

Original Article

HSC-specific knockdown of GGPPS alleviated CCl₄-induced chronic liver fibrosis through mediating RhoA/Rock pathway

Shan-Shan Lai^{2,3}, Xiao Fu^{3,4}, Qi Cheng³, Zi-Han Yu³, En-Ze Jiang³, Dan-Dan Zhao^{3,5}, De-Cai Yu⁴, Yu-Dong Qiu⁴, Xiang Gao⁶, Huang-Xian Ju⁷, Wei Wang⁸, Qing Jiang^{9,10}, Min-Sheng Zhu⁶, Chao-Jun Li³, Bin Xue^{1,3,11,12}

¹Core Laboratory, Sir Run Run Hospital, Nanjing Medical University, Nanjing 211166, China; ²Jiangsu Key Laboratory for Biodiversity and Biotechnology, College of Life Sciences, Nanjing Normal University, Nanjing 210023, China; ³Jiangsu Key Laboratory of Molecular Medicine and School of Medicine, Nanjing University, Nanjing 210093, China; ⁴Department of Hepatobiliary Surgery, The Affiliated Drum Tower Hospital, Medical School of Nanjing University, Nanjing 210093, China; ⁵Research Center, Shanghai Chest Hospital, Shanghai Jiao Tong University, Shanghai 200030, China; ⁶Key Laboratory of Model Animal for Disease Study of Ministry of Education, Model Animal Research Center, Nanjing University, Nanjing 210093, China; ⁷MOE Key Laboratory of Analytical Chemistry for Life Science, School of Chemistry and Chemical Engineering, Nanjing University, Nanjing 210093, China; ⁸National Laboratory of Solid State Microstructures, Department of Physics, Nanjing University, Nanjing 210093, China; ⁹Department of Sports Medicine and Adult Reconstructive Surgery, Drum Tower Hospital, School of Medicine, Nanjing University, Nanjing 210008, China; ¹⁰Joint Research Center for Bone and Joint Disease, Model Animal Research Center (MARC), Nanjing University, Nanjing 210093, China; ¹¹State Key Laboratory of Pharmaceutical Biotechnology, Jiangsu Key Laboratory of Molecular Medicine and School of Medicine, Nanjing University, Nanjing 210093, China; ¹²State Key Laboratory of Natural Medicines, China Pharmaceutical University, Nanjing 210009, China

Received November 12, 2018; Accepted February 7, 2019; Epub April 15, 2019; Published April 30, 2019

Abstract: Hepatic stellate cells (HSCs) play a critical role in the pathogenesis and reversal of liver fibrosis. Targeting HSCs is of great significance in the treatment of hepatic fibrosis, and has attracted wide attention of scholars. Here we demonstrated that expression of geranylgeranyldiphosphate synthase (GGPPS) predominantly increased in HSCs in murine fibrotic liver. HSC-specific knockdown of GGPPS using vitamin A-coupled liposome carrying siRNA-ggpps decreased activation of HSCs and alleviated fiber accumulation *in vivo*. Furthermore, our *in vitro* studies showed that GGPPS was up-regulated during HSCs activation in TGF- β 1-dependent manner. Inhibition of GGPPS suppressed TGF- β 1 induced F-actin reorganization and HSCs activation in LX-2 cells. Further, we found that GGPPS regulated HSCs activation and liver fibrosis possibly by enhancing RhoA/Rock kinase signaling. So its concluded that GGPPS promotes liver fibrosis by activating HSCs, which may represent a potential target for anti-fibrosis therapies.

Keywords: GGPPS, hepatic stellate cell, liver fibrosis, TGF- β 1

Introduction

Liver fibrosis, which is characterized by excessive deposition of extracellular matrix (ECM) proteins, is a main pathologic basis for chronic liver diseases. Advanced liver fibrosis results in cirrhosis, liver failure, and is associated with an enhanced risk of hepatocellular carcinoma (HCC) [1, 2]. There is an increasing evidence that liver fibrosis is reversible [3, 4]. However, advanced cirrhosis may not be completely reversible, and the recovery depends on the etiology and stage of the disease [5].

HSCs play a crucial role in the progression and reversion of liver fibrosis [6]. In normal liver, HSCs maintain a non-proliferative, quiescent phenotype. Following liver injury, HSCs trans-differentiate from vitamin-A-storing cells to myofibroblast-like cells, a kind of proliferative, contractile, inflammatory and chemotactic cells [7]. This activation process is provoked by cytokines, including transforming growth factor- β (TGF- β) 1, platelet derived growth factor (PDGF) and tumor necrosis factor- α (TNF- α). Activated HSCs are characterized by the expression of α -smooth muscle actin (α -SMA), loss of vitamin

GGPPS promotes HSCs activation and liver fibrosis

A and enhanced production of ECM proteins, thereby promoting the fibrosis progression [6]. Although HSCs constitute only approximately 5-8% of the total liver cells, they are the major contributors for liver fibrosis [8]. Elimination of activated HSCs is one of the effective way to reverse liver fibrosis [2]. HSCs are an appealing target for antifibrogenic therapy, however, targeted delivery of antifibrotic agents to HSCs is a major challenge in such therapies. Up to now, at least three mechanisms are effective in HSCs clearing: apoptosis, senescence and reversion [7]. Apoptosis of HSCs during resolution of liver fibrosis results in the decrease in the number of activated HSCs [7]. HSCs undergo senescence during liver fibrosis in rodents and human [9]. Senescence of HSCs results in reduced expression of ECM, increased expression of matrix metalloproteinases (MMPs), thereby facilitating the resolution of fibrosis [9]. Importance of senescence in the resolution of fibrosis was confirmed in mice with ablation of a p53-dependent senescence program in HSCs [10]. Additionally, it is reported that half of the activated HSCs escape apoptosis during regression of liver fibrosis and acquire a phenotype similar to quiescent HSCs [11].

Mevalonate pathway participates in the synthesis of the majority of body cholesterol, and inhibited mevalonate with statins exhibit antifibrotic efficacy in multiple organ fibrosis [12-17]. Several studies have focused on the therapeutic potential of statins in treatment of fibrosis. For example, Rombouts et al. found that lovastatin and simvastatin reduced the protein steady state level of collagens type I, III and IV in primary cultured rat HSCs [12]. Simvastatin suppressed TGF β 1-induced fibronectin in airway fibroblasts, which relied on activation of GGTase I and the availability of geranylgeranylpyrophosphate (GGPP) [18]. However, statins have some unavoidable adverse sides effects, such as muscle problems, an increased risk of diabetes mellitus and increased liver damage [19, 20]. So highly efficient target therapy is urgently needed for treatment of liver fibrosis.

GGPPS is an enzyme critical for catalyzing the synthesis of GGPP in the mevalonate pathway. Our previous reports find that aberrant GGPPS expression is associated with various diseases. For example, GGPPS deletion in β -cell results in typical T2DM β -cell dysfunction through

Rab27A geranylgeranylation [21]. GGPPS deficiency inhibits lipid-induced muscle insulin resistance [22]. In addition, GGPPS is up-regulated during liver injury and may play a critical role during the development of HCC from cirrhosis [23]. Therefore, GGPPS-deficiency promotes HSC activation by cross-talk between hepatocytes and HSCs. However, the role of GGPPS in HSC activation during liver fibrosis and the underlying mechanism is still not fully understood.

In this study, CCl₄-induced mouse liver fibrosis model was established. The role and mechanism of GGPPS in HSC activation and liver fibrosis were investigated. Our findings may provide evidence for a promising therapy for liver fibrosis through targeting GGPPS in HSCs.

Materials and methods

Patient samples

Human liver tissues with cirrhosis were obtained from 6 patients diagnosed with liver cirrhosis in Drum Tower Hospital (Nanjing, China). Normal liver tissues were obtained from 5 age- and sex-matched patients who were diagnosed with gall-stone. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Animals and liver fibrosis model

8-weeks-old male C57BL/6 mice used in this study were obtained from Nanjing Model Animal Research Center (Nanjing, China) and kept in a specific pathogen-free facility with a 12 h/12 h cycle and received human treatment. They were randomly assigned into CCl₄ group (n=21) and olive group (n=5). To induce liver fibrosis, mouse was given two times of injection of CCl₄ (1 μ l/g body weight; mixed with olive oil at the volume ratio of 1:3) (Sigma Chemicals Co., USA) i.p. every week on Tuesday and Friday for consecutive 12 weeks. Control mice received equal volume of olive oil (1 μ l/g body weight). All animal experiments were conducted according to the ethical guidelines of Animal Care and Use Committee at the Model Animal Research Centre of Nanjing University, China.

GGPPS promotes HSCs activation and liver fibrosis

Knockdown of GGPPS in vivo

Liver fibrosis was generated by 12 week treatment of mice with CCl_4 . Subsequently, mice were treated with vitamin A (VA)-coupled liposomes carrying siRNA-ggpps (VA-lip-siRNA-ggpps) or negative control (VA-lip-NC) (0.75 mg/kg by intraperitoneal injection, two times a week) for 3 weeks. Vitamin A were purchased from sigma. Cationic liposomes DC-6-14 were purchased from Hokkaido System Science. SiRNAs directed the mice GGPPS were made by GenePharma (China). Non-Targeting siRNA Pool from GenePharma was used as a negative control. Mice were sacrificed 48 hours after the last CCl_4 injection. The VA-lip-siRNAggpps was prepared as previously describe [24]. The efficacy of VA-lip-siRNAggpps was tested in primary HSCs.

Isolation of primary hepatocytes and hepatic stellate cells

HSCs were isolated from CCl_4 -treated mice before or after GGPPS knockdown. The liver perfusion buffer and liver digest medium were prewarmed to 37°C. The liver was perfused in situ via the inferior vena cava with EGTA buffer while the vena porta was used as outflow until the liver became pale. The perfusion was continued with Type I collagenase (Roche Applied Science) buffer. The liver was carefully removed and dissociated in 10 mL 10% DMEM medium, and then filtered through 70 μm cell strainers and centrifuged for 5 min at 50 \times g at 4°C. The supernatant and precipitate were used to separate the HSCs and hepatocytes in the following operation, respectively.

The supernatant was transferred to a new centrifuge tube and centrifuged for 10 min at 2000 \times g at 4°C, then suspended the precipitate with 2 ml 10% FBS culture medium. A discontinuous density gradient (15%, 10%) was made using OptiPrep™ (Sigma-Aldrich, St. Louis, MO) following the manufacturer's instructions and the cells were centrifuged for 10 min at 2000 \times g at 4°C. The HSCs at the interface were collected.

The precipitate was suspended with 20 ml 40% percoll (Sigma-Aldrich, St. Louis, MO) plus solution, after gentle mix, the cells were centrifuged at 400 \times g for 10 min at 4°C. Hepatocytes were collected after removing the supernatant.

Cell culture and treatment

Human HSC cell line (LX-2) was purchased from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific) containing 10% fetal bovine serum (FBS, Thermo Fisher Scientific) under 37°C containing 5% CO_2 in a humidified atmosphere.

For TGF- β 1 treatment, LX-2 cells were pre-incubated with DMEM containing 0.5% FBS for 24 h, and stimulated with 7.5 ng/ml TGF- β 1 (Sigma-Aldrich) for 24 h. For inhibitors treatment, SB431542 (Sigma-Aldrich, St. Louis, MO), Rapamycin (Sigma-Aldrich, St. Louis, MO) and Fasudil (Sigma-Aldrich, St. Louis, MO) were added in culture medium 2 h before TGF- β 1 stimulation. For the infection of adenovirus, LX-2 cells were collected 48 h after infection with Ad-GFP and Ad-GGPPS. For Membrane association measurements, LX-2 cells were transfected with siRNAggpps or scramble control using lipofectamine RNAi^{MAX} (Thermo Fisher Scientific) in 6-well dishes following the manufacturer's instructions. After stimulated with 7.5 ng/ml TGF- β 1 for 24 h, cells were lysed, homogenized, and ultracentrifuged to fractionate membrane proteins and cytoplasmic proteins as described previously [25]. The ratio of the distribution of RhoA on the membrane to that on the cytoplasm was calculated by Mean gray value using Image J software.

RNA extraction and real-time PCR

Total RNA was isolated from mouse livers or LX-2 cells using Trizol reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. Total RNA was reverse transcribed to complementary DNA using the Reverse Transcription System (Takara, Dalian, China). Real-time PCR was performed using the SYBR Green real-time PCR Master Mix (Toyobo, Osaka, Japan). The reactions were performed on the ABI-7300 (Applied Biosystems, Foster City, CA, USA). Sequences of primers for the reference gene (GAPDH) and interested genes are listed as follows: α -SMA forward (5'-CCTGAAGTACCCGATAGAACAT-3'); α -SMA reverse (5'-TCTCCAGAGTCCAGCACGAT-3'); Collgen1 forward (5'-AGGCCAAGACGAAGACATC-3'); Collgen1 reverse (5'-AGATCACGTCATCGACAACA-3'); GGPPS reverse (5'-CGTCGGAGTTTTGAGTTGTCT-3'); Timp-

GGPPS promotes HSCs activation and liver fibrosis

1 forward (5'-ATTCCGACCTCGTCATCAG-3'); Timp-1 reverse (5'-ATTCCTCACAGCCAACAGTG-3'); GAPDH forward (5'-CCCTTCATTGACCTCAACTACATG-3'); GAPDH reverse (5'-TGGGATTTCCATGATGACAAGC-3').

Western blotting

Tissues were homogenized and the cells were directly lysed with RIPA buffer with protease inhibitors (Roche, Germany). Protein concentration was determined using the bicinchoninic acid protein assay reagents (KeyGen biotech, China). Proteins were separated by SDS-PAGE and transferred to PVDF membrane. After the membrane was incubated with primary antibodies against GGPPS, TGF- β 1, RhoA (Santa Cruz Biotechnology Inc., Santa Cruz, USA), α -SMA (Thermo Fisher Scientific), GAPDH, β -Actin and α -Tubulin (Bioworld Technology, Inc, China), p-Smad, Smad, p-S6, S6, MLC and p-MLC (Cell signaling technology, MA, USA) and HRP-conjugated second antibodies (Betotome Biotechnology, China), and the signal was developed by ECL (Millipore, Switzerland) and visualized by Tanon 5200 imaging system (Tanon, China).

Histological analyses

The isolated mouse liver tissues were fixed in 4% Paraformaldehyde, embedded in paraffin and cut into 5-mm sections. After deparaffinization and hydration, sections were stained in 0.1% (w/v) Sirius Red (Direct Red 80, Sigma-Aldrich) in a saturated aqueous solution of picric acid for 1 h. Then the slides were rinsed out twice for 15 min each in 0.01 NHCl to remove unbound dye. After dehydration, the slides were mounted. Images were photographed under an Olympus BX51 microscope. The percentage of fibrotic area was calculated by Image-Pro Plus 6.0. To assess the GGPPS distribution, the sections were further processed for immunostaining (IF) with rabbit-anti GGPPS antibody (generated by our lab, 1:100 dilution) and mouse-anti α -SMA (Thermo Fisher Scientific, 1:1000 dilution). Alexa Fluor 488-conjugated goat anti mice and Alexa Fluor 594-conjugated goat anti rabbit IgG polyclonal (Invitrogen, Carlsbad, CA, 1:200 dilution) was used as secondary antibodies.

Phalloidin staining

Nontransfected, scramble control- or siRNA_{gpps}-transfected LX-2 cells were trypsinized and

plated on coverslips. LX-2 cells were treated with 7.5 ng/ml TGF- β 1 for 24 h after pre-treated with 0.5% FBS. Cultures were then fixed in 4% Paraformaldehyde during 15 min and cells were permeabilized with 2% Triton-X-100 for 10 min. After three washes in PBS, cells were incubated with FITC-Phalloidine (Sigma-Aldrich, St. Louis, MO) and DAPI for 2 h at room temperature. Coverslips were then mounted in PBS: Glycerol, 1:9, and viewed on Olympus BX51 microscope.

Statistical analysis

The results are expressed as mean \pm standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance using GraphPad Prism 5, and $P < 0.05$ was considered significant.

Results

GGPPS is specifically up-regulated in activated HSCs

Our previous findings suggest that GGPPS was up-regulated in liver injury and HCC [26]. To investigate the GGPPS expression in HSCs, liver samples were collected from mice after model establishment and the expression of GGPPS was examined by immunofluorescence. α -SMA was used as a marker for activated HSCs. As shown in **Figure 1A**, GGPPS was highly expressed in activated HSCs in mice with CCl₄-induced early hepatic fibrosis but not in hepatocytes. Expression of GGPPS was also examined in isolated HSCs from CCl₄- or olive oil-treated mice by western blotting. As expected, GGPPS was obviously higher in HSCs from CCl₄-treated mouse livers than that from control livers (**Figure 1B**). Next, human cirrhotic liver samples and healthy liver samples were collected and the expression of GGPPS was analyzed by western blotting. Similarly, expression of GGPPS was detected in TGF- β 1 and α -SMA highly expressed livers (**Figure 1C**).

HSC-specific GGPPS knockdown in mice inhibits the activation of HSCs and alleviates liver fibrosis

Vitamin A-coupled siRNA was used to specifically knockdown GGPPS in HSCs *in vivo*. To confirm the targeted delivery of siRNA_{gpps} to HSCs, expression of GGPPS was analyzed by real-time PCR in isolated HSCs and hepato-

GGPPS promotes HSCs activation and liver fibrosis

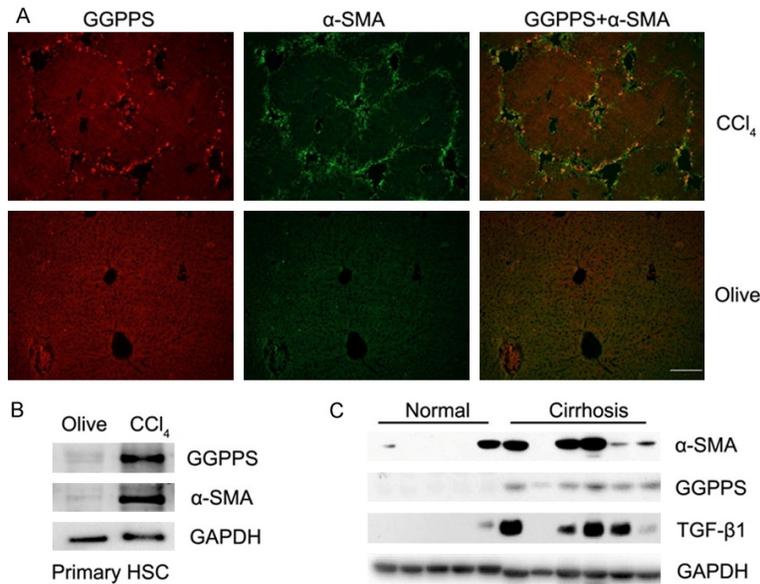


Figure 1. Analysis of GGPPS expression *in vivo*. A. Male C57BL/6 mice were i.p. injected with CCl₄ or olive oil for 12 weeks, and the livers were collected. Immunofluorescence staining against GGPPS (red) and α -SMA (green) was performed in liver sections from CCl₄- or olive oil-treated mice. Scale bar, 50 μ M. B. GGPPS levels in the isolated HSCs from CCl₄- or olive oil-treated mice were determined by western blotting. C. Expression of GGPPS was detected in patients' liver cirrhotic tissues or normal liver tissues by western blotting.

cytes from the CCl₄-induced fibrotic livers. As shown in **Figure 2A**, compared with VA-lip-NC, VA-lip-siRNA_{ggpps} administration significantly down-regulated GGPPS expression in HSCs, but not in hepatocytes.

After being treated with CCl₄ for 12 consecutive weeks, the expression of α -SMA, Collagen I and Timp-1 mRNA in livers were significantly increased compared with that treated with olive oil (**Figure 2B**). However, knockdown of GGPPS in HSCs by VA-lip-siRNA_{ggpps} reduced their expression in a certain degree compared with VA-lip-NC. The α -SMA protein was also decreased after GGPPS siRNA injection, indicating that HSC activation may be suppressed (**Figure 2C**). Similarly, the fibrotic area was smaller in liver specimens from VA-lip-siRNA_{ggpps} treated mice than in VA-lip-NC or olive oil treated specimens as demonstrated by Sirius red staining (**Figure 2D**).

GGPPS participates in TGF- β 1-induced HSCs activation

TGF- β 1 is considered as the most important pro-fibrogenic cytokine and mediates the HSCs activation and ECM production. In order to

investigate whether GGPPS is involved in TGF- β 1 induced HSCs activation, we first examined GGPPS expression in LX-2 cells following TGF- β 1 stimulation. Endogenous expression of α -SMA was increased 24 h after incubation with TGF- β 1 in a dose dependent manner, and the expression of GGPPS showed the similar tendency with that of α -SMA (**Figure 3A**). Since 7.5 ng/ml is sufficient to cause significant increases in α -SMA and GGPPS expression, we selected this dose in subsequent experiments. Moreover, we found that TGF- β 1 can also induce α -SMA and GGPPS expression in a time dependent manner (**Figure 3B**). Overexpression of GGPPS in LX2 cells up-regulated exogenous α -SMA expression (**Figure 3C**). Whereas knockdown of GGPPS by siRNA down regulated

TGF- β 1 induced expression of α -SMA (**Figure 3D**) and the formation of F-actin (**Figure 3E**).

In order to demonstrate whether TGF- β 1 could induce GGPPS expression through the classical TGF- β /smad pathway, SB431542 was used to inhibit the smad2/3 signaling pathway. Unexpectedly, although SB431542 significantly decreased phosphorylated smad2 and smad3 expression, it had no obvious effects on α -SMA or GGPPS expression (**Figure 4A**). Next rapamycin, an inhibitor of mTOR was administrated to determine whether mTOR pathway mediates the up-regulation of GGPPS induced by TGF- β 1. Western blotting results showed that treatment of rapamycin suppressed TGF- β 1-induced up-regulation of GGPPS in a dose-dependent manner (**Figure 4B**). These results imply that TGF- β 1 promotes GGPPS expression through non-classical pathway.

GGPPS mediates HSCs activation through RhoA/ROCK signaling pathway

There may exist a cross-talk between TGF- β /Smad pathway and non-Smad signaling pathway. Therefore, we analyzed whether the Smad pathway could be affected by GGPPS in turn.

GGPPS promotes HSCs activation and liver fibrosis

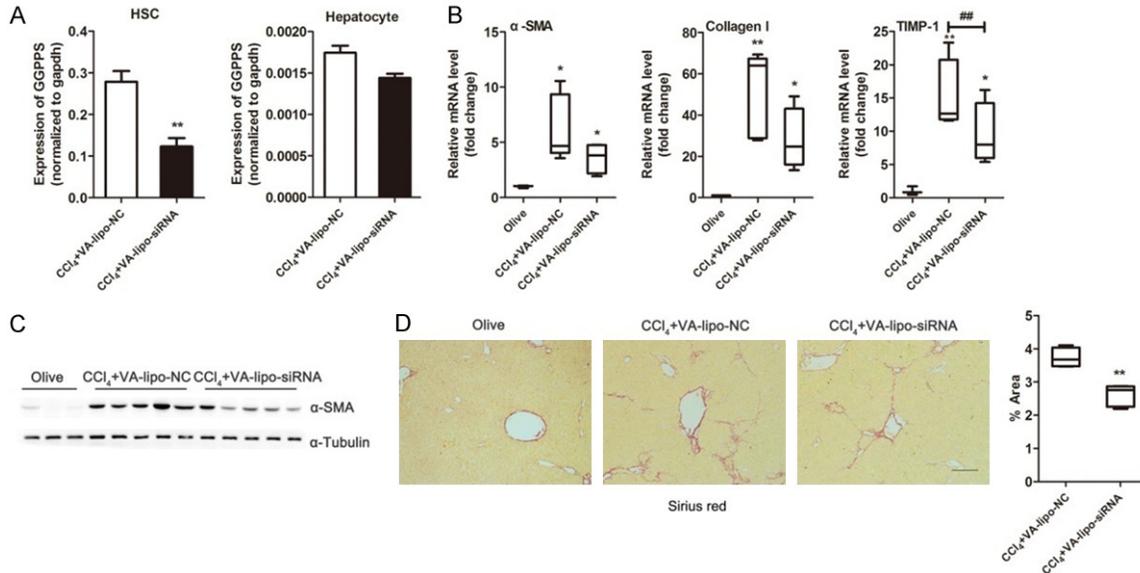


Figure 2. HSCs-specific GGPPS knockdown inhibits HSCs activation and alleviates liver fibrosis. **A.** Expression of GGPPS in HSCs from VA-lip-siRNAaggpps (n=2) or VA-lip-NC (n=2) treated mice were analyzed by real-time PCR. **, P<0.01 vs NC. **B.** Relative expression of α-SMA, collagen I and TIMP-1 mRNA in livers from CCI₄+VA-lipo-siRNAaggpps (n=5), CCI₄+VA-lipo-NC (n=5) or Olive (n=3) treated mice. *, P<0.05; **, P<0.01 vs olive; ##, P<0.01 vs NC. **C.** Expression of α-SMA, GGPPS or α-Tubulin in livers from CCI₄+VA-lipo-siRNAaggpps (n=5), CCI₄+VA-lipo-NC (n=5) or Olive (n=3) treated mice were detected by western blotting. **D.** Liver fibrosis was evaluated by Sirius red staining. Sirius red-positive area was calculated. **, P<0.01. Scale bar, 50 μM.

We found that inhibition of GGPPS in LX-2 cells doesn't affect the phosphorylation of both Smad2 and Smad3 (**Figure 4C**). It has been reported that Rho signaling pathways play an important role in the activation of HSC, and ROCK inhibitor prevents DMN-induced hepatic fibrosis and reduces the α-SMA expression in liver, as well as in vitro [27]. Therefore, we speculated that GGPPS may regulate HSC activation and α-SMA expression through Rho-dependent pathway. We found increased phosphorylation of RhoA/Rock downstream effector-myosin light chain (MLC) along with TGF-β1 treatment in a time-dependent manner (**Figure 4D**). Moreover, the RhoA kinase inhibitor, fadusil, significantly blocked TGF-induced α-SMA expression (**Figure 4E**). In addition, membrane associated RhoA was determined after inhibition of GGPPS. As shown in **Figure 4F**, membrane accumulation of RhoA was increased 24 h after TGF-β treatment, which can be reduced by GGPPS knockdown. These data indicates that GGPPS may promote HSC activation through mediating RhoA geranylgeranylation and activation of RhoA/Rock pathway.

Discussion

In the present study, we found that the expression of GGPPS was increased in activated

HSCs during chronic liver fibrosis. To elucidate the significance of the specific expression of GGPPS in HSC activation and liver fibrosis development, we established a liver fibrosis mice model and specifically knocked down GGPPS in HSCs by using vitamin A coupled liposome to deliver ggpps siRNA to fibrotic liver. Our results suggest that GGPPS aggravate liver fibrosis by promoting HSCs activation though mediating RhoA/ROCK signaling pathway.

The Mevalonate pathway, which synthesizes cholesterol, plays an important role in the activation of HSCs and occurrence of fibrosis, which has been confirmed by the using of HMG CoA reductase inhibitors (statins). Statin can inhibit the activation and proliferation of HSCs, and may be an effective preventative therapy for fibrosis progression and virus-related liver cirrhosis in clinical studies [28-30]. In addition, a recent study has shown that Mevalonate pathway also plays a role in the contraction of activated HSCs [30]. In this experiment, Liu et al. treated HSCs with a traditional Chinese medicine Sodium ferulate (SF) and found that SF inhibited the contraction of activated HSCs through affecting RhoA activation and this inhibition was efficiently reversed by addition of GGPP. Here, our present work further confirmed

GGPPS promotes HSCs activation and liver fibrosis

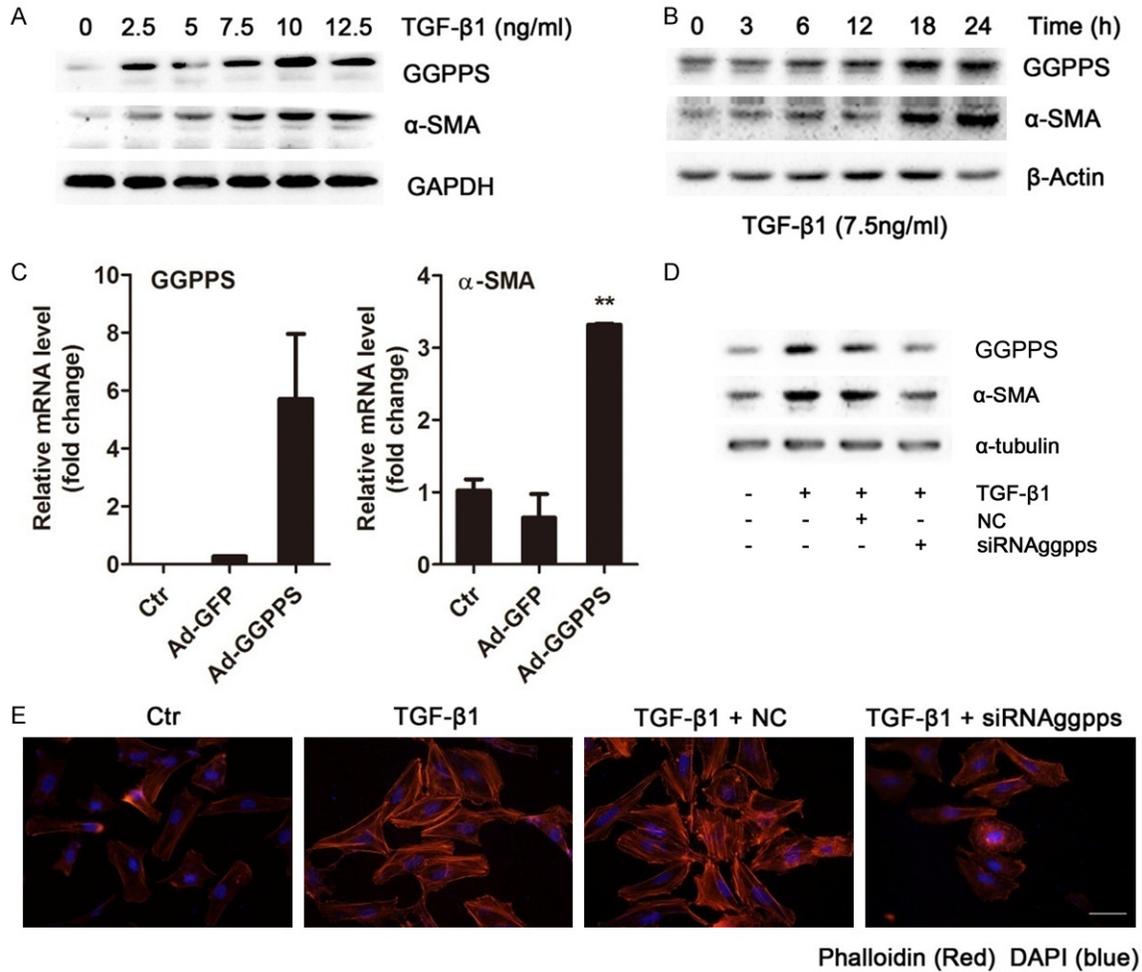


Figure 3. TGF-β mediates HSC activation through GGPPS. A. Western blotting analysis of the expression of α-SMA, GGPPS and GAPDH in response to 24 h exposure to increasing doses of TGF-β1. B. Western blotting analysis the expression of α-SMA, GGPPS and GAPDH at different time after exposure to 7.5 ng/ml TGF-β1. C. mRNA level of GGPPS, α-SMA were analyzed by real-time PCR 24 h after infection with Ad-GGPPS. *P<0.05, **P<0.01 vs Ad-GFP. D. Expression of GGPPS, α-SMA and α-Tubulin were detected by western blotting after 7.5 ng/ml TGF-β1 treatment for 24 h in control or GGPPS knockdown LX-2 cells. E. F-actin was detected after 7.5 ng/ml TGF-β1 treatment for 24 h in control or GGPPS knockdown LX-2 cells. Scale bar, 50 μM.

that Mevalonate pathway is important for HSC activation and fibrosis progress. In mevalonate pathway, GGPPS is responsible for biosynthesis of GGPP, which is required for Rho, Rab and Rap family proteins [31]. Abnormal expression of GGPPS results to metabolic disease [22], Heart failure [32] and reproductive organ development problems [33, 34]. GGPPS is also closely related to liver diseases, such as HCC and liver damage [23, 26]. In combination with our previous study, GGPPS in hepatocytes and HSCs participates in the activation of HSC and liver fibrosis through different mechanisms. Therefore, GGPPS can be used as a target for the treatment of liver diseases.

Liver fibrosis is a reversible wound-healing response regulated by many kinds of liver cells. At early stages of fibrogenic process, wound-healing responses are initiated by liver sinusoidal endothelial cells, platelets, TGF-β and PDGF. As the main fibrogenic cell type in the liver, HSCs become activated as a result of interactions with hepatocytes, macrophages, endothelial cells, cholangiocytes and natural killer cells. HSCs trans-differentiate into myofibroblast-like cells and cause the deposition of ECM proteins. Activation of HSCs is crucial to liver fibrosis, and therapies targeting these cells are promising for treatment of chronic liver diseases. It is reported that in the animal

of vitamin A has also been confirmed by Zhao et al. [36]. Since GGPPS was prominently expressed in HSCs during fibrosis (as shown in our immunofluorescence results), we choose vitamin A to delivery GGPPS siRNA to specifically knockdown GGPPS expression in HSCs. The expression of GGPPS was indeed knocked down in HSCs. Retinol-binding protein 4 (RBP4) is a newly discovered adipokine that produced by hepatocytes and adipocytes, which delivers vitamin A to tissues [40]. Because the existence such Vitamin A binding proteins in hepatocytes, we also detected a slight decrease in GGPPS mRNA expression in primary hepatocytes after VA-lip-siRNaggpps injection. Zhao et al. suggested that target the surface receptor of activated HSCs is also a good method to deliver therapeutic agents, which has a higher specificity and uptake [36]. Taken together, we demonstrated that GGPPS is highly expressed in HSCs and regulates the activation of HSC and liver fibrosis though the RhoA/Rock pathway. Our study provides better understanding of the role of GGPPS in regulating fibrosis progress. Our findings may provide evidence for the development of novel anti-fibrogenic strategies and potent drugs to protect hepatic fibrosis process.

Acknowledgements

This work was supported by the Chinese National Science Foundation (31371373, 3177-1572, 31701234, 31601153), the Nature Science Foundation of Jiangsu Province (BK-20151395, BK20171035, BK20160619), the Open Fund of State Key Laboratory of Natural Medicines (No. SKLNMKF201606), the Natural Science Foundation of the Jiangsu Higher Education Institutions (17KJB180006) and the Fundamental Research Funds for the Central Universities (021414380330).

Disclosure of conflict of interest

None.

Abbreviations

HSC, Hepatic stellate cell; GGPPS, Geranylgeranyldiphosphate synthase; ECM, Extracellular matrix; HCC, hepatocellular carcinoma; TGF- β , transforming growth factor- β ; PDGF, platelet derived growth factor; TNF- α , tumor necrosis factor- α ; α -SMA, smooth muscle α -actin; MMP,

Matrix metalloproteinases; GGPP, geranylgeranylpyrophosphate; MLC, myosin light chain; SF, Sodium ferulate.

Address correspondence to: Bin Xue, Sir Run Run Hospital, Nanjing Medical University; State Key Laboratory of Pharmaceutical Biotechnology, Jiangsu Key Laboratory of Molecular Medicine and School of Medicine, Nanjing University, Nanjing 210093, China. Tel: +86-25-83596845; Fax: +86-25-8359-6845; E-mail: xuebin@nju.edu.cn

References

- [1] Blachier M, Leleu H, Peck-Radosavljevic M, Valla DC and Roudot-Thoraval F. The burden of liver disease in Europe: a review of available epidemiological data. *J Hepatol* 2013; 58: 593-608.
- [2] Zoubek ME, Trautwein C and Strnad P. Reversal of liver fibrosis: from fiction to reality. *Best Pract Res Clin Gastroenterol* 2017; 31: 129-141.
- [3] Marcellin P, Gane E, Buti M, Afdhal N, Sievert W, Jacobson IM, Washington MK, Germanidis G, Flaherty JF, Aguilar Schall R, Bornstein JD, Kitrinos KM, Subramanian GM, McHutchison JG and Heathcote EJ. Regression of cirrhosis during treatment with tenofovir disoproxil fumarate for chronic hepatitis B: a 5-year open-label follow-up study. *Lancet* 2013; 381: 468-475.
- [4] Chang TT, Liaw YF, Wu SS, Schiff E, Han KH, Lai CL, Safadi R, Lee SS, Halota W, Goodman Z, Chi YC, Zhang H, Hindes R, Iloeje U, Beebe S and Kreter B. Long-term entecavir therapy results in the reversal of fibrosis/cirrhosis and continued histological improvement in patients with chronic hepatitis B. *Hepatology* 2010; 52: 886-893.
- [5] Kisseleva T and Brenner DA. Hepatic stellate cells and the reversal of fibrosis. *J Gastroenterol Hepatol* 2006; 21 Suppl 3: S84-87.
- [6] Friedman SL. Mechanisms of hepatic fibrogenesis. *Gastroenterology* 2008; 134: 1655-1669.
- [7] Tsuchida T and Friedman SL. Mechanisms of hepatic stellate cell activation. *Nat Rev Gastroenterol Hepatol* 2017; 14: 397-411.
- [8] Marra F and Pinzani M. Role of hepatic stellate cells in the pathogenesis of portal hypertension. *Nefrologia* 2002; 22 Suppl 5: 34-40.
- [9] Krizhanovsky V, Yon M, Dickins RA, Hearn S, Simon J, Miething C, Yee H, Zender L and Lowe SW. Senescence of activated stellate cells limits liver fibrosis. *Cell* 2008; 134: 657-667.
- [10] Lujambio A, Akkari L, Simon J, Grace D, Tschaharganeh DF, Bolden JE, Zhao Z, Thapar V, Joyce JA, Krizhanovsky V and Lowe SW. Non-

GGPPS promotes HSCs activation and liver fibrosis

- cell-autonomous tumor suppression by p53. *Cell* 2013; 153: 449-460.
- [11] Kisseleva T, Cong M, Paik Y, Scholten D, Jiang C, Benner C, Iwaisako K, Moore-Morris T, Scott B, Tsukamoto H, Evans SM, Dillmann W, Glass CK and Brenner DA. Myofibroblasts revert to an inactive phenotype during regression of liver fibrosis. *Proc Natl Acad Sci U S A* 2012; 109: 9448-9453.
- [12] Rombouts K, Kisanga E, Hellemans K, Wielant A, Schuppan D and Geerts A. Effect of HMG-CoA reductase inhibitors on proliferation and protein synthesis by rat hepatic stellate cells. *J Hepatol* 2003; 38: 564-72.
- [13] Louneva N, Huaman G, Fertala J and Jimenez SA. Inhibition of systemic sclerosis dermal fibroblast type I collagen production and gene expression by simvastatin. *Arthritis Rheum* 2006; 54: 1298-308.
- [14] Li C, Yang CW, Park JH, Lim SW, Sun BK, Jung JY, Kim SB, Kim YS, Kim J and Bang BK. Pravastatin treatment attenuates interstitial inflammation and fibrosis in a rat model of chronic cyclosporine-induced nephropathy. *Am J Physiol Renal Physiol* 2004; 286: F46-57.
- [15] Jaster R, Brock P, Sparmann G, Emmrich J and Liebe S. Inhibition of pancreatic stellate cell activation by the hydroxymethylglutaryl coenzyme A reductase inhibitor lovastatin. *Biochemical Pharmacology* 2003; 65: 1295-1303.
- [16] Saka M, Obata K, Ichihara S, Cheng XW, Kimata H, Noda A, Izawa H, Nagata K and Yokota M. Attenuation of ventricular hypertrophy and fibrosis in rats by pitavastatin: potential role of the RhoA-extracellular signal-regulated kinase-serum response factor signalling pathway. *Clin Exp Pharmacol Physiol* 2006; 33: 1164-1171.
- [17] Essig M, Vrtovnsnik F, Nguyen G, Sraer JD and Friedlander G. Lovastatin modulates in vivo and in vitro the plasminogen activator/plasmin system of rat proximal tubular cells: role of geranylgeranylation and Rho proteins. *J Am Soc Nephrol* 1998; 9: 1377-1388.
- [18] Schaafsma D, McNeill KD, Mutawe MM, Ghavami S, Unruh H, Jacques E, Laviolette M, Chakir J and Halayko AJ. Simvastatin inhibits TGF-beta1-induced fibronectin in human airway fibroblasts. *Respir Res* 2011; 12: 113.
- [19] Bellosta S and Corsini A. Statin drug interactions and related adverse reactions. *Expert Opin Drug Saf* 2012; 11: 933-946.
- [20] Thompson PD, Panza G, Zaleski A and Taylor B. Statin-associated side effects. *J Am Coll Cardiol* 2016; 67: 2395-2410.
- [21] Jiang S, Shen D, Jia WJ, Han X, Shen N, Tao W, Gao X, Xue B and Li CJ. GGPPS mediated Rab27A geranylgeranylation regulates beta-cell dysfunction during type 2 diabetes development via affecting insulin granule docked pool formation. *J Pathol* 2015.
- [22] Tao W, Wu J, Xie BX, Zhao YY, Shen N, Jiang S, Wang XX, Xu N, Jiang C, Chen S, Gao X, Xue B and Li CJ. Lipid-induced muscle insulin resistance is mediated by GGPPS via modulation of the RhoA/rho kinase signaling pathway. *J Biol Chem* 2015; 290: 20086-20097.
- [23] Yu DC, Liu J, Chen J, Shao JJ, Shen X, Xia HG, Li CJ, Xue B and Ding YT. GGPPS1 predicts the biological character of hepatocellular carcinoma in patients with cirrhosis. *BMC Cancer* 2014; 14: 248.
- [24] Sato Y, Murase K, Kato J, Kobune M, Sato T, Kawano Y, Takimoto R, Takada K, Miyanishi K, Matsunaga T, Takayama T and Niitsu Y. Resolution of liver cirrhosis using vitamin A-coupled liposomes to deliver siRNA against a collagen-specific chaperone. *Nat Biotechnol* 2008; 26: 431-442.
- [25] Wang XX, Ying P, Diao F, Wang Q, Ye D, Jiang C, Shen N, Xu N, Chen WB, Lai SS, Jiang S, Miao XL, Feng J, Tao WW, Zhao NW, Yao B, Xu ZP, Sun HX, Li JM, Sha JH, Huang XX, Shi QH, Tang H, Gao X and Li CJ. Altered protein prenylation in Sertoli cells is associated with adult infertility resulting from childhood mumps infection. *J Exp Med* 2013; 210: 1559-1574.
- [26] Chen WB, Lai SS, Yu DC, Liu J, Jiang S, Zhao DD, Ding YT, Li CJ and Xue B. GGPPS deficiency aggravates CCl4-induced liver injury by inducing hepatocyte apoptosis. *FEBS Lett* 2015; 589: 1119-1126.
- [27] Tada S, Iwamoto H, Nakamuta M, Sugimoto R, Enjoji M, Nakashima Y and Nawata H. A selective ROCK inhibitor, Y27632, prevents dimethylnitrosamine-induced hepatic fibrosis in rats. *J Hepatol* 2001; 34: 529-536.
- [28] Ma X, Sun D, Li C, Ying J and Yan Y. Statin use and virus-related cirrhosis: a systemic review and meta-analysis. *Clin Res Hepatol Gastroenterol* 2017; 41: 533-542.
- [29] Rombouts K, Kisanga E, Hellemans K, Wielant A, Schuppan D and Geerts A. Effect of HMG-CoA reductase inhibitors on proliferation and protein synthesis by rat hepatic stellate cells. *J Hepatol* 2003; 38: 564-572.
- [30] Liu J, Peng L, Yang J, Wang M, Xu S, Liu J, Han P, He J, Tian D and Zhou Q. Sodium ferulate reduces portal pressure through inhibition of RhoA/Rho-Kinase and activation of endothelial nitric oxide synthase in cirrhotic rats. *Dig Dis Sci* 2015; 60: 2019-2029.
- [31] Van Aelst L and D'Souza-Schorey C. Rho GTPases and signaling networks. *Genes Dev* 1997; 11: 2295-2322.
- [32] Xu N, Guan S, Chen Z, Yu Y, Xie J, Pan FY, Zhao NW, Liu L, Yang ZZ, Gao X, Xu B and Li CJ. The alteration of protein prenylation induces car-

GGPPS promotes HSCs activation and liver fibrosis

- diomyocyte hypertrophy through Rheb-mTORC1 signalling and leads to chronic heart failure. *J Pathol* 2015; 235: 672-685.
- [33] Diao F, Jiang C, Wang XX, Zhu RL, Wang Q, Yao B and Li CJ. Alteration of protein prenylation promotes spermatogonial differentiation and exhausts spermatogonial stem cells in newborn mice. *Sci Rep* 2016; 6: 28917.
- [34] Jiang C, Diao F, Sang YJ, Xu N, Zhu RL, Wang XX, Chen Z, Tao WW, Yao B, Sun HX, Huang XX, Xue B and Li CJ. GGPP-mediated protein geranylgeranylation in oocyte is essential for the establishment of oocyte-granulosa cell communication and primary-secondary follicle transition in mouse ovary. *PLoS Genet* 2017; 13: e1006535.
- [35] Troeger JS, Mederacke I, Gwak GY, Dapito DH, Mu X, Hsu CC, Pradere JP, Friedman RA and Schwabe RF. Deactivation of hepatic stellate cells during liver fibrosis resolution in mice. *Gastroenterology* 2012; 143: 1073-1083 e1022.
- [36] Zhao Z, Li Y, Jain A, Chen Z, Liu H, Jin W and Cheng K. Development of a peptide-modified siRNA nanocomplex for hepatic stellate cells. *Nanomedicine* 2018; 14: 51-61.
- [37] Greenhalgh SN, Conroy KP and Henderson NC. Cre-activity in the liver: transgenic approaches to targeting hepatic nonparenchymal cells. *Hepatology* 2015; 61: 2091-2099.
- [38] Kocabayoglu P, Lade A, Lee YA, Dragomir AC, Sun X, Fiel MI, Thung S, Aloman C, Soriano P, Hoshida Y and Friedman SL. beta-PDGF receptor expressed by hepatic stellate cells regulates fibrosis in murine liver injury, but not carcinogenesis. *J Hepatol* 2015; 63: 141-147.
- [39] Higashi N, Sato M, Kojima N, Irie T, Kawamura K, Mabuchi A and Senoo H. Vitamin A storage in hepatic stellate cells in the regenerating rat liver: with special reference to zonal heterogeneity. *Anat Rec A Discov Mol Cell Evol Biol* 2005; 286: 899-907.
- [40] Cheng J, Song ZY, Pu L, Yang H, Zheng JM, Zhang ZY, Shi XE and Yang GS. Retinol binding protein 4 affects the adipogenesis of porcine preadipocytes through insulin signaling pathways. *Biochem Cell Biol* 2013; 91: 236-243.