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 *Age*I, *Spe*I, *Bsr*GI and *Xba*I 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 *Age*I and *Spe*I 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 *Spe*I and *Bsr*GI 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 *Bsr*GI and *Xba*I 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-IRES. 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.

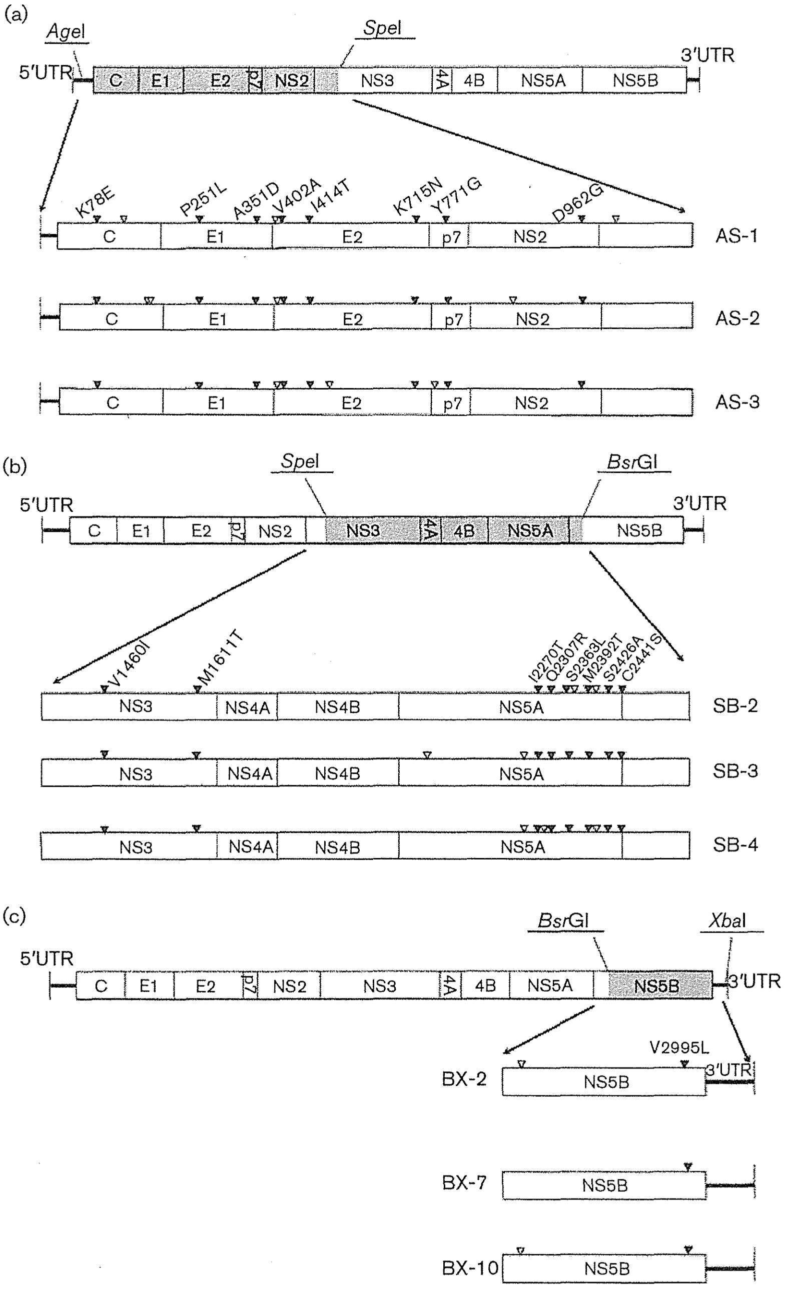

Fig. 1. Genetic mutations causing amino acid substitutions during long-term HCV infection. RT-PCR was performed for HCV RNAs from HuH-7 cells 130 days after JFH-1 infection. PCR products were subcloned into the pBluescript II plasmid. Three clones of (a) the Core to NS2 region between the *Age*I and *Spe*I sites (AS), (b) the NS3 to NS5A region between the *Spe*I and *Bsr*GI sites (SB) and (c) the NS5B to 3′X region between the *Bsr*GI and *Xba*I sites (BX) were subjected to sequencing analysis. ▼ and ▽ represent conservative and non-conservative amino acid substitutions, respectively.

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.

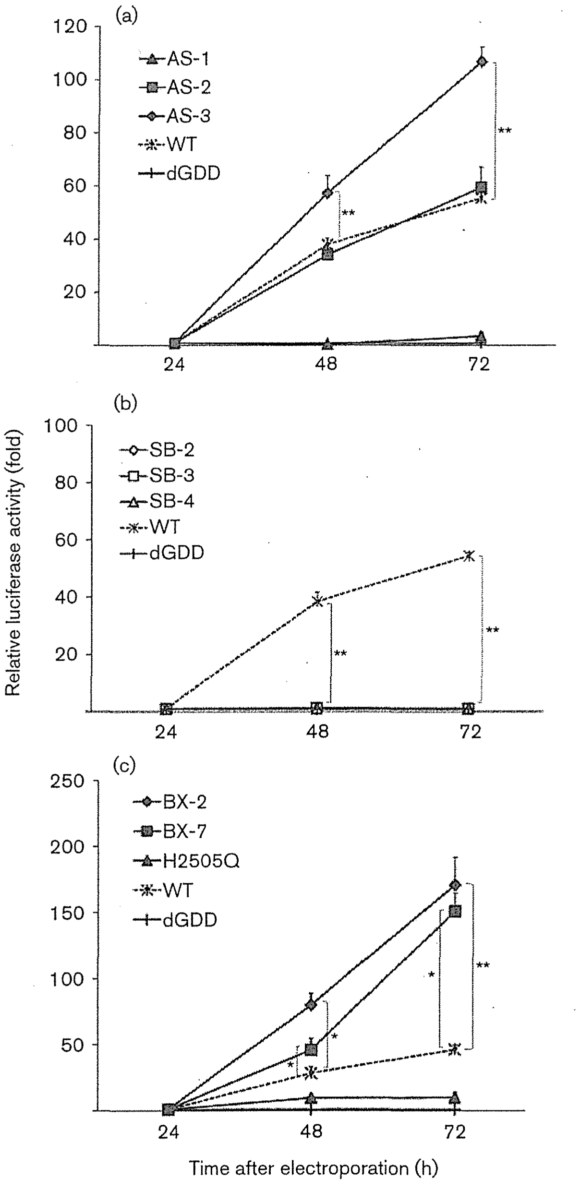

Fig. 2. Effect of amino acid substitutions on HCV RNA replication. (a) The Core to NS2 region; (b) the NS3 to NS5A region; (c) the NS5B to 3′X region. Amino acid substitutions were introduced into pJR/C5B and *in vitro*-synthesized RNAs were electroporated into HuH-7-derived RSc cells. RL activity was determined 24, 48 and 72 h after electroporation. dGDD, Negative control without the GDD motif; WT, wild type. *$P<0.05$; **$P<0.01$.

HCV infection in HuH-7- and Li23-derived cell lines

(a)

Consensus		TFDRLQVLDD**H**YRDVLKE	
JFH-1	2495	TFDRTQVLDA**H**YDSVLKD	2512
O	2472	TFDRLQVLDD**H**YRDVLKE	2489
KAH-5	2472	TFDRLQVLDD**H**YRDVLKE	2489
1B-4	2472	TFDRLQVLDD**H**YRDVLKE	2489
Consensus		PAASRLDLSGWFV**A**GYSGGDIYHS	
JFH-1	2982	PEARLLDLSSWFT**V**GAGGGDIFHS	3005
O	2959	PAASRLDLSGWFV**A**GYSGGDIYHS	2982
KAH-5	2959	PAASRLDLSGWFV**A**GYSGGDIYHS	2982
1B-4	2959	PAASRLDLSGWFV**A**GYSGGDIYHS	2982

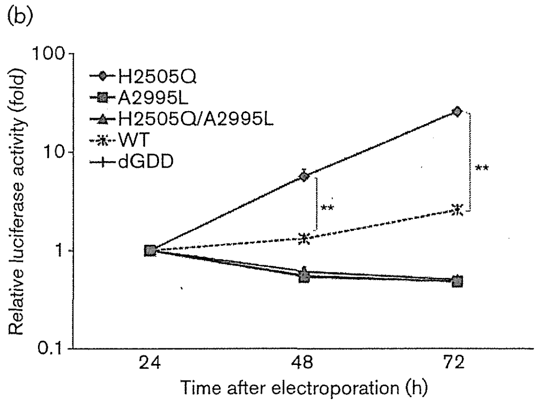

Fig. 3. Effect of amino acid substitutions in NS5B on genotype 1b and 2a HCV RNA replication. (a) Alignment of amino acids at positions 2505 (JFH-1) and 2482 (genotype 1b) and around the adjacent region (upper panel). Alignment of amino acids at positions 2995 (JFH-1) and 2972 (genotype 1b) and around the adjacent region (lower panel). The HCV strains O, KAH5 and 1B-4 belong to genotype 1b. (b) H2505Q and/or V2995L were introduced into the HCV-O subgenomic replicon (pOR/3-5B), and transcribed RNAs were electroporated into RSc cells. RL activities were tested 24, 48 and 72 h after infection. dGDD, Negative control without the GDD motif; WT, wild type. **$P<0.01$.

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

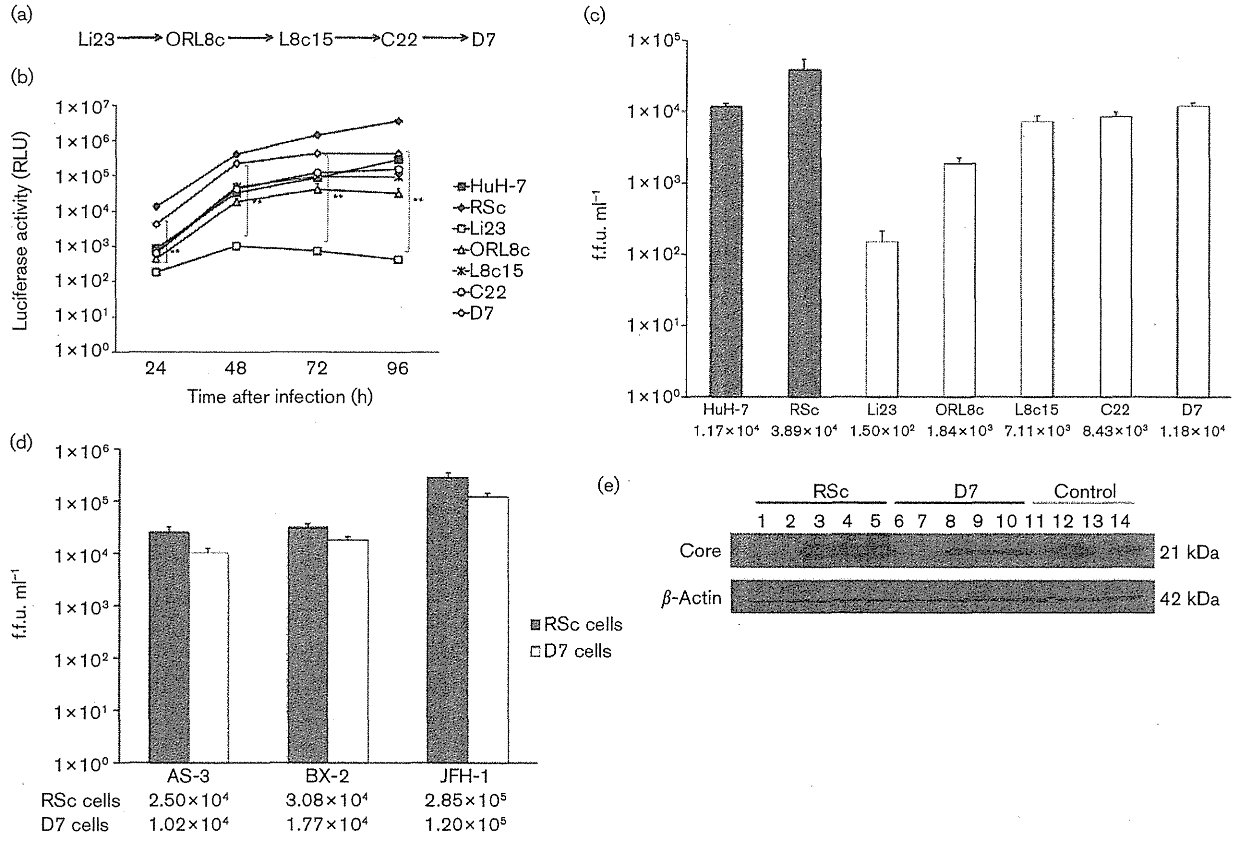

Fig. 4. HCV infection in HuH-7- and Li23-derived cell lines. (a) History of the selection of subclonal Li23-derived cell lines. (b) HuH-7, HuH-7-derived RSc, and Li23-derived ORL8c, L8c15, C22 and D7 cells were inoculated with supernatant from RSc cells replicating JR/C5B/BX-2. $^{**}P<0.01$. (c) f.f.u. 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.

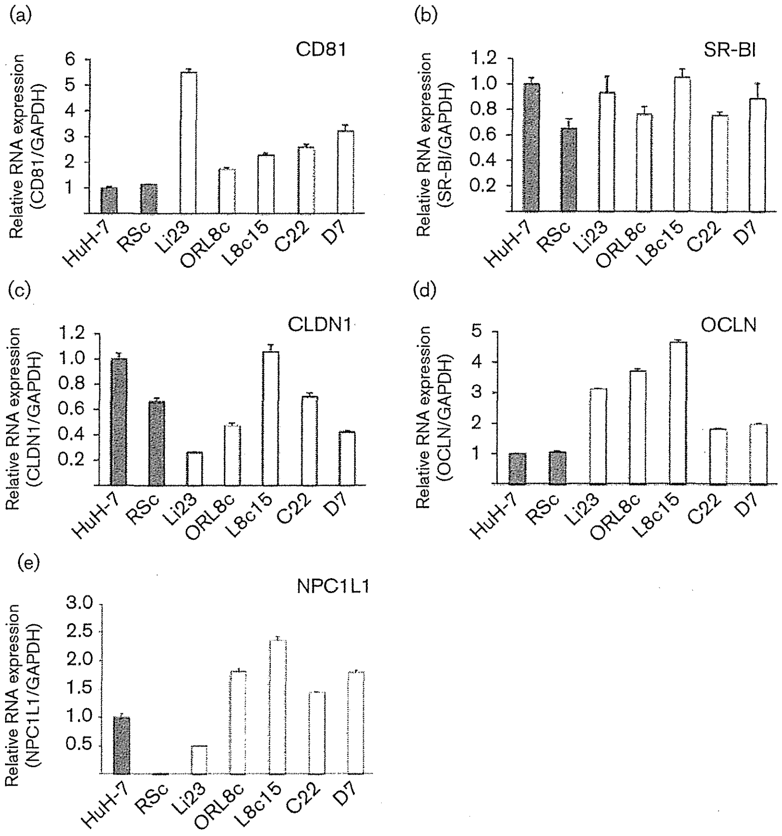

Fig. 5. Expression levels of HCV receptors in HuH-7- and Li23-derived cells. Quantitative RT-PCR was performed for CD81, SR-BI, CLDN1, OCLN and NPC1L1 as described in Methods. Relative expression levels of mRNA are shown, when the expression level of each receptor in HuH-7 was assigned to be 1. GAPDH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

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 2.5×10^4 and 1.0×10^4 in RSc and D7 cells, respectively; those for BX-2 were 3.1×10^4 and 1.8×10^4 in RSc and D7 cells, respectively; and those for authentic JFH-1 were 2.9×10^5 and 1.2×10^5 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 cell lines (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 I (SR-BI), claudin-1 (CLDN1) and occludin (OCLN). We also examined the expression of the recently reported HCV entry factor Niemann–Pick C1-like 1 (NPC1L1) (Sainz *et al.*, 2012). Expression levels of CD81 in Li23 and its subclonal cells were higher than those in HuH-7 and RSc cells (Fig. 5a). Although expression of CD81 in Li23-derived cell lines was lower than that in parental Li23 cells, interestingly the expression levels of CD81 increased during the rounds of selection. There is no difference in the expression of SR-BI among the cell lines tested (Fig. 5b). The expression of CLDN1 in Li23-derived cells was higher than that in parental Li23 cells (Fig. 5c). Expression levels of OCLN in Li23 and its subclonal cells were higher than those in HuH-7 and RSc cells (Fig. 5d). Finally, the expression of NPC1L1 in Li23-derived cell lines was higher than that in parental Li23 cells (Fig. 5e). It is noteworthy that the expression level of NPC1L1 in RSc cells was approximately 2 $\log_{10}$ lower than that in parental HuH-7 cells. Taken together, these results indicate that the expression levels of CLDN1 and NPC1L1 in Li23-derived cells were higher than those in parental Li23 cells.

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 naïve 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 developed a JFH-1 reporter virus assay system using our previously reported line of Li23 cells (Kato *et al.*, 2009).

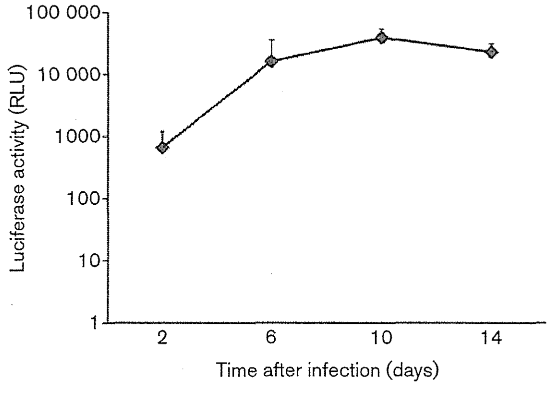

Fig. 6. HCV life cycle in Li23-derived D7 cells. D7 cells were inoculated with the supernatant from D7 cells after infection with JFH-1 with BX-2 mutants. RL activities were tested 2, 6, 10 and 14 days after infection.

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 JHF-1 reporter-assay systems using HuH-7-derived RSc and Li23-derived D7 cells. Expression levels of CLDN1 and NP1C1L 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$(dA)_{23}$ primer. The following primer pairs were employed: to amplify the Core to NS2 region, JFH-1/*Age*I (5′-CCCAAGCTTACCGGTGAGTACACCGGAATTGC-3′) and JFH-1/*Spe*IR (5′-TGCCATGTGCCTTGGATAGGTACG-3′); for the NS3 to NS5A region, JFH-1/*Spe*I (5′-CCCAGGGGTACAAAGTACTAGTGC-3′) and JFH-1/*Bsr*GIR (5′-CCCAAGCTTTACCTTTTTAGCCCTCTGTGAGGC-3′); for the NS5B to 3′X region, JFH-1/*Bsr*GI (5′-CCGCTCGAGACCCTTTGAGTAACTCGCTGTTGC-3′) and JFH-1/*Xba*IR (5′-GCTCTAGACATGATCTGCAGAGAGACCAGTTAC-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 pFGR-JFH-1 (GenBank accession no. AB237837) at the *Age*I and *Pme*I sites. For the first PCR, the primer pair 5′-GCGCCTAGCCATGGCGTTAGTATG-3′ (J5dC) and 5′-AAGCCATGGCCGGCCCTGGGCGACGGTTGGTGTTTCTTTTGG-3′ (J5dCR) was employed to amplify the 5′UTR, and the primer pair 5′-AACCGTCGCCCAGGGCCGGCCATGGCTTCCAAGGTGTACGACCCC-3′ (JRL) and 5′-TCGAAATCTCGTGATGGCAGGTTGG-3′ (JRLR) was employed to amplify the RL region. These first PCR products were used in the second PCR as the templates. For the second PCR, the primer pair J5dC and JRLR was employed to amplify the 5′UTR and RL. KOD-plus DNA polymerase was used for PCR.

The H2505H and/or A2995L mutations were introduced into the HCV-O replicon by QuikChange mutagenesis (Stratagene) as described previously (Ikeda *et al.*, 2002).

Luciferase reporter assay. For the luciferase assay, approximately 1.0–1.5 × 10^4 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.*, 2005). The cells were harvested with *Renilla* lysis reagent (Promega) and subjected to RL assay according to the manufacturer's protocol.

Western blot analysis. Preparation of cell lysates, SDS-PAGE and immunoblotting were performed as described previously (Kato *et al.*, 2003). 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}, 6×10^3 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 LightCycle 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′-AGATCTTCTTCTTCCGCCTCCA-3′) and NPC1L1R (5′-TGCCAG-AGCCGGGTTAAC-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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REFERENCES

Abe, K., Ikeda, M., Dansako, H., Naka, K. & Kato, N. (2007). Cell culture-adaptive NS3 mutations required for the robust replication of genome-length hepatitis C virus RNA. *Virus Res* **125**, 88–97.

Ariumi, Y., Kuroki, M., Abe, K., Dansako, H., Ikeda, M., Wakita, T. & Kato, N. (2007). DDX3 DEAD-box RNA helicase is required for hepatitis C virus RNA replication. *J Virol* **81**, 13922–13926.

Blight, K. J., McKeating, J. A. & Rice, C. M. (2002). Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. *J Virol* **76**, 13001–13014.

Ikeda, M., Yi, M., Li, K. & Lemon, S. M. (2002). Selectable subgenomic and genome-length dicistronic RNAs derived from an infectious molecular clone of the HCV-N strain of hepatitis C virus replicate efficiently in cultured Huh7 cells. *J Virol* **76**, 2997–3006.

Ikeda, M., Abe, K., Dansako, H., Nakamura, T., Naka, K. & Kato, N. (2005). Efficient replication of a full-length hepatitis C virus genome, strain O, in cell culture, and development of a luciferase reporter system. *Biochem Biophys Res Commun* **329**, 1350–1359.

Kambara, H., Fukuhara, T., Shiokawa, M., Ono, C., Ohara, Y., Kamitani, W. & Matsuura, Y. (2012). Establishment of a novel permissive cell line for the propagation of hepatitis C virus by expression of microRNA miR122. *J Virol* **86**, 1382–1393.

Kato, N. (2001). Molecular virology of hepatitis C virus. *Acta Med Okayama* **55**, 133–159.

Kato, N., Hijikata, M., Ootsuyama, Y., Nakagawa, M., Ohkoshi, S., Sugimura, T. & Shimotohno, K. (1990). Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. *Proc Natl Acad Sci U S A* **87**, 9524–9528.

Kato, N., Sugiyama, K., Namba, K., Dansako, H., Nakamura, T., Takami, M., Naka, K., Nozaki, A. & Shimotohno, K. (2003). Establishment of a hepatitis C virus subgenomic replicon derived from human hepatocytes infected in vitro. *Biochem Biophys Res Commun* **306**, 756–766.

Kato, T., Choi, Y., Elmowalid, G., Sapp, R. K., Barth, H., Furusaka, A., Mishiro, S., Wakita, T., Krawczynski, K. & Liang, T. J. (2008). Hepatitis C virus JFH-1 strain infection in chimpanzees is associated with low pathogenicity and emergence of an adaptive mutation. *Hepatology* **48**, 732–740.

Kato, N., Mori, K., Abe, K., Dansako, H., Kuroki, M., Ariumi, Y., Wakita, T. & Ikeda, M. (2009). Efficient replication systems for hepatitis C virus using a new human hepatoma cell line. *Virus Res* **146**, 41–50.

Koutsoudakis, G., Kaul, A., Steinmann, E., Kallis, S., Lohmann, V., Pietschmann, T. & Bartenschlager, R. (2006). Characterization of the early steps of hepatitis C virus infection by using luciferase reporter viruses. *J Virol* **80**, 5308–5320.

Lindenbach, B. D., Evans, M. J., Syder, A. J., Wölk, B., Tellinghuisen, T. L., Liu, C. C., Maruyama, T., Hynes, R. O., Burton, D. R. & other authors (2005). Complete replication of hepatitis C virus in cell culture. *Science* **309**, 623–626.

Lohmann, V., Körner, F., Koch, J., Herian, U., Theilmann, L. & Bartenschlager, R. (1999). Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* **285**, 110–113.

Lohmann, V., Körner, F., Dobierzewska, A. & Bartenschlager, R. (2001). Mutations in hepatitis C virus RNAs conferring cell culture adaptation. *J Virol* **75**, 1437–1449.

Marcello, T., Grakoui, A., Barba-Spaeth, G., Machlin, E. S., Kotenko, S. V., MacDonald, M. R. & Rice, C. M. (2006). Interferons α and λ inhibit hepatitis C virus replication with distinct signal transduction and gene regulation kinetics. *Gastroenterology* **131**, 1887–1898.

Masaki, T., Suzuki, R., Murakami, K., Aizaki, H., Ishii, K., Murayama, A., Date, T., Matsuura, Y., Miyamura, T. & other authors (2008). Interaction of hepatitis C virus nonstructural protein 5A with core protein is critical for the production of infectious virus particles. *J Virol* **82**, 7964–7976.

Mori, K., Ikeda, M., Ariumi, Y. & Kato, N. (2010). Gene expression profile of Li23, a new human hepatoma cell line that enables robust hepatitis C virus replication: comparison with HuH-7 and other hepatic cell lines. *Hepatol Res* **40**, 1248–1253.

Mori, K., Ikeda, M., Ariumi, Y., Dansako, H., Wakita, T. & Kato, N. (2011). Mechanism of action of ribavirin in a novel hepatitis C virus replication cell system. *Virus Res* **157**, 61–70.

Narbus, C. M., Israelow, B., Sourisseau, M., Michta, M. L., Hopcraft, S. E., Zeiner, G. M. & Evans, M. J. (2011). HepG2 cells expressing microRNA miR-122 support the entire hepatitis C virus life cycle. *J Virol* **85**, 12087–12092.

Nakamuta, M., Fujino, T., Yada, R., Aoyagi, Y., Yasutake, K., Kohjima, M., Fukuizumi, K., Yoshimoto, T., Harada, N. & other authors (2011). Expression profiles of genes associated with viral entry in HCV-infected human liver. *J Med Virol* **83**, 921–927.

Nishimura, G., Ikeda, M., Mori, K., Nakazawa, T., Ariumi, Y., Dansako, H. & Kato, N. (2009). Replicons from genotype 1b HCV-positive sera exhibit diverse sensitivities to anti-HCV reagents. *Antiviral Res* **82**, 42–50.

Pietschmann, T., Lohmann, V., Kaul, A., Krieger, N., Rinck, G., Rutter, G., Strand, D. & Bartenschlager, R. (2002). Persistent and transient replication of full-length hepatitis C virus genomes in cell culture. *J Virol* **76**, 4008–4021.

Pietschmann, T., Zayas, M., Meuleman, P., Long, G., Appel, N., Koutsoudakis, G., Kallis, S., Leroux-Roels, G., Lohmann, V. & Bartenschlager, R. (2009). Production of infectious genotype 1b virus particles in cell culture and impairment by replication enhancing mutations. *PLoS Pathog* **5**, e1000475.

Sainz, B., Jr, Barretto, N., Martin, D. N., Hiraga, N., Imamura, M., Hussain, S., Marsh, K. A., Yu, X., Chayama, K. & other authors (2012).

Identification of the Niemann–Pick C1-like 1 cholesterol absorption receptor as a new hepatitis C virus entry factor. *Nat Med* **18**, 281–285.

Tanaka, T., Kato, N., Cho, M. J., Sugiyama, K. & Shimotohno, K. (1996). Structure of the 3′ terminus of the hepatitis C virus genome. *J Virol* **70**, 3307–3312.

Tellinghuisen, T. L., Foss, K. L. & Treadaway, J. (2008). Regulation of hepatitis C virion production via phosphorylation of the NS5A protein. *PLoS Pathog* **4**, e1000032.

Ueda, Y., Mori, K., Ariumi, Y., Ikeda, M. & Kato, N. (2011). Plural assay systems derived from different cell lines and hepatitis C virus strains are required for the objective evaluation of anti-hepatitis C virus reagents. *Biochem Biophys Res Commun* **409**, 663–668.

Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Kräusslich, H. G. & other authors (2005). Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* **11**, 791–796.

Zhong, J., Gastaminza, P., Cheng, G., Kapadia, S., Kato, T., Burton, D. R., Wieland, S. F., Uprichard, S. L., Wakita, T. & Chisari, F. V. (2005). Robust hepatitis C virus infection in vitro. *Proc Natl Acad Sci U S A* **102**, 9294–9299.

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Raloxifene inhibits hepatitis C virus infection and replication

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ABSTRACT

Postmenopausal women with chronic hepatitis C exhibited a poor response to interferon (IFN) therapy compared to premenopausal women. Osteoporosis is the typical complication that occurs in postmenopausal women. Recently, it was reported that an osteoporotic reagent, vitamin D3, exhibited anti-hepatitis C virus (HCV) activity. Therefore, we investigated whether or not another osteoporotic reagent, raloxifene, would exhibit anti-HCV activity in cell culture systems. Here, we demonstrated that raloxifene inhibited HCV RNA replication in genotype 1b and infection in genotype 2a. Raloxifene enhanced the anti-HCV activity of IFN-α. These results suggest a link between the molecular biology of osteoporosis and the HCV life cycle.

1. Introduction

Hepatitis C virus (HCV) belongs to the *Flaviviridae* family and contains a positive single-stranded RNA genome of 9.6 kb. The HCV genome encodes a single polyprotein precursor of approximately 3000 amino acid residues, which is cleaved by the host and viral proteases into at least 10 proteins in the following order: Core, envelope 1 (E1), E2, p7, nonstructural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B [1–3].

The virological study and screening of antiviral reagents for HCV was difficult until the replicon system was developed [4–7]. In 2005, an infectious HCV production system was developed using genotype 2a HCV JFH-1 and hepatoma-derived HuH-7 cells, and the HCV life cycle was reproduced in a cell culture system [8]. We previously developed genome-length HCV reporter assay systems using HuH-7-derived OR6 cells [4]. In OR6 cells, the genotype 1b HCV-O with renilla luciferase (*RL*) replicates robustly. We also developed an HCV JFH-1 reporter infection assay system [9].

HCV infection frequently causes chronic hepatitis (CH) and leads to serious liver cirrhosis and hepatocellular carcinoma. Therefore, HCV infection is a major health problem worldwide. The elimination of HCV by antiviral reagents seems to be the most efficient therapy for preventing the fatal state of the disease. Pegylated-interferon (PEG-IFN) with ribavirin (RBV) is the current standard therapy for CH–C, but its sustained virological response (SVR) rate has remained 40–50%. Recently, a protease inhibitor, telaprevir, improved the SVR rate by up to 60–70% in combination with PEG-IFN/RBV [10]. The response to PEG-IFN/RBV therapy depends on host factors as well as viral factors. Among the host factors, age and gender are known to be associated with the outcome of IFN/RBV therapy [11,12]. Postmenopausal women with CH–C exhibited a poor response to IFN therapy compared to premenopausal women [11]. The decrease in estrogen may affect the response to IFN therapy. Dyslipidemia and osteoporosis are the typical complications in postmenopausal women. We and other groups reported that statins, which are dyslipidemia reagents, inhibited HCV proliferation in vitro and in vivo [13–17]. Recently it was reported that vitamin D3, an osteoporotic reagent, exhibited anti-HCV activity in vitro and in vivo [18–21]. It was also reported that 17β-estradiol inhibited the production of infectious HCV [22]. Taken together, these reports suggest an association between hepatitis C and complications due to the decrease of estrogen.

Raloxifene and tamoxifen are synthetic selective estrogen receptor modulators (SERMs) and are used for breast cancer and osteoporosis, respectively, in clinical settings. The responses of SERMs are mediated by estrogen receptors (ERs), either ERα or ERβ. SERMs exhibit agonistic actions in some tissues but antagonistic actions in others. Both raloxifene and tamoxifen are antagonists in breast and agonists in bone. However, only tamoxifen, and not raloxifene, exhibited agonistic activity in the uterus. It was reported that tamoxifen inhibited HCV RNA replication [23]. However, tamoxifen's agonist action leads to uterine cancer. Raloxifene belongs to an antiosteoporotic reagent and offers the advantage of safety without uterine cancer. Therefore, we decided to investigate whether or not raloxifene would exhibit anti-HCV activity in our developed cell culture systems.

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2. Materials and methods

2.1. Reagents and antibodies

Raloxifene was purchased from LKT Laboratories, Inc. (St. Paul, MN). IFN-α and tamoxifen were purchased from Sigma–Aldrich (St. Louis, MO). Pitavastatin (PTV) was purchased from Kowa Company (Nagoya, Japan). The antibodies used in this study were those specific to HCV Core (CP11, Institute of Immunology, Tokyo, Japan), NS3 (Novocastra Laboratories, Newcastle, UK), and β-actin (Sigma).

2.2. Cell culture and HCV RNAs

HuH-7 cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Invitrogen Life Technology, Carlsbad, CA) supplemented with 10% fetal bovine serum, penicillin, and streptomycin. HuH-7-derived OR6 and sOR cells were genome-length and subgenome HCV (O strain of genotype 1b) RNA harboring cells, respectively and cultured in the above medium supplemented with G418 (0.3 mg/ml; Geneticin, Invitrogen) [4]. HCVs replicating in OR6 and sOR cells contain *RL* and neomycin phosphotransferase (*NPT*) genes after 5′-untranslated region (UTR). HuH-7-derived RSc cells are cured cells, in which HCV RNA was eliminated by IFN-α; they are used for HCV JFH-1 infection [9]. RSc cells are also used for subgenomic JFH-1 RNA (JRN/35B) replication. JRN/35B contains *RL* and *NPT* genes after 5′-UTR.

2.3. RL assay

For the RL assay, 1.5×10^4 OR6 were plated onto 24-well plates in triplicate and cultured for 24 h. The cells were treated with each reagent for 72 h. Then the cells were harvested with *Renilla* lysis reagent (Promega, Madison, WI) and subjected to RL assay according to the manufacturer's protocol.

2.4. WST-1 cell proliferation assay

The cells (2×10^3 cells) were plated onto a 96-well plate in triplicate at 24 h before treatment with each reagent. At 72 h after treatment, the cells were subjected to a WST-1 cell proliferation assay (Takara Bio, Otsu, Japan) according to the manufacturer's protocol.

2.5. Western blot analysis

For Western blot analysis, 4×10^4 cells were plated onto 6-well plates, cultured for 24 h, and then treated with reagent(s) for 72 h and 120 h. Preparation of the cell lysates, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and immunoblotting were then performed as previously described [24]. Immunocomplexes on the membranes were detected by enhanced chemiluminescence assay (Renaissance; Perkin Elmer Life Science, Wellesley, MA).

2.6. HCV infection

RSc cells (1.5×10^4 cells) were plated onto a 24-well plate 24 h before infection. To evaluate the effect of the treatment prior to infection, the cells were first treated with raloxifene for 24 h, then inoculated with reporter JFH-1 (JR/C5B/BX-2) supernatant at a multiplicity of infection (MOI) of 0.2, cultured for 48 h, and subjected to RL assay as described previously [9]. The JR/C5B/BX-2 contains the *RL* gene in the first cistron following the encephalomyocarditis virus-internal ribosomal entry site (*EMCV-IRES*) gene and the open reading frame (ORF) of JFH-1 in the second cistron. To evaluate the effect of the treatment after infection, the cells were inoculated with reporter JFH-1 supernatant at MOI of 0.2, cultured for 72 h, and subjected to RL assay.

3. Results

3.1. Raloxifene inhibited HCV RNA replication

The HCV RNA that replicated in HuH-7-derived OR6 cells was a genome-length HCV with *RL*, *NPT*, and *EMCV-IRES* in the first cistron and the ORF of HCV (O strain of genotype 1b) in the second cistron [4]. OR6 cells could not produce infectious HCV. Therefore, we can monitor the replication step in the HCV life cycle using OR6 cells. Raloxifene inhibited HCV RNA replication in a dose-dependent manner, and its 50% effective concentration (EC_{50}) was 1 μM (Fig. 1A). Raloxifene did not exhibit cytotoxicity to OR6 cells until 2.5 μM (Fig. 1B). Raloxifene also inhibited intracellular Core and NS3 production in a dose- and time-dependent manner (Fig. 1C). The intensities of Core and NS3 in OR6 cells treated with 2.5 μM of raloxifene decreased to almost the level of cells treated with 10 IU/ml of IFN-α at 120 h after treatment. We also examined anti-HCV activity of raloxifene using subgenomic HCV replicon harboring sOR cells. Raloxifene exhibited weak anti HCV activity to sOR cells as compared with OR6 cells (Supplementary Figs. 1A and 1B). These results suggest that raloxifene exhibits anti-HCV activity and decreased the expression levels of HCV proteins more slowly compared to IFN-α.

3.2. Raloxifene enhanced anti-HCV activity of IFN-α

We investigated the anti-HCV activity of raloxifene in combination with a representative anti-HCV reagent, IFN-α. HCV RNA replication decreased in a dose-dependent manner after co-treatment with IFN-α and raloxifene (Fig. 2A). The results were almost similar to the expected effect of raloxifene in combination with IFN-α calculated from the anti-HCV activity of each reagent (Fig. 2B). These results indicate that the anti-HCV activity of raloxifene and IFN-α exhibited additive effect. We also examined the anti-HCV activity of previously reported SERM, tamoxifen. Tamoxifen also exhibited additive anti-HCV activity on HCV RNA replication in combination with IFN-α (Supplementary Figs. 2A–C). These results indicate that raloxifene as well as tamoxifen enhanced the anti-HCV activity of IFN-α. As both raloxifene and IFN-α are clinically used reagents, raloxifene seemed to be a candidate reagent as an add-on treatment to IFN-α in patients with CH–C.

3.3. Raloxifene antagonized anti-HCV activity of statin

We previously reported that statins exhibited anti-HCV activity using the OR6 assay system [14]. Statin is the first-choice reagent for dyslipidemia. As dyslipidemia and osteoporosis are major complications in postmenopausal women, we invested the effect of raloxifene on the anti-HCV activity of PTV. Raloxifene did not enhance the anti-HCV activity of PTV (Fig. 3A). Fig. 3B exhibits the expected anti-HCV activity of co-treatment with raloxifene and PTV calculated from the anti-HCV effect of either raloxifen or PTV alone. Raloxifene exhibited an antagonistic effect on PTV's anti-HCV activity. Raloxifene's antagonistic effect on PTV increased dose-dependently. The co-treatment with raloxifene (2.5 μM) and PTV (0.25, 0.5, and 1 μM) resulted in lower anti-HCV activity than did treatment with raloxifene alone (2.5 μM). These results suggest that we should be careful in the administration of statins with raloxifene to postmenopausal woman with CH–C.

3.4. Raloxifene inhibited infection of genotype 2a HCV

To further investigate the anti-HCV activity of raloxifene, we examined whether or not raloxifene could inhibit HCV infection. For this purpose, we used our recently developed JFH-1 reporter infection assay system [9]. HuH-7-derived RSc's are highly HCV-permissive cell lines. Raloxifene was pretreated at 24 h before HCV infection. The cells were inoculated with HCV JFH-1 virion with *RL* (JR/C5B/BX-2), and

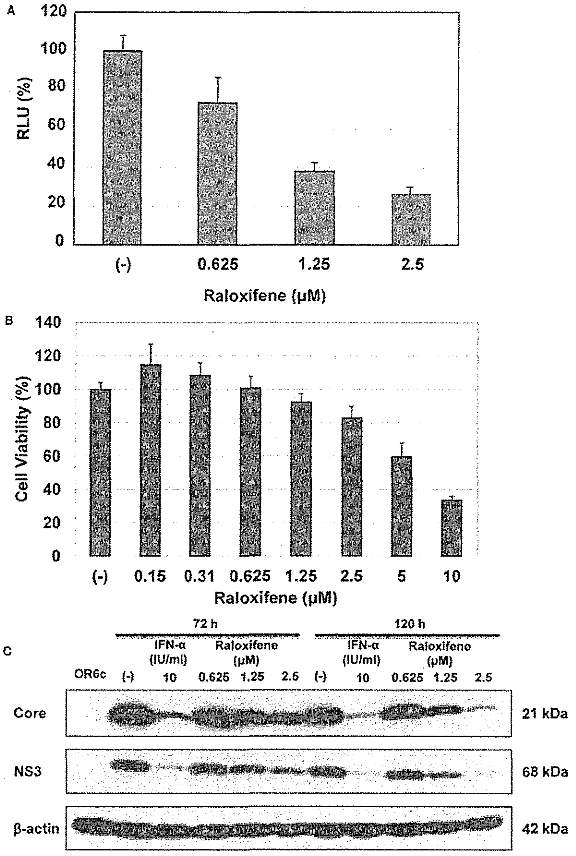

Fig. 1. Raloxifene inhibited HCV RNA replication. (A) Anti-HCV activity of raloxifene in OR6 cells. OR6 cells were treated with raloxifene (0, 0.625, 1.25, and 2.5 μM) for 72 h. Relative RL activity (relative light unit: RLU) for HCV RNA replication is expressed as a percentage of control. Each bar represents the average with standard deviations of triplicate data points. (B) Effect of raloxifene on OR6 cell viability. Cell viability at 72 h after raloxifene treatment (0.15, 0.31, 0.625, 1.25, 2.5, 5, and 10 μM) was determined using WST-1 cell proliferation assay and is expressed as a percentage of control. (C) Raloxifene inhibited HCV proteins. OR6 cells were treated with IFN-α (10 IU/ml) or raloxifene (0, 0.625, 1.25, and 2.5 μM). After 72 or 120 h treatment, the production of Core and that of NS3 were analyzed by immunoblotting using anti-Core and anti-NS3 antibodies, respectively. OR6c cells were cured cells in which HCV RNA was eliminated using IFN-α, and were used as a negative control. β-actin was used as a control for the amount of protein loaded per lane.

the infection was monitored with RL activity at 48 h after infection. As shown in Fig. 4A, raloxifene inhibited HCV infection in RSc cells in a dose-dependent manner. Next we examined the effect of raloxifene after HCV infection. RSc cells were inoculated with HCV JFH-1 virion with RL. After HCV infection, the cells were treated with raloxifene for 72 h and raloxifene's inhibitory effect on post-infection was assessed using the RL assay. Raloxifene inhibited HCV proliferation in a dose-dependent manner when it was added to the cells after infection in RSc cells, although inhibitory effect of raloxifene on JFH-1 HCV RNA replication seemed to be weak compared to the genotype 1b HCV-O RNA replication (Fig. 4B). Raloxifene did not exhibit cytotoxicity to RSc cells until 2.5 μM (Fig. 4C). We found that raloxifene could not inhibit subgenomic JFH-1 HCV (JRN/35B) RNA replication (Fig. 4D). We further examined the inhibitory action of raloxifene around infection step. RSc cells were treated for short time with raloxifene around infection step: for 1, 4, and 4 h before, during, and after inoculation, respectively (Fig. 4E). Raloxifene inhibited JFH-1 infection, when it was treated during inoculation but not just before or after inoculation. In case of genotype 2a JFH-1, raloxifene's anti-HCV activity is mainly due to the inhibition of infection. These results indicate that raloxifene inhibits JFH-1 infection but not its RNA replication.

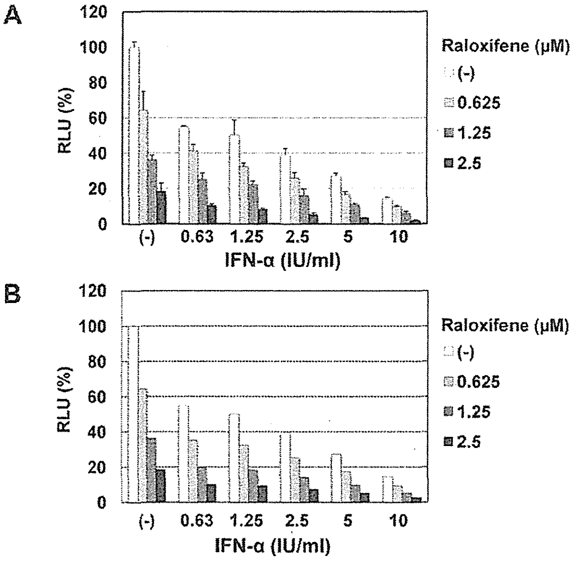

Fig. 2. Raloxifene enhanced the anti-HCV activity of IFN-α. (A) Anti-HCV activity of raloxifene in combination with IFN-α. OR6 cells were co-treated with raloxifene (0, 0.625, 1.25, and 2.5 μM) and IFN-α (0, 0.63, 1.25, 2.5, 5, 10 IU/ml). Relative RL activity is shown as a percentage of control. Each bar represents the average with standard deviations of triplicate data points. (B) Expected anti-HCV activity was calculated based on the results when the cells were treated with only raloxifene or IFN-α.

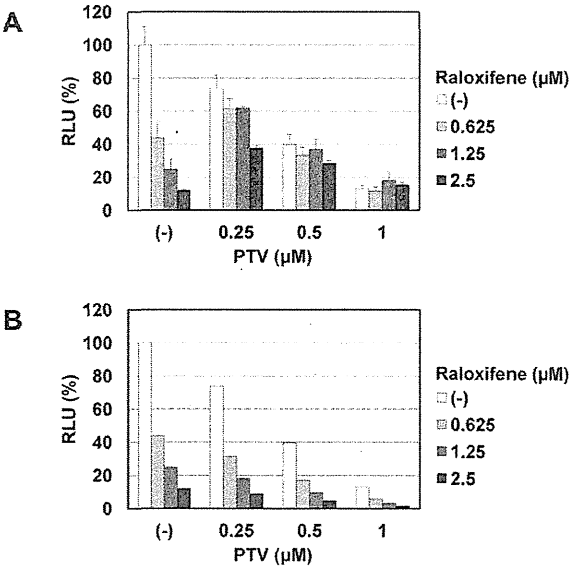

Fig. 3. Statin antagonized the anti-HCV activity of raloxifene. (A) OR6 cells were co-treated with raloxifene (0, 0.625, 1.25, and 2.5 μM) and PTV (0, 0.25, 0.5, and 1 μM). Relative RL activity was shown as a percentage of control. Each bar represents the average with standard deviations of triplicate data points. (B) Expected anti-HCV activity was calculated based on the results when the cells were treated with only raloxifene or PTV.

4. Discussion

In this study, we demonstrated that raloxifene, an osteoporotic reagent, inhibited the replication of genotypes 1b HCV RNA replication and inhibited genotype 2a HCV JFH-1 infection. Raloxifene additively enhanced the anti-HCV activity of IFN-α. On the other hand, raloxifene exhibited an antagonistic effect on statins.

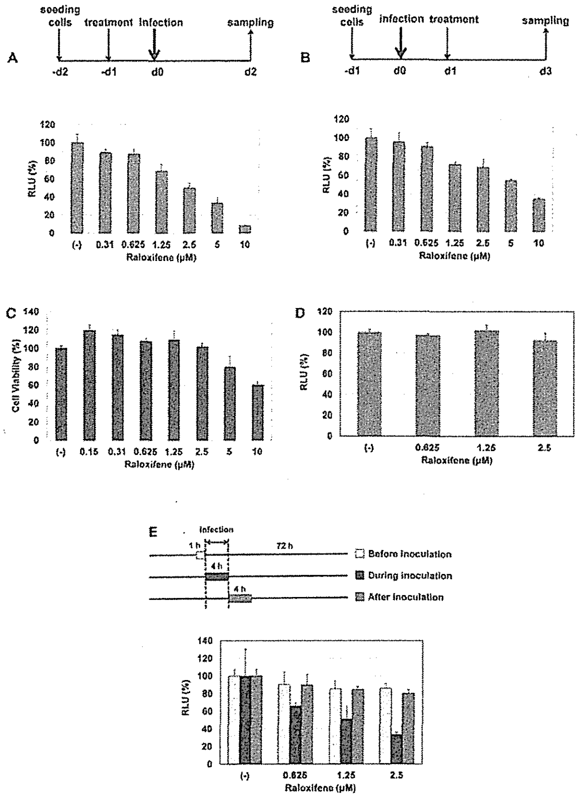

Fig. 4. Raloxifene inhibited genotype 2a HCV infection. (A) Raloxifene inhibited HCV JFH-1 infection. RSc cells were treated with raloxifene (0, 0.31, 0.625, 1.25, 2.5, 5, and 10 μM) 24 h before infection. HCV JFH-1 reporter virion was used as an inoculum after removal of raloxifene. The cells were then infected with reporter JFH-1 virion and cultured for 48 h. The inhibition of HCV infection was assessed by relative RL activity and expressed as a percentage of control. (B) Raloxifene inhibited HCV JFH-1 proliferation after infection. RSc cells were inoculated with HCV JFH-1 reporter virion and cultured for 24 h. Then the cells were treated with raloxifene (0, 0.31, 0.625, 1.25, 2.5, 5, and 10 μM) for 48 h. The inhibitory effect on HCV proliferation after infection was assessed by relative RL activity and expressed as a percentage of control. Each bar represents the average with standard deviations of triplicate data points. (C) Effect of raloxifene on RSc cells viability. Cell viability at 72 h after raloxifene treatment (0.15, 0.31, 0.625, 1.25, 2.5, 5, and 10 μM) was determined using WST-1 cell proliferation assay and is expressed as a percentage of control. (D) Subgenomic JFH-1 RNA (JRN/35B) replicating RSc cells were treated with raloxifene (0, 0.625, 1.25, and 2.5 μM) for 72 h. Relative RL activity for HCV RNA replication is expressed as a percentage of control. Each bar represents the average with standard deviations of triplicate data points. (E) Raloxifene (0, 0.625, 1.25, and 2.5 μM) was treated for 1, 4, and 4 h before, during, and after JFH-1 inoculation to RSc cells at MOI of 0.2, respectively. The cells were then cultured for 72 h. The inhibition of HCV infection was assessed by relative RL activity and expressed as a percentage of control.

PEG-IFN/RBV therapy led to a 40–50% SVR rate among patients with CH–C. Telaprevir with PEG-IFN/RBV increases the effect of PEG-IFN/RBV therapy by 10–20%. However, the major complication of anemia in PEG-IFN/RBV therapy increased when telaprevir was added. Considering that PEG-IFN/RBV-based therapy is less effective on postmenopausal women, an alternative therapy with minimal side effects is needed. Add-on therapy for postmenopausal women may be a candidate for improving the SVR in these patients. We focused on the reagents, which compensate for the lack of estrogen function. Dyslipidemia and osteoporosis are the major complications in postmenopausal women, and these complications are attributable to the decrease in estrogen. Statins are clinically used reagents for dyslipidemia; they inhibit HCV RNA replication in vivo as well as in vitro [13–17]. Therefore, we investigated whether or not raloxifene exhibits anti-HCV activity using genotype 1b HCV RNA replication and genotype 2a infection systems. In the HCV life cycle, raloxifene inhibited genotype 2a HCV infection and genotypes 1b HCV RNA replication. Raloxifene may be a potential reagent with different anti-HCV mechanisms in the HCV life cycle. Further study is needed to clarify these underlying mechanisms.

Recently it was reported that vitamin D3, an osteoporotic reagent, inhibited HCV production in cell culture systems [20,21]. Furthermore, it was reported that vitamin D3 was associated with the effect of therapy for patients with CH–C [18,19]. Statins inhibited HCV RNA replication by suppressing geranylgeranyl pyrophosphate (GGPP) production [14]. Another osteoporotic reagent, bisphosphonate, may possess anti-HCV activity, because it also inhibited the biosynthesis of GGPP in the mevalonate pathway by inhibiting farnesyl pyrophosphate synthetase. Taken together, these findings indicate it is likely that the HCV life cycle is associated with osteoporosis.

Raloxifene and tamoxifen are SERMs for osteoporosis and breast cancer, respectively. Tamoxifen is used for estrogen receptor-positive breast cancer, and it inhibits HCV RNA replication in cell culture [23]. Tamoxifen's anti-HCV activity is associated with ERα. In our study, raloxifene inhibited HCV infection as well as replication. To clarify the multi-potential effects of raloxifene, further study is needed. The incidence of side effects including uterine cancer is lower in raloxifene therapy than in tamoxifen therapy [25]. This is another advantage of raloxifene in clinical use for patients with CH–C.

As for the precise role of ERα or ERβ on the HCV life cycle, we could not reach a clear conclusion because microarray analysis revealed an absence of expression for both ERα and ERβ in OR6 cells (data not shown). Hayashida et al. [22] reported that the most potent physiological estrogen, 17-β-estradiol, inhibited infectious HCV production using HuH-7.5 cells, and that ERα-selective agonist inhibited infectious HCV production whereas ERβ-selective agonist did not. Watashi et al. [23] reported that RNA interference-mediated knockdown of ERα reduced HCV RNA replication. In our study, the anti-HCV activity of raloxifene in infection and replication did not seem attributable to ERα or ERβ. It is not clear why our HuH-7-derived OR6 cells did not express ERα or ERβ. HuH-7 cells were developed in 1982 at Okayama University and distributed worldwide [26]. Recently, Bensadoun et al. [27] reported that the genetic background of the *IL28B* genotype of HuH-7 cells differed among different laboratories. This may be a consequence of the polyploidal nature of hepatoma cells. A similar mechanism might cause the different expression levels of ERα and ERβ. Another ER, GPR30 [28], was expressed in OR6 cells (data not shown; from microarray analysis). GPR30 may be the responsible host factor for anti-HCV activity in OR6 cells. Further study is needed to clarify this issue.

In conclusion, we found that raloxifene inhibited HCV RNA replication in genotype 1b and infection in genotype 2a. Raloxifene additively enhanced the anti-HCV activity of IFN-α. The antagonistic effects of statins and raloxifene will yield information on the clinical use of these reagents. Our results, as well as the reports of vitamin D3's anti-HCV activity, will open new fields of treatment for both osteoporosis and HCV infection.

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Supplementary Material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fob.2012.08.003.

References

[1] Kato N. (2001) Molecular virology of hepatitis C virus. Acta Med. Okayama. 55, 133–159.

[2] Kato N., Hijikata M., Ootsuyama Y., Nakagawa M., Ohkoshi S., Sugimura T. et al. (1990) Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc. Natl. Acad. Sci. USA. 87, 9524–9528.

[3] Tanaka T., Kato N., Cho M.J., Sugiyama K., Shimotohno K. (1996) Structure of the 3' terminus of the hepatitis C virus genome. J. Virol. 70, 3307–3312.

[4] Ikeda M., Abe K., Dansako H., Nakamura T., Naka K., Kato N. (2005) Efficient replication of a full-length hepatitis C virus genome, strain O, in cell culture, and development of a luciferase reporter system. Biochem. Biophys. Res. Commun. 329, 1350–1359.

[5] Ikeda M., Yi M., Li K., Lemon S.M. (2002) Selectable subgenomic and genome-length dicistronic RNAs derived from an infectious molecular clone of the HCV-N strain of hepatitis C virus replicate efficiently in cultured Huh7 cells. J. Virol. 76, 2997–3006.

[6] Lohmann V., Korner F., Koch J., Herian U., Theilmann L., Bartenschlager R. (1999) Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science. 285, 110–113.

[7] Pietschmann T., Lohmann V., Kaul A., Krieger N., Rinck G., Rutter G. et al. (2002) Persistent and transient replication of full-length hepatitis C virus genomes in cell culture. J. Virol. 76, 4008–4021.

[8] Wakita T. (2005) Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. 11, 791–796.

[9] Takeda M., Ikeda M., Ariumi Y., Wakita T., Kato N (2012) Development of hepatitis C virus production reporter assay systems using two different hepatoma cell lines. J. Gen. Virol. 93, 1422–1431.

[10] McHutchison J.G. (2009) Telaprevir with peginterferon and ribavirin for chronic HCV genotype 1 infection. N. Engl. J. Med. 360, 1827–1838.

[11] Hayashi J., Kishihara Y., Ueno K., Yamaji K., Kawakami Y., Furusyo N. et al. (1998) Age-related response to interferon alfa treatment in women vs men with chronic hepatitis C virus infection. Arch. Int. Med. 158, 177–181.

[12] Iwasaki Y. (2006) Limitation of combination therapy of interferon and ribavirin for older patients with chronic hepatitis C. Hepatology. 43, 54–63.

[13] Bader T., Fazili J., Madhoun M., Aston C., Hughes D., Rizvi S. et al. (2008) Fluvastatin inhibits hepatitis C replication in humans. Am. J. Gastroenterol. 103, 1383–1389.

[14] Ikeda M., Abe K., Yamada M., Dansako H., Naka K., Kato N. (2006) Different anti-HCV profiles of statins and their potential for combination therapy with interferon. Hepatology. 44, 117–125.

[15] Ikeda M., Kato N. (2007) Life style-related diseases of the digestive system: cell culture system for the screening of anti-hepatitis C virus (HCV) reagents: suppression of HCV replication by statins and synergistic action with interferon. J. Pharmacol. Sci. 105, 145–150.

[16] Rao G.A., Pandya P.K. (2011) Statin therapy improves sustained virologic response among diabetic patients with chronic hepatitis C. Gastroenterology. 140, 144–152.

[17] Sezaki H. (2009) An open pilot study exploring the efficacy of fluvastatin, pegylated interferon and ribavirin in patients with hepatitis C virus genotype 1b in high viral loads. Intervirology. 52, 43–48.

[18] Abu-Mouch S., Fireman Z., Jarchovsky J., Zeina A.R., Assy N. (2011) Vitamin D supplementation improves sustained virologic response in chronic hepatitis C (genotype 1)-naive patients. World J. Gastroenterol. 17, 5184–5190.

[19] Bitetto D. (2011) Vitamin D supplementation improves response to antiviral treatment for recurrent hepatitis C. Transpl. Int. 24, 43–50.

[20] Gal-Tanamy M., Bachmetov L., Ravid A., Koren R., Erman A., Tur-Kaspa R. et al. (2011) Vitamin D: an innate antiviral agent suppressing hepatitis C virus in human hepatocytes. Hepatology. 54, 1570–1579.

[21] Matsumura, T., Kato, T., Sugiyama, N., Tasaka-Fujita, M., Murayama, A., Masaki, T., Wakita, T., Imawari, M. 25-hydroxyvitamin D(3) suppresses hepatitis C virus production. Hepatology, in press.

[22] Hayashida K., Shoji I., Deng L., Jiang D.P., Ide Y.H., Hotta H. (2010) 17beta-estradiol inhibits the production of infectious particles of hepatitis C virus. Microbiol. Immunol. 54, 684–690.

[23] Watashi K., Inoue D., Hijikata M., Goto K., Aly H.H., Shimotohno K. (2007) Anti-hepatitis C virus activity of tamoxifen reveals the functional association of estrogen receptor with viral RNA polymerase NS5B. J. Biol. Chem. 282, 32765–32772.

[24] Kato N. (2003) Establishment of a hepatitis C virus subgenomic replicon derived from human hepatocytes infected in vitro. Biochem. Biophys. Res. Commun. 306, 756–766.

[25] Runowicz C.D., Costantino J.P., Wickerham D.L., Cecchini R.S., Cronin W.M., Ford L.G. et al. (2011) Gynecologic conditions in participants in the NSABP breast cancer prevention study of tamoxifen and raloxifene (STAR). Am. J. Obstet. Gynecol. 205, 535e1–535e5.

[26] Nakabayashi H., Taketa K., Miyano K., Yamane T., Sato J. (1982) Growth of human hepatoma cells lines with differentiated functions in chemically defined medium. Cancer Res. 42, 3858–3863.

[27] Bensadoun P., Rodriguez C., Soulier A., Higgs M., Chevaliez S., Pawlotsky J.M. (2011) Genetic background of hepatocyte cell lines: are in vitro hepatitis C virus research data reliable. Hepatology. 54, 748.

[28] Revankar C.M., Cimino D.F., Sklar L.A., Arterburn J.B., Prossnitz E.R. (2005) A transmembrane intracellular estrogen receptor mediates rapid cell signaling. Science. 307, 1625–1630.

Virus Research 167 (2012) 74–85

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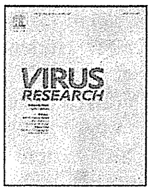

Identification of host genes showing differential expression profiles with cell-based long-term replication of hepatitis C virus RNA

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ABSTRACT

Persistent hepatitis C virus (HCV) infection frequently causes hepatocellular carcinoma. However, the mechanisms of HCV-associated hepatocarcinogenesis and disease progression are unclear. Although the human hepatoma cell line, HuH-7, has been widely used as the only cell culture system for robust HCV replication, we recently developed new human hepatoma Li23 cell line-derived OL, OL8, OL11, and OL14 cells, in which genome-length HCV RNA (O strain of genotype 1b) efficiently replicates. OL, OL8, OL11, and OL14 cells were cultured for more than 2 years. We prepared cured cells from OL8 and OL11 cells by interferon-γ treatment. The cured cells were also cultured for more than 2 years. cDNA microarray and RT-PCR analyses were performed using total RNAs prepared from these cells. We first selected several hundred highly or moderately expressed probes, the expression levels of which were upregulated or downregulated at ratios of more than 2 or less than 0.5 in each set of compared cells (e.g., parent OL8 cells versus OL8 cells cultured for 2 years). From among these probes, we next selected those whose expression levels commonly changed during a 2-year culture of genome-length HCV RNA-replicating cells, but which did not change during a 2-year culture period in cured cells. We further examined the expression levels of the selected candidate genes by RT-PCR analysis using additional specimens from the cells cultured for 3.5 years. Reproducibility of the RT-PCR analysis using specimens from recultured cells was also confirmed. Finally, we identified 5 upregulated genes and 4 downregulated genes, the expression levels of which were irreversibly altered during 3.5-year replication of HCV RNA. These genes may play roles in the optimization of the environment in HCV RNA replication, or may play key roles in the progression of HCV-associated hepatic diseases.

1. Introduction

Hepatitis C virus (HCV) is a causative agent of chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma (HCC) (Choo et al., 1989; Saito et al., 1990; Thomas, 2000). However, the mechanisms of HCV-associated hepatocarcinogenesis and disease progression are still unclear. HCV is an enveloped virus with a positive single-stranded 9.6 kb RNA genome, which encodes a large polyprotein precursor of approximately 3000 amino acid residues. This polyprotein is cleaved by a combination of the host and viral proteases into at least 10 proteins in the following order: Core, envelope 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (Hijikata et al., 1991, 1993; Kato et al., 1990).

The initial development of a cell culture-based replicon system (Lohmann et al., 1999) and a genome-length HCV RNA-replication system (Ikeda et al., 2002) using genotype 1b strains enabled the rapid progression of investigations into the mechanisms underlying HCV replication (Bartenschlager, 2005; Lindenbach and Rice, 2005). Furthermore, these RNA replication systems have been improved such that they have become suitable for the screening of anti-HCV reagents by the introduction of reporter genes such as luciferase (Ikeda et al., 2005; Krieger et al., 2001). Moreover, in 2005, an efficient virus production system using the JFH1 genotype 2a strain was developed using human hepatoma cell line HuH-7-derived cells (Wakita et al., 2005). However, to date, HuH-7-derived cells are used as the only cell culture

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Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; E1, envelope 1; EGF, epidermal growth factor; RT-PCR, reverse transcription-polymerase chain reaction; IFN, interferon; ACSM3, acyl-CoA synthetase medium-chain family member 3; ANGPT1, angiopoietin 1; CDKN2C, cyclin-dependent kinase inhibitor 2C; PLA1A, phospholipase A1 member A; SEL1L3, Sel-1 suppressor of lin-12-like 3; SLC39A4, solute carrier family 39 member 4; TBC1D4, TBC1 domain family member 4; WISP3, WNT1 inducible signaling pathway protein 3; ANXA1, annexin A1; AREG, amphiregulin; BASP1, brain abundant, membrane attached signal protein 1; CIDEC, cell death activator CIDE-3; CPB2, carboxypeptidase B2; HSPA6, heat-shock 70 kDa protein B'; PI3, peptidase inhibitor 3; SLC1A3, solute carrier family 1 member 3; THSD4, thrombospondin type-1 domain-containing protein 4; ICAM-1, intercellular adhesion molecule-1; ALXR, ANXA1 receptor.

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system for robust HCV replication (Bartenschlager and Sparacio, 2007; Lindenbach and Rice, 2005). Most studies of HCV replication or anti-HCV reagents are currently carried out using a HuH-7-derived cell culture system. Therefore, it remains unclear whether or not recent advances obtained from the HuH-7-derived cell culture system reflect the general features of HCV replication or anti-HCV targets. To resolve this issue, we aimed to find a cell line other than HuH-7 that enables robust HCV replication. We recently found a new human hepatoma cell line, Li23, that enables efficient HCV RNA replication and persistent HCV production (Kato et al., 2009b). In that study, we established genome-length HCV RNA replicating cell lines, OL (polyclonal; a mixture of approximately 200 clones), OL8 (monoclonal), OL11 (monoclonal), and OL14 (monoclonal), and characterized them (Kato et al., 2009b). We further developed Li23-derived drug assay systems (ORL8 and ORL11) (Kato et al., 2009b), which are relevant to the HuH-7-derived OR6 assay system (Ikeda et al., 2005). Since we demonstrated that the gene expression profile of Li23 cells was distinct from that of HuH-7 cells (Mori et al., 2010), we expected to find that the host factors required for HCV replication or anti-HCV targets in Li23-derived cells would also be distinct from those in HuH-7-derived cells. Indeed, we found that treatment of the cells with approximately 10 μM (a clinically achievable concentration) of ribavirin, an anti-HCV drug, efficiently inhibited HCV RNA replication in both the Li23-derived ORL8 and ORL11 assay systems, but not in the HuH-7-derived OR6 assay system (Mori et al., 2011). We further demonstrated that more than half of the 26 anti-HCV reagents that have been reported by other groups as anti-HCV candidates using HuH-7-derived assay systems other than OR6 assay system exhibited different anti-HCV activities from those of the previous studies (Ueda et al., 2011). In addition, we observed that the anti-HCV activities evaluated by the OR6 and ORL8 assay systems were also frequently different (Ueda et al., 2011). Furthermore, Li23-derived cells showed epidermal growth factor (EGF)-dependent growth (Kato et al., 2009b)-like immortalized or primary hepatocyte cells (e.g., PH5CH8 (Ikeda et al., 1998)), whereas HuH-7-derived cells can grow in an EGF-independent manner. Our findings, when taken together, suggested that a study using Li23-derived cells might yield unexpected results, since only HuH-7-derived cells are commonly used in a wide range of HCV studies.

Moreover, our findings to date suggested that the long-term replication of HCV RNA may cause irreversible changes in the gene expression profiles of host cells, yielding an environment for facilitative viral replication or progression of a malignant phenotype. To investigate this possibility, we carried out cDNA microarray and/or reverse transcription-polymerase chain reaction (RT-PCR) analyses using Li23-derived cells (OL, OL8, OL11, and OL14) in order to identify host genes for which expression levels were irreversibly altered by the long-term replication of HCV RNA. Here we report the identification of such host genes.

2. Materials and methods

2.1. Cell culture

The Li23 cell line consists of human hepatoma cells from a Japanese male (age 56) was established and characterized in 2009 (Kato et al., 2009b). Li23 cells were maintained in modified culture medium for the PH5CH8 human immortalized hepatocyte cell line (Ikeda et al., 1998), as described previously (Kato et al., 2009b). Genome-length HCV RNA-replicating cells (Li23-derived OL, OL8, OL11, and OL14 cells) were also maintained in the medium for the Li23 cells in the presence of 0.3 mg/mL of G418 (Geneticin, Invitrogen, Carlsbad, CA). Cured cells (OL8c and OL11c cells), from which the HCV RNA had been eliminated by interferon (IFN)-γ treatment (Abe et al., 2007), were cultured in the medium for the Li23 cells. These cells were passaged every 7 days for 3.5 years. In this study, OL, OL8, OL11, OL14, OL8c, and OL11c cells were renamed as OL(0Y), OL8(0Y), OL11(0Y), OL14(0Y), OL8c(0Y), and OL11c(0Y) cells, respectively, to specify the time at which the cells were established. These "0Y" cells of passage number 3 were used in this study. Two-year cultures of OL(0Y), OL8(0Y), OL11(0Y), OL14(0Y), OL8c(0Y), and OL11c(0Y) cells were designated as OL(2Y), OL8(2Y), OL11(2Y), OL14(2Y), OL8c(2Y), and OL11c(2Y) cells, respectively. The 3.5-year cultures of OL8(0Y), OL11(0Y), OL8c(0Y), and OL11c(0Y) cells were designated as OL8(3.5Y), OL11(3.5Y), OL8c(3.5Y), and OL11c(3.5Y) cells, respectively. The cured cells obtained from OL8(2Y) and OL11(2Y) cells by IFN-γ treatment (Abe et al., 2007) were designated as OL8(2Y)c and OL11(2Y)c cells, respectively, and were maintained in the medium for the Li23 cells.

2.2. cDNA microarray analysis

OL(0Y), OL(2Y), OL8(0Y), OL8(2Y), OL11(0Y), OL11(2Y), OL8c(0Y), OL8c(2Y), OL11c(0Y), and OL11c(2Y) cells were cultured in the medium without G418 during a few passages, and then these cells (1×10^6 each) were plated onto 10-cm diameter dishes and cultured for 2 or 3 days. Total RNAs from these cells (approximately 70–80% confluency) were prepared using the RNeasy extraction kit (QIAGEN, Hilden, Germany). As previously described (Kato et al., 2009b; Mori et al., 2010), cDNA microarray analysis was performed by Dragon Genomics Center of Takara Bio. (Otsu, Japan) through an authorized Affymetrix service provider using the GeneChip Human Genome U133 Plus 2.0 Array. Differentially expressed genes were selected by comparing the arrays from the genome-length HCV RNA-replicating cells, and the selected genes were further compared with the arrays from the cured cells (see Fig. 2 for details).

2.3. RT-PCR

We performed RT-PCR in order to detect cellular mRNA as described previously (Dansako et al., 2003). Briefly, total RNA (2 μg) was reverse-transcribed with M-MLV reverse trascriptase (Invitrogen) using an oligo dT primer (Invitrogen) according to the manufacturer's protocol. One-tenth of the synthesized cDNA was used for the PCR. The primers arranged for this study are listed in Table 1.

2.4. Quantitative RT-PCR analysis

The quantitative RT-PCR analysis for HCV RNA was performed using a real-time LightCycler PCR (Roche Diagnostics, Basel, Switzerland) as described previously (Ikeda et al., 2005; Kato et al., 2009b). Quantitative RT-PCR analysis for the mRNAs of the selected genes was also performed using a real-time LightCycler PCR. The primer sets used in this study are listed in Table 1.

2.5. Western blot analysis

The preparation of cell lysates, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and immunoblotting analysis with a PVDF membrane were performed as previously described (Kato et al., 2003). The antibodies used for the O strain in this study were those against Core (CP9, CP11, and CP14 monoclonal antibodies [Institute of Immunology, Tokyo, Japan]; a polyclonal antibody [a generous gift from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science]), E1 and NS5B (a generous gift from Dr. M. Kohara), and NS3 (Novocastra Laboratories, Newcastle upon Tyne, UK). β-Actin antibody (Sigma, St. Louis, MO)

Table 1
Primers used for RT-PCR analysis.

Gene (accession no.)	Direction	Nucleotide sequence (5′–3′)	Products (bp)	Gene (accession no.)	Direction	Nucleotide sequence (5′–3′)	Products (bp)
Acyl-CoA synthetase medium-chain family member 3 (ACSM3; NM_005622)	Forward Reverse	GCATTCAAGTTCTACCCAACCGAC GGCTGCTGACAACAGCTGACTC	258	Brain abundant, membrane attached signal protein 1 (BASP1; NM_006317)	Forward Reverse	GGATGAATGCCAGCTTTCAGACAG ACTGGAACTGCAATGAACGCAGAC	247
Angiopoietin 1 (ANGPT1; NM_001146)	Forward Reverse	ATACAACATCGTGAAGATGGAAGTC CCGTGTAAGATCAGGCTGCTCTG	287	Cell death activator CIDE-3 (CIDEC; NM_022094)	Forward Reverse	GATCTGTACAAGCTGAACCCACAG GACAGGTCGGGATAAGGGATGAG	265
Cyclin-dependent kinase inhibitor 2C (CDKN2C; NM_001262)	Forward Reverse	AAGACCGAACTGGTTTCGCTGTC CATAGAGCCTGGCCAAATCACAG	246	Carboxypeptidase B2 (CPB2; NM_001872)	Forward Reverse	GGAACTGTCTCTAGTAGCCAGTG CAGCGGCAAAAGCTTCTCTACAG	242
Phospholipase A1 member A (PLA1A; NM_015900)	Forward Reverse	GGAGTTTCACTTGAAGGAACTGAG GTTCACTGGTTCAGGTAAGCAGAC	292	Heat shock 70 kDa protein B' (HSPA6; NM_002155)	Forward Reverse	TGAAGCCGAGCAGTACAAGGCTG CTCCCTCTTCTGATGCTCATACTC	235
Sel-1 suppressor of lin-12-like 3 (SEL1L3; NM_015187)	Forward Reverse	ACCTGCACTTGCGGCTTCTCTG AGAGGCATCTGCAGCTGGAGTC	212	Peptidase inhibitor 3 (PI3; NM_002638)	Forward Reverse	GGTTCTAGAGGCAGCTGTCACG CCGCAAGAGCCTTCACAGCAC	276[a]
Solute carrier family 39 member 4 (SLC39A4; NM_017767)	Forward Reverse	GCCTGTTCCTCTACGTAGCACTC GAAGGTGATGTCATCCTCGTACAG	158	Peptidase inhibitor 3 (PI3; NM_002638)	Forward Reverse	GGTTCTAGAGGCAGCTGTCACG GCAGTCAGTATCTTTCAAGCAGC	241[b]
TBC1 domain family, member 4 (TBC1D4; NM_014832)	Forward Reverse	GGAGAGGGCCAATAGCCAACTG AGCTTCCGGAGTTGCTCCACTG	198	Solute carrier family member 3 (SLC1A3; NM_004172)	Forward Reverse	CAATGGCGTGGACAAGCGCGTC CCGACAGATGTCAGCACAATGAC	240
WNT1 inducible signaling pathway protein 3 (WISP3; NM_003880)	Forward Reverse	AGAGATGCTGTATCCCTAATAAGTC CAGGTTCTCTGCAGTTTCTCTGAC	129	Thrombospondin type-1 domain-containing protein 4 (THSD4; NM_024817)	Forward Reverse	TGGAGTCAGTGTTCCATCGAGTG GGGTCACAGAGGTTACTTAGAGTC	275
Annexin A1 (ANXA1; NM_000700)	Forward Reverse	GACTTGGCTGATTCAGATGCCAG AATGTCACCTTTCAACTCCAGGTC	192	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; NM_002046)	Forward Reverse	GACTCATGACCACAGTCCATGC GAGGAGACCACCTGGTGCTCAG	334
Amphiregulin (AREG;.NM_001657)	Forward Reverse	CGGGAGCCGACTATGACTACTC AAGGCAGCTATGGCTGCTAATGC	391				

[a] This primer set was used for RT-PCR analysis.

[b] This primer set was used for quantitative RT-PCR analysis.

was used as the control for the amount of protein loaded per lane. Immunocomplexes were detected by the Renaissance enhanced chemiluminescence assay (PerkinElmer Life Sciences, Boston, MA).

2.6. Statistical analysis

Statistical comparison of the mRNA levels between the various time points was performed using Student's *t*-test. *P* values of less than 0.05 were considered statistically significant.

3. Results

3.1. Efficient replication of genome-length HCV RNA is maintained in long-term cell culture

To prepare specimens for the cDNA microarray analysis, genome-length HCV RNA-replicating OL(0Y), OL8(0Y), OL11(0Y), and OL14(0Y) cells were cultured for 2 years, and were designated as OL(2Y), OL8(2Y), OL11(2Y), and OL14(2Y) cells, respectively. OL8c(0Y) and OL11c(0Y) cells were also cultured for 2 years, and were designated as OL8c(2Y) and OL11c(2Y) cells, respectively. We observed that the growth rates of all cell lines increased in a time-dependent manner, while the appreciable changes of cell shapes were not observed. The doubling time of genome-length HCV RNA-replicating cells (OL(0Y), OL8(0Y), OL11(0Y), and OL14(0Y)) and cured cells (OL8c(0Y) and OL11c(0Y)) was approximately 41 h and 32 h, respectively. After 2-year culture, these values reduced to approximately 28 h and 23 h.

Using the total RNA specimens obtained from genome-length HCV RNA-replicating cells, the levels of genome-length HCV RNAs were examined by quantitative RT-PCR analysis. The results revealed that the levels of the genome-length HCV RNAs had increased in all cases after a 2-year period of HCV RNA replication (Fig. 1). The levels of HCV proteins (Core, E1, NS3, and NS5B) were also examined by Western blot analysis. The E1, NS3, and NS5B were detected in all specimens, except for the Li23 cells, although a little larger size of E1 was additionally detected in the specimens from 2-year culture (Fig. 1). This phenomenon may indicate the appearance of additional N-glycosylation sites by mutations caused during the 2-year replication of the HCV RNA, as observed in a previous report (Mori et al., 2008). However, genetic analysis of HCV RNAs from 2-year culture of OL8, OL11, and OL14 cell series has detected no additional N-glycosylation sites by mutations (Kato et al., unpublished results). Therefore, the mobility change of E1 may be due to the other modifications such as O-glycosylation. In addition, Core was not detected in the cultures of OL11(2Y) cells, even when polyclonal anti-Core antibody was used (Fig. 1). A similar phenomenon was observed in a previous study using HuH-7-derived genome-length HCV RNA-replicating cells (Kato et al., 2009a). In that study, we showed that the Core region was not deleted, but mutated at several positions within the epitopes of the anti-Core antibody (Kato et al., 2009a). The results of genetic analysis using Li23-derived cells as described above (Kato et al., unpublished results) were also similar with those in the previous study using HuH-7-derived cells (Kato et al., 2009a).

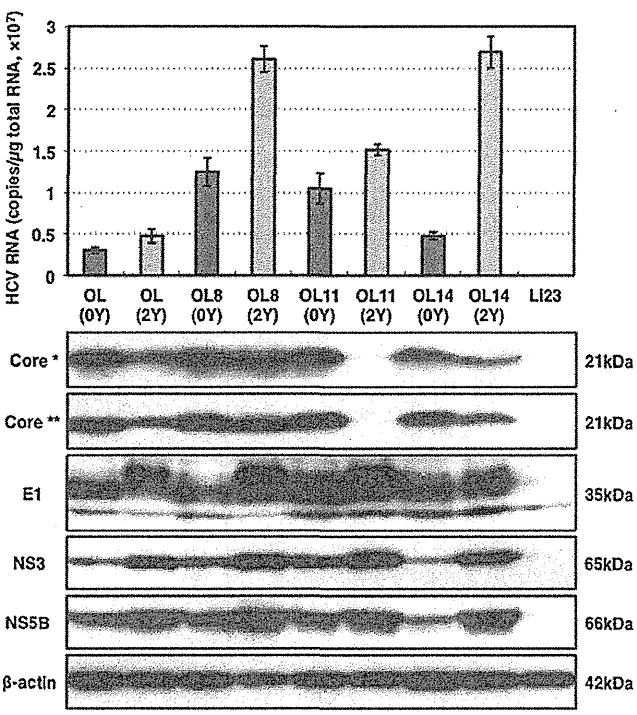

Fig. 1. Characterization of genome-length HCV RNA-replicating cells in long-term cell culture. The upper panel shows the results of a quantitative RT-PCR analysis of intracellular genome-length HCV RNA. Total RNAs from OL(0Y), OL8(0Y), OL11(0Y), and OL14(0Y) cells after 2 years [OL(2Y), OL8(2Y), OL11(2Y), and OL14(2Y)] in culture, as well as total RNAs from the parental OL(0Y), OL8(0Y), OL11(0Y), and OL14(0Y) cells were used for the analysis. Total RNA from Li23 cells was used as a negative control. The lower panel shows the results of the Western blot analysis. Cellular lysates from cells used for quantitative RT-PCR were also used for comparison. HCV Core, E1, NS3, and NS5B were detected by Western blot analysis. β-Actin was used as a control for the amount of protein loaded per lane. A single asterisk indicates that the anti-Core polyclonal antibody was used for detection. A double asterisk indicates that a mixture of three kinds (CP9, CP11, and CP14) of anti-Core monoclonal antibodies was used for detection.

3.2. Selection of genes showing irreversible changes with long-term HCV RNA replication

To identify those genes whose expression levels were irreversibly altered by the long-term replication of HCV RNA, we performed a combination of cDNA microarray and RT-PCR analyses using several Li23-derived cell lines. An outline of the selection process performed in this study is provided in Fig. 2. The first microarray analysis I was carried out by the comparison of OL(0Y) cells versus OL(2Y) cells, OL8(0Y) cells versus OL8(2Y) cells, and OL11(0Y) cells versus OL11(2Y) cells. In this step, we selected those genes whose expression levels commonly showed changes in at least two of three comparative analyses to avoid the bias caused by the difference of cell clonality, since OL(0Y) was a polyclonal cell line, while OL8(0Y) and OL11(0Y) were monoclonal cell lines (Kato et al., 2009b). As regards the selected genes, a microarray analysis II was performed in which OL8c(0Y) cells were compared to OL8c(2Y) cells, and OL11c(0Y) cells were compared to OL11c(2Y) cells. In this step, the genes were excluded from those selected by the microarray analysis I if their expression levels had changed during the 2-year culture of cured cells. As regards the selected genes, we next performed a RT-PCR analysis I to examine the reproducibility of changes in gene expression levels. In this step, we added the results of a new comparative series, OL14(0Y) versus OL14(2Y), to arrive at the judgment to advance to the next step of analysis. We selected genes for which expression levels had changed in more than five of six comparative series (Fig. 2). At the last step, we confirmed by RT-PCR analysis II whether or not the expression levels of the selected genes in OL8(2Y) or OL11(2Y) cells had changed by HCV RNA replication. When the gene expression levels had not changed in two comparative series (OL8(2Y) versus OL8(2Y)c and OL11(2Y) versus OL11(2Y)c), the genes were selected as the candidates exhibiting irreversible changes after 2-year HCV RNA replication.

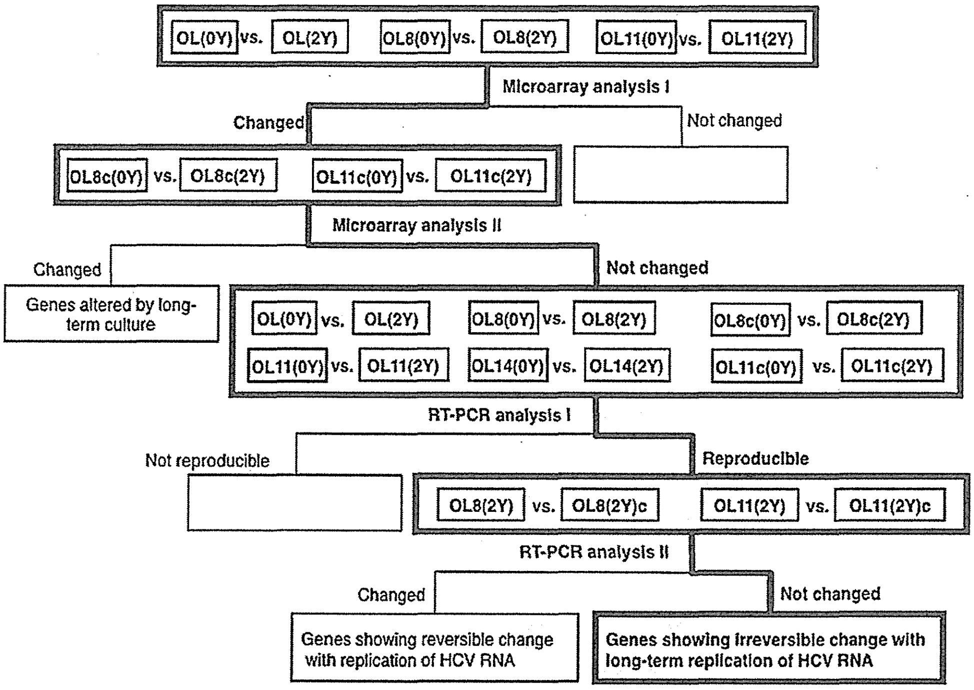

Fig. 2. Outline of selection process performed in this study. To obtain the objective genes, cDNA microarray analyses I and II were performed, and then RT-PCR analyses I and II were also performed.

3.3. Selection and expression profiles of genes showing upregulated expression during long-term HCV RNA replication

The process outlined in Fig. 2 was used to identify those genes that exhibited irreversibly upregulated expression during the 2-year replication of HCV RNA. Microarray analysis I revealed 1912, 1148, and 1633 probes, the expression levels of which were upregulated at a ratio of more than 2 in the case of OL(0Y) cells versus OL(2Y) cells, OL8(0Y) cells versus OL8(2Y) cells, and OL11(0Y) cells versus OL11(2Y) cells, respectively. To avoid the possibility that the genes showing low expression level are selected, the ratios and expression values were used in combination for the selection. As the minimum expression level, more than 100 (actual value of measurement), which was detectable within 30 cycles in RT-PCR analysis, was adopted. From among these probes, we selected those showing ratios of more than 4 with an expression level of more than 100, or those showing ratios of more than 3 with an expression level of more than 200, or those showing an expression level of 1000. By this selection process, 559, 237, and 368 genes (redundant probes excluded) were assigned in the case of OL(0Y) cells versus OL(2Y) cells, OL8(0Y) cells versus OL8(2Y) cells, and OL11(0Y) cells versus OL11(2Y) cells, respectively (Fig. 3A). At this step, we obtained 51 genes as candidates exhibiting upregulation in more than two of three comparisons. Based on the results of the subsequent microarray analysis II, we further selected 14 genes from a total of 51 genes, because the expression levels of the remaining 37 genes increased during the 2-year culture of cured cells (Fig. 3B). The list of these genes was shown in Supplemental Table 1. As regards the 14 selected genes, we performed an RT-PCR analysis I to confirm the results obtained by the cDNA microarray analysis and to examine the status of gene expression in an additional comparison of OL14(0Y) cells versus OL14(2Y) cells. This analysis revealed that the mRNA levels of 6 of 14 genes showed no enhancement in two of four comparative series (data not shown). Therefore, in this step, these 6 genes were excluded from the candidate genes. However, the mRNA levels of the remaining 8 genes (acyl-CoA synthetase medium-chain family member 3 [*ACSM3*], angiopoietin 1 [*ANGPT1*], cyclin-dependent kinase inhibitor 2C [*CDKN2C*], phospholipase A1 member A [*PLA1A*], Sel-1 suppressor of lin-12-like 3 [*SEL1L3*], solute carrier family 39 member 4 [*SLC39A4*], TBC1 domain family member 4 [*TBC1D4*], and WNT1 inducible signaling pathway protein 3 [*WISP3*]) were enhanced in more than three of four comparative series (Fig. 3C). Furthermore, we demonstrated by RT-PCR analysis II that the expression levels of these 8 genes did not return to initial levels, even after elimination of HCV RNA from OL8(2Y) or OL11(2Y) cells (Fig. 3C). It was noteworthy that the mRNA levels of the *ANGPT1* and *PLA1A* genes were enhanced in all comparative series (Fig. 3C).

3.4. Selection and expression profiles of genes showing downregulated expression during long-term HCV RNA replication

To obtain genes showing irreversibly downregulated expression during the 2-year HCV RNA replication period, we performed a selection of genes according to the methods described for the selection of upregulated genes. The first microarray analysis I in this series revealed 1901, 2128, and 1579 probes whose expression levels were downregulated at a ratio of less than 0.5 in the case of OL(0Y) cells versus OL(2Y) cells, OL8(0Y) cells versus OL8(2Y) cells, and OL11(0Y) cells versus OL11(2Y) cells, respectively. As described in Section 3.3, the ratios and expression values were used in combination for the selection. From among these probes, we selected those showing ratios of less than 0.25 with an initial expression level of more than 1000 (actual value of measurement), or those showing ratios of less than 0.33 with an initial expression level of more than 200, or those showing an initial expression level of 100. By this selection process, 828, 622, and 466 genes (redundant probes excluded) were assigned in the case of OL(0Y) cells versus OL(2Y) cells, OL8(0Y) cells versus OL8(2Y) cells, and OL11(0Y) cells versus OL11(2Y) cells, respectively (Fig. 4A). At this step, we obtained 236 genes as candidates showing downregulation in more than two of three comparisons. Based on the results

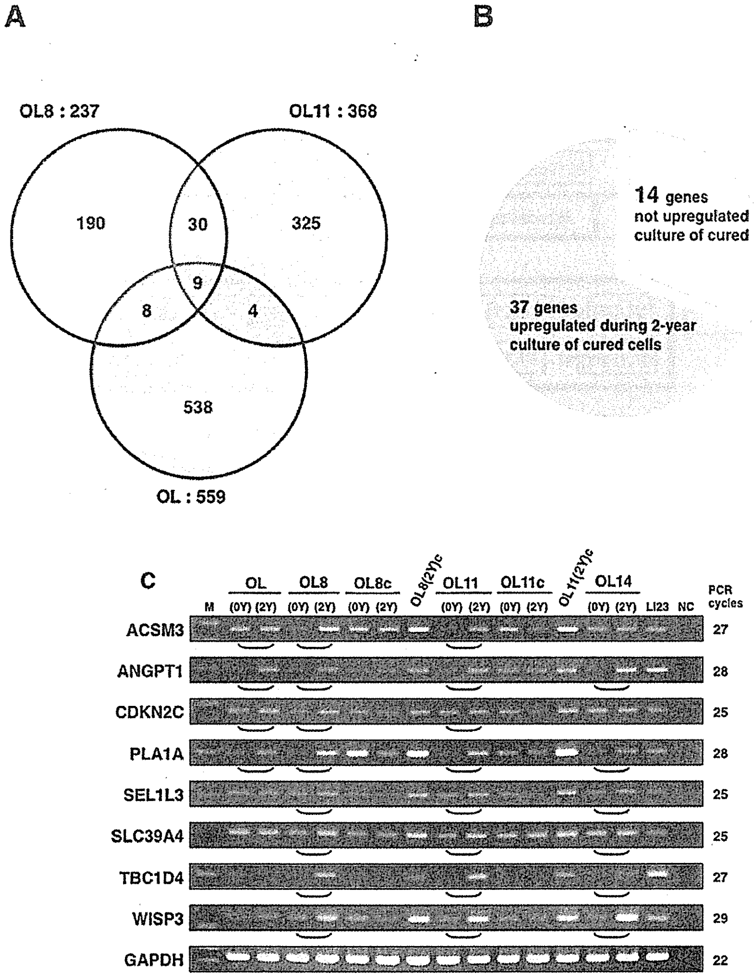

Fig. 3. Identification of genes irreversibly upregulated during 2-year replication of HCV RNA. (A) Upregulated genes obtained by microarray analysis I shown in Fig. 2. Genes whose expression levels were upregulated at ratios of more than 2 in the case of OL(0Y) versus OL(2Y) cells, OL8(0Y) versus OL8(2Y) cells, or OL11(0Y) versus OL11(2Y) cells were selected, and 51 genes upregulated in at least two of three comparisons were obtained. (B) Further selection by microarray analysis II, shown in Fig. 2. Genes whose expression levels were upregulated during 2-year culture (OL8c(2Y) or OL11c(2Y) cells) of the cured OL8c(0Y) or OL11c(0Y) cells were eliminated. (C) Expression profiles of upregulated genes. RT-PCR analyses I and II shown in Fig. 2 were performed as described in Section 2. PCR products were detected by staining with ethidium bromide after separation by electrophoresis on 3% agarose gels. The round parenthesis indicates the comparative series showing the upregulated expression.

of the second microarray analysis II, we were able to select 17 genes from a total of 236 genes, as the expression levels of most of the genes had decreased during the 2-year culture of cured cells (Fig. 4B). The list of these genes was shown in Supplemental Table 2. As regards the 17 selected genes, we performed an initial RT-PCR analysis I to confirm the results obtained by the microarray analysis I and to examine the status of gene expression by additional comparison of OL14(0Y) cells versus OL14(2Y) cells. This analysis revealed that the mRNA levels of 8 of 17 genes showed no suppression in more than two of four comparative series (data not shown). Therefore, these 8 genes were excluded from the candidate genes in this step. However, the mRNA levels of the remaining 9 genes (annexin A1 [*ANXA1*], amphiregulin [*AREG*], brain abundant, membrane attached signal protein 1 [*BASP1*], cell death activator CIDE-3 [*CIDEC*], carboxypeptidase B2 [*CPB2*], heat-shock 70 kDa protein B' [*HSPA6*], peptidase inhibitor 3 [*PI3*], solute carrier family 1 member 3 [*SLC1A3*], and thrombospondin type-1 domain-containing protein 4 [*THSD4*]) were suppressed in more than three of four comparative series (Fig. 4C). Furthermore, we demonstrated by RT-PCR analysis II that the expression levels of these 9 genes did not return to initial levels, even after the elimination of HCV RNA from OL8(2Y) or OL11(2Y) cells (Fig. 4C). It is noteworthy that the mRNA levels of *BASP1*, *CIDEC*, *HSPA6*, and *PI3* genes were suppressed in all comparative series (Fig. 4C).

3.5. Expression profiles of selected genes during 3.5-year replication of HCV RNA

As described above, we selected 8 upregulated genes and 9 downregulated genes, the expression levels of which had irreversibly changed after a 2-year period of HCV RNA replication. However, reproducibility of the RT-PCR analysis using total RNA specimens prepared from independent recultured cells would be needed or arriving at a reliable conclusion. Furthermore, in this context, it would also be important to clarify whether or not these irreversible changes in RNA expression levels remained stable or were further enhanced during HCV RNA replication if the cells were cultured for a period of more than 2 years. Since the OL8(2Y), OL8c(2Y), OL11(2Y), and OL11c(2Y) cells were continuously cultured for a period of up to 3.5 years, they were used as OL8(3.5Y), OL8c(3.5Y), OL11(3.5Y), and OL11c(3.5Y) cells with the recultured OL8(0Y), OL8(2Y), OL8c(0Y), OL8c(2Y), OL11(0Y),