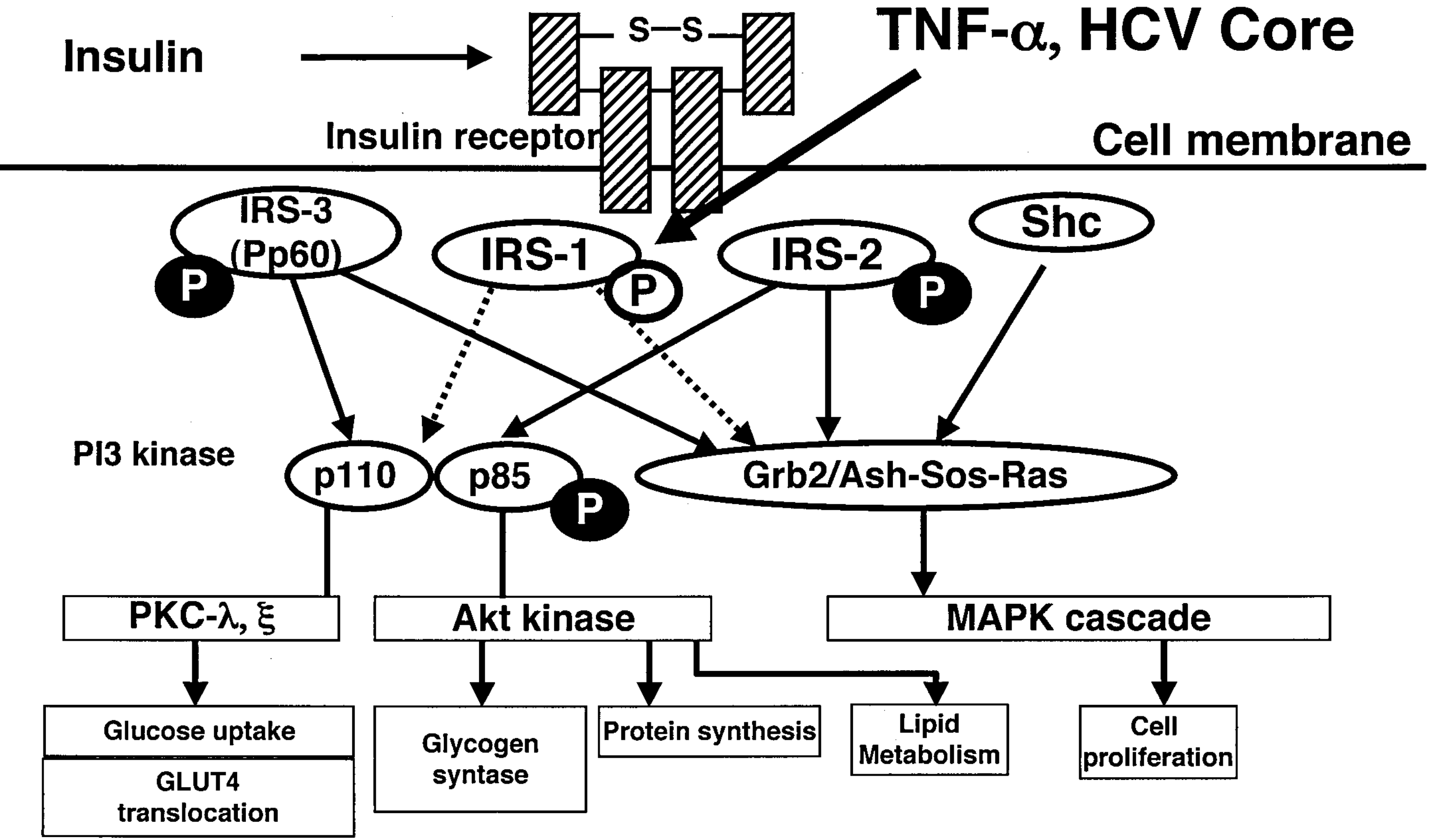
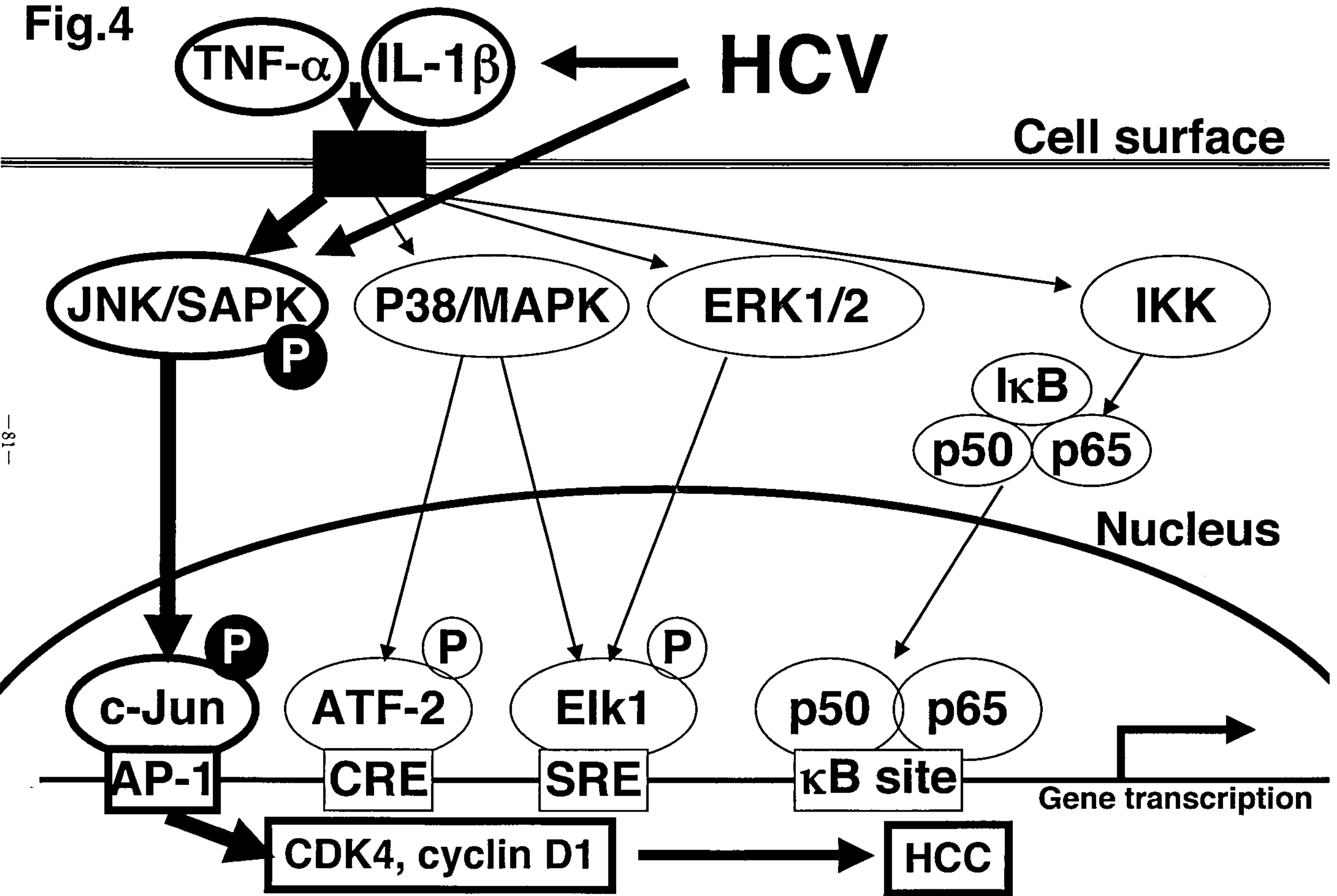


Fig. 3
Insulin
S—S
TNF-α, HCV Core
Insulin receptor
Cell membrane
IRS-3
(Pp60)
P
IRS-1
P
IRS-2
P
Shc
PI3 kinase
p110
p85
P
Grb2/Ash-Sos-Ras
PKC-λ, ξ
Akt kinase
MAPK cascade
Glucose uptake
GLUT4
translocation
Glycogen
syntase
Protein synthesis
Lipid
Metabolism
Cell
proliferation

Fig.4



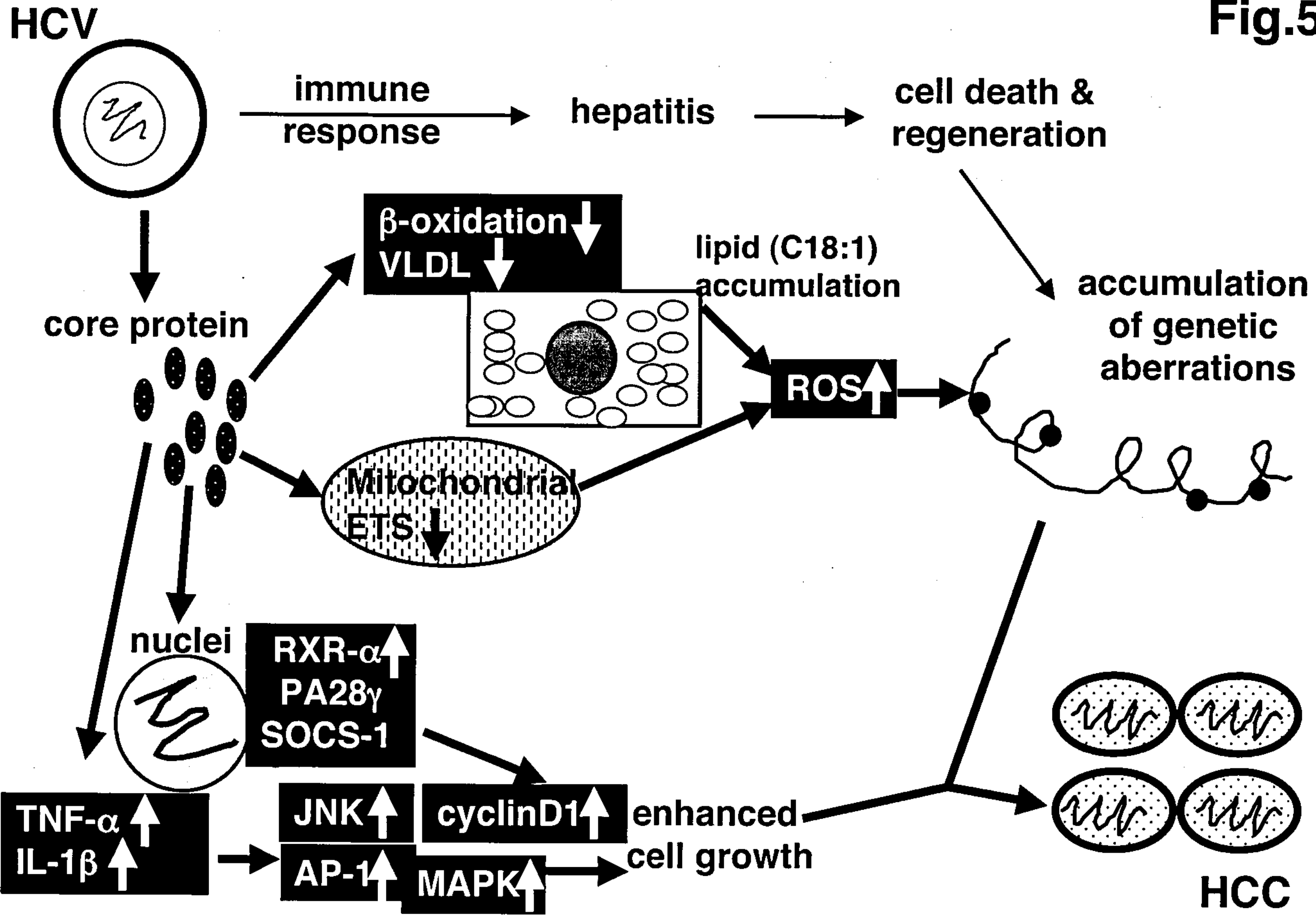
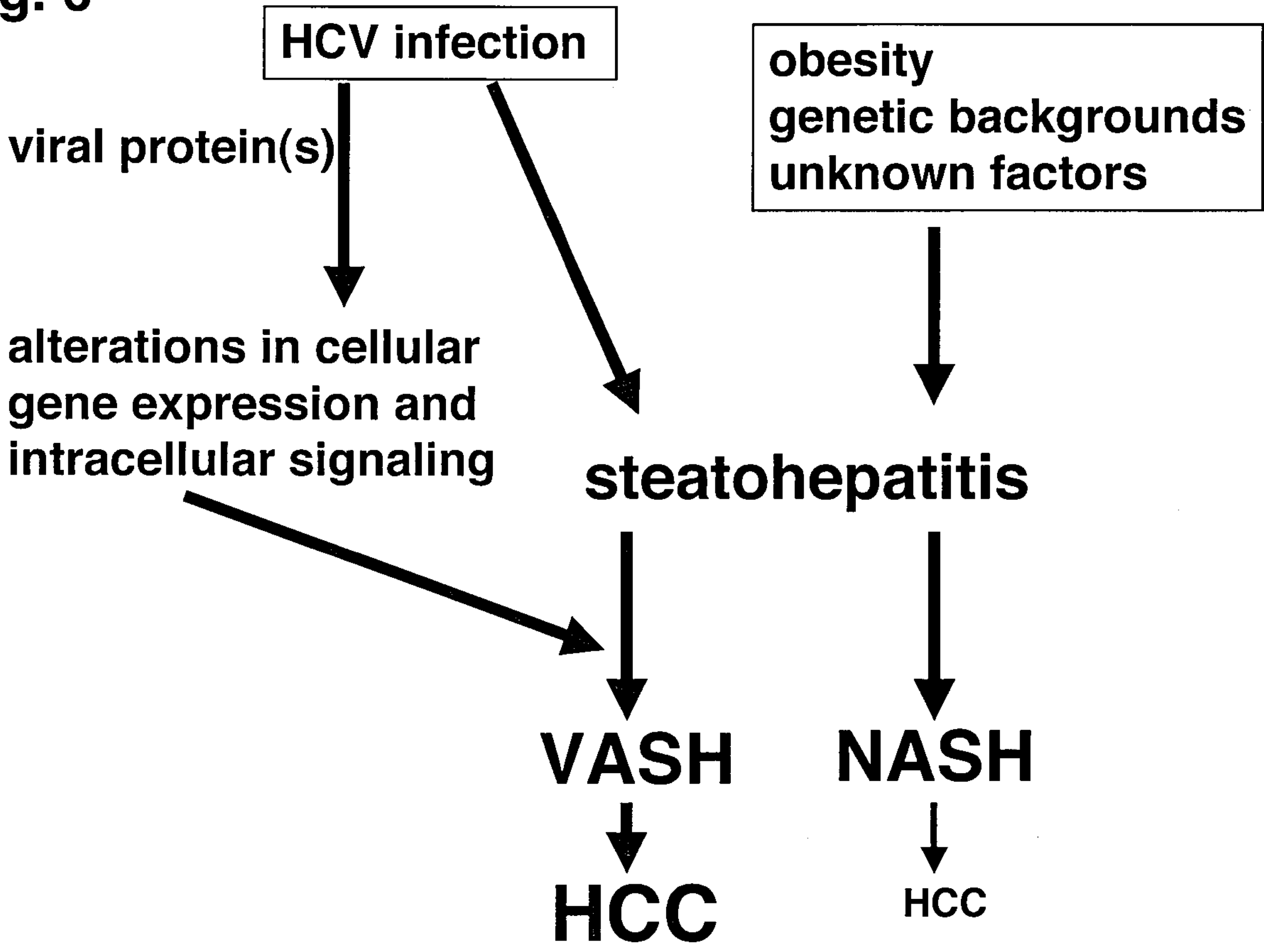


Fig.5
HCV
immune response
hepatitis
cell death & regeneration
core protein
β-oxidation
VLDL
lipid (C18:1) accumulation
accumulation of genetic aberrations
ROS
Mitochondrial ETS
nuclei
RXR-α
PA28γ
SOCS-1
TNF-α
IL-1β
JNK
AP-1
MAPK
cyclinD1
enhanced cell growth
HCC

Fig. 6



Hepatitis C Virus Core Protein Exerts an Inhibitory Effect on Suppressor of Cytokine Signaling (SOCS)-1 Gene Expression

(Running Title: Suppression of SOCS-1 by HCV Core)

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(2,815 words in the text)

ABSTRACT

Background/Aims. Suppressor of cytokine signaling (SOCS)-1, a negative feedback regulator of cytokine signaling pathway, also has a tumor suppressor activity, the silencing of its gene by hypermethylation is suggested to contribute to hepatocarcinogenesis. We studied the effect of the core protein of hepatitis C virus (HCV) on the expression of *SOCS-1* gene.

Methods. HCV core gene transgenic mice, which develop hepatocellular carcinoma late in life, HepG2 cells expressing the core protein, and human liver tissues were analyzed.

Results. The expression of *SOCS-1* gene was significantly suppressed in the liver of core gene transgenic mice and HepG2 cells expressing the core protein, while that of *SOCS-3* gene was conserved. *SOCS-1* expression levels also decreased in HCV-positive human liver tissues. The core protein differentially down-regulated the expression of signal transducer and activator of transcription (STAT) target genes, but rather enhanced STAT1 and STAT3 activation after interleukin-6 stimulation in mouse liver tissues and cells.

Conclusions. HCV core protein down-regulates the expression of *SOCS-1* gene. This is a mechanism leading to *SOCS-1* silencing, an alternative to the hypermethylation of the gene; this effect of the core protein may modulate the intracellular signaling pathway, contributing to the pathogenesis in HCV infection including hepatocarcinogenesis. (199 words)

Key Words: tumor suppressor gene, hepatocellular carcinoma, transgenic mouse, STAT3

INTRODUCTION

Hepatitis C virus (HCV) infection is a major cause of chronic hepatitis. A substantial proportion of patients with chronic hepatitis C eventually develop hepatocellular carcinoma (HCC), which is one of the leading causes of death worldwide [1,2]. Despite the absence of appropriate *in vitro* replication systems or practical infectious animal model systems, the mechanism underlying hepatocarcinogenesis in human HCV infection is gradually clarified. Both the direct and indirect effects of HCV on HCC development are demonstrated [3-6]. The accumulation of gene aberrations, such as the inactivation of tumor suppressor genes or the activation of oncogenes, which are induced through the inflammation-mediated continuous death of hepatocytes followed by regeneration, may be one of the mechanisms underlying hepatocarcinogenesis [3]. On the other hand, the viral gene products are suggested to contribute to the development of HCC by their direct effects on hepatocytes [4]. Such direct effects have been demonstrated by the use of model systems including mice [7-9]. HCV-infected hepatocytes produce viral structural and nonstructural proteins. Some of these confer certain phenotypes to hepatocytes and may be associated with the pathogenesis of HCV infection including the development of HCC. Among such viral proteins, the core protein of HCV has a variety of biological activities, including oncogenic activity, which substantially affects host cellular functions [7-11].

Suppressor of cytokine signaling (SOCS)-1, also called signal transducer and activator of transcription (STAT)-induced STAT inhibitor-1 or Jak binding protein-1, is a negative feedback regulator of cytokine signaling through the Jak/STAT pathway.

SOCS-1 contains the SH2 domain and directly interacts with the kinase domain of Jak to suppress Jak activity. *SOCS-1* gene expression is augmented by various cytokines, such as interferon (IFN)-γ, interleukin (IL)-6 or leukemia inhibitory factor (LIF), resulting in the suppression of the signal transduction downstream pathways of these cytokines [12-14]. Moreover, SOCS-1 has been recently shown to exhibit a tumor suppressor activity. SOCS-1 suppresses the expression of several oncogenes or growth-related genes acting as a negative regulator of cell proliferation: the loss of function of SOCS-1 facilitates tumor progression [15-17]. As a mechanism underlying the loss of function of SOCS-1, a recent study has revealed a frequent silencing of the *SOCS-1* gene by CpG methylation in HCC tissues [18-20]. Alternatively, however, it may be possible that HCV infection, particularly, the proteins that the HCV genome encodes *per se*, may render the *SOCS-1* gene unable to exhibit its function by gene silencing.

We examined such a possibility using a mouse model for HCV infection that is destined to develop HCC [7,9], as well as cultured cells expressing the HCV core protein [21]. The core protein markedly suppressed the expression of the *SOCS-1* gene in both liver tissues and cultured cells. This silencing of the *SOCS-1* gene may be one of explanations for the pathogenicity of HCV in humans.

MATERIALS AND METHODS

Transgenic Mouse and Cell Lines. HCV core gene transgenic mice have been described previously [7]. These mice develop HCC late in life [7, 9]. The mice were cared for according to the institutional guidelines and maintained in a specific pathogen-free state. All the animals received humane care and the study protocol complied with the institution's guidelines for the care and use of experimental animals. HepG2 cell lines expressing the HCV core protein under the control of CAG promoter (Hep39J, Hep396 and Hep397) or a control HepG2 line (Hepswx) carrying the empty vector were described previously [21, 22].

IL-6 Stimulation. For the *in vivo* experiments, 0.05~0.5 μg/g BW murine IL-6 (Diaclone, Besançon, France) was administered into 8 w.o. male mice i.p., and liver tissues were obtained 60 min later. Cultured cells were treated with human IL-6 (Diaclone) at 10~100 ng/ml or IFN-α at 1.0~10.0 ng/ml and then were harvested 60 min later.

Reverse Transcription (RT)-PCR Analysis. Total RNA was extracted from liver tissues or cultured cells before and after the treatment with IL-6 using TRIzol (Invitrogen). RNA was reverse-transcribed using oligo(dT) primers and Superscript II (Invitrogen). Equal amounts of cDNA were then subjected to PCR. The primer pairs used were:

5'-CACTCACTTCCGCACCTTCC-3' (forward) and

5'-TCCAGCAGCTCGAAAAGGCA-3' (reverse) for murine *SOCS-1*,

5'-CACGCACTTCCGCACATTCC-3' (forward) and

5' -TCCAGCAGCTCGAAGAGGCA-3' (reverse) for human *SOCS-1*,

5'-TCACCCACAGCAAGTTTCCCGC-3' (forward) and

5'-GTTGACAGTCTTCCGACAAAGATGC-3' (reverse) for murine *SOCS-3*,

5'- CACGCACTTCCGCACATTCC-3' (forward), and

5'-GTTGACGGTCTTCCGACAGAGATGC-3' (reverse) for human *SOCS-3*.

For the RT-PCR analysis, the quantity of cDNA template and the number of amplification cycles were optimized to ensure that the reaction was terminated during the linear phase of product amplification, so that the semiquantitative comparison of mRNA abundance between different samples was possible [23]. The intensities of the bands were determined using a densitometer. RT-PCR was also done using GAPDH primers to adjust the amounts of RNA in each experiment.

Human Liver Tissue Samples and Real-Time PCR. Nine patients with HCC who had underlying chronic hepatitis C were studied for *SOCS-1* expression in noncancerous tissues. Additional nine patients, who were found to be negative for both HBsAg and anti-HCV at the time of operation, were also studied. The latter patients underwent liver resection for metastatic liver tumors from colon cancer. The experimental protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in *a priori* approval by the Ethics Review Committee for Human Experimentation. Informed consent was obtained from each patient. The noncancerous liver tissues obtained from these patients were immediately frozen and stored at -80°C until further use.

Taqman real-time RT-PCR was performed as described previously [24], using an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA). Primers and the TaqMan probe for *SOCS-1* were as follows:

Forward primer: 5'-CTGGCCCCGGAGCAT-3'

Reverse primer: 5'-GTTGTGTGCTACCATCCTACAGA-3'

Probe: 5'-FAM-CCGGACGCTATGGCCCA-MGB-3'

Primers and probes for *SOCS-3*, *β-actin*, interferon regulatory factor (*IRF*)-1, c-*myc* and *bcl*-X_L genes were purchased from ABI by Assays-on-Demand system.

Methylation Status. The methylation status of the *SOCS-1* gene was analyzed by methylation-specific PCR as described previously [20].

Western Blotting and Immunoprecipitation. Nuclear and cytoplasmic fractions were prepared from HepG2 cells, and Western blotting was performed as described previously [25]. Anti-STAT1 and anti-STAT3 polyclonal antibodies (Cell Signaling Technology, Inc., Beverly, MA), anti-phosphorylated STAT3 (Tyr705) polyclonal antibody (Cell Signaling Technology), anti-phosphorylated STAT3 monoclonal antibody (Upstate Biotechnology Inc., Lake Placid, NY), and anti-protein inhibitor of activated STAT (PIAS)1, anti-PIAS3 and anti-SOCS-1 antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used. Immunoprecipitation was done as described previously using antibodies followed by protein A-Sepharose [26].

Immunocytofluorescence. HepG2 cell lines with or without the core gene were grown overnight on chamber slides and treated with 10 ng/ml human IL-6 for 60 min. Cells were fixed with 4% paraformaldehyde plus methanol, and reacted with the anti-STAT3 antibody followed by incubation with a FITC-labeled secondary antibody.

Statistical Analysis. The results are expressed as means ± S.D. The significance of the difference in means was determined by Mann-Whitney's *U*-test. $P<0.05$ was considered significant.

RESULTS

HCV Core Protein Suppresses SOCS-1 Gene Expression. To examine the impact of the core protein on *SOCS-1* gene expression, we analyzed mRNA expression levels by semi-quantitative RT-PCR in liver tissues from the HCV core gene transgenic and nontransgenic control mice. *SOCS-1* mRNA expression levels in mouse liver tissues of nontransgenic mice were increased in a dose-dependent manner of IL-6, but were only marginal in the liver tissues from the core gene transgenic mice even in those treated with the maximal dose of IL-6 (0.5 μg/g BW, n=5 each) (Fig. 1A & C). In contrast, the expression levels of *SOCS-3* mRNA in the core gene transgenic mice were comparable to or rather higher than those in nontransgenic mice, before and after stimulation with IL-6 (Fig. 1A & E) [27, 28].

We then examined whether or not this observation in mice is reproducible in HepG2 cell lines that constitutively express the core protein. *SOCS-1* gene expression was suppressed in the core-expressing HepG2 cell lines Hep396, Hep397 and Hep39J, even after stimulation with IL-6, while control bulk HepG2 cells or a control Hepswx cell line expressed *SOCS-1* mRNA at high levels (Fig. 1B & D). In contrast, the levels of *SOCS-3* gene expression were similar among the core-expressing HepG2 cell lines and control HepG2 cells, and were augmented by stimulation with IL-6 (Fig. 1B & F). These observations indicate that the core protein selectively suppresses *SOCS-1* gene expression before the translational level. The SOCS-1 protein was not detectable by Western blotting either in the mouse liver or HepG2 cells using currently available anti-SOCS-1antibodies.

These results, obtained in HepG2 cell lines constitutively expressing the core

protein, were then evaluated using a transient expression system. In this system, HepG2 cells were infected with baculovirus expressing the core protein as described previously [29], and *SOCS-1* expression was determined by semiquantitative RT-PCR. The introduction of the core protein selectively suppressed the expression level of *SOCS-1* mRNA even after stimulation with IL-6 (data not shown).

Modulation of expression by the core protein of STAT-target genes other than *SOCS*s was then examined by determining the mRNA levels in mouse liver tissues. Expression of *IRF-1* gene was suppressed in the presence of the core protein under the stimulation with IL-6, while that of c-*myc* was not affected (Fig. 2A & B). The expression of *bcl*-X_L gene was rather augmented by the core protein although the difference was not statistically significant (Fig. 2C).

The methylation status of *SOCS-1* gene was then explored in liver tissues from the core gene transgenic mice by a method described previously [20], to determine whether or not the *SOCS-1* gene expression may be suppressed by hypermethylation. No hypermethylation was observed in the *SOCS-1* gene of the core gene transgenic mice either at the 5'-noncoding region or the CpG island in the coding region (Fig. 3).

In the analysis of *SOCS-1* expression in noncancerous liver tissues from patients with HCV infection, the *SOCS-1* mRNA expression levels were 0.494 ± 0.352 in HCV-positive patients (n=9) and 0.862 ± 0.465 in the control subjects without HCV infection (n=9) (in arbitrary units, p=0.0345). Thus, the SOCS-1 levels in the liver tissues of chronic hepatitis C patients were significantly lower than those of subjects without HCV infection.

The Core Protein Did Not Suppress Phosphorylation of STAT3 or STAT1. The

activation of STAT3 enhances *SOCS-1* expression, thereby forming a negative feedback loop to the STAT3 status [17]. To determine whether or not STAT3 activation is involved in the *SOCS-1* gene suppression in this system, the tyrosine phosphorylation of STAT3 in the mouse liver was examined by Western blotting using an anti-phospho-STAT3 (tyrosine $(Tyr)^{705}$) antibody. At baseline, Tyr^{705} phosphorylation of STAT3 was low in both the core gene transgenic and nontransgenic mice. However, in response to stimulation with IL-6, the levels of Tyr^{705} phosphorylation of STAT3 was higher in the liver tissues from the core gene transgenic mice than that from nontransgenic mice. A representative result is shown in Fig. 4A. Similarly, the levels of Tyr^{705} phosphorylation of STAT3 were higher in HepG2 cell lines expressing the core protein than those in control cells (Fig. 4B). These results observed in HepG2 cells constitutively expressing the core protein were also evaluated in a transient expression system using a recombinant baculovirus, as described above. The Tyr^{705} phosphorylation of STAT3 was enhanced in HepG2 cells infected with baculovirus expressing the core protein compared with mock-infected HepG2 cells (data not shown). The activation of STAT1 was also analyzed using HepG2 cell lines. As shown in Fig. 4C, the levels of STAT1 phosphorylation was higher in HepG2 cells expressing the core protein than in control cells similar to the result on STAT3.

Subcellular Localizations of STAT3 and STAT1. STAT activation by tyrosine phosphorylation results in the migration of STAT from the cytoplasm to the nucleus to bind to genomic DNA, modulating of cellular gene expression. We thereby evaluated the subcellular localization of STAT3 and STAT1 by preparing cytoplasmic and nuclear fractions from HepG2 cells followed by Western blotting. The amounts of

STAT3 in the nuclei of core-expressing HepG2 cells were similar to or slightly larger than those in control HepG2 cells in the presence or absence of IL-6 (Fig. 5A). This result was confirmed by an immunofluorescence study (Fig. 5B). A similar result was obtained in the analysis of STAT1 subcellular localization (Fig. 5C). These observations indicate that the HCV core protein does not inhibit the translocation of STAT3 or STAT1 to the nucleus, and the feedback mechanism is not the cause of *SOCS-1* gene suppression.

Because PIAS3 blocks the nuclear translocation of STAT3 or binding of STAT3 to genomic DNA [30], the expression of PIAS3 was examined by Western blotting. However, there was no significant difference in the levels of PIAS3 between core-expressing HepG2 cells and control HepG2 cells (data not shown). Co-immunoprecipitation analysis was also performed using HepG2 cell lines to know whether or not the core protein affects the association of PIAS1 with STAT1 or PIAS3 with STAT3. However, neither co-immunoprecipitation of STAT1 with anti-PIAS1 antibody nor that of STAT3 with anti-PIAS3 antibody was affected by the presence of the core protein (Fig. 6). We also examined the possibility of the interaction of the core protein with STAT3, which blocks the binding of STAT3 to the promoter of *SOCS-1* gene. For this purpose, a co-immunoprecipitation technique was utilized with whole-cell extracts of core-expressing HepG2 cells. However, no association was observed between these two proteins.

DISCUSSION

In the current study, we demonstrated that the core protein of HCV suppresses the expression of *SOCS-1* mRNA in the liver tissues of mice that develop HCC late in their life [4, 7]. This observation was reproduced in cultured cells that expressed the core protein. This phenomenon may contribute to the modification of the IFN signaling systems in HCV infection, because SOCS-1 and SOCS-3 play central roles in the Jak/STAT pathway as negative feedback regulators [12-14]. In addition, since SOCS-1 also possesses a tumor suppressor activity [15-17], the down-regulation of *SOCS-1* may contribute to hepatocarcinogenesis in HCV infection. It has been reported that the silencing of the *SOCS-1* gene by hypermethylation is associated with the development of HCC [18-20]. Among patients with HCV infection, a major cause of chronic hepatitis worldwide, HCC develops at a very high incidence [1, 2]. Hence, there may be an alternative mechanism of *SOCS-1* silencing to gene methylation in HCV infection. Our current data suggest a possibility of such a mechanism in that HCV *per se* acts as a negative regulator of SOCS-1, a tumor suppressor. The expression levels of *SOCS-1* mRNA in noncancerous liver tissues in chronic hepatitis C patients were also significantly lower than those in HCV-negative subjects, although the "shut-off" of the *SOCS-1* gene observed in the experimental systems was not the case. This may be due to the presence of other factors influencing *SOCS-1* gene expression *in vivo*, including inflammation.

In the exploration of the mechanism underlying the down-regulation of *SOCS-1* expression, we first examined the methylation status of the *SOCS-1* gene in liver tissues from core gene transgenic mice by methylation-specific PCR. Neither the

5'-non-coding region nor the CpG island in the coding region of the *SOCS-1* gene [18-20] was hypermethylated, refuting methylation as a mechanism of SOCS-1 suppression.

We next determined whether or not STAT3, a transcription factor for the *SOCS-1* gene, is involved in the suppression of *SOCS-1* by the core protein: a decreased level or a disturbed phosphorylation of STAT3 may account for the suppression of *SOCS-1*. It was found, however, that STAT3 was rather activated by the core protein, consistent with a previous report [28]. The effect on STAT3 activation by the core protein is yet controversial [31]. Similarly, the activation and nuclear translocation of STAT1 was not disturbed by the presence of the core protein. The core protein differentially affected the expression of STAT-target genes such as *IRF1*, c-*myc* or *bcl*-X_L. The core protein suppressed *IRF1* expression in the mouse liver but did not those of c-*myc* and *bcl*-X_L genes. Regulation of *IRF1* expression is STAT1-dependent in general, although STAT3 is also involved when stimulated by IL-6 [32]. c-*myc* and *bcl*-X_L inductions by IL-6 are chiefly mediated by STAT3 [33]. Thus, the modulation of expression by the core protein may occur in some other STAT-target genes, suggesting somewhere in Jak/STAT signaling pathway including STAT1 activation is impaired by the core protein. However, no defect was identified in the activation and nuclear translocation of STAT1 and STAT3 in the current study. Thus, although we could not define the precise role of the core protein in *SOCS-1* gene suppression, the direct effect of the core protein on the transcription of the gene is the most likely.

In summary, we found that the HCV core protein selectively suppresses *SOCS-1* gene expression in the liver tissues of animals and cultured cells. These

findings may provide a basis for an alternative mechanism of the switch-off of SOCS-1 in the pathogenesis of HCV infection by modulating a tumor suppressor activity or responses to IFNs.

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