spite of efficient replication of HCV RNA in nonhepatic cells, suggesting that liver-specific factors other than miR-122 are involved in HCV assembly. Previous reports suggested that very-low-density lipoprotein (VLDL)-associated proteins, including apolipoprotein B (ApoB), apolipoprotein E (ApoE), and microsomal triglyceride transfer protein (MTTP), play important roles in infectious particle production of HCV (19–23). In addition, Miyanari et al. indicated that lipid droplets (LDs) are crucial organelles for HCV particle assembly (24). These reports suggest that liver-specific lipid metabolism and liver-specific host factors closely participate in assembly of HCV.

Cancer cells are classified into well-differentiated and intermediately and poorly differentiated stages, and these stages have been shown to be strongly related to cancer behaviors, with an immature tumor generally being more aggressive than its more differentiated counterpart. Thus, it is believed that well-differentiated cancer cells maintain the tissue-specific cellular functions and exhibit morphology similar to that of normal cells (25). Permissive cell lines for HCV propagation, including Huh7, HepG2, and Hep3B cells, are derived from well-differentiated hepatocellular carcinoma (HCC) (26, 27). In addition, recent reports indicated 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 (28–30). These results suggest that the hepatic differentiation required for hepatic functions is involved in HCV propagation.

In this study, we identified novel cell lines supporting complete propagation of HCVcc by screening cancer cell lines expressing α-fetoprotein (AFP), which is highly expressed in well-differentiated hepatocellular carcinoma and evaluated in most cancer cell lines (31). These cells exhibit high levels of expression of liver-specific host factors and permit complete propagation of HCVcc without any exogenous expression of liver-specific factors, including receptor molecules, miR-122, and apolipoproteins. Our current study suggests that hepatic differentiation participates in the expression of liver-specific host factors required for complete propagation of HCV.

## MATERIALS AND METHODS

**NextBio Body Atlas.** The NextBio Body Atlas application allows the aggregated analysis of gene expression across various normal tissues, normal cell types, and cancer cell lines. It enables us to investigate the expression of individual genes as well as gene sets. Samples for Body Atlas data are obtained from publicly available studies that are internally curated, annotated, and processed (32). Body Atlas measurements are generated from all available RNA expression studies that used Affymetrix U133 Plus or U133A Genechip arrays for human studies. The results for 128 human tissue samples from 1,067 arrays, those for 157 human cell types from 1,474 arrays, and those for 359 human cancer cell lines from 376 arrays are incorporated. In this study, we screened cell lines expressing a high level of AFP. The details of the analysis protocol developed by NextBio were described previously (32). The raw data used in this application are derived from the GSK Cancer Cell Line data deposited at the National Cancer Institute website (https://array.nci.nih.gov/caarray/project/woost-00041).

**Plasmids.** The cDNA clones of pri-miR-122 and *Aequorea coerulescens* green fluorescent protein (AcGFP) were inserted between the XhoI and XbaI sites of lentiviral vector pCSII-EF-RfA, which was provided by M. Hijikata, and the resulting plasmids were designated pCSII-EF-miR-122 and pCSII-EF-AcGFP, respectively. Plasmids pHH-JFH1-E2p7NS2mt, encoding a cDNA of a full-length RNA of the JFH-1 strain, and pJFH2/AS/mtT4, encoding a cDNA of a full-length RNA of the JFH-2 strain, were described previously (33, 34). pSGR-Con1, which encodes a subgenomic replicon (SGR) of the Con1 strain, was provided by R. Bartenschlager. pIFN-β-Luc and pISRE-Luc carrying a firefly luciferase (Luc) gene under the control of the IFN-β- and IFN-sensitive response element (ISRE) promoters, respectively, were provided by S. Akira. The internal control plasmid encoding a *Renilla* luciferase (pRL-SV40) was purchased from Promega (Madison, WI). The plasmids used in this study were confirmed by sequencing with an ABI Prism 3130 genetic analyzer (Applied Biosystems, Tokyo, Japan).

**Cell lines.** All cell lines were cultured at 37°C under the conditions of a humidified atmosphere and 5% $CO_2$. Human hepatocellular carcinoma-derived Huh7, Hep3B, HepG2, and JHH-4 (JCRB0435) cells, embryonic kidney-derived 293T cells, gastric cancer-derived FU97 (JCRB1074) cells, and ovarian adenocarcinoma-derived OV-90 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum (FCS). JHH-4 and FU97 cells were obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank. OV-90 cells were obtained from the American Type Culture Collection (ATCC). 293T-CLDN cells stably expressing claudin-1 (CLDN1) were established by the introduction of expression plasmid pCAG-pm3 encoding CLDN1 under the control of the CAG promoter. The Huh7-derived cell line Huh7.5.1 was provided by F. Chisari. Huh7 and FU97 cells harboring the SGR of the Con1 strain (Con1-SGR) were prepared as described previously (35) and maintained in DMEM containing 1 mg/ml and 400 μg/ml of G418 (Nacalai Tesque, Kyoto, Japan), respectively.

**Preparation of viruses.** HCVs derived from the genotype 2a JFH-1 strain (HCVcc) were prepared after serial passages of the culture supernatants of Huh7.5.1 cells transfected with pHH-JFH1-E2p7NS2mt into Huh7.5.1 cells (33). HCVs derived from the genotype 2a JFH-2 strain (HCVcc/JFH-2) were prepared by several passages of the culture supernatants of Huh7.5.1 cells electroporated with JFH-2 RNA transcribed *in vitro*. Infectious titers were determined by a focus-forming assay and expressed as focus-forming units (FFU) (15). The vesicular stomatitis virus (VSV) variant NCP12.1 derived from the Indiana strain was provided by M. Whitt. Pseudotype VSVs bearing HCV E1 and E2 glycoproteins (HCVpv), were prepared as described previously (36), and infectivity was assessed by a luciferase assay system (Promega) according to a protocol provided by the manufacturer and expressed in relative light units (RLU).

**Antibodies and drugs.** Mouse monoclonal antibodies to HCV nonstructural protein 5A (NS5A) and β-actin were purchased from Austral Biologicals (San Ramon, CA) and Sigma-Aldrich, respectively. Rabbit anti-HCV core protein was prepared as described previously (37). Mouse anti-E2 polyclonal antibody was also prepared (unpublished data). Anti-human CD81 (hCD81) monoclonal antibody (JS-81) and rabbit anti-scavenger receptor class B type 1 (SR-BI) antibody were purchased from BD Biosciences (Franklin Lakes, NJ) and Novus Biologicals (Littleton, CO), respectively. Rabbit anti-CLDN1 and anti-occludin (OCLN) antibodies, Alexa Fluor 488 (AF488)-conjugated anti-rabbit and -mouse IgG antibodies, and AF594-conjugated anti-rabbit IgG antibodies were purchased from Life Technologies (Carlsbad, CA). Rabbit anti-signal transducer and activators of transcription 2 (STAT2) antibody and anti-calregulin antibody were purchased from Santa Cruz (Santa Cruz, CA). Rabbit anti-IFN regulatory factor 3 (IRF3) antibody was purchased from Abcam (Cambridge, United Kingdom). Phycoerythrin (PE)-conjugated anti-hCD81 and anti-mouse IgG antibodies were purchased from BD Biosciences. Anti-ApoB horseradish peroxidase (HRP)-conjugated antibody was purchased from ALerCHEK (Springvale, ME). The HCV NS3/4A protease inhibitor was purchased from Acme Bioscience (Salt Lake City, UT). Human recombinant alpha IFN (IFN-α) was purchased from PBL Biomedical Laboratories (Piscataway, NJ). BODIPY558/568 lipid probe and 4′, 6-diamidino-2-phenylindole (DAPI) were purchased from Life Technologies and Vector Laboratories, Inc. (Burlingame, CA), respectively. Cyclosporine and CP-346086 were purchased from Sigma-Aldrich. Ribavirin (RBV) was purchased from Tokyo Chemical Industry

(Tokyo, Japan). BMS-790052 and PSI-7977 were purchased from Shanghai Haoyuan Chemexpress (Shanghai, China). BMS-200150 was purchased from ChemStep (Martillac, France). The locked nucleic acid (LNA) targeted to miR-122, miR-122–LNA (5′-CcAttGTcaCaCtCC-3′), and its negative control, control-LNA (Ctrl-LNA) (5′-CcAttCTgaCcCtA C-3′), were purchased from Gene Design (Osaka, Japan); LNAs and DNAs are indicated in capital and lowercase letters, respectively. Sulfur atoms in oligonucleotide phosphorothioates are substituted for nonbridging oxygen atoms. The capital C indicates LNA methylcytosine.

**Transfection and lentiviral gene transduction.** Cells were transfected with the plasmids by using TransIT LT-1 transfection reagents (Mirus, Madison, WI) according to the manufacturer's protocol. LNAs were introduced into cells by Lipofectamine RNAi MAX (Life Technologies). The lentiviral vectors and ViraPower lentiviral packaging mix (Life Technologies) were cotransfected into 293T cells, and the supernatants were recovered at 48 h posttransfection. The lentivirus titer was determined by a lenti-XTM quantitative reverse transcription-PCR (qRT-PCR) titration kit (Clontech, Mountain View, CA), and the expression levels of miR-122 and AcGFP were determined at 48 h postinoculation.

**Quantitative RT-PCR.** HCV RNA levels were determined by a method described previously (38). Total RNA was extracted from cells by using an RNeasy minikit (Qiagen, Valencia, CA), and the first-strand cDNA synthesis and qRT-PCR were performed with TaqMan EZ RT-PCR core reagents and a ViiA7 system (Life Technologies), respectively, according to the manufacturer's protocol. The primers for TaqMan PCR targeted to the noncoding region of HCV RNA were synthesized as previously reported (39). To determine the expression of miR-122, total miRNAs were prepared by using an miReasy minikit (Qiagen), and miR-122 was determined by using the fully processed miR-122-specific RT and PCR primers provided in the TaqMan microRNA assays (Life Technologies) according to the manufacturer's protocol. U6 small nuclear RNA was used as an internal control. Fluorescent signals were analyzed with the ViiA7 system.

**Immunoblotting.** Cells were lysed on ice in lysis buffer (20 mM Tris-HCl [pH 7.4], 135 mM NaCl, 1% Triton X-100, 10% glycerol) supplemented with a protease inhibitor mix (Nacalai Tesque). Culture supernatants of cells incubated for 3 days were used for detection of ApoB. The samples were boiled in loading buffer and subjected to a 5% to 20% gradient SDS-PAGE or 3 to 8% Tris-acetate gel (Thermo Scientific, Waltham, MA). The proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) and reacted with the appropriate antibodies. The immune complexes were visualized with SuperSignal West Femto substrate (Pierce, Rockford, IL) and detected with an LAS-3000 image analyzer system (Fujifilm, Tokyo, Japan).

**Immunofluorescence assay.** Cells cultured on glass slides were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at room temperature for 30 min, permeabilized for 20 min at room temperature with PBS containing 0.2% Triton X-100, washed three times with PBS, and blocked with PBS containing 2% FCS for 1 h at room temperature. Then cells were incubated with PBS containing appropriate primary antibodies at room temperature for 1 h, washed three times with PBS, and incubated with PBS containing AF488- or AF594-conjugated secondary antibodies at room temperature for 45 min. For lipid droplet staining, cells were incubated in medium containing 20 μg/ml BODIPY for 20 min at 37°C, washed with prewarmed fresh medium, and incubated for 20 min at 37°C. The stained cells were covered with Vectashield Mounting Medium containing DAPI (Vector Laboratories Inc., Burlingame, CA) and observed with a FluoView FV1000 laser scanning confocal microscope (Olympus, Tokyo, Japan).

***In vitro* transcription, RNA transfection, and colony formation.** The plasmid pSGR-Con1 was linearized with ScaI and transcribed *in vitro* by using a MEGAscript T7 kit (Life Technologies) according to the manufacturer's protocol. The *in vitro*-transcribed RNA (10 μg) was electroporated into FU97 cells at $10^7$ cells/0.4 ml under conditions of 210 V and 960 μF using a Gene Pulser apparatus (Bio-Rad, Hercules, CA) and plated on DMEM containing 10% FCS. The medium of FU97 cells was replaced with fresh DMEM containing 10% FCS and 400 μg/ml G418 at 24 h postelectroporation. The remaining colonies were cloned by using a cloning ring (Asahi Glass, Tokyo, Japan) or fixed with 4% PFA in PBS and stained with crystal violet at 5 weeks postelectroporation.

**Flow cytometry.** Cultured cells were detached with 0.25% trypsin-EDTA, incubated with PE-conjugated anti-hCD81 antibody or anti-mouse IgG antibody for 1 h at 4°C, washed twice with PBS containing 1% bovine serum albumin (BSA), and analyzed by using a flow cytometry system (FACSCalibur; BD Biosciences).

**Gene silencing.** A commercially available small interfering RNA (siRNA) pool targeting ApoB and ApoE (siGENOME SMARTpool human ApoB and ApoE) and a control nontargeting siRNA were purchased from Dharmacon (Buckinghamshire, United Kingdom) and transfected into JHH-4 and FU97 cells using Lipofectamine RNAi MAX (Life Technologies) according to the manufacturer's protocol.

**Luciferase assay.** Cells seeded onto 24-well plates at a concentration of $5 \times 10^4$ cells/well were transfected with 250 ng of each of the plasmids, stimulated with the appropriate ligands for 24 h at 24 h posttransfection, and lysed in 100 μl of passive lysis buffer (Promega). Luciferase activity was measured in 20-μl aliquots of the cell lysates using a dual-luciferase reporter assay system (Promega). Firefly luciferase activity was standardized with that of *Renilla* luciferase cotransfected with the internal control plasmid pRL-SV40 and was expressed in RLU.

**Neutralization assay.** Huh7, JHH-4, and FU97 cells were pretreated with 10 μg/ml of anti-hCD81 (JS-81) monoclonal antibody for 1 h at 37°C and then inoculated with HCVcc ($1 \times 10^6$ FFU/ml). Anti-E2 monoclonal antibody (10 μg/ml) was incubated with HCVcc ($1 \times 10^6$ FFU/ml) for 1 h and then inoculated into cells. Intracellular HCV RNA levels at 12, 24, 48, and 72 h postinfection were determined by qRT-PCR.

**Buoyant density gradient analysis.** Culture supernatants of Huh7.5.1 and FU97 cells infected with HCVcc at 72 h postinfection were passed through 0.45-μm-pore-size filters and concentrated by a Spin-X Concentrator (100,000-molecular-weight cutoff column; Corning, Lowell, MA). One milliliter of concentrated sample was layered onto the top of a linear gradient formed from 10% to 40% of OptiPrep (Axis-Shield PoC, Oslo, Norway) in PBS and spun at 32,000 rpm for 16 h at 4°C by using an SW41-Ti rotor (Beckman Coulter, Fullerton, CA). Each fraction collected from the top was analyzed by qRT-PCR, focus-forming assay, and immunoblotting.

**Statistical analysis.** The data for statistical analyses are the averages of three independent experiments. Results were expressed as the means ± standard deviations. The significance of differences in the means was determined by Student's *t* test.

## RESULTS

**JHH-4 and FU97 cells express high levels of the liver-specific host factors required for HCV propagation.** AFP is known as a marker for not only well-differentiated HCC (31) but also the early stage of differentiation to hepatocytes in embryonic stem (ES)/iPS cells (29, 40, 41). Generally, well-differentiated cancer cells show better maintenance of their cell-specific functions than poorly differentiated cancer cells. Therefore, we hypothesized that cancer cell lines with high levels of AFP expression retain sufficient hepatic function for HCV propagation. To examine this hypothesis, we first screened cancer cell lines by using the NextBio Body Atlas application and identified the following cell lines that expressed high levels of AFP: Takigawa and FU97 cells derived from gastric cancer, HepG2 and Hep3B cells from hepatocellular carcinoma, Caco-2 cells from colon cancer, and OV-90 cells from ovarian cancer. To evaluate the correlation of AFP expression with the hepatic functions in these cell lines, we examined the expression of liver-specific host factors, including albumin (ALB), ApoB, and ApoE, by using the Web-based NextBio search engine and found

that these cell lines expressed higher levels of the liver-specific genes than the HEK293 cells used as negative controls (Fig. 1A). These results suggested that the expression of AFP was correlated with that of the examined liver-specific host factors in cancer cell lines. Next, to confirm this correlation, the expression levels of ALB, ApoB, ApoE, MTTP, and miR-122 were determined by qPCR in AFP-expressing cell lines, including FU97, OV-90, and HCC-derived Huh7, HepG2, Hep3B, and JHH-4 cells (Fig. 1B). Takigawa cells were difficult to culture, and Caco-2 cells have previously been reported to permit entry and replication of HCV (42, 43); therefore, we excluded these cell lines for further analyses. JHH-4 cells were previously shown to permit a partial propagation of HCV in a three-dimensional cultivation by using a radial-flow bioreactor system upon inoculation with plasma from an HCV carrier (44). In contrast to 293T cells, these AFP-expressing cell lines express high levels of the examined liver-specific host factors, suggesting that these cell lines maintain their hepatic functions. Because previous studies have shown that Huh7, HepG2, and Hep3B cells are susceptible to HCVcc infection, we selected JHH-4, FU97, and OV-90 cells for further investigation as new cell line candidates for HCV propagation. It is well known that hepatocytes and intestinal cells produce ApoB100 and ApoB48, respectively. ApoB100 was detected in the culture supernatants of Huh7, JHH-4, FU97, and OV-90 cells but not in those of 293T cells by immunoblotting (Fig. 1C). These results suggest that FU97 and OV-90 cells are differentiated into hepatocyte-like cells and possess liver-specific functions. The expression of entry receptor molecules for HCV, including hCD81, CLDN1, OCLN, and SR-BI (45–48), in these cell lines was confirmed by immunoblotting and fluorescence-activated cell sorting (FACS) analyses (Fig. 1D and E). To further determine the authenticity of the receptor candidates for HCV entry, HCVpv was inoculated into these cell lines. Although the infectivity of HCVpv to JHH-4 and FU97 cells was comparable to that in Huh7 cells, OV-90 cells did not show any susceptibility to HCVpv infection (Fig. 1F). Cell surface expression of CLDN1 and OCLN was detected in OV-90 cells (data not shown); therefore, lack of susceptibility of OV-90 cells to HCVpv infection might be attributable to lower expression of SR-B1 and hCD81 in OV-90 cells than in other cell lines (Fig. 1D and E). Thus, we selected JHH-4 and FU97 cells for further investigation of HCV propagation.

**JHH-4 and FU97 cells permit HCV propagation.** To examine the susceptibility of JHH-4 and FU97 cells to HCV propagation, HCVcc was inoculated into these cells at a multiplicity of infection (MOI) of 1, and intracellular HCV RNA and infectious titers in the culture supernatants were determined by qRT-PCR and focus-forming assay, respectively. FU97 cells exhibited higher levels of HCV gene expression than JHH-4 cells, and these levels increased continuously until 48 h postinfection; treatment with IFN-α significantly inhibited HCV gene expression in both JHH-4 and FU97 cells (Fig. 2A, left panels). In addition, substantial amounts of infectious particles were detected in the culture supernatants of JHH-4 and FU97 cells infected with HCVcc, in contrast to the lack of infectious particles in the culture supernatants of 293T-CLDN/miR-122 cells infected with HCVcc (Fig. 2A, bar graph). Recent reports have shown that exogenous expression of miR-122 enhances HCV RNA abundances in several hepatic or nonhepatic cell lines (16–18). Therefore, we examined the effect of miR-122 overexpression on HCV RNA abundances in both JHH-4 and FU97 cells. miR-122 was introduced in these cells by a lentiviral vector encoding pri-miR-122, an unprocessed miR-122, and miR-122 expression was confirmed by quantitative PCR (qPCR) analysis (Fig. 2B, bar graph). In contrast to the slight increase of HCV RNA in FU97 cells, JHH-4 cells exhibited a significant increase of HCV RNA, suggesting that the expression level of miR-122 is a key determinant for the efficient propagation of HCV (Fig. 2B, right panels). A previous study has shown that NS5A proteins were localized around the endoplasmic reticulum (ER) membrane, and accumulation of core protein around lipid droplets (LDs) facilitates efficient assembly of infectious particles in Huh7 cells (24). Immunofluorescence microscopy observation revealed that core and NS5A proteins in JHH-4 and FU97 cells infected with HCVcc were detected around LDs and in the ER together with double-stranded RNA (dsRNA), respectively (Fig. 2C). These results suggest that expression of liver-specific factors permits complete propagation of HCVcc in JHH-4 and FU97 cells and that hepatic characteristics play crucial roles in HCV propagation.

**JHH-4 and FU97 cells permit complete propagation of HCVcc without any exogenous expression of host factors crucial for propagation of HCV.** To further characterize the propagation of HCV in JHH-4 and FU97 cells, we examined the effects of the HCV inhibitors on the replication of HCV RNA. Preincubation with anti-HCV E2 antibody and pretreatment of cells with anti-hCD81 monoclonal antibody significantly inhibited HCVcc infection not only in Huh7 cells but also in JHH-4 and FU97 cells, suggesting that hCD81 is also involved in HCV entry into JHH-4 and FU97 cells (Fig. 3A, left panels). To examine the effect of miR-122 expression on HCV RNA abundances, cells were treated with LNA specific to either miR-122 (miR-122–LNA) or a nonspecific LNA (Ctrl-LNA) at 6 h before infection with HCVcc. Treatment with miR-122–LNA but not with Ctrl-LNA significantly reduced the HCV RNA abundances in these cell lines, suggesting that miR-122 also plays a crucial role in the efficient propagation of HCVcc in JHH-4 and FU97 cells (Fig. 3A, right panels). Previous reports showed that treatment with MTTP inhibitors inhibited the production of infectious particles of HCVcc in Huh7 cells (20, 22). Although intracellular HCV RNA levels in Huh7, JHH-4, and FU97 cells were not inhibited by the treatment with MTTP inhibitors, including CP-346086 and BMS-200150 (Fig. 3B, left panels), the production of infectious particles was significantly decreased in these cells (Fig. 3B, right panels). These results suggest that the VLDL secretion pathway also participates in the propagation of HCV in JHH-4 and FU97 cells. Furthermore, it was shown that ApoB and ApoE are involved in the production of HCV particles in Huh7 cells (20–22). To confirm the role of ApoB and ApoE in HCV propagation in JHH-4 and FU97 cells, the expression of ApoB and ApoE was suppressed by siRNAs (Fig. 3C, left panels). The suppression of ApoB and ApoE expression significantly reduced HCV RNA levels in cells infected with HCVcc at an MOI of 1 (Fig. 3C, middle panels) and significantly reduced the infectious titers in the supernatants (Fig. 3C, right panels) at 72 h postinfection. Collectively, these results suggest that the JHH-4 and FU97 cells permit complete propagation of HCVcc without any exogenous expression of the host factors crucial for propagation of HCV, including receptor molecules, miR-122, and VLDL-associated proteins. FU97 cells exhibited higher susceptibility to HCVcc propagation than JHH-4 cells (Fig. 2A), and thus we characterized the FU97 cells in greater detail.

**Establishment of HCV RNA replicon and cured cells by using FU97 cells.** To further examine the characteristics of FU97 cells

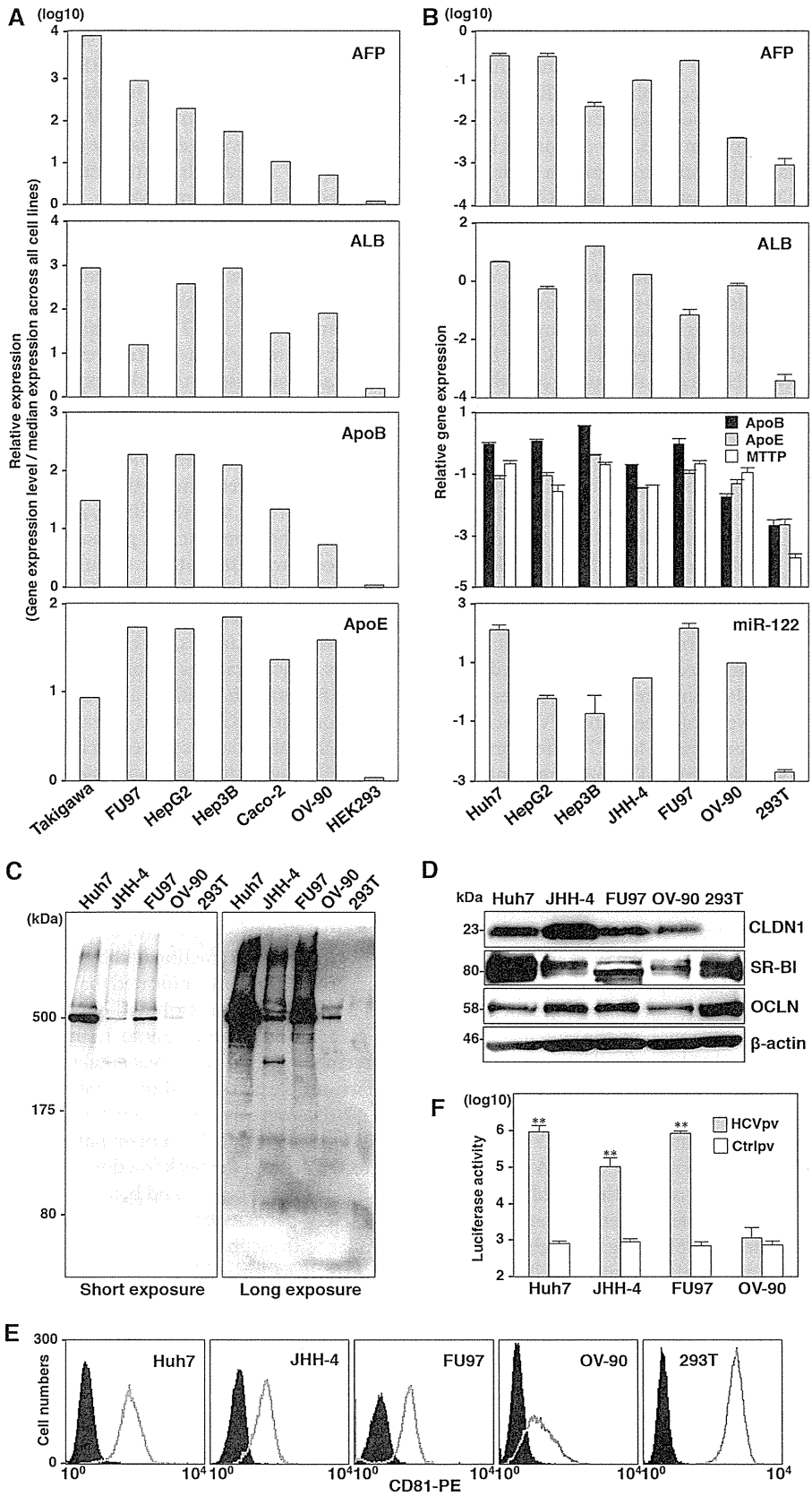



FIG 1 JHH-4 and FU97 cells express high levels of the liver-specific host factors required for HCV propagation. (A) Expression of AFP, ALB, ApoB, and ApoE in cancer cell lines screened by the NextBio Body Atlas application. The expression levels were standardized by the median expression across all cell 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 ($3 \times 10^7$ to $7 \times 10^7$ 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).

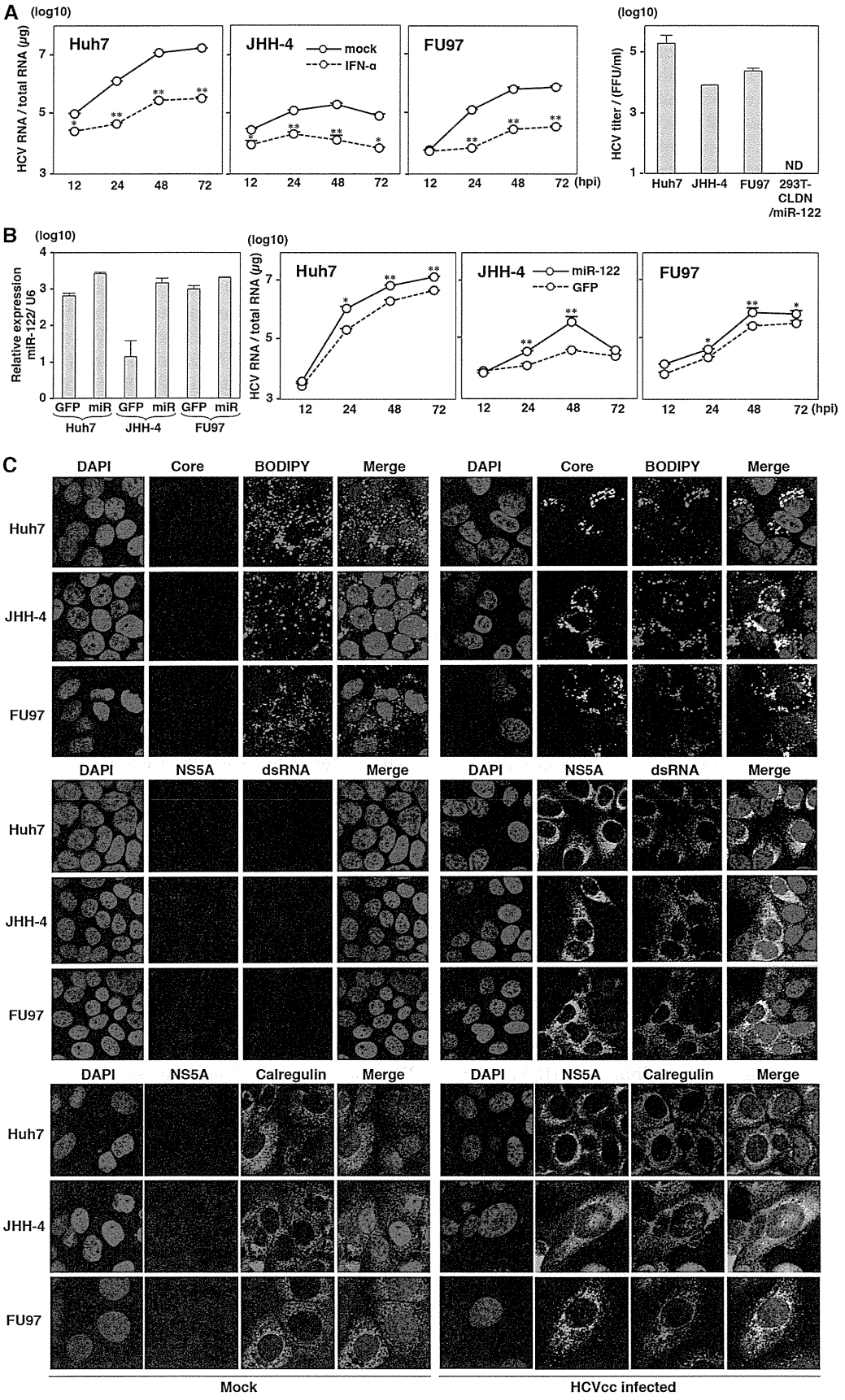
**Cured FU97 cells exhibit normal innate immune response.** It has been shown that one of the reasons for the high susceptibility of the cured cell line, Huh7.5 cells, to HCVcc infection is the impairment of the innate immune responses caused by mutation in RIG-I, a key sensor for viral RNA (50). To 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.


A
(log10)
HCV RNA / total RNA (μg)
Huh7
JHH-4
mock
IFN-α
FU97
12 24 48 72 (hpi)
(log10)
HCV titer / (FFU/ml)
ND
Huh7 JHH-4 FU97 293T-CLDN /miR-122
B
(log10)
Relative expression miR-122/ U6
GFP miR GFP miR GFP miR
Huh7 JHH-4 FU97
(log10)
HCV RNA / total RNA (μg)
Huh7
JHH-4
miR-122
GFP
FU97
12 24 48 72 (hpi)
C
DAPI Core BODIPY Merge
DAPI NS5A dsRNA Merge
DAPI NS5A Calregulin Merge
Huh7
JHH-4
FU97
Mock
HCVcc infected

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 $1.5 \times 10^5$ 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 ($2.5 \times 10^4$ FFU/ml), followed by parental FU97 ($1.2 \times 10^4$ FFU/ml) and Huh7 ($9 \times 10^3$ 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.

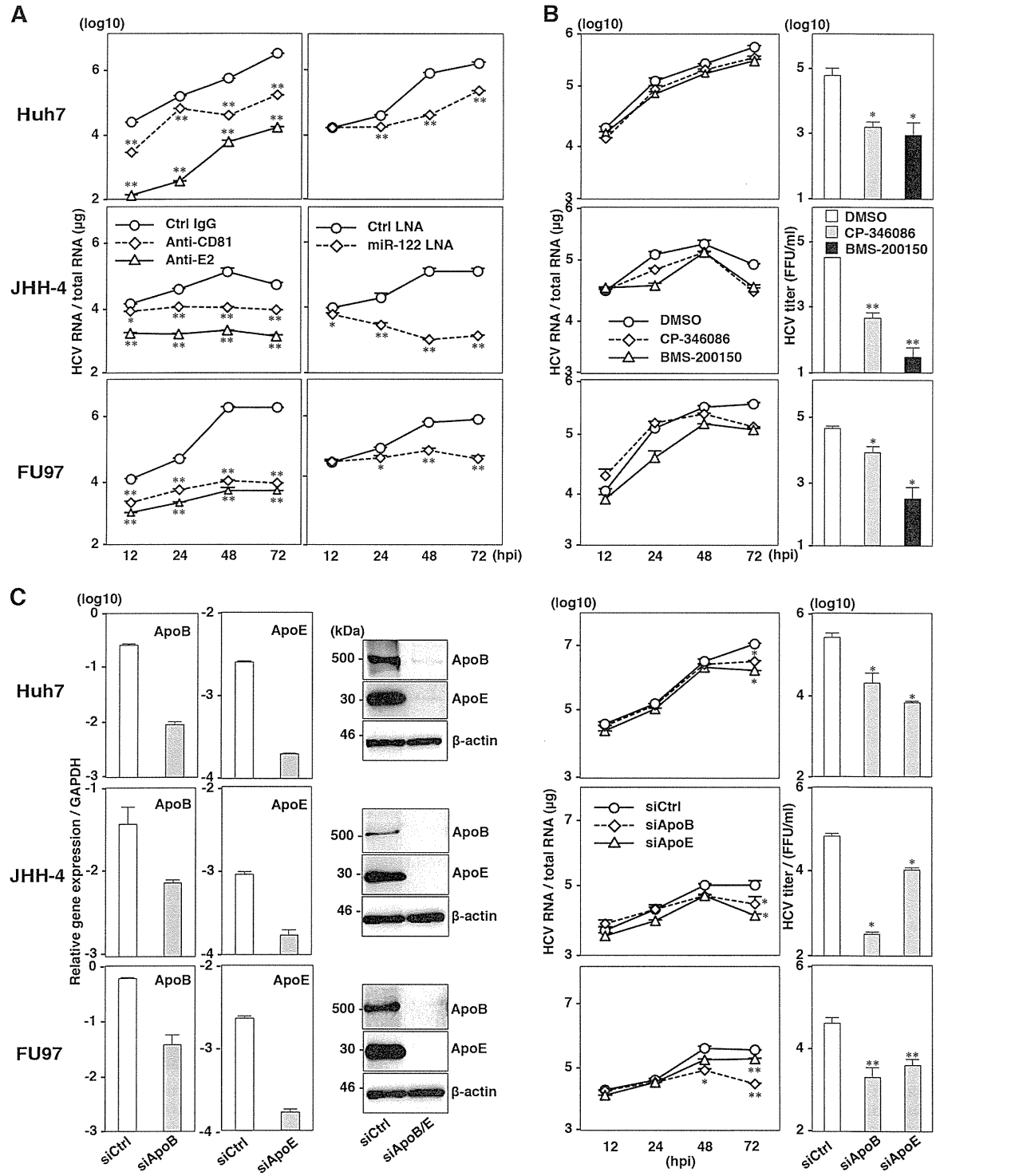



FIG 3 JHH-4 and FU97 cells permit complete propagation of HCVcc without any exogenous expression of host factors crucial for propagation of HCVcc. (A) Effect of inhibitors on the propagation of HCVcc in Huh7, JHH-4, and FU97 cells. (Left panels) HCVcc was preincubated with anti-E2 antibody and inoculated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

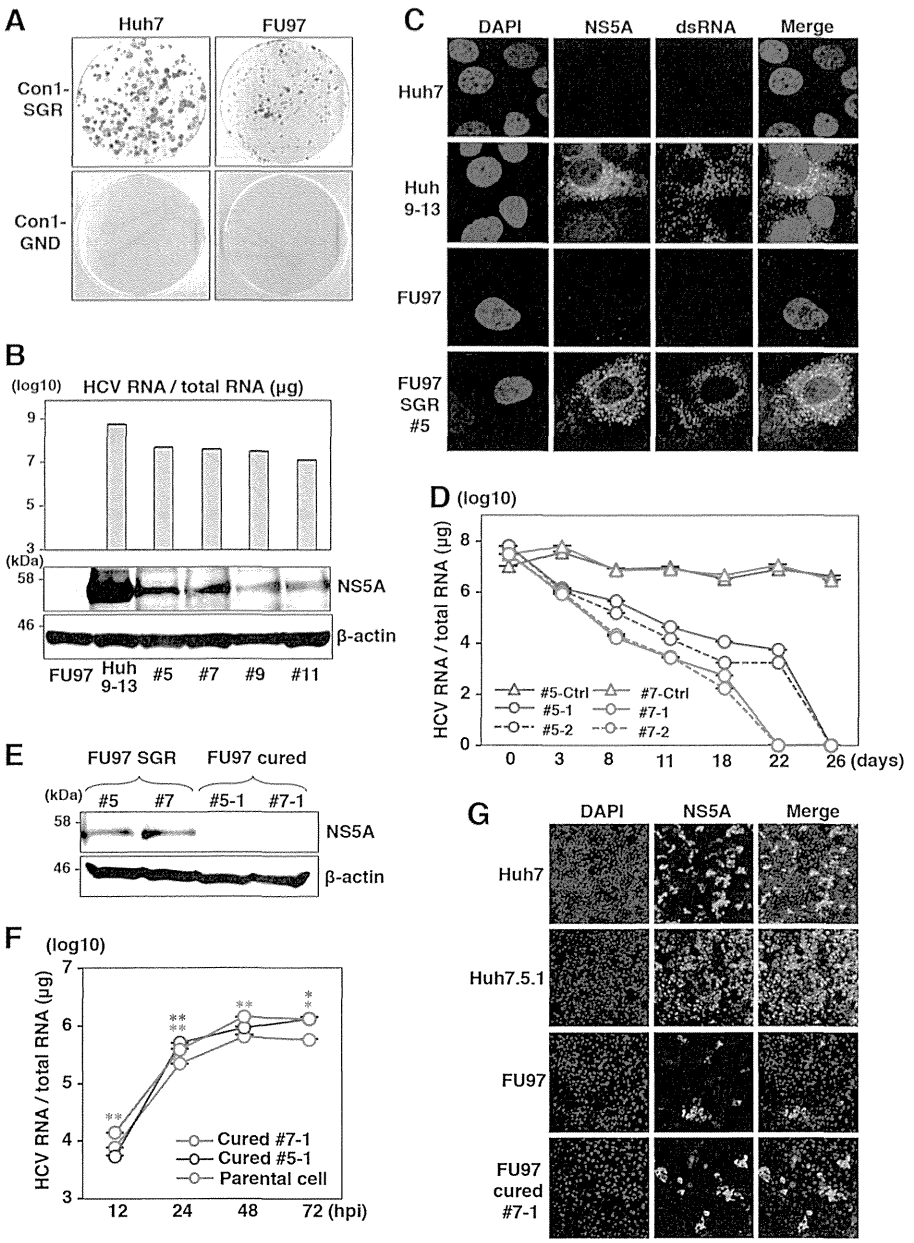



**FIG 4** Establishment of HCV RNA replicon and cured FU97 cells. (A) Wild-type SGR RNA (Con1-SGR) or replication-defective RNA (Con1-GND) of the HCV Con1 strain was electroporated into Huh7 and FU97 cells and replaced with medium containing 1 mg/ml and 400 μg/ml of G418 at 24 h postelectroporation,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

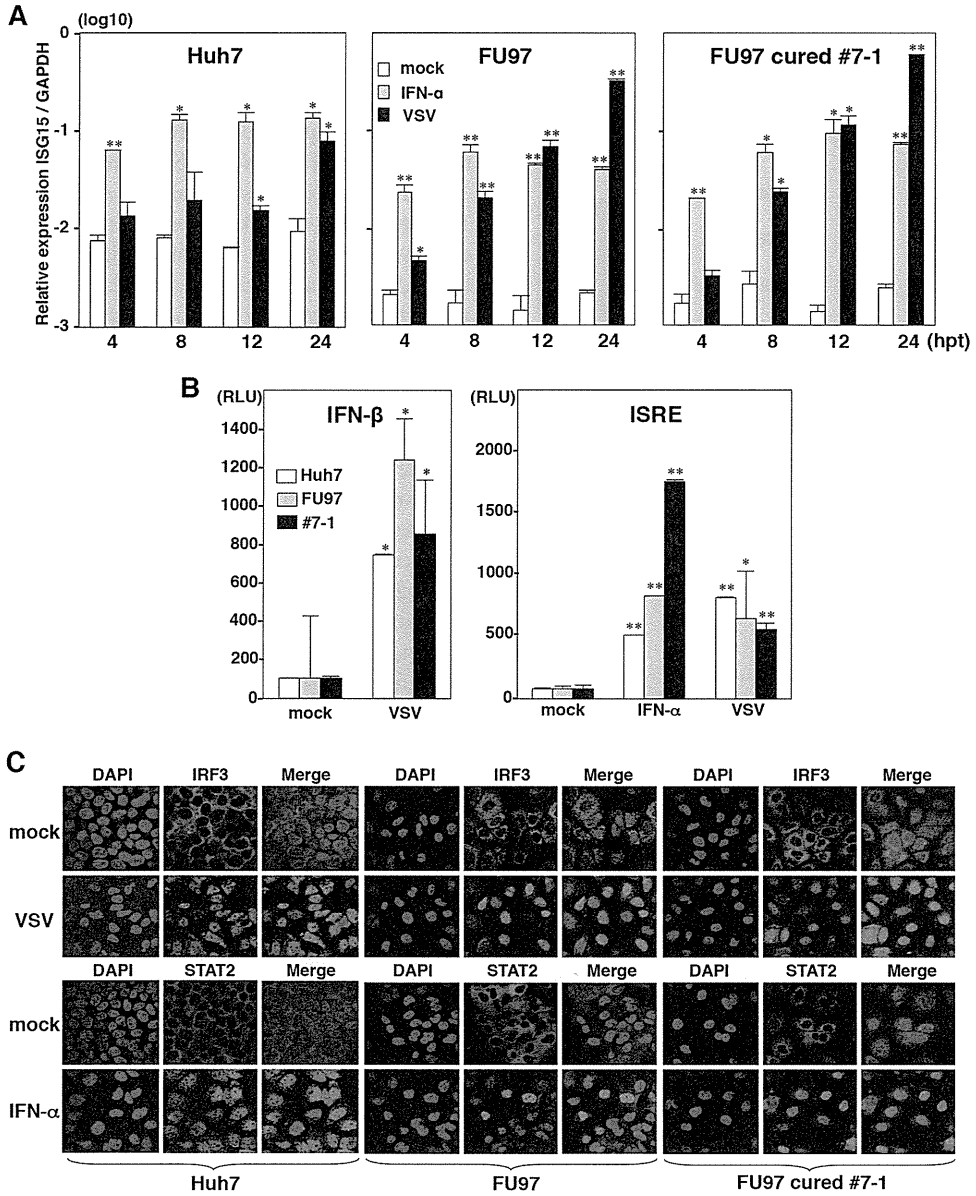



FIG 5 Innate immune response in cured FU97 cells. (A) Huh7, parental, and cured FU97 cells (clone 7-1) were stimulated with 100 IU/ml of IFN-α or infected with VSV. The expression of mRNA of ISG15 at 4, 8, 12, and 24 h posttreatment (hpt) was determined by qPCR and standardized by that of glyceraldehyde-3-phosphate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

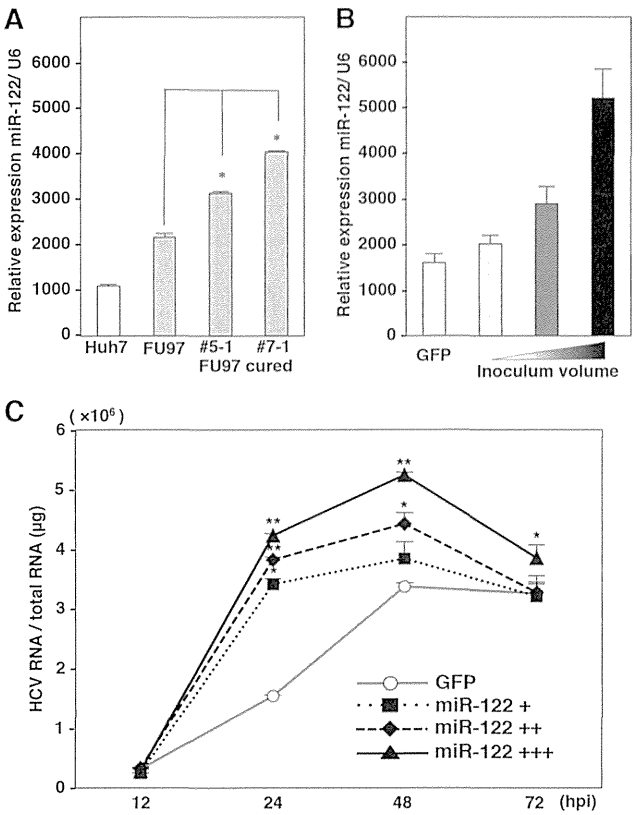



FIG 6 Expression of miR-122 is one of the determinants for HCV RNA abundances. (A) Total RNA was extracted from Huh7 and parental and cured FU97 (clones 5-1 and 7-1) cells, and the relative expression of miR-122 was determined by qPCR. U6 snRNA was used as an internal control. (B) Establishment of FU97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

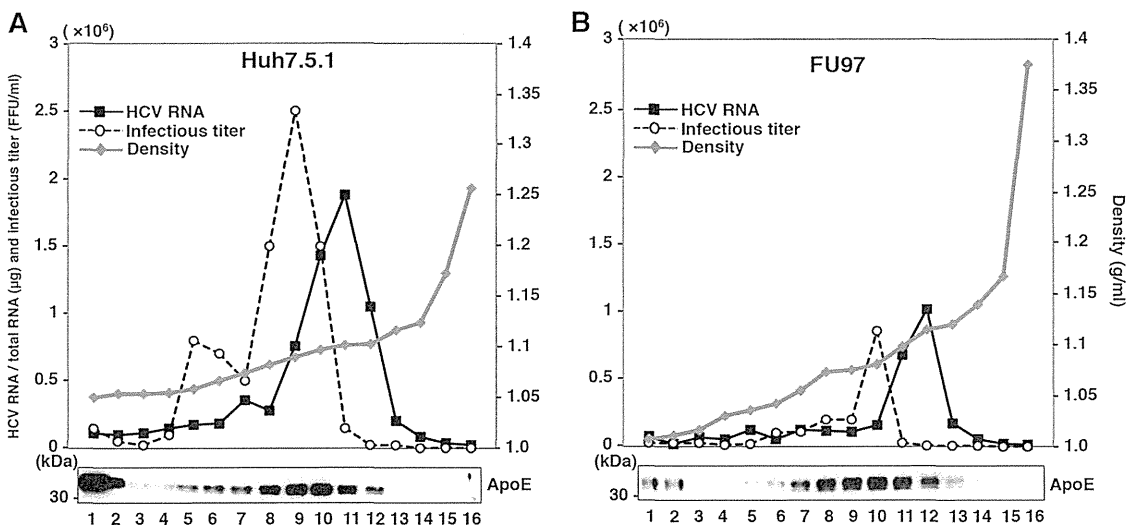



FIG 7 HCV particles produced in FU97 cells exhibit similar characteristics to those in hepatic cells. HCV particles in the culture supernatants of Huh7.5.1 and FU97 cells were harvested at 72 h postinfection with HCVcc and analyzed by using iodixanol density gradient centrifugation. HCV RNA and infectious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

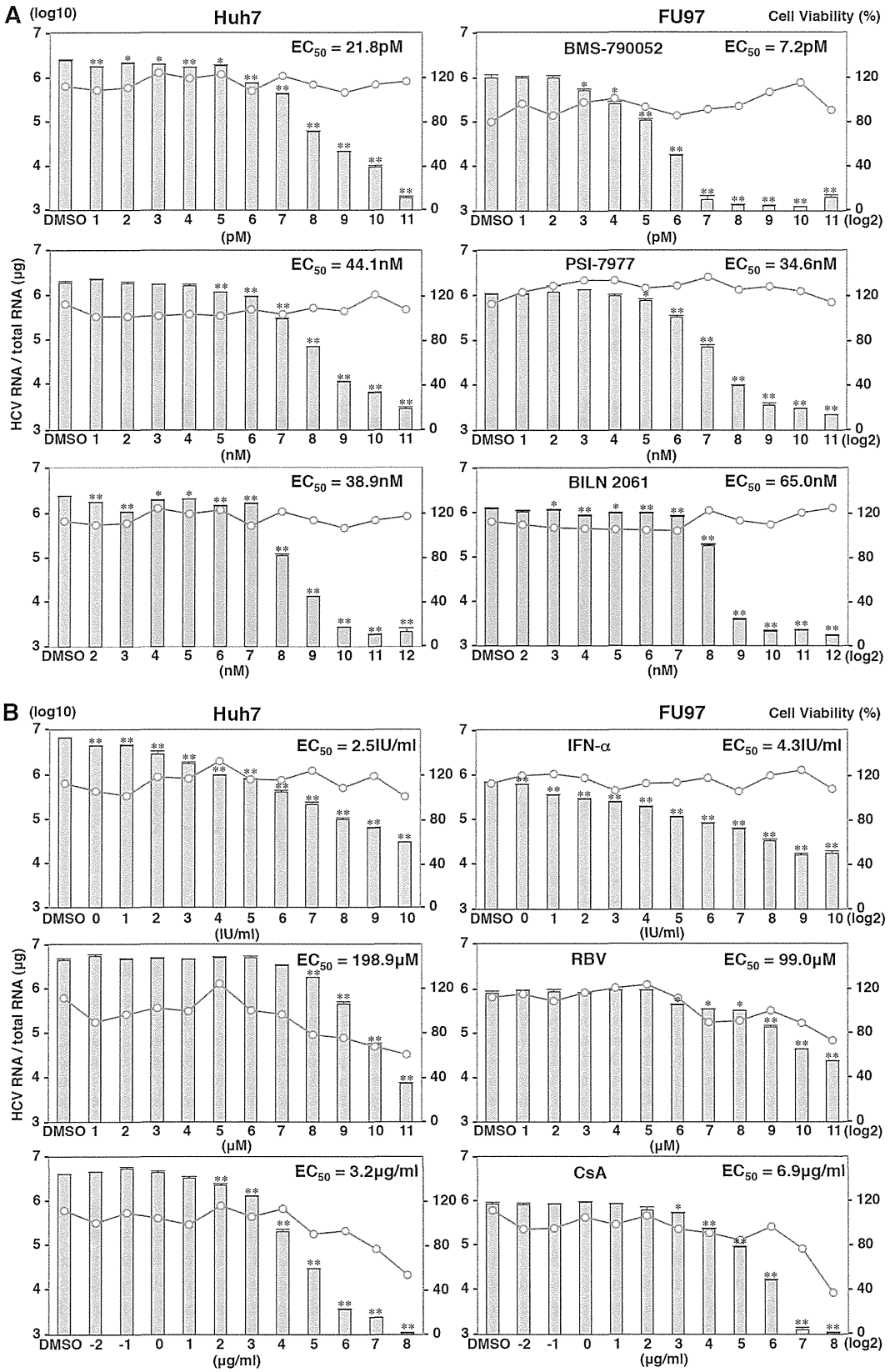



FIG 8 Effects of anti-HCV drugs on the propagation of HCVcc in FU97 cells. (A) Effect of DAAs on the propagation of HCVcc in Huh7 and FU97 cells. Cells infected with HCVcc at an MOI of 1 were treated with BMS-790052, PSI-7977, and BILN 2061 at 3 h postinfection (identifications in right-hand panels).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

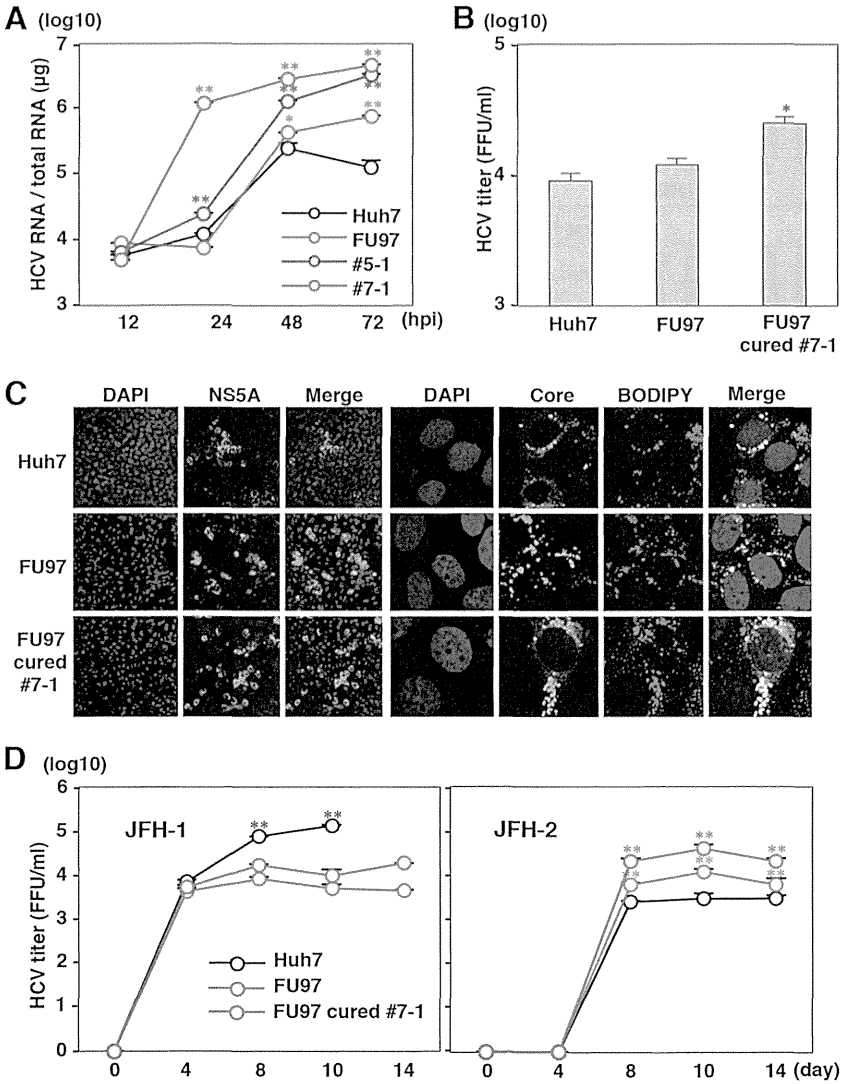



FIG 9 Propagation of HCVcc/JFH-2 in FU97 cells. (A) Huh7, FU97 parental, FU97 cured 5-1, and FU97 cured 7-1 cells were infected with HCVcc/JFH-2 at an MOI of 1, and the intracellular HCV RNA level was determined by qRT-PCR at 12, 24, 48, and 72 h postinfection. (B) Huh7, FU97, and FU97 cured 7-1 cells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

## ACKNOWLEDGMENTS

We thank M. Tomiyama for her secretarial work and M. Ishibashi and Y. Sugiyama for their technical assistance. We also thank M. Hijikata, R. Bartenschlager, S. Akira, F. Chisari, and M. Whitt for providing experimental materials.

This work was supported in part by grants-in-aid from the Japanese Ministry of Health, Labor, and Welfare (Research on Hepatitis), the Japanese Ministry of Education, Culture, Sports, Science, and Technology, the Naito Foundation, and the Takeda Science Foundation.

## REFERENCES

1. **Maasoumy B, Wedemeyer H.** 2012. Natural history of acute and chronic hepatitis C. Best Pract. Res. Clin. Gastroenterol. **26:**401–412. http://dx.doi.org/10.1016/j.bpg.2012.09.009.
2. **Poynard T, Colombo M, Bruix J, Schiff E, Terg R, Flamm S, Moreno-Otero R, Carrilho F, Schmidt W, Berg T, McGarrity T, Heathcote EJ, Gonçales F, Diago M, Craxi A, Silva M, Bedossa P, Mukhopadhyay P, Griffel L, Burroughs M, Brass C, Albrecht J, Epic Study Group.** 2009. Peginterferon alfa-2b and ribavirin: effective in patients with hepatitis C who failed interferon alfa/ribavirin therapy. Gastroenterology **136:**1618–1628.e2. http://dx.doi.org/10.1053/j.gastro.2009.01.039.
3. **Chatel-Chaix L, Germain MA, Götte M, Lamarre D.** 2012. Direct-acting and host-targeting HCV inhibitors: current and future directions. Curr. Opin. Virol. **2:**588–598. http://dx.doi.org/10.1016/j.coviro.2012.08.002.
4. **Jazwinski AB, Muir AJ.** 2011. Direct-acting antiviral medications for chronic hepatitis C virus infection. Gastroenterol. Hepatol. (NY) **7:**154–162. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3079144/.
5. **McHutchison JG, Manns MP, Muir AJ, Terrault NA, Jacobson IM, Afdhal NH, Heathcote EJ, Zeuzem S, Reesink HW, Garg J, Bsharat M, George S, Kauffman RS, Adda N, Di Bisceglie AM, Team PS.** 2010. Telaprevir for previously treated chronic HCV infection. N. Engl. J. Med. **362:**1292–1303. http://dx.doi.org/10.1056/NEJMoa0908014.
6. **Lok AS, Gardiner DF, Lawitz E, Martorell C, Everson GT, Ghalib R, Reindollar R, Rustgi V, McPhee F, Wind-Rotolo M, Persson A, Zhu K, Dimitrova DI, Eley T, Guo T, Grasela DM, Pasquinelli C.** 2012. Preliminary study of two antiviral agents for hepatitis C genotype 1. N. Engl. J. Med. **366:**216–224. http://dx.doi.org/10.1056/NEJMoa1104430.
7. **McPhee F, Friborg J, Levine S, Chen C, Falk P, Yu F, Hernandez D, Lee MS, Chaniewski S, Sheaffer AK, Pasquinelli C.** 2012. Resistance analysis of the hepatitis C virus NS3 protease inhibitor asunaprevir. Antimicrob. Agents Chemother. **56:**3670–3681. http://dx.doi.org/10.1128/AAC.00308-12.
8. **Pelosi LA, Voss S, Liu M, Gao M, Lemm JA.** 2012. Effect on hepatitis C virus replication of combinations of direct-acting antivirals, including NS5A inhibitor daclatasvir. Antimicrob. Agents Chemother. **56:**5230–5239. http://dx.doi.org/10.1128/AAC.01209-12.
9. **Fridell RA, Wang C, Sun JH, O'Boyle DR, Nower P, Valera L, Qiu D, Roberts S, Huang X, Kienzle B, Bifano M, Nettles RE, Gao M.** 2011. Genotypic and phenotypic analysis of variants resistant to hepatitis C virus nonstructural protein 5A replication complex inhibitor BMS-790052 in humans: in vitro and in vivo correlations. Hepatology **54:**1924–1935. http://dx.doi.org/10.1002/hep.24594.
10. **Sarrazin C, Zeuzem S.** 2010. Resistance to direct antiviral agents in patients with hepatitis C virus infection. Gastroenterology **138:**447–462. http://dx.doi.org/10.1053/j.gastro.2009.11.055.
11. **Susser S, Welsch C, Wang Y, Zettler M, Domingues FS, Karey U, Hughes E, Ralston R, Tong X, Herrmann E, Zeuzem S, Sarrazin C.** 2009. Characterization of resistance to the protease inhibitor boceprevir in hepatitis C virus-infected patients. Hepatology **50:**1709–1718. http://dx.doi.org/10.1002/hep.23192.
12. **Vermehren J, Sarrazin C.** 2012. The role of resistance in HCV treatment. Best Pract. Res. Clin. Gastroenterol. **26:**487–503. http://dx.doi.org/10.1016/j.bpg.2012.09.011.
13. **Bukh J.** 2004. A critical role for the chimpanzee model in the study of hepatitis C. Hepatology **39:**1469–1475. http://dx.doi.org/10.1002/hep.20268.
14. **Bukh J.** 2012. Animal models for the study of hepatitis C virus infection and related liver disease. Gastroenterology **142:**1279–1287.e1273. http://dx.doi.org/10.1053/j.gastro.2012.02.016.
15. **Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Kräusslich HG, Mizokami M, Bartenschlager R, Liang TJ.** 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. **11:**791–796. http://dx.doi.org/10.1038/nm1268.
16. **Narbus CM, Israelow B, Sourisseau M, Michta ML, Hopcraft SE, Zeiner GM, Evans MJ.** 2011. HepG2 cells expressing microRNA miR-122 support the entire hepatitis C virus life cycle. J. Virol. **85:**12087–12092. http://dx.doi.org/10.1128/JVI.05843-11.
17. **Kambara H, Fukuhara T, Shiokawa M, Ono C, Ohara Y, Kamitani W, Matsuura Y.** 2012. Establishment of a novel permissive cell line for the propagation of hepatitis C virus by expression of microRNA miR122. J. Virol. **86:**1382–1393. http://dx.doi.org/10.1128/JVI.06242-11.
18. **Fukuhara T, Kambara H, Shiokawa M, Ono C, Katoh H, Morita E, Okuzaki D, Maehara Y, Koike K, Matsuura Y.** 2012. Expression of microRNA miR-122 facilitates an efficient replication in nonhepatic cells upon infection with hepatitis C virus. J. Virol. **86:**7918–7933. http://dx.doi.org/10.1128/JVI.00567-12.
19. **Chang KS, Jiang J, Cai Z, Luo G.** 2007. Human apolipoprotein E is required for infectivity and production of hepatitis C virus in cell culture. J. Virol. **81:**13783–13793. http://dx.doi.org/10.1128/JVI.01091-07.
20. **Gastaminza P, Cheng G, Wieland S, Zhong J, Liao W, Chisari FV.** 2008. Cellular determinants of hepatitis C virus assembly, maturation, degradation, and secretion. J. Virol. **82:**2120–2129. http://dx.doi.org/10.1128/JVI.02053-07.
21. **Huang H, Sun F, Owen DM, Li W, Chen Y, Gale M, Ye J.** 2007. Hepatitis C virus production by human hepatocytes dependent on assembly and secretion of very low-density lipoproteins. Proc. Natl. Acad. Sci. U. S. A. **104:**5848–5853. http://dx.doi.org/10.1073/pnas.0700760104.
22. **Jiang J, Luo G.** 2009. Apolipoprotein E but not B is required for the formation of infectious hepatitis C virus particles. J. Virol. **83:**12680–12691. http://dx.doi.org/10.1128/JVI.01476-09.
23. **Syed GH, Amako Y, Siddiqui A.** 2010. Hepatitis C virus hijacks host lipid metabolism. Trends Endocrinol. Metab. **21:**33–40. http://dx.doi.org/10.1016/j.tem.2009.07.005.
24. **Miyanari Y, Atsuzawa K, Usuda N, Watashi K, Hishiki T, Zayas M, Bartenschlager R, Wakita T, Hijikata M, Shimotohno K.** 2007. The lipid

droplet is an important organelle for hepatitis C virus production. Nat. Cell Biol. 9:1089–1097. http://dx.doi.org/10.1038/ncb1631.
25. Jögi A, Vaapil M, Johansson M, Påhlman S. 2012. Cancer cell differentiation heterogeneity and aggressive behavior in solid tumors. Ups. J. Med. Sci. 117:217–224. http://dx.doi.org/10.3109/03009734.2012.659294.
26. Nakabayashi H, Taketa K, Miyano K, Yamane T, Sato J. 1982. Growth of human hepatoma cells lines with differentiated functions in chemically defined medium. Cancer Res. 42:3858–3863.
27. Slany A, Haudek VJ, Zwickl H, Gundacker NC, Grusch M, Weiss TS, Seir K, Rodgarkia-Dara C, Hellerbrand C, Gerner C. 2010. Cell characterization by proteome profiling applied to primary hepatocytes and hepatocyte cell lines Hep-G2 and Hep-3B. J. Proteome Res. 9:6–21. http://dx.doi.org/10.1021/pr900057t.
28. Schwartz RE, Trehan K, Andrus L, Sheahan TP, Ploss A, Duncan SA, Rice CM, Bhatia SN. 2012. Modeling hepatitis C virus infection using human induced pluripotent stem cells. Proc. Natl. Acad. Sci. U. S. A. 109:2544–2548. http://dx.doi.org/10.1073/pnas.1121400109.
29. Si-Tayeb K, Noto FK, Nagaoka M, Li J, Battle MA, Duris C, North PE, Dalton S, Duncan SA. 2010. Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells. Hepatology 51:297–305. http://dx.doi.org/10.1002/hep.23354.
30. Wu X, Robotham JM, Lee E, Dalton S, Kneteman NM, Gilbert DM, Tang H. 2012. Productive hepatitis C virus infection of stem cell-derived hepatocytes reveals a critical transition to viral permissiveness during differentiation. PLoS Pathog. 8:e1002617. http://dx.doi.org/10.1371/journal.ppat.1002617.
31. Debruyne EN, Delanghe JR. 2008. Diagnosing and monitoring hepatocellular carcinoma with alpha-fetoprotein: new aspects and applications. Clin. Chim. Acta 395:19–26. http://dx.doi.org/10.1016/j.cca.2008.05.010.
32. Kupershmidt I, Su QJ, Grewal A, Sundaresh S, Halperin I, Flynn J, Shekar M, Wang H, Park J, Cui W, Wall GD, Wisotzkey R, Alag S, Akhtari S, Ronaghi M. 2010. Ontology-based meta-analysis of global collections of high-throughput public data. PLoS One 5:e13066. http://dx.doi.org/10.1371/journal.pone.0013066.
33. Masaki T, Suzuki R, Saeed M, Mori K, Matsuda M, Aizaki H, Ishii K, Maki N, Miyamura T, Matsuura Y, Wakita T, Suzuki T. 2010. Production of infectious hepatitis C virus by using RNA polymerase I-mediated transcription. J. Virol. 84:5824–5835. http://dx.doi.org/10.1128/JVI.02397-09.
34. Date T, Kato T, Kato J, Takahashi H, Morikawa K, Akazawa D, Murayama A, Tanaka-Kaneko K, Sata T, Tanaka Y, Mizokami M, Wakita T. 2012. Novel cell culture-adapted genotype 2a hepatitis C virus infectious clone. J. Virol. 86:10805–10820. http://dx.doi.org/10.1128/JVI.07235-11.
35. Pietschmann T, Lohmann V, Kaul A, Krieger N, Rinck G, Rutter G, Strand D, Bartenschlager R. 2002. Persistent and transient replication of full-length hepatitis C virus genomes in cell culture. J. Virol. 76:4008–4021. http://dx.doi.org/10.1128/JVI.76.8.4008-4021.2002.
36. Tani H, Komoda Y, Matsuo E, Suzuki K, Hamamoto I, Yamashita T, Moriishi K, Fujiyama K, Kanto T, Hayashi N, Owsianka A, Patel AH, Whitt MA, Matsuura Y. 2007. Replication-competent recombinant vesicular stomatitis virus encoding hepatitis C virus envelope proteins. J. Virol. 81:8601–8612. http://dx.doi.org/10.1128/JVI.00608-07.
37. Moriishi K, Shoji I, Mori Y, Suzuki R, Suzuki T, Kataoka C, Matsuura Y. 2010. Involvement of PA28γ in the propagation of hepatitis C virus. Hepatology 52:411–420. http://dx.doi.org/10.1002/hep.23680.
38. Fukuhara T, Tani H, Shiokawa M, Goto Y, Abe T, Taketomi A, Shirabe K, Maehara Y, Matsuura Y. 2011. Intracellular delivery of serum-derived hepatitis C virus. Microbes Infect. 13:405–412. http://dx.doi.org/10.1016/j.micinf.2011.01.005.
39. Morris T, Robertson B, Gallagher M. 1996. Rapid reverse transcription-PCR detection of hepatitis C virus RNA in serum by using the TaqMan fluorogenic detection system. J. Clin. Microbiol. 34:2933–2936.
40. Latchman DS, Brzeski H, Lovell-Badge R, Evans MJ. 1984. Expression of the alpha-fetoprotein gene in pluripotent and committed cells. Biochim. Biophys. Acta 783:130–136. http://dx.doi.org/10.1016/0167-4781(84)90004-6.
41. Roelandt P, Obeid S, Paeshuyse J, Vanhove J, Van Lommel A, Nahmias Y, Nevens F, Neyts J, Verfaillie CM. 2012. Human pluripotent stem cell-derived hepatocytes support complete replication of hepatitis C virus. J. Hepatol. 57:246–251. http://dx.doi.org/10.1016/j.jhep.2012.03.030.
42. Mee CJ, Grove J, Harris HJ, Hu K, Balfe P, McKeating JA. 2008. Effect of cell polarization on hepatitis C virus entry. J. Virol. 82:461–470. http://dx.doi.org/10.1128/JVI.01894-07.
43. Wilson GK, Stamataki Z. 2012. In vitro systems for the study of hepatitis C virus infection. Int. J. Hepatol. 2012:292591. http://dx.doi.org/10.1155/2012/292591.
44. Aizaki H, Nagamori S, Matsuda M, Kawakami H, Hashimoto O, Ishiko H, Kawada M, Matsuura T, Hasumura S, Matsuura Y, Suzuki T, Miyamura T. 2003. Production and release of infectious hepatitis C virus from human liver cell cultures in the three-dimensional radial-flow bioreactor. Virology 314:16–25. http://dx.doi.org/10.1016/S0042-6822(03)00383-0.
45. Pileri P, Uematsu Y, Campagnoli S, Galli G, Falugi F, Petracca R, Weiner AJ, Houghton M, Rosa D, Grandi G, Abrignani S. 1998. Binding of hepatitis C virus to CD81. Science 282:938–941. http://dx.doi.org/10.1126/science.282.5390.938.
46. Scarselli E, Ansuini H, Cerino R, Roccasecca RM, Acali S, Filocamo G, Traboni C, Nicosia A, Cortese R, Vitelli A. 2002. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. EMBO J. 21:5017–5025. http://dx.doi.org/10.1093/emboj/cdf529.
47. Evans MJ, von Hahn T, Tscherne DM, Syder AJ, Panis M, Wölk B, Hatziioannou T, McKeating JA, Bieniasz PD, Rice CM. 2007. Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. Nature 446:801–805. http://dx.doi.org/10.1038/nature05654.
48. Ploss A, Evans MJ, Gaysinskaya VA, Panis M, You H, de Jong YP, Rice CM. 2009. Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. Nature 457:882–886. http://dx.doi.org/10.1038/nature07684.
49. Blight KJ, McKeating JA, Rice CM. 2002. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. J. Virol. 76:13001–13014. http://dx.doi.org/10.1128/JVI.76.24.13001-13014.2002.
50. Sumpter R, Loo YM, Foy E, Li K, Yoneyama M, Fujita T, Lemon SM, Gale M. 2005. Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. J. Virol. 79:2689–2699. http://dx.doi.org/10.1128/JVI.79.5.2689-2699.2005.
51. Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, Liu CC, Maruyama T, Hynes RO, Burton DR, McKeating JA, Rice CM. 2005. Complete replication of hepatitis C virus in cell culture. Science 309:623–626. http://dx.doi.org/10.1126/science.1114016.
52. Lindenbach BD, Meuleman P, Ploss A, Vanwolleghem T, Syder AJ, McKeating JA, Lanford RE, Feinstone SM, Major ME, Leroux-Roels G, Rice CM. 2006. Cell culture-grown hepatitis C virus is infectious in vivo and can be recultured in vitro. Proc. Natl. Acad. Sci. U. S. A. 103:3805–3809. http://dx.doi.org/10.1073/pnas.0511218103.
53. Feld JJ, Hoofnagle JH. 2005. Mechanism of action of interferon and ribavirin in treatment of hepatitis C. Nature 436:967–972. http://dx.doi.org/10.1038/nature04082.
54. Frese M, Pietschmann T, Moradpour D, Haller O, Bartenschlager R. 2001. Interferon-alpha inhibits hepatitis C virus subgenomic RNA replication by an MxA-independent pathway. J. Gen. Virol. 82:723–733. http://vir.sgmjournals.org/content/82/4/723.full.
55. Watashi K, Hijikata M, Hosaka M, Yamaji M, Shimotohno K. 2003. Cyclosporin A suppresses replication of hepatitis C virus genome in cultured hepatocytes. Hepatology 38:1282–1288. http://dx.doi.org/10.1053/jhep.2003.50449.
56. Gottwein JM, Scheel TK, Jensen TB, Lademann JB, Prentoe JC, Knudsen ML, Hoegh AM, Bukh J. 2009. Development and characterization of hepatitis C virus genotype 1–7 cell culture systems: role of CD81 and scavenger receptor class B type I and effect of antiviral drugs. Hepatology 49:364–377. http://dx.doi.org/10.1002/hep.22673.
57. Scheel TK, Gottwein JM, Mikkelsen LS, Jensen TB, Bukh J. 2011. Recombinant HCV variants with NS5A from genotypes 1–7 have different sensitivities to an NS5A inhibitor but not interferon-γ. Gastroenterology 140:1032–1042. http://dx.doi.org/10.1053/j.gastro.2010.11.036.
58. Tariq H, Manzoor S, Parvaiz F, Javed F, Fatima K, Qadri I. 2012. An overview: in vitro models of HCV replication in different cell cultures. Infect. Genet. Evol. 12:13–20. http://dx.doi.org/10.1016/j.meegid.2011.10.009.
59. Pietschmann T, Kaul A, Koutsoudakis G, Shavinskaya A, Kallis S, Steinmann E, Abid K, Negro F, Dreux M, Cosset FL, Bartenschlager R. 2006. Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. Proc. Natl. Acad. Sci. U. S. A. 103:7408–7413. http://dx.doi.org/10.1073/pnas.0504877103.

60. **Yi M, Ma Y, Yates J, Lemon SM.** 2007. Compensatory mutations in E1, p7, NS2, and NS3 enhance yields of cell culture-infectious intergenotypic chimeric hepatitis C virus. J. Virol. **81:**629–638. http://dx.doi.org/10.1128/JVI.01890-06.
61. **Yi M, Villanueva RA, Thomas DL, Wakita T, Lemon SM.** 2006. Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells. Proc. Natl. Acad. Sci. U. S. A. **103:**2310–2315. http://dx.doi.org/10.1073/pnas.0510727103.
62. **Li YP, Ramirez S, Jensen SB, Purcell RH, Gottwein JM, Bukh J.** 2012. Highly efficient full-length hepatitis C virus genotype 1 (strain TN) infectious culture system. Proc. Natl. Acad. Sci. U. S. A. **109:**19757–19762. http://dx.doi.org/10.1073/pnas.1218260109.
63. **Saeed M, Gondeau C, Hmwe S, Yokokawa H, Date T, Suzuki T, Kato T, Maurel P, Wakita T.** 2013. Replication of hepatitis C virus genotype 3a in cultured cells. Gastroenterology **144:**56–58.e57. http://dx.doi.org/10.1053/j.gastro.2012.09.017.
64. **Da Costa D, Turek M, Felmlee DJ, Girardi E, Pfeffer S, Long G, Bartenschlager R, Zeisel MB, Baumert TF.** 2012. Reconstitution of the entire hepatitis C virus life cycle in nonhepatic cells. J. Virol. **86:**11919–11925. http://dx.doi.org/10.1128/JVI.01066-12.
65. **Aoki Y, Aizaki H, Shimoike T, Tani H, Ishii K, Saito I, Matsuura Y, Miyamura T.** 1998. A human liver cell line exhibits efficient translation of HCV RNAs produced by a recombinant adenovirus expressing T7 RNA polymerase. Virology **250:**140–150. http://dx.doi.org/10.1006/viro.1998.9361.
66. **Matsuda M.** 2000. Biological behavior of an alpha-phetoprotein-producing gastric cancer (FU97). J. Nara Med. Assoc. **51:**79–89.
67. **Chun H, Kwon SJ.** 2011. Clinicopathological characteristics of alpha-fetoprotein-producing gastric cancer. J. Gastric Cancer **11:**23–30. http://dx.doi.org/10.5230/jgc.2011.11.1.23.
68. **Dabeva MD, Laconi E, Oren R, Petkov PM, Hurston E, Shafritz DA.** 1998. Liver regeneration and alpha-fetoprotein messenger RNA expression in the retrorsine model for hepatocyte transplantation. Cancer Res. **58:**5825–5834.
69. **Kuhlmann WD, Peschke P.** 2006. Hepatic progenitor cells, stem cells, and AFP expression in models of liver injury. Int. J. Exp. Pathol. **87:**343–359. http://dx.doi.org/10.1111/j.1365-2613.2006.00485.x.
70. **Watanabe H.** 1971. Early appearance of embryonic γ-globulin in rat serum during carcinogenesis with 4-dimethylaminoazobenzene. Cancer Res. **31:**1192–1194.
71. **Ishikura H, Fukasawa Y, Ogasawara K, Natori T, Tsukada Y, Aizawa M.** 1985. An AFP-producing gastric carcinoma with features of hepatic differentiation. A case report. Cancer **56:**840–848.
72. **Ishikura H, Kirimoto K, Shamoto M, Miyamoto Y, Yamagiwa H, Itoh T, Aizawa M.** 1986. Hepatoid adenocarcinomas of the stomach. An analysis of seven cases. Cancer **58:**119–126.
73. **Nettles RE, Gao M, Bifano M, Chung E, Persson A, Marbury TC, Goldwater R, DeMicco MP, Rodriguez-Torres M, Vutikullird A, Fuentes E, Lawitz E, Lopez-Talavera JC, Grasela DM.** 2011. Multiple ascending dose study of BMS-790052, a nonstructural protein 5A replication complex inhibitor, in patients infected with hepatitis C virus genotype 1. Hepatology **54:**1956–1965. http://dx.doi.org/10.1002/hep.24609.
74. **Elfiky AA, Elshemey WM, Gawad WA, Desoky OS.** 2013. Molecular modeling comparison of the performance of NS5b polymerase inhibitor (PSI-7977) on prevalent HCV genotypes. Protein J. **32:**75–80. http://dx.doi.org/10.1007/s10930-013-9462-9.
75. **Hinrichsen H, Benhamou Y, Wedemeyer H, Reiser M, Sentjens RE, Calleja JL, Forns X, Erhardt A, Crönlein J, Chaves RL, Yong CL, Nehmiz G, Steinmann GG.** 2004. Short-term antiviral efficacy of BILN 2061, a hepatitis C virus serine protease inhibitor, in hepatitis C genotype 1 patients. Gastroenterology **127:**1347–1355. http://dx.doi.org/10.1053/j.gastro.2004.08.002.
76. **Kato N, Abe K, Mori K, Ariumi Y, Dansako H, Ikeda M.** 2009. Genetic variability and diversity of intracellular genome-length hepatitis C virus RNA in long-term cell culture. Arch. Virol. **154:**77–85. http://dx.doi.org/10.1007/s00705-008-0282-8.
77. **Mori K, Ikeda M, Ariumi Y, Dansako H, Wakita T, Kato N.** 2011. Mechanism of action of ribavirin in a novel hepatitis C virus replication cell system. Virus Res. **157:**61–70. http://dx.doi.org/10.1016/j.virusres.2011.02.005.
78. **Mori K, Hiraoka O, Ikeda M, Ariumi Y, Hiramoto A, Wataya Y, Kato N.** 2013. Adenosine kinase is a key determinant for the anti-HCV activity of ribavirin. Hepatology **58:**1236–1244. http://dx.doi.org/10.1002/hep.26421.
79. **Fukuhara T, Taketomi A, Motomura T, Okano S, Ninomiya A, Abe T, Uchiyama H, Soejima Y, Shirabe K, Matsuura Y, Maehara Y.** 2010. Variants in *IL28B* in liver recipients and donors correlate with response to peg-interferon and ribavirin therapy for recurrent hepatitis C. Gastroenterology **139:**1577–1585.e3. http://dx.doi.org/10.1053/j.gastro.2010.07.058.
80. **Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, Bassendine M, Spengler U, Dore GJ, Powell E, Riordan S, Sheridan D, Smedile A, Fragomeli V, Muller T, Bahlo M, Stewart GJ, Booth DR, George J.** 2009. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat. Genet. **41:**1100–1104. http://dx.doi.org/10.1038/ng.447.
81. **Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, Nakagawa M, Korenaga M, Hino K, Hige S, Ito Y, Mita E, Tanaka E, Mochida S, Murawaki Y, Honda M, Sakai A, Hiasa Y, Nishiguchi S, Koike A, Sakaida I, Imamura M, Ito K, Yano K, Masaki N, Sugauchi F, Izumi N, Tokunaga K, Mizokami M.** 2009. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat. Genet. **41:**1105–1109. http://dx.doi.org/10.1038/ng.449.
82. **Zhou D, Mei Q, Li J, He H.** 2012. Cyclophilin A and viral infections. Biochem. Biophys. Res. Commun. **424:**647–650. http://dx.doi.org/10.1016/j.bbrc.2012.07.024.
83. **Braaten D, Luban J.** 2001. Cyclophilin A regulates HIV-1 infectivity, as demonstrated by gene targeting in human T cells. EMBO J. **20:**1300–1309. http://dx.doi.org/10.1093/emboj/20.6.1300.
84. **Luban J, Bossolt KL, Franke EK, Kalpana GV, Goff Stephen P.** 1993. Human immunodeficiency virus type 1 Gag protein binds to cyclophilins A and B. Cell **73:**1067–1078. http://dx.doi.org/10.1016/0092-8674(93)90637-6.
85. **Tian X, Zhao C, Zhu H, She W, Zhang J, Liu J, Li L, Zheng S, Wen YM, Xie Y.** 2010. Hepatitis B virus (HBV) surface antigen interacts with and promotes cyclophilin a secretion: possible link to pathogenesis of HBV infection. J. Virol. **84:**3373–3381. http://dx.doi.org/10.1128/JVI.02555-09.
86. **Zhao C, Fang CY, Tian XC, Wang L, Yang PY, Wen YM.** 2007. Proteomic analysis of hepatitis B surface antigen positive transgenic mouse liver and decrease of cyclophilin A. J. Med. Virol. **79:**1478–1484. http://dx.doi.org/10.1002/jmv.20945.
87. **Liu X, Sun L, Yu M, Wang Z, Xu C, Xue Q, Zhang K, Ye X, Kitamura Y, Liu W.** 2009. Cyclophilin A interacts with influenza A virus M1 protein and impairs the early stage of the viral replication. Cell Microbiol. **11:**730–741. http://dx.doi.org/10.1111/j.1462-5822.2009.01286.x.
88. **Inoue K, Sekiyama K, Yamada M, Watanabe T, Yasuda H, Yoshiba M.** 2003. Combined interferon α2b and cyclosporin A in the treatment of chronic hepatitis C: controlled trial. J. Gastroenterol. **38:**567–572.
89. **Dorner M, Horwitz JA, Donovan BM, Labitt RN, Budell WC, Friling T, Vogt A, Catanese MT, Satoh T, Kawai T, Akira S, Law M, Rice CM, Ploss A.** 2013. Completion of the entire hepatitis C virus life cycle in genetically humanized mice. Nature **501:**237–241. http://dx.doi.org/10.1038/nature12427.
90. **Ross-Thriepland D, Amako Y, Harris M.** 2013. The C terminus of NS5A domain II is a key determinant of hepatitis C virus genome replication, but is not required for virion assembly and release. J. Gen. Virol. **94:**1009–1018. http://dx.doi.org/10.1099/vir.0.050633-0.
91. **Yang F, Robotham JM, Nelson HB, Irsigler A, Kenworthy R, Tang H.** 2008. Cyclophilin A is an essential cofactor for hepatitis C virus infection and the principal mediator of cyclosporine resistance in vitro. J. Virol. **82:**5269–5278. http://dx.doi.org/10.1128/JVI.02614-07.

JVI
Journals.ASM.org

# Expression of MicroRNA miR-122 Facilitates an Efficient Replication in Nonhepatic Cells upon Infection with Hepatitis C Virus

Takasuke Fukuhara,[a] Hiroto Kambara,[a] Mai Shiokawa,[a] Chikako Ono,[a] Hiroshi Katoh,[a] Eiji Morita,[a] Daisuke Okuzaki,[b] Yoshihiko Maehara,[c] Kazuhiko Koike,[d] and Yoshiharu Matsuura[a]

Department of Molecular Virology[a] and DNA-Chip Developmental Center for Infectious Diseases,[b] Research Institute for Microbial Diseases, Osaka University, Osaka, Japan; Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan[c]; and Department of Gastroenterology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan[d]

**Hepatitis C virus (HCV) is one of the most common etiologic agents of chronic liver diseases, including liver cirrhosis and hepatocellular carcinoma. In addition, HCV infection is often associated with extrahepatic manifestations (EHM), including mixed cryoglobulinemia and non-Hodgkin's lymphoma. However, the mechanisms of cell tropism of HCV and HCV-induced EHM remain elusive, because *in vitro* propagation of HCV has been limited in the combination of cell culture-adapted HCV (HCVcc) and several hepatic cell lines. Recently, a liver-specific microRNA called miR-122 was shown to facilitate the efficient propagation of HCVcc in several hepatic cell lines. In this study, we evaluated the importance of miR-122 on the replication of HCV in nonhepatic cells. Among the nonhepatic cell lines expressing functional HCV entry receptors, Hec1B cells derived from human uterus exhibited a low level of replication of the HCV genome upon infection with HCVcc. Exogenous expression of miR-122 in several cells facilitates efficient viral replication but not production of infectious particles, probably due to the lack of hepatocytic lipid metabolism. Furthermore, expression of mutant miR-122 carrying a substitution in a seed domain was required for efficient replication of mutant HCVcc carrying complementary substitutions in miR-122-binding sites, suggesting that specific interaction between miR-122 and HCV RNA is essential for the enhancement of viral replication. In conclusion, although miR-122 facilitates efficient viral replication in nonhepatic cells, factors other than miR-122, which are most likely specific to hepatocytes, are required for HCV assembly.**

More than 170 million individuals worldwide are infected with hepatitis C virus (HCV), and cirrhosis and hepatocellular carcinoma induced by HCV infection are life-threatening diseases (57). Although therapy combining pegylated interferon (IFN) and ribavirin has achieved a sustained virological response in 50% of individuals infected with HCV genotype 1 (37), a more effective therapeutic modality for HCV infection is needed (46). The establishment of *in vivo* and *in vitro* infection systems has been hampered by the narrow host range and tissue tropism of HCV. Although the chimpanzee is the only experimental animal susceptible to HCV infection, it is difficult to use the chimpanzee in experiments due to ethical concerns (3). Furthermore, robust *in vitro* HCV propagation is limited to the combination of cell culture-adapted clones based on the genotype 2a JFH1 strain (HCVcc) and human hepatoma cell lines, including Huh7, Hep3B, and HepG2 (29, 43, 62).

It is well-known that HCV mainly infects hepatocytes. However, the precise mechanism underlying the liver tropism of HCV has not been clarified. Chronic hepatitis C virus infection is often associated with at least one extrahepatic manifestation (EHM), including mixed cryoglobulinemia, non-Hodgkin's lymphoma, lichen planus, thyroiditis, diabetes mellitus, Sjögren syndrome, and arthritis (19). EHMs are frequently more serious than hepatic disease in some patients and sometimes occur even in patients with persistently normal liver functions (19). Mixed cryoglobulinemia is the most-well-characterized HCV-associated disease and is curable by viral clearance through antiviral therapies (6). Although replication of HCV RNA in peripheral blood mononuclear cells (PBMCs) and neuronal cells at a low level was suggested (64), the biological significance of the extrahepatic replication of HCV, particularly in the development of EHMs, is not well understood.

MicroRNAs (miRNAs) are small noncoding RNAs consisting of 20 to 25 nucleotides that modulate gene expression in plants and animals (1, 24). Most miRNAs negatively regulate translation through the interaction with the 3′ untranslated region (UTR) of mRNA in a sequence-specific manner. miRNA 122 (miR-122) is liver specific, is the most abundantly expressed miRNA in the liver, and represses the translation of several mRNAs (5, 7). Jopling et al. reported for the first time that the inhibition of miR-122 dramatically decreased RNA replication in HCV subgenomic replicon (SGR) cells (28). In addition, several reports revealed that a specific interaction between the seed domain of miR-122 and the complementary sequences in the 5′ UTR of HCV RNA is essential for the enhancement of translation and replication of the HCV genome (21, 25, 27, 36). Endogenous expression levels of miR-122 are significantly higher in Huh7 cells than in other hepatic and nonhepatic cell lines (Fig. 1). In addition, previous reports showed that miR-122 expression enhanced the replication of SGR RNA in human embryonic kidney 293 (HEK293) cells and mouse embryonic fibroblasts (MEFs) (8, 35). Furthermore, it was recently shown that exogenous expression of miR-122 facilitates the efficient propagation of HCVcc in Hep3B and HepG2 cells, which are

Received 4 March 2012 Accepted 10 May 2012
Published ahead of print 16 May 2012
Address correspondence to Yoshiharu Matsuura, matsuura@biken.osaka-u.ac.jp.

doi:10.1128/JVI.00567-12

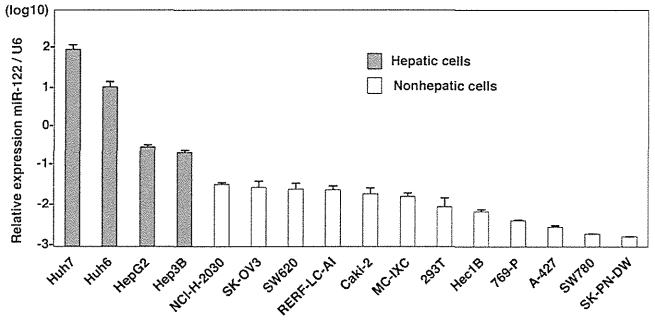



**FIG 1** Endogenous expression levels of miR-122 in hepatic and nonhepatic cells. Total miRNAs were extracted from Huh7, Huh6, HepG2, Hep3B, NCI-H-2030, SK-OV3, SW620, RERF-LC-AI, Caki-2, MC-IXC, 293T, Hec1B, 769-P, A-427, SW780, and SK-PN-DW cells, and the expression levels of miR-122 were determined by qRT-PCR.

nonpermissive for HCVcc propagation (29, 43). These results suggest that the high susceptibility of Huh7 cells to the propagation of HCVcc is attributable to the high expression level of miR-122 and raise the possibility of expanding the HCV host range through the exogenous expression of miR-122 in nonhepatic cells.

In this study, we assessed the effect of miR-122 expression on the replication of HCVcc and SGR RNA in several nonhepatic cell lines. Although the exogenous expression of miR-122 in the cell lines facilitates significant RNA replication through a gene-specific interaction between miR-122 and 5′ UTR of HCV RNA, no infectivity was detected in either the cells or the culture supernatants. The current study suggests that the expression of miR-122 plays an important role in HCV cell tropism, as well as in the possible involvement of nonhepatic cells in EHM, through an incomplete propagation of HCV.

## MATERIALS AND METHODS

**NextBio Body Atlas.** The NextBio Body Atlas application presents an aggregated analysis of gene expression across various normal tissues, normal cell types, and cancer cell lines. It enables us to investigate the expression of individual genes as well as gene sets. Samples for Body Atlas data are obtained from publicly available studies that are internally curated, annotated, and processed (31). Body Atlas measurements are generated from all available RNA expression studies that used Affymetrix U133 Plus or U133A Genechip arrays for human studies. The results corresponding to 128 human tissue samples were incorporated from 1,067 arrays, the results corresponding to 157 human cell types were incorporated from 1,474 arrays, and the results corresponding to 359 human cancer cell lines were incorporated from 376 arrays. Gene queries return a list of relevant tissues or cell types rank ordered by absolute gene expression and grouped by body systems or across all body systems. In the current analysis, we screened for nonhepatic cell lines expressing HCV receptor candidates, including CD81, SR-BI, claudin1 (CLDN1), and occludin (OCLN), or very-low-density lipoprotein (VLDL)-associated proteins, including apolipoprotein E (ApoE), ApoB, and microsomal triglyceride transfer protein (MTTP). A detailed analysis protocol developed by NextBio was described previously (31). The raw data used in this application are derived from the GSK Cancer Cell Line data deposited at the National Cancer Institute website (https://array.nci.nih.gov/caarray/project/woost-00041) and additionally from NCBI Gene Expression Omnibus (GEO) accession number GSE5720 for cell lines SK-OV-3 and SW620.

**Sample collection and RNA extraction for microarray analysis.** Total RNAs extracted from cells were purified by using an miRNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Eluted RNAs were quantified using a Nanodrop ND-1000 (version 3.5.2) spectrophotometer (Thermo Scientific, Wartham, MA). RNA integrity was evaluated using the RNA 6000 LabChip kit and bioanalyzer (Agilent Technologies, Santa Clara, CA). Each RNA that had an RNA integrity number (RIN) greater than 9.0 was used for the microarray experiments.

**Microarray experiment.** Expression profiling was generated using the 4 × 44K whole-human-genome oligonucleotide microarray (version 2.0) G4845A (Agilent Technologies). Each microarray uses 44,495 probes to interrogate 27,958 Entrez gene RNAs. One hundred nanograms of total RNA was reverse transcribed into double-stranded cDNAs by AffinityScript multiple-temperature reverse transcriptase and amplified for 2 h at 40°C. The resulting cDNAs were subsequently used for *in vitro* transcription by the T7 polymerase and labeled with cyanine-3-labeled cytosine triphosphate (Perkin Elmer, Waltham, MA) for 2 h at 40°C using a low-input Quick-Amp labeling kit (Agilent Technologies) according to the manufacturer's protocol. After labeling, the rates of dye incorporation and quantification were measured with a Nanodrop ND-1000 (version 3.5.2) spectrophotometer (Thermo Scientific), and then the cRNAs were fragmented for 30 min at 60°C in the dark. Differentially labeled samples of 1,650 ng of cRNA were hybridized on Agilent 4 × 44K whole-genome arrays (version 2.0; 026652; Agilent Design) at 65°C for 17 h with rotation in the dark. Hybridization was performed using a gene expression hybridization kit (Agilent Technologies) following the manufacturer's instructions. After washing in gene expression washing buffer, each slide was scanned with the Agilent microarray scanner G2505C. Feature extraction software (version 10.5.1.1) employing defaults for all parameters was used to convert the images into gene expression data. Raw data were imported into a Subio platform (version 1.12) for database management and quality control. Raw intensity data were normalized against GAPDH (glyceraldehyde-3-phosphate dehydrogenase) expression levels for further analysis. These raw data have been accepted by GEO (a public repository for microarray data aimed at storing minimum information about microarray experiments [MIAME]).

**Plasmids.** The cDNA clones of wild-type (WT) and mutant (MT) pri-miR-122 and *Aequorea coerulescens* green fluorescent protein (AcGFP) were inserted between the XhoI and XbaI sites of lentiviral vector pCSII-EF-RfA, which was kindly provided by M. Hijikata, and the resulting plasmids were designated pCSII-EF-WT-miR-122, pCSII-EF-MT-miR-122, and pCSII-EF-AcGFP, respectively. Plasmids pHH-JFH1 and pSGR-JFH1, which encode full-length and subgenomic cDNA of the JFH1 strain, respectively, were kindly provided by T. Wakita. pHH-JFH1-E2p7NS2mt contains three adaptive mutations in pHH-JFH1 (53). pHH-JFH1-M1 and pHH-JFH1-M2 were established by the introduction of a point mutation of nucleotide 26 located in site 1 and nucleotide 41 in site 2 of the 5′ UTR of the JFH1 cDNA construct pHH-JFH1. pSGR-Con1, which encodes SGR of the Con1 strain, was kindly provided by R. Bartenschlager. The complementary sequences of miR-122 were introduced into the multicloning site of the pmirGLO vector (Promega, Madison, WI), and the resulting plasmid was designated pmirGLO-compl-miR-122. The plasmids used in this study were confirmed by sequencing with an ABI 3130 genetic analyzer (Applied Biosystems, Tokyo, Japan).

**Cell lines.** All cell lines were cultured at 37°C under the conditions of a humidified atmosphere and 5% $CO_2$. The following cells were maintained in Dulbecco modified Eagle medium (DMEM; Sigma-Aldrich, St. Louis, MO) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum (FCS): human hepatocellular carcinoma-derived Huh7, Hep3B, and HepG2; embryonic kidney-derived HEK293 and 293T; lung-derived RERF-LC-AI, NCI-H-2030, and A-427; kidney-derived Caki-2 and 769-P; neuron-derived MC-IXC and SK-PN-DW; uterus-derived Hec1B; ovary-derived SK-OV3; colon-derived SW620; and urinary bladder-derived SW780 cells. RERF-LC-AI (RCB0444) cells were provided by the RIKEN BRC through the National Bio-Resource Project of MEXT, Japan. Hec1B (JCRB1193) cells were obtained from the JCRB Cell Bank. NCI-H-2030, A-427, Caki-2, 769-P, MC-IXC, SK-PN-DW, SK-OV3, and SW780 cells were obtained from the American Type Culture Collection (ATCC). SW620 cells were kindly provided by C.

Oneyama. 293T-CLDN cells stably expressing CLDN1 were established by the introduction of the expression plasmids encoding CLDN1 under the control of the CAG promoter of pCAG-pm3. The Huh7-derived cell line Huh7.5.1 was kindly provided by F. Chisari. The Huh7OK1 cell line efficiently propagates HCVcc as previously described (45). Huh7, Hec1B, and HEK293 cells harboring Con1- or JFH1-based HCV SGR were prepared according to the method described in a previous report (47) and maintained in DMEM containing 10% FCS and 1 mg/ml G418 (Nakalai Tesque, Kyoto, Japan).

**Antibodies and drugs.** Mouse monoclonal antibodies to HCV nonstructural protein 5A (NS5A) and β-actin were purchased from Austral Biologicals (San Ramon, CA) and Sigma-Aldrich, respectively. Mouse anti-ApoE antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-HCV core protein and NS5A were prepared as described previously (41). Rabbit anti-SR-BI antibody was purchased from Novus Biologicals (Littleton, CO). Rabbit anti-CLDN1 and -OCLN antibodies, Alexa Fluor 488 (AF488)-conjugated anti-rabbit or -mouse IgG antibodies, and AF594-conjugated anti-mouse IgG2a antibodies were purchased from Invitrogen (San Diego, CA). Mouse anti-FKBP8 antibody was described previously (44). Mouse anti-double-stranded RNA (anti-dsRNA) IgG2a (J1 and K2) antibodies were obtained from Biocenter Ltd. (Szirak, Hungary). The HCV NS3/4A protease inhibitor was purchased from Acme Bioscience (Salt Lake City, UT). Human recombinant alpha IFN (IFN-α) and cyclosporine were purchased from PBL Biomedical Laboratories (Piscataway, NJ) and Sigma-Aldrich, respectively. BODIPY 558/568 lipid probe was purchased from Invitrogen. The locked nucleic acid (LNA) targeted to miR-122, LNA-miR-122 (5′-CcAttGTcaCaCtCC-3′), and its negative control, LNA-control (5′-CcAttCTgaCcCtAC-3′) (LNAs are in capital letters, DNAs are in lowercase letters; sulfur atoms in oligonucleotide phosphorothioates are substituted for nonbridging oxygen atoms; the capital C indicates LNA methylcytosine), were purchased from Gene Design (Osaka, Japan) (15).

**Preparation of viruses.** pHH-JFH1-E2p7NS2mt was introduced into Huh7.5.1 cells, HCVcc in the supernatant was collected after serial passages (39), and infectious titers were determined by a focus-forming assay and expressed in focus-forming units (FFUs) (62). Mutant HCVcc was produced from Huh7.5.1 cells expressing MT miR-122 according to the method of a previous report with minor modifications (25). HCVpv, a pseudotype vesicular stomatitis virus (VSV) bearing HCV E1 and E2 glycoproteins, was prepared as previously described (61), and infectivity was assessed by luciferase expression on a Bright-Glo luciferase assay system (Promega), following a protocol provided by the manufacturer and expressed in relative light units (RLUs).

**Lipofection and lentiviral gene transduction.** Cells were transfected with the plasmids by using Trans IT LT-1 transfection reagent (Mirus, Madison, WI) according to the manufacturer's protocol. LNAs were introduced into cells by Lipofectamine RNAiMAX (Invitrogen). The lentiviral vectors and ViraPower lentiviral packaging mix (Invitrogen) were cotransfected into 293T cells, and the supernatants were recovered at 48 h posttransfection. The lentivirus titer was determined by a Lenti-XTM quantitative reverse transcription-PCR (qRT-PCR) titration kit (Clontech, Mountain View, CA), and the expression levels of miR-122 and AcGFP were determined at 48 h postinoculation.

**Quantitative RT-PCR.** HCV RNA levels were determined by the method described previously (18). Total RNA was extracted from cells by using an RNeasy minikit (Qiagen). The first-strand cDNA synthesis and qRT-PCR were performed with TaqMan EZ RT-PCR core reagents and an ABI Prism 7000 system (Applied Biosystems), respectively, according to the manufacturer's protocols. The primers for TaqMan PCR targeted to the noncoding region of HCV RNA were synthesized as previously reported (42). To determine the expression levels of miR-122, total miRNA was prepared by using the miRNeasy minikit, and miR-122 expression was determined by using fully processed miR-122-specific RT and PCR primers provided in the TaqMan microRNA assays according to the manufacturer's protocol. U6 small nuclear RNA was used as an internal control. Fluorescent signals were analyzed with the ABI Prism 7000 system.

**Immunoblotting.** Cells were lysed on ice in lysis buffer (20 mM Tris-HCl [pH 7.4], 135 mM NaCl, 1% Triton X-100, 10% glycerol) supplemented with a protease inhibitor mix (Nacalai Tesque). The samples were boiled in loading buffer and subjected to 5 to 20% gradient SDS-PAGE. The proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) and reacted with the appropriate antibodies. The immune complexes were visualized with SuperSignal West Femto substrate (Pierce, Rockford, IL) and detected with an LAS-3000 image analyzer system (Fujifilm, Tokyo, Japan).

**Immunofluorescence assay.** Cells cultured on glass slides were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at room temperature for 30 min, permeabilized for 20 min at room temperature with PBS containing 0.2% Triton, after being washed three times with PBS, and blocked with PBS containing 2% FCS for 1 h at room temperature. The cells were incubated with PBS containing appropriate primary antibodies at room temperature for 1 h, washed three times with PBS, and incubated with PBS containing AF488- or AF594-conjugated secondary antibodies at room temperature for 1 h. For lipid droplet staining, cells incubated in medium containing 20 μg/ml BODIPY for 20 min at 37°C were washed with prewarmed fresh medium and incubated for 20 min at 37°C. Cell nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole). Cells were observed with a FluoView FV1000 laser scanning confocal microscope (Olympus, Tokyo, Japan).

***In vitro* transcription, RNA transfection, and colony formation.** The plasmids pSGR-Con1 and pSGR-*JFH1* were linearized with ScaI and XbaI, respectively, and treated with mung bean exonuclease. The linearized DNA was transcribed *in vitro* by using a MEGAscript T7 kit (Applied Biosystems) according to the manufacturer's protocol. The *in vitro*-transcribed RNA (10 μg) was electroporated into Hec1B and HEK293 cells at $10^7$ cells/0.4 ml under conditions of 190 V and 975 μF using a Gene Pulser apparatus (Bio-Rad, Hercules, CA) and plated on DMEM containing 10% FCS. The medium was replaced with fresh DMEM containing 10% FCS and 1 mg/ml G418 at 24 h posttransfection. The remaining colonies were cloned by using a cloning ring (Asahi Glass, Tokyo, Japan) or fixed with 4% PFA and stained with crystal violet at 4 weeks postelectroporation.

**Electron microscopy and correlative FM-EM analysis.** Cells were cultured on a Cell Desk polystyrene coverslip (Sumitomo Bakelite) and were fixed with 2% formaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 7% sucrose. Cells were postfixed for 1 h with 1% osmium tetroxide and 0.5% potassium ferrocyanide in 0.1 M cacodylate buffer (pH 7.4), dehydrated in graded series of ethanol, and embedded in Epon812 (TAAB). Ultrathin (80-nm) sections were stained with saturated uranyl acetate and lead citrate solution. Electron micrographs were obtained with a JEM-1011 transmission electron microscope (JEOL). Correlative fluorescence microscopy (FM)-electron microscopy (EM) allows individual cells to be examined both in an overview with FM and in a detailed subcellular-structure view with EM (51). The NS5A was stained and observed in the Hec1B-derived Con1 SGR cells by the correlative FM-EM method as described previously (44).

**Intracellular infectivity.** Intracellular viral titers were determined according to a method previously reported (20). Briefly, cells were extensively washed with PBS, scraped, and centrifuged for 5 min at 1,000 × *g*. Cell pellets were resuspended in 500 μl of DMEM containing 10% FCS and subjected to three cycles of freezing and thawing using liquid nitrogen and a thermo block set to 37°C. Cell lysates were centrifuged at 10,000 × *g* for 10 min at 4°C to remove cell debris. Cell-associated infectivity was determined by a focus-forming assay.

**Statistical analysis.** The data for statistical analyses are averages of three independent experiments. Results were expressed as means ± standard deviations. The significance of differences in the means was determined by Student's *t* test.

**Microarray data accession number.** Access to data concerning this study may be found under GEO experiment accession number GSE32886.

## RESULTS

**Nonhepatic cell lines susceptible to HCVcc by expression of miR-122.** Human CD81 (hCD81), SR-B1, CLDN1, and OCLN are crucial for HCV entry (16, 48, 49, 56). First, we examined the expression of these receptor candidates in nonhepatic cell lines by using the web-based NextBio search engine (Cupertino, CA). Multidimensional scaling was used to visualize the differences in expression patterns of molecules of various tissues, cells, and cell lines from those of hepatic cell lines and primary hepatocytes. We selected nine nonhepatic cell lines as possibly being susceptible to HCVcc infection: NCI-H-2030 (lung), Caki-2 (kidney), 769-P (bladder), A-427 (lung), SK-OV3 (ovary), SW780 (bladder), SW620 (colon), RERF-LC-AI (lung), and Hec1B (uterus) (Fig. 2). In addition, three nonhepatic cell lines previously reported to be susceptible to replication of HCV RNA—that is, SK-PN-DW (neuron), MC-IXC (neuron), and 293T-CLDN (kidney)—were included in this study (8, 17). The expression of each receptor molecule in these 12 nonhepatic cell lines was confirmed by fluorescence-activated cell sorter (FACS) analysis and immunoblotting (Fig. 3A and B). To examine the expression of the functional receptors for HCV entry in these cell lines, we inoculated HCVpv into the cells. Ten of the cell lines (A-427 and SW780 being the exceptions) exhibited various degrees of susceptibility to HCVpv infection (Fig. 3C). Therefore, we examined the possibility of propagation of HCVcc by the expression of miR-122 in these 10 cell lines.

To introduce miR-122 in the cell lines, we employed a lentiviral vector encoding pri-miR-122, an unprocessed miR-122. To confirm the maturation of pri-miR-122 to form functional RNA-induced silencing complexes (RISCs), suppression of the translation of the target mRNA was determined by a dual reporter assay. Translation of a firefly luciferase mRNA containing the sequences complementary to miR-122 in the 3′ UTR was suppressed by infection with the lentivirus encoding pri-miR-122 but not by infection with a control virus (data not shown), suggesting that the pri-miR-122 is processed into a functionally mature miR-122. By using this lentiviral vector, high levels of miR-122 expression were achieved in the 10 cell lines, comparable to the endogenous expression level of miR-122 in Huh7 cells (Fig. 4A).

To examine the effect of the exogenous expression of miR-122 on HCV replication, the nonhepatic cell lines expressing miR-122 were infected with HCVcc at a multiplicity of infection (MOI) of 1, and intracellular viral RNA was determined (Fig. 4B). The expression of miR-122 significantly increased the amount of the HCV genome in Hec1B, 293T-CLDN, MC-IXC, and RERF-LC-AI cells as well as Huh7 cells and slightly increased it in SK-OV3 and NCI-H-2030 cells. Although the levels of viral RNA in SW620, Caki-2, and SK-PN-DW cells upon expression of miR-122 were higher than those in control cells, no increase of viral RNA was observed. No effect of the expression of miR-122 was observed in 769-P cells. Interestingly, naïve Hec1B cells exhibited a delayed increase in viral RNA from 24 to 48 h postinfection, in contrast to the gradual decrease of viral RNA in other cell lines. Replication of HCV RNA in both naïve and miR-122-expressing Hec1B cells was inhibited by treatment with an inhibitor for HCV protease but not by treatment with IFN-α, due to the lack of an IFN receptor (11), whereas treatments with either IFN-α or the protease inhibitor suppressed the replication of HCV in the other cell lines expressing miR-122 (Fig. 4C). These results indicate that exogenous miR-122 expression enhances the replication of HCV even in nonhepatic cells. Hec1B cells exhibit a delayed replication of HCV, and HCV replication was enhanced by the exogenous expression of miR-122. Therefore, in this study we used Hec1B cells to investigate the biological significance of miR-122 on the replication of HCVcc in nonhepatic cells.

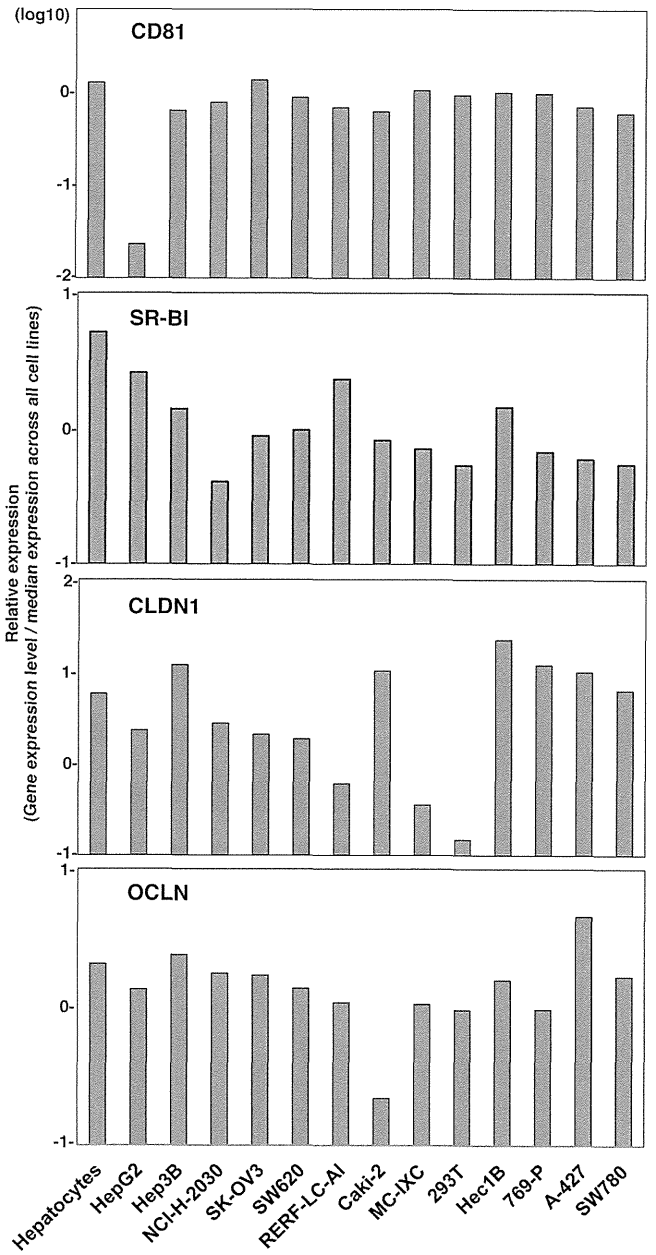



FIG 2 Receptor expression profiling in nonhepatic cells. Relative expression levels of CD81, SR-BI, CLDN1, and OCLN in primary hepatocytes, hepatic cell lines HepG2 and Hep3B, and nonhepatic cells were determined by using the NextBio Body Atlas. Expression levels were standardized by the median expression across all cell lines.

**Expression of miR-122 is essential for enhancing HCV replication in Hec1B cells.** To confirm the specificity of HCV replication in Hec1B cells, HCVcc was preincubated with an anti-HCV E2 monoclonal antibody, AP-33, or Hec1B/miR-122 and Hec1B/