small assay volumes are required for maximum efficiency, in order to minimize expense, space requirements, reagent consumption, and experimental time. The advancement of HTS technologies has made it possible to execute cell-based HTS with 384-well and even 1536-well plates. Use of automated methods for plate filling, compound transfer, and plate reading further maximizes efficiency. With the assistance of sophisticated software tools, screening data can be rapidly analyzed in an automated fashion.[5-7]

In addition to an efficient screening platform, large, diverse libraries of small molecule pertubagens appropriately formatted for this platform are required. The known bioactives library, a collection of 2568 known and proven bioactive molecules, including FDA-approved drugs, was the library screened in this report. Also available were commercially available combinatorial chemistry libraries and several diversity-oriented synthesis (DOS) libraries. One of these DOS libraries, Project Kornberg (a library of over 8000 DOS molecules that links chemists from multiple research groups throughout the US), was also screened using the methodology developed in this article. These results are the subject of a separate report.

Finally, a cell line carrying an accurate and reliable reporter is needed to efficiently screen large small-molecule libraries. To this end, the selection and optimization of the most appropriate HCV replicon cell line is very important. To successfully perform high-density HTS using 384-well plates, several characteristics of an HCV replicon cell line must be taken into account, such as RNA replication levels carrying an easy read-out reporter gene and host cell growth characteristics.

We selected the subgenomic Huh7/Rep-Feo replicon cell line because of its robust level of replication.[8] The level of HCV replication appears to be very high in this replicon cell line, although the precise comparison of replication levels between various replicon cell lines is difficult because of differing conditions in different laboratories.[16-19] We chose a replicon bearing the firefly luciferase gene because it can be easily measured, is very sensitive, and has a suitably long half-life.[8,20] Indeed, we obtained a high S/B ratio (>100) in the 384-well plate format, ensuring the suitability of this system for high-density HTS assays.

To facilitate detection of HCV replication, several replicon cell lines that carry reporter genes, such as beta-lactamase,[21] luciferase,[8,9,20] and secreted alkaline phosphatase (SEAP),[22,23] have also been developed. In fact, a cell-based HTS assay using 384-well plates has been developed with an HCV replicon bearing a beta-lactamase reporter gene.[24] However, this replicon model has several disadvantages. There is a relatively low signal to background. Moreover, additional processing is required to suppress the high background signal. Finally, because the cell line was transiently transfected, it requires extensive preparation prior to screening. *Renilla* luciferase has also been used as a reporter gene, but is not an ideal choice because of its very short signal half-life. Moreover, aspiration and lysis processing steps must be carried out prior to signal detection.[9] On the other hand, replicon cell models bearing a SEAP reporter do not require aspiration and lysis steps.[22,23] These systems, however, either require another viral protein, such as tat, to express the reporter protein or require the action of NS3/4A-specific protease activity.[22,23] Replicons bearing firefly luciferase reporter genes, therefore, appear to be better suited for use in HTS assays.

Many small molecules are cytotoxic, and hepatocyte replicon cell lines are highly sensitive to cytotoxic or cytostatic agents.[8,20] Cytotoxic effects can be mistaken for antiviral activity by decreasing luciferase signal merely by decreasing cell viability and not by decreasing HCV RNA replication, leading to false-positive results. In fact, when we confined our analysis to the reporter gene assay alone, our hit rates were very high, primarily due to false-positive results from cytotoxic compounds. We therefore perform both the reporter gene assay and a cell viability assay in parallel in our primary HTS, a step we view as essential. This counter screen was executed in order to minimize confounding from increased or decreased luciferase signal due to increased or decreased cell viability, respectively.

To verify hit compounds identified from primary screening, an appropriate secondary assay must be performed. We used the subgenomic Huh7/Rep-Feo replicon cell line in our primary screen because of its simplicity, rapidity, robustness, and reproducibility. Its disadvantages include its lack of structural proteins, its specific adaptive mutation, and its firefly luciferase reporter gene. By using a subgenomic replicon, the influence of structural proteins on the antiviral activity of compounds cannot be probed. Furthermore, a cell culture adaptive mutation may affect the biochemical properties of a protein by altering its sensitivity to an antiviral drug.[3,4] Compounds that specifically inhibit firefly luciferase activity can be identified as false-positive antiviral hits. For instance, pifithrin, an antiviral hit in our primary screen, inhibits firefly luciferase, but not *Renilla* luciferase.[25] In the secondary screen, we validated primary hits using a full-length OR6 replicon,[9] thereby ensuring validation in a more authentic viral polyprotein context. This full-length replicon also possesses a cell culture-adaptive mutation and a reporter gene distinct from those found in the subgenomic Huh7/Rep-Feo replicon, thereby minimizing confounding from those factors.

One of the limitations of high-throughput screening is that the generation of adequate dose-response curves for each of the compounds tested in a large primary screen would overwhelm the capacity of even the best screening technology platform by the sheer number of data points generated. Although screening at a single fixed concen-

tration per compound may lead to missing potential hits, it is much more efficient generating the dose-response data only for those compounds identified as hits. We conducted secondary validation screens to generate adequate dose-response curves for our hit compounds. We also performed cell viability assays in our secondary screens to minimize confounding from cytotoxicity.

Corticosteroids have been reported to promote HCV replication. It is thought that they exert their effects via host cellular immunosuppression.[13] However, our results demonstrate the direct proviral effect of corticosteroids in both a subgenomic and a full-length HCV replicon system. These data suggest that corticosteroids are capable of promoting HCV replication independent of their host immunosuppressive activity. Further study of the direct regulation of HCV replication by corticosteroids is warranted.

Strikingly, the statins were found to exhibit powerful antiviral effects. Lovastatin has been shown in an independent model to decrease HCV replication.[12] These compounds are inhibitors of HMG-CoA reductase and shut down cholesterol biosynthesis by preventing the formation of mevalonate from 3-hydroxy-3-methyl-glutaryl CoA. In addition to lowering intracellular levels of sterols, statins also reduce levels of isoprenoids, which are derived from mevalonate. Isoprenoids such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate serve as lipid attachments for a variety of intracellular signaling molecules. It has been reported that inhibition of geranylgeranylation, rather than the synthesis of cholesterol itself, is responsible for the inhibition of HCV RNA replication.[12,26] All statins except pravastatin showed good dose response in the secondary assays, with IC_{50} values from 1–10 μM. The HMG-CoA reductase inhibitors therefore appear to demonstrate potential as antiviral agents.

Recently, Ikeda et al reported anti-HCV profiles of 5 different statins using the OR6 replicon system. They found that fluvastatin exhibited the strongest anti-HCV activity, whereas atorvastatin, simvastatin, and lovastatin had moderate-to-mild anti-HCV activity. Pravastatin did not have significant anti-HCV activity.[27] However, our results show that the anti-HCV activity of the statins in order of decreasing potency was simvastatin, fluvastatin, atorvastatin, mevastatin, lovastatin, and pravastatin. It is likely that the IC_{50} of simvastatin, 1.6 μM, was higher than Ikeda's because our data were obtained at only 48 hours after treatment, as opposed to 72 hours in the Ikeda study. Pravastatin also had anti-HCV activity, although it was very weak (IC_{50}: 238.5 μM, data not shown). Furthermore, as with the study of Ikeda, the anti-HCV effect of the statins, including that of pravastatin, was rescued by addition of geranylgeraniol (data not shown). Because the HCV genome does not encode a geranylgeranylated protein, we hypothesize that a host geranylgeranylated protein must play an important role in HCV replication and that inhibition of the geranylgeranylation of this protein represents a potential strategy for blocking HCV replication. Thus, for statins to exert their anti-HCV effects, they must deplete mevalonate sufficiently to lower the cellular pools of geranylgeranyl pyrophosphates in the replicon cell lines. We speculate that this indirect mechanism of action explains an anti-HCV IC_{50} for statins in the micromolar range, in contrast to their IC_{50} for HMG-CoA reductase in the nanomolar range.[12,28] Potential reasons for pravastatin's observed weaker activity include its higher hydrophilicity compared to the other statins and its lack of metabolism by the cytochrome P450 system.[29]

Although we verified several hit compounds, such as the corticosteroids and the HMG-CoA reductase inhibitors, our validation assays revealed some false positives from our primary screening. Tetrandrine and MY-5445 were antiviral hits in the primary screen that were found to be false positives with significant cytotoxicity in secondary, but not primary, screening. This phenomenon was also observed for lovastatin. These false-positive results may be due in part to the lack of HCV structural proteins in our primary screening system. For example, the expression of HCV core protein has been reported to affect Fas-mediated apoptosis depending on environmental conditions.[30] Thus, it is possible that HCV core, in the presence of small molecules, could lead to apoptosis and cytotoxicity. These findings demonstrate that validation of primary screening results with subgenomic replicon systems should be performed with full-length replicon systems in order to totally assess the potential interactions of these compounds in the authentic viral protein context.

Although our cell-based HTS system represents an effective screening method for identification of potential antiviral agents, there are potential limitations to use of the Huh7/Rep-Feo replicon cell line, which is based on a subgenomic, not full-length, genotype 1b clone. By using the full-length OR6 replicon cell line for our confirmatory secondary assays, we have addressed one particular limitation. With regard to genotype, recent description of a bona fide infectious HCV cell line propagating genotype 2 virus[31,32] will permit assessment of the generalizability of the inhibitory action of these agents against other HCV genotypes. To date, the vast majority of HCV replicons have been developed in Huh7 cells, which appear to be uniquely permissive for these studies. With the eventual development of successful replication models in other hepatocyte cell lines, further testing of these compounds in such lines will also be of interest.

In summary, we have developed a rapid, reliable, reproducible, and validated cell-based HTS method to identify positive and negative regulators of HCV replication. This method can be used not only to identify putative antiviral agents but also cellular regulators of replication. The compounds identified by these screens can be used not

only for medicinal effect but also as a productive approach to the characterization of key viral-host interactions critical to viral replication.

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Hepatitis C virus non-structural proteins responsible for suppression of the RIG-I/Cardif-induced interferon response

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Viral infections activate cellular expression of type I interferons (IFNs). These responses are partly triggered by RIG-I and mediated by Cardif, TBK1, IKKε and IRF-3. This study analysed the mechanisms of dsRNA-induced IFN responses in various cell lines that supported subgenomic hepatitis C virus (HCV) replication. Transfection of dsRNA into Huh7, HeLa and HEK293 cells induced an IFN expression response as shown by IRF-3 dimerization, whilst these responses were abolished in corresponding cell lines that expressed HCV replicons. Similarly, RIG-I-dependent activation of the IFN-stimulated response element (ISRE) was significantly suppressed by cells expressing the HCV replicon and restored in replicon-eliminated cells. Overexpression analyses of individual HCV non-structural proteins revealed that NS4B, as well as NS34A, significantly inhibited RIG-I-triggered ISRE activation. Taken together, HCV replication and protein expression substantially blocked the dsRNA-triggered, RIG-I-mediated IFN expression response and this blockade was partly mediated by HCV NS4B, as well as NS34A. These mechanisms may contribute to the clinical persistence of HCV infection and could constitute a novel antiviral therapeutic target.

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INTRODUCTION

Type I interferon (IFN) plays a central role in eliminating virus, not only following clinical therapeutic application but also as a cellular immune response (Samuel, 2001; Taniguchi & Takaoka, 2002). Hepatitis C virus (HCV) infection is characterized by persistence and replication of the virus in the liver, despite an intact host immune system (Alter, 1997). Indeed, even after administration of the currently most potent IFN reagents, as many as half of the patients are refractory to the treatment and fail to eradicate the virus (Fried *et al.*, 2002). These features have led to speculation that HCV escapes from or attenuates the host antiviral response (Katze *et al.*, 2002).

Cellular antiviral responses are primarily mediated by IFN and IFN-stimulated genes (ISGs), including 2,5-oligoadenylate synthetase, dsRNA-dependent protein kinase R (PKR) and MxA proteins, as well as by as yet uncharacterized genes (Itsui *et al.*, 2006; Stark *et al.*, 1998). A study of experimental chimpanzee HCV infection has shown that various cytokines and chemokines are induced in the liver during the course of acute HCV infection and its clearance, and that a considerable proportion of the genes is induced by type I IFN (Bigger *et al.*, 2001).

Control of expression of ISGs is mediated by binding of type I IFNs to their receptors. Following receptor binding, STAT1 and STAT2 are phosphorylated to form ISGF-3, which translocates to the nucleus and binds the IFN-stimulated response element (ISRE), located in the promoter/enhancer region of ISGs, and activates transcription of ISGs (Samuel,

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2001; Taniguchi *et al.*, 2001; Taniguchi & Takaoka, 2002). ISRE-dependent gene expression is also mediated by binding of the ISRE by molecules such as IRF-1, IRF-3 and IRF-7 (Kanazawa *et al.*, 2004). IRF-3 is a transducer of virus-mediated signalling and plays a critical role in the induction of cellular antiviral responses (Lin *et al.*, 1998; Sato *et al.*, 2000; Taniguchi *et al.*, 2001; Yoneyama *et al.*, 1998). Transcriptional activation and suppression of IRF-3 are inversely correlated with the level of HCV replication *in vitro* (Yamashiro *et al.*, 2006). Following virus infection, IRF-3 is phosphorylated by two cytoplasmic kinases, TBK1 and IKKε (Fitzgerald *et al.*, 2003; Sharma *et al.*, 2003). The phosphorylated IRF-3 forms a homodimer, translocates to the nucleus and predominantly activates expression of the IFN-β gene and certain ISGs (Doyle *et al.*, 2002; Nakaya *et al.*, 2001; Taniguchi & Takaoka, 2002).

RIG-I is a recently identified cytoplasmic DExD/H box RNA helicase that participates in recognition of virus-related dsRNA as a pathogen-related molecular pattern (Yoneyama *et al.*, 2005). RIG-I contains two caspase-recruitment domains (CARDs) in the N terminus and a DExD/H box RNA helicase in the C terminus. MDA5 has been identified as another CARD-containing DExD/H box RNA helicase (Andrejeva *et al.*, 2004). More recently, an adaptor molecule of RIG-I and MDA5, Cardif (also known as IPS-I, MAVS and VISA), has been identified by four independent groups (Kawai *et al.*, 2005; Meylan *et al.*, 2005; Seth *et al.*, 2005; Xu *et al.*, 2005). On association with dsRNA, RIG-I or MDA5 causes conformational changes and homo-oligomerization, and binds the CARD of Cardif (Saito *et al.*, 2007). Cardif subsequently recruits the kinases TBK1 and IKKε, which catalyse phosphorylation and activation of IRF-3 (Yoneyama *et al.*, 1998).

The IRF-3-mediated IFN-β induction pathway could be a target for viruses to counteract antiviral responses and promote their replication in host cells. Ebola virus, bovine viral diarrhea virus (BVDV) and influenza A virus interfere with the activation of IRF-3 through interactions of their virus-encoded proteins (Basler *et al.*, 2003; Schweizer & Peterhans, 2001; Talon *et al.*, 2000). There are several reports that HCV proteins interact with IFN-mediated antiviral systems. The NS5A and E2 proteins have been reported to interfere with the action of IFN by inhibiting the activity of PKR (He & Katze, 2002). It was reported recently that the HCV NS34A protease blocks virus-induced activation of IRF-3, possibly by proteolytic cleavage of Cardif (Foy *et al.*, 2003; Meylan *et al.*, 2005).

The HCV subgenomic replicon is an *in vitro* model that simulates autonomous cellular replication of HCV genomic RNA (Lohmann *et al.*, 1999). Expression of the HCV replicon can be abolished by treatment with small amounts of type I and type II IFNs (Blight *et al.*, 2000; Frese *et al.*, 2002; Guo *et al.*, 2001), suggesting intact IFN receptor-mediated cellular responses. In contrast, viral expression persists in the absence of the exogenous IFN. Baseline expression levels of ISG were substantially decreased in cells expressing the HCV replicon compared with parental Huh7 cells (Kanazawa *et al.*, 2004). These findings led us to speculate that intracellular virus-induced antiviral responses are attenuated or caused to malfunction by the expression of viral proteins.

In this study, we investigated cell lines that support subgenomic HCV replication and HCV cell culture for the dsRNA-induced cellular IFN expression pathway. Here, we report that RIG-I- and Cardif-mediated IFN gene activation is uniformly attenuated in several replicon-expressing cell lines of different lineages and, more importantly, that the HCV NS4B protein is involved in the suppression of antiviral IFN responses.

METHODS

Plasmids. Plasmids pEF-flagRIG-I and ΔRIG-I expressed full-length and C-terminally truncated RIG-I protein, respectively (Yoneyama *et al.*, 2004). The plasmid pER-flagRIG-IKA (RIG-IKA) has a point mutation in the putative ATP-binding site of the RIG-I helicase domain and was used as a negative control for ΔRIG-I and RIG-I full transfection assays. Expression plasmids for full-length Cardif (Cardif), Cardif CARD (CARD) and CARD-truncated Cardif (ΔCARD) were provided by Dr J. Tschopp (University of Lausanne, Switzerland) (Meylan *et al.*, 2005). Expression plasmids for toll-like receptor 3 (TLR3) and TIR domain-containing adaptor inducing IFN-β (TRIF), the transmembrane receptor of dsRNA and the adaptor molecule of TLR3, respectively, were provided by Dr S. Akira (Osaka University, Japan). Plasmids expressing HCV NS345, NS3, NS34A, NS4A, NS4B, NS5A and NS5B were amplified from HCV pCV-J4-L4S (Yanagi *et al.*, 1997) by PCR and subcloned. The DNA fragments were inserted into the vector pcDNA4/TO/*myc*-His (Invitrogen). Nucleotide sequences were confirmed by sequencing. Plasmids TOPO-NS34A (HCV N), TOPO-NS4B (HCV N) and pcDNA-NS4B (HCV JFH1) expressed Myc-tagged NS34A and NS4B proteins derived from the HCV N (Beard *et al.*, 1999) and HCV JFH1 (Wakita *et al.*, 2005) strains, as indicated. Plasmid pISRE-TA-Luc (Invitrogen) contained five copies of consensus ISRE motifs upstream of the firefly luciferase gene. Plasmid pIFNβ-Fluc was constructed by cloning the human IFN-β promoter region, spanning nt −110 to −36, upstream of the firefly luciferase gene of pGL3 Basic (Promega). Plasmid pcDNA3.1 (Invitrogen) was used as an empty vector for mock transfection. pRL-CMV (Promega), which expressed the *Renilla* luciferase protein, was used for correction of transfection efficiency.

Cell culture. HCV strain JFH1-infected Huh7.5.1, Huh7, Huh7.5.1 (kindly provided by Dr F. Chisari, The Scripps Institute, CA, USA; Zhong *et al.*, 2005), HeLa and HEK293 cells were maintained in Dulbecco's modified minimal essential medium (Sigma) supplemented with 2 mM L-glutamine and 10 % fetal calf serum at 37 °C with 5 % CO_2. Cells expressing the HCV replicon were cultured in medium containing 100 μg G418 (Wako) ml^{-1}.

HCV replicon constructs and transfected cell lines. An HCV subgenomic replicon plasmid, pHCVIbneo-delS (designated pRep-N), was derived from an HCV clone of strain N, genotype 1b, and pSGR-JFH1 was derived from HCV JFH1, genotype 2a (Guo *et al.*, 2001; Wakita *et al.*, 2005). These replicons were reconstructed by substituting the neomycin phosphotransferase gene with a fusion gene comprising *Renilla* luciferase and neomycin phosphotransferase to construct pRep-Reo-1b and pRep-Reo-2a, respectively (Tanabe *et al.*, 2004; Yokota *et al.*, 2003). RNA was synthesized from the replicons using T7 polymerase (Promega) and transfected into Huh7,

HeLa and HEK293 cells. After culture in the presence of G418, cell lines stably expressing the replicon were established (Huh7/1bReo, Huh7/2aReo, HeLa/2aReo and 293/2aReo). We have previously reported that firefly luciferase activities of Feo-replicon-expressing cells correlate well with HCV NS3, NS4A and NS5A protein expression levels and with the levels of replicon RNA (Yokota *et al.*, 2003).

Transient transfection. Transient DNA transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. ISRE reporter assays were carried out as previously described (Nakagawa et al., 2004). To analyse IFN expression in HCV JFH1 cell cultures, a total of 1×10^5 Huh7.5.1, JFH-1 infected Huh7.5.1 and IFN-treated Huh7.5.1 cells were seeded into 24-well plates the day before transfection. Plasmids pISRE-TA-Luc and ΔRIG-I (200 ng each) were transfected using 1 μl Lipofectamine 2000. RIG-IKA was used as a control. Luciferase assays were performed on day 3 post-transfection.

For further study, 400 ng of each non-structural protein was added to 1×10^4 Huh7 or HEK293 cells that had been seeded into 96-well plates the day before transfection. pISRE-TA-Luc and ΔRIG-I (40 ng each) were transfected using 0.5 μl Lipofectamine 2000. RIG-IKA was used as a control.

Western blotting. Preparation of the cytoplasmic and nuclear fractions of cell lysates was carried out as described previously (Tanabe *et al.*, 2004). Protein (20 μg) was separated using NuPAGE 4–12% Bis/Tris gels (Invitrogen) and blotted onto an Immobilon PVDF membrane (Roche). The membrane was immunoblotted with anti-IRF-3 (Santa Cruz) and detected by chemiluminescence (BM Chemiluminescence Blotting Substrate; Roche).

RT-PCR. Interleukin (IL)-8 mRNA was detected by RT-PCR as described previously (Itsui *et al.*, 2006). The primers used were IL8-S (5′-GCACAAACTTTCAGAGACAGCAGAGCACAC-3′) and IL8-AS (5′-CAGAGCTGCAGAAATCAGGAAGGCTGCCAA-3′).

Indirect immunofluorescence assay. Cells seeded onto tissue culture chamber slides were fixed with cold acetone. The cells were incubated with anti-protein disulphide isomerase (PDI) or anti-Myc antibodies and subsequently with Alexa 488- or Alexa 568-labelled secondary antibodies. Cells were mounted with VECTA SHIELD Mounting Medium and DAPI (Vector Laboratories) and visualized by fluorescence microscopy (BZ-8000; Keyence).

Luciferase reporter assays. Luciferase activity was measured using a 1420 Multilabel Counter (ARVO MX; PerkinElmer) using a Bright-Glo Luciferase Assay System (Promega) or a Dual Luciferase Assay System (Promega). Assays were carried out in triplicate and the results expressed as means ± SD.

MTS assay. To evaluate cell viabilities, dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium (MTS) assays were performed using a CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega) according to manufacturer's instructions.

Statistical analyses. Statistical analyses were performed using an unpaired, two-tailed Student's *t*-test. *P* values of less than 0.05 were considered to be statistically significant.

RESULTS

IRF-3 dimer formation is attenuated in cells expressing the HCV replicon

In the HCV replicon-expressing cell lines Huh7/Rep-Reo-2a, Hela/Rep-Reo-2a and 293/Rep-Reo-2a, replicon expression levels corresponded well to internal *Renilla* luciferase activities. Expression of the HCV replicon was suppressed by IFN in a dose-dependent manner (data not shown).

Activation of RIG-I or MDA5 induces phosphorylation and homodimerization of IRF-3. Following transfection of poly(I:C) into Huh7, HeLa or HEK293 cells, IRF-3 dimers were detected (Fig. 1). However, in cells supporting HCV replicons, IRF-3 dimer formation was almost completely abolished. These findings showed that expression of HCV proteins blocked activation of dsRNA-mediated IFN expression and that these effects were consistently found in several cell lines of different origin.

The HCV replicon suppresses RIG-I/Cardif-induced IFN responses

ISRE reporter activities did not increase in naïve Huh7, HeLa or HEK293 cells following transfection of poly(I:C), whilst overexpression of full-length RIG-I increased poly(I:C)-mediated ISRE reporter activity in Huh7 and HEK293 cells (data not shown). In RIG-I-overexpressing Huh7 cells, transduction with an HCV replicon abolished the poly(I:C)-induced ISRE activation, and elimination of the replicon by IFN treatment restored these ISRE responses (Fig. 2a). Consistent results were obtained by overexpression of ΔRIG-I, a constitutively active form. Transfection of ΔRIG-I in Huh7 and HEK293 cells induced ISRE activation, whilst these responses were abolished or significantly suppressed in cell lines expressing HCV replicons and were recovered by eliminating the replicon by IFN treatment (data not shown). Similarly, ISRE activation by overexpression of Cardif, an adaptor molecule of RIG-I, was almost completely blocked in replicon-expressing cells and was recovered by eliminating the replicon from the cells (data not shown). The RIG-I-mediated IFN response was

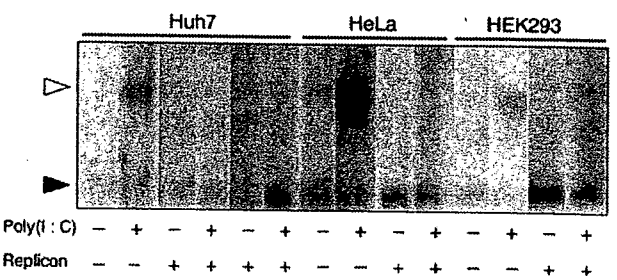

Fig. 1. Double-stranded RNA-induced IRF-3 dimer formation in cell lines that support HCV subgenomic replication. Poly(I:C) was transfected into naïve Huh7, HeLa and HEK293 cells, and into corresponding cell lines expressing the HCV replicon. Six hours after transfection, cell lysates were prepared, separated in polyacrylamide gels and blotted onto PVDF membrane. The membrane was immunoblotted with anti-IRF-3 and visualized by chemiluminescence (see Methods). The positions of the IRF-3 dimer (open arrowhead) and monomer (closed arrowhead) are indicated.

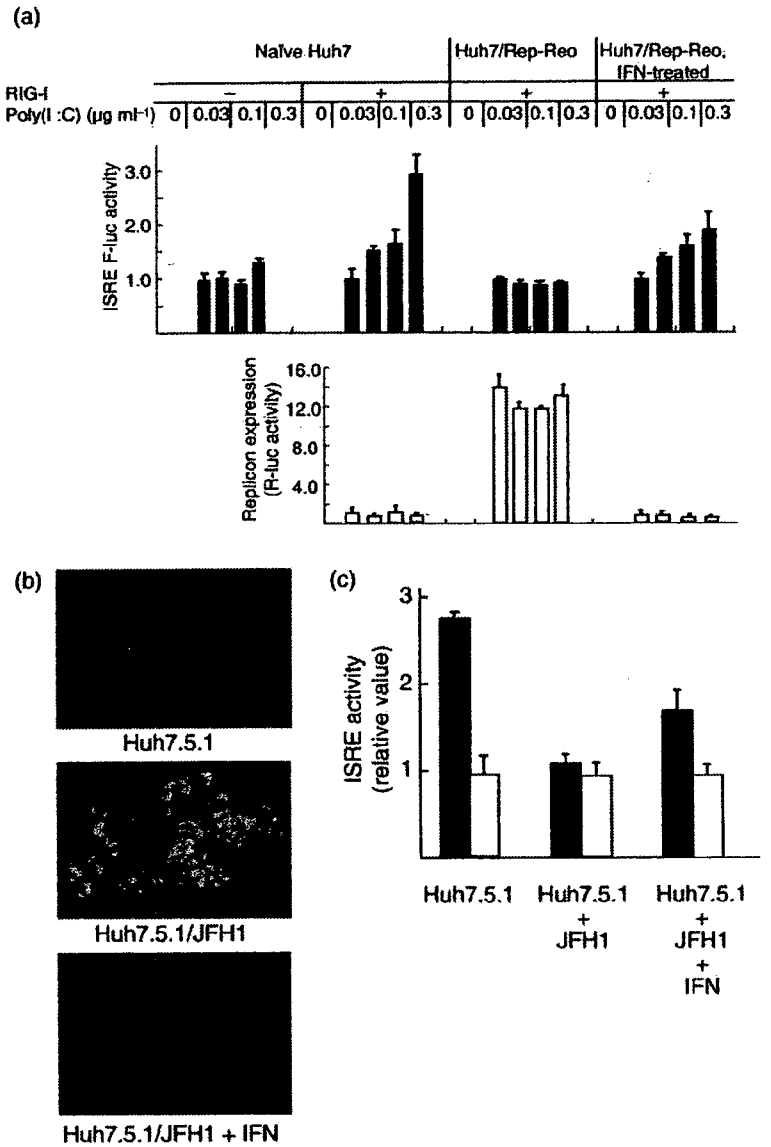

Fig. 2. Suppression of dsRNA-induced, RIG-I-mediated ISRE activation by HCV replication. (a) The HCV replicon suppresses transcriptional activation after poly(I:C) stimulation. The RIG-I expression plasmid and pISRE-TA-Luc were transiently transfected into the cell lines indicated. The following day, the amounts of poly(I:C) indicated were transfected into the corresponding cell lines and dual luciferase assays were carried out 8 h after transfection. Filled bars indicate ISRE-regulated firefly luciferase (F-luc) activities and open bars indicate *Renilla* luciferase (R-luc) activities representing replicon expression levels. In both graphs, scales for the *y*-axis are shown as relative values. Assays were carried out in triplicate and results are shown as means ± SD. (b) Immunofluorescence microscopy results. Huh7.5.1 cells infected with HCV JFH1 (Huh7.5.1/JFH1) and JFH1-infected cells from which the virus had been eliminated by IFN treatment (Huh7.5.1/JFH1 + IFN) were incubated with anti-core primary antibodies followed by Alexa Fluor-conjugated secondary antibody (green). Nuclei were stained with DAPI (blue). (c) ISRE activation by ΔRIG-I overexpression. The plasmid pISRE-TA-Luc was co-transfected with ΔRIG-I (filled bars) or RIG-IKA (empty bars) into naïve Huh7.5.1, Huh7.5.1/JFH1 or Huh7.5.1/JFH1 + IFN cells. Luciferase assays were carried out 8 h after transfection. The *y*-axis indicates ISRE-regulated luciferase activity shown as relative values. Assays were carried out in triplicate and results are shown as means ± SD.

also suppressed in HCV JFH1 virus cell culture. In JFH1-infected Huh7.5.1 cells, ΔRIG-I-induced ISRE reporter activation was significantly suppressed, but was recovered in IFN-treated, virus-eliminated cells (Fig. 2b and c). These results demonstrated that RIG-I- and Cardif-mediated antiviral responses were substantially suppressed by both subgenomic and genomic viral replication in both hepatocyte- and non-hepatocyte-derived host cells.

NS34A and NS4B are responsible for suppressing RIG-I-mediated IFN responses

We next sought to define which HCV proteins were responsible for inhibition of the RIG-I- and IRF-3-mediated IFN induction pathway. We constructed expression plasmids that expressed the non-structural proteins NS345, NS3, NS34A, NS4A, NS4B, NS5A and NS5B (Fig. 3a). We transfected each expression plasmid with simultaneous activation of the RIG-I pathway by overexpression of ΔRIG-I, Cardif, TBK1 and IKKε (Fig. 3b–e). Expression of full-length non-structural (NS345) and NS34A proteins inhibited ISRE activation mediated by expression of RIG-I and Cardif but not that mediated by TBK1 and IKKε. Interestingly, it was found that NS4B also inhibited ISRE activation mediated by expression of RIG-I and Cardif, but not by TBK1 and IKKε. Consistent with Fig. 3(b), overexpression of NS4B significantly suppressed ΔRIG-I-induced activation of the authentic IFN-β promoter (Fig. 3f).

Another group has studied IFN antagonism of flavivirus non-structural proteins and has reported that HCV NS4B did not affect IFN responses (Muñoz-Jordán *et al.*, 2005).

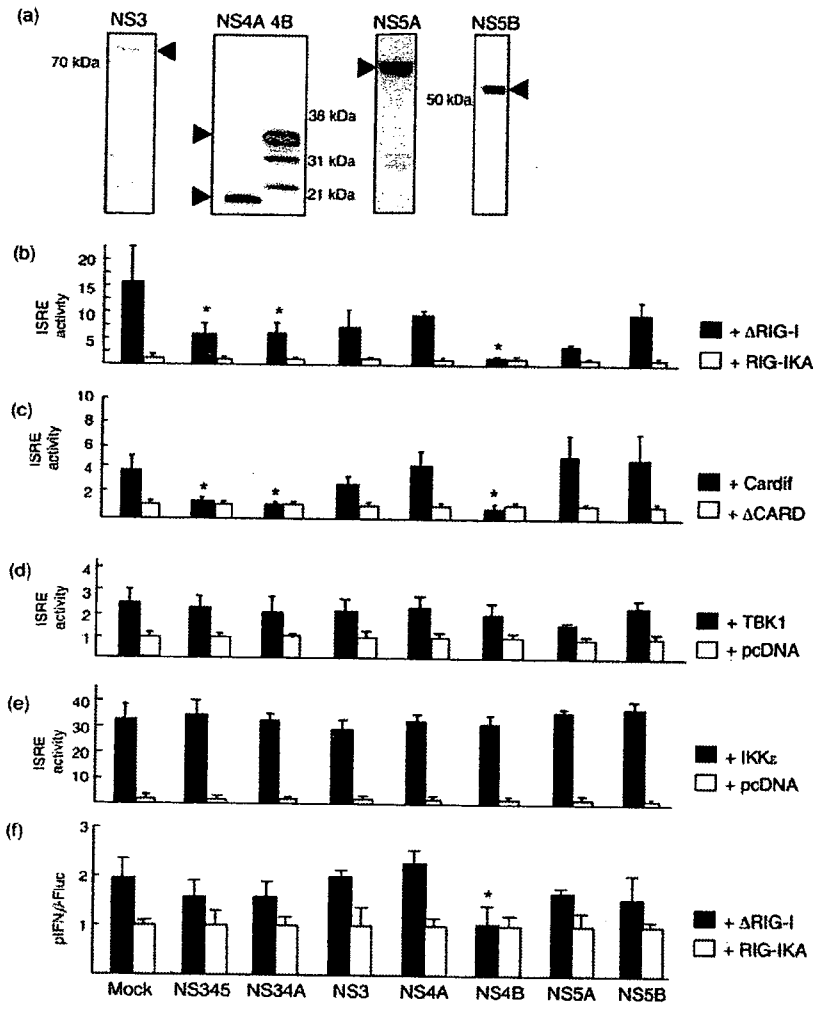

Fig. 3. Co-transfection analyses using plasmids that express individual HCV non-structural proteins. (a) Western blotting. Plasmids expressing the indicated Myc-tagged HCV proteins were transfected into Huh7 cells. Western blotting was carried out using anti-Myc antibody. (b–e) The following plasmids were co-transfected into Huh7 cells: pISRE-TA-Luc, pRL-CMV, the indicated plasmids expressing ΔRIG-I (b), Cardif (c), TBK1 (d) and IKKε (e), and the indicated plasmids expressing individual HCV non-structural proteins. Plasmids RIG-IKA, ΔCARD or pcDNA were used as negative controls as indicated. Twenty-four hours after transfection, luciferase activities were measured. The *y*-axis shows relative values. Assays were carried out in triplicate and results are given as means ± SD. *, $P<0.05$. (f) pIFN-β and pRL-CMV were co-transfected into Huh7 cells, with plasmids expressing individual HCV non-structural proteins and plasmid expressing ΔRIG-I. Luciferase activities were measured 24 h after transfection. The *y*-axis shows relative values. Assays were carried out in triplicate and results are given as means ± SD. *, $P<0.05$. Plasmid RIG-IKA was used as a negative control.

To investigate strain-specific differences in the characteristics of NS4B proteins, we performed co-transfection assays using NS4B expression constructs from HCV N (Beard *et al.*, 1999) and JFH1 (Wakita *et al.*, 2005) strains, as well as HCV strain J4 (Fig. 4a and b). All NS4B constructs suppressed ΔRIG-I- or Cardif-mediated ISRE activation. These results suggested that the above-described effects of NS4B were independent of HCV strain.

NS4B has been reported to induce an unfolded protein response or endoplasmic reticulum (ER) stress through ATF6 or IRE1-X box protein (XBP1) pathways (Zheng *et al.*, 2005). The ER stress induces production of IL-8, which has been reported to interfere with the IFN system (Polyak *et al.*, 2001). Therefore, we detected expression of IL-8 using RT-PCR in cells with and without overexpression of NS4B. As shown in Fig. 4(c), no significant difference was observed in IL-8 mRNA levels among mock-, NS34A- and NS4B-transfected cells. These results showed that NS4B overexpression in the present study did not induce expression of IL-8 and that the IFN-antagonizing effects of NS4B were independent of IL-8.

It has been reported that NS34A suppresses the TLR3-mediated IFN response (Breiman *et al.*, 2005; Ferreon *et al.*, 2005). However, overexpression of HCV non-structural proteins did not suppress ISRE activation that was induced by overexpression of TLR-3 or TRIF (Fig. 5a and b), nor did NS34A from two different HCV strains, J4 and N, show significant suppression of TRIF-mediated ISRE activation (Fig. 5c). Although strain-specific differences might be involved, these data suggest that neither NS34A nor NS4B affect the TLR3-triggered, TRIF-mediated IFN expression signalling pathway.

The NS4B N terminus is involved in inhibition of the RIG-I-mediated pathway

Given the result that NS4B suppressed the RIG-I-mediated IFN expression pathway, we next investigated which domain of NS4B was responsible. We constructed plasmids that expressed truncated NS4B in which the protein-coding frame was truncated at four positions corresponding to the five transmembrane domains (Lundin *et al.*, 2003) (Fig. 6a).

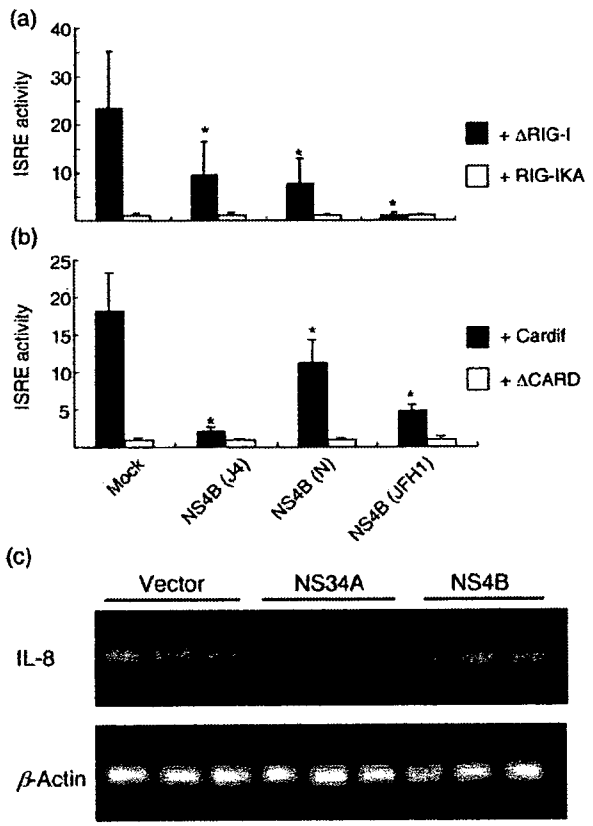

Fig. 4. Co-transfection analyses of HCV NS4B proteins of different origins. NS4B (J4), NS4B (N) and NS4B (JFH1) denote HCV NS4B proteins that were cloned from HCV strains J4, N and JFH1, respectively. The NS4B plasmids indicated were co-transfected with plasmids expressing ΔRIG-I (a) or Cardif (b). Luciferase activities were measured 24 h after transfection. The *y*-axis shows relative values. Assays were carried out in triplicate and results are given as means ± SD. *, $P<0.05$. (c) Semi-quantitative detection of IL-8 mRNA by RT-PCR. cDNA was prepared from Huh7 cells transfected with empty vector or with NS34A or NS4B expression plasmid.

Expression and subcellular localization of NS4B truncated proteins were visualized by indirect immunofluorescence assays (Fig. 7). Each of the NS4B truncated proteins was localized predominantly to the perinuclear rim as dense spots. Some of the spots were similar to the staining of the ER-resident host protein PDI, consistent with previous reports (Lindström *et al.*, 2006; Lundin *et al.*, 2006). These truncated expression plasmids were co-transfected with Cardif expression plasmids into Huh7 cells. As shown in Fig. 6(b), Cardif-mediated ISRE activation was significantly suppressed by co-transfection of NS4Bt1–156 and NS4Bt1–186, as well as full-length NS4B, whilst transfection of NS4Bt90–260 and NS4Bt110–260 did not significantly suppress Cardif-mediated ISRE activation. The shortest construct, NS4Bt131–260, partially retained the ability to reduce ISRE activity. These results suggested that the N-terminal domain of NS4B, which includes aa 1–110, might function directly to suppress RIG-I-mediated IFN expression responses.

DISCUSSION

The recent discovery of cytoplasmic dsRNA sensor molecules has resulted in rapid expansion of knowledge about the IFN-mediated virus defence pathway (Yoneyama *et al.*, 2004). Several reports suggest that viruses target the IFN system to establish replication in the host cells (Kato *et al.*, 2006). We have confirmed that the dsRNA-triggered, IRF-3-mediated IFN activation pathway was blocked in several replicon-supporting cell lines (Fig. 1). Similarly, the dsRNA responses were substantially suppressed in HCV JFH-1 cell culture compared with parental Huh7 cells (Fig. 2b and c). Overexpression analyses showed that RIG-I- and Cardif-mediated ISRE activation was significantly suppressed in HCV replicon-expressing cells, which recovered after elimination of the replicon by IFN treatment (Fig. 2a). In contrast, TBK1- or IKKε-mediated ISRE activation was not suppressed in replicon-expressing cells. Overexpression of individual HCV non-structural proteins revealed that not only NS34A but also NS4B inhibited the ISRE activation signal (Figs 3, 4 and 5). These results suggested that HCV non-structural proteins suppress the IFN induction pathway and that the target host molecule could be Cardif or an unknown adaptor molecule acting between Cardif and TBK1/IKKε.

NS4B protein is a 27 kDa hydrophobic integral membrane protein that is localized in the ER with other non-structural proteins. Studies on other flaviviruses such as Kunjin virus and BVDV support the notion that NS4B may indeed be an essential part of the replication mechanism (Grassmann *et al.*, 2001; Khromykh *et al.*, 2000; Li & McNally, 2001; Qu *et al.*, 2001). These systems have demonstrated that intact NS4B is necessary in a *cis* configuration in the polyprotein for maintaining viral replication (Grassmann *et al.*, 2001; Khromykh *et al.*, 2000). Furthermore, single mutations in NS4B alter the cytopathic effects of BVDV and even mediate changes in the cellular tropism of Dengue virus (Hanley *et al.*, 2003; Qu *et al.*, 2001). In HCV, the search for cell-culture-adaptive mutations in HCV subgenomic replicons has led to the generation of mutations in the NS4B region that confer higher replication levels and resistance to IFNs, as well as broadening the tropism for different cell lines (Lohmann *et al.*, 2003; Sumpter *et al.*, 2004; Zhu *et al.*, 2003). These pieces of evidence may imply that NS4B is not only part of the replication machinery but may also have other functions that enable establishment of viral replication.

NS4B truncation assays showed that RIG-I/Cardif-mediated ISRE activation was significantly suppressed by expression of N terminus-containing constructs (Fig. 6). These results imply that the N-terminal domain of NS4B, which is located between positions 1 and 110, may be essential for suppressing IFN expression responses in host

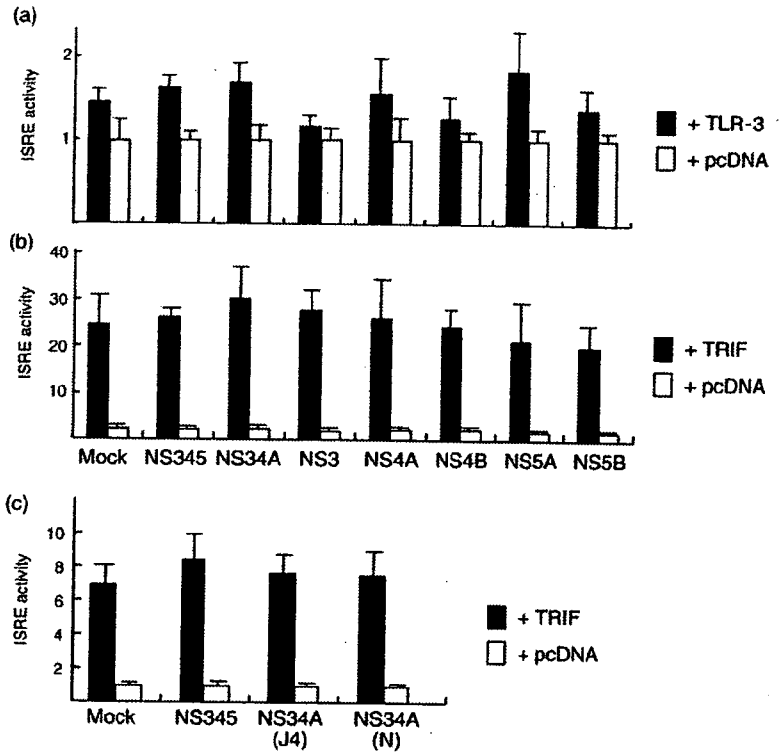

Fig. 5. Co-transfection analyses of HCV non-structural proteins and plasmid expressing TLR-3 or TRIF. (a, b). The following plasmids were co-transfected into Huh7 cells: pISRE-TA-Luc, pRL-CMV, plasmids expressing TLR-3 or TRIF and plasmids expressing individual HCV non-structural proteins, as indicated. Luciferase activities were measured 24 h after transfection. The *y*-axis shows relative values. Assays were carried out in triplicate and results are given as means ± SD. (c) NS34A (J4) and NS34A (N) denote plasmids expressing HCV NS34A derived from HCV strains J4 and N, respectively. The NS4B plasmids indicated were co-transfected with pISRE-TA-Luc, pRL-CMV and plasmids expressing TRIF or pcDNA. Luciferase activities were measured 24 h after transfection. The *y*-axis shows relative values. Assays were carried out in triplicate and results are given as means ± SD.

cells. Lindström *et al.*, (2006) investigated single point mutations in NS4B that negatively affected expression efficiency of the HCV replicon and reported that most of the active mutations were located around the N-terminal domain. A distinctive feature of NS4B is that it requires membrane rearrangement to form its native structure

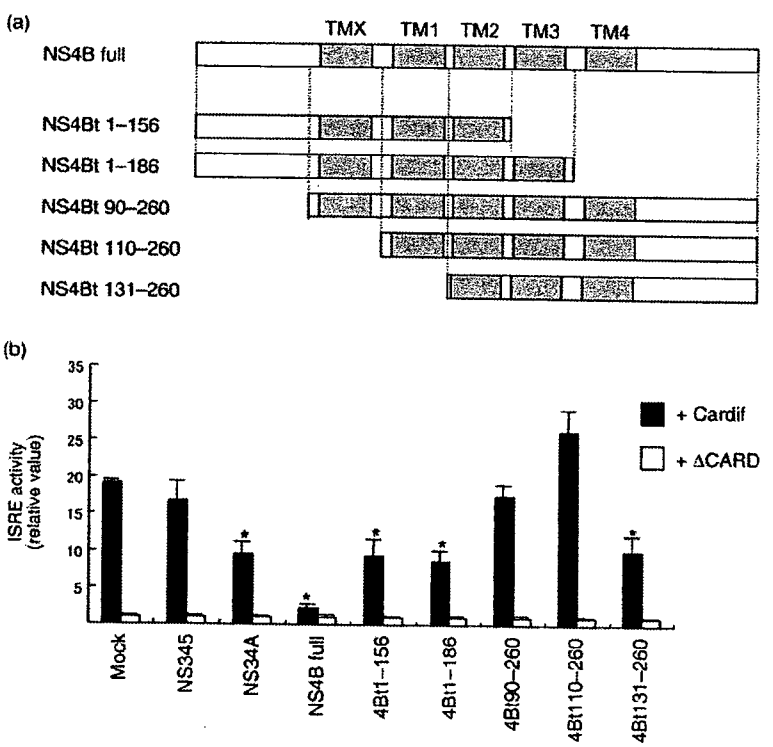

Fig. 6. Co-transfection analyses using truncated NS4B expression constructs. (a) Truncated constructs of NS4B. The protein-coding frame of NS4B was truncated in five constructs corresponding to the five transmembrane domains, as reported by Lundin *et al.* (2003). (b) The truncated NS4B plasmids, pISRE-TA-Luc and the Cardif- or ΔCARD-expressing plasmids indicated were co-transfected into Huh7 cells. Luciferase activities were measured 24 h after transfection. Results are given as means ± SD.

(Lindström *et al.*, 2006; Lundin *et al.*, 2006). The HCV polyprotein is translated from a single reading frame and subjected to proteolytic cleavage by the host signal peptidase and two viral proteases (Grakoui *et al.*, 1993). The mature form of NS4B is localized in the ER and constitutes a subcellular structure called the membrane-associated focus (MAF) (Gretton *et al.*, 2005). Once the NS4B is cleaved, the N-terminal peptide of NS4B is translocated from the cytoplasmic to the luminal side, giving it a fifth transmembrane region (Lundin *et al.*, 2006). The N-terminal amphipathic helix (AH) 1 of NS4B is necessary for this translocation and for MAF formation; NS4B molecules that were truncated at the AH1 lacked the ability to create the MAF, to translocate and to replicate (Elazar *et al.*, 2004; Lindström *et al.*, 2006).

In our assay, NS4Bt131–260 regained the ability to reduce ISRE activity. As we confirmed that all mutants co-localized with the ER, there may be some effect of the N-terminal localization of this mutant. The precise mechanism of NS4B suppression is still not clear and further experiments are needed.

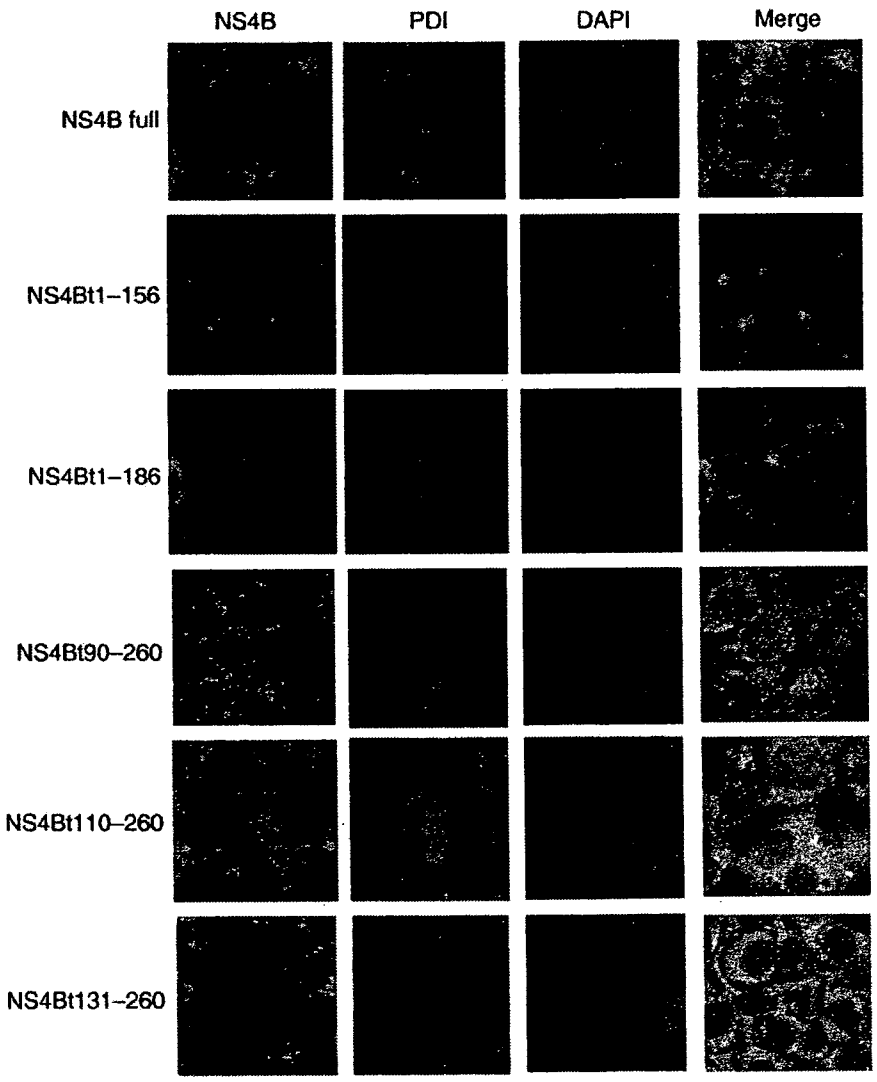

Fig. 7. Indirect immunofluorescence analysis of truncated NS4B proteins. The NS4B constructs indicated were transiently transfected into Huh7 cells. After 48 h, cells were labelled with anti-Myc or anti-PDI antibody. NS4B proteins were immunostained with Alexa Fluor 488-labelled goat anti-mouse IgG, whilst PDI was stained with Alexa Fluor 568-labelled goat anti-rabbit IgG. DAPI staining revealed the nuclear chromatin. Representative immunofluorescence images derived from a number of experiments are shown as four images of a single focal plane of Huh7 cells, showing NS4B proteins (green), PDI (red), DAPI staining (blue) and the superimposed images (merge).

NS4B has been reported to induce an unfolded protein response or ER stress (Zheng *et al.*, 2005). Accumulation of unfolded or misfolded proteins in the ER is detected by three ER sensor proteins, ATF6, IRE-1 and PKR-like ER kinase (PERK), and triggers the unfolded protein response as a stress response and induces expression of molecular chaperon proteins, global shut-off of protein translation and apoptotic cell death (Mori, 2000). Therefore, it may be possible that transgenic overexpression of NS4B induces ER stress and suppresses overall protein synthesis, including that of IFNs. In our experiments, however, NS4B suppressed the ISRE-mediated IFN gene activation but did not suppress non-specific protein expression, as demonstrated by *Renilla* luciferase activity in the control plasmid driven by the herpes simplex thymidine kinase promoter. In addition, the growth and viability of cells that overexpressed NS4B did not differ from untransfected cells or from those transfected with the other HCV proteins. IL-8 overproduction induced by ER stress was not observed in our NS4B-overexpressing cells (Fig. 4c). These findings indicated that the inhibitory effect of NS4B is specific to the IFN induction pathway and is not a non-specific effect through ER stress.

In conclusion, we have shown that dsRNA-induced IFN expression was suppressed by NS4B. These virus–host interactions probably contribute to HCV persistence and to the pathogenesis of HCV-associated liver disease.

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Bone morphogenetic protein-7 and interferon-alpha synergistically suppress hepatitis C virus replicon

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Abstract

Various cytokines contribute to control hepatitis C virus (HCV) viral replication. HCV subgenomic replicon systems have been developed, and cell-cycle-dependent replication has been reported. But the molecules involved in this processes is not totally elucidated. The aim of this study is to investigate the involvement of the bone morphogenetic protein (BMP)-7, a member of TGF-β superfamily, to the *in vitro* HCV replication. BMP-7 dose-dependently suppressed the replication and protein expression from the HCV replicon in Huh7/Rep-Feo cells and was associated with cell-cycle arrest at the G1 phase. These results were consistent with the effect of TGF-β in a previous study. Combination of BMP-7 and interferon-alpha showed a synergic decrease of HCV replication, and was more effective compared to the treatment with interferon-alpha alone. This synergistic effect was also present in HCV-JFH1 virus cell culture. While BMP-7 alone did not stimulate expression of the interferon-stimulated genes (ISGs), it augmented interferon-induced expression of the ISGs independently of the interferon-induced Jak/STAT pathway. Taken together, BMP-7 may constitute a novel molecule to suppress HCV replication.

Keywords: Hepatitis C virus (HCV); Replicon; Bone morphogenetic protein (BMP)-7; TGF-beta superfamily; Transcription; Osteogenic protein (OP)-1; Interferon (IFN); HCV-JFH1 cell culture

More than 170 million people are chronically infected with hepatitis C virus (HCV) worldwide [1]. Most of acute and chronic HCV infections remain asymptomatic, while some develop liver inflammation and fibrogenesis, cirrhosis and hepatocellular malignancy in long years of persistency [2]. Current therapies against HCV infection are based on high-dose administration of IFN in combination with ribavirin [3,4]. However, they are effective in only 30–40% of patients treated and often carry serious adverse effects. These difficulties in eradicating HCV are attributable to such limited treatment options against the virus. Therefore, the search for novel therapeutic agents remains a strong aspiration.

The establishment of autonomously replicating subgenomic HCV-RNA, expressing HCV proteins, much improved the situation of molecular investigation of the pathophysiological mechanisms of HCV replication [5–8]. Although the mechanism has not been identified yet, the replication of HCV genome was highly dependent on cellular proliferation and substantially enhanced in the S phase of the cell cycle [9,10].

Abbreviations: HCV, hepatitis C virus; BMP, bone morphogenetic protein; SNP, single nucleotide polymorphism; ALK, activin-like kinase; BMPR II, BMP receptor type II; IFN, interferon; ISG, interferon-stimulated gene; ISRE, interferon-stimulated response element; MxA, myxovirus resistance-1; 2-5-OAS, 2-5-oligoadenylate synthetase; SMAD, SMA- and MAD-related protein.

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The bone morphogenetic protein (BMP)-7, also known as osteogenic protein (OP)-1 [11], is one of the TGF-β superfamily [12] and inhibits cell growth in several tumor cell lines [13–15], while the rolls in hepatocytes are not clear. BMP-7 efficiently bounds to ALK-2 [16] and mediates the signal effectively with a type-II receptor, BMPR II [17,18]. Because these receptors are not ubiquitously expressed in every cell line [13], responsiveness to the BMP treatment is considered to depend on the expression of the receptors.

In this study, we investigated effects of BMP-7 on HCV replication using two HCV infection and replication models, HCV subgenomic replicon system [19,20], and HCV-JFH1 virus cell culture [21]. Here, we have demonstrated that BMP-7 inhibits HCV replication *in vitro*, potentially by arresting cell cycle at G1 phase. Potential cross talk with IFN-ISRE (interferon-stimulated response element) pathway is also discussed.

Materials and methods

Reagents and equipments. The experimental materials were purchased as in the following suppliers. ISOGEN (Nippon Gene, Tokyo, Japan); SuperScript First-Strand Synthesis System for RT-PCR kit (Invitrogen, Carlsbad, CA); Dulbecco's modified minimal essential medium (Sigma, St. Louis, MO); G418 (Wako, Osaka, Japan); recombinant human Bone morphogenetic protein 7 (R&D Systems, Minneapolis, MN), recombinant human interferon (IFN)-α2 (PBL Biomedical Laboratories, Piscataway, NJ); RNase (Wako); Bright-Glo Luciferase Assay System (Promega, Madison, WI); Lipofectamine 2000 (Invitrogen); pRL-TK vector (Promega); Protein assay dye reagent (Bio-Rad, Hercules, CA); BioPhotometer (Eppendorf, Hamburg, Germany); and LightCycler FastStart DNA Master SYBR Green I mix (Roche Diagnostics GmbH, Germany).

RT-PCR for the BMP receptors. Total RNA was extracted from Huh7/Rep-Feo and parental Huh-7 cells using ISOGEN according to the manufacturer's instructions. Reverse transcription reactions were performed by a two-step PCR method using a SuperScript First-Strand Synthesis System for RT-PCR kit. For the first-strand synthesis, 5 μg total RNA was reverse-transcripted using $Oligo_{dT}$ primers and 1 μl of derived cDNA was used for amplification. PCR cycle conditions were as follows: 30 cycles of a cycle reaction of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, followed by a final extension at 72 °C for 7 min. The primers used to detect mRNA for ALK 2 (ActR-I: BC036748.1) and BMPR II (NM_001204) were ALK2-F: 5′-GAA GTT CTA GAT GAA ACC ATC C-3′ and ALK2-R: 5′-GTT CAC AGT CAT CGA GCG AGG T-3′; BMPR2-F: 5′-CTG GGT AAG AGA GAA GGA ATC T-3′ and BMPR2-R: 5′-CCA TCC AAG CTA TTA CTA AAG T-3′, respectively.

HCV replicon, cell culture, and assays. An HCV replicon harboring cell line, Huh7/Rep-Feo, was used for the *in vitro* replication assay, which expressed a chimeric gene of firefly luciferase and neomycin phosphotransferase. [19,20]. Huh7/Rep-Feo cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 200 μg/ml of G418 and kept at 37 °C under 5% CO_2. Huh7/Rep-Feo cells were seeded 2.5×10^4 per 24-well plates and incubated 20 h before the treatment in a conventional medium consisting of Dulbecco's modified minimal essential medium with 10% fetal bovine serum supplemented with 100 IU of penicillin/ml, 100 μg of streptomycin/ml, and 250 μg/ml of G418 at 37 °C under 5% CO_2. Recombinant human BMP-7 (50–200 ng/ml) or human IFNα-2 (0.5 U/ml) was added as indicated, and the cells were harvested after 24, 48, and 72 h. The luciferase activities were quantified using a luminometer and the Bright-Glo Luciferase Assay System. Assays were performed in triplicate and the results were expressed as means ± standard deviation (SD) as percentages of the controls. Total protein level was measured using according to the manufacturer's instructions. The luciferase activities were standardized by total protein level.

Cell-cycle analysis. Huh7/Rep-Feo replicon cells were incubated with 100 ng/ml of BMP-7 for 72 h. The cellular DNA labeled with PI was incubated with 250 μg/ml of RNase at 37 °C for 30 min and was analyzed using Flow cytometry (FACS; Beckman–Coulter, Fullerton, CA).

Reporter assays. p-55C1BLuc reporter gene containing multiple ISREs was kindly provided by Takashi Fujita (The metropolitan institute of Medical Science, Tokyo, Japan) [22]. pRL-TK vector (*Renilla* luciferase) was used as a control for transfection efficiency. We seeded 5×10^4 Huh7 cells per well. Twenty-four hours later, we transfected 400 ng of p-55C1BLuc and 10 ng of pRL-TK using Lipofectamine 2000. After 4 h incubation, transfection mixture was replaced with the culture medium with or without BMP-7 or IFN. The cells were harvested after 12 h of transfection. The luciferase activities were measured according to the production manual and the values were normalized with *Renilla* luciferase activity.

Quantitative real-time PCR. Cells were treated with 100 ng/ml of BMP-7 or 10 U/ml of INF and were harvested after 48 h. Total RNA was extracted as described above. Real-time PCR was performed on the Mx3005 P (Stratagene, La Jolla, CA) in a total volume of 20 μl containing 1 μl of template cDNA, 2.5 mM of $MgCl_2$, 0.5 μM of each primer, and 2 μl of LightCycler FastStart DNA Master SYBR Green I mix. The temperature program consisted of an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 56 °C for 30 s, and 72 °C for 30 s with a single fluorescence taken at the end of each cycle. Quantitation was based on the measurements of each sample in their log-linear phase and analyzed using Mx3005P software version 1.0. Expressions of the myxovirus resistance-1 (MxA) and 2-5-oligoadenylate synthetase (2-5-OAS) gene mRNA levels were normalized with respect to the glycerol-3-phosphate dehydrogenase (G3PDH) mRNA and expressed as a ratio to the non-treated cells (relative expression). The primers used were as follows: G3PDH-F: 5′-ACC ACA GTC CAT GCC ATC AC-3′; G3PDH-R: 5′-TCC ACC ACC CTG TTG CTG TA-3′; MxA-F: 5′-GCT ACA CAC CGT GAC GGA TAT GG-3′; MxA-R: 5′-CGA GCT GGA TTG GAA AGC CC-3′; 2-5-OAS-F: 5′-TCA GAA GAG AAG CCA ACG TGA-3′; 2-5-OAS-R: 5′-CGG AGA CAG CGA GGG TAA AT-3′.

Analyses of drug synergism. The effects of treatment of Huh7/Rep-Feo cells with and IFN, alone and in combination, were analyzed according to classical isobologram analysis [23,24]. Dose–inhibition curves of IFN and BMP-7 were drawn, with the two drugs used alone or in combination. In each drug combination, the concentrations of IFN and BMP-7 that inhibited HCV replication to 50% (IC_{50}) were plotted against the fractional concentration of IFN and BMP-7 on the *X*- and *Y*-axis, respectively. A theoretical *line of additivity* is drawn between plots of the IC_{50} for either drug that were used alone. The combined effects of the two drugs were considered as 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-JFH1 cell culture. Huh7.5.1 cell line [25] (kindly provided by Dr. Frank V. Chisari) was infected by culture supernatant of HCV-JFH1 cell culture [21]. The infected cells were treated with IFN or BMP-7 or in combination. Virus replication was detected by the immunocytochemistry using anti-HCV-core monoclonal antibody (Kindly provided by Dr. Takaji Wakita).

Statistical analyses. Differences were compared using ANOVA. Basically p values less than 0.05 were considered as statistically significant. A significance of the differences compared to non-treated cells was tested using Dunnett's multiple comparison when appropriate.

Results

BMP-7 suppresses in vitro HCV replication

First, we examined expression of ALK-2 and BMPR II, which are the cell-surface receptors for BMP-7, in Huh7

cells with and without expression of HCV replicon. An RT-PCR analysis showed that ALK-2 and BMPR II genes were expressed in both Huh7 and Huh7/Rep-Feo cells (see the Supplementary Fig. 1). We also confirmed an expression of ALK-6 gene, but failed to detect that of ALK-3 in Huh7/Rep-Feo cells and in Huh-7 (data not shown).

In the Huh7/Rep-Feo cells the reduction of the HCV replication was detectable as luciferase activity [19,20]. To assess the effects of BMP-7, we treated Huh7/Rep-Feo cells with various doses of BMP-7 for 72 h. Time-course analyses showed that BMP-7 suppressed replication of the HCV replicon in a dose-dependent manner (Fig. 1A). When we treated the cells with BMP-7 (50, 100, and 200 ng/ml), HCV genome was significantly suppressed its multiplication compared to that in untreated cells ($p < 0.01$, Fig. 1B). The cell growth, as measured by the amount of total protein, did not significantly change by the treatment, (Fig. 1B). Similarly in the western blotting, treatment of 200 and 400 ng/ml of BMP-7 suppressed HCV-NA5A protein expression levels by 76% and 59%, respectively, without effects of a host protein expression (Fig. 1C). These data indicated that that BMP-7 suppressed expression of HCV replicon without affecting cell viability substantially. We then analyzed the cell-cycle profiles of Huh7/Rep-Feo cells with and without BMP-7 treatment. Seventy-two hours after the cell treatment with 100 ng/ml of BMP-7 significantly increased fraction of cells in the G1 phase than the untreated cells ($p < 0.05$), which was accompanied by decrease of cells in both S and G2/M phases ($p < 0.05$, see the Supplementary Fig. 2).

BMP-7 and IFN have synergistic inhibitory effects on HCV

To determine whether the anti-HCV effects of BMP-7 would be synergic with that of IFN, we treated the replicon cells concomitantly with IFN and BMP-7. As shown in the Fig. 2A, luciferase activities at after 72 h treatment with IFN clearly reduced. A greater reduction of the replication was found when the cells were treated with 100 ng/ml of BMP7 with IFN compared to that of 0.5 U/ml IFN alone ($p < 0.01$).

The synergy of IFN and BMP-7 was evaluated further by classical isobologram analysis [23,24]. The two drugs were administered in combination with fixed ratios adjusted by the IC_{50} of each drug (FIC ratio) at 1:0, 4:1, 2:1, 1:2, 1:4, and 0:1, respectively, and the dose–effect plots were drown (Fig. 2B). Each FIC for IFN and for BMP-7 at 50% inhibition were plotted on the *X*- and *Y*-axis, respectively to generate an isobologram (Fig. 2C). Each plot showing the FIC of each drug ratio fell below the line showing additivity, indicating that the effects of the drug

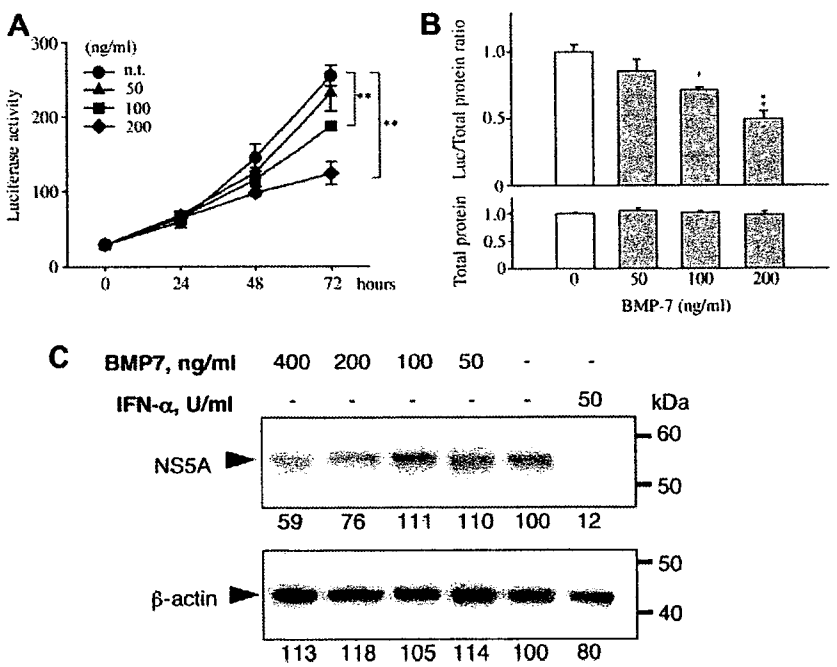

Fig. 1. Suppression of HCV replication and cell growth by BMP-7. (A) Time course in the presence of various concentrations of BMP-7. The cells were cultured in the presence of indicated concentrations of BMP-7 and luciferase activities were measured at 24, 48 and 72 h of treatment. (B) Luciferase activities were standardized with total protein level and showed as ratio to that of non-treated cells. The assays were done in triplicate. Data are indicated as means and SD (standard deviation). n.t., non-treated (negative control). '*' and '**' indicate *p*-values of less than 0.05 and 0.01, respectively. (C) Dose-dependent suppression of HCV protein expression by BMP-7. Expression of HCV protein expression was detected by the Western blotting. Huh7/Rep-Feo cells were cultured in the presence of indicated concentration of BMP-7 or IFN. The cells were harvested after 48 h of treatment. The cell lysate was separated on NuPAGE 4–12% Bis–Tris gels, transferred onto a PVDF membrane, and incubated with a monoclonal anti-NS5A antibody or an anti-β-actin antibody. Optical densitometries were performed, and the values were indicated at the bottom of each blot as a percent of drug-negative control. Positions of the protein size markers are indicated.

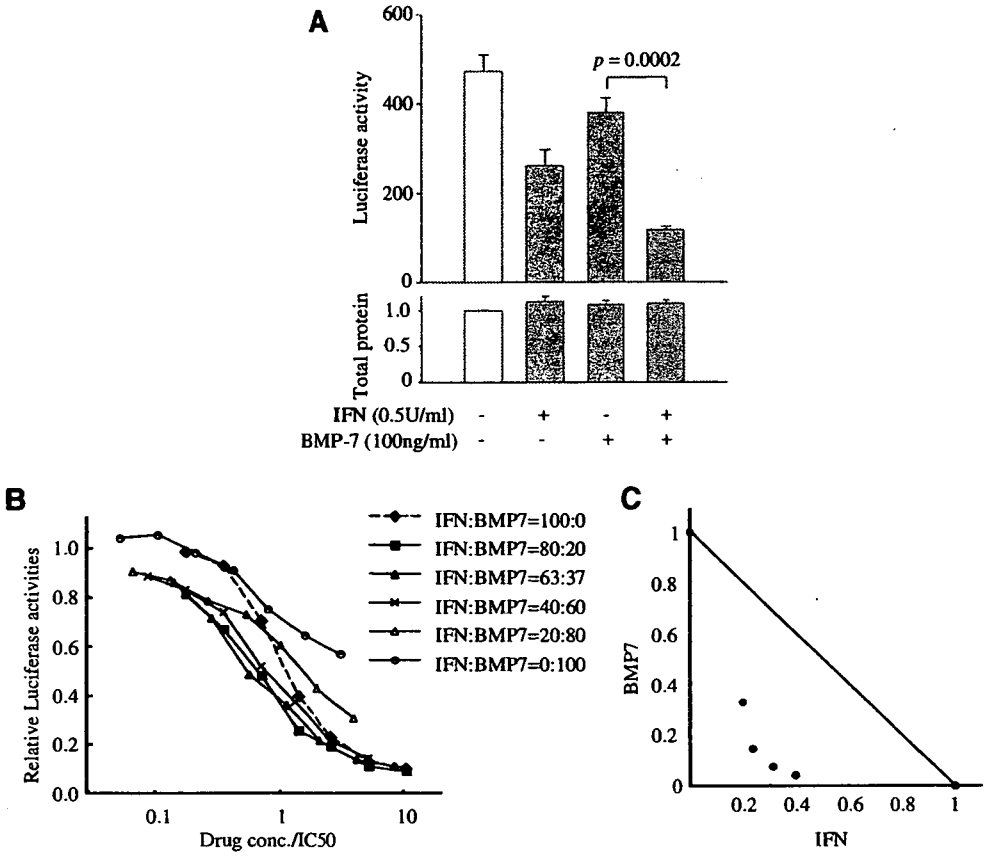

Fig. 2. Effects of combination of BMP-7 and IFN on HCV replication. (A) The suppression of the viral replication measured as luciferase activities. The cells were treated with IFN for 72 h with or without BMP-7. A greater reduction of replication was seen compared the treatment of IFN alone ($p = 0.0002$). (B) Dose–inhibition curves of IFN and BMP-7 that are combined in indicated proportions, adjusted by the IC_{50} of each drug. Assays were done in replicates of five samples, and mean values were plotted. Standard errors were within 15% in all plots (error bars are not shown). (C) Graphical representation of the isobologram analysis. In each drug combination in (B) the concentrations of IFN and BMP-7 that inhibited HCV replication to 50% (IC_{50}) were plotted against the fractional concentration of IFN and BMP-7 on the *X*- and *Y*-axis, respectively. A theoretical *line of additivity* is drawn between the IC_{50} for each drug alone. All of the FIC plots for the BMP-7 and IFN combinations fell below the *line of additivity*, indicating synergy.

combination on intracellular HCV-RNA replication is strongly synergistic. The MTT reduction values at drug concentrations used in this isobologram analysis did not show any significant decreases (data not shown), suggesting that the synergistic action of IFN and BMP-7 on HCV replication is through their pharmacological effects, and not due to augmentation of cytotoxicity.

BMP-7 suppresses HCV-JFH1 virus cell culture

The demonstrated inhibitory effects and the synergistic action of BMP-7 on HCV subgenomic replication were validated further by using HCV-JFH1 cell-culture system [21]. A Huh7.5.1 cell line that was infected by HCV-JFH1 was treated with either BMP-7 or IFN alone or in combination. As shown in the Fig. 3, treatment of the cells with IFN or BMP-7 alone resulted in significant decrease of percents of HCV-core-positive cells ($6.2 \pm 1.8\%$ and $5.7 \pm 0.9\%$, respectively, as compared with $12.3 \pm 2.7\%$ in the untreated cells with the *p*-values of 0.009 and 0.004, respectively). Furthermore, combination treatment of IFN and BMP-7 further decreased HCV-core-positive cells to $2.6 \pm 1.7\%$, which was significantly lower as compared with those treated with IFN and BMP-7 alone (*p*-values of 0.027 and 0.017, respectively).

Signal cross talk between BMP-7 and IFN

The synergic effect of BMP-7 with IFN could be either caused by a further strengthen of IFN-ISRE pathway or by other aberrant pathway(s). To see whether there is a cross talk between IFN-ISRE pathway and BMP-7 signal transduction, we conducted an ISRE reporter assay. We transfected the p-55C1BLuc plasmid in Huh7 cells and cultured with BMP-7 (0, 50, 100, and 200 ng/ml) or with IFN (0, 0.1, 1, and 10 U/ml). After 12 h of incubation, BMP-7 did not activate ISRE-promoter activities (Fig. 4A). These results suggested that the action of BMP-7 on the intracellular replication of HCV replicon was independent of the IFN-ISRE pathway.

Although BMP-7 did not mediate the signal via ISRE, it hampered the multiplication of the viral genome in some

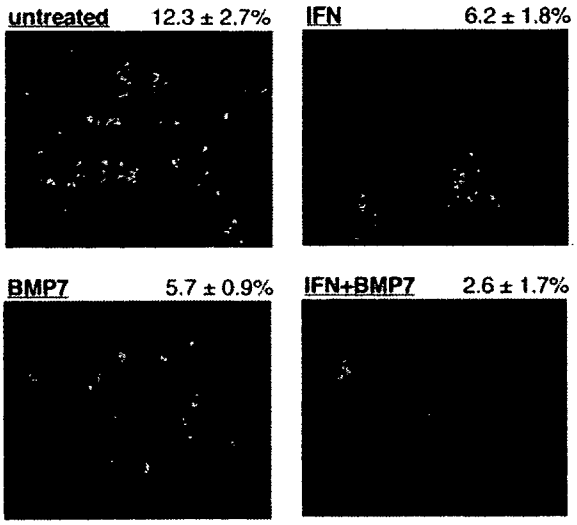

Fig. 3. Effects of BMP-7 and IFN on HCV-JFH1 cell culture. A Huh7.5.1 cell line [25] was infected by culture supernatant of HCV-JFH1 clone [21]. The cells were cultured in the presence of IFN (50 U/ml) or BMP-7 (50 ng/ml), or in combination of both drugs. Forty-eight hours after the culture, immunocytochemistry was performed using anti-HCV-core primary antibody followed by Alexa Fluor 488 anti-mouse IgG antibody. Nuclei were stained with DAPI. The percents of HCV-positive cells were calculated at four different visual fields, and indicated as a mean ± SD on the top of each figure.

way. We examined whether the treatment of the BMP-7 alters the expressions of IFN inducible genes such as MxA and 2-5-OAS. Fig. 4B shows that the exposure of BMP-7 resulted in higher expression levels of MxA and 2-5-OAS genes than non-treated Huh-7/Rep-Feo cells. The induction level brought by BMP-7 was, however, much less than that by IFN.

Discussion

Our results demonstrate that BMP-7 inhibits the intracellular replication of an HCV subgenome in a dose-dependent manner (Fig. 1). This inhibition was associated with cell-cycle arrest of the host Huh7/Rep-Feo cell. Thus, it is possible that the same mechanism that led to cell-cycle arrest may have inhibited the replication of HCV subgenome. This effect was clearly not due to cytotoxic effect of BMP-7, since the cell growth as measured by the total protein level was not changed. One might question the reason why we observed G1 arrest without any change in cell number or total protein. We surmise that BMP-7 first acts on cell-cycle system to arrest them in G1 phase, then after a prolonged treatment the total cell number or protein level may gradually fall off.

Our results are reminiscent of a previous study which reported that TGF-β-1 represses HCV replication *in vitro* according to the cell-cycle arrest in G1 phase [10]. Apparently, TGF-β-1 inhibited HCV replicon and cell-cycle arrest at a lower concentration compared to BMP-7, indicating that BMP-7 is less potent than TGF-β-1. In the same study, BMP-4 was shown not to inhibit HCV replication or cell

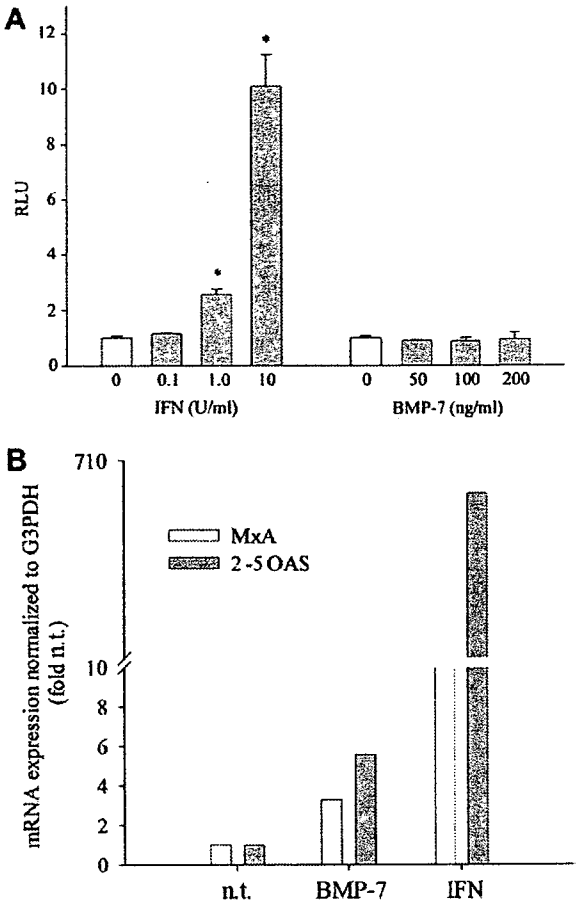

Fig. 4. BMP-7 augments IFN-induced activation of ISRE-dependent gene expression. (A) ISRE-responsive luciferase reporter assay. The Huh7 cells were treated either with 50, 100, 200 ng/ml of BMP-7 or 0.1, 1, 10 U/ml of IFN-alpha 4 h after transfection. Luciferase activities were measured 12 h after transfection and shown as ratio to that of non-treated cells. Error bars indicate the standard deviation (SD). Assay was done in triplicate. A significance of the differences was tested using Dunnett's multiple comparison when appropriate. * indicate p-values of less than 0.01. (B) BMP-7 activates IFN-inducible gene expression. Cells were exposed to BMP-7 (100 ng/ml) or IF (10 U/ml) for 48 h. The mRNA levels were measured by real-time PCR and normalized to G3DPH expression levels. Data are shown as fold ratio to non-treated samples. n.t., non-treated control.

proliferation [10]. However, this experiment was done at a low concentration of BMP-4, and thus the effect of BMP-4 and other BMP family cytokines warrant further study.

This raises an interesting question as to whether the suppressive effect is specific to BMP-7 and TGF-β-1 or whether it is common feature for the TGF-β superfamily cytokines. The difference of the response might be explained by the difference in the expression profiles of cell surface receptors. An interesting aspect of HCV genome and TGF-β is that the HCV core protein activates TGF-β-1 gene expression [26]. In some instance, there is a negative feed back between TGF-β and BMP pathways [27]. More detailed analysis of the BMP-7 pathway in hepatocyte is warranted to reveal the mechanism for repression of HCV replication by BMP-7 stimulation.

Of note is that BMP-7 had a prominent synergic effect with IFN on inhibiting HCV replication *in vitro* (Figs. 2 and 3). The antiviral actions of IFN are mediated by several interferon-stimulated genes (ISGs) [28,29]. An ISG, 2-5-OAS, activates ribonuclease L, which leads to general translational suppression through the degradation of 28S ribosomal RNA [30]. MxA destabilizes cellular RNA, represses general protein synthesis, and leads to apoptotic cell death [31,32]. Our result showed that BMP-7 does not mediate signal directly to the IFN-ISRE pathway (Fig. 4A). Nevertheless, BMP-7 augmented levels of MxA and 2-5-OAS mRNAs that are induced by IFN (Fig. 4B). These results suggest that the synergistic action of BMP-7 and IFN is through augmentation of IFN-induced antiviral gene responses by BMP-7.

Still, it remains to be clarified how the responses of the two drugs interact with each other and which alternative signal pathways are involved. While the canonical signal of BMP-7 employs the SMAD complex partially shared by TGF-β [33], other signals including MAP kinase pathway are also reported for BMP signal transduction [26,27]. So does IFN activate MAP kinase pathways that include p38 and ERK1/2. Thus, it is possible that the cross-talk of these aberrant signal pathways by BMP-7 and IFN may be responsible for the synergistic effect [34].

Considering the clinical situation that no antiviral agent, except IFN, has proven efficacy against HCV, combinations of IFN with certain agents, which suppress HCV replication or augment IFN action, will continue to dominate anti-HCV therapies. Our present study showed substantial synergistic effects of IFN and BMP-7 on HCV replication and demonstrated that the synergistic effects are partly through an action of BMP-7 that augments cellular Jak/STAT responses to IFN. These results suggest that the clinical antiviral effects of IFN treatment may potentially be improved by combination with BMP-7. Further exploring the mechanism of action of BMP-7 is warranted to develop novel anti-HCV chemotherapeutics.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.03.167.

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