

## Supplementary Information

### Tyrosine Kinase SYK is a Potential Therapeutic Target for Liver Fibrosis

Chen Qu<sup>1,2#</sup>, Dandan Zheng<sup>1,2#</sup>, Sai Li<sup>3#</sup>, Yingjun Liu<sup>4#</sup>, Anna Lidofsky<sup>5</sup>, Jacinta A. Holmes<sup>5</sup>, Jianning Chen<sup>6</sup>, Lu He<sup>1,2</sup>, Lan Wei<sup>7</sup>, Yadi Liao<sup>1,2</sup>, Hui Yuan<sup>1,2</sup>, Qimeng Jin<sup>1,2</sup>, Zelong Lin<sup>1,2</sup>, Qiaoting Hu<sup>1,2</sup>, Yuchuan Jiang<sup>1,2</sup>, Mengxian Tu<sup>1,2</sup>, Xijun Chen<sup>1,2</sup>, Weiming Li<sup>1,2</sup>, Wenyu Lin<sup>5</sup>, Bryan C. Fuchs<sup>7</sup>, Raymond T. Chung<sup>5</sup>, and Jian Hong<sup>1,2,5\*</sup>

<sup>1</sup>*Department of Abdominal Surgery, Integrated Hospital of Traditional Chinese Medicine, Southern Medical University, Guangzhou, Guangdong 510315, China*

<sup>2</sup>*Cancer Center, Southern Medical University, Guangzhou, Guangdong 510315, China*

<sup>3</sup>*Department of Pharmacy, Integrated Hospital of Traditional Chinese Medicine, Southern Medical University, Guangzhou, Guangdong 510315, China*

<sup>4</sup>*Department of General Surgery, Affiliated Tumor Hospital of Zhengzhou University, Zhengzhou, Henan 450008, China*

<sup>5</sup>*Gastrointestinal Unit, Department of Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA*

<sup>6</sup>*Department of Pathology, The Third Affiliated Hospital of Sun Yat-Sen University, Guangzhou 510630, China*

<sup>7</sup>*Division of Surgical Oncology, Massachusetts General Hospital Cancer Center and Harvard Medical School, Boston, MA*

#Authors share co-first authorship.

**\*Corresponding authors:** Dr. Jian Hong, Department of Abdominal Surgery, Integrated Hospital of Traditional Chinese Medicine, Southern Medical University, Guangzhou, Guangdong 510135, China. Phone & Fax: (+86 20) 6165 0514; E-mail: [jhong7@smu.edu.cn](mailto:jhong7@smu.edu.cn).

## Supplementary Figure Legends

**Supplementary Figure 1. SYK expression is up-regulated in human liver tissues in patients with intrahepatic cholestasis and non-alcoholic steatohepatitis (NASH).** (A&B) SYK expression was up-regulated in liver biopsies from infants with biliary atresia or other causes of intrahepatic cholestasis (GSE46960), and in NASH patients (GSE48452). (C) SYK immunohistochemistry (IHC) and Sirius red staining were performed in normal, cholestasis and NASH-associated liver fibrotic/cirrhotic tissues. Representative images of SYK and Sirius red staining are shown in the left panel. The right panel shows Ishak fibrosis scores, and SYK staining quantified by IHC in normal, cholestasis and NASH liver tissue. Scale bars, 200  $\mu$ m.

**Supplementary Figure 2. Higher SYK expression is associated with the development of rodent fibrosis.** (A) Higher mRNA expression of SYK and ACTA2 associated with liver fibrosis progression in a CCl<sub>4</sub>-induced fibrosis model given by gavage for 6, 12 or 18 weeks. (B) Positive correlation between SYK and ACTA2 mRNA expression in CCl<sub>4</sub>-induced fibrotic liver tissue in mice. Pearson's correlation analysis was used for statistical analysis. (C) DEN-induced rat fibrosis model, where DEN is administered by intraperitoneal (IP) injection for 8, 12 or 18 weeks. Representative images of liver tissue stained with Sirius red (magnification 40X) and  $\alpha$ -SMA (magnification 200X) for indicated treatment duration. (D&E) Higher mRNA expression of SYK, ACTA2 (D&E) and  $\alpha$ -SMA protein levels (E) was associated with liver fibrosis progression in a time dependent manner. Immunostaining of  $\alpha$ -SMA was detected by IHC. (F) Representative images of H&E (magnification 40X), Trichrome (40X),  $\alpha$ -SMA

(magnification 100X) and SYK (magnification 100X) staining in liver tissues from rats undergoing surgical BDL or sham surgery. **(G)** Higher IHC staining of  $\alpha$ -SMA was associated with SYK staining in a DEN-induced fibrosis rat model compared to sham control. Data presented are means  $\pm$  SD. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

**Supplementary Figure 3. SYK expression in various liver cell types. (A)** SYK expression in bile duct cells. Representative images of immunofluorescent co-staining of CK19 (green) and SYK (red) in normal and HBV-induced fibrotic liver tissue. **(B)** SYK expression in endothelial cells. Representative images of immunofluorescent co-staining of CD31 (green) and SYK (red) in normal and HBV-induced fibrotic liver tissue. **(C)** SYK expression in macrophages. Representative images of immunofluorescent co-staining of CD68 (green) and SYK (red) in normal and HBV-induced fibrotic liver tissue. **(D)** Immunofluorescent co-staining of CD68 (green) and SYK (red) in primary Kupffer cells isolated from normal and CCl<sub>4</sub>-induced fibrotic mouse liver. Scale bars, 10  $\mu$ m. **(E&F)** SYK and activated SYK [p-SYK(Y525/526)] expression in liver tissue from normal and CCl<sub>4</sub>-induced fibrotic mice **(E)**, and in hepatocytes and HSCs isolated from normal or CCl<sub>4</sub>-induced mouse fibrosis liver tissues **(F)**.

**Supplementary Figure 4. HCV or HBV infection is associated with induction of SYK expression. (A&B)** HCV infection in JFH1-infected Huh7.5.1 cells was associated with higher mRNA expression of SYK (A) and *TGF- $\beta$ 1* (B). **(C)** Positive correlation between mRNA levels of SYK and *TGF- $\beta$ 1* in JFH1 infected Huh7.5.1 cells for indicated time points. Pearson's correlation was used for statistical analysis. **(D)** Higher SYK mRNA expression was associated with HBV replication in HepG2.2.1.5

cells. HepG2.2.1.5 is HBV DNA sequences chromosomally integrated into HepG2, HepG2 cells are uninfected controls. Data presented are means  $\pm$  SD. \*\*\*,  $P < 0.001$ .

**Supplementary Figure 5. SYK overexpression does not stimulate Matrigel - induced inactivation of HSCs. (A)** SYK overexpression did not significantly affect protein levels of  $\alpha$ -SMA and PDGFR $\beta$  in LX-2 and TWNT-4 cells cultured on Matrigel. **(B)** Relative mRNA expression of *ACTA2*, *PDGFR $\beta$* , *COL1A1*, *TIMP1*, *PAI-1* and *TGF- $\beta$ 1* in control (Vector) and SYK over-expressed LX-2 and TWNT-4 cells cultured with or without Matrigel. Data presented are means  $\pm$  SD. NS = not significant. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

**Supplementary Figure 6. SYK overexpression promotes fibrosis related gene expression in HSCs. (A&B)** Overexpression of wild type (WT) or catalytically activated (CA) mutant SYK, but not kinase dead (KD) mutant SYK significantly enhanced the expression of fibrosis related genes, including *ACTA2*, *PDGFR $\beta$* , *COL1A1*, *TIMP1*, *PAI-1* and *TGF- $\beta$ 1* by qRT-PCR in LX-2 and TWNT-4 cells. Data presented are means  $\pm$  SD. NS = not significant. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

**Supplementary Figure 7. Two isoforms of SYK, SYK(L) and SYK(S), are both upregulated in CCl<sub>4</sub> and DEN-induced murine liver fibrosis models. (A)** Enhancement of mRNA expression of both *SYK(L)* and *SYK(S)* in CCl<sub>4</sub>-induced mouse liver fibrogenesis in a time dependent manner from 6, 12 to 18 weeks. **(B)** Increasing of mRNA expression of both *SYK(L)* and *SYK(S)* in DEN-induced rat liver fibrosis in a time dependent manner from 8, 12 to 18 weeks. Data presented are means  $\pm$  SD. NS = not significant. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

**Supplementary Figure 8. Overexpression of two SYK isoforms in HSCs enhances fibrotic related gene expression. (A&B)** Overexpression of SYK(L) or SYK(S) increased mRNA expression of *ACTA2*, *PDGFR $\beta$* , *COL1A1*, *TIMP1*, *PAI-1* and *TGF- $\beta$ 1* by qRT-PCR in LX-2 (A) and TWNT-4 (B) cells. Data presented are means  $\pm$  SD. NS=not significant. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

**Supplementary Figure 9. siRNAs to MYB, CBP and MYC do not affect SYK mRNA expression. (A)** si-MYB did not affect SYK mRNA expression in LX-2 and TWNT-4 cells compared to negative control siRNA (si-Ctrl). **(B)** si-CBP did not affect mRNA expression of SYK in LX-2 and TWNT-4 cells compared to si-Ctrl. **(C)** si-MYC did not affect mRNA expression of SYK in LX-2 and TWNT-4 cells compared to si-Ctrl. Data presented are means  $\pm$  SD. NS=not significant. \*\*\*,  $P < 0.001$ .

**Supplementary Figure 10. The anti-fibrotic effects of siRNA targeting SYK and specific transcription factors. (A&B)** siRNAs to SYK, MYB, CBP or MYC significantly reduced fibrosis related gene expression, including *ACTA2*, *PDGFR $\beta$* , *TGF- $\beta$ 1* and *PAI-1* in LX-2 (A) and TWNT-4 (B) cells compared to si-Ctrl. **(C)** siRNAs to SYK, MYB, CBP or MYC significantly reduced the protein levels of TGF- $\beta$ 1 and PAI-1 in cell culture supernatants of LX-2 and TWNT-4 cells as detected by ELISA. Data presented are means  $\pm$  SD. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

**Supplementary Figure 11. SYK overexpression increases fibrosis related cytokines expression in JFH1 HCV infected Huh7.5.1 cells. (A&B)** JFH1 HCV infected Huh7.5.1-JFH1 cells were transfected with vector, wild type (WT) or kinase dead (KD) SYK plasmids for 36 hours. Relative mRNA expression of *CXCL10*, *IL8*,

*CXCL1*, *CXCL2*, *IL9* and *IL18* in the transfected cells were detected by qRT-PCR (A), and protein expression of CXCL10, IL8 and CXCL1 from cell culture supernatants were measured by ELISA (B). Data presented are means  $\pm$  SD. NS=not significant. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

**Supplementary Figure 12. HSCs express higher levels of fibrosis related genes compared to hepatocytes. (A)** HSCs produced higher levels of fibrosis related gene mRNA expression, including *TGF- $\beta$ 1*, *PAI-1*, *TIMP-1* and *COL1A1* compared to Huh7.5.1 cells. **(B-D)** HSCs produced higher levels of fibrosis related proteins, including TGF- $\beta$ 1 (B), PAI-1 (C) and TIMP1 (D) in cell culture supernatant compared to Huh7.5.1 cells. Supernatant cytokine levels were detected by ELISA.

**Supplementary Figure 13. SYK inhibitor PRT062607 attenuates activation and proliferation of HSCs. (A)** Structural formulas of the SYK inhibitors GS-9973 and PRT062607. **(B)** SYK inhibitor (GS-9973 or PRT062607) reduced overexpression of SYK-WT induced p-SYK (Y525/526) protein phosphorylation level by immunoblotting in LX-2 and TWNT-4 cells. **(C)** Inhibition of proliferation by PRT062607 occurs in a dose-dependent manner in LX-2 and TWNT-4 cells. **(D)** 2.0  $\mu$ M PRT062607 for 48 hours reduced protein levels of COL-1, TIMP-1, PAI-1, TGF- $\beta$ 1, LN and HA by ELISA in cell culture supernatants from LX-2 and TWNT-4 cells. Data presented are means  $\pm$  SD. NS=not significant. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

**Supplementary Figure 14. SYK inhibition attenuates liver fibrosis in a CCl<sub>4</sub>-induced mouse fibrosis model. (A)** The left panel shows images of mouse liver and IHC stains of Sirius red (magnification 40X) and  $\alpha$ -SMA (magnification 200X) in normal

liver or liver tissue from CCL<sub>4</sub>-induced fibrotic mice after treatment with vehicle, GS-9973 5 mg/kg or GS-9973 20 mg/kg. The right panel shows Sirius red and  $\alpha$ -SMA IHC staining for each treatment group. **(B)** GS-9973 5 mg/kg decreased CCL<sub>4</sub>-induced protein levels of  $\alpha$ -SMA by immunoblotting in fibrotic liver tissues compared to control mice. **(C)** GS-9973 reduced mRNA expression of *ACTA2*, *TIMP1* and *COL1A1* in fibrotic liver tissue compared to control mice. mRNA levels were measured by qRT-PCR. **(D)** Serum levels of ALT, ALP, TBIL and ALB were compared between different treatment groups. **(E)** On-treatment weight change for each group during the GS-9973 treatment period. Data presented are means  $\pm$  SD. NS=not significant. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

**Supplementary Figure 15. SYK inhibition attenuates liver fibrosis and HCC development in a DEN-induced rat model.** **(A)** Representative images of rat livers, and IHC staining for Sirius red (magnification 40 X) and  $\alpha$ -SMA (magnification 200X) in normal liver or liver tissues from DEN-induced liver fibrotic rats after treatment with vehicle, GS-9973 2.5 mg/kg or 10 mg/kg. **(B)** Sirius red and  $\alpha$ -SMA IHC staining from each treatment group are compared. **(C)** On-treatment weight change for each group during the GS-9973 treatment period. **(D)** Liver/body weight ratio of the different treatment groups. **(E)** GS-9973 significantly reduced the number of HCC tumor nodules (counted if  $> 5$  mm in diameter) compared to vehicle control rats. **(F)** The proportion of smaller tumors (counted if diameter  $< 8$  mm) was significantly decreased by GS-9973 (##,  $P < 0.01$ ). Data presented are means  $\pm$  SD. NS=not significant. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

**Supplementary Figure 16. SYK inhibition attenuates liver fibrosis and jaundice in a BDL-induced liver fibrosis rat model.** (A) Representative images of rat livers, H&E, Sirius red,  $\alpha$ -SMA and SYK stains of normal rat liver or BDL fibrotic rat liver with vehicle, GS-9973 2.5 mg/kg or GS-9973 10 mg/kg. Scale bars, 100  $\mu$ m. (B) GS-9973 significantly reduced the Ishak fibrosis score, and  $\alpha$ -SMA and p-SYK staining compared to vehicle control rats. (C) GS-9973 reduced liver/body weight ratio compared to vehicle control rats. (D) On-treatment weight-change of each group during the GS-9973 treatment period. (E) GS-9973 reduced serum hyaluronic acid levels compared to vehicle control rats. (F) GS-9973 reduced serum ALP, ALT, AST, TBIL, Alb and Glu levels compared to vehicle control rats. Data presented are means  $\pm$  SD. NS=not significant. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

**Supplementary Figure 17. SYK inhibition attenuates Ki-67 expression in murine fibrosis models.** (A) The left panel shows representative images of Ki-67 IHC stains in liver tissue from olive oil or CCl<sub>4</sub> treated mice after treatment with vehicle, GS-9973 5 mg/kg or GS-9973 20 mg/kg. The right panel shows the quantitative result. (B) The left panel shows representative images of Ki-67 staining of liver tissues from sham or BDL rats in each treatment group. The right panel shows the quantitative result. Scale bar, 100  $\mu$ m. Data presented are means  $\pm$  SD. \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ .



## **Supplementary Tables**

**Supplementary Table 1.** PCR array results showing 84 transcription factors regulated by SYK siRNA in LX-2 cells.

**Supplementary Table 2.** Changes in cytokine/chemokine expression by PCR array in JFH1 HCV infected huh7.5.1 cells with SYK siRNA or negative siRNA control.

**Supplementary Table 3.** The hemodynamic effects of GS-9973 treatment on murine.

**Supplementary Table 4.** Real-time polymerase chain reaction primers.

## **Supplementary Materials and Methods**

### **Cell Cultures and Infectious Viruses**

Hep G2, Hep G2.2.1.5, Huh7.5.1, LX-2 and TWNT-4 cells were grown in high-glucose DMEM with 10% FBS, 100 U/mL of penicillin and 100 µg/mL of streptomycin. For HCV infection, the Huh7.5.1 cells were infected with genotype 2a JFH1 HCV virus (0.2 MOI) as previously described.<sup>(1-3)</sup> An inactive phenotype of hepatic stellate cells (HSCs) was induced by culturing HSCs on Matrigel (Corning, Corning, NY).

### **Co-culture of Hepatocytes and HSCs**

A Transwell co-culture system was utilized in this study. LX-2 or TWNT-4 cells were pre-cultured in 12-well plates for 24 hours and Huh7.5.1 cells (with or without JFH1 HCV infection) were seeded in Transwell inserts (Costar, Kennebunk, ME) that were subsequently loaded into the HSC-containing wells. The base of the insert has a membrane with 0.4 µm pores, which permit free exchange of secreted proteins between cell types. The supernatant and cells were collected and analyzed after 48 hours co-culture.

### **Chemicals**

For *in vitro* experiments, GS-9973 (Entospletinib; Selleck Chemicals, Houston, TX) and PRT062607 (P505-15; Selleck Chemicals) were dissolved in DMSO (Sigma-Aldrich, St. Louis, MO) and further diluted to the required concentration. For *in vivo* experiments, a GS-9973 suspension was prepared in 0.5% carboxymethyl cellulose sodium normal saline solution.

## **Human Samples**

One hundred and twenty-seven liver samples with HBV-associated fibrosis/cirrhosis, 30 liver samples with NASH, 7 liver samples demonstrating cholestasis and 3 liver samples with alcohol-induced liver fibrosis/cirrhosis were collected in China. Twenty-two liver samples with HCV-associated fibrosis/cirrhosis were collected at the Massachusetts General Hospital (Boston, USA). Normal liver samples from 36 patients were obtained from individuals who underwent liver resection for benign hepatic lesions (including 24 cases of hepatic hemangioma and 12 cases of focal nodular hyperplasia). All liver samples were obtained under protocols approved by the Integrated Hospital of Traditional Chinese Medicine of Southern Medical University Office for Protection of Human Subjects and the Institutional Review Board for the Massachusetts General Hospital.

## **Murine Liver Fibrosis Models**

**Carbon tetrachloride (CCl<sub>4</sub>)-induced mouse liver fibrosis model.** Six weeks old male C57BL/6 mice (Vital River, Beijing, China) were treated three times a week with 0.1 mL of a 40 percent solution of CCl<sub>4</sub> in olive oil or olive oil alone (vehicle control mice) by oral gavage for 18 weeks. Vehicle control mice and a subset of CCl<sub>4</sub> treated mice received daily gavage of either 5 or 20 mg/kg GS-9973 (Selleck Chemicals, Houston, TX) or vehicle (n=6 for each groups) during weeks 6 through 10. Mice were sacrificed 72h after the final treatment. The liver was harvested, and a section was fixed in 10% buffered formalin for histologic analyses. The remaining portions of the liver were snap frozen and stored at -80°C until use. At the time of sacrifice, a cardiac

terminal blood draw was performed. Blood was allowed to clot, then centrifuged to isolate serum for liver function testing. The serum was stored at -80°C prior to use.

**Diethylnitrosamine (DEN)-induced rat liver fibrosis/HCC model.** Six weeks old male Wistar rats (Vital River) received weekly intraperitoneal (IP) injections of DEN (50 mg/kg, Sigma-Aldrich, St. Louis, MO) or phosphate buffered saline vehicle control for 18 weeks. Vehicle control mice and a subset of DEN treated rats received daily gavage of either 2.5 or 10 mg/kg GS-9973 or vehicle (n=6 for each groups) during weeks 13 through 18. Rats were sacrificed 72h after the final DEN injection. At the time of sacrifice, HCCs were counted and measured. The liver and blood samples were harvested as described above.

**Bile duct ligation (BDL) rat model.** Six weeks old male Wistar rats (Vital River) underwent double ligation of the bile duct or sham surgery (by way of a small laparotomy) under general anesthesia (1% pentobarbital sodium, 40mg/kg, IP). Four days after surgery, the sham and BDL rats were given daily gavage of either 2.5 or 10 mg/kg GS-9973 or vehicle (n=8 for each group) for 14 days. The rats were sacrificed on day 19 after BDL surgery. The liver and blood samples were harvested as described above.

All animal handling and procedures were approved by the Animal Care and Use Committee of Southern Medical University.

### **Hemodynamic studies**

A subset of rats were anesthetized with 1% pentobarbital sodium (40 mg/kg IP). A polyethylene tube filled with saline was inserted into the portal vein and aortaventralis

respectively, and the intravascular portal vein pressure and systemic arterial pressure was measured with a physiological pressure transducer (SP844; Memscap, Crolles Cedex, France), Rats were then sacrificed.

### **Small interfering RNA (siRNA) Transfection**

We utilized siRNAs to knockdown the expression of selected target genes. siRNAs were reverse transfected into cells using Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA). The Dharmacon ON-TARGET plus SMART pool Human siRNAs (GE Healthcare Life Sciences, Pittsburgh, PA) used for gene knockdown were as follows: SYK siRNA, MYC siRNA, MYB siRNA, CBP siRNA, and non-targeting negative control siRNA.

### **Plasmid Construction**

Expression plasmids containing pcDNA3.1-Flag-SYK(L) [also named SYK-WT] and pcDNA3.1-Flag-SYK(S) were constructed as previously described.<sup>(4, 5)</sup> Mutation of SYK kinase dead (KD) and SYK catalytically activated (CA) domains were introduced using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), and verified by DNA sequencing.

The sequence of primers used for KD mutagenesis were as follows <sup>(6)</sup>:

5'-GTGAAAACCGTGGCTGTGCGAATACTGAAAACGAGGC-3' (forward),

5'-GCCTCGTTTTTCAGTATTCGCACAGCCACGGTTTTTCAC-3' (reverse).

The sequence of primers used for CA mutagenesis were as follows:

5'-GTGAAAACCGTGGCTGTGCAAATACTGAAAACGAGGCCAAT-3' (forward),

5'-GCCTCGTTTTTCAGTATTCGCACAGCCACGGTTTTTCACAACTTT-3' (reverse).

### **Lentiviral Vector Production and Infection**

Lentiviral vectors were generated as described.<sup>(7)</sup> Briefly, lentiviral particles were produced by co-transfection of 293T cells with the packaging vectors payload, psPAX2, pRSV-REV and pMD2.G plasmids. Lentiviral supernatants were harvested at 48 and 72 hours after transfection, filtered and stored at -80°C until use. The lentiviral particles were used to infect indicated cells with 8 µg/ml Polybrene (Sigma-Aldrich) and then were selected with 750 µg/ml of G418 (Calbiochem, San Diego, CA).

### **RNA preparation and quantitative RT-PCR (qPCR)**

Total RNA of cell culture samples were isolated by using the Total RNA kit (QIAGEN, Dusseldorf, Germany). Total RNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Inc., DE, USA). Reverse transcription (RT) was performed using the Real Time RT reagent kit (TakaRa Bio Inc., Shiga, Japan). Quantitative RT-PCR (qRT-PCR) was performed using the PowerUp SYBR Green Master Mix (ThermoFisher Scientific, Waltham, MA) and QuantStudio3 system (ThermoFisher Scientific). The amplification conditions were: 94°C 3 min; 40 cycles of 95°C 20 s, 60°C 40 s and 72°C 20 s; and elongation at 72°C for 5 min. The mRNA expression level of target genes was normalized to GAPDH by using the  $2^{-\Delta\Delta C_t}$  method. Primer sequences are available in Supplementary Table 3.

### **PCR Array**

The RT<sup>2</sup> Profiler™ PCR Array of Human Transcription Factors and Human Cytokines & Chemokines (QIAGEN) was used, according to the manufacturer's instructions. The relative gene expression ratio was calculated by the  $\Delta\Delta C_t$  method. The Qiagen qPCR

array analysis web portal was used to assist in the analysis (<http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php>).

### **Immunoblotting and Immunoprecipitation**

Immunoblotting and Immunoprecipitation (IP) were performed as previously described.<sup>(1, 4, 5)</sup> For immunoblotting, equivalent amounts of protein were separated by SDS-PAGE gel electrophoresis and blotted onto nitrocellulose membranes. Primary antibodies (1:500-1,000) were used for immuno-detection with horseradish peroxidase-conjugated secondary antibodies (1:5,000; Santa Cruz Biotechnology, Dallas, TX) and enhanced chemiluminescence reagents (Pierce, Rockford, IL). The primary antibodies were purchased from Santa Cruz Biotechnology [SYK (N-19; rabbit polyclonal), SYK (4D10; mouse monoclonal) and GAPDH (G-9; mouse monoclonal)], ThermoFisher Scientific [c-Myb (rabbit polyclonal),  $\alpha$ -SMA (rabbit polyclonal), HBV-Core (10C6; mouse monoclonal), HCV-Core (goat polyclonal)], Sigma-Aldrich ( $\beta$ -actin and Flag, both mouse monoclonal), GeneTex [PAI-1 (3A120; mouse monoclonal)], Cell Signaling Technology [Cyclin D1 (rabbit polyclonal), phospho-SYK (Tyr525/526; C87C1; rabbit monoclonal), c-Myc (D3N8F; rabbit monoclonal), PDGFR $\beta$  (28E1; rabbit monoclonal)]. For immunoprecipitation, cell lysates were incubated with 1  $\mu$ g of specific antibody at 4°C overnight, followed by the addition of 30  $\mu$ l of protein A/G-conjugated agarose beads (Santa Cruz Biotechnology), which were prewashed with lysis buffer. The precipitates were then washed with ice-cold PBS, resuspended in 6X Laemmli buffer, and resolved by SDS-PAGE, followed by immunoblotting.

### **Chromatin Immunoprecipitation (ChIP) Assay**

The ChIP assay was performed using the Magna CHIP™ chromatin immunoprecipitation kit (Millipore, Billerica, MA) according to the manufacturer's instructions. Briefly, the indicated cells were cross-linked with 1% formaldehyde, lysed and sonicated to obtain adequate fragment sizes of DNA. The DNA was then eluted and purified according to the manufacturer's instructions. ChIP was carried out with the anti-H3K9ac antibody or anti-IgG. PCR (ChIP-PCR) was conducted using the ChIP-eluted DNA as template. The human *CCND1* promoter specific primers used in PCR were 5'-AACGTCACACGGACTACAGG-3' (forward) and 5'-CTCCCTCGCG CTCTTCTG-3' (reverse). Human *MYC* promoter specific primers were 5'-TCATAACG CGCTCTCCAAGT-3' (forward) and 5'-CAGAGCGTGGGATGTTAGTG-3' (reverse).

### **Enzyme-Linked Immunosorbent Assay (ELISA)**

Specific ELISA kits were used to quantitate CXCL10, IL8, CXCL1, COL-I, TIMP1, PAI-1, TGF-β1, laminin (LN) and hyaluronic (HA) in cell culture supernatants according to the manufacturer's instructions. ELISA kits were purchased from R&D Systems (CXCL10, IL8, CXCL1, TIMP1, PAI-1, TGF-β1 and HA), TSZELISA (COL-I) and Millipore (LN).

### **Histology, Immunohistochemistry and Immunofluorescence**

Formalin-fixed, paraffin-embedded liver tissue samples were cut into 4 μm-thick sections and stained with hematoxylin eosin (H&E), Sirius red, Masson's trichrome and immunohistochemistry according to standard procedures. The amount of Sirius red staining was quantified with ImageJ (ImageJ, <http://imagej.net/>). Fibrosis was scored



according to the Ishak scoring system,<sup>(8)</sup> in a blinded fashion by a single expert pathologist. For immunohistochemistry (IHC), liver sections were stained with the following antibodies: SYK (rabbit polyclonal; Abcam, Cambridge, UK), HNF4 $\alpha$  (K9218; mouse monoclonal; Abcam),  $\alpha$ -SMA (1A4; mouse monoclonal; ZSGB-Bio, Beijing, China), phospho-SYK (Tyr525/526; C87C1; rabbit monoclonal; Cell Signaling Technology, Danvers, MA) and Ki-67 (UMAB107; mouse monoclonal; ZSGB-Bio). Both the intensity and extent of immunostaining were taken into consideration when analyzing the data. The intensity of staining was determined by the following rules: 0 for negative; 1 for weak staining; 2 for moderate staining; 3 for strong staining. The staining extent was the percentages of cell staining positive, scored from 0% to 100%. We randomly selected 10 areas from each slice to count the percentage of positive stained cell and to calculate the mean staining extent. The final score was obtained by multiplying these two values (intensity score  $\times$  extent score).<sup>(9)</sup>

For dual immunofluorescence staining, sections or cells fixed with ice-cold methanol were co-stained with SYK (rabbit polyclonal; Abcam) and either HNF4 $\alpha$  (K9218; mouse monoclonal; Abcam),  $\alpha$ -SMA (1A4; mouse monoclonal; ZSGB-Bio), CK19 (A53-B/A2; mouse monoclonal; Abcam), CD31 (mouse monoclonal; Cell Signaling Technology) or CD68 (KP1; mouse monoclonal; Abcam) with detection by the appropriate secondary antibodies labeled with either Alexa Fluor 488 or Alexa Fluor 555, according to the manufacturer's instructions. Nuclei were stained with DAPI.

### **Hydroxyproline Assay**

Hydroxyproline quantification of frozen liver tissue was performed using a colorimetric

hydroxyproline assay kit (Abcam), according to the manufacturer's instructions. The hydroxyproline content was expressed as amount ( $\mu\text{g}$ ) per gram of liver tissue ( $\mu\text{g/g}$ ).

### **Primary Cell Isolation and Culture**

Primary mouse hepatocytes, HSCs and macrophage were isolated and cultured as previous reported.<sup>(10)</sup> Briefly, mouse livers were perfused *in situ* and digested with a buffer containing collagenase (Sigma-Aldrich). The liver was then homogenized under sterile conditions, and further digested *ex vivo* with collagenase solution. The digested liver was filtered through a 70  $\mu\text{m}$  cell strainer, and hepatocytes, HSCs and macrophages were separated by density gradient centrifugation. Primary human HSCs were isolated from healthy donor livers (Triangle Research Labs, Durham, NC) as previous described.<sup>(11)</sup> The isolated HSCs were cultured in high-glucose DMEM with 10% FBS, 100 U/mL of penicillin and 100  $\mu\text{g/mL}$  of streptomycin and maintained at 37°C in a humidified incubator with 5%  $\text{CO}_2$  in air.

### **Cell Proliferation Assay**

LX-2 and TWNT-4 cells were seeded at 4,000 cells per well in 96-well microplates. The cells were treated with varying concentrations of GS-9973 (0.01-20.0  $\mu\text{M}$ ) or PRT062607 (0.01-10  $\mu\text{M}$ ) or DMSO for the indicated time period. Cell viability was measured using the Cell Counting Assay Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) according to the manufacturer's instructions.

### **Liver Function Test**

Serum levels of several biochemical markers were measured to assess liver function and liver injury. The measured biochemical markers included alkaline phosphatase

(ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), albumin (Alb), glucose (Glu) and hyaluronic acid (HA). ALP, ALT, AST, TBIL, Alb and Glu were measured using the Roche Cobas 6000 Analyzer (Roche, Basel, Switzerland). HA was measured using the Bioscience PETFCK96-1 Analyzer (Bioscience, Tianjin, China).

### **Public Dataset**

Gene expression profiles based on gene chips of HBV-infected (GSE38941), HCV-infected (GSE38226), alcoholic hepatitis (GSE28619), cholestasis (GSE46960) and NASH (GSE48452) liver tissues were obtained from the National Cancer for Biotechnology Information Gene Expression Omnibus database (GEO). Expression profiles of these datasets were reanalyzed using R and correlated packages (<http://www.r-project.org/>).

### **Statistical analyses**

The Student *t* test or the Mann-Whitney *U* test were used to compare values between subgroups. The Kruskal-Wallis test followed by post-hoc Dunn-Holland-Wolfe test or Mann-Whitney *U* test were used to assess differences between Ishak fibrosis scores. The Pearson correlation test (two-tailed) was used to calculate the correlation coefficient. Data were expressed as mean  $\pm$  standard deviation (SD) of at least three biological replicates. Statistical significance was declared if  $P < 0.05$ . All analyses were performed using SPSS software (Version 23.0, IBM, Armonk, NY).

## Supplementary References

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