tive real-time polymerase chain reaction (PCR) was performed on an iQ5 Real-Time PCR detection system (Bio-Rad Laboratories). Standard curves using four 10-fold dilutions (1×, 0.1×, 0.01×, 0.001×) were produced to ensure that the amplification efficiencies were similar and in the range of 95% to 105%. The messenger RNA (mRNA) level of each target gene was normalized to the relative amount of the housekeeping gene TBP. The comparative threshold cycles ($C_T$) method, $2^{-\Delta CT}$, was used to calculate the changes in gene expression for each target gene.

***Statistical Analysis.*** Each experiment was performed at least three times. Representative data are shown and are expressed as the mean ± standard deviation. Depending on datasets, statistical analysis was performed using a *t* test, Mann-Whitney test, or analysis of variance. *P* values of $<0.05$ were considered significant.

## Results

***OSM Increases Expression of Functional HIF1α Under Normoxic Conditions.*** Exposure of HepG2 hepatoma cells to OSM for different times led to a profound and transient increase in HIF1α protein levels that lasted up to 24 hours. The up-regulation reached a maximum after about 6 hours of stimulation. As expected, OSM induced STAT3, Erk1/2, and p38 phosphorylation (Fig. 1A). HIF1α was also induced upon stimulation of the human hepatocyte cell line PH5CH8 with OSM (Fig. 1B). Quantitation of western blots showed that upon OSM stimulation, the HIF1α protein is up-regulated by factors of 2.66 ± 0.3 in PH5CH8 cells and 2.7 ± 0.9 in HepG2 cells.

To investigate whether OSM-induced HIF1α is functional, we tested the effect of OSM on HepG2 cells transfected with a HIF1-responsive luciferase reporter gene construct. OSM treatment increased luciferase activity three-fold (Fig. 1C). Thus, OSM-induced HIF1α is transcriptionally active even under normoxic conditions.

***OSM Augments Hypoxia-Dependent HIF1α Induction and Hypoxia-Mediated Target Gene Expression.*** Next, we compared the induction of HIF1α by OSM under normoxia with that induced by hypoxia. In addition, we examined whether OSM may affect the hypoxia-dependent induction of HIF1α. We found that HIF1α levels induced by OSM under normoxia were slightly lower than those induced by hypoxia after 4 and 6 hours of induction, respectively. Interestingly, when cells were treated with OSM under hypoxia, the increase in HIF1α protein levels was higher than under each treatment alone (Fig. 2A). Quantitation of western blots showed that the HIF1α protein is up-regulated by factors of 3.6 ± 0.19 upon hypoxia, 2.7 ± 0.9 upon OSM, and 6.4 ± 1.8 upon combined treatment with hypoxia and OSM.

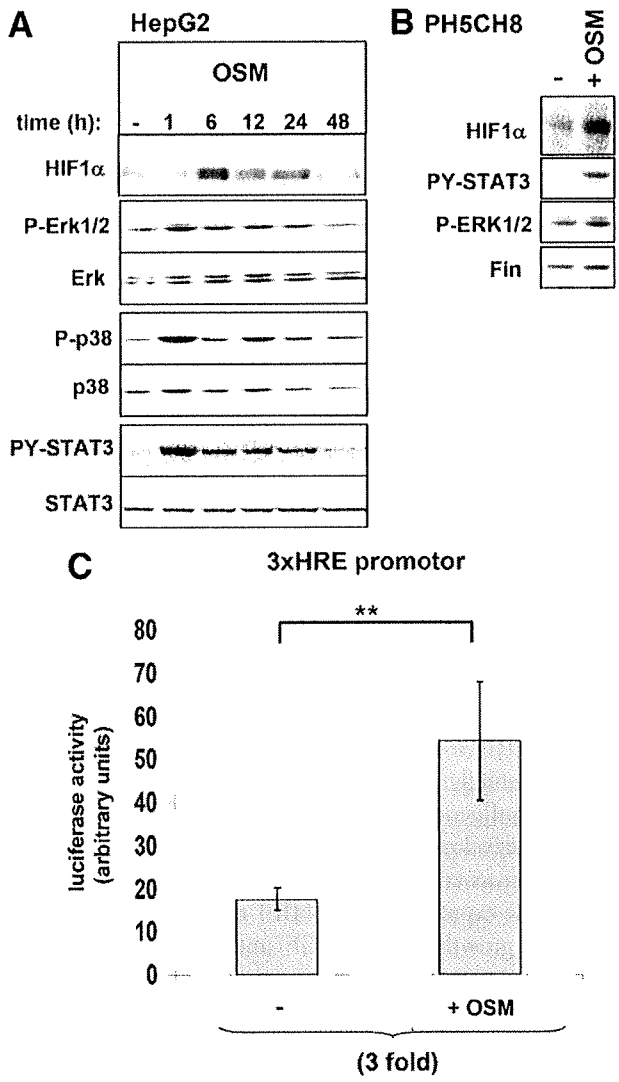


Fig. 1. OSM induces HIF1α protein levels over an extended period, and the induced HIF1α is transcriptionally active. (A) HepG2 cells were treated for the indicated periods with OSM (10 ng/mL). Lysates of the cells were separated via SDS-PAGE, and western blots of the membranes were detected with HIF1α, phospho-STAT3, phospho-Erk1/2, phospho-p38, STAT3, Erk1/2, and p38 antibodies. (B) PH5CH8 cells were stimulated for 4 hours with OSM (10 ng/mL) or left untreated. Western blots of the membranes were detected as described in (A). (C) HepG2 cells were transfected with the luciferase reporter gene plasmids pGL3-EPO-HRE-Luc and the β-galactosidase expression vector pCH110. Twenty-four hours after transfection, the medium was exchanged and the cells were treated for an additional 16 hours with OSM (10 ng/mL) before lysates were prepared, and the reporter gene activity was measured as described in Experimental Procedures. $^{**}P < 0.01$.

We then investigated HIF1α mRNA expression under the same conditions and found that OSM induces HIF1α mRNA levels stronger (3.4-fold) than hypoxia (2.3-fold). Combined treatment with hypoxia and OSM led to an

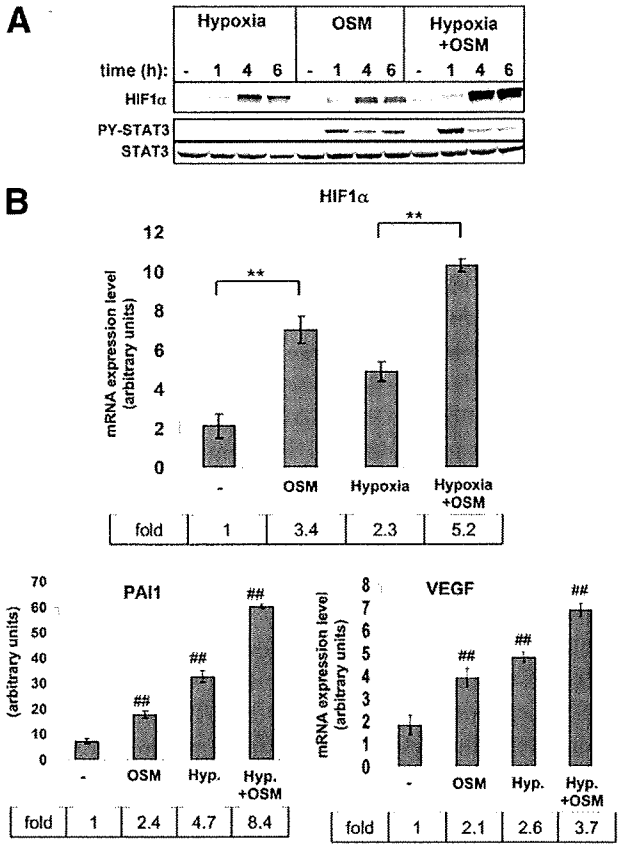


Fig. 2. OSM enhances hypoxia-induced HIF1α, PAI1, and VEGF expression. (A) HepG2 cells were treated for the indicated periods with hypoxic conditions and/or OSM (10 ng/mL). Lysates of the cells were separated via SDS-PAGE, and western blots of the membranes were detected with antibodies directed against HIF1α, phospho-STAT3, and STAT3. (B) HepG2 cells were stimulated for 4 hours with hypoxic conditions and/or OSM (10 ng/mL). RNA was prepared, and HIF1α mRNA levels were analyzed via quantitative PCR. $^{**}P < 0.01$. (C) HepG2 cells were treated as described in (B) and VEGF and PAI1 mRNA levels were analyzed via quantitative PCR. $^{\#\#}P < 0.01$ versus untreated controls.

even higher induction of HIF1α mRNA (5.2-fold) (Fig. 2B). Thus OSM significantly increases HIF1α mRNA under normoxia and hypoxia, which matches with the protein up-regulation seen in the western blots (Fig. 2A). Two important target genes of the hypoxic response, PAI1 and VEGF, were also significantly up-regulated by OSM, and a combined treatment with hypoxia and OSM led to an even stronger induction of PAI1 and VEGF mRNA (Fig. 2C).

***OSM-Mediated Up-Regulation of HIF1α Protein Levels Is Due to De Novo Transcription but not Regulation of Protein Stability.*** To find out whether OSM influences HIF1α protein stability, we aimed to measure HIF1α protein half-life. Therefore, we stimulated HepG2 cells with OSM or $CoCl_2$ (a hypoxia mimetic) for 6 hours to induce a robust HIF1α expression before the translation inhibitor cycloheximide was added for different periods. We found that the OSM-induced HIF1α protein disappeared completely after a 10-minute treatment with cycloheximide (Fig. 3A, right panel). In contrast, $CoCl_2$-induced HIF1α was still well detectable after 1 hour; it disappeared after 3 hours of cycloheximide treatment (Fig. 3A, left panel). There was no difference in HIF1α protein stability between $CoCl_2$ and the combined treatment with OSM and $CoCl_2$. These data show that the OSM-mediated HIF1α up-regulation is not due to an enhanced stability of the protein.

In contrast, experiments with the transcription inhibitor actinomycin D showed that less HIF1α protein was detectable when cells were treated with OSM in the presence of actinomycin D relative to cells treated with OSM alone (Fig. 3B). This was even more remarkable because actinomycin D treatment prevents the STAT3-induced up-regulation of the feedback inhibitor SOCS3, which suppresses STAT3 activation upon OSM. Despite the higher STAT3 activation observed in actinomycin D–treated cells, no HIF1α expression could be observed. Actinomycin D did not affect HIF1α expression in cells exposed to hypoxia, which was expected because hypoxia essentially increases protein stability. Here, the HIF1α

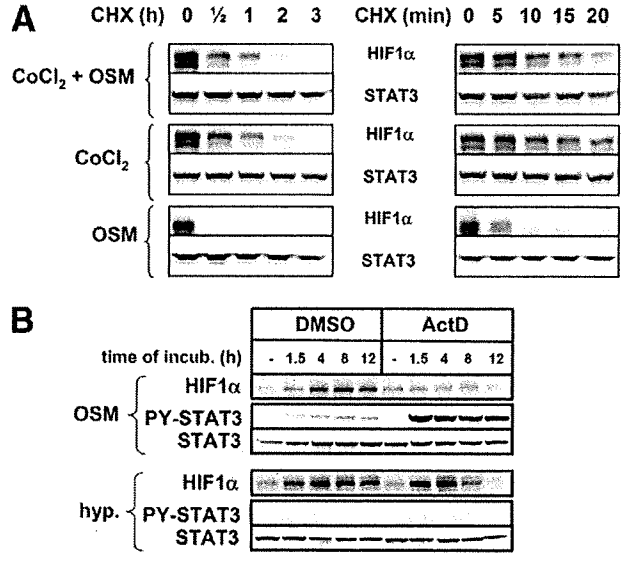


Fig. 3. OSM increases transcription of HIF1α but does not affect HIF1α protein stability. (A) HepG2 cells were stimulated for 6 hours with $CoCl_2$ (50 μM) and/or OSM (10 ng/mL). Cycloheximide (10 μg/mL) was then added for the indicated periods before lysates were prepared. Western blots were detected with HIF1α and STAT3 antibodies. For HIF1α detection, the western blots were exposed so that the band intensity of the untreated lane for all treatments was comparable. (B) HepG2 cells were treated for the indicated periods with OSM (10 ng/mL) or hypoxia (hyp.) in the presence of dimethyl sulfoxide alone or actinomycin D (5 μg/mL). Western blots of lysates separated via SDS-PAGE were detected with HIF1α, phospho-STAT3, and STAT3 antibodies.

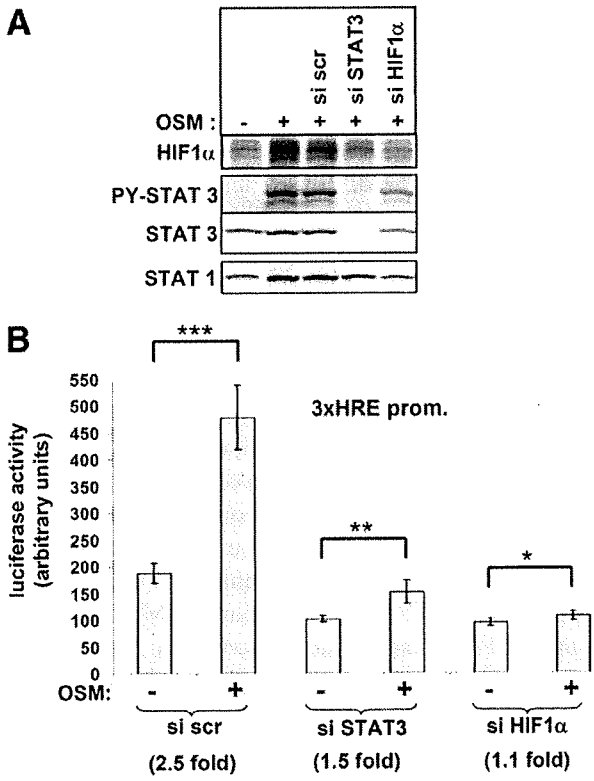


Fig. 4. Knockdown of STAT3 by siRNA decreases HIF1α protein levels and HIF1α-responsive reporter gene activity upon OSM treatment. (A) HepG2 cells were transfected with siRNAs as indicated. Forty-eight hours after transfection, cells were treated for 4 hours with OSM (10 ng/mL). Lysates of the cells were separated via SDS-PAGE, and western blots of the membranes were detected with HIF1α, phospho-STAT3, STAT3 and STAT1. (B) HepG2 cells were transfected with siRNAs as indicated, the luciferase reporter gene plasmids pGL3-EPO-HRE-Luc and the β-galactosidase expression vector pCH110. The cells were treated for additional 16 hours with OSM (10 ng/mL) before lysates were prepared. $***P < 0.001$. $**P < 0.01$. $*P < 0.05$.

levels decreased only after 8 hours, in accordance with the normal half-life of HIF1α under hypoxia (8-10 hours).[22]

In addition, our data in the Supporting Information show that OSM, in contrast to hypoxia, does not affect hydroxylation and ubiquitination (Supporting Fig. 2). Together, these data provide evidence that OSM enhances HIF1α via transcriptional regulation.

***STAT3 Protein Is Crucial for OSM-Induced Up-Regulation of HIF1α.*** After having shown that OSM-mediated up-regulation involves transcriptional regulation, we addressed the role of STAT3, playing a pivotal role in signaling of IL-6–type cytokines. Suppression of STAT3 by siRNA led to a loss of HIF1α expression upon OSM treatment, similar to the HIF1α siRNA used as a positive control (Fig. 4A). The unspecific control siRNA did not show these pronounced effects. Upon transfection of STAT3 or HIF1α siRNAs together with the 3xHRE reporter gene construct, OSM only weakly induced the reporter gene activity, whereas OSM induced reporter gene activity 2.5-fold when the unspecific control siRNA was transfected (Fig. 4B). In addition, the STAT3 inhibitor Stattic or dominant negative STAT3 also inhibited OSM-dependent effects (increase of HIF1α protein and mRNA levels, HRE promotor activity, or target gene induction) (Supporting Figs. 3-5). Furthermore, we provide evidence for the relevance of Erk signaling in OSM-mediated induction of HIF1α protein and activity (Supporting Figs. 3-5).

***OSM-Induced HIF1α Is Crucially Involved in the Transcriptional Regulation of the Genes for VEGF and PAI1.*** To investigate the relevance of HIF1α expression in OSM signal transduction, we examined the effects of HIF1α suppression on the target genes PAI1 and VEGF. We found that the OSM-dependent induction of both the VEGF and PAI1 mRNA were decreased upon HIF1α suppression (Supporting Fig. 6). In addition, the OSM-mediated induction of the VEGF and PAI1 promotor was down-regulated by HIF1α siRNA and by STAT3 siRNA (Fig. 5A), while a control siRNA had no effect. Consistent with this finding, we found that the OSM-dependent induction of the VEGF promoter was reduced by about 50% when a VEGF promoter construct mutated at the HRE was used for transfection (Fig. 5B). Because the robust induction of the VEGF promoter by hypoxia requires the integrity of the HRE and the AP1 site, we also used a construct where the AP1 site was mutated; the OSM-dependent induction of this promoter was reduced by about 25% (Fig. 5B). Importantly, mutation of the previously described STAT3 binding site within the VEGF promoter had no effect on the OSM-dependent induction of reporter gene activity (Fig. 5B). Thus, OSM-induced VEGF transcription seems to be mediated via HIF1 and AP1, rather than by STAT3.

In addition, HIF1 seems to play a crucial role in OSM-mediated activation of the PAI1 promotor, for which a reduction of about 40% was observed when the HRE was mutated (Fig. 5C).

Together, these data indicate that *de novo*–transcribed HIF1α importantly contributes to the OSM-induced VEGF and PAI1 transcription.

## Discussion

The first major finding of the present study is that the cytokine OSM, which activates the STAT3, Erk1/2, and p38 signaling pathways, can induce a robust up-regulation of HIF1α protein levels in hepatocytes and hepatoma cells under normoxic conditions and leads to the formation of transcriptionally active HIF1 complexes. OSM-induced HIF1α protein up-regulation was stronger compared with other cytokines (IL-6, IL-1β, TNF-α, interferon-γ), some of which have been implicated in

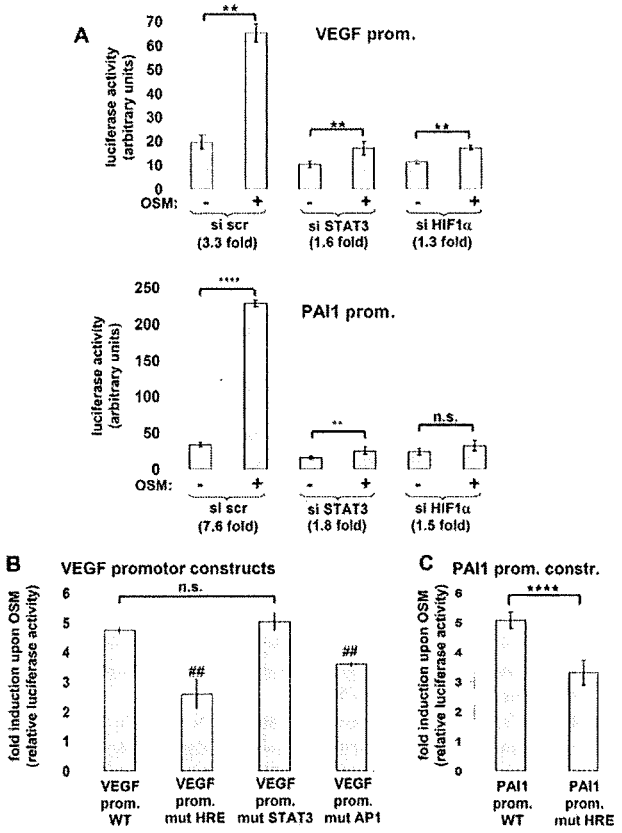


Fig. 5. Regulation of the VEGF and PAI1 gene promotors by OSM. (A) HepG2 cells were transfected with siRNAs as indicated, the luciferase reporter gene plasmids pGL3-VEGF-Luc or pGL3-hPAI1-796 and the β-galactosidase expression vector pCH110, and processed as described in Fig. 4B. $^{****}P < 0.0001$. $^{**}P < 0.01$. n. s., not significant. (B) HepG2 cells were transfected with the luciferase reporter gene plasmids pGL3-VEGF-Luc or the following mutants thereof: pGL3-VEGFmutHRE-Luc (containing a mutated HIF1α binding site), pGL3-VEGFmutSTAT3-Luc (containing a mutated STAT3 binding site), or pGL3-VEGFmutAP1-Luc (containing a mutated AP1 binding site). The β-galactosidase expression vector pCH110 was cotransfected. Twenty-four hours after transfection, the medium was exchanged, and the cells were treated for an additional 16 hours with OSM (10 ng/mL) before lysates were prepared. Values for luciferase activity (relative to β-galactosidase activity) are shown as fold induction compared with untreated samples. $^{\#\#}P < 0.01$ versus wild-type construct. (C) HepG2 cells were transfected with pGL3-hPAI1-796 or with pGL3-hPAI1-796-M2 containing a mutation in the HRE and processed as described in (B). $^{****}P < 0.0001$.

HIF1α up-regulation before (IL-6, IL-1β, TNF-α) (Supporting Fig. 1). Moreover, the OSM-increased HIF1α protein was shown to be involved in the enhanced expression of the HIF1 target genes PAI1 and VEGF.

Our study also shows that OSM and hypoxia differ in their mechanism of HIF1α up-regulation. Regulation of HIF1α activity is complex and under normoxic conditions, HIF1α has an extremely short half-life because it is continuously degraded due to the initial hydroxylation at two proline residues and transcriptional activity is reduced due to hydroxylation of asparagine 803. Hypoxia reduces the activity of the oxygen-utilizing hydroxylases, thereby stabilizing the protein and increasing its transactivity.[5] However, our data provide evidence that OSM does not contribute to an increased stability or increased transactivity as shown in the experiments with the Gal-HIF1α-TADN or TADC gene constructs (Supporting Fig. 2C). Furthermore, we could show that OSM-induced HIF1α was clearly ubiquitinated, which was less the case for hypoxia-stabilized HIF1α (Supporting Fig. 2A,B). We propose that transcriptional mechanisms are responsible for the OSM-mediated HIF1α protein up-regulation because it was inhibitable by the transcriptional inhibitor actinomycin D, whereas this was not the case for hypoxic treatment. The importance of HIF1α regulation at the mRNA level is further supported by findings showing that hepatocyte growth factor, angiotensin-II, lipopolysaccharide, IL-1, thrombin, or hypoxia also enhance HIF1α mRNA levels in different cell types (see Bonello et al.[23] and references therein).

Moreover, our tests with inhibitors for Janus kinases, STAT3, and mitogen-activated protein kinase kinase indicate that these pathways play an important role in OSM-dependent induction of HIF1α mRNA and protein expression (Supporting Fig. 3), HIF1α-dependent reporter gene activity (3x HRE promotor) (Supporting Fig. 4A), as well as in the regulation of the target genes VEGF and PAI1 (Supporting Fig. 5). In contrast, inhibitor tests suggested that p38 mitogen-activated protein kinases or the PI3K/Akt pathway are not involved in OSM-induced HIF1α expression (data not shown).

Experiments with siRNA further revealed that STAT3 plays a crucial role in OSM-regulation of HIF1α protein levels and HIF1α-dependent transcriptional activity. Moreover, dominant negative STAT3 (STAT3-DN) also led to a down-regulation of HIF1α-dependent transcriptional activity (Supporting Fig. 4B). It has been shown that HIF1α transcription is regulated by SP-1[24] and NF-κB transcription factors.[23] In addition to that, the present study indicates that the transcriptional regulator STAT3 appears to be a key player for HIF1α transcription in response to OSM. STAT3 was also found to be involved in HIF1α mRNA expression in tumor cells and tumor-associated myeloid cells.[25]

Our study provides evidence that HIF1α is important for OSM signal transduction. Experiments with HIF1α siRNA showed that the expression of the OSM target genes VEGF and PAI1 involves regulation by HIF1α. OSM and other IL-6–type cytokines have been shown to induce expression of the HIF1 target gene VEGF.[10,12,26-29] The VEGF gene is also considered a STAT3 target gene, because a dominant negative STAT3 reduced[10,27,30,31] and constitutively active forms of

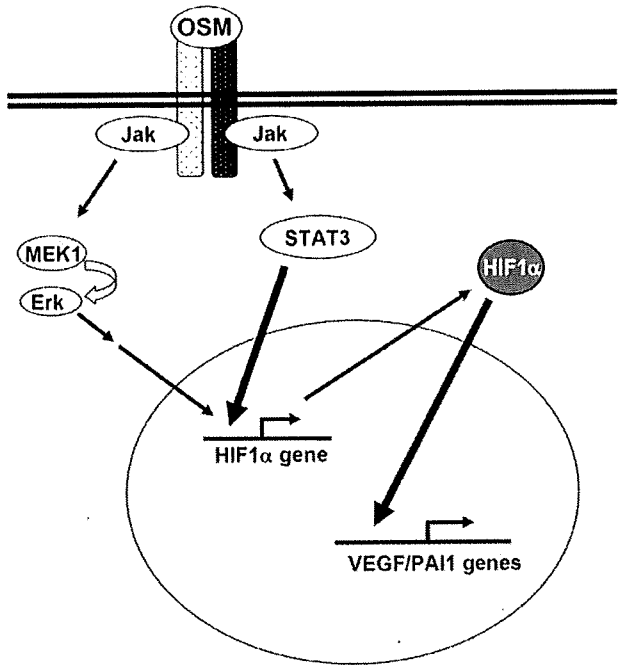


Fig. 6. Schematic representation of VEGF gene regulation by OSM.

STAT3 induced the VEGF promoter,[27,30,31] and a STAT3 binding element was detected at site −848.[30,31] We found that mutation of the described STAT3 binding site in the VEGF promoter at −848 did not affect OSM-dependent induction of reporter gene activity. These data are consistent with those of another study in which deletion of this site also did not affect IL-6–induced reporter gene activity.[27] Instead, our results with the VEGF reporter gene constructs clearly demonstrate a relevance of the HIF1 and AP1 binding elements for OSM-mediated regulation, because the constructs mutated at the HRE and the AP1 sites displayed significantly reduced induction in response to OSM. Thus, we conclude from our data that STAT3 regulation of the VEGF gene may rather be mediated indirectly via Erk1/2 and STAT3-dependent induction of HIF1α transcription. HIF1α then regulates VEGF and PAI1 transcription (Fig. 6), which leads to increased secretion of VEGF and PAI1 proteins (Supporting Fig. 7).

Although the present study was performed with hepatoma cells and nontransformed hepatocytes, it will be interesting to further clarify the role of HIF1α in OSM-mediated signal transduction and regulation of secreted factors involved in tissue remodeling (such as VEGF and PAI1) in processes such as liver development, regeneration, and inflammation.

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# Replicons from genotype 1b HCV-positive sera exhibit diverse sensitivities to anti-HCV reagents

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ABSTRACT

Half of the population of genotype 1 HCV is resistant to current pegylated-interferon-α (PEG-IFN-α) and ribavirin therapy. The resistance to IFN therapy is an urgent problem, especially in patients with genotype 1 HCV infection. However, sensitivities among HCV strains to anti-HCV reagents including IFNs have not been thoroughly addressed. Here, we established three different subgenomic replicons (1B-4, 1B-5, and KAH5 strains) in addition to our previously established replicon (O strain). We comparatively examined the sensitivities of four replicons to IFN-α, IFN-γ, IFN-λ, cyclosporine A, and fluvastatin. Among the replicons, the 1B-4 and KAH5 replicons were the most sensitive and resistant, respectively to IFN-λ ($EC_{50}$: 1.50 ng/ml vs. 8.50 ng/ml) and fluvastatin ($EC_{50}$: 2.82 μM vs. 7.87 μM), although these replicons possessed similar features in terms of genetic distance from the O strain, HCV RNA expression levels, and sensitivity to IFN-α ($EC_{50}$: 1.44 IU/ml vs. 1.37 IU/ml) and cyclosporine A ($EC_{50}$: 0.71 μg/ml vs. 0.96 μg/ml). These replicons are thus useful tools for examining the mechanism of anti-HCV activity, especially in IFN-λ and statins.



## 1. Introduction

Hepatitis C virus (HCV) belongs to *Flaviviridae* family and contains a positive single-stranded RNA genome of 9.6 kb (Kato et al., 1990; Tanaka et al., 1996). The viral genome encodes a single polyprotein of approximately 3010 amino acid residues, which is proteolytically processed by host and viral proteases into at least 10 proteins in the following order: Core, envelope 1 (E1), E2, p7, non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (Kato, 2001). HCV infection frequently causes chronic hepatitis C (CH C) and progresses to fatal cirrhosis and hepatocellular carcinoma. The current standard therapy for CH C is pegylated-interferon-α (PEG-IFN-α) and ribavirin. However, the cure rate of the therapy for the treatment of CH C is limited to approximately 50% (Firpi and Nelson, 2007). The major cause of resistance to this therapeutic approach was observed in genotype 1 HCVs. However, the mechanisms of the diverse sensitivity to IFN therapy among genotype 1 HCVs have remained unclear. Therefore, the development of more effective anti-HCV reagents is an urgent issue.

Since the HCV replicon system was developed by Lohmann et al. (1999), several groups have reported candidate anti-HCV reagents. Statin, a 3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, is one of the well-characterized anti-HCV reagents and its anti-HCV activity has been shown to be due to the inhibition of geranylgeranylation of host proteins (Ikeda et al., 2006; Kapadia and Chisari, 2005; Ye et al., 2003). Cyclosporine A (CsA), an immunosuppressant, is another well-characterized anti-HCV reagent that inhibits HCV RNA replication via its interaction with cyclophilins (CyPs) (Inoue et al., 2007; Nakagawa et al., 2005; Watashi et al., 2003). In addition to type I IFNs (α and β) and type II IFN (γ), recently identified type III IFN (λ) has been reported to possess anti-HCV activity in cell culture (Doyle et al., 2006; Marcello et al., 2006; Robek et al., 2005). Subgenomic HCV replicons have been reported since the breakthrough of the Con1(1b) replicon using different HCV strains: H77 (1a), N (1b), 1B-1 (1b), O (1b), JFH 1 (2a), and AH1 (1b) (Blight et al., 2003; Ikeda et al., 2002, 2005; Kato et al., 2003a,b; Kishine et al., 2002; Lohmann et al., 1999; Mori et al., 2008; Pietschmann et al., 2002). Moreover, a number of groups have examined anti-HCV reagents using the established replicon. However, such studies have been conducted using replicon(s) from only one or two HCV strain(s). To date, there has been no comprehensive study regarding the diverse sensitivities of anti-HCV reagents to genotype 1 HCV replicons from different strains.

To address this issue, we developed three HCV replicons from different genotype 1b HCV positive sera, in addition to our previously reported O strain (Ikeda et al., 2005). Two replicons were

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constructed using HCV-positive sera from healthy carriers (1B-4 and 1B-5) and one replicon was constructed using serum sampled from a case of acute hepatitis C (KAH5). These replicons contained neomycin phosphotransferase (Neo) and *Renilla* luciferase (RL) genes at the first cistron of the replicon with the aim of conducting a stable and highly sensitive reporter assay. In this study of four replicons, we examined the anti-HCV reagents IFN-α, IFN-γ, IFN-λ, CsA, and various statins (pitavastatin (PTV), fluvastatin (FLV), and rosvastatin (RSV)), and we found diverse sensitivities among the replicons. Newly developed replicons will be useful tools for the present study regarding the diverse sensitivities of genotype 1b HCVs to anti-HCV reagents, including IFNs.

## 2. Materials and methods

### 2.1. HCV-positive sera and GeneBank accession numbers

Serum O (previously described as 1B-2), 1B-4, and 1B-5 were derived from an HCV-positive healthy carrier and have been described previously (Ikeda et al., 1997). Serum KAH5 was obtained from a patient with acute hepatitis C (AH C) who provided prior informed consent. The nucleotide sequence data for 1B-4, 1B-5, and KAH5 will appear in the DDBJ, EMBL, and GeneBank nucleotide sequence databases under accession nos. AB442219, AB442220, and AB442222, respectively.

### 2.2. Cell cultures

Three HCV-positive sera (KAH5, 1B-4, and 1B-5 strains) were used for the development of subgenomic replicons with reporter (RL). We first established 9, 4, and 6 replicon harboring clonal cell lines derived from KAH5, 1B-4, and 1B-5 strains, respectively. Then, after characterization for these cell lines, we selected the representative clonal cell lines and designated sKAH5R (clone 6), s1B-4R (clone 2), and s1B-5R (clone 4) as sKAH5R, s1B-4R, and s1B-5R, respectively (Supplemental Figs. 1A, B, and C). sO and O cells were used as subgenomic and genome-length HCV RNA-harboring cells with a Neo gene in the first cistron, as previously described (Kato et al., 2003a; Ikeda et al., 2005). These cells were derived from a hepatoma cell line, HuH-7, and were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, streptomycin, and 0.3 mg/ml of G418 (Geneticin; Invitrogen, Carlsbad, CA). The cells were passaged twice weekly at a 5:1 split ratio. The sequences in the original subgenomic replicons were described above and appeared in the database with indicated accession numbers.

### 2.3. RT-nested PCR

HCV RNAs were prepared from HCV-positive sera (1B-4, 1B-5, and KAH5) using ISOGEN-LS (Nippon Gene Co., Tokyo, Japan) according to the manufacturer's protocol. These RNA samples were used for RT-PCR in order to amplify the NS2 to NS5B region (6.0 kb) of the HCV genomes. RT was performed with the OligodA23 primer, 5′-AAAAAAAAAAAAAAAAAAAAAAA-3′. The primer pair 542: 5′-GTAGAGCCCGTCGTCTTCTCTGACATGGA-3′ and 9388R: 5′-ATGGCCTATTGGCCTGGAGTG-3′ was employed in the first-round PCR (35 cycles). The primer pair 3295X: 5′-ATTATTCTAGACTGACA-TGGAGACCAAGATCATCAC-3′ and 9357RX: 5′-ATTATTCTAGACCCG-TTCACCGGTTGGGGAGCAG-3′, containing the XbaI site (underlined) was employed in the second-round PCR (35 cycles). SuperScript III reverse transcriptase (Invitrogen) and KOD-plus DNA polymerase (Toyobo, Osaka, Japan) were used for RT and PCR, respectively.

### 2.4. Plasmid construction

To construct an HCV replicon with RL and Neo genes, we used a previously described pRN/3-5B/KE plasmid as a cassette vector (Ikeda et al., 2005). Basically, the NS3 to NS5B region was replaced with RT-PCR products from sera with 1B-4, 1B-5, and KAH5 at SpeI (located in NS3) and BsiWI (located in NS5B) sites. The PCR products were further amplified with the primers NS3 SpeI: 5′-ATCA-TCACTAGTCTCACAGGCCGGGACAAGAAAC-3, containing the SpeI site (underlined); and NS5B BsiWI: 5′-CTTGGTCCGTACGGCCCAGTT-GAAGAGGTACTTGC-3′, containing the BsiWI site (underlined). The amplified fragments were digested with SpeI and BsiWI, and were ligated into the pRN/3-5B/KE cassette vector, which was predigested with SpeI and BsiWI.

### 2.5. RNA transcription

Plasmid DNAs were linearized by XbaI digestion and were used for RNA synthesis with T7 MEGAscript (Ambion) as previously described (Kato et al., 2003a).

### 2.6. RNA transfection and G418-resistant cells

Ten micrograms of *in vitro* synthesized HCV replicon RNAs were introduced into HuH-7 derived cells (OR6c cells) by electroporation, and the cells were selected in the presence of G418 (0.3 mg/ml) for 3 weeks as described previously (Mori et al., 2008).

### 2.7. Western blot analysis

The preparation of cell lysates, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting were performed as described previously (Kato et al., 2003a). The antibodies used in this study were those against Core, NS3, NS5A, and NS5B. β-actin antibody (AC-15, Sigma) was used as a control for the amount of protein loaded per lane. Immunocomplexes were detected with the Renaissance enhanced chemiluminescence assay (PerkinElmer Life Science, Boston, MA).

### 2.8. Quantification of HCV RNA

The RNAs were prepared from an HCV replicon RNA replicating cell line, and 2 μg of each total RNA was used for RT-qPCR with 5′-UTR of an HCV-specific primer pair, as described previously (Ikeda et al., 2005). Experiments were conducted in triplicate.

### 2.9. Northern blot analysis

Total RNA was extracted from the cultured cells using an RNeasy Mini Kit according to the manufacturer's protocol (QIAGEN). Three micrograms of total RNA were used for the analysis. HCV-specific RNA and β-actin were detected according to a previously described method (Ikeda et al., 2005).

### 2.10. Reagents

IFN-α and IFN-γ were purchased from Sigma, and CsA was obtained from Calbiochem (San Diego, CA). IFN-λ (IL-29) was purchased from WAKO. PTV was purchased from the Kowa Company, Ltd. (Tokyo, Japan). FLV was purchased from Calbiochem. RSV was obtained from AstraZeneca.

### 2.11. Luciferase reporter assay

For the luciferase assay, $1.0–1.5 \times 10^4$ HCV replicon-harboring cells were plated onto 24-well plates in triplicate and were cultured

for 24 h. The cells were treated with each anti-HCV reagent for 72 h. Then the cells were harvested with *Renilla* lysis reagent (Promega, Madison, WI) and subjected to RL assay according to the manufacturer's protocol. All the luciferase assays were repeated at least three times.

### 2.12. Statistical analysis

Statistical comparison of the luciferase activity in various treatment groups was performed using Student's *t*-test. *P* values of less than 0.05 were considered statistically significant.

## 3. Results

### 3.1. Establishment of four subgenomic replicon-harboring cell lines using different genotype 1b HCV sera

We tried to establish replicon-harboring cells from different HCV-positive sera to assess the sensitivity of anti-HCV reagents among genotype 1b HCV strains. To this end, three sera (1B-4, KAH5, and 1B-5) were used to amplify the NS region of HCV genomes by reverse transcription-polymerase chain reaction (RT-PCR). The dicistronic replicons were designed as shown in Fig. 1A. RL and Neo genes were introduced into the first cistron and translation was driven by the HCV internal ribosomal entry site (IRES) leading to the expression of RL and Neo as a fusion protein. In the second cistron, NS3 to NS5B was translated via the encephalomyocarditis virus (EMCV) IRES (Fig. 1A). We introduced *in vitro*-synthesized HCV replicon RNAs (10 μg) into OR6c cells, in which HCV RNA was eliminated from OR6 cells by IFN-α treatment. After 3 weeks of G418 selection, we obtained HCV replicon-harboring cell colonies, i.e., more than 100 colonies from KAH5 and 20 colonies from 1B-4. However, no colony formation was observed among 1B-5 replicon-RNA-introduced cells. Therefore, we next attempted to perform the electroporation of a 1B-5 replicon with mutations derived from the HCV sequence in s1B-5 replicon-harboring cells, in which the replicating HCV replicon possessed only neomycin-resistant genes in the first cistron (data not shown). The mutations introduced into 1B-5 replicon were E1758D and I1851F in NS4B and R2192W and E2414Q in NS5A. Consequently, we established 9, 4, and 6 replicon-harboring cells from KAH5, 1B-4, and 1B-5, respectively, and confirmed the expression of HCV RNA and proteins. In addition to three replicon RNAs, the previously described ORN/3-5B/KE replicon RNA was also introduced into OR6c cells and selected as sOR in this study (Ikeda et al., 2005). The representative clonal cell lines, which grow healthy and stably expressed abundant HCV proteins, are used in the following experiments (Supplemental Fig. 1A, B, and C). These replicon-harboring cell lines were established from genotype 1b HCV strains: 1B-4, KAH5, O, and 1B-5 and were designated as s1B-4R, sKAH5R, sOR, and s1B-5R, respectively. We confirmed the expression of NS3, NS5A, and NS5B proteins in all replicon-harboring cells (Fig. 1B). The expression levels of HCV RNAs in the replicon-harboring cells were examined for the 5′-UTR by quantitative RT- PCR (RT-qPCR) (Fig. 1C). s1B-4R cells exhibited the highest levels of expression of HCV RNA (approximately $10^8$ copies/μg total RNA), followed by sKAH5R, sOR, and s1B-5R cells (Fig. 1C). All of the replicon-harboring cells expressed HCV RNA at levels greater than at least $4 \times 10^7$ copies/μg total RNA. Northern blot analysis also demonstrated the presence of HCV-specific RNA with a length of approximately 9 kb in the total RNA extracts from four replicon-harboring cells (Fig. 1D). These four genotype 1b HCV replicon reporter systems were established and used for further analyses of sensitivity to anti-HCV reagents.

### 3.2. Diverse activity of various IFN types on HCV replicons

IFN-α belongs to the type I IFN group and is currently used as standard therapy for patients with CH C. Therefore, first we evaluated the activity of IFN-α using the four developed replicons and a reporter assay. The s1B-4R and sKAH5R replicons showed almost equal and moderate sensitivity to IFN-α (50% effective concentration ($EC_{50}$): 1.44 and 1.37 IU/ml, respectively) (Fig. 2). The s1B-5R and sOR replicons, respectively, exhibited the highest ($EC_{50}$: 1.10 IU/ml) and lowest ($EC_{50}$: 2.35 IU/ml) sensitivity to IFN-α among the replicons tested (Fig. 2). We also examined the activity of IFN-α on HCV protein expression levels in these four replicons. The findings from the Western blot analysis of the sensitivity to IFN-α

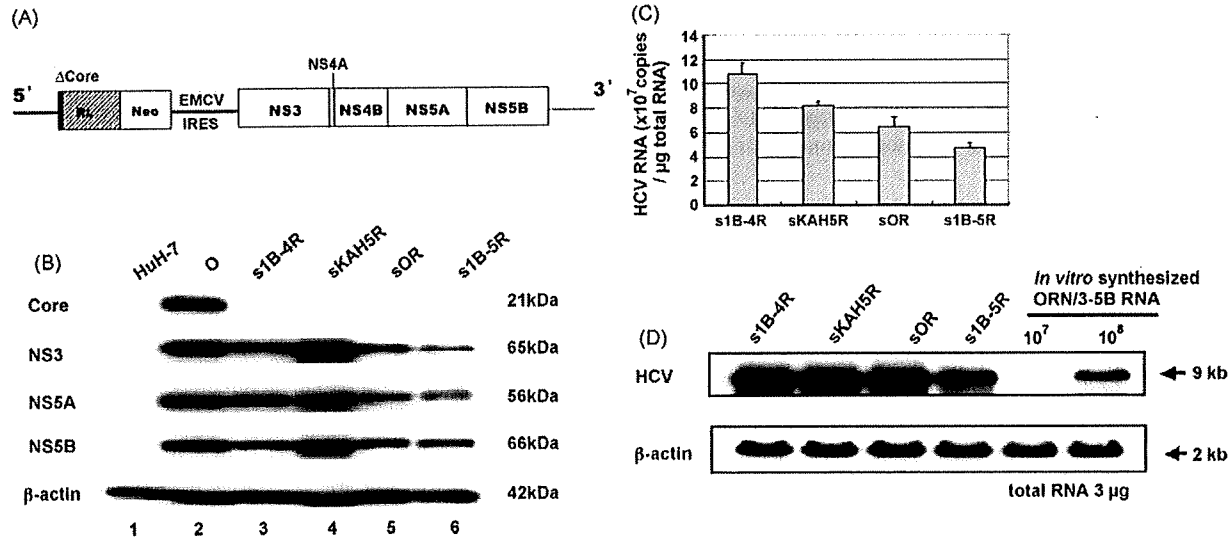


**Fig. 1.** The expression of HCV proteins and HCV RNAs in four replicon-harboring cell lines. (A) Schematic gene organization of subgenomic replicon RNA. The NS3 to NS5B region and 12 N-terminal amino acid residues of the Core (ΔC) are depicted in closed boxes. Untranslated regions, EMCV IRES, RL, and Neo genes are indicated by thin lines, thick line, shaded box, and open box. (B) Western blot analysis of HCV proteins. Production of Core, NS3, NS5A, and NS5B in HuH-7 cells (lane 1), O cells (lane 2), s1B-4R cells (lane 3), sKAH5R cells (lane 4), sOR cells (lane 5), and s1B-5R cells (lane 6) were analyzed by immunoblotting using anti-Core, anti-NS3, anti-NS5A, and anti-NS5B antibodies. (C) RT-qPCR analysis. The levels of HCV RNA in G418-resistant cells were quantified by LightCycler PCR. (D) Northern blot analysis. RNAs from s1B-4R, sKAH5R, sOR, and s1B-5R cells were used for comparison. *In vitro*-synthesized ORN/3-5B RNA was also used for comparative analyses.

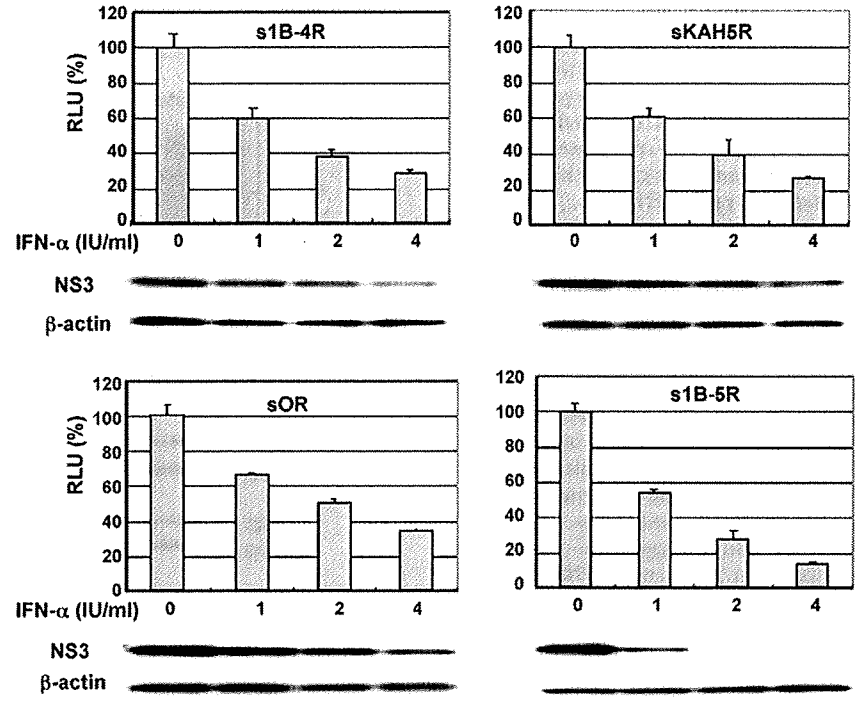


**Fig. 2.** The activity of IFN-α on HCV replicon RNA replication. Reporter assay and Western blot analysis for HCV replicons in IFN-α treatment. s1B-4R cells, sKAH5R cells, sOR cells, and s1B-5R cells were treated with IFN-α (0, 1, 2, and 4 IU/ml) for 72 h. Then, the cells were subjected to RL assay (upper panels) and Western blot analysis for NS3 (lower panels). The percent relative luciferase unit (RLU (%)) was calculated with the RL activity of untreated cells assigned at a value of 100%. The data indicate means ± S.D.s of triplicate samples. All of the luciferase assays were repeated at least three times. β-Actin was used as a control for the amount of proteins loaded per lane.

coincided with the results of the reporter assay. Thus, these results indicated that genotype 1b replicons possess different sensitivities to IFN-α.

Next, we examined the sensitivity of four replicons to type II IFN, IFN-γ, because in our previous study, HCV (genotype 1b, AH1 strain) from a patient with AH C was found to be more resistant to IFN-γ than was HCV-O (Mori et al., 2008). In this study, sKAH5R was also derived from the serum of a patient with AH C. The reporter assay revealed that sKAH5R has the lowest sensitivity to IFN-γ ($EC_{50}$: 2.26 IU/ml) among the replicons tested (Fig. 3). To calculate the $EC_{50}$ of IFN-γ to sKAH5R, we also treated sKAH5R with IFN-γ at 2 and 4 IU/ml for 72 h (data not shown). The $EC_{50}$ of IFN-γ to s1B-4R, sOR,

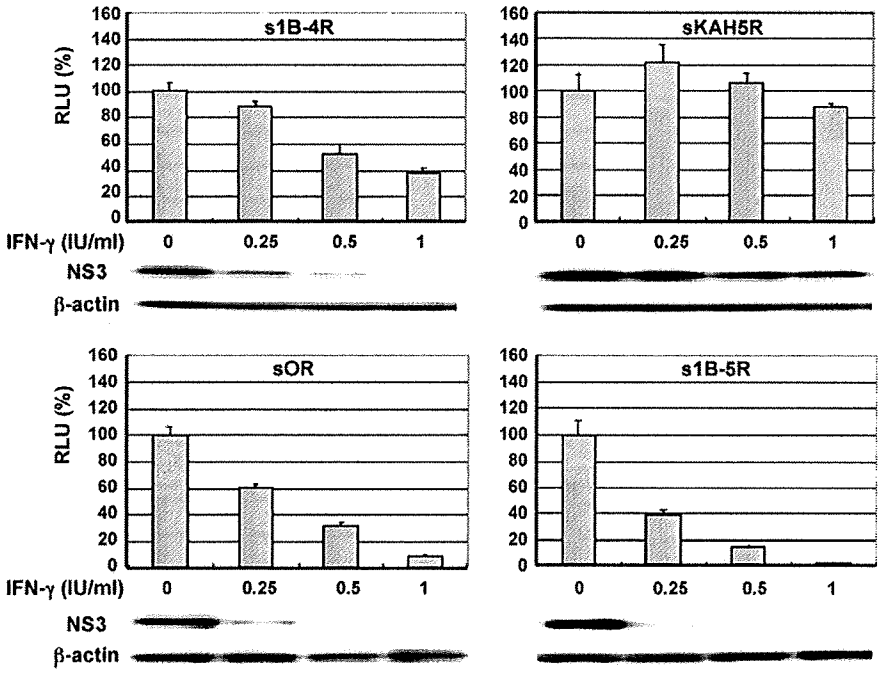


**Fig. 3.** The activity of IFN-γ on HCV replicon RNA replication. Reporter assay and Western blot analysis for HCV replicons in IFN-γ treatment. s1B-4R cells, sKAH5R cells, sOR cells, and s1B-5R cells were treated with IFN-γ (0, 0.25, 0.5, and 1 IU/ml) for 72 h and then the cells were subjected to RL assay (upper panels) and Western blot analysis for NS3 (lower panels). All of the luciferase assays were repeated at least three times.

and s1B-5R was 0.54, 0.33, and 0.21 IU/ml, respectively. The results of Western blot analyses of sensitivity to IFN-γ coincided with those of the reporter assay. Interestingly, again in this study, the HCV RNA derived from the patient with AH C was resistant to IFN-γ, as was the AH1 strain. These results may suggest that AH C in pathologic states of HCV infection may be involved in the IFN-γ resistance feature of the replicon. Further studies will be needed to clarify this issue.

We analyzed a recently identified type III IFN, IFN-λ, in terms of its anti-HCV activity against four HCV replicons. IFN-λ shares the same Jak/Stat signaling pathway with type I IFNs, which express a common set of IFN-stimulating genes (ISGs). However, IFN-λ uses distinct receptors composed of IFNLR1 and IL10R2. Here, sKAH5R and s1B-4R, respectively, exhibited the lowest and highest sensitivities to IFN-λ ($EC_{50}$: 8.25 and 1.50 ng/ml) (Fig. 4A). Additionally, sOR and s1B-5R exhibited moderate sensitivity to IFN-λ ($EC_{50}$: 4.48 and 4.82 ng/ml, respectively) (Fig. 4A). These diverse inhibitory activities of IFN-λ were also confirmed by Western blot analysis (Fig. 4B). Moreover, s1B-4R and sKAH5R showed similar sensitivities to IFN-α. However, it was of note that these replicons exhibited different degrees of sensitivity to IFN-λ, which uses a common Jak/Stat signaling pathway. These results suggest the presence of a complicated antiviral mechanism in type I and III IFNs. Recently, it was reported that IFN-λ in combination with IFN-α or IFN-γ enhanced anti-HCV activity (Pagliaccetti et al., 2008). Therefore, s1B-4R and sKAH5R are useful for the study in combination treatment of IFNs.

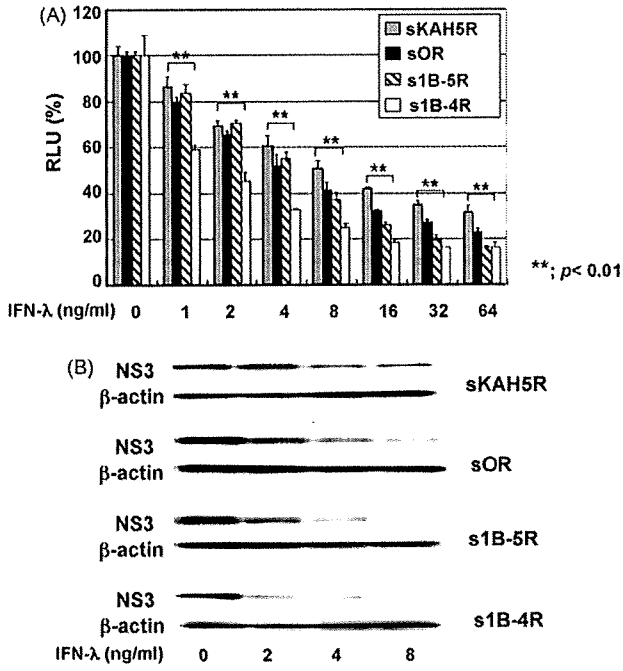


**Fig. 4.** Effects of IFN-λ on HCV replicon RNA replication. (A) Reporter assay and Western blot analysis for HCV replicons in IFN-λ treatment. s1B-4R cells, sKAH5R cells, sOR cells, and s1B-5R cells were treated with IFN-λ (0, 2, 4, 8, 16, 32, and 64 ng/ml) for 72 h, and then the cells were subjected to RL assay (B) and Western blot analysis. Four replicon-harboring cell types were treated with IFN-λ (0, 2, 4, and 8 ng/ml) for 72 h and were subjected to Western blot analysis of NS3. All of the luciferase assays were repeated at least three times.

### 3.3. Diverse effects of PTV but not CsA on HCV replicons

Anti-HCV reagents other than IFNs were examined in terms of their effectiveness in the presence of various replicons. As CsA is a well-characterized anti-HCV reagent, we examined the sensitivities of the replicons to CsA by reporter assay. There were no significant differences in sensitivity to CsA among the replicons (Fig. 5). The $EC_{50}$ of CsA to s1B-4R, sKAH5R, sOR, and s1B-5R was 0.71, 0.96, 1.10, and 0.85 μg/ml, respectively. We also obtained similar results by Western blot analysis. In contrast to the findings of the IFN study, there were no significant differences in sensitivity to CsA among the genotype 1b replicons tested.

Statins, which are HMG-CoA reductase inhibitors, are yet another well-characterized anti-HCV reagent. Therefore, we

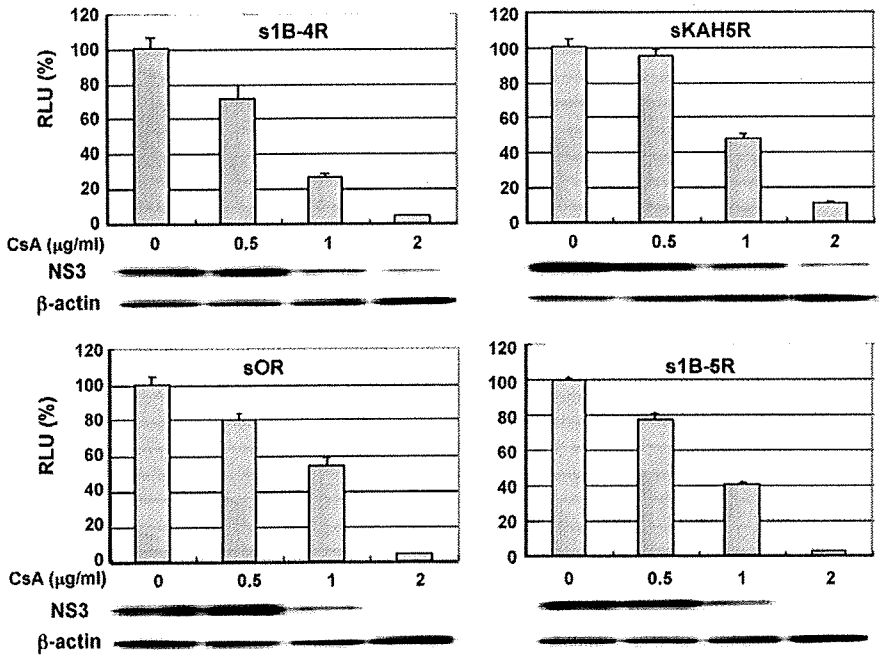


**Fig. 5.** The activity of CsA on HCV replicon RNA replication. Reporter assay and Western blot analysis for HCV replicons in CsA treatment. s1B-4R cells, sKAH5R cells, sOR cells, and s1B-5R cells were treated with CsA (0, 0.5, 1, and 2 μg/ml) for 72 h, and then the cells were subjected to RL assay (upper panels) and Western blot analysis for NS3 (lower panels), as described in Fig. 2. All of the luciferase assays were repeated at least three times.

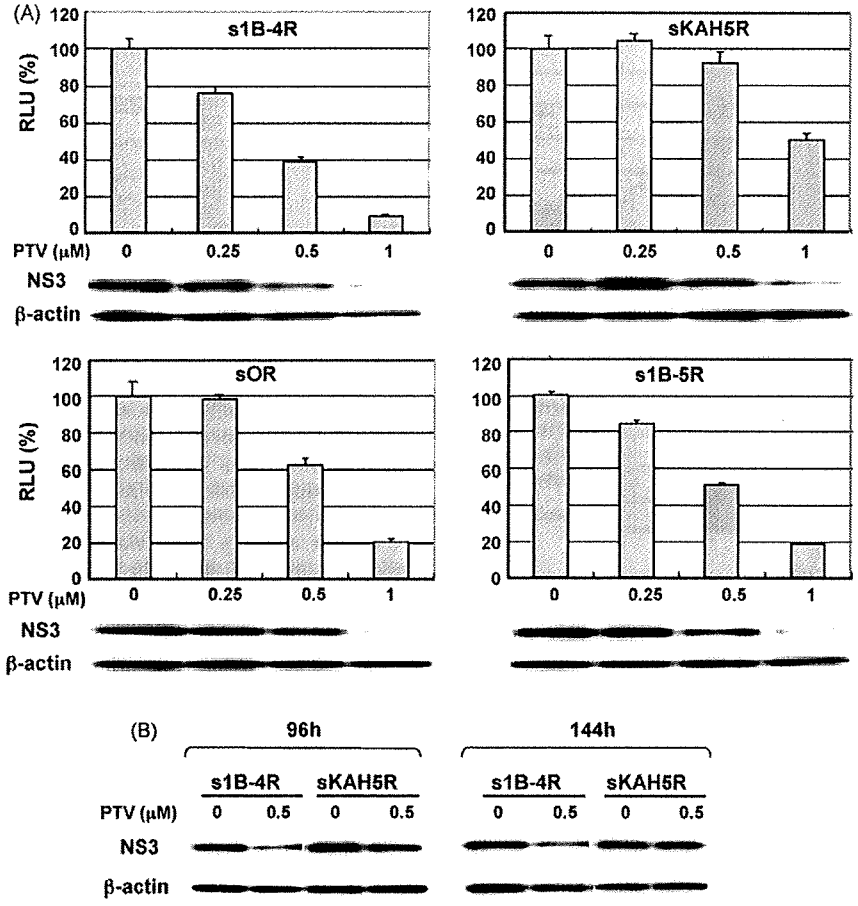


**Fig. 6.** The activity of PTV on HCV replicon RNA replication. (A) Reporter assay and Western blot analysis for HCV replicons in PTV treatment. s1B-4R cells, sKAH5R cells, sOR cells, and s1B-5R cells were treated with PTV (0, 0.25, 0.5, and 1 μM) for 72 h, and then the cells were subjected to RL assay and Western blot analysis, as described in Fig. 2. All of the luciferase assays were repeated at least three times. (B) s1B-4R cells and sKAH5R cells were treated with PTV (0 and 0.5 μM) for 96 and 144 h and were subjected to Western blot analysis.

examined the sensitivity of the replicons to PTV. The reporter assay revealed that sKAH5R has the lowest sensitivity to PTV ($EC_{50}$: 1.00 μM) among the replicons tested (Fig. 6A). The sensitivities of the other replicons to PTV were almost identical, and the $EC_{50}$ values of s1B-4R, sOR, and s1B-5R were 0.40, 0.64, and 0.51 μM, respectively (Fig. 6A). We also obtained similar results by Western blot analysis using cell lysates at 72 h after treatment. The inhibition of HCV protein in s1B-4R persisted until 96 and 144 h after treatment with PTV (0.5 μM) (Fig. 6B). FBL2 is identified as a geranylgeranylated cellular protein required for HCV RNA replication (Wang et al., 2005). Therefore, we examined the expression levels of FBL2 in s1B-4R and sKAH5R. The expression levels of FBL2 mRNA were almost equal between both cells (Supplemental Fig. 2). This result indicates that low sensitivity of sKAH5R to statins is not due to the low expression of FBL2. Previously, we used an OR6 assay system to demonstrate that PTV inhibited genome-length HCV RNA replication, and the $EC_{50}$ of PTV was found to be 0.45 μM (Ikeda et al., 2006; Ikeda and Kato, 2007). The $EC_{50}$ values of PTV in three replicons other than sKAH5R were almost equal to that of PTV in OR6. These results, taken together, suggest that sKAH5R is resistant to PTV as well as to IFN-γ and IFN-λ.

### 3.4. Resistance to statins in a replicon from a patient with AH C

To further confirm that sKAH5R is resistant to statins, we examined the sensitivity of the replicons to FLV and RSV using a reporter assay. Here, sKAH5R exhibited the lowest sensitivity to FLV and RSV (Fig. 7). In the case of sKAH5R, the $EC_{50}$ of FLV was 7.87 μM, and the $EC_{50}$ of RSV exceeded 20 μM, because RSV was toxic to cells at concentrations of more than 20 μM. Moreover, sOR and s1B-5R showed almost equal and moderate sensitivities to both FLV and RSV. It was of note that these results were in agreement with those regarding PTV sensitivity, i.e., s1B-4R exhibited the highest sensitivity to both FLV and RSV. The $EC_{50}$ values of FLV and RSV to s1B-4R were 2.82 and 10.12 μM, respectively. These results suggest that sKAH5R exhibits some resistance, and s1B-4R some sensitivity, to statins. Therefore, these replicons may serve as useful tools for investigating the mechanism of the anti-HCV activity of statins.

### 3.5. Polyclonal KAH5 replicon with a statin-resistant phenotype

sKAH5R replicon cells were found to possess the least sensitivity to statins among the replicon-harboring cells tested. However, the statin-resistant phenotype may be due to cell clonality rather than HCV strain, because the sKAH5R replicon cells used here were a cloned cell line selected from numerous G418-resistant colonies. We thus examined the sensitivity of polyclonal sKAH5R cells to statins, and then compared the results with those obtained using polyclonal s1B-4R cells in order to rule out this possibility. In polyclonal sKAH5R, the $EC_{50}$ values of PTV and FLV were 0.88 and 6.56 μM, respectively (Fig. 8), and the $EC_{50}$ of RSV exceeded 20 μM (Fig. 8), because RSV is toxic to these cells at concentrations of more than 20 μM. In polyclonal s1B-4R, the $EC_{50}$ values of PTV, FLV, and RSV were 0.47, 3.41, and 10.00 μM, respectively (Fig. 8).

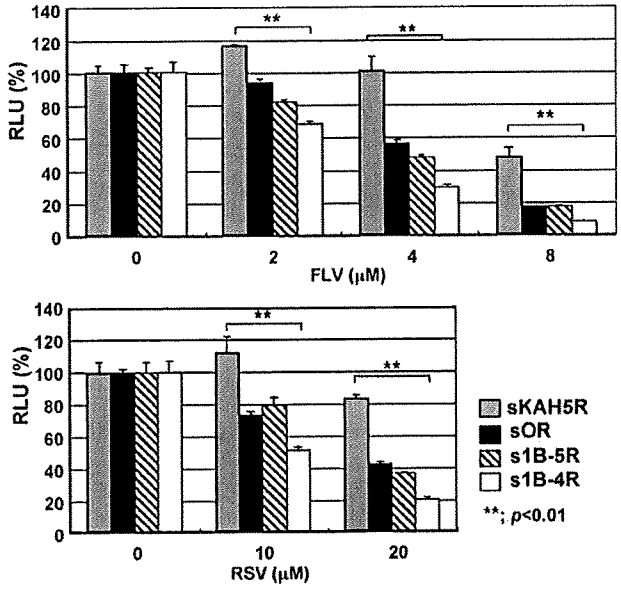


**Fig. 7.** HCV replicons exhibit diverse sensitivities to statins. Reporter assay of the sensitivity of HCV replicons to FLV. sKAH5R cells (light column), sOR cells (dark column) s1B-5R cells (shaded column), and s1B-4R cells (open column) were treated with FLV (0, 2, 4, and 8 μM) for 72 h (upper panel), and then the cells were subjected to an RL assay. A reporter assay of RSV sensitivity to HCV replicons was performed using RSV (0, 10, and 20 μM) (lower panel). All of the luciferase assays were repeated at least three times.

The polyclonal sKAH5R cells exhibited less sensitivity to PTV, FLV, and RSV than did polyclonal s1B-4R cells. These results suggest that the statin-resistant phenotype of sKAH5R is due to the KAH5 strain-specific viral factors rather than to the cell clonality of sKAH5R cells.

### 3.6. Second generation of sKAH5R possessed less sensitive phenotype to PTV than that of s1B-4R

To further demonstrate that the statin-resistant phenotype of sKAH5R is not due to the clonal specificity of the cells, we developed the second generation of sKAH5R and s1B-4R. Total RNAs from sKAH5R and s1B-4R were introduced into naïve OR6c cells. The second generation of sKAH5R and s1B-4R, designated as ssKAH5R and ss1B-4R, respectively, were selected as the polyclonal cells after 3 weeks G418 selection. ssKAH5R revealed less sensitive to PTV than ss1B-4R ($EC_{50}$: 0.76 μM vs. 0.43 μM) (Fig. 9A). These results further support that the viral factor plays the major role in the statin-resistant phenotype of sKAH5R.

On the contrary, there was no significant difference between ssKAH5R and ss1B-4R in the sensitivity to IFN-λ ($EC_{50}$: 4.1 ng/ml vs. 3.5 ng/ml) (Fig. 9B). These results suggest that cellular factors are dominant in the sensitivity to IFN-λ.

**Table 1**
$EC_{50}$ of anti-HCV reagents to HCV replicons.

| | s1B-4R | sKAH5R | sOR | s1B-5R |
|---|---|---|---|---|
| IFN-α (IU/ml) | 1.44 | 1.37 | 2.35 | 1.10 |
| IFN-γ (IU/ml) | 0.54 | 2.26 | 0.33 | 0.21 |
| IFN-λ (ng/ml) | 1.50 | 8.25 | 4.48 | 4.82 |
| CsA (μg/ml) | 0.71 | 0.96 | 1.10 | 0.85 |
| PTV (μM) | 0.40 | 1.00 | 0.64 | 0.51 |
| FLV (μM) | 2.82 | 7.87 | 4.53 | 3.81 |
| RSV (μM) | 10.12 | ND | 17.52 | 17.10 |

ND: not determined.

## 4. Discussion

In the present study, we established an HCV replicon reporter assay system using four genotype 1b HCV strains (1B-4, KAH5, O, and 1B-5). Genotype 1 HCV infection accounts for most cases of resistance to current PEG-IFN-α and ribavirin therapy. However, in most previous reports, anti-HCV reagents have been assessed in terms of their effects using replicon(s) derived from only one or two HCV strain(s). Therefore, in order to further evaluate the anti-HCV activity of various reagents among the genotype 1b HCVs, we performed a comparative study using the present replicon reporter assay system, which was found to a precise, highly sensitive, and time-sparing assay compared to assays involving the quantification of HCV RNA. The $EC_{50}$ values of anti-HCV reagents in four genotype 1b replicons are summarized in Table 1.

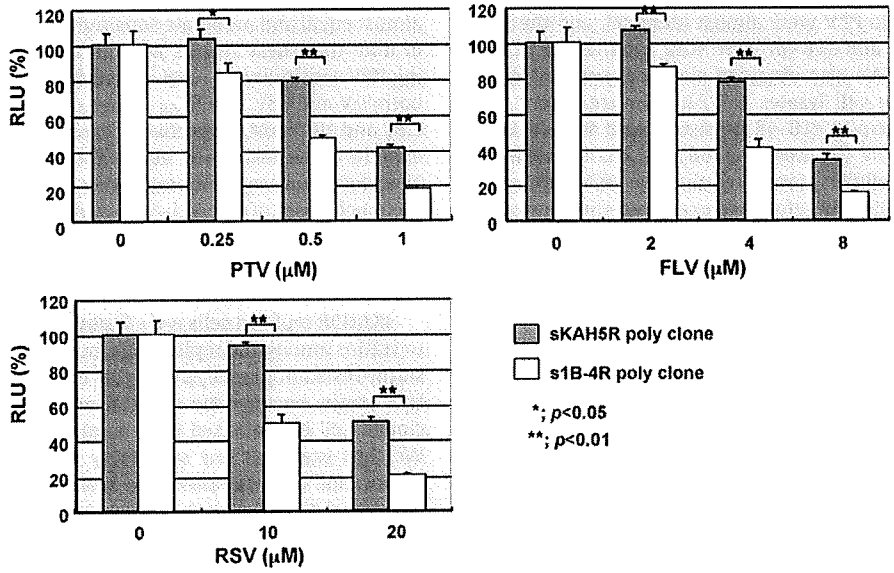


**Fig. 8.** Diverse sensitivities of polyclonal replicons to statins. Reporter assay of the sensitivity of polyclonal sKAH5R and s1B-4R replicons to PTV, FLV, and RSV. Polyclonal sKAH5R cells and polyclonal s1B-4R cells were treated with PTV (0, 0.25, 0.5, and 1 μM), FLV (0, 2, 4, and 8 μM), and RSV (0, 10, and 20 μM) for 72 h and then were subjected to RL assay. All the luciferase assays were repeated at least three times.

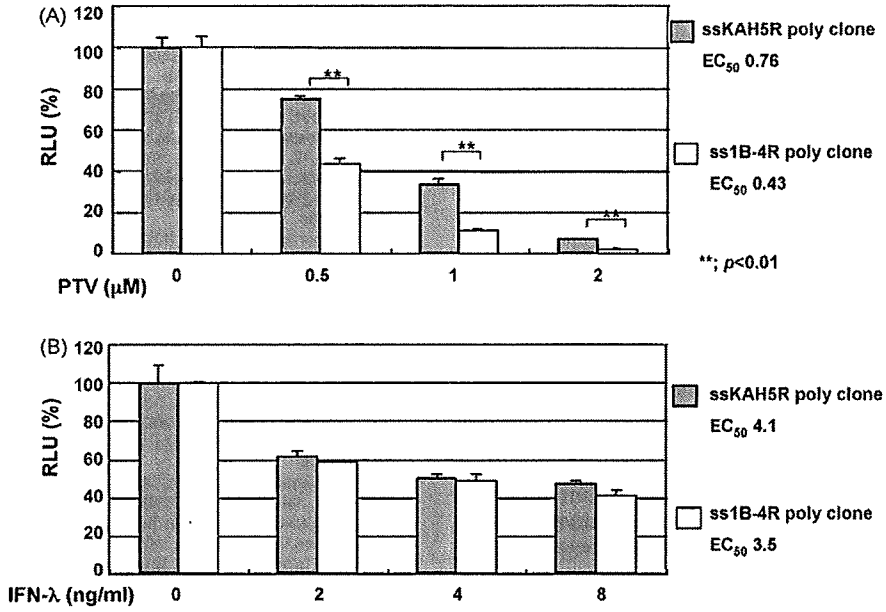


**Fig. 9.** The sensitivities of the second generation of sKAH5R and s1B-4R to PTV and IFN-λ. Polyclonal second generations of sKAH5R (ssKAH5R) and s1B-4R (ss1B-4R) were treated with PTV (0, 0.5, 1, and 2 μM) (A) and IFN-λ (0, 2, 4, and 8 ng/ml) (B) for 72 h and then were subjected to RL assay. All the luciferase assays were repeated at least three times.

Here, sOR exhibited the lowest level of sensitivity to IFN-α ($EC_{50}$: 2.35 IU/ml). In the clinical setting, high titers of HCV RNA are among the determining factors for IFN resistance. However, the sensitivity to IFN-α was found to be greater in the case of s1B-4R and sKAH5R than in sOR, although HCV RNA titers were higher than those of sOR. These results suggest that factor(s) other than the HCV RNA level may be involved in conferring sensitivity to IFN-α, and the genetic background of sOR may serve as a candidate for interpreting differences in IFN-α sensitivity among the genotype 1b HCVs tested. Previously the structural region of HCV was shown to be involved in viral resistance to type I IFN (Taylor et al., 1999). Therefore, the development of genome-length HCV RNA reporter systems from 1B-4, 1B-5, and KAH5R strains in addition to our developed OR6 cells will overcome the limitation of subgenomic replicon and will become powerful tool for the study of anti-HCV reagents including IFNs. Now we are planning to develop the genome-length HCV RNA reporter systems using these three HCV strains.

sKAH5R exhibited the lowest level of sensitivity to IFN-γ ($EC_{50}$: 2.26 IU/ml) among the replicons tested, as it is approximately 10 times more resistant to IFN-γ than s1B-5R ($EC_{50}$: 0.21 IU/ml). KAH5 was the only HCV strain derived from a patient with AH C in the present study. In our previous study, an AH1 strain derived from a patient with AH C also exhibited lower sensitivity to IFN-γ ($EC_{50}$: 1.9 IU/ml) than did O strain ($EC_{50}$: 0.3 IU/ml) in the genome-length HCV RNA replication system (Mori et al., 2008). In both subgenomic and genome-length HCV RNA replication systems, HCV strains from patients with AH C possess less sensitivity to IFN-γ than do HCV strains from healthy carriers. These results suggest that the NS region of HCV derived from AH C may be involved in IFN-γ resistance.

In 2003, IFN-λ was identified by two groups at the same time, and this novel IFN was classified as type III IFN. IFN-λ shares the Jak/Stat signaling pathway with the type I IFNs, although they bind to distinct membrane receptors, i.e., type I IFNs bind to the heterodimer of IFNAR1 and IFNAR2, whereas type III IFNs bind to the heterodimer of IFNLR1 and IL10R2 (Uze and Monneron, 2007). Therefore, we expected to obtain similar IFN-α sensitivity results in the four replicons tested here. However, unexpectedly, the profiles of replicon sensitivity to IFN-α and IFN-λ differed. The sensitivity of s1B-4R to IFN-λ was approximately five times greater than that of sKAH5R, although the sensitivities of these replicons to IFN-α were almost identical. There are several possible interpretations of these unexpected findings. First, an unidentified branched signaling pathway may account for variation in the anti-HCV activity of IFN-λ. Second, expression levels of the receptor for IFN-λ may vary. The second generation replicon assays suggest that the cellular factors may be dominant in the sensitivity to IFN-λ. Further study will be needed to clarify this issue. The anti-HCV activity of IFN-λ has already been reported by several groups using HCV RNA-harboring cells (Doyle et al., 2006; Marcello et al., 2006; Robek et al., 2005). The present study was the first to demonstrate the diverse anti-HCV activities of IFN-λ on HCV replicons.

In the case of CsA, we did not observe any significant differences among the genotype 1b replicons. Using a genome-length HCV RNA replication system, we recently demonstrated that an AH1 strain obtained from an AH C patient showed greater sensitivity than did an O strain (Mori et al., 2008). There are two possible explanations for this high sensitivity to CsA in the replicon derived from the AH C case: first, a high degree of sensitivity to CsA may not be a common feature in HCV from AH C and may instead be strain-dependent; second, a particular structural region of HCV from AH C may be responsible for this high level of sensitivity to CsA. However, further study will be needed to fully account for these findings.

Respectively, sKAH5R and s1B-4R exhibited the lowest ($EC_{50}$: 1.00 μM) and highest ($EC_{50}$: 0.40 μM) levels of sensitivity to PTV among the replicons tested. We also confirmed that sKAH5R and s1B-4R possessed the lowest and highest levels of sensitivity, respectively in both FLV and RSV treatment. Therefore, resistance to statins may be a unique feature of sKAH5R. It should be noted that we obtained these results using polyclonal sKAH5R and polyclonal s1B-4R cells. The polyclonal sKAH5R cells were less sensitive to PTV, FLV, and RSV than were the polyclonal s1B-4R cells. Therefore, the statin-resistant phenotype of sKAH5R cells is due to a KAH5 strain-specific characteristic, rather than to the clonality of the cells. The second generation of replicon harboring cells, ssKAH5R and ss1B-4R, further supported that the viral factor plays the major role in the statin-resistant phenotype of sKAH5R. These two cell lines with contrasting sensitivity to statins promise to be useful for

determining the statin resistance-responsible region of HCV and also for investigating the anti-HCV mechanism of statins in general.

In the present study, we demonstrated the diverse profiles of four HCV replicons to anti-HCV reagents. sKAH5R showed the lowest sensitivity to IFN-γ, IFN-λ, and statins (PTV, RSV, and FLV). In contrast, s1B-4R exhibited the highest level of sensitivity to IFN-λ and statins (PTV, RSV, and FLV). sKAH5R and s1B-4R possessed a sensitive and a resistant phenotype to various anti-HCV reagents. The nucleotide sequences in the NS3-NS5B regions of 1B-4, 1B-5, and KAH5 strains showed differences of 6.5%, 8.6%, and 6.1%, respectively, from those of the O strain. Similarly, the amino acid sequences in the NS3–NS5B regions of 1B-4, 1B-5, and KAH5 strains showed differences of 2.6%, 4.7%, and 2.5%, respectively, from those of the O strain. Phylogenetic analysis revealed that O, 1B-4, and KAH5 strains formed the cluster different from 1B-5 strain (Supplemental Fig. 3). These data indicate that sKAH5R and s1B-4R are at a similar genetic distance from the O strain. These two replicons were also found to possess similar features in terms of HCV RNA expression levels and sensitivity to IFN-α. Therefore, sKAH5R and s1B-4R are expected to be useful tools for comparative analyses of anti-HCV determining factors of HCV, especially as regards IFN-λ and statins.

In conclusion, we established an HCV replicon reporter assay system with four different genotype 1b HCV strains. This replicon system is a useful tool for investigating differences in sensitivity to anti-HCV reagents among genotype 1b HCV strains, and it is expected to increase the rate of resolution of HCV cases otherwise resistant to current IFN therapy.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2009.01.007.

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# HCV replication suppresses cellular glucose uptake through down-regulation of cell surface expression of glucose transporters☆

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See Editorial, pages 845–847

***Background/Aims*: Persistent infection with hepatitis C virus (HCV) causes extrahepatic diseases, including diabetes. We investigated the possible effect(s) of HCV replication on cellular glucose uptake and expression of the facilitative glucose transporter (GLUT) 2 and 1.**

***Methods*: We used Huh-7.5 cells harboring either an HCV subgenomic RNA replicon (SGR) or an HCV full-genomic RNA replicon (FGR), HCV-infected cells, and the respective cells treated with interferon (IFN). We also used liver tissue samples obtained from patients with or without HCV infection.**

***Results*: Glucose uptake and surface expression of GLUT2 and GLUT1 were suppressed in SGR, FGR and HCV-infected cells compared to the control cells. Expression levels of GLUT2 mRNA, but not GLUT1 mRNA, were lower in SGR, FGR and HCV-infected cells than in the control. Luciferase reporter assay demonstrated decreased GLUT2 promoter activities in SGR, FGR and HCV-infected cells. IFN treatment restored glucose uptake, GLUT2 surface expression, GLUT2 mRNA expression and GLUT2 promoter activities. Also, GLUT2 expression was reduced in hepatocytes of liver tissues obtained from HCV-infected patients.**

***Conclusions*: HCV replication down-regulates cell surface expression of GLUT2 partly at the transcriptional level, and possibly at the intracellular trafficking level as suggested for GLUT1, thereby lowering glucose uptake by hepatocytes.**


***Keywords*: Diabetes mellitus; Down-regulation; Glucose uptake; GLUT1; GLUT2; Hepatitis C virus; Hepatocyte; Interferon; Replicon**

## 1. Introduction

Hepatitis C virus (HCV) is a small, enveloped RNA virus, which belongs to the genus *Hepacivirus* within the family *Flaviviridae*. The viral genome consists of single-stranded, positive-sense RNA of 9.6 kb that encodes a polyprotein of about 3000 amino acids. There are six major genotypes of HCV worldwide, with each genotype being further classified into a number of subtypes, such as HCV-1a and -1b [1,2]. The polyprotein is processed by host cellular and viral proteases to yield at least 10 structural and nonstructural (NS) proteins, such

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*Abbreviations:* FGR, full-genome RNA replicon; GLUT, glucose transporter; HBV, hepatitis B virus; HCV, hepatitis C virus; IFN, interferon; SGR, subgenomic RNA replicon.

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as core protein, envelope glycoproteins (E1 and E2), p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B [3,4].

HCV prevails in most parts of the world with an estimated number of about 170 million carriers and, hence, HCV infection is a major global healthcare problem [5]. Persistent infection with HCV causes not only liver diseases, including hepatitis, but also extrahepatic manifestations, such as type 2 diabetes [6–8]. While it has been known that liver cirrhosis impairs the glucose metabolism of the liver, there are some reports showing that HCV-infected patients over 40 years old have an increased risk for type 2 diabetes – three times higher than that for patients without HCV infection [9,10]. These reports imply the possibility that HCV infection directly predisposes the host towards type 2 diabetes. However, the precise mechanism(s) is poorly understood.

Glucose is transported into the cell via various isoforms of the facilitative glucose transporter (GLUT) that are present in most cells. Currently, a total of 14 isoforms have been identified in the GLUT family [11–13]. GLUT2 is expressed tissue-specifically in the liver, pancreatic β-cells, hypothalamic glial cells, retina and enterocytes [14]. On the other hand, GLUT1 is expressed at high levels in all fetal tissues and, in adults, it is widely expressed but most abundant in erythrocytes, endothelial cells of the blood–brain barrier, renal tubules of the kidney, and any kind of malignant cells including hepatocellular carcinoma [13].

In the present study, we demonstrated that HCV infection suppressed hepatocytic glucose uptake through down-regulation of surface expression of GLUT in a human hepatocellular carcinoma-derived cell line Huh-7.5. We also demonstrated that GLUT2 expression in hepatocytes of the liver tissues from HCV-infected patients was lower than in those from patients without HCV infection. We propose that HCV replication decreases glucose uptake and cell surface expression of GLUT, which would eventually lead to glucose metabolism disorder.

## 2. Materials and methods

### 2.1. Cell culture, HCV RNA replication, HCV infection and IFN treatment

A human hepatoma-derived cell line, Huh-7.5, which is highly permissive to HCV RNA replication [15], was kindly provided by Dr. C.M. Rice (The Rockefeller University, New York, NY, USA). The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum.

Huh-7.5 cells stably harboring an HCV-1b subgenomic RNA replicon (referred to as SGR cells, hereafter) were prepared as describe previously [16–18], using pFK5B/2884Gly (a kind gift from Dr. R. Bartenschlager, University of Heidelberg, Heidelberg, Germany). In SGR cells, the HCV subgenomic RNA replicon autonomously replicates to express NS3 to NS5B of HCV (Fig. 1). Cells harboring a full-length HCV-1b RNA replicon derived from pON/C-5B (referred to as FGR cells, hereafter) were described previously [19,20]. In FGR cells, the genome-size HCV RNA replicon autonomously replicates to express all the HCV proteins (the core protein, E1, E2, p7, NS2, NS3 to NS5B).

The pFL-J6/JFH1 plasmid that encodes the entire viral genome of a chimeric strain of HCV-2a, J6/JFH1 [21], was kindly provided by Dr. C.M. Rice. The HCV RNA genome was transcribed *in vitro* from pFL-J6/JFH1 and transfected to Huh-7.5 cells. The virus produced in the culture supernatant was used for infection experiments at multiplicities of infection of 1.0 and cultured for 5 days after virus infection.

In some experiments, SGR and FGR cells, as well as HCV-infected cells at 5 days after virus infection, were treated with 1000 IU/ml of IFN (Sigma, St. Louis, MI, USA) for 10 days to eliminate HCV replication.

### 2.2. Immunofluorescence

Cells were fixed with 3.7% paraformaldehyde and incubated with mouse monoclonal antibody against HCV NS5A (Chemicon International, Inc., Temecula, CA, USA) or HCV core (Abcam, Tokyo, Japan). The cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (MBL Co. Ltd., Nagoya, Japan), and observed under a fluorescent microscope (BX51; Olympus, Tokyo, Japan).

### 2.3. Immunoblotting

Cells were solubilized in lysis buffer as reported previously [22]. The cell lysates were electrophoresed subjected to 8% polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane (Millipore Corp., Billerica, MA, USA). The membranes were incubated with mouse monoclonal antibodies against HCV NS5A or NS3 (Chemicon), followed by incubation with peroxidase-conjugated goat anti-mouse IgG (MBL). The positive bands were visualized by using ECL detection system (GE Healthcare UK Ltd., Buckinghamshire, UK).

### 2.4. Uptake of 2-deoxy-D-glucose and thymidine

Cells cultured in 12-well plates were deprived of serum by incubation in serum-free medium for 12 h. The cells were then pre-incubated for 20 min in 450 μl of KRH (25 mM Hepes, 120 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 1.3 mM $KH_2PO_4$ and 0.1% BSA, pH 7.4). Glucose uptake assay was performed as describe previously [23]. In brief, glucose uptake was initiated by addition of 50 μl of reaction solution (KRH containing 0.5 mM, 0.25 μCi 2-deoxy-D-[1,2-$^3$H]glucose) to each well. As a negative control, 100 μM phloretin was added to reaction solution. After 10 min, transport was terminated by washing the cells with ice-cold KRH buffer containing 100 μM phloretin. The cells were solubilized by 0.1% sodium dodecyl sulfate, and the incorporated radioactivity was measured by liquid scintillation counter (LS6500; Beckman Coulter, Fullerton, CA). In some experiments, GLUT1 and GLUT2 were ectopically expressed by using the pCAGGS expression vector [24] and glucose uptake was measured as described above.

### 2.5. Flow cytometry

To examine cell surface expression of GLUT1 and GLUT2, cells harvested in PBS containing 0.2% EDTA were incubated with rabbit polyclonal antibodies against GLUT1 or GLUT2 (1:200; Alpha Diagnostic International, San Antonio, TX, USA) on ice for 1 h. After being washed, the cells were incubated with FITC-labeled goat anti-rabbit IgG (1:200; BD Pharmingen, Franklin Lakes, NJ, USA) on ice for another 1 h. Analysis was carried out using flow cytometer and a total of 10,000 live cell events were measured. Results were displayed graphically as overlaying histograms demonstrating the shift of the mean FITC staining value.

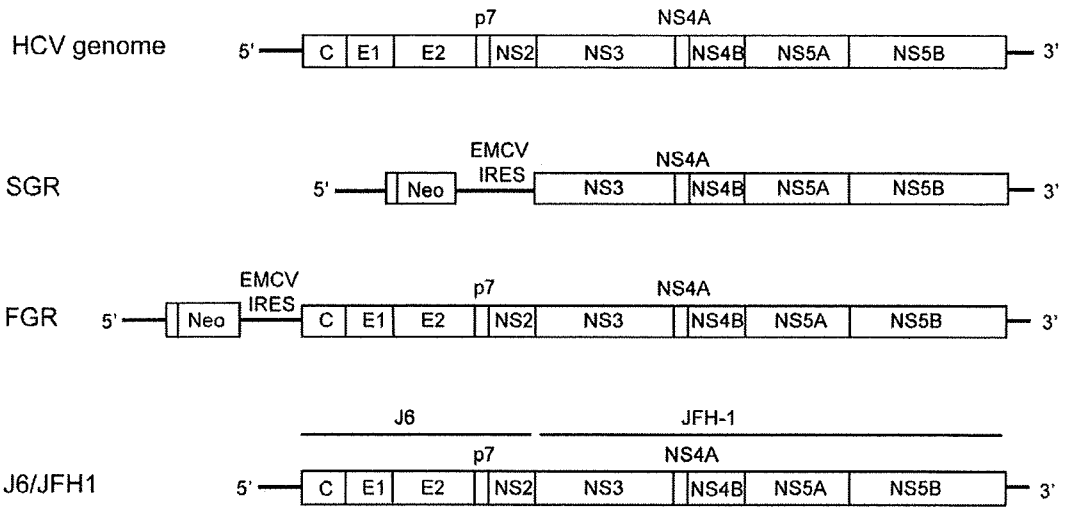


**Fig. 1. The HCV genome and HCV RNA replicons. Schematic diagram of the HCV genome, SGR, FGR and the chimeric HCV J6/JFH1 genome are shown. EMCV IRES, encephalomyocarditis virus internal ribosome entry site; Neo, neomycin-resistance gene.**

### 2.6. Real-time quantitative RT-PCR

Total cellular RNA was isolated using the TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA) and cDNA was generated using QuantiTect Reverse Transcription system (Qiagen, Valencia, CA, USA). Real-time quantitative PCR was performed on a SYBR *Premix Ex Taq* (Takara Bio, Kyoto, Japan) using SYBR green chemistry in ABI PRISM 7000 (Applied Biosystems, Foster, CA, USA). β-Glucuronidase was used as an internal control. The primers used are shown in Table 1.

### 2.7. Luciferase reporter assay

We constructed the human GLUT2 promoter-luciferase reporter gene (pGLUT2-1291Luc) by cloning a 1.6-kb genomic fragment that encompasses the human GLUT2 promoter (−1291 to +308) [14] into the pGL4 vector plasmid (Promega, Madison, WI, USA). pGLUT2-1291Luc thus contains a 1291-bp fragment of the human GLUT2 promoter upstream of the minimal promoter and the coding sequence of the *Photimus pyralis* (firefly) luciferase. pRL-CMV-*Renilla* (Promega) was used as an internal control. Cells were transfected with pGLUT2-1291Luc (1 μg) and pRL-CMV-*Renilla* (10 ng). After 24 h, a luciferase assay was performed by using Dual-luciferase reporter assay system (Promega). Firefly and *Renilla* luciferase activities were measured by Lumat LB 9501 (Berthold, Bad Wildbad, Germany). Firefly luciferase activity was normalized to *Renilla* luciferase activity for each sample.

### 2.8. Immunohistochemistry

Human adult liver autopsy materials and surgically removed liver tissues of patients with HCV- or HBV-associated hepatocellular carcinoma, and those with metastatic liver cancer were obtained with written informed consent. The tissues were fixed with 10% buffered formalin (pH 7.0), embedded in paraffin and sectioned at intervals of 4 μm. Immunohistochemical staining was performed with a DAKO ENVISION+ Kit (Dako, Glostrup, Denmark). In brief, fixed sections were treated with 3% hydrogen peroxide, and were autoclaved at 121 °C for 20 min. Then, the sections were incubated with a blocking solution and then with either anti-GLUT2 rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or normal rabbit IgG (Santa Cruz Biotechnology) as a control. The sections were incubated with horseradish peroxidase-labeled polymer-conjugated goat anti-rabbit IgG, followed by incubation in a chromogenic solution. The sections were then counterstained with hematoxylin and examined with a light microscope. GLUT2 expression levels were arbitrarily determined by two examiners, including a pathologist, in a blinded manner.

### 2.9. Statistical analysis

Results were expressed as mean ± SEM. Statistical significance was evaluated by ANOVA, and statistical significance was defined as $P < 0.05$.

**Table 1**
**Sequences and positions of the primers used in this study.**

| Gene name (GenBank ID) | Primer | Position | PCR product (bp) |
|---|---|---|---|
| GLUT2 | 5′-TGGGCTGAGGAAGAGACTGT-3′ | 279–298 | 461 |
| (J03810) | 5′-AGAGACTGAAGGATGGCTCG-3′ | 739–720 | |
| GLUT1 | 5′-TGAACCTGCTGGCCTTC-3′ | 437–453 | 399 |
| (AK292791) | 5′-GCAGCTTCTTTAGCACA-3′ | 835–819 | |
| HCV NS5B | 5′-ACCAAGCTCAAACTCACTCCA-3′ | 9191–9211 | 119 |
| (AJ238799) | 5′-AGCGGGGTCGGGCACGAGACA-3′ | 9309–9289 | |
| β-glucuronidase | 5′-ATCAAAAACGCAGAAAATACG-3′ | 1747–1767 | 238 |
| (M15182) | 5′-ACGCAGGTGGTATCAGTCTTG-3′ | 1984–1964 | |

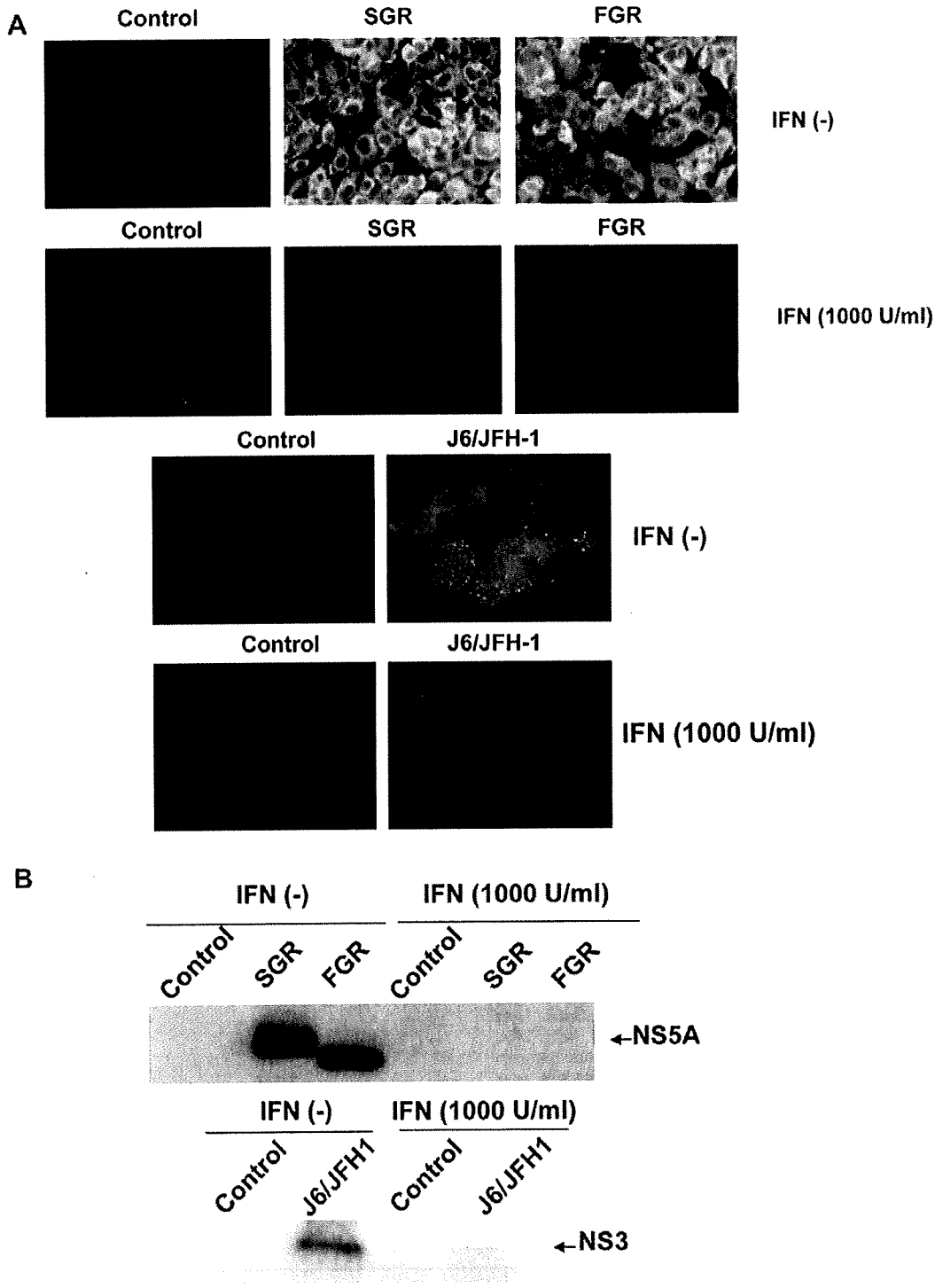


Fig. 2. Expression of HCV proteins in SGR, FGR, HCV-infected cells and the respective cells treated with IFN. (A) Cells were immunostained with anti-NS5A antibody (for SGR, FGR and the control cells) or anti-core antibody (for HCV-infected cells and the control). In parallel, cells were treated with IFN (1000 IU/ml) for 10 days to eliminate HCV replication before being subjected to immunostaining. (B) Cells were analyzed by immunoblotting with anti-NS5A antibody (upper panel) or anti-NS3 antibody (lower panel). In parallel, cells were treated with IFN (1,000 IU/ml) for 10 days to eliminate HCV replication before being subjected to immunoblotting.

## 3. Results

### 3.1. HCV protein expression in SGR, FGR, HCV-infected cells and those treated with IFN

Immunofluorescence analysis revealed that almost all the cells in SGR and FGR cultures, and >90% of the cells in the HCV J6/JFH1-infected culture were positive for HCV antigens (Fig. 2A). Western blot analysis also confirmed HCV protein expression in SGR, FGR and HCV-infected cells (Fig. 2B). In some experiments, HCV replication in SGR, FGR and HCV-infected cells was eliminated by IFN treatment for 10 days (Fig. 2A and B).

### 3.2. Selective suppression of cellular glucose uptake by HCV replication

2-Deoxygluose uptake levels in SGR, FGR and HCV-infected cells were significantly suppressed by about 50–60%, compared with the control Huh-7.5 cells (Fig. 3A and B). On the other hand, thymidine uptake, which was used as a control, did not significantly differ among all the cells tested (data not shown). Moreover, glucose uptake levels in SGR, FGR and HCV-infected cells were restored by IFN treatment (Fig. 3A and B). These results strongly suggest that cellular glucose uptake is selectively suppressed by HCV RNA replication.

### 3.3. Down-regulation of cell surface expression of GLUT2 and GLUT1 by HCV replication

GLUT2 is the principal glucose transporter of hepatocytes *in vivo* while GLUT1 is expressed in a wide variety of cultured cells. We therefore examined cell surface expression of GLUT2 and GLUT1 by flow cytometry analysis. As shown in Fig. 4A, cell surface expression of GLUT2 and GLUT1 was markedly down-regulated in SGR and FGR cells, compared with the control. On the other hand, cell surface expression of transferrin receptor was not significantly suppressed in SGR or FGR, compared with the control, with the result ensuring the specificity of the down-regulation of GLUT2 and GLUT1 cell surface expression in SGR and FGR (Fig. 4A). Moreover, treatment of SGR and FGR cells with IFN restored the surface expression of GLUT2 and GLUT1 (Fig. 4A). These results suggest that HCV RNA replication specifically mediates down-regulation of GLUT2 and GLUT1.

Down-regulation of GLUT2 surface expression was observed also in HCV-infected cells (Fig. 4B). On the other hand, down-regulation of GLUT1 surface expression was only marginal and, compared to that of GLUT2, less evidently observed in HCV-infected cells. As a control, cell surface expression of transferrin receptor did not differ at all between HCV-infected cells and the control. Again, treatment of HCV-infected cells with IFN restored surface expression of GLUT2 (Fig. 4B).

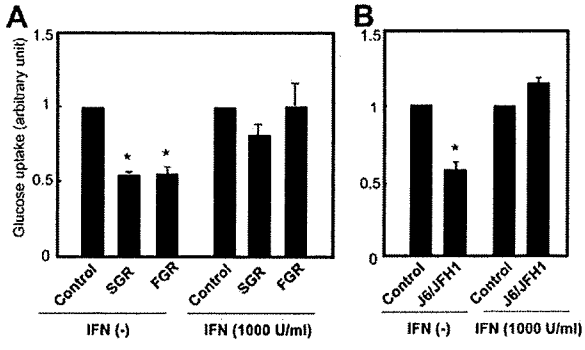


**Fig. 3. Selective suppression of cellular glucose uptake by HCV replication. (A) Uptake of 2-deoxy-D-[1,2-$^3$H] glucose in SGR, FGR and HCV-negative control. In parallel, cells were treated with IFN (1000 IU/ml) for 10 days to eliminate HCV replication before being subjected to glucose uptake analysis. Data represent mean ± SEM of four independent experiments and the values for the control cells were arbitrarily expressed as 1.0. $^*P < 0.01$, compared with the control. (B) Uptake of 2-deoxy-D-[1,2-$^3$H] glucose in J6/JFH-1-infected cells and the uninfected control. In parallel, cells at 5 days after infection were treated with IFN (1000 IU/ml) for 10 days to eliminate HCV replication before being subjected to glucose uptake analysis.**

### 3.4. Proteasomal degradation is not involved in the down-regulation of GLUT2 or GLUT1

Some viruses down-regulate cell surface molecules, such as immunoreceptors and intercellular adhesion molecules, through ubiquitination and proteasomal degradation of the target proteins [25]. To test this possibility, we treated SGR and FGR cells with lactacystin, a potent proteasome inhibitor. While lactacystin treatment enhanced cell surface expression of transferrin receptor, the same treatment did not increase cell surface expression of GLUT2 or GLUT1 in SGR or FGR cells (Fig. 5). This result suggested that down-regulation of cell surface expression of GLUT2 or GLUT1 in HCV-replicating cells was not due to increased degradation through the ubiquitin–proteasome system. The result rather implied the possible involvement of another mechanism(s), e.g., transcriptional suppression and/or impaired intracellular trafficking.

### 3.5. Transcriptional suppression of GLUT2, but not GLUT1, by HCV replication

To examine whether HCV RNA replication suppresses GLUT2 and GLUT1 expression at the transcriptional level, we measured mRNA expression levels by quantitative RT-PCR. The results obtained revealed that GLUT2 mRNA levels were reduced significantly in SGR, FGR and HCV-infected cells, compared to the control (Fig. 6A). It should be noted that the degree of GLUT2 mRNA suppression was greater in FGR