

## REFERENCES

- Abe T, et al. 2007. Hepatitis C virus nonstructural protein 5A modulates the toll-like receptor-MyD88-dependent signaling pathway in macrophage cell lines. *J. Virol.* 81:8953–8966.
- Boisvert J, et al. 2003. Liver-infiltrating lymphocytes in end-stage hepatitis C virus: subsets, activation status, and chemokine receptor phenotypes. *J. Hepatol.* 38:67–75.
- Butera D, et al. 2005. Plasma chemokine levels correlate with the outcome of antiviral therapy in patients with hepatitis C. *Blood* 106:1175–1182.
- Casrouge A, et al. 2011. Evidence for an antagonist form of the chemokine CXCL10 in patients chronically infected with HCV. *J. Clin. Invest.* 121:308–317.
- Diago M, et al. 2006. Association of pretreatment serum interferon gamma inducible protein 10 levels with sustained virological response to peginterferon plus ribavirin therapy in genotype 1 infected patients with chronic hepatitis C. *Gut* 55:374–379.
- Dolganic A, et al. 2003. Hepatitis C virus core and nonstructural protein 3 proteins induce pro- and anti-inflammatory cytokines and inhibit dendritic cell differentiation. *J. Immunol.* 170:5615–5624.
- Dolganic A, et al. 2004. Hepatitis C core and nonstructural 3 proteins trigger Toll-like receptor 2-mediated pathways and inflammatory activation. *Gastroenterology* 127:1513–1524.
- Ebihara T, Shingai M, Matsumoto M, Wakita T, Seya T. 2008. Hepatitis C virus-infected hepatocytes extrinsically modulate dendritic cell maturation to activate T cells and natural killer cells. *Hepatology* 48:48–58.
- Guechot J, et al. 2000. Prognostic value of serum hyaluronan in patients with compensated HCV cirrhosis. *J. Hepatol.* 32:447–452.
- Güntherth U, et al. 1998. Functional involvement of CD44, a family of cell adhesion molecules, in immune responses, tumour progression and haematopoiesis. *Adv. Exp. Med. Biol.* 451:43–49.
- Güntherth U, et al. 1995. Are CD44 variant isoforms involved in human tumour progression? *Cancer Surv.* 24:19–42.
- Harvey CE, et al. 2003. Expression of the chemokine IP-10 (CXCL10) by hepatocytes in chronic hepatitis C virus infection correlates with histological severity and lobular inflammation. *J. Leukoc. Biol.* 74:360–369.
- Helbig KJ, et al. 2009. Differential expression of the CXCR3 ligands in chronic hepatitis C virus (HCV) infection and their modulation by HCV in vitro. *J. Virol.* 83:836–846.
- Helbig KJ, et al. 2004. Expression of the CXCR3 ligand I-TAC by hepatocytes in chronic hepatitis C and its correlation with hepatic inflammation. *Hepatology* 39:1220–1229.
- Herrlich P, et al. 1998. How tumor cells make use of CD44. *Cell. Adhes. Commun.* 6:141–147.
- Heydtmann M, Adams DH. 2009. Chemokines in the immunopathogenesis of hepatitis C infection. *Hepatology* 49:676–688.
- Itoh Y, et al. 2001. Clinical significance of elevated serum interferon-inducible protein-10 levels in hepatitis C virus carriers with persistently normal serum transaminase levels. *J. Viral. Hepat.* 8:341–348.
- Jiang D, et al. 2005. Regulation of lung injury and repair by Toll-like receptors and hyaluronan. *Nat. Med.* 11:1173–1179.
- Jiang D, Liang J, Noble PW. 2007. Hyaluronan in tissue injury and repair. *Annu. Rev. Cell Dev. Biol.* 23:435–461.
- Kaimori A, et al. 2004. Pseudotype hepatitis C virus enters immature myeloid dendritic cells through the interaction with lectin. *Virology* 324:74–83.
- Kawana H, et al. 2008. CD44 suppresses TLR-mediated inflammation. *J. Immunol.* 180:4235–4245.
- Lagging M, et al. 2006. IP-10 predicts viral response and therapeutic outcome in difficult-to-treat patients with HCV genotype 1 infection. *Hepatology* 44:1617–1625.
- Larrubia JR, Benito-Martinez S, Calvino M, Sanz-de-Villalobos E, Parra-Cid T. 2008. Role of chemokines and their receptors in viral persistence and liver damage during chronic hepatitis C virus infection. *World J. Gastroenterol.* 14:7149–7159.
- Larrubia JR, et al. 2007. The role of CCR5/CXCR3 expressing CD8+ cells in liver damage and viral control during persistent hepatitis C virus infection. *J. Hepatol.* 47:632–641.
- Lemon SM. 2010. Induction and evasion of innate antiviral responses by hepatitis C virus. *J. Biol. Chem.* 285:22741–22747.
- Leroy V, et al. 2003. Phenotypic and functional characterization of intrahepatic T lymphocytes during chronic hepatitis C. *Hepatology* 38:829–841.
- Liang J, et al. 2007. CD44 is a negative regulator of acute pulmonary inflammation and lipopolysaccharide-TLR signaling in mouse macrophages. *J. Immunol.* 178:2469–2475.
- Lohmann V, et al. 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* 285:110–113.
- Marukian S, et al. 2008. Cell culture-produced hepatitis C virus does not infect peripheral blood mononuclear cells. *Hepatology* 48:1843–1850.
- McHutchison JG, et al. 2000. Measurement of serum hyaluronic acid in patients with chronic hepatitis C and its relationship to liver histology. Consensus Interferon Study Group. *J. Gastroenterol. Hepatol.* 15:945–951.
- Mielgo A, van Driel M, Bloem A, Landmann L, Güntherth U. 2006. A novel antiapoptotic mechanism based on interference of Fas signaling by CD44 variant isoforms. *Cell Death Differ.* 13:465–477.
- Moriishi K, Matsuura Y. 2003. Mechanisms of hepatitis C virus infection. *Antivir. Chem. Chemother.* 14:285–297.
- Narumi S, et al. 1997. Expression of IFN-inducible protein-10 in chronic hepatitis. *J. Immunol.* 158:5536–5544.
- Okamoto T, et al. 2008. A single-amino-acid mutation in hepatitis C virus NS5A disrupting FKBP8 interaction impairs viral replication. *J. Virol.* 82:3480–3489.
- Patel K, et al. 2003. Clinical use of hyaluronic acid as a predictor of fibrosis change in hepatitis C. *J. Gastroenterol. Hepatol.* 18:253–257.
- Patzwahl R, Meier V, Ramadori G, Mihm S. 2001. Enhanced expression of interferon-regulated genes in the liver of patients with chronic hepatitis C virus infection: detection by suppression-subtractive hybridization. *J. Virol.* 75:1332–1338.
- Polyak SJ, Khabar KS, Rezeiq M, Gretch DR. 2001. Elevated levels of interleukin-8 in serum are associated with hepatitis C virus infection and resistance to interferon therapy. *J. Virol.* 75:6209–6211.
- Ponta H, Sherman L, Herrlich PA. 2003. CD44: from adhesion molecules to signalling regulators. *Nat. Rev. Mol. Cell Biol.* 4:33–45.
- Radkowski M, et al. 2004. Infection of primary human macrophages with hepatitis C virus in vitro: induction of tumour necrosis factor-alpha and interleukin 8. *J. Gen. Virol.* 85:47–59.
- Romero AI, et al. 2006. Interferon (IFN)-gamma-inducible protein-10: association with histological results, viral kinetics, and outcome during treatment with pegylated IFN-alpha 2a and ribavirin for chronic hepatitis C virus infection. *J. Infect. Dis.* 194:895–903.
- Scheibner KA, et al. 2006. Hyaluronan fragments act as an endogenous danger signal by engaging TLR2. *J. Immunol.* 177:1272–1281.
- Shields PL, et al. 1999. Chemokine and chemokine receptor interactions provide a mechanism for selective T cell recruitment to specific liver compartments within hepatitis C-infected liver. *J. Immunol.* 163:6236–6243.
- Shiina M, Rehmann B. 2008. Cell culture-produced hepatitis C virus impairs plasmacytoid dendritic cell function. *Hepatology* 47:385–395.
- Takeuchi O, Akira S. 2010. Pattern recognition receptors and inflammation. *Cell* 140:805–820.
- Ueno T, et al. 1995. Serum hyaluronate predicts response to interferon-alpha therapy in patients with chronic hepatitis C. *Hepatogastroenterology* 42:522–527.
- Wakita T, et al. 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat. Med.* 11:791–796.
- Wang J, Holmes TH, Cheung R, Greenberg HB, He XS. 2004. Expression of chemokine receptors on intrahepatic and peripheral lymphocytes in chronic hepatitis C infection: its relationship to liver inflammation. *J. Infect. Dis.* 190:989–997.
- Wong VS, et al. 1998. Serum hyaluronic acid is a useful marker of liver fibrosis in chronic hepatitis C virus infection. *J. Viral. Hepat.* 5:187–192.
- Zeremski M, et al. 2008. Intrahepatic levels of CXCR3-associated chemokines correlate with liver inflammation and fibrosis in chronic hepatitis C. *Hepatology* 48:1440–1450.

# 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 $\beta$ -Luc and pISRE-Luc carrying a firefly luciferase gene under the control of the beta IFN (IFN- $\beta$ ) 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<sub>2</sub>. 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  $\mu$ 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  $\mu$ g/ml streptomycin, and 10% FCS. Hep3B replicon cells harboring the subgenomic HCV RNA were maintained in DMEM containing 10% FCS, NEAA, and 400  $\mu$ 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- $\mu$ 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

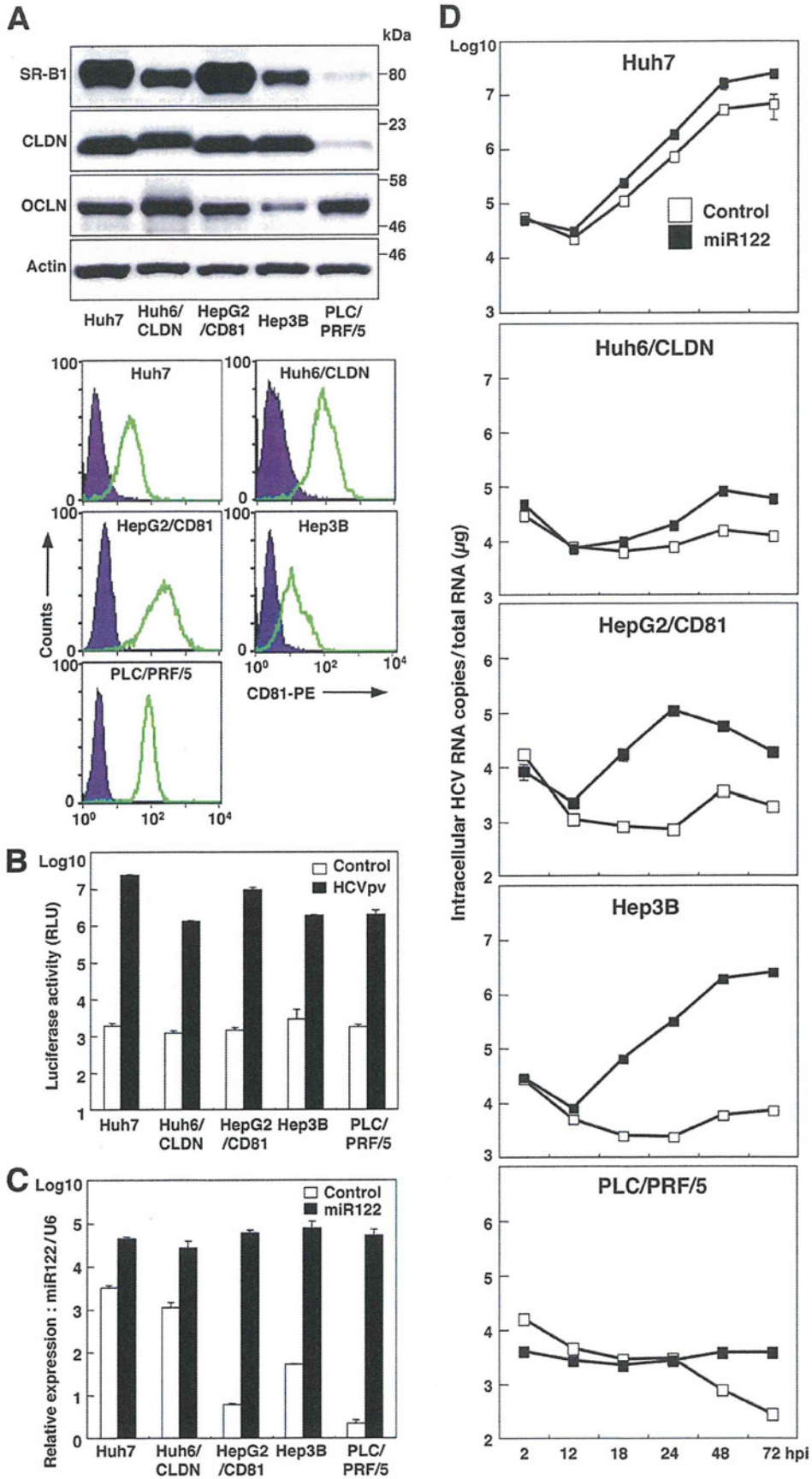
(VSV) variant NCP12.1, derived from the Indiana strain, was kindly provided by M. Whitt. Pseudotype VSVs bearing the HCV E1 and E2 glycoproteins (HCVpv) and VSV G protein (VSVpv) were prepared as described previously (60). The infectivity of the pseudotype viruses was assessed by the expression of luciferase, 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- $\alpha$ 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'-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  $\beta$ -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<sup>6</sup> 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<sup>4</sup> 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.

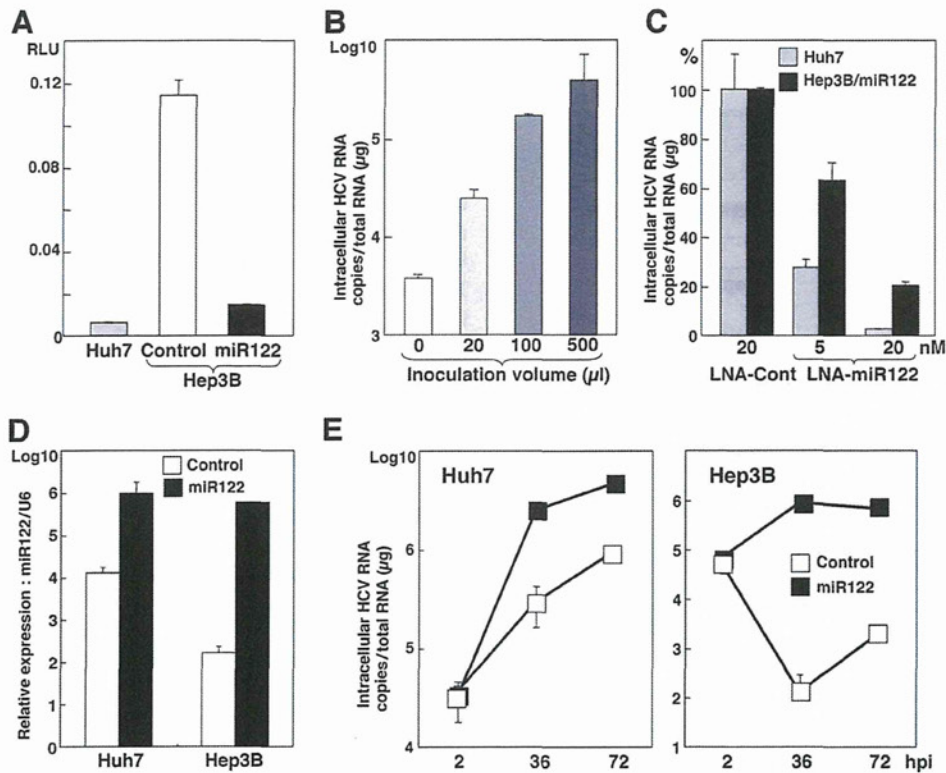


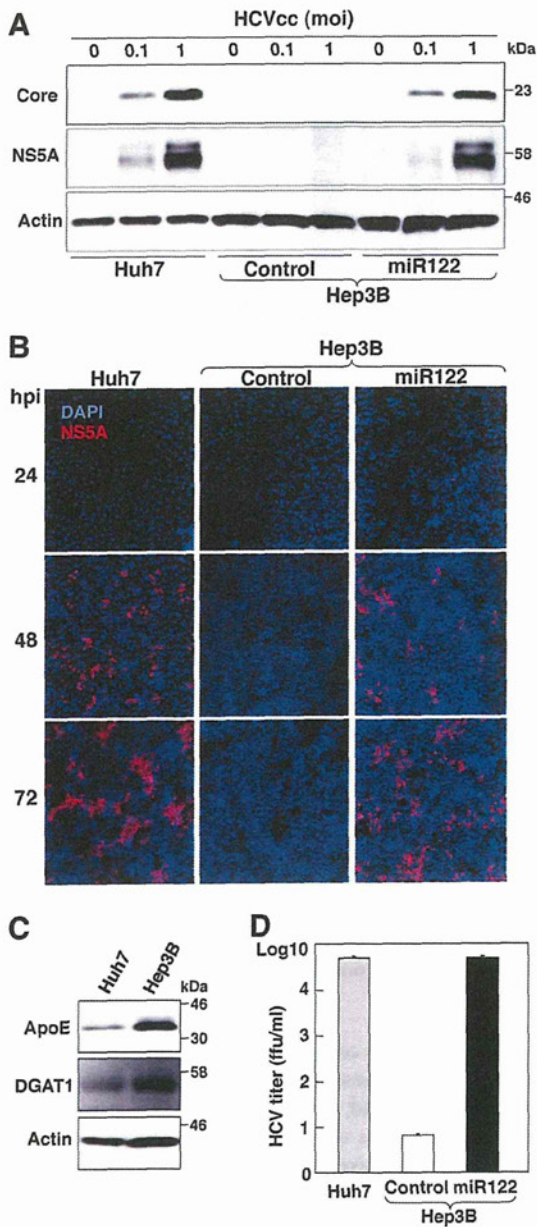
FIG 2 Expression of biologically active miR122 facilitates replication of HCVcc in Hep3B cells. (A) Huh7, Hep3B/Cont, and Hep3B/miR122 cells were transfected with pmirGLO-miR122comp, and luciferase activity was determined at 24 h posttransfection. (B) Hep3B cells were transfected with the lentiviral vector expressing miR122 in a dose-dependent manner and then infected with HCVcc at an MOI of 1 at 48 h posttransduction. 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  $5 \times 10^4$  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- $\alpha$  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

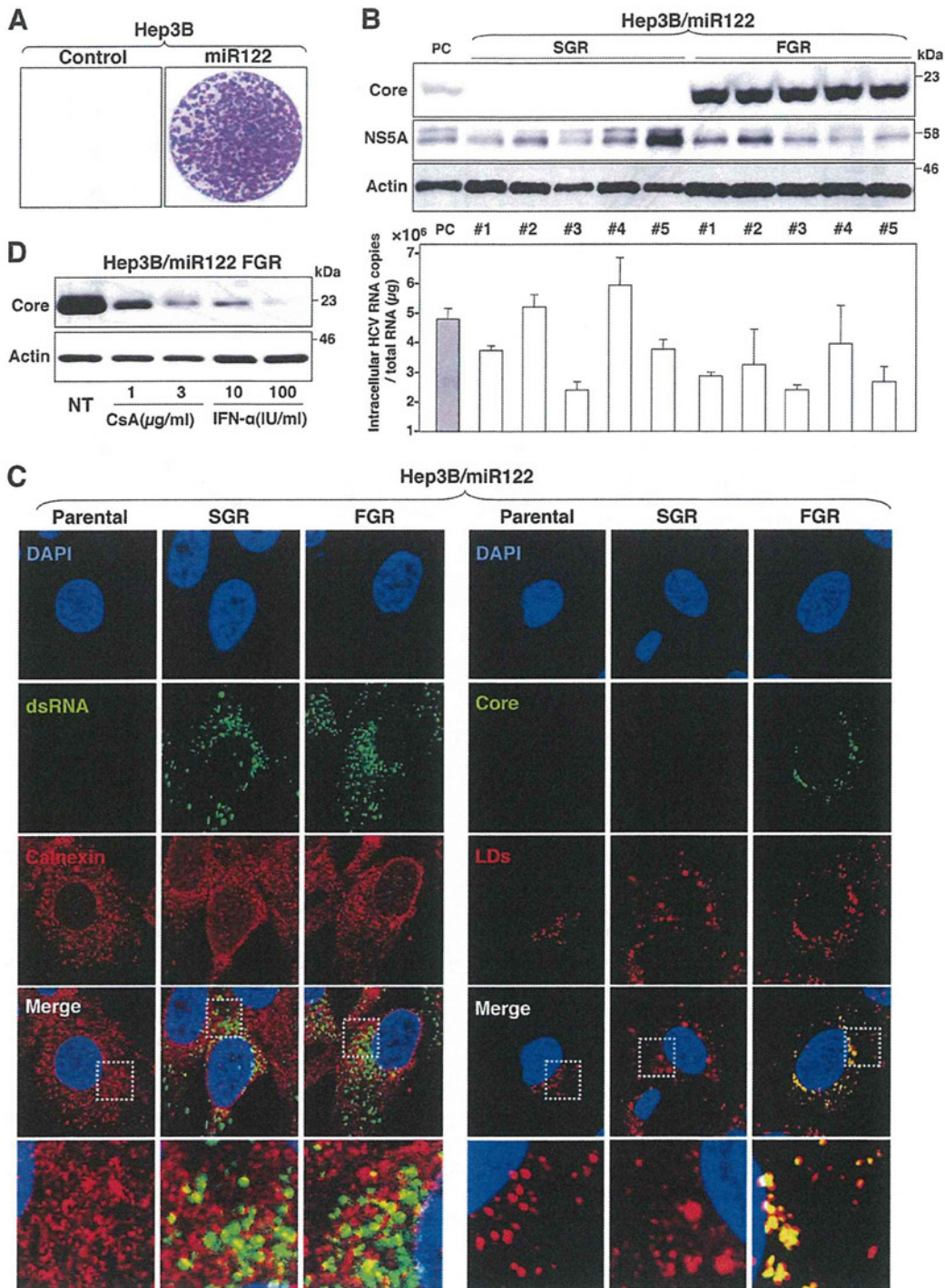


**FIG 3** Establishment of a novel permissive cell line for robust propagation of HCVcc by expression of miR122 in Hep3B cells. (A) Huh7, Hep3B/Cont, and Hep3B/miR122 cells were infected with HCVcc at an MOI of 0.1 or 1, and the levels of expression of viral proteins were determined by immunoblotting 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  $\mu\text{g/ml}$  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- $\alpha$  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.

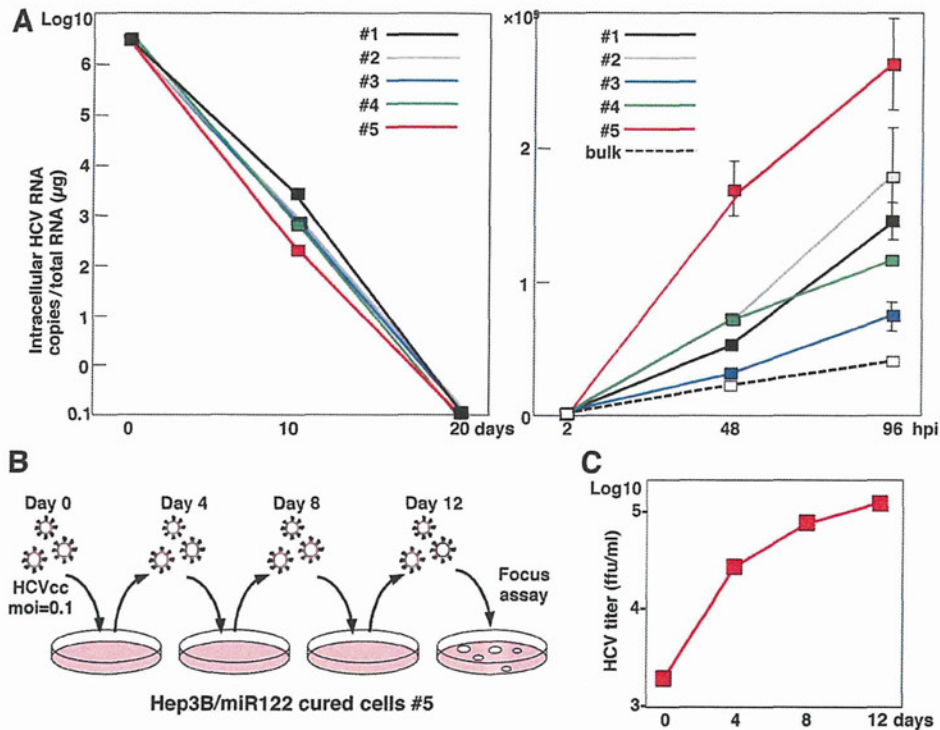
**Elimination of HCV RNA from HCV replicon RNA from Hep3B/miR122 cells enhances propagation of HCVcc.** To establish cured Hep3B/miR122 cells, five clones of the Hep3B/miR122 replicon cells harboring FGR of the JFH1 strain were treated with 100 IU/ml of IFN- $\alpha$  to eliminate viral RNA, and viral 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- $\beta$  (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- $\alpha$ . 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- $\alpha$  (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.

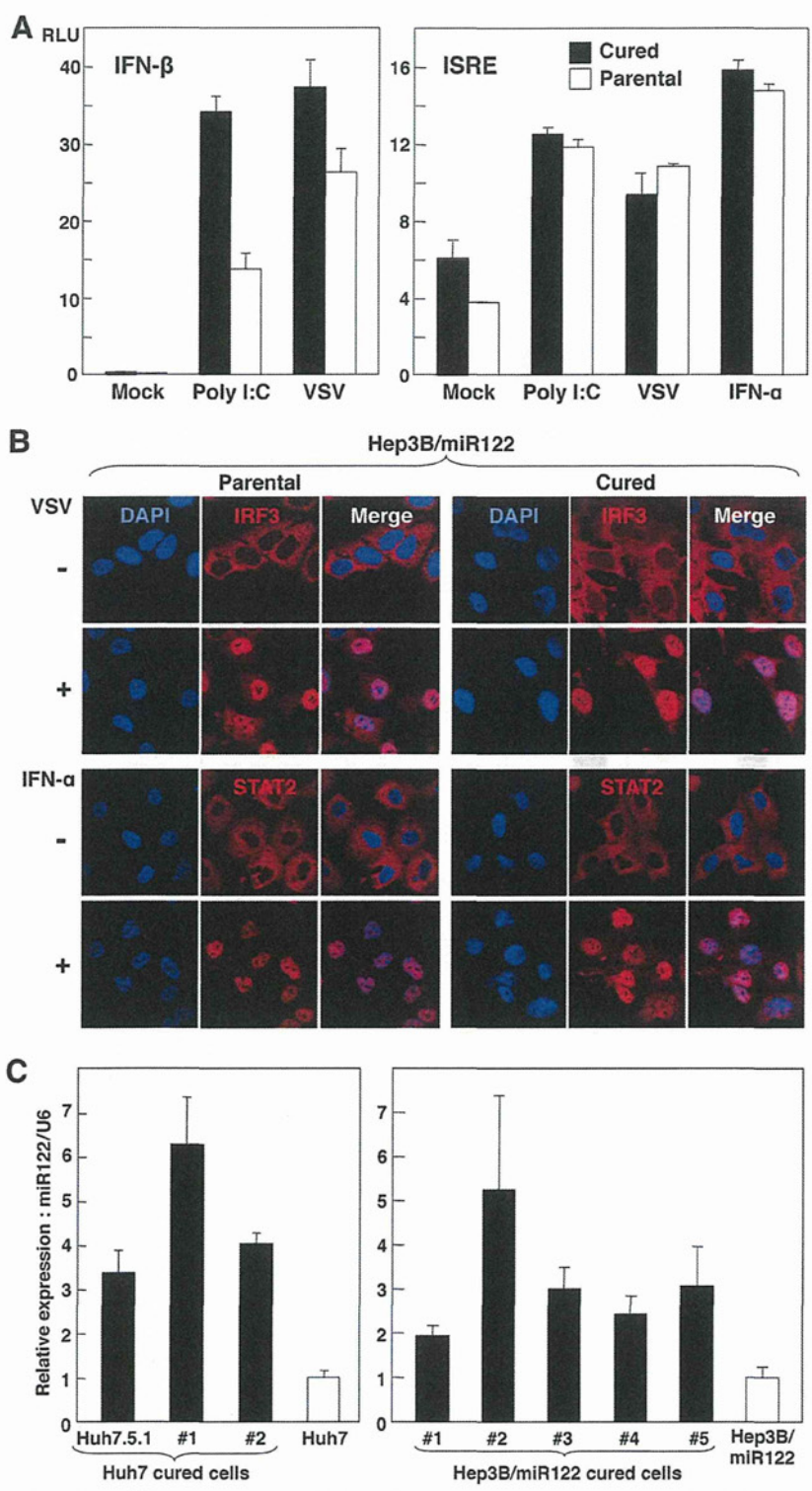
fluorescence analysis. IRF3 and STAT2 in both cured and parental Hep3B/miR122 cells were translocated into the nucleus upon stimulation with VSV and IFN- $\alpha$ , respectively (Fig. 6B). These results suggest that the efficient propagation of HCVcc in the cured Hep3B/miR122 cells might be attributable 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 pIFN $\beta$ -Luc and pRL-TK and then infected with the VSV NCP mutant at an MOI of 0.01 or transfected with 1  $\mu$ g 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- $\alpha$  (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- $\alpha$  (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.

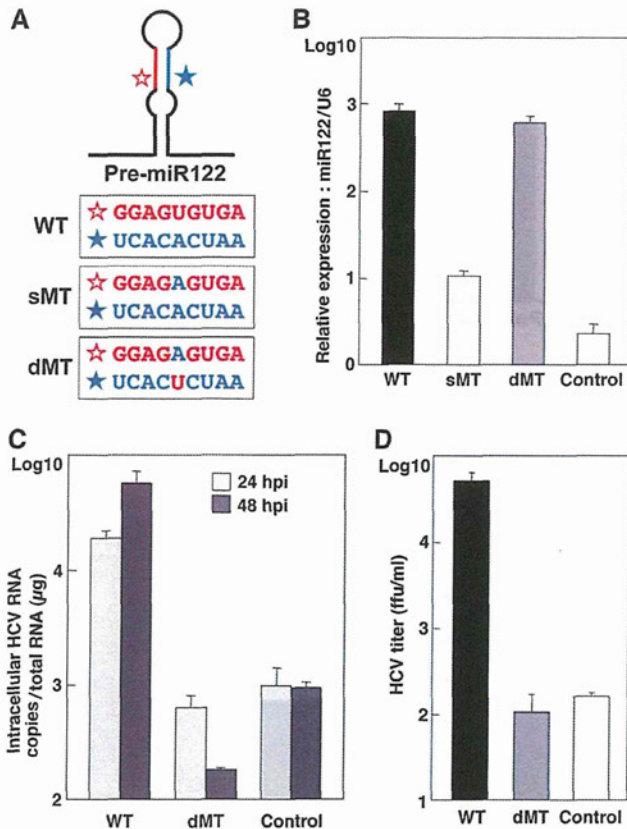


FIG 7 Specific interaction of miR122 with viral RNA is crucial for efficient propagation of HCVcc. (A) Diagram of pre-miR122 and partial nucleotide sequences of wild type (WT) miR122 and mutant miR122 carrying a single mutation (sMT) and double mutations (dMT). (B) Hep3B cells were transfected with lentiviral 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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#### REFERENCES

- Aden DP, Fogel A, Plotkin S, Damjanov I, Knowles BB. 1979. Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. *Nature* 282:615–616.
- Bai S, et al. 2009. MicroRNA-122 inhibits tumorigenic properties of hepatocellular carcinoma cells and sensitizes these cells to sorafenib. *J. Biol. Chem.* 284:32015–32027.
- Bartel DP. 2009. MicroRNAs: target recognition and regulatory functions. *Cell* 136:215–233.
- Blight KJ, McKeating JA, Rice CM. 2002. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. *J. Virol.* 76:13001–13014.
- Burns DM, D'Ambrogio A, Nottrott S, Richter JD. 2011. CPEB and two poly(A) polymerases control miR-122 stability and p53 mRNA translation. *Nature* 473:105–108.
- Castoldi M, et al. 2011. The liver-specific microRNA miR-122 controls systemic iron homeostasis in mice. *J. Clin. Invest.* 121:1386–1396.
- Chang J, et al. 2008. Liver-specific microRNA miR-122 enhances the replication of hepatitis C virus in nonhepatic cells. *J. Virol.* 82:8215–8223.
- Chang J, et al. 2004. miR-122, a mammalian liver-specific microRNA, is processed from her mRNA and may downregulate the high affinity cationic amino acid transporter CAT-1. *RNA Biol.* 1:106–113.
- Chang KS, Jiang J, Cai Z, Luo G. 2007. Human apolipoprotein E is required for infectivity and production of hepatitis C virus in cell culture. *J. Virol.* 81:13783–13793.
- Cormier EG, et al. 2004. CD81 is an entry coreceptor for hepatitis C virus. *Proc. Natl. Acad. Sci. U. S. A.* 101:7270–7274.
- Date T, et al. 2004. Genotype 2a hepatitis C virus subgenomic replicon can replicate in HepG2 and IMY-N9 cells. *J. Biol. Chem.* 279:22371–22376.
- Denard B, et al. 2011. The membrane-bound transcription factor CREB3L1 is activated in response to virus infection to inhibit proliferation of virus-infected cells. *Cell Host Microbe* 10:65–74.
- Ehrhardt M, et al. 18 April 2011. Profound differences of microRNA expression patterns in hepatocytes and hepatoma cell lines commonly used in hepatitis C virus studies. *Hepatology*. [Epub ahead of print.]
- Elmen J, et al. 2008. LNA-mediated microRNA silencing in non-human primates. *Nature* 452:896–899.
- Evans MJ, et al. 2007. Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. *Nature* 446:801–805.
- Farci P, et al. 2000. The outcome of acute hepatitis C predicted by the evolution of the viral quasispecies. *Science* 288:339–344.
- Feigelstock DA, Mihalik KB, Kaplan G, Feinstone SM. 2010. Increased susceptibility of Huh7 cells to HCV replication does not require mutations in RIG-I. *Virology* 407:7–14.
- Feliu A, Gay E, Garcia-Retortillo M, Saiz JC, Forns X. 2004. Evolution of hepatitis C virus quasispecies immediately following liver transplantation. *Liver Transpl.* 10:1131–1139.
- Ge D, et al. 2009. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* 461:399–401.
- Gentzsch J, et al. 2011. Hepatitis C virus complete life cycle screen for identification of small molecules with pro- or antiviral activity. *Antiviral Res.* 89:136–148.
- Gottwein JM, et al. 2009. Development and characterization of hepatitis C virus genotype 1-7 cell culture systems: role of CD81 and scavenger receptor class B type I and effect of antiviral drugs. *Hepatology* 49:364–377.
- Haid S, Windisch MP, Bartenschlager R, Pietschmann T. 2010. Mouse-specific residues of claudin-1 limit hepatitis C virus genotype 2a infection in a human hepatocyte cell line. *J. Virol.* 84:964–975.
- Henke JI, et al. 2008. microRNA-122 stimulates translation of hepatitis C virus RNA. *EMBO J.* 27:3300–3310.
- Herker E, et al. 2010. Efficient hepatitis C virus particle formation requires diacylglycerol acyltransferase-1. *Nat. Med.* 16:1295–1298.
- Hughes MG, Jr, et al. 2005. HCV infection of the transplanted liver: changing CD81 and HVR1 variants immediately after liver transplantation. *Am. J. Transplant.* 5:2504–2513.
- Huntzinger E, Izaurralde E. 2011. Gene silencing by microRNAs: contributions of translational repression and mRNA decay. *Nat. Rev. Genet.* 12:99–110.
- Jangra RK, Yi M, Lemon SM. 2010. Regulation of hepatitis C virus translation and infectious virus production by the microRNA miR-122. *J. Virol.* 84:6615–6625.
- Jopling CL, Schutz S, Sarnow P. 2008. Position-dependent function for a tandem microRNA miR-122-binding site located in the hepatitis C virus RNA genome. *Cell Host Microbe* 4:77–85.
- Jopling CL, Yi M, Lancaster AM, Lemon SM, Sarnow P. 2005. Modulation of hepatitis C virus RNA abundance by a liver-specific microRNA. *Science* 309:1577–1581.
- Kato N, et al. 2009. Efficient replication systems for hepatitis C virus using a new human hepatoma cell line. *Virus Res.* 146:41–50.
- Lanford RE, et al. 2010. Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. *Science* 327:198–201.
- Laskus T, et al. 2004. Analysis of hepatitis C virus quasispecies transmission and evolution in patients infected through blood transfusion. *Gastroenterology* 127:764–776.
- Lauring AS, Andino R. 2010. Quasispecies theory and the behavior of RNA viruses. *PLoS Pathog.* 6:e1001005.
- Lavanchy D. 2009. The global burden of hepatitis C. *Liver Int.* 29(Suppl. 1):74–81.
- Lin LT, et al. 2010. Replication of subgenomic hepatitis C virus replicons in mouse fibroblasts is facilitated by deletion of interferon regulatory factor 3 and expression of liver-specific microRNA 122. *J. Virol.* 84:9170–9180.
- Lindenbach BD, et al. 2005. Complete replication of hepatitis C virus in cell culture. *Science* 309:623–626.
- Lohmann V, et al. 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* 285:110–113.
- Machlin ES, Sarnow P, Sagan SM. 2011. Masking the 5' terminal nucleotides of the hepatitis C virus genome by an unconventional microRNA-target RNA complex. *Proc. Natl. Acad. Sci. U. S. A.* 108:3193–3198.
- Masaki T, et al. 2010. Production of infectious hepatitis C virus by using RNA polymerase I-mediated transcription. *J. Virol.* 84:5824–5835.
- Miyazari Y, et al. 2007. The lipid droplet is an important organelle for hepatitis C virus production. *Nat. Cell Biol.* 9:1089–1097.
- Moradpour D, Penin F, Rice CM. 2007. Replication of hepatitis C virus. *Nat. Rev. Microbiol.* 5:453–463.
- Moriishi K, Matsuura Y. 2007. Evaluation systems for anti-HCV drugs. *Adv. Drug Deliv. Rev.* 59:1213–1221.

43. Moriishi K, Matsuura Y. 2007. Host factors involved in the replication of hepatitis C virus. *Rev. Med. Virol.* 17:343–354.
44. Moriishi K, Matsuura Y. 2003. Mechanisms of hepatitis C virus infection. *Antivir. Chem. Chemother.* 14:285–297.
45. Moriishi K, et al. 2010. Involvement of PA28gamma in the propagation of hepatitis C virus. *Hepatology* 52:411–420.
46. Narbus CM, et al. 2011. HepG2 cells expressing microRNA miR-122 support the entire hepatitis C virus life cycle. *J. Virol.* 85:12087–12092.
47. Park JG, et al. 1995. Characterization of cell lines established from human hepatocellular carcinoma. *Int. J. Cancer* 62:276–282.
48. Pileri P, et al. 1998. Binding of hepatitis C virus to CD81. *Science* 282: 938–941.
49. Ploss A, et al. 2009. Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. *Nature* 457:882–886.
50. Ploss A, et al. 2010. Persistent hepatitis C virus infection in microscale primary human hepatocyte cultures. *Proc. Natl. Acad. Sci. U. S. A.* 107: 3141–3145.
51. Randall G, et al. 2007. Cellular cofactors affecting hepatitis C virus infection and replication. *Proc. Natl. Acad. Sci. U. S. A.* 104:12884–12889.
52. Roberts AP, Lewis AP, Jopling CL. 2011. miR-122 activates hepatitis C virus translation by a specialized mechanism requiring particular RNA components. *Nucleic Acids Res.* 39:7716–7729.
53. Russell RS, et al. 2008. Advantages of a single-cycle production assay to study cell culture-adaptive mutations of hepatitis C virus. *Proc. Natl. Acad. Sci. U. S. A.* 105:4370–4375.
54. Scarselli E, et al. 2002. The human scavenger receptor class B type 1 is a novel candidate receptor for the hepatitis C virus. *EMBO J.* 21:5017–5025.
55. Seow TK, Liang RC, Leow CK, Chung MC. 2001. Hepatocellular carcinoma: from bedside to proteomics. *Proteomics* 1:1249–1263.
56. Skalsky RL, Cullen BR. 2010. Viruses, microRNAs, and host interactions. *Annu. Rev. Microbiol.* 64:123–141.
57. Sumpter R, Jr, et al. 2005. Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. *J. Virol.* 79:2689–2699.
58. Suppiah V, et al. 2009. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. *Nat. Genet.* 41: 1100–1104.
59. Tanaka Y, et al. 2009. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat. Genet.* 41:1105–1109.
60. Tani H, et al. 2007. Replication-competent recombinant vesicular stomatitis virus encoding hepatitis C virus envelope proteins. *J. Virol.* 81: 8601–8612.
61. Targett-Adams P, Boulant S, McLauchlan J. 2008. Visualization of double-stranded RNA in cells supporting hepatitis C virus RNA replication. *J. Virol.* 82:2182–2195.
62. Thomas DL, et al. 2009. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. *Nature* 461:798–801.
63. Vannucchi AM, et al. 1993. Effects of cyclosporin A on erythropoietin production by the human Hep3B hepatoma cell line. *Blood* 82:978–984.
64. Wakita T, et al. 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat. Med.* 11:791–796.
65. Walters KA, et al. 2006. Host-specific response to HCV infection in the chimeric SCID-beige/Alb-uPA mouse model: role of the innate antiviral immune response. *PLoS Pathog.* 2:e59.
66. Windisch MP, et al. 2005. Dissecting the interferon-induced inhibition of hepatitis C virus replication by using a novel host cell line. *J. Virol.* 79: 13778–13793.
67. Wong N, et al. 2005. Transcriptional profiling identifies gene expression changes associated with IFN-alpha tolerance in hepatitis C-related hepatocellular carcinoma cells. *Clin. Cancer Res.* 11:1319–1326.
68. Yi M, Villanueva RA, Thomas DL, Wakita T, Lemon SM. 2006. Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells. *Proc. Natl. Acad. Sci. U. S. A.* 103: 2310–2315.
69. Yoneyama M, et al. 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat. Immunol.* 5:730–737.
70. Zhong J, et al. 2005. Robust hepatitis C virus infection in vitro. *Proc. Natl. Acad. Sci. U. S. A.* 102:9294–9299.
71. Zhu H, et al. 2007. Hepatitis C virus triggers apoptosis of a newly developed hepatoma cell line through antiviral defense system. *Gastroenterology* 133:1649–1659.

## 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.

**Keywords** HCV · miR-122 · Lipid metabolism

### 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.

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## 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

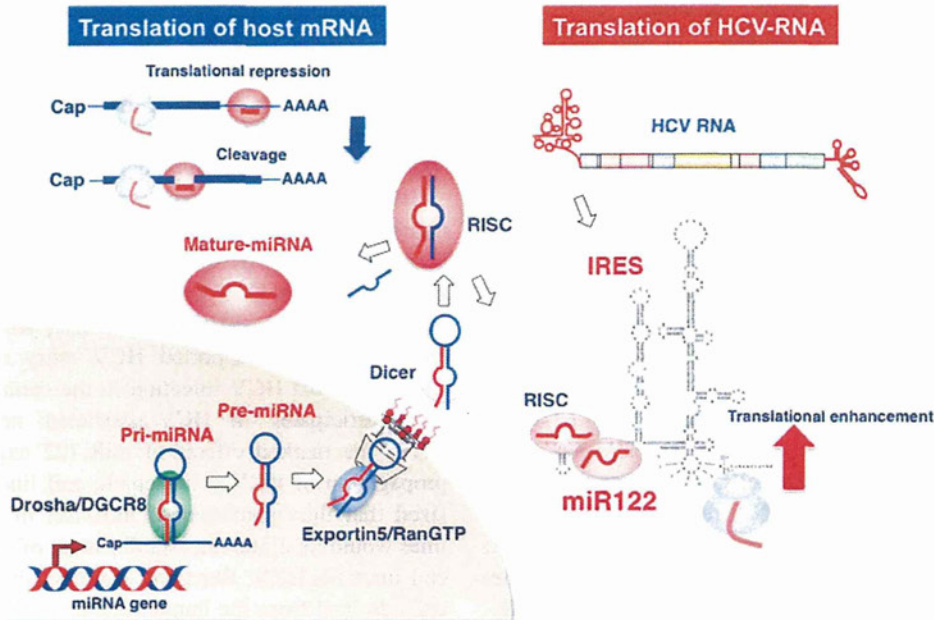
miR-122 is a liver-specific microRNA and is the microRNA most abundantly expressed in the liver [29–31]. Although Li et al. [32] have suggested that hepatocyte nuclear factor 4 alpha (HNF4A) positively regulates the expression of miR-122, the details on the tissue specificity of miR-122 expression have 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



**Fig. 1** miR-122 enhances the translation of hepatitis C virus (HCV) RNA. Primary miRNA (*pri-miRNA*) transcribed by RNA polymerase II in the nucleus is processed into precursor miRNA (*pre-miRNA*) by Drosha and DiGeorge syndrome critical region protein 8 (*DGCR8*). Pre-miRNA is exported into the cytoplasm by nucleocytoplasmic shuttle protein exportin 5, processed to 22nt by dicer, and then incorporated into argonaute proteins to form the RNA-induced

silencing complex (*RISC*). The passenger strand of miRNA (*blue*) is degraded and the guide strand (*red*) is matured in the *RISC*. Generally, miRNA represses the translation of host mRNA by binding to its 3'untranslated region (3'UTR). In contrast, liver-specific miR-122 binds to two sites in the 5'UTR of the HCV genome and enhances its translation and replication. *GTP* Guanosine-5-triphosphate, *IRES* internal ribosomal entry site

fully elucidated yet, Henke et al. [43], by using polymerase defective viral RNA, showed that miR-122 stimulated the translation of HCV RNA by enhancing the association of ribosomes at an early initiation stage. They concluded that miR-122 might contribute to HCV liver tropism at the level of translation. Wilson et al. [44] showed that knockdown of Ago2 in SGR cells and HCVcc-infected cells attenuated HCV replication, and that knockdown of Ago2 also reduced the translation of the polymerase defective HCV RNA. Shimakami et al. [45] showed that miR-122 stabilized viral RNA and reduced its decay in concert with Ago2, and that miR-122-dependent stabilization of HCV RNA was not observed in Ago2-knockout murine embryonic fibroblasts. These results suggest that Ago2 is required for the efficient enhancement of both the translation and replication of HCV. On the other hand, Machlin et al. [41] have suggested that the 3' overhang binding of miR-122 to the 5' end of the HCV genome participates in circumvention from the recognition by the cytoplasmic RNA sensor, RIG-I. It is feasible to speculate that miR-122 has other functions in the HCV life cycle, in addition to the stabilization of viral RNA and evasion from the host's innate immune response.

### Establishment of new permissive cell lines for HCV propagation by the expression of miR-122

The lack of immunocompetent small animal models and cell culture systems to support the propagation of HCV in patient sera has hampered both the understanding of the HCV life cycle and the development of antiviral drugs [46]. HCV replicon cells in which the HCV genome autonomously replicates, and pseudotype viruses bearing HCV E1 and E2 glycoproteins were established to assess viral replication and entry, respectively [47, 48]. Afterwards, an infectious HCV derived from the JFH1 strain of genotype 2a (HCVcc) was developed [7, 8]. On the basis of the data obtained from these in vitro systems, the HCV life cycle has been clarified, and host factors involved in HCV propagation have been identified as therapeutic targets for chronic hepatitis C [46]. However, the robust propagation of HCVcc in well-characterized human liver cell lines other than Huh7 had not been successful until recently. Chang et al. [49] showed that the exogenous expression of miR-122 facilitated the replication of HCV RNA in kidney-derived HEK-293 cells. In addition, Lin et al. have demonstrated that the expression of miR-122 and depletion of interferon regulatory factor 3 (IRF-3) permit replication



of the HCV genome in mouse fibroblasts [50]. These results suggest that the expression of miR-122 might facilitate the efficient replication of HCVcc not only in hepatic cells but also in nonhepatic cells. In fact, the expression level of miR-122 in Huh7 cells has been shown to be higher than that in other hepatic cell lines, including Huh6, HepG2, and Hep3B cells [10]. Recently, two groups reported that miR-122 expression facilitated the efficient propagation of HCVcc in human hepatic cell lines [10, 11]. Narbus et al. [11] showed that HepG2 cells stably expressing CD81 and miR-122 supported efficient replication and the production of infectious particles. Interestingly, internal ribosomal entry site (IRES)-dependent translation of HCV exhibited a slight (1.4–2.1-fold) increase by the expression of miR-122 in HepG2 cells compared with that in parental cells, suggesting that miR-122 is required for efficient RNA replication but not in translation in HepG2 cells upon infection with HCVcc. Kambara et al. [10] established a novel permissive cell line for the propagation of HCVcc by the expression of miR-122 in Hep3B cells. miR-122 expression facilitated the efficient propagation of HCVcc and the establishment of HCV replicon cells in Hep3B cells. In addition, “cured” Hep3B cells established by the elimination of HCV RNA from the Hep3B replicon cells facilitated the efficient propagation of HCVcc compared to parental cells. Interestingly, the expression of miR-122 in the “cured” Hep3B cells was significantly higher than that in the parental cells. In addition, Ehrhardt et al. [51] have shown that the expression levels of miR-122 in Huh7-derived cured cells, including Huh7.5 and Huh-Lunet cells, are significantly higher than those in parental Huh7 cells. Collectively, these results suggest that miR-122 is a key determinant of the efficient replication of HCVcc in hepatic cell lines.

#### **Expression of miR-122 facilitates the efficient replication of HCV in nonhepatic cells**

In clinical studies, negative strands of HCV genome have been detected in nonhepatic tissues of chronic hepatitis C patients, suggesting the possibility of extrahepatic propagation of HCV [52–56]. In addition, HCV replication was detected in peripheral blood mononuclear cells (PBMCs) of patients with occult HCV infection [57]. Roque-Afonso et al. [52] showed that highly divergent variants of HCV were detectable in PBMCs, but not in plasma or in liver, suggesting the possibility of the extrahepatic propagation of HCV. Furthermore, previous reports have suggested that recurrences of HCV infection after antiviral treatment or liver transplantation were attributable to chronic infection of HCV in extrahepatic tissues [58]. Collectively, these results might suggest a correlation between extrahepatic

HCV replication and the development of EHM, including mixed cryoglobulinemia and non-Hodgkin lymphoma, which are frequently observed in chronic hepatitis C patients. However, details of the extrahepatic propagation of HCV have not been studied owing to the lack of an appropriate experimental model [59, 60].

HCV replicon cells have been established in several nonhepatic cell lines. Kato et al. [61] established JFH1-based SGR cells by using HeLa and HEK293 cells, suggesting that the HCV genome can replicate in nonhepatic cells. In addition, Fletcher et al. [62] showed that brain endothelial cells supported HCV entry and replication, suggesting that HCV infection in the central nervous system participates in HCV-associated neuropathologies. Given the marked effects of miR-122 expression on the propagation of HCVcc in hepatic cell lines, we hypothesized that the expression of miR-122 in nonhepatic cell lines would facilitate the establishment of novel permissive cell lines for HCV. Recently, we have shown that Hec1B cells derived from the human uterus exhibited a low level of viral replication and the exogenous expression of miR-122 significantly enhanced replication upon infection with HCVcc [63]. In addition, an miR-122-specific inhibitor for miR-122 called locked nucleic acid (LNA-miR-122) inhibited the enhancement of HCVcc replication in Hec1B cells expressing miR-122, while the basal replication of HCVcc in parental Hec1B cells was resistant to the treatment. These results suggest that Hec1B cells permit HCV replication in an miR-122-independent manner and the exogenous expression of miR-122 enhances viral replication. In this report, cured Hec1B cells established by the elimination of HCV RNA from Hec1B replicon cells exhibited more potent replication of HCVcc than the parental cells. As seen in the cured Hep3B cells, the expression levels of miR-122 in the Hec1B cured cells were significantly higher than those in the parental cells [63]. Taken together, these results show that the expression of miR-122 facilitates the replication of HCVcc in nonhepatic cells.

#### **Viral assembly in nonhepatic cells**

Previous reports have shown that the production of VLDL is involved in the formation of infectious HCV particles [15, 16]. Apolipoprotein B (ApoB), apolipoprotein E (ApoE), and microsomal triglyceride transfer protein (MTTP) have major roles in the secretion of VLDL. Gastaminza et al. [15] have demonstrated that ApoB and MTTP are cellular factors essential for the efficient assembly of infectious HCV particles. They concluded that HCV acquired hepatocyte tropism through utilization of the VLDL secretory pathway. On the other hand, studies by

other groups have demonstrated that infectious HCV particles are highly enriched in ApoE, which is a major determinant of HCV infectivity and production [64]. In their reports, small interfering RNA (siRNA)-mediated knockdown of ApoB and treatment with MTTP inhibitors exhibited no significant effect on the infectivity and production of HCV, suggesting that ApoE but not ApoB is required for viral assembly. In addition, Mancone et al. [65] have shown that apolipoprotein A-I (ApoA-I) is required for the replication of HCV and the production of infectious particles. Collectively, these results suggest that several VLDL-associated proteins are involved in HCV assembly.

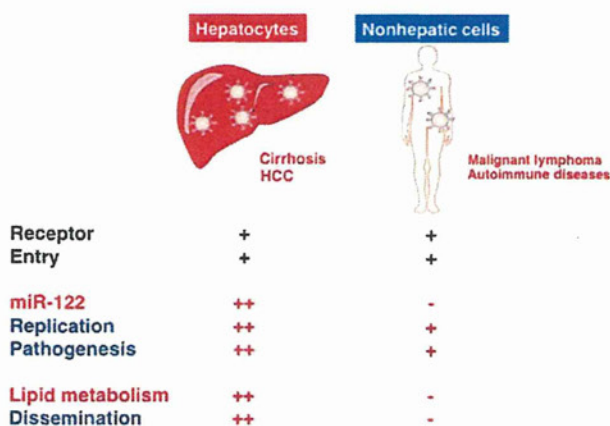
In our recent report, the viral assembly process was shown to be impaired in nonhepatic cells exogenously expressing miR-122, in spite of the efficient replication of the HCV genome [63]. Interestingly, low but substantial infectious titers were detected in hepatic Hep3B cells upon infection with HCVcc, even though the RNA replication was lower than that in nonhepatic Hec1B cells expressing miR-122. The expression levels of VLDL-associated proteins, including ApoE, ApoB, and MTTP, in nonhepatic cell lines were significantly lower than those in hepatic cell lines, suggesting that lack of expression of VLDL-associated proteins is one of the reasons for the inability of nonhepatic cells to produce infectious particles. Miyanari et al. [66] showed that lipid droplets (LDs) were required for the formation of infectious particles via interaction between the core protein and viral RNA. Interestingly, only a small amount of LDs was detected in nonhepatic cells, including Hec1B and HEK293T cells, compared with the amount in hepatic cell lines, suggesting that a low level of LD formation is also involved in the impairment of infectious particle formation in nonhepatic cells [63]. Taken together, these findings suggest the possibility that the reconstitution of functional lipid metabolism in nonhepatic cells facilitates the production of infectious particles.

### Tropism of HCV infection

In many cases, the cell tropism of viral infection is defined by the expression of virus-specific receptors. The expression of CD4 and chemokine receptors has an important role in the determination of the lymphotropism of human immunodeficiency virus infection [67]. In measles virus infection, the signaling lymphocyte activation molecule is a determinant of lymphotropism [68, 69]. Previous reports have shown that human CD81, scavenger receptor class B1 (SR-B1), Claudin1 (CLDN1), and Occludin (OCLN) are crucial for HCV entry [70–73]. Although murine cells cannot permit HCV entry, the exogenous expression of

human-derived receptor candidates in murine cells has been shown to facilitate HCV entry, suggesting that HCV-specific receptors participate in the determination of the cell tropism of HCV [74, 75]. However, previous reports have also revealed that HCV receptor candidates were highly expressed in many nonhepatic tissues [62, 76], and our recent report has demonstrated that many nonhepatic cells permit the entry of HCV pseudotypes [63]. In addition, many reports have suggested the possibility of HCV replication in extrahepatic sites such as PBMCs and neuronal cells [55, 62], suggesting that host factors other than receptors could be involved in the tissue tropism of HCV.

Although previous reports have shown that host factors such as VAMP-associated protein (VAP)-A, VAP-B, cyclophilin A, FK506 binding protein 8, and heat shock protein 90 participate in HCV replication, these molecules are unlikely to participate in the determination of the liver tropism of HCV, owing to their ubiquitous expression [46, 77–79]. As described above, miR-122 is abundantly expressed specifically in hepatocytes and is essential for the efficient replication of HCV. In addition, a recent report showed that hepatocyte-like cells derived from induced pluripotent stem cells (iPSCs) expressed high levels of miR-122 and supported the entire life cycle of HCVcc, suggesting that miR-122 might be one of the most critical determinants of the liver tropism of HCV infection [80, 81]. On the other hand, VLDL-associated proteins,



**Fig. 2** HCV replication in hepatocytes and nonhepatic cells. Chronic HCV infection induces liver cirrhosis and hepatocellular carcinoma (HCC), and is also often associated with the development of extrahepatic manifestations (EHM) such as malignant lymphoma and autoimmune diseases. Not only hepatocytes but also nonhepatic cells express major HCV receptors, including CD81, SR-B1, CLDN1, and OCLN. In hepatocytes, functional expression of miR-122 and lipid metabolism facilitate the efficient propagation of HCV. In contrast, the lack of expression of miR-122 and very-low-density lipoprotein (VLDL)-associated proteins might be associated with the incomplete propagation of HCV in nonhepatic cells. Low levels of HCV replication in nonhepatic cells may participate in the development of EHM

including ApoB, ApoE, and MTTP, are specifically expressed in hepatic cells, and no infectious particles are produced in nonhepatic cells such as Hec1B and 293T-CLDN cells [63]. Collectively, these data suggest that the VLDL-producing system is involved in the liver tropism of HCV.

Although HCV can internalize not only into hepatocytes but also into nonhepatic cells through receptor-mediated endocytosis, miR-122 expression and functional lipid metabolism in hepatocytes facilitate the efficient replication and assembly of HCV (Fig. 2). On the other hand, lack of expression of miR-122 and VLDL-associated proteins might be associated with the incomplete propagation of HCV in nonhepatic cells (Fig. 2).

## Conclusion

Recent progress in HCV research has revealed that the tissue tropism of HCV is reliant on the expression of liver-specific miR-122 and a functional lipid metabolism rather than being reliant on the expression of entry receptors. However, the molecular mechanisms of the enhancement of viral replication induced by the interaction of miR-122 with the 5'UTR of HCV and the assembly of viral particles via VLDL-producing machinery remain unknown. In addition, the participation of nonhepatic cells in the development of EHM has been suggested, through an incomplete or low level of HCV replication. Elucidation of the liver tropism of HCV will provide a clue to the development of new antiviral drugs for the treatment of chronic hepatitis C and could lead to an understanding of the pathogenesis of EHM induced by HCV infection.

**Conflict of interest** The authors declare that they have no conflicts of interest.

## References

- Seeff LB. Natural history of chronic hepatitis C. *Hepatology*. 2002;36:S35–46.
- Hartridge-Lambert SK, Stein EM, Markowitz AJ, Portlock CS. Hepatitis C and non-Hodgkin lymphoma: the clinical perspective. *Hepatology*. 2012;55:634–41.
- Calleja JL, Albillos A, Moreno-Otero R, Rossi I, Cacho G, Domper F, et al. Sustained response to interferon-alpha or to interferon-alpha plus ribavirin in hepatitis C virus-associated symptomatic mixed cryoglobulinaemia. *Aliment Pharmacol Ther*. 1999;13:1179–86.
- Gumber SC, Chopra S. Hepatitis C: a multifaceted disease. Review of extrahepatic manifestations. *Ann Intern Med*. 1995;123:615–20.
- Galossi A, Guarisco R, Bellis L, Puoti C. Extrahepatic manifestations of chronic HCV infection. *J Gastrointest Liver Dis*. 2007;16:65–73.
- Bukh J. A critical role for the chimpanzee model in the study of hepatitis C. *Hepatology*. 2004;39:1469–75.
- Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med*. 2005;11:791–6.
- Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, Liu CC, et al. Complete replication of hepatitis C virus in cell culture. *Science*. 2005;309:623–6.
- Jopling CL, Yi M, Lancaster AM, Lemon SM, Sarnow P. Modulation of hepatitis C virus RNA abundance by a liver-specific microRNA. *Science*. 2005;309:1577–81.
- Kambara H, Fukuhara T, Shiokawa M, Ono C, Ohara Y, Kamitani W, et al. Establishment of a novel permissive cell line for the propagation of hepatitis C virus by expression of microRNA miR122. *J Virol*. 2012;86:1382–93.
- Narbus CM, Israelow B, Sourisseau M, Michta ML, Hopcraft SE, Zeiner GM, et al. HepG2 cells expressing microRNA miR-122 support the entire hepatitis C virus life cycle. *J Virol*. 2011;85:12087–92.
- Sainz B Jr, Barretto N, Yu X, Corcoran P, Uprichard SL. Permissiveness of human hepatoma cell lines for HCV infection. *J Virol*. 2012;9:30.
- Fukuhara T, Tani H, Shiokawa M, Goto Y, Abe T, Taketomi A, et al. Intracellular delivery of serum-derived hepatitis C virus. *Microbes Infect*. 2011;13:405–12.
- Lanford RE, Hildebrandt-Eriksen ES, Petri A, Persson R, Lindow M, Munk ME, et al. Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. *Science*. 2010;327:198–201.
- Gastaminza P, Cheng G, Wieland S, Zhong J, Liao W, Chisari FV. Cellular determinants of hepatitis C virus assembly, maturation, degradation, and secretion. *J Virol*. 2008;82:2120–9.
- Cun W, Jiang J, Luo G. The C-terminal alpha-helix domain of apolipoprotein E is required for interaction with nonstructural protein 5A and assembly of hepatitis C virus. *J Virol*. 2010;84:11532–41.
- Lee RC, Feinbaum RL, Ambros V. The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell*. 1993;75:843–54.
- Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell*. 2009;136:215–33.
- Huntzinger E, Izaurralde E. Gene silencing by microRNAs: contributions of translational repression and mRNA decay. *Nat Rev Genet*. 2011;12:99–110.
- Hutvagner G, Simard MJ. Argonaute proteins: key players in RNA silencing. *Nat Rev Mol Cell Biol*. 2008;9:22–32.
- Pfeffer S, Zavolan M, Grasser FA, Chien M, Russo JJ, Ju J, et al. Identification of virus-encoded microRNAs. *Science*. 2004;304:734–6.
- Boss IW, Renne R. Viral miRNAs and immune evasion. *Biochim Biophys Acta*. 2011;1809:708–14.
- Ziegelbauer JM. Functions of Kaposi's sarcoma-associated herpesvirus microRNAs. *Biochim Biophys Acta*. 2011;1809:623–30.
- Umbach JL, Kramer MF, Jurak I, Karnowski HW, Coen DM, Cullen BR. MicroRNAs expressed by herpes simplex virus 1 during latent infection regulate viral mRNAs. *Nature*. 2008;454:780–3.
- Nachmani D, Stern-Ginossar N, Sarid R, Mandelboim O. Diverse herpesvirus microRNAs target the stress-induced immune ligand MICB to escape recognition by natural killer cells. *Cell Host Microbe*. 2009;5:376–85.
- Rouha H, Thurner C, Mandl CW. Functional microRNA generated from a cytoplasmic RNA virus. *Nucl Acids Res*. 2010;38:8328–37.
- Perez JT, Varble A, Sachidanandam R, Zlatev I, Manoharan M, Garcia-Sastre A, et al. Influenza A virus-generated small RNAs regulate the switch from transcription to replication. *Proc Natl Acad Sci USA*. 2010;107:11525–30.

28. Hussain M, Torres S, Schnettler E, Funk A, Grundhoff A, Pijlman GP, et al. West Nile virus encodes a microRNA-like small RNA in the 3' untranslated region which up-regulates GATA4 mRNA and facilitates virus replication in mosquito cells. *Nucl Acids Res.* 2012;40:2210–23.
29. Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T. Identification of tissue-specific microRNAs from mouse. *Curr Biol.* 2002;12:735–9.
30. Chang J, Provost P, Taylor JM. Resistance of human hepatitis delta virus RNAs to dicer activity. *J Virol.* 2003;77:11910–7.
31. Chang J, Nicolas E, Marks D, Sander C, Lerro A, Buendia MA, et al. miR-122, a mammalian liver-specific microRNA, is processed from hcr mRNA and may downregulate the high affinity cationic amino acid transporter CAT-1. *RNA Biol.* 2004;1:106–13.
32. Li ZY, Xi Y, Zhu WN, Zeng C, Zhang ZQ, Guo ZC, et al. Positive regulation of hepatic miR-122 expression by HNF4alpha. *J Hepatol.* 2011;55:602–11.
33. Burns DM, D'Ambrogio A, Nottrott S, Richter JD. CPEB and two poly(A) polymerases control miR-122 stability and p53 mRNA translation. *Nature.* 2011;473:105–8.
34. Castoldi M, Vujic Spasic M, Altamura S, Elmen J, Lindow M, Kiss J, et al. The liver-specific microRNA miR-122 controls systemic iron homeostasis in mice. *J Clin Invest.* 2011;121:1386–96.
35. Bai S, Nasser MW, Wang B, Hsu SH, Datta J, Kutay H, et al. MicroRNA-122 inhibits tumorigenic properties of hepatocellular carcinoma cells and sensitizes these cells to sorafenib. *J Biol Chem.* 2009;284:32015–27.
36. Esau C, Davis S, Murray SF, Yu XX, Pandey SK, Pear M, et al. miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. *Cell Metab.* 2006;3:87–98.
37. Qiu L, Fan H, Jin W, Zhao B, Wang Y, Ju Y, et al. miR-122-induced down-regulation of HO-1 negatively affects miR-122-mediated suppression of HBV. *Biochem Biophys Res Commun.* 2010;398:771–7.
38. Wang S, Qiu L, Yan X, Jin W, Wang Y, Chen L, et al. Loss of microRNA 122 expression in patients with hepatitis B enhances hepatitis B virus replication through cyclin G(1)-modulated P53 activity. *Hepatology.* 2012;55:730–41.
39. Zhou J, Yu L, Gao X, Hu J, Wang J, Dai Z, et al. Plasma microRNA panel to diagnose hepatitis B virus-related hepatocellular carcinoma. *J Clin Oncol.* 2011;29:4781–8.
40. Jopling CL, Schutz S, Sarnow P. Position-dependent function for a tandem microRNA miR-122-binding site located in the hepatitis C virus RNA genome. *Cell Host Microbe.* 2008;4:77–85.
41. Machlin ES, Sarnow P, Sagan SM. Masking the 5' terminal nucleotides of the hepatitis C virus genome by an unconventional microRNA-target RNA complex. *Proc Natl Acad Sci USA.* 2011;108:3193–8.
42. Shimakami T, Yamane D, Welsch C, Hensley L, Jangra RK, Lemon SM. Base pairing between hepatitis C virus RNA and microRNA 122 3' of its seed sequence is essential for genome stabilization and production of infectious virus. *J Virol.* 2012;86:7372–83.
43. Henke JI, Goergen D, Zheng J, Song Y, Schuttler CG, Fehr C, et al. MicroRNA-122 stimulates translation of hepatitis C virus RNA. *EMBO J.* 2008;27:3300–10.
44. Wilson JA, Zhang C, Huys A, Richardson CD. Human Ago2 is required for efficient microRNA 122 regulation of hepatitis C virus RNA accumulation and translation. *J Virol.* 2011;85:2342–50.
45. Shimakami T, Yamane D, Jangra RK, Kempf BJ, Spaniel C, Barton DJ, et al. Stabilization of hepatitis C virus RNA by an Ago2-miR-122 complex. *Proc Natl Acad Sci USA.* 2012;109:941–6.
46. Moriishi K, Matsuura Y. Host factors involved in the replication of hepatitis C virus. *Rev Med Virol.* 2007;17:343–54.
47. Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science.* 1999;285:110–3.
48. Bartosch B, Dubuisson J, Cosset FL. Infectious hepatitis C virus pseudo-particles containing functional E1–E2 envelope protein complexes. *J Exp Med.* 2003;197:633–42.
49. Chang J, Guo JT, Jiang D, Guo H, Taylor JM, Block TM. Liver-specific microRNA miR-122 enhances the replication of hepatitis C virus in nonhepatic cells. *J Virol.* 2008;82:8215–23.
50. Lin LT, Noyce RS, Pham TN, Wilson JA, Sisson GR, Michalak TI, et al. Replication of subgenomic hepatitis C virus replicons in mouse fibroblasts is facilitated by deletion of interferon regulatory factor 3 and expression of liver-specific microRNA 122. *J Virol.* 2010;84:9170–80.
51. Ehrhardt M, Leidinger P, Keller A, Baumert T, Diez J, Meese E, et al. Profound differences of microRNA expression patterns in hepatocytes and hepatoma cell lines commonly used in hepatitis C virus studies. *Hepatology.* 2011;54:1112–3.
52. Roque-Afonso AM, Ducoulombier D, Di Liberto G, Kara R, Gigou M, Dussaix E, et al. Compartmentalization of hepatitis C virus genotypes between plasma and peripheral blood mononuclear cells. *J Virol.* 2005;79:6349–57.
53. Zehender G, De Maddalena C, Bernini F, Ebranati E, Monti G, Pioltelli P, et al. Compartmentalization of hepatitis C virus quasispecies in blood mononuclear cells of patients with mixed cryoglobulinemic syndrome. *J Virol.* 2005;79:9145–56.
54. Blackard JT, Kemmer N, Sherman KE. Extrahepatic replication of HCV: insights into clinical manifestations and biological consequences. *Hepatology.* 2006;44:15–22.
55. Laskus T, Operskalski EA, Radkowski M, Wilkinson J, Mack WJ, deGiacomo M, et al. Negative-strand hepatitis C virus (HCV) RNA in peripheral blood mononuclear cells from anti-HCV-positive/HIV-infected women. *J Infect Dis.* 2007;195:124–33.
56. Fletcher NF, Wilson GK, Murray J, Hu K, Lewis A, Reynolds GM, et al. Hepatitis C virus infects the endothelial cells of the blood-brain barrier. *Gastroenterology.* 2012;142:634–43 e6.
57. Castillo I, Rodriguez-Inigo E, Bartolome J, de Lucas S, Ortiz-Movilla N, Lopez-Alcorocho JM, et al. Hepatitis C virus replicates in peripheral blood mononuclear cells of patients with occult hepatitis C virus infection. *Gut.* 2005;54:682–5.
58. Laskus T, Radkowski M, Wilkinson J, Vargas H, Rakela J. The origin of hepatitis C virus reinfecting transplanted livers: serum-derived versus peripheral blood mononuclear cell-derived virus. *J Infect Dis.* 2002;185:417–21.
59. Ito M, Masumi A, Mochida K, Kukihara H, Moriishi K, Matsuura Y, et al. Peripheral B cells may serve as a reservoir for persistent hepatitis C virus infection. *J Innate Immun.* 2010;2:607–17.
60. Ramirez S, Perez-Del-Pulgar S, Carrion JA, Costa J, Gonzalez P, Massaguer A, et al. Hepatitis C virus compartmentalization and infection recurrence after liver transplantation. *Am J Transpl.* 2009;9:1591–601.
61. Kato T, Date T, Miyamoto M, Zhao Z, Mizokami M, Wakita T. Nonhepatic cell lines HeLa and 293 support efficient replication of the hepatitis C virus genotype 2a subgenomic replicon. *J Virol.* 2005;79:592–6.
62. Fletcher NF, Yang JP, Farquhar MJ, Hu K, Davis C, He Q, et al. Hepatitis C virus infection of neuroepithelioma cell lines. *Gastroenterology.* 2010;139:1365–74.
63. Fukuhara T, Kambara H, Shiokawa M, Ono C, Katoh H, Morita E, et al. Expression of microRNA miR-122 facilitates an efficient replication in nonhepatic cells upon infection with hepatitis C virus. *J Virol.* 2012;86:7918–33.