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Hepatitis C Virus Core Protein Stimulates Fibrogenesis in Hepatic Stellate Cells Involving the Obese Receptor

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ABSTRACT

Hepatitis C virus core protein (HCVcp), which is secreted by infected cells, is reported as an immunomodulator in immune cells. However, the effects of HCVcp on hepatic stellate cells (HSCs), the key cells in liver fibrosis, still remain unclear. In this study, we investigated the effects of HCVcp on obese receptor (ObR) related downstream signaling pathways and fibrogenic gene expression in HSCs. LX-2, a human HSC line, was incubated with HCVcp. Inhibitors and short interfering RNAs were used to interrogate the mechanisms of HCVcp action on HSCs. HCVcp (20–100 ng/ml) concentration-dependently stimulated α-smooth muscle actin (α-SMA) protein expression and mRNA expression of *α-SMA*, *procollagen α2(I)* and *TGF-β1* genes, with a plateau of 220% of controls at 100 ng/ml. HCVcp induced mRNA and protein expression of ObR. Blocking of Ob-Rb with a neutralizing antibody inhibited phosphorylation of signal transducer and activator of transcription 3 (STAT3) and AMPKα stimulated by HCVcp. Furthermore, knockdown of Ob-Rb down-regulated HCVcp-induced STAT3, AKT, and AMPKα phosphorylation, and reversed HCVcp-suppressed mRNA expression of matrix metalloproteinase (*MMP*)-1, peroxisome proliferator-activated receptor (*PPAR*)γ and sterol regulatory element binding protein-1c (*SREBP-1c*) genes. AMPKα signaling blockade reversed HCVcp-suppressed *SREBP-1c* mRNA expression. HCVcp stimulated reactive oxygen species formation and $gp91^{phox}$ (a component of NADPH oxidase) protein expression, together with AKT phosphorylation, leading to suppression of *PPARγ* and *SREBP-1c* genes. Our results provide a new finding that HCVcp induced ObR-dependent Janus Kinase (JAK) 2–STAT3, AMPKα, and AKT signaling pathways and modulated downstream fibrogenetic gene expression in HSCs. J. Cell. Biochem. 114: 541–550, 2013.

KEY WORDS: HEPATITIS C VIRUS CORE PROTEIN; OXIDATIVE STRESS; HEPATIC STELLATE CELL; OBESE RECEPTOR B (OB-RB); AMP-ACTIVATED PROTEIN KINASE (AMPK); JANUS KINASE 2 (JAK2); SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION 3 (STAT3)

Hepatitis C virus (HCV) infects about 170 million people worldwide, leading to a series of complications including steatosis, cirrhosis, and hepatocellular carcinoma [Lavanchy, 2008; Sheikh et al., 2008]. Liver fibrosis, the initial step of cirrhosis, is characterized with the excessive accumulation of extracellular matrix (ECM) protein [Hernandez-Gea and Friedman, 2011]. In liver injury, hepatic stellate cells (HSCs), the main fibrogenic cells, produce ECM to repair the damage [Friedman, 2010]. The mechanisms of HCV-induced liver fibrosis are not fully clarified. Recent studies suggest that HCV non-structural genes are associated with up-regulation of transforming growth factor β1 (TGF-β1) and other profibrogenic factors in infected hepatocytes [Anja, 2005], and co-culture of stable HepG2–HCVcp with HSCs results in HSC activation [Shin et al., 2005]. HCVcp-transfected hepatocytes are also shown to secrete interleukin-8, resulting in activation of HSCs [Clément et al., 2010].

HCVcp, a 21-kDa structural protein localized in hepatocytes, has been shown to exert various biologic functions including apoptosis,

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Contributions to the work: Ching-Fen Wu performed the experiments, analyzed the data, and drafted the manuscript. Yun-Lian Lin and Yi-Tsau Huang designed the study, supervised the work, and revised the manuscript.

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oxidative stress, and immunomodulation [Lai, 2002; Irshad and Dhar, 2006]. The introduction of HCVcp increases mRNA expression of *IL-6*, *gp130*, and *leptin receptor* genes and activates signal transducer and activator of transcription 3 (STAT3) signaling pathways in hepatocytes [Basu et al., 2006; Kawamura et al., 2006]. Aside from intracellular localization, HCVcp is secreted into bloodstream, and detectable in the plasma of infected patients [Tanaka et al., 1996]. Recent reports reveal that HCVcp triggers toll-like receptor 2 (TLR-2)-mediated MyD88-dependent NF-κB pathways in monocytes and inhibits IFN-α production in plasmacytoid dendritic cells [Dolganiuc et al., 2004, 2006]. HCVcp is shown to impair T-cell functions by binding to gC1qR [Yao et al., 2004]. In addition, it has been shown that HCVcp stimulates HSC proliferation [Bataller et al., 2004]. Recently, one study reports that blocking of TLR2 prevents HCVcp-induced profibrogenic actions of HSCs [Coenen et al., 2011]. However, the impact of free HCVcp on HSCs and its profibrogenic function has not been fully studied.

Leptin receptor (Ob receptor) has six isoforms, but only the long form (Ob-Rb) is the functional form to activate signaling pathways. The Ob-Rb isoform modulates most of the biological actions of leptin via activation of the Janus kinase 2 (JAK2)/STAT3 pathway [Israel and Chua, 2010]. During liver injury, JAK/STAT signaling is a critical pathway for stimulation of inflammation-associated genes and ECM degradation [Yoshimura et al., 2007]. In addition, induction of JAK–STAT signaling plays a crucial role in hepatic fibrogenesis with HSC transdifferentiation [Lakner et al., 2010], inhibition of matrix metalloproteinase (MMP)-1, and up-regulation of tissue inhibitor of metalloproteinase (TIMP)-1 in HSCs [Cao et al., 2004, 2007]. Upon binding to Ob-Rb, leptin is demonstrated to enhance pathways involving reactive oxygen species (ROS) production, phosphorylation of JAK2–STAT3, and activation of PI3K/AKT [Frühbeck, 2006; Marra, 2007]. In metabolic steatosis, high levels of serum leptin are observed and reported to be associated with hepatic fibrogenesis. Because chronic HCV infection is associated with both hepatic steatosis and fibrosis, we speculated that free HCVcp might stimulate hepatic fibrogenesis similar to leptin. Hence, the aim of this study is to elucidate the effects of HCVcp on HSCs.

MATERIALS AND METHODS

MATERIALS

Recombinant HCVcp (amino acids 2–192, 22 kDa) was obtained from AbD Serotec (Oxford, UK). Diphenyleneiodonium (DPI) was purchased from Sigma (St Louis, MO, USA). AG 490, LY 294002, and leptin were purchased from Calbiochem (San Diego, CA, USA). Rabbit anti-mouse obese receptor (Ob-Rb, long isoform of obese receptor) IgG was obtained from ADI (San Antonio, TX, USA).

CULTURE OF CELL LINES

LX-2, an immortalized human HSC line, was a kind gift from Dr. S. L. Friedman, Mount Sinai School of Medicine, NY, USA. LX-2 cells were cultured in Dulbecco's modified Eagle's medium containing 2% fetal bovine serum (FBS).

ROS MEASUREMENT

The seeded cells were incubated with 8-μM CM-H2DCF-DA (Invitrogen, Carlsbad, CA, USA) for 1 h at 37°C before treatment with HCVcp. ROS levels were detected by measuring fluorescence at 485 nm for excitation and 530 nm for emission using Flex station (Molecular Devices Corp., Sunnyvale, CA, USA), as previously reported by us [Tsai et al., 2010].

WESTERN BLOTTING ANALYSIS

A whole cell protein sample (20 μg) was subjected to SDS–PAGE with 10% resolving gel. The gel with separated proteins was then transferred onto Immobilon-PVDF membrane (Millipore, Bedford, MA, USA). The primary antibodies used in Western blotting included p-AMPKα (Tyr-172), AMPKα, p-STAT3 (Tyr-705), STAT3, p-JAK2 (Tyr-1007/1008) and JAK2 (Cell Signaling Technology, Beverly, MA, USA), $gp91^{phox}$ and α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Blots were developed using ECL detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK). The densities of representative immunoblots were quantified and normalized to α-tubulin.

SHORT INTERFERING RNA ANALYSIS

Trans IT-TKO® transfection reagent (Mirus, Madison, WI, USA) was used to transfect the following short interfering RNAs (siRNAs) into LX-2, respectively: On-Target plus SMART pool human STAT3 and ObR siRNA, and On-Target plus non-targeting pool siRNA (Thermo Scientific, Waltham, MA, USA). The non-targeting pool siRNA was used as a negative control for transfection. The transfection procedure was performed according to the manufacturer's protocol.

REAL-TIME POLYMERASE CHAIN REACTION (PCR)

Total RNA was extracted by TRIzol® according to the manufacturer's protocol. One microgram RNA was reversely transcribed to cDNA by using RevertAid™ First Strand cDNA Synthesis kit (Fermentas, Burlington, CA, USA). The mRNA levels were analyzed in triplicates with the use of Smart Quant Green Master mix (Protech, Taiwan) by LightCycler® 480 Real-Time PCR System (Roche, Indianapolis, IN, USA) and normalized to GAPDH mRNA expression. The sequences of the primers used for real-time PCR were listed in Table I.

STATISTICS

Data were shown as mean ± standard errors of the mean. The results were obtained from three independent experiments, and statistical analysis was performed by using one-way ANOVA. $P < 0.05$ was considered as statistically significant.

RESULTS

EFFECTS OF HCVcp ON α-SMA AND FIBROGENIC GENE EXPRESSION IN LX-2 CELLS

HCV infection has been reported to be associated with TGF-β1 up-regulation and ROS production [Otani et al., 2005; Lin et al., 2010], which are thought to be important mediators for HCV-induced liver fibrosis [Friedman, 2010; Hernandez-Gea and Friedman, 2011]. Up-regulation of α-SMA is a key marker of HSC activation [Friedman,

TABLE I. Primer Sequences

	Forward (5′–3′)	Reverse (5′–3′)
TIMP-1	TGA CAT CCG GTT CGT CTA CA	TGA TGT GCA AGA GTC CAT CC
MMP-1	GAT CAT CGG GAC AAC TCT CCT	TCC GGG TAG AAG GGA TTT GTG
MMP-2	GCT GGG AGC ATG GCG ATG GAT ACC	GGA CAG AAG CCG TAC TTG CCA TCC
SREBP-1c	GCG GAG CCA TGG ATT GCA C	CTC TTC CTT GAT ACC AGG CCC
STAT3	GCT TCC TGC AAG AGT CGA AT	ATT GGC TTC TCA AGA TAC CTG
Ob-Rb	AGG ACG AAA GCC AGA GAC AA	AAA TGC CTG GGC CTC TAT CT
Ob-Rt	CCA CCA TTG GTA CCA TTT CC	GGC ATT CAT GTT CAT TGC AG
PPARγ	GAG CCC AAG TTT GAG TTT GC	GGC GGT CTC CAC TGA GAA TA
MCP-1	CGC GAG CTA TAG AAG AAT CAC	TTG GGT TGT GGA GTG AGT GT
TGF-β	TCT CTC CAA CCT GCC ACA GA	GAT CGC GCC CAT CTA GGT T
Procollagen α2(I)	GTC CCT GAA GTC AGC TGC TAT	TGG GAC AGT CCA GTT CTT CAT
GAPDH	CAA TGA CCC CTT CAT TGA CC	GAT CTC GCT CCT GGA AGA TG

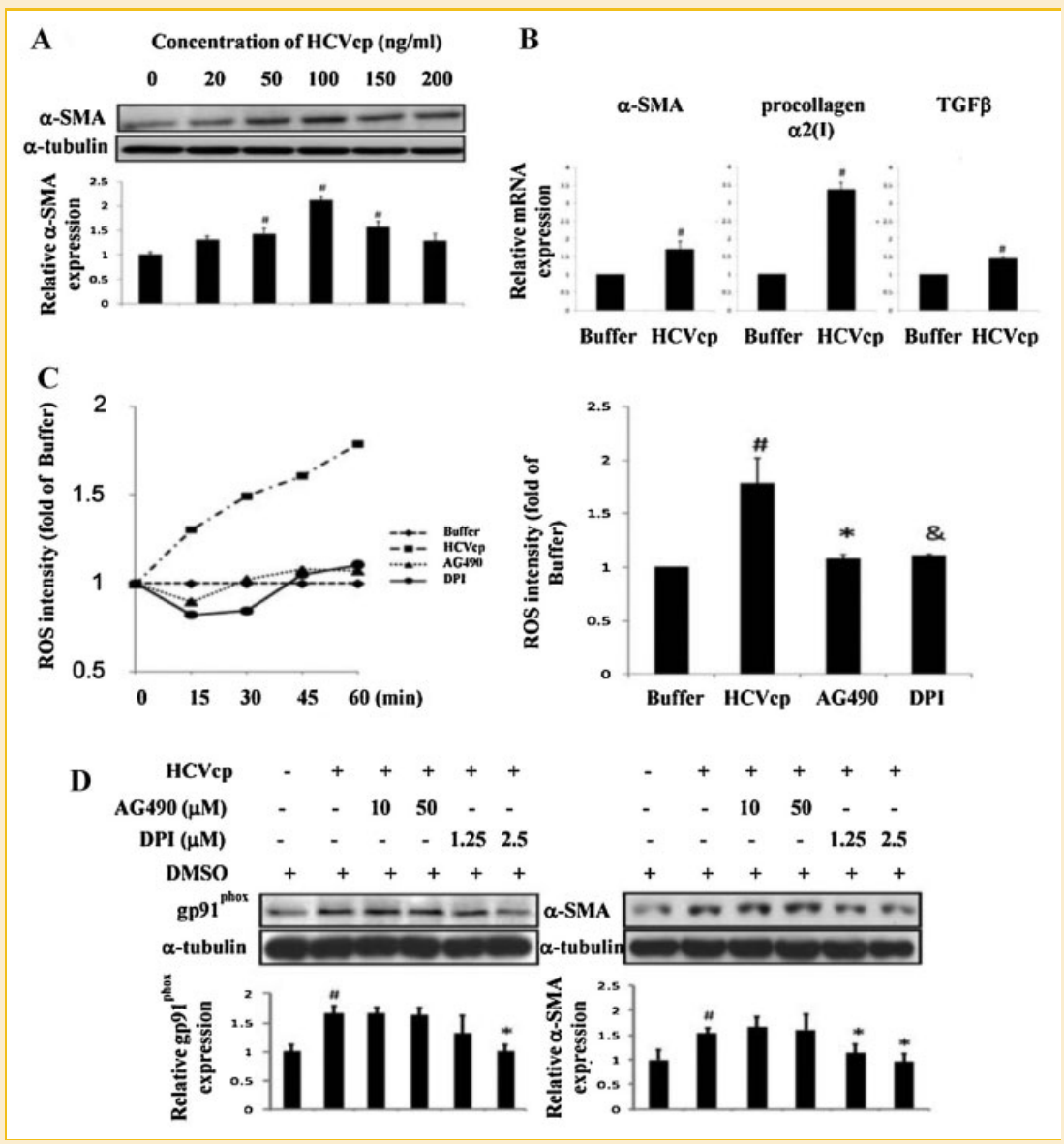

Fig. 1. HCVcp induced elevation of ROS, α-SMA, and fibrogenic genes in LX-2 cells. A: Cells were treated with different concentrations of HCVcp for 24 h and α-SMA protein level was detected using Western blot. B: Cells were treated with 100 ng/ml HCVcp for 24 h. Ten microliters of production from reverse transcription, primers for *α-SMA*, *procollagen α2(I)*, *TGF-β1* genes, and Smart Quant Green Master mix were subjected to real-time PCR. C: Cells were cultured for 24 h, and then treated or untreated with DPI (2.5 μM) or AG490 (50 μM) for 1 h. The DCFDA-stained LX-2 cells were treated with HCVcp, and signals were recorded every 15 min. Right panel represented numerical expression of relative signals at 1 h. D: Cells were pretreated with AG490 (10 and 50 μM) or DPI (1.25 and 2.5 μM) for 1 h prior to stimulation of HCVcp for 2 h for detection of $gp91^{phox}$ and 24 h for α-SMA. The expression of $gp91^{phox}$ and α-SMA was assessed by Western blotting analysis. #, $P < 0.05$ compared with untreated cells; * and &, $P < 0.05$ compared with HCVcp treatment.

2010]. Therefore, we first investigated whether HCVcp promoted fibrogenesis in LX-2 cells. Treatment of HCVcp (20–200 ng/ml) for 24 h increased α-SMA expression in LX-2 cells, reaching a maximal expression of 2.2-fold at 100 ng/ml of HCVcp, which was chosen for later experiments (Fig. 1A). Up-regulation of α-SMA by HCVcp was inhibited by the NADPH oxidase inhibitor, DPI (Fig. 1D), suggesting an important role of oxidative stress in HSC activation. As shown in Figure 1B, mRNA expression of *α-SMA*, *procollagen α2(I)*, and *TGF-β1* genes was also significantly stimulated by HCVcp.

EFFECT OF HCVcp ON INTRACELLULAR ROS FORMATION IN LX-2 CELLS

Whether HCVcp stimulated ROS production in LX-2 cells was determined. HCVcp (0–60 min) time-dependently enhanced ROS

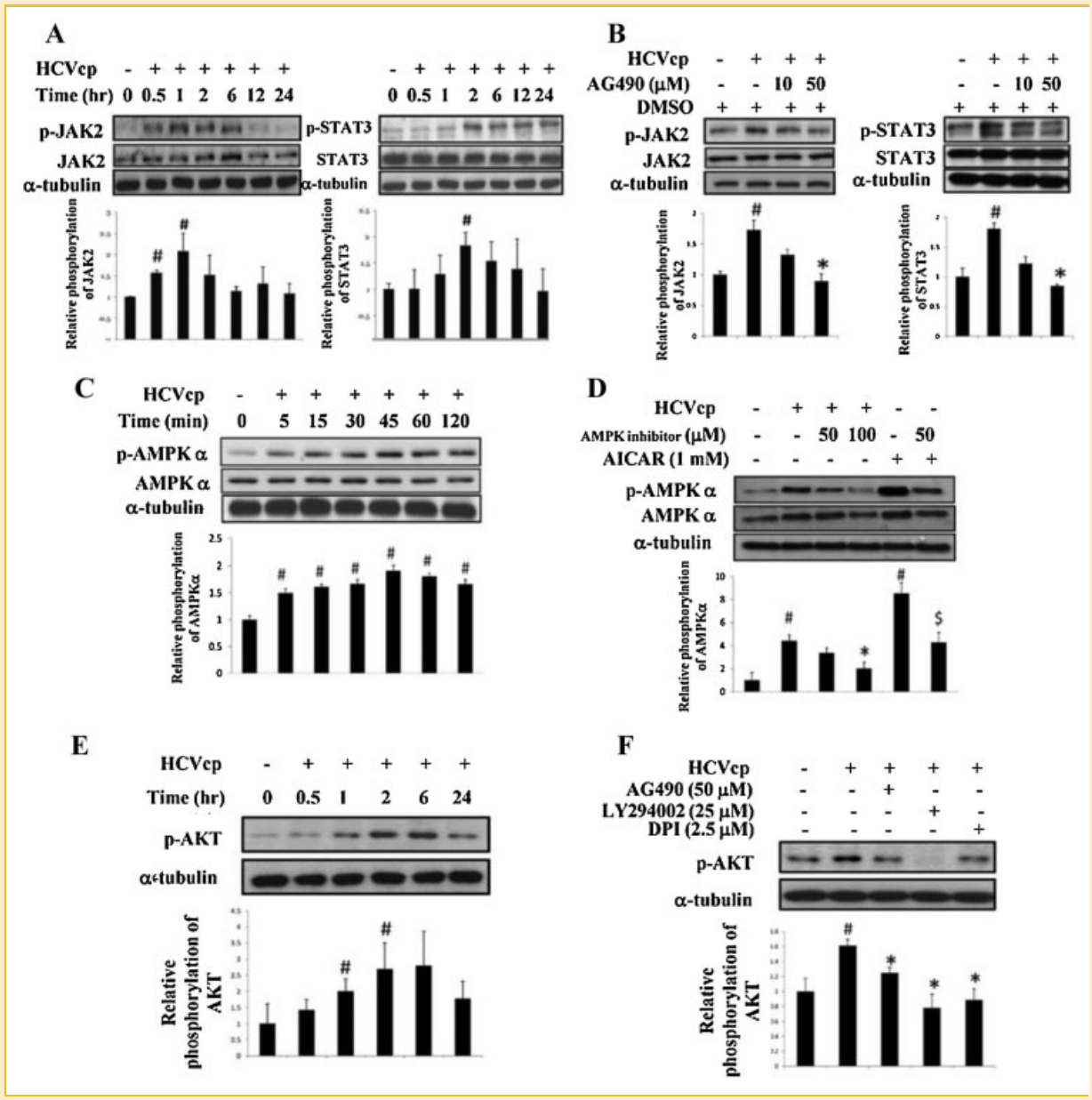

Fig. 2. HCVcp initiated JAK2–STAT3, AKT, and AMPKα phosphorylation. A: LX-2 cells were treated with HCVcp at the indicated time. Blots were detected with p-JAK2, p-STAT3, JAK2, and STAT3 antibodies. A maximum phosphorylation of JAK2 and STAT3 was detected at 1 and 2 h, respectively. B: Pretreatment with or without AG490 (10 and 50 μM) for 1 h prior to treatment with HCVcp for 1 and 2 h to detect phosphorylation of JAK2 and STAT3, respectively. C: LX-2 cells were treated with HCVcp at the indicated time. In the Western blot, a plateau phosphorylation of AMPKα was observed at 45 min after HCVcp treatment. D: Pretreatment with or without AMPK inhibitor compound C (50 and 100 μM) for 1 h prior to treatment with HCVcp or AICAR (1 mM) for 45 min. E: LX-2 cells were incubated with HCVcp at the indicated time (0, 0.5, 1, 2, 6, and 24 h). Time 0 represents untreated cells. The blots were detected with p-AKT antibody. F: Cells were pretreated with or without AG490 (50 μM), DPI (2.5 μM), or LY294002 (25 μM) 1 h prior to treatment with HCVcp for 2 h. Cells were extracted, and the lysates were analyzed for AKT phosphorylation. #, $P < 0.05$ compared with untreated cells; *, $P < 0.05$ compared with HCVcp treatment; $, $P < 0.05$ compared with AICAR treatment.

production in DCFDA-loaded LX-2 cells, which was blocked by DPI and a JAK inhibitor, AG490 (Fig. 1C). HCVcp also up-regulated protein expression of gp91^{phox} (a component of NADPH oxidase) and α-SMA, which was reversed by DPI, but not AG490 (Fig. 1D), implying that JAK–STAT signaling was not directly responsible for modulating expression of gp91^{phox} and α-SMA. Taken together, we demonstrated that HCVcp directly stimulated *α-SMA*, *procollagen α2(I)*, and *TGF-β1* gene expression, and ROS production in HSCs, and the profibrogenic role of free HCVcp was characterized.

EFFECTS OF HCVcp ON JAK2–STAT3, AMPKα, AND AKT SIGNALING PATHWAYS IN LX-2 CELLS

Western blotting analysis was used to evaluate the role of HCVcp in JAK2–STAT3, AMPKα, and AKT signaling. Phosphorylation of JAK2 and STAT3 was significantly elevated after HCVcp treatment at 1 and 2 h, respectively, which was inhibited by pretreatment of AG490 for 1 h (Fig. 2A and B). Similarly, HCVcp enhanced AMPKα phosphorylation, peaking at 45 min, which was significantly suppressed by the AMPK inhibitor, compound C. Compound C

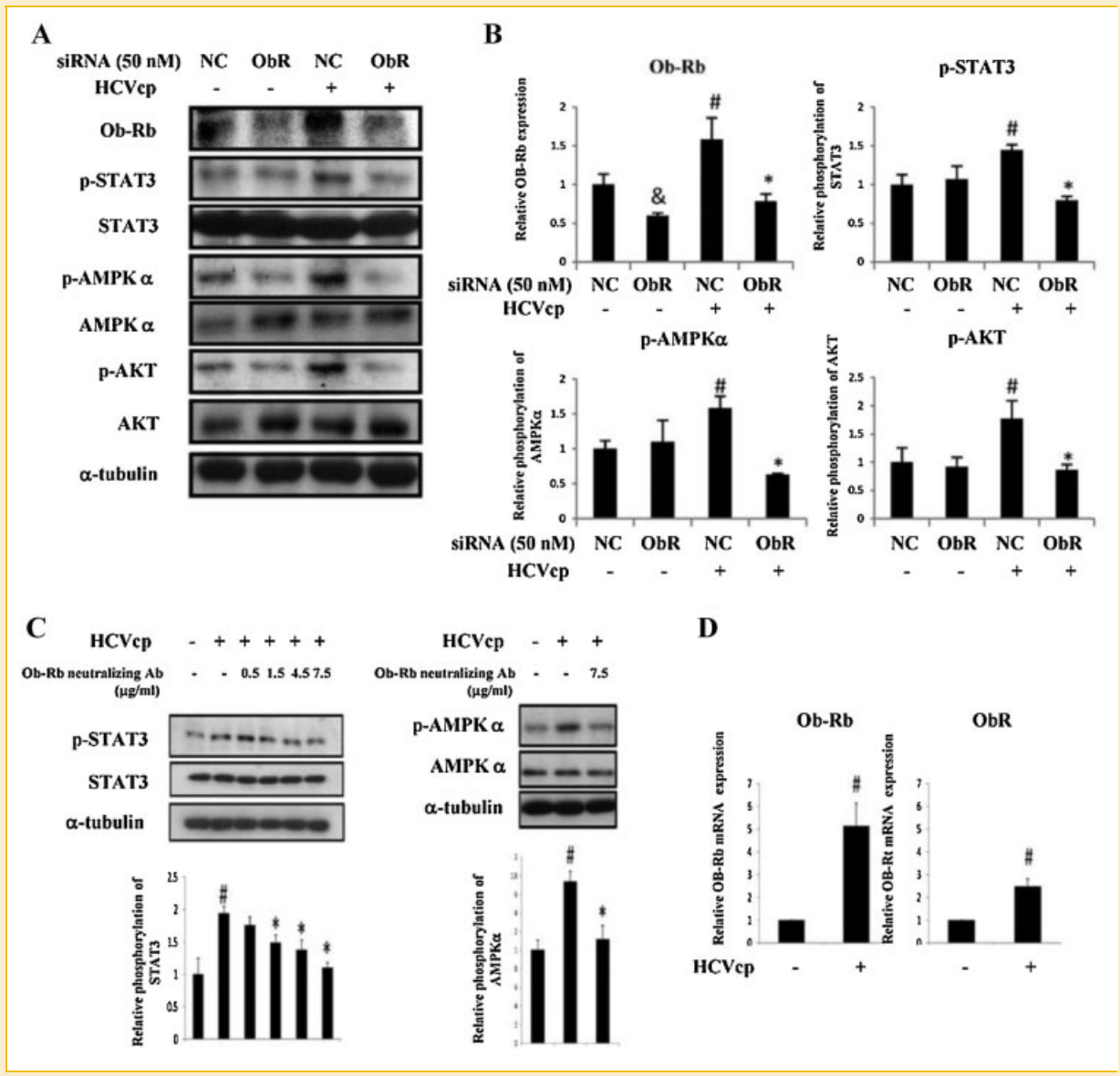

Fig. 3. HCVcp activated STAT3, AKT, and AMPKα phosphorylation via Ob-Rb. A: LX-2 cells were transfected with non-specific control (NC) and ObR siRNA, respectively. After 24 h of transfection and 72 h of incubation with fresh medium containing 2% FBS, cells were treated with HCVcp for 2 h. Cell lysates were collected and subjected to Western blotting to detect phosphorylation and total protein expression of STAT3, AKT, and AMPKα. B: Numerical expression of phosphorylation levels. The signals of representative immunoblots (ObR) were quantified and normalized to α-tubulin, whereas p-STAT3, p-AMPKα, and p-AKT were quantified and normalized to STAT3, AMPKα, and AKT, respectively. C: Cells were incubated with different concentrations of Ob-Rb-neutralizing antibody for 1 h prior to HCVcp for 45 min and 2 h, respectively. The lysates were collected to detect AMPKα and STAT3 phosphorylation. D: Cells were treated with HCVcp for 24 h. The mRNA levels of Ob-Rb and Ob-R were determined by real-time PCR. Details were described in Materials and Methods Section. #, $P < 0.05$ compared with untreated cells; *, $P < 0.05$ compared with HCVcp treatment.

also inhibited AICAR (an AMPK activator)-stimulated AMPKα phosphorylation (Fig. 2C and D). Activated PI3-kinase/AKT pathway has been implicated in regulating cell proliferation, collagen secretion, and prevention of apoptosis in HSCs [Tsai et al., 2010]. In this study, HCVcp treatment for 1–6 h induced AKT phosphorylation (Fig. 2E), which was reversed by AG490, LY 294002 (PI-3K inhibitor) and DPI (Fig. 2F). These results demonstrated that HCVcp activated JAK2–STAT3, AMPKα, and AKT signaling pathways.

Ob-Rb MEDIATED HCVcp-INDUCED JAK2–STAT3, AMPKα, AND AKT SIGNALING PATHWAYS IN LX-2 CELLS

It has been suggested that the JAK2–STAT3, AMPKα, and AKT signaling pathways related to metabolic regulation are activated by leptin via Ob-Rb [Ceddia, 2005]. Therefore, we hypothesized that the function of HCVcp was similar to leptin and we further investigated whether HCVcp-induced JAK2–STAT3, AMPKα, and AKT signaling pathways were mediated through the obese receptor. To perform a

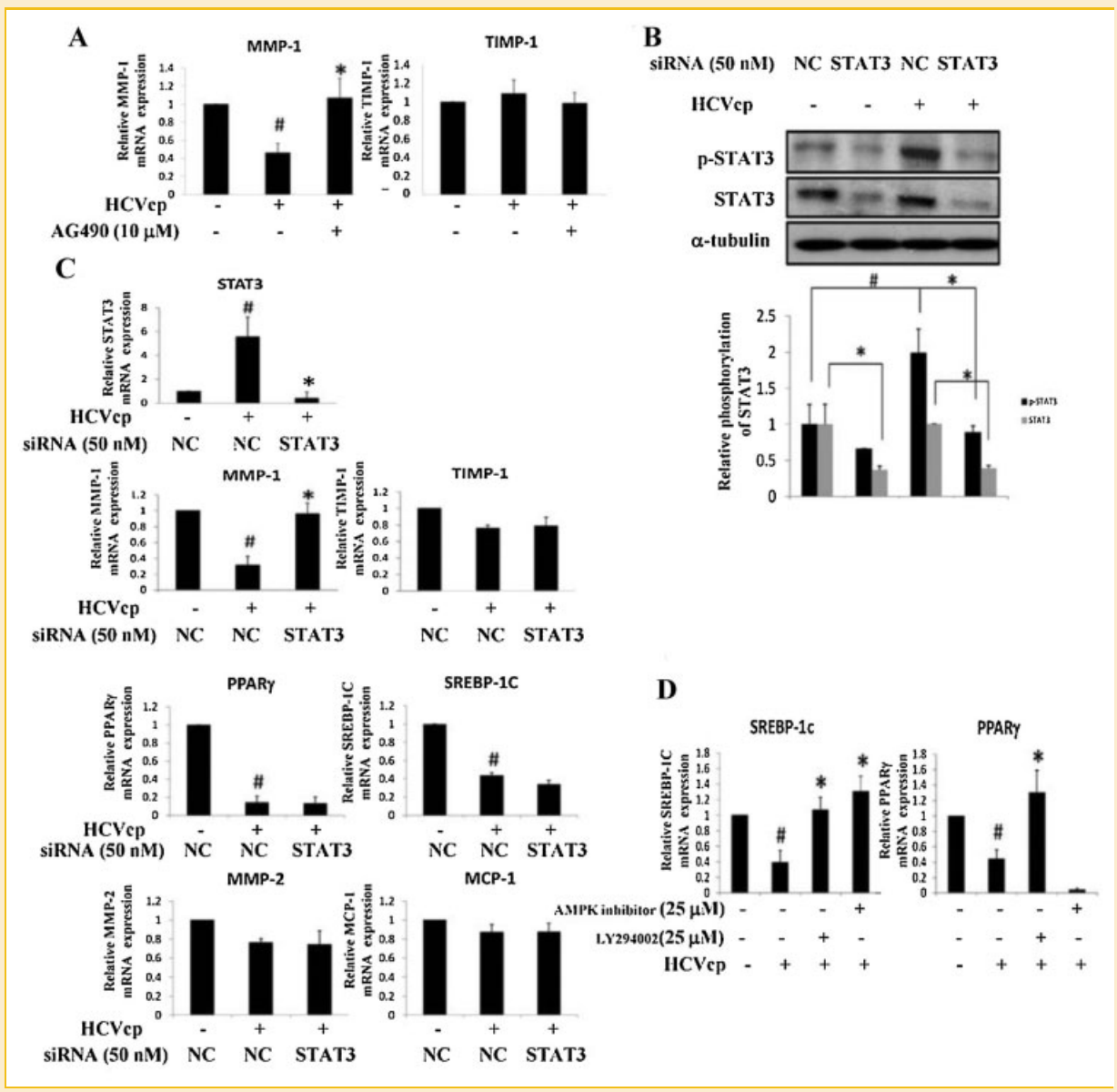

Fig. 4. Effects of small interfering RNA and specific inhibitors on HCVcp-induced gene expression in LX-2 cells. A: Cells were incubated in the absence or presence of AG490 for 1 h, and then treated with HCVcp for 18 h. Real-time PCR was performed to analyze mRNA expression of *MMP-1* and *TIMP-1*. B: LX-2 cells were transfected with non-specific control (NC) and STAT3 siRNA, respectively. After 24 h of transfection and 48 h incubation with fresh medium containing 2% FBS, cells were treated with HCVcp for 2 h. Cell lysates were collected and subjected to Western blotting to detect expression of p-STAT3 and STAT3. C: After transfection with siRNA, cells were treated with HCVcp for 18 h. The mRNA levels of *STAT3*, *MMP-1*, *TIMP-1*, *MMP-2*, *MCP-1*, *PPARγ*, and *SREBP-1c* were determined by real-time PCR. #, $P < 0.05$ compared with cells with NC siRNA and without HCVcp treatment; *, $P < 0.05$ compared with cells with HCVcp treatment. D: Cells were incubated in the absence or presence of AMPK inhibitor and LY294002, respectively. After 1 h of incubation, cells were treated with HCVcp for 18 h, and mRNA expression of *PPARγ* and *SREBP-1c* were analyzed. #, $P < 0.05$ compared with untreated cells; *, $P < 0.05$ compared with HCVcp treatment.

genomic blockage of Ob-Rb, siRNA for Ob-Rb was used to examine phosphorylation of STAT3, AMPKα, and AKT. The Western blots showed that knockdown of *Ob-Rb* gene down-regulated phosphorylation of STAT3, AMPKα, and AKT (Fig. 3A and B). Furthermore, after incubation of an Ob-Rb neutralizing antibody, HCVcp-induced phosphorylation of AMPKα and STAT3 was suppressed (Fig. 3C). In addition, HCVcp stimulated mRNA expression of *ObR* and *Ob-Rb* genes (Fig. 3D). Taken together, these data suggested that HCVcp activated phosphorylation of JAK2–STAT3, AMPKα, and AKT through an Ob-Rb-dependent pathway.

EFFECTS OF JAK INHIBITOR AND STAT3 KNOCKDOWN ON HCVcp-SUPPRESSED *MMP-1* mRNA EXPRESSION

It has been reported that liver fibrosis is associated with suppression of MMP-1 and stimulation of TIMP-1, leading to impaired degradation of ECM [Benyon et al., 1996]. To assess the effect of HCVcp-induced JAK2–STAT3 signaling on transcription of *MMP-1* and *TIMP-1* genes, STAT3 was knocked down by siRNA transfection in LX-2 cells incubated with HCVcp (Fig. 4B). The STAT3 siRNA was effective in suppressing HCVcp-stimulated STAT3 mRNA and protein expression levels, as compared with non-specific control siRNA. Both STAT3 siRNA and AG490 reversed HCVcp-suppressed *MMP-1* mRNA expression, but not *TIMP-1* gene (Fig. 4A and C), suggesting that HCVcp suppressed *MMP-1* mRNA expression in LX-2 via JAK2–STAT3 phosphorylation, and modulation of *TIMP-1* mRNA expression might not be associated with HCVcp.

EFFECTS OF AMPKα AND PI-3K INHIBITORS ON *SREBP-1c* AND *PPARγ* mRNA EXPRESSION LEVELS IN LX-2 CELLS

Peroxisome proliferator-activated receptor-γ (PPARγ) has been shown to reverse HSC activation and maintain HSC quiescence [Zhou et al., 2009]. It has been demonstrated that PPARγ was regulated by the PI3K/AKT pathway in rat HSCs [Marra, 1999]. Recent studies report that sterol regulatory element binding protein-1c (SREBP-1c) and PPARγ are regulated through AMPK signaling in hepatocytes [You et al., 2004; Kohjima et al., 2008]. Hence, we further investigated the effects of HCVcp on mRNA expression of *SREBP-1c* and *PPARγ* genes. HCVcp-down-regulated *SREBP-1c* expression in LX-2 cells was reversed by the PI-3K inhibitor (LY294002) and the AMPK inhibitor (compound C), but not by STAT3 knockdown (Fig. 4C and D). Inhibition of PPARgamma caused by HCVcp was reversed by LY294002. The results suggested that down-regulation of *SREBP-1c* and *PPARγ* genes by HCVcp was modulated through AKT and/or AMPK signaling, not STAT3. In addition, our results showed that HCVcp did not affect expression of *MCP-1* and *MMP-2* genes (Fig. 4C).

EFFECTS OF *Ob-Rb* KNOCKDOWN ON *MMP-1*, *SREBP-1c*, AND *PPARγ* mRNA EXPRESSION LEVELS IN LX-2 CELLS

To confirm the role of Ob-Rb on the expression of *MMP-1*, *SREBP-1c*, and *PPARγ* genes, Ob-Rb was knocked down by siRNA transfection in LX-2 cells. The ObR siRNA was effective in suppressing HCVcp-stimulated Ob-Rb mRNA expression, as compared with non-specific control siRNA. HCVcp-suppressed mRNA expression of *MMP-1*, *SREBP-1c*, and *PPARγ* genes was reversed by *Ob-Rb* knockdown (Fig. 5), suggesting that HCVcp modulated expression of *MMP-1*, *SREBP-1c*, and *PPARγ* genes via Ob-Rb.

EFFECTS OF HCVcp ON mRNA EXPRESSION OF *LEPTIN* AND *IL-6* GENES IN LX-2 CELLS

Leptin, which is produced by *ob* gene, has been shown as a specific agonist for the ObR–JAK2–STAT3 signaling pathway.

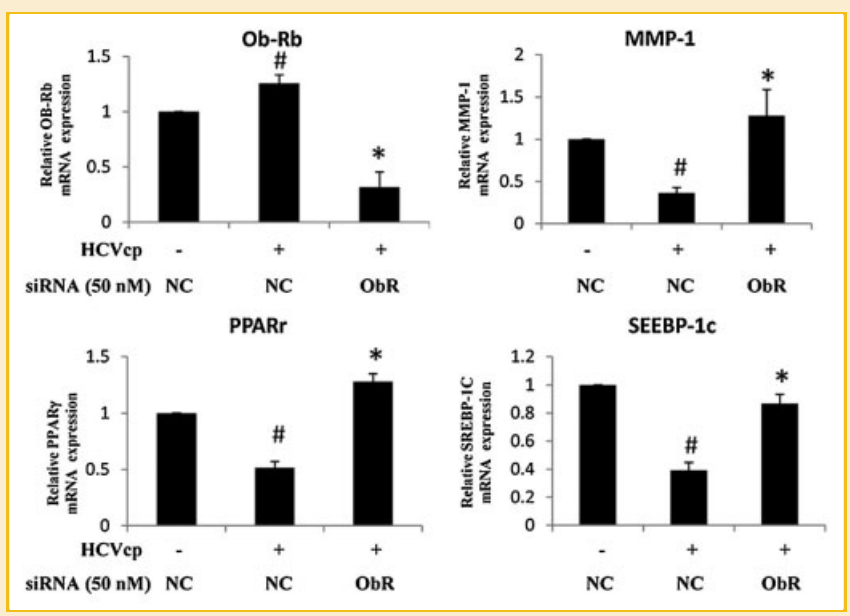

Fig. 5. Knockdown of *Ob-Rb* gene on HCVcp-induced gene expression in LX-2 cells. LX-2 cells were transfected with non-specific control (NC) and ObR siRNA, respectively. After 24 h of transfection, cells were treated with HCVcp for 18 h. The mRNA levels of *Ob-Rb*, *MMP-1*, *PPARγ*, and *SREBP-1c* were determined by real-time PCR. #, $P < 0.05$ compared with cells with NC siRNA and without HCVcp treatment; *, $P < 0.05$ compared with cells with HCVcp treatment.

IL-6 activates JAK2–STAT3 signaling and is reportedly increased in response to HCVcp in hepatocytes [Ni et al., 2004; Machida et al., 2010]. Both cytokines are shown to be up-regulated in hepatic fibrosis. To investigate whether the effects of HCVcp on JAK2–STAT3 signaling involved IL-6 and leptin, we examined the effects of HCVcp on mRNA expression of *IL-6* and *leptin* genes in a time-course study. The results showed that HCVcp significantly stimulated *leptin* and *IL-6* mRNA expression at the incubation time of 18 h, but not earlier (Fig. 6).

DISCUSSION

Ob-Rb, which is the functional form of leptin receptor, is present in various organs including the liver. It is widely known that Ob-Rb mediates most of the biological actions of leptin via stimulation of JAK2–STAT3 and AMPK pathways [Marra and Bertolani, 2009]. According to the previous reports, HCVcp has been shown to induce inflammation responses in monocytes and fibrogenesis in HSCs by interacting with TLR2, indicating that TLR2 is the main target recognized by HCVcp [Dolganiuc et al., 2004; Coenen et al., 2011]. In the present study, we showed that HCVcp-induced fibrogenesis involving the obese long form receptor Ob-Rb on HSCs. HCVcp stimulated JAK2–STAT3, AMPKα, and AKT phosphorylation via Ob-Rb and suppressed transcription of *MMP-1*, *PPARγ*, and *SREBP-1c* genes (Fig. 7). To our knowledge, the present study is the first report describing the involvement the obese receptor in HSC activation by HCVcp.

Ob-Rb and downstream AMPKα and AKT signaling pathways play important roles in energy homeostasis [Gorska et al., 2010].

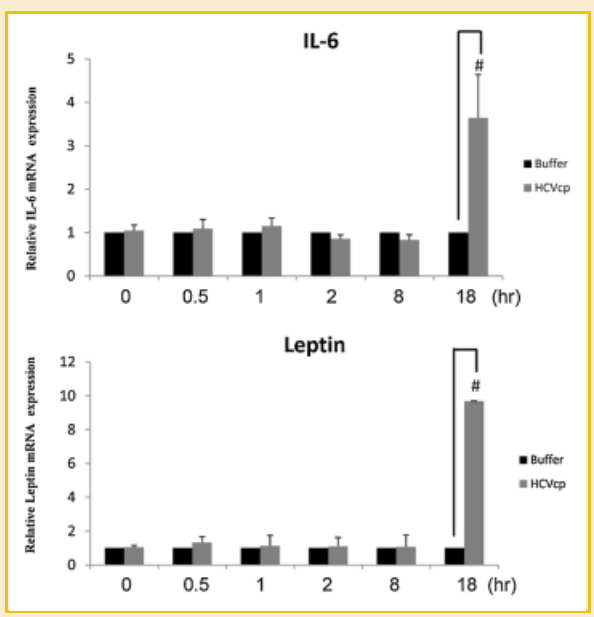

Fig. 6. Effects of HCVcp on *IL-6* and *leptin* gene expression in LX-2 cells. Cells were treated with HCVcp at the indicated time (0, 0.5, 1, 2, 8, and 18 h). The mRNA levels of *IL-6* and *leptin* were detected by real-time PCR. #, $P < 0.05$ compared with untreated cells at the same indicated time.

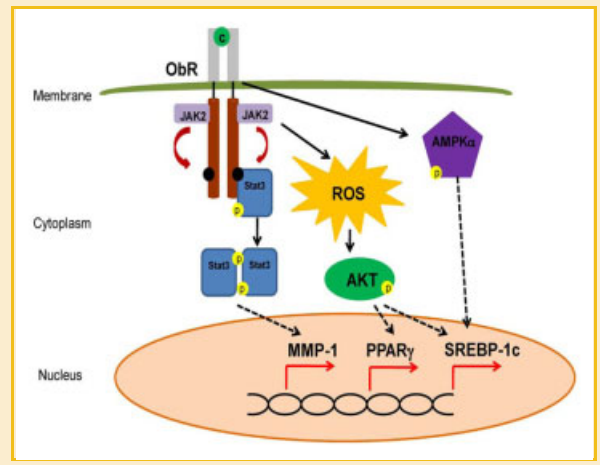

Fig. 7. Schematic model of HCVcp-induced Ob-Rb-mediated ROS formation, JAK2/STAT3 and AMPKα signaling in hepatic fibrogenesis. Here we demonstrated that binding of HCVcp to Ob-Rb initiated JAK2–STAT3 and AMPKα signaling, which led to suppression of MMP-1 and SREBP-1c, respectively. JAK2-mediated ROS formation stimulated AKT phosphorylation and downstream PPARγ and SREBP-1c mRNA suppression. (--|, inhibition; →, stimulation)

HSC activation is characterized by loss of intracellular lipid droplets, and transcriptional factors such as SREBP-1c and PPARγ are proposed as regulators in lipogenesis [Tsukamoto et al., 2006; Friedman, 2008]. Our results show that suppression of *SREBP-1c* and *PPARγ* gene transcription by HCVcp was reversed by blocking Ob-Rb and modulated by AMPKα and AKT in HSCs. It suggests that HCVcp regulated SREBP-1c and PPARγ function via Ob-Rb, leading to HSC activation. In addition, HCVcp decreased the degradation of ECM by suppressing *MMP-1* mRNA expression via Ob-Rb–JAK2–STAT3 pathways, suggesting the involvement of the leptin receptor in the profibrogenic action of HCVcp, as Ob-Rb blockade by using neutralizing anti-Ob-Rb antibody and siRNA for knockdown of *Ob-Rb* gene would negate HCVcp effects (Fig. 3). However, the mechanisms about whether and how HCVcp directly binds to Ob-Rb still need to be investigated.

In Figure 6, IL-6 and leptin were not obviously stimulated by HCVcp prior to 18 h of stimulation. Whereas, our results show that phosphorylation of JAK2 and STAT3 was stimulated by HCVcp within 30 min and 1 h, peaking at 1 and 2 h, respectively (Fig. 2A and B). Taken together, our results suggest that HCVcp initiated JAK2–STAT3 signaling with involvement of IL-6 and leptin only at later stages of signaling loops.

As shown in Figure 1, HCVcp-induced mRNA expression of *α-SMA*, *procollagen α2(I)*, and *TGF-β1* genes, leading to activation and fibrogenesis of HSCs. In addition, HCVcp-induced ROS production was mediated by JAK2 (Fig. 1). Nevertheless, whether JAK2 modulates HCVcp-induced mRNA expression of *α-SMA*, *procollagen α2(I)*, and *TGF-β1* genes still remains to be examined. HCVcp-stimulated leptin might play a role in the induction of *α-SMA*, *procollagen α2(I)*, and *TGF-β1* genes, as shown by a previous study implicating fibrogenic action of leptin in HSCs [Wang et al., 2009].

MMP-2 and MCP-1 are known as chemoattractants for HSCs and migration of activated HSCs is an important step in development of liver fibrosis [Benyon et al., 1996; Frühbeck, 2006; Friedman, 2008]. In our results, HCVcp did not induce *MMP-2* or *MCP-1* mRNA expression in LX-2 cells (Fig. 4C), implying that HCVcp activation of HSCs might be independent of cell migration.

AMPK is considered pivotal both as a sensor in cellular energy dynamics and as a regulator of systemic metabolic homeostasis [Viollet et al., 2009]. To date, the role of AMPK phosphorylation in HSC activation is still controversial. Up-regulation of AMPK has been shown to inhibit proliferation of HSCs [Adachi and Brenner, 2008]. Leptin has been reported to up- or down-regulate AMPK signaling by different groups [Handy et al., 2010; Tang and Chen, 2010]. Hence, the role of AMPK signaling on HSC activation needs further investigation.

Although the HCVcp concentrations we used (20–200 ng/ml) in the experiments were higher than plasma concentrations in the infected patients, it is possible that the in situ concentrations of HCVcp in the liver are much higher than those in the peripheral circulation.

In conclusion, our study for the first time demonstrated a novel role of HCVcp on HSC activation by promoting oxidative stress, Ob-Rb-dependent AMPKα, AKT, and JAK2–STAT3 signaling, contributing to hepatic fibrogenesis. These findings provide additional evidence for HCVcp-induced hepatic fibrogenesis.

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