Development of hepatitis C virus production reporter-assay systems using two different hepatoma cell lines

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A hepatitis C virus (HCV) infection system was developed previously using the HCV JFH-1 strain (genotype 2a) and HuH-7 cells, and this cell culture is so far the only robust production system for HCV. In patients with chronic hepatitis C, the virological effects of pegylated interferon and ribavirin therapy differ depending on the HCV strain and the genetic background of the host. Recently, we reported the hepatoma-derived Li23 cell line, in which the JFH-1 life cycle is reproduced at a level almost equal to that in HuH-7-derived RSc cells. To monitor the HCV life cycle more easily, we here developed JFH-1 reporter-assay systems using both HuH-7- and Li23-derived cell lines. To identify any genetic mutations by long-term cell culture, HCV RNAs in HuH-7 cells were amplified 130 days after infection and subjected to sequence analysis to find adaptive mutation(s) for robust virus replication. We identified two mutations, H2505Q and V2995L, in the NS5B region. V2995L but not H2505Q enhanced JFH-1 RNA replication. However, we found that H2505Q but not V2995L enhanced HCV RNA replication of strain O (genotype 1b). We also selected highly permissive D7 cells by serial subcloning of Li23 cells. The expression levels of claudin-1 and Niemann–Pick C1-like 1 in D7 cells are higher than those in parental Li23 cells. In this study, we developed HCV JFH-1 reporter-assay systems using two distinct hepatoma cell lines, HuH-7 and Li23. The mutations in NS5B resulted in different effects on strains O and JFH-1 HCV RNA replication.

INTRODUCTION

Hepatitis C virus (HCV) infection frequently causes chronic hepatitis and leads to liver cirrhosis and hepatocellular carcinoma. Elimination of HCV by antiviral reagents seems to be the most efficient therapy to prevent fatality.

HCV belongs to the family Flaviviridae and contains a positive ssRNA genome of 9.6 kb. The HCV genome encodes a single polyprotein precursor of approximately 3000 aa, which is cleaved by host and viral proteases into at least 10 proteins in the following order: Core, envelope 1 (E1), E2, p7, non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A and NS5B (Kato, 2001; Kato et al., 1990; Tanaka et al., 1996).

Evaluation of anti-HCV reagents was difficult before the development of the HCV replicon system (Lohmann et al., 1999). The HCV replicon system enabled investigation of anti-HCV reagents and the cellular factors involved in HCV RNA replication. Following introduction of the replicon system, genome-length HCV RNA-replication systems and reporter-assay systems were also developed (Ikeda et al., 2002, 2005; Lohmann et al., 2001; Pietschmann et al., 2002). In 2005, an HCV infection system was developed using the genotype 2a JFH-1 strain (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). The JFH-1 infection system has been used to study not only viral RNA replication, but also virus infection and release. This HCV cell-culture system was developed using the human hepatoma cell line HuH-7 and, thus far, HuH-7 is the only cell line to exhibit robust HCV production. Therefore, we intended to test the susceptibility of various other cell lines to HCV RNA replication. We reported previously that the hepatoma cell line Li23 supports robust HCV RNA replication and is also susceptible to authentic JFH-1 infection (Kato et al., 2009). Microarray analysis
revealed that HuH-7 and Li23 cells exhibited distinct gene-expression profiles (Mori et al., 2010). For example, we identified three genes (New York oesophageal squamous cell carcinoma 1, β-defensin-1 and galectin-3) showing Li23-specific expression. Using HuH-7 and Li23 cells in combination with HCV strain O (genotype 1b), we developed drug-assay systems (OR6 and ORL8, respectively) by introducing the Renilla luciferase (RL) gene (Ikeda et al., 2005; Kato et al., 2009). We found and reported that the sensitivities to anti-HCV reagents were different between the HuH-7 and Li23 assay systems; for example, the Li23 assay system was 10 times more sensitive to ribavirin than the HuH-7 assay system (Mori et al., 2011). Methotrexate showed very strong anti-HCV activity in the Li23 assay system, although it showed very weak anti-HCV activity in the HuH-7 assay system (Ueda et al., 2011). These results encouraged us to develop a JFH-1 reporter-assay system using HuH-7 and Li23 cells. This JFH-1 reporter-assay system not only facilitated monitoring of virus infection and release steps, but also provided us with new information that could be missed in these steps when using only a HuH-7 assay system. However, increasing the size of the viral genome by introducing exogenous genes [RL and the encephalomyocarditis virus internal ribosomal site (EMCV-ires)] reduced the efficiency of HCV RNA replication. To overcome this issue, we tried to improve the efficiency of HCV RNA replication by introducing adaptive mutations and by subcloning the parental cells.

Here, we developed JFH-1 HCV production reporter-assay systems in HuH-7- and Li23-derived cells using adaptive mutations and subcloned cells, which monitor the life cycle of HCV with luciferase activity. We also tested the effect of the mutations in NS5B from the JFH-1 strain on RNA replication of the specific genotype 1b O strain.

RESULTS

HCV mutations caused by long-term cell culture

The efficiency of HCV RNA replication depends on viral genetic mutations, host cells and viral genome size. For development of the HCV reporter-assay system, use of a longer viral genome reduced the efficiency of virus replication. To compensate for this issue, we tried to introduce adaptive mutations into the JFH-1 genome. We examined the viral sequences of JFH-1 130 days after infection of HuH-7-derived RSc cells. We performed RT-PCR for three parts of the viral genome: Core to NS2, NS3 to NS5A, and NS5B to 3'X. These three parts were separated by the Agel, SpeI, BsrGI and XbaI sites on the viral genome. We introduced PCR products into the cloning vector and three independent clones were subjected to sequencing analysis.

In the Core to NS2 region between the Agel and SpeI sites (designated AS), there were eight common mutations with amino acid substitutions: lysine to glutamate at aa 78 (K78E) in Core, P251L and A351D in E1, V402A, I414T and K715N in E2, Y771C in p7, and D962G in NS2 (Fig. 1a). In the NS3 to NS5A region between SpeI and BsrGI sites (designated SB), there were eight common mutations with amino acid substitutions: V1460I and M1611T in NS3, and I2270T, Q2307R, S2363L, M2392T, S2426A and C2441S in NS5A (Fig. 1b). In the NS5B to 3’X region between the BsrGI and XbaI sites (designated BX), there was only one common mutation with an amino acid substitution, V2995L in NS5B (Fig. 1c). The determined sequences were studied further to enhance HCV RNA replication in the JFH-1 reporter assay.

Effect of genetic mutations on HCV RNA replication

To monitor the virus life cycle more easily, we constructed dicistronic JFH-1 with a reporter gene, pJR/C-5B. The first cistron contained the RL gene and was translated by the HCV-IRES. The second cistron contained the JFH-1 ORF and was translated by the EMCV-RES. This construct facilitated monitoring of all steps of the virus life cycle by quantification of RL activity. However, the use of a longer viral genome resulted in lower replication efficiency. We tested the effect on HCV RNA replication of amino acid substitution caused during long-term cell culture.

The amino acid substitution clusters from three independent clones in Core to NS2 (AS-1, AS-2, AS-3) were introduced into pJR/C-5B. In vitro-transcribed HCV RNA was introduced into HuH-7-derived RSc cells, and RL activities were monitored 24, 48 and 72 h after electroporation (Fig. 2a). AS-3 exhibited higher replication efficiency than the wild type (WT). However, the replication efficiency of AS-2 was almost equal to that of the WT, and AS-1 exhibited lower replication efficiency than the WT. AS-3 possessed the highest replication efficiency among the tested JFH-1 mutants: at 72 h, the luciferase value of this clone was approximately 100 times that at 24 h.

The three pJR/C-5B constructs with mutations in NS3 to NS5A (SB-2, SB-3 and SB-4) were transcribed and introduced into RSc cells to compare the efficiency of HCV RNA replication (Fig. 2b). Unexpectedly, RL activity was not increased over 72 h after electroporation and exhibited a pattern similar to that of JFH-1 without the GDD motif. This result indicates that the mutation in NS3 to NS5A exhibited a negative effect on HCV RNA replication.

Finally, we tested the effect of the mutations in the NS5B region on HCV RNA replication. BX-2 contains two mutations with amino acid substitution (H2505Q and V2995L) and BX-7 contains only V2995L (Fig. 2c). JFH-1 with mutation(s) of BX-2 or BX-7 exhibited strong enhancement of HCV RNA replication. These results indicate that V2995L works as a strong replication-enhancing mutation (REM) in JFH-1 HCV RNA replication.
Mutations in NS5B enhanced HCV RNA replication differently in genotypes 1b and 2a

V2995L in NS5B is a common substitution, occurring in three clones, and H2505Q is conserved in two clones (BX-2 and BX-10). We examined the corresponding amino acids at positions 2995 and 2505 in genotype 1b replication-competent HCV strains O, 1B-4 and KAH5 (Fig. 3a) (Nishimura et al., 2009). The histidine at aa 2505 in JFH-1 is conserved in O, 1B-4 and KAH5 at the corresponding position, aa 2482. The valine at aa 2995 in JFH-1 is an alanine in O, 1B-4 and KAH5 at the corresponding position, aa 2972 (Fig. 3a). It is not clear whether the adaptive mutation found in genotype 2a is effective in genotype 1b HCV. Therefore, we investigated the effect of V2995L and/or H2505Q substitution on genotype 1b HCV RNA replication. We introduced substitutions V2995L and/or H2505Q into the subgenomic replicon, pOR/3-5B (HCV-O). In contrast to the case of JFH-1, H2505Q but not V2995L enhanced HCV-O RNA replication (Fig. 3b). These results indicate that the mutations in NS5B derived from JFH-1 functioned differently in genotype 1b HCV-O RNA replication.
HCV infection in HuH-7- and Li23-derived cell lines

As well as viral genetic mutations, the choice of host cells is important for the efficiency of HCV RNA replication. Cured cells in which HCV RNAs were eliminated by IFN-α, such as HuH-7.5, HuH-7.5.1 and our RSc cells, exhibit higher replication efficiency than their parental HuH-7 cells (Ariumi et al., 2007; Blight et al., 2002; Zhong et al., 2005). Therefore, we examined whether subcloned Li23 cells might enhance HCV RNA replication. We performed serial subcloning of Li23 cells from Li23-derived ORL8c cells by the limiting-dilution method (Fig. 4a). ORL8c cells are a cured cell line in which genome-length HCV RNAs were eliminated by interferon (IFN) treatment (Kato et al., 2009). The subclonal Li23-derived cell lines were selected from among 50–100 independent single cells in 96-well plates by three-round limiting dilution from ORL8c cells (Fig. S1a, available in JGV Online). First, L8c15 cells were selected from their parental ORL8c cells by limiting dilution. Then, C22 cells were selected from their parental L8c15 cells by limiting dilution. Finally, D7 cells were selected from C22 cells by limiting dilution (Fig. S1b). Together, these steps resulted in the...
selection of three subclonal cell lines that respectively exhibited the strongest replication efficiency in each round of selection. The lineages of the selected cell lines after three rounds of subcloning were designated L8c15, C22 and D7 cells, respectively.

We tested the subcloned cells for their HCV infectivities in comparison with those of HuH-7 and HuH-7-derived RSc cells. We reported previously that RSc cells could strongly support HCV replication and production (Kato et al., 2009). Li23 and its derived ORL8c, L8c15, C22 and D7 cell lines were infected using the supernatant from RSc cells replicating JR/C-5B with BX-2 mutations at an m.o.i. of 0.2 (Fig. 4b, c). RL activities were determined 24, 48, 72 and 96 h after infection and f.f.u. ml⁻¹ were determined 48 h after infection. The efficiency of HCV infectivity was highest in D7 cells, followed in order by C22, L8c15 and Li23 cells. HCV RNA replication in D7 cells was almost equal to that in RSc cells. These results suggest that the subcloned cell lines exhibit higher susceptibility to HCV infection than their parental cells.

Next, we further characterized the susceptibility of D7 cells to HCV infection in comparison with RSc cells, because D7 cells exhibited the highest susceptibility to HCV infection among the Li23-derived cell lines. D7 cells also exhibited the highest production and release of Core into the supernatant among the parental C22-derived subclonal

**Fig. 4.** HCV infection in HuH-7- and Li23-derived cell lines. (a) History of the selection of subclonal Li23-derived cell lines. (b) HuH-7, HuH-7-derived RSc, and Li23-derived ORL8c, L8c15, C22 and D7 cells were inoculated with supernatant from RSc cells replicating JR/C5B/BX-2. **P<0.01. (c) f.f.u. ml⁻¹ values were determined 48 h after infection of HuH-7- and Li23-derived cells with HCV using the supernatant from RSc cells replicating JR/C5B/BX-2. (d) f.f.u. ml⁻¹ values were determined 48 h after infection of RSc or D7 cells with HCV using the supernatant from RSc cells replicating JR/C5B/AS-3 or JR/C5B/BX-2. Supernatant from authentic JFH-1-replicating RSc cells was used as a positive control. (e) Core expression levels in RSc or D7 cells were determined 1, 2, 3 and 4 days after infection with JFH-1 with BX-2 mutations. Lanes: 1 and 6, mock-infected cells; 2 and 7, cells 1 day after infection; 3 and 8, cells 2 days after infection; 4 and 9, cells 3 days after infection; 5 and 10, cells 4 days after infection; 11 and 12, OR6c and OR6 cells, respectively; 13 and 14, ORL8c and ORL8 cells, respectively. OR6 and ORL8 were used as positive controls; OR6c and OR8c were used as negative controls. β-Actin was used as a control for the amount of protein loaded per lane.
cells (Fig. S1b). The susceptibility of the HCV reporter-assay system to HCV infection was examined using HuH-7- and Li23-derived cells. Supernatants from RSc cells replicating JR/C-5B with AS-3 or BX-2 mutations were used as inocula. The supernatant from authentic JFH-1-replicating RSc cells was used as a positive control. RSc and D7 cells were inoculated with each HCV-containing supernatant and f.f.u. ml⁻¹ were determined 48 h after infection. As shown in Fig. 4(d), the values of f.f.u. ml⁻¹ for AS-3 were 2.5 × 10⁴ and 1.0 × 10⁴ in RSc and D7 cells, respectively; those for BX-2 were 3.1 × 10⁴ and 1.8 × 10⁴ in RSc and D7 cells, respectively; and those for authentic JFH-1 were 2.9 × 10⁵ and 1.2 × 10⁵ in RSc and D7 cells, respectively. These results indicate that the infectivities of these three inocula were almost equal in RSc and D7 cells.

Next we examined Core expression after infection of RSc and D7 cells with HCV, as D7 cells exhibited the highest infectivity among the Li23-derived cells (Fig. 4e). Core was detected 2, 3 and 4 days after infection of the supernatant from RSc cells infected by JR/C-5B with BX-2. Although Core expression in D7 cells was slightly weaker than that in RSc cells, the signal of Core in HCV-infected D7 cells was equal to that in stable ORL8 cells. These results suggest that the JFH-1 reporter-assay system in Li23 cells is useful not only for the RL assay, but also for Core expression.

Expression of HCV receptors in parental and subclonal hepatoma cell lines

We tested expression of the HCV receptors CD81, scavenger receptor class B member 1 (SR-BI), CLDN1, OCLN and NPC1L1 as described in Methods. Relative expression levels of mRNA are shown, when the expression level of each receptor in HuH-7 was assigned to be 1. GAPDH was used as an internal control. Experiments were done in triplicate.

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**Fig. 5.** Expression levels of HCV receptors in HuH-7- and Li23-derived cells. Quantitative RT-PCR was performed for CD81, SR-BI, CLDN1, OCLN and NPC1L1 as described in Methods. Relative expression levels of mRNA are shown, when the expression level of each receptor in HuH-7 was assigned to be 1. GAPDH was used as an internal control. Experiments were done in triplicate.
Life cycle of the HCV reporter-assay system in Li23-derived cells

We investigated whether D7 cells produce infectious HCV. First, D7 cells were inoculated using the supernatant from RSc cells replicating JR/C5B with BX-2, and the supernatant was stored at 17 days after infection. Then, the supernatant derived from the D7 cells was used as an inoculum for reinfection of naive D7 cells. RL activities were determined 2, 6, 10 and 14 days after reinfection (Fig. 6). RL activity was increased after reinfection in D7 cells and reached a plateau 10 days after reinfection. These data indicate that the JFH-1 reporter-assay system is also useful for monitoring the HCV life cycle in Li23-derived cell lines.

DISCUSSION

In this study, we developed an HCV production reporter-assay system using two distinct hepatoma cell lines, HuH-7 and Li23. Robust HCV RNA replication and virus production were achieved by the introduction of REMs into the structural region or the NS5B region. These REMs were obtained from JFH-1-infected long-term cultured cells. The two REMs in NS5B (H2505Q and V2995L substitutions) derived from JFH-1 had different effects on replication of genotype 1b HCV-O RNA and genotype 2a JFH-1 RNA. Furthermore, the subcloned Li23-derived D7 cells produced by serial limiting dilution supported this HCV production reporter-assay system.

Several groups have reported JFH-1 reporter virus systems (Koutsoudakis et al., 2006; Marcello et al., 2006; Pietschmann et al., 2002; Wakita et al., 2005). However, robust reporter virus production was limited within the study using HuH-7-derived cells. Therefore, we attempted to develop a JFH-1 reporter virus assay system using our previously reported line of Li23 cells (Kato et al., 2009).

The introduction of RL and EMCV-IRES genes into the HCV gene lengthened the genome of HCV by approximately 1.9 kb and led to a reduction in the efficiency of HCV RNA replication. To overcome this disadvantage, we adopted the following strategies: (i) introduce the REMs; (ii) select cloned Li23-derived cells with a highly permissive host condition by the serial limiting-dilution method. For the first purpose, we performed sequence analyses for HCV RNA from JFH-1-infected RSc cells. Mutations in the region from Core to NS2 or NS5B enhanced HCV RNA replication. However, combination of mutations from two different regions reduced HCV RNA replication (Fig. S2). The reason for this may be that these two mutation clusters were obtained from distinct RT-PCR-amplified clones and they were not necessarily located on the same viral genome. It has been reported that the combination of REMs exhibited an antagonistic effect on HCV RNA replication (Lohmann et al., 2001). For the second purpose, we selected highly permissive Li23-derived clonal cells by the limiting-dilution method. We obtained three Li23-derived subclonal cell lines, L8c15, C22 and D7, in order from parental Li23-derived ORL8c cells. The efficiency of infectivity was highest in D7 cells, followed in order by C22, L8c15 and Li23 cells. D7 cells were highly permissive for infection of HCV with NS5B mutations.

As shown in Fig. 3(a), the histidine at aa 2505 in JFH-1 was conserved in the replication-competent O, 1B-4 and KAH5 strains at the corresponding position, aa 2482. The valine at aa 2995 in JFH-1 was alanine in strains O, 1B-4 and KAH5 at the corresponding position, aa 2972. The REMs in genotype 1b HCV were usually obtained by selection with neomycin after HCV RNA electroporation. Pietschmann et al. (2009) reported that REMs impaired infectious virus production. Most REMs are located in the NS3 and NS5A regions (Abe et al., 2007; Blight et al., 2002; Lohmann et al., 2001; Pietschmann et al., 2002). NS5A is a key molecule for virus production, and REMs affect the phosphorylation status of NS5A and the interaction with Core (Kato et al., 2008; Masaki et al., 2008; Tellinghuisen et al., 2008). In contrast, our REMs in NS5B were obtained in JFH-1-infected long-term cell culture without drug selection. Taking this information into account, we considered that H2505Q in NS5B might not interfere with genotype 1b virus production. We attempted to apply this REM from genotype 2a to genotype 1b and found that H2505Q enhanced replication of the genotype 1b HCV-O replicon. We are currently investigating whether our NS5B REM could enhance genotype 1b HCV production. As for the substitution at aa 2995 in JFH-1 (aa 2972 in genotype 1b), we should be careful in interpretation, because the backgrounds at this position are different between genotypes 2 and 1. Analysis of an HCV database (http://s2as02.genes.nig.ac.jp/) revealed that the consensus amino acids at position 2995 in genotype 2 and at 2972 in genotype 1 were valine and alanine, respectively. Furthermore, alanine and valine are not found at position 2995 in genotype 2 or at 2972 in genotype 1, respectively. These observations...
indicate that amino acid substitution between alanine and valine at these positions may be lethal for HCV of both genotypes. The amino acid at position 2995 in genotype 2 (2972 in genotype 1) is just upstream of a cis-acting replication element in NS5B. Therefore, the nucleotide at this position may affect the HCV RNA replication. To clarify this issue, further study will be needed.

A comparative study using HuH-7- and Li23-based JFH-1 reporter-assay systems would be expected to reveal new information on virus entry and release steps, because the backgrounds of these cells are different. Our recent study of these cells revealed the difference in sensitivities to anti-HCV reagents including ribavirin and methotrexate (Mori et al., 2011; Ueda et al., 2011). Furthermore, the IL28B genotype was different between HuH-7 and Li23 cells. The IL28B genotype (rs8099917) of HuH-7 cells renders them resistant to pegylated IFN and ribavirin, and Li23 cells are sensitive to pegylated IFN and ribavirin (M. Ikeda and N. Kato, unpublished data).

Recently, it was reported that stable expression of miR122 enhanced JFH-1 HCV production in Hep3B and HepG2 (Kambara et al., 2012; Narbus et al., 2011). It is noteworthy that the expression of miR122 in Li23-derived cells was almost the same as that in HuH-7 cells (Fig. S3). High-level expression of miR122 in Li23 cells may be one of the reasons that Li23 cells can support HCV production as robust as that in HuH-7 cells among the hepatocyte-derived cell lines. Interestingly, the expression levels of miR122 are higher in ORL8c, L8c15 and D7 cells, but not in C22 cells, than those in parental Li23 cells (Fig. S3). This result suggests that the expression level of miR122 may partly contribute to the fitness of HCV replication and production.

So far, we have only little information regarding the mechanism by which subclonal cells support HCV replication and production more efficiently than the parental cells. In this study, we found that the expression levels of CLDN1 and NPC1L1 in Li23-derived subclonal cells were higher than those in the parental Li23 cells. These results suggest that a high expression level of these entry factors in Li23-derived subclonal cells may contribute to enhanced virus entry. In the course of the experiment to determine the expression levels of NPC1L1 in HuH-7- and Li23-derived cell lines, we found that RSc cells expressed a very low level of NPC1L1 compared with the parental HuH-7 cells. Possible mechanisms for this are: (i) very low-level expression of NPC1L1 is sufficient for HCV entry; (ii) an unknown entry factor compensates for NPC1L1 in the entry step in RSc cells. Further study will be needed to clarify this issue.

In summary, we have developed JFH-1 reporter-assay systems using HuH-7-derived RSc and Li23-derived D7 cells. Expression levels of CLDN1 and NPC1L1 were higher than those in the parental Li23 cells. We found different effects of REMs (V2995L and H2505Q) in NS5B on virus RNA replication in genotype 2a and 1b HCV strains. These findings will become useful tools for the study of the life cycle of HCV.

**METHODS**

**Cell cultures.** RSc and ORL8c cells were derived from the cell lines HuH-7 and Li23, respectively, as described previously (Kato et al., 2009). L8c15, D7 and C22 cells were selected from ORL8c, L8c15 and C22 cells, respectively, by limiting dilution. HuH-7 and RSc cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies) supplemented with 10% FBS (Life Technologies). Li23-derived cell lines were maintained in F12 medium (Life Technologies) and DMEM (1:1 in volume) supplemented with 1% FBS and epidermal growth factor (50 ng ml⁻¹; PeproTech, Inc.) as described previously (Kato et al., 2009).

**RT-PCR and sequencing analysis.** RSc cells were infected with cell-culture-grown HCV (HCVcc) and cultured for 130 days. Total RNAs from these cells were prepared using an RNeasy extraction kit (Qiagen). These RNA samples were used for RT-PCR in order to amplify the Core to NS2 (4.0 kb), NS3 to NS5A (3.6 kb) and NS5B to 3′X (1.9 kb) regions. Reverse transcription was performed with an oligo(dA)₂₉ primer. The following primer pairs were employed: to amplify the Core to NS2 region, JFH-1/ AgeI (5′-CCCAAATGTTACGCGTGAATTACGGGAAATGC-3′) and JFH-1/SpeI (5′-TGCCAGTGTGCAATGTTAGC-3′); for the NS3 to NS5A region, JFH-1/Spel (5′-CCGAGGATCAATTAGCTAGTG-3′) and JFH-1/ BsrGI (5′-CCCAGGATTTCACTTTTACCCCATCGTGTGAGGC-3′); for the NS5B to 3′X region, JFH-1/BsrGI (5′-CCGCTCGAGGACCGTTCGATTTTGAACTCAGGTGTTGC-3′) and JFH-1/XhoR (5′-GCTCTAGAATCCGATCTCGAGAGACGACGATAC-3′). SuperScript III reverse transcriptase (Invitrogen) and KOD-plus DNA polymerase (TOYOBO) were used for reverse transcription and PCR, respectively. PCR products were ligated into pBluescript II (Fermentas) and three independent clones were subjected to sequencing analysis.

**Plasmid construction.** pJR/C-5B plasmid is a dicistronic HCV JFH-1 construct. The RL gene and HCV ORF were introduced into the first and second cistrons, respectively. To construct this plasmid, we fused the JFH-1 5′UTR with the RL gene by overlap PCR, and the PCR products were ligated into pGFR-JFH-1 (GenBank accession no. AB237837) at the AgeI and Pmdl sites. For the first PCR, the primer pair 5′-GCCCTACGACCTAGTATG-3′ (J5dC) and 5′-AACGATGGCCCGGCGGCCTCGTTGTGTCTTTTTGG-3′ (J5dCR) was employed to amplify the 5′UTR, and the primer pair 5′-GCTCAGGACCGTGATTTTGAGTAACTCGCTGTTGC-3′ and 5′-GCCATGATTTTGATGAACTCGCTGTTGC-3′ (JFH-1/XhoR) was used for amplification of this region. The resulting PCR products were ligated into pBluescript II vector (Strategene) and subcloned into the XhoI and SpeI sites of pENTR/D-TOPO vector (Invitrogen). The plasmid obtained was designed as pENTR/D-TOPO-HCV-RL. After excision of the RL region from the pENTR/D-TOPO-HCV-RL vector, the RL region was ligated into the pRC-HCV vector (GenBank accession no. AB056301) at the NotI and XhoI sites. The resulting plasmid was named pRc-HCV-RL.

**Luciferase reporter assay.** For the luciferase assay, approximately 10⁴ HCV-harbouring cells were plated onto 24-well plates in triplicate and were cultured for 24–96 h after electroporation or infection, as described previously (Ikeda et al., 2002). The cells were harvested with RIPA lysis reagent (Promega) and subjected to RL assay according to the manufacturer’s protocol.

**Western blot analysis.** Preparations of cell lysates, SDS-PAGE and immunoblotting were performed as described previously (Kato et al., archived).

**Immunohistochemistry.** Immunohistochemical staining was performed as described previously (Ikeda et al., 2002). The antibodies used in this study were Core (CP11; Institute of Immunology, Tokyo, Japan) and β-actin (AC-15; Sigma) antibodies. Immunocomplexes were detected with a Renaissance enhanced chemiluminescence assay (PerkinElmer Life Science).
HCV infection and determination of f.f.u. To determine f.f.u. ml$^{-1}$, 6 x 10$^5$ cells were plated onto a 96-well plate 24 h before infection. The supernatant of HCV RNA-replicating cells was diluted serially and was used as an inoculum. Forty-eight hours after infection, the cells were fixed and Core was stained with anti-Core antibody and HRP-conjugated mouse anti-IgG antibody. Then, the expression of Core was visualized with a DAB substrate kit (DAKO). Culture supernatants and cells were collected for quantification of Core by ELISA (Mitsubishi Kagaku Bio-Clinical Laboratories).

Quantitative RT-PCR analysis. Quantitative RT-PCR analysis for HCV receptors was performed using real-time LightCycler PCR (Roche Diagnostics) as described previously (Ikeda et al., 2005). The primer pairs for CD81, SR-BI, CLDN1 and OCLN were reported previously (Nakamuta et al., 2011). The primer pair NPC1L1 (5’-AGATCTTCTTCTCGGCTCCA-3’) and NPC1L1R (5’-TGCCAGAGCCGGTGAAC-3’) was used for NPC1L1.

Statistical analysis. Luciferase activities were compared statistically between the various treatment groups using Student’s t-test. P-values of <0.05 were considered statistically significant. The mean ± SD was determined from at least three independent experiments.

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