

with the difference between first generation control and IFN-α-resistant cells. Together with these studies, the present results suggest that cellular factors play a predominant role in the IFN-resistance phenotype in cell culture.

The present study is the first report on the establishment of IFN-resistant genome-length HCV RNA-harboring cells, because previous IFN-resistant cells were established using subgenomic replicons.^{21,22,32,33} Recently, it was reported that the 70th AA (Q or H) and 91st AA (M) in the core region are responsible for the

resistance to PEG IFN and ribavirin therapy.²³ Core AA (70th/91st) of the original HCV-O sequence in OR6 is (Q/L). Therefore, the original HCV-O sequence is a partially resistant type based on this criterion of the core sequence (70th/91st). We could not detect any change in these AA positions in the core region after IFN treatment in IFN-resistant cells. To clarify the role of the core sequence (70th/91st) in IFN resistance, we are now planning to make the HCV-O with wild and mutant type sequences at 70th/91st in the core region. These HCV will provide us with important information regard-

Figure 5 The prevention of the relapse of hepatitis C virus (HCV) in interferon (IFN)- α -resistant cells. (a) Schema of the relapse cell culture assay. The control cells (OR6(C)) or the IFN- α -resistant cells (OR6(R)) were treated with IFN- α alone or in combination with IFN- γ , FLV, or CsA for 72 h. Then the cells were cultured for 0, 24, 48, and 72 h for luciferase assay and for 120 h for western blot analysis. (b and c) Luciferase assay and western blot analysis for the cells treated with IFN- α . The control cells were treated with IFN- α (250 IU/mL) for 72 h and subjected to luciferase assay. The IFN- α -resistant cells were treated with IFN- α at 250 IU/mL and 1000 IU/mL for 72 h and subjected to luciferase assay. The cells treated with the conditions above were further cultured for 48 h and subjected to western blot analysis using the core antibody. OR6c was used as a negative control (lane 1). OR6(C) and OR6(R) treated with IFN- α under the conditions shown above were also subjected to western blot analysis (lanes 2–4). (d and e) Luciferase assay and western blot analysis for the cells treated with IFN- α alone or in combination with IFN- γ . OR6(R) were treated with IFN- α (250 IU/mL and 1000 IU/mL) alone, or IFN- α (250 IU/mL) and IFN- γ (4 IU/mL) and subjected to luciferase assay and western blotting analysis. (f and g) Luciferase assay and western blot analysis for the cells treated with IFN- α alone or in combination with FLV. OR6(R) were treated with IFN- α (250 IU/mL and 1000 IU/mL) alone, or IFN- α (250 IU/mL) and FLV (10 μ M) and subjected to luciferase assay and western blot analysis. (h and i) Luciferase assay and western blot analysis for the cells treated with IFN- α alone or in combination with CsA. OR6(R) were treated with IFN- α (250 IU/mL and 1000 IU/mL) alone, or IFN- α (250 IU/mL) and CsA (0.5 μ g/mL) and subjected to luciferase assay and western blot assay. (j) Time course of HCV RNA levels before and after withdrawal of anti-HCV reagents in IFN- α resistant cells. OR6(R) were treated with IFN- α (250 IU/mL and 1000 IU/mL) alone, or IFN- α (250 IU/mL) in combination with IFN- γ (4 IU/mL), FLV (10 μ M), or CsA (0.5 μ g/mL) for 72 h. Then, anti-HCV reagents were removed and the cells were further cultured for 72 h.



ing IFN-resistant mechanism related to core sequences. The IFN-sensitivity-determining region (ISDR) in NS5A has also been reported as the region responsible for IFN resistance.³⁴ There is no AA substitution in ISDR in the original HCV-O sequence. Therefore, the original HCV-O sequence is a resistant-type based on the criterion of the ISDR. More recently, El-Shamy *et al.*³⁵ reported that the sequence variation in IFN/ribavirin resistant-determining region (IRRDR) (2334–2379 AA in NS5A) could correlate with SVR on IFN/ribavirin therapy. In the original HCV-O sequence, there were four AA substitutions in IRRDR. Therefore, original HCV-O sequence is a resistant-type based on the criterion of the IRRDR. We could not detect any mutations in NS5A in IFN-resistant cells. In a previous study using subgenomic 1B-1 and Con1 replicon, AA substitutions were frequently seen in NS5A.^{21,22} The NS3 is another region in which frequent mutations occurred in 1B-1 and Con1 replicons with the IFN-resistance phenotype. The mutations P1115L and E1202G in NS3 have been identified in HCV sequences of OR6(C) cells and OR6(R) cells, respectively. We previously reported that these mutations are adaptive mutations, and that E1202G has a stronger enhancing effect on HCV RNA replication than P1115L.³⁶ This difference in the effects of adaptive mutations might be at least partly responsible for the diverse sensitivity of HCV to IFN- α . In the present study, we couldn't identify mutations responsible for IFN-resistance, because the effect of the viral mutations on IFN-resistance seems to be not so strong compared with the cellular factor and the possible combinations of mutations are so large.

The advantages of using the reporter HCV RNA system are its precision and high-sensitivity for monitoring HCV RNA replication. The system is especially useful for the detection of HCV RNA replication at near undetectable levels. The monitoring of HCV relapse after combination treatment with IFN- α and anti-HCV reagents is a good example of the use of this assay system. Therefore, we tried to develop a relapse cell culture model using the established IFN- α -resistant cells. The IFN- α -resistant HCV RNA-harboring cells are less sensitive to IFN- α and IFN- β than control cells. However, both cell lines possessed similar sensitivity to IFN- γ , CsA, and FLV. The luciferase assay revealed that 250 and 1000 IU/mL of IFN- α was needed to prevent the relapse of HCV in control cells and IFN- α -resistant cells, respectively. Four IU/mL of IFN- γ , 0.5 μ g/mL of CsA, or 5 μ M of FLV could help to prevent the relapse of HCV in combination with 250 IU/mL of IFN- α . These results suggest that IFN- γ , CsA, and FLV are good partners with IFN- α in combination therapy to prevent the relapse of IFN- α -resistant HCV.

The relapse model in the present study corresponds with a relapse just after the end of IFN treatment in clinical therapy. The evaluation for the relapse required only 72 h by luciferase assay. As we described in the first section of the Results, we need an additional 3 weeks to confirm the complete elimination of HCV or SVR in the cell culture model by colony formation assay. To further evaluate the complete elimination, of HCV, a more sensitive colony formation assay is needed. For example, although 4 weeks of IFN- α treatment at 10 IU achieved the inhibition of the relapse, G418-resistant colonies

emerged after 3 weeks. We are currently planning a study on the SVR cell culture model using established IFN- α -resistant HCV RNA-harboring cells.

To use our cell culture system as the relapse model, there are some limitations derived from the differences between cell culture and humans: (i) SVR in patients with CHC is assessed by the serum HCV RNA. However, our system monitors the intracellular HCV RNA levels; and (ii) HuH-7 cells lack several innate immunities, such as TRL3 in cell surface. To date, we don't have a genotype 1b HCV infection system and cell lines other than HuH-7 cells, which support robust HCV RNA replication. The development of a genotype 1b HCV infection system and cell line will overcome the limitation and improve our present relapse model.

In conclusion, we established IFN- α -resistant, HCV-harboring cells from genome-length HCV RNA-harboring OR6 cells. In addition, we developed a relapse model using the IFN- α -resistant HCV RNA-harboring cells. The anti-HCV reagents, which exhibited a mechanism different from IFN- α efficiently, could prevent the relapse of HCV in IFN- α -resistant HCV RNA-harboring cells. This cell culture model should prove useful for the study of relapse after IFN therapy.

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Hypoxia-Inducible Factor 1 α Is Up-Regulated by Oncostatin M and Participates in Oncostatin M Signaling

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The interleukin-6–type cytokine oncostatin M (OSM) acts via the Janus kinase/signal transducer and activator of transcription pathway as well as via activation of mitogen-activated protein kinases and is known to critically regulate processes such as liver development and regeneration, hematopoiesis, and angiogenesis, which are also determined by hypoxia with the hypoxia-inducible factor 1 α (HIF1 α) as a key component. Here we show that treatment of hepatocytes and hepatoma cells with OSM leads to an increased protein level of HIF1 α under normoxic and hypoxic conditions. Furthermore, the OSM-dependent HIF1 α increase is mediated via Janus kinase/signal transducer and activator of transcription 3 and mitogen-activated protein kinase kinase/extracellular signal-regulated kinase 1/2 pathways. OSM-mediated HIF1 α up-regulation did not result from an increase in HIF1 α protein stability but from increased transcription from the *HIF1 α* gene. In addition, we show that the OSM-induced *HIF1 α* gene transcription and the resulting enhanced HIF1 α protein levels are important for the OSM-dependent vascular endothelial growth factor and plasminogen activator inhibitor 1 gene induction associated with several diseases. **Conclusion:** HIF1 α levels increase significantly after treatment of hepatocytes and hepatoma cells with OSM, and HIF1 α contributes to OSM downstream signaling events, pointing to a cross-talk between cytokine and hypoxia signaling in processes such as liver development and regeneration. (HEPATOLOGY 2009;50:253-260.)

Abbreviations: Erk, extracellular signal-regulated kinase; HIF, hypoxia-inducible factor; HIF1 α , hypoxia-inducible factor 1 α ; HRE, hypoxia response element; IL, interleukin; mRNA, messenger RNA; OSM, oncostatin M; PAI1, plasminogen activator inhibitor 1; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; siRNA, small interfering RNA; STAT, signal transducer and activator of transcription; TNF- α , tumor necrosis factor α ; VEGF, vascular endothelial growth factor.

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Oncostatin M (OSM) is an interleukin (IL)-6–type cytokine produced by monocytes and macrophages, T cells, and several other cell types. OSM receptors are widely expressed and are composed of the common signal transducer gp130 in complex with the LIFR or the OSMR. OSM has pleiotropic effects that in part overlap with those of other IL-6–type cytokines; examples include inflammation, neurogenesis, regulation of cell proliferation, and fibrosis.¹⁻³ In addition, OSM plays a crucial role in the orchestration of hematopoiesis and liver development.³

Upon OSM-induced receptor clustering, Janus kinases—mainly Janus kinase 1—are activated, phosphorylate tyrosines within the receptor that recruit other signaling proteins with matching SH2 domains such as signal transducers and activators of transcription (STATs) or adapter proteins for the mitogen-activated protein kinases to the receptor. The major signaling cascades activated by OSM include STAT3 as well as the extracellular signal-regulated kinase (Erk) 1/2 and p38 pathways.²⁻⁴

Several processes such as hematopoiesis, angiogenesis, liver development, metabolism, inflammation, and tu-

morigenesis are also crucially influenced by the ambient oxygen tension of the tissue. Hypoxia-inducible factors (HIFs) act as master regulators for the expression of genes essential in a hypoxic microenvironment. The best characterized factor is HIF1, which regulates more than 100 genes. HIF1 is formed by dimerization of the oxygen-sensitive hypoxia-inducible factor 1 α (HIF1 α) subunit with the constitutively expressed beta-subunit, which is also known as ARNT. Under normoxic conditions, the HIF1 α subunit is hydroxylated and quickly degraded via the proteasome. Under hypoxic conditions, the activity of the hydroxylases is reduced, and HIF1 α protein is stabilized and can bind to hypoxia-response elements (HREs) within the regulatory areas of HIF target genes and efficiently recruit cofactors.⁵

In addition to hypoxia, HIF1 α has also been shown to be up-regulated under normoxia in response to growth factors, thrombin, lipopolysaccharide, angiotensin II, insulin, or the cytokines IL-1 and tumor necrosis factor α (TNF- α).⁶ Although several details have been unraveled regarding the ability of hypoxia to stabilize HIF1 α , the mechanisms by which those factors (especially cytokines) induce HIF1 α have not been fully elucidated.

Interestingly, OSM has been described to orchestrate the hypoxia-influenced processes of hematopoiesis, angiogenesis, liver development, and regeneration.^{3,7} This suggests a possible cross-talk of the OSM and hypoxia signaling pathways. The expression of the HIF1 target genes vascular endothelial growth factor (VEGF) and plasminogen activator inhibitor 1 (PAI1)—which are crucial for angiogenesis and tissue remodeling, respectively—can also be up-regulated by the cytokine OSM.⁸⁻¹² OSM supports *in vitro* differentiation of fetal hepatic cells into liver-like structures, which is paralleled by enhanced VEGF expression.¹¹ Moreover, OSM mediates differentiation of oval cells into hepatocytes.¹³ OSM plays a crucial, nonredundant role in liver regeneration, as shown for OSM receptor knock-out mice after partial hepatectomy or CCl₄ treatment,¹⁴ and OSM gene therapy attenuates liver damage induced by dimethylnitrosamine.¹⁵ Interestingly, HIF1 α is also expressed during liver regeneration,¹⁶ and HIF1 α supports the growth of hepatoma cells *in vivo* and *in vitro*.^{17,18}

Because both OSM and HIF1 α play a pivotal role in liver-related processes (development, regeneration, carcinogenesis), the aim of the present study was to investigate whether the OSM signaling pathway has an impact on the HIF1 system in hepatoma cells and hepatocytes.

Materials and Methods

Cell Culture and Reagents. HepG2 hepatoma cells were maintained in DMEM/NUT-MIX-F12 medium

(Lonza) supplemented with 10% fetal bovine serum (PAA), 100 mg/L streptomycin, and 60 mg/L penicillin (Cytogen). The human hepatocyte cell line PH5CH8 has been described.¹⁹ Cells were grown at 37°C in a water-saturated atmosphere at 5% CO₂. Hypoxia treatment was performed at 37°C in a water-saturated atmosphere at 5% CO₂ and 6% oxygen. HepG2 cells were transfected using the Fugene reagent (Roche) according to the manufacturer's recommendations. Cotransfections of small interfering RNA (siRNA) and reporter gene constructs are described in the Supporting Information. Human recombinant OSM was obtained from Peprotech. Actinomycin D and cycloheximide were obtained from Calbiochem. Stattic²⁰ was from Sigma.

Western Blot Analysis and Antibodies. All steps of cell lysis and immunoprecipitation were performed at 4°C using ice cold buffers. Cells were lysed on a dish with lysis buffer containing 30 mM Tris/HCl (pH 6.7), 5% glycerol, 2.5% mercaptoethanol, and 1% sodium dodecyl sulfate. The lysates were further analyzed via sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting. Antibodies against HIF1 α , STAT3, STAT1, and Fin13 were obtained from BD Transduction Laboratories. Antibodies against phospho-STAT3, phospho-STAT1, Erk1/2, phospho-Erk1/2, p38, and phospho-p38 were obtained from Cell Signaling. ECL signals were detected as described.²¹ Before re-probing, blots were stripped as described.⁴

Reporter Gene Assays. HepG2 cells were transfected with 1 μ g of the β -galactosidase control plasmid (pCH110, Amersham Biosciences) and 1.5 μ g of the respective reporter gene construct. Twenty-four hours after transfection, the cells were treated with the different stimuli as described in the figure legends. Cell lysis and luciferase assays were performed using the Promega luciferase assay system (Promega, Madison, WI) (see Supporting Information for further details). All experiments were performed at least in triplicate, and biological triplicates were also performed within one experiment. Luciferase activity values were normalized to transfection efficiency monitored by the cotransfected β -galactosidase expression vector. For some experiments the luciferase activity values after OSM stimulation were additionally normalized to values from control unstimulated cells.

Quantitative Real-Time Polymerase Chain Reaction. The exact protocol is described in the Supporting Information. Total RNA was extracted using the RNeasy Mini Kit (Macherey Nagel) according to the manufacturer's instructions. The concentration of isolated RNA was measured using a NanoDrop spectrophotometer. One microgram of total RNA was reverse-transcribed with a ThermoScript RT-PCR System (Invitrogen). Quantita-

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 ($1\times$, $0.1\times$, $0.01\times$, $0.001\times$) 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, $2^{-\Delta C_T}$, 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 \pm 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 HIF1 α Under Normoxic Conditions. Exposure of HepG2 hepatoma cells to OSM for different times led to a profound and transient increase in HIF1 α 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). HIF1 α 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 HIF1 α 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 HIF1 α 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 HIF1 α is transcriptionally active even under normoxic conditions.

OSM Augments Hypoxia-Dependent HIF1 α Induction and Hypoxia-Mediated Target Gene Expression. Next, we compared the induction of HIF1 α by OSM under normoxia with that induced by hypoxia. In addition, we examined whether OSM may affect the hypoxia-dependent induction of HIF1 α . We found that HIF1 α 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 HIF1 α protein levels was higher than under each treatment alone (Fig. 2A). Quantitation of western blots showed that the HIF1 α protein is up-regulated by factors of 3.6 ± 0.19

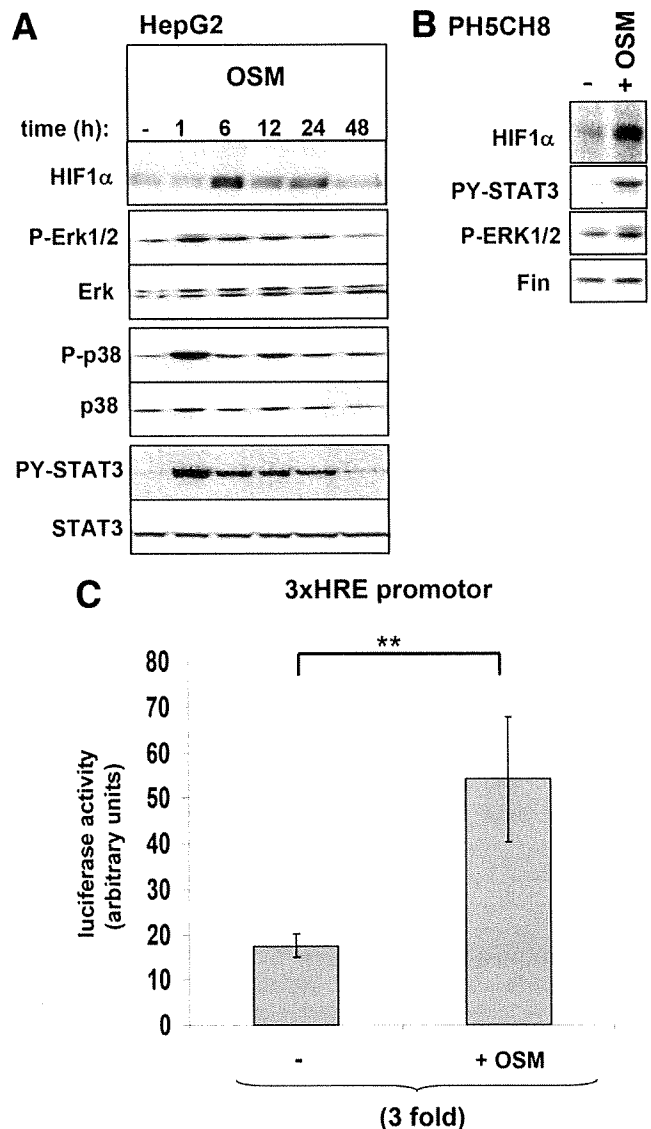


Fig. 1. OSM induces HIF1 α protein levels over an extended period, and the induced HIF1 α is transcriptionally active. (A) HepG2 cells were treated for the indicated periods with OSM (10 ng/mL). Lysates of the cells were separated via SDS-PAGE, and western blots of the membranes were detected with HIF1 α , 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. $**P < 0.01$.

upon hypoxia, 2.7 ± 0.9 upon OSM, and 6.4 ± 1.8 upon combined treatment with hypoxia and OSM.

We then investigated HIF1 α mRNA expression under the same conditions and found that OSM induces HIF1 α mRNA levels stronger (3.4-fold) than hypoxia (2.3-fold). Combined treatment with hypoxia and OSM led to an

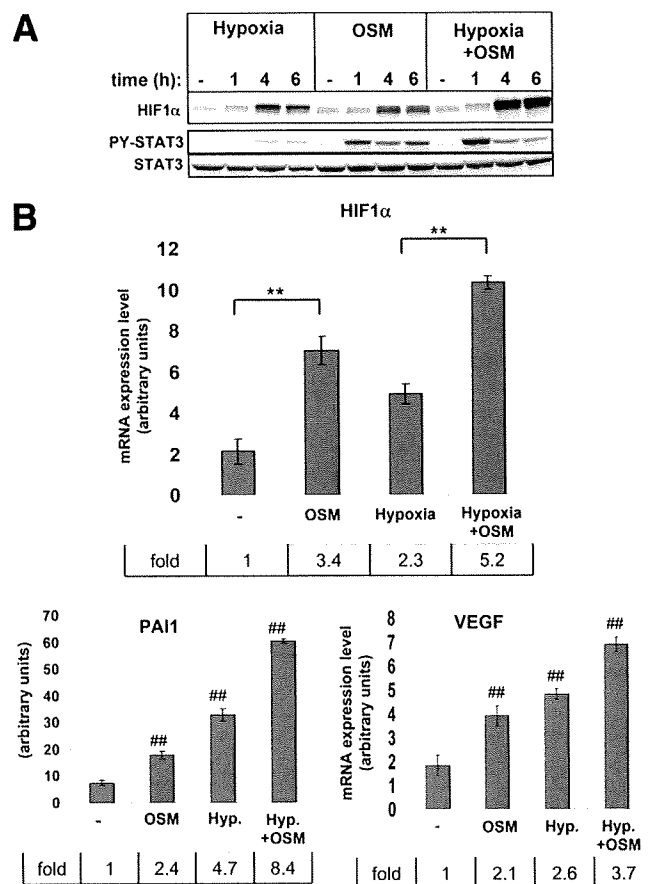


Fig. 2. OSM enhances hypoxia-induced HIF1 α , PAI1, and VEGF expression. (A) HepG2 cells were treated for the indicated periods with hypoxic conditions and/or OSM (10 ng/mL). Lysates of the cells were separated via SDS-PAGE, and western blots of the membranes were detected with antibodies directed against HIF1 α , 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 HIF1 α 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 HIF1 α mRNA (5.2-fold) (Fig. 2B). Thus OSM significantly increases HIF1 α 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 HIF1 α Protein Levels Is Due to De Novo Transcription but not Regulation of Protein Stability. To find out whether OSM influences HIF1 α protein stability, we aimed to measure HIF1 α protein half-life. Therefore, we stimulated HepG2 cells with OSM or CoCl₂ (a hypoxia mimetic) for 6 hours to induce a robust HIF1 α expression before the

translation inhibitor cycloheximide was added for different periods. We found that the OSM-induced HIF1 α protein disappeared completely after a 10-minute treatment with cycloheximide (Fig. 3A, right panel). In contrast, CoCl₂-induced HIF1 α was still well detectable after 1 hour; it disappeared after 3 hours of cycloheximide treatment (Fig. 3A, left panel). There was no difference in HIF1 α protein stability between CoCl₂ and the combined treatment with OSM and CoCl₂. These data show that the OSM-mediated HIF1 α up-regulation is not due to an enhanced stability of the protein.

In contrast, experiments with the transcription inhibitor actinomycin D showed that less HIF1 α 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 HIF1 α expression could be observed. Actinomycin D did not affect HIF1 α expression in cells exposed to hypoxia, which was expected because hypoxia essentially increases protein stability. Here, the HIF1 α

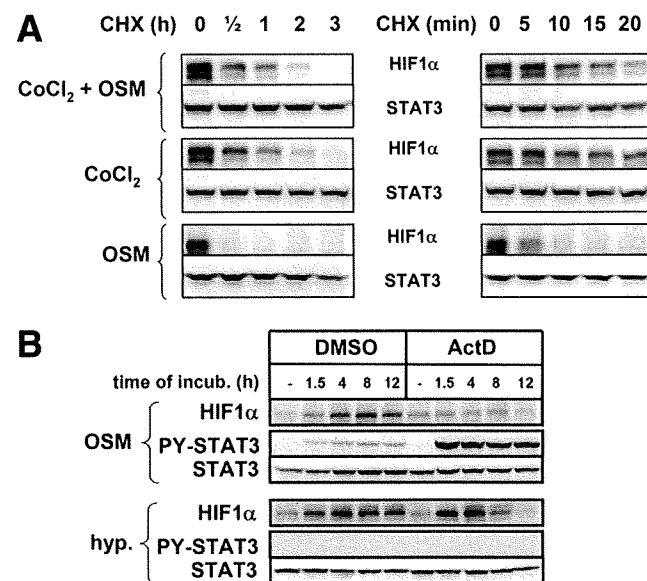


Fig. 3. OSM increases transcription of HIF1 α but does not affect HIF1 α protein stability. (A) HepG2 cells were stimulated for 6 hours with CoCl₂ (50 μ M) and/or OSM (10 ng/mL). Cycloheximide (10 μ g/mL) was then added for the indicated periods before lysates were prepared. Western blots were detected with HIF1 α and STAT3 antibodies. For HIF1 α 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 μ g/mL). Western blots of lysates separated via SDS-PAGE were detected with HIF1 α , phospho-STAT3, and STAT3 antibodies.

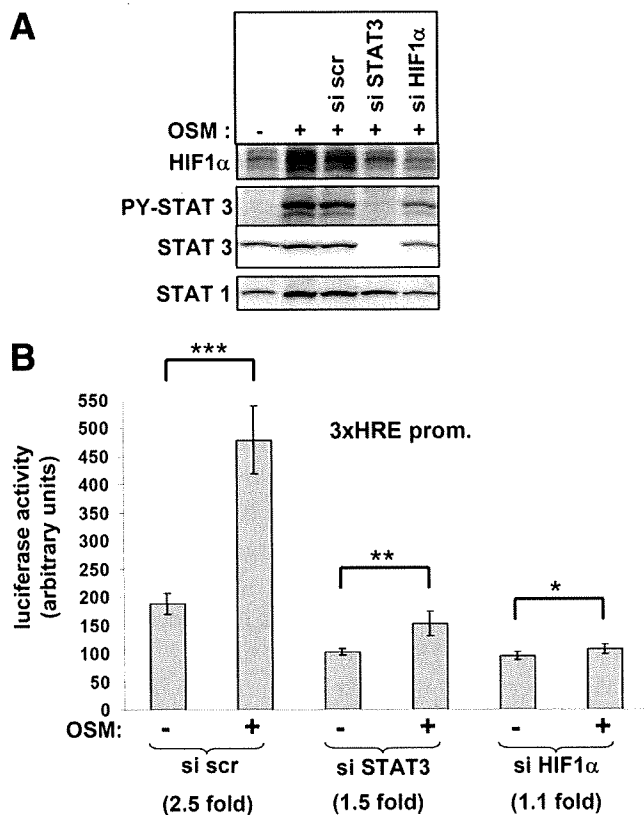


Fig. 4. Knockdown of STAT3 by siRNA decreases HIF1 α protein levels and HIF1 α -responsive reporter gene activity upon OSM treatment. (A) HepG2 cells were transfected with siRNAs as indicated. Forty-eight hours after transfection, cells were treated for 4 hours with OSM (10 ng/mL). Lysates of the cells were separated via SDS-PAGE, and western blots of the membranes were detected with HIF1 α , 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 HIF1 α 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 HIF1 α via transcriptional regulation.

STAT3 Protein Is Crucial for OSM-Induced Up-Regulation of HIF1 α . 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 HIF1 α expression upon OSM treatment, similar to the HIF1 α siRNA used as a positive control (Fig. 4A). The unspecific control siRNA did not show these pronounced effects. Upon transfection of STAT3 or HIF1 α 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 HIF1 α protein and mRNA levels, HRE promoter activity, or target gene induction) (Supporting Figs. 3–5). Furthermore, we provide evidence for the relevance of Erk signaling in OSM-mediated induction of HIF1 α protein and activity (Supporting Figs. 3–5).

OSM-Induced HIF1 α Is Crucially Involved in the Transcriptional Regulation of the Genes for VEGF and PAI1. To investigate the relevance of HIF1 α expression in OSM signal transduction, we examined the effects of HIF1 α 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 HIF1 α suppression (Supporting Fig. 6). In addition, the OSM-mediated induction of the VEGF and PAI1 promoter was down-regulated by HIF1 α 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 promoter, for which a reduction of about 40% was observed when the HRE was mutated (Fig. 5C).

Together, these data indicate that *de novo*-transcribed HIF1 α 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 HIF1 α protein levels in hepatocytes and hepatoma cells under normoxic conditions and leads to the formation of transcriptionally active HIF1 complexes. OSM-induced HIF1 α protein up-regulation was stronger compared with other cytokines (IL-6, IL-1 β , TNF- α , interferon- γ), some of which have been implicated in

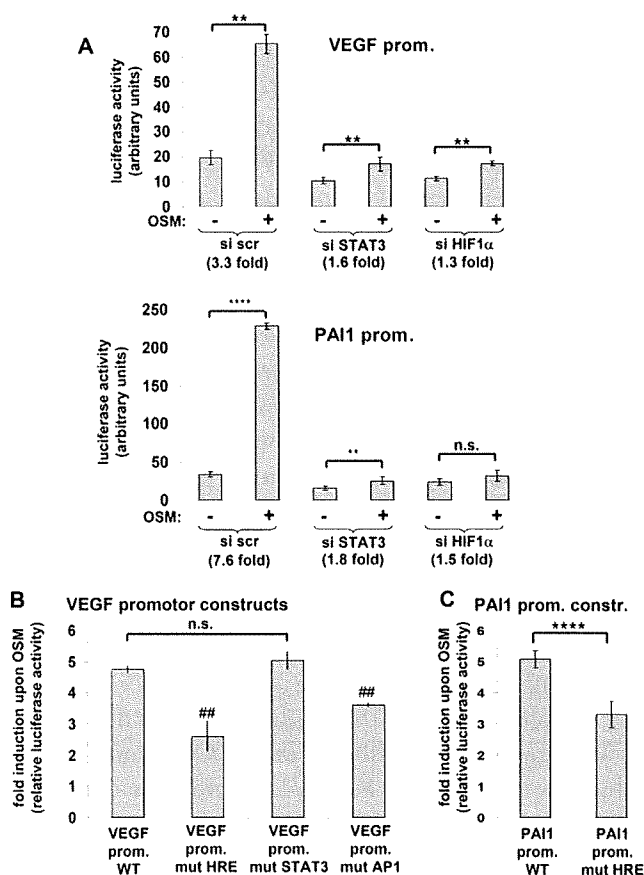


Fig. 5. Regulation of the VEGF and PAI1 gene promoters by OSM. (A) HepG2 cells were transfected with siRNAs as indicated, the luciferase reporter gene plasmids pGL3-VEGF-Luc or pGL3-hPAI1-796 and the β -galactosidase expression vector pCH110, and processed as described in Fig. 4B. **** $P < 0.0001$. ** $P < 0.01$. 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 HIF1 α 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). **** $P < 0.0001$.

HIF1 α up-regulation before (IL-6, IL-1 β , TNF- α) (Supporting Fig. 1). Moreover, the OSM-increased HIF1 α 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 HIF1 α up-regulation. Regulation of HIF1 α activity is complex and under normoxic conditions, HIF1 α 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-HIF1 α -TADN or TADC gene constructs (Supporting Fig. 2C). Furthermore, we could show that OSM-induced HIF1 α was clearly ubiquitinated, which was less the case for hypoxia-stabilized HIF1 α (Supporting Fig. 2A,B). We propose that transcriptional mechanisms are responsible for the OSM-mediated HIF1 α 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 HIF1 α 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 HIF1 α 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 HIF1 α mRNA and protein expression (Supporting Fig. 3), HIF1 α -dependent reporter gene activity (3x HRE promoter) (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 HIF1 α expression (data not shown).

Experiments with siRNA further revealed that STAT3 plays a crucial role in OSM-regulation of HIF1 α protein levels and HIF1 α -dependent transcriptional activity. Moreover, dominant negative STAT3 (STAT3-DN) also led to a down-regulation of HIF1 α -dependent transcriptional activity (Supporting Fig. 4B). It has been shown that HIF1 α transcription is regulated by SP-1²⁴ and NF- κ B transcription factors.²³ In addition to that, the present study indicates that the transcriptional regulator STAT3 appears to be a key player for HIF1 α transcription in response to OSM. STAT3 was also found to be involved in HIF1 α mRNA expression in tumor cells and tumor-associated myeloid cells.²⁵

Our study provides evidence that HIF1 α is important for OSM signal transduction. Experiments with HIF1 α siRNA showed that the expression of the OSM target genes VEGF and PAI1 involves regulation by HIF1 α . 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

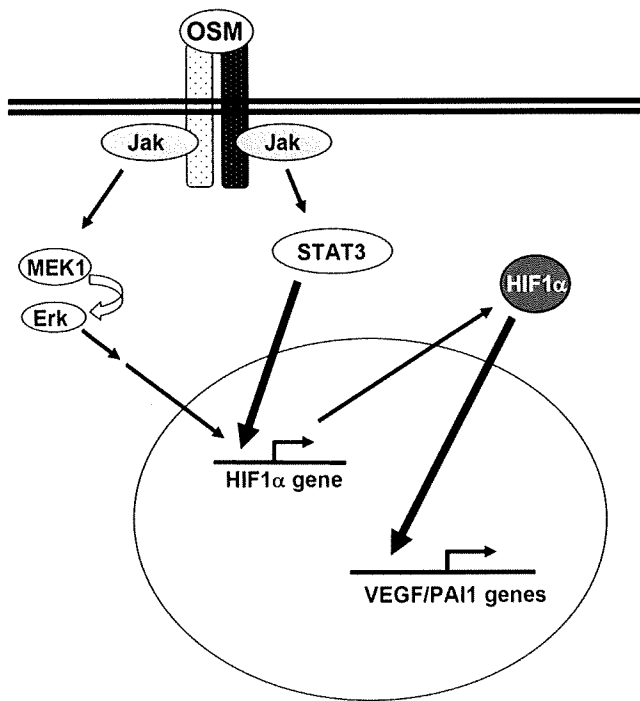


Fig. 6. Schematic representation of VEGF gene regulation by OSM.

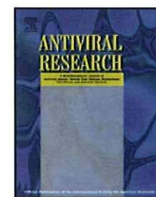
STAT3 induced the VEGF promoter,^{27,30,31} and a STAT3 binding element was detected at site -848 .^{30,31} We found that mutation of the described STAT3 binding site in the VEGF promoter at -848 did not affect OSM-dependent induction of reporter gene activity. These data are consistent with those of 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 HIF1 α transcription. HIF1 α 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 HIF1 α 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 (EC₅₀: 1.50 ng/ml vs. 8.50 ng/ml) and fluvastatin (EC₅₀: 2.82 μ M vs. 7.87 μ M), although these replicons possessed similar features in terms of genetic distance from the O strain, HCV RNA expression levels, and sensitivity to IFN- α (EC₅₀: 1.44 IU/ml vs. 1.37 IU/ml) and cyclosporine A (EC₅₀: 0.71 μ g/ml vs. 0.96 μ g/ml). These replicons are thus useful tools for examining the mechanism of anti-HCV activity, especially in IFN- λ and statins.

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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'-AAAAAAAAAAAAAAAAAAAAA-3'. The primer pair 542: 5'-GTAGAGCCCGTCGCTCTCTGACATGGA-3' and 9388R: 5'-ATGGCCTATTGGCCTGGAGTG-3' was employed in the first-round PCR (35 cycles). The primer pair 3295X: 5'-ATTATTCTAGACTGACATGGAGACCAAGATCATCAC-3' and 9357RX: 5'-ATTATTCTAGACCCGTTACCCGGTTGGGGAGCAG-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'-CTTGGTCCGTACGCCCGCCAGTTGAAGAGGTACTTGC-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 μ g 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, $1.0\text{--}1.5 \times 10^4$ 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 μ g) 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/ μ g 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 4×10^7 copies/ μ g 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 (EC₅₀): 1.44 and 1.37 IU/ml, respectively) (Fig. 2). The s1B-5R and sOR replicons, respectively, exhibited the highest (EC₅₀: 1.10 IU/ml) and lowest (EC₅₀: 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

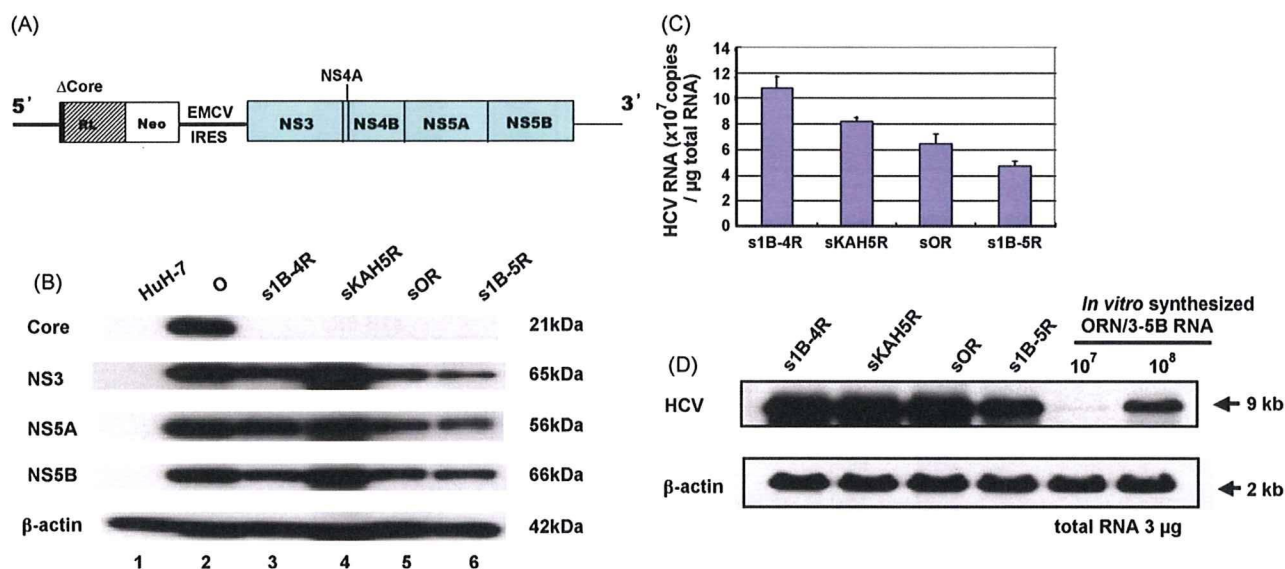


Fig. 1. The expression of HCV proteins and HCV RNAs in four replicon-harboring cell lines. (A) Schematic gene organization of subgenomic replicon RNA. The NS3 to NS5B region and 12 N-terminal amino acid residues of the Core (Δ) are depicted in closed boxes. Untranslated regions, EMCV IRES, RL, and Neo 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.

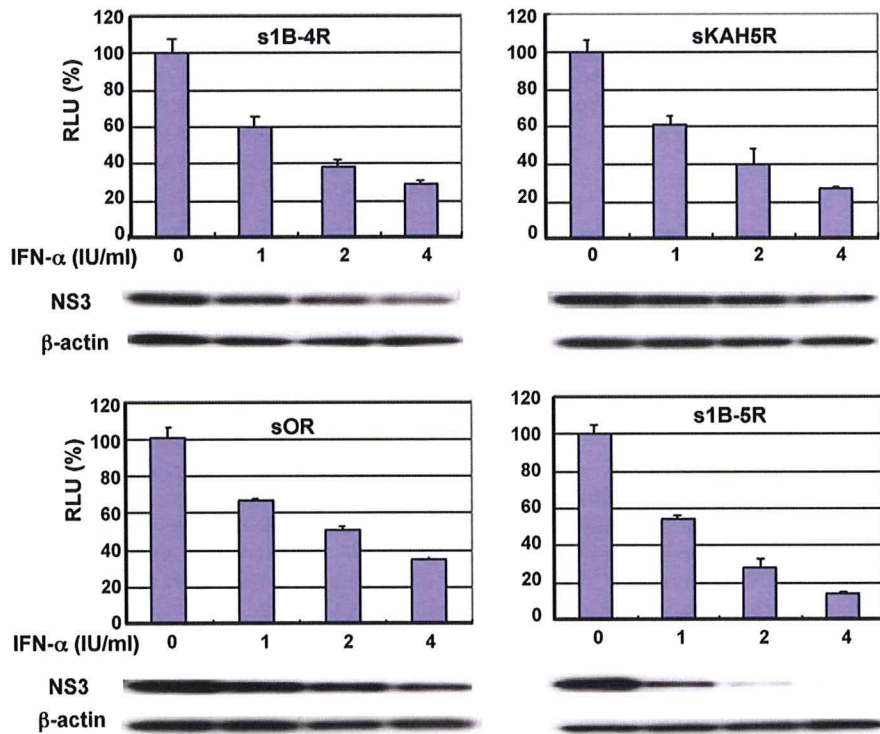


Fig. 2. The activity of IFN- α on HCV replicon RNA replication. Reporter assay and Western blot analysis for HCV replicons in IFN- α treatment. s1B-4R cells, sKAH5R cells, sOR cells, and s1B-5R cells were treated with IFN- α (0, 1, 2, and 4 IU/ml) for 72 h. Then, the cells were subjected to RL assay (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 \pm 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,

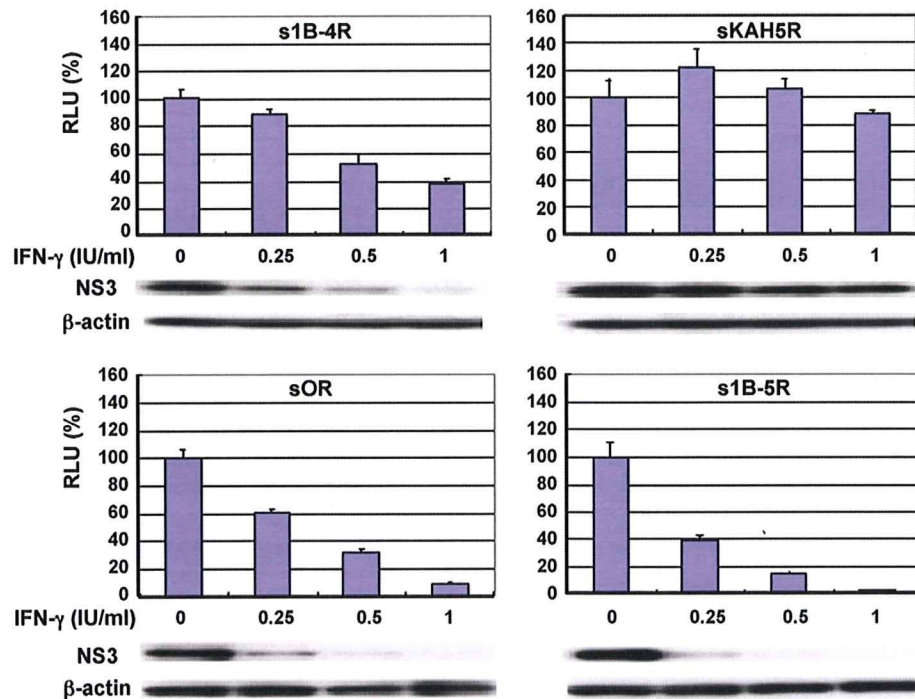


Fig. 3. The activity of IFN- γ on HCV replicon RNA replication. Reporter assay and Western blot analysis for HCV replicons in IFN- γ treatment. s1B-4R cells, sKAH5R cells, sOR cells, and s1B-5R cells were treated with IFN- γ (0, 0.25, 0.5, and 1 IU/ml) for 72 h and then the cells were subjected to RL 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.

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 μ g/ml, respectively. We also obtained similar results by Western blot analysis. In contrast to the findings of the IFN study,

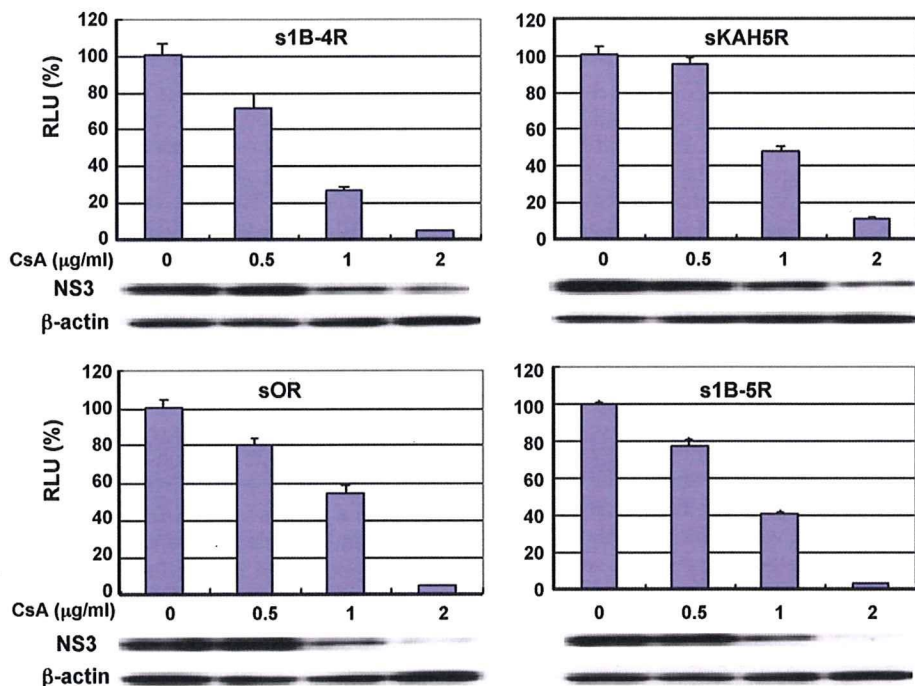


Fig. 5. The activity of CsA on HCV replicon RNA replication. Reporter assay and Western blot analysis for HCV replicons in CsA treatment. s1B-4R cells, sKAH5R cells, sOR cells, and s1B-5R cells were treated with CsA (0, 0.5, 1, and 2 μ g/ml) for 72 h, and then the cells were subjected to RL assay (upper 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.

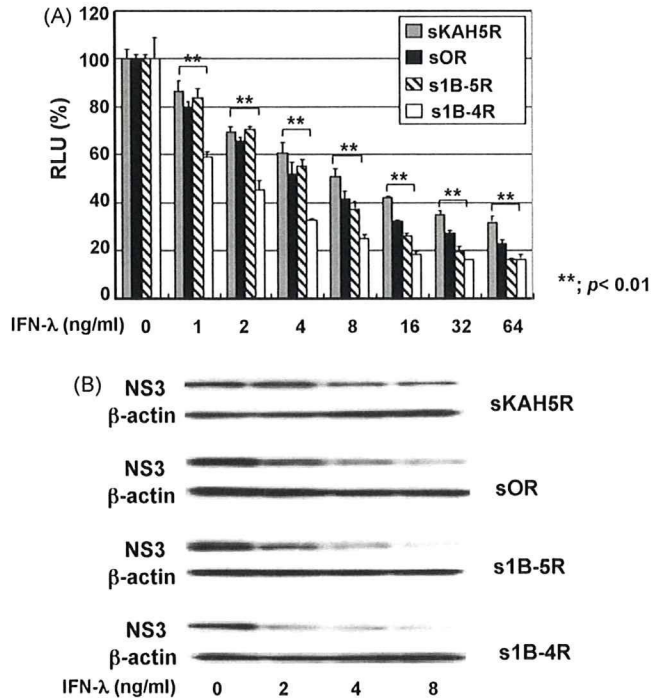


Fig. 4. Effects of IFN- λ on HCV replicon RNA replication. (A) Reporter assay and Western blot analysis for HCV replicons in IFN- λ treatment. s1B-4R cells, sKAH5R cells, sOR cells, and s1B-5R cells were treated with IFN- λ (0, 2, 4, 8, 16, 32, and 64 ng/ml) for 72 h, and then the cells were subjected 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.

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

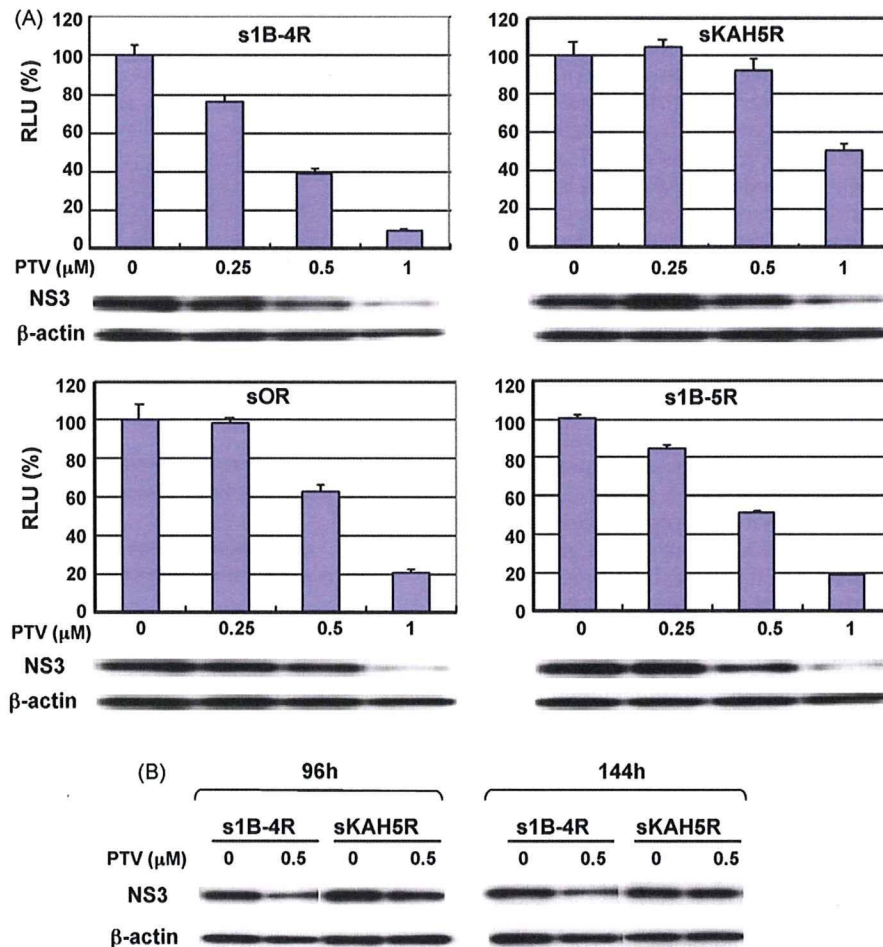


Fig. 6. The activity of PTV on HCV replicon RNA replication. (A) Reporter assay and Western blot analysis for HCV replicons in PTV treatment. s1B-4R cells, sKAH5R cells, sOR cells, and s1B-5R cells were treated with PTV (0, 0.25, 0.5, and 1 μM) for 72 h, and then the cells were subjected to RL assay 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).

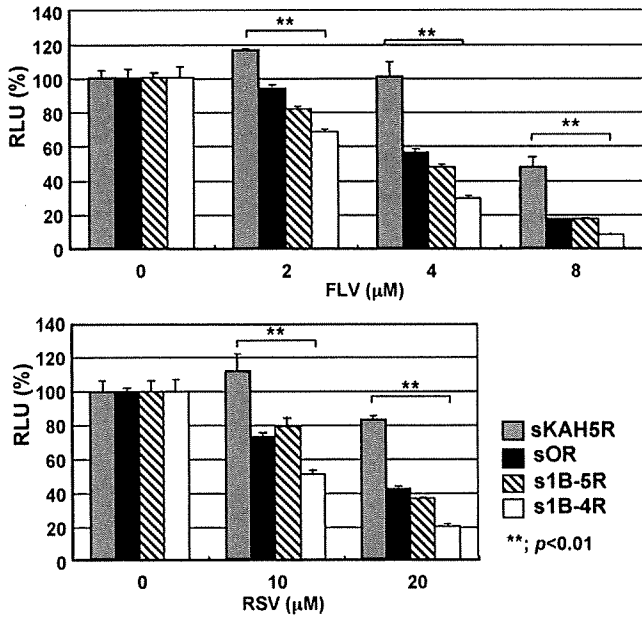


Fig. 7. HCV replicons exhibit diverse sensitivities to statins. Reporter assay of the sensitivity of HCV replicons to FLV. sKAH5R cells (light column), sOR cells (dark column) s1B-5R cells (shaded column), and s1B-4R cells (open column) were treated with FLV (0, 2, 4, and 8 μM) for 72 h (upper panel), 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 devel-

Table 1
EC₅₀ 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 ($\mu\text{g/ml}$) | 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.

oped 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 (EC₅₀: 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 (EC₅₀: 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 .

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 EC₅₀ values of anti-HCV reagents in four genotype 1b replicons are summarized in Table 1.

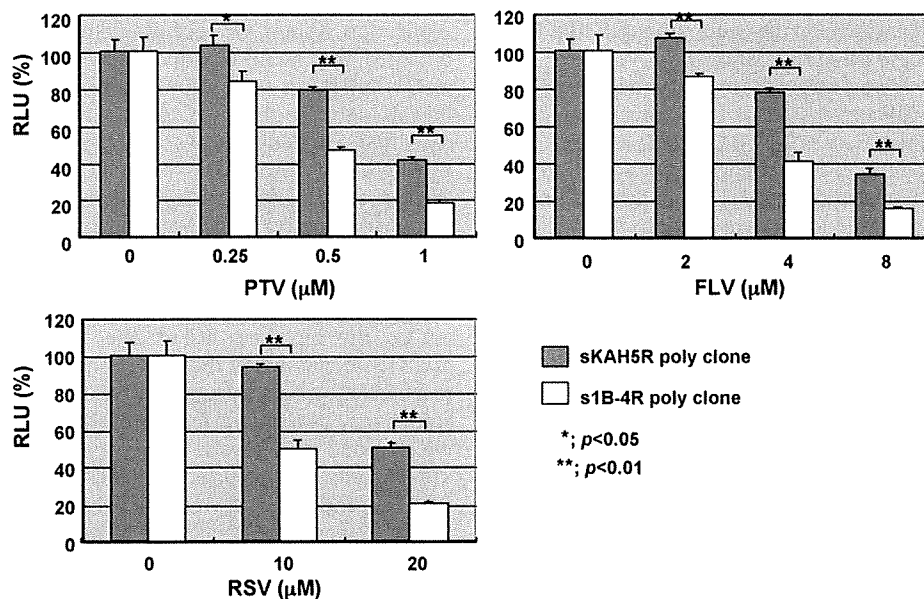


Fig. 8. Diverse sensitivities of polyclonal replicons to statins. Reporter assay of the sensitivity of polyclonal sKAH5R and s1B-4R replicons to PTV, FLV, and RSV. Polyclonal sKAH5R cells and polyclonal s1B-4R cells were treated with PTV (0, 0.25, 0.5, and 1 μM), FLV (0, 2, 4, and 8 μM), and RSV (0,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

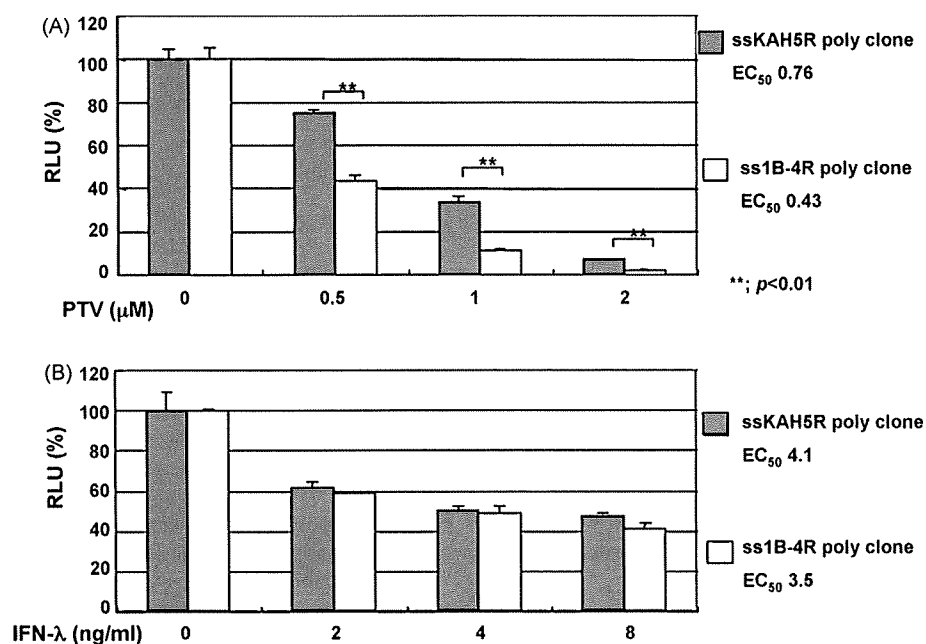


Fig. 9. The sensitivities of the second generation of sKAH5R and s1B-4R to PTV and IFN-λ. Polyclonal second generations of sKAH5R (ssKAH5R) and s1B-4R (ss1B-4R) were treated with PTV (0, 0.5, 1, and 2 μM) (A) and IFN-λ (0, 2, 4, and 8 ng/ml) (B) for 72 h and then were subjected to RL assay. All the luciferase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