

FIG 14 Innate immune responses in parental and cured Hec1B/miR-122 cells. Parental and cured Hec1B/miR-122 cells were stimulated with 100 U of IFN- α (A) or VSV (B). The expression levels of IFN-stimulated gene 15 (ISG15) were determined by qRT-PCR at 2, 12, and 24 h posttreatment.

ability of miR-122 to promote the growth of a laboratory strain of HCV (HJ3-5) is dependent upon its direct interaction with both seed sequence-binding sites in the 5' UTR and that the binding to site 1 is more important for efficient replication than the binding to site 2 (25). Recently, it was shown that the binding of miR-122 to the 5' UTR of the HCV genome masks the 5'-terminal sequences of the viral genome through the 3' overhanging nucleotides of miR-122 (36). It is necessary to evaluate the importance of this enhancement mechanism on mutant HCVcc infection in Hec1B cells.

In summary, we demonstrated that HCV is capable of replicating at a low level in nonhepatic cells and that exogenous expression of miR-122 facilitates efficient viral replication but not the production of infectious particles, probably due to the lack of hepatocytic lipid metabolism in nonhepatic cell lines. These results suggest that miR-122 plays a crucial role in determination of the cell tropism of HCV and the possible involvement of incomplete propagation of HCV in the development of EHM in hepatitis C patients.

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CD44 Participates in IP-10 Induction in Cells in Which Hepatitis C Virus RNA Is Replicating, through an Interaction with Toll-Like Receptor 2 and Hyaluronan

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The mechanisms of induction of liver injury during chronic infection with hepatitis C virus (HCV) are not well understood. Gamma interferon (IFN- γ)-inducible protein 10 (IP-10), a member of the CXC chemokine family, is expressed in the liver of chronic hepatitis C (CHC) patients and selectively recruits activated T cells to the sites of inflammation. Recently, it was shown that a low plasma concentration of IP-10 in CHC patients was closely associated with the outcome of antiviral therapy. In this study, we examined the role of the Toll-like receptor (TLR) pathway on IP-10 production in cells replicating HCV. Among the CXC chemokines, the expression of IP-10 was specifically increased in cells replicating HCV upon stimulation with conventional TLR2 ligands. The enhancement of IP-10 production upon stimulation with TLR2 ligands in cells replicating HCV induced CD44 expression. CD44 is a broadly distributed type I transmembrane glycoprotein and a receptor for the glycosaminoglycan hyaluronan (HA). In CHC patients, the expression of HA in serum has been shown to increase in accord with the progression of liver fibrosis, and HA also works as a ligand for TLR2. In the present study, IP-10 production upon HA stimulation was dependent on the expression of TLR2 and CD44, and a direct association between TLR2 and CD44 was observed. These results suggest that endogenous expression of HA in hepatocytes in CHC patients participates in IP-10 production through an engagement of TLR2 and CD44.

Hepatitis C virus (HCV) infects 170 million people worldwide and frequently leads to the development of cirrhosis and hepatocellular carcinoma (32). The current combination therapy of pegylated interferon (IFN) and ribavirin is effective in fewer than 50% of patients infected with HCV of genotype 1. Histological analyses of the liver biopsy specimens of chronic hepatitis C (CHC) patients have revealed the infiltration of mononuclear cells, including T and B lymphocytes, natural killer (NK) and NKT cells, and virus-specific cytotoxic T lymphocytes (2, 26, 42, 47). Long-term infection by HCV is associated with progressive infiltration of the liver parenchyma by the mononuclear cells, fibrosis, cirrhosis, and, finally, the development of hepatocellular carcinoma. Although the factors that regulate the recruitment of mononuclear cells and the other components of the inflammatory response to the HCV-infected liver cells are not well characterized, it has been hypothesized that chemokines and other inflammatory cytokines play fundamental roles in the immune cell recruitment.

Chemokines, small chemotactic cytokines (approximately 8 to 10 kDa) that act to guide leukocytes to sites of inflammation, are important determinants of the development of intrahepatic inflammation in chronic HCV infection (16). Although chemokines play crucial roles in viral elimination, persistent expression of chemokines may induce tissue damage and inflammation in chronic infection. CXCR3 is a receptor for the CXC chemokines, including IP-10 (also known as CXCL10), MIG (also known as CXCL9), and I-TAC (also known as CXCL11). Recent studies have shown that the CXCR3 ligands are elevated in the livers and sera of CHC patients (12–14, 17, 33, 36, 40, 49), and IP-10 was shown to correlate with treatment response. In addition, several studies suggested a significant association between the expression

of the CXC chemokines and the development of progressive liver injury in CHC patients (23, 49). In CHC patients, these chemokines are expressed in hepatocytes, hepatic stellate cells, and sinusoidal endothelial cells (12, 14, 33, 42, 49), and the majority of intrahepatic mononuclear cells express CXCR3, suggesting that the CXC chemokine network plays a pivotal role in the migration of mononuclear cells to the liver and in the subsequent intrahepatic inflammation.

Among chemokines, IP-10 plays a central role in liver inflammation, and it is expressed in the liver of hepatitis C patients (12, 33, 42). Several independent studies indicate that elevated plasma levels of IP-10 predict the failure of combination therapy (3, 5, 40). In addition, a recent study suggests that IP-10 in the plasma of many hepatitis C patients is cleaved by DPP4 (also known as CD26) and that the truncated IP-10 works as an IP-10 receptor antagonist (4). In contrast to these clinical observations, little is known about the expression of the CXC chemokines in cells replicating HCV.

Production of the inflammatory chemokines upon viral infec-

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tion is also crucial for the induction of innate immunity through the activation of pattern recognition receptors (PRR), including Toll-like receptors (TLR) and RIG-I-like receptors (RLR) (44). TLR play an important role in the recognition of a wide variety of pathogens and their components, while RLR, including RIG-I and MDA5, sensitize cells in response to double-stranded RNA (dsRNA) generated by viral infection or to poly(I·C). Various pro-inflammatory cytokines and chemokines are activated by the PRR through the translocation of transcription factors, such as IFN regulatory factor (IRF) and NF- κ B, into the nucleus and binding to their cognate promoter elements together with other transcription factors.

In this study, we have examined the role of the TLR pathway on the production of the CXC chemokines in human liver cell lines replicating HCV RNA. Among the CXC chemokines, IP-10 production was specifically enhanced in cells replicating HCV upon stimulation with conventional TLR2 ligands. Moreover, we identified CD44, a receptor for the glycosaminoglycan hyaluronan (HA) (38), as a molecule involved in IP-10 production in the HCV-replicating cells. The cell surface expression of CD44 was also upregulated in the cells harboring HCV replicons of genotypes 1b and 2a, and IP-10 production was enhanced in cells replicating HCV upon stimulation with HA. Importantly, HA also works as a ligand for TLR2 (41), and HA expression in serum is increased in CHC patients in accord with the progression of liver fibrosis (9, 30, 35, 45, 48). These results suggest that the production of IP-10 is enhanced by endogenous HA in hepatocytes in CHC patients through an interaction with TLR2 and CD44.

MATERIALS AND METHODS

Cells and viruses. Huh7OK1 cells that exhibit high susceptibility to infectious cell culture-adapted HCV clone (HCVcc) propagation (34), Huh7 cells, and 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (FCS). Huh7 cells harboring an HCV subgenomic RNA replicon of genotype 1b (Con1 strain) (28) or 2a (JFH1 strain) were cultured in DMEM supplemented with 10% FCS, 1 mg/ml G418 (Nacalai Tesque, Kyoto, Japan), and nonessential amino acids. Human normal hepatocytes and human liver sinusoidal endothelial cells were purchased from TaKaRa Bio, Inc. (Shiga, Japan) and maintained in primary hepatocytes and endothelial selective medium (TaKaRa Bio), respectively. Huh7 cells harboring a Japanese encephalitis virus (JEV) subgenomic RNA replicon (Nakayama strain) were cultured in DMEM supplemented with 10% FCS and 1 μ g/ml puromycin (InvivoGen, San Diego, CA). The cells were cultured at 37°C in a humidified atmosphere with 5% CO₂. The infectious RNA of the JFH1 strain was introduced into Huh7OK1 cells, and the infectious titers were expressed as focus-forming units (FFU) (46).

Plasmids and reagents. The cDNA fragment encoding CD44 was kindly provided by U. Günthert (University of Basel, Switzerland) (31) and subcloned into pcDNA3.1-C-myc-His (Invitrogen, Carlsbad, CA). The N-terminal or C-terminal deletion mutant of CD44 was amplified by PCR using *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA) and subcloned into pcDNA3.1-C-myc-His. The cDNA fragment encoding the full-length human TLR2 was amplified by reverse transcription (RT)-PCR from total RNA of THP-1 cells and subcloned into pFlagCMV-1 (Sigma). The C-terminal deletion mutant of TLR2 was amplified by PCR using *Pfu* Turbo DNA polymerase (Stratagene) and subcloned into pFlagCMV-1. All PCR products were confirmed by sequencing with an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Tokyo, Japan). Lipopolysaccharide (LPS) derived from *Salmonella enterica* subsp. *enterica* serovar Minnesota (Re-595) and peptidoglycans (PGN) derived from *Staphylococcus aureus* were purchased from Sigma. FSL-1 (a synthetic lipopeptide derived from *Mycoplasma salivarium*), Pam2CSK

(a tripalmitoylated lipopeptide), Pam3CSK (a tripalmitoylated lipopeptide), phosphorothioate-stabilized human CpG (hCpG) oligodeoxynucleotides (ODN 2006) (TCG-TCG-TTT-TGT-CGT-TTT-GTC-GTT), R-837, and poly(I·C) were purchased from InvivoGen. Purified hyaluronan from human umbilical cords was purchased from Calbiochem (Darmstadt, Germany). The recombinant human alpha interferon (IFN- α) and IFN- γ were purchased from PBL Biomedical Laboratories (New Brunswick, NJ). The Quantikine ELISA (enzyme-linked immunosorbent assay) human CXCL10/IP-10 immunoassay was purchased from R&D Systems, Inc. (Minneapolis, MN). The HCV NS3/-4A protease inhibitor BILN2061 was purchased from Acme Bioscience (Belmont, CA). Lack of contamination of endotoxin (<0.01 endotoxin units/ml) in the reagents, including virus stocks, recombinant proteins, and all ligands, was confirmed by using a Pyrodict endotoxin measure kit (Seikagaku Co., Tokyo, Japan).

Binding of recombinant CD44s to TLR2 or ligands. To generate a C-terminal deletion mutant of TLR2 (comprised of amino acid residues from 1 to 587) or CD44 (comprised of amino acid residues from 1 to 223), each of the cDNAs encoding N-terminally FLAG-tagged TLR2 (TLR2 Δ TM) or C-terminally His-tagged CD44 (CD44 Δ TM) were subcloned into pFastBac (Invitrogen), and recombinant baculoviruses possessing the cDNA were produced by using a Bac-to-Bac baculovirus expression system according to the manufacturer's instructions (Invitrogen). At 3 days after infection, TLR2 Δ TM and CD44 Δ TM proteins were purified from the culture supernatants and cell lysates by using column chromatography with nickel-nitrilotriacetic acid beads (Qiagen, Valencia, CA) and anti-FLAG M2 affinity gel (Sigma) according to the respective manufacturer's instructions. The protein concentrations were determined by using a Micro BCA (bicinchoninic acid) protein assay kit (Pierce, Rockford, IL). One hundred microliters of TLR2 Δ TM (20 μ g/ml), HA (50 μ g/ml), PGN (40 μ g/ml), LPS (40 μ g/ml), or bovine serum albumin (BSA) (50 μ g/ml) was added to a 96-well microtiter plate (Nunc Maxisorp P/N; Nalge Nunc International, Rochester, NY) and incubated at 4°C overnight. Nonspecific binding was blocked by incubating with phosphate-buffered saline (PBS) containing 2% FCS (PBSF) at room temperature for 1 h, followed by washing with PBS containing 0.02% Tween 20 (PBST) and incubation with various concentrations of CD44 Δ TM in the PBSF at room temperature for 1 h. The wells were washed with PBST and incubated for 1 h with an antihexahistidine monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, and then supplemented with *o*-phenylenediamine after washing with PBST. The binding of the CD44 Δ TM to TLR2 Δ TM, PGN, LPS, or BSA was determined by measuring the absorbance at 450 nm.

Immunoprecipitation and immunoblotting. Cells were transfected with the plasmids by the lipofection method, harvested at 48 h posttransfection, washed three times with ice-cold PBS, and suspended in 0.4 ml lysis buffer containing 20 mM Tris-HCl (pH 7.4), 135 mM NaCl, 1% Triton X-100, 1% glycerol, and protease inhibitor cocktail tablets (Roche Molecular Biochemicals, Mannheim, Germany). Cell lysates were incubated for 30 min at 4°C and centrifuged at 14,000 \times g for 15 min at 4°C. The supernatant was immunoprecipitated with mouse monoclonal anti-FLAG M2 and protein G-Sepharose 4B fast flow beads (Amersham Pharmacia Biotech, Franklin Lakes, NJ), and the precipitates were washed with Tris-buffered saline containing 20 mM Tris-HCl (pH 7.4), 135 mM NaCl, and 0.05% Tween 20 (TBST). The proteins bound to the beads were boiled in 20 μ l of sample buffer, subjected to sodium dodecyl sulfate-12.5% polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene difluoride membranes (Millipore, Tokyo, Japan). These membranes were blocked with TBST containing 5% skim milk and incubated with mouse monoclonal anti-FLAG M2 or anti-hexahistidine monoclonal antibody (Santa Cruz) at room temperature for 1 h and then with horseradish peroxidase-conjugated anti-mouse IgG antibody at room temperature for 1 h. The recombinant proteins were analyzed by SDS-12.5% PAGE under reducing conditions, stained with GelCord blue

stain reagent (Pierce), and detected by immunoblot analysis using anti-hexahistidine monoclonal antibody (Santa Cruz), anti-FLAG M2 monoclonal antibody, anti-human CD44 monoclonal antibody (clone 3C-11; Cell Signaling Technology, Inc., Beverly, MA), or anti-human TLR2 polyclonal antibody (clone TLR2.1; Santa Cruz). The stable knockdown clones (1×10^5 cells/well) were stimulated with FSL-1 ($1 \mu\text{g/ml}$), and degradation of $\text{I}\kappa\text{B}\alpha$ and expression of CD44 and β -actin were determined by immunoblotting using antibodies specific to $\text{I}\kappa\text{B}\alpha$ (Cell Signaling), CD44 (clone 3C-11), and β -actin (Sigma). The immune complexes and cell lysates were visualized with Super Signal West femto substrate (Pierce) and detected by using an LAS-3000 image analyzer system (Fujifilm, Tokyo, Japan).

DNA microarray analysis. Total RNA was extracted from Huh7 and HCV subgenomic replicon-harboring cells stimulated or not with FSL-1 ($1 \mu\text{g/ml}$) for 24 h by using an RNeasy minikit (Qiagen) and purified by using a QuickPrep mRNA purification kit (GE Healthcare Life Science, Little Chalfont, United Kingdom). Differentially expressed genes were screened with the use of a DNA microarray system, the human genome U133 plus 2.0 (Kurabo, Osaka, Japan). Data were analyzed by using the GeneChip operating software version 1.4 (Affymetrix 690036) with Microarray Suite version 5.0 (MAS 5.0). Differentially expressed genes were extracted using DNA microarray viewer (Kurabo), and then hierarchical clustering was performed by using Avadis 4.3 prophetic software (Strand Life Sciences, Bangalore, India).

Stable knockdown cell clones. The short interfering RNA (siRNA) sequences of the sense strands targeted to human CD44 (5'-GGAAAUGGUGCAUUUGGUGdTdT-3'), human TLR2 (5'-GCCUUGACCUGUCAACAAdTdT-3'), and human MyD88 (5'-GGAGGAUUGCCAAAAGUAUdTdT-3') were designed by using pSilencer Expression Vectors Insert Design Tool (Ambion, Austin, TX) and were introduced into the BamHI and HindIII sites of pSilencer 2.1 U6 Puro vector (Ambion). To establish stable knockdown cell clones, cells were transfected with the plasmids and drug-resistant clones were selected by treatment with puromycin (InvivoGen) at a final concentration of $1 \mu\text{g/ml}$.

Real-time PCR. Total RNA was prepared from cells by using an RNeasy minikit (Qiagen), and the first-strand cDNA was synthesized by using a ReverTra Ace (TOYOBO, Osaka, Japan) and oligo(dT)₂₀ primer. Each cDNA was determined by using Platinum SYBR green quantitative PCR (qPCR) SuperMix UDG (Invitrogen) according to the manufacturer's protocol. Fluorescent signals were analyzed with an ABI PRISM 7000 (Applied Biosystems). The human IP-10, MIG, I-TAC, interleukin-8 (IL-8), HCV internal ribosome entry site (IRES), CD44, TLR1, TLR2, TLR4, TLR6, TLR7, TLR9, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes were amplified using the primer pairs 5'-GGCCATCAAGAATTTACTGAAAGCA-3' and 5'-TCTGTGTGGTCCATCCTTGGAA-3', 5'-TTAAATCTGGCCACAGACAACTC-3' and 5'-GCAGCCAAGTCGGTTAGTGGA-3', 5'-CTTTCATGTTACGACATTTCTACTCC-3' and 5'-CCTATGCAAAGACAGCGTCCCTC-3', 5'-CCCAAGGACGGAGACTTCGAT-3' and 5'-GAAACTTGCTGTGGGTGACCAT-3', 5'-GAGTGTCTGTCAGCCTCCA-3' and 5'-CAC TCGCAAGCACCTATCA-3', 5'-AACCTTGCAACATTGCCTGA-3' and 5'-GCTTCCAGAGTTACGCCCTTGA-3', 5'-GGAGCAATGCTGCTGTTC-3' and 5'-GCCAATATGCCTTTGTTATCCTG-3', 5'-GAAAGCTCCAGCAGGAACATC-3' and 5'-GAATGAAGTCCCCTTATGAAGACA-3', 5'-AGGATGATGCCAGGATGATGTC-3' and 5'-TCAGGTCCAGGTCTTGGTTAG-3', 5'-CCTGGCAAGAGCATTGTGGA-3' and 5'-TCGTAATGGCACTCACTCTG-3', 5'-TCTTCAACCAGACCTCTACATCCA-3' and 5'-GGAACATCCAGAGTGACATCACAG-3', 5'-GGGACCTCGAGTGTGAAGCA-3' and 5'-CTGGAGCTCACAGGGTAGGAA-3', and 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCTGTTGCTGTA-3', respectively. The expression of the mRNA of each gene was normalized to that of GAPDH.

Reporter assay. Cells seeded onto 12-well plates at a concentration of 1.5×10^5 cells/well were transfected with 100 ng of each of the plasmids encoding the luciferase gene under the control of the IP-10, endothelial-

leukocyte adhesion molecule (ELAM), and IFN-stimulated response element (ISRE) promoters and stimulated with 500 $\mu\text{g/ml}$ of HA or 250 ng/ml of IFN- α at 24 h posttransfection. Luciferase activity was determined with the dual-luciferase reporter assay system (Promega, Inc., Madison, WI) and the *Renilla* luciferase reporter gene was simultaneously transfected as an internal control.

Gene silencing by siRNA. siRNAs targeted to the endogenous human CD44 (5'-GGAAAUGGUGCAUUUGGUGdTdT-3') and negative control no. 1, which exhibits no downregulation of any human genes, were purchased from Ambion. HCV replicon-harboring cells were transfected with 100 nM siRNA by using siFactor (B-Bridge International, Sunnyvale, CA) according to the manufacturer's protocol.

Flow cytometry. The cell surface expression of CD44 and CD81 on Huh7 cells, cells harboring subgenomic replicons of JEV and HCV of genotypes 1b (Con1 strain) and 2a (JFH1 strain), and Huh7OK1 cells infected with HCVcc (JFH1) was analyzed by flow cytometry (Becton Dickinson, Mountain View, CA). Cells were washed twice in PBSF and incubated with mouse monoclonal antibodies to human CD44 (clone BU52; Ancell, Inc., Bayport, MN) and human CD81 (BD Biosciences). Mouse IgG1 and IgG2b (BD Biosciences) were used as isotype controls. Cells were washed with PBSF and incubated with the phycoerythrin (PE)-conjugated anti-mouse IgG monoclonal antibody.

Statistical analysis. Results were expressed as means \pm standard deviations. The significance of differences in the means was determined by Student's *t* test.

RESULTS

Expression of IP-10 is specifically increased in cells replicating HCV upon stimulation with conventional TLR2 ligands. To examine the involvement of TLR signaling pathways in IP-10 production in cells replicating HCV, we determined the level of IP-10 mRNA in the HCV replicon-harboring and Huh7 cells upon stimulation with various TLR ligands. Among the TLR ligands we examined, PGN derived from bacterial components induced a significant enhancement of IP-10 production in the replicon-harboring cells but not in Huh7 cells (Fig. 1A). A dose-dependent induction of IP-10 was observed in the replicon-harboring cells treated with PGN (Fig. 1B). The enhancement of IP-10 production was also observed in the replicon-harboring cells in response to other TLR2 ligands, including FSL-1, Pam2CSK, and Pam3CSK, which are responsive to each of the heterodimers of TLR2/-6 and TLR1/-6, in contrast to the inhibition of IP-10 production in the replicon-harboring cells upon stimulation with poly(I-C) (Fig. 1C), which was probably due to interference in the dsRNA-dependent signaling pathway by NS3/-4A protease as described previously (25). The expression of TLR2 was confirmed not only in Huh7 and HCV replicon-harboring cells but also in primary human normal hepatocytes and human liver sinusoidal endothelial cells (Fig. 1D). To further examine the expression of each of the CXC chemokines in the HCV replicon-harboring cells, the level of mRNA of the CXC chemokines was determined by real-time PCR upon stimulation with PGN or IFN- γ . Although treatment with IFN- γ suppressed the replication of HCV and induced the expression of IP-10, MIG, and I-TAC in the replicon-harboring cells, stimulation with PGN induced the expression of IP-10 but not of MIG and I-TAC in the replicon-harboring cells (Fig. 1E).

We next examined the effect of HCV infection on the expression of CXC chemokines in cells upon stimulation with PGN. Huh7OK1 cells were established by the elimination of HCV RNA from the replicon-harboring cells by treatment with IFN (34). Huh7OK1 cells are highly permissive to HCVcc (JFH1 strain) in-

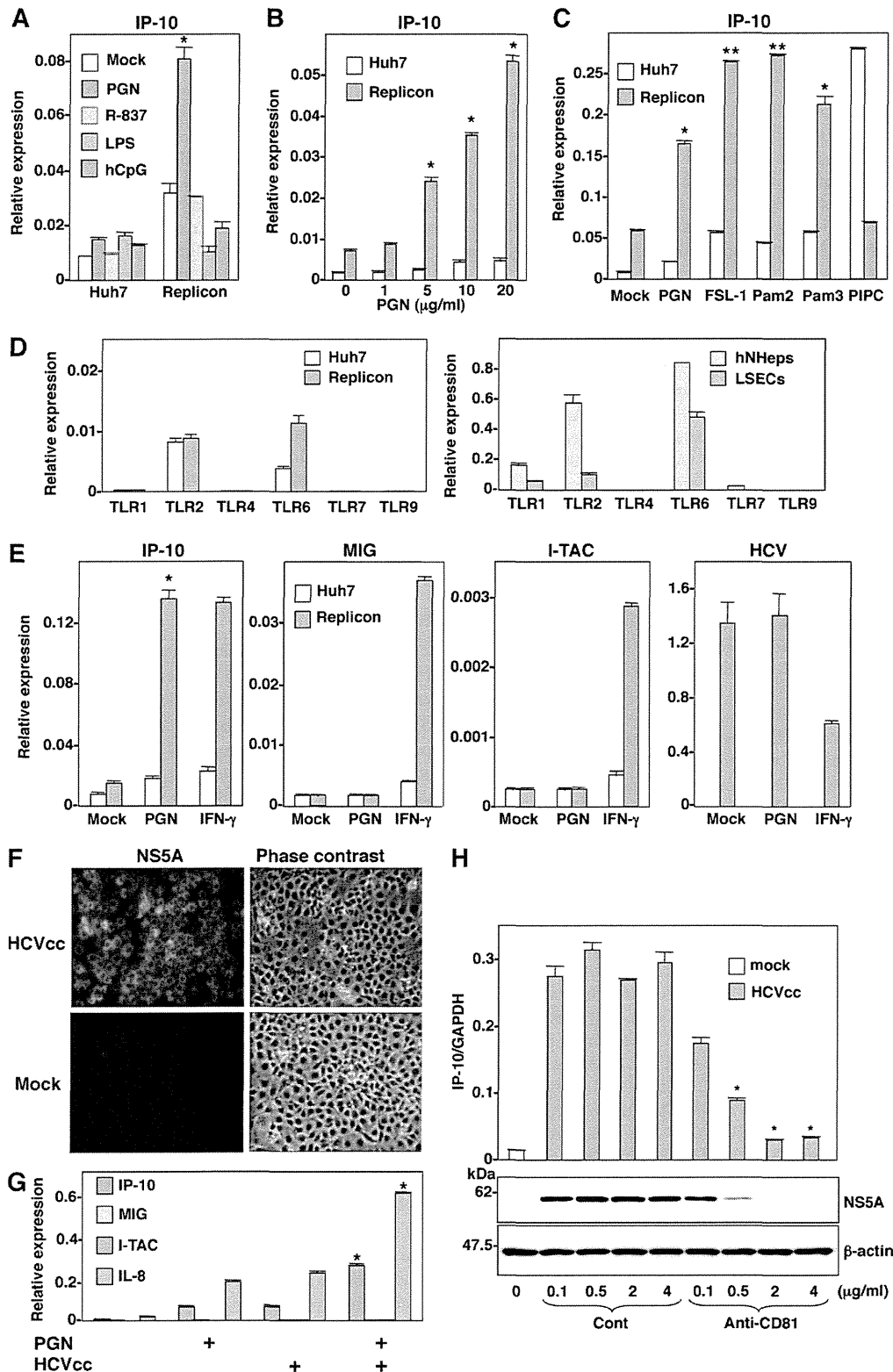


FIG 1 Enhancement of IP-10 production in the HCV-replicating cells upon stimulation with TLR2 ligands. (A) Huh7 and HCV replicon-harboring cells were stimulated with 20 $\mu\text{g/ml}$ of PGN, 10 $\mu\text{g/ml}$ of R-837, 20 $\mu\text{g/ml}$ of LPS, or 10 $\mu\text{g/ml}$ of hCpG, and the levels of IP-10 mRNA were determined by real-time PCR at 24 h after stimulation. (B) Huh7 and HCV replicon-harboring cells were stimulated with various concentrations of PGN, and the levels of IP-10 mRNA were determined at 24 h after stimulation. (C) Huh7 and HCV replicon-harboring cells were stimulated with 20 $\mu\text{g/ml}$ of PGN, 1 $\mu\text{g/ml}$ of FSL-1, 1 $\mu\text{g/ml}$ of Pam2CSK (Pam2), 1 $\mu\text{g/ml}$ of Pam3CSK (Pam3), or 50 $\mu\text{g/ml}$ of poly(I:C) (PIPC), and the level of IP-10 mRNA was determined at 24 h after stimulation. (D) Total RNA was extracted from Huh7 cells, HCV replicon-harboring cells, primary human normal hepatocytes (hNHeps), and human liver sinusoidal endothelial cells (LSECs) cells, and expression levels of

fection, and most of the cells were infected within 7 days postinfection (Fig. 1F). Expression of IP-10 was induced in Huh7OK1 cells upon either stimulation with PGN or infection with HCVcc and was synergistically increased by costimulation (Fig. 1G). IL-8 is an inflammatory chemokine involved in HCV pathogenesis and a marker of the prognosis of CHC patients treated with IFN (37). In addition to IP-10, the production of IL-8 was also enhanced in Huh7OK1 cells infected with HCVcc upon stimulation with PGN (Fig. 1G). IP-10 production in Huh7OK1 cells upon infection with HCVcc was inhibited by pretreatment of cells with an anti-CD81 antibody in a dose-dependent manner in accord with the suppression of NS5A expression (Fig. 1H). These results indicate that the expression of IP-10 is specifically increased in cells replicating HCV upon stimulation with conventional TLR2 ligands.

Expression of CD44 is enhanced in cells replicating HCV and is associated with an increase of IP-10 production in response to TLR2 ligands. To determine the mechanism of enhancement of IP-10 production in cells replicating HCV upon stimulation with TLR2 ligands, the gene expression profiles in Huh7 and the replicon-harboring cells at 24 h after stimulation with a TLR2 ligand (FSL-1) were assessed by DNA microarray analysis. A variety of TLR2 ligand-inducible genes were regulated in the replicon-harboring cells upon FSL-1 stimulation (Fig. 2A). Among them, we focused on CD44 as a candidate molecule for participation in the enhancement of IP-10 production due to the significant enhancement of this molecule in the HCV replicon-harboring cells. CD44 is an adhesion molecule, a broadly distributed type I transmembrane glycoprotein, and a receptor for the glycosaminoglycan hyaluronan (HA). It has been shown that CD44 plays an important role in a variety of immunologic functions, including the adhesion, differentiation, homing, and activation of leukocytes and T cell extravasation to sites of inflammation (38). To confirm the data from the DNA microarray analysis, we examined the expression of CD44 in HCV replicon-harboring cells stimulated with TLR2 ligands. The expression of CD44 mRNA was upregulated in the replicon-harboring cells but not in Huh7 cells and was further enhanced by stimulation with FSL-1 or Pam3CSK in accord with the increase of IP-10 expression (Fig. 2B). Immunoblot analysis confirmed the enhancement of endogenous CD44 expression in the replicon-harboring cells (Fig. 2C). Furthermore, the cell surface expression of CD44 was also upregulated in the HCV replicon-harboring cells of genotypes 1b (Con1 strain) and 2a (JFH1 strain) but not in Huh7 and JEV subgenomic replicon-harboring cells (Fig. 2D), consistent with the enhancement of CD44 expression upon stimulation with FSL-1 (Fig. 2E). Furthermore, the expression of CD44 in the replicon-harboring cells was reduced by treatment with an HCV protease inhibitor (BILN2061), suggesting that enhancement of CD44 is dependent on the replication of HCV (Fig. 2F). These results suggest that the expression of CD44 is enhanced in HCV replicon-harboring cells autonomously rep-

licating the HCV genome and is associated with an increase of IP-10 production in response to TLR2 ligands.

CD44 does not participate in the IP-10 production upon stimulation with TLR2 ligands derived from bacterial components. To further examine the involvement of CD44 in IP-10 production through the TLR2 signaling pathway, we assessed the effect of knockdown of CD44 expression on the IP-10 production in HCV replicon-harboring cells. Although transduction of small interfering RNA (siRNA) targeted to an ectodomain of CD44 which is well conserved among the CD44 variant isoforms suppressed the expression of CD44, IP-10 production upon stimulation with FSL-1 in the replicon-harboring cells exhibited no reduction (Fig. 3A). Similarly, the stable knockdown of CD44 in the HCV replicon-harboring cells did not show a reduction of IP-10 production upon stimulation with FSL-1 (Fig. 3B), suggesting that CD44 expression is not involved in IP-10 production upon stimulation with the conventional TLR2 ligands derived from bacterial components. Furthermore, the degradation of I κ B α upon stimulation with FSL-1 was not affected by the stable knockdown of CD44 in the replicon-harboring cells (Fig. 3C). These results suggest that CD44 is not involved in the immune activation by stimulation with TLR2 ligands derived from bacterial components in cells replicating HCV RNA.

HA participates in IP-10 production in cells replicating HCV. Previously, it has been shown that a low-molecular-weight HA derived from the human umbilical cord acts as a TLR2 ligand in primary murine macrophage cells (41). Serum HA is derived from lymphocytes, fibroblasts, and hepatic stellate cells in the liver, and elevation of serum HA is an indicator for hepatic fibrosis and dysfunction of sinusoidal endothelial cells, because most HA is degraded in the hepatic sinusoidal endothelial cells (22). To determine the role of HA in IP-10 production in human hepatoma cell lines, we established Huh7OK1 cell lines stably expressing siRNA targeted to CD44, TLR2, or MyD88. The IP-10 production upon stimulation with HA was severely impaired by the knockdown of either gene (Fig. 4), suggesting that IP-10 production upon stimulation with HA is totally dependent upon the CD44-TLR2-MyD88 axis.

The production of IP-10 in the replicon-harboring cells was enhanced in both mRNA and protein levels by stimulation with HA (Fig. 5A). In addition to IP-10, the HCV replicon-harboring cells induced the expression of IL-8 but not of other CXCR3 ligands, including MIG and I-TAC, upon stimulation with HA (Fig. 5B). The enhancement of IP-10 production by stimulation with HA was also observed in the HCV replicon-harboring cells derived from other genotypes but not in the parental Huh7 cells and JEV replicon-harboring cells (Fig. 5C). The stable knockdown of CD44 in the HCV replicon-harboring cells significantly suppressed IP-10 production upon stimulation with HA but not with IFN- α and IFN- γ (Fig. 5D). The promoter region of IP-10 in-

TLR mRNA were determined by real-time PCR. (E) Huh7 and HCV replicon-harboring cells were stimulated with 20 μ g/ml of PGN or 250 ng/ml of IFN- γ , and the IP-10, MIG, or I-TAC mRNA level and HCV IRES RNA level were determined by real-time PCR at 24 h after stimulation. (F) Huh7OK1 cells infected with HCVcc at a multiplicity of infection (MOI) of 1 and incubated for 7 days were fixed with 4% paraformaldehyde-PBS, permeabilized with 0.25% saponin, and immunostained with an anti-NS5A monoclonal antibody. (G) Huh7OK1 cells infected with HCVcc were stimulated with 20 μ g/ml of PGN at 6 days postinfection, and the IP-10, MIG, I-TAC, or IL-8 mRNA level was determined by real-time PCR at 24 h after stimulation. (H) Huh7OK1 cells were treated with various amounts of antibodies against isotype control IgG (Cont) or human CD81 for 2 h at 37°C and infected with HCVcc at an MOI of 1. Levels of IP-10 mRNA and expression of NS5A were determined at 6 days postinfection by real-time PCR and immunoblotting, respectively. Data from real-time PCR were normalized to the amount of GAPDH mRNA. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$) versus the results for control cells or mock-infected cells.

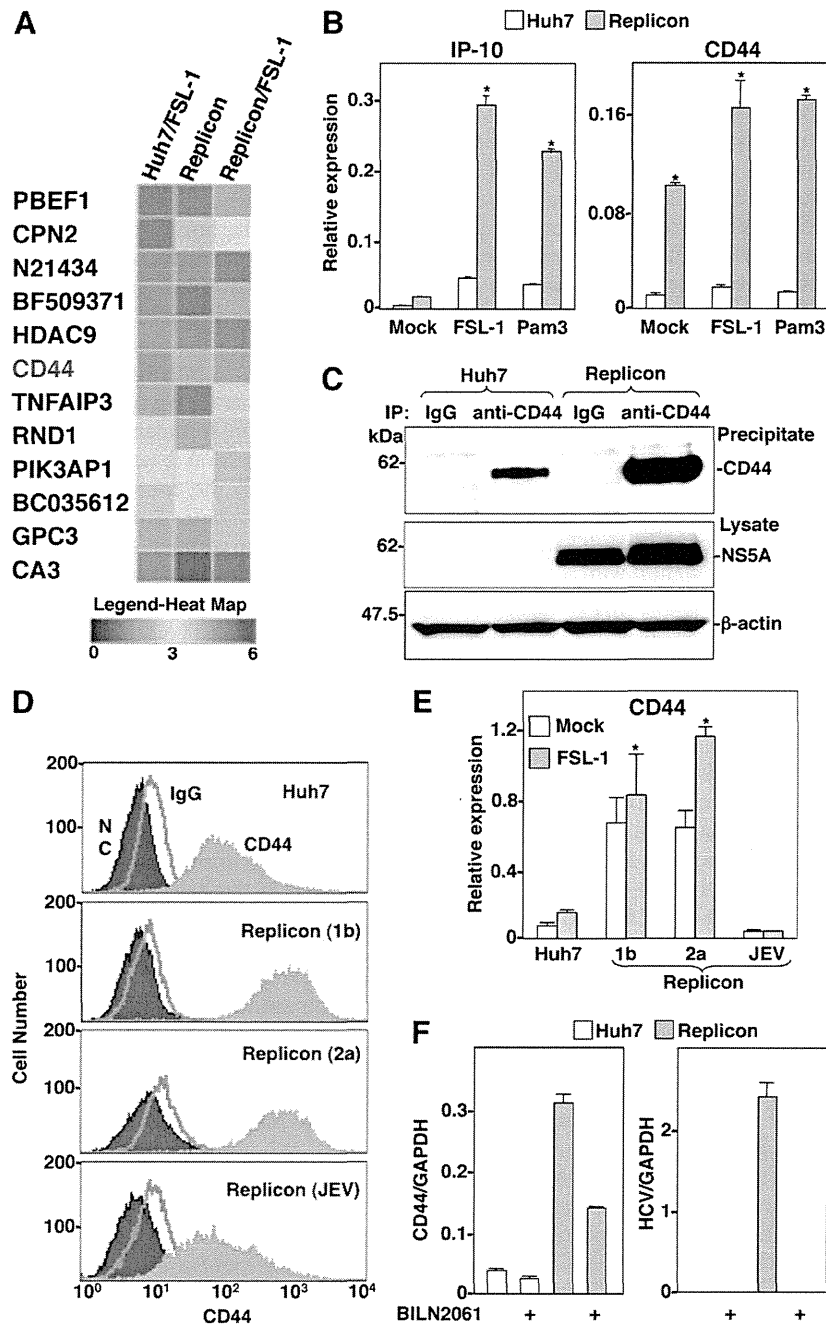


FIG 2 Expression of CD44 in HCV replicon-harboring cells is upregulated in accord with IP-10 production in response to TLR2 ligands. (A) Twelve genes in Huh7 and HCV replicon-harboring cells treated or not treated with FSL-1 were selected, and the resulting heat map is shown. PBEF1, pre-B-cell colony enhancing factor 1; CPN2, carboxypeptidase N, polypeptide 2; N21434, full-length insert cDNA YQ07B06; BF509371, unannotated protein; HDAC9, histone deacetylase 9; TNFAIP3, tumor necrosis factor, alpha-induced protein 3; RND1, Rho family GTPase 1; PIK3AP1, phosphoinositide-3-kinase adaptor protein 1; BC035612, *Homo sapiens* clone IMAGE:4183247 mRNA; GPC3, glypican 3; CA3, carbonic anhydrase III, muscle specific. (B) Huh7 and HCV replicon-harboring cells were stimulated with 1 μ g/ml of FSL-1 or 1 μ g/ml of Pam3CSK (Pam3), and the mRNA levels of IP-10 and CD44 were determined by real-time PCR at 24 h after stimulation. (C) Expression of CD44, NS5A, and β -actin in Huh7 and HCV replicon-harboring cells was determined by immunoblotting. IP, immunoprecipitation. (D) Cell surface expression of CD44 on Huh7 cells, HCV replicon-harboring cells derived from genotype 1b (Con1 strain) and 2a (JFH1 strain), and JEV replicon-harboring cells was determined by flow cytometry. The filled histograms of purple and orange indicate results for unstained (NC) and stained cells, respectively. Blue lines indicate results for isotype control. (E) Huh7 cells, HCV replicon-harboring cells (Con1 and JFH1 strains), and JEV replicon-harboring cells were stimulated with 1 μ g/ml of FSL-1, and the level of CD44 mRNA was determined by real-time PCR at 24 h after stimulation. (F) Huh7 and HCV replicon-harboring cells were treated with 100 nM HCV protease inhibitor (BILN2061), and RNA levels of CD44 and HCV were determined at 72 h posttreatment. Data from real-time PCR were normalized to the amount of GAPDH mRNA. Asterisks indicate significant differences (*, $P < 0.05$) versus the results for control cells.

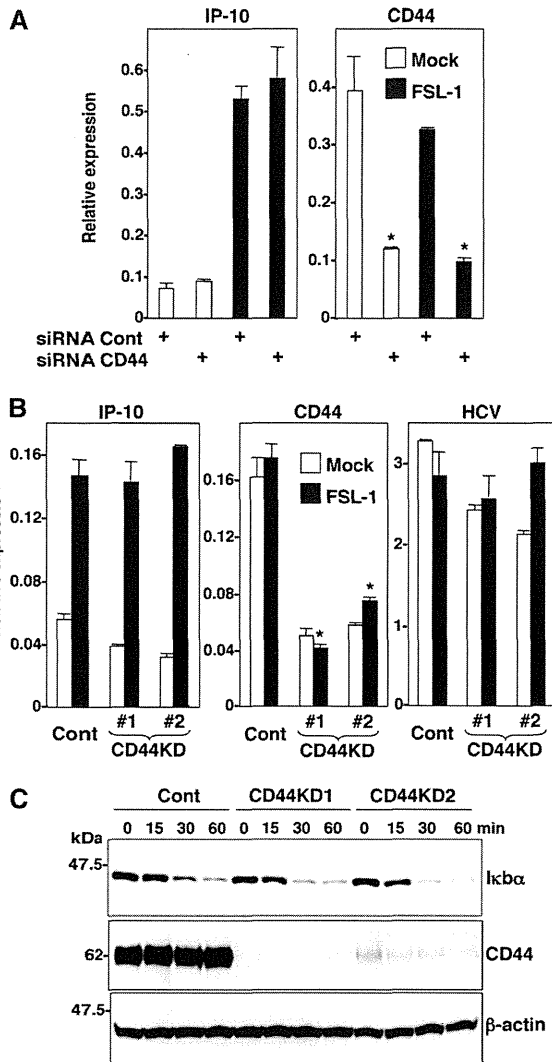


FIG 3 Effect of CD44 silencing on IP-10 production in replicon-harboring cells upon stimulation with TLR2 ligands derived from bacterial components. (A) HCV replicon-harboring cells were transfected with siRNA targeted to CD44 gene or control siRNA at a final concentration of 100 nM and stimulated with 1 μ g/ml FSL-1 at 72 h posttransfection. mRNA levels of IP-10 and CD44 were determined by real-time PCR at 24 h after stimulation. (B) Stable knockdown (KD) cell clones based on the HCV replicon-harboring cells expressing siRNA targeted to CD44 gene (CD44KD #1 and #2) or control siRNA (Cont) were stimulated with 1 μ g/ml of FSL-1. mRNA levels of IP-10, HCV IRES, and CD44 were determined by real-time PCR at 24 h after stimulation. Data from real-time PCR were normalized to the amount of GAPDH mRNA. Asterisks indicate significant differences (*, $P < 0.05$) versus the results for cells transfected with control siRNA. (C) The stable knockdown cell clones based on the HCV replicon-harboring cells were stimulated with 1 μ g/ml of FSL-1 for the times indicated, and expression of I κ B α , CD44, and β -actin was determined by immunoblotting.

cludes ISRE and two NF- κ B-binding regions that are different from those of other CXCR3 ligands (13). The reporter activation assay revealed that IP-10 production in the HCV replicon-harboring cells upon stimulation with HA is dominantly regulated by an NF- κ B-dependent pathway (Fig. 5E). Furthermore, activation of the IP-10 promoter upon stimulation with HA but not with IFN- α was suppressed in the stable CD44 knockdown cells (Fig. 5F).

We next examined the IP-10 expression in cells infected with

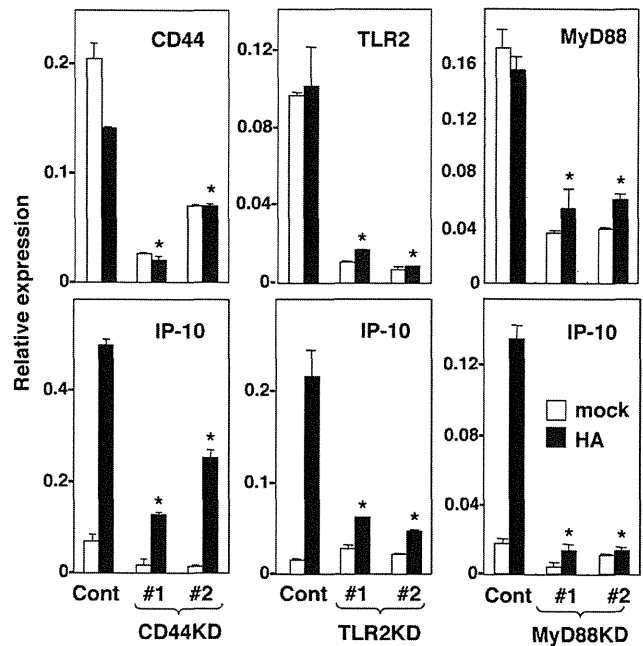


FIG 4 HA induces IP-10 production in human hepatoma cell lines through the TLR2-CD44-MyD88 axis. The stable knockdown cell clones based on the Huh7OK1 cells expressing siRNA targeted to the CD44, TLR2, or MyD88 gene (#1 and #2) or control siRNA (Cont) were stimulated with 500 μ g/ml of HA. mRNA levels of IP-10, CD44, TLR2, and MyD88 genes were determined by real-time PCR at 24 h after stimulation. Data from real-time PCR were normalized to the amount of GAPDH mRNA. Asterisks indicate significant differences (*, $P < 0.05$) versus the results for cells transfected with control siRNA.

HCVcc upon stimulation with HA. Although no significant increase in the cell surface expression and a slight increase of transcription of CD44 were observed in cells infected with HCVcc (Fig. 6A), IP-10 was induced in cells infected with HCVcc or treated with HA and was additively enhanced by costimulation with HCVcc and HA (Fig. 6B). Furthermore, IP-10 production upon costimulation with HCVcc and HA was decreased in the CD44 knockdown cells (Fig. 6C), whereas the expression of CD81 and viral propagation was not affected by the knockdown of CD44 (Fig. 6D and E). Collectively, these results suggest that the expression of IP-10 was also enhanced in cells infected with HCVcc upon stimulation with HA.

CD44 and TLR2 interact through their extracellular domains. To gain more insight into the IP-10 production by stimulation with HA through TLR2 and CD44, we determined the regions responsible for the interaction between CD44 and TLR2. The CD44 gene contains at least 20 exons, and various isoforms are generated through variable splicing of the internal 10 exons (Fig. 7A) (38). The wild-type and a mutant CD44 lacking the intracellular domain (CD44 Δ TM; consists of amino acid residues from 1 to 223) but not a mutant missing the extracellular domain (CD44 Δ EC; consists of amino acid residues from 223 to 361) were coprecipitated with TLR2 by immunoprecipitation analysis (Fig. 7B). A TLR2 mutant lacking the transmembrane region (TLR2 Δ TM; consists of amino acid residues from 1 to 587) but not a mutant missing the extracellular domain (TLR2 Δ EC; consists of amino acid residues from 588 to 784) exhibits a weak but substantial interaction with CD44 (Fig. 7C), indicating that CD44

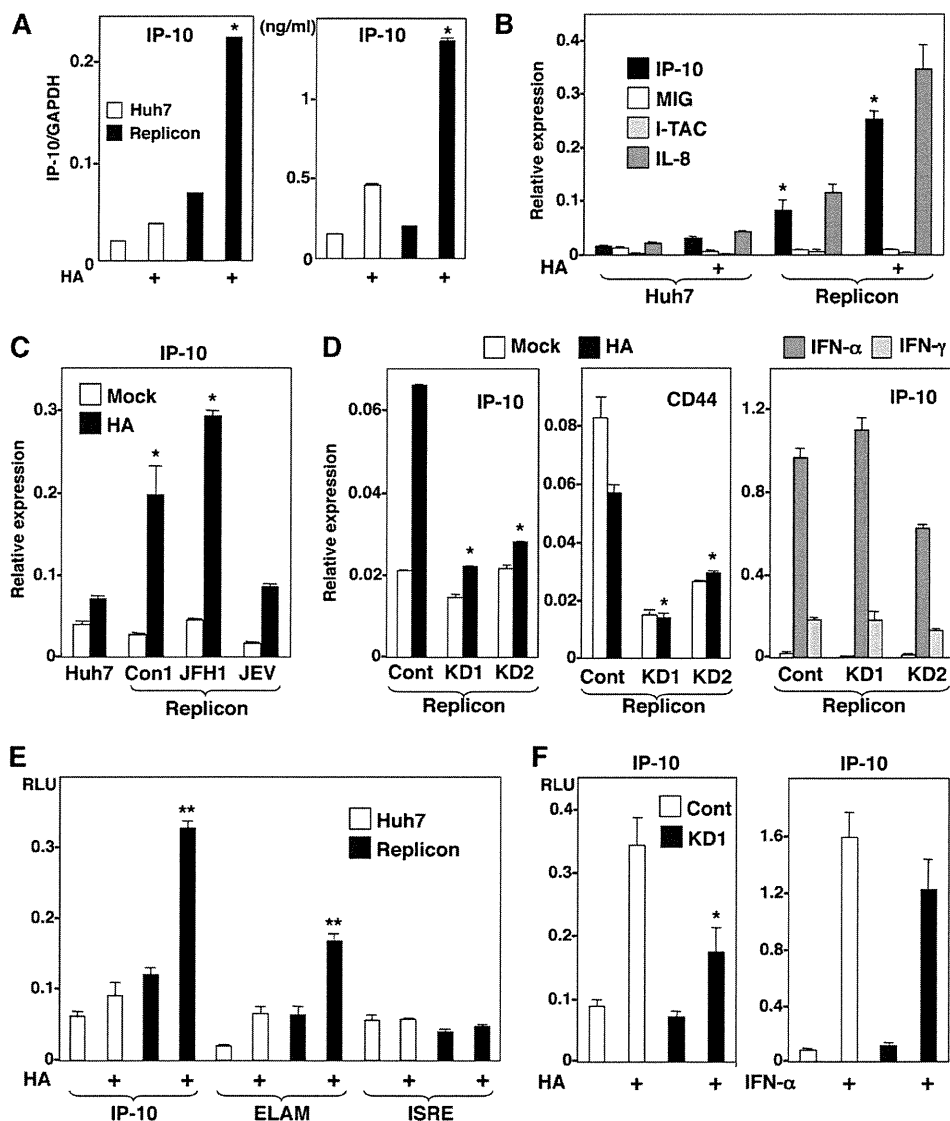


FIG 5 HA participates in IP-10 production in cells replicating HCV. (A) Huh7 and HCV replicon-harboring cells transfected with a plasmid encoding a FLAG-tagged TLR2 were stimulated with 500 $\mu\text{g/ml}$ of HA, and IP-10 mRNA levels (left) and production of IP-10 in culture supernatants (right) 24 h after stimulation were determined by real-time PCR and sandwich ELISA, respectively. (B) Huh7 and HCV replicon-harboring cells were stimulated with 500 $\mu\text{g/ml}$ of HA, and mRNA levels of IP-10, MIG, I-TAC, and IL-8 were determined by real-time PCR at 24 h after stimulation. (C) Huh7 cells, HCV replicon-harboring cells (Con1 and JFH1 strains), and JEV replicon-harboring cells were stimulated with 500 $\mu\text{g/ml}$ of HA, and the level of IP-10 mRNA was determined by real-time PCR at 24 h after stimulation. (D) Stable knockdown cell clones based on the HCV replicon-harboring cells expressing siRNA targeted to the CD44 gene (KD1 and KD2) or control siRNA (Cont) were stimulated with 500 $\mu\text{g/ml}$ of HA or 250 ng/ml of IFN- α and IFN- γ . mRNA levels of IP-10 and CD44 were determined by real-time PCR at 24 h after stimulation. (E) Huh7 and HCV replicon-harboring cells were transfected with each of the reporter plasmids encoding a firefly luciferase gene under the control of the IP-10, ELAM, or ISRE promoter together with a plasmid encoding a *Renilla* luciferase gene under the thymidine kinase (TK) promoter and stimulated with 500 $\mu\text{g/ml}$ HA at 24 h posttransfection. Relative luciferase units (RLU) were determined after standardization with the expression of *Renilla* luciferase at 24 h after stimulation. (F) Stable knockdown cell clones based on the HCV replicon-harboring cells expressing siRNA targeted to the CD44 gene (KD1) or control siRNA (Cont) were transfected with a reporter plasmid encoding a firefly luciferase gene under the control of the IP-10 promoter together with a plasmid encoding a *Renilla* luciferase gene under the TK promoter and stimulated with 500 $\mu\text{g/ml}$ HA or 250 ng/ml IFN- α at 24 h posttransfection. Data from real-time PCR were normalized to the amount of GAPDH mRNA. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$) versus the results for control cells or cells transfected with control siRNA.

and TLR2 interact through their extracellular domains. Interestingly, the interaction between CD44 and TLR2 was enhanced by stimulation not only with HA but also PGN (Fig. 7D), although a TLR2 ligand (FSL-1) induces IP-10 production in cells replicating HCV through a CD44-independent pathway, as shown in Fig. 3. To further clarify the direct interaction between CD44 and TLR2,

the extracellular domains of His-tagged CD44 (CD44 Δ TM) and FLAG-tagged TLR2 (TLR2 Δ TM) were expressed in insect cells. Purified samples were examined by Coomassie staining and immunoblotting (Fig. 7E). The CD44 Δ TM applied in coats to the microplates exhibited binding to TLR2 Δ TM but not to BSA in a dose-dependent manner (Fig. 7F, left). Furthermore, both PGN

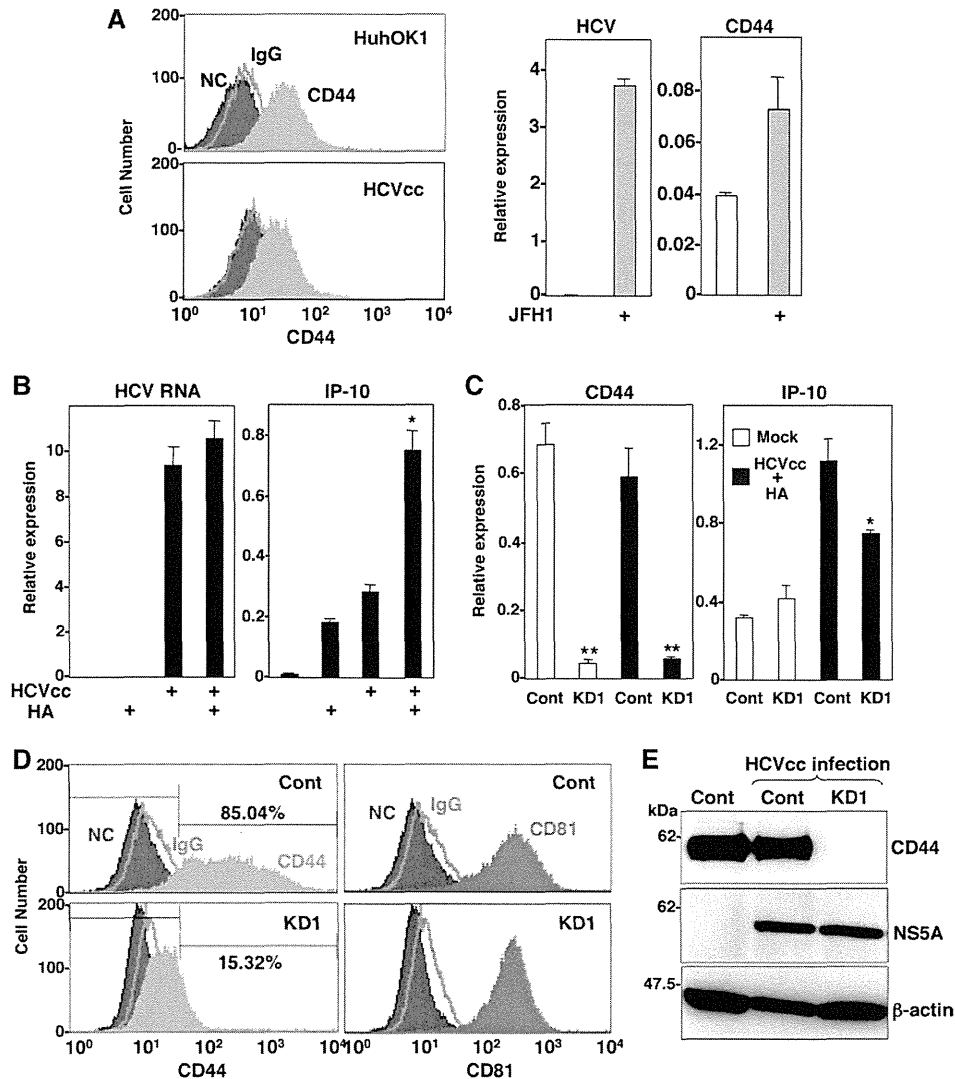


FIG 6 Enhancement of IP-10 production in HCVcc-infected cells upon stimulation with HA. (A) Left, cell surface expression of CD44 on Huh7OK1 cells infected with HCVcc at an MOI of 1 and incubated for 7 days was determined by using antibody that recognizes an ectodomain of CD44 (BU52 clones) and analyzed by flow cytometry. The filled histograms of purple and orange indicate unstained and stained cells, respectively. Blue lines indicate isotype control. Right, levels of CD44 mRNA and HCV RNA in Huh7OK1 cells infected with HCVcc. (B) Huh7OK1 cells infected with HCVcc at an MOI of 1 were stimulated with 500 $\mu\text{g/ml}$ HA at 6 days posttransfection, and IP-10 mRNA and HCV RNA were determined at 24 h after stimulation. The filled histograms of purple, orange, and green indicate results for unstained, CD44-positive, and CD81-positive cells, respectively. Blue lines indicate results for isotype control. (C) Stable knockdown cell clones based on Huh7OK1 cells expressing siRNA targeted to CD44 gene (KD1) or control siRNA (Cont) and infected with HCVcc at an MOI of 1 were stimulated with 500 $\mu\text{g/ml}$ HA at 6 days postinfection, and mRNA levels of CD44 and IP-10 at 24 h after stimulation were determined by real-time PCR. (D) Cell surface expression of CD44 and CD81 on the KD1 and control siRNA cells upon infection with HCVcc at an MOI of 1 was determined by flow cytometry at 7 days postinfection. The filled histograms of purple, orange, and green indicate results for unstained, CD44-positive, and CD81-positive cells, respectively. Blue lines indicate results for isotype control. (E) The KD1 and control siRNA cells were infected with HCVcc at an MOI of 1, and expression of CD44, NS5A, and β -actin at 7 days postinfection was determined by immunoblotting. Data from real-time PCR were normalized to the amount of GAPDH mRNA. Asterisks indicate significant differences ($*P < 0.05$) versus the results for cells transfected with control siRNA or mock-infected cells.

and HA also bound to CD44 ΔTM in a dose-dependent manner, in contrast to a weak interaction with LPS (Fig. 7F, right). These results suggest that IP-10 is induced in cells replicating HCV upon stimulation with HA through an engagement of the extracellular domains of CD44 and TLR2.

DISCUSSION

It has been shown that the expression of CXC chemokines is closely linked to the outcome of antiviral therapy in CHC patients. Successful antiviral therapy is associated with an increase in circu-

lating CXCR3⁺ CD8⁺ T cells and the reduction of IP-10 and MIG expression in serum (24). A high level of IP-10 in the plasma of CHC patients has been shown to be an important negative prognostic biomarker of combination therapy with pegylated IFN and ribavirin (3, 5, 40). Furthermore, a recent study suggests that the truncated IP-10 processed by an endogenous DPP4 in the plasma of CHC patients works as an IP-10 receptor antagonist (4). However, the molecular mechanisms of the production of IP-10 in CHC patients have not yet been characterized.

In this study, we suggested that CD44 is involved in the IP-10

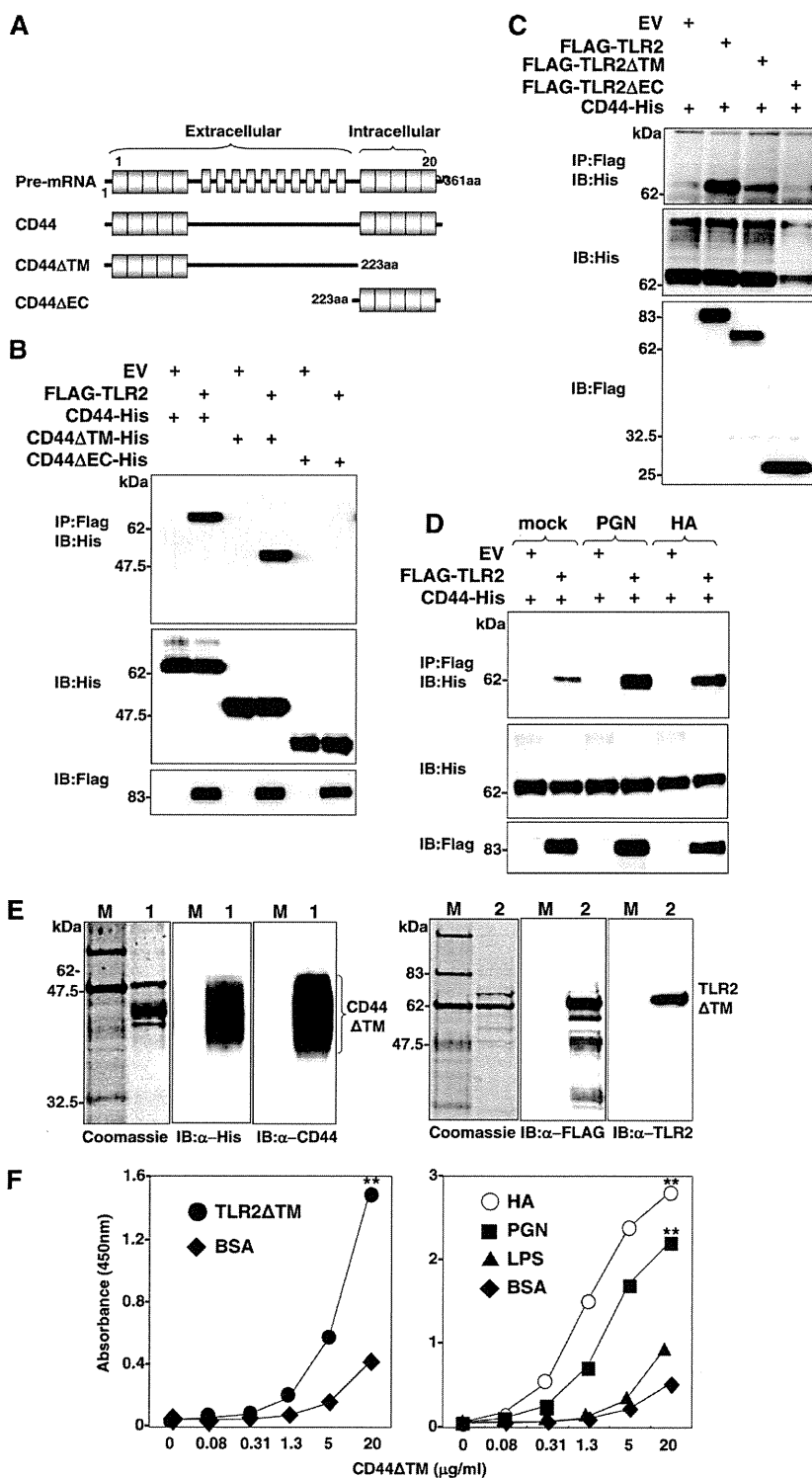


FIG 7 CD44 and TLR2 interact through their extracellular domains. (A) Structures of CD44 and its mutants used in this study. (B) FLAG-TLR2 was coexpressed with CD44-His, its mutants, or empty vector (EV) in 293T cells and immunoprecipitated with anti-FLAG antibody, and the precipitates were determined by immunoblotting (IB) with anti-His antibody. (C) CD44-His was coexpressed with FLAG-TLR2, its mutants, or empty vector in 293T cells and subjected to immunoprecipitation and immunoblotting using the appropriate antibodies. (D) Flag-TLR2 was coexpressed with CD44-His in 293T cells, stimulated with 20 $\mu\text{g}/\text{ml}$ PGN or 500 $\mu\text{g}/\text{ml}$ HA at 36 h posttransfection, and subjected to immunoprecipitation and immunoblotting using the appropriate antibodies at 24 h after stimulation. (E) Purified CD44 ΔTM (lanes 1) and TLR2 ΔTM (lanes 2) were examined by Coomassie staining and immunoblotting using antitag and specific antibodies. M denotes molecular mass markers. (F) Microtiter wells were coated with 20 $\mu\text{g}/\text{ml}$ TLR2 ΔTM (closed circles), 50 $\mu\text{g}/\text{ml}$ HA (open circles), 40 $\mu\text{g}/\text{ml}$ PGN (closed squares), 40 $\mu\text{g}/\text{ml}$ LPS (closed triangles), or 50 $\mu\text{g}/\text{ml}$ BSA (closed diamonds) at 4°C overnight and then incubated with the indicated concentrations of CD44 ΔTM at room temperature for 1 h. The binding of CD44 ΔTM was determined by measuring the absorbance at 450 nm. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$) versus the results for wells treated with BSA.

production upon stimulation with HA through an engagement with TLR2 and that the enhancement of CD44 expression was observed in the HCV replicon-harboring cells of genotypes 1b and 2a but not in cells infected with HCVcc (genotype 2a, JFH1 strain). We do not know the reason why CD44 expression was enhanced in the replicon-harboring cells but not in cells infected with HCVcc, despite the identical origin of the viral genome. Continuous replication of the HCV genome might be required for the enhancement of CD44 expression in the replicon-harboring cells autonomously replicating the HCV genome, in contrast to HCVcc-infected cells exhibiting distinct cytopathic effects. To clarify the role of CD44 in the IP-10 production in cells infected with HCV in more detail, we have to await the establishment of a robust and reliable *in vitro* replication system of various HCV genotypes, especially genotypes 1b and 1a, which are associated with progressive liver injury and persistent infection.

The cellular sources of CXCR3 ligands in CHC patients would be liver parenchymal cells, hepatic stellate cells, and sinusoidal endothelial cells within the liver and infiltrated immunocompetent cells, such as lymphocytes, macrophages, and dendritic cells. We have shown previously that production of IP-10 was enhanced in the macrophage cell lines stably expressing HCV NS5A proteins in response to various TLR ligands, in contrast to the impairment of most proinflammatory cytokines and chemokines (1; also unpublished data). Although replication of HCV in the immunocompetent cells is conflicting (8, 20, 29, 39, 43), it might be feasible to speculate that IP-10 is produced in the immunocompetent cells of CHC patients.

Upon tissue injury, high-molecular-weight HA, a ubiquitously distributed extracellular matrix component, is degraded into low-molecular-weight HA, which in turn activates an inflammatory response, although the precise receptor targeted for this response is still controversial (19). On the other hand, it has been reported that CD44 is dispensable for chemokine production by stimulation with HA in macrophages (18). Interestingly, in HCV-replicating cells, IP-10 production upon stimulation with HA but not with FSL-1 requires CD44. These results suggest that IP-10 production by stimulation with endogenous TLR2 ligands may be regulated by at least two different pathways in hepatocytes of CHC patients, through CD44-dependent and -independent pathways in response to HA and ligands derived from the intestinal microbiota, respectively. The increase of HA expression in accord with the progression of liver fibrosis in CHC patients may participate in the CD44-dependent IP-10 induction. On the other hand, HCV core and NS3 proteins have been shown to induce immune activation in immunocompetent cells through a TLR2-dependent signaling pathway, suggesting that HCV proteins also participate in immune activation as exogenous ligands (6, 7). We tried to neutralize the IP-10 induction in the HCV replicon-harboring cells by using monoclonal antibodies against CD44 and TLR2. However, these antibodies exhibited no significant inhibition of IP-10 production upon stimulation with HA (data not shown), probably due to lack of inhibition of the interaction between ligands and receptors. Furthermore, pretreatment with PGN exhibited no effect on the binding of HA to CD44 (data not shown), suggesting that the TLR2 agonist and HA bind to different regions of CD44. Further studies are needed to clarify the relationship between TLR2 and CD44 for IP-10 production in the HCV-replicating cells.

In contrast to our observations, it has been reported that the

induction of CXC chemokines, particularly I-TAC, was significantly enhanced in HCV-replicating cells following stimulation with either IFN- γ or TNF- α and that stimulation with both had a synergistic effect (14). Although we confirmed that the expression of all of the CXC chemokines was significantly induced by stimulation with IFN- γ alone and costimulation with TNF- α in the HCV replicon-harboring cells (Fig. 1E and data not shown), only IP-10 was induced by stimulation with PGN or HA, suggesting that IP-10 is produced in HCV-replicating cells in a ligand-specific manner. The synergistic increase of I-TAC by the activation of IRF3 through a dsRNA-dependent signaling pathway has also been reported (13); however, it is difficult to reconcile the selective increase of I-TAC production by the dsRNA-mediated innate immune response because of the inhibition of the signaling pathway by the HCV NS3/4A protease (25). Our data indicated that IP-10 production induced by HA or PGN is dependent upon the TLR2-MyD88-NF- κ B axis, suggesting that the activation of NF- κ B upon stimulation with HA plays a crucial role in the IP-10 production in cells replicating HCV. Although both the IP-10 and I-TAC promoters contain the ISRE, an increase in IP-10 production from stimulation with HCV RNA (5' untranslated region), poly(I-C), IFN- γ , or TNF- α was not observed (13). Among the CXC chemokines, only IP-10 has two NF- κ B-binding elements in the promoter, and the activation of IP-10 by stimulation with HA was mainly regulated by NF- κ B but not ISRE in cells replicating HCV (Fig. 5E). These results strongly supported our notion that the selective increase of IP-10 production by stimulation with HA is dominantly regulated by the activation of NF- κ B in the HCV-replicating cells.

CD44 variants have been implicated in many biological processes, including hematopoiesis, chronic inflammation, and metastatic spread of cancer cells (10, 38), and are useful markers in the diagnosis and prognosis of the progression of human tumors (11, 15). In chronic HCV infection, HA has been shown to be involved in HCV pathogenesis, while the participation of the specific CD44 variants has not been studied yet. The CD44v8 to -v10 variants have been shown to directly associate with TLR2 through the cytoplasmic domain and negatively regulate the inflammatory response in macrophages and mouse embryonic fibroblasts (21). Furthermore, it has been shown that the expression of CD44 contributes to the suppression of TLR4-mediated inflammation through the induction of the negative regulator in alveolar and peritoneal macrophages (27). The expression of TLR and CD44 variants varies among cell types, and the expression pattern of the molecules might determine the inflammatory response in cells infected with HCV. Further studies are needed to clarify the involvement of each of the CD44 variants in the pathogenesis of HCV.

Intervention to reduce the expression of endogenous HA and to inhibit the interaction between CD44 and TLR2 may provide a novel therapeutic measure for CHC patients exhibiting no response to the current pharmaceutical intervention.

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Establishment of a Novel Permissive Cell Line for the Propagation of Hepatitis C Virus by Expression of MicroRNA miR122

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The robust cell culture systems for hepatitis C virus (HCV) are limited to those using cell culture-adapted clones (HCV in cell culture [HCVcc]) and cells derived from the human hepatoma cell line Huh7. However, accumulating data suggest that host factors, including innate immunity and gene polymorphisms, contribute to the variation in host response to HCV infection. Therefore, the existing *in vitro* systems for HCV propagation are not sufficient to elucidate the life cycle of HCV. A liver-specific microRNA, miR122, has been shown to participate in the efficient replication of HCV. In this study, we examined the possibility of establishing a new permissive cell line for HCV propagation by the expression of miR122. A high level of miR122 was expressed by a lentiviral vector placed into human liver cell lines at a level comparable to the endogenous level in Huh7 cells. Among the cell lines that we examined, Hep3B cells stably expressing miR122 (Hep3B/miR122) exhibited a significant enhancement of HCVcc propagation. Surprisingly, the levels of production of infectious particles in Hep3B/miR122 cells upon infection with HCVcc were comparable to those in Huh7 cells. Furthermore, a line of “cured” cells, established by elimination of HCV RNA from the Hep3B/miR122 replicon cells, exhibited an enhanced expression of miR122 and a continuous increase of infectious titers of HCVcc in every passage. The establishment of the new permissive cell line for HCVcc will have significant implications not only for basic HCV research but also for the development of new therapeutics.

Hepatitis C virus (HCV) infects over 170 million people worldwide and frequently leads to persistent infection, which in turn can lead to chronic hepatitis, cirrhosis, and hepatocellular carcinoma (34). HCV belongs to the *Flaviviridae* family and has a single-stranded positive RNA genome of approximately 9.6 kb. The genome of HCV is translated into a single polyprotein at the endoplasmic reticulum (ER) membrane and is then cleaved by host- and virus-encoded proteases, resulting in 10 structural and nonstructural proteins (41, 44). Due to the lack of a small-animal model and an efficient cell culture system, efforts to understand the HCV life cycle as well as development of anti-HCV drugs have been hampered (42). In a major breakthrough, HCV replicon cells, in which HCV RNA autonomously replicates, were established by Lohmann et al. (37). Afterwards, the infectious HCV in cell culture (HCVcc), based on the genotype 2a JFH1 strain in combination with the human hepatocellular carcinoma cell line Huh7, was developed (36, 64, 70). On the basis of the results obtained with these *in vitro* systems, the life cycle of HCV was clarified, and substantial progress has been made in screening host factors involved in HCV propagation as well as anti-HCV drug candidates (20, 51). Among them, a liver-specific microRNA (miRNA), miR122, has been shown to be one of the most important host factors for HCV replication.

miRNAs are small noncoding RNAs that consist of 20 to 25 nucleotides and modulate gene expression in plants and animals (3, 26). Most miRNAs negatively regulate translation through interaction with the 3' untranslated region (UTR) of mRNA in a sequence-specific manner. Some of them have been shown to play important roles in the viral life cycle (56). Interestingly, miR122 has been shown to bind to HCV 5' UTRs and to enhance translation and replication of HCV RNA (23, 28, 29, 38, 52). In addition, enhancement of HCVcc propagation through the direct interaction of miR122 with HCV 5' UTR has been demonstrated (27). Recently, intravenous administration of the locked nucleic acid (LNA) complementary to miR122 was shown to suppress the

propagation of HCV in chimpanzees chronically infected with HCV, suggesting that miR122 is a promising therapeutic target for chronic hepatitis C (31).

It has been shown that HCV exploits various host factors to form a replication complex for efficient replication (43). *In vitro* propagation of HCV is limited to Huh7 cells and their derivatives, and thus, it is important to confirm the data obtained in Huh7 cells by using other human liver cell lines, because the patterns of gene expression vary among cell lines. Although establishment of an HCV replicon system based on liver cell lines has been reported (11, 66), robust propagation of HCVcc in well-characterized human liver cell lines other than Huh7 cells has not succeeded yet. The gene expression profile of mice xenotransplanted with human hepatocytes from different donors inoculated with a single source of HCV revealed that host factors contributed to the variation in host response to HCV infection, including the activation of innate antiviral signaling pathways (65). Furthermore, gene polymorphism in interleukin 28B (IL-28B) was shown to be associated with natural clearance (62) and response to combination therapy with interferon (IFN) and ribavirin (19, 58, 59). Therefore, the solely available *in vitro* propagation system for HCVcc, employing Huh7-derived cells, is not sufficient. The establishment of alternative HCV strains and permissive cell lines is needed to elucidate molecular mechanisms of propagation and pathogenesis of HCV in more detail.

Although there have been several attempts to generate chime-

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ric HCVs based on the JFH1 strain (21) and an infectious clone of genotype 1a, H77S, that produces fewer infectious particles than the genotype 2a JFH1 strain (68), propagation of HCV was still limited to Huh7 cells. Exogenous expression of miR122 has been shown to support HCV RNA replication in a human embryonic kidney epithelial cell line and mouse embryonic fibroblasts (7, 35), and we therefore thought that the possibility of complete propagation of HCVcc in various human liver cell lines by the expression of miR122 needed to be examined. Among the cell lines that we examined, Hep3B cells, which were established from human liver tumor biopsy samples in 1976 (1) and have been well characterized as model liver cells in various fields of research (47, 55, 63, 67), were shown to support the efficient propagation of HCVcc comparable to that in Huh7 cells by the expression of miR122. Establishment of novel cell culture systems through the exogenous expression of miR122 provides a clue to understanding the precise roles of miR122 in the life cycle of HCV.

MATERIALS AND METHODS

Plasmids. The cDNA clones of wild-type miR122 (WT-miR122), single mutant miR122 (sMT-miR122), double mutant miR122 (dMT-miR122), *Aequorea coerulescens* green fluorescent protein (AcGFP), and claudin-1 (CLDN) were inserted between the XhoI and XbaI sites of a lentiviral vector, pCSII-EF-RfA, which was kindly provided by M. Hijikata, and the resulting plasmids were designated pCSII-EF-WT-miR122, pCSII-EF-sMT-miR122, pCSII-EF-dMT-miR122, pCSII-EF-AcGFP, and pCSII-EF-Claudin1, respectively. pHH-JFH1 was kindly provided by T. Wakita (39). pHH-JFH1-E2p7NS2mt contains three adaptive mutations in pHH-JFH1 (53). pFGR-JFH1 and pSGR-JFH1 encoded a full-length and a subgenomic cDNA of the JFH1 strain, respectively. The complementary sequence of miR122 was inserted into the PmeI site of the pmirGLO vector (Promega, Madison, WI), and the resulting plasmid was designated pmirGLO-miR122comp. pIFN β -Luc and pISRE-Luc carrying a firefly luciferase gene under the control of the beta IFN (IFN- β) and interferon-sensitive response element (ISRE) promoters, respectively, were kindly provided by T. Kawai and S. Akira. The internal control plasmid encoding a *Renilla* luciferase (pRL-TK) was purchased from Promega. The plasmids used in this study were confirmed by sequencing with an ABI Prism 3130 genetic analyzer (Applied Biosystems, Tokyo, Japan).

Cells. All cell lines were cultured at 37°C under the condition of a humidified atmosphere and 5% CO₂. The human embryonic kidney 293T cell line and hepatocellular carcinoma cell lines Huh7, Huh6/CLDN, HepG2/CD81, Hep3B, and PKC/PRL/5 were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO) supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10% fetal calf serum (FCS). HepG2/CD81 cells were generated as described previously (60). Huh6 cells were transduced with a lentiviral vector expressing claudin-1, and the resulting cells were designated Huh6/CLDN. The Huh7-derived cell line Huh7.5.1 was kindly provided by F. Chisari and was maintained in DMEM containing nonessential amino acids (NEAA), 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10% FCS. Hep3B replicon cells harboring the subgenomic HCV RNA were maintained in DMEM containing 10% FCS, NEAA, and 400 μ g/ml G418 (Nakalai Tesque, Kyoto, Japan).

Viruses. pHH-JFH1-E2p7NS2mt was transfected into Huh7.5.1 cells, and the culture supernatants were collected after serial passages. The infectivity of HCVcc was determined by focus-forming assay and expressed in focus-forming units (FFU) (64). The lentiviral vectors and ViraPower lentiviral packaging mix (Invitrogen, San Diego, CA) were cotransfected into 293T cells, and the supernatants were recovered at 48 h posttransfection. The culture supernatants were centrifuged at 1,000 \times g for 5 min and cleared through a 0.45- μ m-pore-size filter. The lentivirus titer was determined by a Lenti-X quantitative reverse transcription (qRT)-PCR titration kit (Clontech, Mountain View, CA). The vesicular stomatitis virus

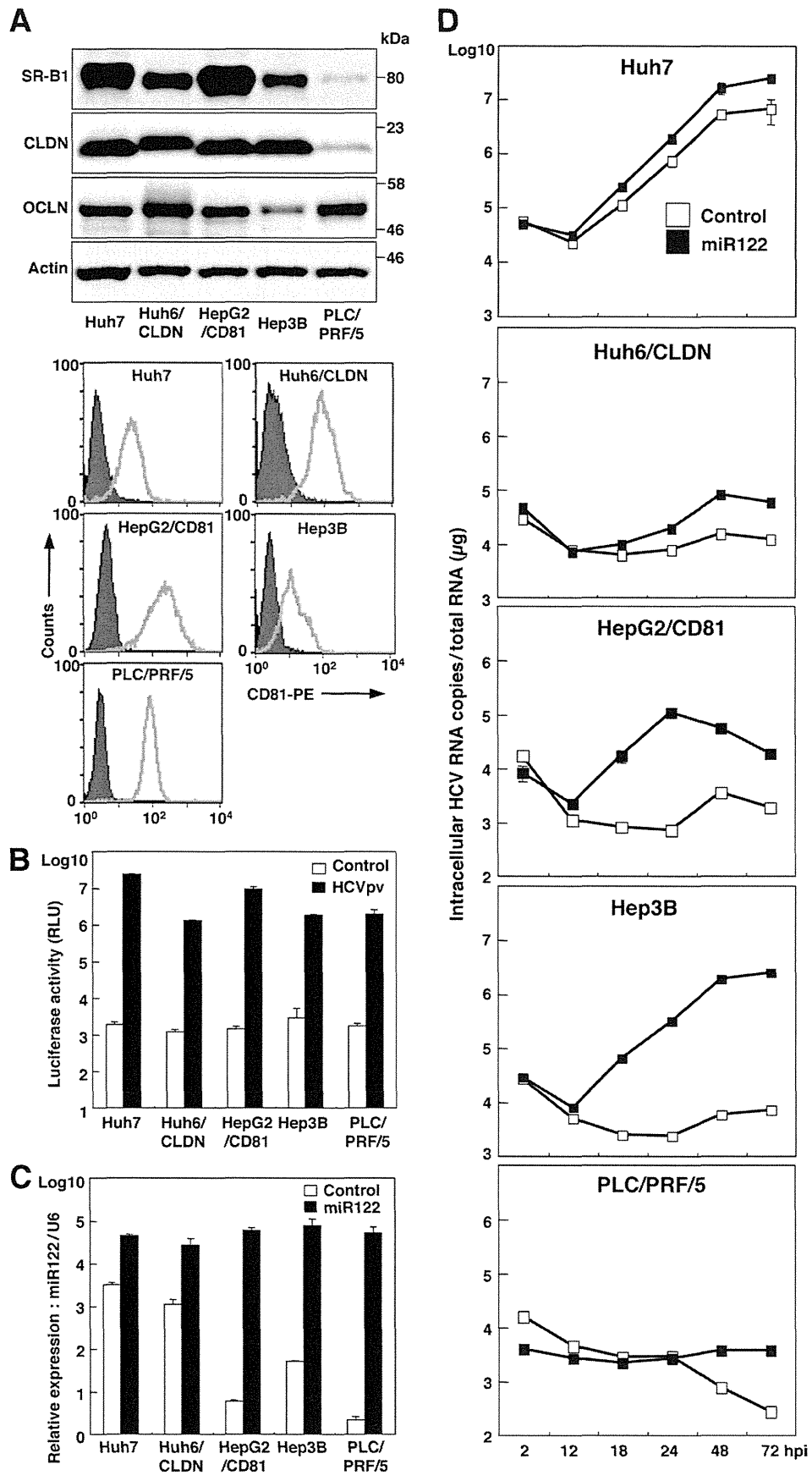
(VSV) variant NCP12.1, derived from the Indiana strain, was kindly provided by M. Whitt. Pseudotype VSVs bearing the HCV E1 and E2 glycoproteins (HCVpv) and VSV G protein (VSVpv) were prepared as described previously (60). The infectivity of the pseudotype viruses was assessed by the expression of luciferase, determined by a Bright-Glo luciferase assay system (Promega) following a protocol provided by the manufacturer and expressed in relative light units (RLU).

Reagents and antibodies. Cyclosporine (CsA) and human recombinant IFN- α 2 were purchased from Sigma and R&D Systems (Minneapolis, MN), respectively. BODIPY 558/568 lipid probe was purchased from Invitrogen. Poly(I-C) was purchased from InvivoGen (San Diego, CA). LNAs complementary to miR122 (LNA-miR122; 5'-CcAttGTcaCaCtCC-3') and its negative control (LNA-Cont; 5'-CcAttCTgaCtCtAC-3') (LNA in capital letters, DNA in lowercase letters; sulfur atoms in oligonucleotide phosphorothioates are substituted for nonbridging oxygen atoms; capital C indicates LNA methylcytosine) (14) were purchased from Gene Design (Osaka, Japan). miScript miRNA mimics hsa-miR122 and its negative control were purchased from Qiagen (Valencia, CA). Mouse monoclonal antibodies to HCV NS5A and β -actin were purchased from Austral Biologicals (San Ramon, CA) and Sigma, respectively. Mouse anti-apolipoprotein E (anti-ApoE), rabbit anti-diacylglycerol acyltransferase 1 (DGAT1), rabbit anti-signal transducer and activators of transcription 2 (anti-STAT2), and rabbit anti-IFN regulatory factor 3 (anti-IRF3) antibodies were purchased from Santa Cruz (Santa Cruz, CA). Rabbit anti-HCV core protein was prepared as described previously (45). Phycoerythrin (PE)-conjugated anti-human CD81 (anti-hCD81) and anti-mouse IgG antibodies were purchased from BD Biosciences (Franklin Lakes, NJ). Mouse anti-double-stranded RNA (anti-dsRNA) IgG2a (J1 and K2) antibodies were from Biocenter Ltd. (Szirak, Hungary). Alexa Fluor 488 (AF488)-conjugated anti-mouse and -rabbit IgG and AF594-conjugated anti-rabbit IgG antibodies were from Invitrogen.

Quantitative RT-PCR. For quantitation of HCV RNA, total RNA was prepared from cells by using an RNeasy minikit (Qiagen). The synthesis of a first-stranded cDNA and quantitative RT-PCR were performed using TaqMan EZ RT-PCR core reagents and an ABI Prism 7000 system (Applied Biosystems) according to the manufacturer's protocol. For quantitation of miRNA, total RNA was prepared from cells by using an miRNeasy minikit (Qiagen), and miR122 was estimated by using miR122-specific RT primers and amplified using specific primers provided in the TaqMan MicroRNA assays (Applied Biosystems) according to the manufacturer's protocol. U6 small nuclear RNA (snRNA) was used as an internal control. Fluorescent signals were analyzed by an ABI Prism 7000 system (Applied Biosystems).

Transfection and immunoblotting. Cells were transfected with the plasmids by using *Trans* IT LT-1 (Mirus, Madison, WI) or Lipofectamine 2000 (Invitrogen) according to the manufacturers' protocols. Cells were lysed on ice in Triton 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 sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) and reacted with primary antibody and then secondary horseradish peroxidase-conjugated antibody. The immunocomplexes were visualized with Super Signal West Femto substrate (Pierce, Rockford, IL) and detected by using an LAS-3000 image analyzer (Fujifilm, Tokyo, Japan).

Indirect immunofluorescence assay. Cells cultured on glass slides were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 30 min. After washing three times with PBS, the cells were permeabilized for 20 min at room temperature with PBS containing 0.25% saponin and blocked with phosphate buffer containing 2% bovine serum albumin (BSA) for 1 h at room temperature. The cells were incubated with blocking buffer containing mouse anti-dsRNA, rabbit anti-NS5A, rabbit anti-core, rabbit anti-IRF3, or rabbit anti-STAT2 at room temperature for 1 h, washed three times with PBS, and incubated



with blocking buffer containing appropriate AF488-conjugated and AF594-conjugated secondary antibodies at room temperature for 1 h. Finally, the cells were washed three times with PBS and observed with a FluoView FV1000 laser scanning confocal microscope (Olympus, Tokyo, Japan).

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

***In vitro* transcription, RNA transfection, and colony formation.** The plasmids pSGR-JFH1 and pFGR-JFH1 were linearized with XbaI 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 Hep3B cells at 10⁶ cells/0.4 ml under conditions of 270 V and 960 µF using a Gene Pulser apparatus (Bio-Rad, Hercules, CA) and plated on DMEM containing 10% FCS and NEAA. The medium was replaced with fresh DMEM containing 10% FCS, NEAA, and 400 µg/ml G418 at 24 h posttransfection. The remaining colonies were fixed with 4% paraformaldehyde and stained with crystal violet at 1 month postelectroporation.

Luciferase assay. Cells were seeded onto 24-well plates at a concentration of 5 × 10⁴ cells/well and transfected with 250 ng of each of the plasmids. At 24 h posttransfection, cells were stimulated with the appropriate ligands for 24 h and then 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-TK and was expressed as RLU.

RESULTS

Expression of miR122 facilitates replication of HCVcc in various liver cell lines. The robust *in vitro* cell culture systems for HCV use the HCV genotype 2a isolate JFH1 and Huh7-derived cell lines (64). To expand the host range of HCVcc to gain more insight into the host-virus interaction, we examined the effect of expression of miR122, a liver-specific microRNA that was shown to be crucial for the efficient replication of HCV (27–29, 38, 52), in several well-characterized liver cell lines: Huh6, HepG2, Hep3B, and PLC/PRF/5. Although hCD81, SR-B1, claudin-1 (CLDN), and occludin (OCLN) are known to be crucial for entry of HCVcc (15, 48, 49, 54), the Huh6 and HepG2 cell lines express little or no CLDN and hCD81 (10, 22), respectively. Therefore, CLDN and hCD81 were exogenously expressed in the cell lines, and the resulting lines were designated Huh6/CLDN and HepG2/CD81, respectively. Expression of the receptor molecules in the cell lines was confirmed by immunoblot and fluorescence-activated cell sorter (FACS) analyses (Fig. 1A). To further examine the susceptibility to HCV infection, pseudotyped VSV bearing the HCV envelope protein, HCVpv, was inoculated into these cell lines. Significant expression of luciferase was observed in these cell lines upon infection with HCVpv but not upon infection with the con-

trol virus (Fig. 1B), suggesting that the liver cell lines express functional receptors required for entry of HCV. To determine the effect of miR122 on the replication of HCVcc, we next assessed the level of miR122 in the liver cell lines by qRT-PCR. Although miR122 is highly expressed in the liver (13), the expression level of miR122 varied among the liver cell lines (Fig. 1C, white bars). To examine the effect of the exogenous expression of miR122 in the liver cell lines on the replication of HCVcc, miR122 was expressed in the cell lines by the lentiviral vector. The expression level of miR122 in the liver cell lines, including Huh7 cells, was shown to be upregulated to a significantly greater extent than that in Huh7 cells alone (Fig. 1C, black bars). To examine the effect of miR122 on the replication of HCV, HCVcc was inoculated into the cell lines (Fig. 1D). Although Huh7 cells exhibited an efficient HCV replication, a slight enhancement of the replication was observed by the expression of miR122. No HCV replication was observed in PLC/PRF/5 cells irrespective of miR122 expression. Hep3B and HepG2/CD81 cells exhibited a significant enhancement of HCV replication by the expression of miR122, in contrast to a slight increase in Huh6/CLDN cells. Notably, HCV RNA levels were drastically increased by more than 300-fold at 72 h postinfection in Hep3B cells by the expression of miR122, suggesting that Hep3B is the most suitable cell line for investigating the biological significance of miR122 on the propagation of HCV and for establishing a permissive cell line for HCVcc. Therefore, we used Hep3B cells overexpressing miR122 (Hep3B/miR122 cells) for further experiments.

Expression of biologically active miR122 facilitates replication of HCVcc in Hep3B cells. To confirm the activity of endogenously and exogenously expressed miR122 to suppress the translation in cells, a pmirGLO vector carrying the complementary sequence of miR122 under the luciferase gene was transfected into Huh7 cells, Hep3B cells expressing AcGFP (Hep3B/Cont), and Hep3B/miR122 cells. Suppression of luciferase expression was observed in Huh7 and Hep3B/miR122 cells but not in Hep3B/Cont cells (Fig. 2A), suggesting that miR122 exogenously expressed in Hep3B cells is as biologically active as that endogenously expressed in Huh7 cells. To determine the effect of miR122 on the propagation of HCVcc, Hep3B cells were infected with the lentiviral vector expressing miR122 and then inoculated with HCVcc. The levels of HCV RNA in Hep3B cells upon infection with HCVcc were increased in proportion to the amount of lentiviral vector (Fig. 2B). Recently, an inhibitor for miR122, SPC3649, which is an LNA in which 2' oxygen and 4' carbon are connected via methylene units, has been shown to possess potent anti-HCV activity in chimpanzees chronically infected with HCV (31). We next examined the effect of LNA on the replication of HCVcc in Huh7 and Hep3B/miR122 cells. HCV RNA replication in Huh7 and Hep3B/miR122 cells was significantly and dose-dependently decreased by treatment with LNA-miR122 but not treatment with LNA-Cont (Fig. 2C). We further investigated the effect of the

FIG 1 Expression of miR122 facilitates replication of HCVcc in various liver cell lines. (A) Human liver cell lines Huh7, Huh6/CLDN, HepG2/CD81, Hep3B, and PLC/PRF/5 were lysed and subjected to immunoblotting using appropriate antibodies. The expression levels of hCD81 in the liver cell lines were determined by flow cytometry. (B) The human liver cell lines were inoculated with HCVpv or control virus and washed three times after 2 h of incubation. Luciferase activities were determined at 24 h postinfection. (C) The cell lines were transduced with lentiviral vectors expressing miR122 or AcGFP as a control. After serial passages, total RNA was extracted from the cells and relative expression of miR122 was determined by qRT-PCR by using U6 snRNA as an internal control. (D) The cells expressing miR122 or control were infected with HCVcc at an MOI of 1. Total RNA was extracted from the cells at the indicated time and subjected to qRT-PCR analysis. The data are representative of three independent experiments. Error bars indicate the standard deviation of the mean.

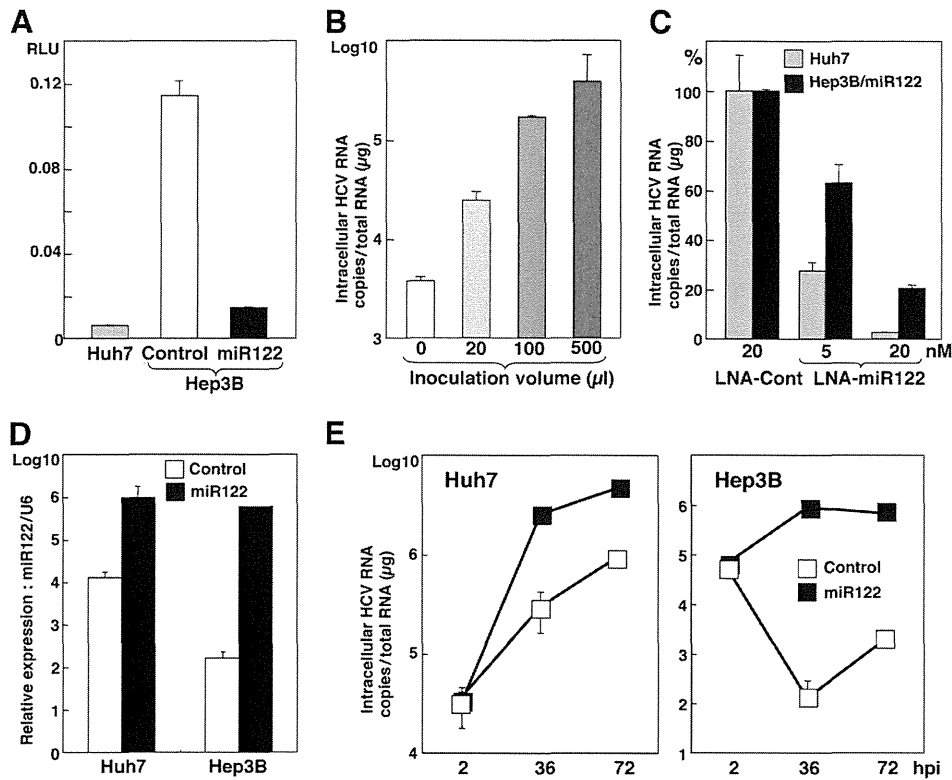


FIG 2 Expression of biologically active miR122 facilitates replication of HCVcc in Hep3B cells. (A) Huh7, Hep3B/Cont, and Hep3B/miR122 cells were transfected with pmirGLO-miR122comp, and luciferase activity was determined at 24 h posttransfection. (B) Hep3B cells were transfected with the lentiviral vector expressing miR122 in a dose-dependent manner and then infected with HCVcc at an MOI of 1 at 48 h posttransfection. Total RNA was extracted from the cells at 72 h postinfection and subjected to qRT-PCR. (C) LNA-Cont (20 nM) or LNA-miR122 (5 nM or 20 nM) was introduced into Hep3B/miR122 cells and infected with HCVcc at an MOI of 1 at 12 h posttransfection. Total RNA was extracted from the cells at 24 h postinfection and subjected to qRT-PCR. (D) Huh7 and Hep3B cells were transfected with mimic miR122 (20 nM) or a negative control (20 nM), and total miRNA was determined by qRT-PCR at 24 h posttransfection. (E) Huh7 and Hep3B cells were transfected with mimic miR122 (20 nM) or a negative control (20 nM) and infected with HCVcc at an MOI of 1 at 12 h posttransfection. Total RNA was extracted from the cells at the indicated time (hpi, hours postinfection) and subjected to qRT-PCR.

mimic miR122, the synthetic double-stranded RNA oligonucleotides that mimic endogenous miRNA function, on the propagation of HCV. Huh7 and Hep3B cells transfected with mimic miR122 but not those transfected with the negative control exhibited a high level of expression of miR122 (Fig. 2D) and enhanced RNA replication upon infection with HCVcc (Fig. 2E). Collectively, these results clearly indicate that expression of biologically active miR122 plays a crucial role in the replication of HCV in Hep3B cells.

Establishment of a novel permissive cell line for robust propagation of HCVcc by expression of miR122 in Hep3B cells. We next examined the possibility of establishing a permissive cell line for the robust propagation of HCVcc by the expression of miR122 in Hep3B cells. Huh7, Hep3B/miR122, and Hep3B/Cont cells were infected with HCVcc, and the levels of expression of HCV NS5A and core proteins were assessed by immunoblotting at 72 h postinfection. Expression of the viral proteins in Hep3B/miR122 cells was almost comparable to that in Huh7 cells, in contrast to no expression in Hep3B/Cont cells (Fig. 3A). Small foci stained by immunofluorescence assay appeared at 24 h postinfection in Hep3B/miR122 and Huh7 cells but not in Hep3B/Cont cells and grew into large foci at 72 h postinfection, indicating that infectious particles are generated in Hep3B/miR122 cells and the progeny particles expand infection to the neighboring cells (Fig. 3B). The

morphology of Hep3B cells is completely different from that of Huh7 cells, and thus, these results are not due to contamination of Huh7 cells. DGAT1 and ApoE have been shown to play crucial roles in the recruitment of core protein to the lipid droplets and viral infectivity, respectively (9, 24). Higher levels of expression of ApoE and DGAT1 were detected in Hep3B cells than in Huh7 cells (Fig. 3C). Furthermore, the concentration of infectious particles recovered in the culture supernatant of Hep3B/miR122 cells infected with HCVcc at a multiplicity of infection (MOI) of 1 at 72 h postinfection was approximately 5×10^4 FFU/ml, which was comparable to that in Huh7 cells, and was in clear contrast to the significantly lower titer in Hep3B/Cont cells (less than 10 FFU/ml). These results clearly indicate that expression of miR122 in Hep3B cells enables the establishment of a novel permissive cell line for the robust propagation of HCVcc.

Establishment of an HCV RNA replicon in Hep3B/miR122 cells. It has been shown that “cured” cells established through the elimination of the HCV genome from replicon cells by treatment with IFN- α exhibited more potent propagation of HCVcc than the original Huh7 cells (4). To establish a cured cell line derived from Hep3B/miR122 cells for further improvement of HCVcc propagation, we first established HCV replicon cells in Hep3B/miR122 cells. *In vitro*-transcribed sub- or full-genomic HCV RNA of the JFH1 strain was electroporated into Hep3B/miR122 and

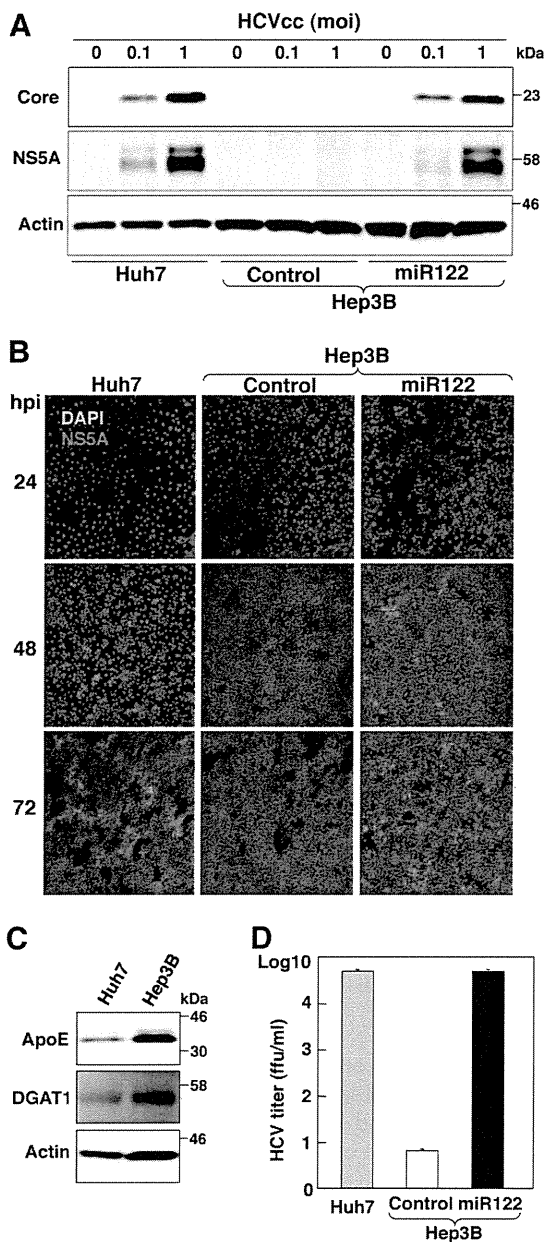


FIG 3 Establishment of a novel permissive cell line for robust propagation of HCVcc by expression of miR122 in Hep3B cells. (A) Huh7, Hep3B/Cont, and Hep3B/miR122 cells were infected with HCVcc at an MOI of 0.1 or 1, and the levels of expression of viral proteins were determined by immunoblotting using appropriate antibodies at 72 h postinfection. (B) Huh7, Hep3B/Cont, and Hep3B/miR122 cells were infected with HCVcc at an MOI of 1 and incubated with 1% methylcellulose in DMEM containing 5% FCS for the indicated time. Cells were fixed with 4% paraformaldehyde and subjected to indirect immunofluorescence assay using anti-NS5A antibody, followed by AF594-conjugated anti-rabbit IgG (red). Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; blue). (C) Huh7 and Hep3B cells were lysed and subjected to immunoblotting using appropriate antibodies. (D) Huh7, Hep3B/Cont, and Hep3B/miR122 cells were infected with HCVcc at an MOI of 1, the culture supernatants were collected at 72 h postinfection, and the viral titers of the supernatants were determined by focus-forming assay using Huh7.5.1 cells.

Hep3B/Cont cells, the cells were cultured with 400 μ g/ml of G418 for 1 month, and subgenomic replicon (SGR) and full-genomic replicon (FGR) cells were established. Hep3B/miR122 cells electroporated with viral RNA generated a large number of colonies, in contrast to the complete absence of colony formation in Hep3B/Cont cells (Fig. 4A). High levels of HCV RNA comparable to those in the Huh7 cells harboring SGR of the JFH1 strain were detected in Hep3B/miR122 cells harboring either SGR or FGR of the JFH1 strain (Fig. 4B, lower). Expression of NS5A was detected in all of the clones of Hep3B/miR122 cells harboring either SGR or FGR, and that of the core protein was detected in all of the FGR clones (Fig. 4B, upper). HCV core protein and RNA were shown to localize mainly on the lipid droplets and on the cytoplasmic face of ER, respectively (40, 61). Immunofluorescence analyses revealed that dsRNA was colocalized with calnexin, an ER marker, in both SGR and FGR cells and HCV core protein was colocalized with lipid droplets in the FGR cells, as previously described (Fig. 4C). Treatment of Hep3B/miR122 cells harboring an FGR of the JFH1 strain with either CsA or IFN- α decreased the expression of core protein in a dose-dependent manner (Fig. 4D), suggesting that the Hep3B/miR122 replicon cells can be used for screening antiviral compounds for HCV.

Elimination of HCV RNA from HCV replicon RNA from Hep3B/miR122 cells enhances propagation of HCVcc. To establish cured Hep3B/miR122 cells, five clones of the Hep3B/miR122 replicon cells harboring FGR of the JFH1 strain were treated with 100 IU/ml of IFN- α to eliminate viral RNA, and viral RNA was gradually decreased and completely eliminated at 20 days post-treatment (Fig. 5A, left). We then examined the sensitivity of the cured cell clones for propagation of HCVcc. All of the cured cell clones exhibited enhancement of propagation of HCVcc, especially clone 5, which achieved a level of replication of HCVcc more than 6-fold higher than that in the parental Hep3B/miR122 cells (Fig. 5A, right). To examine the effect of serial passage of HCVcc in the cured Hep3B/miR122 cells, HCVcc was inoculated into the cured cells at an MOI of 0.1, and the culture supernatants harvested at 4 days postinfection were reinoculated into the naïve cured cells (Fig. 5B). Infectious titers in the culture supernatants were continuously increased in accord with the number of passages (Fig. 5C). These results indicate that a novel cell line capable of complete propagation of HCVcc was established by the introduction of miR122 and the curing process, as in the case of Huh7 cells by using Hep3B cells.

Cured Hep3B/miR122 cells facilitate efficient propagation of HCVcc through enhanced expression of miR122. It has been reported that one of the reasons for the high susceptibility of the cured cell line Huh7.5 to the propagation of HCVcc is the disruption of the innate immune responses caused by mutation in RIG-I, a key sensor for viral RNA in the cytoplasm (57, 69). To examine the innate immune response in the cured Hep3B/miR122 cells, reporter plasmids encoding the luciferase gene under the control of either the IFN- β (Fig. 6A, left) or ISRE (Fig. 6A, right) promoter were transfected into the cured or parental Hep3B/miR122 cells and stimulated with poly(I-C), VSV, or IFN- α . Activation of these promoters in the cured Hep3B/miR122 cells was not impaired but rather was enhanced upon stimulation with poly(I-C) or VSV compared with that in the parental cells. To further assess the authenticity of viral RNA recognition and ISG induction pathways in the cured Hep3B/miR122 cells, nuclear localization of IRF3 and STAT2 upon stimulation was determined by immuno-