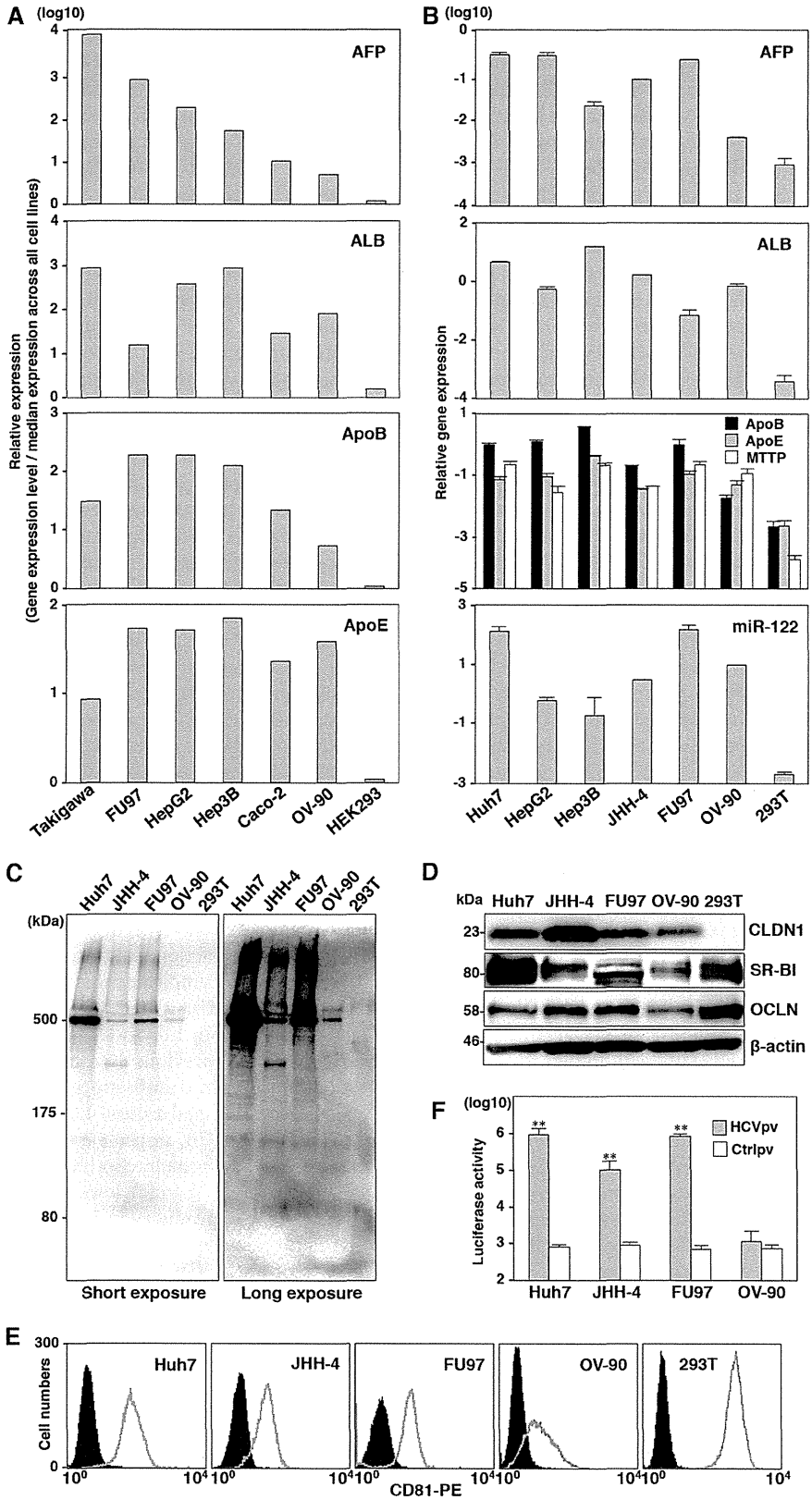


FIG 1 JHH-4 and FU97 cells express high levels of the liver-specific host factors required for HCV propagation. (A) Expression of AFP, ALB, ApoB, and ApoE in cancer cell lines screened by the NextBio Body Atlas application. The expression levels were standardized by the median expression across all cell lines. (B) Expression of AFP, ALB, ApoB, ApoE, MTTP, and miR-122 in AFP-expressing cell lines including HepG2, Hep3B, FU97, and OV-90 cells identified by NextBio

with respect to HCV RNA replication, *in vitro*-transcribed subgenomic HCV RNA of the Con1 strain was electroporated into Huh7 and FU97 cells and cultured in medium containing G418 for a month, and then subgenomic replicon (SGR) cells of the Con1 strain were established (Fig. 4A). Replication of HCV RNA in four clones of the FU97 replicon cells was examined by qRT-PCR and immunoblotting. All clones contained a high level of HCV RNA (3×10^7 to 7×10^7 copies/μg total RNA) (Fig. 4B, upper panel), and the NS5A protein was also detected (Fig. 4B, lower panel). We examined the localization of NS5A and dsRNA in clone 5 of FU97 SGR cells by immunofluorescence analysis. Colocalization of NS5A with dsRNA was observed in clone 5, suggesting that the replication complex required for viral RNA replication was generated in the FU97 SGR cells (Fig. 4C). It has been shown that the infectivity of HCVcc in the cured cells that were established by elimination of the viral genome by treatment with antivirals from Huh7 replicon cells is significantly higher than that in parental Huh7 cells (49). To establish FU97 cured cells, two clones of FU97 replicon 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 BMS-790052 and 100 nM BILN 2061 (clones 5-2 and 7-2) to eliminate viral RNA. Viral RNA was gradually decreased and was completely eliminated at 26 days posttreatment in four clones (Fig. 4D), and elimination of NS5A expression in cured cells was confirmed by immunoblot analysis (Fig. 4E). Next, to examine the susceptibility of the cured cells to the propagation of HCVcc, FU97 cured cell clones (clones 5-1 and 7-1) and parental FU97 cells were infected with HCVcc at an MOI of 1. The cured cells are more permissive to HCV infection, resulting in increased HCV RNA (Fig. 4F) and NS5A abundances (Fig. 4G) compared to the parental cells. These results suggest that susceptibility of the cured FU97 cells to the propagation of HCVcc is higher than that of parental cells, as seen in previous studies using hepatic and nonhepatic cells (17, 18, 49).

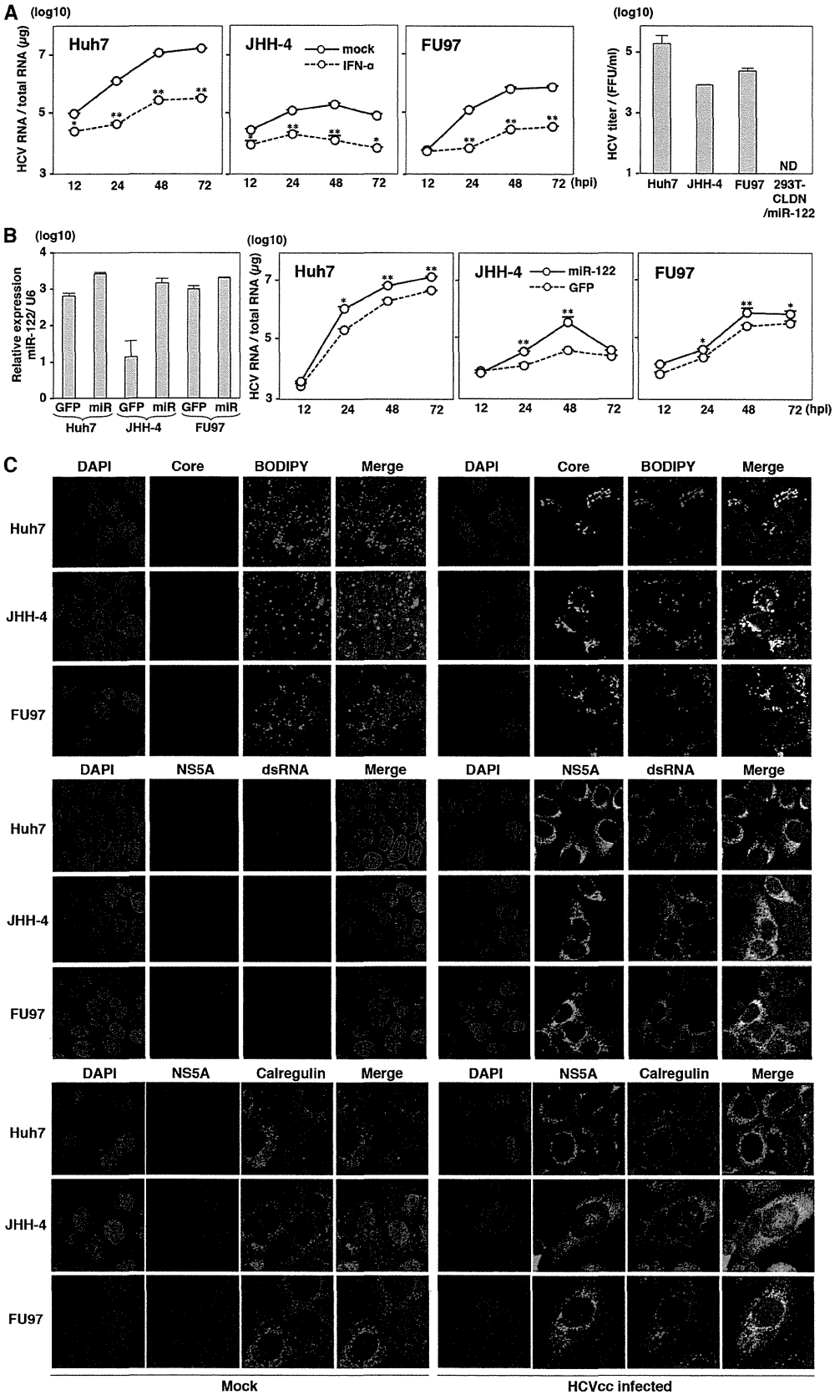
Cured FU97 cells exhibit normal innate immune response. It has been shown that one of the reasons for the high susceptibility of the cured cell line, Huh7.5 cells, to HCVcc infection is the impairment of the innate immune responses caused by mutation in RIG-I, a key sensor for viral RNA (50). To examine the involvement of the innate immune response in the enhancement of HCVcc propagation in the cured FU97 cells, the expression levels of IFN-stimulated gene 15 (ISG15) were determined upon stimulation with IFN-α or infection with VSV. Expression of ISG15 was significantly increased in both parental and cured FU97 cells by treatment with IFN-α or infection with VSV (Fig. 5A). To further confirm the innate immune responses in the cured FU97 cells, reporter plasmids encoding the luciferase gene under the control of either the IFN-β (Fig. 5B, left) or ISRE (Fig. 5B, right) promoter were transfected into both parental and cured FU97 cells and treated with IFN-α or inoculated with VSV. Activation of these promoters in the cured cells was comparable to that in the parental cells. To further assess the authenticity of viral RNA recognition and ISG induction pathways in the cured cells, nuclear localization of IRF3 and STAT2 upon stimulation was determined by immunofluorescence analysis. IRF3 and STAT2 in both parental and cured FU97 cells were translocated at similar levels into the nucleus upon infection with VSV or treatment with IFN-α (Fig. 5C). These results suggest that the efficient propagation of HCVcc in the FU97 cured cells is attributable to reasons other than impairment of innate immunity.

Expression of miR-122 is one of the determinants for HCV RNA abundances. We hypothesized that HCV replicon cells are capable of surviving in the presence of G418 by amplification of the viral genome through the enhancement of miR-122 expression, and cured FU97 cells acquired the ability to propagate HCVcc due to the high-level expression of miR-122. Our previous study also suggested that the expression levels of miR-122 in Huh7, Hep3B, and Hec1B cured cells were higher than those in parental cells (17, 18). To test this hypothesis, the expression levels of miR-122 in the cured FU97 cells were compared with those in parental cells. Interestingly, the cured FU97 cell clones exhibited a 1.8-fold increase in miR-122 expression (Fig. 6A). These results suggested that the efficient propagation of HCVcc in the cured FU97 cells was attributable to enhanced expression of miR-122 rather than the impairment of the innate immunity. To further confirm the correlation between the expression of miR-122 and HCV RNA abundances, we established FU97 cell lines expressing various concentrations of miR-122 by using a lentiviral vector (Fig. 6B), and HCV RNA abundances in these cell lines upon infection with HCVcc were determined by qRT-PCR (Fig. 6C). HCV RNA abundances increased in accord with the expression of miR-122, suggesting that expression of miR-122 is one of the determinants for HCV RNA abundances in cells infected with HCVcc.

HCV particles produced in FU97 cells exhibit similar characteristics to those in hepatic cells. To examine the characteristics of viral particles produced in FU97 cells, HCV particles recovered from the culture supernatants of Huh7.5.1 and FU97 cells infected with HCVcc were fractionated by buoyant density gradient analysis. Previous reports indicated that viral RNA and infectious particles were broadly distributed, with peaks in fractions from 1.13 to 1.14 g/ml and from 1.09 to 1.10 g/ml, respectively (51, 52). In agreement with the previous data, major peaks of HCV RNA and infectious particles in culture supernatants of both Huh7.5.1 and FU97 cells were detected around 1.10 g/ml and 1.09 g/ml, respectively (Fig. 7A and 7B, upper panels). Furthermore, ApoE was detected around the peak fractions of infectivity in both Huh7.5.1 and FU97 cells (Fig. 7A and B, lower panels). These results suggest that HCV particles produced in FU97 cells exhibit characteristics similar to those in hepatic cells.

Effects of anti-HCV drugs on the propagation of HCV in FU97 cells. To determine the difference in the efficacies of antivirals on the HCV propagated in Huh7 and FU97 cells, three DAAs, i.e., BMS-790052, PSI-7977, and BILN 2061 targeting NS5A,

Body Atlas and Huh7, JHH-4, and 293T cells was determined by qPCR. The relative expression of AFP, ApoB, ApoE, MTTP, and ALB mRNA was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, and that of miR-122 was normalized to that of U6 snRNA. (C) Secretion of ApoB in the culture supernatants of Huh7, JHH-4, FU97, OV-90, and 293T cells was determined by immunoblotting by using anti-ApoB antibody. The molecular mass of ApoB100 secreted from hepatocyte is about 500 kDa. (D) Expression of CLDN1, SR-BI, and OCLN in these cell lines was determined by immunoblotting. (E) Expression of hCD81 in the cell lines was determined by flow cytometry. (F) HCVpv-bearing HCV envelope proteins and control virus (Ctrlpv) were inoculated into the cell lines, and luciferase activities were determined at 24 h postinfection. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$) versus the results for control virus.



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.

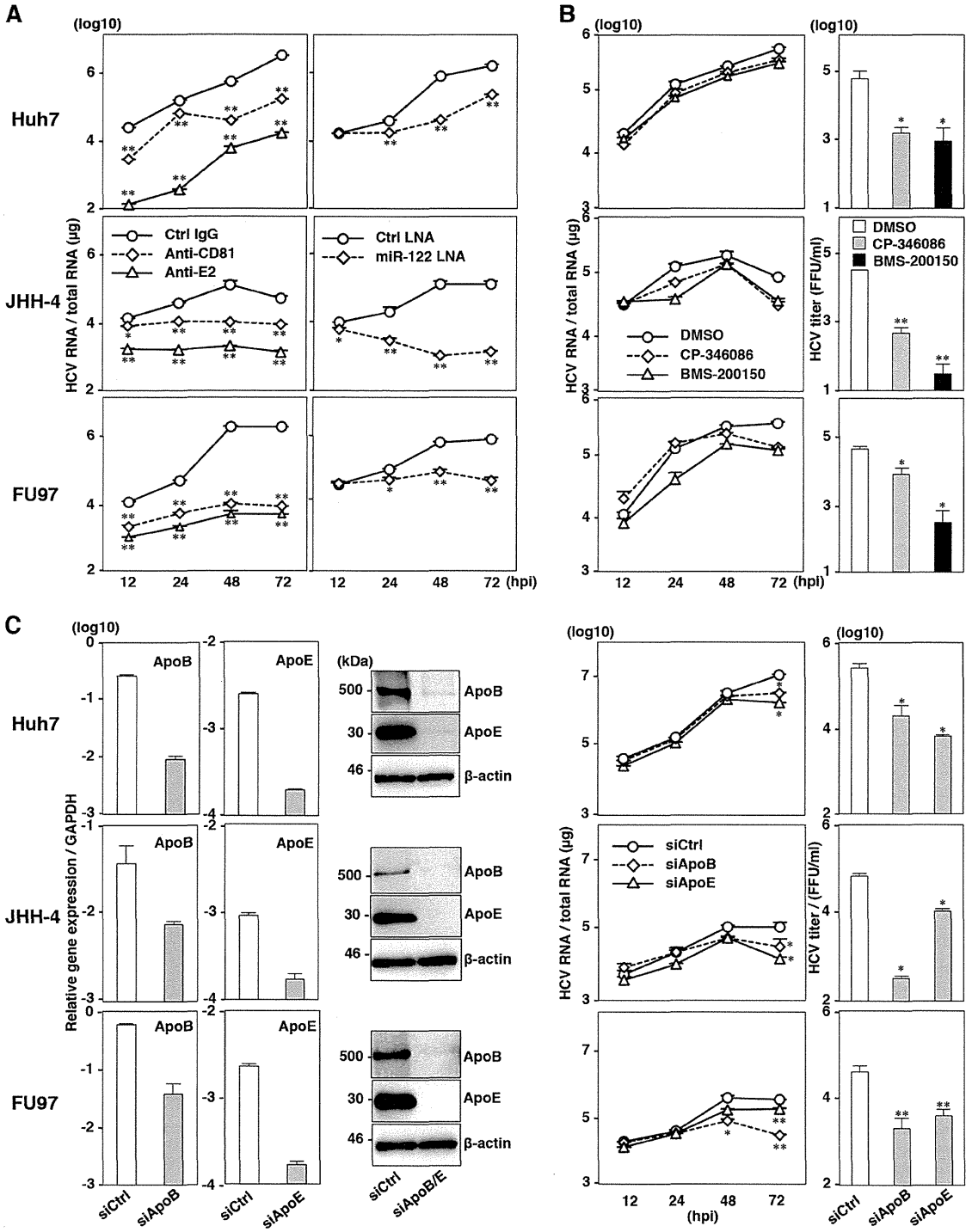


FIG 3 JHH-4 and FU97 cells permit complete propagation of HCVcc without any exogenous expression of host factors crucial for propagation of HCVcc. (A) Effect of inhibitors on the propagation of HCVcc in Huh7, JHH-4, and FU97 cells. (Left panels) HCVcc was preincubated with anti-E2 antibody and inoculated 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.

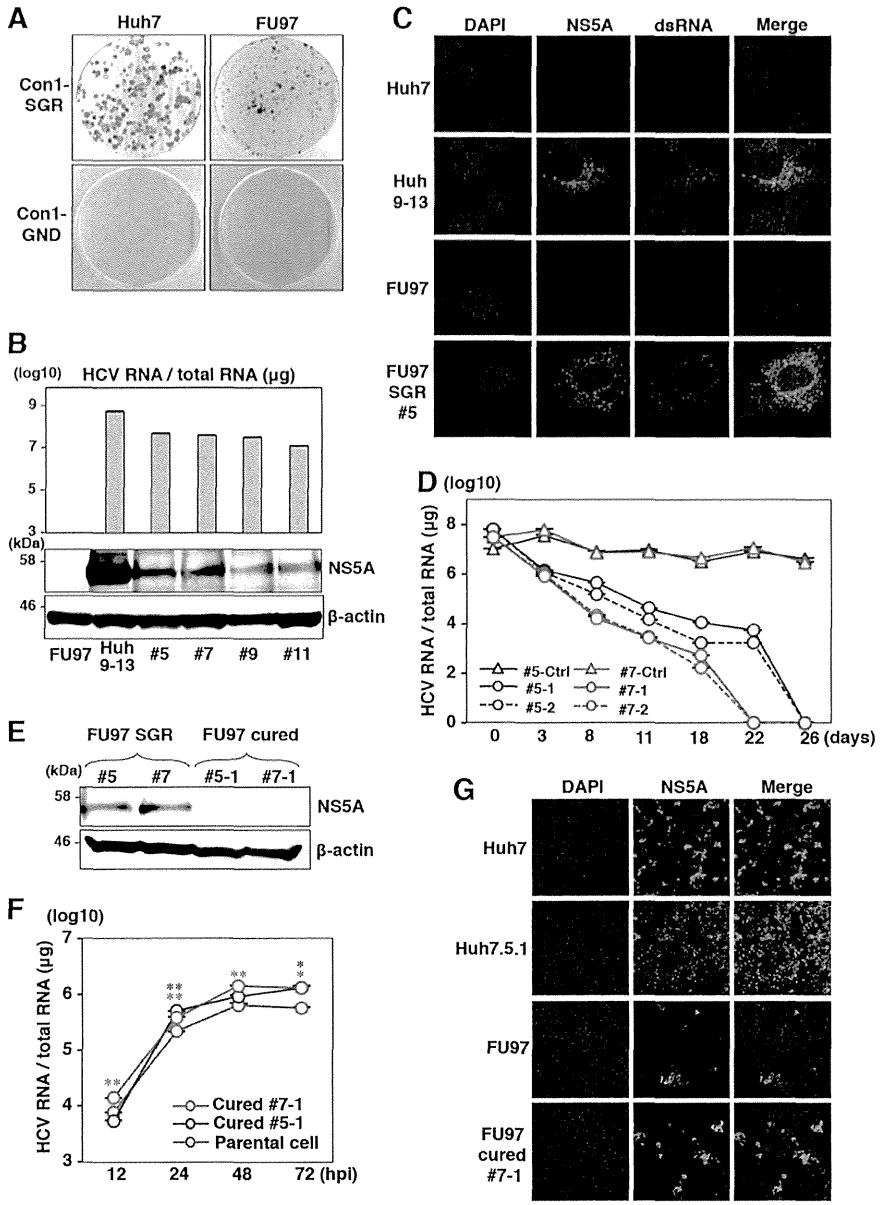


FIG 4 Establishment of HCV RNA replicon and cured FU97 cells. (A) Wild-type SGR RNA (Con1-SGR) or replication-defective RNA (Con1-GND) of the HCV Con1 strain was electroporated into Huh7 and FU97 cells and replaced with medium containing 1 mg/ml and 400 μg/ml of G418 at 24 h postelectroporation, respectively. 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

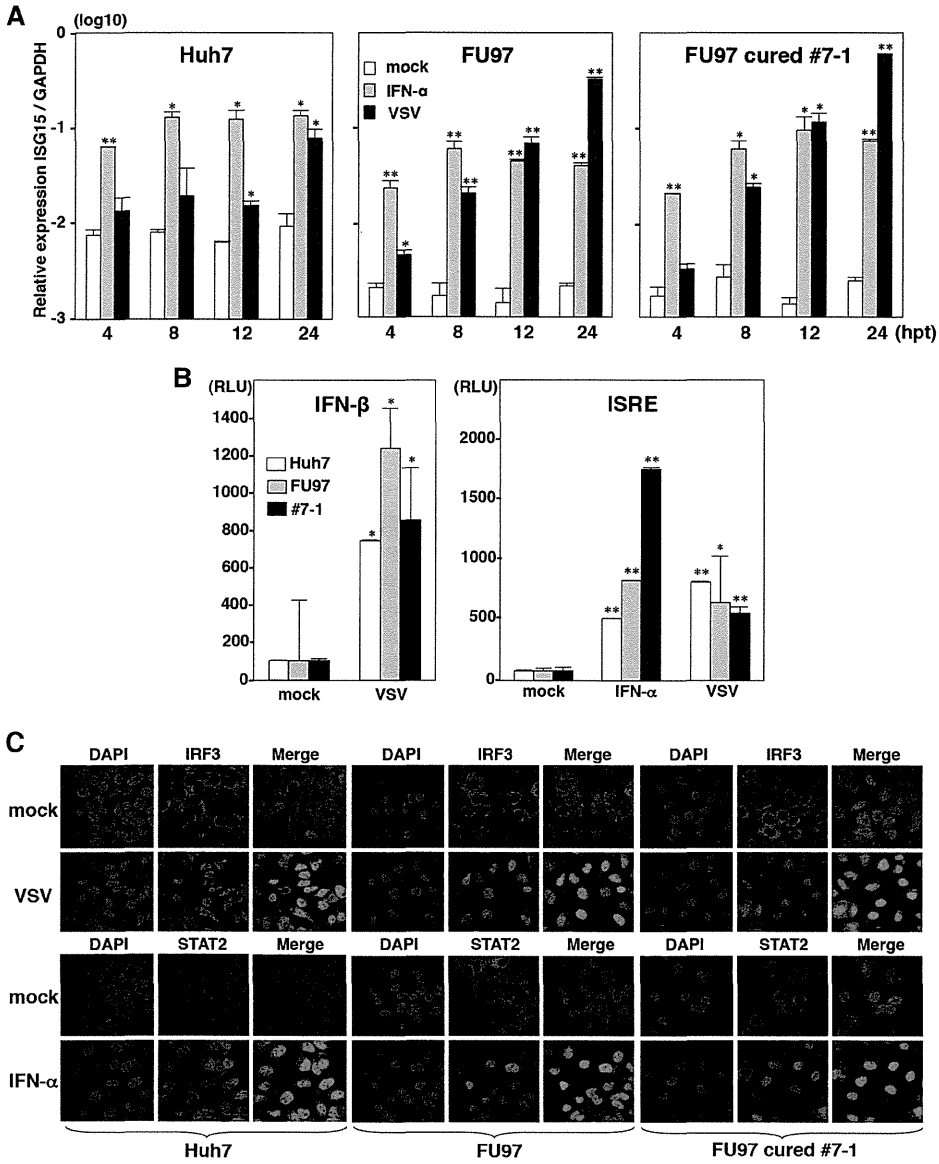


FIG 5 Innate immune response in cured FU97 cells. (A) Huh7, parental, and cured FU97 cells (clone 7-1) were stimulated with 100 IU/ml of IFN-α or infected with VSV. The expression of mRNA of ISG15 at 4, 8, 12, and 24 h posttreatment (hpt) was determined by qPCR and standardized by that of glyceraldehyde-3-phosphate 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

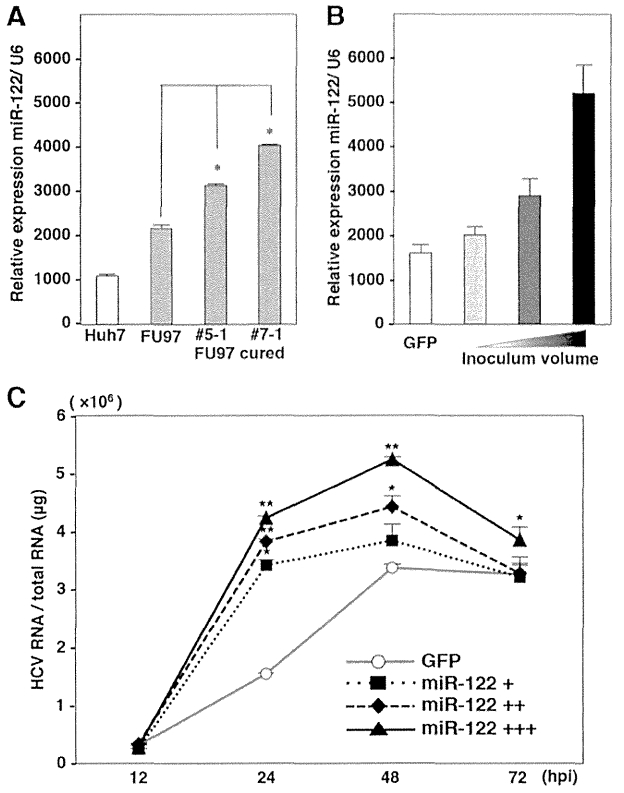


FIG 6 Expression of miR-122 is one of the determinants for HCV RNA abundances. (A) Total RNA was extracted from Huh7 and parental and cured FU97 (clones 5-1 and 7-1) cells, and the relative expression of miR-122 was determined by qPCR. U6 snRNA was used as an internal control. (B) Establishment of FU97 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-

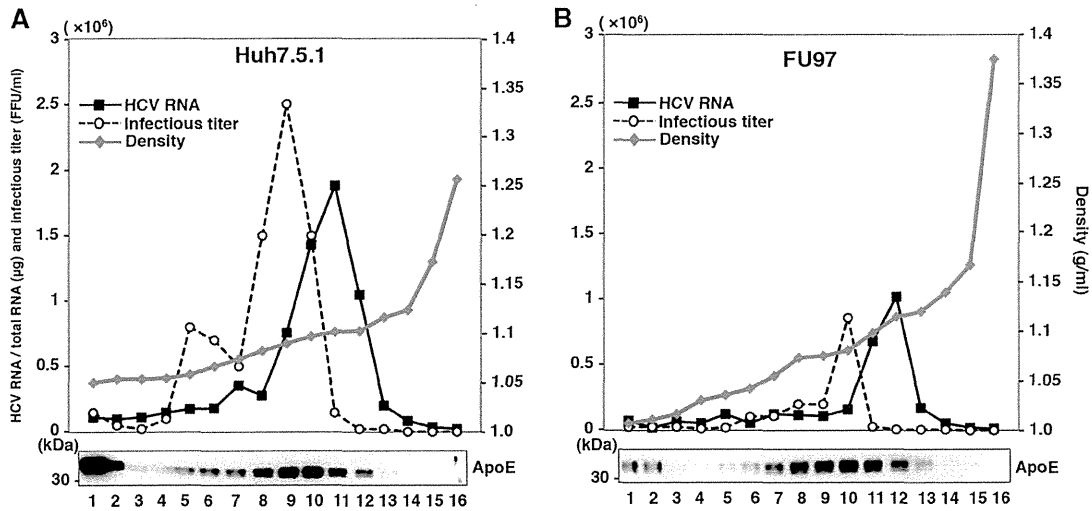


FIG 7 HCV particles produced in FU97 cells exhibit similar characteristics to those in hepatic cells. HCV particles in the culture supernatants of Huh7.5.1 and FU97 cells were harvested at 72 h postinfection with HCVcc and analyzed by using iodixanol density gradient centrifugation. HCV RNA and infectious 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).

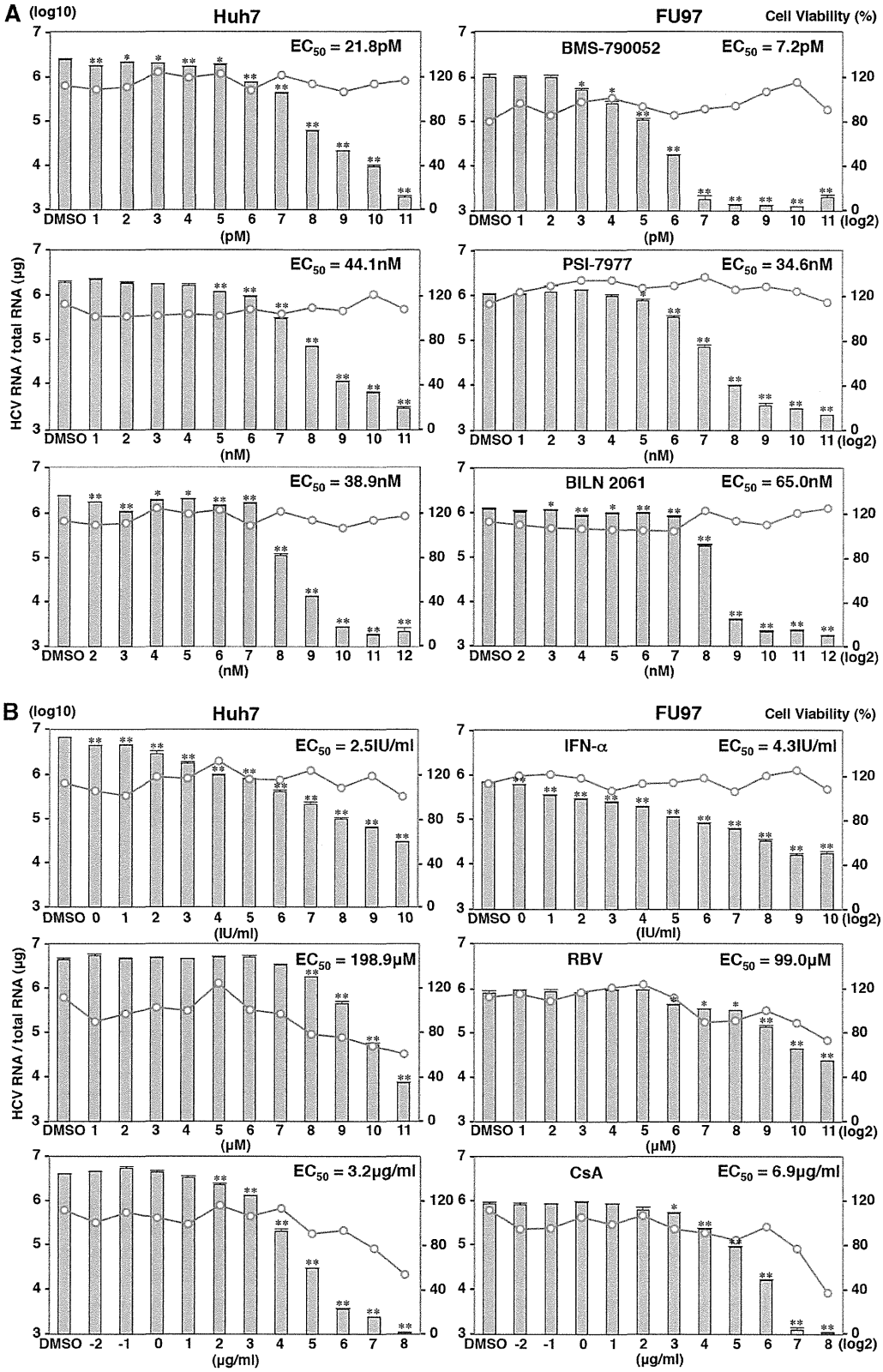


FIG 8 Effects of anti-HCV drugs on the propagation of HCVcc in FU97 cells. (A) Effect of DAAs on the propagation of HCVcc in Huh7 and FU97 cells. Cells infected with HCVcc at an MOI of 1 were treated with BMS-790052, PSI-7977, and BILN 2061 at 3 h postinfection (identifications in right-hand panels). (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.

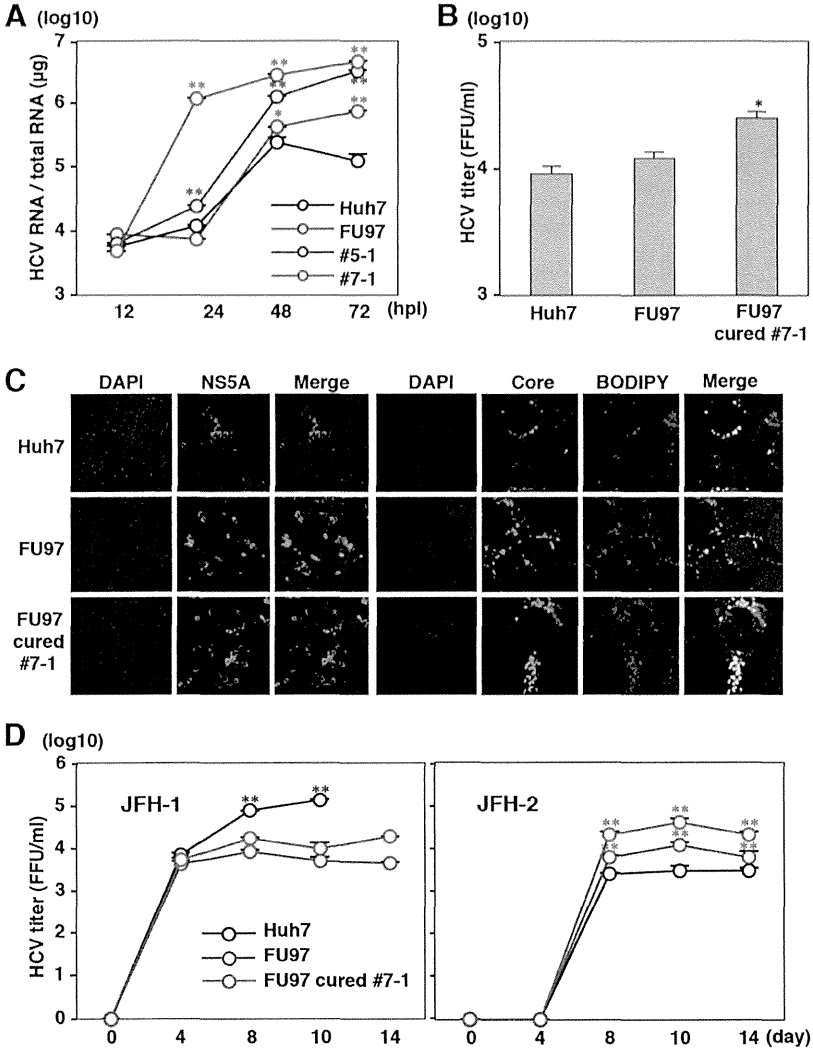


FIG 9 Propagation of HCVcc/JFH-2 in FU97 cells. (A) Huh7, FU97 parental, FU97 cured 5-1, and FU97 cured 7-1 cells were infected with HCVcc/JFH-2 at an MOI of 1, and the intracellular HCV RNA level was determined by qRT-PCR at 12, 24, 48, and 72 h postinfection. (B) Huh7, FU97, and FU97 cured 7-1 cells 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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Title Page

Factors associated with the effect of interferon-α sequential therapy in order to discontinue nucleos(t)ide analogue treatment in patients with chronic hepatitis B

Short title: NUCs/IFNα sequential therapy

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Abbreviations:

HBV: Hepatitis B virus, NUC: nucleos(t)ide analog, IFNα: interferon-α, LAM: lamivudine, ADV: adefovir dipivoxil, ETV: entecavir, HCC: Hepatocellular carcinoma, HBsAg: hepatitis B s antigen, HBcrAg: hepatitis B core related antigen, HBeAg: hepatitis B e antigen, peg-IFNα: pegylated interferon-α

Title page	3	pages
Abstract	1	page
Text	11	pages
Acknowledgements	1	page
References	4	pages
Figure legends	1	page
Tables	4	pages
Figures	3	pages

Abstract

Aim: The factors associated with the outcome of sequential therapy with interferon-α (IFNα) in order to halt nucleos(t)ide analogue (NUC) maintenance treatment for chronic hepatitis B were analyzed. ***Methods:*** A total of 50 patients with chronic hepatitis B who underwent IFNα sequential therapy for cessation of NUCs were enrolled retrospectively. The subjects received NUCs plus IFNα for 4 weeks followed by IFNα alone for 24 weeks. Natural IFNα of 6-million-unit doses was administered 3 times a week. A successful response to NUCs/IFNα sequential therapy was defined as serum hepatitis B virus (HBV) DNA below 4.0 log copies/ml, serum alanine aminotransferase (ALT) below 30 IU/L, and hepatitis B e antigen negativity at 24 months after completing the treatment. ***Results:*** Multivariate analysis revealed that hepatitis B surface antigen (HBsAg) of ≥3.0 log U/ml (P <0.002) and hepatitis B core-related antigen (HBcrAg) of ≥4.5 log U/ml (P <0.003) at the start of IFNα administration were significant factors associated with a 24-month non-response. Maximal levels of ALT and HBV DNA during the follow-up period after completing IFNα therapy were significantly related (P <0.001), and receiver operating characteristic analysis showed that both maximal ALT (P <0.001) and HBV DNA (P <0.001) were significantly related to the final 24-month response. ***Conclusions:*** The combinational use of HBsAg and HBcrAg levels may be useful to predict the 24-month outcome of NUCs/IFNα sequential therapy. Maximal levels of ALT and HBV DNA during post-treatment follow-up might also help monitor responses to IFNα sequential therapy.

Keywords: nucleos(t)ide analogues, interferon-α, sequential therapy, hepatitis B surface antigen, hepatitis B core-related antigen

Introduction

Hepatitis B virus (HBV) infection is a widespread health problem with an estimated 350 to 400 million carriers worldwide. Prolonged infection with HBV can cause chronic hepatitis, which may eventually develop into liver cirrhosis and hepatocellular carcinoma (HCC).[1-3] Currently available antiviral treatments for hepatitis B include nucleos(t)ide analogues (NUCs) and interferon-α (IFNα).[4] NUCs are orally administered and are associated with low rates of adverse effects. Although treatment with NUCs, such as lamivudine (LVD), adefovir dipivoxil (ADV), and entecavir (ETV), induces virological and biochemical responses in most patients, NUC therapy also carries the risk of drug-resistance. Furthermore, patients with hepatitis B are required to undergo extended treatment with NUCs because early discontinuance often leads to relapse.[5, 6] In contrast, the remission of chronic hepatitis B by IFNα is prolonged, but is achieved only in a small percentage of patients.

Serfaty et al.[7] conducted a pilot study on sequential therapy using LVD and IFNα and concluded that this treatment could induce a sustained virologic response in patients with chronic hepatitis B who did not respond to IFNα alone. However, ensuing reports[8-12] were unable to confirm such a cooperative effect. Since the clinical backgrounds of the enrolled patients also differed among the above reports, it has become necessary to clarify the factors associated with the outcome of IFNα sequential therapy in order to estimate its clinical significance.

We previously analyzed patients with chronic hepatitis B who ceased NUC therapy and showed that lower hepatitis B surface antigen (HBsAg) and hepatitis B core-related antigen (HBcrAg) levels were associated with a favorable clinical outcome in subjects negative for hepatitis B e antigen (HBeAg) and HBV DNA at NUC discontinuation.[13, 14] Although we identified patients in whom NUCs could be safely

halted with high reliance, such patients accounted for a relatively minor percentage. Therefore, we conducted the present study to analyze the effect of IFNα sequential therapy on successfully stopping NUCs.

This report retrospectively analyzes the factors associated with outcome of IFNα sequential therapy following NUC treatment. As the subjects were followed long-term, treatment responses at 24 months after stopping IFNα were evaluated and compared with those at 6 and 12 months.

Patients and Methods

Patients

A total of 50 patients with chronic hepatitis B who underwent IFNα sequential therapy in order to halt NUC therapy between May, 2002 and September, 2010 were enrolled. Subjects received NUC plus IFNα for 4 weeks followed by IFNα alone for 20 weeks (Fig. 1). Natural IFNα (Sumiferon, Sumitomo Dainippon Pharma Co., Ltd., Tokyo) at a dose of 6 million units was administered 3 times a week. Doses were reduced to 3 million units during exceptional circumstances, such as side effects. All patients completed 24 weeks of IFNα administration and received over 80% of the scheduled dose. Patients were recruited retrospectively from 8 hospitals across Japan (Shinshu University Hospital, National Hospital Organization Nagasaki Medical Center, Toranomon Hospital, Hiroshima University Hospital, Chiba University Hospital, The Hospital of Hyogo College of Medicine, Kumamoto Shinto General Hospital, and Teine Keijinkai Hospital). The demographic data of the subjects are presented in Table 1. The median age at NUC cessation was 35 years. Approximately three-fourths of the patients were men. Genotype C HBV was predominant as has earlier been reported for Japan.[15] Eighty-six percent of patients began NUC therapy

with LVD and 14% did so with ETV. The duration of NUC administration ranged from 4 to 121 months. The follow-up period was defined as the point of stopping IFNα administration up until the last visit or to when NUCs were re-administered due to reactivation of hepatitis B. NUCs were recommenced in 25 (50%) of the 50 patients enrolled. Among them, 17 were treated before judgment of the 24-month response to sequential therapy. All patients requiring re-administration of NUCs possessed ALT levels of over 80 IU/l and HBV DNA levels of over 5.8 log copies/ml at or just before the point of NUC re-continuation, which fulfilled the established requirements for restarting NUCs.[13, 14, 16]

HBsAg was confirmed to be positive on at least two occasions at least 6 months apart in all patients before NUC treatment. Tests for hepatitis C and human immunodeficiency virus antibodies were all negative. Patients complicated with HCC or signs of hepatic failure at the cessation of NUC administration were excluded from the study. No such complications were observed during follow-up.

With few exceptions, patients were seen at least once a month during the first year of follow-up, at least once every 3 months during the second year, and at least once every 6 months afterwards. No patient developed HCC or hepatic failure during the follow-up period. Stored serum samples were kept frozen at -20°C or below until assayed. This study was approved by the Ethics Committees of all participating institutions (approval reference 1117 for Shinshu University Hospital, 24085 for National Hospital Organization Nagasaki Medical Center, 758 for Toranomon Hospital, 321 for Hiroshima University Hospital, 934 and 977 for Chiba University Hospital, 779 for The Hospital of Hyogo College of Medicine, 411 for Kumamoto Shinto General Hospital, and "Analysis of efficacy of IFN to stop NUCs in patients with chronic hepatitis B" for Teine Keijinkai Hospital).