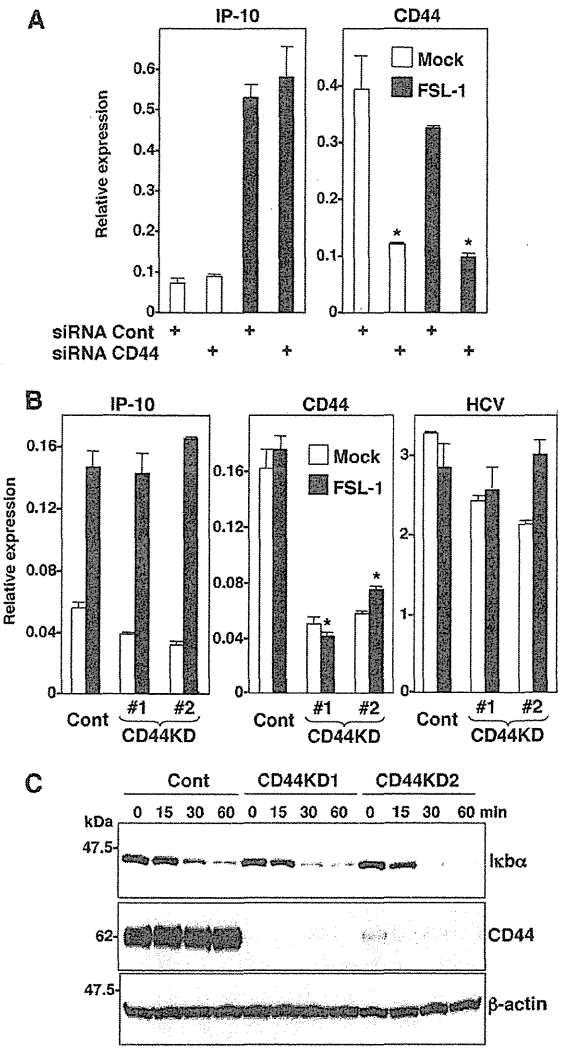

FIG 3 Effect of CD44 silencing on IP-10 production in replicon-harboring cells upon stimulation with TLR2 ligands derived from bacterial components. (A) HCV replicon-harboring cells were transfected with siRNA targeted to CD44 gene or control siRNA at a final concentration of 100 nM and stimulated with 1 μg/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 μg/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 μg/ml of FSL-1 for the times indicated, and expression of IκBα, CD44, and β-actin was determined by immunoblotting.

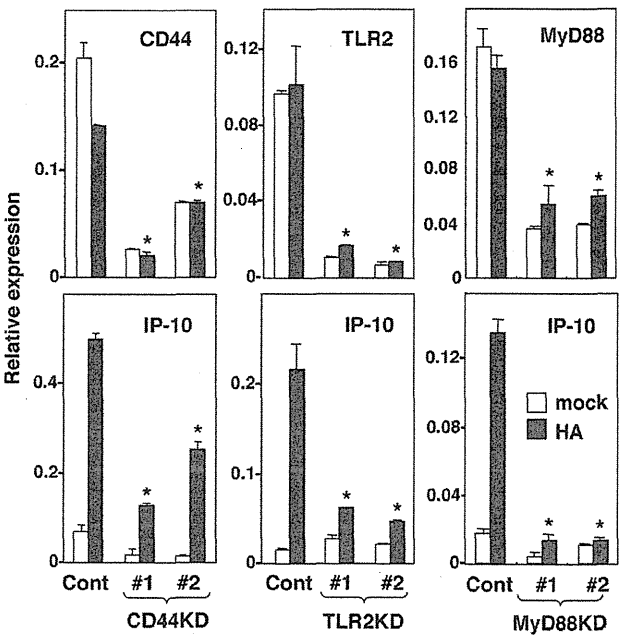

FIG 4 HA induces IP-10 production in human hepatoma cell lines through the TLR2-CD44-MyD88 axis. The stable knockdown cell clones based on the Huh7OK1 cells expressing siRNA targeted to the CD44, TLR2, or MyD88 gene (#1 and #2) or control siRNA (Cont) were stimulated with 500 μg/ml of HA. mRNA levels 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.

cludes ISRE and two NF-κB-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-κB-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 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 (CD44ΔTM; consists of amino acid residues from 1 to 223) but not a mutant missing the extracellular domain (CD44ΔEC; 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 (TLR2ΔTM; consists of amino acid residues from 1 to 587) but not a mutant missing the extracellular domain (TLR2ΔEC; consists of amino acid residues from 588 to 784) exhibits a weak but substantial interaction with CD44 (Fig. 7C), indicating that CD44

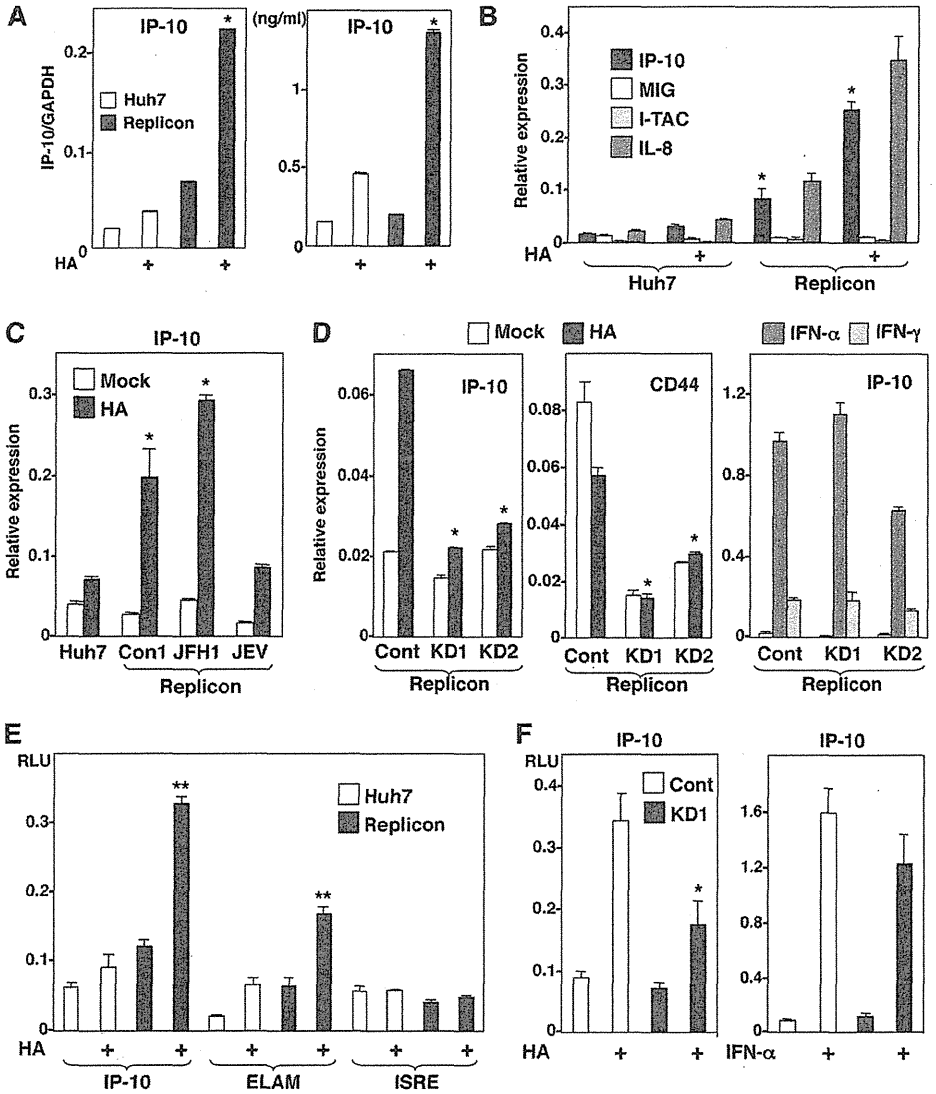

FIG 5 HA participates in IP-10 production in cells replicating HCV. (A) Huh7 and HCV replicon-harboring cells transfected with a plasmid encoding a FLAG-tagged TLR2 were stimulated with 500 μg/ml of HA, and IP-10 mRNA levels (left) and production of IP-10 in culture supernatants (right) 24 h after stimulation were determined by real-time PCR and sandwich ELISA, respectively. (B) Huh7 and HCV replicon-harboring cells were stimulated with 500 μg/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 μg/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 μg/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 μg/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 μg/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 (CD44ΔTM) and FLAG-tagged TLR2 (TLR2ΔTM) were expressed in insect cells. Purified samples were examined by Coomassie staining and immunoblotting (Fig. 7E). The CD44ΔTM applied in coats to the microplates exhibited binding to TLR2ΔTM but not to BSA in a dose-dependent manner (Fig. 7F, left). Furthermore, both PGN

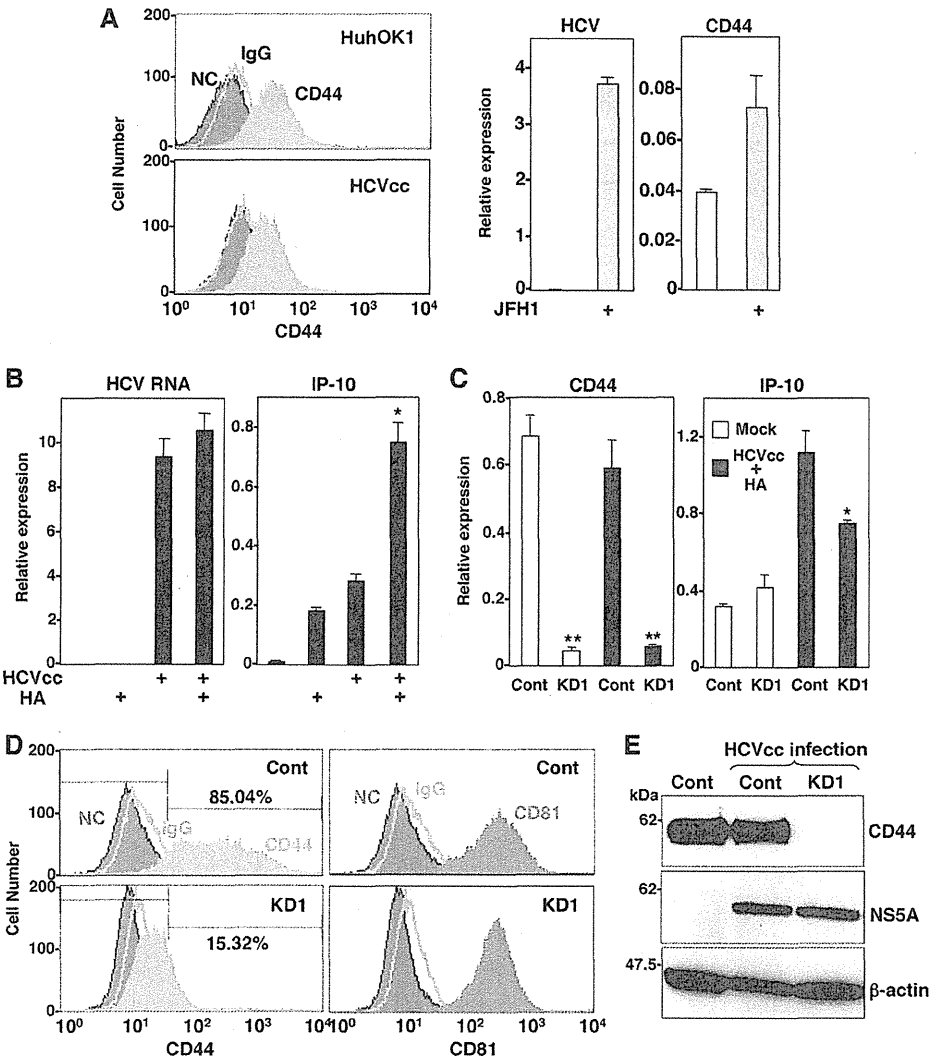

FIG 6 Enhancement of IP-10 production in HCVcc-infected cells upon stimulation with HA. (A) Left, cell surface expression of CD44 on Huh7OK1 cells infected with HCVcc at an MOI of 1 and incubated for 7 days was determined by using antibody that recognizes an ectodomain of CD44 (BU52 clones) and analyzed 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 μg/ml HA at 6 days posttransfection, and IP-10 mRNA and HCV RNA were determined at 24 h after stimulation. (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 μg/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 CD44ΔTM 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 circulating 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

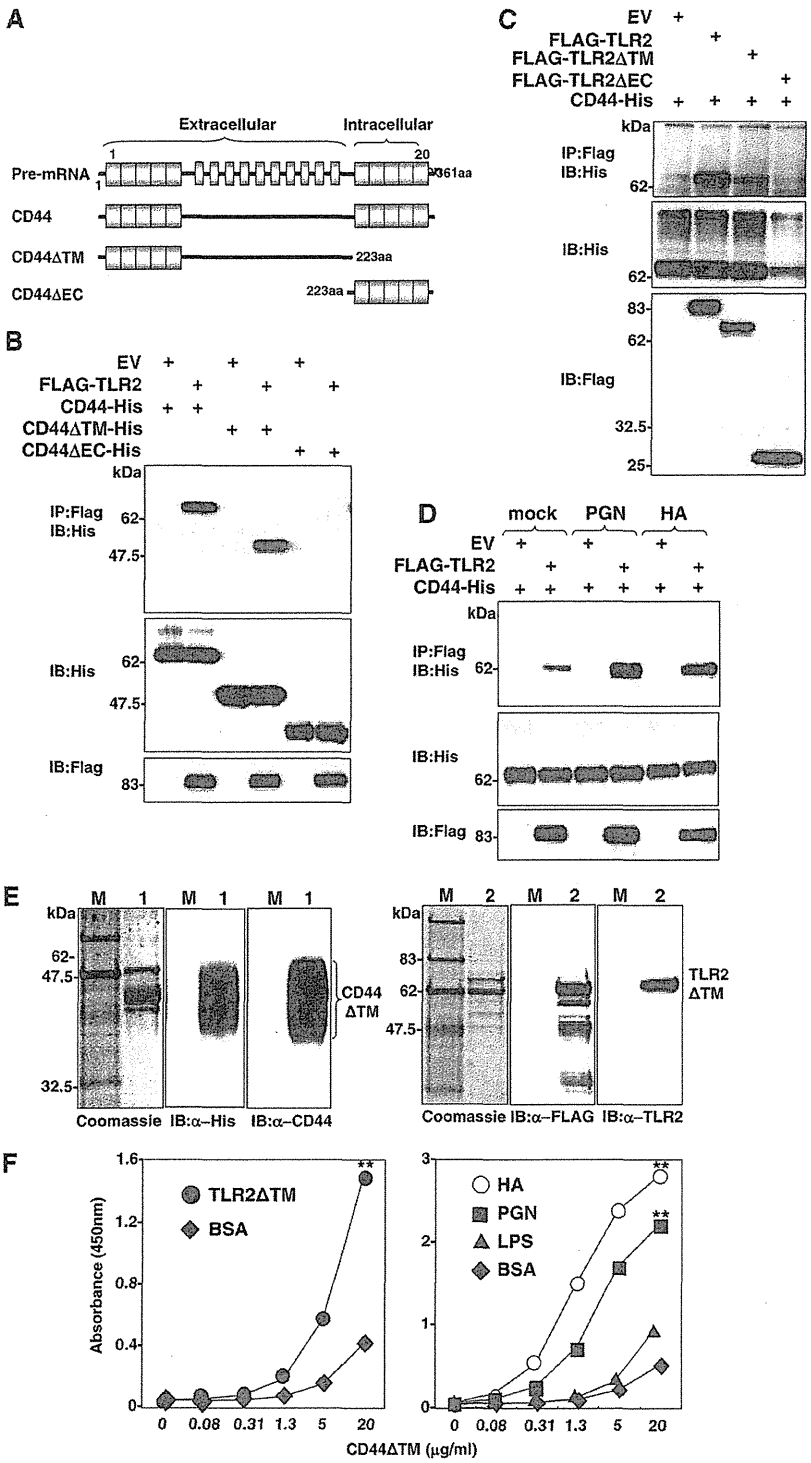

FIG 7 CD44 and TLR2 interact through their extracellular domains. (A) Structures of CD44 and its mutants used in this study. (B) FLAG-TLR2 was coexpressed with CD44-His, its mutants, or empty vector (EV) in 293T cells and immunoprecipitated with anti-FLAG antibody, and the precipitates were determined 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 μg/ml PGN or 500 μg/ml HA at 36 h posttransfection, and subjected to immunoprecipitation and immunoblotting using the appropriate antibodies at 24 h after stimulation. (E) Purified CD44ΔTM (lanes 1) and TLR2ΔTM (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 μg/ml TLR2ΔTM (closed circles), 50 μg/ml HA (open circles), 40 μg/ml PGN (closed squares), 40 μg/ml LPS (closed triangles), or 50 μg/ml BSA (closed diamonds) at 4°C overnight and then incubated with the indicated concentrations of CD44ΔTM at room temperature for 1 h. The binding of CD44ΔTM 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-κB axis, suggesting that the activation of NF-κB 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-κB-binding elements in the promoter, and the activation of IP-10 by stimulation with HA was mainly regulated by NF-κB 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-κB 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. pIFNβ-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% CO_2. 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 μg/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 μg/ml streptomycin, and 10% FCS. Hep3B replicon cells harboring the subgenomic HCV RNA were maintained in DMEM containing 10% FCS, NEAA, and 400 μg/ml G418 (Nakalai Tesque, Kyoto, Japan).

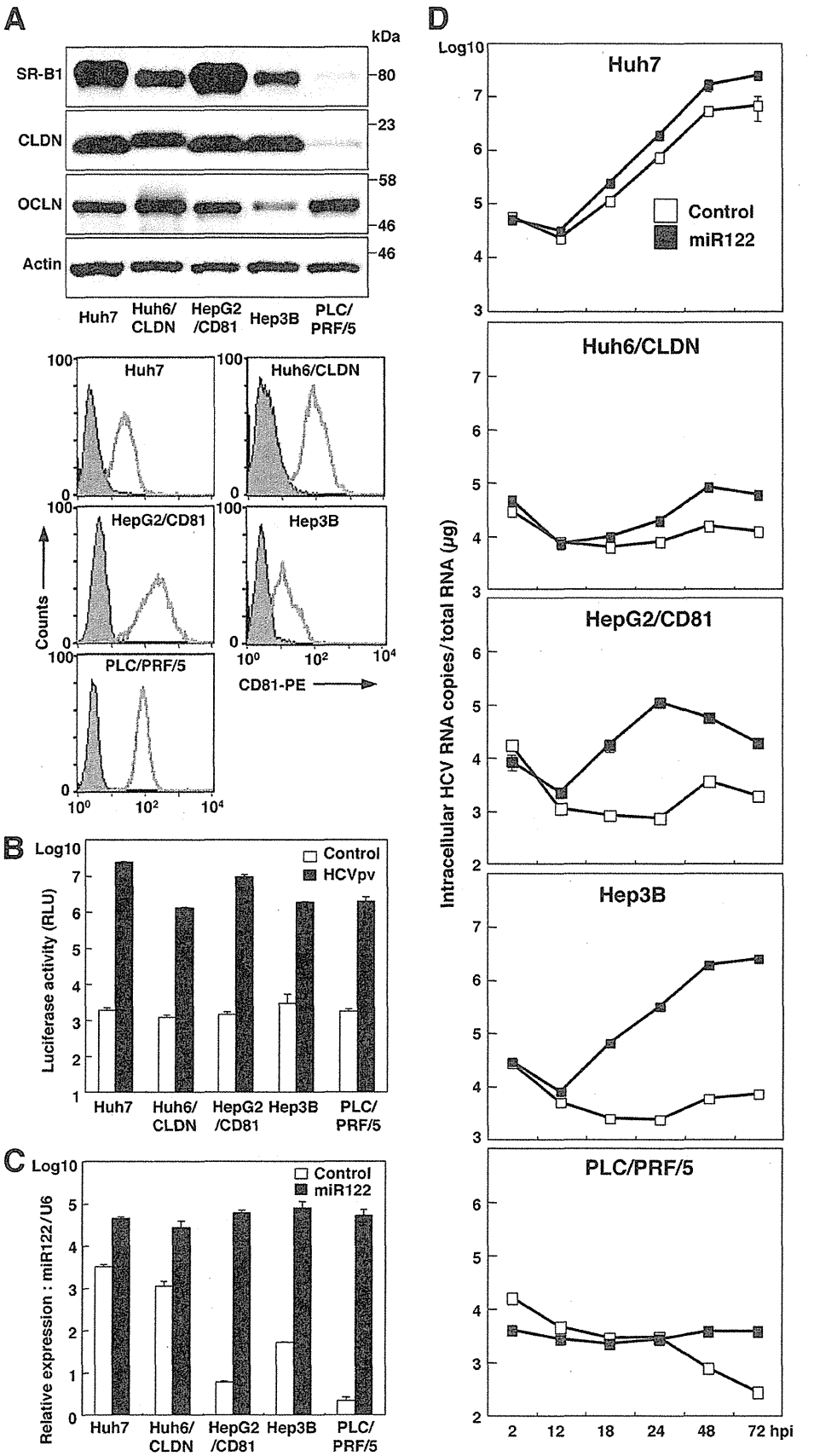
Viruses. pHH-JFH1-E2p7NS2mt was transfected into Huh7.5.1 cells, and the culture supernatants were collected after serial passages. The infectivity of HCVcc was determined by focus-forming assay and expressed in focus-forming units (FFU) (64). The lentiviral vectors and ViraPower lentiviral packaging 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-μm-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-α2 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′-CcAttCTgaCcCtAC-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 10^6 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 5×10^4 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 control 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.

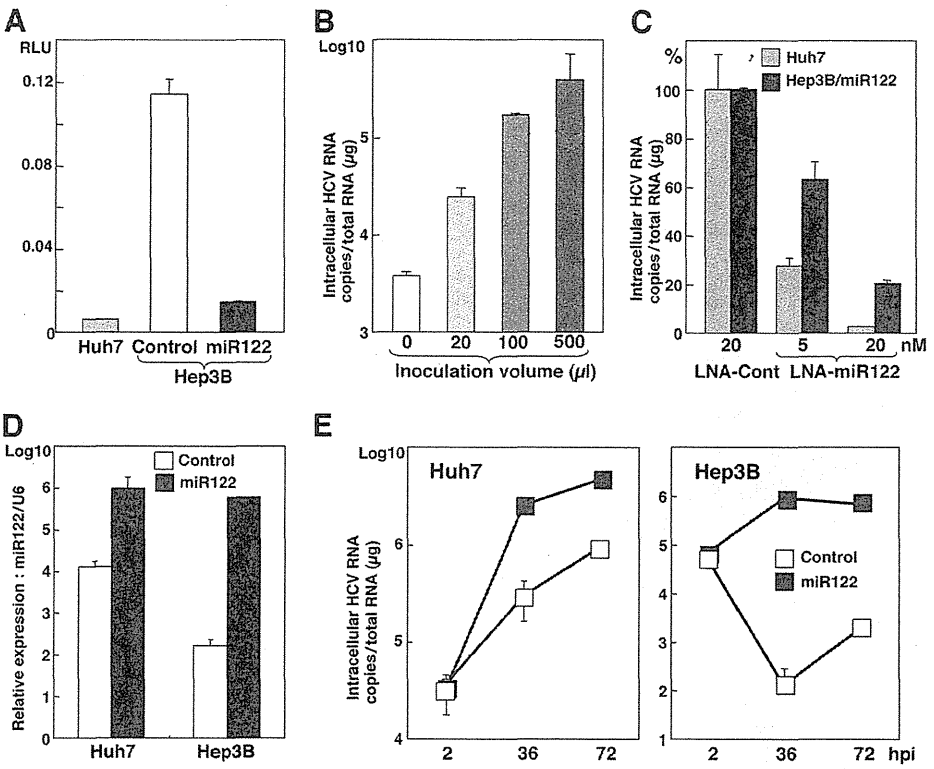


FIG 2 Expression of biologically active miR122 facilitates replication of HCVcc in Hep3B cells. (A) Huh7, Hep3B/Cont, and Hep3B/miR122 cells were transfected with pmirGLO-miR122comp, and luciferase activity was determined at 24 h posttransfection. (B) Hep3B cells were transduced with the lentiviral vector expressing miR122 in a dose-dependent manner and then infected with HCVcc at an MOI of 1 at 48 h postransduction. 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

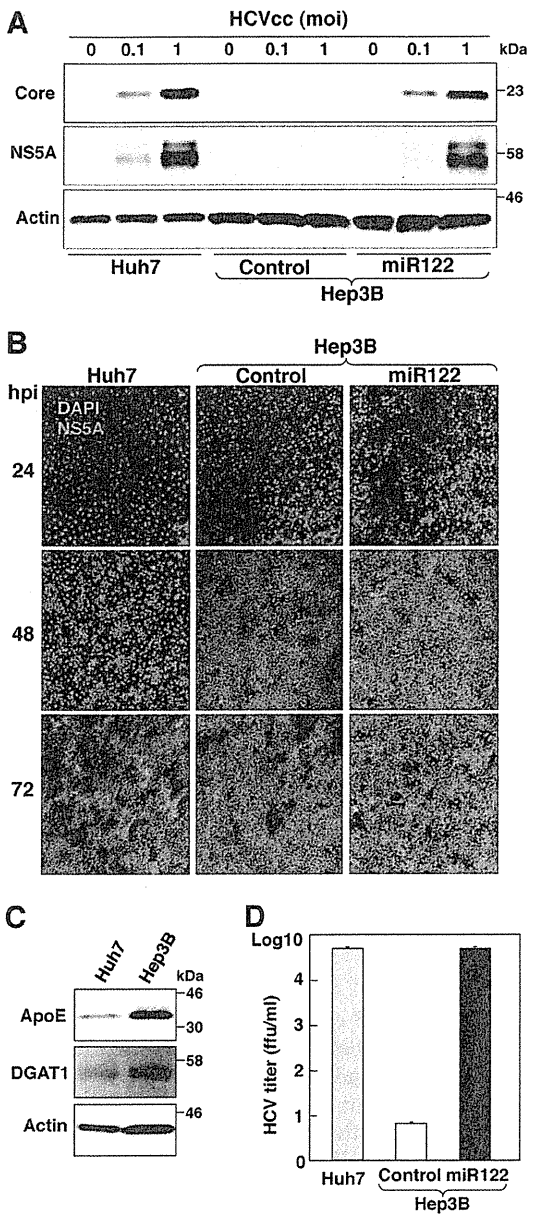


FIG 3 Establishment of a novel permissive cell line for robust propagation of HCVcc by expression of miR122 in Hep3B cells. (A) Huh7, Hep3B/Cont, and Hep3B/miR122 cells were infected with HCVcc at an MOI of 0.1 or 1, and the levels of expression of viral proteins were determined by immunoblotting using 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 μg/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 posttreatment (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-

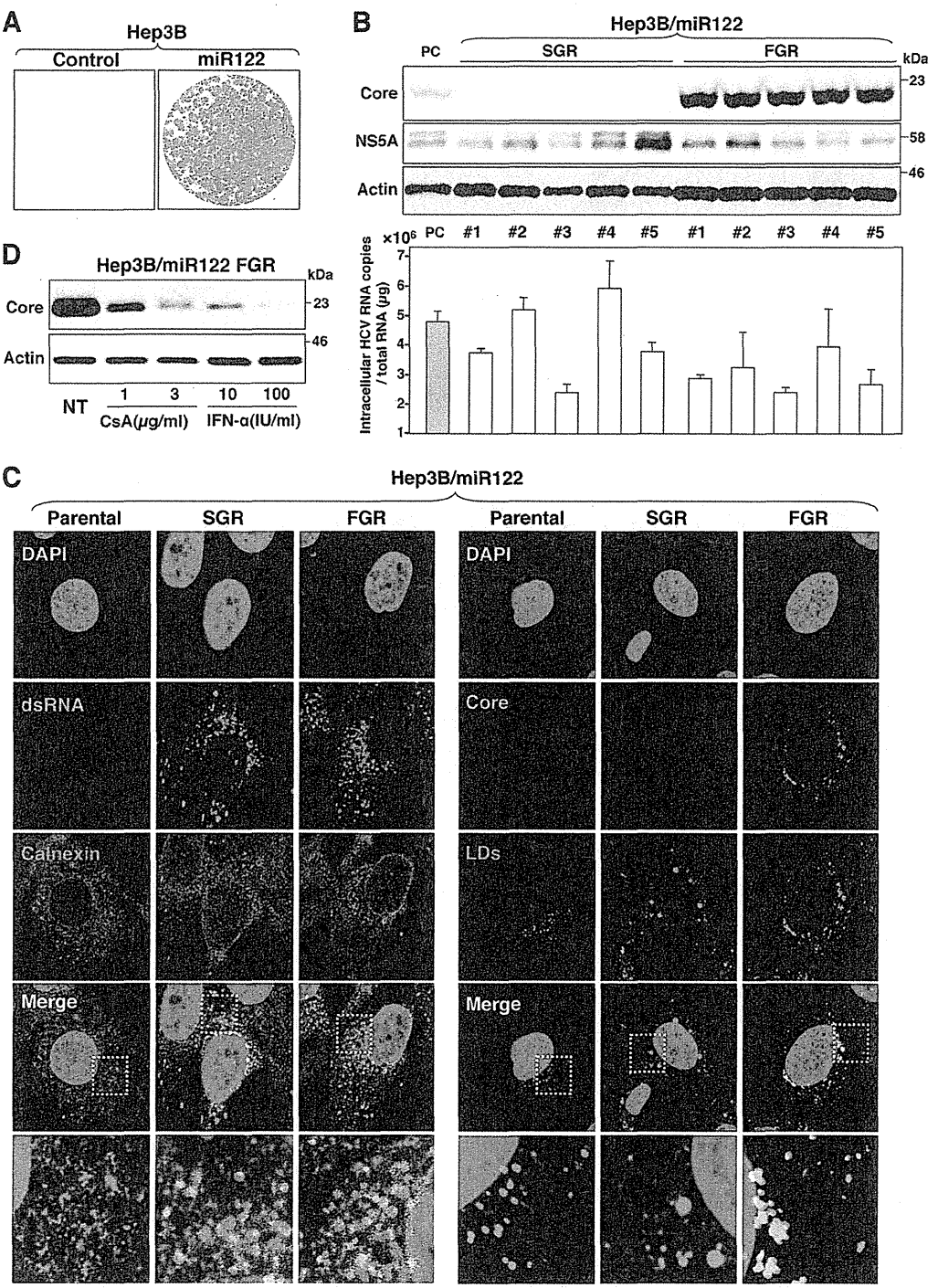


FIG 4 Establishment of an HCV RNA replicon in Hep3B/miR122 cells. (A) Full-genomic replicon RNA of HCV was electroporated into Hep3B/Cont and Hep3B/miR122 cells, and the medium was replaced with DMEM containing 10% FCS and 400 μg/ml G418 at 24 h posttransfection. Colony formation was determined as indicated in Materials and Methods. (B) (Upper) Sub- and full-genomic HCV replicons (SGR and FGR) in Hep3B/miR122 cells were subjected to immunoblotting using the appropriate antibodies. Huh7.5.1 cells infected with HCVcc were used as a positive control (PC). (Lower) Intracellular HCV copy number in replicon clones. SGR in Huh7 cells was used as a positive control. (C) SGR and FGR in Hep3B/miR122 cells were fixed with 4% paraformaldehyde and subjected to indirect immunofluorescence assay using the appropriate antibodies. Lipid droplets (LDs) were stained red with BODIPY. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (blue). The boxed regions in the merged images are magnified. (D) Hep3B/miR122 FGR cells were treated with DMEM containing 10% FCS and the indicated concentrations of CsA and IFN-α and then subjected to immunoblotting using appropriate antibodies at 48 h posttransfection. NT, no treatment.

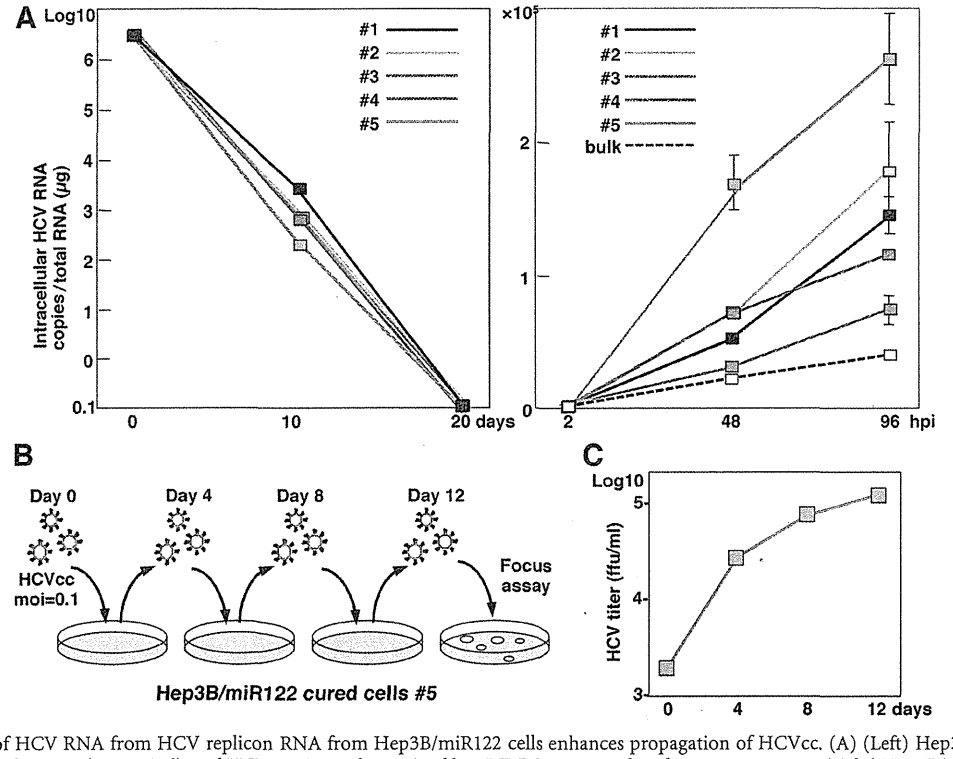


FIG 5 Elimination of HCV RNA from HCV replicon RNA from Hep3B/miR122 cells enhances propagation of HCVcc. (A) (Left) Hep3B/miR122 FGR cell clones were treated with IFN-α (100 IU/ml), and HCV RNA was determined by qRT-PCR at 10 and 20 days posttreatment; (right) Hep3B/miR122 parental cells (bulk) and the cured cells were infected with HCVcc at an MOI of 0.1, and HCV RNA was determined by qRT-PCR at 48 and 96 h postinfection. (B) Schematic diagram of the experimental procedure for serial passage of HCVcc in Hep3B/miR122 cured cells. The cured cells were infected with HCVcc at an MOI of 0.1. (C) The infectious titers in the culture supernatants of the Hep3B/miR122 cured cells were determined at the indicated time points by focus-forming assay using Hep3B/miR122 cells.

fluorescence analysis. IRF3 and STAT2 in both cured and parental Hep3B/miR122 cells were translocated into the nucleus upon stimulation with VSV and IFN-α, respectively (Fig. 6B). These results suggest that the efficient propagation of HCVcc in the cured Hep3B/miR122 cells might be attributable to reasons other than impairment of the innate immune response. Therefore, we hypothesized that the Hep3B/miR122 cells harboring the HCV genome are capable of surviving in the presence of a high concentration of G418 by amplification of the viral genome through enhancement of miR122 expression and that once HCV RNA was eliminated, the cured cells would acquire the ability to propagate HCV due to the high expression of miR122. To test this hypothesis, the levels of miR122 in both Huh7- and Hep3B/miR122-derived cured cells were compared with those in the parental cells. Intriguingly, both cured cell lines exhibited a significant increase of miR122 expression (approximately 2- to 6-fold) in comparison with that in the parental cells (Fig. 6C). These results suggest that the efficient propagation of HCVcc in the cured Hep3B/miR122 cells was partially attributable to an enhanced expression of miR122, rather than an impairment of the signaling pathway of innate immunity.

Specific interaction of miR122 with viral RNA is crucial for efficient propagation of HCVcc. To evaluate the effect of a specific interaction of miR122 with the target sequence in the 5′ UTR of HCV RNA on the enhancement of viral propagation, we generated two mutant pre-miR122s: sMT-miR122 has a substitution of uridine to adenosine, and dMT-miR122 carries an additional complementary substitution of adenosine to uridine to stabilize the expression. These substitutions have been shown to abrogate interaction with the target sequence (27) (Fig. 7A). A high level of expression of dMT-miR122 comparable to that of WT-miR122 was detected in Hep3B cells, in contrast to the low level of expression of sMT-miR122 (Fig. 7B). As described above, the expression level of miR122 in Hep3B cells was significantly lower than that in Huh7 cells (Fig. 1B). Taking advantage of this low level of miR122 expression, WT-miR122 and dMT-miR122 were exogenously expressed in Hep3B cells by the lentiviral vector to assess the importance of the specific interaction of miR122 with viral RNA. Not only intracellular viral RNA levels but also infectious titers in the culture supernatants were enhanced by the expression of WT-miR122, but they were not enhanced by the expression of dMT-miR122 (Fig. 7C and D). These results suggest that specific interaction of miR122 with the 5′ UTR of HCV is crucial for the efficient replication and propagation of HCV.

DISCUSSION

Most miRNAs utilize the normal RNA interfering pathway and repress translation of the target mRNAs (3, 26). For instance, miR122 targets the 3′ UTR of the cytoplasmic polyadenylation element binding protein (CPEB) (5), hemochromatosis (*Hfe*) and hemojuvelin (*Hjv*) (6), a disintegrin and metalloprotease family 10 (ADAM10) (2), and cationic amino transporter 1 (CAT-1) (8) and represses their translation. In contrast, HCV uniquely exploits the liver-specific miR122 to stimulate viral translation (23, 27–29,

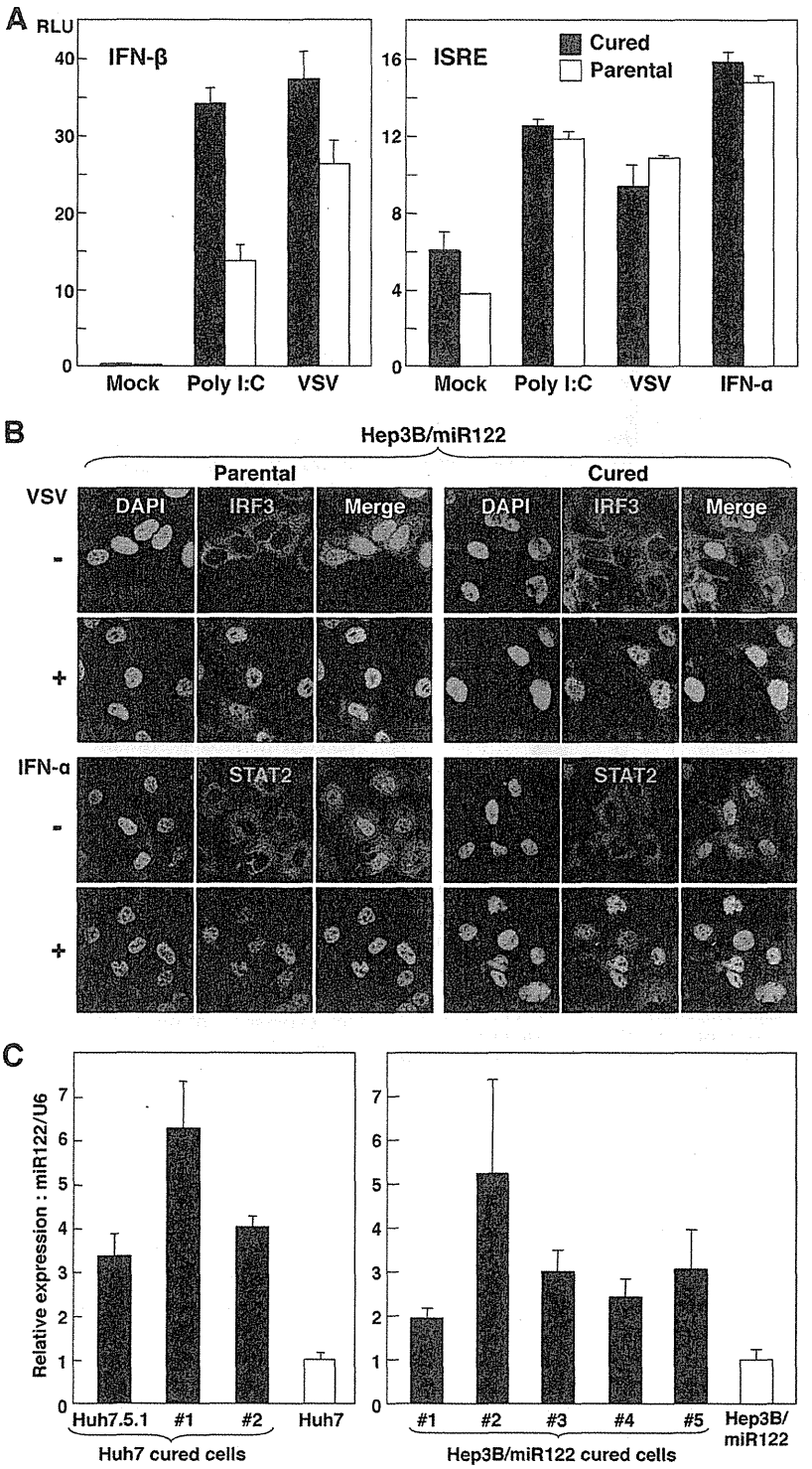


FIG 6 Cured Hep3B/miR122 cells facilitate efficient propagation of HCVcc through enhanced expression of miR122. (A) (Left) Hep3B/miR122 parental cells and cured cells of clone 5 were cotransfected with pIFNβ-Luc and pRL-TK and then infected with the VSV NCP mutant at an MOI of 0.01 or transfected with 1 μg of poly(I·C) at 24 h posttransfection, and luciferase activities were determined at 48 h posttreatment; (right) the cells were cotransfected with pISRE-Luc and pRL-TK and then infected with VSV at an MOI of 0.01 or treated with IFN-α (100 IU/ml) at 24 h posttransfection, and luciferase activities were determined at 48 h posttreatment. (B) (Upper) Hep3B/miR122 parental cells and the cured cells were infected with VSV at an MOI of 0.01, fixed with 4% phosphonoformic acid at 18 h postinfection, and subjected to indirect immunofluorescence assay using rabbit anti-IRF3 antibody, followed by AF488-conjugated anti-rabbit IgG (red); (lower) the cells were treated with IFN-α (100 IU/ml), fixed with 4% paraformaldehyde at 1 h postinfection, and subjected to indirect immunofluorescence assay using rabbit anti-STAT2 antibody, followed by AF488-conjugated anti-rabbit IgG (red). Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (blue). (C) Total RNA was extracted from parental Huh7 and Hep3B/miR122 cells and their cured cells, and the relative expression of miR122 was determined by qRT-PCR by using U6 snRNA as an internal control.

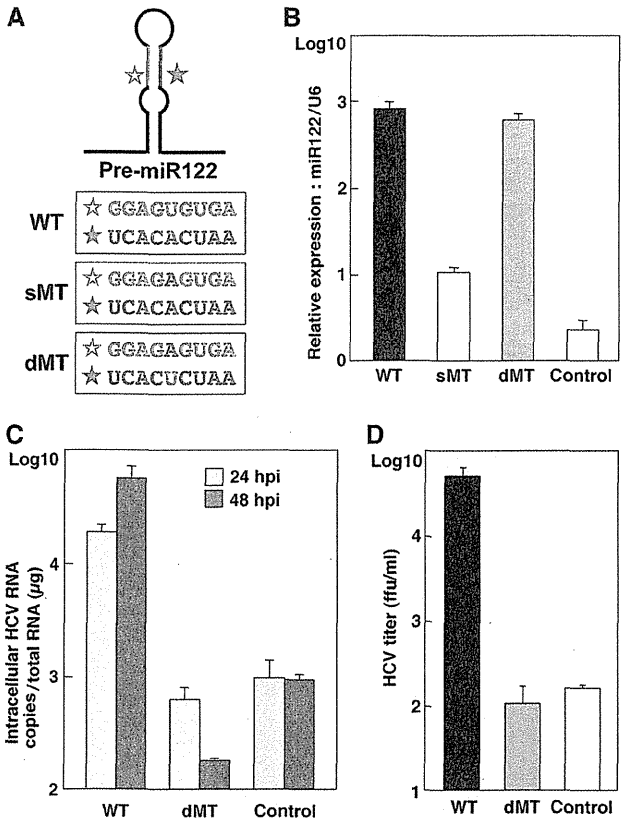


FIG 7 Specific interaction of miR122 with viral RNA is crucial for efficient propagation of HCVcc. (A) Diagram of pre-miR122 and partial nucleotide sequences of wild type (WT) miR122 and mutant miR122 carrying a single mutation (sMT) and double mutations (dMT). (B) Hep3B cells were transduced with lentiviral vectors expressing either WT-, sMT-, or dMT-miR122 or with a control, and the relative expression of miR122 was determined by qRT-PCR by using U6 snRNA as an internal control. (C) Hep3B cells expressing WT- or dMT-miR122 or the control cells were infected with HCVcc at an MOI of 1, and the level of HCV RNA was determined by qRT-PCR at 24 and 48 h postinfection. (D) 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.

38, 52). In this study, we assessed the possibility of establishment of human liver cell lines that are susceptible to HCVcc propagation through exogenous expression of miR122 by a lentiviral vector. Although Huh7 cells and their derived cell lines are highly susceptible to propagation of HCVcc, they intrinsically express an abundant amount of miR122. Among the cell lines that we investigated, Hep3B cells exhibit a high sensitivity to HCVcc propagation by expression of miR122 compared to that of Huh7 cells, whereas no sensitivity to HCVcc was observed in the parental Hep3B cells. Therefore, the Hep3B cell line was suggested to be an ideal tool to investigate miR122 function in the life cycle of HCV.

RNA viruses replicate in host cells with high error rates, generating a broad population diversity, which allows rapid adaptation to new environments (33). HCV propagates in the liver of patients with quasispecies heterogeneity and transmits to a new host through contaminated blood or blood products (16). It is known that the complexity of HCV clones significantly decreases during transmission through a genetic bottleneck, resulting in a more homogeneous population. This selection of certain clones is mainly caused by the host factors required for viral replication and immune pressure in a new host and is involved in the early phase of HCV infection in the new environment (18, 25, 32). A sole cell line, Huh7, has been employed in most of the experiments for *in vitro* studies of entry, RNA replication, and particle formation of HCV. Therefore, it has not been possible to assess propagation of HCVcc in human liver cell lines other than Huh7 cells and transmission of HCVcc to liver cell lines of different origins. The establishment of a novel human liver cell line, Hep3B/miR122, for propagation of HCVcc would help to generate new insights into the mutual interaction between HCV and human hepatocytes. Although we are not able to evaluate the effects of the acquired immunity on the induction of the adaptive mutations in cell culture systems, we can assess the host factors involved in the generation of the adaptive mutations by using two different human liver cell lines that support continuous propagation of HCVcc. Further studies are needed to determine the adaptive mutations in the HCV genome by passage in either Hep3B/miR122 or Huh7 cells and in one after the other.

At least seven major HCV genotypes and numerous subtypes have been identified (21), but laboratory strains capable of replicating *in vitro* are limited (36, 64, 68, 70). It is important to establish cell lines that permit the complete propagation of a wide range of HCV genotypes for further understanding of the life cycle of HCV. Although the partial replication of serum-derived HCV in primary hepatocytes in a specialized culture system has been reported (50), development of a simpler and more user-friendly system is required for promotion of research on HCV. It might be feasible to establish new cell culture systems for not only various genotypes of infectious HCV clones but also serum-derived HCV by the expression of miR122 in various human liver cell lines.

While preparing the manuscript, Narbus et al. reported that the expression of miR122 enhances HCV replication in HepG2/CD81 cells (46). Our data also demonstrated that the expression of miR122 increased HCV replication in HepG2/CD81 cells, as shown in Fig. 1D. However, the impact of miR122 expression on the production of infectious particles in HepG2/CD81 cells is significantly lower than that in Huh7 cells (46). Although LH86 (71) and Li23 (30) cell lines derived from human hepatocellular carcinoma have been shown to permit propagation of HCVcc, these cell lines are not well characterized. In contrast, the Hep3B cell line has been utilized in a wide range of research fields for a long time, resulting in the accumulation of many sources of data from genomic and proteomic analyses (1, 47, 55, 63, 67). Moreover, the Hep3B cell line is available from the major cell banks all over the world, which should readily allow reevaluation of the findings in this study. Comparison of the experimental data on HCVcc propagation between Huh7 and Hep3B/miR122 cells might provide a clue to understanding the host factors crucial for the efficient propagation of HCV in human liver cells.

The higher susceptibility to HCVcc propagation of the cured cells derived from Huh7 cells than the parental cells was suggested to be attributable to impairment of the innate immune response (57). However, this is not the only reason for efficient propagation of HCVcc in the Huh7-based cured cell lines (17). It has been shown that cured cell lines, such as Huh7.5.1 and Huh7-Lunet, express a higher level of miR122 than the parental Huh7 cells (13), suggesting that upregulation of miR122 in the cured cells participates in the efficient propagation of HCVcc. However, the level of

miR122 expression in the cured Hep3B cells was not necessarily correlated with the replication efficiency of HCVcc in the present work (Fig. 6C). Most recently, Denard et al. reported that the expression of CREB3L1/OASIS, which specifically prevents division of virus-infected cells, in cured Huh7 cells was reduced compared to that in the parental cells (12), suggesting that CREB3L1/OASIS is also involved in the enhancement of HCVcc propagation in the cured cells.

In this study, we have shown that expression of miR122 confers susceptibility to human liver cell lines for the efficient propagation of HCVcc. Elimination of the HCV genome from the replicon cells of Hep3B/miR122 cells enhanced propagation of HCVcc in accord with the increment of miR122 expression, and propagation of HCVcc in the cured cells was continuously increased in every passage. Furthermore, the interaction between HCV RNA and miR122 was shown to be specific for production of infectious particles in Hep3B/miR122 cells. The establishment of a new permissive cell line for HCVcc allows us not only to investigate the biological function of miR122 on the life cycle of HCV but also to develop novel therapeutics for chronic hepatitis C.

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REVIEW

Role of miR-122 and lipid metabolism in HCV infection

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Abstract Hepatitis C virus (HCV) exhibits a narrow host range and a specific tissue tropism. Mice expressing major entry receptors for HCV permit viral entry, and therefore the species tropism of HCV infection is considered to be reliant on the expression of the entry receptors. However, HCV receptor candidates are expressed and replication of HCV-RNA can be detected in several nonhepatic cell lines, suggesting that nonhepatic cells are also susceptible to HCV infection. Recently it was shown that the exogenous expression of a liver-specific microRNA, miR-122, facilitated the efficient replication of HCV not only in hepatic cell lines, including Hep3B and HepG2 cells, but also in nonhepatic cell lines, including Hec1B and HEK-293T cells, suggesting that miR-122 is required for the efficient replication of HCV in cultured cells. However, no infectious particle was detected in the nonhepatic cell lines, in spite of the efficient replication of HCV-RNA. In the nonhepatic cells, only small numbers of lipid droplets and low levels of very-low-density lipoprotein-associated proteins were observed compared with findings in the hepatic cell lines, suggesting that functional lipid metabolism participates in the assembly of HCV. Taken together, these findings indicate that miR-122 and functional lipid metabolism are involved in the tissue tropism of HCV infection. In this review, we would like to focus on the role of miR-122 and lipid metabolism in the cell tropism of HCV.

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Introduction

More than 170 million individuals worldwide are chronically infected with hepatitis C virus (HCV), and the cirrhosis and hepatocellular carcinoma (HCC) induced by HCV infection are life-threatening diseases [1]. On the other hand, HCV infection sometimes induces extra-hepatic manifestations (EHM), including mixed cryoglobulinemia and non-Hodgkin lymphoma [2–5]. The mechanisms of the pathogenesis and cell tropism of HCV have not been fully elucidated yet owing to the lack of an appropriate infection model. Although chimpanzees are susceptible to HCV infection, the use of these animals to study experimental infection is ethically problematic, and no other animal model with susceptibility to HCV infection has been established [6]. Furthermore, robust in vitro HCV propagation has been limited to the combination of cell-culture-adapted clones based on the genotype 2a JFH1 strain (HCVcc) and human liver cancer-derived Huh7 cells [7, 8]. The expression of a liver-specific microRNA, miR-122, has been shown to dramatically enhance the translation and replication of HCV-RNA [9]. Recently, several reports have shown that the exogenous expression of miR-122 facilitates the efficient replication of viral RNA in several hepatic and nonhepatic cell lines [10–13]. Of note, the clinical application of a specific inhibitor of miR-122 to chronic hepatitis C patients is now in progress [14]. In addition, it has been shown that liver-specific expression of very-low-density lipoprotein (VLDL)-associated proteins is involved in the assembly of infectious HCV particles [15, 16]. This review will focus on the role of miR-122 expression and lipid metabolism in HCV infection.

microRNA and virus infection

miRNAs were first identified by Lee et al. [17] and since that time a great number of miRNAs have been registered in the miRNA database. miRNA incorporated into RNA-induced silencing complex (RISC) interacts with a target mRNA via a specific recognition element. RISC contains argonaute 2 (Ago2), Dicer, and TAR RNA binding protein (TRBP) [18, 19]. In humans, Ago2 plays a pivotal role in the repression of translation of target genes [20]. It is now commonly believed that miRNAs play important roles in cell homeostasis, and that abnormality of miRNA expression participates in the development of several diseases, including viral infections [18, 19]. miRNAs encoded by Epstein–Barr virus (EBV) were identified in 2004 [4, 21], and over 200 viral miRNAs have been reported in several DNA viruses, especially in herpesviruses [22, 23]. Previous reports have shown that viral miRNAs participate in viral propagation by regulating the host gene expression [22, 23]. Many viral miRNAs suppress the host gene expression involved in innate and acquired immunities and enhance viral propagation [22, 24, 25]. Most RNA viruses replicate in the cytoplasm, and thus it had been believed that RNA viruses do not encode viral miRNAs. Rouha et al. [26] showed that an RNA virus, the tick-borne encephalitis virus, is capable of producing functional miRNA by the insertion of an miRNA element into viral RNA. Actually, it has been shown that virus-derived small RNAs emerge by infection with RNA viruses, including influenza virus and West Nile virus [27, 28]. These data suggest that both viral-encoded and host gene-derived miRNAs are involved in the regulation of viral propagation.

Liver-specific microRNA, miR-122

miR-122 is a liver-specific microRNA and is the microRNA most abundantly expressed in the liver [29–31]. Although Li et al. [32] have suggested that hepatocyte nuclear factor 4 alpha (HNF4A) positively regulates the expression of miR-122, the details on the tissue specificity of miR-122 expression have not been fully elucidated yet. miR-122 targets the 3′untranslated region (3′UTR) of the mRNAs of cytoplasmic polyadenylation element binding protein (CPEB), hemochromatosis (Hfe), hemojuverin (Hjv), disintegrin, and metalloprotease family 10 (ADAM10) and represses their translation [33–35]. miR-122 activates the translation of p53 mRNA through the suppression of CPEB and participates in cellular senescence [33]. Through the inhibition of Hfe and Hjv, miR-122 participates in iron metabolism [34]. Esau et al. [36] showed that miR-122 positively regulated lipid metabolism through the reduction of the mRNAs of lipid-associated proteins, and that inhibition of miR-122 expression attenuated liver steatosis in high-fat-fed mice, suggesting that miR-122 may be an attractive therapeutic target for metabolic diseases. miR-122 has also been shown to be involved in the propagation of hepatitis viruses, including hepatitis B virus (HBV) and HCV [9, 37, 38]. Wang et al. [38] have revealed that miR-122 suppresses cyclin G1, and this factor is known to enhance the replication of HBV by inhibiting the binding of p53 to HBV enhancer elements. In other reports, a low level of miR-122 expression in plasma was significantly associated with the incidence of HBV-related HCC [39]. These results suggest that miR-122 expression inhibits the propagation and pathogenesis of HBV. On the other hand, miR-122 expression enhances the propagation of HCV through genetic interaction with the 5′UTR of the HCV genome [9]. It is interesting to note that the effects of miR-122 expression on viral propagation are different between HBV and HCV.

miR-122 expression and HCV infection (Fig. 1)

Jopling et al. [9] reported for the first time that the inhibition of miR-122 dramatically decreased RNA replication in HCV replicon cells harboring subgenomic (SGR) or fullgenomic (FGR) viral RNA. They identified the 21 nucleotide (nt) of the miR-122 binding site in the 5′ end of the 5′UTR of HCV RNA. In addition, lack of enhancement of HCV replication by the expression of a mutant miR-122 incapable of binding to the 5′UTR was canceled by the introduction of a complementary mutation in the 5′UTR, suggesting that direct interaction of miR-122 with the 5′UTR is crucial for the enhancement of HCV replication. In subsequent reports, they identified a second adjacent miR-122 binding site in the 5′UTR [40]. Furthermore, ectopic expression of the mutant miR-122 rescued the replication of an HCV RNA possessing mutations in both miR-122 binding sites, suggesting that the interaction of miR-122 with both sites in the 5′UTR is required to augment viral replication. In addition, Machlin et al. [41] have revealed that not only the seed sequence but also nucleotides located at the positions of 15 and 16 in miR-122 are required for the enhancement of HCV replication. Interestingly, nucleotides 15 and 16 are not required for the conventional microRNA function of miR-122, suggesting that the conventional machinery of miR-122 is not involved in the miR-122-dependent enhancement of HCV replication. A recent study showed that the interaction of miR-122 with the 5′UTR of HCV was also required for the efficient production of infectious particles in cell culture [42].

Although the precise mechanisms of the miR-122-mediated enhancement of HCV replication have not been