PPARα signaling affects HCV replication

We next examined the potential role of PPARα signaling on HCV proliferation by monitoring HCV replication in 2D-HuS-E/2 cells that had been infected with HCV-RC5 and subsequently treated with the PPARα agonist fenofibrate [14] or the PPARα antagonist MK886 [14] (Fig. 3B). As outlined in Fig. 3A, a dose-dependent increase in HCV replication was observed in fenofibrate-treated cells. In contrast, a dose-dependent decrease in HCV proliferation was observed in the presence of MK886. Similarly, treatment with MK886 reduced HCV proliferation in 3D/TGP-HuS-E/2 cells (Fig. 3C). The response of HCV proliferation in response to fenofibrate and MK886 treatment was also analyzed in LucNeo#2 cells that contained HCV replicon RNA (LNMH14) derived from the HCV-1b genome (Fig. 4A). Luciferase expression in these cells represented replication of the HCV replicon [6] and, as shown in Fig. 4A, luciferase activity in the cells treated with fenofibrate or MK886 also showed either enhancement or suppression of replicon proliferation, respectively. In addition, the increased HCV replication following fenofibrate treatment was completely abolished when treated with MK886 simultaneously. As MK886 is known to induce apoptosis when administered in high doses [15], the cell viability was examined using the XTT assay. There were no significant effects on cell viability after treatment with fenofibrate. Although MK886 resulted in a minor reduction in XTT values when high doses (10–15 μM) were administered, this reduction was not statistically significant when compared to its effect on HCV replication (Fig. 4B). This result suggests that PPARα signaling is required for HCV replication and that suppression of PPARα signaling has an anti-HCV effect.

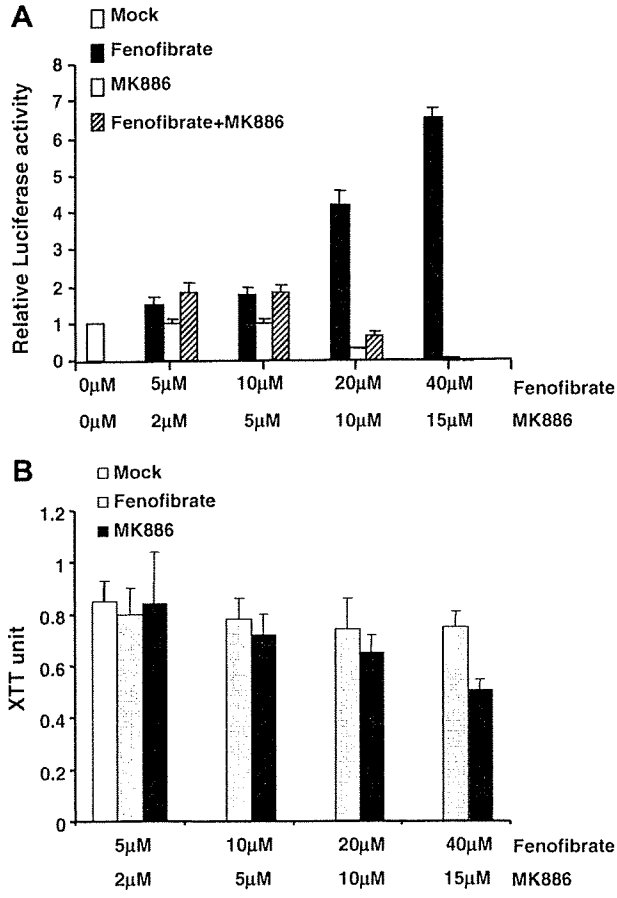

Fig. 4. The effects of PPARα agonists and antagonists on the replication of HCV subgenomic replicons. (A) LucNeo#2 cells containing a HCV subgenomic replicon termed LNMH14, were mock treated or treated with fenofibrate, MK886, or a combination of both fenofibrate and MK886 at the indicated concentrations for 2 days. Luciferase activity derived from the replicon was then measured as an indicator of HCV replication [7]. (B) Following treatment with fenofibrate and Mk886, LucNeo#2 cells were cultured for 2 days and cell viability measured using the XTT assay (Roche, Mannheim, Germany).

Discussion

In the current study, we demonstrated that immortalized hepatocyte HuS-E/2 cells cultured in 3D/TGP support the infection and replication of natural HCV derived from patient sera. Unlike recombinant HCVs, which have been required to adapt to sublines of HuH-7 cells [16], the population of the natural HCV is fairly polymorphic, demonstrating different responses to a variety of anti-viral agents [17,18]. The 3D/TGP-HuS-E/2 cells have the advantage of being a small-scale 3D cultured cells, which are cultured in 12-well plates at a density of 1×10^5/well, that allow the study of both viral and cellular events. In the current study, it demonstrated a 2 log increase in susceptibility to natural HCV infection and replication when compared to conventional 2D culture systems. Thus it offers an important advantage in the study of natural HCV infection and replication, and the response of natural HCV to anti-HCV drugs.

As the ability of HuS-E/2 cells to support infection and replication of natural HCV was greatly altered by the culture conditions, it is likely that the culture system described in our study will provide important information in regards to the cellular factors that support the HCV life cycle. The microarray study showed that the expression of some genes related to the PPARα signaling pathway were upregulated in the 3D cultured HuS-E/2 cells. Using both PPARα signaling agonists and antagonists, PPARα signaling was shown to affect infection and proliferation of natural HCV. PPARα is a ligand-activated transcription factor that is primarily expressed in tissues with high lipid metabolism including the liver, where it functions as one of three major nuclear receptors and is essential for its normal function [19]. Similar to a part of our data, a negative effect on HCV replication was previously observed in the replicon-bearing cells treated with siRNA for PPARα, with only 50% reduction of HCV-RNA [20]. In this study, even a large dose of PPARα agonist enhanced natural HCV replication in the 2D-HuS-E/2 cells for three times, despite the 2 logs enhancement of HCV proliferation in 3D/TGP culture. This implies that additional factors activated in 3D/TGP-HuS-E/2 cells may be required for the efficient HCV proliferation. Further analysis of the microarray data may provide us with further information on factors that may prove useful in the development of anti-HCV drugs.

In conclusion, the novel *in vitro* culture system combining TGP and immortalized hepatocytes described in this study demonstrated efficient support of natural HCV infection and replication. This system may be used in future virological studies to define new anti-HCV strategies. It may also prove useful for the specific design of effective individual therapy according to patient-specific strains.

Acknowledgments

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Identification of cellular and viral factors related to anti-hepatitis C virus activity of cyclophilin inhibitor

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We have so far reported that an immunosuppressant cyclosporin A (CsA), a well-known cyclophilin (CyP) inhibitor (CPI), strongly suppressed hepatitis C virus (HCV) replication in cell culture, and that CyPB was a cellular cofactor for viral replication. To further investigate antiviral mechanisms of CPI, we here developed cells carrying CsA-resistant HCV replicons, by culturing the HCV subgenomic replicon cells for 4 weeks in the presence of CsA with G418. Transfection of total RNA from the isolated CsA-resistant cells to naïve Huh7 cells conferred CsA resistance, suggesting that the replicon RNA itself was responsible for the resistant phenotype. Of the identified amino acid mutations, D320E in NS5A conferred the CsA resistance. The replicon carrying the D320E mutation was sensitive to interferon-α, but was resistant to CsA and other CPIs including NIM811 and sanglifehrin A. Knockdown of individual CyP subtypes revealed CyP40, in addition to CyPA and CyPB, contributed to viral replication, and CsA-resistant replicons acquired independence from CyPA for efficient replication. These data provide important evidence on the mechanisms underlying the regulation of HCV replication by CyP and for designing novel and specific anti-HCV strategies with CPIs. (*Cancer Sci* 2009; 100: 1943–1950)

Hepatitis C virus (HCV) is a leading cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC), and affects an estimated 170 million people worldwide.[1] The current standard therapy for patients infected with HCV is the combination treatment with pegylated interferon and ribavirin.[2,3] However, approximately half of individuals infected with HCV are unable to reach sustained virological response following such treatment. In addition, several side effects have been reported, which hinder continued treatment and impair the regimen efficacy. Thus, the development of novel anti-HCV strategies is essential for the treatment of infected individuals.

We have previously reported that a well-known immunosuppressant cyclosporin A (CsA) strongly suppressed the replication of HCV *in vitro*, in a manner independent of the interferon (IFN) signal transduction pathway.[4] Cyclophilin B (CyPB), a cellular target of CsA, was subsequently revealed to facilitate viral replication via the regulation of the RNA binding ability of NS5B.[5] Thus CyP, in addition to viral proteins including NS3 protease and NS5B polymerase, can also prove useful as a molecular target for antiviral strategies. Indeed, the non-immunosuppressive CsA analogs NIM811, DEBIO-025, and SCY635 have been observed to exert strong inhibitory effects on HCV replication, and these compounds are now in clinical trial.[6–8] Thus, it is crucial to deepen understanding of the anti-HCV actions of cyclophilin inhibitor (CPI) in order to maximize the efficacy of the agent. CPIs also need to face challenges such as side effects and drug resistance, which was observed as barrier to successful treatment in cases of human immunodeficiency virus (HIV),[9–12] and further clarification of the mechanism of CPI's anti-HCV activities is vital for the development of stronger and more specific therapeutic drug types. For this purpose, we here established and characterized the resistant replicon to CPIs using the subgenomic replicon system. We found that D320E, a mutation in NS5A, conferred resistance to CsA on the replicon, while additional mutations in NS3, Q86R and I252T seen in our CsA-resistant clone affected the replication fitness positively and negatively, respectively. The CsA-resistant replicons with the D320E mutation showed cross-resistance to other CPIs, NIM811 and sanglifehrin A (SFA), which were thus verified to suppress HCV replication through targeting CyP, and those resistant replicons were inhibited by treatment with IFNα as effectively as the wild type. Knockdown of individual CyP subtypes in the wild-type and CsA-resistant replicon cells revealed that CyP40, besides CyPA and CyPB, played important roles in HCV replication, and CyPA was related to the CsA-resistance. These results are important for elucidating additional mechanisms of the regulation of HCV replication by CyP and also for designing novel and specific anti-HCV strategies with CPI.

Materials and Methods

Compounds. CsA and IFNα were purchased from Merck Biosciences (San Diego, CA, USA) and Otsuka Pharmaceutical (Tokyo, Japan), respectively. NIM811 and SFA were generously provided by Novartis (Basel, Switzerland).

Cell culture. MH14 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum, nonessential amino acids (Invitrogen), and L-glutamine (Invitrogen) in the presence of 700 μg/mL G418 (Invitrogen).

Establishment of cell clones. We established each cell clone along with the outline shown in Figure 1. CsR#4, CsR#10, and CsR#11 cells were established through the selection of MH14#12 cell colonies in the presence of 1000 μg/mL G418 and 2 μg/mL CsA. CsR#11-2 and CsR#11-3 cells were established from Huh7 cells transfected with total RNAs extracted from CsR#11 cells in the presence of 700 μg/mL G418. Q86R, D320E, Q86R/D320E, and Q86R/I252T/D320E cells were produced by 700 μg/mL G418 selection of Huh7 cells transfected with 5 μg RNA transcribed from pMH14 carrying the individual mutations Q86R in NS3 and D320E in NS5A, double mutations Q86R in NS3 and D320E in NS5A, and triple mutations Q86R in NS3, I252T in NS3, and D320E in NS5A, respectively.

Colony formation assay. MH14 cells were treated with either CsA or NIM811 in the presence of 700 μg/mL G418 for 4 weeks, followed by fixation and staining with crystal violet.

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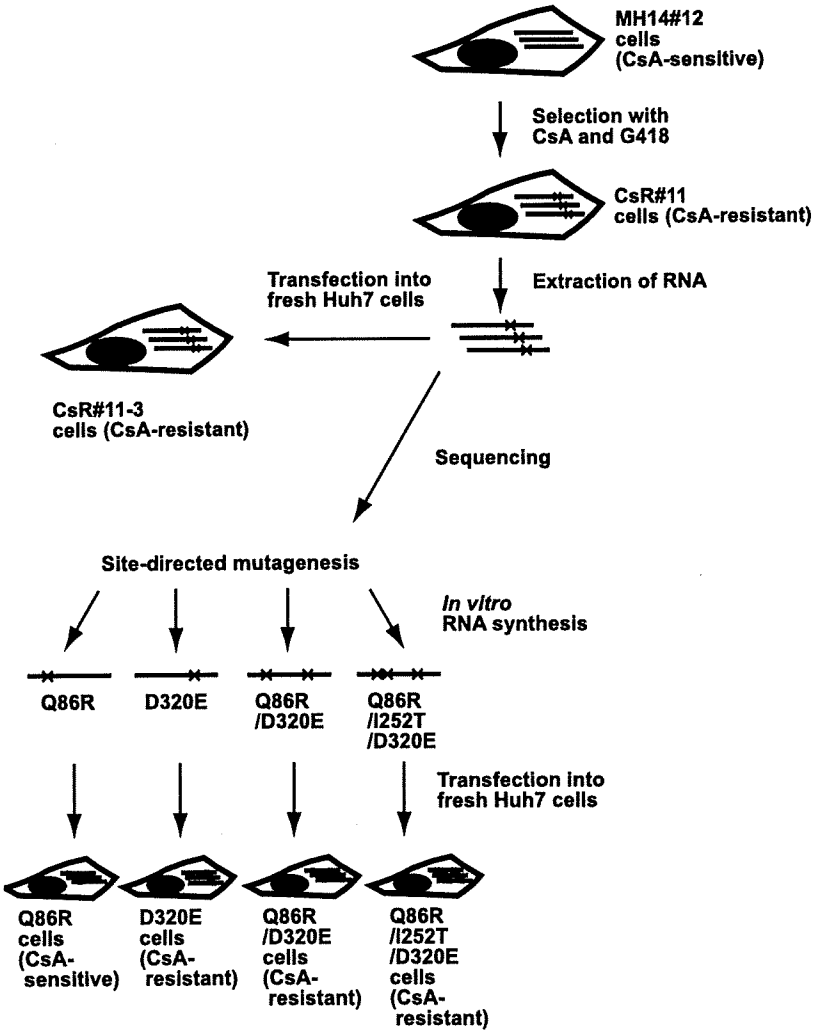

Fig. 1. Schematic diagram outlining the production of individual cell clones carrying hepatitis C virus (HCV) subgenomic replicons. MH14#12 cells, carrying wild-type HCV subgenomic repicon, were treated with 2 μg/mL cyclosporin A (CsA) in the presence of 1000 μg/mL G418 and CsR#11 cells were selected. Total RNA was extracted from CsR#11 cells and transduced into naïve Huh7 cells to select CsR#11-3 cells, and sequencing of the replicon RNA in CsR#11 cells identified mutations, Q86R in NS3, I252T in NS3, and D320E in NS5A. Site-directed mutagenesis followed by *in vitro* RNA synthesis generated HCV replicon RNA carrying Q86R, D320E, Q86R/D320E, and Q86R/I252T/D320E mutations. Transduction of the RNA into naïve Huh7 cells resulted in the production of Q86R, D320E, Q86R/D320E, and Q86R/I252T/D320E cells. The sensitivity of each replicon clone to CsA is presented as 'CsA-resistant' or 'CsA-sensitive'.

Real-time RT-PCR analysis. The 5′-non-translated region of HCV-RNA was quantified using an ABI Prism 7500 sequence detector (Applied Biosystems, Foster City, CA, USA), as previously described.[4]

Replicon sequencing. Total RNA from replicon cells was extracted with sepasol-RNA I Super (Nacalai Tesque, Kyoto, Japan) and subjected to RT-PCR reaction using super script III (Invitrogen). The products were then amplified by dividing the whole HCV region into approximately 300 bp using appropriate primer sets, and the sequence of the entire region encoding non-structural proteins was determined.

Plasmid construction. The Q86R and I252T mutations in NS3 and the D320E mutation in NS5A were generated via site-directed mutagenesis using the following primer sets: Q86R (S) 5′-AGGACCTCGTCGGCTGGCGGGCGCC-3′ plus Q86R (AS) 5′-GGCGCCCGCCAGCCGACGAGGTCCT-3′, I252T (S) 5′-AACACCAGAACTGGGGTAAGGACCA-3′ plus I252T (AS) 5′-TGGTCCTTACCCCAGTTCTGGTGTT-3′, and D320E (S) 5′-GAGTATAATCCTCCACTGCTAGAGC-3′ plus D320E (AS) 5′-GCTCTAGCAGTGGAGGATTATACTC-3′, respectively. The PCR products carrying either Q86R in NS3, I252T in NS3, or D320E in NS5A were inserted into the NotI-MluI and MluI-XbaI sites of pMH14, respectively. The resultant plasmids were termed pMH14 (Q86R), pMH14 (I252T), and pMH14 (D320E) respectively. The double mutant carrying both Q86R and D320E mutations was produced by exchanging the MluI-XbaI region of pMH14 (Q86R) with that of pMH14 (D320E), and termed pMH14 (Q86R/D320E). The triple mutant carrying Q86R, I252T, and D320E was produced by exchanging the Not-MluI region of pMH14 (D320E) with the fragments amplified by the primer set, I252T (S) plus I252T (AS), using pMH14 (Q86R) as templates for the PCR reaction. Sequence analysis of the resultant plasmids was also undertaken for confirmation of the mutations.

***In vitro* RNA synthesis.** Wild-type and mutant RNA of pMH14 was prepared by *in vitro* transcription using the MEGAscript T7 kit (Ambion, Austin, TX, USA), as described previously.[13]

Electroporation and colony formation. 8×10^6 cells suspended in 400 μL of cytomix buffer (120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4, 25 mM HEPES, 2 mM EGTA, and 5 mM $MgCl_2$, together with 2 mM ATP, 5 mM reduced form of glutathione, and 1.25 % DMSO) were electroporated at 250 V, 950 μF with either 100 μg total RNA extracted from replicon cells or 5 μg RNA transcribed *in vitro* from the HCV replicon construct cDNA. Cells were then treated with 1000 μg/mL G418 for 4 weeks following electroporation.

RNAi. Validated siRNAs against CyPB were purchased from Invitrogen. SiRNA duplexes against CyPA (siCyPA161, 5′-UCUGUGAAAGCAGGAACCCUU-3′; siCyPA285, 5′-GAUG

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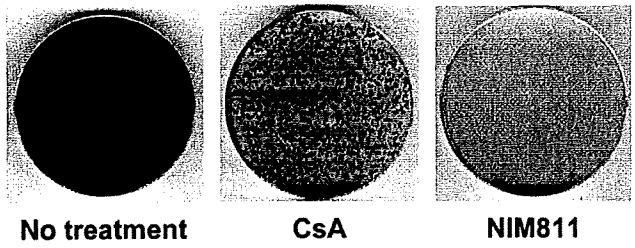

Fig. 2. Cyclophilin (CyP) inhibitor (CPI)-resistant colony emergence. MH14#12 cells were treated either without (left panel) or with 2 μg/mL CPIs, cyclosporin A (CsA) (middle panel), and NIM811 (right panel), in the presence of 700 μg/mL G418. Following 4 weeks in culture, cells were fixed and stained with crystal violet.

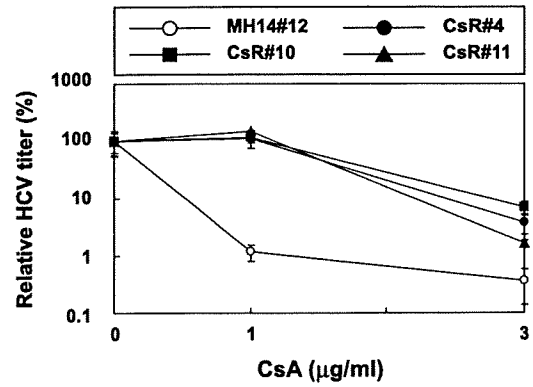

Fig. 3. Cyclosporin A (CsA) responses of the hepatitis C virus (HCV) replicon clones surviving the selection with CsA and G418. MH14#12 cells and three MH14#12-derived cell clones that survived double selection with G418 and CsA, CsR#4, CsR#10, and CsR#11, were treated with 1 and 3 μg/mL CsA for 7 days, and the HCV-RNA titers were measured by real-time RT-PCR. The data represent the percentage of HCV-RNA level in cells either untreated or treated with CsA, and the dots represent the means of three independent experiments.

CCAGGACCCGUAUGCUU-3′; siCyPA459, 5′-CUUCUUG CUGGUCUUGCCAUU-3′) were synthesized (Yahima Pure Chemicals, Osaka, Japan). SiRNAs against CyP40 were purchased from Invitrogen (siCyP40-3) and from Ambion (siCyP40-4). Pre-designed siRNAs, siCyPC, siCyPE, siCyPF, and siCyPG were obtained from Ambion. Transfection was performed using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) with 20 nM siRNAs in the absence of CsA according to the manufacturer's protocol.

Reverse transcription–polymerase chain reaction (RT-PCR) analysis. RT-PCR was performed as described previously[4] using the following primer sets: 5′-TGTTCTTCGACATTGCCGTC-3′ and 5′-CAGTCTTGGCAGTGCAGATG-3′ to detect mRNA for CyPA, 5′-TCTCCGAACGCAACATGAAG-3′ and 5′-CTGCGA TGATCACATCCTTC-3′to detect mRNA for CyPB, 5′-GGCGCA CTTGTGTTTTCTTC-3′ and 5′-TGCCATAGTGCTTCAGCTTG-3′ to detect mRNA for CyPC, 5′-TTTCGTGCACTGTGTACAGG-3′ and 5′-TTGGCTCTATCTGCTGTCTC-3′ to detect mRNA for CyP40, 5′-AGAGGAAGTGGACGACAAAG-3′ and 5′-GATGTCCATGTACACCTGAG-3′ to detect mRNA for CyPE, 5′-TGGAGCTGAAGGCAGATGTC-3′ and 5′-ACGTGACCG AACACAACATG-3′ to detect mRNA for CyPF, 5′-GAGTTGT CTCTTTCACAGAG-3′ and 5′-AACTGAGTATCCGTACCTCC-3′ to detect mRNA for CyPG, and 5′-ATGGGGAAGGTGAA GGTCGG-3′ and 5′-TGGAGGGATCTCGCTCCTGG-3′ to detect glyceraldehydes-3-phosphate dehydrogenase (GAPDH).

Results

Resistance emergence against individual CPIs. We have previously demonstrated the robust anti-HCV activities of CPIs, and it was reported that CPI significantly decreased HCV viral load in HCV-infected patients.[14,15] The problem of the drug-resistant HCV variants, hence, should be assessed *in vitro*, considering that practical efficacies of these inhibitors with long-term effectiveness are required in patients. In the first step of this study, we investigated the emergence of drug resistant replicon against CPIs. We treated MH14#12 cells, Huh7 cells carrying wild-type MH14 replicon with 2 μg/mL CsA, or the non-immunosuppressive analog NIM811 in the presence of 700 μg/mL G418 for 4 weeks. To visualize the appearance of drug-resistant clones, we stained cells after the selection. We observed colonies resistant to CsA, while we obtained few colonies under the treatment with the same concentration of NIM811 (Fig. 2).

Isolation and characterization of replicon cells resistant to CsA. To characterize the CsA-resistant HCV, we isolated the resistant clones following selection with 2 μg/mL CsA and 1000 μg/mL G418 for 4 weeks. We obtained several clones (named CsR cells), and examined their CsA responses. In contrast to the wild-type MH14#12 replicon cell, which showed an approximately 2-log reduction of HCV-RNA level by treatment with 1 μg/mL CsA for 7 days, all the clones isolated (the results of three representative clones, CsR#4, CsR#10, and CsR#11 cells are shown here) demonstrated resistant phenotypes against CsA with no significant reduction of HCV-RNA by CsA treatment at 1 μg/mL (Fig. 3). The resistance of these clones was thought to arise as a result of (1) mutations on the HCV-RNA genome or (2) alterations in cellular factors. To test the first possibility, we investigated whether HCV-RNA itself in CsR#11 could induce the CsA resistance to naïve cells. Fresh Huh7 cells were transfected with total RNA, including HCV replicon RNA, extracted from CsR#11 cells or MH14#12 cells as controls and cultured for 3 weeks in the presence of G418 (Fig. 1). The resulting colonies were isolated and propagated individually (named cell clones from total RNA of wild-type MH14#12, MH14#12-1, MH14#12-4, and MH14#12-5 cells, and those from CsR#11, CsR#11-2, CsR#11-3, and CsR#11-5 cells). The HCV-RNA titer in MH14#12-derived cells was reduced approximately to 100th by treatment with 1 μg/mL CsA for 7 days (Fig. 4). In contrast, cell clones generated from CsR#11 cells retained a normal HCV titer level after treatment with CsA, indicating that they had lost their sensitivity to CsA. Thus, it was suggested that the CsA-resistant profile in CsR#11 cells was attributed to its HCV-RNA.

D320E mutation in NS5A confers HCV replicon resistance to CsA. In order to identify the mutation in the HCV genome that resulted in the resistance to CsA, HCV subgenomic RNA isolated from CsR#11 cells was sequenced across the subgenomic region encoding non-structural proteins. We found three specific base changes that resulted in amino acid alteration including changes from glutamine to arginine, and isoleucine to threonine at positions 86 (Q86R) and 252 (I252T) in NS3, respectively, and a change from aspartic acid to glutamic acid at position 320 (D320E) in NS5A. Given that all these three mutations, Q86R, I252T, and D320E, were retained in every replicon in CsR#11-2, CsR#11-3, and CsR#11-5 cells, it is likely that they are inherited from CsR#11 cells and are associated with the acquired CsA-resistant phenotype. To examine this possibility, we synthesized replicon RNA carrying all these three mutations and established cells carrying these replicons. The resultant cell clones were named Q86R/I252T/D320E-1 and -3 cells. Treatment of these cells with 1 μg/mL CsA decreased the HCV titer only

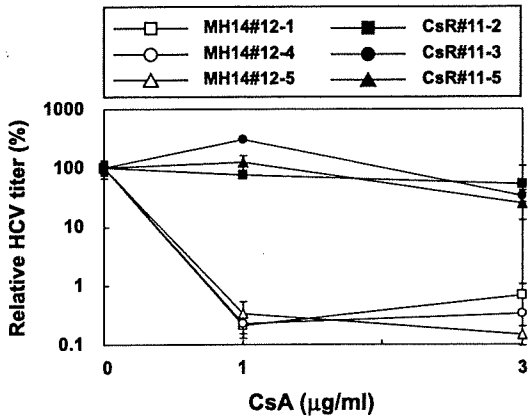

Fig. 4. Hepatitis C virus (HCV) RNA alteration contributed to cyclosporin A (CsA)-resistance. Total RNA extracted from CsA-resistant CsR#11 cells or that from wild-type MH14#12 cells as a control was transfected into Huh7 cells. Colonies established after 4-week selection with G418 were isolated, propagated individually, and tested for CsA response. Three cell clones derived from MH14#12 cells, MH14#12-1, MH14#12-4, and MH14#12-5 cells, and three cell clones from CsR#11 cells, CsR#11-2, CsR#11-3, and CsR#11-5 cells, were treated with 1 and 3 μg/mL CsA for 7 days. HCV-RNA titers were quantified by real-time RT-PCR analysis. The dots represent the means of three independent experiments.

by 1 log, in contrast to the wild-type MH14 clone, in which CsA decreased HCV-RNA by more than 2 logs under the same experimental condition (Fig. 5b). Thus, these mutations were demonstrated to confer CsA resistance; in addition to this, some cellular factors in Huh7 cells may also play minor roles in modulating the CsA sensitivities, given the result that Q86R/I252T/D320E cell clones were relatively sensitive to CsA compared with CsR#11-derived cell clones as shown in Figure 4. We next aimed to determine which of the three mutations, Q86R/I252T/D320E, was responsible for the CsA resistant phenotype, and individual mutations were engineered back into the wild-type MH14 replicon and stable replicon cells were produced as described above. Among three single amino acid mutations, the I252T mutation in NS3 resulted in a significant reduction in replication fitness (Fig. 5a), and almost failed to produce cell colonies. Cell clones harboring MH14 with both Q86R and D320E mutations, Q86R/D320E-2 and Q86R/D320E-3 cells, showed reduced sensitivity to CsA that was comparable to the levels in Q86R/I252T/D320E cells, suggesting Q86R and/or D320E mutation(s) was enough to confer the resistance. Subsequently, we treated the replicon cell clones carrying MH14 with either Q86R or D320E mutation alone, Q86R (Q86R-1 and -4 cells) and D320E (D320E-1 and -2 cells) cells, with CsA for 1 week. The titer of Q86R replicons was reduced to less than 100th by CsA treatment at a concentration of 1 μg/mL, similar to the wild type. In contrast, HCV replicon with D320E mutation in NS5A exhibited reduced sensitivity to CsA, resulting in little reduction of HCV-RNA by the treatment with 1 μg/mL CsA (Fig. 5b). Q86R mutation considerably enhanced colony

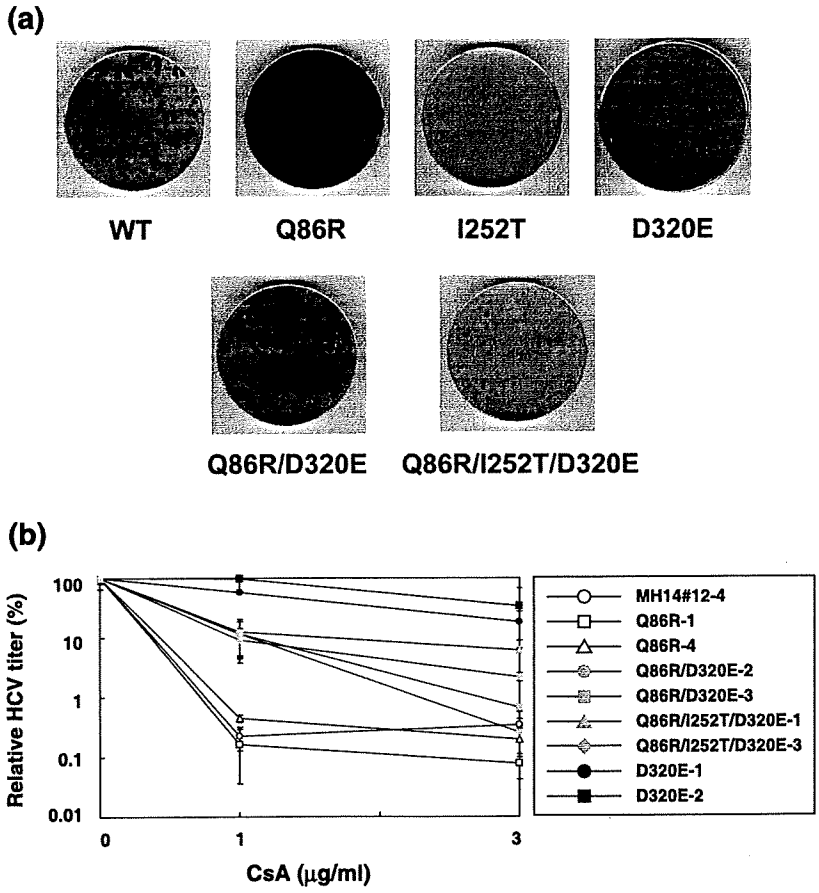

Fig. 5. The amino acid mutation D320E in NS5A conferred the cyclosporin A (CsA)-resistance to hepatitis C virus (HCV) replicons. (a) Colony formation assay for replicons carrying mutations. Five-microgram replicon RNA carrying the mutation(s), Q86R in NS3, I252T in NS3, D320E in NS5A, Q86R and D320E, or Q86R, I252T and D320E, or wild-type RNA transcribed *in vitro* were transduced into Huh7 cells. After culture with G418 for 4 weeks, colonies were stained with crystal violet. (b) Cell clones with replicons carrying indicated mutations were treated with 1 and 3 μg/mL CsA for 7 days. HCV-RNA titers were quantified by real-time RT-PCR analysis. The dots represent the means of three independent experiments. MH14#12-4, wild-type replicon; Q86R-1 and Q86R-4, replicon with Q86R mutation; D320E-1 and D320E-2, replicon with D320E mutation; Q86R/D320E-2 and Q86R/D320E-3, replicon with both Q86R and D320E mutations; Q86R/I252T/D320E-1 and Q86R/I252T/D320E-3, replicon with all three mutations, Q86R, I252T, and D320E.

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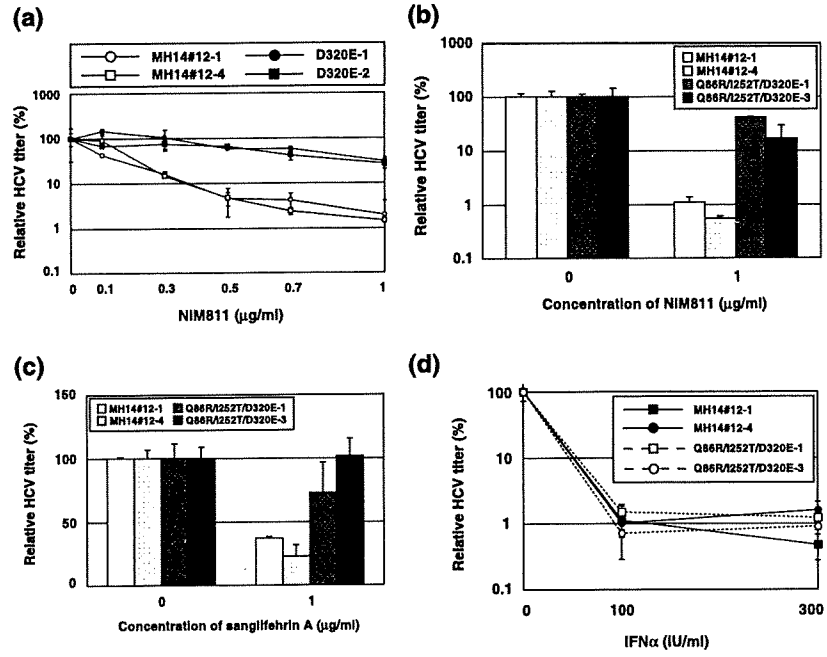

Fig. 6. Cyclosporin A (CsA)-resistant replicons demonstrated cross resistance to additional cyclophilin (CyP) inhibitors, NIM811 and sanglifehrin A (SFA), but not interferon (IFN)-α. MH14#12-1, MH14#12-4, D320E-1 and D320E-2 cells were treated with NIM811 at 0.1, 0.3, 0.5, 0.7 and 1 μg/mL (a), and MH14#12-1, MH14#12-4, Q86R/I252T/D320E-1, and Q86R/I252T/D320E-3 cells were treated with 1 μg/mL NIM811 (b), 1 μg/mL SFA (c), and 100 and 300 IU/mL IFN-α (d) for 7 days. The amounts of hepatitis C virus (HCV) RNA were quantified by real-time RT-PCR analysis. The data represent the means of three independent experiments.

formation efficiency and the D320E mutation showed little significant effect on the efficiency (Fig. 5a). Thus, the D320E mutation in NS5A was suggested as a sufficient factor to induce HCV replicon resistance to CsA, while the Q86R mutation was likely not to contribute to the resistance but to augment the efficiency of HCV replication itself.

The point mutation in NS5A conferred resistance to CPIs. Next, we examined cross-resistance between CsA and other CPIs or IFNα using the CsA-resistant replicon we produced above. Treatment with 0.1–1 μg/mL NIM811 for 7 days showed that the response to NIM811 of D320E-1 and -2 cells was less compared with that of MH14#12-1 and -4 cells, indicating that a CsA-resistant clone also acquired NIM811 resistance (Fig. 6a). A similar result was seen using Q86R/I252T/D320E cells (Fig. 6b). We then tested the anti-HCV activity of SFA, an additional CPI possessing distinct chemical backbone from those of cyclosporins.[16,17] Treatment with 1 μg/mL SFA reduced HCV replication in the wild-type cells, MH14#12-1, and -4 cells; however, it did not significantly reduce replication in Q86R/I252T/D320E cells (Fig. 6c). These results demonstrate that the CsA-resistant cells described in this study were also resistant to additional CPIs, confirming that these two compounds exerted anti-HCV effects via targeting CyP. Finally, we treated Q86R/I252T/D320E cell clones with 100 and 300 IU/mL IFNα for 7 days, and HCV-RNA titers were reduced by 2 logs in both clonal cell lines examined, Q86R/I252T/D320E-1 and Q86R/I252T/D320E-3 cells, as well as in wild-type MH14#12-derived cells, MH14#12-1, and MH14#12-4 cells (Fig. 6d). These results suggested no cross-resistance between CsA and IFNα, consistent with the previous report that the anti-HCV activity of CsA was independent of the IFNα signaling pathway.[18]

The role of CyP subtypes in HCV replication. We have previously reported that CyPB played a significant role in the efficient replication of HCV and CsA inhibited CyPB-mediated regulation of HCV replication. We have also suggested the involvement of other CyP subtypes in HCV replication.[19] To gain further insight into mechanisms underlying the anti-HCV properties of CPIs, we examined the roles of individual CyP subtypes in HCV replication in the wild-type MH14#12-1 and -4 replicon cells. In order to achieve this we knocked down CyPB with siRNAs (Fig. 7d), siCyPB-1 and -2, and found that this procedure reduced the amount of replicons to approximately half the initial level (Fig. 7c), a result consistent with the previous reports. Knockdown of CyPC, CyPE, CyPF, and CyPG (Fig. 7b) did not significantly affect the viral replication under these experimental conditions (Fig. 7a). Some groups have also suggested a role of CyPA in HCV replication.[20,21] Then, we synthesized individual siRNAs reported so far to be effective against CyPA, siCyPA-161, siCyPA-285, and siCyPA-459, and transfected them using a reagent with low cytotoxic activity to knock down endogenous CyPA (Fig. 7d). As shown in Figure 7c, the siRNAs directed against CyPA reduced HCV titers in MH14#12-1, and -4 cells. We previously observed that knockdown of CyPA little affected HCV replication in MH14 cells.[5] Here, by using a new transfection reagent with less cytotoxicity and higher knockdown efficiency, we observed the effect of CyPA knockdown on HCV replication, which suggests that CyPA-mediated regulation of HCV replication is strictly influenced by CyPA's expression level and cellular condition. Under this experimental condition, our RNAi experiments also displayed that knockdown of CyP40 (Fig. 7g), alternatively known as peptidylprolyl isomerase D (NM_005038), decreased the HCV titer (Fig. 7f) without significant cytotoxic effects, presenting CyP40 as additional cellular factor required for HCV replication.

CyPA was related to the CsA-resistant phenotype. We next asked which CyP subtype among CyPA, B, and 40 was related to the CsA resistance observed in our clones. To answer this question, we performed RNAi experiments in the CsA-resistant cell lines, CsR#11-2 and CsR#11-3 cells. Transfection of these cells with specific CyPB or CyP40 siRNAs resulted in the reduction of each subtype (Fig. 7d,g) and decreased the amount of HCV-RNA in CsR#11-derived cells and wild-type MH14#12-derived cells by approximately 50% (Fig. 7c,f). Thus, CyPB and CyP40 were likely to play roles in viral replication, even in the CsA-resistant cells. However, relative HCV titers were not reduced by CyPA knockdown in these CsA-resistant cells in contrast to the case with the wild-type replicon cells (Fig. 7c). A similar resistant phenotype to CyPA knockdown was observed in D320E

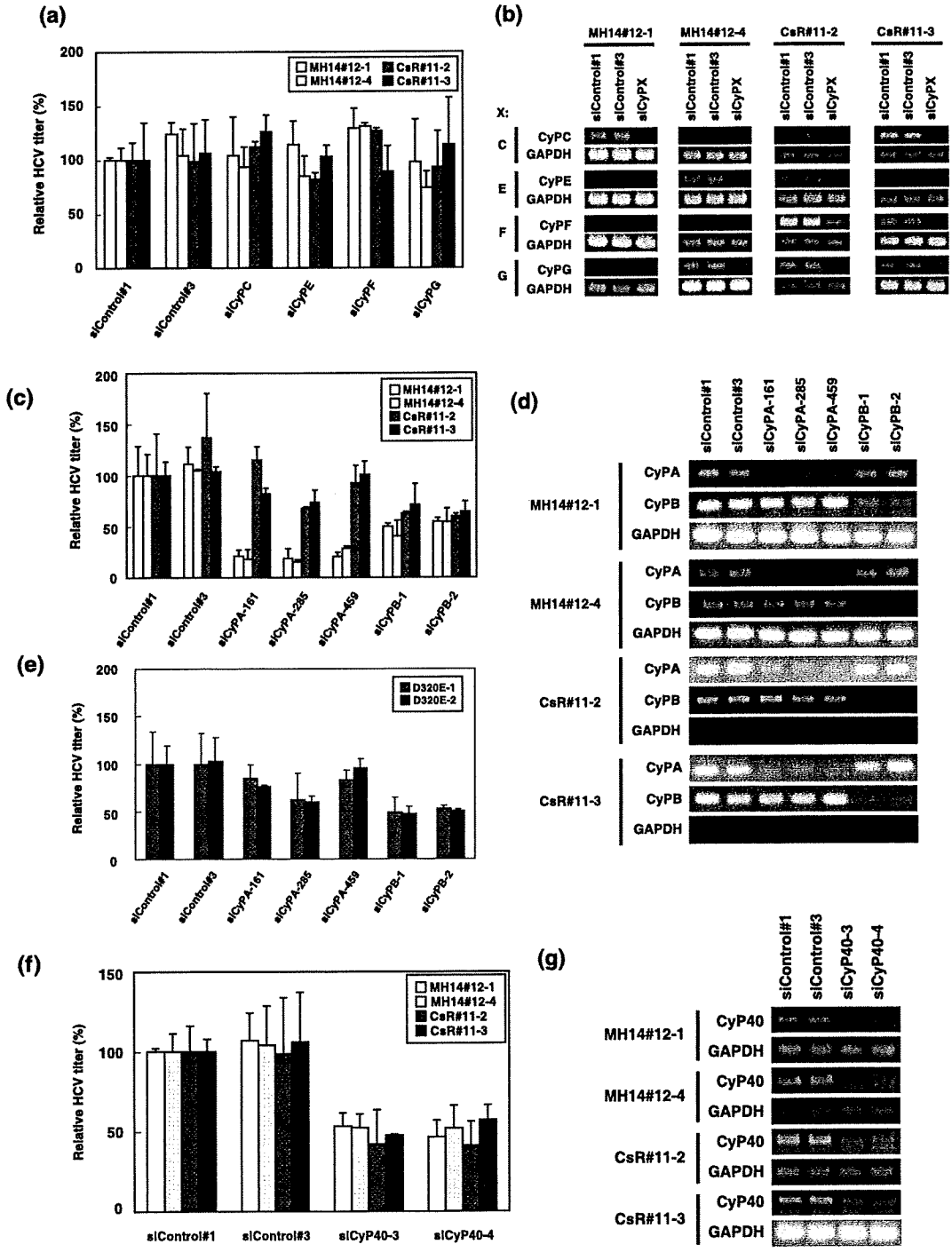

Fig. 7. Cyclophilin (CyP) subtypes related to anti-hepatitis C virus (HCV) effect of CyP inhibitor. MH14#12-derived cells, MH14#12-1 and MH14#12-4 cells, and CsR#11-2 and CsR#11-3 cells, were transfected with siRNAs specific for CyPC (siCyPC), CyPE (siCyPE), CyPF (siCyPF), and CyPG (siCyPG) (a); or those specific for CyPA (siCyPA-161, siCyPA-285, and siCyPA-459) and CyPB (siCyPB-1 and siCyPB-2) (c); or those specific for CyP40 (siCyP40-3 and siCyP40-4) (f); or randomized siRNA controls (siControl#1 and siControl#3). D320E cells were also transfected with the above siRNAs specific for either CyPA or CyPB (e). At 5 days post-transfection, the levels of HCV-RNA were quantified by real-time RT-PCR analysis. The mRNA levels of individual CyP subtypes, CyPC, CyPE, CyPF, and CyPG (CyPX corresponds to each CyP subtype indicated on the left side of the panels) (b), or CyPA and CyPB (d), or CyP40 (g) were measured using glyceraldehydes-3-phosphate dehydrogenase (GAPDH) as internal controls by RT-PCR analysis at 5 days post-transfection. The data represent the means of three independent experiments.

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cell clones (Fig. 7e), showing that CyPA was related to the CsA-resistance conferred by D320E mutation. The CsA-resistant clones obtained in this study were likely to have acquired CyPA independence for efficient HCV replication.

Discussion

Given that CPIs suppressed HCV viral load in cell culture and in patients with chronic hepatitis C,[14,15] CPIs are expected to be new anti-HCV agents. It is important to further reveal the factors related to CPI's anti-HCV activities, thinking over the practical use of CPIs with maximized efficacy and high specificity facing challenges such as side effects and the emergence of resistance to them in clinical settings. Here, we isolated and characterized a variant resistant to CPIs using a HCV subgenomic replicon system. A mutation in NS5A, D320E, was shown to confer the CPI-resistance to HCV replicon, resulting in CyPA independence for efficient viral replication. In addition, assessment of a wide range of CyP subtype knockdown experiments found CyP40 to be a new contributor to HCV replication.

Of the mutations identified, Q86R substitution in NS3 dramatically enhanced the capacity of replication. This mutation was observed as compensatory mutation[22] following the selection of replicons resistant to protease inhibitors SCH503034[23] and SCH6.[24] In addition, this mutation also appeared following the passaging of replicon cells in the absence of drug pressure.[25,26] In actuality, this mutation did not contribute to CsA resistance in the replicon cells (Fig. 5b), and thus was thought to be an adaptive mutation similar to that suggested in previous reports. I252T mutation in NS3, on the other hand, severely reduced the replicative fitness of HCV. The significance of I252T mutation under CsA treatment remains to be studied. The alteration of amino acid residue in NS5A, D320E, resulted in the conversion of HCV replicon to that of the CsA-resistant phenotype. There have been no reports of a link between NS5A and individual CyP subtype in the context of HCV replication, though mutations in NS5A were found to be keys for the acquisition of CsA resistance.[27] We have previously reported that CyPB was important for viral replication, but NS5A did not interact with CyPB in MH14 cells.[5] Indeed, in cells harboring replicons with D320E mutation, CyPB was found to contribute to viral replication but was not related to CsA resistance, as knockdown of CyPB diminished the viral titer to approximately half, similar to the case of the wild type. Therefore, other CyP molecules crucial for viral replication were suggested to be involved in the phenomenon of the CsA resistance. CyPA is another CyP subtype recently published to be critical for HCV replication in connection with viral polymerase.[20,21] Our CsA-resistant replicon cells displayed resistance to CyPA knockdown when compared to wild-type replicon, suggesting that CyPA participated in the replication process and the CsA resistance was due in part to resistance to CyPA inhibition. Therefore, it might be possible that NS5A functions coordinated with CyPA for viral replication and D320E mutation could contribute to enhancement of the relation. But NS5A was unable to bind CyPA *in vitro*.[5] NS5A might be regulated by CyPA associated with other cellular or viral factors during HCV replication. The fact that the D320 falls upon one of the two discontinuous domains needed for the interaction with NS5B to functionally modulate it[28,29] lead us to presume influence of NS5A on the reported NS5B–CyPA interaction.[21] In addition to CyPA and CyPB, which have been published to be cellular factors required for HCV replication, the results suggested that another CyP subtype, CyP40, contributed to viral replication. Acting as a molecular chaperone, it is conceivable that CyP40 directly interacts with viral proteins to boost their functions, similar to CyPA and CyPB. Heat shock protein (Hsp) 90 is a well-known chaperone forming complex with CyP40. Recently, Hsp90 was shown to be harnessed by HCV NS5A via the FK-506 binding protein 8 (FKBP8) bridge. FKBP8 is a homologous immunophilin of CyP40 that is required for viral replication.[30] This result led to the hypothesis that CyP40 serves as a linker between viral proteins and Hsp90. CyP40 is also known to associate with estrogen receptor (ESR) and we have published that ESRα escorted NS5B to replication complex (RC).[31] We also speculate CyP40 connected to ESRα may be important for the recruitment or functional reinforcement of viral and cellular factors for HCV replication in RC. Among these CyP subtypes, CyPA dependency was suggested to be one of the determinants of CsA sensitivity. Interestingly, CyPB and CyP40 play significant role in HCV replication even in CsA-resistant replicon cells. Another CPI, NIM811, is also likely to target CyPA, at least in part, to suppress HCV replication, given the cross-resistance of CsA-resistant replicon to MIN811. However, there is still also the possibility that other CyPs mediate anti-HCV effect of NIM811, which needs to be elucidated in future study.

Understanding the profile of CPI-resistance mutations in the HCV genome and the viral and cellular factors involved will aid in the progression of CPI-centered strategies preparing for the problem of drug resistance. In addition, the cells harboring CPI-resistant replicons established here may prove beneficial for further characterization of resistance mechanisms and for the screening of novel compounds with the potential of clinical application to defeat CPI-resistant variants. Also, CyP40 as a contributor to HCV replication could become another specific antiviral target. The information arising from this study is expected to contribute to the successful use of CPIs against a liver carcinogen, HCV.

Acknowledgments

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Strain-Dependent Viral Dynamics and Virus-Cell Interactions in a Novel *In Vitro* System Supporting the Life Cycle of Blood-Borne Hepatitis C Virus

Hussein Hassan Aly,[1,2] Yue Qi,[3] Kimie Atsuzawa,[4] Nobuteru Usuda,[4] Yasutsugu Takada,[5] Masashi Mizokami,[6] Kunitada Shimotohno,[7] and Makoto Hijikata[1,3]

We developed an *in vitro* system that can be used for the study of the life cycle of a wide variety of blood-borne hepatitis C viruses (HCV) from various patients using a three-dimensional hollow fiber culture system and an immortalized primary human hepatocyte (HuS-E/2) cell line. Unlike the conventional two-dimensional culture, this system not only enhanced the infectivity of blood-borne HCV but also supported its long-term proliferation and the production of infectious virus particles. Both sucrose gradient fractionation and electron microscopy examination showed that the produced virus-like particles are within a similar fraction and size range to those previously reported. Infection with different HCV strains showed strain-dependent different patterns of HCV proliferation and particle production. Fluctuation of virus proliferation and particle production was found during prolonged culture and was found to be associated with change in the major replicating virus strain. Induction of cellular apoptosis was only found when strains of HCV-2a genotype were used for infection. Interferon-alpha stimulation also varied among different strains of HCV-1b genotypes tested in this study. *Conclusion:* These results suggest that this *in vitro* infection system can reproduce strain-dependent events reflecting viral dynamics and virus-cell interactions at the early phase of blood-borne HCV infection, and that this system can allow the development of new anti-HCV strategies specific to various HCV strains. (HEPATOLOGY 2009;50:689-696.)

Hepatitis C virus (HCV) is a serious problem worldwide, with 3% of the world's population chronically infected.[1] Chronic infection with HCV may lead to high rates of liver cirrhosis and hepatocellular carcinoma.[2] Because the HCV standard therapy is still insufficient for treating many patients,[3] the development of more effective and less toxic anti-HCV agents is desired. The virological studies required to reach this goal need reproducible and efficient HCV proliferation in cell culture. An *in vitro* infection system using recombinant HCV-JFH1 was developed. In this system, HuH7 cells transfected with *in vitro*-synthesized JFH1-RNA were

Abbreviations: 2D, two-dimensional; 2D-HuS-E/2, HuS-E/2 cells cultured in two-dimensional condition; 3D, three-dimensional; 3D/HF, 3D hollow fibers; 3D-HuS-E/2, HuS-E/2 cells cultured in three-dimensional condition in the hollow fibers; HCV, hepatitis C virus; IFN-α, interferon alpha; LDH, lactate dehydrogenase; p.i., postinfection; RFB, radial-flow bioreactor; RT-PCR, reverse transcription polymerase chain reaction.

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shown to secrete infectious viral particles.[4] This system, however, requires the combination of HuH-7-derived cell lines and JFH1-based constructs, limiting its usefulness for studying other HCV strains. Because HuH-7 cells cannot support the complete life cycle of blood-borne HCV (bbHCV) derived from clinical samples,[5] this system is insufficient for studying all the events related to bbHCV infection.

Many researchers have attempted to develop an *in vitro* system for bbHCV.[6-8] These current systems, however, are still insufficient due to their low efficiency for infectivity and replication of bbHCV. Working toward this same goal, we recently established immortalized primary human hepatocyte cell lines by transducing them with E6 and E7 genes from the human papilloma virus 18.[5,9] As expected, we observed improved infection and replication of bbHCV especially in one of these cell lines (HuS-E/2 cells) that showed a similar expression profile to that of human primary hepatocytes, but this strategy did not improve production of infectious particles.

Recently, a hybrid artificial liver support system was developed using animal hepatocytes cultured in a three-dimensional hollow fiber (3D/HF) system. This bioartificial liver showed several characteristic features of liver tissue for more than 4 months.[10-12] By growing our HuS-E/2 cells in a similar 3D culture[5] the gene expression profile was improved to more closely match that of human primary hepatocytes. Because the 3D cell culture condition more closely reproduces the *in vivo* environment of hepatocytes,[13] culturing these cells in this manner may support the entire HCV life cycle.

In this study we utilized this small 3D culture system and showed it to be ideal for culturing HuS-E/2 cells for the study of bbHCV infection. Using this system we are now able to study the variable patterns of the life cycle of different bbHCV strains as well as HCV-related cellular events.

Materials and Methods

Cell Culture. HuS-E/2 cells were cultured as previously described.[5] For the 3D/HF system, HuS-E/2 suspension was injected into the lumen of HF (Toyobo, Osaka, Japan) made from cellulose acetate and containing pores for nutrients and waste exchange (Supporting Fig. 1). The bundles were centrifuged to induce organoid formation. The cells in the fibers were cultured in 12-well plates (two capillary bundles per well) with gentle rotation using serum-free medium (Toyobo) in a CO_2 incubator at 37°C. The number of cells was adjusted to 3 × 105 cells per two-capillary bundle at the start of each experiment.

RNA Experiments. Total RNA was extracted from two-dimensional (2D) cultured cells, patient sera, or from 100 times concentrated culture medium as previously described.[4,5] For cells cultured in the 3D/HF, sterile scissors were used to cut each fiber into small pieces (1 mm^2 each), which were then solubilized in Sepasol RNA-1 (Nacalai Tesque, Kyoto, Japan). RNA was then extracted according to the manufacturer's protocol. Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed as described.[5]

HCV Infection. HCV infection experiments were carried out using sera from HCV patients. The amount of each inoculum was adjusted so as to add similar amount of HCV-RNA to the medium of the cells. After 24 hours, the cells were washed three times with phosphate-buffered saline (PBS) and cultured for the designated times. To assess the passage of infectivity, 12 mL of culture medium from the primary infected cells was collected, concentrated 100 times by filtration through Amicon Ultra-15, Ultracel-10K filters (Millipore, Carrigtwohill, Cork, Ireland), and 40 μL concentrated medium/well was used to infect naïve HuS-E/2 cells. All experiments were done with approval of the Ethical Committee of Kyoto University. Informed consent from patients was required for this approval.

Cloning and Sequencing. To amplify the complementary DNA (cDNA) fragment corresponding to hypervariable region 1 (HVR-1),[14] a nested RT-PCR was performed using Superscript III (Invitrogen, Carlsbad, CA) and PrimeSTAR HS DNA Polymerase (Takara, Tokyo, Japan). Reaction conditions were adjusted according to the manufacturer's protocol. Primers used were previously described[15] and are shown in Supporting Table 1. PCR products were then purified and cloned using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen). Ten recombinant clones were randomly isolated for each PCR product and sequenced as described.[16]

Quantitative Detection of HCV Core and Interferon alpha (IFN-α) Protein by Enzyme-Linked Immunosorbent Assay (ELISA). The culture medium of infected cells was collected and concentrated 100 times as previously mentioned for the detection of HCV-core, or used directly for detection of IFN-α. HCV core protein was quantified using the Trak-C Core ELISA (Ortho Clinical Diagnostics, Neckargemünd, Germany). IFN-α was quantified using the Human IFN-A ELISA kit (PBL Biomedical Laboratories, Piscataway, NJ). Light absorbance was then measured using a Wallac 1420 multilabel counter (PerkinElmer Life Science, Waltham, MA).

Cytotoxicity Assay. Culture medium was collected from HCV-infected cells and used for measuring lactate dehydrogenase (LDH) levels using an LDH cytotoxicity

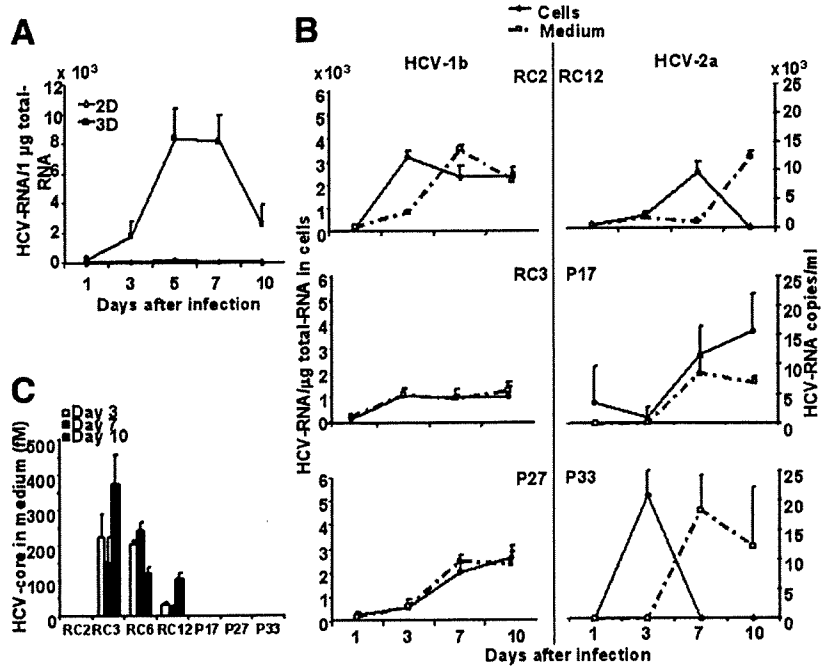

Fig. 1. Infection and proliferation of bbHCV in 3D-HuS-E/2 cells. (A) The quantity of HCV genomic RNA in 1 µg total RNA of 2D- or 3D-HuS-E/2 cells infected with HCV-RC6 was determined at each timepoint after infection by real-time RT-PCR analysis. (B) 3D-HuS-E/2 cells were infected with HCV-1b-containing sera: RC2, RC3, and P27; or HCV-2a-containing sera: 4: RC12, P17, P33. The quantity of HCV genomic RNA in the infected cells was determined as in (A). The culture medium from the last 2 days at each timepoint was collected, concentrated, and the amount of HCV-RNA (B) or HCV-core (C) was measured. Data represent the mean ± standard deviation (SD) of three independent experiments.

detection kit (Takara Biomedicals). Light absorbance was then measured as described above.

Sucrose Density Gradient. The culture medium of the infected cells was collected, concentrated 500 times, and loaded onto a 20%-50% (wt/vol) sucrose gradient containing 50 mM PBS, 100 mM NaCl, and 1 mM EDTA, followed by centrifugation at 100,000*g* for 16 hours at 4°C in a SW41Ti rotor (Beckman, Fullerton, CA). The gradient was fractionated into 31 fractions that were used for HCV-RNA and core detection and HCV infection into naïve cells as described above.

Electron Microscopy. The 1.12 g/mL fraction obtained by the sucrose density gradient showed the secondary infection activity as analyzed by transmission electron microscopy. The fraction was ultracentrifuged and the almost all supernatant was removed. The residual 10 µL of the solution was directly applied to a formvar-carbon grid for negative staining with 1% uranyl acetate solution and observed with an electron microscope (JEOL1010, JEOL, Tokyo, Japan).

Results

HuS-E/2 Cells Cultured in 3D/HF System Are Highly Permissive for Infection and Proliferation of bbHCV. We compared the ability of HuS-E/2 cells cultured in the 3D/HF system (3D-HuS-E/2 cells) to those cultured as a monolayer (2D-HuS/E2 cells) to reproduce infection by HCV genotype 1b (HCV-RC6), derived from patient serum (RC6). The HCV-RC6 RNA levels in the 3D-HuS/E2 cells were significantly higher at all timepoints (Fig. 1A), showing that the 3D/HF system greatly improves the proliferation of bbHCV in HuS-E/2 cells. We observed that both the early stages of infection and the continuous replication of HCV-RC6 in HuS-E/2 cells was improved by 3D/HF culture when the culture conditions were changed after the infection from 3D/HF to 2D and vice versa (Supporting Fig. 2).

As reported,[17] blocking CD81, an HCV-supposed entry receptor, during infection significantly impaired HCV proliferation into 3D-HuS-E/2 cells (Supporting Fig. 3), suggesting that CD81 is essential for HCV infectivity in 3D-HuS-E/2 cells. Although the expression level of CD81 mRNA in 3D-HuS-E/2 cells was observed, no significant change from 2D-HuS/E2 cells was found (data not shown), indicating that the quantity of CD81, at least, is not responsible for the improvement.

We then examined whether this system can be used for proliferation of six different bbHCV samples, three of which are HCV-1b (HCV-RC2, HCV-RC3, and HCV-P27) and three HCV-2a genotypes (HCV-RC12, HCV-P17, and HCV-P33) (Fig. 1B). Proliferation of HCV-RNA in the cells was seen in all six cases, suggesting that this system can be widely used for analysis of infection and proliferation of bbHCV strains. HCV-RNA and HCV-core were also detected in the culture medium (Fig. 1B). Different HCV strains showed variable patterns of proliferation and HCV-core secretion into the medium. Although HCV-core was detected from day 3 onward when

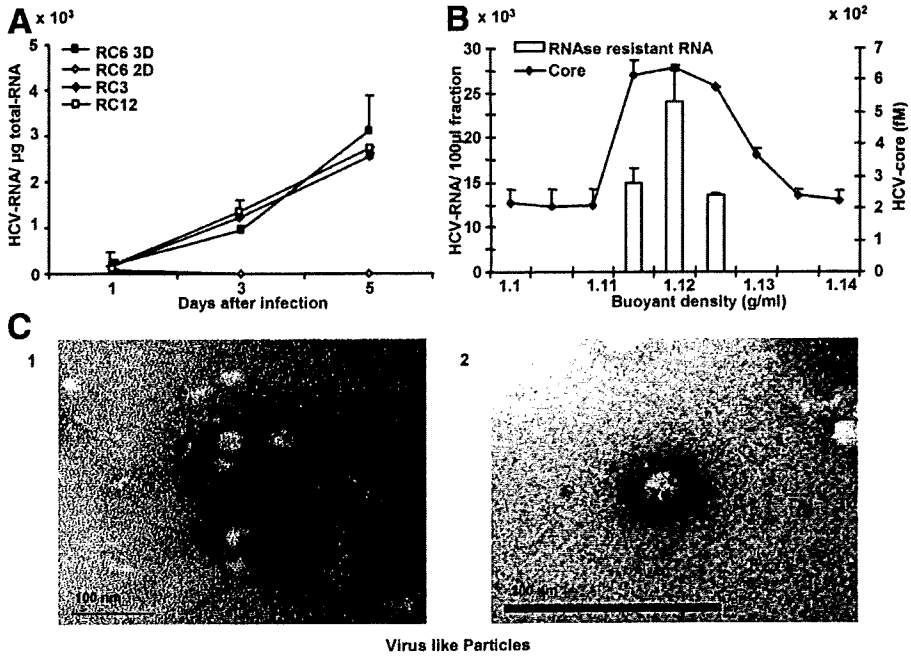

Fig. 2. Production of infectious virus-like particles from 3D-HuS-E/2 cells infected with different HCV strains. (A) The culture medium of 3D-HuS-E/2 cells infected with HCV-RC3 or HCV-RC6 was collected from days 5 to 7 p.i. and for HCV-RC12 from days 23 to 25 p.i. The culture medium of 2D-HuS-E/2 cells infected with HCV-RC6 was also collected from days 5 to 7 p.i., and used to treat naïve 3D-HuS-E/2 cells. The quantity of HCV genomic RNA in 1 μg of total cellular RNA was determined as in Fig. 1. (B) The concentrated culture medium of 3D-HuS-E/2 cells infected with HCV-RC3 was collected from days 5 to 7 p.i., and fractionated by ultracentrifugation with a 20%-50% sucrose density gradient. HCV-core protein and the RNase A-resistant HCV-RNA in the different fractions were quantitatively analyzed using an HCV-core ELISA kit and real-time RT-PCR, respectively. Data represent the mean ± SD of three independent experiments. (C) Photomicrograph showing negatively stained virus-like particles from the culture medium of HCV-RC3-infected 3D-HuS-E/2 cells (arrowheads, panels 1 and 2). The arrows indicate the spike-like structures found on the surface of the virus-like particles (panel 2).

RC3, RC6, and RC12 were used for infection, it was undetectable when RC2, P17, P27, and P33 sera were used, similar to 2D-HuS-E/2 cells infected with HCV-RC6 (Fig. 1C).

Production of Infectious Particles from 3D-HuS-E/2 Cells Infected with bbHCV. The culture media from 2D or 3D-HuS/E2 cells infected with RC6 serum (Fig. 1A) were collected from days 5 to 7 postinfection (p.i.), concentrated, and inoculated into naïve 3D-HuS-E/2 cell culture media. HCV-RNA's proliferation in the infected cells was only detected when using the culture medium from 3D-HuS-E/2 cells and not 2D-HuS-E/2 cells (Fig. 2A). Media collected from HCV-RC3 at days 5 to 7 and from HCV-RC12 from days 23 to 25 p.i. were also able to infect naïve cells (Fig. 2A). These data suggested the production and secretion of infectious virus-like particles. To investigate this further, biophysical analysis was performed. The culture medium of HCV-RC3 infected 3D-HuS-E/2 cells at day 7 p.i. was fractionated using a sucrose density gradient after RNase A treatment. HCV core was detected in the 1.11 to 1.14 g/mL fractions; similarly, the nuclease-resistant HCV RNA peaked in the 1.12 g/mL fraction (Fig. 2B). Furthermore, only the 1.12 g/mL fraction was able to infect naïve cells as examined above (data not shown). This fraction was pelleted by ultracentrifugation and examined by electron microscopy with negative staining. We observed 33-nm to 45-nm diameter spherical particles (Fig. 2C, panel 1) with spike-like structures from 7-9 nm in length on the surface (Fig. 2C, panel 2), consistent with HCV morphology reported previously in HCV patients.[18] These were detected in the sample collected from HCV-RC3-treated but not mock-treated 3D-HuS-E/2 cells. These data suggest that production of infectious virus-like particles occurs in 3D-HuS-E/2 cells infected with some bbHCV strains. It is therefore likely that 3D-HuS-E/2 cells can be used to reproduce nearly all steps in the HCV life cycle.

Prolonged Culture of HCV-Infected Cells in the 3D Hollow Fiber System. For HCV-RC6-infected cells (Fig. 3A), the amount of HCV-RNA in the cells fluctuated during the 30-day culture period. The levels of both HCV-RNA and HCV-core in the medium showed a similar pattern of fluctuations that peaked on days 5 and 20 p.i. Unlike RC6, the pattern of HCV-RNA levels in the medium of RC12-infected cells showed a negative

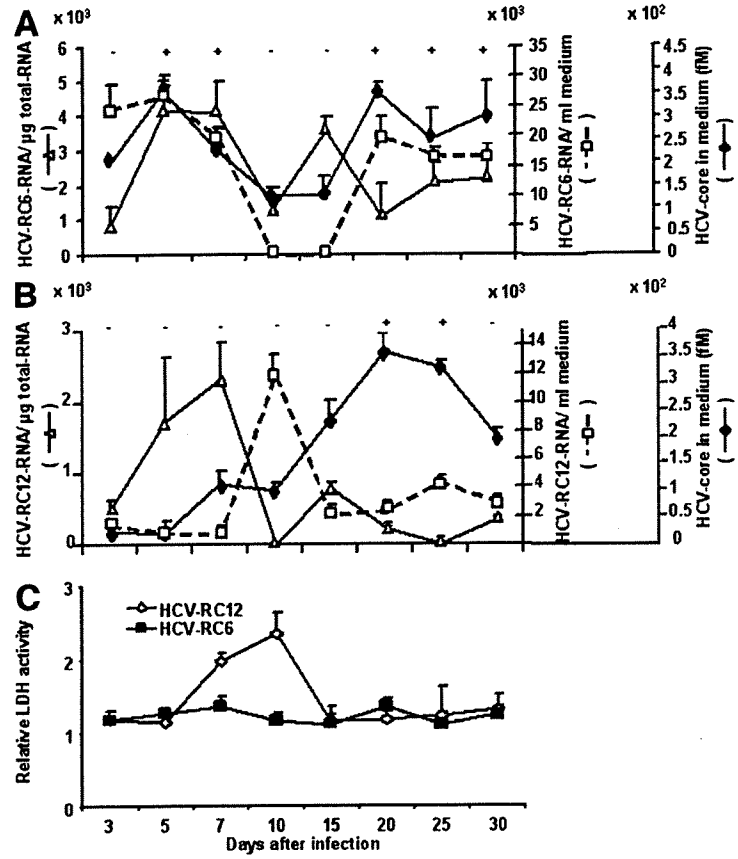

Fig. 3. Prolonged culture of HCV-infected cells in the 3D/HF system. After infection with HCV-RC6 (A) and HCV-RC12 (B), 3D-HuS-E/2 cells were cultured for 30 days with a medium change every 2 days. The HCV-RNA in the cells and medium as well as the HCV-core in the medium were quantitatively analyzed at the designated timepoints as in Fig. 1. Culture media were also used to treat naïve 3D-HuS-E/2 cells to examine the secondary infection as in Fig. 2. (+) and (−) indicate detection or no detection of secondary infection. (C) Culture media of HCV-RC6 and HCV-RC12 infected cells collected at each timepoint were used for the detection of LDH levels released from dead cells. LDH levels were normalized to uninfected cells cultured for the same time. Data represent the mean ± SD of three independent experiments.

correlation with that detected in the cells. This was clearly seen on day 10 p.i., when a sharp increase and decrease of HCV-RNA in the medium and the cells, respectively, was observed (Fig. 3B). Similarly, the amount of HCV-core detected in the medium throughout the culture was not correlated with RNA levels in the medium. Instead, core levels were very low in the first 10 days, at which time levels increased, reaching a peak on day 20 p.i. (Fig. 3B). Culture media from cells infected with HCV-RC6 from days 5 to 7 and 20 to 30 p.i. (Fig. 3A) and that from HCV-RC12 from days 20 to 25 p.i. showed passage of infectivity (Fig. 3B). All culture media showing infectivity appeared to have a high amount of HCV-core protein.

Clonal Changes in HCV During Prolonged Culture. In order to perform a populational analysis to understand the fluctuating pattern seen during HCV proliferation, two sera with limited HCV variants, HCV-RC6 (two major strains) and -RC12 (single major strain) from immunosuppressed liver transplantation patients with recurrent HCV were used in the previous prolonged infection experiment. The variants' composition was analyzed by single-strand confirmation polymorphism analysis for HCV-HVR1 (Supporting Fig. 4). RC6 serum (Fig. 4A) showed two different major sequences, HCV-RC6-1 and -2 strains, which constituted 60% and 40%, respectively, and shared 85% homology. In cells infected with HCV-RC6 the nucleotide sequence of HVR1 on day 5 showed 97% homology to HCV-RC6-1, and on day 20 p.i. it showed 97% homology to HCV-RC6-2. These data suggest selection of the dominant HCV strain in the cells over time. For RC12 (Fig. 4B), the nucleotide sequence on day 5 p.i. had only one nucleotide difference from that of the HCV from the original serum. The sequence from day 20 p.i. was four nucleotides different from that from the serum, and five different from the cells on day 5 p.i. These data indicated that each peak of HCV-RNA that appeared in the cells infected with RC12 serum included primarily a single HCV strain with a slightly different genomic sequence. This suggests that the periodic appearance of HCV-RNA peaks in the cells infected with a particular HCV strain is a result of selection and/or mutation of HCV strains during the prolonged culture period.

Cellular Response Induced by bbHCV Infection. At day 10 p.i., HCV-RNA levels in the culture medium rose and RNA levels in 3D-HuS-E/2 cells infected with HCV-RC12 dropped (Figs. 1B, 3B). To determine if this was caused by a cytotoxic effect of HCV infection, LDH levels were measured in the culture medium of HCV-RC6- and

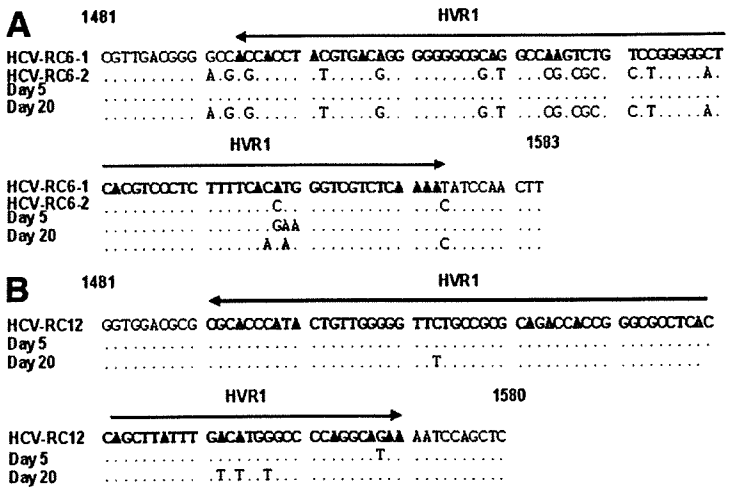

Fig. 4. Comparison of HCV-HVR1 sequences in the serum used for infection and the HCV replicating in the cells on days 5 and 20 after infection of HCV-RC6 (A) or HCV-RC12 (B). Nucleotide numbering was based on HCV-J1 sequence (GenBank Access. No. D10749). Three additional nucleotides were found at the 5′-terminal end of the E2 regions of all RC6 sequences. The major sequence present in the serum used for infection is shown in the upper row in each panel. Dots represent the identical nucleotides.

HCV-RC12-infected 3D-HuS-E/2 cells. LDH activity showed a strong correlation with HCV-RNA levels in the medium on day 10 p.i. in HCV-R12-infected cells (Fig. 3B), suggesting a cytotoxic effect of HCV-RC12 that was not observed in the case of HCV-RC6 (Fig. 3A,C). To determine if this HCV infection-mediated cytotoxicity is due to apoptosis, as with other viruses belonging to the Flaviviridae family,[19] the involvement of caspase was examined using the caspase inhibitor z-VAD-fmk. A significant dose-dependent reduction in HCV-RNA levels in the medium and LDH activity (Fig. 5A,B) was found, whereas no significant effect was observed on the viability of noninfected cells (Fig. 5B) or intracellular HCV-RNA levels (Fig. 5A). This suggested that the cytotoxic effect of HCV infection is mediated by apoptosis. It is noteworthy that HCV-induced cytopathicity was also found when HCV-P17 and HCV-P33 samples were used for infection (both are HCV-2a genotype) and was not reproduced in any of the HCV-1b genotype samples used in this work (Fig. 5C).

After infection with HCV-RC6, no cytotoxicity was detected that might have inhibited HCV-RC6-1 proliferation in the cells. However, HCV-RC6-2 RNA replaced HCV-RC6-1 RNA during prolonged culture. To assess a

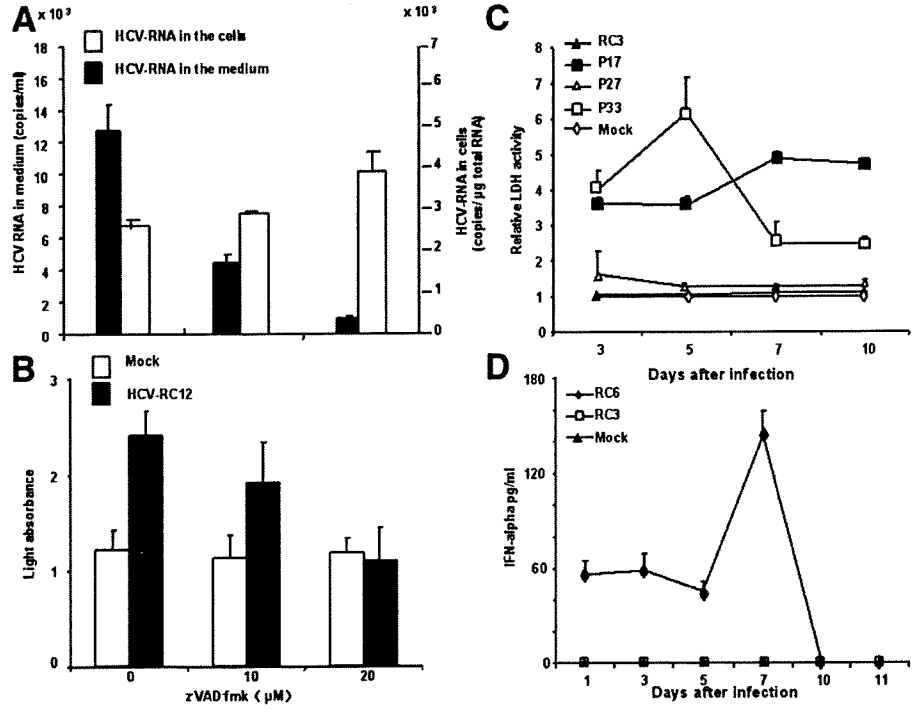

Fig. 5. Cellular response of 3D-HuS-E/2 cells infected with bbHCV. 3D-HuS-E/2 cells infected with HCV-RC12 and mock-treated cells were cultured for 10 days in the presence of z-VAD-fmk (0, 10, and 20 μM). (A) HCV-RNA in the cells and medium on day 10 was measured as in Fig. 1. (B) LDH levels in the medium on day 10 after infection with HCV-RC12 was measured as in Fig. 3. (C) Culture media of HCV-RC3, HCV-P17, HCV-P27, HCV-P33, and mock-infected cells collected at designated points were used for the detection of LDH levels. (D) IFN-α levels in the culture media of HCV-RC6, HCV-RC3, and mock-infected cells collected at each designated timepoint were measured by ELISA. Data represent the mean ± SD of three independent experiments.

possible role of the innate-immune response in this phenomenon, the production of IFN-α in the medium was measured during the first 11 days p.i. IFN-α production was detected as early as day 1 p.i., reached a peak at day 7 p.i., and was then rapidly lost (Fig. 5D). These data suggest that HCV-RC6-1 infection induced the innate-immune response of the cells, possibly leading to suppression of its proliferation. In contrast to HCV-RC6-1, HCV-RC3 did not show any stimulation of IFN-α production upon infection in the first 10 days, showing a possible strain-dependent evasion from the host defense within the same genotype.

Discussion

In this study we report the development of a novel system that reproduces bbHCV infection, proliferation, and production of infectious virus. The most recent models used in the study of the life cycle of HCV infection are based on subclones of HuH-7 cells infected with JFH1 recombinant virus or its derivatives.[4] HuH-7 cells and its subclones, however, do not support the entire life cycle of the bbHCVs present in patients' blood.[5] Moreover, HCV has considerable diversity and variability. It is generally classified into six major genotypes and more than 100 subtypes.[20] This huge pool of natural HCV variants causes a wide variety of diseases, including chronic hepatitis, cirrhosis, and hepatocellular carcinoma.[21] JFH1, however, is a single isolate of HCV genotype 2a that was originally derived from a patient with rare fulminant hepatitis.[4] We suggest that our newly established system has an important advantage because it supports the entire life cycle of a variety of HCV strains and genotypes.

Due to the lack of some *in vivo* factors, including host immune response, *in vitro* systems may not completely reproduce the *in vivo* situation. However, *in vitro* experimental systems seem to be important to simplify particular events from the complex situation *in vivo*. From that standpoint, our cell culture system is likely reproducing the early event of HCV infection in the absence of host-immune responses and supporting whole life cycle of the blood-borne HCV. Several *in vitro* hepatocyte culture systems have been reported to be useful for studying the infection and replication of bbHCV.[5-8,22] Only the radial-flow bioreactor (RFB) 3D culture system demonstrated production of infectious viruses.[22] In our studies we observed not only the enhancement of HCV replication, but also the production of infectious HCV particles in the medium using the 3D/HF system. These data suggest that some structure of the cell mass formed by the 3D culture system, most likely the polar character, is essential for the life cycle of bbHCV. The RFB system is composed of a dedicated device containing 1×10^9 FLC4 cells with a culture area of 2.7 m^2.[22] It can only be used to study HCV particle production in the medium and not the cellular events that accompany the HCV life cycle. In contrast, because cells grown in our 3D/HF system are cultured in 12-well plates at a density of 3×10^5/fiber, it is much simpler to study both viral and cellular events.

The production of infectious particles was not detected with infection by different HCV strains, despite detecting equivalent levels of HCV-RNA in the cells (Fig. 1B,C). Delayed production of infectious particles was also observed in cells infected with HCV-RC12 after prolonged culture. A similar delay was also observed in the RFB system.[22] Considering the relative stability of HuS-E/2 cells[5] and the relatively high frequency of the change in HCV population in the cells,[16] it is likely that mutation of the HCV genome and/or selection of clones during prolonged culture improved the productivity of infectious particles. A marked improvement of infectious particle production by substitution of the structural proteins of the genome was also reported in the recombinant HCV production system.[23] The lack of production of infectious particles soon after infection may serve to avoid an early strong response from the host immune system, and demonstrates a novel mechanism of latent infection by HCV. Although they may not be associated with plasma components as those present *in vivo*, HCV virus-like particles produced by this system showed a close resemblance to those isolated from infected HCV patients because they showed the same size[18] and were within the fraction range.[24] They may help in the study of viral and cellular factors required for particle production and the possible receptors utilized for infection with different HCV strains.

Fluctuation in HCV proliferation was observed during the prolonged culture of 3D-HuS-E/2 cells infected with bbHCV (Fig. 3A,B), consistent with previous reports in other culture systems.[6,22] This fluctuation was associated with a change in viral quasispecies, suggesting that an HCV strain having a growth advantage proliferates selectively and dominantly in these culture conditions. Because the progressive emergence of each dominant strain was only temporary, it is highly likely that the infection and proliferation of such an HCV strain is suppressed by cellular mechanism(s). Our results suggest that there are actually two cellular mechanisms functioning to do this. The first is the involvement of the innate immune system, as evidenced by the secretion of IFN-α during the first week of infection (Fig. 5D). This is the first report of secretion of IFN-α from cultured cells infected with bbHCV. Although recent reports suggest that stimulation of the IFN pathway by HCV infection could be impaired by HCV NS3-4a proteinase-mediated cleavage of IPS-

1,[25] our results suggest that not all bbHCVs possess a host cell suppressive function. The second mechanism is HCV-induced cell death (Fig. 3C). Almost all the studies reporting HCV-induced apoptosis used hepatocellular carcinoma cell lines.[26,27] Because it has been established that the inability to undergo apoptosis is essential for the development of cancer,[28-30] our use of immortalized, noncancerous HuS-E/2 hepatocytes may make it possible to reproduce the physiological response of the cells to bbHCV infection more closely. Although HCV-induced apoptosis was not found when HCV-1b was used for infection, it was found in all cases where HCV-2a was used, suggesting a higher cytopathic tendency of the HCV-2a genotype. HCV proliferation was continuously found even after the suppression of the first peak of RNA production during prolonged culture. How HCV survives under those conditions is still unknown. Further studies to clarify the molecular mechanisms involving the HCV-cell interaction can be done using this novel 3D culture system that reproduces the infection of a variety of bbHCVs.

In conclusion, we have established a new *in vitro* culture system that can support the entire life cycle of a variety of HCV isolates and genotypes. Although this *in vitro* model system may not completely reproduce the *in vivo* situation, we believe it is the first *in vitro* system showing HCV strain-dependent virus/cell interaction including induction of cellular apoptosis and/or evasion from cellular innate immune response, which may make it a good tool for analysis of virus/host interaction together with the development of new anti-HCV strategies for the different bbHCV strains.

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TORC2, a Coactivator of cAMP-response Element-binding Protein, Promotes Epstein-Barr Virus Reactivation from Latency through Interaction with Viral BZLF1 Protein*[S]

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Reactivation of the Epstein-Barr virus from latency is dependent on expression of the viral BZLF1 protein. The BZLF1 promoter (Zp) normally exhibits only low basal activity but is activated in response to chemical inducers such as 12-*O*-tetradecanoylphorbol-13-acetate and calcium ionophore. We found here that Transducer of Regulated cAMP-response Element-binding Protein (CREB) (TORC) 2 enhances Zp activity 10-fold and more than 100-fold with co-expression of the BZLF1 protein. Mutational analysis of Zp revealed that the activation by TORC is dependent on ZII and ZIII *cis* elements, binding sites for CREB family transcriptional factors and the BZLF1 protein, respectively. Immunoprecipitation, chromatin immunoprecipitation, and reporter assay using Gal4-luc and Gal4BD-BZLF1 fusion protein indicate that TORC2 interacts with BZLF1, and that the complex is efficiently recruited onto Zp. These observations clearly indicate that TORC2 activates the promoter through interaction with the BZLF1 protein as well as CREB family transcriptional factors. Induction of the lytic replication resulted in the translocation of TORC2 from cytoplasm to viral replication compartments in nuclei, and furthermore, activation of Zp by TORC2 was augmented by calcium-regulated phosphatase, calcineurin. Silencing of endogenous TORC2 gene expression by RNA interference decreased the levels of the BZLF1 protein in response to 12-*O*-tetradecanoylphorbol-13-acetate/ionophore. Based on these results, we conclude that Epstein-Barr virus exploits the calcineurin-TORC signaling pathway through interactions between TORC and the BZLF1 protein in reactivation from latency.

Epstein-Barr virus (EBV)[3] is a human γ-herpesvirus that predominantly establishes latent infection in B lymphocytes. Only a small percentage of infected cells switch from the latent stage into the lytic cycle and produce progeny viruses. Although the mechanism of EBV reactivation *in vivo* is not fully understood, it is known to be elicited by treatment of latently infected B cells with some chemical or biological reagents, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), calcium ionophore, sodium butyrate, or immunoglobulin (Ig). Stimulation of the EBV lytic cascade by any of those reagents leads to the expression of two presumed viral immediate-early genes, BZLF1 and BRLF1. The BZLF1 protein is a transcriptional activator that shares structural similarities to the basic leucine zipper (b-Zip) family transcriptional factors, and BZLF1 expression alone can trigger the entire reactivation cascade (1–3).

Expression of the BZLF1 gene is tightly controlled at the transcriptional level. The BZLF1 promoter (Zp) normally exhibits low basal activity usually and is activated in response to TPA or the other reagents described above. The minimal sequence of Zp necessary for the activation by the inducers is 233 bp in length (4). The region harbors at least three types of *cis* regulatory elements, referred to as ZI, ZII, and ZIII. Four copies of the ZI element (ZIA-D) are distributed within the minimal Zp. The myocyte enhancer factor 2D binds to ZIA, ZIB, and ZID (5), whereas Sp1 or Sp3 can bind to ZIA, ZIC, and ZID (6). A single ZII element is located near TATA, sharing homology with binding sites for the cyclic AMP-response element-binding protein (CREB) or the AP-1 family transcriptional factor (7, 8). Two copies of the ZIII element (ZIIIA, B) are bound by the BZLF1 protein. Previous studies have demonstrated that both ZI and ZII elements are necessary for the initial activation of the promoter by TPA/ionophore or IgG(2). Then, the expressed BZLF1 protein joins to further activate Zp by binding to the ZIIIA and B elements (9).

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[3] The abbreviations used are: EBV, Epstein-Barr virus; Zp, BZLF1 promoter; CREB, cyclic AMP-response element (CRE)-binding protein; TORC, Transducer of Regulated CREB; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; b-Zip, basic leucine zipper; CBP, CREB-binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IP, immunoprecipitation; IB, immunoblotting; ChIP, chromatin immunoprecipitation; siRNA, small interfering RNA; RT, reverse transcription; CMV, cytomegalovirus.

Transducer of Regulated CREB (TORC) 1, 2, and 3 were identified from a lymphocyte cDNA library as a family of CREB co-activators that bind to CREB and enhance CRE-mediated transcription in an Ser-133 phosphorylation-independent manner (10, 11). It was reported recently that TORCs are activated by the calcium-regulated phosphatase, calcineurin (12, 13). Dephosphorylation of TORC by the phosphatase triggers release from 14-3-3 proteins and translocation from cytoplasm to nucleus. Interestingly, the activation of EBV Zp is blocked by calcineurin inhibitors, such as cyclosporin A or FK506 (14). Based on these studies, we hypothesized that TORCs might be involved in the transcriptional activation of Zp, leading to a switch from latent state to the lytic replication.

In the present study we show that TORC1, -2, and -3 can all enhance Zp, especially with co-expression of BZLF1. TORCs activate the promoter through interaction not only with CREB but also the BZLF1 protein. We also provide evidence that the activation of the promoter by TORC2 is up-regulated by calcium-regulated phosphatase, calcineurin. These results indicate involvement of TORCs in EBV reactivation from latency.

EXPERIMENTAL PROCEDURES

Cell Culture and Antibodies—HEK293T, EBV-Bac-293, and GTC-4 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum. EBV-293 cells were prepared by transfection with EBV-Bac DNA (15) into HEK293 cells subcloned in our laboratory (16) followed by hygromycin selection. GTC-4 is a cell line established from an EBV-positive gastric cancer by Dr. M. Tajima (Teikyo University) (17). Akata, B95-8, and Tet-BZLF1/B95-8 cells were maintained in RPMI1640 as described previously (18, 19). To induce lytic EBV replication in Tet-BZLF1/B95-8 cells, a tetracycline derivative, doxycycline, was added to the culture medium at a final concentration of 2 μg/ml. The mouse anti-FLAG, hemagglutinin, -BZLF1, and -GAPDH antibodies were from Sigma, Roche Applied Science, Dako A/S, and Ambion, respectively. Rabbit anti-PCNA and -TORC2 antibodies were from Oncogene and Calbiochem, respectively, and rabbit anti-BMRF1 and -BALF5 antibodies have been reported previously (20). The anti-tubulin antibody was purchased from Cell Signaling. Horseradish peroxidase-linked goat antibodies to mouse or rabbit IgG were from Amersham Biosciences. Horseradish peroxidase (HRP)-linked goat antibody to rat IgG was obtained from Jackson ImmunoResearch, and TrueBlot HRP anti-mouse and rabbit IgG were from eBioscience.

Plasmid Construction—The pZp-luc reporter plasmid was constructed by inserting the minimal sequence of Zp (from −221 to +12) prepared by PCR into XhoI and HindIII sites of pGL4.10 (Promega). Primer sequences for the PCR were 5′-TAGCCTCGAGGCCATGCATATTTCAACTGG-3′ (forward), 5′-GCCAAGCTTCAAGGTGCAATGTTTAGTGAG-3′ (reverse). Point mutations in the minimal Zp were introduced by PCR using following primers: mZII, 5′-TCACAGAGGAGGCTGGTGCC-3′ (forward), 5′-TGAATTCGTTTGGGACGTGC-3′ (reverse); mZIII, 5′-GCACCGCTAATGTACCTCATAG-3′ (forward), 5′-CTGTGAATTCTGCATAGTTTC-3′ (reverse). Expression plasmids for TORC3 and CREB1A have been reported elsewhere (21–23). TORC1 and TORC2 genes were amplified and cloned into EcoRI and XhoI sites of pcHA (22, 24) using the following primers: TORC1, 5′-AAAGAATTCATGGCGACTTCGAACAATCCGCGG-3′ (forward), 5′-AAACTCGAGTCACAGGCGGTCCATCCGGAAGGT-3′ (reverse), TORC2, 5′-AAAGAATTCATGGCGACGTCGGGGGCGAACGGG-3′ (forward), 5′-AAACTCGAGTCATTGGAGCCGGTCACTGCGGAA-3′ (reverse). The pCRE-Luc and pRL-TK reporter plasmids were obtained commercially (Stratagene). For the pcDNABZLF1 expression plasmid, the BZLF1 gene was cloned into pcDNA3 at BamHI and XhoI sites. The sequence in the b-Zip domain (amino acids 200–227) was deleted to generate pcDNAdBZLF1. To prepare the expression vector for Gal4-BZLF1fusion protein, the BZLF1 sequence was recloned into EcoRI and XbaI sites of the pM vector (Clontech) after PCR using the primers 5′-CCGGAATTCATGATGGACCCAAACTCGAC-3′ (forward) and 5′-CTTATCTAGATTAGAAATTTAAGAGATCCTCG-3′ (reverse). The pGal4-luc reporter plasmid has been reported previously (21).

Transfection and Luciferase Assay—Plasmid DNA was transfected into HEK293T or EBV-293 cells using Lipofectamine 2000 reagent (Invitrogen). The total amounts of plasmid DNAs were standardized by the addition of an empty vector, pcDNA3. Proteins were extracted from cells with the lysis buffer supplied in a Dual Luciferase Reporter Assay System (Promega) kit, and luciferase activities were measured using the kit. The counts for firefly luciferase were normalized to those for renilla luciferase. GTC-4 and Akata cells were electronically transfected using a Microporator (Digital Bio).

Immunoprecipitation (IP) and Immunoblotting (IB)—For IP, cells were lysed in 0.2% Nonidet P-40 buffer (10 mM Tris-HCl, pH 7.8, 100 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, and protease and phosphatase inhibitor mixture). After centrifugation, lysates were precleared with protein G-Sepharose (Amersham Biosciences), mixed with antibody, and then incubated for 1 h. Immunocomplexes were recovered by incubating with G-Sepharose for 1 h, and the resin was washed 5 times with the same buffer. Samples were subjected to SDS-PAGE followed by IB with the indicated antibodies as described previously (24).

Chromatin IP (ChIP) Assay—ChIP assays were performed essentially as described (Upstate Biotechnology, Inc.) with formaldehyde cross-linked chromatin from 1×10^6 cells for each reaction. Cells were lysed, and chromatin was sonicated to obtain DNA fragments with an average length of 300 bp. After centrifugation, the chromatin was diluted 10-fold with ChIP dilution buffer and precleared with protein A-agarose beads containing salmon sperm DNA (Upstate). Anti-FLAG IgG or normal rabbit IgG were added to the sample and incubated overnight with rotation. The immune complexes were collected by the addition of the protein A-agarose beads, and DNA was purified using a QIAquick PCR purification kit (Qiagen) after uncoupling of the cross-linking and proteinase K digestion. The recovered DNA was amplified by PCR using primers specific for Zp, 5′-TAGCCTCGAGGCCATGCATATTTCAACTGG-3′ and 5′-GCCAAGCTTCAAGGTGCAATGTTTAGTGAG-3′, and for the EBNA-1 open reading frame, 5′-GTCATCATCATCCGGGTCTC-3′ and 5′-TTCGGGTTGGAACCTCCTTG-3′. The PCR products were then analyzed