver fibrosis via the PI3K-Akt signaling pathway. Journal of Cellular Biochemistry. 121(2): p. 1431–1440. (2019)

Figures

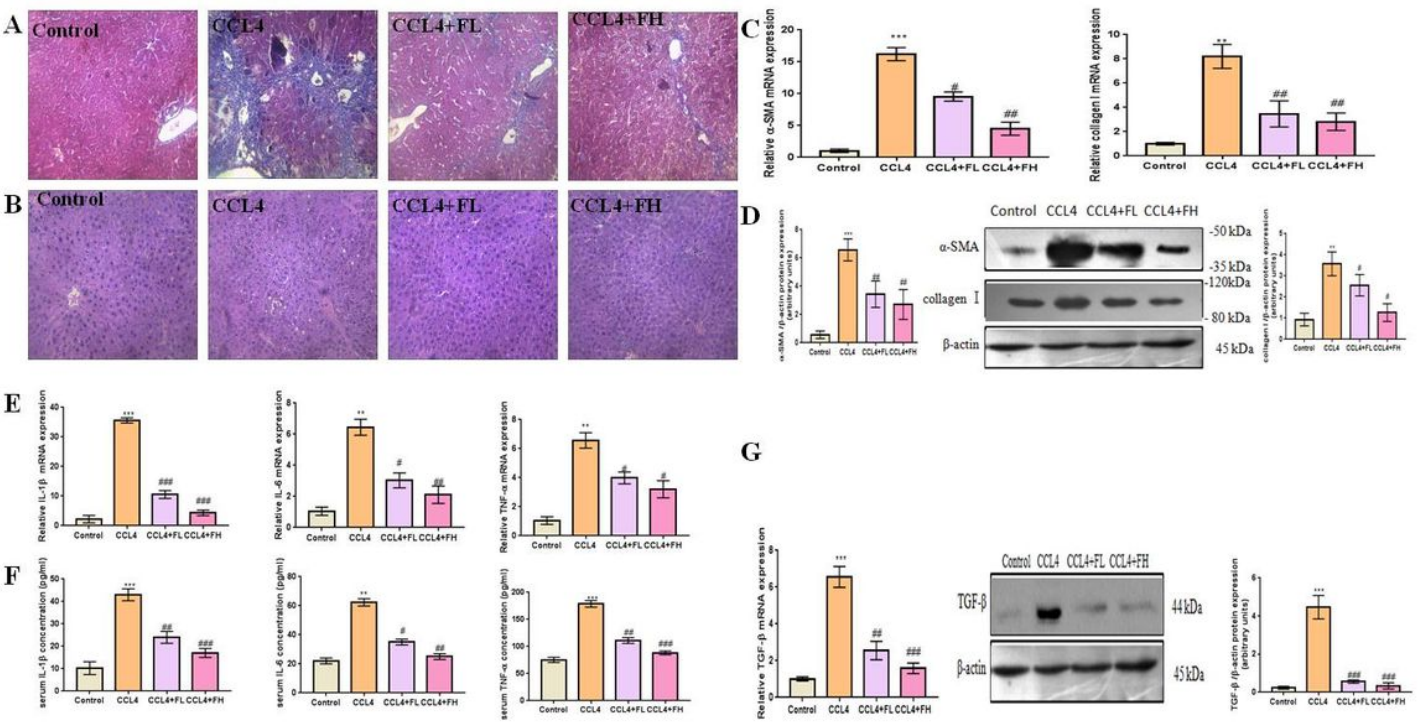

Figure 1

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. RT-PCR and western blotting were used to measure liver fibrosis related protein expression. CCL4 means model group, FL means the low dose of treatment with FGF21. FH means the high dose of treatment with FGF21. (A, B) Respresentative Masson staining (×200) and HE staining (×200) of liver sections from CCL4 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.

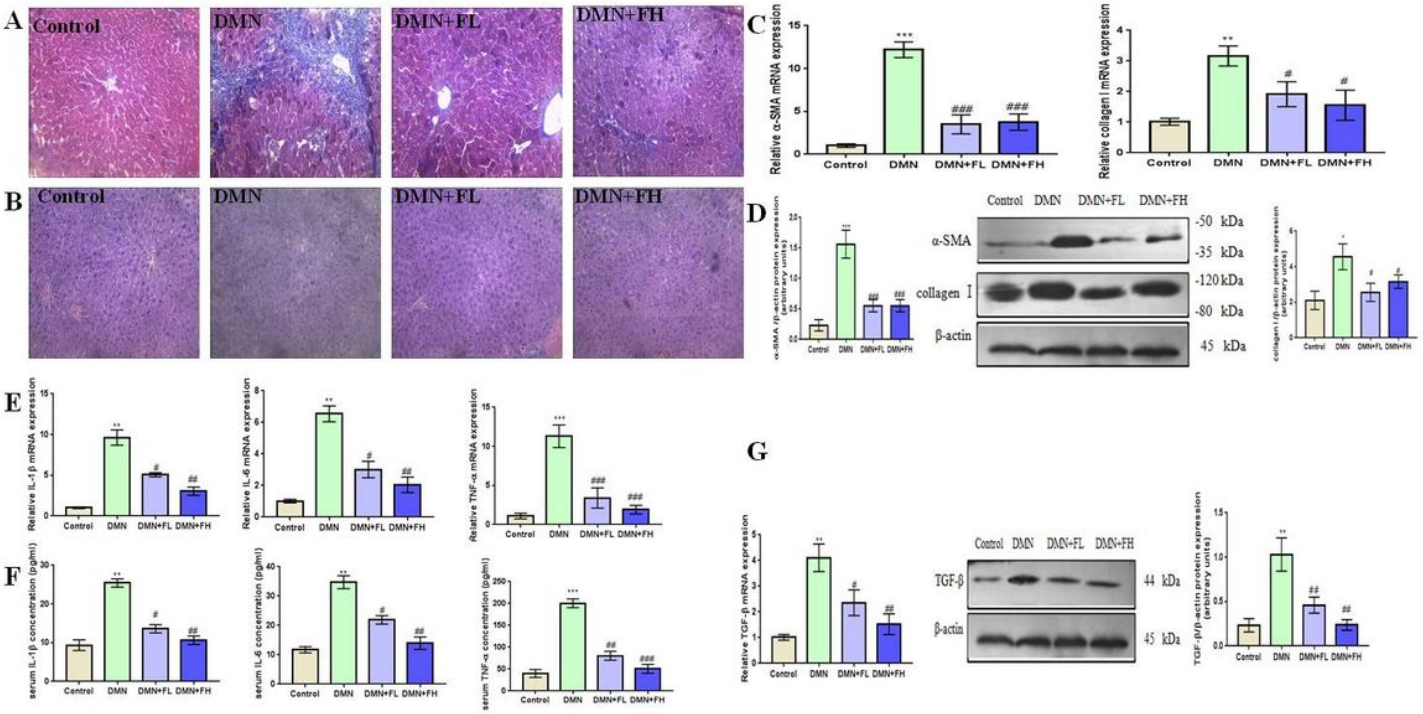

Figure 2

FGF21 ameliorates DMN-induced hepatic fibrosis in mice. DMN means model group, FL means the low dose of treatment with FGF21, FH means the high dose of treatment with FGF21. (A, B) Respresentative Masson staining (×200) and HE staining (×200) of liver sections from DMN model mice. Results revealed that distortion of the normal architecture and an extensive deposition of collagen were observed in model mice. whereas, there were no significant changes in treatment groups. RT-PCR and Western blotting were used to analyze the mRNA and protein levels of α-SMA and collagenI in the livers of each group mice. (C) The mRNA expression levels of α-SMA and collagenI in each group. (D) The protein expression levels 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 DMN-induced model mice The bands were analyzed with Image J. Experiments were done in triplicate. 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.

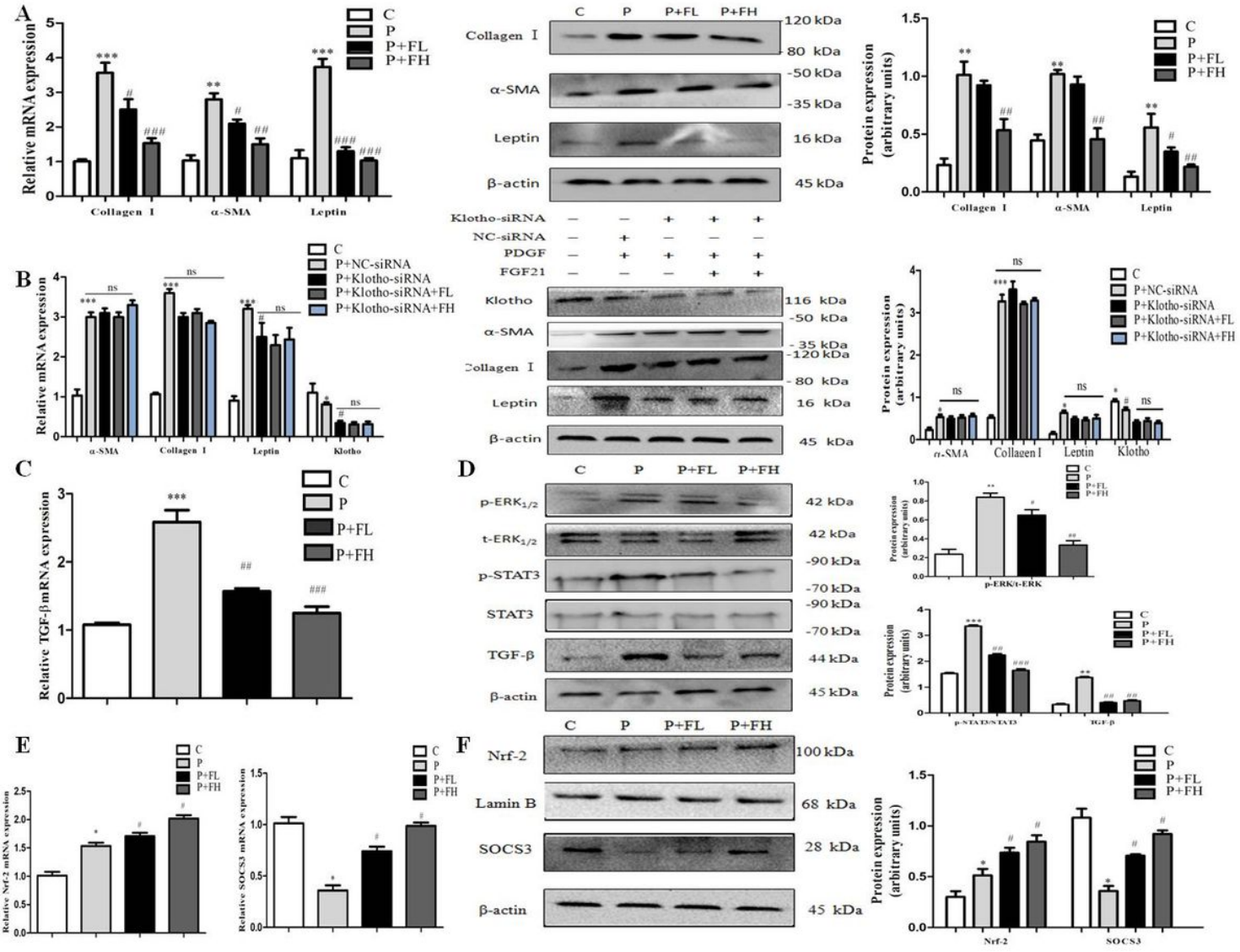

Figure 3

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 the low dose of treatment with FGF21, FH means the high dose of treatment with 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, collagenI/β-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.

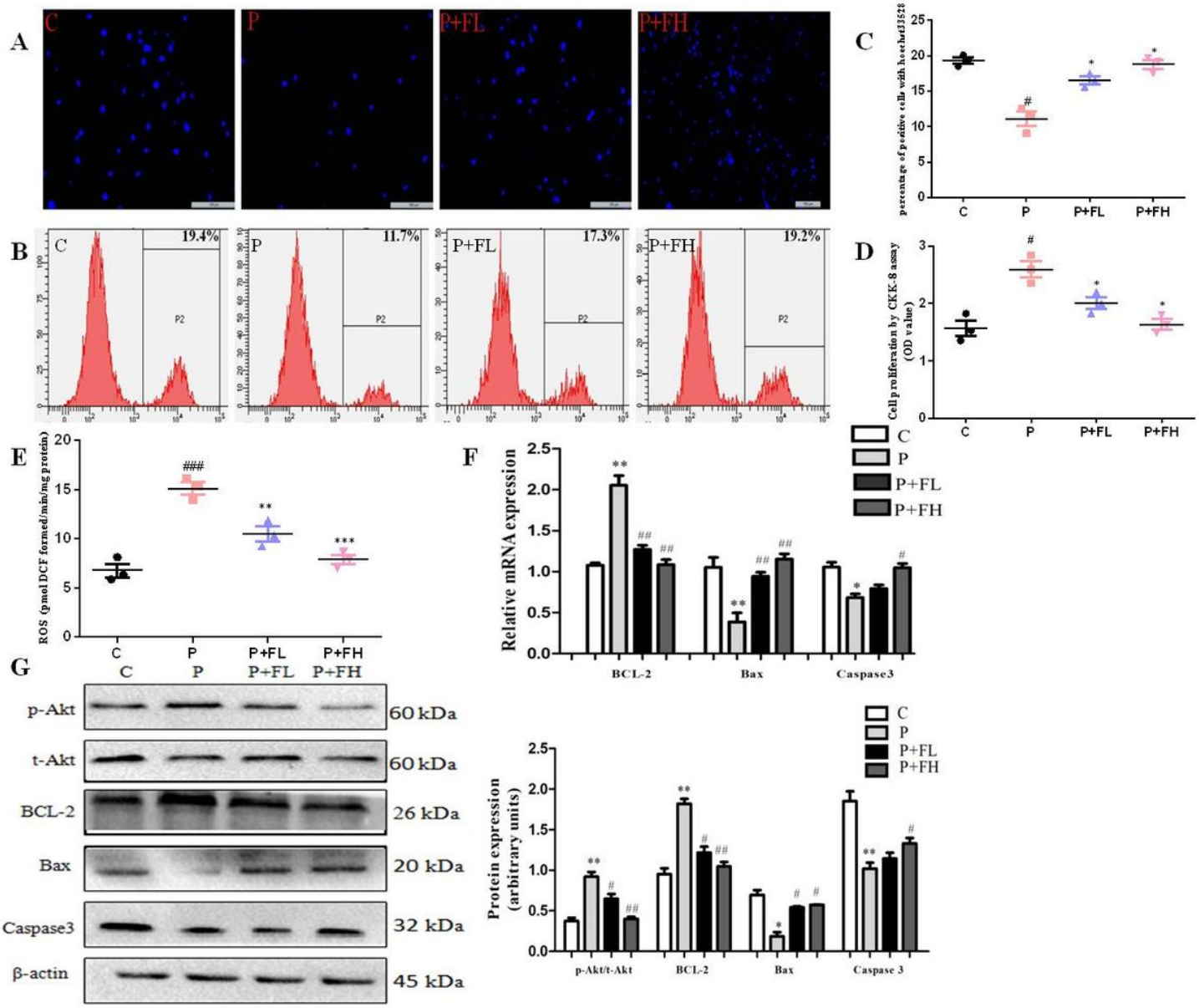

Figure 4

FGF21 attenuates cell proliferation, renders HSCs sensitive to apoptosis and reduces the level of ROS. (A, B) Hotecest33528 staining and Flow cytometry analysis of cell apoptosis in each group. (C) The perentage of cell apoptosis in each group. (D). CCK-8 ananlysis of cell proliferation in each group. (E) The level of ROS was evaluated by ROS Assay Kit in each group. Experiments were done in triplicate. (F). RT-PCR analysis of Bcl-2, Bax and caspase 3 level in the liver of each group. (G). Western blot analysis of Bcl-2, Bax, caspase 3, p-AKT and t-AKT in the liver of each group. The bands were analyzed with Image J. All data represent mean ± SD, Experiments were done in triplicate. #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.

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