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Original Paper

PTPRO-Associated Hepatic Stellate Cell Activation Plays a Critical Role in Liver Fibrosis

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Abstract
Background/Aims: PTPRO (protein tyrosine phosphatase, receptor type O) is implicated in diverse physiological and pathological processes in cancer and hepatic ischemia/reperfusion injury, although little is known about its role in hepatic fibrosis. ***Methods:*** Here, by using genetically deficient mice, we reported that PTPRO knockout ($PTPRO^{-/-}$) significantly attenuated liver injury, release of inflammatory factors, tissue remodeling, and liver fibrosis in two experimental mouse models of fibrogenesis induced by bile-duct ligation or carbon tetrachloride administration. ***Results:*** However, we proved that PTPRO expression was strongly downregulated in clinical and experimental liver fibrosis specimens. Further investigations revealed that stimulation of primary hepatic stellate cells (HSCs) and hepatocytes with specific activator platelet-derived growth factor (PDGF)-BB increased PTPRO transcription in HSCs but had the opposite effect in primary hepatocytes. More importantly, synthetic short hairpin RNA targeting PTPRO significantly neutralized PDGF-BB-induced HSC proliferation and myofibroblast marker expression through downregulated phosphorylation of extracellular signal-regulated kinase (ERK) and AKT. ***Conclusion:*** These observations confirm that PTPRO plays a critical role in liver fibrogenesis by affecting PDGF signaling in HSC activation and might be developed into a feasible therapeutic approach for the treatment of chronic fibrotic liver diseases.

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Introduction

Liver fibrosis is a pathological process arising from continuous wound-healing responses to liver injury, which encompasses infection-related hepatic fibrosis, metabolic-type fibrosis, and biliary-type fibrosis. As the injury proceeds, the extracellular matrix (ECM) such as collagen and fibronectin accumulates and forms a fibrous scar to nodules of regenerating hepatocytes, which predisposes to liver failure and hepatocellular carcinoma [1]. Hepatic stellate cells (HSCs) resident in the space between hepatocytes and liver sinusoidal endothelial cells (LSECs) are viewed as a predominant cellular source of ECM when activated [2, 3]. They normally store vitamin (retinoid) droplets in their cytoplasm, and are differentiated by injury-associated immunological processes into fibrogenic myofibroblasts that produce ECM and affect the function of hepatocytes and LSECs [4, 5]. Fibrosis progression and regression need specific and complicated signaling pathways, including chemokines and cytokines such as platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β and leptin [6-8]. Many recent studies have focused on the early events in liver fibrosis, such as immune interactions with interleukin (IL)-17 and IL-33 [9-11], but how they support fibrosis and activate HSCs requires verification.

Protein-tyrosine phosphatases (PTPs) can act in an opposite but equally important manner to protein-tyrosine kinases (PTKs), as a key part of post-translational modification strategies to regulate protein activities [12]. Protein tyrosine phosphatase receptor type O (PTPRO), also named glomerular epithelial protein 1 (GLEPP), is an important member of the receptor-type PTPs family that is expressed in humans, rats and rabbits. There are two types of PTPRO, full-length PTPRO (PTPRO-FL) expressed in kidney, brain, lung, liver and breast, and truncated PTPRO (PTPROt) which is predominantly expressed in B lymphocytes and macrophages [13]. PTPRO has been implicated in several biological processes, including neuronal growth and branching [14, 15], mammary epithelial cell morphogenesis [16], and as a biomarker in various types of carcinoma [17-20]. Previous research from our center has indicated that PTPRO expression promoted by estrogen receptor α contributes to the sex difference in incidence of hepatocellular carcinoma [21]. Furthermore, we also reported that PTPRO played a bidirectional role in hepatic ischemia/reperfusion (I/R) injury by affecting signal transducer and activator of transcription (STAT)3 activity [22]. However, the role of PTPRO in liver fibrosis is still unclear. Here, we identified PTPRO as a key mediator of hepatic fibrosis, and further explored the idea of therapeutic modulation of PTPRO in hepatic inflammation and fibrosis.

Materials and Methods

Patient samples

All human liver samples were collected at First Affiliated Hospital of Nanjing Medical University, China (Table 1). Informed consent was obtained from the patients before the specimens were obtained. Cirrhosis specimens (n=18) were collected from patients undergoing liver transplantation for end-stage liver cirrhosis. Normal liver tissue (n=8) was collected from patients receiving hepatoectomy for hepatic cavernous hemangioma. Liver samples were analyzed by quantitative real-time PCR, or fixed in 4% paraformaldehyde followed by paraffin embedding for immunoblotting.

Mouse models

Specific pathogen-free, male C57BL/6 mice, aged 8–10 weeks were obtained from the Experimental Animal Center of Nanjing Medical University. PTPRO knockout (KO) C57BL/6 mice were kindly donated by Dr. Bixby, University of Miami, USA and bred in the Model Animal Research Center of Nanjing University. Carbon tetrachloride (CCl_4; 0.6 g/kg) was administered intraperitoneally twice weekly for 4 weeks, mixed with corn oil (or oil for control), and the mice were sacrificed 3 days after the final dose, when transaminase levels should return to near normal, as described previously [23]. For bile duct ligation (BDL), mice were anesthetized with 1% pentobarbital. After midline laparotomy, the common bile duct was dissociated and ligated twice with 6-0 silk sutures, and the abdomen was closed. Sham operation was performed similarly,

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Table 1. Clinical characteristics. Data are presented as mean (minimum-maximum). ALT, alanine aminotransferase; HBV, hepatitis B virus (Others are alcoholic cirrhosis)

Characteristics	Fibrotic Liver	Control Liver
N	18	8
Sex	20 male	7 male
Mean age (minimum–maximum), y	52.2 (40-59)	44.1 (32-55)
HBV infected, n	16	3
Mean ALT level (minimum-maximum), IU/l	50.8(32.5-68.3)	29.6 (13.6-43.3)
Mean total bilirubin level (minimum-maximum)	15.6 (4.2-34.1)	10.1 (5.1-17.6)
Mean serum albumin (minimum-maximum), g/l	32.5 (25-42)	36.2 (32-45)
Complicated with hepatocellular carcinoma, n	9	0

except that the bile duct was not ligated [24]. To eliminate the influence of other complications, animals were sacrificed 3 or 14 days after BDL. Serum and liver were prepared for other research. Indicated groups of BDL challenged mice were observed until 21 days for overall survival rate analysis,

Microscopic investigation of liver after BDL or CCl4 treatment
Fragments of liver tissues were fixed in 4% buffered formaldehyde. Sections (3 μm) of liver were cut and stained with hematoxylin and eosin (H&E). Liver H&E sections were analyzed for I/R injury under a Leica DM4000 B Upright Research Microscope (100×).

Measurement of plasma alanine aminotransferase (ALT) activity
Blood was obtained by cardiac puncture at the time of sacrifice for analysis of serum ALT as an index of hepatocellular injury. Measurements of serum ALT were made using a diagnostic kit (Sigma, St. Louis, MO, USA).

HSCs and hepatocytes isolation and restimulation assay
Primary mouse HSCs were isolated from livers of C57BL/6 mice, as described previously, with modification [10]. Livers were perfused *in situ* with 45 ml Gibco Liver Perfusion Media (Invitrogen, Carlsbad, CA, USA) followed by 45 ml Gibco Liver Digestion Media (Invitrogen). The liver digests were filtered through a cell strainer and washed with Gey's Balanced Salt Solution (GBSS) (Sigma) containing DNase I (2 mg/ml, Roche Diagnostics). The homogenate was centrifuged at 25 g for 5 min at room temperature to remove the hepatocytes. The supernatant was transferred to a new tube and centrifuged at 400 g for 10 min at 4°C. The cell pellet was resuspended in 5 ml 15% OptiPrep (Sigma), and loaded carefully with 5 ml of 11.5% OptiPrep and 2 ml GBSS. After centrifugation at 1400 g for 17 min at 4°C, the cell fraction in the GBSS and 11.5% OptiPrep interphase was gently aspirated, mixed with GBSS, and centrifuged at 1400 g for 10 min at 4°C. After another wash, the final cell pellet was resuspended in Dulbecco's Modified Eagle's Medium (DMEM) containing penicillin, streptomycin and 10% fetal bovine serum (FBS) (complete medium), and then plated on uncoated plastic at a density of 5×10^6 per 10-cm diameter plate. After the first 24 h, nonadherent cells and debris were removed by washing. Cell viability was >90% as assessed by Trypan blue exclusion. Purity was 90–95% as assessed by light microscopy. Primary murine hepatocytes were isolated from male C57BL/6 mice aged 6–8 weeks, as previously reported [22]. Isolated HSCs and hepatocytes were placed into the 96 well plate (8×10^4 cells/well) in DMEM and exposed to recombinant (rm) PDGF-BB (Peprotech, UK) (5ng/ml) [25] . After 12 h incubation at 37°C, cells were harvested for other tests.

RNA interference analysis
PTPRO silencing by mouse PTPRO small hairpin RNA (shRNA) (5'-GCG CTC ATA CCG AAT GTC AAT CTC GAG ATT GAC ATT CGG TAT GAGCGC-3') or scrambled sequences (5'-TGACAGTCGATTGATGTGATT-3') designed and synthesized by Wuhan Genesial Corporation (Wuhan, China) was tested in HSCs. PTPRO levels following shRNA silencing were analyzed by RT-PCR and immunoblotting using anti-PTPRO antibodies. Such analysis revealed that the PTPRO-shRNA silenced PTPRO expression by ~90% at a relatively low concentration (50 nmol/l) using Lipofectamine 2000 (Invitrogen) for three independent transfection experiments.

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Table 2. Primer used

Genes	Forward(5'-3')	Reverse(5'-3')
PTPRO(Mouse)	ATTGGACTGGATATTCCGCACT	CAGGATGTTTGTGTAGCGGTTT
PTPRO(Human)	ATGACTTCAGCCGTGTGAGAT	GGGTGGCAATATACTCCTGGG
α-SMA	GTCCCAGACATCAGGGAGTAA	TCGGATACTTCAGCGTCAGGA
Col-1α1	TTCACCTACAGCACGCTTGTG	GATGACTGTCTTGCCCCAAGTT
MMP-2	CAAGTTCCCCGGCGATGTC	TTCTGGTCAAGGTCACCTGTC
TIMP-1	GCAACTCGGACCTGGTCATAA	CGGCCCGTGATGAGAAACT
TGF-β	CTCCCGTGGCTTCTAGTGC	GCCTTAGTTTGGACAGGATCTG
GAPDH(Mouse)	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
GAPDH(Human)	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

Real-time quantitative PCR

RNA was isolated from mouse or human liver tissue by homogenization and purification using TRIzol reagent (Invitrogen). RNA was isolated from HSCs using RNeasy. Following DNase treatment and reverse transcription, real-time quantitative PCR of mouse and human samples was performed for 40 cycles of 15 s at 95°C and 1 min at 60°C using a one-step RT-PCR kit (TaKaRa, Dalian, China). The specific primers used are summarized in Table 2. Quantification was performed by comparing the Ct values of each sample with a standard curve and normalization to GAPDH. Values were expressed as fold induction in comparison with untreated or sham controls.

Immunohistochemistry

Immunohistochemistry of α-smooth muscle actin (SMA) was performed using 5-μm paraffin sections. The sections were deparaffined in xylene and rehydrated in alcohol and distilled water. Antigen retrieval was performed by heating sections in solution (10 mM citrate buffer, pH 6.0) in a pressure cooker. H_2O_2 (3%) was used to eliminate endogenous peroxidase. Slides were washed with Tris-buffered saline (TBS)–Triton three times and once with TBS for 5 min. Nonspecific binding was blocked using 5% goat serum for 30 min. The blocking buffer was removed and sections were incubated with a polyclonal α-SMA antibody (AB5694; Abcam, Cambridge, UK). Control experiments were incubated with rabbit serum replacing the first antibody. After incubation overnight at 4°C and washing, the sections were incubated with biotinylated goat anti-rabbit IgG antibody. After washing, peroxidase-coupled antibody was applied for 30 min at room temperature. Bound antibody was detected with 3, 3'-diaminobenzidine tetrachloride (DAB) (Sigma–Aldrich, St. Louis, MO, USA). All sections were counterstained with hematoxylin. Image-ProPlus version 6.0 was used to evaluate the areas of positive α-SMA as computer-assisted semi-quantitative.

Collagen detection assay

Supernatants of liver homogenate samples were collected from mice; cell medium were obtained from 5 ng/ml rmPDGF-BB restimulation for 12 h (1×10^5 cells/ml in 6-well plates). The collagen concentration was measured using the Sircol assay according to the manufacturer's recommended procedure (S1000, Biocolor, Carrickfergus, UK). Masson's trichrome stain and hydroxyproline assay were used to focus on the change in hepatic collagen content *in vitro* and *in vivo*. The detailed methods were according to the manufacturer's recommended procedure (HT15; Sigma–Aldrich and KGT030-2; KeyGEN Biotech, Nanjing, China).

Cytotoxicity and viability assays

Cell viability was assessed using the MTT reduction assay. Formation of blue formazan was measured spectrophotometrically from the metabolism of MTT by mitochondrial dehydrogenases that are active only in live cells. Isolated HSCs were seeded in 96-well plates (1×10^4 cells per well) and then incubated in DMEM containing penicillin, streptomycin and 10% FBS for 24 h. The cells were pretreated with various concentrations of rmPDGF-BB for 12 h, and MTT (5 mg/ml) was added to each of the wells, and the plate was incubated for an additional 1 h at 37°C. The medium was removed, and the intracellular formazan product was dissolved in 250 μl DMSO. The absorbance of each well was measured at 540 nm using a microplate reader. OD values from untreated control cells were designated as 100% viability. Triplicate specimens were
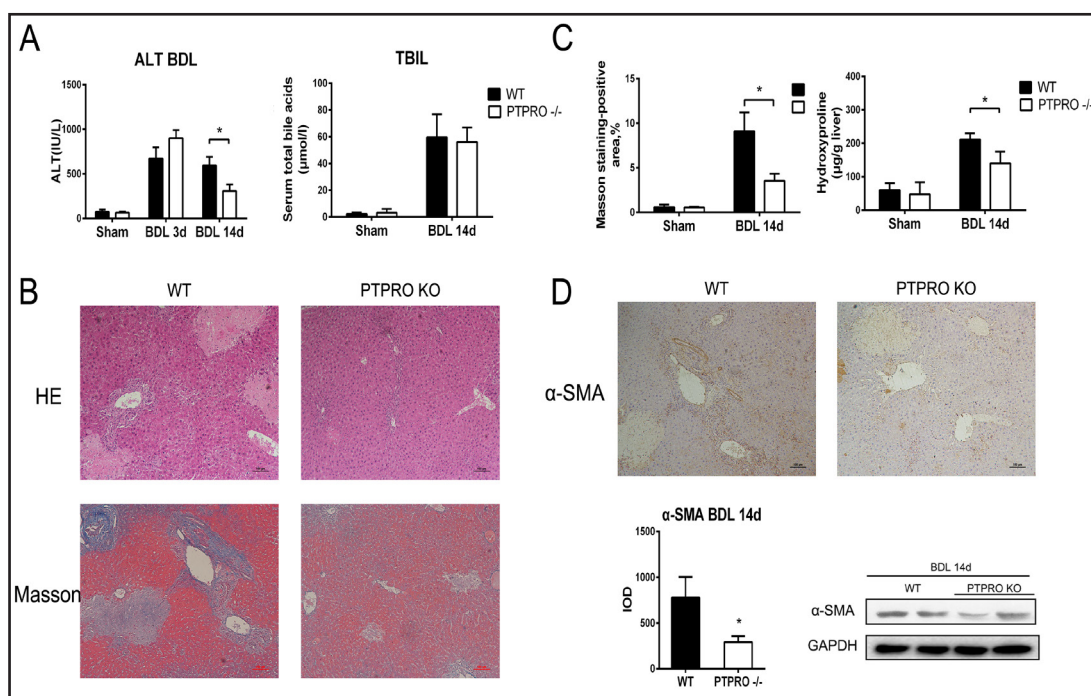
Fig. 1. PTPRO is required for hepatic fibrogenesis in BDL. Mice underwent BDL and were sacrificed 3 or 14 days later. (A) Serum ALT values and total bilirubin (TBil) were measured 3 and 14 days after BDL from WT and PTPRO$^{-/-}$ mice (n=8 per group) compared to each sham-operated control (n=4). TBil in BDL mice was almost identical, indicating a similar cholestatic effect of BDL between the groups of animals. (B) H&E showed that the WT group had more widespread bile infarctions and cholestatic hepatitis than the PTPRO$^{-/-}$ group had. Fibrillar collagen was determined by Masson staining (Collagen fibers stained as blue and cytoplasm stained as red, original magnification, 100×). (C) Quantification of positive area by Masson staining (left). Hydroxyproline quantification indicated that collengen deposition was increased in BDL-treated liver (right). (D) Expression of α-SMA was measured by immunohistochemistry. Western blotting of α-SMA between the two groups. Average integrated optical density (IOD) was obtained by analyzing five fields for each slide evaluated by Image-Pro Plus software (version 5.0) for immunohistochemical staining (*$P<0.05$).

repeated three times independently. Comparisons between groups were performed by one-way ANOVA followed by the Newman–Keuls post test.

Western blotting

Total cell (or tissue) protein was extracted on ice by homogenization and the concentration was determined using the Bradford method with a Bio-Rad protein assay, Protein extract (20 mg/lane) was separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). After the blockade of nonspecific protein binding with 5% milk or serum, the membrane was incubated at a dilution with different antibodies. Immunoblotting was performed using antibodies against PTPRO (#12161; Proteintech Group, Chicago, IL), α-SMA, rabbit monoclonal antibodies against ERK1/2 (#9102), phospho-ERK1/2 (#9101s), AKT (#4691), and phospho-AKT (T308) (#9275) (Cell Signaling Technology, Danvers, MA, USA) at a dilution of 1:1000. Blots were reprobed with mouse GAPDH antibody (Cell Signaling Technology). Horseradish-peroxidase-linked anti-rabbit and anti-goat antibodies were used as secondary antibodies. The results were exposed to autoradiography film (Kodak XAR Film) after the enhanced chemiluminescence light method.

Statistical analysis

All data was expressed as mean ± SEM. Student t tests were used to calculate statistical significance for data sets following normal distribution. Log-rank test were used to analyze the survival rate. $P<0.05$ was considered statistically significant. Some figures were prepared by GraphPad Prism 6.

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Results

BDL-induced chronic inflammation and liver fibrosis is PTPRO dependent

To test the functional importance of PTPRO in hepatic fibrosis, paired PTPRO KO mice and littermate WT mice were used in two kinds of models as mentioned above. In the model of BDL, total bilirubin (TBil) in challenged mice was measured, indicating a similar cholestatic effect of BDL between WT and PTPRO KO mice. The level of alanine aspartate aminotransferase in serum of mice confirmed the liver injury induced by BDL. ALT activity was reduced in PTPRO KO mice compared with WT mice at 14 days but there was no significant difference at 3 days (Fig. 1A). H&E staining showed that mouse liver at 14 days after BDL demonstrated severe cholestatic hepatitis with widespread bile infarctions, bile ductular proliferation, and hepatocellular damage, whereas in PTPRO$^{-/-}$ mice there was less biliary injury (Fig. 1B). Furthermore, Masson's trichrome staining and hydroxyproline quantification indicated significant reduction of collagen deposition in PTPRO$^{-/-}$ mice (Fig. 1B, 1C). Moreover, α-SMA, a cardinal marker for HSC activation, was increased in BDL-challenged mice as compared to sham-operated mice. By using immunohistochemistry and western blotting, we showed that α-SMA immunoreactivity was increased in the sinusoids of BDL WT mice but reduced in BDL PTPRO$^{-/-}$ mice (Fig. 1D).

PTPRO deficiency protects against CCl4-induced chronic hepatic injury and fibrosis

PTPRO deficiency largely protected mice from BDL-associated liver injury and fibrosis, therefore, another model was introduced to confirm the role of PTPRO in chemically induced liver fibrosis. CCl$_4$ was injected into C57BL/6 or PTPRO KO mice at 0.6 g/kg twice weekly. After 4 weeks, mice were sacrificed. The severity of liver injury in the PTPRO KO group was
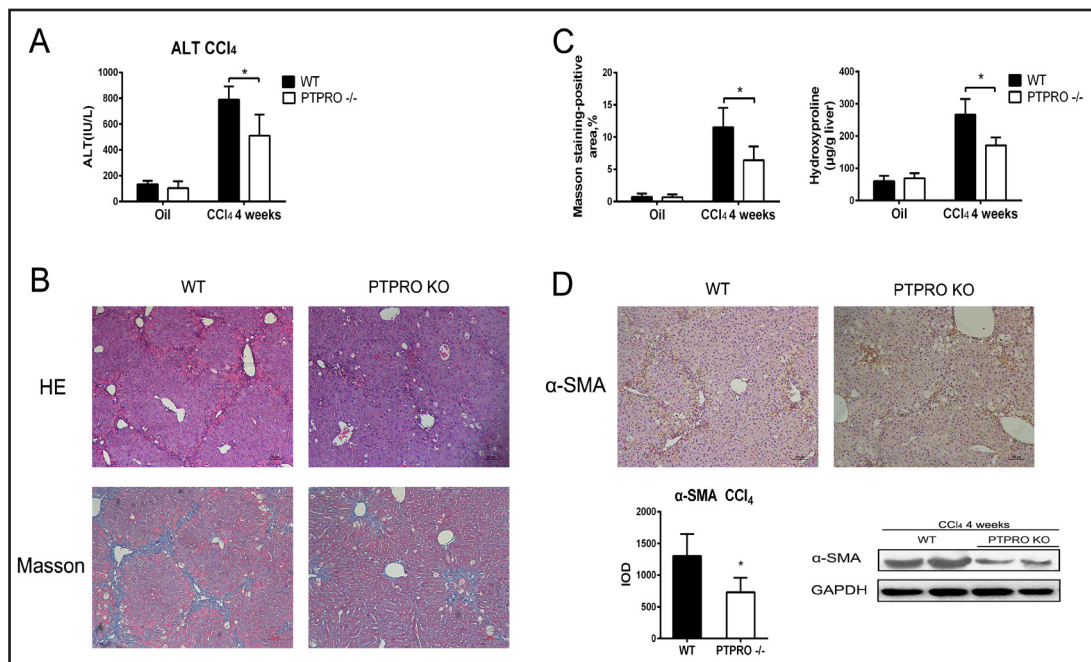

Fig. 2. PTPRO is required for hepatic fibrogenesis in CCl$_4$ treatment. C57BL/6 and PTPRO$^{-/-}$ mice were administered intraperitoneally 0.6 g/kg CCl$_4$ (diluted in corn oil) or oil alone twice weekly for 4 weeks, and mice were sacrificed at 48 h after the final challenge. (A) Serum ALT values were measured from WT and PTPRO$^{-/-}$ mice. (B) Representative H&E (original magnification, 100×) and collagen deposition in liver sections (Masson trichrome, original magnification, 100×) from C57BL/6 and PTPRO$^{-/-}$ mice injected with CCl$_4$ or oil are shown. (C) Quantification of positive area of IOD by Masson staining and quantification of hydroxyproline content. (D) Expression of α-SMA was measured by immunohistochemistry while IOD in D was obtained, and expression of α-SMA was measured by western blotting.
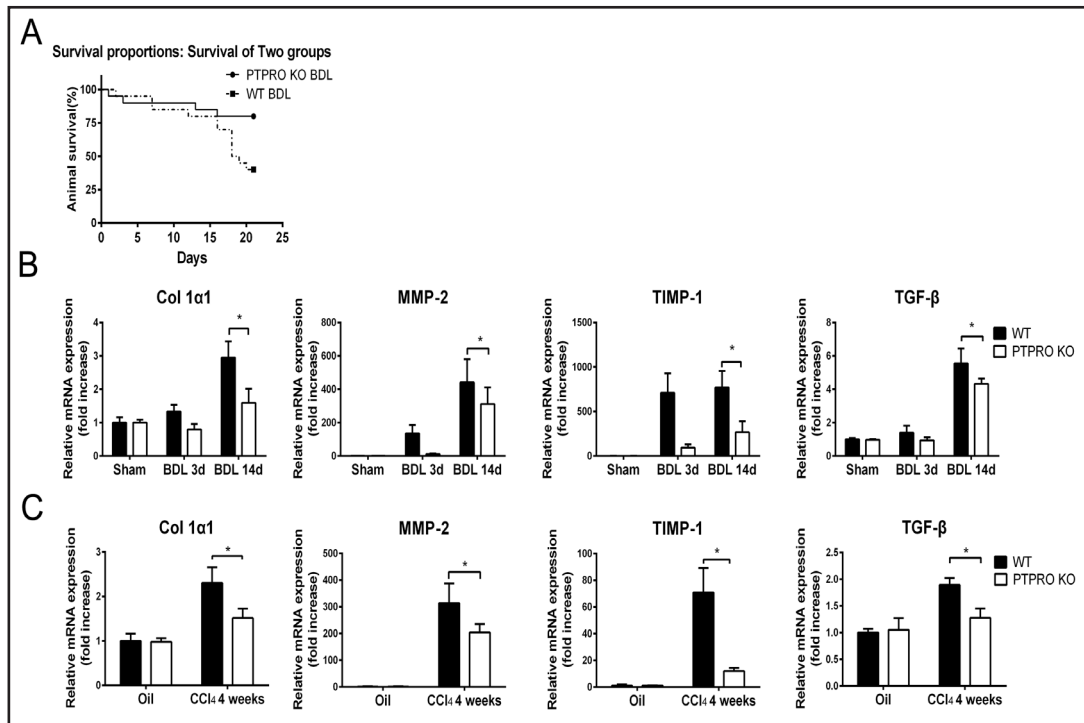
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Fig. 3. Inflammatory cytokine expression and animal survival following liver fibrosis are dependent upon PTPRO. (A) Animal survival following BDL was enhanced in PTPRO$^{-/-}$ mice (n=20). Initially on day 14 after BDL, 85% of PTPRO$^{-/-}$ mice were alive, whereas 80% of the WT animals survived cholestatic liver injury and fibrosis. However on day 21 after BDL, 80% of PTPRO$^{-/-}$ mice were still alive compared to only 40% in the WT group (log-rank test, P=0.04, 95% CI=1.206–8.588). (B) Expression of profibrogenic markers Col-1α1, MMP-2, TIMP-1 and TGF-β was determined by quantitative real-time PCR in BDL or sham-operated mice, which indicated that BDL-induced liver inflammation and fibrosis are PTPRO dependent. (C) Expression of those factors as mentioned above in CCl$_4$-treated mice (*P<0.05).

relatively milder, by analyzing serum ALT level (Fig. 2A). Both Masson's and H&E staining of the liver sections showed that 4 weeks repeated CCl$_4$ injection resulted in obvious vesicular degeneration and uniform fibrous tissue deposition in WT mice, which was attenuated in PTPRO KO mice (Fig. 2B). As for the quantitative grading, the Masson staining-positive area and hepatic hydroxyproline content of the PTPRO$^{-/-}$ mice were significantly lower than in the WT group (Fig. 2C). This suggested that PTPRO-deficient mice showed reduced accumulation of collagen and had a protective role in CCl$_4$-induced hepatic fibrosis. These findings were confirmed by the reduction in Immunocytochemical staining and western blotting for α-SMA (Fig. 2D).

Inflammatory cytokine expression and animal survival levels following liver fibrosis are dependent upon PTPRO

Although PTPRO deficiency largely protected the mice from BDL- and CCl$_4$-induced chronic liver injury and liver fibrosis, the exact mechanisms underlying this protective capacity remains unclear. Therefore, we investigated the inflammatory cytokine expression profiles in C57BL/6 and PTPRO$^{-/-}$ mice challenged in these two models. We first examined the overall survival in BDL-challenged mice. By day 21 after BDL, PTPRO$^{-/-}$ mice (n=20) exhibited longer overall survival than the WT group (n=20) (80% vs 40%, respectively, log-rank test P=0.04, 95% CI=1.206–8.588) (Fig. 3A). Considering that the PTPRO deletion is not liver specific, the organ-specific effects responsible for enhanced animal survival could not be specifically delineated. However, because PTPRO KO reduces liver injury, hepatoprotection from BDL likely and partly contributes to the improved survival.

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Fig. 4. PTPRO downregulated in murine and human liver fibrosis. Fibrotic and normal liver tissues were collected from patients undergoing partial hepatectomy for early-stage hepatocellular carcinoma or hepatic hemangioma. (A) PTPRO mRNA transcript level was downregulated more in human fibrotic liver (n=18) than normal liver (n=8). BDL (n=8) and CCl_4 injection (n=8) were performed to induce liver fibrosis in WT mice. (B and C) Expression of PTPRO and α-SMA in mouse liver was tested by western blotting or quantitative PCR, and the results were shown as fold changes compared with each control (n=4) (Data were from independent samples and expressed as mean ± SEM, *$P<0.05$ by Student t tests).
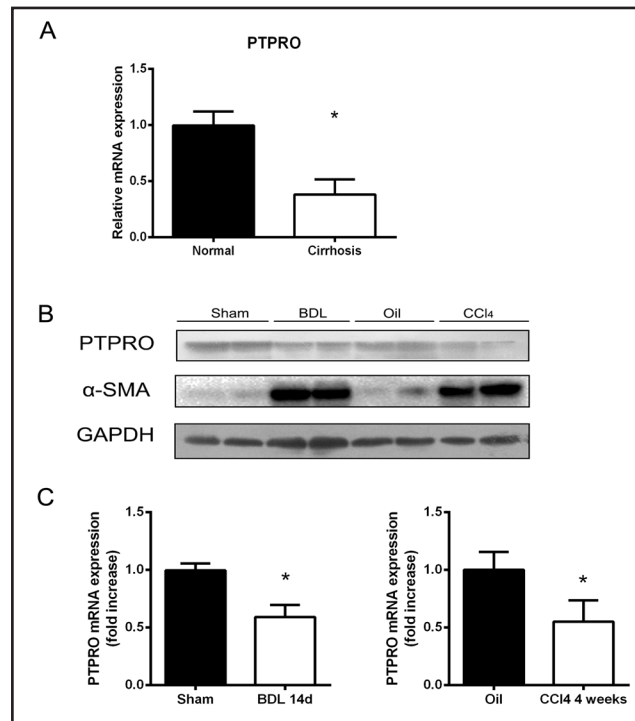
Second, to ascertain if HSC activation was also associated with enhanced hepatic fibrogenesis, mRNA for profibrogenic markers, such as collagen type 1 α1 (Col1α1) was significantly lower in BDL- or CCl_4-challenged PTPRO$^{-/-}$ mice. Tissue inhibitor of metalloproteinase (TIMP)-1, matrix metalloproteinase (MMP)-2 and another important fibrosis- related factor, transforming growth factor (TGF)-β1, also exhibited the same trend (Fig. 3B and 3C). These experiments confirmed that PTPRO plays an important role in BDL- and CCl_4-induced liver fibrosis, and PTPRO deficiency may decrease the degree of liver fibrosis.

PTPRO is downregulated in mouse and human fibrotic liver

Since PTPRO deficiency largely protect the mice from liver, we collected cirrhotic (n=18) and normal (n=8) liver tissue for real-time PCR for clinical verification. We found that PTPRO mRNA in patient samples was strongly downregulated in comparison with normal liver ($P<0.05$; Fig. 4A). To determine whether PTPRO played a critical role in liver fibrogenesis *in vivo*, we induced two different experimental fibrogenesis models by BDL and CCl_4 administration in wild-type (WT) mice. α-SMA, a cardinal marker for HSC activation, was upregulated in fibrosis specimens, indicating that fibrosis was induced in both models. We found marked reduction of PTPRO expression in fibrotic liver at the mRNA and protein levels, suggesting that PTPRO participates in pathological progress (Fig. 4B, 4C).

Effect of PTPRO in HSC and hepatocyte activation and downstream signaling

Previous research has shown that PTPRO is significantly downregulated in clinical fibrotic liver specimens and experimental fibrotic mouse liver, while PTPRO KO largely protects mice from BDL- and CCl_4-induced chronic liver injury and fibrogenesis. To address this paradox, we clarified the role of PTPRO in liver fibrosis at the cellular level.

Transactivation of quiescent HSCs is thought to be a key step in collagen deposition and ECM accumulation in liver cirrhosis [3]. During HSC activation, numerous pathways are activated as paracrine or autocrine signaling. PDGF is the classic cytokine which could be released during hepatocellular damage and trigger downstream signaling by PI3K/AKT, ERK and other pathways [25, 26]. A recent study found that PDGF is a vital direct mitogen and information regulator for hepatocytes [27, 28]. We first validated that alteration of
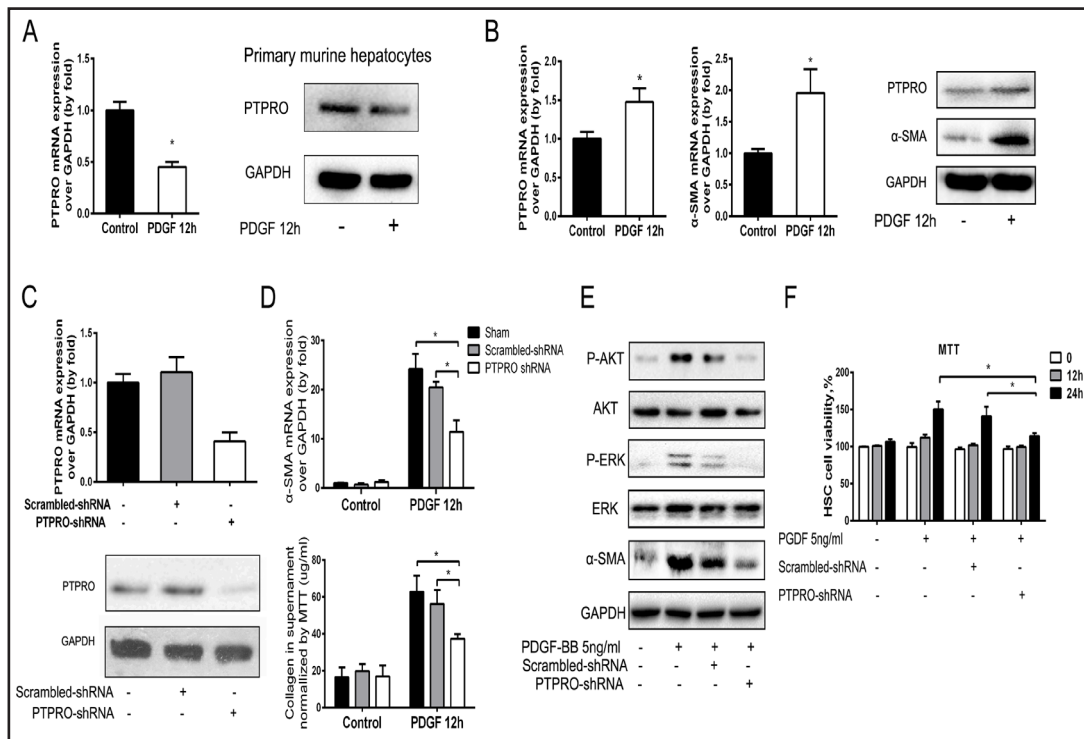
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Fig. 5. Effect of PTPRO in HSC activation and downstream signaling. Mouse HSCs were isolated from naïve C57BL/6 mice and stimulated as previously described. (A) RNA and protein of PTPRO in isolated hepatocytes after PDGF-BB administration for 12 h. (B) After 12 h restimulation, PDGF-treated HSCs were analyzed for PTPRO and α-SMA at the RNA and protein levels, compared with original HSCs. (C) Twenty-four hours PTPRO-shRNA transfection resulted in a significant decrease in PTPRO expression in comparison with the same cells transfected with scrambled-shRNA, by quantitative real-time PCR and western blotting. (D) Under rmPDFG-BB (5 ng/ml) treatment for 12 h, α-SMA transcription in primary HSCs was attenuated by PTPRO-shRNA transfection. The same trend was observed for soluble collagen assessed by Sircol assay. The collagen concentration was standardized by MTT viability. (E) PDGF-BB administration activated HSCs through intracellular AKT and ERK phosphorylation, while PTPRO-shRNA significantly reduced kinase activation and subsequently attenuated α-SMA production. (F) PTPRO-shRNA decreased HSC proliferation at 12 and 24 h after rmPDGF-BB treatment, as compared to the scrambled-siRNA transfected and naive cells (*$P<0.05$).

PTPRO gene expression during 12 h stimulation of primary hepatocytes and HSCs with nonlethal concentrations of PDGF-BB. PTPRO transcription was significantly decreased in hepatocytes during PDGF-BB administration, while the opposite effect was seen in HSCs (Figure 5A and 5B).

To establish the critical role of PTPRO in activated HSCs, shRNA specific for mouse PTPRO (PTPRO-shRNA) was used to knockdown PTPRO expression. As illustrated in Fig. 5C, PTPRO-shRNA induced a significant decrease in PTPRO levels in HSCs after 24 h transfection, in comparison to the cells transfected with scrambled shRNA (Fig. 5C). Moreover, we observed a strong increase of α-SMA transcription and collagen release in PDGF-BB-treated primary HSCs and supernatant, respectively, which indicated that PDGF-BB-activated HSCs could be the main sources of ECM and collagen. PTPRO knockdown also reduced HSC activation by controlling expression of α-SMA and soluble collagen (Fig. 5D).

Next, we determined if silencing PTPRO affected the activity of PI3K and ERK by analyzing the protein levels of p-AKT and p-ERK in HSCs after PTPRO downregulation. Western blotting showed a decrease in both p-AKT and p-ERK in activated HSCs transfected with PTPRO-shRNA in comparison to the scrambled-shRNA-treated cells after PDGF-BB

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restimulation (Fig. 5E). These data suggest that PTPRO affects HSC activation through p-AKT and p-ERK signaling pathways, which may be attributed to the PI3K pathway [29]. The viability of HSCs after 24 h incubation with PDGF-BB and PTPRO-shRNA decreased in the MTT assay, as compared to cell treated with PDGF-BB and transfected with scrambled siRNA (Fig. 5F). This indicated that knockdown of PTPRO could neutralize PDGF-BB-induced HSC proliferation, while no significant differences were observed after 12 h incubation.

Discussion

Although liver fibrosis is a critical stage in the mortality of chronic liver diseases, the pathological mediator for fibrogenesis has not been fully clarified [30]. A key event leading to liver damage is the transition of quiescent HSCs into activated myofibroblasts [31]. Therefore, inhibition of HSC activation/proliferation results primarily from recruitment of inflammatory cells or subsequent production of cytokines, and blockade of ECM formation is a key strategy for therapeutic intervention.

PTPRO is an important member of the receptor-type PTP family, which is implicated in several biological processes. Low or aberrant expression of PTPRO has been shown in many types of human cancer, including lung cancer, hepatocellular carcinoma, and breast cancer [18, 32, 33]. Research also shows that PTPRO inhibitors can significantly reduce the pathophysiological severity in a mouse model of allergic dermatitis and inflammatory bowel disease [34].. Other PTPs play a regulatory role in fibrosis. For instance, PTP-α promotes profibrotic signaling pathways in lung fibroblasts through control of cellular responsiveness to TGF-β [35]. Orthovanadate as a PTP inhibitor suppresses HSC activation and liver fibrosis in rats [36]. PTEN (phosphatase and tension homolog) is a critical tumor suppressor in various types of cancer, whose reduction is followed by activation of the PI3K/AKT and ERK pathways, resulting in HSC activation [37]. How PTPRO acts in liver fibrosis is still unknown. In our current study, we utilized gene-deficient mice and demonstrated that PTPRO expression was reduced in mouse and human fibrotic liver tissue, and that PTPRO KO or knock-down plays a critical role in emolliating collagen deposition and HSC activation via PDGF/AKT and ERK pathways.

We demonstrated mice deficient in PTPRO displayed a significant reduction in hepatic inflammation and fibrogenesis in two different models. At the meantime, the expression of PTPRO in both human cirrhotic tissue and mouse fibrosis models are downregulated. So, hepatocytes and HSCs were investigated separately *in vitro*. We found that PDGF-BB stimulation reduced PTPRO expression in hepatocytes but increased its production in HSCs. Considering that hepatocytes comprise the majority of hepatic cells, downregulated PTPRO in whole liver homogenate might not reflect the actual situation in different cell types. Although the mechanism underlying this phenomenon is still unclear, we further demonstrated that inhibition of PTPRO had profound antifibrogenic effects and improved survival in BDL mice.

PTPRO plays a critical role in aberrant carcinogenesis, therefore, we tried to verify the exact consequences in BDL- and CCl$_4$-induced chronic liver injury and fibrosis. BDL operation and CCl$_4$ injection contained autoimmune activation and acute inflammation stimulate, which are the well simulate for human primary biliary cirrhosis and hepatitis B virus infection associated liver fibrosis [38, 39]. We found that PTPRO deficiency largely protected mice from repeated challenges that cause liver injury, profibrotic cytokine expression, and fibrosis with collagen deposition. TGF-β as the classical profibrotic factor enhancing collagen and ECM deposition through the Smad2 and Smad3 pathway is PTPRO dependent [40]. Although further investigation on the relation between TGF-β and PTPRO is lacking, PTPRO KO is assumed to have a protective effect in liver cirrhosis.

PDGF is a potent inducer of HSC and hepatocyte activation and proliferation [27, 41]. It has been shown that both the ERK and PI3K/AKT pathways are markedly activated following stimulation with PDGF and other growth factors and subsequently promote HSC activation

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[29, 42]. Mouse models simulate a complex pathophysiological syndrome including chronic inflammation injury and the multilineage antimitochondrial response that causes human cirrhosis [38, 43]. Considering that viability of HSCs isolated from PTPRO$^{-/-}$ mice is not satisfied for the following test and PTPRO deletion is not liver specific, which could affect experiment result, we utilized HSCs isolated from livers of C57BL/6 mice following shRNA treatment to minimize the above influence. We found that PTPRO expression was enhanced by PDGF, and knockdown of PTPRO with shRNA strongly inhibited PDGF-induced proliferation and α-SMA expression, which suppressed the phosphorylation of PDGF/AKT and PDGF/ERK simultaneously in activated HSCs. PTPRO expression was notably reduced in the primary murine hepatocytes after treatment with PDGF for 12 h. However hepatic fibrogenesis is a complex response mediated by many different cell populations, with ECM accumulation representing the final step. It is conceivable that other cells such as Kupffer cells and hepatocytes are also involved during the wound-healing response [26]. PTPRO plays a dual role in hepatic I/R injury, and can act as a marker of acute podocyte injury in rat nephrosis [22]. PTPRO expression attenuates STAT3 activity, which in hepatocytes plays a protective role in preventing liver fibrosis [44, 45]. Research has also found that up-regulation of STAT3 phosphorylation is dependent on Kupffer-cell-derived IL-6 secretion [46]. PTPRO expression *in vivo* may also be an indirect cooperating with a direct mechanism in HSC activation and progression of liver fibrosis. The level of PTPRO expression could also be multi-azimuth controlled by HSCs, Kupffer cells and hepatocytes *in vivo*, which could account for the different trends between PDGF-induced HSC activation *in vitro* and liver homogenate of human and mouse model tissues. Interestingly, we have recently shown that PTPRO expression inhibits progression of hepatocellular carcinoma through downregulating STAT3 [21]. Since liver fibrosis are the precancerous lesion of hepatocellular carcinoma, PTPRO may served as an pro-fibrotic and an anti-oncogenic role at the same time. We speculate that PTPRO may play a different role depending on the cell type, for instance hepatocytes or HSC. As we shown, PTPRO dependent HSC activation contributes to the ECM and collagen deposition but PTPRO attenuated STAT3 activation inhibits HCC progression. On the other hand, the role of PTPRO in immune regulation and surveillance are also important in HCC development which need more meticulous research.

As research continues, liver fibrosis is associated with a growing number of factors including hypoxia and immunoregulation [47, 48]. PDGF, an important signaling pathways involved in hypoxia associated liver fibrosis and HSC activation and proliferation [49], which are currently developed as new antifibrotic drugs target, and have many breakthroughs, including brivanib and nilotinib [50, 51]. Sodium orthovanadate is a PTP inhibitor and a pivotal regulator of profibrotic mechanisms that affect the PDGF pathway [36, 52]. This study shows the positive role of PTPRO in liver fibrosis, which makes a major contribution to understanding the pharmacology of these drugs and drives the discovery of new drugs.

Although the findings suggested a conviced role of PTPRO in regulation of liver fibrosis, more work is necessary to clarify the detail of the signaling pathway and translate this into therapy that alleviates the burden and susceptibility of these disorders in humans.

Disclosure Statement

All authors have contributed substantially to this work and declare no conflict of interest.

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