

FIG 5 Neutralization of HCVcc infection in Hec1B cells by specific antibodies. HCVcc was preincubated with anti-E2 antibody (AP-33) and inoculated into Hec1B/Cont and Hec1B-miR-122 cells. Cells were preincubated with anti-human CD81 antibody and inoculated with HCVcc. Intracellular HCV RNA levels at 2, 18, 24, and 48 h postinfection were determined by qRT-PCR.

To further confirm the specificity of the enhancement of HCV replication by the expression of miR-122, Huh7, Hec1B/miR-122, and Hec1B/Cont cells were treated with LNAs that were either specific to miR-122 (LNA-miR-122) or nonspecific (LNA-control) at 6 h before infection with HCVcc. Although the treatment with LNA-miR-122 inhibited the enhancement of viral RNA replication in Huh7 and Hec1B/miR-122 cells in a dose-dependent manner, no inhibition of viral replication was observed in Hec1B/Cont cells (Fig. 6C). These results suggest that Hec1B cells permit HCV replication in a miR-122-independent manner and exogenous expression of miR-122 enhances viral replication.

Specific interaction between miR-122 and the 5' UTR of HCV is required for HCV replication. To determine the effect of the specific interaction between miR-122 and the 5' UTR of the HCV genome on the enhancement of RNA replication, we generated MT pri-miR-122 carrying a substitution of uridine to adenosine in the seed domain and an additional complementary substitution of adenosine to uridine to stabilize the loop structure of pri-miR-122 (Fig. 8A). A high expression level of MT miR-122, comparable to that of WT miR-122, was introduced into Hec1B cells by infection with lentiviral vectors (Fig. 8B). To determine the specificity of miR-122 on the replication of HCV, Hec1B cells expressing either WT or MT miR-122 were inoculated with HCVcc at an MOI of 1. Enhancement of HCV replication was observed in Hec1B cells by the expression of WT but not that of MT miR-122, suggesting that the sequence specificity of miR-122 with the 5' UTR of HCV is crucial for the efficient replication of HCV (Fig. 8C). To further confirm the effect of the specificity of interaction between miR-122 and the binding sites in the 5' UTR of HCV on the enhancement of HCV replication, we generated two mutant viruses, HCVcc-M1 and HCVcc-M2, carrying complementary substitutions in the miR-122-binding site 1 alone and in both sites 1 and 2 in the 5' UTR of HCV, respectively (Fig. 8D). Recently, Jangra et al. demonstrated that the propagation of a mutant HCVcc bearing mutations in sites 1 and 2 in the 5' UTR was rescued by the expression of MT miR-122 in Huh7.5 cells (25). We confirmed that the propagation of HCVcc-M1 and HCVcc-M2 in Huh7.5.1 cells was rescued by the expression of MT miR-122 but not of WT miR-122, although the recovery of infectious titers of HCVcc-M2 was significantly lower than the recovery of infectious titers of HCVcc-M1 (Fig. 8E). Next, to examine the interaction between miR-122 and the HCV genome in Hec1B cells, we inoculated HCVcc or mutant viruses into Hec1B cells expressing either or

both WT and MT miR-122 and determined the replication of HCV RNA by qRT-PCR (Fig. 8F). Expression of WT and MT miR-122 in Hec1B cells permits replication of HCVcc and HCVcc-M2, respectively, although the enhancing effects differed. On the other hand, the expression of both WT and MT miR-122 is required for the replication of HCVcc-M1, because MT and WT miR-122 bind to sites 1 and 2 in the 5' UTR of this virus, respectively. Interestingly, a low level of HCVcc-M1 replication was also observed in Hec1B cells expressing either WT or MT miR-122, in contrast to the requirement of the corresponding miR-122 for the replication of HCVcc and HCVcc-M2. These results suggest that the specific interaction between miR-122 and the 5' UTR of HCV is crucial for the replication of HCV.

Viral particle formation in hepatic and nonhepatic cells. These data suggest that miR-122 expression facilitates replication of HCV RNA in nonhepatic cells. Recently, we have shown that expression of miR-122 facilitates infectious particle formation of HCVcc in a hepatoma cell line, Hep3B (29). To examine the effect of miR-122 expression on particle formation in nonhepatic cells, intracellular and extracellular viral RNA levels in cells infected with HCVcc were determined. Intracellular RNA replication in the hepatic cell lines, including Huh7.5.1 and Hep3B/miR-122, was increased up to 72 h postinfection with HCVcc, whereas in nonhepatic cell lines, including 293T-CLDN/miR-122 and Hec1B/miR-122, such replication was comparable to that in the hepatic cell lines until 24 h postinfection but reached a limit at this point (Fig. 9A). In spite of no clear increase of intracellular HCV RNA in Hep3B/Cont cells upon infection with HCVcc (Fig. 9A), subtle but substantial production of infectious particles was detected in the culture supernatants at 72 h postinfection, in contrast to no production of infectious particles in those of the nonhepatic cell lines (Fig. 9B). Furthermore, no focus formation was observed in Hec1B/miR-122 cells upon infection with HCVcc, in contrast to the many foci in Huh7.5.1 cells (Fig. 9C), and no infectivity was detected even in the lysates of Hec1B/miR-122 cells infected with HCVcc (Fig. 9D). These results suggest that not only the replication efficiency of viral RNA but also other factors are involved in the assembly of HCV and that the viral assembly process is impaired in Hec1B/miR-122 cells infected with HCVcc, in spite of the efficient replication of HCV RNA.

It was previously shown that lipid droplets, diacylglycerol *O*-acyltransferase 1 (DGAT1), and apolipoproteins B and E play an important role in the assembly of HCV particles (10, 22, 40). To understand the molecular mechanisms underlying the low efficiency of infectious particle formation in nonhepatic cells, we first examined the subcellular localization of lipid droplets and HCV core protein in Hec1B/miR-122 cells infected with HCVcc. Although the core protein was detected around the lipid droplets, as seen in Huh7.5.1 cells, only a small amount of lipid droplets was detected in Hec1B/miR-122 cells infected with HCVcc compared with the amount detected in Huh7.5.1 cells (Fig. 9E), suggesting that the low level of lipid droplet formation is involved in the impairment of infectious particle formation in nonhepatic cells.

Next we examined the expression patterns of molecules involved in lipid metabolism by using cDNA microarray and qPCR analyses. Although expression levels of low-density lipoprotein receptor (LDLR), sterol regulatory element-binding protein 1c (SREBP1c), SREBP2, and DGAT1 in nonhepatic Hec1B and 293T cells were comparable to those in hepatic Huh7 and Hep3B cells, those of VLDL-associated proteins, including ApoE, ApoB, and

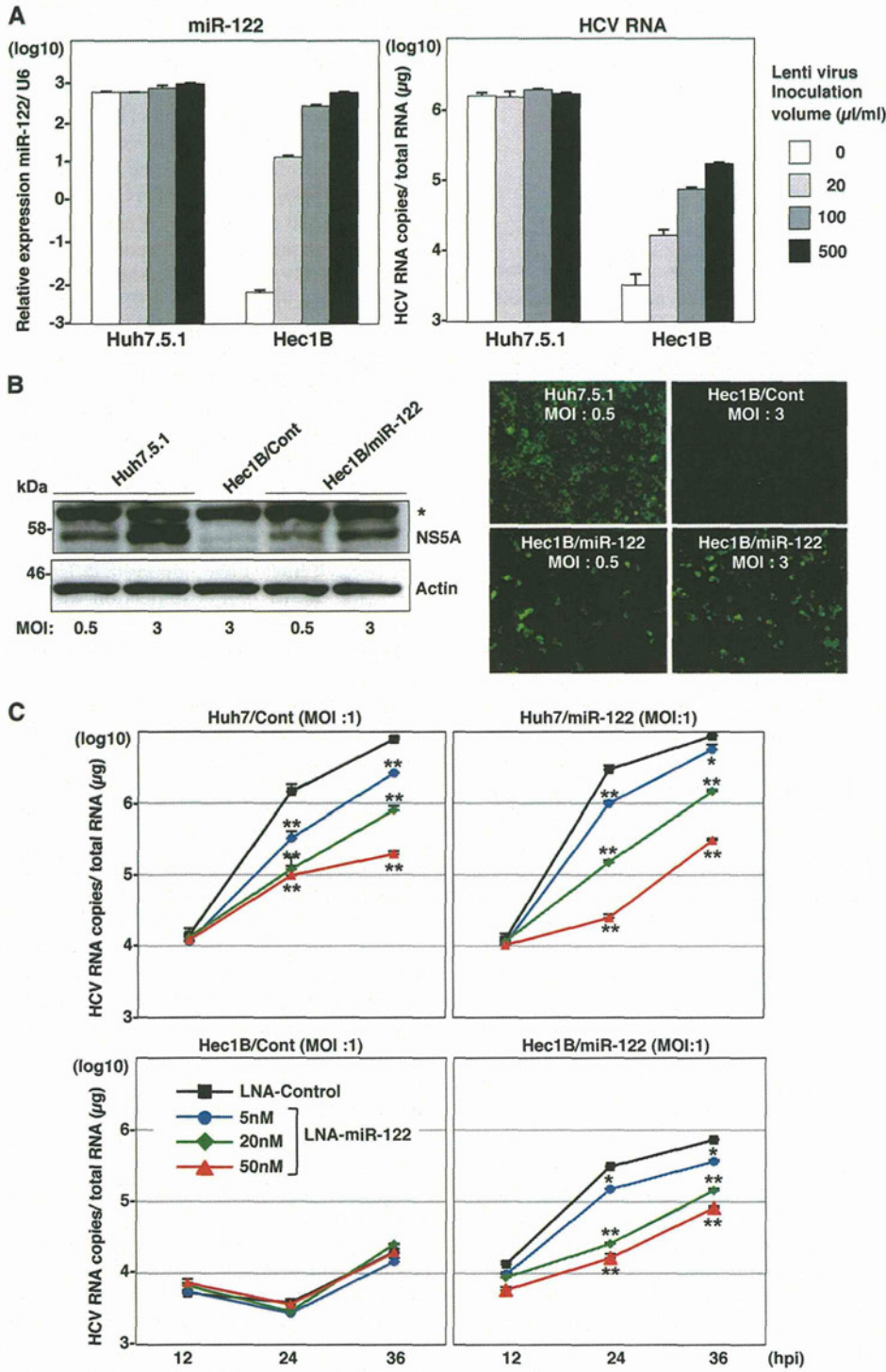


FIG 6 Expression of miR-122 is essential for the enhancement of HCV replication in the Hec1B cells. (A) Huh7.5.1 and Hec1B cells were transduced with lentiviral vectors expressing miR-122 in a dose-dependent manner and infected with HCVcc at an MOI of 1. Intracellular miR-122 and HCV RNA were determined at 24 h postinfection by qRT-PCR. (B) Huh7.5.1 and Hec1B/miR-122 cells were infected with HCVcc at an MOI of 0.5 or 3 and subjected to immunoblotting and immunofluorescence analyses using anti-NS5A antibodies at 48 h postinfection. The asterisk indicates nonspecific bands. (C) LNAs specific to miR-122 at a final concentration of 5 nM, 20 nM, or 50 nM and control (LNA alone at 50 nM) were introduced into Huh7/Cont, Huh7/miR-122, Hec1B/miR-122, and Hec1B/Cont cells by using Lipofectamine RNAiMAX transfection reagent and infected with HCVcc at an MOI of 1 at 6 h posttransfection. Intracellular HCV RNA levels were determined by qRT-PCR at 12, 24, and 36 h postinfection. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$) versus the results for control cells.

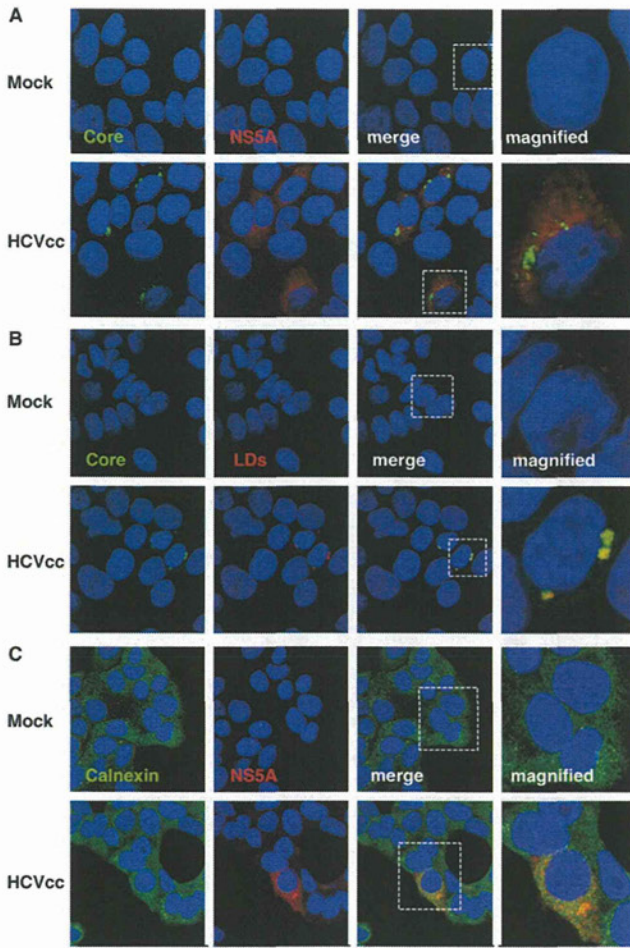


FIG 7 Subcellular localization of core and NS5A proteins in Hec1B/miR-122 cells infected with HCVcc. Hec1B/miR-122 cells infected with or without HCVcc at an MOI of 1 were fixed with 4% PFA at 48 h postinfection and stained with appropriate antibodies to core and NS5A proteins (A), core protein and lipid droplets (B), and NS5A and calnexin (C). The boxes in the merged images were magnified, and the images are displayed on the right.

MTTP, in nonhepatic cells were significantly lower than those in hepatic cells (Fig. 10). Collectively, these results suggest that intracellular functional lipid metabolism, including the biosynthesis of lipid droplets and the production of VLDL, participates in the assembly of HCV.

Establishment of HCV replicon in Hec1B/miR-122 cells. It was previously shown by using RNA replicon cells based on the JFH1 strain that expression of miR-122 enhanced the translation of HCV RNA in HEK293 cells and MEFs (8, 35). We tried to establish HCV replicon cells based on genotype 1b Con1 and genotype 2a JFH1 strains in Hec1B/miR-122 and HEK293 cells stably expressing miR-122 (HEK293/miR-122). To examine the colony formation efficiency of the HCV RNAs of the Con1 and JFH1 strains, SGR RNA was electroporated into the cell lines and selected by G418 for 3 weeks. Expression of miR-122 in Hec1B cells significantly enhanced the colony formation of SGR of the Con1 strain (Fig. 11A), suggesting that the expression of miR-122 in Hec1B cells supports the efficient replication of SGR. HCV replication in 20 replicon clones established by the transfection with

SGR RNA of the Con1 strain in Hec1B/miR-122 cells was examined by qRT-PCR and immunoblotting. All clones contained high levels of HCV RNA (3×10^6 to 5×10^7 copies per μg of total RNA) (Fig. 11B), and expression of NS5A was well correlated with the levels of HCV RNA in the clones (Fig. 11C). Two replicon clones (clones 2 and 10) in Hec1B/miR-122 cells exhibiting high levels of RNA replication and NS5A expression further confirmed the high level of expression of NS5A by immunofluorescent microscopy (Fig. 11D). These results suggest that expression of miR-122 facilitates the efficient replication of SGR of at least two HCV genotypes in Hec1B cells.

Our previous reports showed that HCV NS proteins were colocalized with dsRNA and cochaperone molecules, FK506-binding protein 8 (FKBP8), in dot-like structures on the ER membrane of Huh7 replicon cells (59). Colocalization of NS5A with dsRNA or FKBP8 was observed in the dot-like structures in not only Huh7 SGR cells but also Hec1B/miR-122 SGR cells (Fig. 12A), suggesting that the dot-like structure required for efficient viral replication is also generated in Hec1B/miR-122 replicon cells. It has been shown that HCV replication induces the formation of convoluted membranous structures, called membranous webs, in Huh7 cells (13, 45). FM-EM techniques revealed the localization of NS5A on the convoluted structures in Hec1B/miR-122 replicon cells (Fig. 12B). These results suggest that the replication complex required for viral replication was also generated in the Hec1B/miR-122 replicon cells, as was seen in the Huh7 replicon cells.

miR-122 is a crucial determinant of HCVcc propagation. It has been shown that the infectivity of HCVcc in cured cells, established when IFN treatment induces the elimination of the viral genome from the Huh7 replicon cells harboring an HCV RNA, is significantly higher than that in parental Huh7 cells (2, 66). Therefore, we tried to establish Hec1B-based cured cells from the Con1 SGR clones harboring a high copy number of HCV RNA. Treatment with cyclosporine and the protease inhibitor of HCV suppressed NS5A expression in Hec1B/miR-122 SGR clone 2 in a dose-dependent manner (Fig. 13A), whereas no reduction was observed by the IFN treatment due to a lack of an IFN receptor, as shown in Fig. 4C. It was reported that monotherapy by the HCV protease inhibitor induces the emergence of resistant breakthrough viruses (34, 55). Therefore, we treated five Hec1B/miR-122 SGR clones (clones 2, 5, 10, 14, and 16) with 1 $\mu\text{g}/\text{ml}$ cyclosporine and 100 nM protease inhibitor for HCV. Viral RNA was determined by qRT-PCR every 5 days posttreatment. Elimination of viral RNA was achieved in four clones (clones 2, 5, 10, and 14) within 20 days posttreatment (Fig. 13B). Replication of HCV RNA in the cured cells infected with HCVcc at an MOI of 0.5 was 2- to 30-fold higher than that in parental cells at 24 h postinfection (Fig. 13C). In addition, replication of HCV RNA in cured clone 2 infected with HCVcc at an MOI of 0.1 was comparable to that in Huh7.5.1 cells until 24 h postinfection (Fig. 13D). Expression of NS5A was significantly increased in cured clone 2 compared to that in the parental Hec1B/miR-122 cells (Fig. 13E and F). It was previously shown that the increased permissiveness of Huh7-derived cured cells, Huh7.5 cells, is attributable to a mutation in the RIG-I gene (58). To examine the innate immune response in the parental and cured Hec1B/miR-122 cells, induction of IFN-stimulated gene 15 (ISG15) was determined upon stimulation with IFN- α or VSV. Although induction of ISG15 was not observed in either parental or cured cells upon stimulation with IFN- α due to a lack of an IFN receptor (11) (Fig. 14A), it was

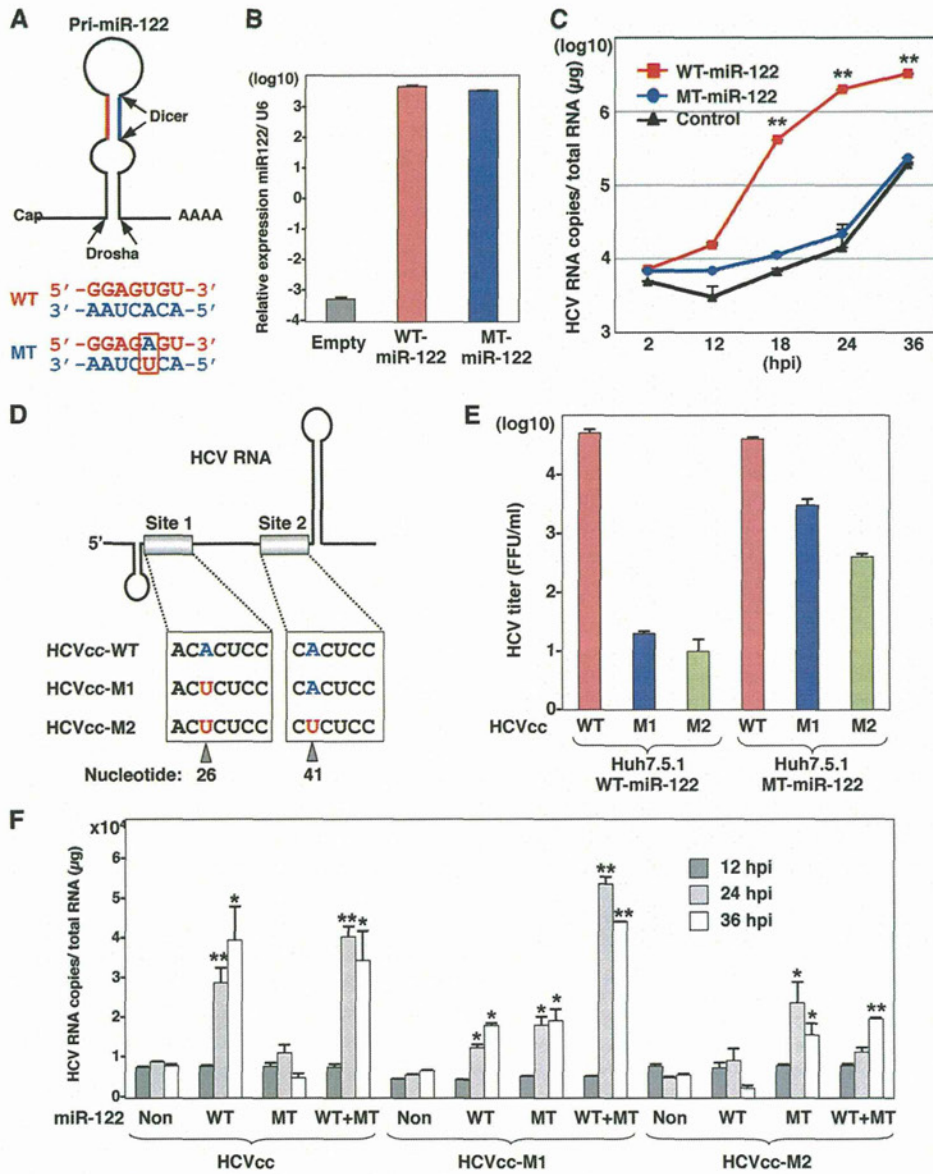


FIG 8 Specific interaction between miR-122 and the 5' UTR of HCV is required for HCV replication. (A) Structures of pri-miR-122 and the nucleotide sequences of WT and MT miR-122, which has a substitution of uridine to adenosine in the seed domain and an additional complementary substitution of adenosine to uridine for stable expression. (B) WT or MT miR-122 was introduced into Hec1B cells by a lentiviral vector, and miR-122 expression levels were determined by qRT-PCR. (C) HCVcc was inoculated into Hec1B cells expressing either WT or MT miR-122 and control cells at an MOI of 1, and the intracellular HCV RNA levels were determined by qRT-PCR. (D) Diagram of mutant viruses HCVcc-M1 and HCVcc-M2 carrying complementary substitutions in the miR-122-binding site 1 alone and both sites 1 and 2 in the 5' UTR of HCV, respectively. (E) Viral RNA of HCVcc, HCVcc-M1, or HCVcc-M2 was electroporated into Huh7.5.1 cells expressing either WT or MT miR-122, and infectious titers of the viruses recovered in the culture supernatants at 72 h postinfection of the second passage were determined by a focus-forming assay in cells expressing either WT or MT miR-122. Red, blue, and green bars, infectious titers of HCVcc, HCVcc-M1, and HCV-M2, respectively. (F) HCVcc, HCVcc-M1, or HCV-M2 was inoculated into Hec1B cells expressing either or both WT and MT miR-122 at an MOI of 0.5, and intracellular HCV RNA levels were determined at 12, 24, and 36 h postinfection by qRT-PCR. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$) versus the results for control cells.

detected in both cells infected with VSV (Fig. 14B). Therefore, other mechanisms should be involved in the enhancement of permissiveness of Hec1B-derived cured cells. Ehrhardt et al. showed that the expression levels of miR-122 in Huh7-derived cured cells, including Huh7.5, Huh7.5.1, and Huh7-Lunet cells, are significantly higher than those in parental Huh7 cells (14). In addition, our recent study indicated that levels of ex-

pression of miR-122 in the cured Huh7 and Hep3B/miR-122 cells were higher than those in parental cells (29). Levels of expression of miR-122 in the Hec1B-based cured cell clones are also higher than those in parental Hec1B/miR-122 cells (Fig. 13G). These results suggest that a high level of miR-122 expression is a crucial determinant of high susceptibility to HCVcc propagation in the cured cells.

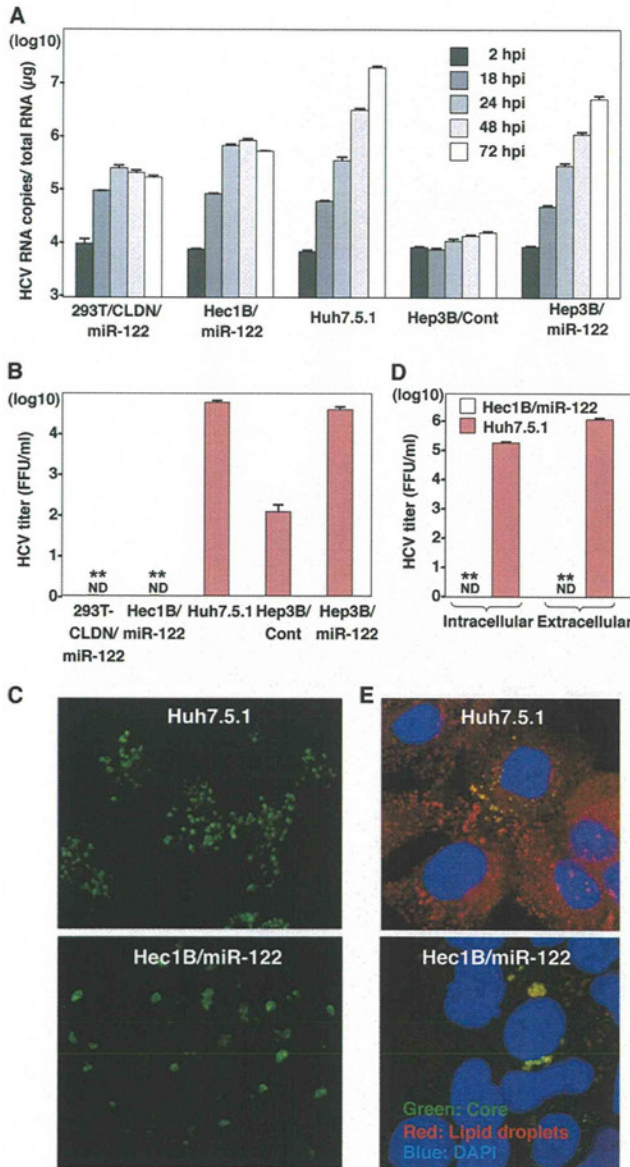


FIG 9 Viral particle formation in hepatic and nonhepatic cells. (A) HCVcc was inoculated into 293T-CLDN/miR-122, Hec1B/miR-122, Hep3B/Cont, and Hep3B/miR-122 cells at an MOI of 1 and into Huh7.5.1 cells at an MOI of 0.1. HCV RNA levels (copies/ μ g) in cells at 2, 18, 24, 48, and 72 h postinfection were determined by qRT-PCR. (B) HCVcc was inoculated into 293T-CLDN/miR-122, Hec1B/miR-122, Hep3B/Cont, and Hep3B/miR-122 cells at an MOI of 1 or into Huh7.5.1 cells at an MOI of 0.1, and infectious titers in the culture supernatants were determined at 72 h postinfection by a focus-forming assay in Huh7.5.1 cells. ND, not determined. (C) Huh7.5.1 and Hec1B/miR-122 cells were infected with HCVcc at MOIs of 0.1 and 1, respectively, incubated with 1% methylcellulose in DMEM containing 10% FCS for 72 h, fixed with 4% PFA, and subjected to immunofluorescence analysis using anti-NS5A antibody. (D) Hec1B/miR-122 and Huh7.5.1 cells were infected with HCVcc at MOIs of 1 and 0.1, respectively, and infectious titers in cells and supernatants were determined by a focus-forming assay at 72 h postinfection. (E) Huh7.5.1 and Hec1B/miR-122 cells were infected with HCVcc at MOIs of 0.1 and 1, respectively, fixed with 4% PFA, and subjected to immunofluorescence assay using anti-core protein antibody (green). Lipid droplets and cell nuclei were stained with BODIPY (red) and DAPI (blue), respectively. Asterisks indicate significant differences (**, $P < 0.01$) versus the results for Huh7.5.1 cells.

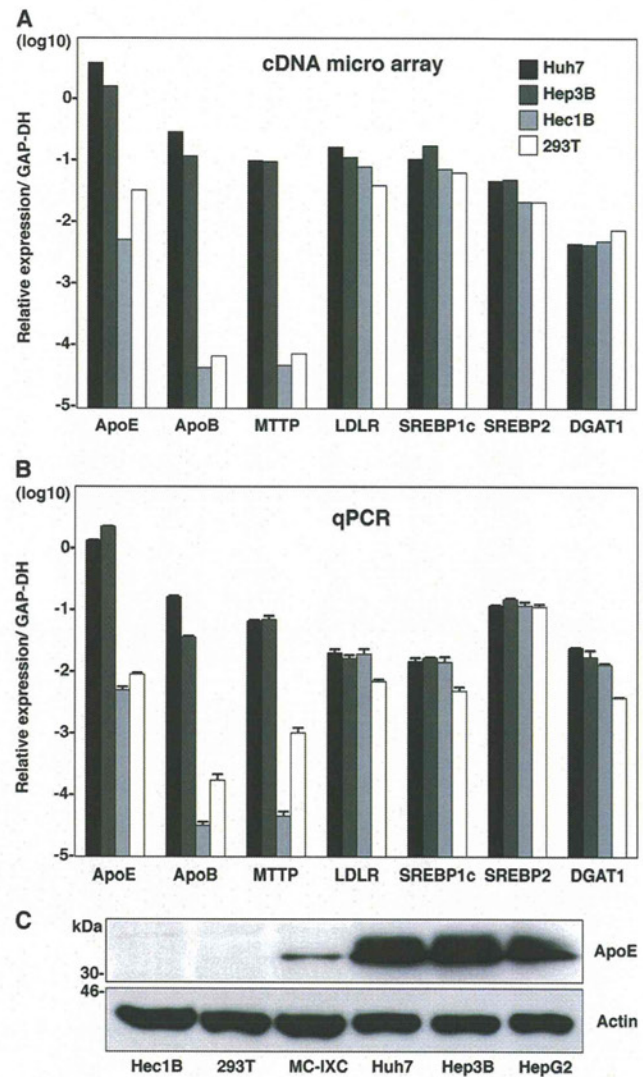


FIG 10 Expression of lipid metabolism-associated proteins in hepatic and nonhepatic cells. (A) Expression levels of ApoE, ApoB, MTTp, LDLR, SREBP1c, SREBP2, and DGAT1 were compared among hepatic (Huh7 and Hep3B) and nonhepatic (Hec1B and 293T) cells using cDNA microarray analyses. (B) Total RNA was extracted from the cells, and expression levels of ApoE, ApoB, MTTp, LDLR, SREBP1c, SREBP2, and DGAT1 gene were determined by qPCR. (C) Nonhepatic (Hec1B, 293T, and MC-IXC) and hepatic (Huh7, Hep3B, and HepG2) cells were subjected to immunoblotting using anti-ApoE antibody.

DISCUSSION

Although multiple epidemiological studies have revealed that HCV infection induces several EHMs, they have not well elucidated the molecular mechanisms of the EHMs induced by HCV infection (19). Indeed, HCVcc does not infect PBMCs (38). It has been shown that two neuroepithelioma cell lines permit HCVcc infection at low levels (17) and lymphotropic strains or quasispecies of HCV exist in infected individuals (12, 52). Furthermore, many molecules involved in the entry, replication, and assembly of HCVcc have been identified, although these molecules are not sufficient to explain the liver tropism of HCV. Recently, a liver-specific microRNA, miR-122, was shown to facilitate the efficient

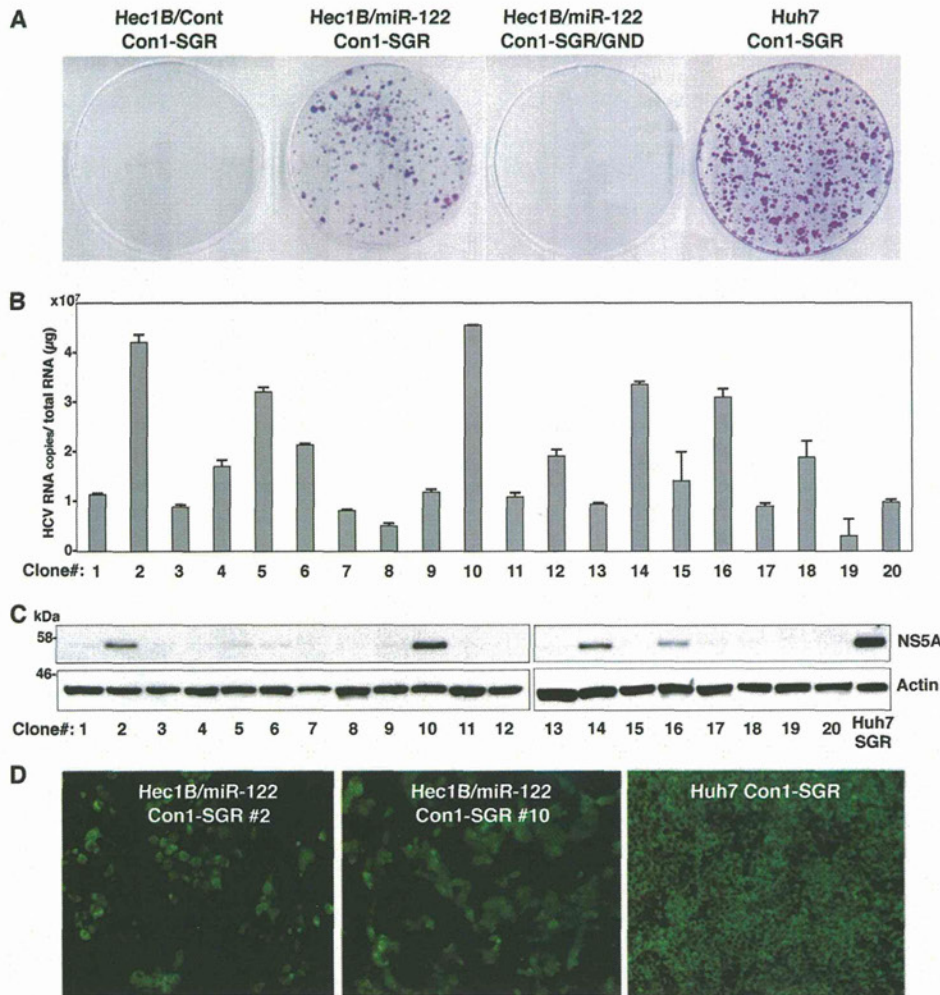


FIG 11 Establishment of Con1-based HCV replicon cells by using Hec1B cells. (A) WT or replication-defective SGR RNA of the HCV Con1 strain was electroporated into Hec1B/Cont, Hec1B/miR-122, and Huh7 cells, and the medium was replaced with DMEM containing 10% FCS and 1 mg/ml G418 at 24 h postelectroporation. Colonies were stained with crystal violet after 3 weeks of selection with G418. (B) Total RNAs of 20 selected clones were extracted and subjected to qRT-PCR. (C) The 20 SGR clones were subjected to immunoblotting using anti-NS5A antibody. Huh7-derived Con1-based SGR cells were used as a positive control. (D) NS5A proteins in SGR clones 2 and 10 were stained with appropriate antibodies and examined by fluorescence microscopy. Huh7-derived Con1-based SGR and parental Hec1B cells were used for positive and negative controls, respectively.

replication of HCV through a specific interaction with the complementary sequences in the 5' UTR of HCV RNA (21, 25, 27, 36). In addition, exogenous expression of miR-122 facilitates the replication of SGR of the JFH1 strain in HEK293 cells (8) and the propagation of HCVcc in HepG2 and Hep3B nonpermissive hepatoma cells (29, 43), suggesting that the expression of miR-122 is required for the efficient replication of HCV. However, HCV replicon cells have also been established in HeLa and L190 cells derived from stellate cells in which no exogenous miR-122 is expressed (30, 63). In this study, naïve Hec1B cells also exhibited a low level of replication upon infection with HCVcc (Fig. 4B), and this replication was resistant to treatment with an inhibitor of miR-122, LNA-miR-122 (Fig. 6C), suggesting that miR-122 expression is not a necessary condition but is required for the enhancement of HCV replication and that HCV is capable of replicating in nonhepatic cells in an miR-122-independent manner. Although the application of miR-122-specific LNAs to chronic

hepatitis C patients is now in progress (32), further studies are needed to clarify the mechanisms underlying the miR-122-independent replication of HCV in more detail.

Although the importance of receptor-mediated entry in the cell tropism of HCV has been evaluated (16, 65), cDNA microarray databases, including the NextBio search engine, revealed that HCV receptor candidates, including hCD81, SR-BI, CLDN1, and OCLN, are highly expressed in many nonhepatic tissues. In addition, our current data and previous reports demonstrated that many nonhepatic cells permitted the entry of the pseudotype viruses bearing HCV envelope proteins, suggesting that other host factors must be involved in the cell tropism of HCV to human hepatocytes (4, 17, 54, 61). The data in this study suggest that miR-122 expression and functional lipid metabolism play crucial roles in the determination of an efficient propagation of HCV *in vitro*. On the other hand, previous studies showed the compartmentalization of genetic variation in HCV between hepatic and

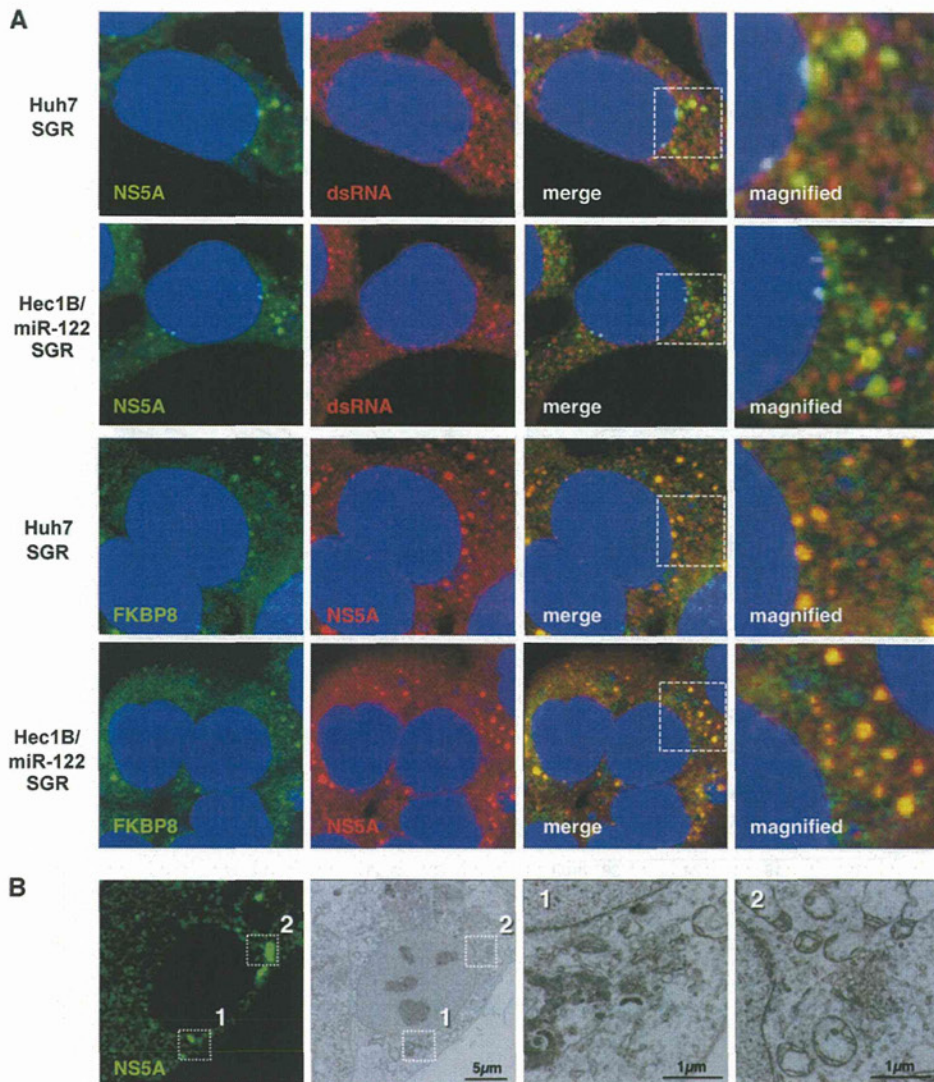


FIG 12 Replication complex in Hec1B/miR-122 replicon cells. (A) Huh7 and Hec1B/miR-122 cells harboring the Con1 SGR RNA were fixed, permeabilized, and stained with antibodies to NS5A and dsRNA or FKBP8. The boxed areas in the merged images were magnified, and the images are displayed on the right. (B) Hec1B-derived Con1 SGR cells were stained with anti-NS5A antibody. Identical fields were observed under EM by using the correlative FM-EM technique. The boxed areas are magnified, and the images displayed on the right.

nonhepatic tissues, suggesting that HCV is capable of replicating in nonhepatic tissues expressing either miR-122, ApoE, ApoB, or MTTP (52). Collectively, these results suggest that entry receptors, miR-122, and functional lipid metabolism are mainly involved in the regulation of internalization, RNA replication, and assembly of HCV, respectively, and are important factors in determining the cell tropism of HCV to hepatocytes. On the other hand, it might be feasible to speculate that EHMs observed in chronic hepatitis C patients are caused by an incomplete miR-122-independent propagation of HCV in nonhepatic cells.

In spite of the efficient replication of HCV in Hec1B and 293T-CLDN cells expressing miR-122, no infectious particle was detected, in contrast to the case with hepatic cells (Fig. 9), suggesting the involvement of liver-specific host factors and/or machineries in the assembly of infectious particles. In general, the liver plays a major role in lipid metabolism, such as in fatty acid and lipopro-

tein syntheses (9), and many reports have indicated the involvement of lipid metabolism, especially triglyceride metabolism, in the assembly and budding of HCV particles. Lipid droplets, MTTP, ApoB, and ApoE have been shown to participate in the assembly and secretion of infectious particles of HCVcc in Huh7 cells (20, 23, 26, 40). In the current analyses, there were fewer lipid droplets and the expression levels of ApoE, ApoB, and MTTP were lower in nonhepatic cells than in hepatic cells. Although minus-strand HCV RNA and viral proteins were detected in nonhepatic cells (33, 60), it was shown that the recurrence of HCV after liver transplantation for patients with HCV-induced liver diseases was mainly caused by HCV variants generated in the liver but not in nonhepatic tissues (50). These results support the notion that replication of HCV RNA in nonhepatic cells is unlikely to be a reservoir for persistent infection, due to the lack of infectious particle formation.

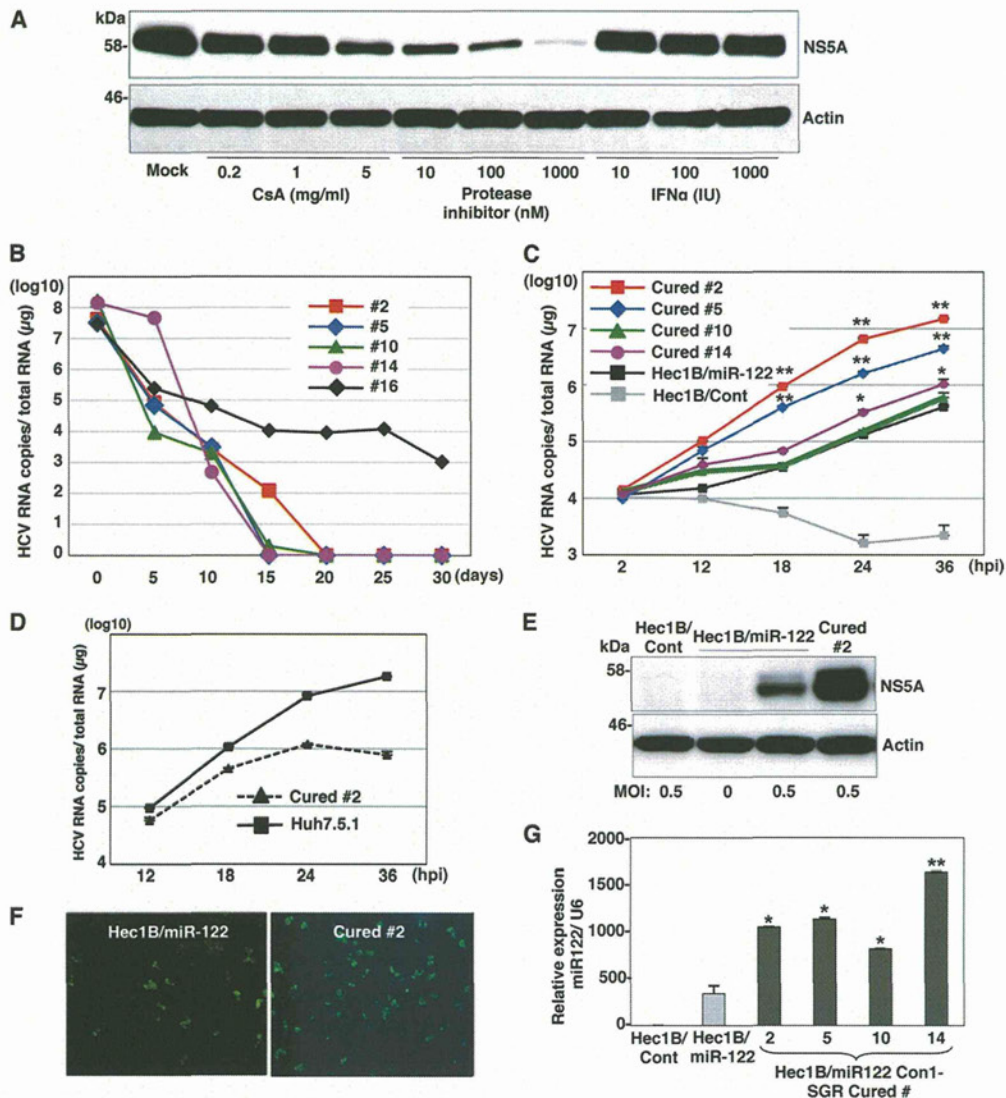


FIG 13 miR-122 is a crucial determinant for the efficient replication of HCVcc and replicon RNA. (A) Hec1B Con1 replicon clone 2 was treated with stepwise concentrations of cyclosporine (CsA; 0.2, 1, and 5 μ g/ml), NS3/4A protease inhibitor (10, 100, and 1,000 nM), or IFN- α (10, 100, and 1,000 IU) and subjected to immunoblotting using anti-NS5A antibody at 48 h posttreatment. (B) Five Con1-based SGR clones were treated with the combination of 1 μ g/ml cyclosporine and 100 nM HCV protease inhibitor to eliminate the HCV genome. Intracellular HCV RNA levels at 5, 10, 15, 20, 25, and 30 days posttreatment were determined by qRT-PCR analysis. (C) HCVcc was inoculated with Hec1B/Cont, parental Hec1B/miR-122, and Hec1B-based cured cells (clones 2, 5, 10, and 14) at an MOI of 1. Intracellular HCV RNA levels at 2, 12, 18, 24, and 36 h postinfection were determined by qRT-PCR analysis. (D) HCVcc was inoculated into Huh7.5.1 and Hec1B/miR-122 cured clone 2 cells at an MOI of 1. Intracellular HCV RNA levels were determined by qRT-PCR at 12, 24, 36, and 48 h postinfection. (E and F) Hec1B/Cont, parental Hec1B/miR-122, and cured cells of clone 2 were infected with HCVcc at an MOI of 0.5. After 48 h, the cells were subjected to immunoblotting and immunofluorescence analyses using appropriate antibodies. (G) Total miRNAs were extracted from Hec1B/Cont (white), parental Hec1B-miR-122 cells (gray), and four cured cell clones (black). miR-122 expression levels in these cells were determined by qRT-PCR analysis. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$) versus the results for parental Hec1B/miR-122 cells.

The endogenous expression of miR-122 is hardly detected in Hec1B cells, in contrast to the abundant expression of miR-122 in Huh7 cells. Therefore, more accurate analyses of the biological significance of the interaction between miR-122 and the 5' UTR on the replication of HCVcc in Hec1B could be possible by introducing mutations not only into viruses but also into miR-122. Replication of HCVcc and a mutant virus bearing two mutations in the 5' UTR (HCVcc-M2) was observed in Hec1B cells expressing WT and MT miR-122, respectively, although the level of replication was lower in cells infected with HCVcc-M2 than in those

infected with HCVcc, probably due to the mutations in the 5' UTR (Fig. 8F). In contrast, a mutant virus (HCVcc-M1) bearing a mutation in site 1 alone exhibited efficient replication in Hec1B cells expressing both WT and MT miR-122 comparable to the replication level of the wild-type virus in cells expressing WT miR-122. Furthermore, the replication level of HCVcc-M1 was low in Hec1B cells expressing either WT or MT miR-122, suggesting that interaction between miR-122 and either of the seed sequence-binding sites in the 5' UTR has an equal ability to enhance the replication of the HCV genome. However, it was shown that the

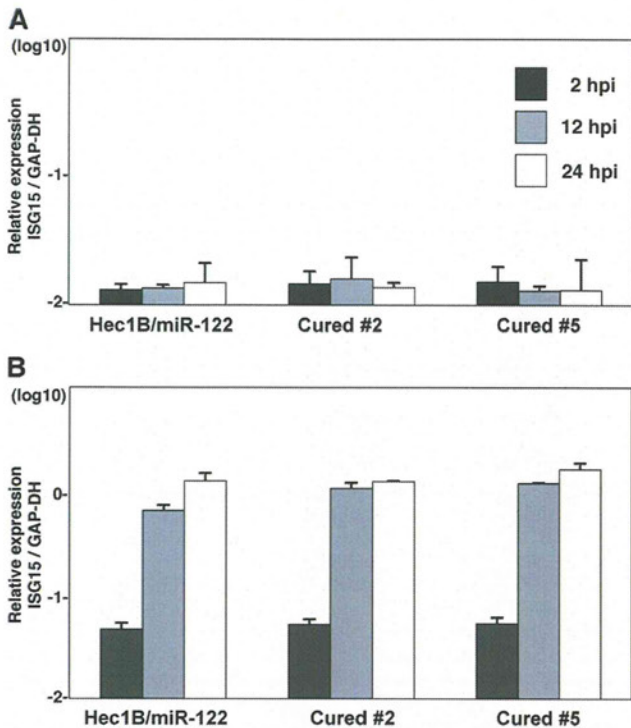


FIG 14 Innate immune responses in parental and cured Hec1B/miR-122 cells. Parental and cured Hec1B/miR-122 cells were stimulated with 100 U of IFN- α (A) or VSV (B). The expression levels of IFN-stimulated gene 15 (ISG15) were determined by qRT-PCR at 2, 12, and 24 h posttreatment.

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

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

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