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Original Article

Lyn kinase enhanced hepatic fibrosis by modulating the activation of hepatic stellate cells

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Abstract: The non-selectivity of tyrosine kinase inhibitors is the leading cause of drug withdrawals, and limits their application in anti-fibrosis. The role of Src tyrosine kinase Lyn in hepatic fibrosis remains elusive. In this study, we aimed to elucidate the role of Lyn kinase in the pathogenesis of hepatic fibrosis. Through examining Lyn-transgenic (Lyn TG) mice treated with CCl4 (carbon tetrachloride), we determined whether Lyn kinase is involved in the pathogenesis of hepatic fibrosis. On top of that, we also investigated the role of Lyn in the activation of hepatic stellate cells (HSCs) *in vitro*. Here, we showed that Lyn kinase was highly expressed in liver fibrosis upon CCl4 treatment. Meanwhile, Lyn TG mice showed that perivascular infiltration of mononuclear cells, and the markers of liver injury and hepatocytes apoptosis were significantly increased in liver tissue after CCl4 treatment. In comparison with wild-type (WT) mice after CCl4 treatment, we found that the fibrotic score in liver tissues of Lyn TG mice with the same treatment went up dramatically, so did the gene expression of fibrotic markers. In addition, over-expression of Lyn kinase drastically promoted the expression of HSCs activation markers *in vivo or in vitro*. Additionally, the Src-specific inhibitor PP2 significantly suppressed the increased expression of integrin αvβ3 in TGF-β1-induced HSCs, and PP2 further induced HSC apoptosis in TGF-β1-treated cells. These results collectively indicated that Lyn kinase is implicated in the pathogenesis of hepatic fibrosis through the modulating of HSC activation.

Keywords: Lyn kinase, hepatic fibrosis, hepatic stellate cells, integrin αvβ3

Introduction

Hepatic cirrhosis is the most common cause of death worldwide. Patients with cirrhosis are at the highest risk of developing hepatic carcinoma [1]. Reversible hepatic wound-healing responses are accompanied by the development of liver fibrosis and progressive loss of liver function [2]. Generally speaking, hepatic fibrosis is characterized by excessive accumulation and reduced degradation of extracellular matrix (ECM) [3]. ECM is produced by myofibroblasts, which serve as the principal effector cells of fibrogenesis and accumulate in the injured liver [4]. The accumulation of ECM proteins and the aggregation of collagen together contribute to eventually developing into cirrhosis [5]. At the same time, activation of hepatic stellate cells (HSCs) is one of the potential sources for these myofibroblasts [6]. HSCs are activated into proliferative and migratory myofibroblasts [7]. Morphological alteration of activated HSCs is associated with increased expression of markers such as collagen type I (Col1α1), smooth muscle actin α (SMAα) and desmin [8]. At present, effective strategies for hepatic fibrosis include inhibiting HSC activation and accelerating the clearance of activated HSCs [9].

It has been shown that members of the tyrosine kinase family play key roles in the control of cell survival, activation and differentiation. Tyrosine kinases include receptor tyrosine kinases and non-receptor tyrosine kinases. Tyrosine kinases are involved in the process of hepatic fibrosis. Previous studies have shown that tyrosine kinases were associated with liver fibrosis by regulating HSCs [10]. The expression levels of receptor tyrosine kinases including platelet-

derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor (VEGFR), and fibroblast growth factor receptor (FGFR) were significantly increased during the process of hepatic fibrosis development. And activated HSCs were associated with increased expression of many tyrosine kinases [11]. Src family tyrosine kinase, one member of the non-receptor tyrosine kinase family, modulates relative signaling pathways after activation in the cytoplasm using a different regulatory mechanism because of a lack of extracellular and transmembrane domains [12]. It was demonstrated that Src inhibitor almost completely prevented the VEGF-induced angiogenic pathway [13].

Recent evidence has suggested that tyrosine kinase inhibitors (TKIs) might be a novel approach to targeting activated HSCs. However, the hepatotoxicity of TKIs is the leading cause of drug withdrawals, thus limiting their application in anti-fibrosis. It is pivotal to develop selective TKIs that specifically target HSCs [10]. Lyn kinase was involved in hepatocytic apoptosis through regulating the activation of STAT1 [14]. However, the effects of Lyn kinase on liver fibrosis are less clear. In this study, we observed high expression of Lyn kinase in a CCl4-induced murine model of liver fibrosis. Over-expression of Lyn kinase improved liver damage and the degree of liver fibrosis by HSC activation in a murine model. *In vitro* studies, Over-expression of Lyn kinase in HSCs via lentivirus-delivery triggered HSC activation. Additionally, Lyn kinase inhibitor caused the apoptosis of activated HSCs. In a nutshell, our study confirmed that Lyn kinase leaded to liver fibrosis by activating HSCs, and the evidence indicated that Lyn might be a potential target for hepatic fibrosis.

Methods

Animals and experimental protocol

Lyn-transgenic mice (Lyn TG) on a C57BL/6J genetic background were acquired from kept in the Inflammation and Allergic Diseases Research Unit of Affiliated Hospital of Southwest Medical University (Luzhou, China). Genotyping was performed by western blotting. The same C57BL/6J genetic backgrounds as wild type (WT) mice were obtained from Tengxin biotechnology Co., Ltd. (Chongqing, China). The Lyn TG mice and WTC57BL/6 mice were maintained under specific pathogen-free conditions. All of the animal experiments used in this study were approved according to the guidelines of the Committee of Animal Experiments Center of Chongqing Medical University. Liver fibrosis was induced by intraperitoneal injection ofCCl4 (1 ml/kg, diluted 1:10 with corn oil) three times per week for 10 weeks. The mice were sacrificed 40 hours after the last CCl4 dose, as previously described [15].

Histology

Tissue specimens were fixed in 10% buffered formalin and embedded in paraffin. Sections (4 μm) of the specimens were stained with standard hematoxylin-eosin staining (H&E), Masson trichrome staining and Sirius red staining. H&E staining was used to observe morphology. Masson trichrome staining and Sirius red staining were used to visualize collagen and fibrotic fibers, respectively. The degree of fibrosis was scored using previously described methods [16]. A score ranging from 0 to 4 was applied to each observed histological assessment for fibrosis degree, with approximately 4 areas scored in total.

Cell culture, viral infection and transfection

A human hepatic stellate cell line (LX-2) was obtained from American Type Culture Collection (ATCC; Manassas, USA) and kept in the Inflammation and Allergic Diseases Research Unit of Affiliated Hospital of Southwest Medical University (Luzhou, China). LX-2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) at 37°C in 5% CO_2 and grown until 70% confluence. HSCs were activated by TGF-β1 (5 ng/ml) *in vitro* using previously described methods [17]. Infectious lentiviral vector-containing particles expressing Lyn were obtained from Cyagen Biosciences (Taichang, China). After the LX-2 cells reached 85% confluence, the medium was replaced with serum-free DMEM culture medium. The lentiviral vector expressing Lyn was used at 10^8 particles per well.

Immunohistochemical staining

Liver tissues were sectioned at 4 μm. The specimens including tissues or cells were fixed with ice-cold methanol and 0.25% Triton X-100 for 10 minutes at room temperature. After block-

Table 1. Primer sequences used in this paper

Gene	Symbol	Primer sequences
mLyn	Sense	TGCGAAGTTCCCTATCAAGTGG
	Antisense	CATCTGCGTTGGTTCTCCCT
mMMP2	Sense	AACGGTCGGGAATACAGCAG
	Antisense	CCACCCATGGTAAACAAGGC
mTGF-beta1	Sense	TTTGGAGCCTGGACACACAGTACA
	Antisense	TGTGTTGGTTGTAGAGGG CAAGGA
mCOL1α1	Sense	GAGGCCTCCCAGAACATCAC
	Antisense	CGATCTCGTTGGATCCCTGG
mGAPDH	Sense	CTTTGGCATTGTGGAAGGGC
	Antisense	CAGGGATGATGTTCTGGGCA
hLyn	Sense	CCCTGTGCATGGTGATGTTATT
	Antisense	GGCTTCTGTGGCTTGGGACTA
hGAPDH	Sense	CCAGCAAGAGCACAAGAGGA
	Antisense	GAGATTCAGTGTGGTGGGGG

ing with 1% BSA (Sigma-Aldrich) containing 0.05% Tween 20 for 1 hour, the specimens were then incubated with anti-Lyn antibody (Santa Cruz, CA, sc-15, AB_2281450), anti-integrin αvβ3 antibody (Bioss, China, bs-1310R), anti-α-SMA antibody (Bioss, China, bs-0189R), anti-desmin antibody (Bioss, China, bs-1026R), and anti-COL1α1 antibody (Bioss, China, bs-10423R). Isotype serum was used as a negative control. FITC-conjugated goat anti-mouse or TRITC-conjugated anti-mouse secondary Abs (Santa Cruz, CA, USA)was used to detect the primary Abs. 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI; Invitrogen, USA) was used for nuclear counter-staining. Tissue apoptosis were detected with a One Step TUNEL Apoptosis Assay Kit (C1089, Beyotime, China). The specimens were analyzed using a Leica SP5 confocal microscope (Leica, Germany).

Real-time quantitative PCR

Total RNA was isolated using TRIzol Reagent (Thermo Scientific, USA). RNA was quantified by an Epoch multi-volume spectrophotometer system (Biotech, USA). Then, cDNA was prepared using PrimeScript™ RT Master Mix (Takara, China). The mRNA level was determined by real-time PCR using a Light Cycler 480 (Roche Diagnostics, USA) with SYBR Advantage qPCR Premix (Clontech, USA). The ΔΔcycle threshold method was used for the calculation of relative changes in mRNA with normalization to GAPDH. The primer sequences are listed in **Table 1**, using previously described methods [18].

Tissue protein extraction, ELISA and western blot analysis

Specimens were extracted with radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate) with optional protease inhibitor cocktail (Fisher Scientific, USA). The concentrations of proteins were quantified using an Epoch multi-volume spectrophotometer system (Biotech, USA). For ELISA (enzyme linked immunosorbent assay), the cytokine levels of TGF-β1 were determined in triplicate from each animal using kits (Beijing Yonghui Biotechnology Co., Ltd., Beijing, China), according to the manufacturer's instructions. For western blot analysis, the extracts were loaded onto an SDS-PAGE gel for electrophoresis and then were transferred to microporous polyvinylidene difluoride (PVDF) membrane. After blocking with 5% skim milk in Tris-buffered saline/Tween 20, the PVDF membrane was incubated with anti-Lyn and anti-β-actinantibodies (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated IgG secondary antibodies (Cell Signaling Technologies, USA) were applied to detect antibody binding. The bands were detected using a PierceECL Western Blotting kit (Pierce Biotechnology, USA).

Apoptosis detection

LX-2 cells were seeded into 6-well plates and maintained in DMEM containing 10% fetal calf serum (FCS) at 37°C in 5% CO_2 and was grown until 70% confluence. The medium was then replaced with a new medium containing TGF-β1 (5 ng/ml) or PP2 (4-Amino-5-(4-chlorohyenyl)-7-(t-butyl) pyrazolo [3, 4-d] pyrimidine, 40 ng/ml). After treatment for 4 hours with PP2 inhibitors, the medium containing TGF-*β*1 (10 ng/ml) was added to be incubated overnight (72 hours) at 37°C. Apoptotic cells of LX-2 were detected with an FITC Annexin Apoptosis Detect kit (BD Pharmingen, USA) by Becton-Dickenson BD FACSAria II flow cytometry, according to the manufacturer's manual.

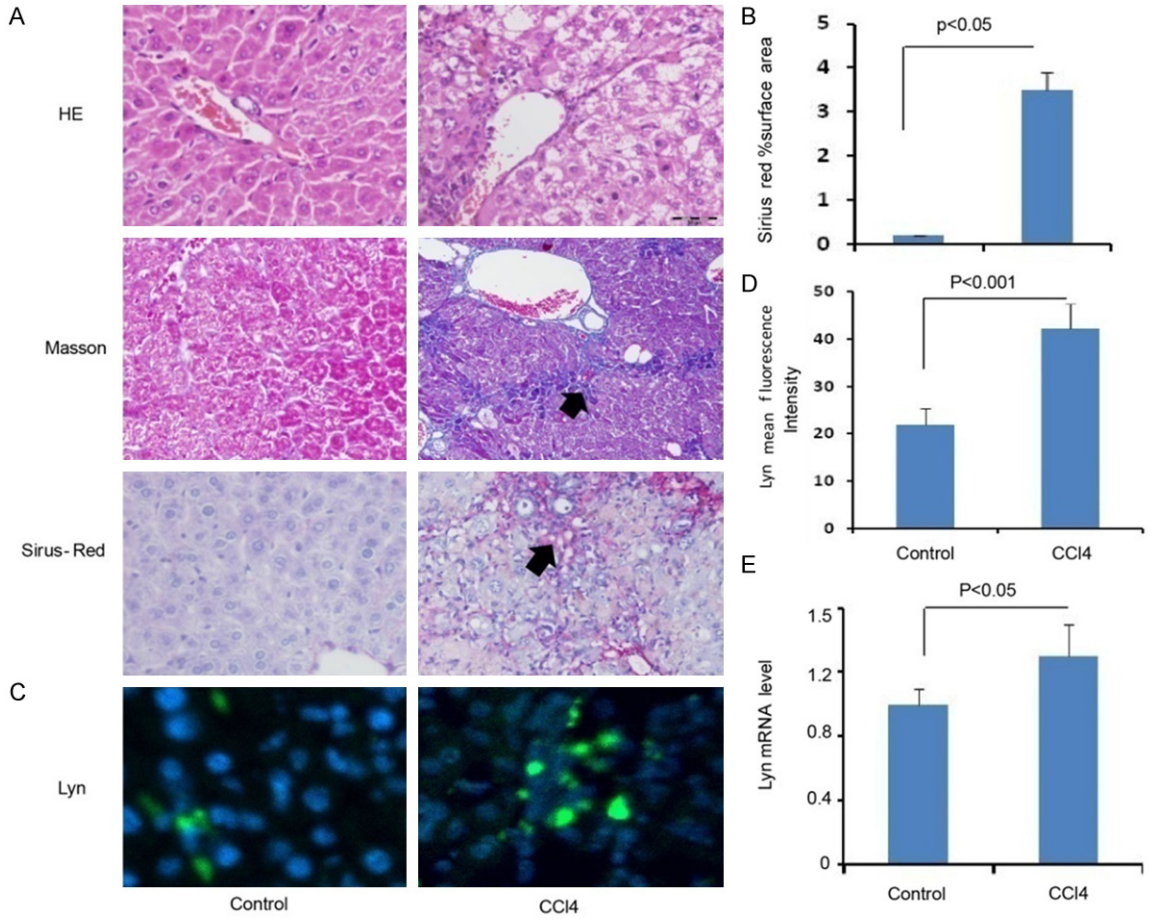

Figure 1. Lyn is highly expressed in CCl4-induced liver fibrosis. (A) H&E, Masson trichrome and Sirius red staining of collagen in liver sections from C57BL/6J wild-type livers. Arrowheads indicate positive areas of Masson trichrome staining and Sirius red staining (×200 magnifications). (B) The percentage of fibrotic area was calculated with Sirius red-positive fibrotic area. (C) Liver sections were stained with Lyn antibody. The specimens were detected using an SP5 Leica confocal microscope. (D) The fluorescence intensity of Lyn as shown in (C). (E) The transcription levels of Lyn were quantified by real-time PCR analysis. All of the data are presented as the *mean* ± *s.d*. One-way ANOVA (Tukey-Kramer post-test or Dunnett's T3) was used for Statistical analysis.

Statistical analysis

All of the statistical analyses were conducted using SPSS software, version 17.0 (Chicago, IL, USA). All of the data are expressed as the *mean* ± *s.d*. Statistical significance was evaluated using one-way ANOVA by Dunnett's test for multiple comparisons. Statistical differences between 2 groups were analyzed using Student's unpaired t test. The level of significance was defined as a *p value* less than 0.05.

Results

Lyn kinase is highly expressed in liver fibrosis upon CCl4 treatment

Previous studies have shown that CCl4 administration induced liver damage and liver fibrosis through various mechanisms [19]. Lyn kinase might be involved in STAT1-mediating hepatocytic apoptosis induced by hepatitis C virus and human immunodeficiency virus envelope proteins [14]. However, the roles of Lyn kinase in liver damage and liver fibrosis remained unclear. To investigate the role of Lyn kinase in CCl4-induced liver damage and liver fibrosis, we observed the morphology and degree of fibrosis in liver in CCl4-treated mice. After CCl4 injection, Sirius red staining showed collagen deposition and fibrotic fibers in CCl4-treated mice (**Figure 1A**). The Sirius red-positive fibrotic area was significantly greater in CCl4-treated mice than in control mice (**Figure 1B**, *P<0.05*). Confocal microscopic analyses revealed increased expression of Lyn kinase in CCl4-treated mice (**Figure 1C**). The fluorescence

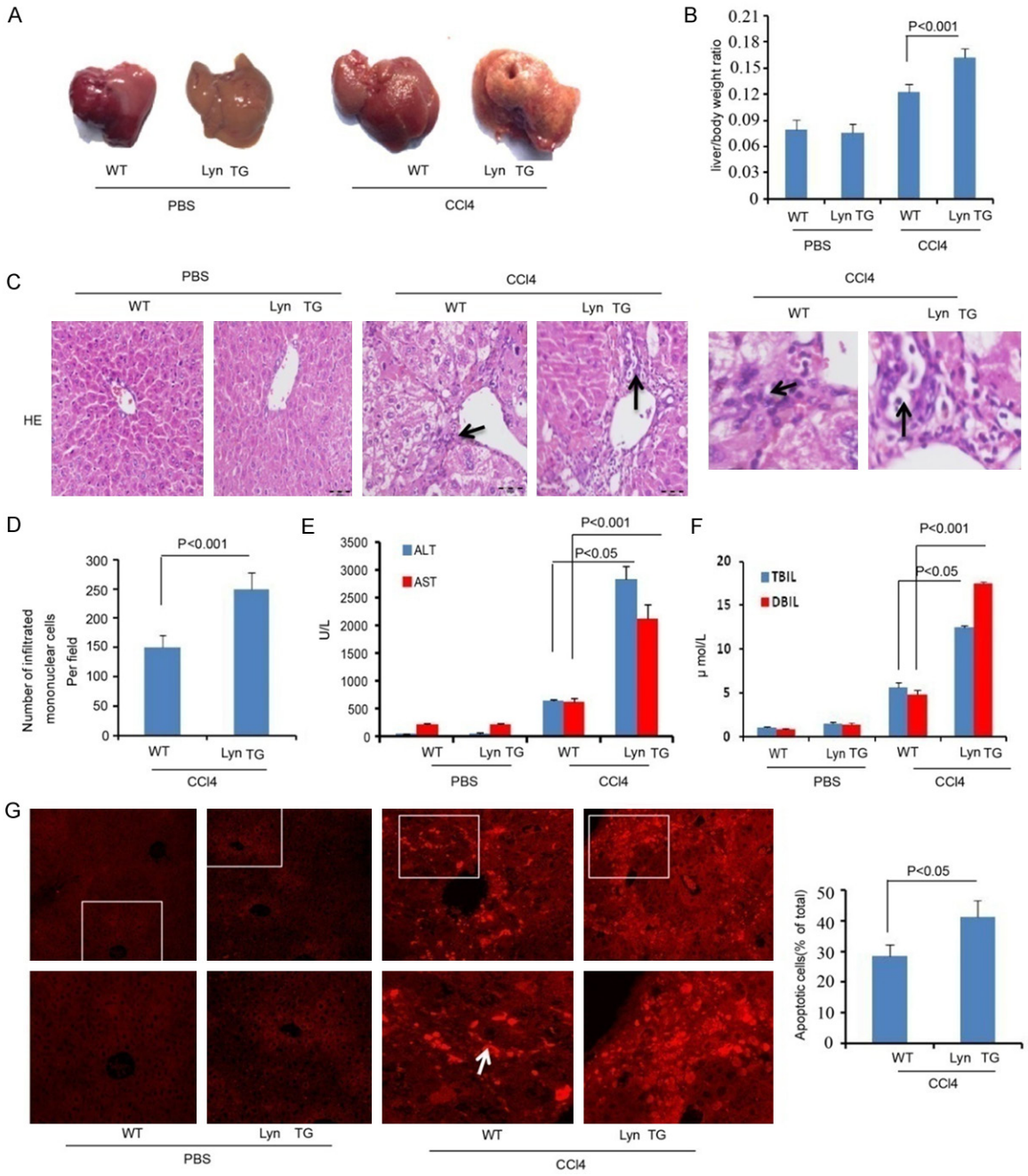

Figure 2. Increased liver injury and in hepatocyte apoptosis Lyn TG mice upon CCl4 treatment. A: Macroscopic images of mouse livers in WT mice and Lyn TG mice after administration of CCl4. B: The ratio of liver weight to body weight was calculated in WT mice and Lyn TG mice after administration of CCl4. C: H&E staining of the liver tissues from WT mice and Lyn TG mice after administration of CCl4. Arrowheads indicate the infiltration of mononuclear cells. D: The numbers of infiltrated cells were calculated in perivascular infiltration. E: Serum ALT and AST activities in WT mice and Lyn TG mice after administration of CCl4. F: Serum TBIL and DBIL in WT mice and Lyn TG mice after administration of CCl4. G: Hepatocyte apoptosis was quantified by TUNEL assay. Apoptotic cells were stained with cyanine 3 (white arrowheads, ×200 magnification). TUNEL-positive nuclei were calculated by counting nuclei in 6 random areas. All of the data are presented as the *mean* ± *s.d*. One-way ANOVA (Tukey-Kramer post-test or Dunnett's T3) was used for Statistical analysis.

intensities of Lyn increased by approximately 2.03-fold in CCl4-treated mice, compared to that in control mice (**Figure 1D**, *P<0.001*). Quantitative real-time PCR revealed a robust

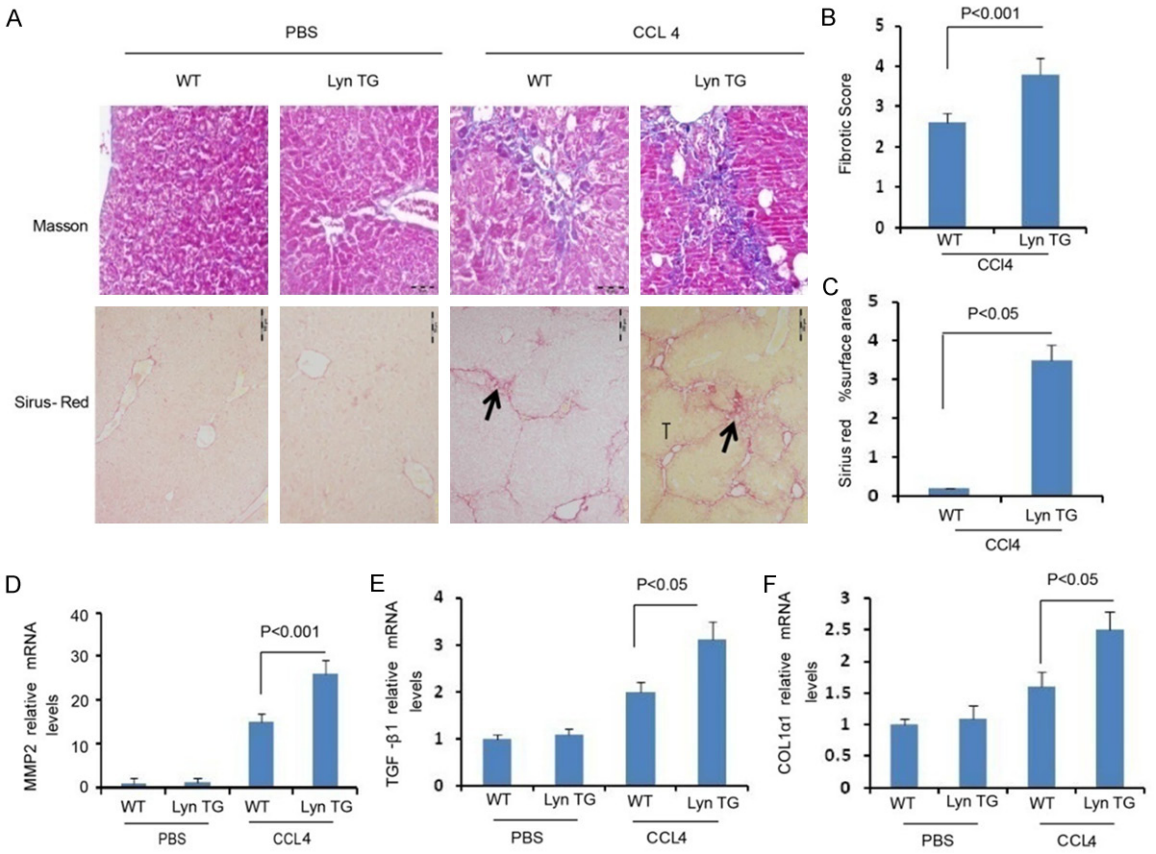

Figure 3. Increased liver fibrosis in Lyn TG mice upon CCl4 treatment. A: Masson trichrome and Sirius red staining of collagen in liver sections from WT mice and Lyn TG mice after administration of CCl4. Arrowheads indicate positive areas with Masson trichrome staining (×200 magnification) and Sirius red staining (×100 magnification). B: The degree of liver fibrosis was scored. C: The percentage of fibrotic area was calculated by the Sirius red-positive fibrotic area. D: The transcription levels of fibrotic genes MMP2 were quantified by real-time PCR analysis. E: The transcription levels of fibrotic genes TGF-β1 were quantified by real-time PCR analysis. F: The transcription levels of fibrotic genes COL1α1 were quantified by real-time PCR analysis. All of the data are presented as the *mean* ± s.*d*. One-way ANOVA (Tukey-Kramer post-test or Dunnett's T3) was used for Statistical analysis.

increase in Lyn transcript levels in CCl4-treated mice, compared to control mice (**Figure 1E**; *P<0.05*). These results indicated that Lyn kinase was highly expressed in liver fibrosis upon CCl4 treatment.

Lyn kinase increases liver injury upon CCl4 treatment

To further investigate the biological significance of Lyn kinase in liver fibrosis, we investigated liver damage using a transgenic approach. We generated Lyn TG mice. Following CCl4 injection, we examined the ratio of liver weight to body weight. CCl4 administration significantly increased the liver-to-body weight ratio in CCl4-treated Lyn TG mice and WT mice, compared to that in control Lyn TG mice and WT mice. In addition, the liver-to-body weight ratio of Lyn TG mice was significantly higher than that of WT mice after CCl4 injection (**Figure 2A** and **2B**; *P<0.001*). HE staining analyses revealed that the histological impact of Lyn transgenic, perivascular infiltration of mononuclear cells were increased in CCl4-treated Lyn TG mice, compared to that in CCl4-treated WT mice (**Figure 2C** and **2D**, arrowheads; **Figure 2D**, *P<0.001*). We also assessed liver injury by measuring serum ALT and AST levels in the mice. Compared with those in WT mice, serum ALT and AST levels in Lyn TG mice showed approximately 4.2-fold and 3.1-fold increases after administration of CCl4 (**Figure 2E**, *P<0.05* and *P<0.001*, respectively). After administration of CCl4, significant increases in TBIL and DBIL levels were also found in Lyn TG mice and WT mice, compared to those in control mice. As observed with Lyn TG mice, TBIL and DBIL levels showed

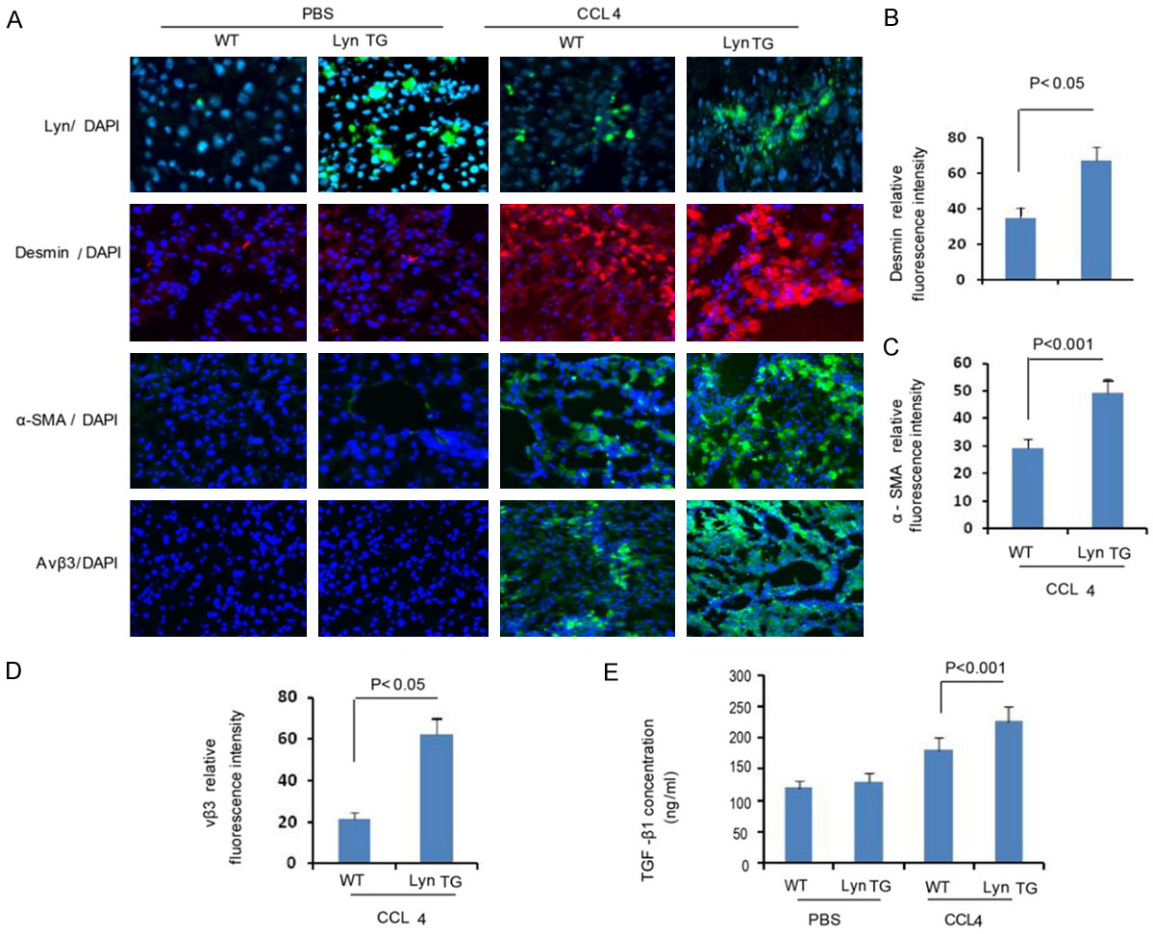

Figure 4. Increased activation of HSCs in Lyn TG mice upon CCl4 treatment. A: Representative immunofluorescence photomicrographs of HSC activation marker (Avβ3, α-SMA and desmin) expression in liver tissues from WT mice and Lyn TG mice after administration of CCl4 (×200 magnification). B: The fluorescence intensity of desmin in 8 random fields. C: The fluorescence intensity of α-SMA in 8 random fields. D: The fluorescence intensity of Avβ3 in 8 random fields. E: TGF-β1 levels in the liver tissues were determined by ELISA. All of the data are presented as the *mean* ± *s.d*. One-way ANOVA (Tukey-Kramer post-test) was used for Statistical analysis.

approximately 2.23-fold and 3.64-fold increases compared with those in WT mice after administration of CCl4 (**Figure 2F**; *P<0.05* and *P<0.001*, respectively). Apoptosis detection showed that CCl4-induced liver injury was associated with hepatocyte apoptosis in CCl4-treated Lyn TG mice and WT mice. Importantly, Lyn TG mice exhibited marked hepatocyte apoptosis compared to WT mice after administration of CCl4 (**Figure 2G**; *P<0.05*). These observations indicated that liver damage processes were enhanced in Lyn TG mice.

Lyn kinase increases liver fibrosis upon CCl4 treatment

To further evaluate the potential role of Lyn kinase in liver fibrosis, Masson trichrome and Sirius red staining were performed on liver sections. After CCl4 injection, Lyn TG mice and WT mice showed collagen deposition as revealed by both Masson trichrome staining and Sirius red staining. No liver fibrosis was observed in the control Lyn TG mice or WT mice (**Figure 3A**). However, CCl4-treated Lyn TG mice showed serious collagen deposition and fibrosis, compared to that in CCl4-treated WT mice. The fibrotic score increased by approximately 1.46-fold in Lyn TG mice, compared to that in WT mice after administration of CCl4 (**Figure 3B**, *P<0.001*). We also observed a significant increase in the Sirius red-positive fibrotic area in Lyn TG mice compared to that in WT mice after administration of CCl4 (**Figure 3C**, *P< 0.05*). COL1α1, α-SMA, and matrix metallo-

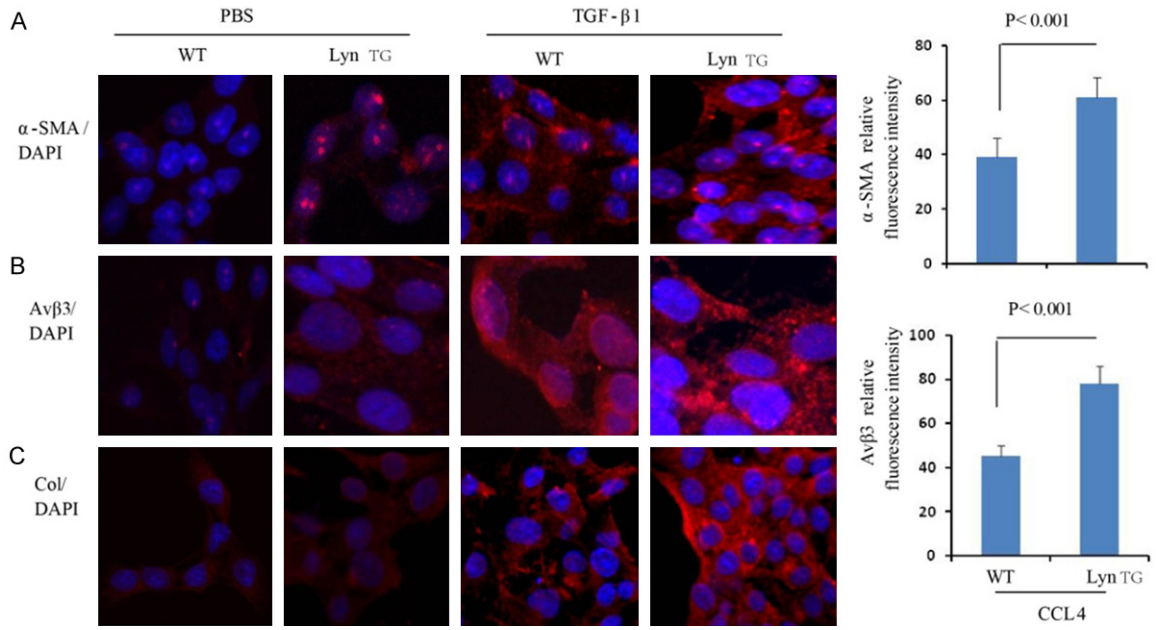

Figure 5. Lyn kinase increased the activation of HSCs *in vitro*. Immunohistochemical staining for HSC activation marker (Avβ3, α-SMA and COL1α1) expression in TGF-*β*1-induced LX-2 and $Lyn^{+/+}$ LX-2. A: Representative photomicrographs and fluorescence intensity of α-SMA in cells (×400 magnification). B: Representative photomicrographs and fluorescence intensity of Avβ3 in cells (×400 magnification). C: Representative photomicrographs and fluorescence intensity of COL1α1 in cells (×400 magnification). All of the data are presented as the *mean* ± *s.d*. One-way ANOVA (Tukey-Kramer post-test) was used for Statistical analysis. LX-2 cell lines transfected with lentiviral vector expressing Lyn were described as $Lyn^{+/+}$ cells. LX-2 cell lines transfected with control lentiviral vector were described as WT cells.

proteinase 2 (MMP2) are involved in major fibrotic genes [20]. TGF-β1 is also part of the critical gene expression of fibrotic markers [10]. To investigate the effects of Lyn in major fibrotic genes in CCl4-treatedmice, the transcript levels of TGF-β1 and MMP2 in liver tissue were determined by quantitative real-time PCR. The transcript levels of MMP2, TGF-β1 and COL1α1 showed significant increases in Lyn TG mice compared to those in WT mice after administration of CCl4 (**Figure 3D-F**; *P<0.001, P<0.001 and P<0.05,* respectively). These results indicated that over-expression of Lyn increased liver fibrosis in mice.

Lyn kinase increases integrin Avβ3, α-SMA and desmin in liver fibrosis upon CCl4 treatment

Previous studies have confirmed that activated HSCs play pivotal roles in the onset and progression of liver fibrosis. Integrin αvβ3 and desmin have been identified as biomarkers of activated HSCs [2]. We determined the levels of Lyn in liver tissue using western blotting. A predominant increase was observed in the levels of Lyn in Lyn TG mice, compared to WT mice (**Figure 4A**). Consistent with previous results, integrin αvβ3 and desmin were upregulated in CCl4-treated Lyn TG mice and WT mice. Surprisingly, we found that the immunofluorescence intensities of integrin desmin and αvβ3 showed 1.91-fold and 3.01-fold increases, respectively, in Lyn TG mice compared to those in WT mice after CCl4 treatment (**Figure 4B** and **4D**; *P<0.05, and P<0.001, respectively*). α-SMA expression is also regarded as a marker of hepatic stellate cell activation [1]. Confocal microscopic analyses revealed that the expression of α-SMA was predominantly increased in CCl4-treated Lyn TG mice and WT mice (**Figure 4C**, *P<0.05)*. We further observed that Lyn TG mice exhibited increased Lyn expression in the liver, compared to WT mice (Supplementary Figure 1A and 1B). Previous studies have shown that TGF-β1 induces the activation of HSCs [20]. To investigate whether Lyn regulates TGF-β1 expression in the pathogenesis of liver fibrosis, we determined the levels of TGF-β1 in liver tissue by ELISA. We also found that TGF-β1 was upregulated in CCl4-treated Lyn TG mice, com-

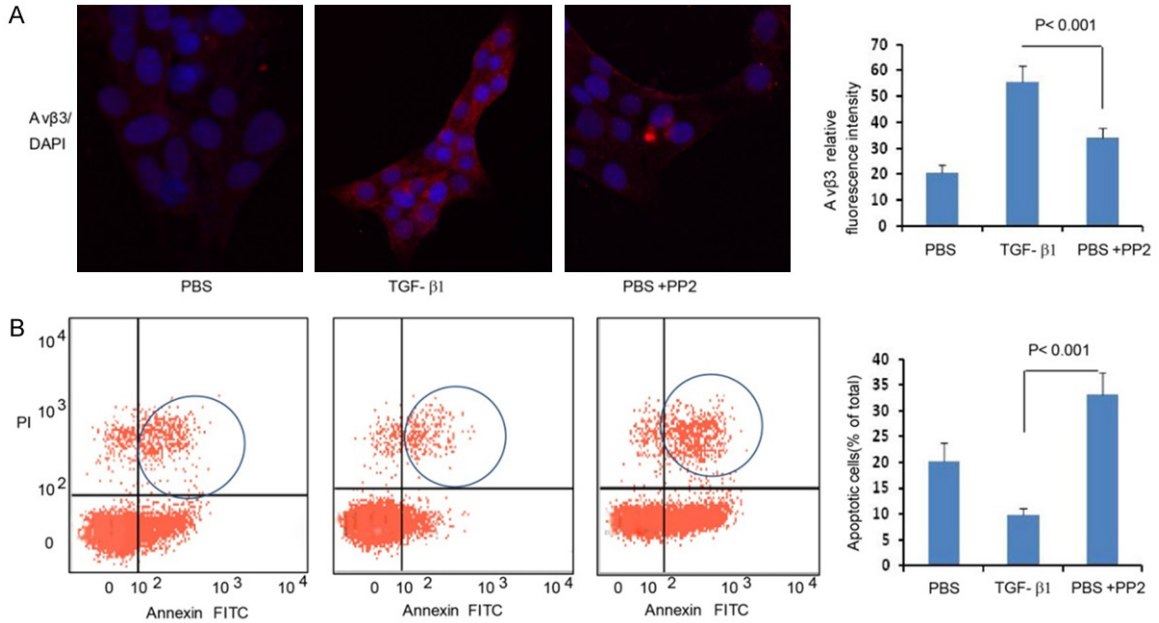

Figure 6. The Src-specific inhibitor PP2 suppressed HSC activation. A: Immunohistochemical staining for HSC activation marker (Avβ3) expression in TGF-β1-induced LX-2 and $Lyn^{+/+}$ LX-2 in the presence of PP2 (×400 magnification). The fluorescence intensity of Avβ3 in cells was measured. B: Apoptotic cells of LX-2 were detected by flow cytometry. All of the data are presented as the mean ± s.d. One-way ANOVA (Tukey-Kramer post-test) was used for Statistical analysis.

pared to that in CCl4-treated WT mice (**Figure 4E**, *P<0.001*). Altogether, these results indicated that over-expression of Lyn kinase escalated the activation of HSCs.

Lyn kinase increased TGF-β1-induced activation of HSCs

To further investigate the role of Lyn in TGF-β1-induced activation of HSCs, we assessed the biomarkers of activated LX-2 cell lines *in vitro*. Similar results were seen in LX-2 cell lines with TGF-β1 treatment, and confocal microscopic analyses found that the expression of α-SMA, integrin αvβ3 and COL1α1 were localized in cytoplasmic areas. LX-2 cell lines were transfected with lentiviral vector expressing Lyn at 10^8 particles per well in 6-well plates. The immunofluorescence intensities of α-SMA showed about 1.56-fold increases in $Lyn^{+/+}$ cells compared to that in WT cells after TGF-β1 treatment (**Figure 5A**, *P<0.001*). The immunofluorescence intensities of integrin αvβ3 increased by approximately 1.73-fold in TGF-β1-treated $Lyn^{+/+}$ cells and WT cells (**Figure 5B**, *P<0.001*). A predominant increase was also found in COL1α1 expression in TGF-β1-treated $Lyn^{+/+}$ cells, compared to TGF-β1-treated WT cells (**Figure 5C**). Together, these results showed that over-expression of Lyn enhanced TGF-β1-induced activation of HSCs *in vitro*.

The Src-specific inhibitor PP2 suppressed HSC activation

To further evaluate the function of Lyn in liver fibrosis, the activation of HSCs under the stimulation of TGF-β1 was analyzed using the Src-specific inhibitor PP2. The fluorescence intensities of integrin αvβ3 significantly decreased when treated with Src-specific inhibitor PP2, compared with that in TGF-β1-induced HSCs (**Figure 6A**, *P<0.001*). Previous studies have shown that suppressing the activation of HSCs might be a potential target for the treatment of hepatic fibrosis by modulating the phenotype of HSCs and inducing the apoptosis of HSCs [21]. To investigate the effects of Lyn deficiency in HSC apoptosis, we treated activated LX-2 cell lines with the Src-specific inhibitor PP2, and quiescent LX-2 cell lines with PBS served as the controls. Consistent with previous findings [22], TGF-β1 decreased LX-2 cell line apoptosis in TGF-β1-treated cells, compared with that in control cells. We next measured apoptosis in LX-2 cell line. We found that apoptotic cells in

the presence of PP2 in TGF-β1-treated cells was significantly increased compared with those in the absence of PP2 (**Figure 6B**, *P<0.001*). Hence, PP2 significantly suppressed LX-2 cell line activation and increased its apoptosis.

Discussion

Hepatic cirrhosis is an increasingly common cause of morbidity and mortality in more developed countries. One-year survival in patients with cirrhosis ranges from 1% to 57%, depending upon the clinical stage. Hepatic cirrhosis has been regarded as a single and terminal disease stage, with an inevitably poor prognosis [23, 24]. Hepatic cirrhosis has also been regarded as a developing and progressive stage of liver fibrosis. Therefore, prevention and early intervention should be new concepts in the management of patients with cirrhosis. In the present study, the principal findings provided new insights regarding the role of Lyn kinase in liver fibrosis. On the basis of that, our data showed that Lyn kinase might contribute to hepatic fibrogenesis by promoting the activation of HSCs.

The degree of liver fibrosis was conventionally elevated by means of histological examination using Masson's trichrome stain, accompanied by hepatic expression of α-sma and evaluation of the ALT/AST serum levels. Collagen proportionate area is superior to other histological methods for classifying cirrhosis and determining prognosis. Quantification of fibrosis with collagen proportionate area has important prognostic implications [25]. In this study, we used a CCl4-induced injury model of liver damage in mice. The model indicated that liver tissues showed collagen deposition in CCl4-induced mice. Analysis of liver fibrotic tissue showed that Lyn kinase was increased compared with the control liver tissues. Hence, the over expression of Lyn kinase was associated with hepatic fibrogenesis.

As a key upstream kinase, Lyn mediated nerve injury [26]. Lyn played a critical role in the process of cerebral ischemia/reperfusion injury through integrin αvβ3 [27]. After demonstrating the relevance of Lyn kinase in liver fibrosis, we utilized Lyn TG mice to investigate how Lyn kinase drives fibrosis development. Firstly, we evaluated the histological impact of Lyn over-expression on liver injury, and CCl4-treated Lyn TG mice exhibited a significant increase in perivascular infiltration of mononuclear cells. Then, we also observed increased liver injury in CCl4-treated Lyn TG mice. Previous studies have shown that liver fibrosis is a wound-healing process in response to reversible injury to hepatocytes. Ongoing hepatocyte apoptosis or necrosis leads to HSCs transforming to myofibroblasts [28]. Here, we further identified that Lyn over-expression significantly increased hepatocyte apoptosis of CCl4-induced liver injury in mice. Our data revealed a novel role for Lyn in a liver injury mouse model. Other than that, we further found that over-expression of Lyn was associated with more pronounced liver injury in CCl4-treated Lyn TG mice, compared to CCl4-treated WT mice.

Fibrosis has been linked to wound healing and repairing. The longer that damage persists, the more ECM is deposited [29]. Some reports have suggested that Src family kinases were involved in upstream signaling of DNA damage checkpoints [30]. As a member of the Src family, Lyn kinase can regulate epithelial-mesenchymal transition in breast cancer. The Lyn gene was also found to be the key nodule in wound healing [31]. So, we further investigated whether the pathological process of liver fibrosis could also be affected by Lyn kinase. In the current study, we showed that Lyn over-expression enhanced the collagen proportionate area in CCl4-induced liver injury in mice. Moreover, fibrotic gene expression was partly increased by Lyn over-expression in CCl4-induced liver injury in mice. It has been known that TGF-β1 is a key player in the activation of myofibroblasts and in proliferation control in liver disease. During liver injury and the regeneration process, HSCs do express and secrete TGF-β1, thus inducing the activation of quiescent HSCs [32]. Therefore, these data indicated that Lyn over-expression increased TGF-β1 in liver injury.

HSC accounts for 5%-8% of the cells in the liver. The activated HSC is the crucial cell type responsible for the excess deposition of collagen [33, 34]. HSCs could be identified as a base in the expression of desmin in the quiescent state and α-SMA in the activated state in animal models [34]. Desmin, belonging to the type III intermediate filament protein family,

maintains the stability of the cellular structure. The present study indicated that Lyn over-expression increased the expression of desmin compared with that in WT mice. These findings were consistent with previous studies in which the expression of desmin was strongly increased during HSC activation [35, 36]. Previous studies have confirmed that activated HSCs were the main cell types expressing integrin αvβ3, which was upregulated and was correlated strongly with the progression of liver fibrosis [37]. Integrin αvβ3 is a therapeutic candidate for liver fibrosis so that neutralization of integrin αvβ3 prevented the activation of the PI3K/pAkt/NFκB-signaling cascade and collagen-I production [38]. We further identified that Lyn over-expression significantly affected the expression of integrin αvβ3 in CCl4-treated Lyn TG mice. Likewise, we also found that the activated markers of HSCs (desmin, α-SMA and integrin αvβ3) were associated with TGF-β1 upregulation in CCl4-treated Lyn TG mice.

Lyn tyrosine kinase electively associates with integrin αvβ3. Knockdown of Lyn resulted in oligodendrocyte apoptosis [27]. Lyn is required to drive integrin αvβ3-dependent progenitor proliferation of oligodendrocytes [39]. In injured livers, integrin αvβ3 regulates HSCs, promoting HSC proliferation while also protecting cells [40]. To attempt to clarify the impact of Lyn in HSC activation, Lyn kinases were over-expressed with Lyn lentivirus delivery. Notably, we confirmed that the over-expression of Lyn increased the expression of α-SMA and integrin αvβ3 in TGF-β1-induced Lyn TGHSCs. We also observed a robust increase in the levels of collagen-I in TGF-β1-induced Lyn TGHSCs. In Lyn-deficient cells, increased integrin αvβ3 was significantly reduced by inhibiting Lyn with PP2 in TGF-β1-induced HSCs. Our findings indicated that Lyn kinases could be involved in the impact of HSC activation on hepatic fibrosis.

Collectively, we conclude that over-expression of Lyn in mice was involved with exacerbated liver injury and hepatocyte apoptosis. We further found that over-expression of Lyn significantly increased the degree of liver fibrosis associated with HSC activation *in vivo*. Our results also confirmed that Lyn increased TGF-β1-induced HSC activation and the inhibitor of Lyn suppressed TGF-β1-induced HSC activation *in vitro*. Therefore, our studies indicated that Lyn kinases could be a novel therapeutic candidate for treating hepatic fibrosis.

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Disclosure of conflict of interest

None.

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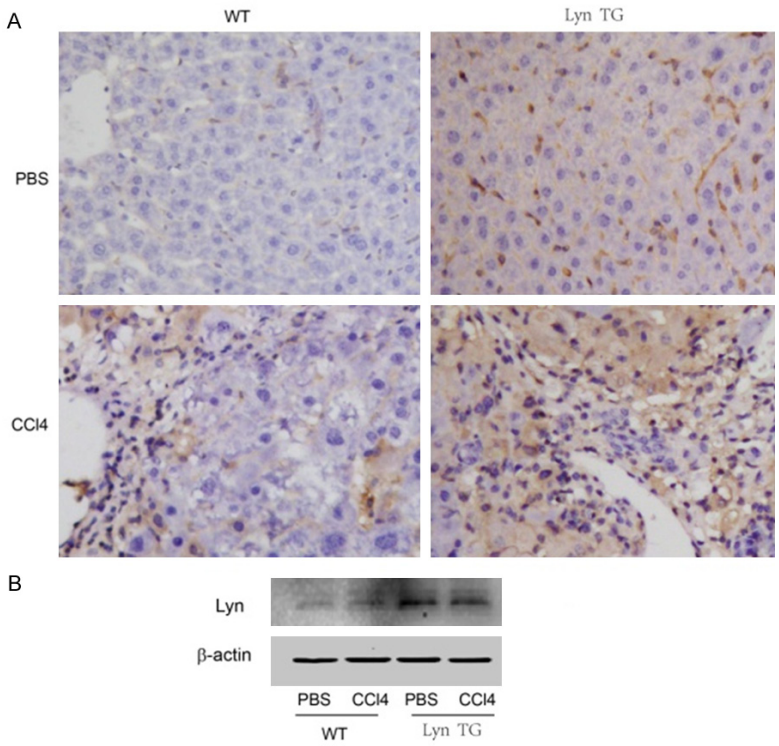

Supplementary Figure 1. The expression of Lyn in liver tissue in mice. A: Representative immunohistochemical staining for Lyn in the liver tissues from WT mice and Lyn TG mice after administration of CCl4 (original magnification: 200×). B: Representative Western blots of Lyn and β-actin in liver tissue.