NS5B, and NS3/4A, respectively, were treated with various concentrations at 3 h postinfection with HCVcc, and the intracellular HCV RNA level was determined by qRT-PCR at 48 h postinfection. Treatment with these DAAs inhibited the HCV RNA level in a dose-dependent manner in both Huh7 and FU97 cells (Fig. 8A, bar graphs) and exhibited no cell toxicity at all even at the highest dose (Fig. 8A, line graphs). The inhibitory effects of BMS-790052 (Fig. 8A, top graphs) on the propagation of HCVcc in FU97 cells were higher than those in Huh7 cells, and the 50% effective concentration (EC_{50}) values of BMS-790052 against propagation of HCVcc in FU97 and Huh7 cells were 7.2 and 21.8 pM, respectively ($P < 0.05$). On the other hand, the antiviral effects of BILN 2061 (Fig. 8A, bottom graphs) on the propagation of HCVcc in FU97 cells were lower than those in Huh7 cells, and EC_{50}s of BILN 2061 against propagation of HCVcc in FU97 and Huh7 cells were 65.0 and 38.9 nM, respectively ($P < 0.01$). PSI-7977 showed almost equivalent inhibitory effects to HCV propagated in FU97 and Huh7 cells, and the EC_{50}s of PSI-7977 against propagation of HCVcc in FU97 and Huh7 cells were 34.6 and 44.1 nM, respectively (Fig. 8A, middle graphs). These results suggest that the antiviral effect of DAAs on the propagation of HCVcc varied between Huh7 and FU97 cells.

Next, we examined the efficacy of IFN-α, RBV, and cyclosporine, which are inhibitors for HCV targeting host factors involved in the propagation of HCVcc (53–55), on the propagation of HCVcc in Huh7 and FU97 cells. Cells were treated with various concentrations of the reagents at 3 h postinfection with HCVcc, and the level of intracellular HCV RNA was determined by qRT-PCR at 48 h postinfection. In contrast to the treatment with DAAs, both Huh7 and FU97 cells exhibited cell toxicity by the treatment with RBV and cyclosporine but not with IFN-α at higher concentrations (Fig. 6B, line graphs). The inhibitory efficacies of IFN-α (Fig. 8B, top graphs) and cyclosporine (Fig. 8B, bottom graphs) on the propagation of HCVcc in FU97 cells were lower than those in Huh7 cells, and the EC_{50}s of IFN-α against propagation of HCVcc in FU97 and Huh7 cells were 4.3 and 2.5 IU/ml, ($P < 0.05$), respectively; those of cyclosporine were 6.9 and 3.2 μg/ml ($P < 0.01$), respectively. On the other hand, the antiviral effect of RBV on the propagation of HCVcc in FU97 cells was higher than that in Huh7 cells, and the EC_{50}s of RBV against propagation of HCVcc in FU97 and Huh7 cells were 99.0 and 198.9 μM, respectively ($P < 0.05$) (Fig. 8B, middle graphs). These results suggest that the efficacies of anti-HCV drugs targeting host factors involved in the infection of HCV were also different between Huh7 and FU97 cells.

FU97 cells exhibit higher susceptibility to HCVcc/JFH-2 propagation than Huh7 cells. HCVcc/JFH-2 was cloned from a patient with fulminant hepatitis and exhibited efficient propagation in Huh7 cured cells (34). *In vitro*-transcribed RNA of pJFH2/AS/mtT4 encoding a full-length JFH-2 strain was electroporated into Huh7.5.1 cells, and HCVcc/JFH-2 of 1.5×10^5 FFU/ml was recovered in the supernatants after serial passages. To examine the susceptibility of FU97 cells to the propagation of HCVcc/JFH-2, cells were infected with HCVcc/JFH-2 at an MOI of 1, and the intracellular HCV RNA level was determined by qRT-PCR. Intracellular HCV RNA in parental and cured FU97 cells increased until 72 h postinfection, while it reached a peak at 48 h postinfection in Huh7 cells, and the highest HCV RNA level was observed in the cured FU97 clones upon infection with HCVcc/JFH-2 (Fig. 9A). Infectious titers in the culture supernatants at 72 h postinfection with HCVcc/JFH-2 were also highest in the cured FU97 7-1 cells (2.5×10^4 FFU/ml), followed by parental FU97 (1.2×10^4 FFU/ml) and Huh7 (9×10^3 FFU/ml) cells (Fig. 9B). Next, we examined the expression and subcellular localization of HCV proteins in cells infected with HCVcc/JFH-2 by immunofluorescence analysis. Expression of NS5A in cells upon infection with HCVcc/JFH-2 was highest in the cured FU97 7-1 cells, followed by parental FU97 cells, and that in Huh7 cells was low (Fig. 9C, left panels). Core protein was detected around LDs in cells infected with HCVcc/JFH-2, as seen in those infected with the HCVcc/JFH-1 strain (Fig. 9C, right). To further confirm the efficient propagation of HCVcc/JFH-2 in FU97 cells, *in vitro*-transcribed viral RNAs of the JFH-1 and JFH-2 strains of HCVcc were electroporated into Huh7, FU97, and cured FU97 cells. Although the infectious titers of the JFH-1 strain in FU97 cells were lower than those in Huh7 cells, those of the JFH-2 strain in FU97 and cured FU97 cells were significantly higher than those in Huh7 cells (Fig. 9D). These results suggest that FU97 cells are more susceptible to propagate HCVcc/JFH-2 than Huh7 cells.

DISCUSSION

Several reports have shown that hepatic differentiation is involved in the susceptibility of ES/iPS cells to HCVcc infection (28, 30, 41). In addition, in hepatic cancer cell lines, including Huh7, HepG2, and Hep3B, cells derived from not poorly but well-differentiated HCC permit complete propagation of HCVcc (15–17), suggesting that hepatic differentiation is closely related to the susceptibility of cells to HCVcc propagation. In this study, we identified two cell lines susceptible to HCVcc infection by the screening of cancer cell lines expressing AFP as a marker of hepatic differentiation. HCC-derived JHH-4 cells and gastric cancer-derived FU97 cells permit complete propagation of HCVcc without any exogenous expression of the host factors required for HCVcc propagation, including HCV receptor candidates, miR-122, and apolipoproteins. In particular, FU97 cells exhibited higher susceptibility to HCVcc/JFH-2 infection than Huh7 cells, suggesting that FU97 cells would be useful tools for further HCV analyses.

Although HCV has been classified into seven major genotypes and a series of subtypes (56, 57), the *in vitro* infection model had been restricted to the JFH-1 strain based on the genotype 2a until

FIG 2 JHH-4 and FU97 cells permit HCV propagation. (A) Intracellular HCV RNA levels in Huh7, JHH-4, and FU97 cells inoculated with HCVcc at an MOI of 1, treated with 100 IU/ml of IFN-α or untreated (mock), were determined by qRT-PCR at 12, 24, 48, and 72 h postinfection (hpi). Infectious titers in the culture supernatants of Huh7, JHH-4, FU97, and 293T-CLDN/miR-122 cells infected with HCVcc at an MOI of 1 were determined by a focus-forming assay at 72 h postinfection (bar graph). (B) Exogenous expression of miR-122 in Huh7, JHH-4, and FU97 cells by lentiviral vector (bar graph). Total cellular miRNA extracted from the cells was subjected to qRT-PCR. U6 was used as an internal control. Intracellular HCV RNA in Huh7, JHH-4, and FU97 cells inoculated with HCVcc at an MOI of 1 was determined by qRT-PCR at 12, 24, 48, and 72 h postinfection. Solid and broken lines indicate HCV RNA abundances in miR-122-expressing and GFP-expressing control cells, respectively. (C) Huh7, JHH-4, and FU97 cells were infected with HCVcc at an MOI of 1, fixed with 4% PFA, and subjected to immunofluorescence analyses by using antibodies against core, NS5A, dsRNA, and calregulin. Lipid droplets and cell nuclei were stained by BODIPY and DAPI, respectively. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$) versus the results for control cells.

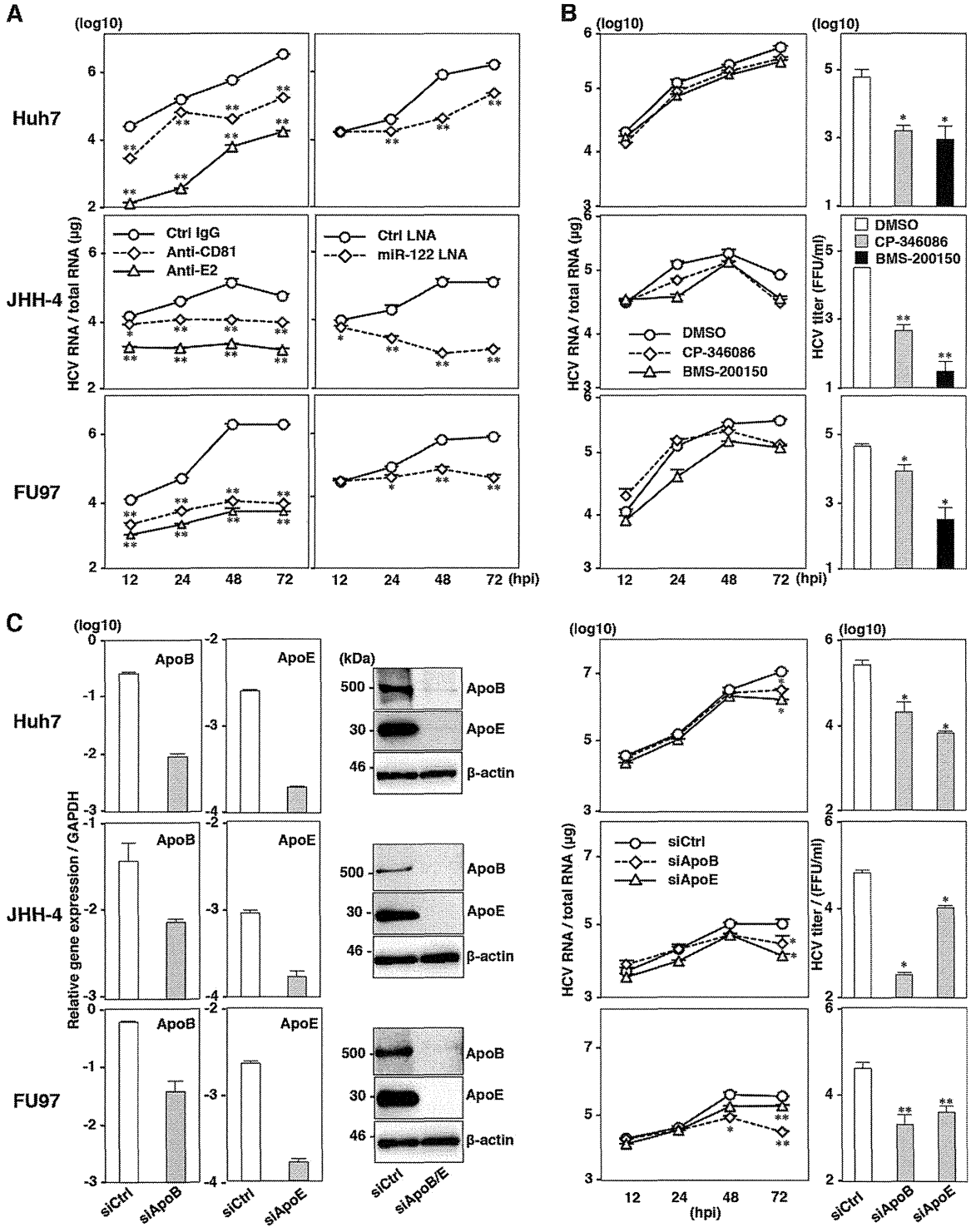

FIG 3 JHH-4 and FU97 cells permit complete propagation of HCVcc without any exogenous expression of host factors crucial for propagation of HCVcc. (A) Effect of inhibitors on the propagation of HCVcc in Huh7, JHH-4, and FU97 cells. (Left panels) HCVcc was preincubated with anti-E2 antibody and inoculated into cells. Cells were preincubated with anti-hCD81 antibody or isotype control antibody (Ctrl IgG) and then infected with HCVcc. (Right panels) Cells were infected with HCVcc and treated with miR-122-LNA (30 nM) or Ctrl-LNA (30 nM) at 6 h postinfection. (B) Huh7, JHH-4, and FU97 cells infected with HCVcc at an MOI of 1 were treated with dimethyl sulfoxide (DMSO) or MTTP inhibitor, CP-346086 (5 μM)or BMS-200150 (10 μM), at 3 h postinfection. Intracellular HCV RNA in cells at 12, 24, 48, and 72 h postinfection was determined by qRT-PCR (left panels). Infectious titers in the culture supernatants of cells infected with HCVcc at an MOI of 1 and treated with 5 μM CP-346086, 10 μM BMS-200150, or dimethyl sulfoxide alone (DMSO) at 3 h postinfection were determined at 72 h postinfection by a focus-forming assay (right graphs). (C) mRNA and protein expression levels of ApoB and ApoE (left panels) in Huh7, JHH-4, and FU97 cells at 48 h posttransfection with siRNA targeting either ApoB or ApoE or a control siRNA (siApoB, siApoE, or siCtrl, respectively) were determined by qRT-PCR and immunoblotting, respectively. Huh7, JHH-4, and FU97 cells were infected with HCVcc at an MOI of 1 at 6 h posttransfection with siRNA targeting either ApoB or ApoE or a control siRNA (siApoB, siApoE, or siCtrl, respectively) (right panels). Intracellular HCV RNA at 12, 24, 48, and 72 h postinfection and infectious titers in the culture supernatants at 72 h postinfection were determined by qRT-PCR and focus-forming assay, respectively. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$) versus the results for control cells.

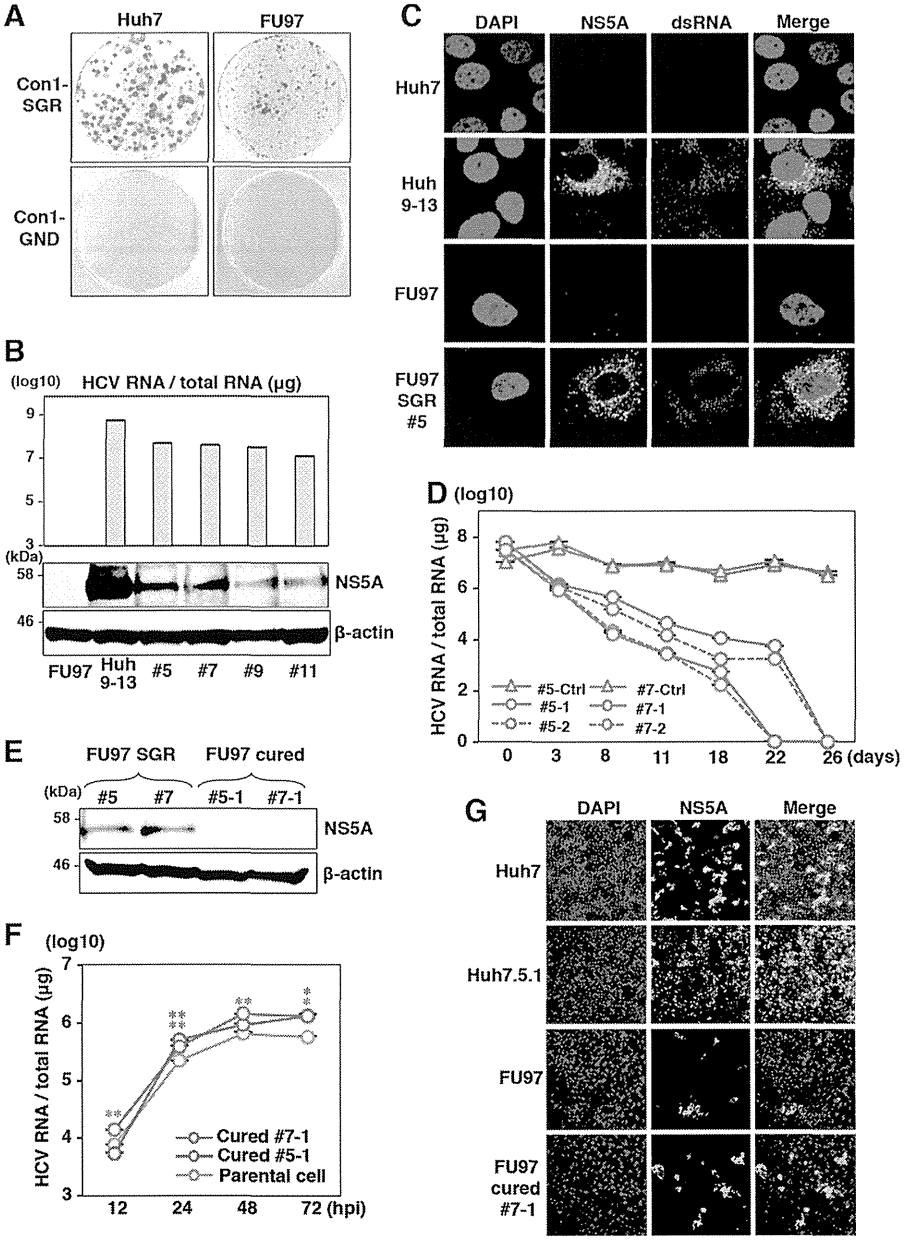

FIG 4 Establishment of HCV RNA replicon and cured FU97 cells. (A) Wild-type SGR RNA (Con1-SGR) or replication-defective RNA (Con1-GND) of the HCV Con1 strain was electroporated into Huh7 and FU97 cells and replaced with medium containing 1 mg/ml and 400 μg/ml of G418 at 24 h postelectroporation, respectively. Colonies were stained with crystal violet at 30 days postselection. (B) Four clones derived from FU97 SGR cells (clones 5, 7, 9, and 11) were subjected to qRT-PCR after extraction of total RNA (upper panel) and to immunoblotting using anti-NS5A antibody (lower panel). Huh9-13 cells, which were Huh7-derived Con1-SGR cells, were used as a positive control. (C) Huh9-13 cells, Huh7 parental cells, FU97-derived Con1-SGR cells (FU97 SGR, clone 5), and FU97 parental cells were fixed in 4% PFA and subjected to immunofluorescence assay using anti-NS5A and anti-dsRNA antibodies. Cell nuclei were stained by DAPI. (D) Elimination of HCV RNA from FU97-derived Con1-SGR cells. Two clones derived from FU97 SGR cells (clones 5 and 7) were treated with a combination of either 100 IU/ml of IFN-α and 100 nM BILN 2061 (clones 5-1 and 7-1) or 10 pM of BMS-790052 and 100 nM BILN 2061 (clones 5-2 and 7-2) to eliminate the HCV genome. Clones 5-Ctrl and 7-Ctrl are negative controls, untreated with anti-HCV drugs. Intracellular HCV RNA at 3, 8, 11, 18, 22, and 26 days posttreatment was determined by qRT-PCR. (E) The expression levels of NS5A in FU97 SGR cells (clones 5 and 7) and in FU97 cured cells (clones 5-1 and 7-1) were determined by immunoblot analysis using anti-NS5A antibody. (F) FU97 cured cells (clone 5-1 and clone 7-1) and parental cells were infected with HCVcc at an MOI of 1; the levels of intracellular HCV RNA at 12, 24, 48, and 72 h postinfection were determined by qRT-PCR. (G) The expression of NS5A in Huh7, Huh7.5.1, FU97, and cured FU97 clone 7-1 was determined by immunofluorescence analysis at 72 h postinfection by using anti-NS5A antibody. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$) versus the results for control cells.

recently (15). To clarify the pathogenesis of HCV depending on the genotypes, the establishment of cell-culture-adapted clones derived from various genotypes is essential (58). Viable JFH1-based intergenotypic recombinants, containing genotype-specific structural proteins, p7 and the complete or partial NS2, were generated for various genotypes of HCV (56, 59, 60). Although robust propagation systems of full-length HCV infectious clones of the H77 strain (genotype 1a) (61), TN strain (1a) (62), JFH-2 strain

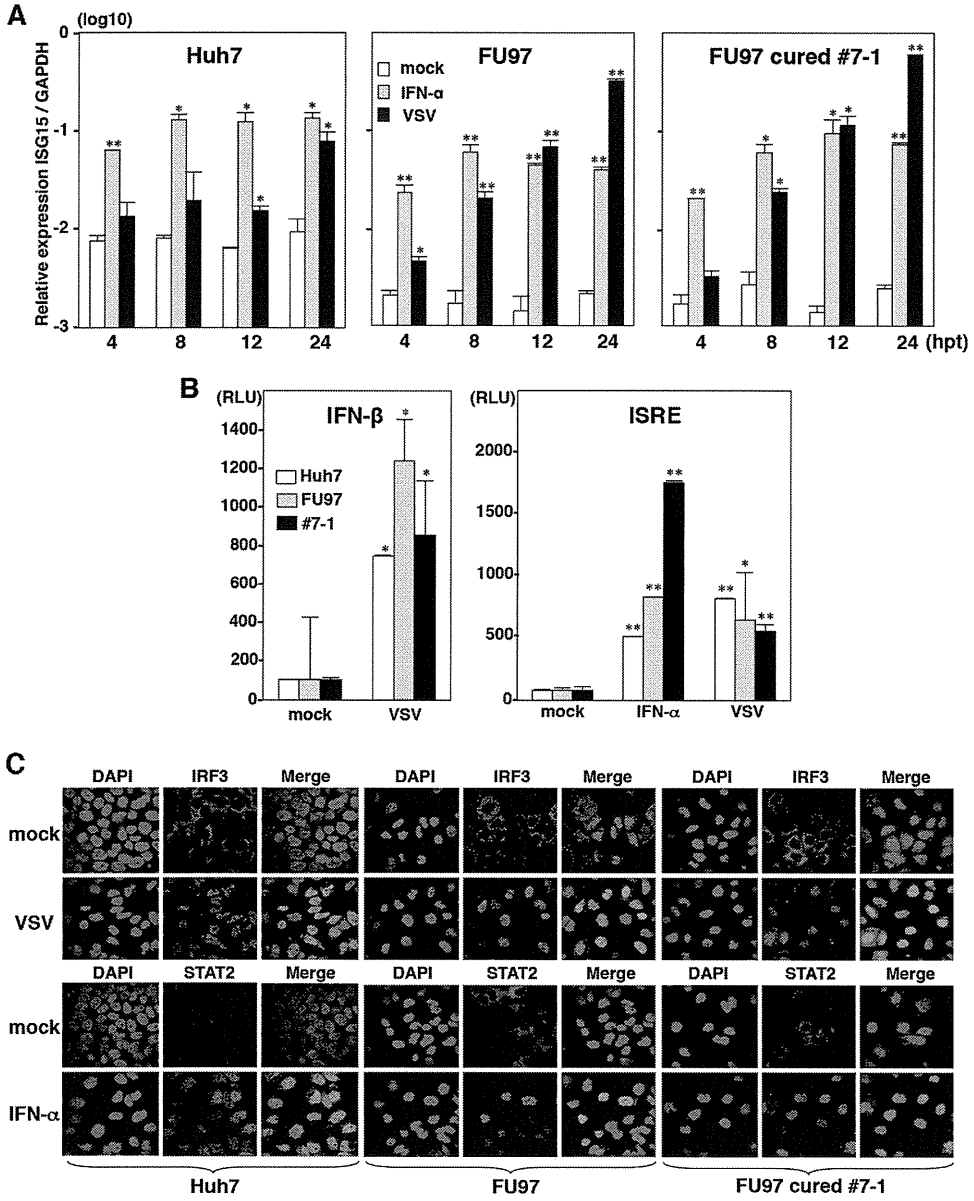

FIG 5 Innate immune response in cured FU97 cells. (A) Huh7, parental, and cured FU97 cells (clone 7-1) were stimulated with 100 IU/ml of IFN-α or infected with VSV. The expression of mRNA of ISG15 at 4, 8, 12, and 24 h posttreatment (hpt) was determined by qPCR and standardized by that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (B) Huh7, parental FU97, and cured FU97 (clone 7-1) cells cotransfected with pIFN-β-Luc and pRL-SV40 were infected with VSV at an MOI of 1 at 24 h posttransfection (left). Cells cotransfected with pISRE-Luc and pRL-SV40 were infected with VSV at an MOI of 1 or stimulated with 100 IU/ml of IFN-α at 24 h posttransfection (right). Luciferase activities were determined at 24 h posttreatment. (C) Huh7, parental FU97, and cured FU97 (clone 7-1) cells were infected with VSV at an MOI of 1 or stimulated with 100 IU/ml of IFN-α, fixed with 4% PFA at 18 h posttreatment, and subjected to immunofluorescence assay using anti-IRF3 and -STAT2 antibodies. Cell nuclei were stained by DAPI. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$) from the results for control cells.

(2a) (34), and S310 strain (3a) (63) were established, the construction of infectious clones of other genotypes has not succeeded yet.

Because permissive cell lines for HCVcc infection *in vitro* had been limited to Huh7 cells due to cell tropism and the narrow host range (13, 14), the establishment of a novel cell culture system supporting HCV propagation is needed for further HCV analyses. Previous reports have demonstrated that HepG2, Hep3B, and HEK293 cells permit HCVcc propagation (16, 17, 64). However, exogenous expression of host factors is necessary for complete propagation of HCVcc in these cell lines. In HepG2 and Hep3B cells, overexpression of miR-122 is essential for efficient replication of HCV RNA (16, 17). In HEK293 cells, the exogenous expression of CLDN1, miR-122, and ApoE was required for infectious particle formation upon infection with HCVcc (64). On the other hand, JHH-4 and FU97 cells permit complete propagation of HCVcc without any exogenous expression of the host factors required for propagation of HCVcc. JHH-4 cells grown in a three-dimensional radial-flow bioreactor were successfully infected following inoculation with plasma from an HCV carrier and transfection of HCV RNA transcribed from full-length cDNA (44). In

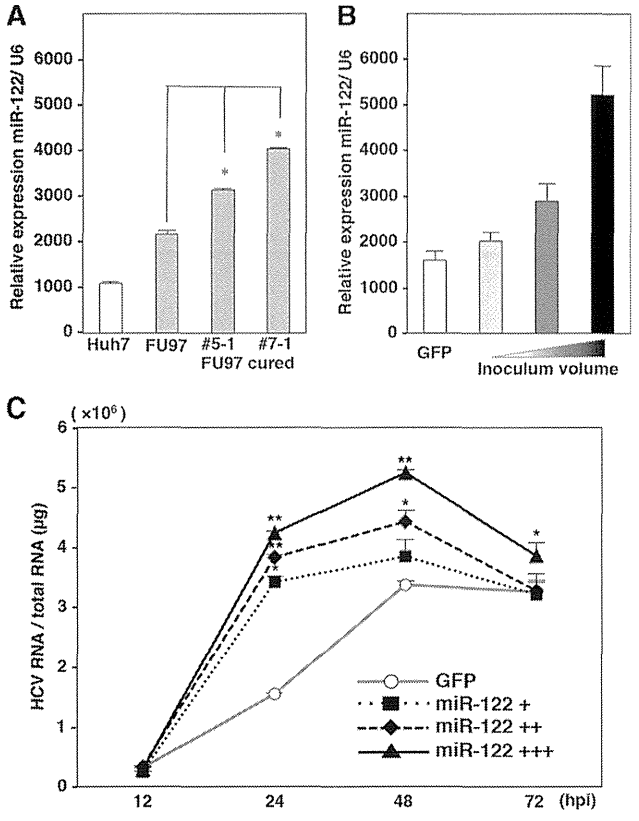

FIG 6 Expression of miR-122 is one of the determinants for HCV RNA abundances. (A) Total RNA was extracted from Huh7 and parental and cured FU97 (clones 5-1 and 7-1) cells, and the relative expression of miR-122 was determined by qPCR. U6 snRNA was used as an internal control. (B) Establishment of FU97 cell lines stably expressing various concentration of miR-122 by infection with a lentiviral vector. FU97 cells infected with lentiviral vector to express GFP were used as a control. (C) FU97 cell lines expressing various concentrations of miR-122 were infected with HCVcc at an MOI of 1, and HCV RNA abundances were determined at 12, 24, 48, and 72 h postinfection (hpi) by qRT-PCR. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$) versus the results for control cells.

addition, JHH-4 cells were suggested to possess some host factors involved in the enhanced translation of HCV RNA (64, 65). Furthermore, high susceptibility of FU97 cells to HCVcc/JFH-2 infection compared to Huh7 cells raises the possibility of using FU97 cells for the propagation of HCVcc derived from other genotypes, including the H77, TN, and S310 strains.

AFP-producing gastric cancer (AFPGC) cell lines, FU97 and Takigawa cells (66), which were identified by using a cDNA array database, were shown to express high levels of liver-specific factors. AFPGC is a rare case and exhibits a worse prognosis and the characteristics of early hepatic metastasis (67). It is hypothesized that production of AFP, which is suppressed in mature hepatocytes, is induced in HCC by the dedifferentiation of cancer cells or the increase in oval cells in the oncogenic pathway (68). Oval cells are believed to be capable of producing AFP, are candidates for hepatic stem cells, have bipotentiality to differentiate into hepatocytes and bile duct epithelial cells, and play an important role in liver regeneration (69, 70). These hypotheses suggest that cancer cells acquired a new function, such as the ability to produce AFP through an alteration in differentiation status. Although the mechanism of AFP production in gastric cancer remains unknown, hepatic dedifferentiation might be induced in gastric cancer. Furthermore, previous reports have proposed the concept of "hepatoid adenocarcinoma" based on the differentiation of AFPGC into hepatocyte-like cells (71, 72), suggesting that FU97 and Takigawa cells obtained the hepatocyte-like characteristics required for HCV propagation through dedifferentiation during the oncogenic process. In addition, recent studies demonstrated that hepatocyte-like cells derived from induced pluripotent stem cells (iPS cells/iPSCs) express high levels of miR-122 and VLDL-associated proteins and support propagation of HCVcc and HCV derived from patient serum (28–30). These results suggest that hepatic differentiation required for hepatic functions plays crucial roles in HCV propagation. In accord with these observations, our data suggest that cancer cell lines differentiated into hepatocyte-

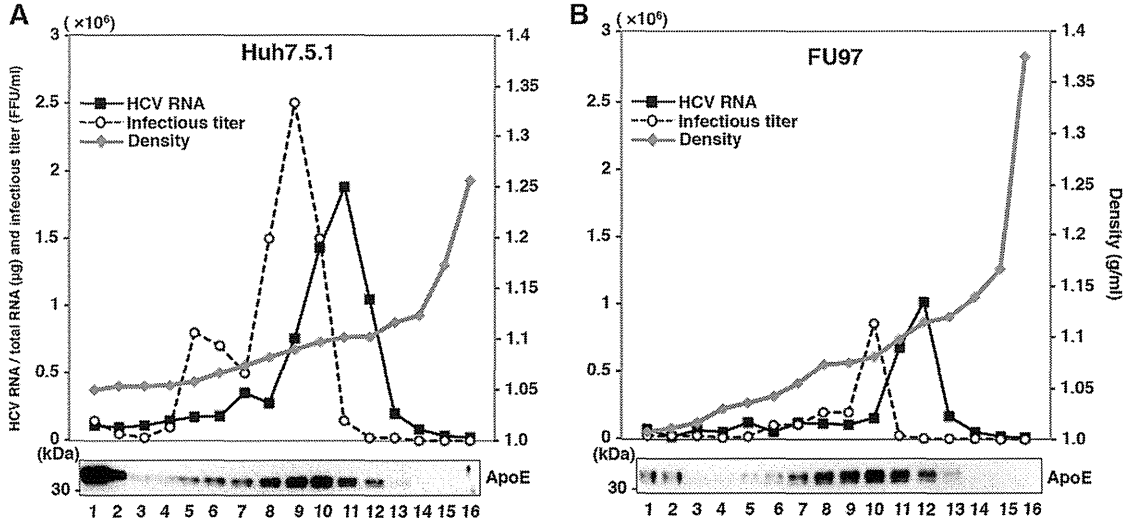

FIG 7 HCV particles produced in FU97 cells exhibit similar characteristics to those in hepatic cells. HCV particles in the culture supernatants of Huh7.5.1 and FU97 cells were harvested at 72 h postinfection with HCVcc and analyzed by using iodixanol density gradient centrifugation. HCV RNA and infectious titers of each fraction were determined by qRT-PCR and focus-forming assay, respectively. Buoyant density was plotted for each fraction (upper panels). Expression of ApoE in each fraction was detected by immunoblotting using anti-ApoE antibody (lower panels).

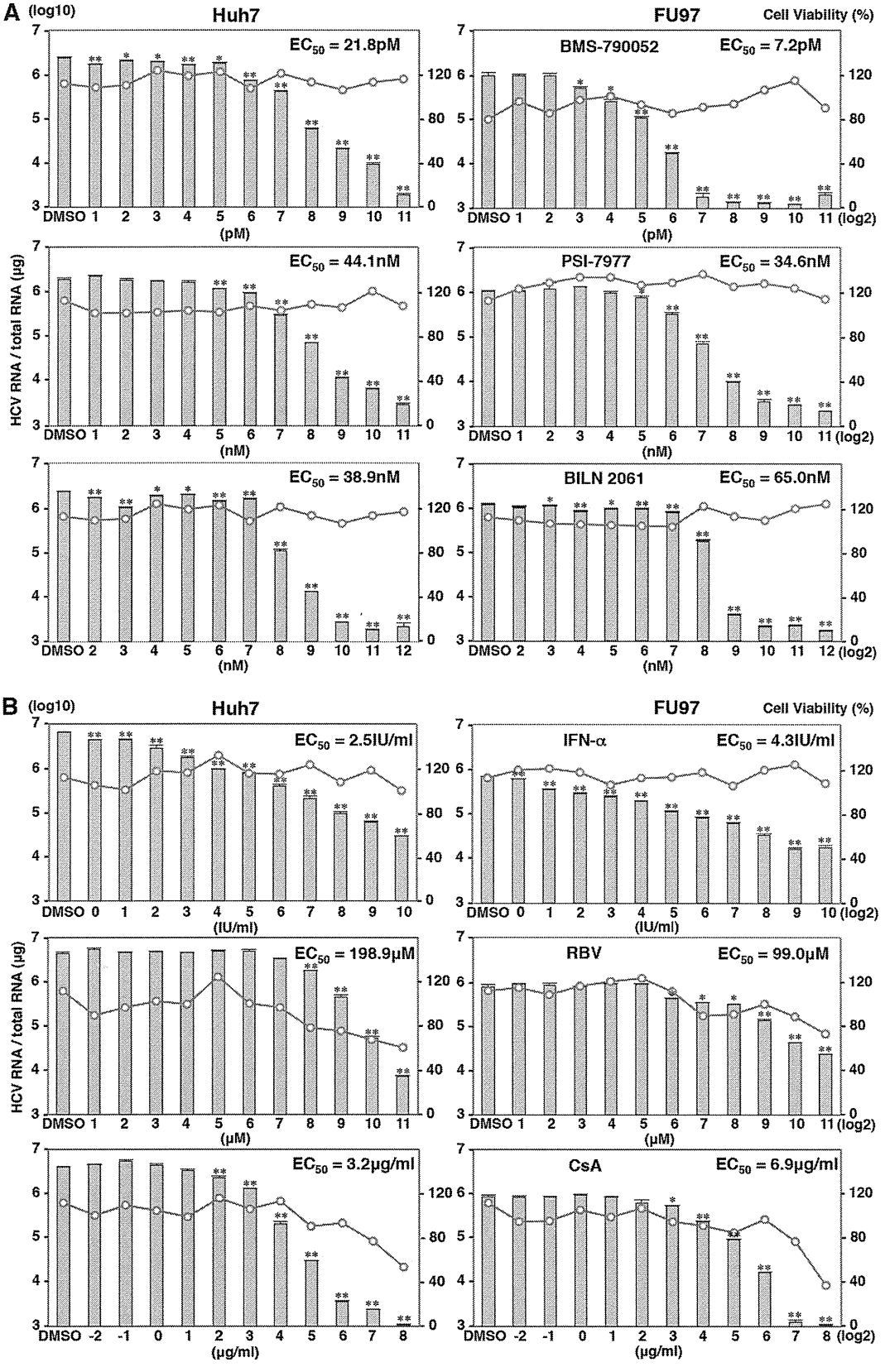

FIG 8 Effects of anti-HCV drugs on the propagation of HCVcc in FU97 cells. (A) Effect of DAAs on the propagation of HCVcc in Huh7 and FU97 cells. Cells infected with HCVcc at an MOI of 1 were treated with BMS-790052, PSI-7977, and BILN 2061 at 3 h postinfection (identifications in right-hand panels). (B) Effect of HCV inhibitors targeting host factors on the propagation of HCVcc in Huh7 and FU97 cells. Cells infected with HCVcc at an MOI of 1 were treated with IFN-α, RBV (middle), and cyclosporine (CsA) at 3 h postinfection (identifications in right-hand panels). Intracellular HCV RNA levels were determined by qRT-PCR at 48 h postinfection (bar graphs), and cell viability was determined as a percentage of the viability of cells treated with 0.1% dimethyl sulfoxide (DMSO) at 48 h posttreatment (line graphs). From the assay results, the 50% effective concentration (EC_{50}) of each reagent was determined. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$) versus the results for control cells.

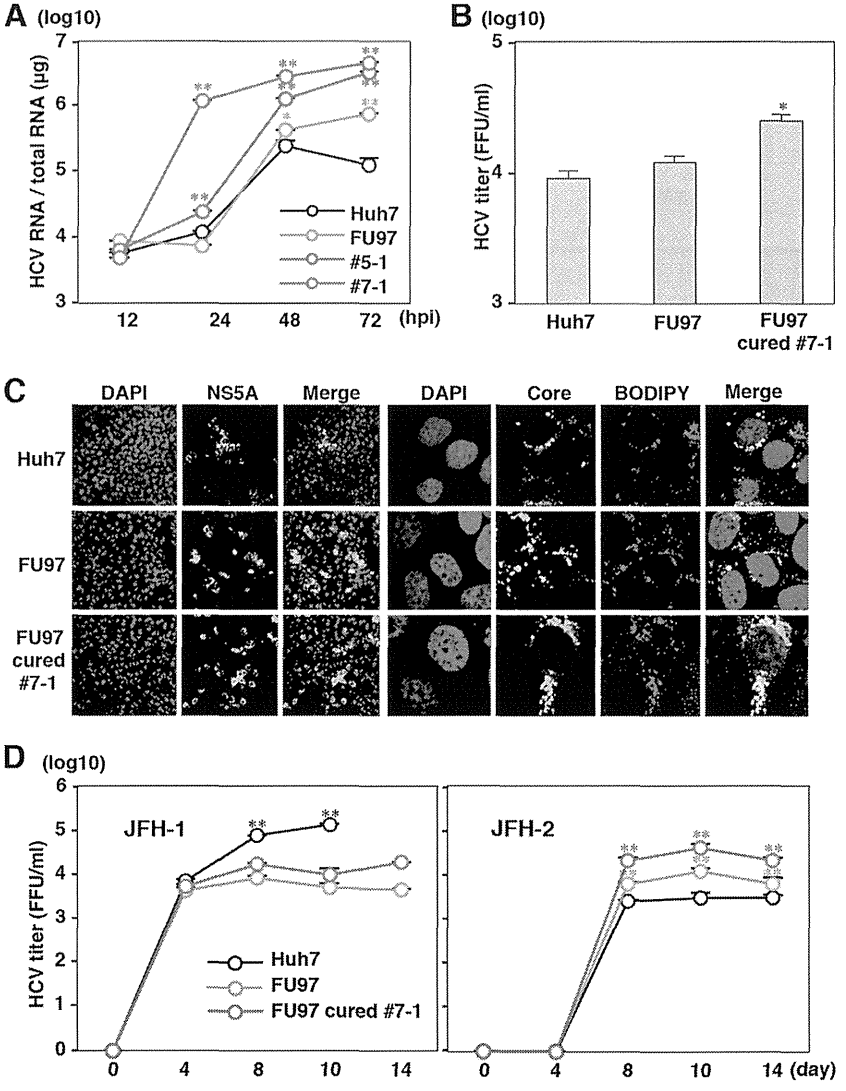

FIG 9 Propagation of HCVcc/JFH-2 in FU97 cells. (A) Huh7, FU97 parental, FU97 cured 5-1, and FU97 cured 7-1 cells were infected with HCVcc/JFH-2 at an MOI of 1, and the intracellular HCV RNA level was determined by qRT-PCR at 12, 24, 48, and 72 h postinfection. (B) Huh7, FU97, and FU97 cured 7-1 cells were infected with HCVcc/JFH-2 at an MOI of 1, and infectious titers in the culture supernatants were determined by focus-forming assay. (C) Huh7, FU97, and FU97 cured 7-1 cells were infected with HCVcc/JFH-2 at an MOI of 1, fixed with 4% PFA at 72 h postinfection, and subjected to immunofluorescence assay using antibodies against NS5A or core. Lipid droplets and cell nuclei were stained with BODIPY and DAPI, respectively. (D) *In vitro*-transcribed JFH-1 and JFH-2 RNAs were electroporated into Huh7, FU97, and FU97 cured 7-1 cells. The infectious titers of JFH-1 and JFH-2 in the culture supernatants from these cells were determined by focus-forming assay up to 14 days postransduction. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$) versus the results for control cells.

like cells to gain hepatic functions could permit complete propagation of HCVcc.

Treatment with DAAs including BMS-790052 (NS5A inhibitor) (73), PSI-7977 (NS5B polymerase inhibitor) (74), and BILN 2061 (NS3/4A protease inhibitor) (75) inhibited propagation of HCV in both Huh7 and FU97 cells infected with HCVcc without any cell toxicity. Antiviral effects of BMS-790052 and BILN 2061 were significantly different between Huh7 and FU97 cells, suggesting that efficacies of DAAs are varied, depending on cell lines. Although anti-HCV drugs targeting host factors including IFN-α, RBV, and cyclosporine also inhibited propagation of HCVcc in a dose-dependent manner in both Huh7 and FU97 cells, treatment with RBV and cyclosporine produced cell toxicity at higher concentrations than treatment with DAAs. Although the antiviral mechanism of RBV against HCV has not been well elucidated yet (53), inhibitory effects of RBV against HCV infection were significantly higher in Li23 cells than those in Huh7 cells (76, 77), and RBV also exhibited a low inhibitory effect upon infection with HCVcc in Huh7 cells compared to that in FU97 cells. Although adenosine kinase (ADK) was shown to be a determinant for the sensitivity of RBV (78), the expression levels of ADK in Huh7 and FU97 cell lines were comparable (data not shown).

The *IL28B* genotype is associated with the sensitivity of IFN treatment for chronic hepatitis C patients (79–81), and patients with the minor *IL28B* genotype exhibit lower susceptibility to the treatment than those with major genotypes. Although FU97 cells showed lower sensitivity to the IFN-α treatment than Huh7 cells, FU97 and Huh7 cells possess major and minor *IL28B* genotypes

(data not shown), respectively. Furthermore, induction of ISG15 by treatment with IFN-α was almost comparable between Huh7 and FU97 cells (Fig. 5A), and expression levels of IFN-α receptor in the cell lines were the same (data not shown), suggesting the involvement of other factors in the difference in the IFN responses between FU97 and Huh7 cells.

Cyclophilins possess peptidyl-prolyl *cis/trans* isomerase (PPIase) activity and are involved in protein folding and assembly. Cyclophilin A (CypA), the most abundant cyclophilin, localizes in the cytoplasm and interacts with the immunosuppressive drug cyclosporine (82). In addition, CypA has been shown to be involved in the propagation of human immunodeficiency virus (83, 84), hepatitis B virus (85, 86), influenza A virus (87), and HCV (88). Replication of HCV RNA was inhibited by suppression of the PPIase activity of CypA by treatments with cyclosporine, mutation in the active site of CypA, and knockdown of CypA (55, 89–91). The same level of CypA expression in Huh7 and FU97 cells (data not shown) suggests that the difference in inhibitory effect of cyclosporine in the cell lines may be attributable to other reasons, such as a difference in PPIase activity of CypA in these cell lines. The differences in the efficacy of anti-HCV drugs between Huh7 and FU97 cells were small; however, FU97 cells have the possibility to possess antiviral activity different from that of Huh7 cells.

In summary, we identified novel permissive cell lines for complete propagation of HCVcc without any artificial manipulation. In particular, gastric cancer-derived FU97 cells exhibited a much higher susceptibility to HCVcc/JFH-2 infection than observed in Huh7 cells, suggesting that FU97 cells would be useful for further investigation of the HCV life cycle, as well as the development of therapeutic agents for chronic hepatitis C.

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Note

Establishment of Hepatitis E Virus Infection-Permissive and -Nonpermissive Human Hepatoma, PLC/PRF/5, Subclones

Running title: Nonpermissive subclones for HEV

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Abstract

PLC/PRF/5 cells showed limited permissiveness, meaning that almost all subclones were permissive, although some subclones did not exhibit permissiveness for hepatitis E virus (HEV) infection. In this study, we performed the single-cell cloning of PLC/PRF/5 and characterized heterogeneous subclones. Notably, the efficiency of intracellular virus replication did not correlate with the permissiveness for HEV infection. However, the virus-like particles bound these nonpermissive subclones on various levels as well as permissive subclones, suggesting that these subclones have some deficiencies in the attachment and entry steps of infection. Our data would be useful for investigation of HEV life cycle.

Keywords: Attachment and entry, Cloning cell lines, Hepatitis E virus (HEV), Nonpermissive

Hepatitis E virus (HEV) is responsible for acute and enterically transmitted hepatitis in the developing world (1), accounting for more than 50% of acute viral hepatitides in young adults, with case fatality of at least 1% in regular patients and up to 20% in pregnant women (2-4). Recent epidemiological studies revealed the widespread prevalence of HEV and anti-HEV antibodies in humans and several animal species worldwide, including developed countries (5-9). HEV has only one serotype, but at least four genotypes, with genotypes 1 and 2 (G1 and G2) exclusively infecting humans, and genotypes 3 and 4 (G3 and G4) additionally infecting swine and several other mammalian species (5, 7, 6). Other genotypes have been reported as causative agents only in wild boar (10, 11).

HEV is a single-stranded positive-sense RNA virus that belongs to the genus *Hepevirus* in the family *Hepeviridae* (12). The virions exhibit distinct buoyant densities in feces (1.26-1.27 g/mL) and in circulating blood (1.15-1.16 g/mL), differences that might be associated with the virions' cellular membrane composition (13). The 7.2-kb genome contains three partially overlapping open reading frames (ORFs) (14). *ORF1* encodes a nonstructural polyprotein that provides RNA-dependent RNA polymerase (RdRp) activity and possibly other functions (15-17). The ORF2 and ORF3 proteins are believed to be encoded by individual subgenomic RNAs generated during replication (18). *ORF2* encodes the viral capsid protein (14, 19).

Before the establishment of high-efficiency HEV cell culture systems, *in vitro* generation of HEV virus-like particles (HEV-LPs) from modified capsid proteins in insect cells (20, 21) or *in vivo* propagation in nonhuman primates were the most useful models for the study of HEV (22).

In 2007, efficient HEV cell-culture propagation systems were established by using PLC/PRF/5, a hepatocarcinoma cell line, and A549, an adenocarcinomic human alveolar basal epithelial cell line (23). The replication efficiencies of these new systems were several orders of magnitude higher than those of previous

culturing systems (24-33, 23). The increased availability of HEV reagents provided by the new culturing systems has permitted many groups to construct HEV infectious clones, thereby facilitating multiple recent reverse genetic studies (34-38).

We first characterized PLC/PRF/5 cells, which was obtained from the Japanese Collection of Research Bioresources (Cell number: JCRB0406, Lot number: 01272003), for HEV infection in our laboratory. The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (FCS), 100 U/mL of penicillin G, and 100 μg/mL of streptomycin at 37°C in a humidified 5% CO_2 atmosphere. Separate 35-mm^2 confluent cultures of the PLC/PRF/5 cells on treated culture plates (Becton, Dickinson and Company) were infected with G3-HEV83-2-27 strain. As a negative control, the same amount and number of cells were not infected but were cultured for the same interval. One set of plates from each group (infected and uninfected) was stained at weekly intervals to provide a time course for testing HEV antigen expression level, implying viral growth. Specifically, at each time interval, separate plates were fixed with 4% formamide, permeabilized with 0.5% Triton X-100 (SIGMA) in phosphate-buffered saline, and then stained with rabbit polyclonal anti-G3-HEV-LPs antibody (21) and finally with Alexa488-conjugated secondary antibody (Life Technologies). Immunofluorescent images of each plate were recorded using a BIOREVO BZ-9000 (KEYENCE). The intensities of immunofluorescence were observed to gradually increase, reflecting the gradual growth of HEV and spread indicating the production of HEV progeny virion only in the HEV-infected cells, in contrast to the uninfected cells. In the infected cells, distinct distributions of intensities were observed with the passage of time (data not shown). To reveal the unique permissiveness of each subclones, single-cell cloning of the host cell line was performed by the limiting dilution method.

In order to obtain subclones, PLC/PRF/5 cells were diluted with medium to a density of 10 cells/mL and seeded at 100 μL/well in 96-well plates. Ninety-eight clones were isolated from the original PLC/PRF/5 cell line. Each clone was designated based on the source plate number - well number (ex. 3-7). The infectious virus G3-HEV83-2-27 was used for experimental infection (38). The cells were infected after initial growth of the stocked subclones, then assayed for HEV antigen expression; spent medium were tested by HEV antigen ELISA using rabbit polyclonal anti-G3-HEV-LP antibody (21) and susceptibility was defined as optical density at 492 nm exceeding 0.2 compared to a pre immune serum. In order to determine the susceptibility of each subclone, cells were infected with G3-HEV83-2-27 strain at the concentration of 2.7×10^6 copies/well (Corning Coster cell culture plate 6 well). At 3 days after inoculation, the culture medium was replaced with fresh maintenance medium consisting of 50% DMEM and 50% Medium 199 (Invitrogen) containing 2% FCS, 30 mM $MgCl_2$, 100 U/mL of penicillin G, and 100 μg/mL streptomycin. Subsequently, the spent medium was collected and replaced with fresh maintenance medium every 3 or 4 days. Antigen ELISA analysis was used to monitor virus production from infected subclones.

Subclones could be divided into three groups (A to C, respectively) based on the pattern of antigen production: A) early antigen secretion (secreted levels observed primarily in the first 3 weeks) (Fig. 1a), B) antigen secreted from Day 14 to 63 (Fig. 1b), or C) antigen not secreted until the end of this study (Fig. 1c). All of the 73 Group-A clones showed susceptibility; almost half of the Group-A clones showed earlier growth. In ELISA testing, the Group-A clones yielded ODs of > 0.2 within 2-3 weeks, indicating that these subclones were highly permissive for HEV infection (Fig. 1a). In the 13 Group-B clones, antigen production yielded ODs of < 0.2 through the first three weeks; all of these subclones showed slower growth than that of Group-A clones, suggesting that the Group-B subclones were moderately permissive for HEV infection. This intermediate permissivity may have

derived from the deletion or partial down-regulation of some non-essential host factors that promote growth efficiency of HEV infection (Fig. 1b). None of the 12 Group-C lines supported HEV infection; these isolates were defined as nonpermissive clones (Fig. 1c). To address the possibility that these properties changed during long-term culturing, representative Group-C subclones were repeatedly re-subcloned and cultured. Even after three months, the Group-C-derived clones still did not support HEV infection (data not shown), demonstrating that these lines remained nonpermissive.

To confirm the non-permissiveness of Group-C PLC/PRF/5 subclones for HEV infection, the corresponding isolates were infected with the G3-HEV83-2-27 virus strain and cultured supernatants were collected periodically. Two inoculated nonpermissive clones (3-13 and 4-29) did not show any antigen production (Fig. 1c under the horizontal black line, Fig. 2a under the horizontal black line). Two other clones, 4-9 belonging to Group B (moderately permissive) and 4-21 belonging to Group A (high permissive), demonstrated permissivity when assessed under the same conditions. Time-course quantification of Subclone 4-21 showed a slight increase of the amount of HEV capsid antigen in the medium collected 7 days post-infection, while the amount of antigen produced by Subclone 4-9 rose starting at 21 days, that is, 14 days later than the start of antigen secretion by 4-21 (Fig. 2a over the horizontal black line).

In order to investigate the HEV replication capability after viral cell entry step among subclones, an infectious clone, which was established from the G3-HEV83-2-27 strain, was used for experimental transfection as described previously (38). Starting at 2 days post-transfection, the spent medium was replaced with maintenance medium every 3 or 4 days. To monitor virus production from the transfected cells, HEV antigen production was determined by ELISA analysis. To estimate the activity of HEV replication in each of four representative

subclones, transfection by the G3-HEV83-2-27 strain was compared between the permissive and nonpermissive PLC/PRF/5 subclones. The replication patterns of the four clones were very similar (Fig. 2b).

The binding affinities of the four subclones for S-VLPs (20, 21) were tested using flow cytometry. The S-VLPs were obtained from a baculovirus expression system using an ORF2 that encoded a truncated capsid protein that lacked the normal N- and C-termini (20, 21). PLC/PRF/5 subclones were washed once with flow cytometry buffer (growth medium for the PLC/PRF/5 cells as above) and then incubated for 30 min at 37°C with 50 ng/mL S-VLPs per 100 μL flow cytometry buffer. These subclones were washed and stained for 30 min at 37°C with 0.1 μL polyclonal anti-G3-HEV-LP antibody per 100 μL flow cytometry buffer (21). After washing with flow cytometry buffer, we incubated the subclones for 30 min at 37°C with secondary antibodies conjugated to FITC (Dako). The cells were washed and analyzed using a FACSCalibur (BD Biosciences) and FlowJo software (Treestar). For each subclone, flow cytometric analysis was performed following incubation with S-VLPs, rabbit polyclonal anti-G3-HEV-LP antibody, or rabbit preimmune serum, followed by incubation with fluorescently-labeled secondary antibody. In comparison with the negative controls, all subclones showed similar levels of S-VLP binding affinity regardless of permissiveness, with approximately 30 to 60% of cells exhibiting fluorescent labeling (Fig. 2c).

In this study, we showed that PLC/PRF/5 cells uniquely presented a limited permissiveness, suggesting that the cells are mixture of permissive and nonpermissive subclones for HEV infection, and that PLC/PRF/5 subclones exhibited various levels of permissivity to HEV virus infection, ranging from strong permissiveness to complete nonpermisiveness, as expected from the limited permissiveness of the parental cell line. While the permissiveness to infection differed among the PLC/PRF/5 subclones, these efficiencies did not correlate with the efficiency of intracellular virus replication. Additionally, nonpermissive subclones retained the ability to bind

HEV-LPs. We infer that these nonpermissive subclones may have some deficiencies in the entry steps of HEV infection after attachment.

We showed that the PLC/PRF/5 subclones exhibited various sensitivities for HEV infection. Akazawa et al. (39) reported a similar phenomenon, with the expression of CD81 (a member of the tetraspanin family that plays a critical role in hepatitis C virus infection (40)) correlating with the permissiveness of Huh7 subclones for HCV infection. Based on that result, those researchers suggested that some unknown receptors might be involved in the permissiveness of Huh7 subclones for HCV infection.

HEV replication efficiencies differed slightly among the PLC/PRF/5 subclones, with efficiencies correlating modestly with the permissiveness to infection; moreover, almost all the remaining subclones of PLC/PRF/5 cells showed similar responses for HEV infection and replication (data not shown). High-level antigen secretion was observed in clones that exhibited rapid increases in HEV antigen production, indicating the efficacy of genome replication and probably reflecting differences in host factors related to HEV life cycle machinery. Akazawa et al. showed that subclones lacking CD81 expression were nonpermissive for HCV infection, while the same subclones still exhibited reduced HCV replication, implying slight differences for HEV replication in permissive subclones and nonpermissive subclones.

S-VLPs bound these subclones at different percentages, suggesting that these subclones might be impaired in HEV entry, that is, in steps occurring after attachment. Heparan sulfate proteoglycans are required for cellular binding of the HEV ORF2 capsid protein and for viral infection (41). This first attachment factor also is used by other viruses (42-44). On the other hand, forced expression of the receptor for enterovirus 71 increased the permissivity of low-permissivity subclones (45). Considered in combination with the findings of the present

study, these data suggest that the differences in S-VLP binding (ranging from 30 to 60%) might reflect differences in the expression levels of unknown receptor(s) and co-receptor(s).

PLC/PRF/5 cells showed limited permissiveness during long-term culturing for HEV infection. Therefore, we cloned and characterized the PLC/PRF/5 cells in an attempt to clarify the reason(s) for the unique character of HEV infection. The observed diversities of viral propagation phenotypes among the subclones imply potential applications for the various subclones in further characterizing the hepatitis E viral life cycle.

In conclusion, we showed the limited permissiveness of PLC/PRF/5 cells, indicating that the cells could be considered quasispecies. To address this variation, we cloned and characterized subclones from our cell line, and showed different susceptibility among subclones. Our data suggest that determination and comparison of gene and protein expression levels in permissive and nonpermissive subclones may be of use for identification of host factors that contribute to the attachment and entry steps in the HEV life cycle.

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Disclosure

None of the authors has any conflict of interest associated with this study.