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The metabolic regulator small heterodimer partner contributes to the glucose and lipid homeostasis abnormalities induced by hepatitis C virus infection

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ABSTRACT

Background: Chronic hepatitis C virus (HCV) infection can predispose the host to metabolic abnormalities. The orphan nuclear receptor small heterodimer partner (SHP; NR0B2) has been identified as a key transcriptional regulatory factor of genes involved in diverse metabolic pathways. The protective effects of SHP against HCV-induced hepatic fibrosis have been reported. However, the exact mechanisms of its role on metabolism are largely unknown. We investigated the role of hepatic SHP in regulating glucose and lipid homeostasis, particularly in the metabolic stress response caused by HCV infection.

Materials and methods: Gluconeogenesis and lipogenesis levels and SHP expression were measured in HCV-infected cells, as well as in liver samples from HCV-infected patients and persistently HCV-infected mice.

Results: We demonstrated that SHP is involved in gluconeogenesis via the acetylation of the Forkhead box O (FoxO) family transcription factor FoxO1, which is mediated by histone deacetylase 9 (HDAC9). Meanwhile, SHP regulates lipogenesis in the liver via suppressing the induction of sterol regulatory element-binding protein-1c (SREBP-1c) expression by the SUMOylation of Liver X receptor α (LXRα) at the SREBP-1c promoter. In particular, SHP can be strongly reduced upon stimulation, such as by HCV infection. The SHP expression levels were decreased in the livers from the CHC patients and persistently HCV-infected mice, and a negative correlation was observed between the SHP expression levels and gluconeogenic or lipogenic activities, emphasizing the clinical relevance of these results.

Conclusions: Our results suggest that SHP is involved in HCV-induced abnormal glucose and lipid homeostasis and that SHP could be a major target for therapeutic interventions targeting HCV-associated metabolic diseases.

1. Introduction

Hepatitis C virus (HCV) infection is the leading cause of viral hepatitis, which may not only cause steatosis but also accelerate the development of type 2 diabetes mellitus in predisposed individuals [1,2]. Evidence suggests that HCV-related glucose and lipid metabolism disorders have a remarkable clinical impact on the prognosis of HCV infection and quality of life for patients [3]. It is important to determine the pathogenesis of metabolic alterations and pathogenetic mechanisms in HCV infection.

Metabolic syndrome is a widespread disorder comprising a group of correlated clinical features with insulin resistance as a common pathogenic determinant; it can lead to type 2 diabetes mellitus, nonalcoholic fatty liver disease, and many types of cancer, including hepatocellular carcinoma [4–8].

Glucose and lipid metabolism are regulated jointly to balance energy use and storage for the maintenance of blood glucose concentrations within a narrow range [9]. High glucose levels, for example, after ingestion of carbohydrates, trigger the secretion of insulin, which stimulates glucose uptake and utilization and promotes glycogen and fatty acid synthesis in the liver. Lipogenesis is executed through a series of enzymes that are regulated for metabolic homeostasis to adapt to changing nutritional and hormonal conditions [10]. Hepatocytes play an essential role in the regulation of blood glucose levels through the coordinated regulation of carbohydrate and lipid metabolism, including

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hepatic gluconeogenesis and lipogenesis [11]. Gluconeogenesis is largely regulated at the transcriptional level by controlling the rate-limiting enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase). The expression of these enzymes is controlled by the hormonal modulation of transcription factors and coactivators, including Forkhead box O (FoxO) proteins [12]. To regulate hepatic lipogenesis, the active nuclear fragment of sterol regulatory element-binding protein-1c (SREBP-1c) increases the transcription of genes encoding acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) [13]. Perturbations in the regulation of hepatic carbohydrate and lipid metabolism lead to the development of many metabolic-related diseases [6]. It has been shown that HCV infection can perturb crucial lipid and glucose pathways in the liver through multiple mechanisms. For example, HCV promotes hepatic glucose production in a FoxO1-dependent pathway [14]. The nonstructural protein 5A (NS5A) protein enhances gluconeogenesis via the induction of Akt-JNK signalling pathway [15]. HCV modulates lipid regulatory factor Angiopoietin-like 3 gene expression through the blockage of the transactivation of liver X receptors (LXRs), which are among the most important nuclear receptors shown to regulate the metabolism of lipids [16,17].

An atypical orphan nuclear receptor small heterodimer partner (SHP; NR0B2) has become the focus of many studies due to its important role in the regulation of metabolic homeostasis through interactions with nuclear receptors and transcription factors [18]. The function of SHP in the liver involves bile acid synthesis from cholesterol, hepatic lipogenesis, gluconeogenesis, and hepatic tumourigenesis [19]. SHP predominantly functions as a repressor by interacting with nuclear receptors. A functional interaction between SHP and the HCV NS5A protein has been reported [20]. The overexpression of SHP reverses the profibrogenic features of HCV-infected cells by decreasing TGF-beta and fibrotic gene expression [21]. Therefore, it is of great interest and importance to understand the biology of SHP in hepatic metabolic homeostasis regulation, particularly in carbohydrate and lipid metabolism, and determine whether SHP influences metabolic alterations in HCV-infected hepatocytes.

Different types of posttranslational modifications, such as phosphorylation, acetylation, and SUMOylation, regulate the transcriptional activation and/or stability of nuclear receptors. Small Ubiquitin-like Modifier (or SUMO) proteins constitute a family of small proteins covalently attached to and detached from other proteins in cells to modify their function [22]. SUMOylation is a post-translational modification involved in various cellular processes, including nuclear-cytosolic transport, transcriptional regulation, protein stability, etc. [23,24].

Here, we report that SHP regulates hepatic gluconeogenesis via the HDAC9-mediated deacetylation of FoxO1 and lipogenesis via the suppression of the induction of SREBP-1c expression by the SUMOylation of LXRα at the SREBP-1c promoter. Moreover, both HCV-infected patients and persistently HCV-infected transgenic mice exhibited down-regulated SHP in their livers and induced gluconeogenic and lipogenic activity. These findings provide insight into the possible mechanisms underlying the development of HCV-induced abnormal glucose and lipid homeostasis.

2. Materials and methods

2.1. Patients and biopsies

All patients, their relatives, or both provided written informed consent for the use of their clinical and pathological information for research purposes and its storage in the hospital's database. The Ethics Committee of the First Hospital of Jilin University approved the methods and experimental protocols used in the present study, which were performed in accordance with the ethical standards of our institutional research committee and the tenets of the 1964 Declaration of Helsinki, its amendments, or comparable ethical standards. The study methods were performed in accordance with the approved guidelines. All human tissue samples were collected from the Liver Unit of the First Hospital of Jilin University. The human liver tissue samples were obtained from 37 HCV-infected patients via fine needle biopsy. The normal human liver tissue was obtained from either spare donor tissue intended for transplantation or normal liver tissue resected from patients with benign hepatic tumours. The diagnosis of the patients with chronic HCV infection and the analysis of all biopsies were based on standard serological assays and the presence of abnormal serum aminotransferase levels for at least six months. All HCV patients tested positive for HCV antibodies based on a third-generation ELISA test. HCV infection was confirmed through the detection of circulating HCV RNA using an HCV PCR-based assay (Qiagen). At the time of biopsy, the liver tissue (2–3 mm) was immediately frozen in TRIzol and stored at −80 °C. The fasting glucose and insulin levels were measured on the days of the biopsy in the fasting state (12 h). Insulin resistance was assessed by the HOMA-IR score [homeostasis model assessment, calculated as (fasting insulin × fasting glucose)/22.5]. FLEXMAP 3D quantification (Luminex, Austin, TX) of the concentrations of insulin was also performed.

2.2. Cells and virus

Human hepatoma Huh7 cells (kindly provided by Frank Chisari) were cultured as previously described [25]. The virus production and infection were performed as previously described [25]. Mock-infected controls were generated in parallel to the virus infections. For the in vitro infection, Huh7 cells (1×10^6) were infected with JFH-1 for the indicated times with the indicated multiplicities of infection (MOI).

2.3. Metabolite profiling

The extraction of the intracellular metabolites and nuclear magnetic resonance (NMR) analysis were performed as previously described [26].

2.4. Plasmids, transfection and luciferase reporter assays

PEPCK-Luc (kindly provided by Prof. Akiyoshi Fukamizu) was constructed by cloning the 671-bp upstream fragments into the promoter-luciferase reporter vector pGL3-basic [27]. The constructs expressing SREBP1c-Luc were purchased from Stratagene. The pRL-SV40-Renilla reporter plasmid (Promega) served as a transfection control. The luciferase reporter assays were performed using a Dual-Luciferase Assay System from Promega as previously described [28]. The plasmids expressing FoxO1, SHP and LXRα were generated from the pXJ40-HA vector with the indicated primers (Supplementary Table S1) and transfected into Huh7 cells (at 1 μg or 5 μg) using electrotransfection (Invitrogen).

2.5. Real-time PCR and Western blotting

The RNA isolation, cDNA synthesis, and quantitative PCR analysis were performed with the indicated primers (Supplementary Table S1) as previously described [25]. The samples were normalized against the actin internal control using the $2-\Delta\Delta Ct$ method, compared as arbitrary units, and represented as the mean ± SEM. Western blotting was performed as previously described [25]. Briefly, the protein lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Millipore). The membranes were blocked with nonfat milk for 1 h and incubated with primary antibodies overnight at 4 °C. The following antibodies were used: SHP, 1:1000, ab186874; NS3, 1:1000, ab13830; SREBP-1c, 1:1000, ab28481; HDAC9, 1:1000, ab18970; anti-SUMO2, 1:1000, ab233222 (Abcam); FoxO1, 1:1000, 9454; PEPCK, 1:1000, 12940; Flag, 1:1000, 2368s; HA, 1:1000, 3724s; Acetylated-Lysine, 1:500, 9681 (Cell Signalling Technology); FAS, 1:1000, 10624-2-AP; GAPDH,

1:5000, 60004-1-Ig; β-Actin, 1:5000, 60008-1-Ig (Proteintech); and Acetyl-FoxO1, 1:500, sc49437 (Santa Cruz Biotechnology). Then, the membranes were incubated with goat anti-rabbit (1:50000, Jackson, 111-035-003) or anti-mouse (1:50000, 115-035-003, Jackson) horseradish peroxidase-conjugated secondary antibodies for 1 h at 37 °C, followed by ECL detection reagent (Pierce). β-actin or GAPDH was used as a loading control.

2.6. RNA interference

For the knockdown experiments, 50 pmol small interfering RNA (siRNA) specific for SHP (Qiagen, GS9734) and a negative control (Qiagen, 1027310) were transfected into Huh7 cells via Lipofectamine 2000 transfection reagent. Briefly, the siRNA was diluted in serum-free Opti-MEM media (Gibco) and incubated for 5 min at room temperature. The two solutions were gently mixed and incubated together for 30 min at room temperature. After the incubation, the complex was added to the plated cells. All assays were performed at least 48 h after the RNA interference transfection.

2.7. Lentiviral transduction and establishment of stable cell lines

The Lentivirus System (purchased from Addgene, http://www.addgene.org) was used for the overexpression assays. The lentiviruses were produced as previously described. In brief, HEK293T cells were co-transfected with the pWPI-HA-SHP plasmid and psPAX2 and pMD2.G packaging plasmids at a 4:3:1 ratio. Seventy-two hours after transfection, the supernatant was collected and applied to infect the target cells in the presence of polybrene (5 μg/ml). The infected cells were selected by puromycin for 7 days before the experiments were conducted. Huh7 cells were used for the generation of cell lines with stably overexpressing SHP protein.

2.8. Glucose production assay

The production of glucose was measured using an Amplex® Red Glucose/Glucose oxidase assay kit (Invitrogen) according to the manufacturer's instructions [29]. Glucose production was normalized to the total cellular protein, which was quantified by using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific).

2.9. Measurement of cellular triacylglycerol (TAG) and TAG secretion

The total content of cellular TAG was extracted by using a tissue/cell triacylglycerol assay kit according to the manufacturer's instruction (Applygen Technologies Inc., Beijing, China), and the data were obtained using a microtiter plate reader (Bio-Rad Laboratories Inc.). The total TAG content was normalized to the total cellular protein content, which was quantified by using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific). Triglyceride secretion was measured following a 24-h incubation of the cells with ^{14}C glycerol (PerkinElmer Life Sciences) at a final concentration of 2.85 mCi/ml. The lipids extracted from the media were separated by thin layer chromatography, and the radioactivity associated with triglycerides was quantified.

2.10. Co-immunoprecipitation and chromatin immunoprecipitation (ChIP)

The co-immunoprecipitation assays were performed as previously described [25]. The ChIP analysis was performed following the procedures described by Daftari et al. with the appropriate primers (Supplementary Table S1) [30].

2.11. PEPCK, FAS and ACC activity assay

The PEPCK enzyme activity was assayed using an NADH-coupled system [31]. The FAS activity was estimated by calculating the NADPH oxidation as previously described [32]. The ACC activity was assessed using a luminescent ADP detection assay (ADP-Glo Kinase Assay Kit; Promega) that measures enzymatic activity by quantifying the ADP produced during the enzymatic first half-reaction as previously described [33].

2.12. Transgenic mice and animal study design

Transgenic C/O^{Tg} mice harbouring both human CD81 and OCLN genes were constructed as previously described [34]. We used 8- to 12-week old age- and gender-matched mice for the in vivo experiments. The information of the HCV-infected ($n = 36$) and mock-infected ($n = 12$) C/O^{Tg} mice raised in synchronization is provided in Supplementary Table S2, and more information has been previously reported [34]. The animal studies were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University.

2.13. Statistical analysis

The data are presented as the means ± SEM. The statistical analysis was carried out using Student's *t*-test to compare two groups or ANOVA to compare multiple groups. The differences were considered significant at $p < 0.05$ (*, $p < 0.05$; **, $p < 0.01$; ***, and $p < 0.001$). The statistical analyses were performed using GraphPad Prism.

3. Results

3.1. HCV infection downregulates host SHP expression in an HCV-dependent manner

The modulation of SHP expression by HCV infection was analysed, and the *SHP* mRNA levels were downregulated in a time- and dose-dependent manner following HCV infection in parallel with the increasing HCV RNA levels (Fig. 1A). In addition, the *SHP* mRNA levels demonstrated no significant changes in the mock-infected cells (data not shown). A similar SHP downregulation was observed at the protein level (Fig. 1B). The SHP mRNA and protein levels were also decreased across a panel of JFH-1-based intergenotypic HCV clones containing the structural regions of diverse HCV genotypes (Fig. 1C and D) ($p < 0.05$), indicating that the HCV-dependent SHP downregulation was not genotype-specific. Furthermore, the treatment with 2′-*C*-methyladenosine (2′CM, an HCV inhibitor targeting the viral NS5B protein) dramatically increased the HCV and SHP levels in the HCV-infected cells ($p < 0.01$), whereas the SHP expression levels in the control cells were unaffected (Fig. 1E and F), suggesting that the SHP reduction was HCV-dependent. Then, we sought to identify the viral component involved in the downregulation of SHP and found that the HCV proteins core, E1, reduced SHP expression in the Huh7 cells (Supplementary Fig. 1), implying that HCV structure proteins are essential for the downregulation of SHP. Furthermore, the role of SHP in the HCV life cycle was analysed.

The HCV-infected cells transfected with siRNAs targeting the SHP sequence and two combinations of siRNAs (siSHP-1 + 2 + 5 and siSHP-1 + 3 + 4) exhibited significantly decreased SHP expression levels compared to the controls, whereas comparable expression levels of the NS3 protein were observed (Supplementary Fig. 2A). Again, silencing SHP expression or overexpressing SHP did not affect the HCV RNA levels in the cells and supernatant or the viral titre in the supernatant (Supplementary Fig. 2B and C).

These results suggest that HCV infection can downregulate SHP expression in an HCV-dependent manner, whereas SHP silencing could not influence HCV replication.

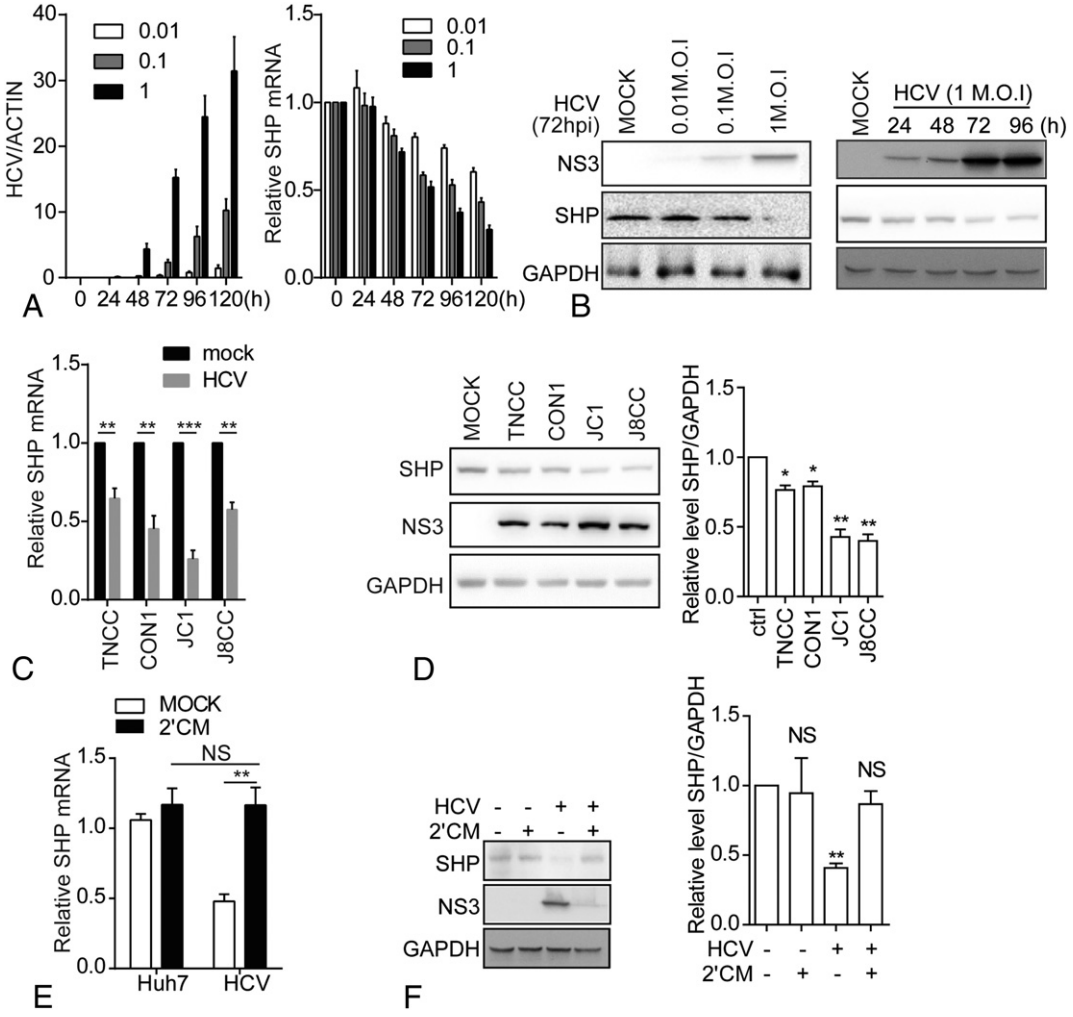

Fig. 1. HCV infection downregulates host SHP expression. HCV RNA, SHP mRNA (A), and SHP protein (B) levels in mock- and HCV-infected Huh7 cells at the indicated multiplicities of infection (MOI) and time points (hours post infection, hpi). HCV RNA, SHP mRNA (C), and SHP protein (D) levels in a panel of JFH-1-based inter-genotypic HCV clones containing the structural regions of diverse HCV genotypes (0.1 MOI) compared with those in Huh7 control cells. HCV RNA and relative intracellular *SHP* mRNA (E) and SHP protein levels (F) in mock- and HCV-infected (1.0 MOI) cells treated with 2′CM (10 mM, 96 h). Quantified data of *SHP* levels normalized to β-actin (C, E). Histogram (D, F right panel) representing a densitometric analysis performed to quantify the relative intensity of the bands detected by Western blotting. GAPDH was used as a loading control. The data are presented as the mean ± SEM of three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

3.2. Effect of hepatic SHP overexpression or inhibition on glucose and lipid metabolism

To gain insight into the metabolic signature associated with SHP, we used an NMR analysis to examine the role of SHP in the metabolites involved in carbohydrate and lipid metabolism in SHP depletion cells. In the SHP-knockdown cells, the intermediates of glycolysis were elevated. Consistent with this pattern, the SHP-knockdown cells exhibited decreased pyruvate concentrations ($p < 0.05$), which were accompanied by higher levels of intracellular glucose ($p < 0.01$), compared to the control cells (Supplementary Fig. 3A). Regarding the lipid profile, the SHP inhibition increased the triacylglycerol (TAG) (neutral lipids) ($p < 0.01$), phosphatidylcholine (PC) ($p < 0.01$), phosphatidylethanolamine (PE) ($p < 0.05$), phosphatidylserine (PS) (phospholipids) ($p < 0.001$), and lysophospholipid (LPC) ($p < 0.01$) levels (Supplementary Fig. 3B). The metabolic profile of the SHP-knockdown cells demonstrated a clear shift towards gluconeogenesis metabolism and lipogenesis.

Subsequently, we investigated whether HCV-induced gluconeogenesis and lipogenesis were SHP-dependent. The SHP overexpression attenuated glucose production following HCV infection, whereas the SHP depletion increased production ($p < 0.01$) (Fig. 2A). The intracellular TAG contents were significantly decreased in the SHP overexpressed HCV-infected cells ($p < 0.01$); however, the secreted TAG demonstrated no significant changes (Fig. 2B). Consistently, the SHP overexpression reduced the expression levels and enzymatic activity of PEPCK ($p < 0.01$), G6Pase ($p < 0.01$), ACC ($p < 0.05$) and FAS ($p < 0.01$) in both the Huh7 and HCV-infected cells (Fig. 2C, Fig. 2D, and Supplementary Fig. 4A). The SHP inhibition increased the expression levels of the gluconeogenic and lipogenic enzymes ($p < 0.05$) (Fig. 2E, Fig. 2F and Supplementary Fig. 4B).

Overall, these results demonstrate that SHP plays roles in regulating gluconeogenesis and lipid synthesis in HCV-promoted gluconeogenesis and lipogenesis.

3.3. Involvement of SHP-dependent FoxO1 acetylation in gluconeogenesis and LXRα SUMOylation in lipogenesis

In support of the changes in the PEPCK expression levels, the hepatic SHP knockdown increased the FoxO1 transcriptional activity and PEPCK promoter activity but reduced the FoxO1 transcriptional activity in the SHP-overexpressed cells (Fig. 3A and B). The immunofluorescent staining also showed similar results (Fig. 3C). Our previous study indicated that HCV infection promoted the activation of the metabolic transcription factor FoxO1 by the deacetylation of nuclear FoxO1. Thus, we examined the

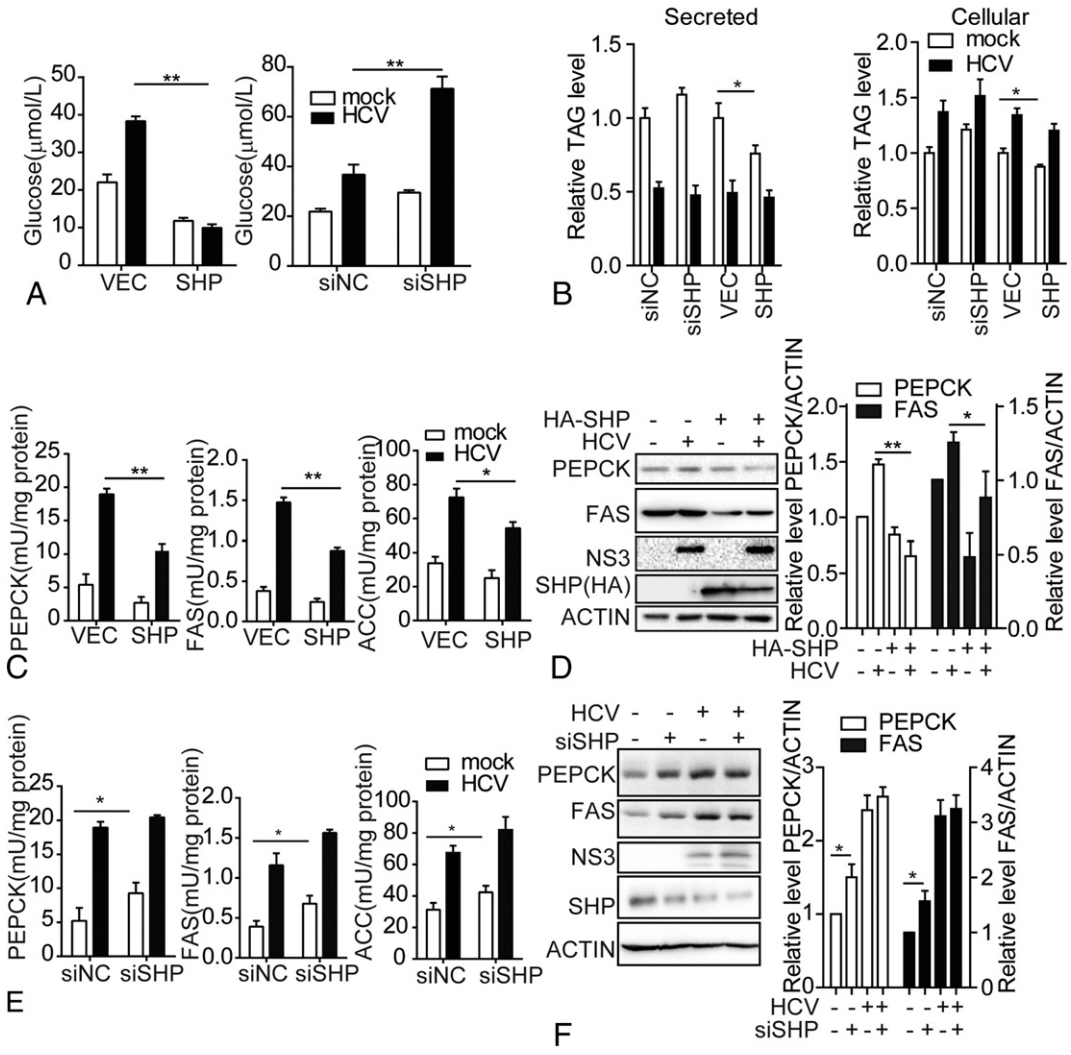

Fig. 2. SHP mediates HCV-induced gluconeogenesis and lipogenesis. Relative cellular glucose production (A) and intracellular TAG content or TAG secretion (B) in mock- and HCV-infected (1.0 MOI) cells with overexpressed SHP or pre-transfection with a combination of siSHP-1 + 2 + 5 for 48 h at 96 hpi. PEPCK, FAS, and ACC enzymatic activity (C). PEPCK, FAS, and NS3 protein levels (D) in mock- and HCV-infected (1.0 MOI) cells with overexpressed SHP at 96 hpi. PEPCK, FAS, and ACC enzymatic activity (E). PEPCK, FAS, and NS3 protein levels (F) in mock- and HCV-infected (1.0 MOI) cells pre-transfected with a combination of siSHP-1 + 2 + 5 for 48 h (D) at 96 hpi. Histogram (D, F right panel) representing a densitometric analysis performed to quantify the relative intensity of the bands detected by Western blotting. Actin was used as a loading control. The data are presented as the mean ± SEM of three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

relationship among HDAC9, SHP and FoxO1. As shown in Fig. 3D, the overexpression of SHP disrupted the HDAC9-FoxO1 association and further increased the acetylation of FoxO1 and gluconeogenic gene expression, whereas the SHP depletion showed the opposite effects.

SREBP-1c is a major regulator involved in lipogenesis. The SHP overexpression significantly attenuated the promoter activity and expression levels of SREBP-1c in the hepatocytes, whereas the SHP inhibition increased these levels (Fig. 4A and B). An LXRα binding site in the SREBP-1c promoter activates SREBP-1c transcription in the presence of LXRα agonists [35]. We attempted to ascertain the specific effects of SHP on the transcriptional activity of LXRα and its underlying mechanisms. First, we observed that LXRα co-immunoprecipitated with SHP in hepatocytes (Fig. 4C). In general, SUMOylation might be responsible for the inhibitory actions of nuclear receptors on the transcription of target genes [36]. We found that HCV infection attenuated the SUMOylation of LXRα (Fig. 4D). To further investigate whether SHP participates in the SUMOylation of LXRα, the excessive or defective expression of SHP in Huh7 cells was transfected with SUMO2, UBC9 and Flag-LXRα and then subjected to an immunoprecipitation analysis. The SUMOylation of LXRα was increased following the SHP overexpression. This effect of SHP on LXRα SUMOylation was additionally confirmed following the SHP knockdown (Fig. 4E). Under these conditions, the chromatin immunoprecipitation analysis further revealed that SREBP-1c was indeed a target of LXRα and that LXRα binding to SREBP-1c was decreased following the SHP overexpression (Fig. 4E).

3.4. Downregulated SHP gene expression in liver biopsies from HCV-infected patients

We further analysed our experimental findings in vivo and examined SHP expression in liver biopsy samples from HCV-infected patients. The demographic and clinicopathological characteristics of the 37 biopsies obtained from HCV-infected patients and 10 biopsies from normal control patients included in the study are shown in Supplementary Table S3. We observed a statistically significant reduction in SHP expression in the liver biopsies of the HCV-infected patients ($p < 0.001$) (Fig. 5A). In the group of 37 HCV-infected patients, the SHP mRNA expression levels were negatively associated with the HCV viral loads in both the liver ($p = 0.0007$) (Fig. 5B) and serum ($p = 0.0042$)

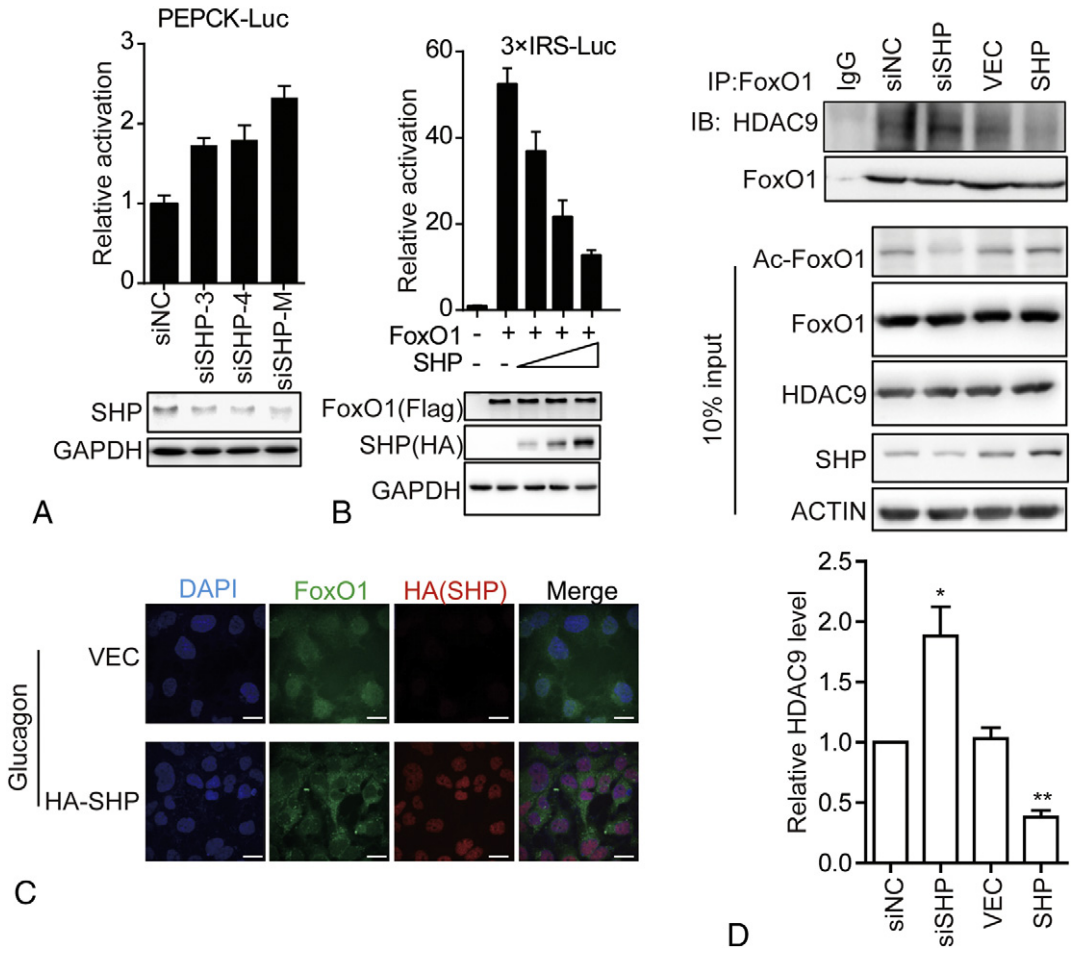

Fig. 3. SHP-dependent FoxO1 acetylation is involved in hepatic gluconeogenesis. (A) Promoter activity of PEPCK in SHP-overexpressing or SHP-knockdown cells. Knockdown of SHP by siRNA was validated by Western blotting. (B) IRE-Luc activity was detected following FoxO1 overexpression and by the dose response upon SHP induction. The data are presented as the mean ± SEM of three independent experiments. Overexpression of indicated proteins validated by Western blotting. (C) Endogenous FoxO1 protein was detected by immunofluorescence in SHP-overexpressed Huh7 cells. Scale bars: 10 μm. (D) Endogenous FoxO1 was immunoprecipitated from the indicated cell lysates and immunoblotted with the indicated antibodies (HDAC9 and CBP). The whole cell lysate used for immunoprecipitation was immunoblotted with the indicated antibodies. Goat anti-rabbit IgG or rabbit anti-mouse was used as a negative control. Representative example of a Western blot of immunoprecipitation is shown. Histogram representing a densitometric analysis performed to quantify the relative intensity of the indicated bands detected by Western blotting. The data are presented as the mean ± SEM of triplicate experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

(Fig. 5C). Furthermore, in the HCV-infected subjects, both the degree of PEPCK gene induction ($p = 0.0015$) and enzymatic activities ($p = 0.0015$) were negatively correlated with the SHP mRNA expression levels (Fig. 5D and E). The degree of SHP induction was correlated, but not significantly, with the HOMA-IR values in the HCV-infected subjects ($p = 0.3019$) (Supplementary Fig. 5A). In accordance with the in vitro results, the ACC enzymatic activity was increased in the HCV-infected patients ($p < 0.001$) and found to be negatively correlated with the SHP mRNA expression levels ($p < 0.0001$) (Fig. 5F and G).

3.5. Exaggerated gluconeogenic and lipogenic response via SHP downregulation in persistent HCV-infected mice

We further demonstrated the relevance of our clinical and in vitro findings in transgenic ICR mice (C/O^{Tg}) that specifically expressed human CD81 and OCLN in their hepatocytes [34]. Consistent with the data observed in the clinical study, SHP mRNA expression continuously increased during the 10 mpi period in the chronically HCV-infected C/O^{Tg} mice (Fig. 6A). Additionally, the PEPCK mRNA levels in the liver samples from the C/O^{Tg} mice were significant increased following HCV infection (Fig. 6B). Furthermore, both the PEPCK mRNA levels ($p = 0.0579$) and enzymatic activities were negatively correlated with the SHP mRNA expression levels in the HCV-infected C/O^{Tg} mice ($p = 0.0007$) (Fig. 6C and D). The degree of SHP induction was negatively correlated with the HOMA-IR values in the HCV-infected C/O^{Tg} mice ($p = 0.0406$) (Supplementary Fig. 5B). Regarding lipogenesis, the ACC enzymatic activity was increased in the HCV-infected mice ($p < 0.001$) and found to be negatively associated with the SHP mRNA expression levels ($p = 0.0123$) (Fig. 6E and F).

4. Discussion

Previous reports have addressed the role of SHP in metabolic regulation; the exact mechanisms of action are largely unknown, particularly following HCV infection. In this study, we report that SHP regulates hepatic gluconeogenesis via the deacetylation of FoxO1, which is mediated by HDAC9. Specifically, SHP expression can be strongly reduced upon HCV infection, indicating that SHP plays a major role in the development of HCV-associated exaggerated gluconeogenic responses. By examining the regulation of SREBP-1c expression by SHP, we found that HCV infection may lead to abnormal lipid homeostasis through SHP downregulation, resulting in decreased SUMOylation of LXR, transactivation of the DNA-binding activity of LXR, and association with SREBP1c and increased hepatic lipogenesis. These findings are summarized in Fig. 7. These results highlight a plausible molecular mechanism of HCV-induced abnormal glucose and lipid homeostasis.

SHP interacts with conventional nuclear receptors and transcription factors and negatively regulates the expression of target genes involved

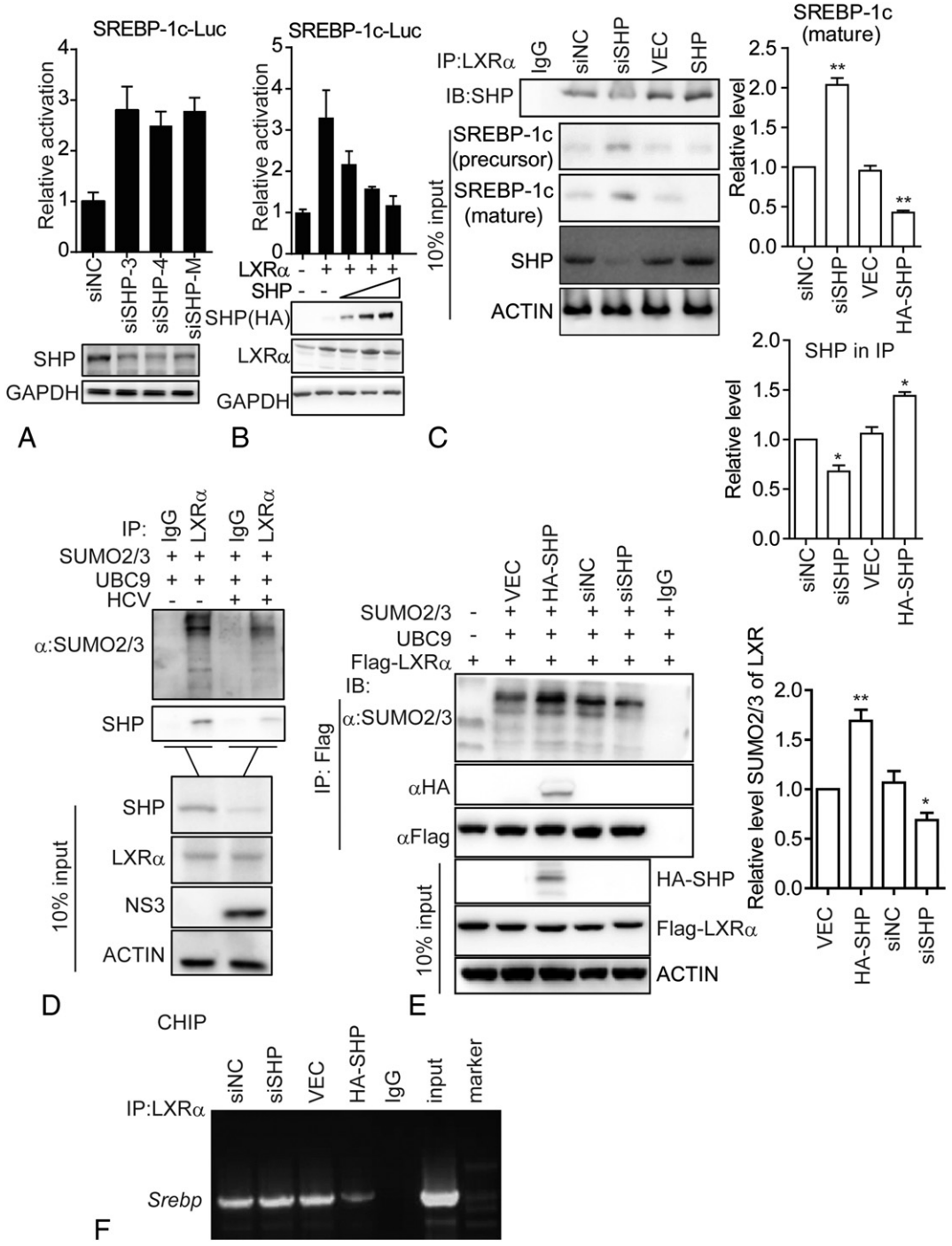

Fig. 4. SHP-dependent LXRα SUMOylation in hepatic lipogenesis. (A) Promoter activity of SREBP-1c in SHP-knockdown cells. Knockdown of SHP by siRNA was validated by Western blotting. (B) SREBP-1c promoter activity was detected following LXRα overexpression and by the dose response upon SHP induction. Overexpression of the indicated proteins validated by Western blotting. The data are presented as the mean ± SEM of three independent experiments. (C) Endogenous LXRα was immunoprecipitated from the indicated cell lysates and immunoblotted with SHP antibodies. The whole cell lysate used for immunoprecipitation was immunoblotted with the indicated antibodies. Goat anti-rabbit IgG or rabbit anti-mouse was used as a negative control. (D) Western blot analysis showing the SUMOylation levels in HCV-infected Huh7 cells pre-transfected with SUMO2/3, Ubc9, and Flag-tagged LXRα. (E) Western blot analysis showing the SUMOylation levels of the indicated epitope-tagged substrates in HA-SHP or SHP siRNA pre-transfected Huh7 cells cotransfected with SUMO2/3, Ubc9, and Flag-tagged LXRα. Representative example of a Western blot of immunoprecipitation is shown. Histogram (C, E) representing a densitometric analysis performed to quantify the relative intensity of the indicated bands detected by Western blotting. The data are presented as the mean ± SEM of triplicate experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. (F) Chromatin immunoprecipitation assays with an antibody against LXRα were used to detect LXRα bound to the proximal SREBP-1c promoter in Huh7 cells pre-transfected with HA-SHP or SHP siRNA. Rabbit IgG was used as a negative control. The data are presented as the mean ± SEM of three independent experiments.

in glucose homeostasis and hepatic lipid metabolism. Many studies have shown that SHP represses transcription factor-mediated transactivation by inhibiting DNA binding, competing for binding to coactivators of nuclear receptors, and recruiting corepressors [37–39]. Indeed, we found that the overexpression of SHP represses the transcriptional activity of FoxO1 and the gluconeogenic gene promoters by disrupting the CBP-FoxO1 association, which is consistent with previous studies [40].

Distinct from the canonical mechanisms, we also found that the overexpression of SHP reduced the acetylation of FoxO1 by disrupting

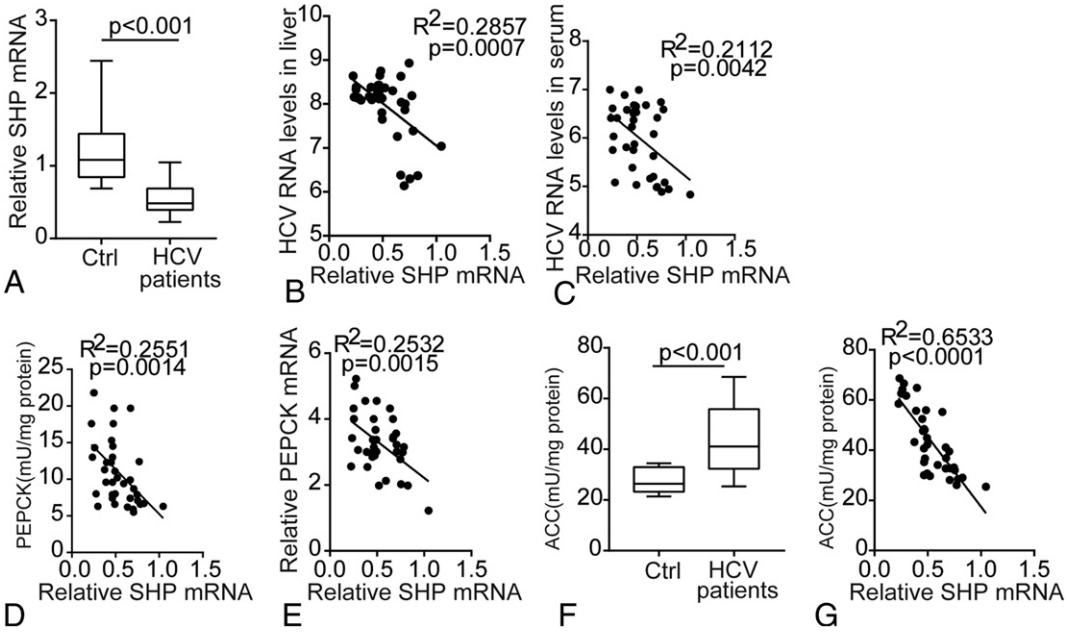

Fig. 5. Downregulated SHP gene expression in liver biopsies from HCV-infected patients. Box plot diagrams showing SHP mRNA (A) and ACC1 enzymatic activity (F) in liver biopsies of 10 normal control patients and 37 patients with chronic hepatitis C (supplementary Table 3). Negative correlation between the SHP mRNA levels in the liver and HCV virus load in the liver (B), HCV virus load in serum (C), PEPCK mRNA levels in the liver (D), PEPCK enzymatic activity in the liver (E), or ACC enzymatic activity in the liver (G). The data are presented as the mean ± SEM and calculated from supplementary Table 3.

the association of HDAC9-FoxO1, which is consistent with our previous study [41]. In addition, SHP mediates LXR-dependent metabolic regulation in hepatocytes by regulating the SUMOylation of LXR. The transcriptional activation and/or stability of nuclear receptors are regulated by different types of posttranslational modifications, such as acetylation and SUMOylation [42]. Several recent studies have reported the mechanisms involved in the SUMO-modification of LXRα responsible for its anti-inflammatory transrepression effects in macrophages and astrocytes [36]. Notably, we described a specific metabolic regulation mechanism of LXRα. SUMOylated LXRα, which was directly modified by SHP, was prevented from binding the promoters of target genes, thereby inhibiting SREBP1c-mediated metabolic gene expression.

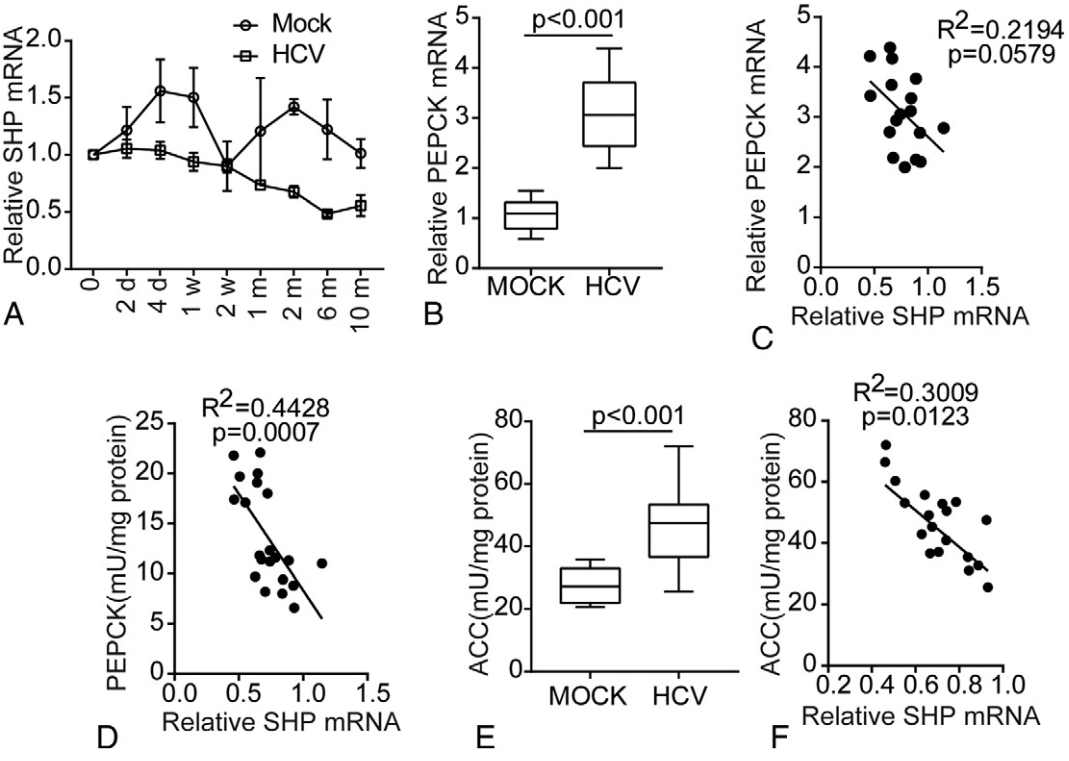

Fig. 6. Exaggerated gluconeogenic and lipogenic response via SHP downregulation in persistent HCV-infected mice. Male and female chronic HCV-infected C/O^{Tg} mice (1 mpi) and mock-infected mice were fasted overnight. (A) Intracellular SHP mRNA levels in the livers of C/O^{Tg} mice mock-infected or infected with HCV for the indicated time. Box plot diagrams showing the PEPCK mRNA levels (B) and ACC1 enzymatic activity (E) in the livers of mock-infected or HCV-infected C/O^{Tg} mice. Negative correlation between SHP mRNA levels in the liver and PEPCK mRNA levels (C), PEPCK enzymatic activity in the liver (D) and ACC enzymatic activity in the liver (F). The data are presented as the mean ± SEM.

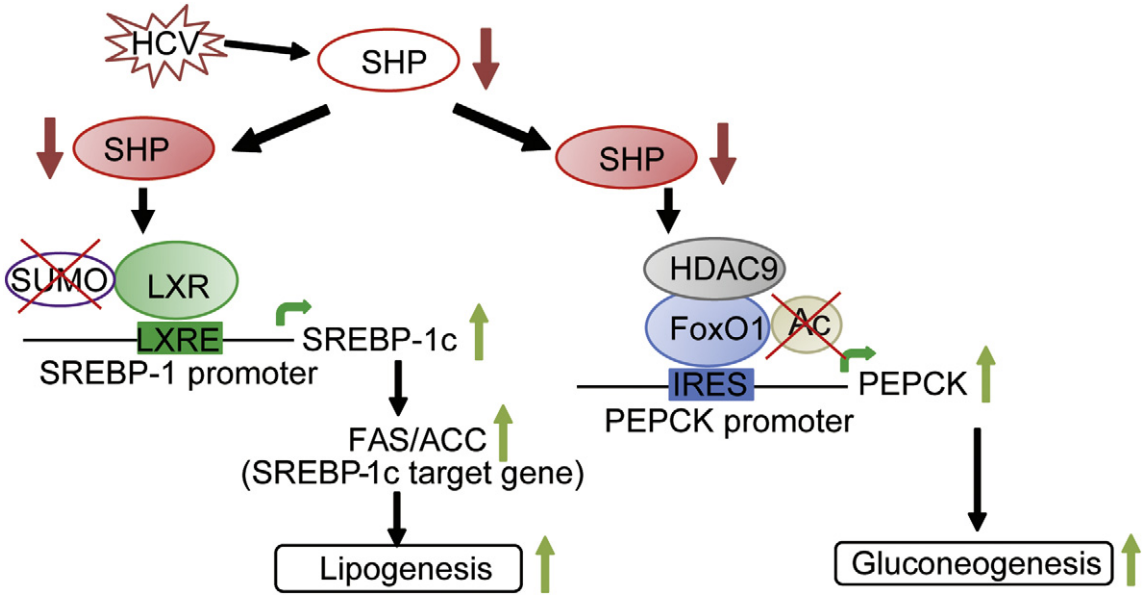

Fig. 7. A hypothetical model illustrating how SHP regulates both lipogenesis and gluconeogenesis following HCV infection.

Recent studies have suggested that virus-induced changes in a host's metabolism can be detrimental to its life cycle [43]. Our clinical and animal studies show that the SHP expression levels were elevated in the liver tissues obtained from the HCV-infected patients and chronic HCV-infected rodent models, which was accompanied by increased gluconeogenic and lipogenic activity. The downregulation of SHP, increased PEPCK, FAS, and ACC enzymatic activities and negative correlation between these findings further confirm the regulation of gluconeogenesis and lipogenesis by SHP following HCV infection. Notably, the livers of insulin-resistant, HCV-associated metabolically abnormal mice showed selective insulin resistance, suggesting a branch point in the insulin signalling pathway. Insulin loses its ability to block glucose production (it fails to suppress PEPCK and other gluconeogenesis genes); however, insulin retains its ability to stimulate fatty acid synthesis (continued enhancement of genes involved in lipogenesis) [44]. Our experiment described the bifurcation point in SHP regulation in the liver, which may account for the following important paradox following HCV infection: One pathway leads to increased gluconeogenesis through FoxO1, and the other pathway leads to an increase in lipogenesis through SREBP-1c.

Recently, retinoid-related molecules, including 4-[3-(1-adamantyl)-4-hydroxyphenyl]-3-chlorocinnamic acid (3Cl-AHPC), were shown to bind SHP and increase SHP activity in the repression of SHP metabolic target genes in human primary hepatocytes [45]. These data further support the finding that SHP may act as a ligand-regulated receptor in metabolic pathways. The modulation of SHP activity by synthetic ligands may be a useful therapeutic strategy for suppressing hepatic gluconeogenesis and lipogenesis, further lowering blood glucose and lipid storage in HCV-associated metabolic syndrome.

Ethics statement

All patients and/or their relatives provided written informed consent for their clinical and pathological information to be used for research and to be stored in the hospital database; this study, including its methods and experimental protocols, was approved by the Ethical Committee of the First Hospital of Jilin University. All procedures in our study were performed in accordance with the ethical standards of our institutional research committee and the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study methods were carried out in accordance with the approved guidelines.

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Author contributions

The study was designed by Q.W. and JZ.C.; Experiments in HCV-infected cells were performed by JZ.C., Y.Z., Q.T. and Y.Z. Experiments in replicon cells were performed by Q.W., M.G. and Y.Z. Patients' studies were performed by JZ.C. Transgenic mice and animal study was performed by JZ.C., Y.Z., Z.Y. Experimental analysis was performed by Q.W., JZ.C., Z.L. and JQ. N.; and the manuscript was written by Q.W. Q.W. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Declaration of competing interest

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.metabol.2019.153954.

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