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Analysis of Interferon Signaling by Infectious Hepatitis C Virus Clones with Substitutions of Core Amino Acids 70 and 91▽§

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Substitution of amino acids 70 and 91 in the hepatitis C virus (HCV) core region is a significant predictor of poor responses to peginterferon-plus-ribavirin therapy, while their molecular mechanisms remain unclear. Here we investigated these differences in the response to alpha interferon (IFN) by using HCV cell culture with R70Q, R70H, and L91M substitutions. IFN treatment of cells transfected or infected with the wild type or the mutant HCV clones showed that the R70Q, R70H, and L91M core mutants were significantly more resistant than the wild type. Among HCV-transfected cells, intracellular HCV RNA levels were significantly higher for the core mutants than for the wild type, while HCV RNA in culture supernatant was significantly lower for these mutants than for the wild type. IFN-induced phosphorylation of STAT1 and STAT2 and expression of the interferon-inducible genes were significantly lower for the core mutants than for the wild type, suggesting cellular unresponsiveness to IFN. The expression level of an interferon signal attenuator, SOCS3, was significantly higher for the R70Q, R70H, and L91M mutants than for the wild type. Interleukin 6 (IL-6), which upregulates SOCS3, was significantly higher for the R70Q, R70H, and L91M mutants than for the wild type, suggesting interferon resistance, possibly through IL-6-induced, SOCS3-mediated suppression of interferon signaling. Expression levels of endoplasmic reticulum (ER) stress proteins were significantly higher in cells transfected with a core mutant than in those transfected with the wild type. In conclusion, HCV R70 and L91 core mutants were resistant to interferon *in vitro*, and the resistance may be induced by IL-6-induced upregulation of SOCS3. Those mechanisms may explain clinical interferon resistance of HCV core mutants.

Hepatitis C virus (HCV) is one of the most important pathogens causing liver-related morbidity and mortality. Approximately 3% of the worldwide population is infected with HCV, which represents 170 million people, and 3 million to 4 million individuals are newly infected each year (33, 47, 62). There is no therapeutic or prophylactic vaccine available for HCV. Antiviral treatment has been shown to improve liver histology and decrease the incidence of hepatocellular carcinoma in chronic hepatitis C (CHC) (17, 64). Current therapies for CHC consist of treatment with pegylated interferon (peg-IFN), which acts both as an antiviral and as an immunoregulatory cytokine, and ribavirin (RBV), an antiviral prodrug that interferes with RNA metabolism (16, 31). However, less than 50% of patients infected with HCV genotype 1 treated in this way achieve a sustained virological response (SVR) or a cure of the infection (14, 16). Given this situations, gaining a detailed understanding of the molecular mechanisms of interferon (IFN) resistance has been a high priority in academia and industry.

The response to peg-IFN-plus-RBV treatment is affected by several viral and host factors, including age, gender (22, 23), grade of liver fibrosis (21, 42), HCV genotype, and serum viral load (14, 59). Several viral genetic factors influence treatment outcomes, including mutations in NS5A-interferon sensitivity determining region (ISDR) (13, 38) and the core region (4, 6). Akuta et al. reported that HCV-core amino acid substitutions at positions 70 and 91 are significantly correlated with poor responses to peg-IFN-plus-RBV therapy (6) and with increased hepatocarcinogenesis (2, 3). Furthermore, it was reported recently that the core amino acid 70 and amino acid 91 substitutions are associated with a poor response to peg-IFN, RBV, and telaprevir combination therapy, respectively (1). However, the underlying molecular mechanisms of such distinct biological properties of the core 70/91 mutations are poorly understood.

In this study, we have analyzed virus infection and replication kinetics and response to interferon treatment using the HCV-JFH1 cell culture system (HCVcc) (60, 65). We constructed HCVcc expressing virus with substitutions of core amino acid 70 and amino acid 91 (R70Q, R70H, and L91M). The core mutant HCV clones were compared in terms of intracellular replication, infectious virus production, and sensitivity to alpha interferon (IFN-α). Here we have shown that the differences in sensitivity to IFN are attributable to upregulated overexpression of the cellular interferon signal attenuator SOCS3 and that this upregulation is caused by overexpression of interleukin-6 (IL-6).

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MATERIALS AND METHODS

Reagents. Recombinant human IFN-α2b was from Schering-Plough (Kenilworth, NJ). Beta-mercaptoethanol was from Wako (Osaka, Japan). Antibodies used were SOCS3 and SOCS1, which were from Cell Signaling (Beverly, MA), HCV core (Abcam, Cambridge, MA), NS5A (BioDesign, Saco, ME), GRP78, GADD153/CHOP (Santa Cruz Biotechnology, Santa Cruz, CA), disulfide isomerase (PDI) (Stressgen Biotechnologies, Victoria, British Columbia, Canada), and beta-actin antibody (Sigma). Secondary antibodies were peroxidase-labeled anti-mouse, anti-rabbit antibody (GE Healthcare, Connecticut), donkey anti-goat IgG-horseradish peroxidase (HRP) antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and Alexa 405-labeled goat anti-mouse and Alexa 568-labeled donkey anti-rabbit IgG antibodies (Invitrogen, Carlsbad, CA).

Cells and cell culture. Huh7 cells were maintained in Dulbecco's modified minimal essential medium (DMEM) (Sigma Chemical Co, St. Louis, MO) supplemented with 2 mmol/liter L-glutamine and 10% fetal bovine serum at 37°C under 5.0% CO_2.

Sequence analyses. Nucleotide sequences were read from both strands using BigDye Terminator cycle sequencing ready reaction kits (Applied Biosystems, Foster City, CA) and an automated DNA sequencer (ABI Prism 310 genetic analyzer; Applied Biosystems).

Establishment of mutant HCV clones. In order to introduce various mutations into the core region of JFH1, plasmid pJFH1full was digested with EcoRI and BsiWI, and then the DNA fragment encompassing nucleotides 1 to 456 was subcloned into the pGEM-T Easy vector (Promega, Madison, WI). The following mutations were introduced into the DNA fragment in the subcloning vector by site-directed mutagenesis (Quick-Change II site-directed mutagenesis kit; Stratagene): R70Q, R70H, L91M, and GKPG77-80KKKK. Finally, the EcoRI-BsiWI fragments were subcloned back into the parental plasmid, pJFH1full.

In vitro RNA synthesis and transfection. Full-length HCV expression plasmids were as follows: pJFH1full, which encodes the full-length HCV-JFH1 sequence (60), pR70Q, pR70H, pL91M, and p7780K. These plasmids were linearized at their 3′ ends and used as templates for HCV RNA synthesis using the RiboMax large-scale RNA production system (Promega, Madison, WI). After DNase I (RQ-1 RNase-free DNase; Promega) treatment, the transcribed HCV RNA was purified using Isogen reagent (Nippon Gene, Tokyo, Japan). For the RNA transfection, Huh7 cells were washed twice in phosphate-buffered saline (PBS), and 5×10^6 cells were suspended in Opti-MEM I (Invitrogen, Carlsbad, CA) containing 10 μg of HCV RNA, transferred into a 4-mm electroporation cuvette, and finally subjected to an electric pulse (1,050 μF and 270 V) using the Easy Jet system (EquiBio, Middlesex, United Kingdom). After electroporation, the cell suspension was left for 5 min at room temperature and then incubated under normal culture conditions in a 10-cm-diameter cell culture dish. Forty-eight hours after transfection, the levels of HCV replication and viral protein expression were detected by real-time PCR and Western blotting.

HCVcc infection analyses. Huh7 cells were plated on 12-well plates at a density of 1.2×10^4 cells per well. Supernatants from HCV RNA-transfected cells were inoculated onto each well at a titer of 8×10^5 copies/well (quantified by real-time reverse transcriptase PCR [RT-PCR]). Forty-eight hours after infection, various amounts of interferon were added, and the cells were harvested after 72 h of the interferon treatment (48).

RNA extraction, cDNA synthesis, and real-time RT-PCR analysis. For the detection of HCV RNA in culture supernatant, the supernatant was passed through a 0.45-μm filter (Millex-HA, Millipore, Bedford, MA) and stored at −80°C until use. Protocols and primers for the real-time RT-PCR analysis of HCV RNA have been described previously (48). For the detection of endogenous mRNAs, total cellular RNA was isolated using an RNeasy Mini kit (Qiagen, Valencia, CA). Two micrograms of total cellular RNA was used to generate cDNA from each sample using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Expression of mRNA was quantified using the TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA) and the ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA).

Luciferase assays. Luciferase activities were measured using a luminometer (Lumat LB9501; Promega) using the Dual-Luciferase reporter assay system (Promega). Assays were performed in triplicate.

Western blot analysis. Western blotting was carried out as described previously (24, 53, 63). Briefly, 10 mg of total cell lysate was separated using NuPAGE 4%–12% Bis-Tris gels (Invitrogen) and blotted onto a polyvinylidene fluoride (PVDF) Western blotting membrane (Roche). The membrane was incubated with the primary antibodies followed by a peroxidase-labeled anti-IgG antibody and visualized by chemiluminescence using the ECL Western blotting analysis system (Amersham Biosciences, Buckinghamshire, United Kingdom).

Immunohistochemistry. HCV-transfected Huh7 cells were cultured on 18-mm round micro cover glasses (Matsunami, Tokyo, Japan). For detection of HCV core, lipid droplet, and endoplasmic reticulum (ER), cells were fixed with cold acetone for 15 min. The cells were incubated with the primary antibodies for 1 h at 37°C. The fluorescent secondary antibodies were Alexa 405 goat anti-mouse and 568 donkey anti-rabbit IgG antibodies (Invitrogen, Carlsbad, CA). Lipid droplets (LDs) were visualized by using Bodipy 493/503 dye (Invitrogen). Cells were mounted with Vecta Shield mounting medium and 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA) and visualized by using a confocal laser scanning microscope (FV10i; Olympus, Tokyo, Japan).

Calculation of 50% effective concentrations (EC_{50}). The EC_{50} was calculated as the concentration of IFN required for 50% reduction in HCV RNA expression. We used the probit regression analysis to obtain values.

Statistical analyses. Statistical analyses were performed by using Welch's *t* test. *P* values of less than 0.05 were considered statistically significant.

RESULTS

HCV core 70/91 mutants show resistance to IFN treatment. First, we investigated sensitivity to IFN treatment of the HCV core mutant R70Q, R70H, and L91M virus clones and compared them to the wild type. The wild type and core mutants were transfected into Huh7 cells, which were cultured in the presence of various concentrations of IFN-α for 48 h. RNA was extracted from the cells and culture supernatant, and the level of HCV RNA was quantified by real-time RT-PCR. Although the levels of supernatant HCV RNA did not differ between the wild type and core mutants (Fig. 1A), the levels of cellular HCV RNA showed that all three core mutants were significantly resistant to IFN compared to the wild type, with EC_{50}s of 5.0 IU/ml, 48 IU/ml, 32 IU/ml, and 47 IU/ml for the R70Q, R70H, L91M, and mutants and the wild type, respectively (Fig. 1B). To exclude the possible effects on interferon signaling by the input HCV RNA, we performed interferon sensitivity analyses by HCVcc infection. As shown in Fig. 1C, the interferon sensitivities of HCV core mutants and the wild type were consistent with the results of HCV RNA transfection. Similarly, according to Western blotting, the core mutants were more resistant to IFN treatment than the wild type (Fig. 1D).

Core mutants show decreased secretion of viral particles. To determine the mechanisms underlying the resistance to interferon, we compared baseline virus expression levels in cells and culture supernatants. The three core mutants, carrying R70Q, R70H, and L91M, expressed significantly higher levels of intracellular HCV RNA than the wild type, as well as the 7780K clone. (Fig. 2A). 7780K was a negative-control clone that lacked virus particle secretion (37). On the contrary, these core mutants released significantly smaller amounts of HCV RNA into the culture supernatant than the wild type, as well as the negative-control 7780K clone. (Fig. 2B). Consistent with the HCV RNA data, Western blotting showed that cellular HCV core protein levels were higher for the core amino acid 70/91 mutants than the wild type (Fig. 2C). These results suggested that the core 70/91 mutant clones were partially defective in the secretion of infectious virus particles.

Subcellular localization of wild-type and mutant core proteins and lipid droplets. It has been reported that HCV core protein localizes on the cellular LD membrane and may mediate encapsidation of viral genomic RNA and subsequent virus assembly (35, 36). Therefore, we visualized the subcellular localization of wild-type and mutant core proteins in rela-

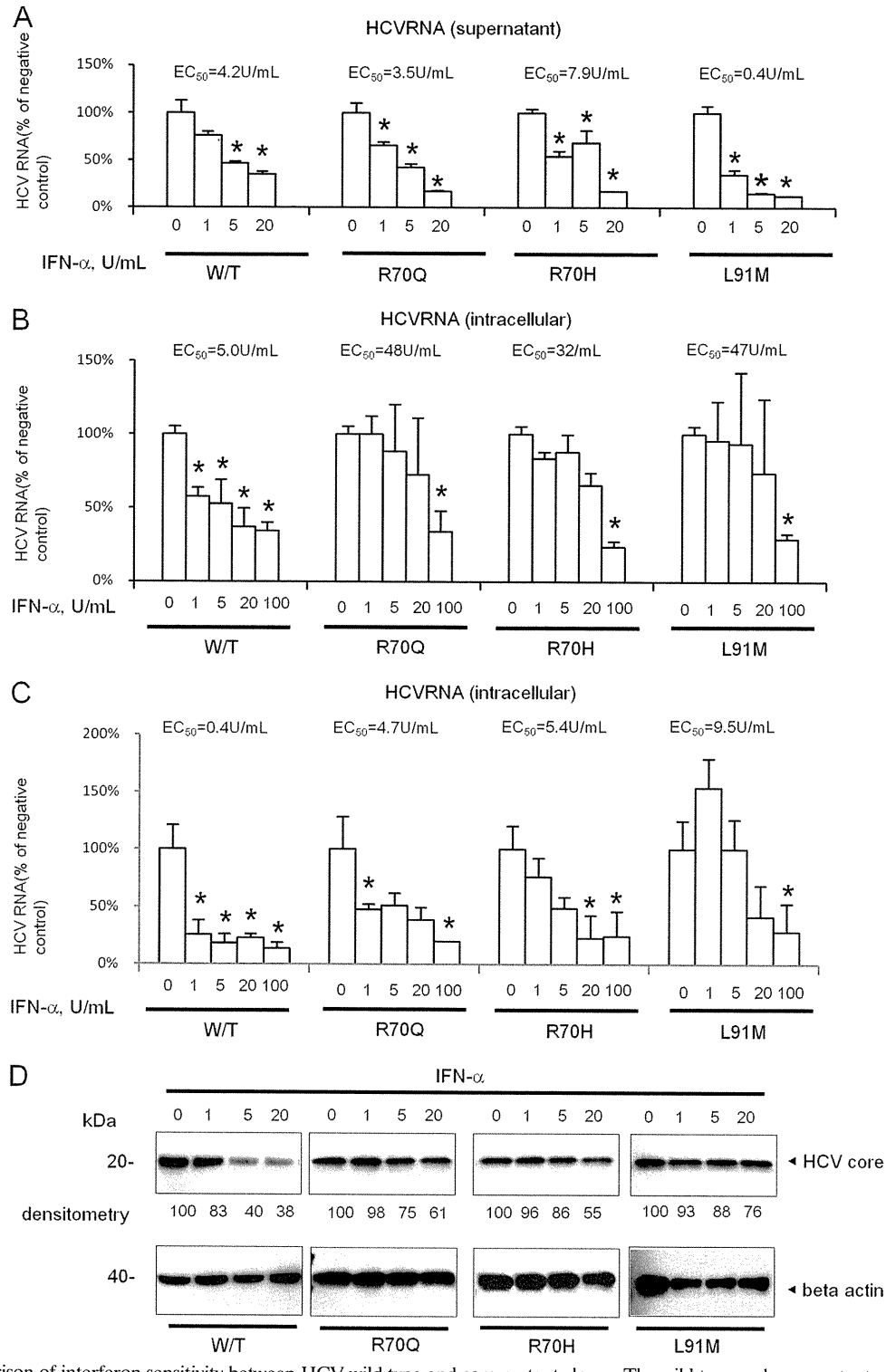

FIG. 1. Comparison of interferon sensitivity between HCV wild type and core mutant clones. The wild type and core mutants were transfected into Huh7 cells and cultured in the presence of IFN-α2b at concentrations ranging from 0 to 100 U/ml. (A) The culture supernatant of HCV-transfected Huh7 cells was collected 72 h after transfection, and the levels of HCV core antigen in the culture supernatant were measured. The values are displayed as percentages of those for the IFN-untreated control. The experiments were repeated three times, and representative results are shown. (B) Expression of intracellular HCV RNA. Cellular RNA was harvested at 72 h posttransfection. HCV RNA was quantified by real-time RT-PCR. The values are displayed as percentages of those for the IFN-untreated control. (C) Expression of intracellular HCV RNA. Cellular RNA was harvested at 72 h postinfection. HCV RNA was quantified by real-time RT-PCR. The values are displayed as percentages of those for the IFN-untreated control. In panels A through C, asterisks indicate *P* values of less than 0.05, compared to results for the interferon-negative control. (D) Western blotting was performed to assess intracellular suppression of HCV core protein. Ten micrograms of harvested cell lysates were subjected to Western blotting using anti-HCV core antibodies. Densitometry of core protein was performed, and results are shown as percentages of the results for an IFN-negative sample.

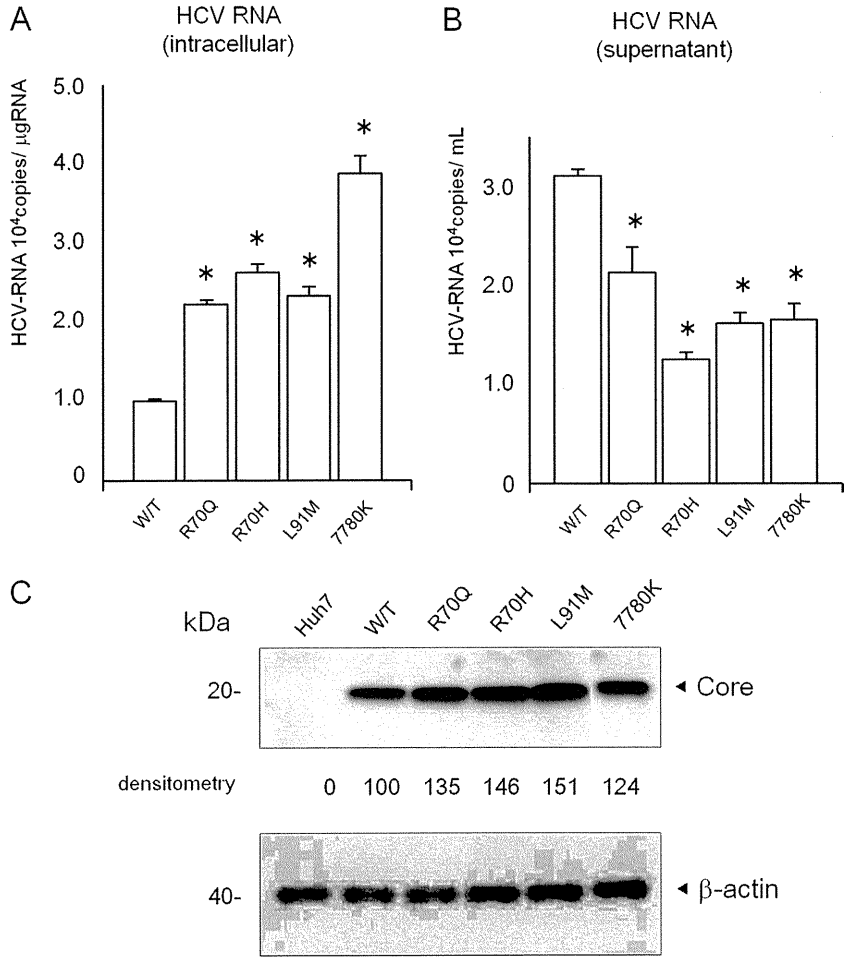

FIG. 2. Analysis of intracellular and supernatant HCV RNA levels in core 70/91 mutants. *In vitro*-transcribed mutant and wild-type RNAs were transfected into Huh7 cells. Three days after transfection, RNA was extracted from cells (A) or culture supernatant (B) and quantified by real-time RT-PCR. Asterisks indicate *P* values of less than 0.05 compared to results for the wild type. (C) Western blotting. Expression of core proteins in HCV-transfected cells. Total cellular protein was prepared from HCV RNA-transfected cells, and Western blotting was performed using anticore and anti-beta-actin antibodies. Densitometry was performed, and results are shown as percentages of that for an HCV-negative sample.

tion to that of LDs and the ER by indirect immunofluorescence and confocal microscopy. Consistent with previous reports, core proteins were colocalized with LDs but not with an ER-located protein, PDI, in the HCV-transfected cells (see the figure in the supplemental material). There were no obvious differences in colocalization of core and LDs or core and ER between the wild type and mutant core proteins.

Induction of interferon-stimulated genes following treatment of HCV-transfected cells with interferon. To investigate the mechanism of the relative IFN resistance of the core 70/91 mutants, as demonstrated in Fig. 1, we analyzed the cellular IFN signaling pathway. First, we assessed the expression and IFN-mediated induction of the mRNA transcripts of the IFN-stimulated genes (ISGs), encoding P56, double-stranded RNA-dependent protein kinase R (PKR), and 2′,5′-oligoadenylate synthetase (25AS), which mediate direct antiviral effects on HCV expression (24, 25). Cellular expression of PKR, P56, and 25AS was substantially increased in HCV-transfected cells, as well as naive cells, following IFN treatment. However, the levels of induction were significantly lower in the three HCV core mutant-transfected cells than in wild-type-transfected cells (Fig. 3A, B, and C). We next detected IFN-induced phosphorylation of STAT1 and STAT2 in the mutant and wild-type HCV-expressing cells. Our previous experiments showed that the levels of phosphorylated STAT1 and STAT2 (pSTAT1 and pSTAT2, respectively) increased within minutes of the addition of IFN and decreased subsequently at 8 h (25). Therefore, we detected pSTAT1 and pSTAT2 levels before and at 15 min after the addition of IFN. As shown in Fig. 3D and E, levels of pSTAT1 and pSTAT2 were lower in core mutant-transfected and -infected cells after IFN treatment than in wild-type-transfected cells and naive cells. These findings indicate that the differences in sensitivity to interferon of core mutant clones and the wild type were associated with attenuation of the cellular IFN signaling pathway.

SOCS3 is upregulated in core mutant clones-transfected, IFN-resistant cells. We examined next the effects of HCV replication on the expression of SOCS1 and SOCS3, proteins that suppress IFN receptor-mediated signaling (50, 58). There was no significant difference in expression levels of SOCS1

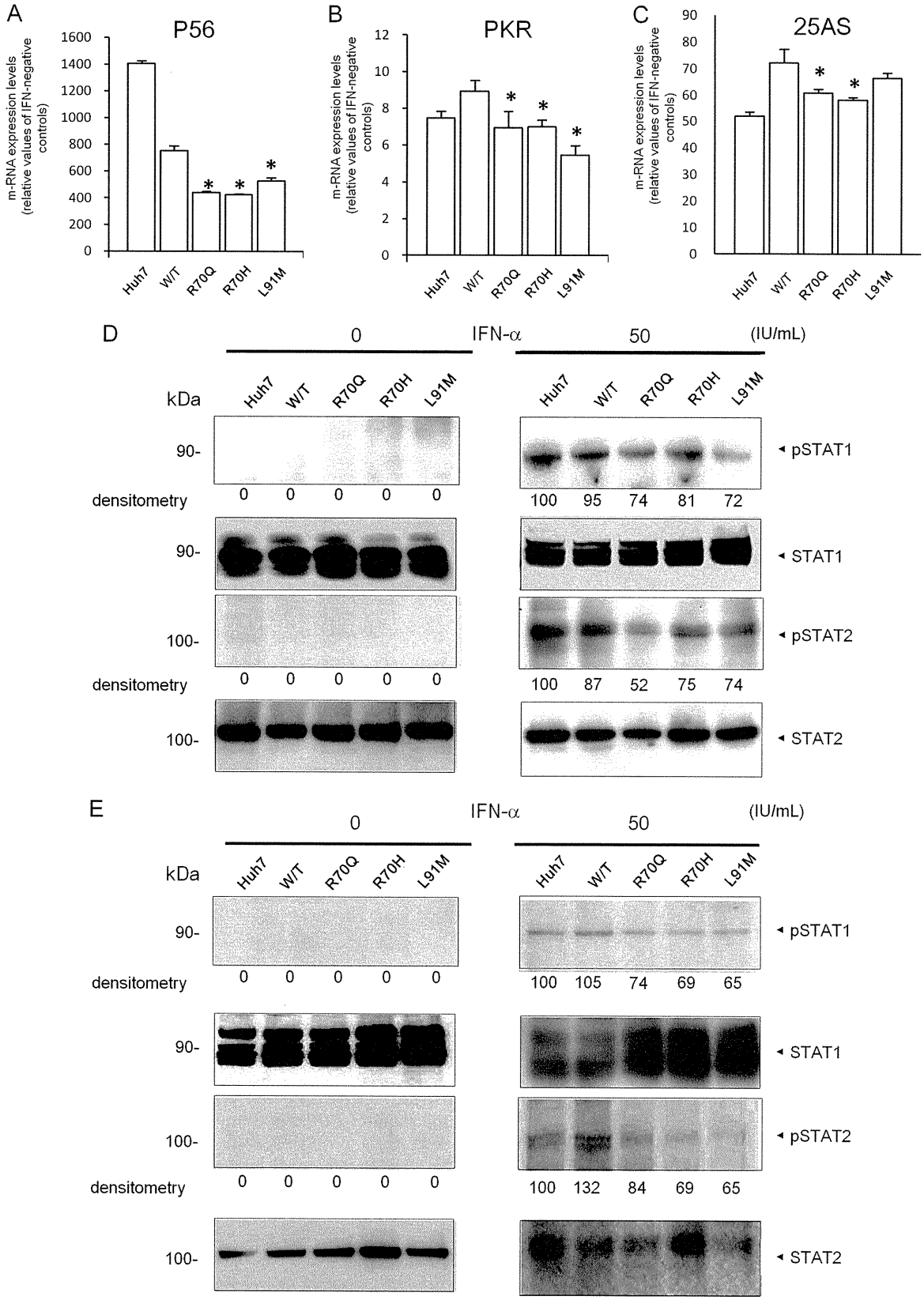

FIG. 3. Interferon-induced expressional induction of the ISGs, P56, PKR, and 25AS in Huh7 cells transfected or infected with wild-type and core mutant JFH1 clones. Two days posttransfection, cells were treated with 50 IU/ml of IFN-α. After 8 h, total cellular RNA was extracted and mRNAs of P56 (A), PKR (B), or 25AS (C) were quantified by real-time RT-PCR analyses. The values are displayed as ratios of IFN-untreated control values. Experiments were repeated three times, and representative results are shown. Asterisks indicate *P* values of less than 0.05 compared to results for the wild type. (D) Western blotting. Expression of total and phosphorylated STAT1 and STAT2 proteins in cells transfected with the wild type and core mutant HCV clones. (E) Western blotting. Expression of total and phosphorylated STAT1 and STAT2 proteins in cells infected with the wild type and core mutant HCV clones. Densitometries for pSTAT1 and pSTAT2 were performed, and results are shown as percentage of results for HCV-negative samples.

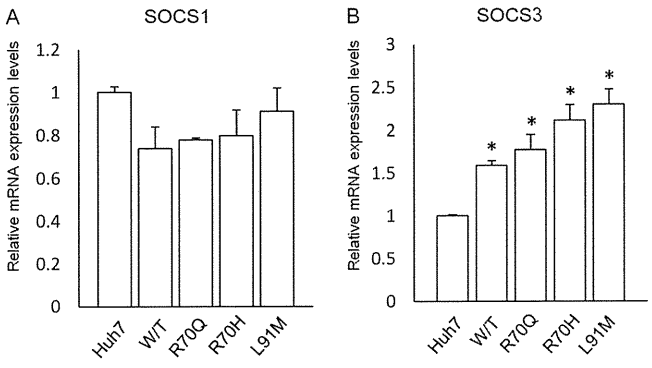

FIG. 4. Effects of core mutant HCV on SOCS1 and SOCS3 expression in Huh7 cells. Expression levels of SOCS1 (A) or SOCS3 (B) in Huh7 cells transfected with the wild type or the core mutant JFH1. Three days posttransfection, total cellular RNA was isolated and the mRNA was quantified by real-time RT-PCR analyses. The experiments were repeated three times, and representative results are shown. The values are displayed as values relative to beta-actin levels. Each experiment was repeated three times, and the representative results are shown. Asterisks indicate *P* values of less than 0.05 compared to results for the wild type.

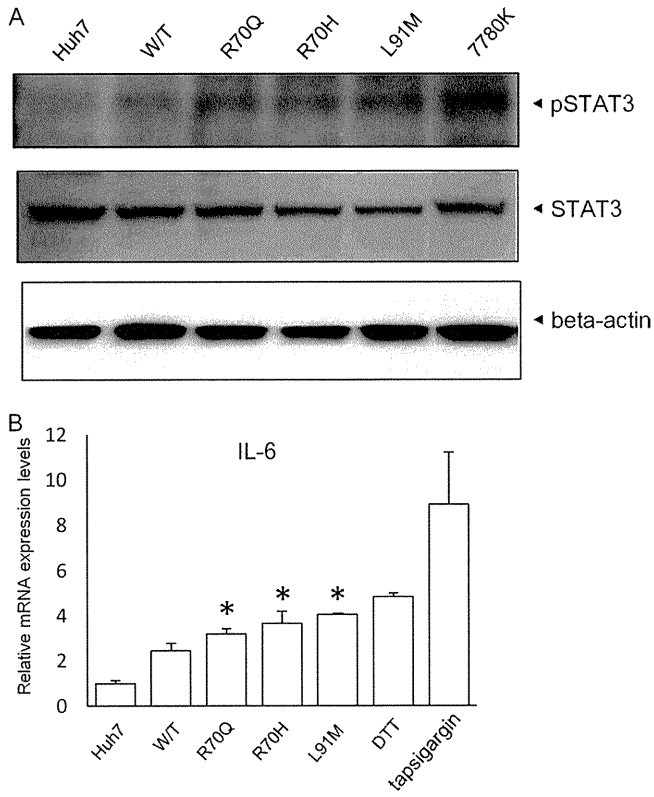

FIG. 5. Expression of phosphorylated STAT3 and IL-6 in cells transfected with the wild type and core mutant HCV-JFH1 clones. (A) Western blotting. Expression of total and phosphorylated STAT3 and beta-actin proteins in cells transfected with the wild type or core mutant HCV clones. (B) Two days posttransfection, total cellular RNA was extracted and mRNAs of IL-6 were quantified by real-time RT-PCR analyses. The values are displayed as the ratio of values of the HCV-untreated control. Asterisks indicate *P* values of less than 0.05 compared to results for the wild type.

mRNA between cells transfected with the wild type and the core mutant clones. In contrast, the SOCS3 mRNA expression level was significantly higher in core mutant-transfected cells than in wild-type-transfected cells (Fig. 4A and B). It is known that SOCS3 is induced principally by phosphorylated STAT3 (pSTAT3) (18) and that interleukin-6 (IL-6) is a strong inducer of pSTAT3 via receptor-mediated Janus kinase activation in the liver (41, 51). On that basis, we investigated whether overexpression of SOCS3 is associated with increased pSTAT3 and with overproduction of IL-6. The pSTAT3 level was significantly higher in core mutant-transfected cells than in JFH1-transfected cells and naive Huh7 cells (Fig. 5A). Moreover, cellular IL-6 mRNA expression was significantly higher in core mutant-transfected cells than in wild-type-transfected cells (Fig. 5B). These findings suggested that upregulation of cellular SOCS3 is associated with the resistance to IFN of the core 70/91 mutant HCV clones and that this effect is mediated partly by overproduction of IL-6.

UPRs are enhanced in core mutant-transfected cells. We have reported that HCV causes direct cytopathic effects on host cells and that these effects are mediated by HCV-induced unfolded protein responses (UPRs) (48). Therefore, we detected the expression of UPR-related proteins, GRP78 and CHOP, in cells expressing wild-type HCV and the core 70/91 mutants. As shown in Fig. 6, HCV-transfected cells showed higher expression levels of GRP78 and CHOP than untransfected cells. Furthermore, cells transfected with HCV core 70/91 mutant clones expressed larger amounts of GRP78 and CHOP than the wild-type-transfected cells. Because IL-6 is principally expressed following UPR induction (Fig. 5B), these data indicate that HCV-induced UPR may be involved in the IFN resistance of core mutant clones.

DISCUSSION

In this study, we used a virus cell culture system to investigate the characteristics of R70Q, R70H, and L91M HCV core mutant viruses, which were clinically resistant to peg-IFN-plus-RBV treatment, and found that these core mutant clones showed resistance to IFN *in vitro*, consistent with the clinical findings (Fig. 1). These differences in the IFN sensitivity of the core mutant clones led us conduct a series of experiments to investigate the molecular mechanisms of IFN-related response pathways. We found that IFN-α receptor-mediated signaling was attenuated in wild-type HCV-infected and core mutant-infected cells compared to that in uninfected cells and that the suppression of IFN signaling was more potent for core mutant clones than for the wild type. The differences in the interferon-mediated antiviral effects were demonstrated further by the difference in the induction rates of IFN-inducible P56, PKR, and 25AS mRNAs (Fig. 3A, B, and C) and IFN-induced phosphorylation of STAT1 and STAT2 (Fig. 3D and E). Furthermore, the expression levels of an interferon signal attenuator, SOCS3, were significantly higher in core mutant-transfected cells than in wild-type-transfected cells. Moreover, cellular expression of IL-6, which induces SOCS3 expression through phosphorylation of STAT3 (18, 41), was significantly higher in the core mutant-transfected cells than in wild-type-transfected cells (Fig. 5A). Taking all these things together, it is suggested strongly that the IFN resistance of core mutant clones is due to

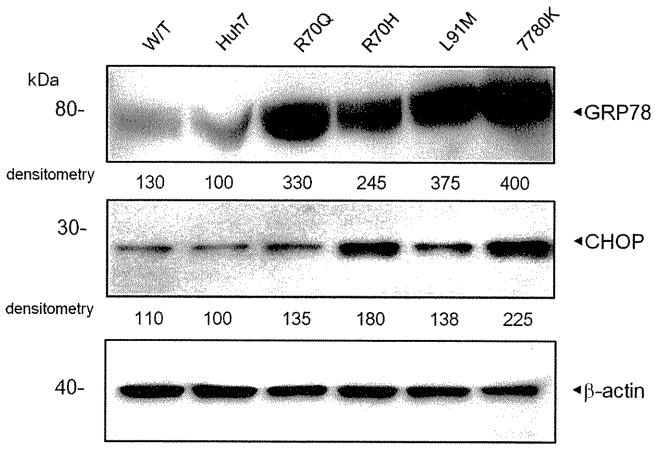

FIG. 6. Expression of GRP78 and CHOP UPR genes in cells transfected with the wild type and core mutant HCV-JFH1 clones. Western blotting was performed to assess UPR following transfection with HCV core mutants. Ten micrograms of harvested cell lysates were subjected to Western blotting using anti-GRP78 and anti-CHOP antibodies. Densitometries for GRP78 and CHOP were performed, and results are shown as percentages of results for uninfected cells.

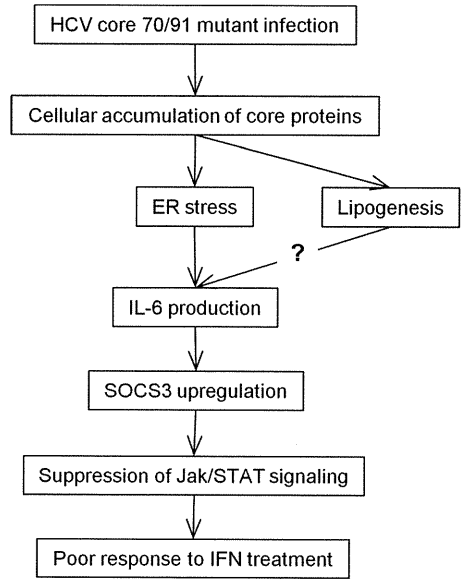

FIG. 7. Schematic diagram of signaling pathway involved in HCV core mutant infection and IFN resistance.

SOCS3-mediated attenuation of IFN responses and that, more importantly, upregulation of cellular IL-6 is attributable to emergence of IFN resistance (Fig. 7).

Miyanari et al. demonstrated that core protein, which is localized in LD-associated membrane, recruits HCV nonstructural (NS) proteins and replication complexes to LD and that this recruitment is critical for producing infectious viruses (35). Furthermore, Masaki et al. reported that the NS5A protein interacts with core at its C-terminal serine cluster and this NS5A-core interaction is crucial for the production of virus particle (32). In this study, there was no difference between the core mutants and the wild-type virus in terms of the pattern of colocalization of core protein with LDs and also the ER membrane (see the figure in the supplemental material). These results suggest that the core amino acid substitutions at positions 70 and 91 do not alter the characteristics of the core protein in terms of subcellular localization. Murray et al. conducted a comprehensive alanine substitution scan of the core protein to search for domains that are essential for virion production. They showed that substitutions of amino acids 70 and 91 spared but slightly decreased the capacity for virus particle production (37), which is consistent with our present results. Those mutations may cause accumulation of virus and core protein in the LDs and ER membrane and may elicit UPRs and IFN resistance.

Type I IFNs and their responsive ISGs are the principal mediators of host defense against virus infections, including HCV (10, 26, 44). Upon binding of IFNs to their receptors, IFNAR1 and IFNAR2, Janus kinases (Jak)1 and 2 phosphorylate STAT1 and STAT2 to form ISGF-3, which translocates to the nucleus and activates transcription of ISGs (46, 54, 55). Members of the SOCS family are potent inhibitors of type I and type III IFN-induced activation of the Jak-STAT pathway and subsequent expression of ISGs (58). HCV, on the other hand, counteracts such IFN-mediated antiviral pathways through its interaction with various steps of IFN signaling. The HCV NS5A and E2 proteins interfere with the action of IFN by inhibiting the activity of PKR (20, 56). NS5A also induces expression of IL-8 and attenuates expression of ISGs (40).

HCV core protein has been reported to interfere with the antiviral actions of IFN. Core protein binds the STAT1-SH domain (29) and destabilizes STAT1 (28) to block IFN signaling. Blindenbacher et al. (8) showed that STAT signaling was strongly inhibited in the hepatocytes of HCV core transgenic mice. Bode et al. showed that HCV core protein induced SOCS3 expression and inhibited tyrosine phosphorylation of STAT1 in HepG2 cells (9). In this study, we used full-length HCV cell culture and found that SOCS3 expression is upregulated at different rates, depending on the genetic sequences of HCV strains, and that these differences in SOCS3 expression are associated with sensitivity to IFN. These results indicate that the IFN resistance of HCV-infected cells is mediated by overexpression of SOCS3, which may be upregulated by HCV proteins, as previously reported (9, 27). Only one amino acid difference, R70Q, R70H, or L91M, might have affected cellular responses to interferon.

IL-6 is the principal activator of STAT3 in hepatocytes (18, 41). It has been reported that plasma IL-6 levels are elevated in CHC patients (30). Basu et al. have conducted DNA microarray analyses in HCV core-expressing cells and demonstrated that genes including those encoding IL-6 and STAT3 were upregulated by core protein (7). Consistent with these findings, we found that cellular IL-6 expression levels were elevated in HCV-transfected cells in the order (from lowest to highest levels) untransfected, wild type, and then core mutants, which correlated well with SOCS3 expression (Fig. 4B) and with cellular responses to IFN (Fig. 1B and C). The inducers of IL-6 remain to be clarified. IL-6 is secreted in response to cellular steatosis and insulin resistance (45). Hepatic steatosis is found in 70% of CHC patients (57) and those with obesity; steatosis or insulin resistance is refractory to IFN treatment (43). Such patients show higher levels of hepatic SOCS3 ex-

pression than those without obesity or insulin resistance (34, 61). We reported previously that a series of genes involved in fatty acid and cholesterol synthesis are upregulated in HCV replicon-expressing and HCV-JFH1-infected cells and increased cellular LDs (39). Such lipogenic cellular processes may be the cause of the upregulated expression of IL-6. Alternatively, UPRs may produce IL-6. Chen et al. have reported that UPRs are coupled with TNF-α and IL-6 production in human macrophages (11). In this study, transfection of Huh7 cells by HCV induced the expression of UPR genes, and their expression levels were significantly higher in mutant core protein-transfected cells than in wild type-transfected cells (Fig. 6).

The differences in ISG expression levels between the HCV wild type and core mutants were significant but small (Fig. 3A, B, and C). As shown in Fig. 3D, and E and 4B, the interclone differences in pSTAT and SOCS3 were significant but relatively small, which may explain the small differences in ISG levels. Similarly, the clinical difference in interferon treatment outcomes between core 70/91 mutants and wild types are significant but are around the sustained viral clearance rates of 32.4% versus 53.5% in core 70 or 91 mutants and wild types, respectively (19), which might be consistent with our present results.

In clinical settings, IFN resistance of the core amino acid 70/91 mutants has been reported for genotype 1b strains (5). At present, there is no report that these mutations are associated with IFN treatment responses to other genotypes, including genotype 2a, which we used in this study. Because HCV strains other than genotypes 1 and 4 are generally sensitive to IFN, the core 70/91 mutations might not affect final treatment outcomes. We have conducted preliminary experiments using genotype 1b infectious clones with low levels of replication and found that these mutations did not significantly affect sensitivity to IFN in culture. It may be necessary to investigate IFN sensitivity when efficient cell culture systems have been developed for HCV genotype 1.

In addition to the poor virological responses of HCV core amino acid 70/91 mutants to peg-IFN-plus-RBV treatment (4, 6, 12), patients infected with the core mutants showed increased incidence of hepatocellular malignancies (2, 15, 49). It has been reported that the HCV core R70 but not L91 mutant frequently causes steatosis and increased hepatic oxidative stress (52). It is possible that core 70/91 mutations not only induce IFN resistance but also may cause other pathophysiological conditions, such as carcinogenesis and disorders of lipid metabolism.

In conclusion, our study demonstrates that the IFN resistance of HCV core mutants may be, for the most part, determined by cellular expression levels of SOCS3 and IL-6. Therapeutic targeting of IL-6 potentially may be a key to targeting IFN resistance and improving antiviral chemotherapeutics against HCV.

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Inhibitory Effect of a Triterpenoid Compound, with or without Alpha Interferon, on Hepatitis C Virus Infection▽†

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A lack of patient response to alpha interferon (α-IFN) plus ribavirin (RBV) treatment is a major problem in eliminating hepatitis C virus (HCV). We screened chemical libraries for compounds that enhanced cellular responses to α-IFN and identified a triterpenoid, toosendanin (TSN). Here, we studied the effects and mechanisms of action of TSN on HCV replication and its effect on α-IFN signaling. We treated HCV genotype 1b replicon-expressing cells and HCV-J6/JFH-infected cells with TSN, with or without α-IFN, and the level of HCV replication was quantified. To study the effects of TSN on α-IFN signaling, we detected components of the interferon-stimulated gene factor 3 (ISGF3), phosphorylated signal transducer and activator of transcription 1 (STAT1), and STAT2 by Western blotting analysis; expression levels of mRNA of interferon regulatory factor 9 using real-time reverse transcription-PCR (RT-PCR); and interferon-stimulated response element reporter activity and measured the expression levels of interferon-inducible genes for 2′,5′-oligoadenylate synthetase, MxA, protein kinase R, and p56 using real-time RT-PCR. TSN alone specifically inhibited expression of the HCV replicon (50% effective concentration = 20.6 nM, 50% cytotoxic concentration > 3 μM, selectivity index > 146). Pretreatment with TSN prior to α-IFN treatment was more effective in suppressing HCV replication than treatment with either drug alone. Although TSN alone did not activate the α-IFN pathway, it significantly enhanced the α-IFN-induced increase of phosphorylated STATs, interferon-stimulated response element activation, and interferon-stimulated gene expression. TSN significantly increased baseline expression of interferon regulatory factor 9, a component of interferon-stimulated gene factor 3. Antiviral effects of treatment with α-IFN can be enhanced by pretreatment with TSN. Its mechanisms of action could potentially be important to identify novel molecular targets to treat HCV infection.

Hepatitis C virus (HCV) is one of the most important pathogens causing acute and chronic hepatitis, liver cirrhosis, and hepatocellular malignancies (29). Alpha interferon (α-IFN) combined with ribavirin (RBV) is the standard treatment for HCV infection (6, 10). However, virus elimination rates are about 50% among treated patients, and therapy is often accompanied by substantial side effects (6, 44). It was recently reported that genetic polymorphisms of the *IL28B* gene, which codes for lambda IFN, are critical for predicting responses to α-IFN plus RBV therapy (8, 35, 38). Patients with minor variants of *IL28B*, who comprise ~50% of Caucasian, 25% of Asian, and ~70% of African populations, showed poor responses to α-IFN treatment. Although new specific anti-HCV drugs are under development, many of them require combined use with α-IFN and RBV (26). Taken together, current difficulties in eliminating HCV are mostly attributable to the limited treatment options and to the limited activity of α-IFN against the virus. For this reason, the development of safe and effective agents that enhance antiviral actions against HCV has been a strong motivation in academia and industry.

To search for a new agent which enhances the effect of α-IFN, we used interferon-stimulated response element (ISRE) reporter screening. We screened a chemical library (60,500 compounds) for compounds that enhance ISRE activity when they are used in combination with α-IFN, using ISRE reporter screening, and identified several compounds that increased the ISRE reporter activities when they are used in combination with α-IFN and that did not show cytotoxicity. Among the hit compounds, toosendanin (TSN; $C_{30}H_{38}O_{11}$; molecular weight = 574) (Fig. 1), which is a triterpenoid derivative extracted from the bark of *Melia toosendan* Sieb et Zucc, was the strongest in enhancing α-IFN-induced ISRE reporter activation and the expression of interferon-stimulated genes (ISGs). TSN has been used as an anthelmintic vermifuge against ascaris (31). Although TSN has some other biological effects against toxin-producing anaerobic bacteria and against carcinoma cells (32, 45), antiviral activity has not been reported.

In this study, we showed, using an HCV replicon system, that TSN, with or without α-IFN, inhibits HCV replication in a cultured human hepatoma Huh7 cell line and that the combination of TSN and α-IFN shows synergistic effects on viral replication. We have investigated the mechanisms of action of

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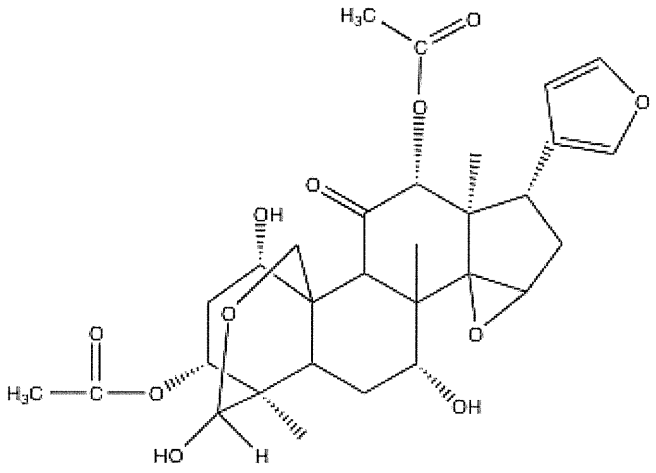

FIG. 1. Chemical structure of toosendanin.

TSN further and show that TSN induced activation of a component of interferon-stimulated gene factor 3 (ISGF3).

MATERIALS AND METHODS

Reagents. Alpha interferon was from Otsuka (Tokushima, Japan). TSN was from APIN Chemicals (Oxon, United Kingdom). Purity was over 77.32%. The designated concentration was achieved through dilution with cell culture medium (the final concentration of dimethyl sulfoxide [DMSO] in the medium was less than 0.3%). Beta-mercaptoethanol was from Wako (Osaka, Japan). The TSN used in this study was solubilized in DMSO.

Cells and cell culture. The human hepatoma cell line Huh7 was maintained in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum at 37°C under 5% CO_2. To maintain cell lines carrying an HCV subgenomic replicon (Huh7/Rep-Feo), G418 (Nakalai Tesque, Kyoto, Japan) was added to the culture medium at a final concentration of 500 μg/ml.

HCV subgenomic replicon construct. The HCV subgenomic replicon plasmid pRep-Feo expresses a fusion gene comprising the firefly luciferase and neomycin phosphotransferase (37, 43). RNA was synthesized *in vitro* from the plasmid and transfected into Huh7 cells. After culture in the presence of G418, cell lines stably expressing the replicon were established.

Reporter constructs. We analyzed the effects of TSN, with or without α-IFN, on signal transduction of ISRE and nuclear factor-kappaB (NF-kappaB). A plasmid, pCIneo-Rluc-IRES-Fluc, was constructed to analyze HCV internal ribosome entry site (IRES)-mediated translation efficiency (23). Plasmids pISRE-TA-Luc and pNF-kappaB-Luc (Clontech Laboratories, Franklin Lakes, NJ) contained consensus motifs upstream of the firefly luciferase gene. A plasmid, pTA-Luc (Clontech), which lacks the enhancer element, was used to determine the background. Plasmid pRL-CMV (Promega, Madison, WI), which expresses the Renilla luciferase protein, was used for normalization of transfection efficiency (17).

ISRE reporter screening. Huh7 cells were seeded in 384-well plates at a density of 3.0×10^3 cells/well. An ISRE-responsive firefly luciferase reporter was introduced using Lipofectamine 2000 (Invitrogen). Five hours after transfection, the cells were treated with 60,500 compounds from chemical libraries at a concentration of 3 μg/ml for 24 h and then treated with α-IFN at a concentration of 3 IU/ml. Six hours later, cells were lysed, and luciferase activities were quantified using a Steady Glo luciferase assay kit (Promega). The compounds were stored in 100% DMSO, and thus, the final concentration of DMSO was 0.3%. *Z'* factors were calculated as reported previously (46).

Luciferase assays and measurements of antiviral activity. Huh7/Rep-Feo cells were cultured with various concentrations of compound, such that the final DMSO concentration was 0.1%. Levels of HCV replication were quantified by internal luciferase assay after 48 h of culture. Luciferase activities were quantified using a luminometer (Promega) and the Bright-Glo luciferase assay system (Promega). Assays were performed in triplicate, and the results are expressed as mean percentage of the controls ± standard deviation (SD). The 50% effective concentration (EC_{50}) values were calculated using the probit method (2, 33). The determination of EC_{50}s was performed three times, and EC_{50}s are presented as means ± SDs for each compound.

MTS assays. To evaluate cell viability, dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium (MTS) assays were performed using a Cell Titer 96 Aqueous One Solution cell proliferation assay (Promega) as previously reported (18, 22). Huh7/Rep-Feo cells and HCV-J6/JFH1-infected Huh7 cells were seeded in 96-well plates at a density of 8.0×10^3 cells/well. After treatment, to analyze the therapeutic index with the same concentration of the drug and administration time, 20 μl/well of Cell Titer 96 Aqueous One Solution reagent was added to the cells cultured in a 96-well plate, the plate was incubated at 37°C for 60 min, and then the absorbance at 490 nm was recorded with a 96-well plate reader. The cells were analyzed when the growth became confluent. Cell viability was expressed as the concentration required for 50% cytotoxicity (CC_{50}). The drug selectivity index was calculated as CC_{50}/EC_{50}. All experiments were performed in triplicate.

Analyses of drug synergism. The effects of treatment of Huh7/Rep-Feo cells with α-IFN, alone and in combination with TSN, were analyzed by using isobologram analysis as described previously (27, 37). Dose inhibition curves of α-IFN and TSN were drawn with the two drugs used alone or in combination. In each drug combination, EC_{50}s for α-IFN and TSN were plotted against the fractional concentration of α-IFN and TSN on the *x* and *y* axes, respectively. A theoretical line of additivity is drawn between plots of the EC_{50} for either drug that was used alone. The combined effects of the two drugs were considered additive, synergistic, or antagonistic if the plots of the drug combination were located on the line, below, or above the line of additivity, respectively.

HCV-J6/JFH1 cell culture. HCV-J6/JFH1 (21), which is a recombinant of HCV-JFH1 (42), was used. *In vitro*-synthesized HCV-J6/JFH1 RNA was transfected into naïve Huh7 cells (48), and the cells were cultured in the presence of drugs (34). Cellular viral RNA expression levels were measured using a real-time reverse transcription-PCR (RT-PCR) system.

Real-time RT-PCR analysis. Real-time RT-PCR was carried out as described previously (7). Total cellular RNA was extracted from cultured cells using Isogen (Nippon Gene, Tokyo, Japan), reverse transcribed, and subjected to real-time RT-PCR analyses. Expression of mRNA was quantified using TaqMan Universal PCR master mix, an ABI 7500 real-time PCR system (Applied Biosystems, CA), and a QuantiTect SYBR green PCR kit (Qiagen, CA). Some primers have been described elsewhere (30, 34). The primers used were -S (5'-TTT GAA ACA TCA AAG TTT TTC ACA GAC CTA-3'), -AS (5'-CAC AGT CAA GGT CCT TAG TAT TTC AGA TGT-3'), p56-S (5'-ACT TCG GAG AAA GGC ATT AGA TCT GGA AAG-3'), p56-AS (5'-TAA GGA CCT TGT CTC ACA GAG TTC TCA AAG-3'), Viperin-S (5'-GCT ACC AAG AGG AGA AAG CA-3'), Viperin-AS (5'-TTG ATC TTC TCC ATA CCA GC-3'), ISG20-S (5'-CTA CGA CAC GTC CAC TGA CAG G-3'), ISG20-AS (5'-CAT CGT TGC CCT CGC ATC TTC-3'), IRF9-S (5'-GCA GCA GCA GCC CTG AGC CAC AGG AAG TTA-3'), IRF9-AS (5'-TTA CCT GGA ACT TCG GTG GGG GGC CCA GGC-3'), IFNAR1-S (5'-CTT TCA AGT TCA GTG GCT CC-3'), IFNAR1-AS (5'-CAT CAG ATG CTT GTA CGC GGA G-3'), IFNAR2-S (5'-GCC AGA ATG CCT TCA TCG TCA G-3'), and IFNAR2-AS (5'-GTG AGT TGG TAC AAT GGA GTG G-3').

Western blotting. Twenty micrograms of total cell lysate was separated by SDS-PAGE and blotted onto a polyvinylidene fluoride Western blotting membrane. The membrane was incubated with the primary antibodies, followed by incubation with a peroxidase-labeled anti-IgG antibody, and were visualized by chemiluminescence using an enhance chemiluminescence Western blotting analysis system (Amersham Biosciences, Buckinghamshire, United Kingdom). The antibodies used were mouse anti-NS5A (BioDesign, ME), rabbit anti-signal transducer and activator of transcription 1 (anti-STAT1) p84/p91, rabbit anti-phospho-STAT1 (Tyr 701), rabbit anti-STAT2, rabbit anti-phospho-STAT2 (Tyr 690) (Santa Cruz, CA), and anti-beta-actin antibody (Sigma). NIH image software was used to analyze the densitometry of the Western blot analysis. Quantification of STAT phosphorylation was done using NIH image software, and the results correspond to the ratio between the phosphorylated STAT1 (p-STAT1) or p-STAT2 amount and the STAT1 or STAT2 amount normalized to the amount for the control without α-IFN and TSN. The results correspond to the ratio between the NS5A amount and the beta-actin amount normalized to the amount for the control without α-IFN and TSN.

Statistical analyses. Statistical analyses were performed using Student's *t* test. *P* values of less than 0.05 were considered statistically significant.

RESULTS

ISRE reporter screening. At the primary screening (n = 1), we defined a 1.5-fold induction in response to α-IFN to be a hit compound, and the hit rate was about 1%. At the secondary screening (n = 4), we selected the compound whose cps were 2 SDs larger than that for the drug used as a negative control, and the hit compound rate was 0.2% of the original library. Both assays were highly reproducible, and reflecting this, the Z' factor (46) for the ISRE reporter screen was 0.97.

TSN has activity against HCV RNA replication. Huh7/Rep-Feo cells were cultured with various concentrations of TSN, and the effect was measured using a luciferase assay. TSN caused a marked suppression of HCV RNA replication in a dose-dependent manner (Fig. 2A). The EC_{50} of TSN was 20.6 nM. In contrast, MTS assays showed that treatment with TSN had little effect on cellular viability and replication, with a CC_{50} of over 3 μM and a selectivity index of more than 146. These results indicated that TSN had an effect against HCV RNA replication when it was used alone and that the effect was specific for HCV replication and not attributable to nonspecific cytotoxicity (Fig. 2B). Similarly, by Western blotting (Fig. 2C), the expression of HCV NS5A protein was shown to be reduced by corresponding amounts following treatment with TSN. To determine whether TSN suppresses HCV IRES-dependent translation, we used a Huh7 cell line that had been stably transfected with pCIneo-Rluc-IRES-Fluc (Fig. 2D). Treatment of these cells with TSN resulted in no significant change of the internal luciferase activities at concentrations of TSN that suppressed expression of the HCV replicon.

TSN increased ISRE reporter activity with α-IFN. Because we identified TSN originally through ISRE reporter-based drug screening, we analyzed the effects of TSN on the cellular responses to α-IFN following pretreatment with TSN. First, we treated ISRE-TA-Luc-transfected Huh7 cells with TSN and α-IFN simultaneously or pretreated the cells with 10 to 100 nM TSN at 24 or 48 h prior to α-IFN treatment. Luciferase assays were performed 6 h after addition of α-IFN at concentrations of 0.1 to 100 IU/ml (Fig. 3A and B). Treatment with TSN alone did not increase ISRE reporter activity. Similarly, simultaneous treatment with TSN and α-IFN did not enhance α-IFN-induced ISRE reporter activation more than treatment with α-IFN alone. In contrast, pretreatment with TSN 24 or 48 h before addition of α-IFN significantly increased ISRE activation compared to that achieved by treatment with α-IFN alone (Fig. 3A). On the basis of these results, we performed the subsequent experiments with addition of TSN 24 h before α-IFN treatment.

We next quantified the expression levels of ISGs, including those for 2′,5′-oligoadenylate synthetase (25AS), MxA, protein kinase R, p56, viperin, and ISG20, which encode proteins with direct antiviral activity (14, 15, 25). Naïve Huh7 cells were treated with TSN for 24 h, followed by treatment with 100 IU/ml α-IFN for 24 h. The expression of each ISG was significantly elevated in a dose-dependent manner following pretreatment with TSN and α-IFN stimulation (Fig. 3C). These results indicated that TSN pretreatment significantly enhanced the cellular response to α-IFN-induced, ISRE-regulated expression of ISGs.

It has been reported that α-IFN receptor-mediated signaling

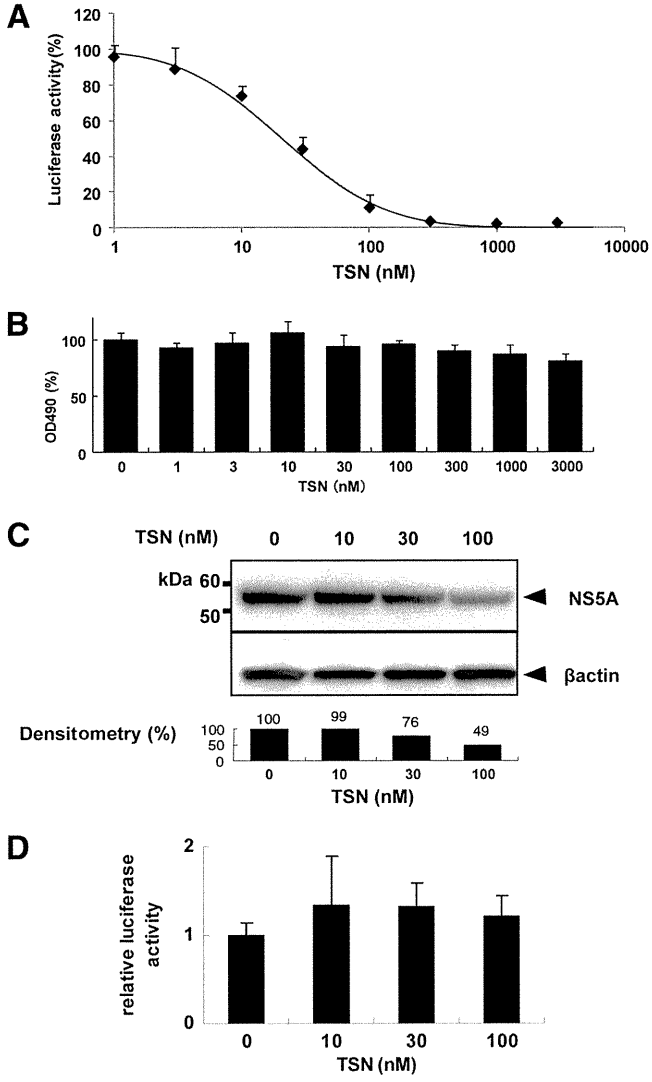

FIG. 2. Effect of TSN on expression of HCV replicon. (A) HCV replicon cells were treated with various concentrations of TSN for 48 h. Replication levels of HCV RNA were analyzed by luciferase assay. Bars indicate luciferase activities relative to that of the drug-negative control. (B) Cell viability was determined by MTS assay. Bars indicate the value relative to that of the drug-negative control. (C) Western blotting analyses. The expression of NS5A and beta-actin was detected using anti-NS5A and anti-beta-actin antibodies. Densitometry of NS5A protein was performed, and the result is indicated as a percentage of the result for the drug-negative control. The assay was repeated three times, and a representative result is shown. (D) A bicistronic reporter gene plasmid, pCIneo-Rluc-IRES-Fluc, was transfected into Huh7 cells. The cells were cultured with TSN at the concentrations indicated, and dual luciferase activities were measured after 24 h of treatment. Values are displayed as ratios of Fluc to Rluc. In panels A, B, and D, the assays were done in triplicate and repeated three times. Error bars indicate means ± SDs.

cross talks with several alternative pathways, including the NF-kappaB, gamma IFN, phosphatidylinositol 3-kinase (PI3K), and mitogen-activated protein kinase (MAPK) pathways (9, 16, 24, 28). Therefore, we analyzed the effect of TSN on the signaling pathways indicated above. Cells were transfected with various reporter plasmids, including NF-kappaB, gamma

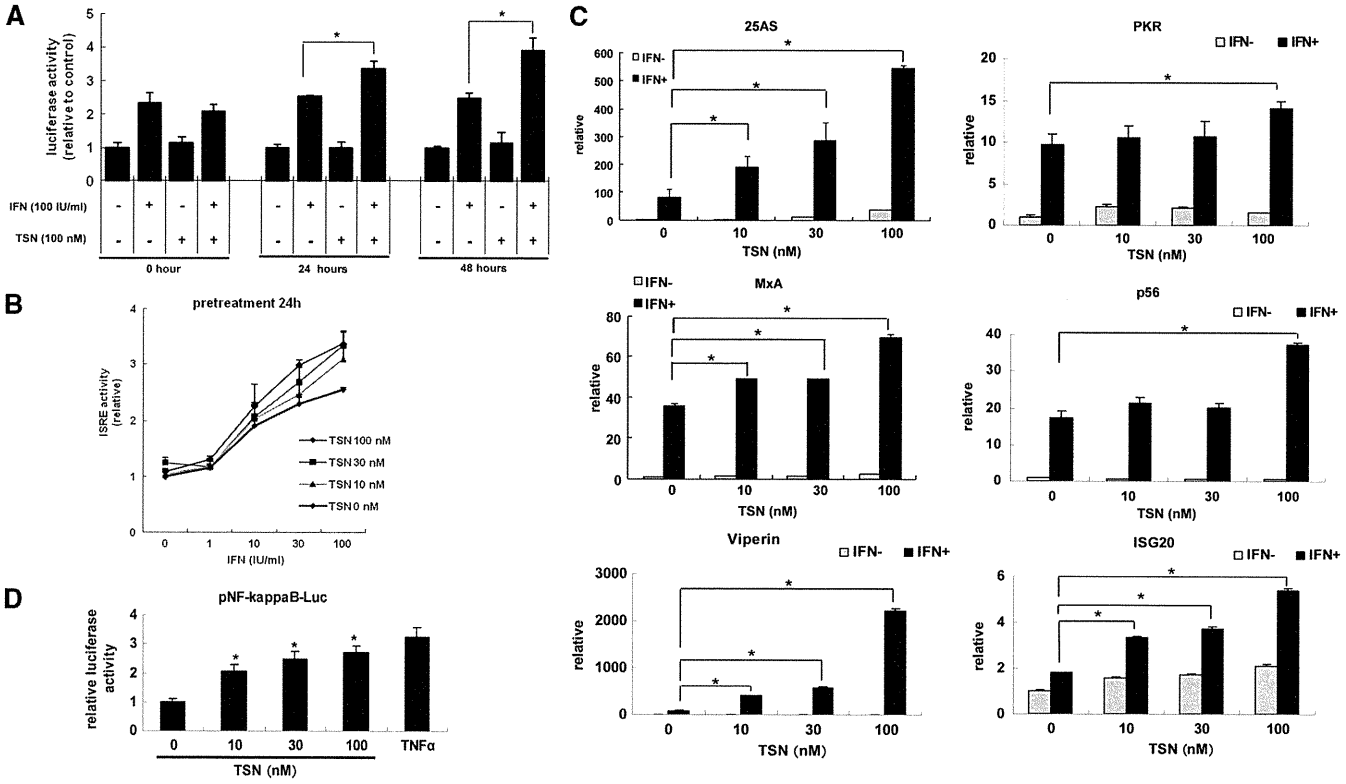

FIG. 3. ISRE reporter screening and aberrant pathway of α-IFN. (A) Pretreatment with TSN. Huh7 cells transfected with a reporter gene (pISRE-Luc and pRL-CMV) were pretreated with TSN (0 or 100 nM) for 0, 24, or 48 h, followed by treatment with α-IFN (0 or 100 IU/ml). Six hours later, the relative ISRE-luciferase activity ($n = 4$) was determined as described in Materials and Methods. The data are expressed as means ± SDs and are a representative example of the data from three similar experiments. (B) Pretreatment with TSN at the concentrations indicated for 24 h, followed by treatment with α-IFN (0 to 100 IU/ml). The ISRE reporter assay was performed as described for panel A. (C) Type I IFN-induced antiviral ISG expression in Huh7 cells. Huh7 cells were treated with TSN for 24 h, followed by treatment with α-IFN at 100 IU/ml for 24 h. The total cellular RNA was then isolated for real-time RT-PCR analysis of the mRNAs of 25AS, MxA, p56, viperin, and ISG20. Beta-actin was used as a control. The data are expressed as means ± SDs and are a representative example of data from three similar experiments. *, $P < 0.05$. (D) Analysis of aberrant pathways of α-IFN signaling under the influence of TSN. Promoter activities of NF-kappaB were analyzed by luciferase reporter assays. These cells were transfected with pNF-kappaB-TA-Luc, pTA-Luc which lacks the enhancer element and which was used as a negative control, and pRL-CMV to normalize transfection efficiency. At 24 h after transfection with these reporters, treatment with TSN (0, 10, 30, 100 nM) was carried out. After 24 h, the relative levels of induction of NF-kappaB activity for each treatment were calculated. TNF-α (50 ng/ml), which was used as a positive control for NF-kappaB, was added 6 h before analysis. The assays were done in triplicate and repeated three times. Error bars indicate means ± SDs. *, $P < 0.05$.

interferon activation site (GAS), or activator protein 1 (AP1) Fluc plasmids. The reporter activities were measured after culture with or without TSN. As shown in Fig. 3D, there was no significant effect of TSN on GAS or AP1 reporter activities (data not shown). In contrast, NF-kappaB reporter activity was significantly elevated by TSN in a dose-dependent fashion.

Synergistic inhibitory effects of TSN and α-IFN on the replicon. We next assessed the effects of TSN combination with α-IFN on the intracellular replication of the HCV genome. Huh7/Rep-Feo cells were treated with various concentrations of TSN (0, 0.01, and 0.03 μg/ml) and α-IFN (0 to 100 IU/ml). Replication of the HCV replicon was suppressed by pretreatment with TSN, followed by treatment with α-IFN, in a dose-dependent manner (Fig. 4A; see Fig. S1 in the supplemental material). The EC_{50} of α-IFN in the absence of TSN was 7.61 IU/ml, while that after pretreatment with 0.03 μg/ml (41 nM) TSN was 3.16 IU/ml. These results indicated that pretreatment with TSN before α-IFN treatment is more effective in inhibiting HCV replication than treatment with α-IFN alone.

Subsequently, we conducted the following assay to determine whether TSN and α-IFN have a synergistic inhibitory effect on the replicon. The relative dose-inhibition curves of α-IFN were plotted for several concentrations of TSN and α-IFN. The curves shifted to the left with increasing concentrations of TSN (Fig. 4B), demonstrating that HCV replication was considerably reduced by the combination compared with that by either TSN or α-IFN alone. An MTS-based cell viability assay did not show significant cytotoxicity from TSN (Fig. 4C). Western blot analysis and densitometry of each blot showed results essentially identical to those from the luciferase assay (Fig. 4D).

We used isobologram analysis to determine whether the anti-HCV effect of TSN is synergistic with that of α-IFN (27, 37). Huh7/Rep-Feo cells were treated with a combination of α-IFN and TSN at an EC_{50} ratio of 1:0, 2:3, 1:4, or 0:1, and the dose-effect plots were drawn (Fig. 4E). The fractional EC_{50}s for α-IFN and TSN were plotted on the x and y axes, respectively, to generate an isobologram (Fig. 4F). Each plot showing

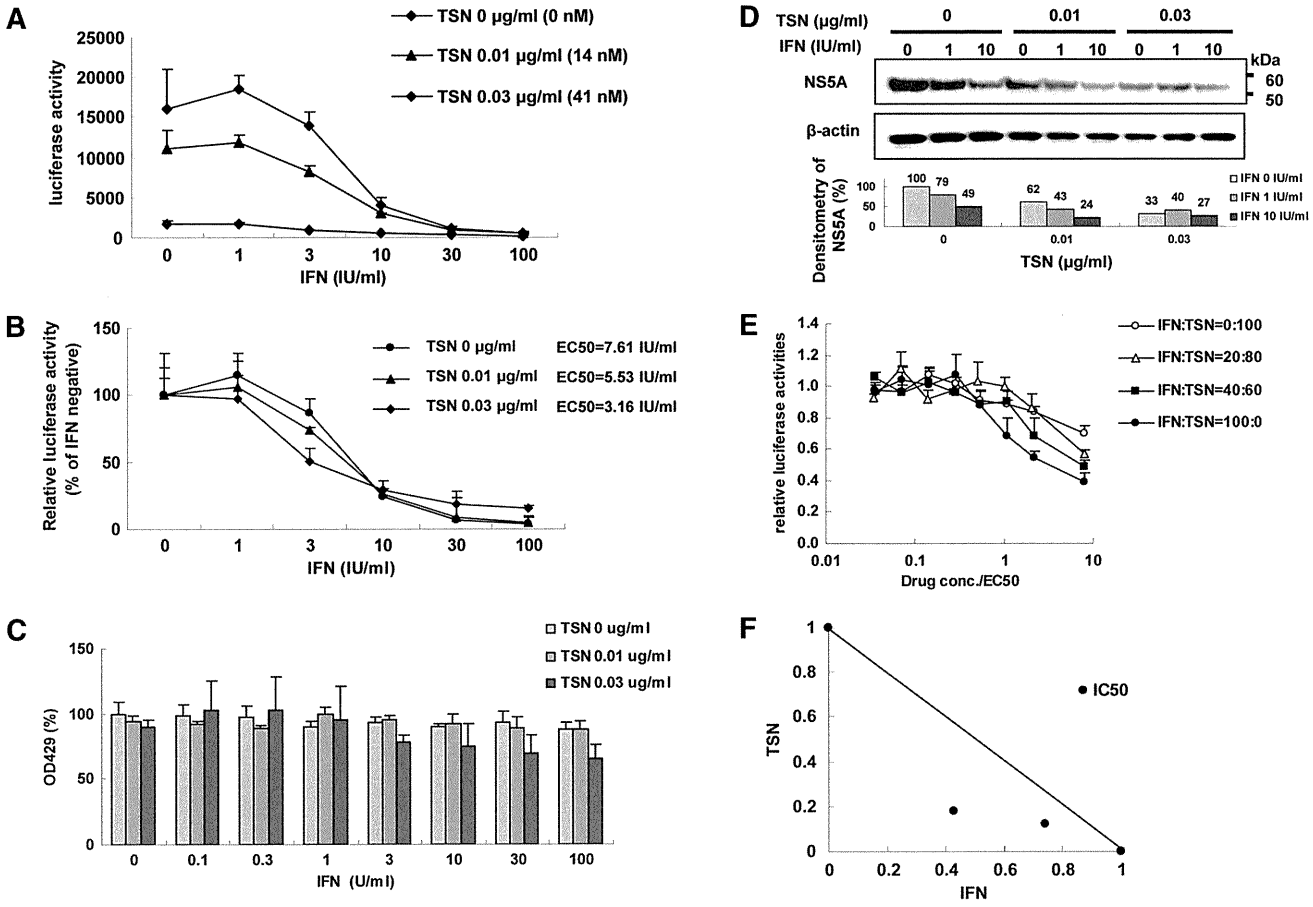

FIG. 4. Suppression of HCV RNA replication by TSN combined with α-IFN. (A and B) Luciferase activity (A, absolute value; B, relative value). Huh7/Rep-Feo cells, which constitutively express an HCV replicon, enable the quantification of replication levels through the measurement of luciferase activity. Absolute and relative dose-response curves in the presence of 24 h of pretreatment of various concentrations of TSN (0, 0.01, 0.03 μg/ml) and α-IFN (0, 100 IU/ml). (A) Bars indicate luciferase activities. (B) Bars indicate luciferase activities relative to the activity of each α-IFN-negative control. Luciferase assays were performed in triplicate. Error bars indicate means ± SDs. (C) MTS assay of Huh7/Rep-Feo cells cultured with the indicated concentrations of TSN and α-IFN. The assays were done in triplicate and repeated three times. Error bars indicate means ± SDs. (D) Western blotting. Ten micrograms of total cellular protein was separated by polyacrylamide gel electrophoresis and transferred onto the membrane. Monoclonal anti-NS5A antibody or an anti-beta-actin antibody was used as the primary antibody. Densitometry of NS5A or beta-actin protein was performed and the result is indicated as a percentage of that for the drug-negative control. The assay was repeated three times, and representative results are shown. (E) Dose-inhibition curves of α-IFN and TSN when they were combined at the indicated ratios, adjusted by the EC_{50} of the individual drug. Assays were done in triplicate, and mean values were plotted and indicated as means ± SDs. (F) Graphical representation of the isobologram analysis. For each drug combination in panel E, the EC_{50}s of α-IFN and TSN for inhibition of HCV replication were plotted against the fractional concentrations of α-IFN and TSN, which are indicated on the *x* and *y* axes, respectively. A theoretical line of additivity is drawn between the EC_{50} for each drug alone. All of the fractional EC_{50} plots for the TSN and α-IFN combinations fell below the line of additivity, indicating synergy.

the fractional EC_{50} of each drug ratio fell below the line showing additivity, indicating that the effect of the drug combination on intracellular HCV RNA replication was synergistic. The MTS values at the drug concentrations used in this isobologram analysis did not show any significant decrease, suggesting that the synergistic action of α-IFN and TSN on HCV replication is through their pharmacological effects and is not due to augmentation of cytotoxicity.

Suppression of HCV-J6/JFH1 infection by pretreatment of TSN with α-IFN. The inhibitory effects of pretreatment with TSN prior to α-IFN treatment demonstrated on HCV subgenomic replication were validated further using HCV-J6/JFH1 cell culture (21, 42). Various concentrations of TSN and α-IFN were added to HCV-J6/JFH1-infected Huh7 cells, and intracellular HCV RNA was quantified after 48 h of incubation. As shown in Fig. 5A, TSN with or without α-IFN suppressed expression of intracellular HCV RNA in a dose-dependent manner. The EC_{50}s of α-IFN with TSN at 0, 10, and 30 nM were 4.71 IU/ml, 3.83 IU/ml, and 3.52 IU/ml, respectively. An MTS-based cell viability assay did not show significant cytotoxicity from TSN (Fig. 5B). These data indicate that pretreatment with TSN also augmented the α-IFN effect on the JFH1 system.

TSN upregulates ISGF3 in combination with α-IFN. Subsequently, we performed experiments to investigate the mechanisms of action of TSN. First, we quantified expression of alpha/beta IFN receptor subunit (IFNAR) 1 and IFNAR2 and the effect of TSN. Real-time RT-PCR analysis showed no

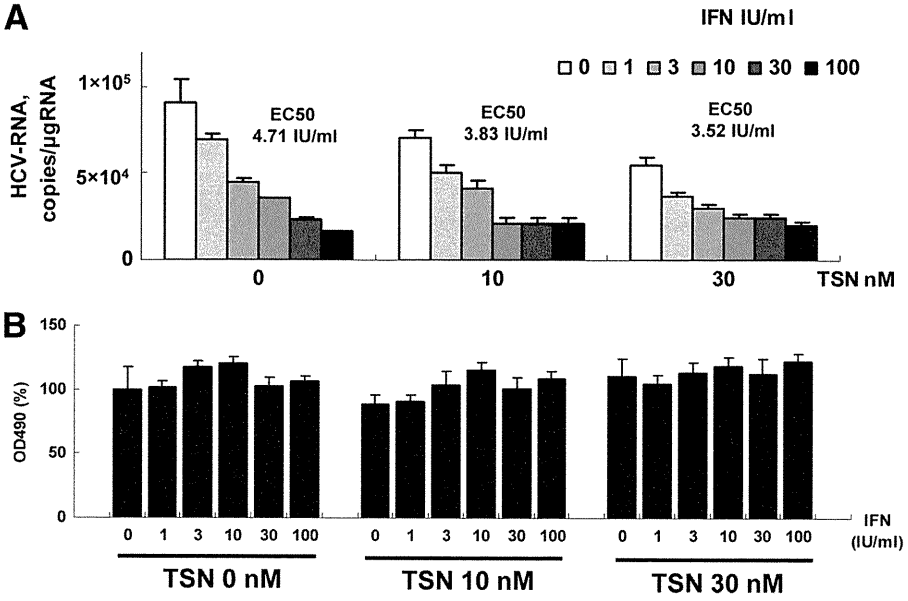

FIG. 5. Suppression of full HCV-J6/JFH1 replication by pretreatment of TSN with α-IFN. Ten micrograms of HCV-J6/JFH1 RNA was transfected into Huh7 cells. At 48 h after transfection, cells were pretreated with TSN for 24 h, followed by treatment with α-IFN (0, 1, 3, 10, 30, 100 IU/ml). At 48 h after α-IFN addition, cells were harvested. (A) Real-time RT-PCR analysis; (B) effect of pretreatment TSN with α-IFN on cell viability. MTS assays were performed 48 h after culture in the presence of pretreatment TSN with α-IFN. Bars indicate values relative to that of the drug-negative control. In panels A and B, the assays were done in triplicate and repeated three times. Error bars indicate means ± SDs.

change in levels of IFNAR1 and IFNAR2 mRNA expression with or without TSN (Fig. 6).

Next, we investigated the ISGF3 components, STAT1, and STAT2, using Western blotting, and interferon regulatory factor 9 (IRF9), using real-time RT-PCR. Huh7 cells were treated with various concentrations of TSN or 0.01% DMSO. Twenty-four hours after TSN treatment, 100 IU/ml of α-IFN was added, and STATs and IRF9 were detected. Western blot analysis demonstrated that phosphorylated STAT1 and STAT2 levels were increased more by treatment with α-IFN and TSN than by α-IFN treatment alone (Fig. 7A and B). In addition, IRF9 mRNA expression was significantly higher following pretreatment with TSN prior to α-IFN therapy than by α-IFN monotherapy (Fig. 8). These findings are consistent with the hypothesis that TSN activates ISGF3 components in combination with α-IFN.

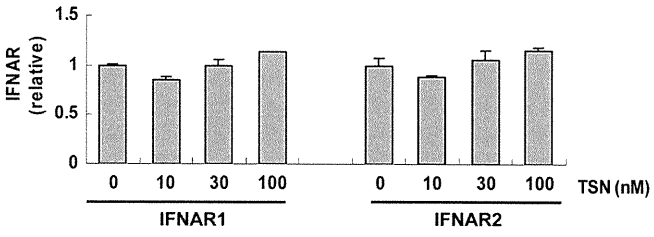

FIG. 6. IFNAR expression. Huh7 cells were pretreated with TSN for 24 h, followed by treatment with 100 IU/ml α-IFN for 6 h. The total cellular RNA was then isolated for real-time RT-PCR analysis of the mRNAs of IFNAR1 and IFNAR2. The assays were done in triplicate and repeated three times. The data are shown as means ± SDs.

DISCUSSION

In this study, we investigated the molecular actions of TSN on HCV replication and on α-IFN-mediated cellular antiviral responses. Treatment of cells expressing an HCV subgenomic replicon with TSN alone specifically inhibited HCV replication with a selectivity index of more than 146 (Fig. 2). In addition, pretreatment of cells with TSN prior to addition of α-IFN augmented α-IFN receptor-mediated, ISRE-regulated gene expression (Fig. 3). Consistent with these findings, TSN pretreatment significantly enhanced the suppressive effects of α-IFN on the HCV replicon and HCV cell culture (Fig. 4 and 5). Finally, we demonstrated that the α-IFN-enhancing effects of TSN are through increased transcriptional activation of a component of ISGF3 (Fig. 7 and 8). Taken together, out results demonstrate that TSN is potentially an effective antiviral agent when it is used alone and especially when it is used in combination with α-IFN and that screening for such α-IFN-enhancing agents may identify promising antiviral therapeutics. Because TSN treatment alone or simultaneous treatment with TSN and α-IFN did not increase ISRE activity or augment α-IFN-mediated ISRE activation, TSN may affect α-IFN sensitivity by upregulating molecules that affect α-IFN receptor-mediated signaling without activating ISRE signaling directly.

Type I interferon plays a central role in eliminating viruses through its innate antiviral activity or following therapeutic application. Binding of α-IFNs to their receptors activates the Jak-STAT pathway to form a complex with ISGF3, which translocates to the nucleus, binds the ISRE located in the promoter/enhancer region of the ISGs, and activates expression of ISGs (28, 39, 40). In this study, we demonstrated that TSN enhanced α-IFN effects by upregulating ISGF3, which

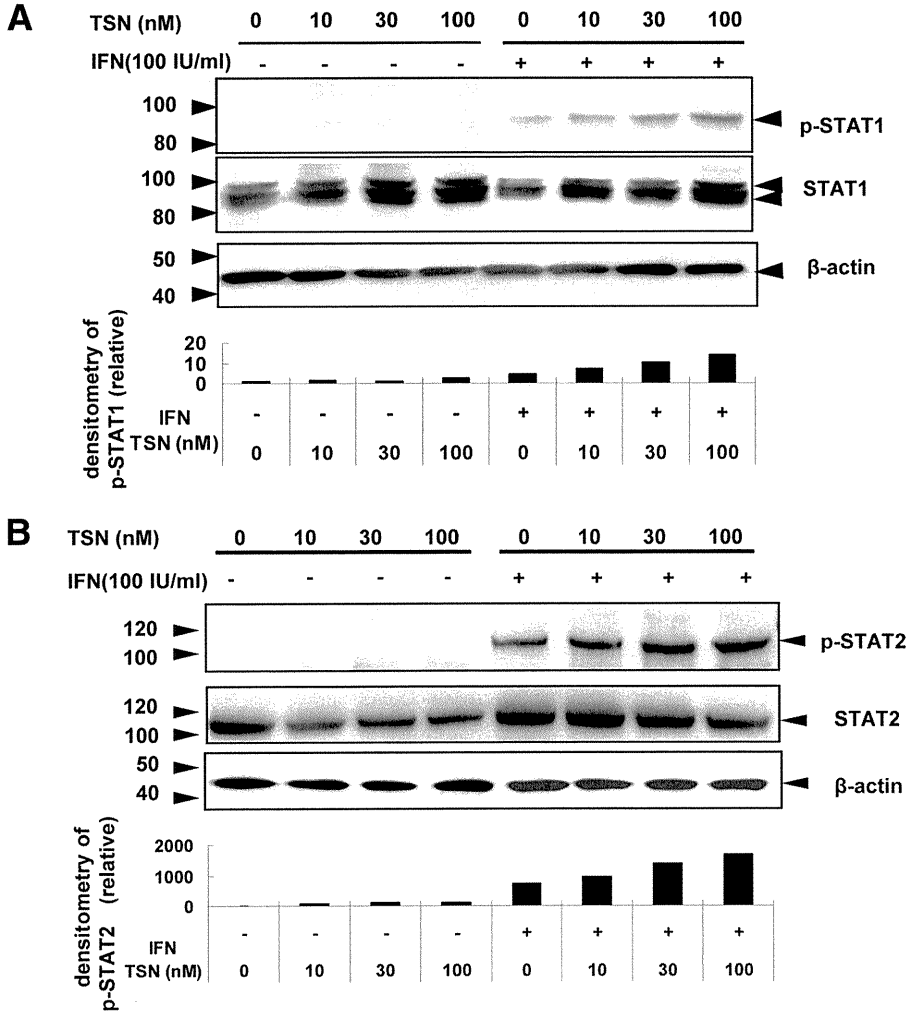

FIG. 7. TSN with α-IFN treatment of Huh7 cells increases phosphorylation of STAT1 and STAT2. (A) Western blotting. Alteration in the distribution of α-IFN-induced phosphorylation of STAT1 and STAT2 by TSN. Huh7 cells were treated with TSN or 0.01% DMSO for 24 h. After that, the cells were stimulated by 100 IU/ml α-IFN for 30 min. Cells were harvested, and the resulting lysates were analyzed for phosphorylated and total STAT1 or STAT2. The relative amounts of phosphorylated STAT1 or STAT2 were normalized to the amount of total STAT1 or STAT2 and expressed relative to the amount for the drug-negative control. The assay was repeated three times, and a representative result is shown.

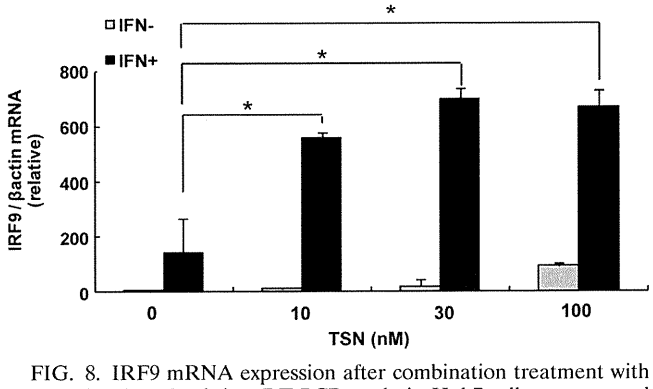

FIG. 8. IRF9 mRNA expression after combination treatment with TSN and α-IFN. Real-time RT-PCR analysis. Huh7 cells were treated with TSN for 24 h. After 6 h, the cells were stimulated by α-IFN (100 IU/ml). We used the method described in the legend to Fig. 6 to analyze the mRNA of IRF9. The assays were done in triplicate and repeated three times. Error bars indicate means ± SDs. *, $P < 0.05$.

may cancel the suppressive effect of HCV gene products on the α-IFN signaling pathway.

In our study, it was not proved that increasing ISRE activities had direct relevance to inhibition of HCV replication. In Fig. 3C, we showed that TSN with α-IFN treatment had elevated the level of expression of mRNA of ISGs. Previous studies suggested that overexpression of known ISGs inhibited HCV replication in HCV replicon-containing Huh7 cells (13, 14). These findings may support the possibility that TSN had the potential to augment the α-IFN effect.

Other than the canonical Jak/STAT-mediated α-IFN signaling pathway, several alternative α-IFN pathways have been reported, including the NF-kappaB, gamma IFN, PI3K, and MAPK pathways (9, 16, 24, 28). We carried out reporter assays using NF-kappaB, AP1, and GAS reporter plasmid constructs and treated the cells with TSN. As shown in Fig. 3D, TSN activated NF-kappaB-regulated gene expression significantly. NF-kappaB is a sequence-specific transcription factor which

regulates the expression of numerous cellular and viral genes and plays important roles in inflammation, innate immune responses, tumorigenesis, and cell survival (3, 19). Activation of NF-kappaB is principally regulated by tumor necrosis factor alpha (TNF-α), Toll-like receptors (TLRs), and RIG-I, which may possibly be associated with the molecular mechanisms of TSN monotherapy. Horsmans et al. (12) and Agrawal and Kandimalla (1) reported that TLR7, -8, and -9 agonists have the ability to modulate TLR-mediated immune responses in targeting a broad range of disease vectors, including HCV, alone or in combination with other therapeutic agents. These reports support the hypothesis that activation of NF-kappaB may be one of the mechanisms of action of TSN.

It has been reported that TSN exhibits cytotoxic/antiproliferative potential at high concentrations (36, 47). In our study, the selectivity index of TSN against HCV was sufficient to ascertain that the antiviral effects are not simply due to the cytotoxicity of TSN. A recent study showed that a triterpenoid compound, dammarenolic acid, inhibits retrovirus, human immunodeficiency virus, simian immunodeficiency virus, murine leukemia virus, and respiratory syncytial virus infections *in vitro* (4) (5). We have analyzed the effects of dammarenolic acid on antiviral actions on Huh7/Rep-Feo cells, cytotoxicity, and ISRE reporter activation. However, dammarenolic acid did not inhibit HCV replication or enhanced α-IFN-induced ISRE activity (data not shown). These findings suggest that the anti-HCV and α-IFN enhancer effects are distinctive features of TSN among triterpenoid compounds. Hiasa et al. have reported that ME3738, a triterpenoid saponin, suppressed HCV replication through production of endogenous beta interferon (11). ME3738 is now in clinical trials for treatment of HCV-infected patients. Taking these findings together, despite reports on the cell-suppressive effect of triterpenoids, properly selected or designed compounds might be used as drugs against HCV infection.

Because the mechanisms of action of these triterpenoid compounds against these viruses are poorly understood, further investigation of the mechanism of action of TSN on HCV may be valuable to implement antiviral strategies against other viruses. It would be important to assess drug resistance after continuous treatment with TSN. There is no *in vitro* or *in vivo* report on resistance of TSN or cellular attenuation of responses to TSN. Such information, if any is found, would help elucidate the mechanism of action of TSN.

Given the current situation of limited therapeutic options against HCV, the search for more potent and less toxic antiviral drugs is needed to improve clinical anti-HCV chemotherapeutics. Several direct antiviral agents with activity against HCV are currently undergoing clinical trials. These include NS3 protease inhibitors and NS5B polymerase inhibitors (41). However, the frequent emergence of drug-resistant viruses is a major weakness of such agents (20). Our results indicate that TSN is also effective at suppressing HCV infection and replication. Future studies with TSN, its derivatives, and other chemicals that target the α-IFN pathway could be directed toward developing a new class of antiviral treatment regimens and drugs.

ACKNOWLEDGMENTS

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Original Article

Studies on virus kinetics using infectious fluorescence-tagged hepatitis C virus cell culture

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Aim: Studies of the complete hepatitis C virus (HCV) life cycle have become possible with the development of a HCV-JFH1 cell culture system.

Methods: In this study, we constructed two fluorescence protein-tagged recombinant JFH1 virus clones, JFH1-EYFP and JFH1-AsRed, as well as two corresponding clones with adaptive mutations, JFH1-EYFP mutant and JFH1-AsRed mutant, that and were as effective as JFH1 in producing infectious virus particles, and investigated their viral infection life cycles.

Results: After infection of the fluorescence-tagged mutant viruses, infected cells increased exponentially. In cells, EYFP or AsRed and NS5A were expressed as a fusion protein and co-localized in core proteins. The rate of the cell–cell spread was dependent on the cell densities with a maximum of $10^{2.5}$/day. Treatment of cells with interferon or a protease inhibitor suppressed expansion of virus-positive cells.

Conclusion: Taken together, these results indicate that fluorescence-tagged HCV is a useful tool to study virus infection life cycles and to assist in the search for novel antiviral compounds.

Key words: AsRed, confocal laser microscopy, HCV-JFH1 cell culture, protease inhibitor, yellow fluorescence protein

INTRODUCTION

HEPATITIS C VIRUS (HCV) infection is characterized frequently by chronic inflammation of the liver, leading to decompensated liver cirrhosis and hepatocellular cancers.[1] Interferon (IFN)-α has been the mainstay of HCV therapy.[2] However, the most effective therapy, pegylated IFN plus ribavirin in combination, can eliminate HCV from only half of the patients treated[3,4] and often is accompanied by substantial side-effects.[5,6] These difficulties in eliminating the virus are attributable mostly to the limited treatment options.[7]

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Hepatitis C virus belongs to the family Flaviviridae. The viruses have positive-strand RNA genomes of approximately 10 kb that encode polyproteins of approximately 3000 amino acids. The protein is post-translationally processed by cellular and viral proteases into at least 10 mature proteins. The viral non-structural (NS) proteins accumulate in the endoplasmic reticulum (ER) and they direct genomic replication and viral protein synthesis.[8,9] Studies of the HCV life cycle and the development of new drugs have long been hampered by the lack of cell culture systems. These problems have been greatly overcome by the development of the HCV subgenomic replicon[10] and HCV-JFH1 cell culture[11] systems.

After the development of HCV-JFH1 cell culture, many variations of reporter protein-tagged HCV systems have been described.[12–15] These reporter systems, however, feature poor or absent virus propagation, secretion and re-infection. The C-terminal end of the NS5A region, which has been used for insertion of