2004). Although most of the above mechanisms were proposed based on studies using HuH-7 (human hepatoma cell line)-derived cells, which are currently used as the only cell culture system for robust HCV replication, the effective concentrations (50–1000 μM) of RBV were much higher than the clinically achievable concentrations (Feld and Hoofnagle, 2005; Feld et al., 2010; Lau et al., 2002; Pawlotsky et al., 2004; Thomas et al., 2011; Zhou et al., 2003). Indeed, our HuH-7-derived cell assay system (OR6) (Ikeda et al., 2005; Naka et al., 2005), in which genome-length HCV RNA (O strain of genotype 1b) encoding renilla luciferase (RL) efficiently replicates, also showed that the 50% effective concentration (EC_{50}) of RBV was approximately 100 μM (Naka et al., 2005).

Recently, we found a new human hepatoma cell line, Li23, that enables robust HCV RNA replication (Kato et al., 2009). We showed by microarray analysis that Li23 cells possessed expression profiles rather different from those in HuH-7 cells (Kato et al., 2009), and that the expression profile of Li23 cells was distinct from those of frequently used other hepatoma cell lines (Mori et al., 2010). We further developed Li23-derived cell culture assay systems (ORL8 and ORL11) in which genome-length HCV RNA (O strain of genotype 1b) encoding RL efficiently replicates (Kato et al., 2009). Here, we unexpectedly observed through the use of these cell culture assay systems the first evidence of anti-HCV activity of RBV at clinically achievable concentrations, and obtained the convincing data that the anti-HCV mechanism of RBV is mediated through the inhibition of IMPDH.

2. Materials and methods

2.1. Cell cultures

HuH-7-derived cells harboring an HCV replicon or genome-length HCV RNA were maintained with medium containing G418 (0.3 mg/ml) as described previously (Ikeda et al., 2005). Li23-derived polyclonal sORL8 and sORL11 cells harboring an HCV replicon were established by the transfection of ORN/3-5B/QR,KE,SR RNA (Kato et al., 2009) into the cured OL8 and OL11 cells, respectively. Li23-derived cells harboring an HCV replicon or genome-length HCV RNA were maintained as described previously (Kato et al., 2009). Cured cells, from which the HCV RNA had been eliminated by IFN treatment, were also maintained as described previously (Kato et al., 2009).

2.2. RL assay

RL assay was performed as described previously (Ikeda et al., 2005; Kato et al., 2009). The experiments were performed at least in triplicate.

2.3. Reagents

RBV was kindly provided by Yamasa (Chiba, Japan). Human IFN-α, vitamin E (VE), phloridzin dihydrate, *S*-(4-nitrobenzyl)-6-thioinosine (NBMPR), and mycophenolic acid (MPA) were purchased from Sigma–Aldrich (St. Louis, MO). Cyclosporine A (CsA) was purchased from Calbiochem (San Diego, CA). Guanosine and adenosine were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.4. Cell viability

Cell viability was examined by the method described previously (Kato et al., 2009). The experiments were performed in triplicate.

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 in this study were those against Core (CP11; Institute of Immunology, Tokyo, Japan), NS5B (a generous gift from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science), signal transduction and activator of transcription 1 (STAT1) and phospho-STAT1 (Tyr701) (BD Transduction Laboratories, Lexington, KY) and equilibrative nucleoside transporter 1 (ENT1) (Abgent, San Diego, CA). β-actin antibody (Sigma–Aldrich) was used as the control for the amount of protein loaded per lane. Immunocomplexes were detected by using a Renaissance enhanced chemiluminescence assay (Perkin Elmer Life Sciences, Boston, MA).

2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from the cultured cells was extracted with an RNeasy Mini Kit (Qiagen, Valencia, CA). RT-PCR was performed by a method described previously (Dansako et al., 2003) using the following primer pairs: IFN-stimulated gene (ISG) 15 (346 bp), 5′-GCCTTCCAGCAGCGTCTGGC-3′ and 5′-GCAGGCGCAGATTCATGAACACGG-3′; IFN regulatory factor 7 (IRF7) (221 bp), 5′-AGCTGCGCTACACGGAGGAACTG-3′ and 5′-CCACCAGCTCTTGGAAGAAGAC-3′; IFN-gamma-inducible protein-10 (IP-10) (111 bp), 5′-GGCCATCAAGAATTTACTGAAAGCA-3′ and 5′-TCTGTGTGGTCCATCCTTGGAA-3′; ENT1 (382 bp), 5′-GAGTTTCA-GTCTCCAACTCTCAG-3′ and 5′-GCATCGTGCTCGAAGACCACAG-3′; ENT2 (306 bp), 5′-CTTGTGTTGGTCTTCACAGTCAC-3′ and 5′-GGTGATGAAGTAGGCATCCTGTG-3′; ENT3 (350 bp), 5′-GTCTTCTTCATCACCAGCCTCATC-3′ and 5′-GTGCTGAGGTAGCCGT-TGCTGAG-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (334 bp), 5′-GACTCATGACCACAGTCCATGC-3′ and 5′-GAGGAGACCACCTGGTGCTCAG-3′.

2.7. Infection of cells with secreted HCV

The inoculum was prepared from HCV-JFH1 (Wakita et al., 2005)-infected HuH-7-derived RSc cells (Ariumi et al., 2007; Kato et al., 2009) at 5 days postinfection and then stored at −80 °C after filtering through a 0.20-μm filter (Kurabo, Osaka, Japan) until use. ORL8c or RSc cells (each 5×10^4) were cultured for 24 h before infection. The cells were infected with 50 μl (equivalent to a multiplicity of infection of 0.05–0.1) of inoculum and maintained for several days until RBV treatment.

2.8. Quasispecies analysis of HCV RNA

ORL8 cells were treated with or without RBV (50 μM) for 72 h. Total RNA from the cultured cells was extracted with an RNeasy Mini Kit (Qiagen). To amplify genome-length HCV RNA, RT-PCR was performed separately in two fragments using KOD-plus DNA polymerase (Toyobo) as described previously (Ikeda et al., 2005; Kato et al., 2003). The two PCR products (the 6.0 kb region covering 5′-untranslated region (5′-UTR) to NS3 and the 6.1 kb region covering NS2 to NS5B) were subcloned into the *Xba*I site of pBR322MC, and sequence analysis of the region encoding RL to the neomycin-resistance gene (Neo^R) (1953 nts), NS5A (1341 nts), or NS5B (1773 nts) was performed as described previously (Ikeda et al., 2005). Synonymous and nonsynonymous substitutions at variance with the parental ORN/C-5B/QR,KE,SR sequences (Kato et al., 2009) were determined. To examine the error frequency of KOD-plus DNA polymerase in the PCR amplification, PCR using the plasmid containing ORN/C-5B/QR,KE,SR sequences was performed separately in the

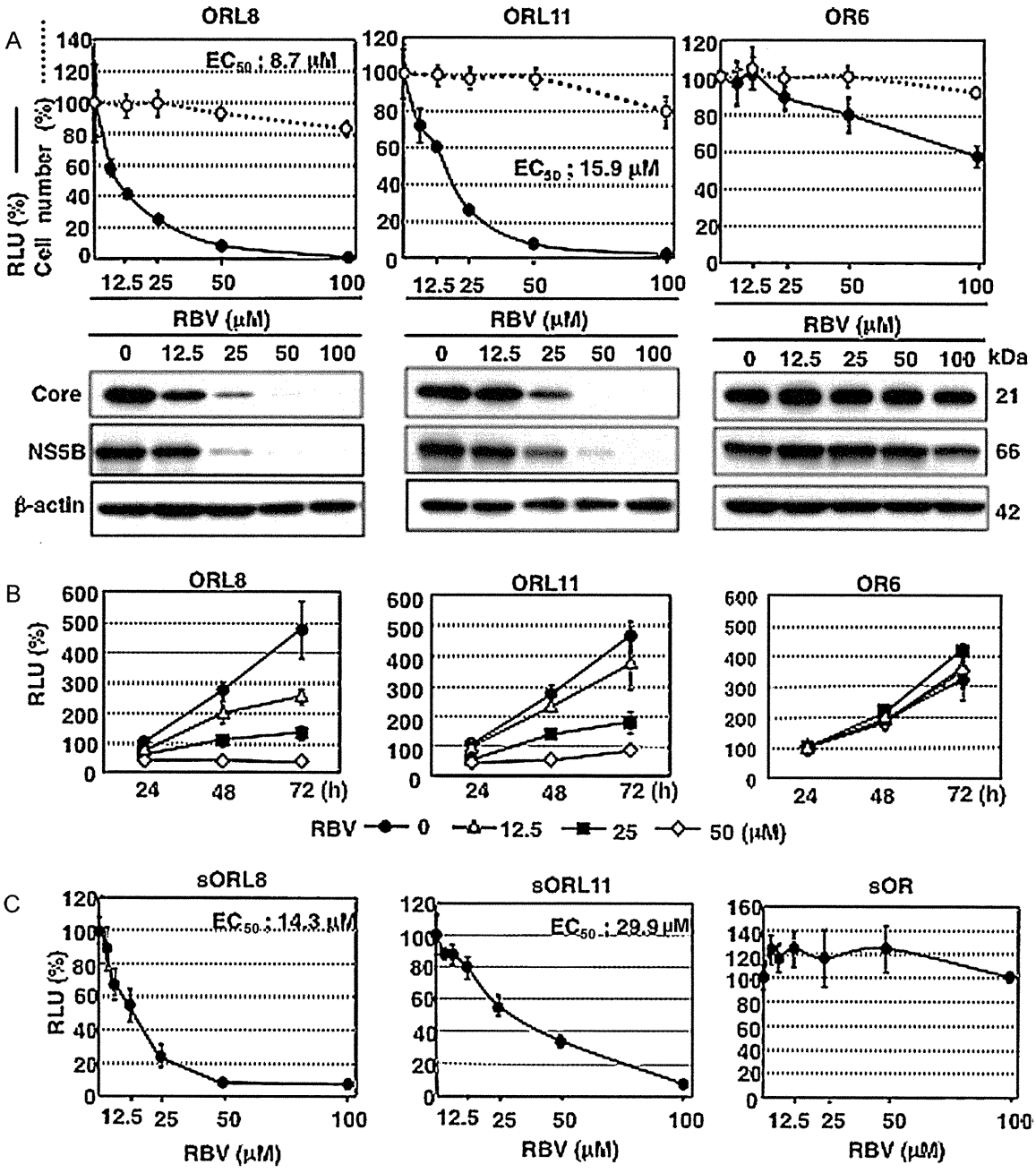

Fig. 1. Anti-HCV activity of RBV detected in the ORL8 and ORL11 system. (A) RBV sensitivities on genome-length HCV RNA replication in ORL8, ORL11, and OR6. The ORL8, ORL11 and OR6 cells were treated with RBV for 72 h, and then an RL assay (bold line in the upper panel) was performed. The relative luciferase activity (RLU) (%) calculated at each point, when the level of luciferase activity in non-treated cells was assigned to be 100% is presented here. The cell number (dotted line in the upper panel) at each concentration was determined as described in Section 2. Western blot analysis of RBV-treated ORL8, ORL11, and OR6 cells for HCV proteins, Core and NS5B, was also performed (lower panel). (B) Time-dependent anti-HCV activity of RBV. The ORL8, ORL11, and OR6 cells were treated with RBV, and an RL assay was performed at 24, 48, and 72 h after the treatment. The RLU (%) calculated at each time point, when the luciferase activity of non-treated cells at 24 h was assigned to be 100%, is shown. (C) Anti-HCV activity of RBV was observed in Li23-derived replicon assay systems (sORL8 and sORL11), but not in HuH-7-derived replicon assay system (sOR). RBV treatment and RL assay were performed as described for panel A.

two parts (6.0 kb covering 5′-UTR to NS3 and 6.1 kb covering NS3 to NS5B) and the two PCR products were subcloned for the sequence analysis as described above.

2.9. RNA interference and quantitative RT-PCR

SiRNA duplexes targeting the coding regions of human IMPDH1 (Dhamacon; catalog no. M-009687-01) and IMPDH2 (Dharmacon; catalog no. M-004330-02) were chemically synthesized. SiRNA duplex non-targeting (Dharmacon; catalog no. D-001206-13) was also used as a control. ORL8 cells were transfected with the indicated siRNA duplexes using Oligofectamine (Invitrogen) (Dansako et al., 2007). Extraction of total RNA and quantitative RT-PCR analysis for HCV RNA were performed by real-time LightCycler PCR as described previously (Ikeda et al., 2005).

2.10. Statistical analysis

Statistical comparison of the luciferase activities between the treatment groups and controls was performed using the Student's

t-test. *P* values of less than 0.05 were considered statistically significant.

3. Results

3.1. Anti-HCV activity of RBV was clearly observed in the Li23-derived assay systems, but not in the HuH-7-derived assay system

Recently we demonstrated that Li23-derived assay systems (ORL8 and ORL11), in which genome-length HCV RNA (O strain of genotype 1b) encoding RL robustly replicates, were frequently more sensitive to anti-HCV reagents such as IFNs and statins than the corresponding HuH-7-derived assay system (OR6) (Kato et al., 2009). Since we had observed a marginal anti-HCV activity of RBV in OR6 system, we assumed that the anti-HCV activity of RBV might also be illuminated by ORL8 or ORL11 system. Indeed, marked differences were observed between OR6 and both of the other assay systems: RBV at clinically achievable concentrations effectively inhibited HCV RNA replication in both ORL8 and ORL11, but not in OR6 (Fig. 1A). The EC_{50} values of RBV in ORL8, ORL11, and OR6 were 8.7, 15.9, and >100 μM, respectively, without suppression of cell growth (upper panels in Fig. 1A). These pronounced differences in the anti-HCV activity of RBV were confirmed by Western blot analysis (lower panels in Fig. 1A). In addition, time course assays revealed that the anti-HCV activity of RBV was dose- and time-dependent in ORL8 and ORL11, but not in OR6 (Fig. 1B). We next examined the activity of RBV using polyclonal cell-based assay systems (sORL8, sORL11, and sOR (Ikeda et al., 2005)) harboring HCV replicon RNA. The results revealed that the EC_{50} values of RBV in sORL8 and sORL11 were 14.3 and 29.9 μM, respectively, whereas RBV showed no anti-HCV activity in sOR (Fig. 1C), suggesting that the anti-HCV activity of RBV was not either a clone-specific or genome-length HCV RNA-specific phenomenon. Moreover, we demonstrated by Western blot (upper panel of Fig. 2) and quantitative RT-PCR (lower panel of Fig. 2) analyses that RBV suppressed HCV RNA replication in HCV-JFH1-infected ORL8c cells, but not in HCV-JFH1-infected RSc cells, which HCV could infect and efficiently replicate within (Ariumi et al., 2007; Kato et al., 2009). These results also indicate that only the Li23-derived assay system can illuminate the anti-HCV activity of RBV.

3.2. An ENT inhibitor cancelled anti-HCV activity of RBV

As one possible explanation for the pronounced differences in RBV activity between the Li23- and HuH-7-derived assay systems, we considered that the efficiencies in the cellular uptake of RBV might have differed between the two types of cells. To date, two families of nucleoside transporter proteins have been identified: the ENT family (ENT1, ENT2, and ENT3) and the concentrative nucleotide transporter (CNT) family (CNT1, CNT2, and CNT3) (Pastor-Anglada et al., 2005). Two recent reports showed that ENT1 and CNT3 might be responsible for RBV uptake in HuH-7 cells (Ibarra and Pfeiffer, 2009), and that ENT1, but not ENT2 or CNTs, is a major RBV uptake transporter in human hepatocytes (Fukuchi et al., 2010). To test these points, we first examined the effects of an ENT inhibitor, NBMPR, and a CNT inhibitor, phloridzin dihydrate, on the anti-HCV activity of RBV (50 μM; 90% effective concentration [EC_{90}]) in ORL8 system. The results revealed that 5 μM NBMPR partially attenuated the anti-HCV activity of RBV in ORL8 (Fig. 3A). The marginal activity of RBV was also not changed in OR6 system treated with these transporter inhibitors (data not shown). A significant dose-dependency of the cancellation by NBMPR was also observed in ORL8 (Fig. 3B). Since we observed a lack of expression of CNT family members in ORL8 cells (data not shown), these

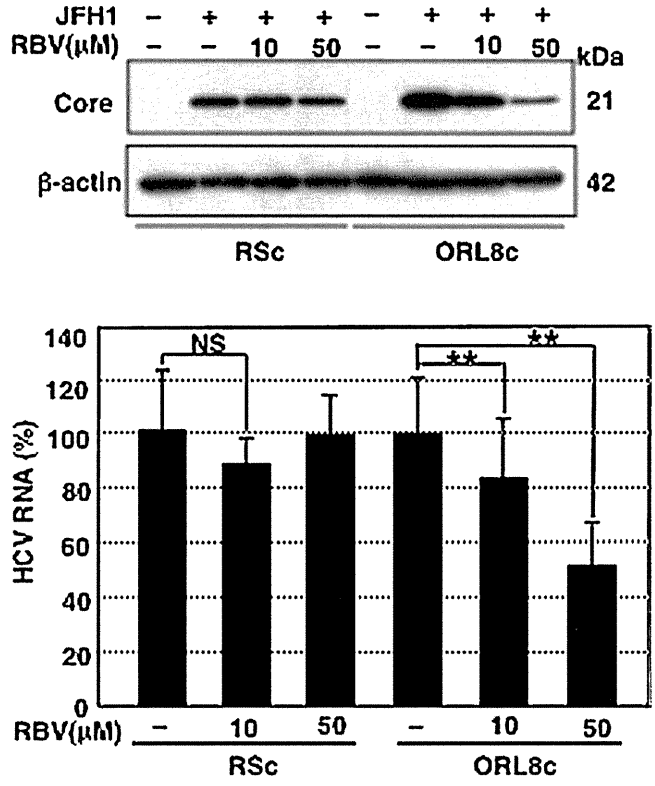

Fig. 2. RBV inhibited HCV production in JFH1-infected ORL8c cells, but not in JFH1-infected RSc cells. JFH1-infected ORL8c and RSc cells were treated with RBV for 72 h, and subjected to Western blot analysis using anti-Core or β-actin antibody (upper panel), and to quantitative RT-PCR analysis (lower panel). Asterisks indicate significant differences compared to the control treatment. $^{**}P < 0.01$; NS, not significant.

results suggest that cellular uptake of RBV is mediated by ENT member(s). Accordingly, we next examined the levels of ENT mRNAs in ORL8 and OR6 cells. However, the expression levels of ENT1, ENT2, and ENT3 mRNAs were comparable between ORL8 and OR6 cells (Fig. 3C). In addition, sequence analysis of ENT1, ENT2, and ENT3 mRNAs (data not shown) and Western blot analysis of ENT1 protein (Fig. 3D) revealed no differences between the two cell lines. These results suggest that the expression levels of ENT members are not associated with the differences in RBV activity.

3.3. RBV did not act as a mutagen in HCV RNA replication

Since the suppressive effect of RBV on HCV RNA replication was clearly observed in ORL8 system, we expected that ORL8 cells would be suitable for analysis of the anti-HCV mechanism of RBV. In regard to the anti-HCV mechanism of RBV, several groups have proposed that RBV (50–400 μM) acts as an RNA mutagen and induces error catastrophe in HCV RNA replication (Contreras et al., 2002; Zhou et al., 2003). Therefore, we first examined whether or not error catastrophe theory is involved in the anti-HCV activity of RBV observed in ORL8 system. To test the mutagenic effect of RBV, ORL8 cells were treated with or without RBV (50 μM; EC_{90} level in ORL8 system) for 72 h, and then genome-length HCV RNA from the ORL8 cells was amplified by RT-PCR. We performed HCV quasispecies analysis by sequencing of RL to the Neo^R, NS5A, and NS5B regions using at least 10 independent clones for each region. To estimate the mutation rate, the total number of mutations and the ratio of nonsynonymous to synonymous mutations in each region were determined by comparison with the parental HCV sequences (Kato et al., 2009). The results revealed that the overall mutation rate and the ratio of nonsynonymous to synonymous mutations in each

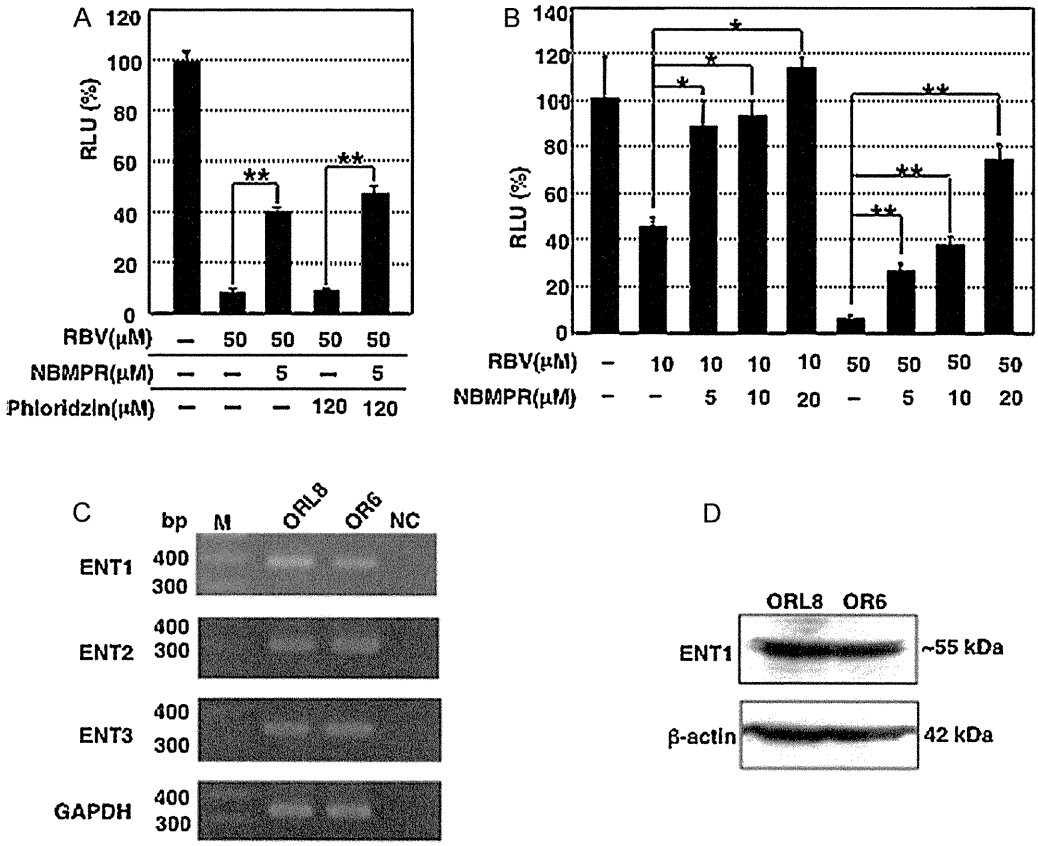

Fig. 3. An ENT inhibitor abolished anti-HCV activity of RBV. (A) An ENT inhibitor, NBMPR, canceled the anti-HCV activity of RBV in ORL8. ORL8 cells were pretreated with NBMPR and/or phloridzin dihydrate for 30 min, and then treated with RBV for 72 h, after which an RL assay was performed. Asterisks indicate significant differences compared to the control treatment. $^{**}P < 0.01$. (B) Dose-dependent cancellation by NBMPR of the activity of RBV. ORL8 cells were pretreated with NBMPR for 30 min, and then treated with RBV for 72 h, after which an RL assay was performed. Asterisks indicate significant differences compared to the control treatment. $^{*}P < 0.05$; $^{**}P < 0.01$. (C) RT-PCR analysis of ENTs. Total RNAs prepared from ORL8 and OR6 cells were subjected to RT-PCR using the primer sets for ENT1, ENT2, ENT3, and GAPDH as described in Section 2. RT-PCR products were detected by staining with ethidium bromide after 3% agarose gel electrophoresis. (D) Western blot analysis of ORL8 and OR6 cells for ENT1. The primary antibody used was ENT1. β-actin was used as a control for the amount of protein loaded per lane.

region were not increased irrespective of the presence or absence of RBV treatment (Table 1). To confirm that mutation frequencies given in Table 1 are overwhelmingly above the error level associated with the PCR, we sequenced independent five clones (6.0 kb covering 5′-UTR to NS3 and 6.1 kb covering NS3 to NS5B) obtained by PCR using KOD-plus DNA polymerase and a plasmid containing the parental HCV sequences (Kato et al., 2009) as a template. No mutations were detected in these sequenced clones, indicating that KOD-plus DNA polymerase possesses extremely high fidelity, and suggesting that the mutations obtained in the present study are not produced by the errors associated with the PCR. Therefore, these results indicate that RBV does not act as a mutagen in HCV RNA replication in ORL8 cells, and suggest that the anti-HCV activity of RBV (EC_{50}; 8.7 μM) observed in ORL8 system is not due to the induction of error catastrophe in the HCV RNA genome.

3.4. RBV did not activate the IFN-signaling pathway

Regarding HCV, Liu et al. (Liu et al., 2007) have reported that RBV (40–500 μM) enhances the IFN-signaling pathway in in vitro cell culture systems. Furthermore, a recent report showed that RBV improved early responses to PEG-IFN through enhanced IFN signaling in the treatment of patients with chronic hepatitis (Feld et al., 2010). In that study, it was shown that the RBV concentration in patients at day 3 was correlated with IP-10 induction at 12 h, but only in patients with an adequate first phase viral decline (Feld et al., 2010). Therefore, we expected that RBV would enhance the IFN-signaling pathway in our new cell culture system. Accordingly, we first examined the effect of RBV in combination with IFN-α on HCV RNA replication using ORL8 system. OR6 system was also used for purpose of comparison. The results showed that RBV had an additive effect in decreasing HCV RNA replica-

Table 1
Mutation frequencies in RL-Neo^R, NS5A, and NS5B regions.

Region	Condition	Total no. of clones	Total no. of mutations	Nonsynonymous/synonymous substitutions (ratio)
RL-Neo^R (1953 nts)	Control	12	59	39/20 (1.95)
	RBV (50 μM)	12	49	31/18 (1.72)
NS5A (1341 nts)	Control	10	35	24/11 (2.18)
	RBV (50 μM)	10	36	24/12 (2.00)
NS5B (1773 nts)	Control	10	10	3/7 (0.43)
	RBV (50 μM)	10	9	2/7 (0.29)

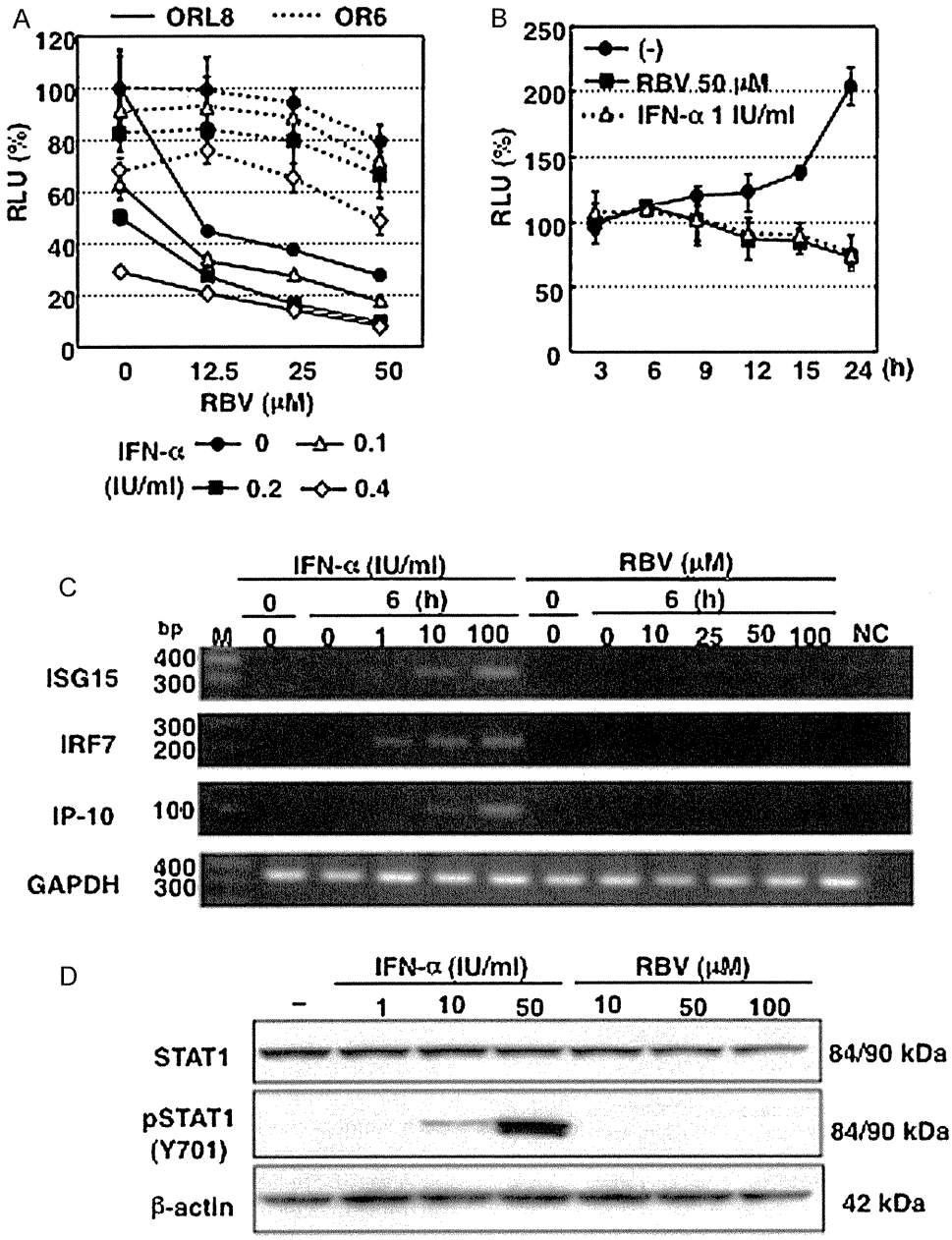

Fig. 4. RBV did not activate the IFN-signaling pathway in ORL8 cells. (A) Additive effect of RBV in combination with IFN-α. ORL8 and OR6 cells were treated with a combination of IFN-α and RBV for 72 h, after which the RL assay was performed. (B) Time course assay of the anti-HCV activity of RBV or IFN-α. ORL8 cells were treated with RBV or IFN-α, and an RL assay was performed at 3, 6, 9, 12, 15, and 24 h after treatment. Presented here is the RLU (%) calculated at each point, when the RL activity of non-treated cells at 3 h was assigned to be 100%. (C) ISGs were not induced by RBV treatment. ORL8 cells were treated with IFN-α or RBV for 6 h, and then the total RNAs extracted from the cells were subjected to RT-PCR using the primer sets for ISG15, IRF7, IP-10, and GAPDH as described in Section 2. RT-PCR products were detected by staining with ethidium bromide after 3% agarose gel electrophoresis. (D) Phosphorylation of STAT1 was not induced by RBV treatment. ORL8 cells were treated with IFN-α or RBV for 30 min, and subjected to Western blot analysis using anti-STAT1, anti-phospho-STAT1(Y701), and anti-β-actin antibodies.

tion in both assay systems, but its activity was greater in ORL8 than in OR6 (Fig. 4A). A comparative time course assay using RBV or IFN-α demonstrated that RBV- and IFN-α-treated ORL8 cells had the same anti-HCV kinetics, leading to decreased RL activity at 9 h after treatment (Fig. 4B). These results suggest that RBV induces some anti-HCV signaling pathway, such as an IFN-signaling pathway, rather than inducing IFN or directly inhibiting RNA replication.

We next examined the ability of RBV to activate ISGs. RT-PCR analysis revealed that RBV treatment (6 h) did not cause an induction of representative ISGs, ISG15, IRF7, and IP-10, in ORL8 cells, although even treatment (6 h) with 1 IU/ml (ISG15 and IRF7) or 10 IU/ml (IP-10) of IFN-α could induce these ISGs (Fig. 4C). Similar results were also obtained in OR6 cells and Huh7.5 cells (data not shown). In addition, enhancement of these ISGs was also not observed in the ORL8 cells co-treated with IFN-α and RBV (data not shown). Furthermore, we examined the phosphorylation status of STAT1 after RBV treatment. The results revealed that RBV treatment (up to 100 μM for 30 min) did not induce the phosphorylation of STAT1 in ORL8 cells, although phosphorylation of STAT1 was observed even after the treatment with 10 IU/ml of IFN-α (Fig. 4D). Together, these results indicate that RBV does not activate the IFN-signaling pathway.

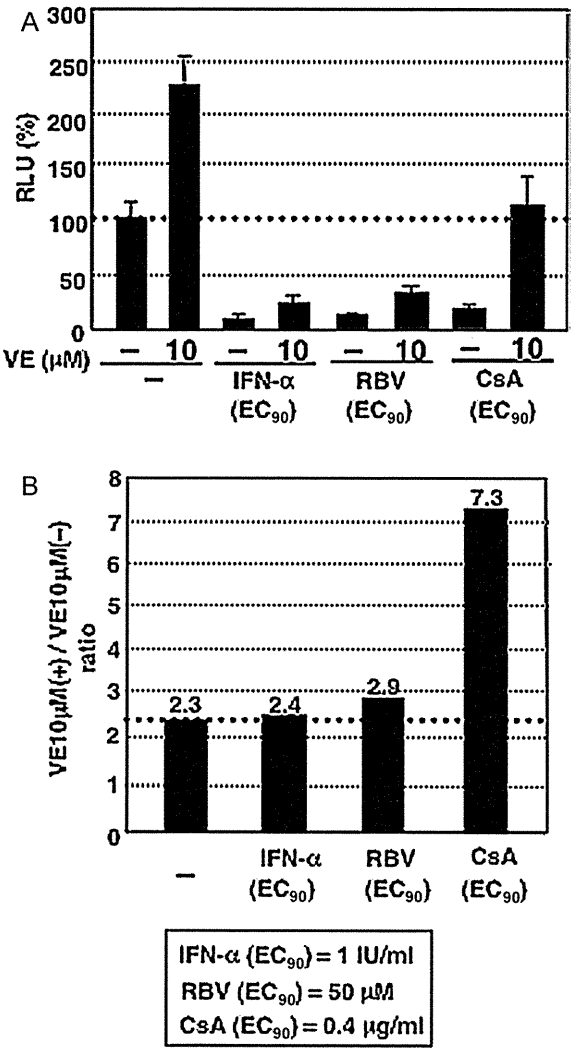

Fig. 5. The anti-HCV activity of RBV was not canceled by addition of VE. (A) Effects of VE on IFN-α, RBV, and CsA at the EC_{90}. ORL8 cells were treated with control medium (–), IFN-α, RBV, or CsA in either the absence or presence of VE for 72 h, and then an RL assay was performed. (B) The ratio of RL activity in the presence of VE to the RL activity in the absence of VE. The above ratio was calculated from the data of panel A. The horizontal line indicates the promotive effect of VE alone on HCV RNA replication as a baseline.

3.5. RBV did not induce the oxidative stress or subsequent anti-HCV status

Recently we reported that the antioxidant VE negated the antiviral activities of a broad range of anti-HCV reagents, including CsA, and demonstrated the involvement of the MEK-ERK1/2-signaling pathway in the anti-HCV status induced by oxidative stress (Yano et al., 2007, 2009). Therefore, we next expected that RBV induces oxidative stress. Accordingly, we examined the effect of VE on RBV, IFN-α, or CsA at the EC_{90} level in ORL8 system. Although the anti-HCV activity of CsA was canceled to a significant level by VE, the inhibitory effects of RBV and IFN-α were hardly influenced by co-treatment with VE (Fig. 5A). We normalized these results by dividing the RL value obtained in the presence of VE by that in the absence of VE as described previously (Yano et al., 2007) (Fig. 5B). The value of RBV was almost the same as that of IFN-α or control, although the value of CsA was somewhat higher (7.3) which was consistent with previous findings (Yano et al., 2007). These results suggest that induction of oxidative stress is not associated with the activity of RBV detected in ORL8 system.

3.6. Guanosine dose-dependently attenuated the anti-HCV activity of RBV

Previously, using a qualitative colony-forming efficiency (CFE) assay of an HCV RNA replicon, Zhou et al. (2003) showed that RBV (50 μM) reduced the CFE by 2-fold in HuH-7 cells, although 10 μM RBV did not result in a significant change in CFE. In that study, when exogenous guanosine, but not adenosine, which would replenish GTP pools via the salvage pathway, was co-administered with RBV, the RBV-induced CFE reduction was partially cancelled (Zhou et al., 2003). From this result, the authors suggested that IMPDH inhibition and subsequent lowering of GTP pools contribute to the observed reduction in CFE. However, they failed to observe the any suppressive effects of the IMPDH inhibitors MPA and Merimepodib (MMPD)/VX-497 on HCV RNA replication (Zhou et al., 2003). Conversely, Henry et al. showed that MPA exerted anti-HCV activity on HCV RNA replication in HuH-7-derived cells (Henry et al., 2006). Therefore, in order to resolve these controversial results, we initially examined the anti-HCV activity of MPA in ORL8 and OR6 systems. The results revealed that MPA strongly inhibited HCV RNA replication in both systems without suppression of cell growth. The EC_{50} values of MPA in the ORL8 and OR6 were 0.29 and 0.57 μM, respectively (Fig. 6A). Dose-dependent cancellation by guanosine, but not by adenosine, of the activity of MPA, was observed in both systems (Fig. 6B and data not shown for OR6 system). These results suggest that the depression of GTP induced by inhibition of IMPDH decreases the level of HCV RNA replication. From these results, we expected that anti-HCV activity of RBV observed in ORL8 might also have been associated with the inhibition of IMPDH. Indeed, significant dose-dependent cancellation by guanosine, but not by adenosine, of the anti-HCV activity of RBV (10 μM) was observed in ORL8 (Fig. 6C). ORL11 also showed a similar cancellation by guanosine (data not shown). The suppressive effect of guanosine on the activity of RBV in ORL8 was confirmed by Western blot analysis (Fig. 6D). These results suggest that the anti-HCV activity of RBV at clinically achievable concentrations in ORL8 is mediated through the inhibition of IMPDH by RBV.

3.7. IMPDH is required for HCV RNA replication

To confirm the involvement of IMPDH on HCV RNA replication, the endogenous expression of IMPDH was suppressed by siRNA specific to IMPDH. Since IMPDH has two isoforms, IMPDH1 and IMPDH2, which share 84% amino-acid homology (Wang et al., 2008), we prepared IMPDH1- and/or IMPDH2-knockdown ORL8 cells. The effective knockdown of IMPDH1 and/or IMPDH2 in ORL8 cells was confirmed by quantitative RT-PCR (Fig. 7A). We observed that the levels of HCV RNA replication in these knockdown cells were notably reduced compared with the control cells without suppression of cell growth (Fig. 7B). These results suggest that IMPDH is crucial for the maintenance of HCV RNA replication. Taken together, these results indicate that the inhibitory activity of RBV on HCV RNA replication in Li23-derived cells is mediated through the inhibition of IMPDH by RBV.

4. Discussion

In this study, using novel Li23-derived cell culture assay systems, we demonstrated for the first time that RBV at clinically achievable concentrations efficiently inhibited HCV RNA replication, and clarified that its anti-HCV activity was mediated by the inhibition of IMPDH.

To date, several mechanisms as described above have been proposed based on the results of studies using an HuH-7-derived cell culture system (Feld and Hoofnagle, 2005; Feld et al., 2010; Lau

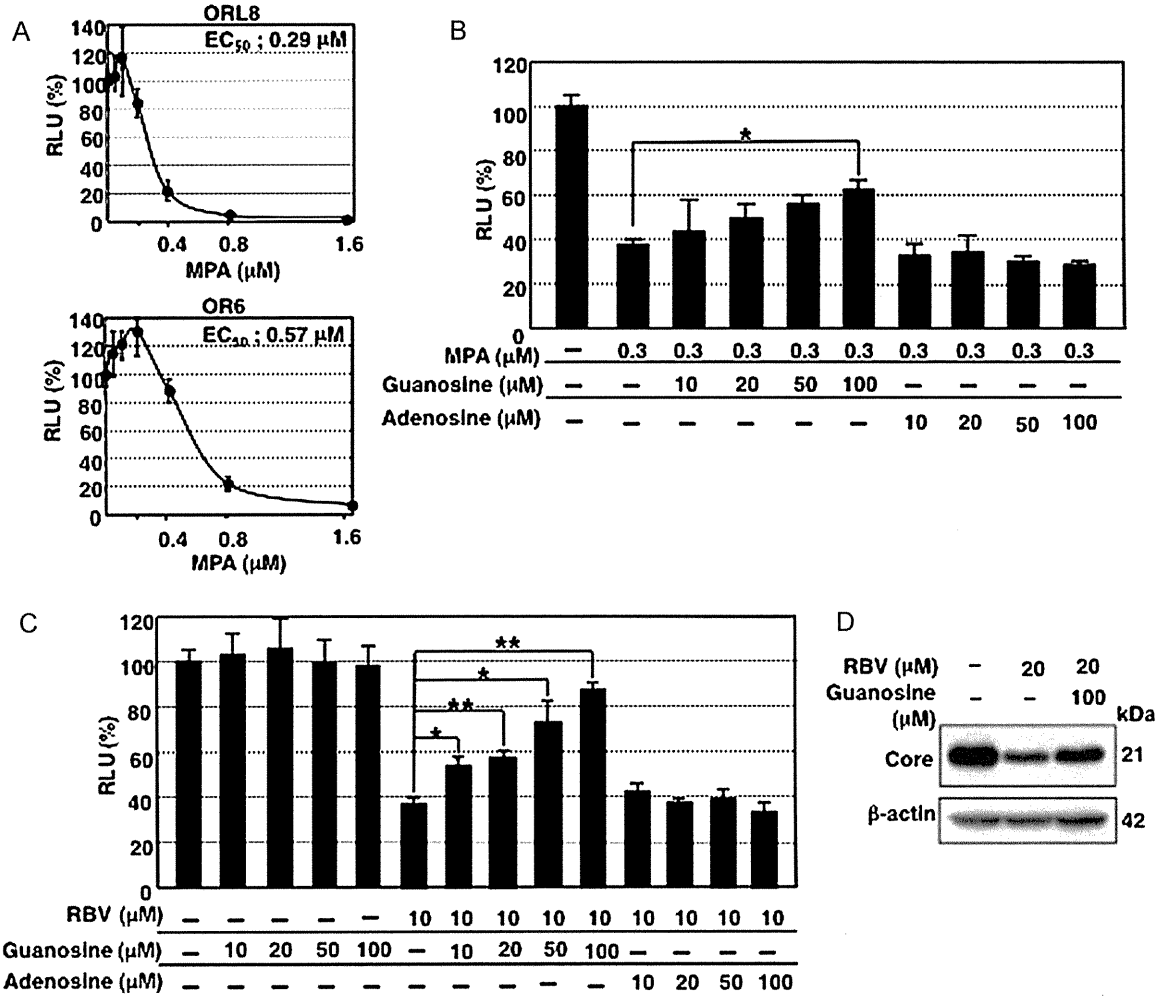

Fig. 6. Guanosine canceled the anti-HCV activity of RBV in ORL8 system. (A) Anti-HCV activity of MPA in ORL8 and OR6. The ORL8 and OR6 cells were treated with MPA for 72 h, and then RL assay was performed. (B) Effect of guanosine or adenosine on MPA in ORL8 system. ORL8 cells were treated with MPA alone or in combination with guanosine or adenosine for 72 h, and then RL assay was performed. Asterisk indicates a significant difference compared to the control treatment. $^{*}P<0.05$. (C) Effect of guanosine or adenosine on RBV in ORL8 system. ORL8 cells were treated with RBV alone or in combination with guanosine or adenosine for 72 h, and then the RL assay was performed. Asterisks indicate significant differences compared to the control treatment. $^{*}P<0.05$; $^{**}P<0.01$. (D) Effect of guanosine on RBV in ORL8 system. ORL8 cells were treated with RBV alone or in combination with guanosine for 72 h, and subjected to Western blot analysis using anti-Core and β-actin antibodies.

et al., 2002; Thomas et al., 2011; Zhou et al., 2003). Although the effective concentrations (50–1000 μM) of RBV in those studies were much higher than the clinically achievable concentrations (5–14 μM) (Feld et al., 2010; Pawlotsky et al., 2004; Tanabe et al., 2004), the effective concentration of RBV in this study was close to the clinically achievable concentrations. Furthermore, it is noteworthy that the replication of a different HCV strain (JFH1 of genotype 2a) in the Li23-derived cell culture system, but not in the HuH-7-derived cell culture system, was also suppressed with RBV at the concentration of 10 μM (Fig. 1C). These results demonstrate that the Li23 cell-derived assay system is a more sensitive biosensor of RBV than the HuH-7 cell-derived assay system.

The finding that RBV remarkably inhibited HCV RNA replication in our new assay systems led us to analyze the anti-HCV mechanism of RBV. In this study, we evaluated several possible anti-HCV mechanisms of RBV, as described above. Regarding the induction of error catastrophe by RBV, we obtained no evidence that RBV (even at 50 μM) acted as a mutagen in HCV RNA replication. Therefore, we could not explain the mechanism underlying the suppression of HCV RNA replication by RBV according to the theory of error catastrophe. In addition, no increasing mutation rate of HCV RNA in patients receiving RBV monotherapy or a combination of RBV plus IFN-α was observed in a previous clinical study (Chevaliez and Pawlotsky, 2007). In consideration of all these findings, we suggest that the clinically achievable concentrations of RBV do not act as a mutagen in HCV RNA replication. Indeed, our previous study using the replicon cell culture system demonstrated that RBV treatment (6 months at 5 and 25 μM) did not accelerate the mutation rate or increase the genetic diversity of the HCV replicon (Kato et al., 2005). In regard to the effect of RBV on the IFN system, we obtained no evidence that RBV (even at 50 μM) induced ISGs (ISG15, IRF7, and IP-10) or phosphorylation of STAT1 even in the cells co-treated with IFN-α and RBV (data not shown). On the other hand, very recently Thomas et al. (Thomas et al., 2011) reported that RBV treatment (500 μM) resulted in the induction of a distinct set of ISGs including ISG15, IRF7, and IRF9, using HuH-7-derived cell line Huh7.5.1. In that study, they demonstrated that the induction of these ISGs was mediated by a novel mechanism different from those associated with IFN signaling and double stranded RNA sensing pathway, and concluded that the effect of RBV on ISG regulation is IFN-independent. However, in our cell culture system, which is highly sensitive to RBV, the induction of ISG15 and IRF7 by RBV was not observed (Fig. 4C). This kind of controversial results may be dependent on the difference of cell lines used in both studies, since recent microarray analysis revealed that the expression profiles of Li23 and HuH-7 cells, both of which possess an environment

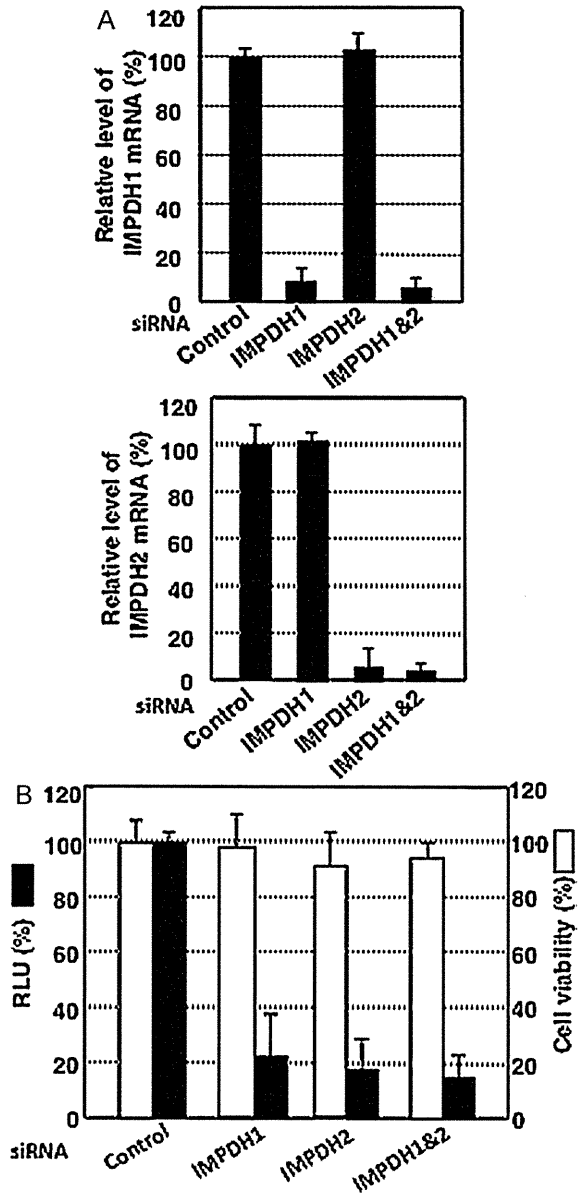

Fig. 7. IMPDH is required for HCV RNA replication. (A) Inhibition of IMPDH1 and IMPDH2 expression by siRNA in ORL8 cells. ORL8 cells were transfected with 8 nM siRNA targeting for IMPDH1 and/or IMPDH2. After 72 h, the expression levels of IMPDH1 and IMPDH2 mRNAs were determined by the quantitative RT-PCR. Experiments were done in triplicate. (B) Suppression of HCV RNA replication in IMPDH1- and/or IMPDH2-knockdown ORL8 cells. The RLU (%) calculated, when the luciferase activity of the cells treated with control siRNA was assigned to be 100%, is shown. The cell viability was determined as described in Section 2.

for robust HCV replication, differed considerably (Kato et al., 2009; Mori et al., 2010). However, Thomas et al. (2011) observed that the addition of guanosine to the medium could block RBV-induced ISGs induction. Therefore, further additional studies would be needed to resolve the differences of results obtained from both studies.

The highlight in this study is that a Li23-derived cell culture system clearly demonstrated an association between the suppression of HCV RNA replication by RBV and IMPDH inhibition by RBV. Although RBV is known to be an IMPDH inhibitor (Lau et al., 2002), it had been considered that such inhibitory activity would not contribute to the anti-HCV activity of RBV, because of the marginal antiviral effect of RBV in HuH-7-derived HCV RNA replicating cells (Naka et al., 2005; Tanabe et al., 2004; Zhou et al., 2003). Although Zhou et al. (2003) previously showed that exogenous guanosine cancelled the RBV-induced CFE reduction using an HuH-7-based HCV replicon system, they did not observe any dose-dependent reversion of the adverse effect of RBV by the addition of guanosine. However, in our Li23-based HCV replication assay system, we observed a near complete cancellation of the activity of RBV in the dose-dependent manner of guanosine (Fig. 6C). This finding indicated that anti-HCV activity of RBV might be mediated through the inhibition of IMPDH by RBV. Indeed, we could demonstrate that HCV RNA replication was notably suppressed in IMPDH-knockdown ORL8 cells (Fig. 7B). Taken together, these results revealed that the Li23-derived assay system was superior to HuH-7-derived assay system in order to clarify the anti-HCV mechanism of RBV.

The remarkable effect of RBV observed in this study was considered to be due to the difference in the cell lines used, because Li23-derived cells possessed rather different gene expression profiles from those in HuH-7-derived cells (Kato et al., 2009; Mori et al., 2010). As one of the possibilities, we examined the expression status of nucleoside transporters (ENT family) involved in cellular uptake of RBV or ATP-binding cassette transporters, including multidrug resistance 1, which is involved in cellular excretion. However, the mRNA levels of these transporters were almost the same in both types of cells (Fig. 3C). Although unfortunately we failed to clarify the mechanism underlying the remarkable differences in the activity of RBV in both types of cells, we observed that the anti-HCV activity of RBV was completely canceled by NBMPR, an ENT inhibitor, suggesting that RBV is taken by ENT member(s) at least in ORL8 cells. This finding supports the recent report describing the involvement of ENT1 on cellular uptake of RBV (Fukuchi et al., 2010; Ibarra and Pfeiffer, 2009). Therefore, a comparative analysis regarding the functions of ENT member(s) derived from both types of cells will be needed. As the other possibility, the differences of activities or expression levels of IMPDH in OR6 and ORL8 cells may contribute to the remarkable effect of RBV observed in ORL8 cells.

On the other hand, it has been known that rapid reduction of the intracellular level of GTP occurs when RBV inhibits IMPDH (Feld and Hoofnagle, 2005). Therefore, it is assumed that the decrease of GTP would lead to a suppression of HCV replication. To date, several studies (Lohmann et al., 1999; Luo et al., 2000; Simister et al., 2009) have shown that high concentration of GTP (approximately 500 μM corresponding to the intracellular concentration) is required for the efficient de novo initiation of RNA synthesis by HCV NS5B RdRp. In addition, Simister et al. (2009) showed that change from 500 μM to 100 μM of GTP concentration decreased a log of the NS5B RdRp activity. From these studies, we expect that the inhibition of IMPDH by RBV may cause rapid decrease of intracellular GTP concentration, resulting in the suppression of de novo RNA synthesis by NS5B. Before our assumption, MMPD/VX-497 has developed as an inhibitor of IMPDH, and it has been shown to exert anti-HCV activity (EC_{50}; 0.39 μM) in an HCV replicon system (Marcellin et al., 2007). However, MMPD/VX-497 monotherapy of patients with chronic hepatitis C had no effect on HCV RNA levels (Marcellin et al., 2007) just as, in another study, RBV monotherapy had no effect on HCV RNA levels in patients with chronic hepatitis C (Di Bisceglie et al., 1995). Although we showed that the EC_{50} value of RBV in this study was equivalent to the clinically achievable concentrations (Feld et al., 2010; Pawlotsky et al., 2004; Tanabe et al., 2004), we considered that the effective concentration for a reduction of HCV RNA levels in monotherapy would be less than the EC_{90} value. However, an IMPDH inhibitor at EC_{50} would be effective in combination with IFN-α as an adjuvant. Indeed, combination therapy with IFN-α and MMPD/VX-497 was effective in previously untreated patients with chronic hepatitis C (McHutchison et al., 2005). However, a recent study (Rustgi et al., 2009) showed that the addition of MMPD/VX-497 to PEG-IFN-α and RBV combination

therapy in patients who had been nonrespondent to PEG-IFN-α and RBV combination therapy did not increase the proportion of patients who achieved an SVR. Since we showed that RBV also acted as an IMPDH inhibitor in the present study, it would seem to be a reasonable result that MMPD/VX-497 had no significant effect on patients who were nonresponsive to combination therapy with PEG-IFN-α and RBV.

In conclusion, we clarified the anti-HCV mechanism of RBV in a new HCV cell culture system. The fact that anti-HCV activity of RBV was mediated by the inhibition of IMPDH would provide a clue to the mechanism of the increase of SVR by the current standard combination therapy with PEG-IFN-α and RBV. In addition, our findings should also be useful for the screening and development of new anti-HCV drugs, which inhibit IMPDH, with reduced side effects, including anemia.

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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

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ABSTRACT

Persistent hepatitis C virus (HCV) infection causes chronic liver diseases and is a global health problem. HuH-7 hepatoma-derived cells are widely used as the only cell-based HCV replication system for HCV research, including drug assays. Recently, using different hepatoma Li23-derived cells, we developed an HCV drug assay system (ORL8), in which the genome-length HCV RNA (O strain of genotype 1b) encoding renilla luciferase replicates efficiently. In this study, using the HuH-7-derived OR6 assay system that we developed previously and the ORL8 assay system, we evaluated 26 anti-HCV reagents, which other groups had reported as anti-HCV candidates using HuH-7-derived assay systems other than OR6. The results revealed that more than half of the reagents showed different anti-HCV activities from those in the previous studies, and that anti-HCV activities evaluated by the OR6 and ORL8 assays were also frequently different. In further evaluation using the HuH-7-derived AH1R assay system, which was developed using the AH1 strain of genotype 1b, several reagents showed different anti-HCV activities in comparison with those evaluated by the OR6 and ORL8 assays. These results suggest that the different activities of anti-HCV reagents are caused by the differences in cell lines or HCV strains used for the development of assay systems. Therefore, we conclude that plural HCV assay systems developed using different cell lines or HCV strains are required for the objective evaluation of anti-HCV reagents.

1. Introduction

Hepatitis C virus (HCV) infection frequently causes chronic hepatitis, which often leads to liver cirrhosis and hepatocellular carcinoma. Since approximately 170 million people are infected with HCV worldwide, HCV infection is a serious global health problem [1]. Although the combination of pegylated-interferon (PEG-IFN) and ribavirin is the standard therapy worldwide, only half of the patients receiving this treatment exhibit a sustained virologic response [2]. HCV is an enveloped virus with a positive single-stranded RNA virus of the *Flaviviridae* family. The HCV genome encodes a large polyprotein precursor of approximately 3000 amino acids, which is cleaved into 10 proteins in the following order: Core, envelope 1 (E1), E2, p7, non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B [3,4].

To date, HuH-7 hepatoma-derived cells are used as the only cell culture system for robust HCV replication in HCV research, including drug assays. We have also developed a HuH-7-derived drug assay system (OR6), in which genome-length HCV RNA (O strain of genotype 1b derived from an HCV-positive blood donor) encoding renilla luciferase (RL) efficiently replicates [5]. Recently, we found a new human hepatoma cell line, Li23, that enables robust HCV RNA replication [6], and we showed that the gene expression profile of Li23 cells was distinct from that of HuH-7 cells, although both cell lines had similar liver-specific expression profiles [7]. In that study, we identified three genes (New York esophageal squamous cell carcinoma 1, β-defensin-1, and galectin-3) showing Li23-specific expression profiles by a comparative analysis using several other hepatic cell lines [7]. We further developed Li23-derived drug assay systems (ORL8 and ORL11), which are relevant to the HuH-7-derived OR6 assay system [6]. During the process of evaluating the ORL8 and ORL11 assay systems using anti-HCV reagents such as IFNs, we noticed that these assay systems were frequently more sensitive to anti-HCV reagents than the OR6 assay system [6]. Furthermore, we recently found that ribavirin at clinically achievable concentrations (approximately 10 μM) effectively inhibited HCV RNA replication in both the ORL8 and ORL11 assay systems, but not in the OR6 assay system [8]. This finding led to the clarification of the anti-HCV mechanism of ribavirin, and we demonstrated that ribavirin's anti-HCV activity was mediated by the inhibition of inosine monophosphate dehydrogenase, a key enzyme in the guanosine biosynthetic pathway [8]. From these findings, we supposed that the anti-HCV reagents reported to date might show different activities among the different drug assay systems. To test this assumption, we evaluated 22 anti-HCV reagents that were reported using HuH-7-derived assay systems other than OR6, using the OR6 and ORL8 assay systems. Four additional

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reagents predicted by antiviral activity other than HCV were also evaluated. Furthermore, a recently developed HuH-7-derived AH1R assay system (AH1 strain of genotype 1b derived from a patient with acute hepatitis) (Mori et al., in preparation) was also used for the evaluation. Here, we report that plural assay systems derived from different cell lines and different HCV strains are required for the objective evaluation of anti-HCV reagents.

2. Materials and methods

2.1. Cell cultures

HuH-7-derived OR6 and AH1R cells were maintained in medium containing G418 (0.3 mg/ml) as described previously [5]. Li23-derived ORL8 cells were also maintained in medium containing G418 (0.3 mg/ml) as described previously [6].

2.2. Reagents

Acetylsalicylic acid, cephalotaxine, clemizole, crucumin, isoliquiritigenin, nitazoxanide, and tizoxanide were purchased from Sigma–Aldrich (St. Louis, MO). Cantharidin, 2′-deoxy-5-fluorouridine, griseofulvin, guanazole, homoharringtonine, resveratrol, and Y7632 were purchased from WAKO Pure Chemical Industries, Ltd. (Osaka, Japan). Artemisinin and bisindoly maleimide 1 were purchased from Alexis Biochemicals (San Diego, CA). Artesunate and silibinin A were purchased from Lkt Laboratories (St. Paul, MN). Esomeprazole and nelfinavir were purchased from Toronto Research Chemicals (North York, ON, Canada). Cinanserin hydrochloride and HA1077 were purchased from Tocris Bioscience (Bristol, UK). 6-Azauridine was purchased from MP Biomedicals (Solon, OH). Carvedilol was purchased from Calbiochem (San Diego, CA). Hemin was purchased from Alfa Aesar (Ward Hill, MA). Methotrexate was purchased from Tokyo Chemical Industry (Tokyo, Japan). Cinanserin hydrochloride, guanazole, HA11077, and Y27632 were dissolved in the culture medium for Li23-derived cells. Artesunate was dissolved in 0.5% $NaHCO_3$ solution. Other reagents were dissolved in dimethyl sulfoxide.

2.3. RL assay

RL assay was performed as described previously [6]. Briefly, the cells were plated onto 24-well plates (2×10^4 cells per well) in triplicate and then treated with each reagent at several concentrations for 72 h. After treatment, the cells were subjected to luciferase assay using the RL assay system (Promega, Madison, WI). From the assay results, the 50% effective concentration (EC_{50}) of each reagent was determined.

2.4. WST-1 cell proliferation assay

The cells were plated onto 96-well plates (1×10^3 cells per well) in triplicate and then treated with each reagent at several concentrations for 72 h. After treatment, the cells were subjected to the WST-1 cell proliferation assay (Takara Bio, Otsu, Japan) according to the manufacturer's protocol. From the assay results, the 50% cytotoxic concentration (CC_{50}) of each reagent was determined.

2.5. Western blot analysis

The preparation of cell lysates, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and immunoblotting analysis were performed as previously described [9]. The antibodies used in this study were those against HCV Core (CP11; Institute of Immunology, Tokyo, Japan) and β-actin (AC-15, Sigma–Aldrich) as the control for the amount of protein loaded per lane. Immunocomplexes were detected with the Renaissance enhanced chemiluminescence assay (Perkin–Elmer Life Sciences, Boston, MA).

2.6. Selective index (SI)

The SI value of each reagent was determined by dividing the CC_{50} value by the EC_{50} value.

3. Results

3.1. Evaluation of 26 reagents for anti-HCV activity using OR6 and ORL8 assay systems

To obtain candidates for the evaluation of anti-HCV activity using OR6 and ORL8 assay systems, we first searched the literature in the PubMed database using the key words (HCV or hepatitis C) and (inhibit or antiviral or suppress or block); this yielded approximately 4500 reports published between January 2003 and April 2010. From these results, we further selected the reports in which the EC_{50} values of reagents were determined or estimated by the HuH-7-derived HCV assay systems using the Con-1 strain (genotype 1b) [10], N strain (genotype 1b) [11], or HCV JFH-1 strain (genotype 2a) [12]. We finally chose 22 commercially available reagents for the evaluation of anti-HCV activity using OR6 and ORL8 assay systems. Four reagents predicted from the antiviral activity (hepatitis B virus, cytomegalovirus, etc.) other than HCV were also included in the evaluation study. The 26 selected reagents and their references are listed in Supplementary Table S1.

For each of the 26 reagents, we determined the EC_{50} value by RL assay and the CC_{50} value by WST-1 assay using the OR6 or ORL8 assay system, and calculated the SI value by dividing the CC_{50} value by the EC_{50} value. For each reagent, we first compared the EC_{50} value obtained from the OR6 or ORL8 assay with that of the previous study. Consequently, we classified the 26 reagents into five classes, A to E (Table 1). Eight reagents (methotrexate, artemisinin, artesunate, clemizole, hemin, 6-azauridine, acetylsalicylic acid, and isoliquiritigenin with the order of the SI value in the ORL8 assay) belonged to class A, in which the EC_{50} value obtained by either the OR6 or ORL8 assay was less than one-third of that in the previous study (Supplementary Table S1 and Table 1). Artesunate, an artemisinin-derivative possessing antiviral activity against cytomegalovirus, herpesvirus, Epstein-Barr virus etc., was included in class A by the comparison with the data on anti-cytomegalovirus activity. In this class, we especially noticed that methotrexate (an anti-cancer drug) showed very strong anti-HCV activity (EC_{50} 0.1 μM; $CC_{50} > 200$ μM; SI > 2000) in the ORL8 assay (upper panel in Fig. 1A and Table 1), whereas methotrexate showed very weak anti-HCV activity ($EC_{50} > 200$ μM; $CC_{50} > 200$ μM) in the OR6 assay as well as in a previous report [13] (upper panel in Fig. 1A and Table 1). This drastic difference was confirmed by Western blot analysis (lower panels in Fig. 1A). These results indicate that only the ORL8 assay is drastically sensitive to methotrexate, and suggest that the anti-HCV activity of methotrexate depends on the types of hepatic cells. The comparison of the EC_{50} values of other reagents belonging to class A revealed that the ORL8 assay was more sensitive than the OR6 assay (1.9–15-fold) to artemisinin, artesunate, clemizole, acetylsalicylic acid, and 6-azauridine, and conversely the OR6 assay was more sensitive than the ORL8 assay (2–2.5-fold) to hemin and isoliquiritigenin (Table 1). Furthermore, the CC_{50} values of clemizole and 6-azauridine also differed more than twofold between the OR6 and OR8 assays (Table 1). These results suggest that the anti-HCV activities of these reagents are affected by the kind of assay systems used. Especially, we noticed that artemisinin and artesunate (antimalarial drugs) showed higher SI values in the

Table 1
Anti HCV activities of 26 reagents evaluated in this study.

	Assay	[a]		OR6		ORL8		AHIR	
	Cell origin	HuH-7		HuH-7		Li23		HuH-7	
	HCV strain	Con-1, N, JFH-1, etc.		O		O		AH1	
Class	Reagent	$\frac{CC_{50}}{EC_{50}}$	SI	$\frac{CC_{50}}{EC_{50}}$	SI	$\frac{CC_{50}}{EC_{50}}$	SI	$\frac{CC_{50}}{EC_{50}}$	SI
A	Methotrexate	$\frac{>100}{>100}$	–	$\frac{>200}{>200}$	–	$\frac{>200}{0.1}$	>2000	$\frac{170}{>200}$	<0.9
A	Artemisinin	$\frac{>177}{>78}$	>2.3	$\frac{380}{81}$	4.7	$\frac{370}{23}$	16	$\frac{310}{5.3}$	58
A	Artesunate[b]	$\frac{>15}{3.9}$	>3.8	$\frac{6.1}{2.3}$	2.7	$\frac{3.4}{0.22}$	15	$\frac{4}{0.81}$	4.9
A	Clemizole	$\frac{>20}{8}$	>2.5	$\frac{11}{22}$	0.5	$\frac{22}{2.0}$	11	$\frac{7.3}{>25}$	<0.3
A	Hemin	$\frac{>52}{22}$	>2.4	$\frac{10}{1.2}$	8.3	$\frac{18}{2.4}$	7.5	$\frac{7.2}{1.1}$	6.5
A	6-Azauridine	$\frac{>100}{100}$	>1.0	$\frac{10}{5.7}$	1.8	$\frac{1.5}{0.37}$	4.1	$\frac{14}{3.3}$	4.2
A	Acetylsalicylic acid	$\frac{8^d}{4^d}$	2.0	$\frac{2.6^d}{1.6^d}$	1.6	$\frac{2.4^d}{0.83^d}$	2.9	ND	–
A	Isoliquiritigenin	$\frac{<24}{24}$	<1.0	$\frac{12}{3.9}$	3.1	$\frac{15}{9.8}$	1.5	ND	–
B	Nelfinavir	$\frac{>10}{9.9}$	>1.0	$\frac{26}{11}$	2.4	$\frac{68}{12}$	5.7	ND	–
B	2′-Deoxy-5-fluorouridine	$\frac{<15}{15}$	<1.0	$\frac{31}{32}$	1.0	$\frac{36}{14}$	2.6	$\frac{13}{86}$	0.2
B	Resveratrol	$\frac{>10}{10}$	>1.0	$\frac{35}{4.3}$	8.1	$\frac{42}{16}$	2.6	$\frac{76}{9.9}$	7.7
B	Cantharidine[c]	$\frac{3.5}{0.3}$	12	$\frac{1.5}{0.28}$	5.4	$\frac{1.8}{0.69}$	2.6	ND	–
B	Homoharringtonine[c]	$\frac{0.5}{30^e}$	17	$\frac{38^e}{18^e}$	2.1	$\frac{0.11}{45^e}$	2.4	$\frac{22^e}{19^e}$	1.2
B	Crucumin	$\frac{>15}{15}$	>1.0	$\frac{18}{14}$	1.3	$\frac{19}{11}$	1.7	ND	–
B	Griseofulvin	$\frac{207}{6.1}$	34	$\frac{16}{4.4}$	3.6	$\frac{14}{8.6}$	1.6	ND	–
B	Cinanserin hydrochloride	$\frac{>10}{>10}$	–	$\frac{33}{25}$	1.3	$\frac{39}{35}$	1.1	ND	–
B	Cephalotaxine[c]	$\frac{>100}{60}$	>1.7	$\frac{35}{29}$	1.2	$\frac{38}{47}$	0.8	$\frac{4.8}{41}$	0.1
C	Tizoxanide	$\frac{15}{0.15}$	100	$\frac{11}{2.4}$	4.6	$\frac{24}{9.6}$	2.5	ND	–
C	Nitazoxanide	$\frac{38}{0.21}$	181	$\frac{11}{2.8}$	3.9	$\frac{17}{9.2}$	1.8	$\frac{7.2}{2.2}$	3.3
D	Guanazole	$\frac{<100}{>100}$	<1.0	$\frac{200}{>200}$	<1.0	$\frac{170}{>200}$	<0.9	$\frac{173}{>200}$	<0.9
D	HA1077	$\frac{50}{15}$	3.3	$\frac{>50}{>50}$	–	$\frac{>50}{>50}$	–	$\frac{>50}{>50}$	–
E	Bisindoly maleimide 1	$\frac{ND}{5}$	–	$\frac{8.1}{6.2}$	1.3	$\frac{15}{15}$	1.0	$\frac{14}{9.1}$	1.5
E	Esomeprazole	$\frac{ND}{>10}$	–	$\frac{67}{67}$	1.0	$\frac{27}{27}$	1.0	$\frac{20}{25}$	0.8
E	Y27632	$\frac{>50}{50}$	>1.0	$\frac{>80}{>80}$	–	$\frac{>80}{>80}$	–	$\frac{39}{>80}$	<0.5
E	Carvedilol	$\frac{17}{4.5}$	3.8	$\frac{4.4}{3.7}$	1.2	$\frac{6.6}{8.8}$	0.8	$\frac{6.3}{6.2}$	1.0
E	Silibinin A	$\frac{ND}{23}$	–	$\frac{12}{85}$	0.1	$\frac{26}{89}$	0.3	$\frac{28}{96}$	0.3

ND, not determined.
[a] Assay used in previous reports.
[b] Reported as anti-cytomegalovirus reagent.
[c] Reported as anti-hepatitis B virus reagent. EC_{50} and CC_{50} values are indicated by the order of μM except 'd' (μM) and 'e' (nM).

ORL8 assay than previously reported [14,15]. The anti-HCV profiles of artemisinin and artesunate in the OR6 and ORL8 assays are shown in Fig. 1B and Supplementary Fig. 1A, respectively. In addition, the comparison of SI values revealed that the OR6 assay was more sensitive to hemin and isoliquiritigenin than the HuH-7-derived assays (Con-1 and N strains) used in the previous reports (Supplementary Table S1), suggesting that the HCV strains used in the assay systems affect the evaluation of anti-HCV reagents.

Nine reagents (nelfinavir, 2′-deoxy-5-fluorouridine, resveratrol, cantharidin, homoharringtonine, crucumin, griseofulvin, cinanserin hydrochloride, and cephalotaxine with the order of SI value in the ORL8 assay) were placed in class B, in which the EC_{50} values obtained by the OR6 and ORL8 assays were similar (more than one-third to less than threefold) to those in the previous study (Table 1 and Supplementary Table S1). Cantharidin, homoharringtonine, and cephalotaxine, all of which possess anti-hepatitis B virus activity, were placed in class B by the comparison with the data on anti-hepatitis B virus activity (Supplementary Fig. 1).

Tizoxanide and nitazoxanide belonged to class C, in which the EC_{50} values obtained by both the OR6 and ORL8 assays were more than threefold higher than in the previous study (Table 1 and Supplementary Table S1). Guanazole and HA1077 were placed in class D, in which there was no anti-HCV activity in both the OR6 and ORL8 assays (Table 1). No anti-HCV activity of guanazole and HA1077 was also confirmed by Western blot analysis (data not shown). Lastly, five reagents (Bisindoly maleimide 1, esomeprazole, Y27632, carvedilol, and silibinin A) were placed in class E, in which pro-HCV activity was exhibited in both OR6 and ORL8 assays. We unexpectedly observed that these reagents enhanced the HCV RNA replication level. As a

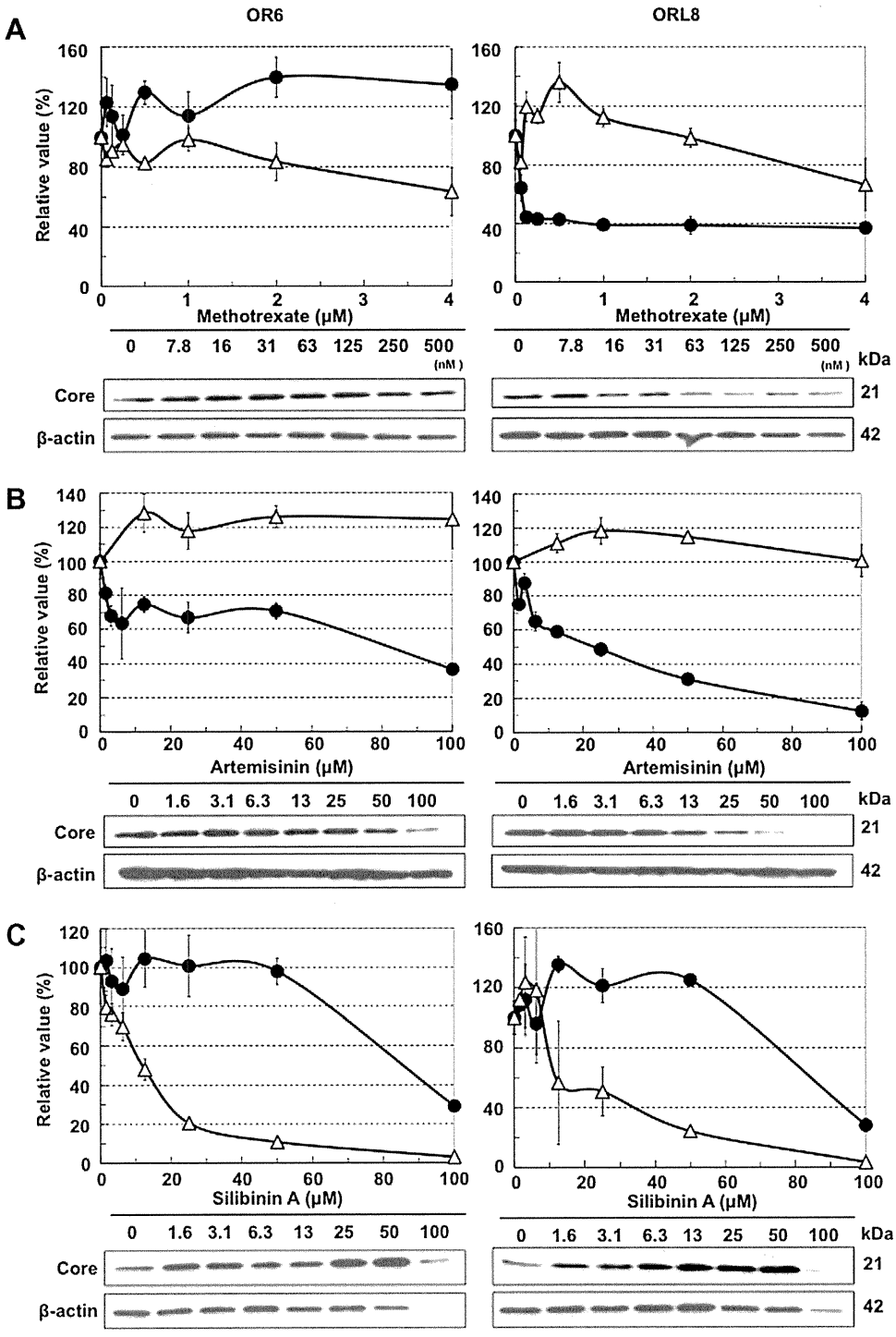

Fig. 1. Anti-HCV profiles of representative reagents in the OR6 and ORL8 assay systems. (A) Methotrexate sensitivities on genome-length HCV RNA replication in the OR6 and ORL8 assay systems. OR6 and ORL8 cells were treated with methotrexate for 72 h, followed by RL assay (black circle in the upper panel) and WST-1 assay (open triangle in the upper panel). The relative value (%) calculated at each point, when the level in nontreated cells was assigned to 100%, is presented here. Western blot analysis of the treated cells for the HCV Core was also performed (lower panel). (B) Artemisinin sensitivities on genome-length HCV RNA replication in the OR6 and ORL8 assay systems. RL assay, WST-1 assay, and Western blot analysis were performed as described in (A). (C) Silibinin A sensitivities on genome-length HCV RNA replication in the OR6 and ORL8 assay systems. RL assay, WST-1 assay, and Western blot analysis were performed as described in (A).

representative reagent, pro-HCV profiles of silibinin A are shown in the upper panel of Fig. 1C. These pro-HCV profiles were confirmed by Western blot analysis (lower panels in Fig. 1C for silibinin A and data not shown for the other reagents). Since the anti-HCV activity of silibinin A was detected by the HCV replicon assay system using the Con-1 strain [14], the converse effects obtained by our assay systems using the O strain may be due to the difference in HCV strains. In summary, the differences in anti-HCV activities observed among HuH-7- and Li23-derived assay systems used in this study and the other HuH-7-derived assay systems used in the previous studies suggest that the activities of anti-HCV reagents differ depending on which HCV strains and cell lines are used in the evaluation assays.

3.2. Evaluation of 18 reagents for anti-HCV activity using AH1R assay system

We previously established a HuH-7-derived cell line (AH1), which harbors genome-length HCV RNA (AH1 strain of genotype 1b) derived from a patient with acute hepatitis [16]. To further examine the effect of the HCV strain on anti-HCV reagent activity, we developed an AH1R assay system that is based on the AH1 cell line and that corresponds to the OR6 assay system (Mori et al., in preparation).

Using the AH1R assay system, we further evaluated the anti-HCV activities of 18 reagents, which showed differential anti-HCV activity between the OR6 and ORL8 assays, or showed either no anti-HCV activity or pro-HCV activity in both the OR6 and ORL8 assays. The results of the evaluation are shown in Table 1. The comparisons of the data obtained by the OR6 and AH1R assays revealed that the difference in the EC_{50} value from reagent to reagent was held within the range of one-third to threefold. However, we noticed that the EC_{50} value (5.3 μM) of artemisinin in the AH1R assay was remarkably lower than that (81 μM) in the OR6 assay (Supplementary Fig. 2 and Table 1), suggesting that artemisinin's anti-HCV activity differs depending on the HCV strain. Furthermore, the results of the AH1R assay revealed that cephalotaxine, belonging to class B, would be recategorized into class D. In summary, some reagents showed differential anti-HCV activities between the HuH-7-derived OR6 (O strain) and AH1R (AH1 strain) assay systems, although most of the reagents showed similar levels of anti-HCV activity in both assays. Taking together the results of the previous and present studies, we conclude that plural assay systems derived from different cell lines and HCV strains are needed for the objective evaluation of anti-HCV reagents.

4. Discussion

In the present study, we demonstrated for the first time that a Li23-cell-derived drug assay system, not a HuH-7-derived system, was important to use for the objective evaluation of anti-HCV reagents. In addition, we demonstrated that assay systems derived from different HCV strains were also necessary for the objective evaluation of anti-HCV reagents.

Among the 26 reagents evaluated by our assay systems, methotrexate showed the most drastic differences between the HuH-7- and Li23-derived assay systems in terms of anti-HCV activity. Although methotrexate showed very weak anti-HCV activity in the HuH-7-derived assay (Con-1 strain) used in a previous study [13] as well as in our OR6 and AH1R assays (O and AH1 strains), the ORL8 assay revealed very strong anti-HCV activity (SI > 2000). Such drastic differences in both assays suggest that some host factor or factors required for HCV RNA replication are different between these two cell lines, although the anti-HCV target of methotrexate is unclear. Since methotrexate is currently used as an anti-cancer drug or anti-rheumatic drug and its EC_{50} value for HCV RNA replication is 0.1 μM, it may be a potential candidate for enhancing the effects of the current combination therapy of PEG-IFN and ribavirin.

The anti-HCV activities of two antimalarial drugs, artemisinin and its derivative artesunate, are interesting. Although Paeshuyse et al. [14] showed that artemisinin possessed weak or moderate anti-HCV activity using a HuH-7- or HuH-6-derived subgenomic HCV replicon system, artemisinin's anti-HCV mechanism was unclear. On the other hand, Efferth et al. [15] reported that artesunate, the most studied artemisinin-derivative for the treatment of severe malaria, possessed antiviral activity against Epstein–Barr virus, human cytomegalovirus, human herpesvirus 6A, herpes simplex virus 1, and so on, except for HCV with the low micromolar range, although artesunate's precise antiviral mechanism was ambiguous. Therefore, we supposed, and our assay systems clearly detected, that both artemisinin and artesunate possess anti-HCV activity. Especially, the AH1R assay was the most sensitive to artemisinin (EC_{50} 5.3 μM), and the ORL8 assay was the most sensitive to artesunate (EC_{50} 0.22 μM). Preliminary experiments for the anti-HCV mechanisms of these reagents showed that they did not activate the IFN-signaling pathway (data not shown), and that they did not induce the oxidative stress (data not shown) as observed in the treatment with a broad range of anti-HCV reagents, including cyclosporine A [8,17]. Further studies are needed to clarify the anti-HCV mechanisms of these reagents. Since the largest SI value of artemisinin was 58 in the AH1R assay and that of artesunate was 16 in the ORL8 assay, these reagents may be also useful for the treatment of patients with chronic hepatitis.

In this study, we demonstrated that many anti-HCV reagents showed differential anti-HCV activities among different assay systems (OR6, ORL8, and AH1R) on HCV RNA replication. These results suggest that reliance on only a single assay system may lead to an incorrect evaluation of anti-HCV candidates. Therefore, we propose that plural assay systems derived from different cell lines and HCV strains should be used in order to evaluate anti-HCV candidates. Furthermore, plural assay systems derived from at least two different cell origins would be also useful for the screening of anti-HCV candidates.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.05.061.

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Expression of human factors CD81, claudin-1, scavenger receptor, and occludin in mouse hepatocytes does not confer susceptibility to HCV entry

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ABSTRACT

No suitable mouse model is available for studying chronic liver disease caused by hepatitis C virus (HCV). CD81, claudin-1, scavenger receptor class B type I, and occludin were recently reported to be the important factors in HCV entry into hepatocytes. We made transgenic mice (Alb-CCSO) expressing the four human proteins and examined whether HCV from a patient serum or HCV pseudoparticles (HCVpp) were capable of infecting them. HCV was not detected in the mouse serum after injecting the mice with HCV from a patient serum. We also found no indications of HCVpp entry into primary hepatocytes from Alb-CCSO mice. In addition, HCV-infectible Hep3B cells were fused with HCV-resistant primary mouse hepatocytes and the fused cells showed 35-fold lower infectivity compared to wild-type Hep3B cells, indicating that primary mouse hepatocytes have the inhibitory factor(s) in HCVpp entry. Our results suggest that the expression of the human factors does not confer susceptibility to HCV entry into the liver.

Hepatitis C virus (HCV) causes a chronic liver disease and affects an estimated 3% of the world population. No vaccine is available for preventing HCV, and the current treatment is often ineffective. The study of HCV has been hampered by the lack of suitable animal models. Chimpanzees are the only available *in vivo* experimental systems, but their use is limited by ethical concerns and high costs. HCV infection and replication in human hepatocytes has been confirmed in chimeric mice, the first reported mouse model (19). Although various factors, such as CD81, claudin-1 (CLDN1), scavenger receptor class B type I (SR-BI), and occludin (OCLN) have been reported to mediate HCV infection (8, 11, 21, 22), CD81 and OCLN must be human proteins to render mouse cells permissive to HCV infection (22). CD81 alone is insufficient to confer susceptibility to HCV in CD81 transgenic mice (16). CD81 and SR-BI interact with the E2 glycoprotein of HCV and have been suggested to play an initial role in attachment of the virus to cells (21, 23). After attachment, receptor-virus complexes are required to enter the cells as CD81-CLDN1-HCV complexes (13, 15) and OCLN-HCV complexes (2). Although HCV is known to enter cells via a pH- and clathrin-dependent endocytic pathway (3, 6, 14, 17, 26), the precise mechanisms are not fully elucidated. In the present study, we generated CD81, CLDN1, SR-BI, OCLN transgenic mice and examined whether HCV infect the liver using HCV from a patient serum and HCV pseudoparticles (HCVpp).

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MATERIALS AND METHODS

Generation of transgenic mice. The animal procedures were approved by the experimental animal ethics committee at Hamamatsu University School of Medicine. The transgenic expression cassettes for human CD81, CLDN1, SR-BI, and OCLN were constructed as follows. The four genes were cloned from human liver total RNA (Ambion, Austin, TX) by RT-PCR using four sets of primers: 5'-ATGGG AGTGGAGGGCTGCAC-3' and 5'-CTCAGTACAC GGAGCTGTTCC-3' for CD81, 5'-ATGGCCAACG CGGGGCTGCA-3' and 5'-GTCACACGTAGTCTT TCCCGC-3' for CLDN1, 5'-ATGGGCTGCTCCGC CAAAGCG-3' and 5'-CTACAGTTTTGCTTCCTG CAGCAC-3' for SR-BI, 5'-CCATGTCATCCAGGC CTCTTG-3' and 5'-CAACTTGGCATCAGCCTTCT ATG-3' for OCLN. The PCR products were ligated into the pGEM-T easy vector (Promega, Madison, WI). The nucleotide sequence of the insert was confirmed by sequencing. The EGFP cDNA in pAlb-EGFP (28) was then replaced by CD81, CLDN1, SR-BI, or OCLN cDNA. Each expression cassette was purified to remove the vector fragment. A mixture of Alb-CD81, Alb-CLDN1, and Alb-SR-BI fragments (molar ratio = 2 : 1 : 1) or the Alb-OCLN fragment was microinjected into fertilized eggs to generate the Alb-CCS mice and Alb-O mice, respectively (Fig. 1A). Transgenes were confirmed by Southern blot and PCR analysis.

CAG-DsRed2 mice carrying the DsRed2 expression vector controlled by the CAG promoter were obtained via a DsRed2-expressing ES cell-contributing chimera mouse produced by the following method. An aggregation of 10–15 ES cells with two (C57BL/6 × DBA/2) F1 eight-cell-stage embryos, from which the zona pellucida had been removed with Tyrode's solution (Sigma-Aldrich, St. Louis, MO), was placed on a plastic dish and cultured overnight. The ES cells and eight-cell embryos developed into a single blastocyst, which was transferred to the uterus of a pseudopregnant female ICR mouse. The genetic background of the parent ES cell line, E14.1, was the 129/Ola strain. Male chimera mice were then bred with C57BL/6 female mice and the germ-line transmission of the ES cells confirmed by the agouti coat color of the offspring.

Protein detection in transgenic mouse liver. The liver was homogenized in PBS containing protease inhibitor cocktail (Sigma). The homogenate was centrifuged at 1,000 × *g* for 5 min at 4°C, and the pellet was dissolved in 5 volumes of NP-40 buffer (20 mM Tris-HCl pH 8.0, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, and 2 mM EDTA) at 4°C for 1 h with rotation. After centrifugation at 18,500 × *g* for 20 min, the supernatant was used for protein analysis. The protein concentration was measured using the BCA method (Thermo Fisher Scientific, Waltham, MA). Proteins in the extracts were separated by SDS-PAGE under reducing or non-reducing conditions and transferred to a PVDF membrane (GE Healthcare, UK). The membranes were incubated first with one of four human-specific monoclonal antibodies: anti-CD81 (Millipore, Billerica, MA), anti-CLDN1 (Abnova, Taiwan), anti-SR-BI (BD Bio-

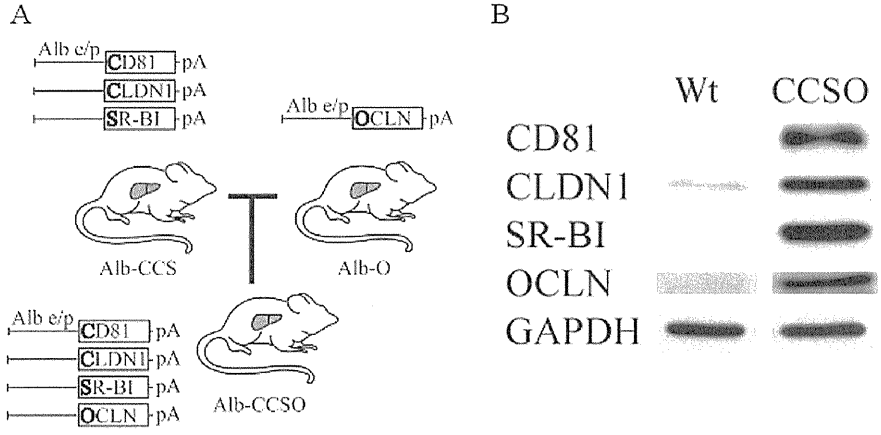

Fig. 1 Generation of CD81, CLDN1, SR-BI, and OCLN transgenic mice (CCSO). (A) Schematic representation of constructs used to generate transgenic mice. All genes were constructed under the control of the albumin enhancer and promoter (Alb e/p). (B) Western blot analysis of the CD81, CLDN1, SR-BI, and OCLN proteins in wild-type (Wt) and CCSO mice. GAPDH protein was used as a loading control. Note that the anti-human CLDN1 antibody cross-reacted with the mouse Cldn1 protein as a background in the wild-type liver.

sciences, Franklin Lakes, NJ), or anti-OCLN (Abnova). The membranes were then incubated with HRP-conjugated anti-mouse IgG antibody (Dako, Denmark). The signals of specific proteins were detected using the ECL kit (Perkin-Elmer, Boston, MA). Polyclonal anti-GAPDH antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a loading control.

Binding of E2 protein to liver sections. To obtain the E2 protein expression vector, the E2 protein region of genotype 1b (J1) was amplified by PCR using pCAGGSc60-p7 (generously provided by Dr. Yoshiharu Matsuura) as a template and the following oligonucleotides as primers: 5'-ATGGATGCA ATGAAGAGAGGGCTCTGCTGTGTGCTGCTGC TGTGTGGAGCAGTCTTCGTTTCGGCTAGCCAT ACCCGCGTGACGGGG-3' and 5'-CTAGTGATGG TGGTGATGGTGTGCTCTATCCCTGTCCTCC-3'. This plasmid DNA encoded the signal peptide of the tissue plasminogen activator (tPA) protein at the N-terminus, the E2 protein from amino acid 384 to 660 which does not contain a membrane spanning region in the middle, and 6 × His tag at the C-terminus as previously described (9, 18, 20, 24). The PCR product was cloned into the pIRES2-EGFP vector (Clontech, Palo Alto, CA) to obtain the CMV-tPA/E2ΔH6 plasmid DNA. The nucleotide sequence was confirmed by sequencing.

The CMV-tPA/E2ΔH6 plasmid DNA was mixed with polyethylene imine (PEI) at a DNA:PEI ratio of 1 : 3 (w/w), and 0.5 mL of the mixture was added to HepG2 cells in 5 mL of culture medium in a 10-cm dish, which was then cultured in a CO_2 incubator for 4 h. The cells were then washed with PBS and cultured in DMEM without fetal bovine serum (FBS). The culture supernatant was collected 48 h after transfection and subjected to Ni-NTA His • Bind Resin (Merck, Germany) according to the manufacturer's instructions. The eluted E2 protein was concentrated using Amicon Ultra-4 (Millipore).

Cryostat liver sections 5-μm-thick were fixed in 1 : 1 acetone-chloroform (v/v) for 30 min at room temperature and used immediately after brief air drying. Sections were blocked with 10% goat serum in PBS for 30 min at room temperature and then incubated with the purified E2 protein overnight at 4°C. The next day, after washing with PBS, the sections were incubated with mouse monoclonal anti-hepatitis C virus E2 antibody (Abcam, UK) for 1 h at room temperature and then with Alexa Fluor 594 goat anti-mouse IgG antibody (Invitrogen, Carlsbad, CA). Sections were counter-stained with DAPI (Dojindo, Japan).

Immunofluorescence microscopy. The staining method was described previously (5). Cryostat liver sections 5-μm-thick were fixed in acetone-chloroform for 30 min at room temperature and used immediately after brief air drying. Sections were blocked with 10% goat serum in PBS for 30 min at room temperature and then incubated with the mouse monoclonal anti-CD81 or anti-SR-BI antibodies at 10 μg/mL in 10% goat serum in PBS overnight at 4°C. The next day, after rinsing with PBS, the sections were incubated with Alexa Fluor 594 goat anti-mouse IgG antibody for 30 min. The sections were then rinsed with PBS and counter-stained with DAPI.

Quantification of HCV RNA in sera using real-time RT-PCR. A written informed consent was obtained from the patient from whom serum was obtained. HCV (genotype 1b) from the patient's serum was injected into the mouse tail vein (0.25 mL/mouse). Two weeks after injection, sera were collected from the injected mice and HCV RNA isolated from the serum using the QIAamp Viral RNA Mini Kit (Qiagen, Germany). The number of RNA copies was analyzed by real-time RT-PCR as described previously (27) using the Step One Plus Real-Time PCR System (Applied Biosystems, Bedford, MA). In this system, the detection limit was 10 copies.

Pseudoparticle production and infection. Murine leukemia virus (MLV)-based HCVpp were generated as described previously (1). Briefly, 8.1 μg of the Gag-Pol packaging construct, 8.1 μg of the transfer vector construct (luciferase), and 2.7 μg of the JFH-1 glycoprotein construct (pcDNAΔC-E1-E2, a kind gift from Dr. Thomas Pietschmann) were co-transfected into 293T cells using the calcium phosphate precipitation method. Supernatants containing the HCVpp were collected 40 h after transfection and passed through a 0.45-μm filter. The HCVpp were pelleted through 20% sucrose cushions by ultracentrifugation using a Beckman Coulter SW 27 rotor at 25,000 rpm for 2 h at 4°C.

Hepatocytes were isolated by the two-step perfusion method described previously (10). The isolated liver cells were suspended in DMEM containing 10% FBS, 100 IU/mL penicillin G, and 100 μg/mL streptomycin sulfate and centrifuged at 50 × g for 5 min at 4°C. This procedure was repeated four times. The purified hepatocytes were plated in a 24-well-plate (2.5×10^5 cells) and cultured overnight in a CO_2 incubator. For infection assays with HCVpp, Hep3B cells were used as a positive control. Hep3B cells were prepared in 24-well-plates (2.0×10^4 cells) in

DMEM with 10% FBS on the same day as the isolation of the primary hepatocytes. On the next day, the cells were infected with HCVpp overnight. The medium was changed the next day and the cells cultured for an additional 48 h. The luciferase activity of the cell extract was measured according to the manufacturer's instructions (Promega).

Fusion of mouse and human hepatocytes. To establish a stable EGFP-expressing Hep3B cell line, Hep3B cells were transfected with pEGFP-N1 DNA (Clontech) and cloned in DMEM containing G418 (0.4 mg/mL) using the cylinder technique. Mouse hepatocytes were isolated from the CAG-DsRed2 mice using the two-step perfusion method as described above. The DsRed2-labelled hepatocytes and EGFP-expressing Hep3B cells were fused (1.4×10^7 cells each) using PEG4000 as described previously (12). The fused cells were resuspended in DMEM with 10% FBS and subjected to sorting with BD FACSAria (BD Biosciences). DsRed2 and EGFP double-positive cells were used for the HCVpp infection assay as described above.

RESULTS

Generation of transgenic mice

The expression of albumin starts in early fetal hepatocytes (E12) and peaks in adult hepatocytes at 4 weeks (25). Therefore, the expression of the transgene was thought to have no crucial effect on liver development and we got apparently healthy and fertile mice. For Alb-CCS mice, 25 founder mice were generated, six of which were positive for transgene integration. Two of these six mice were positive for the three human proteins (CD81, CLDN1, and SR-BI). For Alb-O mice, 90 founder mice were generated, eight of which were positive for transgene integration. Two of these eight mice were positive for the OCLN protein. We chose mice expressing higher amounts of the proteins and crossed the Alb-CCS and Alb-O mice, finally obtaining Alb-CCSO mice. The protein expression was determined by Western blot (Fig. 1B). The anti-human CLDN1 antibody cross-reacted with the mouse protein, but human CLDN1 was prominent in Alb-CCSO mice. We also detected the large amount of human CLDN1 mRNA in addition to the mouse Cldn1 mRNA in the liver of Alb-CCSO mice using RT-PCR analysis (data not shown).

Binding of soluble E2 protein to the liver section

The truncated soluble E2 protein has been reported to bind the CD81 and SR-BI proteins (21, 23). Therefore, soluble E2 protein was expected to bind to liver sections from Alb-CCSO mice. After incubating the liver sections with the purified E2 protein, mouse monoclonal anti-E2 antibody, and Alexa Fluor 594 goat anti-mouse IgG antibody, the cell-cell borders were clearly stained (Fig. 2A, right). In contrast, a background level of E2 binding was detected in wild-type liver (Fig. 2A, left), indicating that E2 binding was prominent in liver sections from Alb-CCSO mice.

Next, we tried to detect CD81 and SR-BI proteins in Alb-CCSO mice. The CD81 and SR-BI proteins were mainly stained in the cell-cell borders (Fig. 2B, right panels). In wild-type liver, little staining was observed using antibodies against human proteins (Fig. 2B, left panels). The results indicate that E2 protein can bind the CD81 and SR-BI proteins in Alb-CCSO mice, and that the site of the E2 binding is similar to that of CD81 and SR-BI proteins. We also tried to detect the CLDN1 and OCLN proteins in Alb-CCSO mice. CLDN1 was detected mainly in cell-cell junction, but OCLN was difficult to be determined due to a low specificity and a high background of anti-human OCLN antibody (data not shown).

Infection of mice with HCV from patient serum

Because the E2 protein specifically bound the Alb-CCSO liver sections, we infected the Alb-CCSO mice with HCV from patient serum. HCV has been shown to infect the human hepatocytes in the chimeric liver of immunocompromised mice and maintain HCV in the mouse serum from 1 to 5 weeks post-infection (19). Immunocompetent mice, such as the Alb-CCSO mice, can elicit an immune response; therefore, the long-term persistence of HCV infection is not expected. To test this assumption, we injected HCV from a patient (5.7 Log IU/mL) into Alb-CCSO mice ($n = 5$) and wild-type mice ($n = 5$), and collected the mouse serum 2 weeks after infection. The RNA copy number in the injected sample was approximately 1.2×10^5 copies, but the copy numbers for the mouse serum samples were under the detection level (Table 1). This finding indicates that the expression of the four human factors in mouse hepatocytes did not confer susceptibility to HCV infection and production *in vivo*.

HCVpp infection of primary hepatocytes from Alb-CCSO mice

The process of HCV infection has several steps. The first step is HCV entry into hepatocytes. The second

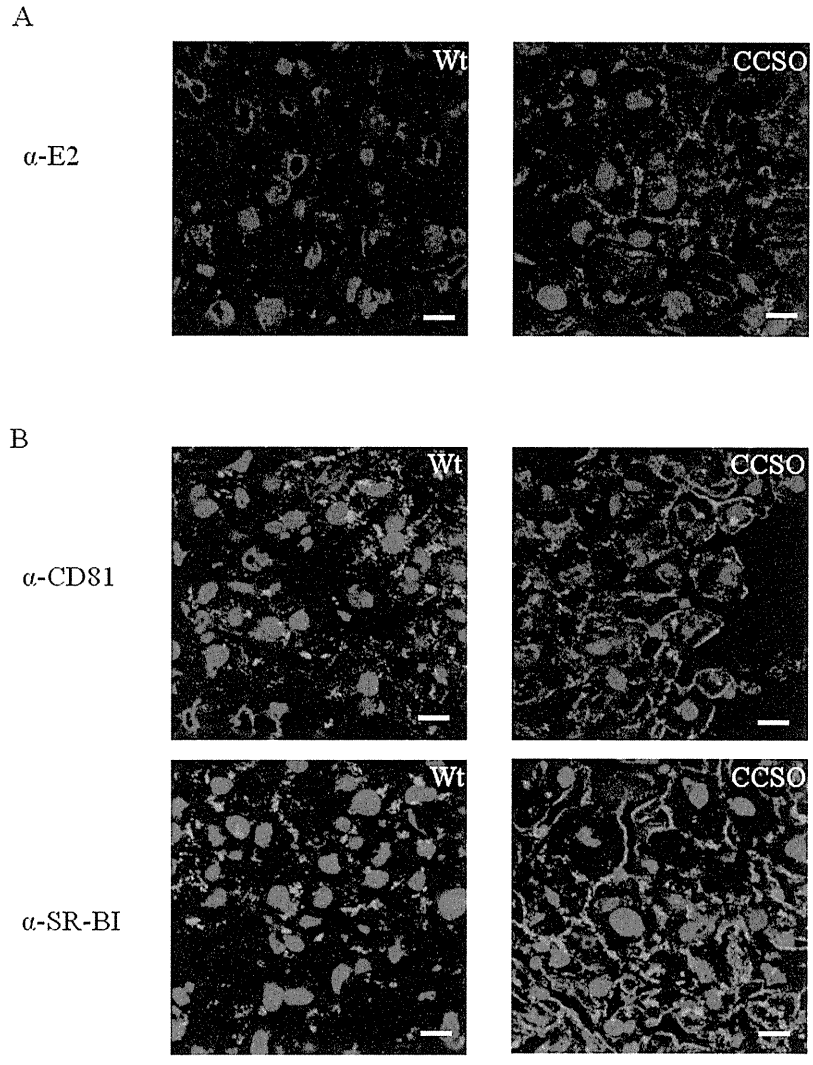

Fig. 2 **Soluble E2 protein bound to liver sections from Alb-CCSO mice.** (A) Binding assay of the E2 protein and Alb-CCSO hepatocytes. The liver sections from wild-type (left) and CCSO (right) mice were incubated with the truncated E2 protein. Bound E2 was visualized using anti-E2 primary antibody and Alexa Fluor 594 conjugated secondary antibody (red). The section was counter-stained with DAPI (blue). (B) Localization of CD81 and SR-BI proteins in the Alb-CCSO liver. Cryostat liver sections from wild-type (left) and CCSO (right) mice were incubated with mouse monoclonal anti-CD81 (upper) and anti-SR-BI (lower) antibodies, and then with Alexa Fluor 594 goat anti-mouse IgG antibody. CD81 and SR-BI are shown in red. The non-specific background staining was observed around nuclei. The section was counter-stained with DAPI (blue). Scale bar represents 10 μm.

Table 1 HCV RNA copy numbers in sera

Wild-type mice (n = 5)	< 10 copies/100 μL
CCSO mice (n = 5)	< 10 copies/100 μL
Injected human serum	1.2×10^5 copies/250 μL

step is the synthesis of viral proteins and replication of the genomic RNA. The last step is virus assembly and budding from the host cells. HCV could not infect the Alb-CCSO mice as shown above, but HCV can enter the mouse hepatocytes expressing the four human factors. To determine this hypothesis, we prepared HCVpp as described above. The HCVpp we used have HCV E1/E2 proteins in the envelope instead of MLV envelope protein, and a luciferase expression unit in the RNA genome. To determine the specificity of HCVpp entry, we also made a pseudoparticle harbouring the VSV-G protein (positive control) and no envelope psudoparticle (negative control) and infected Hep3B cells (data not shown).

Primary hepatocytes were isolated using the collagenase perfusion method. The next day, cells were