

Original Article

Blocking follistatin-like 1 attenuates liver fibrosis in mice by regulating transforming growth factor-beta signaling

Xiao-Hua Zhang^{1*}, Yong Chen^{1*}, Bin Li¹, Ji-Yong Liu¹, Chong-Mei Yang¹, Ming-Ze Ma²

Departments of ¹Gastroenterology, ²Infectious Diseases, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, Shandong Province, China. *Equal contributors.

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Abstract: Aim: To elucidate the effect of inhibiting follistatin-like 1 on liver fibrosis and activation of hepatic stellate cells in mice. Methods: We generated a follistatin-like 1 neutralizing antibody that can inhibit TGF- β 1-induced expression of collagen1 α 1 in primary mouse liver fibroblasts. All of the mice in our study were induced with carbon tetrachloride and thioacetamide. In addition, primary hepatic stellate cells from mice were isolated from fresh livers using density gradient separation. The degree and extent of fibrosis in mouse livers from the different groups were evaluated by Sirius Red and Masson staining. The effect of the follistatin-like 1 neutralizing antibody on proliferation and migration of hepatic stellate cells was detected using CCK-8 and Transwell assays, respectively. Results: Expression of follistatin-like 1 in human cirrhotic liver tissue was higher than that in normal liver tissue. Blocking follistatin-like 1 resulted in a delay of primary hepatic stellate cell activation and down-regulation of the migratory capacity of hepatic stellate cells. Blocking follistatin-like 1 also down-regulated TGF-beta signaling in primary hepatic stellate cells from mice. Finally, inhibition of follistatin-like 1 attenuated liver fibrosis and liver function damage *in vivo*. Conclusions: Inhibiting follistatin-like 1 attenuates liver fibrosis and causes a delay in hepatic stellate cell activation. The effect of follistatin-like 1 on liver fibrosis is mainly attributed to its role in regulating TGF-beta signaling.

Keywords: Follistatin-like 1, liver fibrosis, transforming growth factor-beta, hepatic stellate cells

Introduction

Liver fibrosis is a common chronic liver disease that is characterized by the accumulation of collagen and activation of Hepatic Stellate Cells (HSCs) in the liver [1]. Liver fibrosis is a common precursor of liver cirrhosis and even hepatocellular carcinoma [1]. Liver cirrhosis is irreversible and can only be effectively cured with liver implantation [2]. Therefore, preventing progression from liver fibrosis to liver cirrhosis can be highly beneficial to patients with chronic liver disease.

TGF-beta signaling plays a central role in the progression of liver fibrosis and activation of HSCs [3]. Thus, preventing TGF-beta signaling may theoretically result in the blockage of liver fibrosis progression [3]. Nevertheless, directly blocking TGF-beta signaling has been shown to

cause many unacceptable adverse effects in animal trials [4]. Based on this, it is important to find new targets that can influence TGF-beta signaling as well as liver fibrosis progression.

Follistatin-like 1 (FSTL1) was initially identified as a TGF-beta-induced gene that encodes the secreted protein Fstl1 [5]. Dong et al. reported that FSTL1 can regulate fibroblast activation by affecting TGF-beta signaling and bone morphogenetic protein signaling [6]. Lung fibrosis in mice was attenuated *in vivo* by a FSTL1 neutralizing antibody [7]. The results above suggested that FSTL1 may play a role in the progression of liver fibrosis due to similar mechanisms in the pathogenesis of pulmonary fibrosis and liver fibrosis.

We conducted this study and found that blocking FSTL1 led to attenuation of liver fibrosis in carbon tetrachloride-treated mice. In addition,

we confirmed the effect of FSTL1 on TGF- β signaling in mouse liver tissues.

Materials and methods

Clinical samples

All human normal liver tissues and cirrhotic liver tissues were provided by the Department of Liver Surgery at Shandong Provincial Hospital. All the human tissues were obtained with informed content, and approved by the Ethical Committee of Shandong Provincial Hospital.

Cell culture

Rat HSC-T6 cells were purchased from Millipore Corporation. These cells were cultured in Dulbecco's-modified Eagle medium (DMEM) supplemented with 10% fetal calf serum and 1% antibiotics at 37°C in a Thermo incubator containing 5% carbon dioxide.

Experimental animals

Female BALB/c mice were purchased from Shandong University Laboratory Animal Center and used to produce neutralizing antibodies. In addition, C57BL/6J mice were purchased from Shandong University Laboratory Animal Center and used for carbon tetrachloride-induced liver fibrosis. All of the mice in our study were housed and fed in specific-pathogen free (SPF) conditions and received humane care. We ensured that all animals in our study received humane care, and all study protocols complied with the institution's guidelines.

Carbon tetrachloride- and TAA-induced liver fibrosis model

C57BL/6J mice were injected with carbon tetrachloride (CCl₄) (0.5 μ L per gram body weight) in 25% olive oil twice per week at equal intervals for eight weeks. Another group of C57BL/6J mice were injected with thioacetamide (TAA) (0.2 mg/g body weight) in double distilled water three times per week at equal intervals for eight weeks.

Primary hepatic stellate cell isolation from mice

C57BL/6J mice were sacrificed after anesthesia, and their livers were removed from the

abdominal cavities. The fresh livers were perfused with a digestive solution comprising 0.1% collagenase, 0.25% pronase E and 0.01% DNase and mechanically dissected. The digested livers were incubated in the same digestive solution at 37°C for approximately 25 to 30 minutes. The suspension was then removed and filtered through an iron mesh with 100 μ m pores. The filtered suspension was centrifuged through a Nycodenz gradient (Axis-Shield) at 8.2% concentration. The isolated primary HSCs were resuspended in DMEM supplemented with 20% fetal calf serum and 1% antibiotics and cultured at 37°C overnight in an environment containing 5% carbon dioxide. After 24 hours, cell debris and non-adherent cells were removed.

Generation of FSTL1-neutralizing antibodies

FSTL1 neutralizing antibody generation was performed using conventional hybridoma techniques. His-tag fusion constructs of FSTL1 (which encoded amino acids 37-306 of the full-length mouse FSTL1 protein, NP_009016) were cloned into PET28 vectors and expressed according to previously established protocols. The purified His-tag fusion protein was used as an antigen to immunize BALB/c mice for generation of anti-FSTL1 monoclonal antibodies. Afterwards, one monoclonal antibody was identified that effectively blocked TGF- β 1-induced expression of Collagen1 α 1 in hepatic fibroblasts and this mAb was used as the primary FSTL1 neutralizing antibody for all subsequent animal experiments.

Immunohistochemical and Sirius Red staining

Paraffin-embedded liver tissue from mice and humans were sliced into 5- μ m thick sections followed by deparaffinization and step-wise rehydration in preparation for IHC and Sirius Red staining. For Sirius Red staining, the sections were stained by Sirius Red for approximately three minutes and dehydrated step-wise. For immunohistochemical staining, the sections were incubated with 0.3% hydrogen peroxide for thirty minutes and blocked with 10% bovine serum albumin. The sections were then incubated using antibody targeting FSTL1 (1:200, Abcam) at 4°C overnight and labeled by HRP-conjugated secondary mouse antibody (Abcam) at room temperature for approximately one hour. Afterwards, the sections were incubated with a DAB substrate liquid (Thermo) and

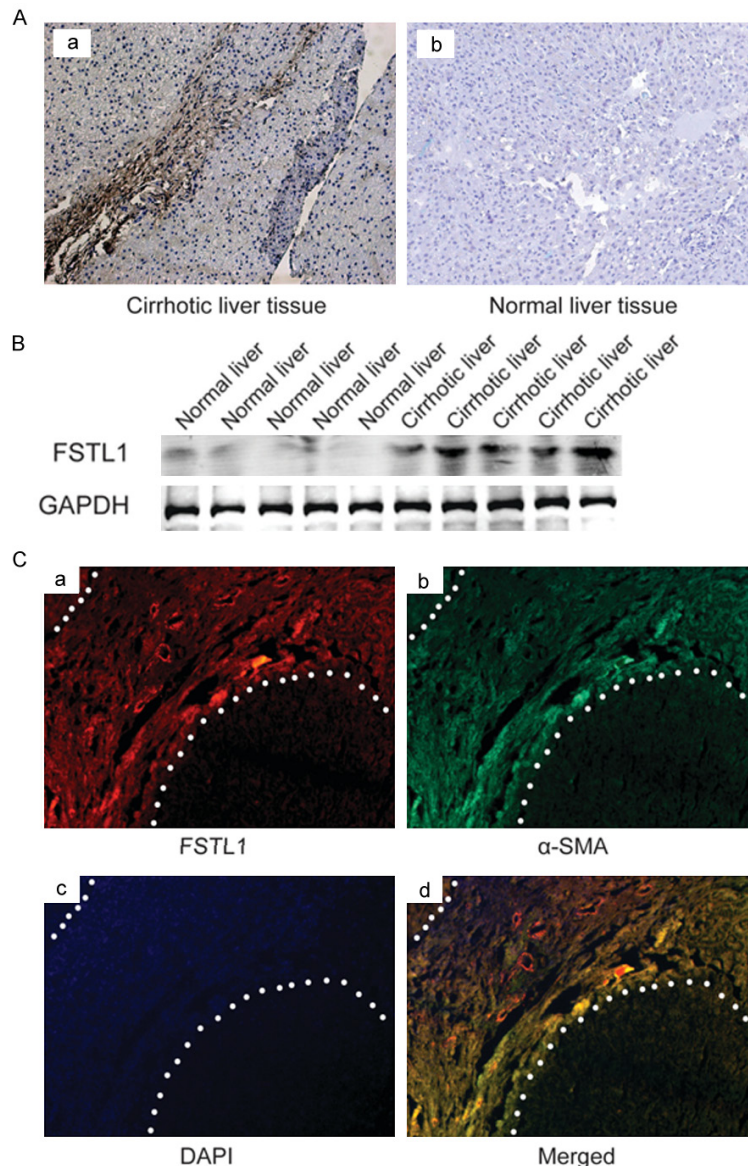


Figure 1. (A) IHC images of FSTL1 expression in human cirrhotic liver tissues (a) and normal liver tissues (b). FSTL1 expression was mainly localized to cirrhotic lesions of liver tissues; (B) Western blot assay showed that FSTL1 protein expression was higher in human cirrhotic liver tissue than in normal liver tissue; (C) ICC assay showed that localization of FSTL1 expression (a) in liver tissue was highly similar to that of α -SMA expression (b).

stained with hematoxylin for two minutes. All the sealed slides were imaged and recorded on a microscope manufactured by Carl Zeiss.

Masson staining

The slides were deparaffinized and rehydrated step-wise before Masson staining. The slides were first stained with Weigert hematoxylin for 5 minutes and washed with water. The slides were then stained with acid ponceau for six

minutes followed by a quick immersion into 2% glacial acetic acid. Afterwards, the slides were immersed in 1% phosphomolybdic acid for approximately three minutes prior to immediate staining with Aniline Blue for five minutes. The slides were subsequently immersed into 0.2% glacial acetic acid briefly and dehydrated step-wise until they were sealed with neutral balsam.

Immunofluorescence staining

For cell staining, primary mouse HSCs were seeded onto rounded coverslips in 24-well plates and incubated at 37°C for approximately seven days in an environment containing 5% CO₂. For F-actin staining, cells were incubated with phalloidin-FITC (Sigma) for seventy minutes at room temperature. For α -SMA staining, cells were incubated with α -SMA antibody (Sigma) for seventy-five minutes at room temperature and subsequently treated with Alexa Fluor 594-conjugated secondary antibody. The nuclei were stained using DAPI (Sigma), and the immunofluorescence images were recorded using a fluorescence microscope (Carl Zeiss).

Western blotting

Cells were lysed in lysis solution (50 mM Tris-HCl, 150 mM NaCl, 1% Triton-X 100, 1 mM

MgCl₂, 1 mM PMSF) and boiled for five minutes. The proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane that was blocked using 1% bovine serum albumin in TBS. The NC membrane was incubated with antibodies targeting FSTL1 (Abcam), Smad2 (Cell Signaling Technology, CST), phospho-Smad2 (CST), Smad3 (CST), phospho-Smad3 (CST), Smad4 (CST), and GAPDH (Sigma) followed by the addition of fluorescence-conjugated secondary antibodies. All the fluo-

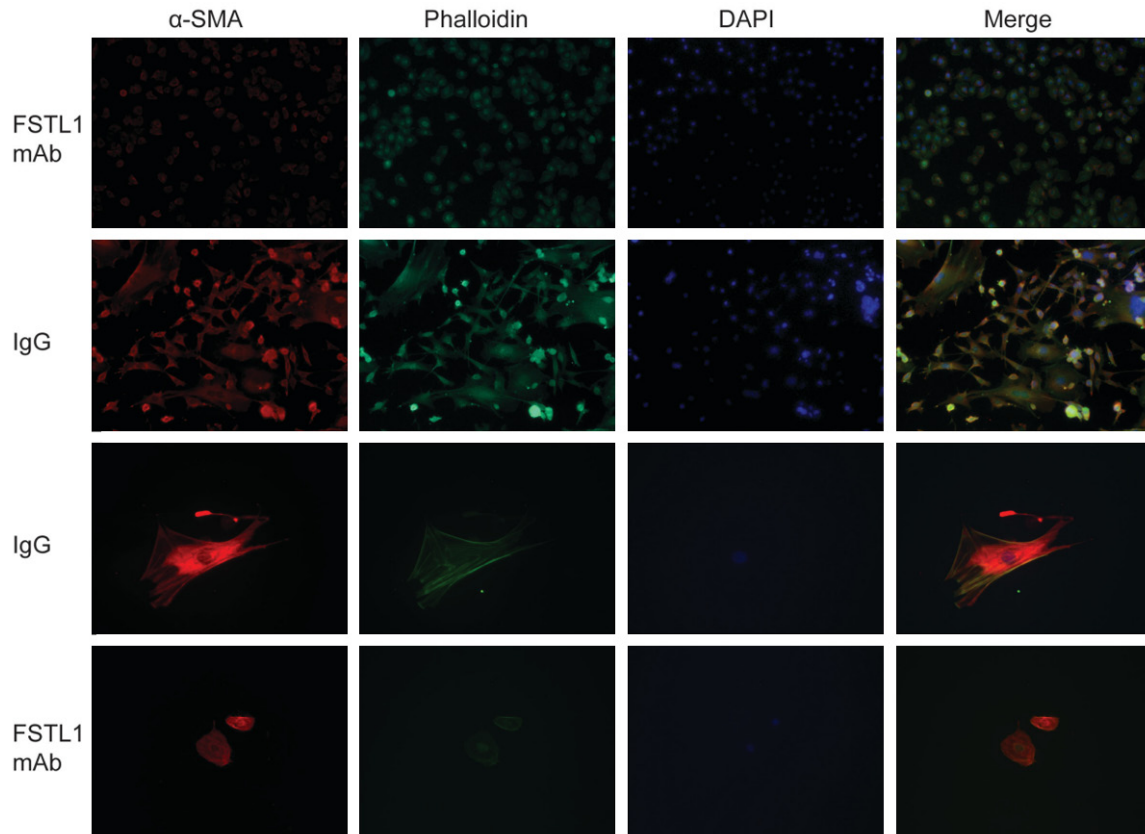


Figure 2. Primary HSCs isolated from mice were fixed at five days. ICC images showed that the activation of HSCs treated with IgG was faster than that of HSCs treated with FSTL1.

rescence signals were captured on an Odyssey imaging system (LI-COR).

Cell migration assay

Cell migration assays were performed using Transwell chambers (purchased from Millipore). Primary hepatic stellate cells (5×10^4 /well) from mice were suspended in 200 μ L serum-free DMEM and seeded into the upper chamber; 700 μ L of DMEM supplemented with 10% fetal bovine serum was added into the lower chamber. The migrated cells were fixed using paraformaldehyde and stained with 0.1% crystal violet. The images were recorded through a microscope and at least three fields per sample were photographed and counted.

Cell counting kit (CCK-8) assay

Primary mouse hepatic stellate cells were seeded into 96-well plates at a density of 4000 cells per 100 μ L and incubated at 37°C in an environment containing 5% CO₂. The CCK-8 re-

agent (10 μ L) was added into each well, and the plates were incubated at 37°C for one to two hours. The absorbance at 450 nm was detected using a microplate reader and recorded for statistical analysis.

Statistical analysis

All the data are presented as the mean \pm standard error of the mean (SEM). Statistical analysis was performed using SPSS 16.0 software. Two-tailed Student's t-test was used for comparisons between groups. A *P* value less than 0.05 was considered statistically significant.

Results

FSTL1 was up-regulated in cirrhotic liver tissues compared with normal liver tissues

We first detected the expression of FSTL1 in human liver tissues using IHC and found that expression of FSTL1 in cirrhotic liver tissue was significantly higher than that in normal liver

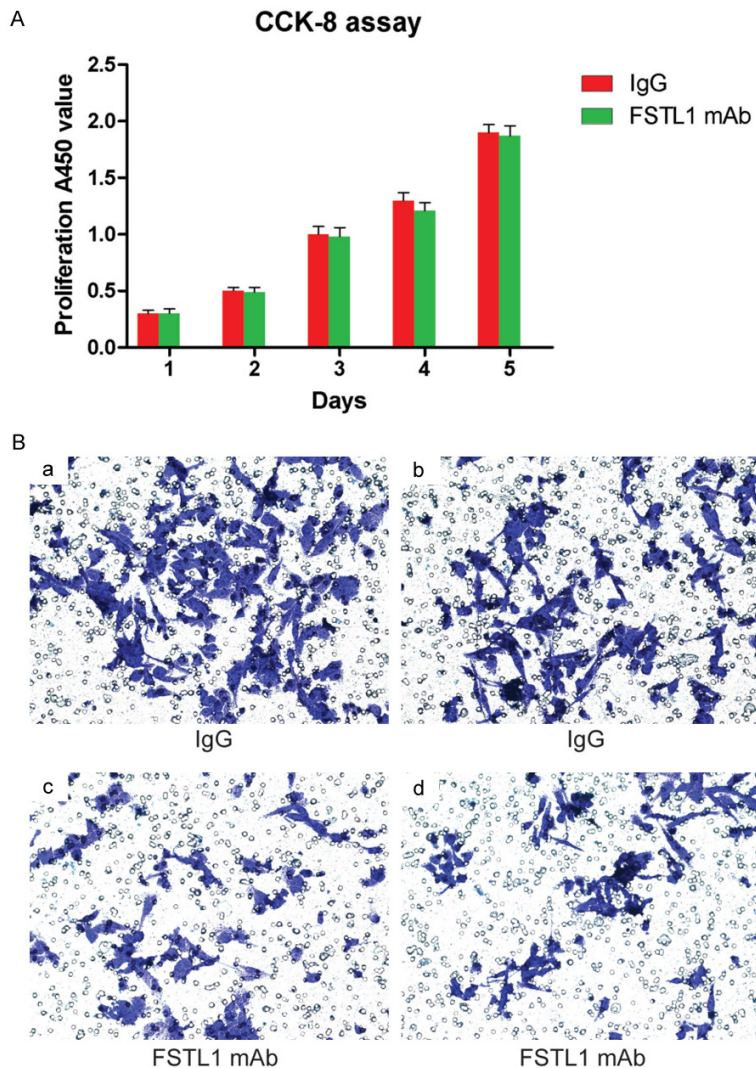


Figure 3. (A) Treatment of HSCs with an FSTL1 mAb had no significant effect on cell proliferation *in vitro*; (B) Transwell assay showed that HSC-T6 cells treated with the FSTL1 mAb (c, d) exhibited reduced migration compared with cells treated with IgG (a, b) *in vitro*.

(**Figure 1A**). In addition, expression of FSTL1 was mainly localized in cirrhotic lesions of liver tissue, which indicated a correlation between FSTL1 and liver cirrhosis (**Figure 1A**). The Western blot assay showed that the protein levels of FSTL1 in cirrhotic liver tissue were remarkably higher than those in normal liver tissues (**Figure 1B**). Immunofluorescence staining revealed that the localization of FSTL1 expression was nearly coincident with α -SMA, which reflects activation of HSCs (**Figure 1C**). These results indicated that FSTL1 may play an important role in activation of HSCs and liver cirrhosis.

FSTL1 mAb inhibited the activation of mouse HSCs in vitro

α -SMA expression is closely correlated with HSC activation and is considered an HSC activation marker. We successfully isolated primary HSCs from mouse livers and subjected the cells to aberrant treatment for five to seven days. Primary HSCs were fixed at five days and stained for immunofluorescence imaging. The results showed that the cell morphology of HSCs treated with FSTL1 mAb were different from HSCs treated with control IgG. The FSTL1 mAb-treated HSCs showed a lower activation state compared with cells in the IgG group (**Figure 2**). The α -SMA expression levels in IgG-treated HSCs were significantly higher than those in the FSTL1 mAb-treated group (**Figure 2**).

FSTL1 mAb inhibited HSC migration but not proliferation

The CCK-8 assay revealed that the FSTL1 mAb exerted no influence on proliferation of primary mouse hepatic stellate cells (**Figure 3A**). However, primary mouse hepatic stellate cells incubated with FSTL1 mAb showed a lower migratory capacity compared

with IgG-treated HSCs (**Figure 3B**). Compared with quiescent HSCs, activated HSCs had higher capacities of migration, contraction, and vitality. From this experiment, we show the inhibitory effect of FSTL1 mAb on the migration of HSCs.

FSTL1 mAb attenuated liver fibrosis in mice

Sirius Red and Masson staining are considered as standard methods for evaluating liver fibrosis. The two methods can accurately show collagen accumulation in fibrotic liver tissues. From the Sirius Red staining, we revealed that FSTL1 mAb-treated fibrotic mice had attenuat-

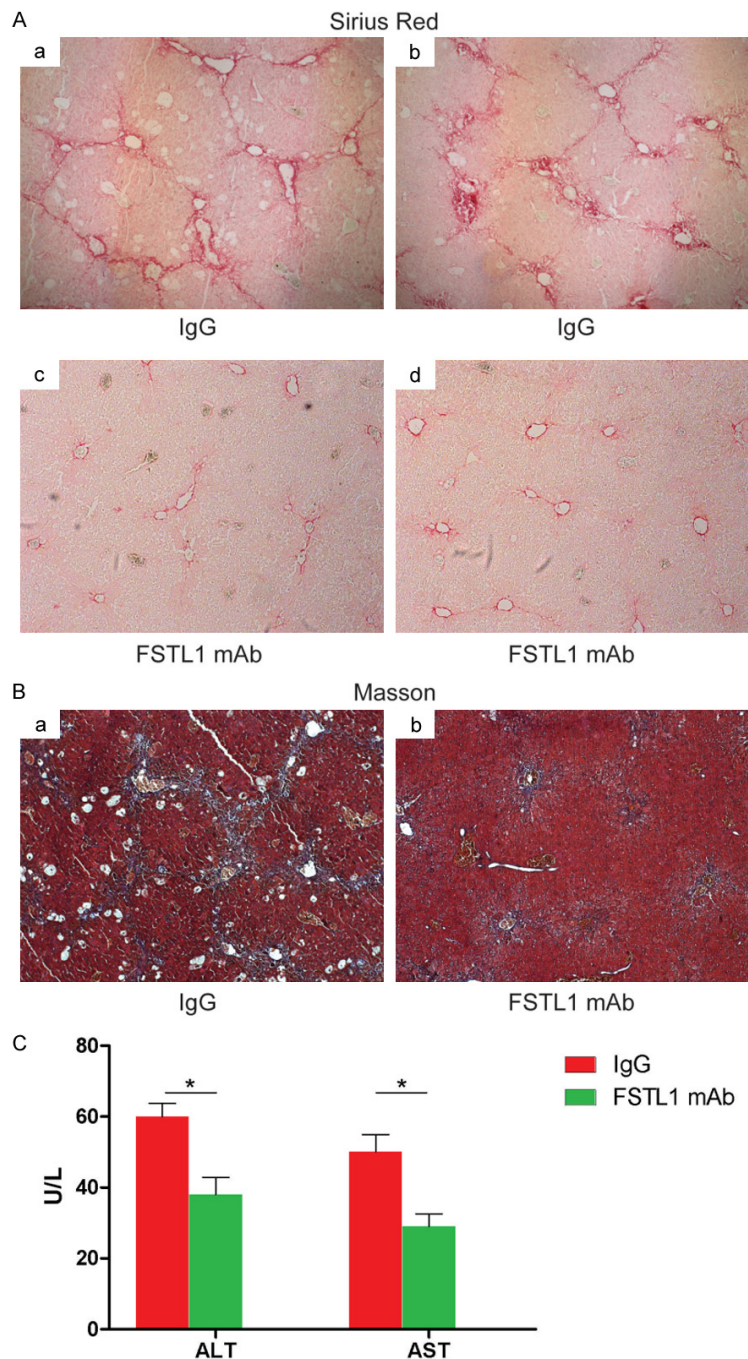


Figure 4. (A) Sirius Red staining showed that CCl₄-induced mice treated with an FSTL1 mAb (c, d) exhibited reduced collagen accumulation, and attenuation of liver fibrosis compared with CCl₄-induced mice treated with IgG (a, b) *in vivo*; (B) Masson staining showed that treatment of CCl₄-induced mice with the FSTL1 mAb (b) resulted in attenuation of liver fibrosis *in vivo*; (C) The ALT and AST detection results showed that treatment of mice with the FSTL1 mAb resulted in attenuation of liver injury caused by CCl₄.

ed liver fibrosis compared with that of IgG-treated fibrotic mice regardless of the induction conditions (i.e., CCl₄ vs. TAA) (Figures 4A, 5A).

From the Masson staining, we showed that collagen accumulation area in IgG-treated mice was significantly larger than that in the FSTL1 mAb-treated group (Figures 4B, 5B). Liver injury can usually be evaluated by detecting ALT and AST levels in the serum, which reflects the degree of liver injury in both humans and mice. The results showed that ALT and AST levels were significantly lower in FSTL1 mAb-treated mice compared with those in the IgG-treated group (Figures 4C, 5C). The results indicate that the degree of liver injury in FSTL1 mAb-treated mice was ameliorated compared with IgG-control group under CCl₄-induced or TAA-induced conditions (Figures 4C, 5C).

FSTL1 mAb inhibited Collagen1A1 and α -SMA expression in the fibrotic livers from CCl₄-or TAA-treated mice

We performed qPCR on CCl₄- and TAA-induced fibrotic mice liver tissues in order to detect expression of Collagen1A1 and α -SMA in the different groups. Both collagen1A1 and α -SMA are up-regulated in fibrotic and cirrhotic liver tissues and are positively correlated with the severity of liver fibrosis. In addition, α -SMA is an activation marker of HSCs, and collagen1A1 is strongly expressed in activated HSCs. Our results show that expression of α -SMA and collagen1A1 are down-regulated due to FSTL1 mAb treatment both in CCl₄-induced and TAA-induced fibrotic livers (Figure 6).

Moreover, we observed that expression of TGF-beta is down-regulated by FSTL1 mAb treatment in CCl₄-induced and TAA-induced fibrotic liver tissues (Figure 6).

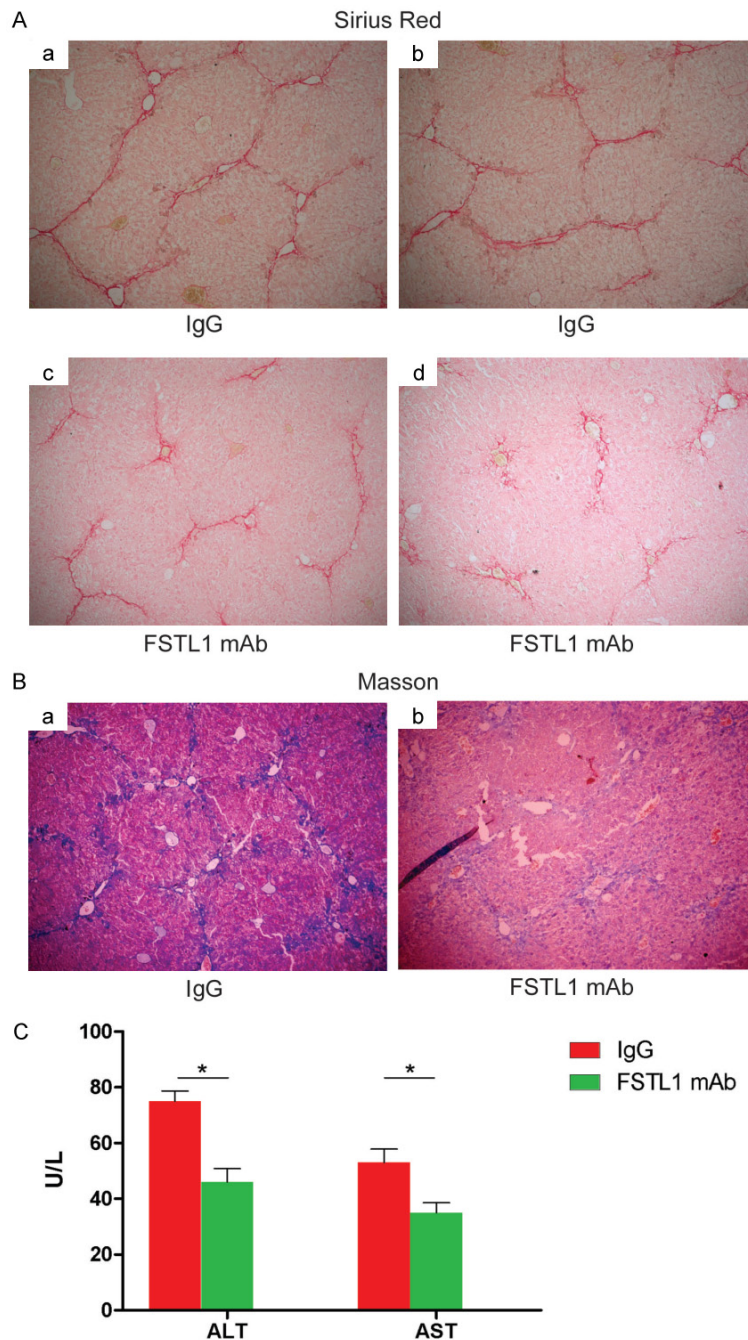


Figure 5. (A) Sirius Red staining showed that TAA-induced mice treated with an FSTL1 mAb (c, d) exhibited reduced collagen accumulation and attenuation of liver fibrosis compared with TAA-induced mice treated with IgG (a, b) *in vivo*; (B) Masson staining showed that treatment of mice with the FSTL1 mAb (b) resulted in attenuation of liver fibrosis induced by TAA *in vivo*; (C) The ALT and AST detection results showed that treatment of mice with the FSTL1 mAb resulted in attenuation of liver injury induced by TAA.

FSTL1 modulated HSC activation and liver fibrosis by facilitating TGF-beta signaling

We used primary mouse hepatic stellate cells to detect signaling. The hepatic stellate cells

treated with FSTL1 mAb showed lower phospho-Smad2 and phospho-Smad3 levels, which reflect TGF-beta signaling activity, compared with IgG-treated primary mouse hepatic stellate cells (**Figure 7**). This result indicates that FSTL1 may play a role in accelerating HSC activation and liver fibrosis by facilitating the TGF-beta signaling pathway.

Discussion

Liver fibrosis occurs during the early stages of liver cirrhosis and is mainly characterized by intrahepatic structural rearrangement, hepatic cell regeneration, and excess accumulation of collagen [8-10]. Liver fibrosis is a reversible process until the development of liver cirrhosis. Progressive liver fibrosis causes increased morbidity and mortality in patients with chronic liver disease [11, 12]. The therapeutic methods for liver fibrosis are limited and have minimal effectiveness to date [13].

Hepatic stellate cells (HSCs), also known as fat-storing cells, vitamin A storing cells [14], or Ito cells, play a central role in the progression of liver fibrosis [15, 16]. HSCs are a major source of extracellular matrix proteins during the liver fibrosis process [17, 18]. HSCs are intralobular connective tissue cells that presenting either myofibroblast-like [19] or lipocytephenotypes [20] when converting from a quiescent state to an activated state [21]. All of the profibrotic factors that target HSCs affect the progression of liver fibrosis [22-24]. Activated HSCs can lead to excess secretion of extracellular matrix, which participates in intrahepatic structural rearrangement [25] and contributes to the increase of hepatic sinusoid pressure via self-

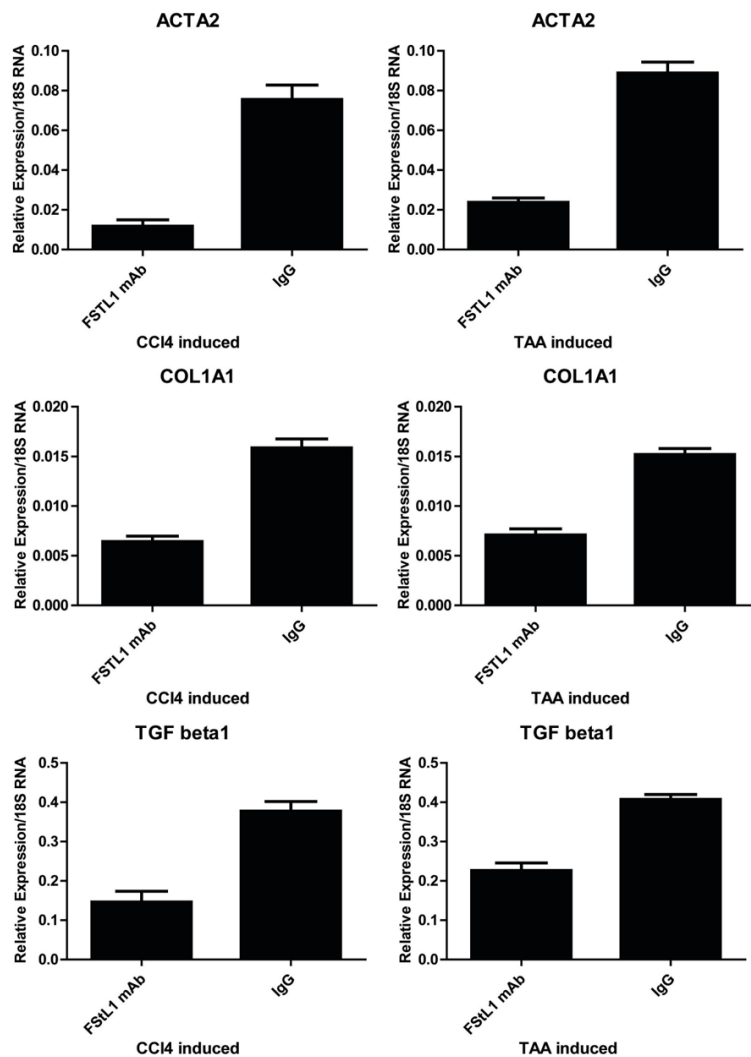


Figure 6. qPCR assay showed that in both CCI₄- and TAA-induced fibrotic liver tissues, the expression of α -SMA, collagen1A1, and TGF-beta are down-regulated in mice treated with an FSTL1 mAb compared with that in mice treated with IgG.

contraction [26-28]. Activation of HSCs is regulated by many cytokines such as tumor necrosis factor- α (TNF- α) [29], transforming growth factor-beta (TGF-beta) [30], insulin-like growth factor-1 (IGF-1) [31], hepatic growth factor (HGF) [32], platelet-derived growth factor (PDGF) [33], and endothelin-1 (ET-1) [34]. Among these cytokines, TGF-beta [35] and related cell signaling pathways play a central role in regulating the activation of HSCs [36]. TGF-beta and related cell signals can accelerate HSC activation [37] as well as enhance HSC proliferation [38], migration [39], contraction [40], and secretion of ECM proteins [41]. Therefore, TGF-beta and other related cell signals are ideal therapeutic targets in treating

liver fibrosis [42]. However, directly blocking TGF-beta signaling causes severe adverse effects in animal models and cannot be instituted in clinical trials [42].

FSTL1 was initially identified as a TGF-beta-induced gene that plays a key role during lung development [43]. FSTL1 was shown to regulate TGF-beta signaling and lead to fibroblast activation in pulmonary fibrosis [44]. Inhibiting FSTL1 with a neutralizing antibody attenuates lung fibrosis *in vivo* [44]. According to a previous report, FSTL1 can modulate myofibroblast activation by facilitating TGF-beta1 signaling in a BMP4-independent manner [44, 45]. In pulmonary fibrosis studies, FSTL1 was found to bind TGF-beta1 and positively regulate TGF-beta/Smad signaling *in vitro* [44, 45]. Pulmonary fibrosis studies have reported that FSTL1 promotes TGF-beta1-induced phosphorylation of Smad2/3, expression of α -SMA, and production of type I collagen [44, 45]. In addition, it has been reported that targeting FSTL1 results in the prevention of bone tumor metastasis. FSTL1 can also protect

cardiomyoblasts from injury by modulating Smad1/5/9 signaling [46]. FSTL1 is produced by cells of mesenchymal origin and has been shown to play an important role in inflammatory diseases [46, 47]. FSTL1 promotes the synthesis of pro-inflammatory cytokines and chemokines both *in vitro* and *in vivo* [47] and has been reported to mediate collagen-induced arthritis in mice and to be up-regulated in rheumatoid arthritis [46, 47]. Moreover, FSTL1 was shown to play an important role in cellular proliferation and apoptosis in lung cancer cells [46, 47]. The mechanism of pulmonary fibrosis is similar to that of liver fibrosis to some extent [47, 48]. These FSTL1 studies in pulmonary fibrosis and other diseases are enlightening. Because of

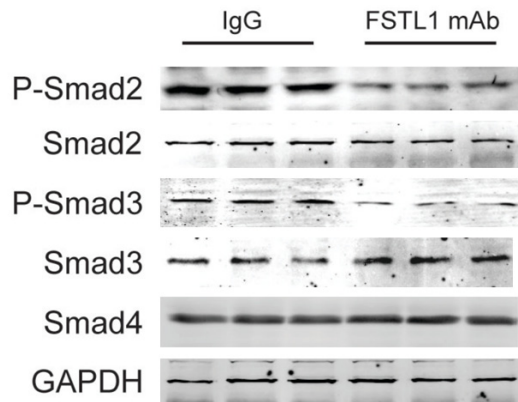


Figure 7. Western blot assay shows that treatment of mouse primary hepatic stellate cells with an FSTL1 mAb resulted in the inhibition of Smad2 and Smad3 phosphorylation.

the reported pro-fibrotic effect of FSTL1 in pulmonary fibrosis [46-49], we chose FSTL1 as a target for preventing liver fibrosis.

In our study, we present evidence for a pro-fibrotic effect of FSTL1 in liver fibrogenesis. Blocking FSTL1 with a neutralizing antibody remarkably reduced liver injury and attenuated liver fibrosis in the CCl₄-induced liver fibrosis mouse model. The activation of primary mouse HSCs treated with the FSTL1 neutralizing antibody was slower compared with that of the IgG-treated control group. We found that the FSTL1 mAb had no effect on HSC proliferation but reduced the migratory capacity of HSCs *in vitro*. We also showed that FSTL1 regulated liver fibrosis *in vivo* via TGF- β signaling. Activation of TGF- β signaling has already been shown to play a central role in regulating the progression of tissue fibrosis. According to previous reports and our study results, positive regulation of FSTL1 on TGF- β signaling can be recognized as the main mechanism to explain the progressive effect of FSTL1 on liver fibrosis.

In summary, our research supports a role for FSTL1 as a therapeutic target in attenuating liver fibrosis. In our study, no severe adverse effects were caused by inhibition of FSTL1 in either liver fibrosis mouse model. We confirmed positive effects of FSTL1 on HSC activation and liver fibrosis.

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Disclosure of conflict of interest

None.

Abbreviations

HSC, Hepatic Stellate cells; TNF- α , tumor necrosis factor- α ; TGF- β , Transforming growth factor- β ; IGF-1, Insulin-like growth factor-1; HGF, Hepatic growth factor; PDGF, Platelet derived growth factor; ET-1, Endothelin-1; FSTL1, Follistatin-like 1; BMP4, Bone Morphogenetic Protein4; α -SMA, α -Smooth Muscle Antigen; mAb, monoclonal Antibody; IHC, Immunohistochemical stain; TAA, Thioacetamide.

Address correspondence to: Ming-Ze Ma, Department of Infectious Diseases, Shandong Provincial Hospital Affiliated to Shandong University, 324 Jing Wu Road, Jinan 250021, Shandong Province, China. Tel: +86-13793179830, +86-531-68778250; Fax: +86-531-87902348; E-mail: qipengmay@163.com

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