PTP1B confers liver fibrosis by regulating the activation of hepatic stellate cells

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Abstract

Liver fibrosis is a reversible wound-healing response to chronic hepatic injuries. Activation of hepatic stellate cells (HSCs) plays a pivotal role in the development of hepatic fibrosis. The currently accepted mechanism for the resolution of liver fibrosis is the apoptosis and inactivation of activated HSCs. Protein tyrosine phosphatase 1B (PTP1B), a prototype of non-receptor protein tyrosine phosphatase, is proved to be a vital modulator in cardiac fibrogenesis. However, the precise role of PTP1B on liver fibrosis and HSC activation is still unclear. **Our study showed that the expression of PTP1B was elevated in fibrotic liver but reduced after spontaneous recovery.** Moreover, stimulation of HSC-T6 cells with transforming growth factor- β 1 (TGF- β 1) resulted in a dose/time-dependent increase of PTP1B mRNA and protein. Co-incubation of HSC-T6 cells with PTP1B-siRNA inhibited the cell proliferation and activation induced by TGF- β 1. Additionally, both mRNA and protein of PTP1B were dramatically decreased in inactivated HSCs after treated with adipogenic differentiation mixture (MDI). Overexpression of PTP1B **hindered** the inactivation of HSC-T6 cells induced by MDI. These observations revealed a regulatory role of PTP1B in liver fibrosis and implied PTP1B as a potential therapeutic target.

Keywords: Protein tyrosine phosphatase 1B (PTP1B), Liver fibrosis, Hepatic stellate cell (HSC), activation, inactivation

Abbreviations

HSCs, hepatic stellate cells; PTP1B, protein tyrosine phosphatase 1B; TGF- β 1, transforming growth factor- β 1; MDI, adipogenic differentiation mixture; ECM, extracellular matrix; qHSCs, quiescent HSCs; α -SMA, α -smooth muscle actin; coll α 1, type I collagen; aHSCs, activated HSCs; iHSCs, inactivated phenotype; PTPN1, protein tyrosine phosphatase non-receptor type 1; PTPs, protein tyrosine phosphatases; NAFLD, non-alcoholic fatty liver disease; ALT, alanine aminotransferase; AST, aspartate aminotransferase;

1. Introduction

Hepatic fibrosis, characterized by progressive deposition of extracellular matrix (ECM) components, is a common pathological consequence of chronic liver injuries induced by hepatitis virus, toxins, drugs, ethanol, auto-immune disorders and so on. The persisting fibrogenesis is widely recognized as the major driving force that eventually leads to liver cirrhosis and hepatic failure. Although experimental studies have revealed targets to prevent fibrosis progression in rodents, **there is still a long way to find standard treatment for liver fibrosis in human.** (Bataller and Brenner, 2005; Novo *et al.*, 2014). Numerous studies have **concluded** that the activation of quiescent hepatic stellate cells (HSCs) is **the** defining event in the development and maintenance of liver fibrosis (Hellerbrand, 2013b). Under physiological conditions, HSCs are located in the subendothelial space of Disse and exhibit a quiescent phenotype, characterized by storage of retinoid and lipid droplets and expression of adipogenic genes (Puche *et al.*, 2013). Upon liver insults, quiescent HSCs (qHSCs) lose the lipid droplets and obtain a myofibroblastic phenotype with remarkable up-regulation of α -smooth muscle actin (α -SMA) and ECM proteins enriched in type I collagen (coll α 1), which is referred to as the activation of HSCs (Mallat and Lotersztajn, 2013). **The ongoing accumulation of collagens distorted** the parenchyma and vascular architecture of liver, **and eventually impaired its differentiated function.** Therefore, **characterizing the underlying mechanisms and inhibiting the activation of HSCs are of great significance for attenuating liver fibrosis.** Many researchers have clearly demonstrated that hepatic fibrosis is reversible. Withdrawal of the etiological source of the chronic injury could reverse hepatic fibrosis in patients with Hepatitis C, Hepatitis B and NASH and in experimental rodent fibrosis models (Kisseleva and Brenner, 2013; Liu *et al.*, 2013; Ramachandran *et al.*, 2015). The currently accepted mechanism for the resolution of liver fibrosis is the apoptosis of activated HSCs (aHSCs) (Iredale *et al.*, 1998). However, some studies have indicated that half of the activated HSCs escape apoptosis during regression of liver fibrosis but revert to a

previously unrecognized inactivated phenotype (iHSCs) with decreased fibrotic genes, such as collagens, α -SMA and so on.(Kisseleva *et al.*, 2012; Troeger *et al.*, 2012). Hongyun She and co-workers (She *et al.*, 2005) have demonstrated that treatment of activated HSC with an adipocyte differentiation cocktail (MDI) could induce morphologic and biochemical reversal of activated HSC to quiescent HSC. These findings confirm the plasticity of HSCs and establish a new framework model for identifying novel molecular mechanisms for the resolution of liver fibrosis (Tsukamoto, 2005; Zhang *et al.*, 2014).

Protein tyrosine phosphatase 1B (PTP1B), encoded by the protein tyrosine phosphatase non-receptor type 1 (PTPN1) gene, is a ubiquitous and abundant intracellular enzyme and the prototype of non-receptor protein tyrosine phosphatases (PTPs) (Charbonneau *et al.*, 1989; Brown-Shimer *et al.*, 1990). For a long time, it has been known as a pivotal regulator and therapeutic target in metabolic diseases such as obesity and type 2 diabetes by promoting insulin resistance, lipogenesis and endoplasmic reticulum stress(Bence, 2010). Consistently, many pharmacological inhibitors of PTP1B have been identified and have elicited promising properties for the management of these conditions (Zhang and Lee, 2003; Panzhinskiy *et al.*, 2013). Recently, it has received renewed attention in organ fibrotic diseases. Pharmacological inhibition or gene deletion of PTP1B significantly prevented the deposition of collagen in heart and reduced cardiac fibrosis and hypertrophy (Gomez *et al.*, 2012). However, whether PTP1B is implicated in hepatic fibrogenesis remains unclear and the underlying functional role of PTP1B in regulating cell fate of HSCs is obscure. While, by integrating the recent studies, we know that PTP1B presents

notable pharmacological functions in the development of acetaminophen-induced liver injury (Sangwan *et al.*, 2006; Mobasher *et al.*, 2013), non-alcoholic fatty liver disease (NAFLD)(Shimizu *et al.*, 2003; Ugi *et al.*, 2009) and even hepatocellular carcinoma(Zheng *et al.*, 2012), implying its essential roles in liver diseases. Hence, in the present study, we tried to investigate the link between PTP1B and liver fibrosis. For this purpose, we analyzed the expression of PTP1B in fibrotic and recovery liver tissues and in activated and inactivated HSCs, respectively. Functionally, we probed the role of PTP1B in the proliferation and activation of TGF- β 1-treated HSCs and effect of PTP1B on the inactivation of aHSCs induced by MDI *in vitro*. These studies will update our understanding of PTP1B in liver fibrosis and provide valuable information about developing therapeutic strategies against fibrosis.

2. Materials and methods

2.1. Animals, mouse models of liver fibrosis

Normal male KunMing (4-week-old) mice were obtained from the Experimental Animal Center of Anhui Medical University used for CCl₄ liver fibrosis model. The animal experimental procedures were reviewed and approved by the University Animal Care and Use Committee. Mice were randomly divided into three groups (twelve mice per group) including control group, model group and recovery group. Hepatic fibrosis was generated by biweekly intraperitoneal injection of CCl₄ (20% in olive oil) at a dose of 0.01 ml/g/mouse for 4 weeks. Control mice were treated intraperitoneally with the same volume of olive oil at the same time intervals. The reversal model was made by 6 weeks' normal feeding after CCl₄ cessation.

2.2. Cell culture

The HSC-T6 cell line was obtained from Shanghai Fumeng Gene Biological Corporation (Shanghai, China). The HSC-T6 cells were maintained in Dulbecco's

modified Eagle's medium (DMEM, USA) supplemented with 10% fetal bovine serum, 100U/ml penicillin, 100mg/ml streptomycin, and 2mM_L-glutamine and incubated at 37°C at an atmosphere of 5% CO₂.

2.3. Total RNA isolation and real-time quantitative PCR (RT-qPCR)

Total RNA was isolated from liver tissues and HSC-T6 cells using TRIZOL reagents (Invitrogen, USA), and the first-strand cDNA was synthesized using Thermoscript RT-PCR synthesis kit (Fermentas, Canada) according to the manufacturer's instructions. Real-time quantitative PCR analyses for mRNA of α -SMA, Coll α 1, PTP1B and β -actin were carried out under a standard protocol using the following primers listed in **Table 1**. PCR was performed at 94 °C for 5 min, followed by 35 cycles of amplification at 94 °C for 40 s, 51 °C or 58 °C for 40 s and 72 °C for 1 min. The band intensities were measured by densitometry and were normalized with β -actin. The fold-change for mRNA relative to β -actin was determined by the formula: $2^{-\Delta\Delta C_t}$. All experiments were performed in triplicate and repeated at least three times.

2.4. RNA interference analysis.

Small interfering RNA (siRNA) oligonucleotides against PTP1B gene or scrambled sequences were designed and synthesized by the Shanghai Gena Pharma Corporation (Shanghai, China). The sequences were as follows: PTP1B-siRNA (sense: 5'-GCCUCUUACUGAUGGACAATT-3' and antisense: 5'-UUGUCCAUCAGUAAGAGGCTT-3'); Scrambled-siRNA (sense: 5'-UUCUCCGAACGUGUCACGUTT3-3' and antisense: 5'-ACGUGACACGUUCGGAGAATT-3'). HSC-T6 cells were cultured in antibiotics-free DMEM for 24h and then subjected to transfection with siRNA using LipofectamineTM2000 (Invitrogen, USA) according to the manufacturer's protocol. Knockdown efficiency was determined by RT-qPCR and Western blot analysis. The culture medium was changed 6h after transfection, and TGF- β 1 (Peprotech, USA) was added at a concentration of 10ng/ml.

2.5. Cell proliferation assays

Cell proliferation assay was determined by standard 3-(4, 5-dimethylthiazol-2-yl)-2, 4-diphenyl-tetrazolium bromide (MTT) assay. Briefly, the cells were seeded at a density of 5×10^3 cells per well in 96-well culture plates and incubated overnight to allow for cell attachment, after which they were transfected with PTP1B-siRNA or Scrambled-siRNA as described above. After culture, 5mg/ml MTT (Sigma, USA) was added and incubated at 37 °C for another 4 h; thereafter, the medium was replaced and the formazan crystals were dissolved in 150µl dimethyl sulfoxide(DMSO). The optical density (OD) was determined with Thermomax microplate reader (Bio-Tek EL, USA) at 490 nm wavelength. All experiments were performed in triplicate and repeated at least three times.

2.6. Cell cycle and apoptosis analysis

For cell cycle analysis, we performed Cell Cycle and Apoptosis Analysis Kit (Beyotime, China). The cells were washed for three times by cold PBS, and then cells were fixed in 70% ethanol in PBS at -20 °C for 24 h. After fixation, cells were washed with cold PBS and stained with 0.5 ml of propidium iodide (PI) staining buffer at 37 °C for 30 min in the dark. Flow cytometric analysis (FACS) was performed on a BDLSR (BD Biosciences), and data fitting was made with the Flow data analysis software package (TreeStar, USA). All experiments were performed in triplicate and repeated at least three times.

For apoptosis analysis, quantification of apoptotic cells was performed with Annexin-V-FITC Apoptosis Detection Kit (BestBio, China) according to the manufacturer's instruction. Cells were discriminated into viable cells, dead cells, early apoptotic cells and apoptotic cells. Analyses were performed on BD FACSVerse (BD Biosciences). Apoptosis was analyzed with FACS using FlowJo data analysis software package (TreeStar, USA). The percentage of apoptotic cells were compared to control groups from each experiment. All experiments were performed in triplicate and repeated at least three times.

2.7. MDI treatment

In vitro activation of HSC-T6 was achieved by co-culturing HSC-T6 cells with TGF- β 1 (10ng/ml) for 48h. After that, they were treated with the adipogenic differentiation mixture (MDI, 0.5mM isobutylmethylxanthine (Sigma-Aldrich, St Louis, MO, USA), 1 μ M dexamethasone(Sigma-Aldrich, St Louis, MO, USA), and 1 μ M insulin(Sigma-Aldrich, St Louis, MO, USA)) and incubated for 48h as described by She H *et al*(She *et al.*, 2005).

2.8. Transfection of HSC-T6 cells with PTP1B plasmid.

Plasmid construction pCDNA3.1 Flag-rat-PTP1B was obtained from Addgene bank (Addgene plasmid#35988). The plasmid pCDA3.1-PTP1B was first constructed by Anna Huttenlocher and colleagues (Cortesio *et al.*, 2008). We acknowledge the principal investigators sincerely. HSC-T6 cells were activated by TGF- β 1 (10ng/ml, 24h), and then were transfected with pCDNA3.1 Flag-rat-PTP1B to induce the ectopic expression of PTP1B, and empty pCDNA3.1 vector (pCDNA3.1-control) as a control. After 6h, the culture medium was removed and replaced by MDI.

2.9. Western blot analysis

Cells were lysed with RIPA lysis buffer (Beyotime, China) according to the manufacturer's instruction. Whole extracts were prepared, and protein concentration was detected using a BCA protein assay kit (Boster, China). Total protein (30 or 50 mg) from samples was separated by sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a PVDF (polyvinylidene fluorid) membrane (Millipore Corp, Billerica, MA, USA). After the proteins were fully transferred, the membranes were incubated in TBST (Tris-buffered Saline with Tween 20) containing 5 % skim milk at 37 $^{\circ}$ C for 3 h, and then incubated with primary antibodies diluted in Primary Antibody Dilution Buffer (Beyotime, China) at 4 $^{\circ}$ C overnight. Rabbit polyclonal anti- α -SMA (Proteintech, USA) was diluted at 1:500, Rabbit polyclonal anti -PTP1B and Coll α 1 (Boster, China) were diluted 1:200; mouse monoclonal anti- β -actin was diluted 1:200. HRP-conjugated anti-mouse and

anti-rabbit were used as secondary antibodies correspondingly secondary antibodies. After washing four times with TBST (Boster, China), the protein blots were detected using the ECL Chemiluminescent Kit (ECL, Thermo Scientific). All experiments were performed in triplicate and repeated at least three times.

2.10. Immunohistochemistry

Liver tissues were fixed in 10% neutral buffered formalin solution, embedded in paraffin, and stained for routine histology. The slides were dewaxed in xylene and dehydrated in alcohol, antigen retrieval was achieved by microwaving in citric saline for 15 min. Thin sections were deparaffinized and treated with 0.3% hydrogen peroxide for 15 min to block endogenous peroxidase activity. The sections were further blocked by 2% bovine serum albumin followed by incubation with primary antibody against α -SMA (1:200) and PTP1B (1:50) for 16 h at 4 °C. After rinsing, the sections were incubated with biotinylated secondary antibody for 60 min at room temperature. The expression of α -SMA and PTP1B were visualized by 3,3'-diaminobenzidine tetrahydrochloride (DAB) staining. The sections were counter stained with Mayer's hematoxylin for 30 s, dehydrated, and PTP1B and α -SMA positive areas within the fibrotic region were then observed.

2.11. Double immunofluorescence staining

For double immunofluorescence staining, sections were blocked with 10% normal serum blocking solution in order to avoid unspecific staining. Then, the sections were incubated with rabbit polyclonal primary antibodies for PTP1B (1:50) and mouse monoclonal primary antibodies for α -SMA (1:100). Sections were incubated with both primary antibodies over-night at 4°C, followed by a mixture of anti-mouse cmc-3(1:200) and anti-rabbit FITC (1:200) conjugated secondary antibodies for 2 h at room temperature. Then the stained sections were examined with a Leica sp5 laser confocal microscope (Germany).

2.12. Serum biochemistry

Blood was taken from the abdominal aorta, centrifuged at 2,000 g for 10 minutes,

and stored at -80°C. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined by alanine aminotransferase/aminotransferase Assay Kits (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions.

2.13. Statistical analysis

Data were presented as the mean $\pm$ SD. Comparisons between groups were made by use of one-way ANOVA with SPSS 17.0 software. For changes in mRNA or protein levels, mRNA (relative expression) and protein (densitometric values) to respective housekeeping controls were compared. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Up-regulation of PTP1B in the fibrotic livers from CCl₄-treated mice and down-regulation in the spontaneous recovery livers.

To determine the expression of PTP1B in the development and recovery of hepatic fibrosis, we performed immunohistochemistry assays on the liver tissues. Histologically, hematoxylin eosin (H&E) staining and Masson Trichrome staining showed that normal lobular architecture with central veins and radiating hepatic cords in the vehicle group. Treatment with CCl₄ resulted in prominent hepatic steatosis, necrosis and formation of regenerative nodules and fibrotic septa. After spontaneous recovery of 6 weeks, the liver exhibited weakened steatosis and necrosis and attenuated deposition of collagen (**Fig.1a**). Moreover, serum ALT and AST levels were obviously **elevated** in treated mice and down-regulated after spontaneous recovery (**Fig.1b**). These data indicated that the fibrosis and recovery model have been successfully established. Furthermore, as showed in **Fig.2a**, PTP1B immunostaining signal was increased in fibrotic liver tissue from CCl₄-treated mice compared to that from vehicle-treated groups; but the signal was decreased in recovery liver tissues. Consistently, myofibroblast marker α -SMA was extensively stained in fibrotic livers and scarcely stained in recovery liver tissues. Moreover, both

RT-qPCR and Western blot analysis demonstrated that expression of PTP1B was elevated in fibrotic livers but declined after spontaneous recovery. (Fig.2b, c). Additionally, the results of immunofluorescence in mice samples indicated that PTP1B (green) was highly expressed in α -SMA positive cells (red) in the fibrotic area, indicating HSCs may be one of main cell types which express PTP1B (Fig. 2d). These data indicate that PTP1B is induced in the experimentally injured liver and reduced after spontaneous recovery, and this was correlated with the expression of α -SMA and Coll α 1, suggesting a functional role of PTP1B in regulating HSCs fates.

3.2. Increasing expression of PTP1B during HSC-T6 activation induced by TGF- β 1.

In chronic liver injury, the activation of quiescent stellate cells into myofibroblast-like cells is a pivotal step for progression of hepatic fibrosis(Hellerbrand, 2013a). Stimulation of HSCs by TGF- β 1 is believed to be the key fibrogenic response in liver fibrosis because the expression of α -SMA and collagens can be dramatically up-regulated by TGF- β 1 (Shen *et al.*, 2003). To investigate the involvement of PTP1B in activation of HSCs, we first examined the expression of PTP1B mRNA and protein in cultured rat HSC-T6 cells treated with TGF- β 1. As illustrated in Fig. 3a, treatment of HSC-T6 cells with TGF- β 1 at the concentration of 0, 5, 10 and 15ng/ml for 24 h led to the elevated expression of α -SMA and Coll α 1. In addition, the levels of α -SMA and Coll α 1were also up-regulated in HSC-T6 cells stimulated with TGF- β 1 (10ng/ml) for 0, 12, 24 and 48 h (Fig.3b). Meanwhile, both mRNA and protein levels of PTP1B were elevated upon TGF- β 1in a dose- and time-dependent manner (Fig.3a, 3b). These findings convincingly suggested that PTP1B was up-regulated according to the progression of HSC-T6 cell activation and it may play an important role in regulating the proliferation and function of the myofibroblasts.

3.3 Blockade of PTP1B inhibited the TGF- β 1-induced proliferation of HSC-T6 cells.

In order to investigate the roles of PTP1B in regulating TGF- β 1-induced HSC proliferation, siRNA specific for rat PTP1B (PTP1B-siRNA) was used to knockdown the PTP1B expression. As showed in **Fig. 4a**, PTP1B-siRNA induced a significant decrease of PTP1B mRNA and protein levels in HSC-T6 cells after 24 h transfection. The MTT assay indicated that the viability of HSC-T6 cells after incubation with TGF- β 1 and PTP1B-siRNA was significantly decreased as compared to the Scrambled-siRNA transfected cells (**Fig. 4b**). Next, to understand whether the reduced cell proliferation was due to cell cycle arrest or apoptosis, we used Flow cytometric analyses to measure the effect of PTP1B on induction of cell cycle and apoptosis. By cell cycle analysis, we found that inhibition of PTP1B resulted in increased percentage of the activated HSCs in G0/G1 phase and decreased population of cells in S phases (**Fig.4c**). However, inhibition of PTP1B did not have significant effect on cell apoptosis (**Fig 4d**). Given these results, loss of PTP1B could inhibit proliferation of activated HSCs induced by TGF- β 1, at least in part, via promoting cell cycle arrest without influencing cell apoptosis

3.4. Blockade of PTP1B decreased TGF- β 1-induced expressions of Coll α 1 and α -SMA in HSC-T6 cells.

To elucidate the functional role of PTP1B during activation of HSC-T6 cells, the effects of siRNA-induced silence of PTP1B on the expressions of Coll α 1 and α -SMA were determined by RT-qPCR and Western blot. The results demonstrated that transfection of PTP1B-siRNA significantly inhibited Coll α 1 and α -SMA mRNA expression in TGF- β 1-treated HSC (**Fig. 5a**). Additionally, **Western** blot results determined that inhibition of PTP1B resulted in a significant decrease in protein levels of Coll α 1 and α -SMA compared with Scrambled-siRNA-transfected HSCs upon TGF- β 1 (**Fig. 5b**). Taken together, these data indicate that blockade of PTP1B decreased the expression of α -SMA and the deposition of Coll α 1 in activated HSCs.

3.5. Decreased expression of PTP1B in inactivated HSC-T6 cells upon MDI treatment.

An important characteristic of the recovery of liver fibrosis is the reversal of myofibroblast-like phenotype to a quiescent-like phenotype (Kisseleva and Brenner, 2013; Liu *et al.*, 2013). Treatment of activated HSCs with an adipocyte differentiation cocktail/mixture can lead to morphologic and biochemical reversal of activated to quiescent-like cells (She *et al.*, 2005). To confirm that changes of PTP1B gene expression during HSC inactivation also occur in vitro, we examined the expression levels of PTP1B in inactivated HSCs exposed to MDI. Inactivated HSC-T6 cells after treatment of MDI exhibited obviously decreased mRNA levels of Col1 α 1 and α -SMA (**Fig.6a**). Consistently, the protein levels of Col1 α 1 and α -SMA notably declined after MDI treatment 48h (**Fig.6b**). These data suggested that the HSC-T6 cells had been successfully reverted to the state of quiescent-like phenotype. Interestingly, compared with activated cells, mRNA level of PTP1B was significantly reduced (**Fig.6a**). Additionally, the results of Western blot confirmed the expression of PTP1B during the inactivation of myofibroblast (**Fig.6b**). All together, the data indicated that PTP1B was down-regulated in inactivated HSCs induced by MDI treatment and it may participate in regulating the reversal process of activated HSCs.

3.6. Over-expression of PTP1B abolished the inactivation of HSC-T6 cells induced by MDI.

In order to explore the function role of PTP1B in the process of HSC inactivation, the effects of PTP1B plasmid on expressions of Col1 α 1 and α -SMA were determined by RT-qPCR and Western blot. As presented in **Fig.7**, transfection with pCDA3.1-PTP1B plasmid significantly increased the mRNA and protein expression of PTP1B. The RT-qPCR results suggested that the introduction of PTP1B significantly abolished the MDI-induced down-regulation of Col1 α 1 and α -SMA mRNA levels (**Fig.7a**). Additionally, Western blot was performed to evaluate the protein levels and confirmed that over-expression of PTP1B notably hindered the inactivation of HSC-T6 cells demonstrated by increased Col1 α 1 and α -SMA protein expressions compared with pCDA3.1-control-transfected inactivated HSCs (**Fig.7b**).

Totally, these results suggesting that PTP1B obstructed the inactivation of activated HSCs and may prove to be a potential target to reverse hepatic fibrosis.

4. Discussion

Liver fibrosis is a common outcome of virtually all chronic liver diseases including chronic HCV and HBV infection, alcohol abuse and non-alcoholic fatty liver disease (NAFLD). It is often considered intractable not only because sustainable development of liver fibrosis may eventually lead to cirrhosis or even cancer, but no drug has yet emerged as an effective anti-fibrotic agent in humans (Bataller and Brenner, 2005). Hepatic fibrosis is characterized by extensive deposition of ECM proteins, mostly type I and III collagens. Numerous studies have concluded that hepatic myofibroblasts, mainly originated from hepatic stellate cells, are the principal source of ECM during liver fibrogenesis (Hernandez-Gea and Friedman, 2011). Therefore, inhibition of HSC activation and blockage of ECM production are important strategies for therapeutic intervention. For a long time, hepatic fibrosis was thought to be an irreversible process because of the collapse of the hepatic parenchyma and its substitution with scars (Popper and Uenfriend, 1970). Notably, recent findings have underlined that established liver fibrosis can be reversed upon cessation of etiological agents. Reversal of liver fibrosis is principally due to the apoptosis and inactivation of activated HSCs. Therefore, it will be of great significance for the recovery of liver fibrosis to identify the underlying mechanism and explore approaches to control the fate of myofibroblasts.

Here, our study provided the initial evidence that PTP1B was dramatically increased in the fibrotic liver tissues from CCl₄-treated mice *in vivo* but decreased after spontaneous recovery. And its expression strongly correlated with the HSC activation process as measured by the induction of α -SMA and Coll α 1. Changes in the expression of PTP1B during HSC activation also occurred *in vitro*. The expression of PTP1B was upregulated dose/time-dependently in response to TGF- β 1, correlating with a gradual increase of α -SMA and Coll α 1 levels. As the first purified PTP,

PTP1B is a central player in negatively regulating insulin and leptin signaling in mice. Therefore, it has been proposed as a therapeutic target against metabolic diseases such as obesity and type 2 diabetes(Bakke and Haj, 2015). Nevertheless, the function of PTP1B is not restricted to metabolic regulation. In the last few decades, numerous study focused on functions of PTP1B has uncovered its fundamental role in liver injuries(Chen *et al.*, 2015). Genetic disruption of PTP1B in mice conferred resistance to acute liver failure induced by acetaminophen (Mobasher *et al.*, 2013) and anti-Fas antibody Jo-2(Sangwan *et al.*, 2006), as proved by increased survival rate, attenuated hepatocyte death, lower level of serum ALT and **reduced inflammatory cytokines**. *In vitro* studies shown that reduction of PTP1B could impair apoptosis of hepatocyte after treatment with TGF- β 1 or after growth factor withdrawal and lead to a more pro-survival phenotype of hepatocytes. Moreover, PTP1B deficiency could protect mice from steatosis and improve hepatic regeneration during non-alcoholic fatty liver disease, strongly suggesting the crucial role of PTP1B in the development of liver damage (Shimizu *et al.*, 2003; Waring *et al.*, 2003; Ugi *et al.*, 2009). However, the exact role of PTP1B in liver fibrosis, the outcome of liver inflammation and steatosis, has not been illuminated. Even so, a lot of evidence has indicated the potential role of PTP1B in promoting the development of fibrosis. Gene deletion of PTP1B significantly **reduced chronic heart failure (CHF)-induced cardiac fibrosis and hypertrophy**. Correspondingly, chronic pharmacological inhibition of PTP1B by the PTP1B inhibitor AS279 obviously inhibited collagen deposition and improved cardiac dysfunction and remodeling, implying the critical function of PTP1B in fibrotic diseases (Gomez *et al.*, 2012). Therefore, the present study was designed to reveal whether PTP1B plays an essential role in the formation and resolution of hepatic fibrosis.

With respect to liver fibrosis, activated HSCs are the principal cell type that promotes ECM deposition and the induction of Coll α 1 and α -SMA is considered as markers of stellate cells activation. Hence, in the present study, HSC-T6 cells were

transfected with PTP1B-siRNA followed by exposure to TGF- β 1 in order to study the effect of PTP1B on stellate cell activation and collagen deposition. **Experimental results showed that blockage of PTP1B remarkably suppressed the expression of α -SMA and Coll α 1, indicating that loss of PTP1B could prevent the activation of HSCs and alleviate the excess collagen deposition.** Furthermore, the MTT assay showed that transfection of PTP1B-siRNA caused a significant inhibition of cell proliferation of activated HSCs upon TGF- β 1 treatment. By cell cycle analysis, we further found that loss of PTP1B increased percentage of activated HSC in G0/G1 phase, accompanied by a significant reduction of cell numbers in the S phase. However, the results from flow cytometry and apoptosis assay indicated that transfection of PTP1B-siRNA had no impact on the apoptosis of activated HSCs. Taken together, these data suggested that blockage of PTP1B could inhibit activation and proliferation of HSC induced by TGF- β 1 without inducing apoptosis of HSCs.

As is known to all, the inactivation of activated HSCs is analogous to the process of preadipocyte differentiation. Treatment of activated HSC with the adipocyte differentiation mixture (MDI) or ectopic expression of PPAR- γ and SREBP-1c, all of which are known to induce preadipocyte differentiation, coordinately cause reversal of activated HSC to quiescent cells (She *et al.*, 2005). Several studies have shown that PTP1B is involved in the process of preadipocyte differentiation. Over-expression of PTP1B in preadipocytes delayed adipocyte differentiation, in contrast, blockage of PTP1B accelerated the differentiation (Miranda *et al.*, 2010; Matsuo *et al.*, 2011; Song *et al.*, 2013), indicating the potential importance of PTP1B in HSC inactivation. Indeed, the expression of PTP1B was decreased in inactivated HSC-T6 cells upon MDI treatment. This finding was consistent with previous study in which PTP1B decreased progressively during differentiation of preadipocytes (Song *et al.*, 2013). Our experiments further demonstrated that overexpression of PTP1B **hindered** the inactivation of HSC-T6 cells induced by MDI, as manifested by restoration of Coll α 1 and α -SMA mRNA and protein expressions.

Collectively, our findings in the present study suggested that PTP1B may play a contributory role in liver fibrosis. To our knowledge, this research may be the first report involved in the expression and role of PTP1B in liver fibrosis. In this study, blockade of PTP1B inhibits TGF- β -induced HSC-T6 cell activation and proliferation, while over-expression of PTP1B hinders the inactivation of HSC-T6 cells (Fig.8). These results indicate the potential of PTP1B as a therapeutic target for liver fibrosis. Pharmacological agents that inhibit PTP1B might be a perspective regarding treatment of liver fibrosis. Therefore, designing and developing PTP1B inhibitors with extremely high potency and selectivity and directing the agents to the location of interest are of great significance for remedying fibrosis. Nevertheless, the fundamental mechanism of PTP1B in the setting of liver fibrosis thus far is incompletely understood. Next, studies applied *in vitro* and *in vivo* techniques will be conducted urgently to explore the mechanism, especially the substrates and signaling pathways that regulated by PTP1B. Looking to the future, we will have a better picture of the complex function and mechanism of PTP1B in liver fibrosis, which could be of great significance in predicting the pace of disease progression and developing the targeted therapeutic medicines.

Acknowledgments

This project was supported by the Key Program of National Science Foundation of China (NO: 81273526, 81473268), Anhui provincial key Scientific and technological project (1301042212), Natural Science Foundation of Anhui Province (1308085MH145), Specialized Research Fund for the Doctoral Program of Higher Education (20123420120001).

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Figure Captions:

Fig.1 Detection of liver pathology of hepatic fibrosis, n=12 per group. a. Pathology observation of the experimental mouse liver sections stained with hematoxylin eosin (H&E) staining and Masson staining. Representative views from each group are presented ($\times 100$). b. Serum ALT and AST levels of mice were determined. ^{**} $p < 0.01$

vs control; [#]*p*<0.05, ^{##}*p*< 0.01 vs model.

Fig.2 Up-regulation of PTP1B in the fibrotic livers from CCl₄-treated mice and down-regulation in the spontaneous recovery livers, n=12 per group. a. The level of PTP1B and α -SMA were analyzed by immune-histochemistry in control, model and recovery livers. Representative views from each group are presented ($\times 100$). b. The expression of PTP1B, Coll α 1 and α -SMA mRNA was assessed by Real-time PCR. The results are expressed as relative expression against negative control expression. The graph represents mRNA expression as mean $\pm$ SD from three independent experiments. ^{**}*p*<0.01 vs control; [#]*p*<0.05, ^{##}*p*< 0.01 vs model. c. The expression of PTP1B, Coll α 1 and α -SMA protein was assessed by Western blot. The results are expressed as relative expression against negative control expression. Representative blots of three independent experiments are shown. ^{**}*p*<0.01 vs control; [#]*p*<0.05, ^{##}*p*< 0.01 vs model. d. Double immunofluorescence staining was performed to determine the colocalization of PTP1B (green) and α -SMA (red) in CCl₄-induced mouse liver fibrosis models. Representative views from each group are presented ($\times 100$).

Fig.3 Increased expression of PTP1B during HSC activation induced by TGF- β 1. a. HSC-T6 cells were stimulated with different concentrations of TGF- β 1 (0, 5, 10 ng/ml) for 24 h. The mRNA level of PTP1B, Coll α 1 and α -SMA were examined by Real-time PCR; the protein level of PTP1B, Coll α 1 and α -SMA were assessed by Western blot. The results are expressed as relative expression against negative control expression. Data represent the mean $\pm$ SD from three independent experiments. ^{*}*p* < 0.05; ^{**}*p* < 0.01 vs 0ng/ml group. b. HSC-T6 cells were stimulated with TGF- β 1 (10ng/ml) for 0, 12, 24 and 48h. The mRNA level of PTP1B, Coll α 1 and α -SMA were examined by Real-time PCR; the protein level of PTP1B, Coll α 1 and α -SMA were assessed by Western blot. The results are expressed as relative expression against negative control expression. Data represent the mean $\pm$ SD from three

independent experiments. * $p < 0.05$; ** $p < 0.01$ vs 0h group.

Fig.4 Effect of PTP1B on cell proliferation, cell cycle and apoptosis in TGF- β 1-treated HSC-T6 cells. a. Reduced expression of PTP1B in HSC-T6 cells by PTP1B-siRNA. The results are expressed as relative expression against control expression. Data represent the mean $\pm$ SD from three independent experiments. ** $p < 0.01$ vs Scrambled-siRNA group. b. Inhibition of PTP1B significantly decreased cell proliferation of TGF- β 1-treated HSC-T6 cells. Proliferation of HSC-T6 cells was tested by MTT assay. Data represent the mean $\pm$ SD from three independent experiments. * $p < 0.05$ vs TGF- β 1+Scrambled-siRNA group. c. Effect of PTP1B on cell cycle in TGF- β 1-treated HSC-T6 cells. The results are expressed as relative expression against control expression without treatment. Representative images of three independent experiments are shown. $^{##}p < 0.01$ vs TGF- β 1+Scrambled-siRNA group. d. Effect of PTP1B on apoptosis in TGF- β 1-treated HSC-T6 cells. The results are expressed as relative expression against control expression without treatment. One representative experiment of the three independent experiments is demonstrated.

Fig.5 Blockade of PTP1B decreased TGF- β 1-induced expressions of α -SMA and Coll1 α 1 in HSC-T6 cells. After PTP1B-siRNA or Scrambled-siRNA transfection, then exposure HSC-T6 cells to TGF- β 1 (10ng/ml) for 24h. (a)Real-time PCR were performed to examine the mRNA level of PTP1B, Coll1 α 1 and α -SMA. (b) Western blot was performed to assess the protein level of PTP1B, Coll1 α 1 and α -SMA. The results are expressed as relative expression against negative control expression. Data represent the mean $\pm$ SD from three independent experiments. ** $p < 0.01$ vs Control; $^{##}p < 0.01$ vs Scrambled-siRNA.

Fig.6 Decreased expression of PTP1B in inactivated HSC-T6 cells. HSC-T6 cells were activated by TGF- β 1 (10ng/ml, 24h), then they were treated with MDI for 48h to

be inactivated. The mRNA level of PTP1B, Colla1 and α -SMA were examined by Real-time PCR (a); the protein level of PTP1B, Colla1 and α -SMA were assessed by Western blot (b). The results are expressed as relative expression against negative control expression. Data represent the mean $\pm$ SD from three independent experiments. $*p<0.05$, $**p<0.01$ vs control; $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs TGF- β 1.

Fig.7 Over-expression of PTP1B reversed the inactivation of HSC-T6 cells induced by MDI. HSC-T6 cells were activated by TGF- β 1 (10ng/ml, 24h), then they were transfected with pCDNA3.1-PTP1B or pCDNA3.1-control plasmid. After that, cells were treated with MDI for 48h to be inactivated. The mRNA level of PTP1B, Colla1 and α -SMA were examined by Real-time PCR (a); the protein level of PTP1B, Colla1 and α -SMA were assessed by Western blot (b). The results are expressed as relative expression against negative control expression. Data represent the mean $\pm$ SD from three independent experiments. $*p<0.05$, $**p<0.01$ vs TGF- β 1; $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs pCDNA3.1-control.

Fig.8 Overview of PTP1B in liver fibrosis.

Figures

Fig. 1

Fig.2

Fig.3

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b

Fig.4

Fig.5

a

b

Fig.6

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a

Fig.7

b

Fig.8