The small heterodimer partner inhibits activation of hepatic stellate cells via autophagy

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Abstract

Background. Hepatic fibrosis is a health concern worldwide, and it is of great importance to develop effective therapeutic targets. The small heterodimer partner (SHP) is a regulator of lipid and bile acid metabolism in the liver.

Objectives. The objective of this study was to investigate the contribution of SHP to hepatic fibrosis and the underlying mechanism.

Material and methods. An in vivo rat model of hepatic fibrosis was created through treatment with carbon tetrachloride. We used arginine-glycine-aspartic acid-poly (ethylene glycol)-polyethyleneimine (RGD-PEG-PEI) for the specific transfer of SHP into hepatic stellate cells (HSC). The level of gene expression was detected using quantitative real-time polymerase chain reaction (qRT-PCR). The LX2 cell line was selected for the in vitro assay. Artificial activation of LX2 in vitro was conducted through treatment with platelet-derived growth factor-BB (PDGF-BB), and autophagy was activated using rapamycin. Gain and loss of function assays were performed using a SHP-expressing plasmid or siRNA-SHP. Both qRT-PCR and western blotting were utilized to detect the level of gene expression.

Results. RGD-PEG-PEI-mediated the specific transduction of SHP into HSC in the liver and effectively increased the expression of SHP in the rat liver. After treatment with RGD-PEG-PEI-SHP, downregulation of liver fibrosis-associated genes was observed. The results of the in vitro assay indicated that SHP attenuated the stimulating effect of PDGF-BB on the activation of LX2 cells. Overexpression of SHP leads to significant downregulation of HSC activation-associated molecular factors, including α -smooth muscle actin, tissue inhibitor of metalloproteinase-1, and type I collagen. Conversely, increased expression of these molecules could be observed following knockdown of SHP. Furthermore, SHP affected fibrosis by inhibiting autophagy activated through treatment with rapamycin in LX2 cells. Overexpression of SHP may prevent liver fibrogenesis through inhibition of autophagy in HSC.

Conclusions. The SHP may prevent liver fibrogenesis through inhibition of autophagy in HSC. A SHP-targeting therapy-based anti-fibrosis strategy possesses potential for application to the treatment of liver fibrosis.

Key words: hepatic stellate cells, autophagy, liver fibrosis, small heterodimer partner

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Introduction

Liver cirrhosis is a severe chronic disease, mainly caused by chronic hepatitis B virus and hepatitis C virus infections, alcohol abuse, and non-alcoholic steatohepatitis.¹ It affects 1–2% of the population worldwide. Currently, there are no effective clinical therapies for liver cirrhosis, except for the management of the primary cause or liver transplantation.²

Hepatic fibrosis is the pre-pathological stage of cirrhosis, which may last for nearly 10 years prior to progressing into cirrhosis. Nevertheless, it is a reversible process and can be attenuated or reversed through the use of effective therapies.³ Hepatocytes possess marked regenerative capability, and the replication of hepatocytes can easily replace the necrotic hepatocytes. However, chronic and sustained damage may impair the regenerative capability of hepatocytes, activating hepatic stellate cells (HSC) to induce liver fibrogenesis through secretion of α -smooth muscle actin (α -SMA), tissue inhibitors of metalloproteinase 1 (TIMP1) and type I collagen. Activation of HSC is a pivotal step in the development of hepatic fibrosis.⁴ Recent studies have suggested the reversibility of hepatic fibrosis,⁵ and several HSC-targeting therapies have been proposed as attractive strategies for the treatment of liver fibrosis, such as inhibition of HSC activation, promotion of HSC phenotypic conversion, promotion of HSC apoptosis, and induction of HSC senescence.⁶ It was reported that the Yes-associated protein and hedgehog inhibitors can suppress myofibroblastic activity by reducing aerobic glycolysis in HSC.⁷ Yang et al. confirmed the protective role of heme oxygenase-1 in inhibiting fibrosis by enhancing nuclear transcription factor kappa B signaling pathway-mediated apoptosis of HSC.⁸ Senescence of activated HSC may be achieved through the upregulation of p53 and p21 by interleukin 10 (IL-10).⁹ Collectively, this evidence indicated that suppression of HSC activation may be a feasible therapeutic target against hepatic fibrosis.

Autophagy, a process of degradation of “old” or “broken” organelles, is utilized by cells to maintain homeostasis and survival during stress.¹⁰ Loss of HSC lipid droplet (LDs), a typical feature of the activation of liver stellate cells, was observed during the process of HSC activation; it is accompanied by an increased level of autophagy.¹¹ Selective reduction of autophagy activity (e.g., treatment with autophagy inhibitor 3-methyladenine or inhibition of the autophagy-related gene autophagy related-5 (Atg5)) can lead to a significant increase in the number and size of LDs in HSC, causing LDs to accumulate and impairs HSC activation.^{12,13} These results suggest that autophagy may act as energy supplier for the activation of HSC by breaking down and reutilizing LDs.¹⁴ Thoen et al. also confirmed the essential contribution of autophagy to liver cirrhosis by inducing the activation of HSC.¹⁵

As a unique member of the nuclear receptor (NR) superfamily, small heterodimer partner (SHP) does not contain

a conserved DNA binding domain. However, its dimerization and ligand-binding domain confers critical ability for directly binding to multiple NRs and exert its function as a transcriptional inhibitor of gene expression.¹⁶ Several mechanisms may be utilized by SHP to repress the transcription of NR target genes. Interaction between SHP and the activation function-2 domain of NRs, mediated by functional LXXXLL-related motifs, can lead to direct competition with coactivator binding.¹⁷ It was also reported that the SHP-RNs complex of the DNA promoter region may be dissociated, as a result of the interaction between SHP and NRs.¹⁸ In addition, SHP may exert a direct inhibitory effect on transcription by recruiting a conventional corepressor to its C terminus, which contains a strong transcriptional repression domain. The *SHP* gene is expressed in a variety of tissues, with the liver being the most SHP-rich organ.¹⁹ Aberrant expression of SHP is associated with numerous diseases, such as obesity and diabetes,²⁰ lipodystrophy syndromes,²¹ and cancer.²² However, the relationships between SHP, autophagy and liver fibrosis remain to be elucidated.

Binding to the type VI collagen receptor on the HSC surface confers circular polypeptide C*GRGDSPC* the ability to specifically target HSC.²³ Our research team previously synthesized the novel cyclic peptide C*GAGASPK* with a more stable spatial conformation. This peptide contains an arginine-glycine-aspartic acid (RGD) sequence and can bind to activated HSC.²⁴ Polyethyleneimine (PEI) can be ingested by cells through phagocytosis, endocytosis and pinocytosis, and combined with DNA molecules through electrostatic interaction. Modification of the transfection complex using poly(ethylene glycol) (PEG) can transform it into a sterically stable nanoparticle, shielding change to reduce the interaction with plasma components.²⁵ For the elucidation of the regulatory effect of SHP on liver fibrosis in vivo, we utilized RGD-PEG-PEI as a polymer carrier to transport a plasmid overexpressing the *SHP* gene into HSC in the liver of rats. An in vitro assay was also utilized to confirm the regulatory effects of SHP on the activation of HSC, and further analyze the mechanism involved in this process.

Material and methods

Chemicals and antibodies

Rabbit anti-SHP (1/200 dilution; Novus, St. Charles, USA) was used for immunohistochemistry. The following antibodies were used for western immunoblotting: β -actin and α -SMA (A5316 and A5228, respectively) (Sigma-Aldrich, St. Louis, USA), type I collagen, P62, Atg12, and LC3-I/II (Cell Signaling Technology Inc, Danvers, USA). Polyvinylidene difluoride membranes, 4–15% Tris hydrochloride gels (BioRad, Richmond, USA), enhanced chemiluminescence reagents (Denville Scientific Inc, Metuchen,

USA), and a protease inhibitor cocktail (Shengyi, Shanghai, China) were used. Human recombinant platelet-derived growth factor-BB (PDGF-BB) and rapamycin (Beyotime Biotechnology, Shanghai, China) were used to study the effect of activation and autophagy in LX2 cells. For quantitative real-time polymerase chain reaction (qRT-PCR), the RNAeasy Kit (Qiagen, Valencia, USA) and High Complementary DNA (cDNA) Reverse Transcription kit (Applied Biosystems, Foster City, USA) were utilized.

Animals

All experimental protocols were approved by the Committee of Ethics of Animal Experiments at Zhongshan Hospital, Shanghai, China (approval No. 20188223). Male, specific pathogen-free Norway rats (age: 6 weeks) were provided by the Experimental Animal Center of Shanghai Medical College of Fudan University (China). The animals were housed in groups at 20 ± 5°C (55% ± 5% humidity) with free access to standard chow and water. A total number of 20 rats were used in this study (animal No. 20180006013073). The study protocol potentially caused slight suffering to the animals; however, animals that lost >30% of the initial body weight were euthanized after being deeply anesthetized with pentobarbital at a dose of 40 mg/kg.

Animal models

Liver fibrosis was induced in rats through the administration of carbon tetrachloride (CCl₄).²⁶ RGD-PEG-PEI-SHP was synthesized as previously described.²⁷ Animals were randomly divided into the following 3 groups: sham (n = 6), CCl₄ (n = 7) and CCl₄+RGD-PEG-PEI-SHP (n = 7). Animals in the control group were treated with 0.9% saline twice weekly for 8 weeks. In the CCl₄ group, rats received intraperitoneally CCl₄ (700 µL/kg of body weight) dissolved in olive oil twice weekly for 2 weeks. In the RGD-PEG-PEI-SHP group, the rats received intraperitoneally CCl₄ (700 µL/kg of body weight) dissolved in olive oil twice weekly for 2 weeks. After 1 week, rats received RGD-PEG-PEI-SHP (concentration: 50 µg/mL; dosage: 0.5 mL/100 g) at weeks 4 and 6. All animals were sacrificed at week 8. The content of liver hydroxyproline and serum hyaluronic acid was measured through routine clinical biochemistry. Additionally, samples obtained from each liver were stored in a –80°C refrigerator for qRT-PCR analysis.

Isolation of total mRNA and assessment of gene expression through qRT-PCR

The TRIzol reagent (Takara Biochemicals, Kyoto, Japan) was utilized to extract total mRNA from cells or rat tissue according to the instruction provided by the manufacturer. Following the removal of genomic DNA from the extracted RNA, the PrimeScript Reverse Transcription Reagent Kit

Table 1. The primer sequences of qRT-PCR

Gene	Primer sequence (5'-3')
<i>α-SMA</i>	Forward: CTCTGGACGCACAACCTGGCATC Reverse: CACGCTCAGCAGTAGTAACGAAGG
<i>SHP</i>	Forward: GTGCCAGCATACTCAAGAAG Reverse: TGGGGTCTGTCTGGCAGTT
<i>ATG3</i>	Forward: ACATGGCAATGGGCTACAGG Reverse: CTGTTTGACCCGCTTAGCA
<i>ATG9</i>	Forward: AGCGTGAGCTGACAGAGTGAC Reverse: AGCGTGAGCTGACAGAGTT
<i>ATG12</i>	Forward: CTGCTGGCGACACCAAGAAA Reverse: CGTGTTCTGCTACTGCCC
<i>ATG14</i>	Forward: TTCAGAGGCATAATCGCAAAC Reverse: CCAGACGCTCATAATGACTTCTT
<i>PDGF-β</i>	Forward: CTCGATCCGCTCCTTTGATGA Reverse: CGTTGGTGCGGCTATGAG
<i>Col1A1</i>	Forward: GTGCGATGACGTGATCTGTGA Reverse: CGGTGGTTTCTTGGTCGGT
<i>TIMP1</i>	Forward: ACCACCTTATACCAAGCGTTATGA Reverse: GGTGTAGACGAACCGGATGTC
<i>MMP2</i>	Forward: CCCACTGCGGTTTTCTCGAAT Reverse: CAAAGGGGTATCCATCGCCAT
<i>Fibronectin</i>	Forward: TGGAACTTCTACCAAGTGCGAC Reverse: TGT CTT CCC ATC ATC GTA ACA C
<i>TGF-β1</i>	Forward: AGCTGCGCTTGACAGATTA Reverse: AGCCCTGTATTCCGTCTCT
<i>MMP2</i>	Forward: ACAAGTGGTCCGCGTAAAGT Reverse: GTAAACAAGGCTTCATGGGGG
<i>β-actin</i>	Forward: CACTGTCGAGTCGCGTCC Reverse: TCATCCATGGCGAAGTGGTG

qRT-PCR – quantitative real-time polymerase chain reaction.

with gDNA Eraser (Takara Biochemicals) was used to reverse-transcribe the extracted RNA into single-stranded cDNA. Quantitative RT-PCR was performed to measure the levels of cDNA using the ABI PRISM 7500 PCR Sequence Detection System (Applied Biosystems) according to the instructions provided by the manufacturer of the Prime Script RT-PCR Kit (Takara Biochemicals). After denaturation for 30 s at 95°C, the samples were subjected to 40 cycles of treatment, consisting of 5 s at 94°C and 32 s at 60°C. A melting curve was generated to test the specificity of primers. Glyceraldehyde-3-phosphate dehydrogenase (GADPH) was used as an internal reference. The primers used are listed in Table 1.

Western blotting

Tissue was dissolved in 1 mL of radioimmunoprecipitation assay (RIPA) buffer (Beyotime Biotechnology), and the total amount of protein was quantified using the Bradford Protein Assay (Beyotime Biotechnology). Protein homogenates were boiled for 15 min at 100°C. For western blotting, proteins (50 µg) were loaded on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, and transferred onto polyvinylidene fluoride (PVDF)

membranes at 100 V for 70 min. Membranes were blocked with 5% bovine serum albumin (BSA) solution for 1 h prior to incubation with SHP (1:2,000; Santa Cruz Biotechnology, Santa Cruz, USA), α -SMA (1:1,000; Santa Cruz Biotechnology) and β -actin (1:1,000; Santa Cruz Biotechnology) antibodies overnight at 4°C. Membranes were washed using Tris-buffered saline with Tween 20 (TBST 20) and probed with appropriate secondary antibodies (labeled with alkaline phosphatase) for 1 h. The ImageJ software (National Institutes of Health, Bethesda, USA) was used to determine the intensity of protein bands.

siRNA-mediated downregulation of SHP

LX2 cells, an immortalized human HSC line,²⁸ were cultured at 37°C in an atmosphere of 5% CO₂ in Dulbecco's Essential Medium (Gibco BRL Life Technologies, Waltham, USA) containing 10% fetal bovine serum (FBS), antibiotics (penicillin/streptomycin) and 2 mM L-glutamine. SHP knockdown was performed according to the approved guidelines. LX2 cells were transfected with targeted or control siRNA; the sense sequence for siRNA-SHP was 5-UAGAAAGCAAGCCAGGAAGAUGACC-3 (Jima Company, Shanghai China). After trypsinization, LX2 cells were seeded into six-well plates at a density of 2×10^5 per well and cultured overnight. Cells were transfected with Lipofectamine 3000 Transfection Reagent (L3000; Invitrogen, Waltham, USA) for 48 h according to the protocol provided by the manufacturer. Transfected cells were cultured for at least 24 h and 48 h for RNA and western blotting analysis, respectively.

Overexpression of SHP in LX2 cells

The overexpression plasmid of SHP (NM-021969, rTA3 and ORF-200bp) was constructed by GeneChem Co. Ltd (Shanghai, China). LX2 cells were seeded into six-well plates. After 24–48 h of incubation, the transfection mixture was removed and replaced with fresh medium. The SHP cDNA was obtained through PCR amplification, subcloned into a vector, and transfected with a SHP-expressing plasmid or negative control using Lipofectamine 3000 (Invitrogen), according to the instructions provided by the manufacturer.

Activation and autophagy in LX2 cells

LX2 HSC were seeded into six-well plates (3×10^5 cells per plate) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. After 24 h, the cells were starved in serum-free medium for 12 h, followed by incubation with PDGF-BB (20 ng/mL) for 48 h. Rapamycin, a macrolide antibiotic, is widely used for inducing autophagy in a variety of cell types. In this study, we used rapamycin (0.5 ng in 0.5 μ L 0.1% dimethyl sulfoxide; Alexis Biochemicals, San Diego, USA) to activate autophagy in LX2 HSC. Briefly,

LX2 cells were initially starved in serum-free medium for 12 h, and subsequently incubated with rapamycin for 24 h. The expression of related genes was analyzed through western blotting and qRT-PCR.

Statistical analysis

All animal data represent at least 6 independent replicates and is expressed as mean $\pm$ standard deviation (SD). Data for LX2 cells represent the average of 3–6 replicates and is expressed as mean $\pm$ SD. Analysis of variance (ANOVA) or Student's t-test were used for statistical comparisons. Statistical tests were performed using the INSTAT statistical software (GraphPad Software, San Diego, USA). In all cases, $p < 0.05$ denoted statistical significance.

Results

RGD-PEG-PEI-SHP complex prevents the development of liver fibrosis in the CCl₄-treated model

The RGD-PEG-PEI carrier specifically transported the plasmid overexpressing SHP into HSC in the liver. Treatment with CCl₄ was an established model for liver fibrosis, characterized by inflammation and necrosis.²⁹ Liver immunohistochemistry and western blotting showed that the expression of SHP in the RGD-PEG-PEI-SHP group was significantly increased than that observed in the sham group. In comparison with the sham group and RGD-PEG-PEI-SHP group, the CCl₄ group showed significantly higher levels of hydroxyproline in the liver and hyaluronic acid in the serum (Fig. 1A and B). The western blotting of liver showed that the expression of SHP in the RGD-PEG-PEI-SHP group was significantly increased than that observed in the sham group. In contrast, this expression was decreased in the model group (Fig. 1C and D). These findings indicated that the SHP-targeting drug-loaded complex effectively increased the expression of SHP in liver tissue.

Quantitative RT-PCR analysis of the mRNA levels of SHP, α -SMA, TIMP1, collagen α 1(I), transforming growth factor- β 1 (TGF- β 1), and matrix metalloproteinase 2 (MMP2) (Fig. 1E–J) in whole liver homogenates showed that the expression of some profibrogenic markers (TGF- β 1 and α -SMA) in the RGD-PEG-PEI-SHP group was significantly reduced ($p < 0.005$ vs CCl₄ group). Moreover, in the RGD-PEG-PEI-SHP group, the expression of the *SHP* gene increased by 1.7-fold ($p < 0.005$ vs the sham group), consistent with previous histological results. The expression of the TIMP1 and collagen α 1(I) genes in the RGD-PEG-PEI-SHP group was significantly induced ($p < 0.05$ vs CCl₄ group). However, there was no statistically significant difference in the expression of the *MMP2* gene observed in the RGD-PEG-PEI-SHP group ($p > 0.05$ vs CCl₄ group). These results suggest that the RGD-PEG-PEI-SHP

Fig. 1. RGD-PEG-PEI-SHP complex prevents the development of liver fibrosis in the CCl₄ treatment model. A and B. Liver hydroxyproline content and serum hyaluronic acid content in rats. C. Protein expression of SHP in the liver was determined with western blotting and shown as a histogram. D. Relative gene expression of SHP, α -SMA, TGF- β 1, TIMP1, Col1a1, and MMP2 was measured with qRT-PCR. Data is presented as the mean $\pm$ SD; n = 6; *p < 0.05; **p < 0.01; ***p < 0.001 vs the sham group

complex can significantly improve the degree of liver fibrosis in the rat model induced by CCl₄, and is not associated with significant hepatotoxicity.

SHP regulates the expression of fibrosis-related genes in HSC

In vivo studies have indicated that SHP prevents the development of liver fibrosis. Therefore, we sought to confirm whether SHP prevents fibrosis mainly by inhibiting

the activation of HSC. For this purpose, we initially silenced the expression of SHP in LX2 cells using an anti-SHP siRNA, and subsequently stably overexpressed the SHP gene through the transfection of the plasmid. Quantitative RT-PCR and western blotting demonstrated that the 3 siRNAs of SHP successfully downregulated the expression of SHP in LX2 cells (Fig. 2A,B). It is worth noting that si-SHP-3 exhibited the highest inhibition efficiency, reaching 87% (n = 6; p < 0.0001 vs sham group). We also overexpressed the SHP using transfected with the plasmid (Fig. 2C,D). The α -SMA,

Fig. 2. The SHP silencing and overexpression in LX2 cells. Anti-SHP siRNA was used as described in the Material and methods section. **A.** Western blotting analysis of SHP obtained from LX2 cells treated with different sequences of siRNA and shown as a histogram. **B.** mRNA expression of SHP was measured using qRT-PCR. **C.** Western blotting analysis of SHP obtained from LX2 cells treated with the plasmid. **D.** mRNA expression of SHP was measured with qRT-PCR. Data is presented as the mean $\pm$ SD; $n = 6$; ** $p < 0.01$; *** $p < 0.001$ vs the control group

collagen $\alpha 1(I)$ and fibronectin are 3 key markers of HSC activation and liver fibrosis. In addition, TGF- β , PDGF, etc., could change the phenotype of HSC and induce their activation. The results of the western blotting analysis showed that knockdown of the *SHP* gene increased the expression of type I collagen and α -SMA (Fig. 3A). We found that the gene expression of collagen $\alpha 1(I)$ and *TIMP1* also increased, following treatment with si-RNA LX2 (Fig. 3B).

Similarly, qRT-PCR analysis of collagen $\alpha 1(I)$, TGF- $\beta 1$, PDGF- β , and α -SMA in si-SHP LX2 cells showed different degrees of increase in mRNA levels compared with control (Fig. 3C).

In contrast, the gene expression of MMP2 decreased by approx. 50% (Fig. 3B; $p < 0.005$ vs sham group). We overexpressed the *SHP* gene to further investigate the role of SHP in regulating the expression of hepatic fibrosis-related genes. Western blotting analysis demonstrated that the relative expression of SHP protein in total lysates obtained from LX2 cells transfected with the plasmid was significantly enhanced compared to that measured in LX2 cells transfected with an empty plasmid vector. These findings were consistent with the results of the qRT-PCR analysis (Fig. 2C,D). Following overexpression of the *SHP* gene, the expression of both fibrosis-related and HSC activation-related genes was decreased, which is contrary to the results observed after silencing the *SHP* gene (Fig. 3D–F). It is particularly noteworthy that the increased expression of SHP could significantly promote a two-fold change in the expression of the *MMP2* gene (Fig. 3E, $n = 3$, $p < 0.01$ vs sham group). Thus, the results clarified that the *SHP* gene exerts a regulatory effect on fibrosis-related genes.

SHP inhibits the PDGF-BB-induced activation of LX2 cells

A large number of studies have confirmed that PDGF-BB can be used to induce the activation of HSC in vitro.^{26,28}

We treated LX2 cells with PDGF-BB (20 ng/mL) for 48 h to induce HSC activation. The 3 groups were the following: normal LX2 cells (control group), LX2 cells of the PDGF-BB intervention group and LX2 cells overexpressing the *SHP* gene from the PDGF-BB intervention group. The western blotting analysis showed that, after treatment of LX2 cells with PDGF-BB (20 ng/mL), the levels of type I collagen and α -SMA were significantly increased compared with those reported in the control group, indicating that PDGF-BB promotes the activation of LX2 cells (Fig. 4A). Although the expression of SHP decreased, the difference was not statistically significant. In the LX2-overexpressing SHP group, the levels of type I collagen and α -SMA were also decreased in SHP-overexpressing LX2 cells compared with those observed in cells subjected to treatment with PDGF-BB alone ($p < 0.05$ vs the PDGF-BB group).

The HSC activation is often accompanied by the increased expression of TGF- $\beta 1$, PDGF- β and fibronectin, which is closely related to liver fibrosis. Quantitative RT-PCR results showed that the levels of α -SMA, TGF- $\beta 1$, PDGF- β , and fibronectin were significantly increased in LX2 cells treated with PDGF-BB, compared with those noted in the control group (Fig. 4B–E). This treatment was also performed in the SHP overexpression group. The gene expression of α -SMA, TGF- $\beta 1$, PDGF- β , and fibronectin exhibited different degrees of recovery compared with those recorded in the sham group. These results indicate that SHP inhibits the activation of LX2 cells treated with PDGF-BB.

SHP inhibits the activation of LX2 cells via autophagy

Recent studies have shown that liver fibrosis is closely related to the enhancement of autophagy.^{30–32} Previous results showed that overexpression of SHP increased the expression of genes involved in the inhibition of fibrosis and

Fig. 3. The SHP regulates the expression of fibrosis-related genes in HSC. Anti-SHP siRNA was used as described in the Materials and methods section. A. LX2 cells were incubated with SHP siRNA, and the protein expression of SHP and α -SMA were determined with western blotting and shown as a histogram. B and C. Relative gene expression of ColA1, MMP1, MMP2, α -SMA, TGF- β 1, PDGF- β 1, and fibronectin was measured using qRT-PCR. The SHP overexpression was sufficient to downregulate the expression of type I collagen and upregulate α -SMA in LX2 cells. D. Protein expression of SHP and α -SMA was determined through western blotting and shown as a histogram. E and F. Relative gene expression of ColA1, MMP1, MMP2, α -SMA, TGF- β 1, PDGF- β 1, and fibronectin was measured with qRT-PCR. Data are presented as the mean $\pm$ SD; $n = 6$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs the control group

activation of HSC. Therefore, we inferred that SHP can act as an anti-fibrosis factor by inhibiting autophagy. To test this hypothesis, we compared the expression of autophagy-related genes in LX2 cells overexpressing SHP. The western blotting analysis showed that the expression of α -SMA was decreased in the SHP group compared with the control group. Furthermore, the expression of autophagy-related genes *Atg12* and *LC3-I/II* was downregulated, whereas that of *P62* was significantly increased (Fig. 5A). Quantitative RT-PCR results showed that the mRNA levels of *Atg3*, *Atg9*, *Atg12*, and *Atg14* in the SHP-overexpressing group were higher than those measured in the control group (Fig. 5B).

To further confirm whether SHP inhibits the activation of LX2 cells through autophagy, we treated wild-type LX2

cells and LX2 cells overexpressing SHP with rapamycin as previously described. After treatment with rapamycin (24 h), western blotting showed that the levels of *Atg12*, and *LC3-I/II* were significantly increased, whereas those of *p62* were decreased compared with the control group. These findings indicated that autophagy in LX2 cells was enhanced in response to treatment with rapamycin. However, in the LX2 cells overexpressing SHP, autophagy was significantly inhibited after treatment with rapamycin compared with the autophagy observed in the rapamycin-alone group (Fig. 5C). Quantitative RT-PCR results were consistent with those of western blotting (Fig. 5D,E). Therefore, this evidence shows that SHP mainly affects fibrosis by inhibiting autophagy in LX2 cells.

Fig. 4. The SHP inhibits PDGF-BB-induced activation of LX2 cells. LX2 cells were treated with PDGF-BB (20 ng/mL) as mentioned in the Materials and methods section. A. Protein expression of SHP, type I collagen, and α-SMA was determined with western blotting and shown as a histogram. B–E. Relative gene expression of ColA1, fibronectin, PDGF-β1, and TGF-β1 was measured with qRT-PCR. Data is presented as mean ± SD; n = 6; *p < 0.05; **p < 0.01; ***p < 0.001 vs the blank control group

Discussion

Liver fibrosis is a common process of liver self-repair in response to chronic liver injury, characterized by the typical manifestation of deposition of extracellular matrix (ECM).³³ Currently, effective treatments for clinical application are not available, thereby posing a great threat to human health. Several types of cell impairment can also be observed in the fibrotic liver, such as hepatocyte apoptosis and necrosis, recruitment of inflammatory cells,

remodeling of liver sinusoid endothelial cells, and activation of HSC.^{34,35} Activated HSCs are regarded as the main source of ECM in the fibrotic liver. Activated HSC can produce collagen and ECM to replace parenchymal tissue caused by scarring. Moreover, inhibition of HSC activation plays a critical role in the prevention and treatment of fibrosis. Extensive efforts were conducted to explore the mechanism involved in the activation of HSC, proposing the concept that liver fibrosis may be a reversible process. Specifically targeting molecules required for HSC

Fig. 5. The SHP inhibits the activation of LX2 cells through autophagy. Overexpression of SHP was achieved through transfection of a plasmid. A. Protein expression of P62, α-SMA, ATG12, LC3-I/II, and SHP was determined with western blotting and shown as a histogram. B. Relative gene expression of Atg3, Atg9, Atg12, and Atg14 was measured with qRT-PCR. LX2 cells were treated with rapamycin (5 μM) as mentioned in the Material and methods section. C. Protein expression of Atg12, P62, SHP, and LC3-I/II was determined with western blotting and shown as a histogram. D–E. Relative gene expression of SHP and Atg3 was measured using qRT-PCR. Data is presented as mean ±SD; n = 6; *p < 0.05; **p < 0.01; ***p < 0.001 vs the blank control group.

activation may be a feasible therapeutic strategy against liver fibrosis.

In the present study, we explored the regulatory effect of SHP in the activation of HSC both in vivo and in vitro, and further analyzed the underlying mechanism. A rat liver fibrosis model induced using CCl₄ was applied to further determine the biological effect of SHP in liver fibrosis in vivo. An RGD-PEG-PEI-mediated specific transduction of SHP into rat liver HSC effectively increased the expression of SHP. After treatment with RGD-PEG-PEI-SHP, downregulation of liver fibrosis-associated genes was observed. These results confirmed that SHP inhibits the activation of HSC, further reversing the progress of liver fibrosis in vivo.

The human LX2 cell line was selected as an in vitro model to further evaluate the inhibitory effect of SHP

on the activation of HSC, as well as the underlying mechanisms. The SHP mimics were transduced into LX2 cells to increase its expression, whereas siRNA-SHP was used to inhibit the expression of SHP. We found that overexpression of SHP leads to significant downregulation of the mRNA and protein levels of HSC activation-associated molecular factors, such as α-SMA, TIMP1 and type I collagen. In contrast, increased expression of these molecules can be observed after knockdown of SHP, suggesting that SHP can inhibit the activation of HSC. The PDGF-BB is the strongest mitogenic cytokine of HSC, commonly utilized as a stimulator of HSC activation in the liver fibrosis model.³⁶ Our study evaluated the effect of SHP on HSC activation induced by PDGF-BB, suggesting that it attenuates the stimulatory effect of PDGF-BB on HSC activation. This finding further elucidated the inhibitory role of SHP

in the activation of HSC. Additionally, the Cell Counting Kit-8 assay also confirmed that SHP could notably suppress growth in LX2 cells. Matrix metalloproteinases are a group of proteolytic enzymes that degrade the ECM. The activities of these enzymes are closely related to the expression of their specific inhibitors (TIMPs). The MMP2 is the main enzyme that reduces extracellular matrix collagen, while TIMP1 inhibits most MMPs. These results suggested that SHP ameliorates liver fibrosis by inhibiting HSC activation and suppressing HSC proliferation to reduce the number of these cells.

Autophagy is a complex metabolic process utilized by cells to degrade their misfolding proteins and damaged organelles, contributing to the maintenance of cellular homeostasis during the destruction of intracellular pathogens and nutrient deprivation.³⁷ Activation of autophagy in HSC contributed to the transformation of the fat-rich phenotype into an activated myofibroblast phenotype. Autophagy promotes the digestion of LDs in resting HSC, which is the activation and fibrosis of HSC. In recent years, the regulatory role of autophagy in the activation of HSC attracted considerable attention owing to the observation that autophagy could break down LDs – an established feature of HSC activation. However, there is a contradictory effect of autophagy on liver fibrosis. Autophagy can act as an inhibitor of HSC activation, attenuating liver fibrosis.³⁸ Several studies have suggested the role of autophagy as an inducer of HSC activation. Accumulating evidence suggests that autophagy plays an energy-providing role in the activation of HSC, and inhibition of autophagy in HSC can attenuate the development of liver fibrosis.³⁹ Hernandez-Gea et al. also reported the role of autophagy in aggravating liver fibrosis by degrading LDs to provide energy for HSC.¹³ Furthermore, the enhancement of autophagy in HSC promoted cell proliferation and the production of type I collagen through hypoxia-inducible factors and the TGF- β /Smad signaling pathway.⁴⁰ These results suggest that autophagy may be a double-edged sword in liver fibrosis. In the present study, we explored the relationship between SHP, autophagy and liver fibrosis. In vitro testing demonstrated that SHP can inhibit autophagy in HSC, as shown by the decreased expression of autophagy marker proteins (e.g., LC3-II or Atg12) and increased expression of P62 (which is negatively correlated with autophagy).⁴¹ These results indicate that SHP may partially inhibit the activation of HSC by attenuating autophagy in these cells.

Conclusions

The results of our study suggest that overexpression of SHP may prevent liver fibrogenesis through inhibition of autophagy in HSC. A SHP-targeting therapy-based anti-fibrosis strategy possesses potential for application to the treatment of liver fibrosis.

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