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Novel permissive cell lines for complete propagation of hepatitis C virus

DOCTOR OF PHILOSOPHY

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ABSTRACT

Hepatitis C virus (HCV) is a major etiologic agent of chronic liver diseases. Although the HCV lifecycle has been clarified by studying laboratory strains of HCV derived from the genotype 2a JFH-1 strain (HCVcc), the mechanisms of particle formation have not been elucidated. Recently, we showed that exogenous expression of a liver-specific microRNA, miR-122, in nonhepatic cell lines facilitates efficient replication but not particle production of HCVcc, suggesting that liver-specific host factors are required for infectious particle formation. In this study, we screened human cancer cell lines for expression of the liver-specific α-fetoprotein by using a cDNA array database, and identified liver-derived JHH-4 cells and stomach-derived FU97 cells, which express liver-specific host factors comparable to Huh7 cells. These cell lines permit not only replication of HCV RNA, but also particle formation upon infection with HCVcc, suggesting that hepatic differentiation participates in the expressions of liver-specific host factors required for HCV propagation. HCV inhibitors targeting host and viral factors exhibited different antiviral efficacies between Huh7 and FU97 cells. Furthermore, FU97 cells exhibited higher susceptibility for propagation of HCVcc derived from the JFH-2 strain than Huh7 cells. These results suggest that hepatic differentiation participates in the expression of liver-specific host factors required for a complete propagation of HCV.

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INTRODUCTION

More than 170 million individuals worldwide are infected with hepatitis C virus (HCV), and the cirrhosis and hepatocellular carcinoma induced by HCV infection are life-threatening diseases (1). Current standard therapy combining peg-interferon (IFN) and ribavirin (RBV) has achieved a sustained virological response (SVR) in 50% of individuals infected with HCV genotype 1 (2). Recently, directly acting antiviral (DAA) agents have been applied in a clinical setting (3). An SVR rate of over 80% has been realized by combination therapy with peg-IFN, RBV and NS3/4A inhibitors in genotype 1 patients (4, 5). In addition, several DAAs, including inhibitors for NS3/4A protease, NS5A, and NS5B polymerase are currently in clinical trials. Several reports have shown that *in vitro* replication of HCV RNA is significantly inhibited by treatment with daclatasvir (NS5A inhibitor) and asunaprevir (NS3 protease inhibitor), and these two DAAs are also effective for patients infected with genotype 1 HCV who showed no response to previous therapy with peg-IFN-α and RBV (6-8). On the other hand, it has been shown that drug-resistant breakthrough viruses emerge during treatment with DAAs (9-12). Therefore, identification of host factors crucial for the propagation of HCV is an important task for the development of novel therapeutics for chronic hepatitis C with a low frequency of emergence of drug-resistant viruses.

The establishment of an *in vivo* infection model has been hampered by the narrow host range and tissue tropism of HCV. Although chimpanzees are the only experimental animals susceptible to HCV infection, it is difficult to use a chimpanzee model of experimental infection due to ethical concerns (13, 14). In addition, *in vitro* infection models have also been restricted to the combination of cell culture-adapted clones based on the genotype 2a JFH-1 strain (HCVcc) and human hepatoma cell lines, including Huh7 (15). Recently, several reports have shown that the exogenous expression of microRNA-122 (miR-122) facilitates the efficient propagation of HCVcc in HepG2 and Hep3B cells, which are nonpermissive for propagation of HCVcc (16, 17). Furthermore, we reported that nonhepatic cell lines, including Hec1B cells derived from uterine endometrial adenocarcinoma, also permit replication of HCV RNA by exogenous expression of miR-122 (18). These reports indicate that miR-122 is one of the most important determinants for liver tropism of HCV infection. Interestingly, formation of infectious particles was not observed in spite of an 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 for 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-, intermediate-, 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 the 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 hepatic differentiation required for the hepatic functions is involved in HCV propagation.

In this study, we identified novel cell lines supporting a complete propagation of HCVcc by the screening of cancer cell lines expressing α-fetoprotein (AFP), which is highly expressed in the well-differentiated hepatocellular carcinoma and evaluated in most of the 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 a 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 1067 arrays, those for 157 human cell types from 1474 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 an SGR of the Con1 strain, was provided by R. Bartenschlager. pIFN-β-Luc and pISRE-Luc carrying a firefly luciferase gene under the control of the IFN-β and IFN sensitive response element (ISRE) promoter, 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 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 an 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 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) following 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). Anti-human CD81 (hCD81) monoclonal antibody (JS-81) and rabbit anti-scavenger receptor class B type1

(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 Flour 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, UK). Phycoerythrin (PE)-conjugated anti-hCD81 and anti-mouse IgG antibodies were purchased from BD Biosciences. Anti-ApoB HRP conjugate antibody was purchased from ALerCHEK (Springvale, ME). The HCV NS3/4A protease inhibitor was purchased from Acme Bioscience (Salt Lake City, UT). Human recombinant α IFN (IFN-α) was purchased from PBL Biomedical Laboratories (Piscataway, NJ). BODIPY558/568 lipid probe and DAPI (4’, 6-diamidino-2-phenylindole) were purchased from Life Technologies and Vector Laboratories Inc. (Burlingame, CA), respectively. Cyclosporin A (CsA) 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, LNA-miR-122 (5’-CcAttGTcaCaCtCC-3’) and its negative control, LNA-control (5’-CcAttCTgaCcCtAC-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 Trans IT 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 post-transfection. 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 post-inoculation.

Quantitative RT-PCR.

HCV RNA levels were determined by the 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 a 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 5% to 20% gradient SDS-PAGE or 3-8% 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 pre-warmed 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 post-electroporation. 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 post-electroporation.

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% 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 control nontargeting siRNA were purchased from Dharmacon (Buckinghamshire, UK) 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 5×10^4 cells/well were transfected with 250 ng of each of the plasmids, stimulated with the appropriate ligands for 24 h at 24 h post-transfection 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 pre-treated with 10 µg/ml of anti-hCD81 (JS-81) monoclonal antibody for 1 h at 37°C and then inoculated with HCVcc (1×10^6 FFU/ml). Anti-E2 monoclonal antibody (10 µg/ml) was incubated with HCVcc (1×10^6 FFU/ml) for 1 h and then inoculated into cells. Intracellular HCV RNA levels at 12, 24, 48, and 72 h post-infection were determined by qRT-PCR.

Buoyant density gradient analysis.

Culture supernatants of Huh7.5.1 and FU97 cells infected with HCVcc at 72 h post-infection were passed through 0.45m pore size filters and concentrated by Spin-X Concentrator (100k-Da molecular weight cutoff column, Corning, Lowell, MA). One ml 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℃ by using 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 average of three independent experiments. Results were expressed as the means ± standard deviation. 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 ES/iPS cells (29, 40, 41). Generally, well-differentiated cancer cells show better maintenance of their cell-specific functions compared to 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 compared to 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 expressions 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 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 FACS analyses (Figs. 1D and 1E). 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 that in other cell lines (Figs. 1D and 1E). 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 were 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 those in JHH-4 cells, and these levels increased continuously until 48 h post-infection, and the treatment with IFN-α significantly inhibited the 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, right, 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 were introduced in these cells by a lentiviral vector encoding pri-miR-122, an unprocessed miR-122, and miR-122 expression was confirmed by qPCR analysis (Fig. 2B, left, 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 ER membrane, and accumulation of core protein around lipid droplets (LDs) facilitates efficient assembly of infectious particles in Huh7 cells (24). Immunofluorescence microscopic 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 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 hepatic characteristics play crucial roles on 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 to Huh7 cells, but also to 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 the HCV RNA abundances, cells were treated with LNA specific to either miR-122 (miR-122-LNA) or nonspecific (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 the 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 was 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 the 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 post-infection. 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 with respect to HCV RNA replication, *in vitro* transcribed subgenomic HCV RNA of the Con1 strain was electroporated into Huh7 and FU97 cells and cultured in medium containing G418 for a month, and then subgenomic replicon (SGR) cells of the Con1 strain were established (Fig. 4A). Replication of HCV RNA in four clones of the FU97 replicon cells was examined by qRT-PCR and immunoblotting. All clones contained a high level of HCV RNA (3×10^7 to 7×10^7 copies /μg total RNA) (Fig. 4B, upper panel) and the NS5A protein was also detected (Fig. 4B, lower panel). We examined the localization of NS5A and dsRNA in clone #5 of FU97 SGR cells by immunofluorescence analysis. Co-localization 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 of BILN 2061 (clones #5-1 and #7-1) or 10 pM of BMS-790052 and 100 nM of BILN 2061 (clones #5-2 and #7-2) to eliminate viral RNA. Viral RNA was gradually decreased and completely eliminated at 26 days post-treatment 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 the 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 of miR-122 expression (Fig. 6A). These results suggested that the efficient propagation of HCVcc in the cured FU97 cells was attributable to an 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 concentration of miR-122 by using 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 consistent with the previous data, major peaks of HCV RNA and infectious particles in both culture supernatants of Huh7.5.1 and FU97 cells were detected around 1.10 g/ml and 1.09 g/ml, respectively (Figs. 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 7B, lower panels). These results suggest that HCV particles produced in FU97 cells exhibit similar characters to those in hepatic cells.

Effects of anti-HCV drugs on the propagation of HCV in FU97 cells.

To determine the difference in the efficacy of antivirals on the HCV propagated in Huh7 and FU97 cells, three DAAs, i.e., BMS-790052, PSI-7977 and BILN 2061 targeting NS5A, NS5B, and NS3/4A, respectively, were treated with various concentrations at 3 h post-infection of HCVcc, and intracellular HCV RNA level was determined by qRT-RCR at 48 h post-infection. 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 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 hands, 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} values 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} values 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 antiviral effect of DAAs on the propagation of HCVcc was varied between Huh7 and FU97 cells.

Next, we examined the efficacy of IFN-α, RBV, and CsA, 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 post-infection of HCVcc, and the level of intracellular HCV RNA was determined by qRT-RCR at 48 h post-infection. In contrast to the treatment with DAAs, both Huh7 and FU97 cells exhibited cell toxicity by the treatment with RBV and CsA but not with IFN-α at higher concentrations (Fig. 6B, line graphs). The inhibitory efficacies of IFN-α (Fig. 8B, top graphs) and CsA (Fig. 8B, bottom graphs) on the propagation of HCVcc in FU97 cells were lower than those in Huh7 cells, and the EC_{50} values of IFN-α against propagation of HCVcc in FU97 and Huh7 cells were 4.3 and 2.5 IU/ml, ($P < 0.05$) and those of CsA 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} values 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 efficacy of anti-HCV drugs targeting host factors involved in the infection of HCV was 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 an efficient propagation in Huh7 cured cells (34). *In vitro* transcribed RNA of pJFH2/AS/mtT4 encoding a full-length JFH-2 strain was electroporated into Huh7.5.1 cells, and HCVcc/JFH-2 of 1.5×10^5 FFU/ml was recovered in the supernatants after serial passages. To examine the susceptibility of FU97 cells to the propagation of HCVcc/JFH-2, cells were infected with HCVcc/JFH-2 at an MOI of 1, and intracellular HCV RNA level was determined by qRT-PCR. Intracellular HCV RNA in parental and cured FU97 cells increased until 72 h post-infection, while it reached a peak at 48 h post-infection 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 post-infection with HCVcc/JFH-2 were also highest in the cured FU97 #7-1 cells (2.5×10^4 FFU/ml), followed by parental FU97 (1.2×10^4 FFU/ml) and Huh7 (9×10^3 FFU/ml) cells (Fig. 9B). Next, we examined the expression and subcellular localization of HCV proteins in cells infected with HCVcc/JFH-2 by immunofluorescence analysis. Expression of NS5A in cells upon infection with HCVcc/JFH-2 was highest in the cured FU97 #7-1 cells, followed by parental FU97 cells, and that in Huh7 cells was low (Fig. 9C, left panels). Core protein was detected around LDs in cells infected with HCVcc/JFH-2, as seen in those infected with the HCVcc/JFH-1 strain (Fig. 9C, right). To further confirm the efficient propagation of HCVcc/JFH-2 in FU97 cells, *in vitro* transcribed viral RNAs of the JFH-1 and JFH-2 strains of HCVcc were electroporated into Huh7, FU97 and cured FU97 cells. Although the infectious titers of the JFH-1 strain in FU97 cells were lower than those in Huh7 cells, those of the JFH-2 strain in FU97 and cured FU97 cells were significantly higher than those in Huh7 cells (Fig. 9D). These results suggest that FU97 cells are more susceptible to propagate HCVcc/JFH-2 than Huh7 cells.

DISCUSSION

Several reports have shown that hepatic differentiation is involved in the susceptibility of ES/iPS cells to HCVcc infection (28, 30, 41). In addition, in hepatic cancer cell lines, including Huh7, HepG2 and Hep3B, cells derived from not poorly- but well-differentiated HCC permit complete propagation of HCVcc (15-17), suggesting that hepatic differentiation is closely related to the susceptibility of cells to HCVcc propagation. In this study, we identified two cell lines susceptible to HCVcc infection by the screening of cancer cell lines expressing AFP as a marker of hepatic differentiation. HCC-derived JHH-4 cells and gastric cancer-derived FU97 cells permit a 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 7 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 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 (1a) (61), TN strain (1a)

(62), JFH-2 strain (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 a 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 a 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 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 worse prognosis and the characteristic 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 of 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 sera (28-30). These results suggest that hepatic differentiation required for hepatic functions plays crucial roles on HCV propagation. In consistent with these observations, our data suggest that cancer cell lines differentiated into hepatocyte-like cells to gain hepatic functions could permit a 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 CsA also inhibited propagation of HCVcc in a dose-dependent manner in both Huh7 and FU97 cells, treatment with RBV and CsA exhibited cell toxicity at higher concentrations in contrast to that with DAAs. Although the antiviral mechanism of RBV against HCV has not been well elucidated yet (53), inhibitory effects of RBV against HCV infection was 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 level of ADK in Huh7 and FU97 cell lines was 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 the treatment with IFN-α was almost comparable between Huh7 and FU97 cells (Fig. 5A), and expression of IFN-α receptor in the cell lines was same level (data not shown), suggesting that the involvement of other factors in the difference of the IFN response between FU97 and Huh7 cells.

Cyclophilins possess peptidyl-prolyl cis/trans isomerase (PPIase) activity and are involved in the protein folding and assembly. Cyclophilin A (CypA), the most abundant cyclophilin, localizes in the cytoplasm and interacts with the immunosuppressive drug CsA (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 CsA, 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 CsA in the cell lines may be attributable to other reasons such as 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 possibility to possess antiviral activity different from Huh7 cells.

In summary, we identified novel permissive cell lines for a 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 was observed in Huh7 cells, suggesting that FU97 cells would be useful for further investigation of the HCV lifecycle, as well as the development of therapeutic agents for chronic hepatitis C.

FIGURES

Figure.1

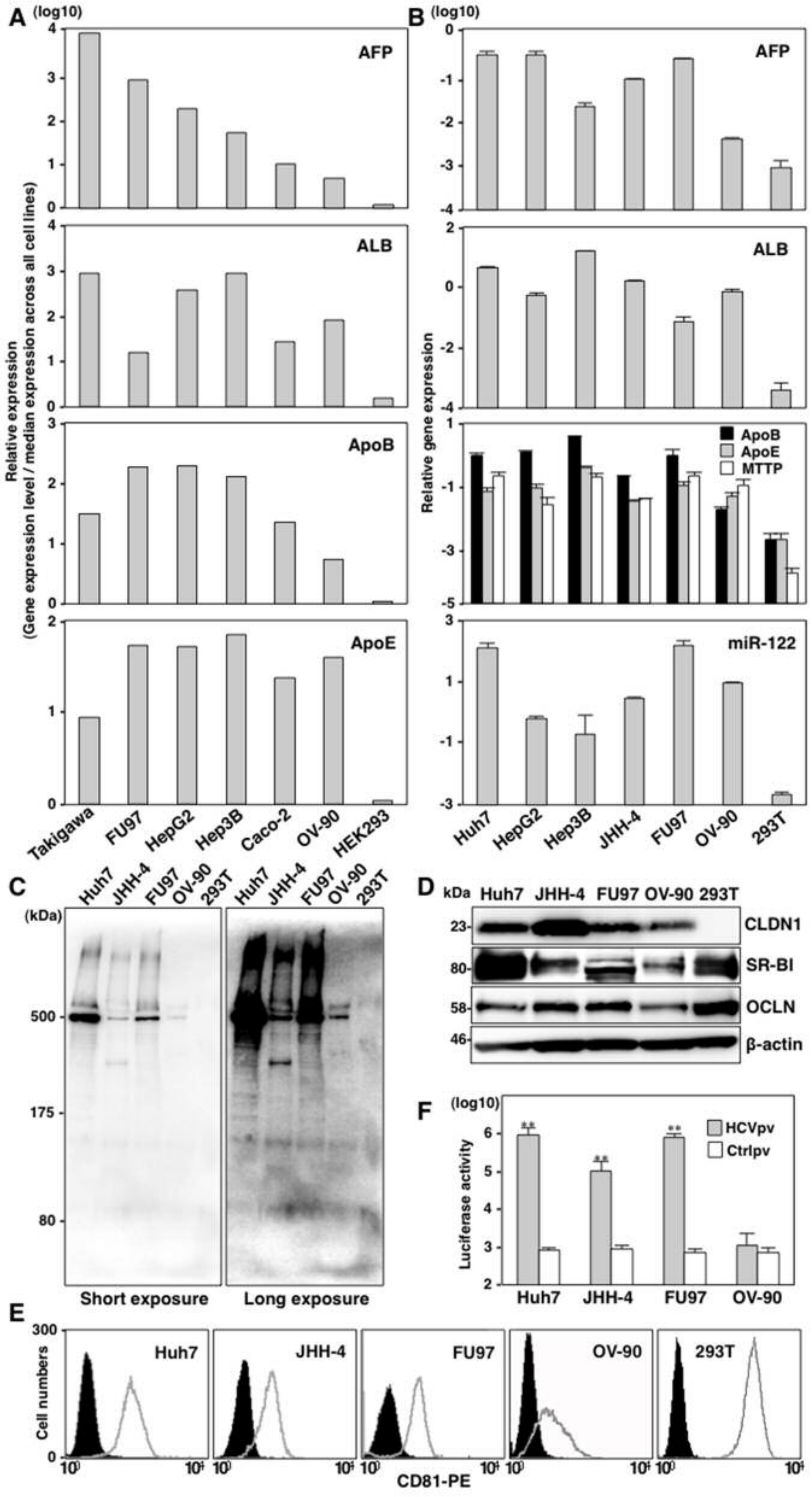


Figure.2

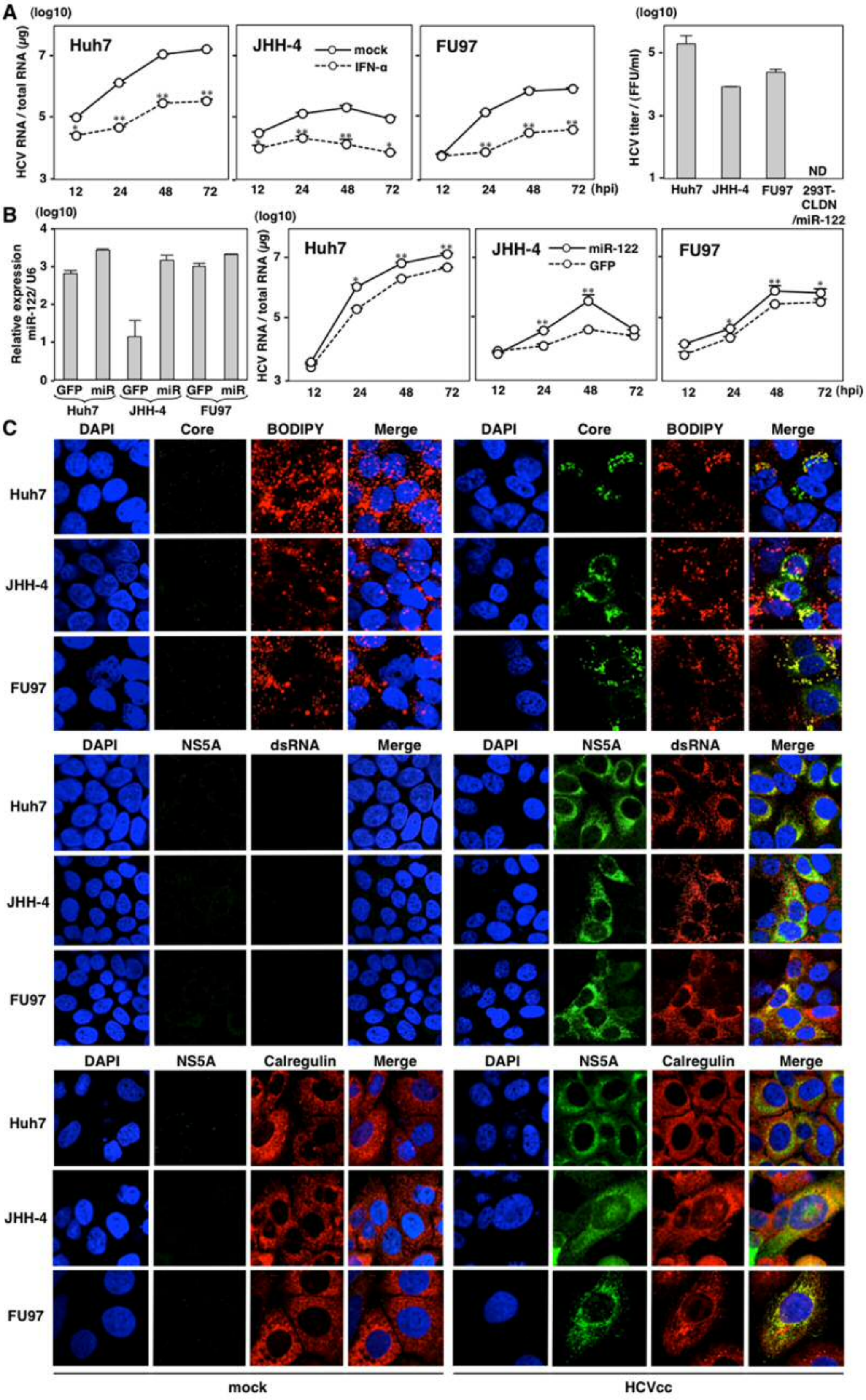

A
(log10)
HCV RNA / total RNA (μg)
3
5
7
Huh7
JHH-4
FU97
mock
IFN-α
*
**
12
24
48
72
(hpi)
(log10)
HCV titer / (FFU/ml)
1
3
5
Huh7
JHH-4
FU97
293T-CLDN/miR-122
ND
B
(log10)
Relative expression miR-122/ U6
0
1
2
3
4
GFP
miR
Huh7
JHH-4
FU97
(log10)
HCV RNA / total RNA (μg)
Huh7
JHH-4
FU97
miR-122
GFP
12
24
48
72
(hpi)
C
DAPI
Core
BODIPY
Merge
NS5A
dsRNA
Calregulin
Huh7
JHH-4
FU97
mock
HCVcc

Figure.3

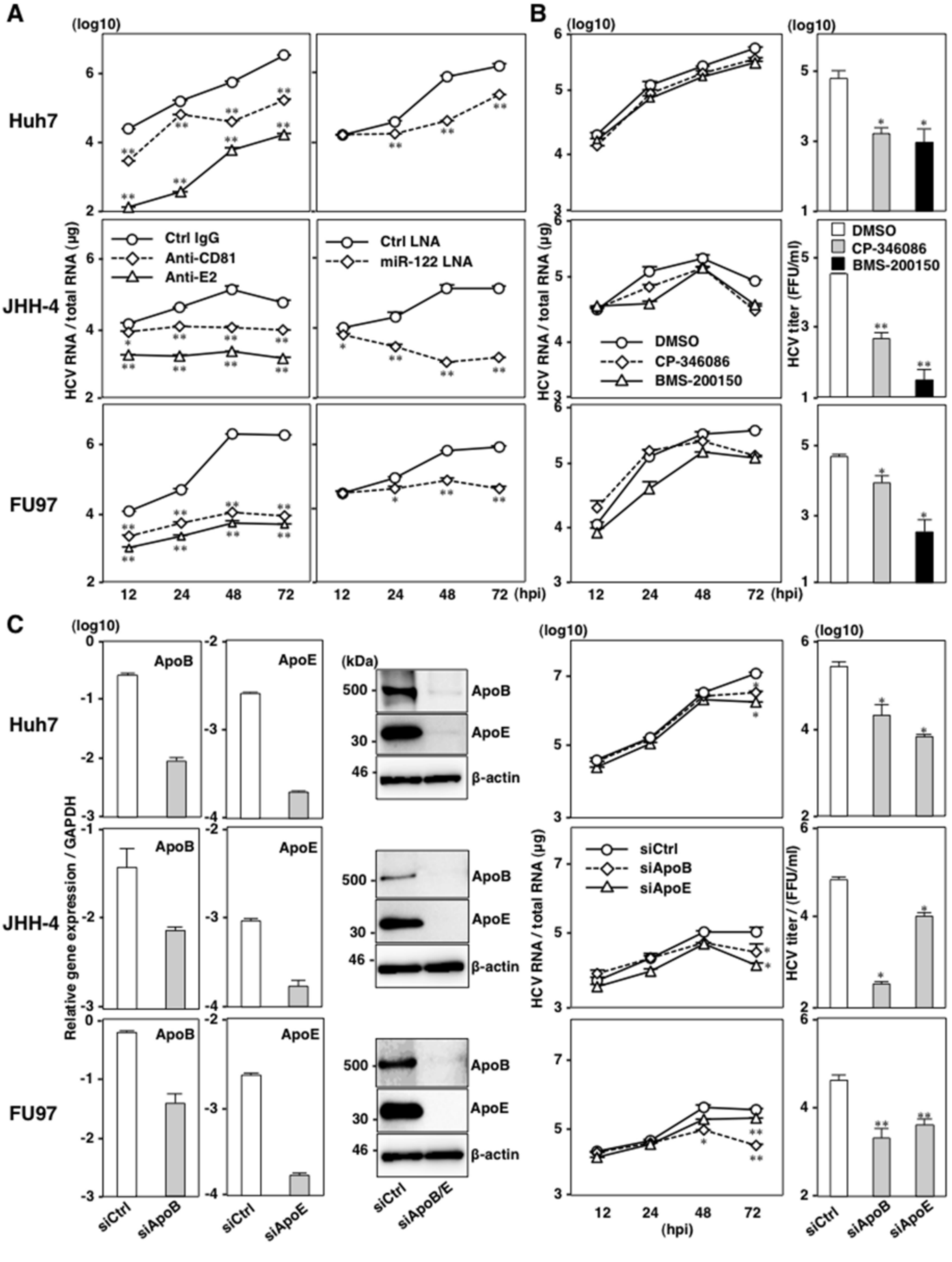

A
B
C
(log10)
Huh7
JHH-4
FU97
HCV RNA / total RNA (μg)
Ctrl IgG
Anti-CD81
Anti-E2
Ctrl LNA
miR-122 LNA
*
**
12
24
48
72
(hpi)
DMSO
CP-346086
BMS-200150
HCV titer (FFU/ml)
Relative gene expression / GAPDH
ApoB
ApoE
(kDa)
500
30
46
β-actin
siCtrl
siApoB
siApoE
siApoB/E
HCV titer / (FFU/ml)

Figure.4

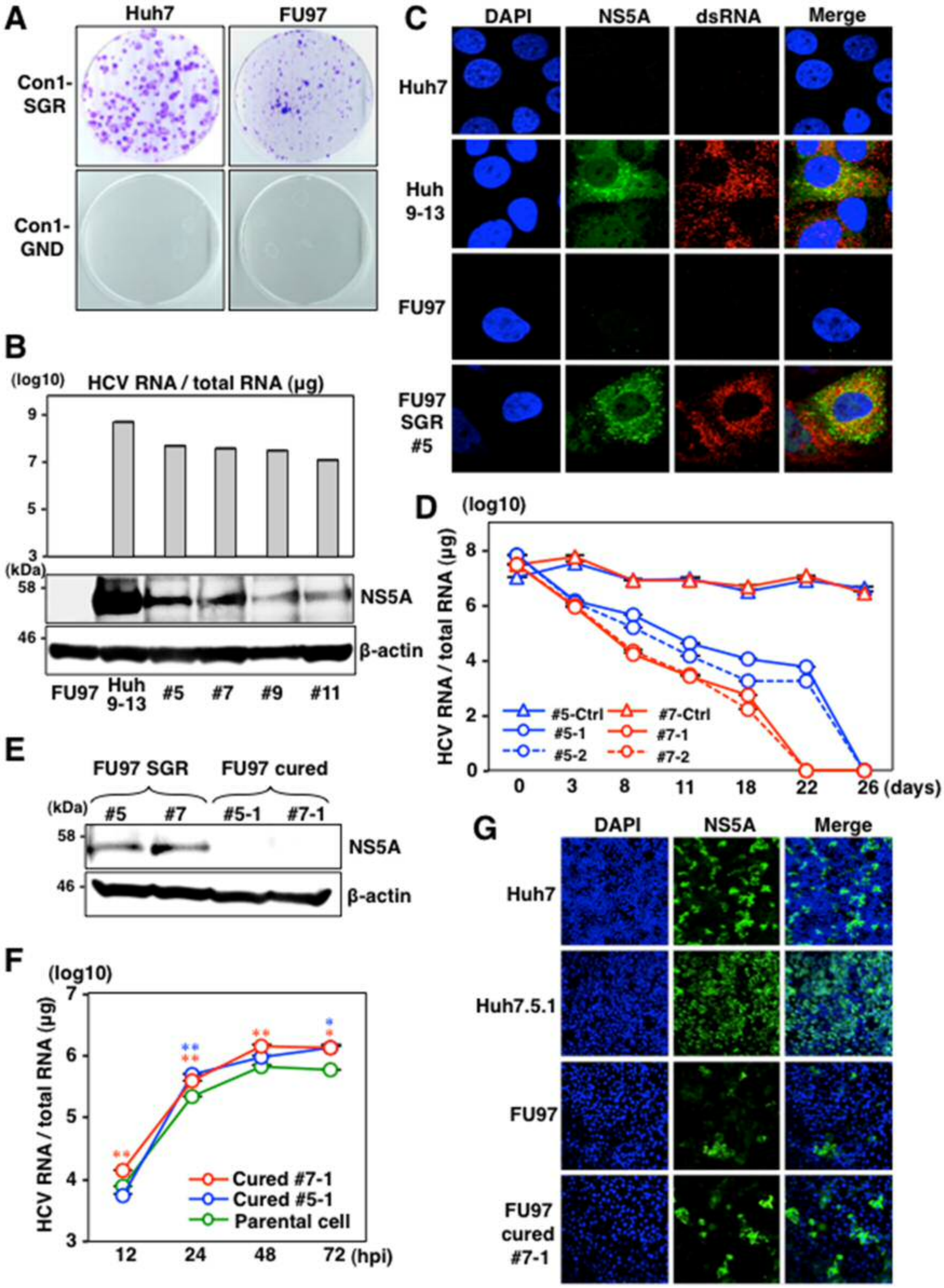

A
Huh7
FU97
Con1-SGR
Con1-GND
B
(log10)
HCV RNA / total RNA (μg)
9
7
5
3
(kDa)
58
NS5A
46
β-actin
FU97
Huh 9-13
#5
#7
#9
#11
C
DAPI
NS5A
dsRNA
Merge
Huh7
Huh 9-13
FU97
FU97 SGR #5
D
(log10)
HCV RNA / total RNA (μg)
8
6
4
2
0
#5-Ctrl
#5-1
#5-2
#7-Ctrl
#7-1
#7-2
0
3
8
11
18
22
26 (days)
E
FU97 SGR
FU97 cured
(kDa)
#5
#7
#5-1
#7-1
58
NS5A
46
β-actin
F
(log10)
HCV RNA / total RNA (μg)
7
6
5
4
3
**
**
**
**
*
*
Cured #7-1
Cured #5-1
Parental cell
12
24
48
72 (hpi)
G
DAPI
NS5A
Merge
Huh7
Huh7.5.1
FU97
FU97 cured #7-1

Figure.5

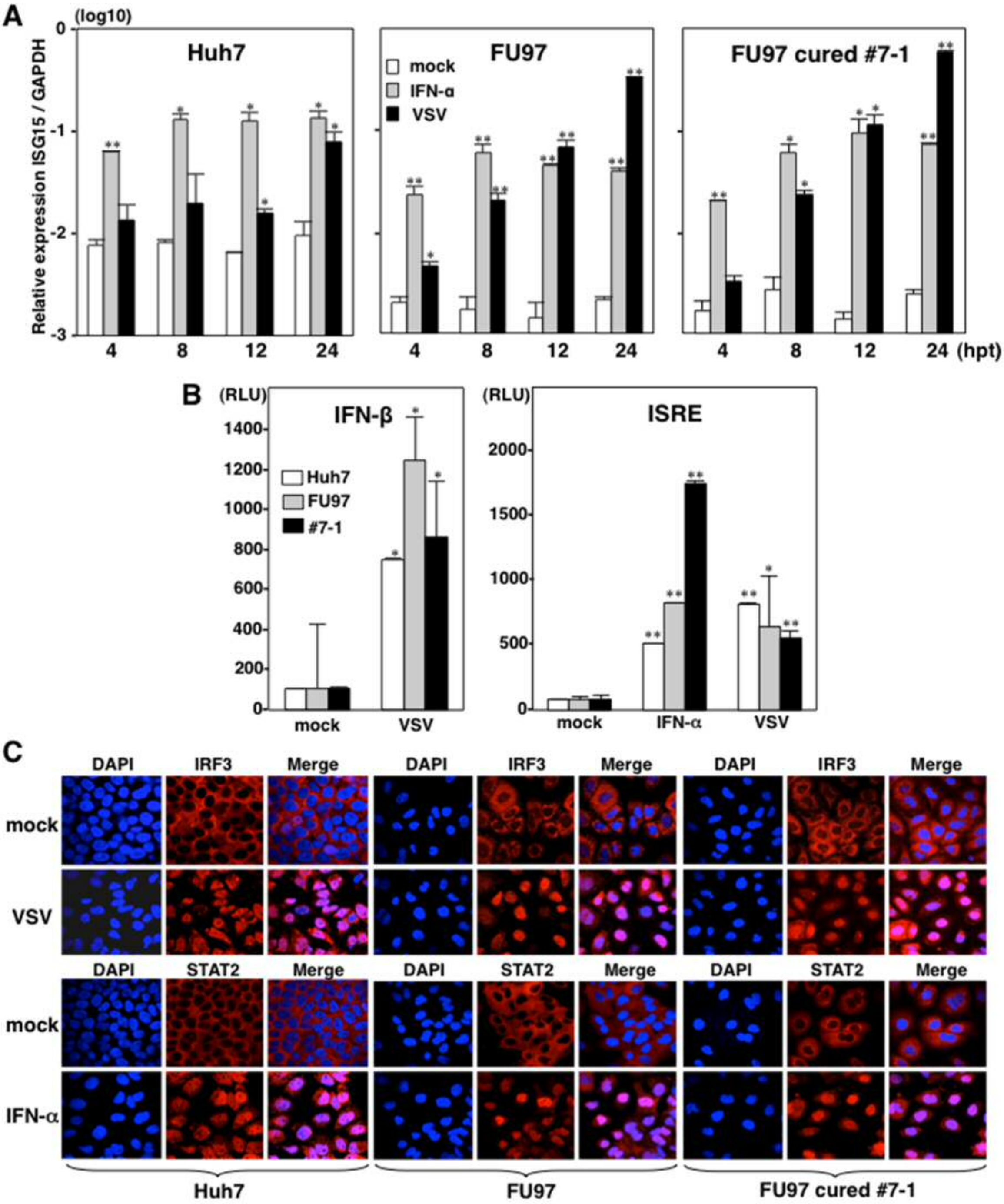

A
(log10)
Relative expression ISG15 / GAPDH
0
-1
-2
-3
Huh7
FU97
FU97 cured #7-1
□ mock
▨ IFN-α
■ VSV
4
8
12
24
24 (hpt)
B
(RLU)
IFN-β
1400
1200
1000
800
600
400
200
0
□ Huh7
▨ FU97
■ #7-1
mock
VSV
ISRE
2000
1500
1000
500
IFN-α
C
DAPI
IRF3
Merge
STAT2
mock
VSV
IFN-α
Huh7
FU97
FU97 cured #7-1

Figure.6

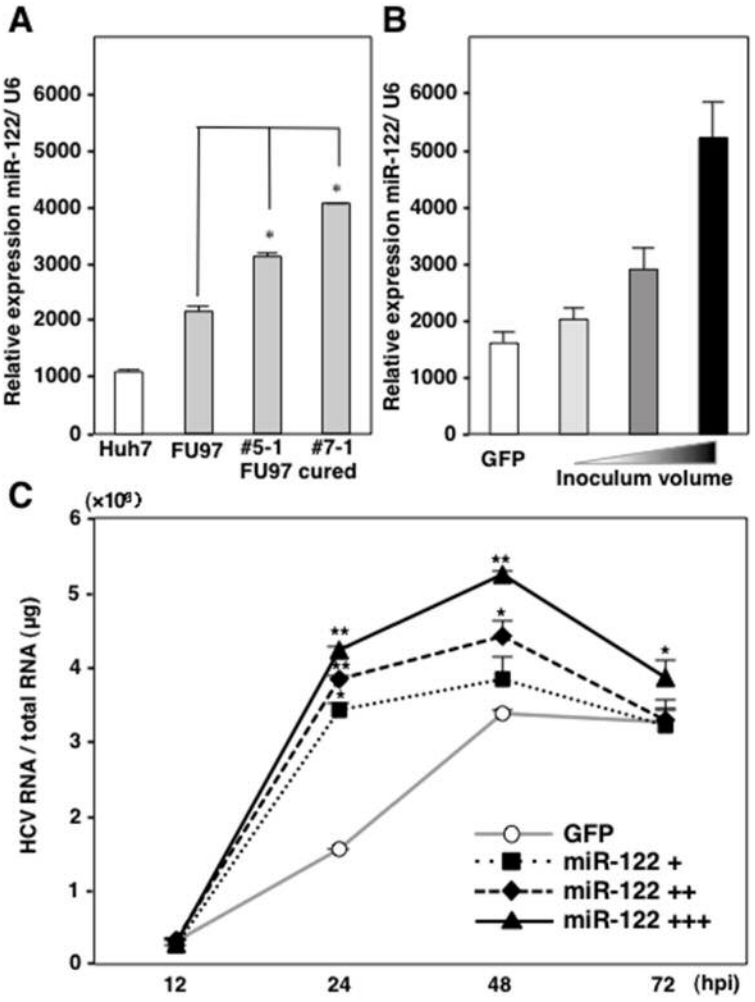
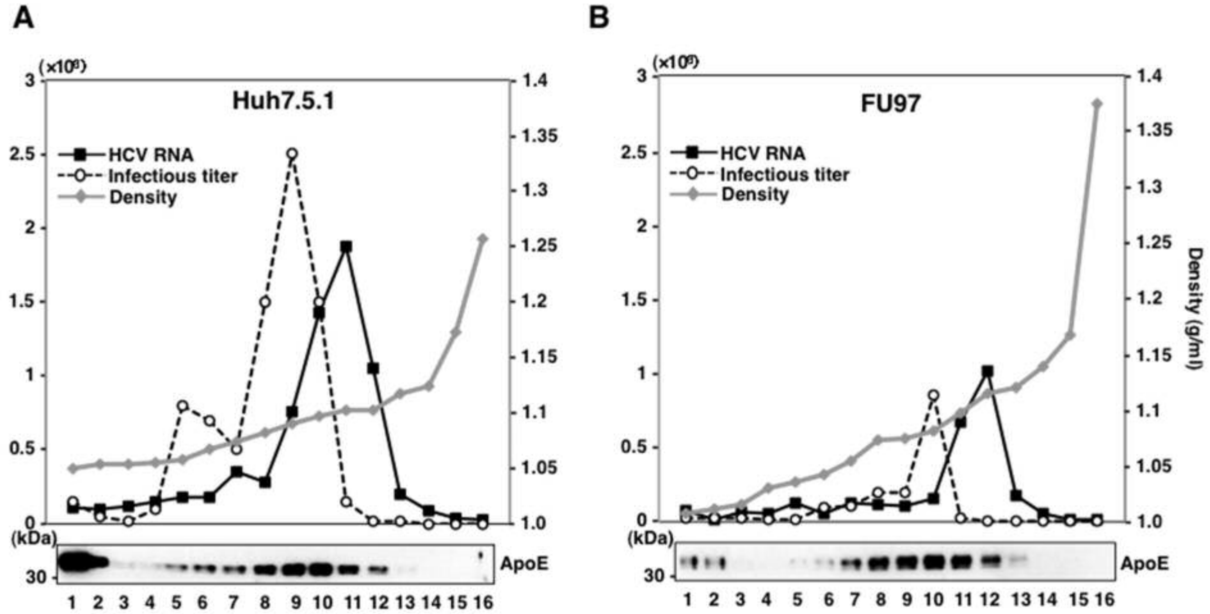

A
Relative expression miR-122/ U6
0
1000
2000
3000
4000
5000
6000
*
*
Huh7
FU97
#5-1
#7-1
FU97 cured
B
Relative expression miR-122/ U6
0
1000
2000
3000
4000
5000
6000
GFP
Inoculum volume
C
$(\times 10^{8})$
HCV RNA / total RNA (µg)
0
1
2
3
4
5
6
12
24
48
72
(hpi)
**
**
*
**
*
*
GFP
miR-122 +
miR-122 ++
miR-122 +++

Figure.7



A
B
Huh7.5.1
FU97
(×10³)
HCV RNA / total RNA (µg) and infectious titer (FFU/ml)
Density (g/ml)
HCV RNA
Infectious titer
Density
0
0.5
1
1.5
2
2.5
3
1.0
1.05
1.1
1.15
1.2
1.25
1.3
1.35
1.4
(kDa)
30
ApoE
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Figure.8

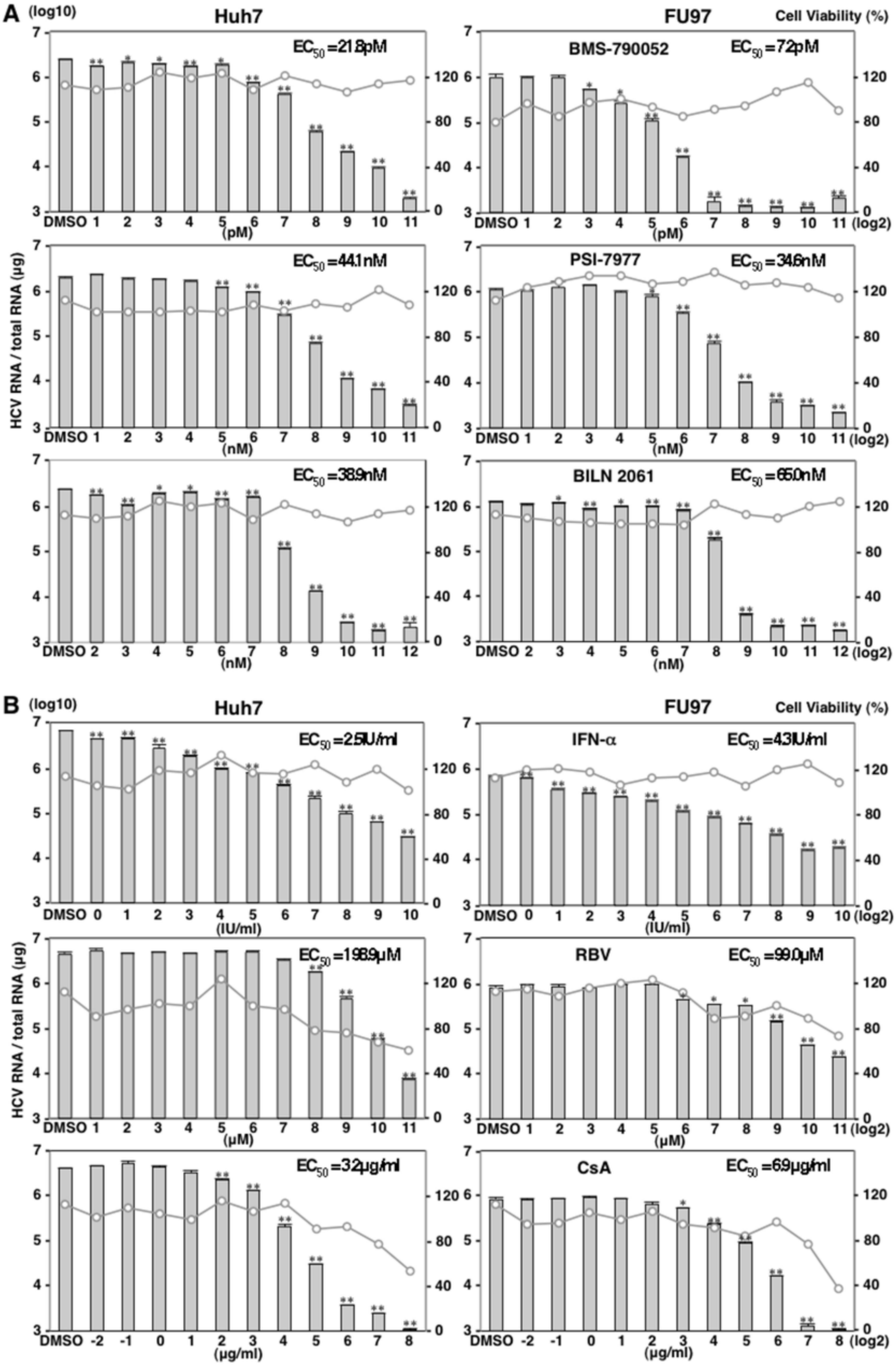

A
(log10)
Huh7
FU97
Cell Viability (%)
HCV RNA / total RNA (μg)
EC$_{50}$ =21.8pM
BMS-790052
EC$_{50}$ =7.2pM
DMSO 1 2 3 4 5 6 7 8 9 10 11
(pM)
(log2)
EC$_{50}$ =44.1nM
PSI-7977
EC$_{50}$ =34.6nM
(nM)
EC$_{50}$ =38.9nM
BILN 2061
EC$_{50}$ =65.0nM
DMSO 2 3 4 5 6 7 8 9 10 11 12
(nM)
B
(log10)
Huh7
FU97
Cell Viability (%)
HCV RNA / total RNA (μg)
EC$_{50}$ =2.5IU/ml
IFN-α
EC$_{50}$ =4.3IU/ml
DMSO 0 1 2 3 4 5 6 7 8 9 10
(IU/ml)
EC$_{50}$ =198.9μM
RBV
EC$_{50}$ =99.0μM
DMSO 1 2 3 4 5 6 7 8 9 10 11
(μM)
EC$_{50}$ =3.2μg/ml
CsA
EC$_{50}$ =6.9μg/ml
DMSO -2 -1 0 1 2 3 4 5 6 7 8
(μg/ml)
(log2)
7 6 5 4 3
0 40 80 120
*
**

Figure.9

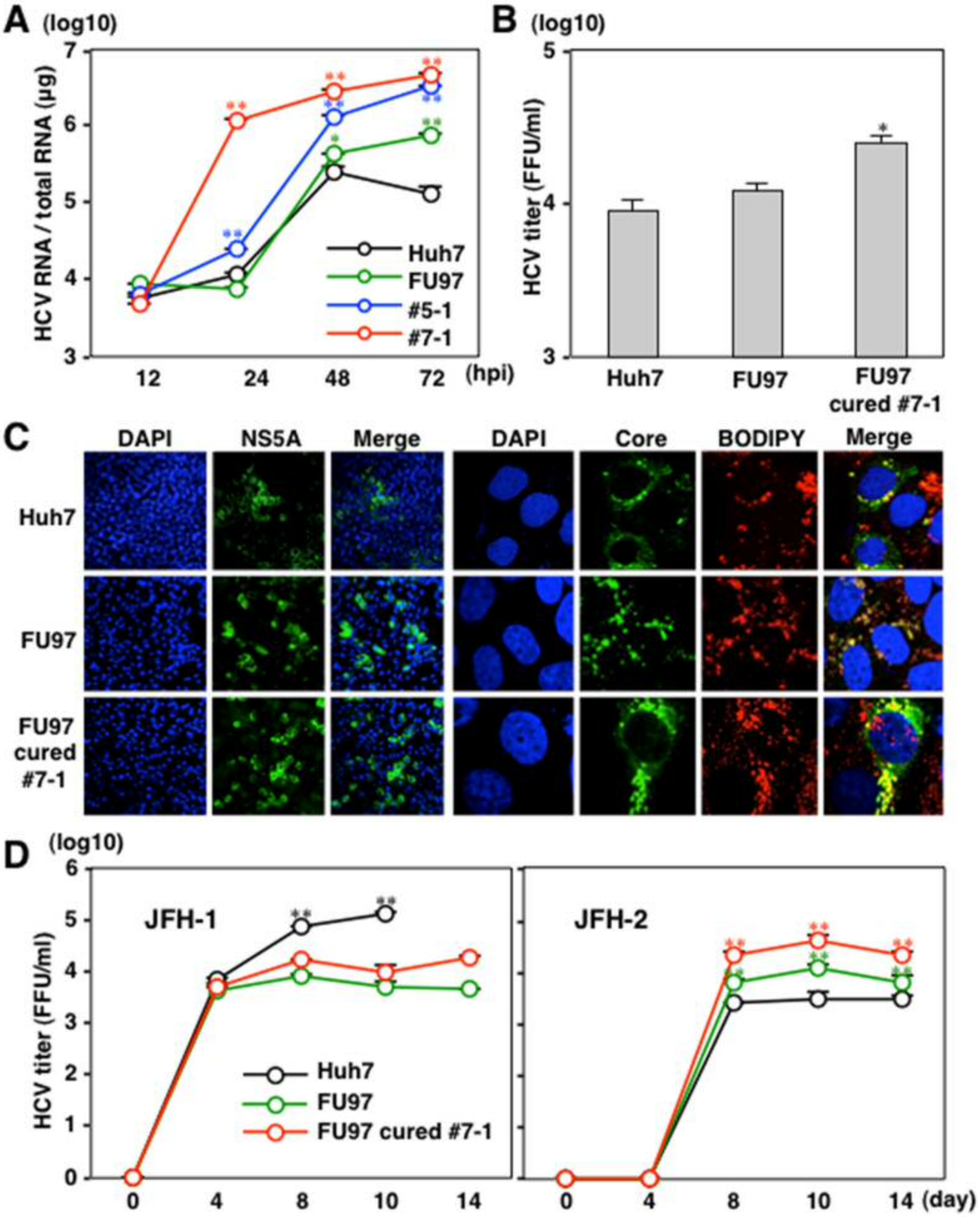

A
(log10)
HCV RNA / total RNA (µg)
7
6
5
4
3
12
24
48
72
(hpi)
Huh7
FU97
#5-1
#7-1
B
(log10)
HCV titer (FFU/ml)
5
4
3
Huh7
FU97
FU97 cured #7-1
C
DAPI
NS5A
Merge
DAPI
Core
BODIPY
Merge
Huh7
FU97
FU97 cured #7-1
D
(log10)
HCV titer (FFU/ml)
JFH-1
JFH-2
Huh7
FU97
FU97 cured #7-1
0
4
8
10
14
(day)

FIGURE LEGENDS

Figure 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, OV-90 cells identified by NextBio 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 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. Molecular weight 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 (gray bars) and control virus (white bars) were inoculated into the cell lines, and luciferase activities were determined at 24 h post-infection. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$) versus the results for control virus.

Figure 2. JHH-4 and FU97 cells permit HCV propagation.

(A) Intracellular HCV RNA levels in Huh7 (left), JHH-4 (middle) and FU97 (right) cells inoculated with HCVcc at an MOI of 1, treated with (broken lines)/without (solid lines) 100 IU/ml of IFN-α were determined by qRT-PCR at 12, 24, 48, and 72 h post-infection. 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 post-infection (right, bar graph). (B) Exogenous expression of miR-122 in Huh7, JHH-4 and FU97 cells by lentiviral vector (left, 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 (left), JHH-4 (middle) and FU97 (right) cells inoculated with HCVcc at an MOI of 1 was determined by qRT-PCR at 12, 24, 48, and 72 h post-infection. 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.

Figure 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 pre-incubated with anti-E2 antibody and inoculated into cells (solid line with triangles). Cells were pre-incubated with anti-hCD81 antibody (dashed line with diamonds) or isotype control antibody (solid line with circles) and then infected with HCVcc. (right panels) Cells were infected with HCVcc and treated with miR-122-LNA (30 nM, dashed line with diamonds) or Ctrl-LNA (30 nM, solid line with circles) at 6 h post-infection. (B) Huh7, JHH-4, and FU97 cells infected with HCVcc at an MOI of 1 were treated with DMSO (solid line with circles) or MTTP inhibitors, CP-346086 (5 μM, dashed line with diamonds) or BMS-200150 (10 μM, solid line with triangles) at 3 h post-infection. Intracellular HCV RNA in cells at 12, 24, 48, and 72 h post-infection 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 of CP-346086 (gray bars), 10 μM of BMS-200150 (black bars) or DMSO alone (white bars), at 3 h post-infection were determined at 72 h post-infection by a focus-forming assay (right panels). (C) (left two panels) mRNA and protein expression of ApoB and ApoE in Huh7, JHH-4 and FU97 cells at 48 h post-transfection with either siApoB, siApoE or siCtrl were determined by qRT-PCR and immunoblotting, respectively. (right two panels) Huh7, JHH-4 and FU97 cells were infected with HCVcc at an MOI of 1 at 6 h post-transfection with either siApoB (dashed line with diamonds), siApoE (solid line with triangles), or siCtrl (solid line with circles). Intracellular HCV RNA at 12, 24, 48, and 72 h post-infection and infectious titers in the culture supernatants at 72 h post-infection 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.

Figure 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 post-electroporation, respectively. Colonies were stained with crystal violet at 30 days post-selection. (B) Four clones derived from FU97-SGR cells (#5, #7, #9 and #11) were subjected to qRT-PCR after extraction of total RNA (upper, bar graph) 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 (#5 and #7) were treated with a combination of either 100 IU/ml of IFN-α and 100 nM of BILN 2061 (#5-1 and #7-1) or 10 pM of BMS-790052 and 100 nM of BILN 2061 (#5-2 and #7-2) to eliminate the HCV genome. Clone #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 post-treatment was determined by qRT-PCR. (E) The expressions of NS5A in FU97-SGR cells (clones #5 and #7) and in FU97 cured cells (clones #5-1 and #7-1) were determined by immunoblotting analysis using anti-NS5A antibody. (F) FU97 cured cells (blue line ; clone #5-1, red line ; clone #7-1) and parental cells (green line) were infected with HCVcc at an MOI of 1, the levels of intracellular HCV RNA at 12, 24, 48, and 72 h post-infection were determined by qRT-PCR. (G) The expression of NS5A in Huh7, Huh7.5.1, FU97, and cured FU97 clones #7-1 was determined by immunofluorescence analysis at 72 h post-infection by using anti-NS5A antibody. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$) versus the results for control cells.

Figure 5. Innate immune response in cured FU97 cells.

(A) Huh7, parental and cured FU97 cells (#7-1) were stimulated with 100 IU/ml of IFN-α (gray bars) or infected with VSV (black bars). The expression of mRNA of ISG15 at 4, 8, 12, and 24 h post-treatment was determined by qPCR and standardized by that of GAPDH. (B) Huh7 (white bars), parental FU97 (gray bars) and cured FU97 cells (#7-1, black bars) co-transfected with pIFNβ-Luc and pRL-SV40 were infected with VSV at an MOI of 1 at 24 h post-transfection (left). Cells co-transfected 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 post-transfection (right). Luciferase activities were determined at 24 h post-treatment. (C) Huh7 (left), parental FU97 (middle) and cured FU97 cells (#7-1, right) 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 post-treatment, and subjected to immunofluorescence assay using anti-IRF3 and -STAT2 antibodies. Cell nuclei were stained by DAPI.

Figure 6. Expression of miR-122 is one of the determinants for HCV RNA abundances.

(A) Total RNA was extracted from Huh7, parental and cured FU97 cells (clones #5-1 and #7-1) and the relative expression of miR-122 was determined by qRT-PCR. U6 snRNA was used as an internal control. (B) Establishment of FU97 cell lines stably expressing various concentration of miR-122 by the infection with lentiviral vector. FU97 cells infected with lentiviral vector to express GFP are used as control. (C) FU97 cell lines expressing various concentration 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 post-infection by qRT-PCR. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$) versus the results for control cells.

Figure 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 post-infection with HCVcc and analyzed by using iodixanol density gradient centrifugation. HCV RNA (solid line with squares) and infectious titers (broken line with open circles) of each fraction were determinant by qRT-PCR and focus-forming assay, respectively. Buoyant density (gray line) was plotted for each fraction (A and B, upper panels). Expression of ApoE in each fraction was detected by immunoblotting using anti-ApoE antibody (A and B, lower panels).

Figure 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 (left panels) and FU97 (right panels) cells. Cells infected with HCVcc at an MOI of 1 were treated with BMS-790052 (top), PSI-7977 (middle), and BILN 2061 (bottom) at 3 h post-infection. (B) Effect of HCV inhibitors targeting host factors on the propagation of HCVcc in Huh7 (left panels) and FU97 (right panels) cells. Cells infected with HCVcc at an MOI of 1 were treated with IFN-α (top), RBV (middle), and CsA (bottom) at 3 h post-infection. Intracellular HCV RNA level was determined by qRT-PCR at 48 h post-infection (bar graphs) and cell viability was determined as a percentage of the viability of cells treated with 0.1% DMSO at 48 h post-treatment (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.

Figure 9. Propagation of HCVcc/JFH-2 in FU97 cells.

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

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ACHIEVEMENTS

Publications

Involvement of ceramide in the propagation of Japanese encephalitis virus.

Hideki Tani, Mai Shiokawa, Yuuki Kaname, Hiroto Kambara, Yoshio Mori, Takayuki Abe, Kohji Moriishi, Yoshihatu Matsuura. Journal of Virology, 84(6), 2798-2807, 2010.

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Novel permissive cell lines for a complete propagation of hepatitis C virus.

Mai Shiokawa, Takasuke Fukuhara, Chikako Ono, Satomi Yamamoto, Toru Okamoto, Noriyuki Watanabe, Takaji Wakita, Yoshiharu Matsuura.（投稿中）

Meetings

不死化ヒト肝細胞株であるHc細胞に対する患者血清由来HCVの持続感染システム

塩川舞、福原崇介、後藤志典、二宮彰紀、谷英樹、阿部隆之、森石恆司、松浦善治、第58回日本ウイルス学会学術集会、2010年11月8日.

Involvement of human liver-specific factors in a complete propagation of hepatitis C virus.

塩川舞、福原崇介、小野慎子、岡本徹、松浦善治、第11回あわじしま感染症・免疫フォーラム、2012年9月12日.

C型肝炎ウイルスの粒子産生に関与するヒト肝臓特異因子の解析と新規感受性細胞株の樹立

塩川舞、福原崇介、小野慎子、山本聡美、岡本徹、松浦善治、第60回日本ウイルス学会学術集会、2012年11月4日.

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INVOLVEMENT OF HUMAN LIVER-SPECIFIC FACTORS IN A COMPLETE PROPAGATION OF HEPATITIS C VIRUS.

Mai Shiokawa, Takasuke Fukuhara, Chikako Ono, Toru Okamoto, Yoshiharu Matsuura. 32nd Annual Meeting American Society for Virology, 22, 7, 2013.

C型肝炎ウイルスに感受性を示す新しい細胞株の性状

塩川舞、福原崇介、小野慎子、山本聡美、岡本徹、松浦善治、第61回日本ウイルス学会学術集会、2013年11月10日．

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