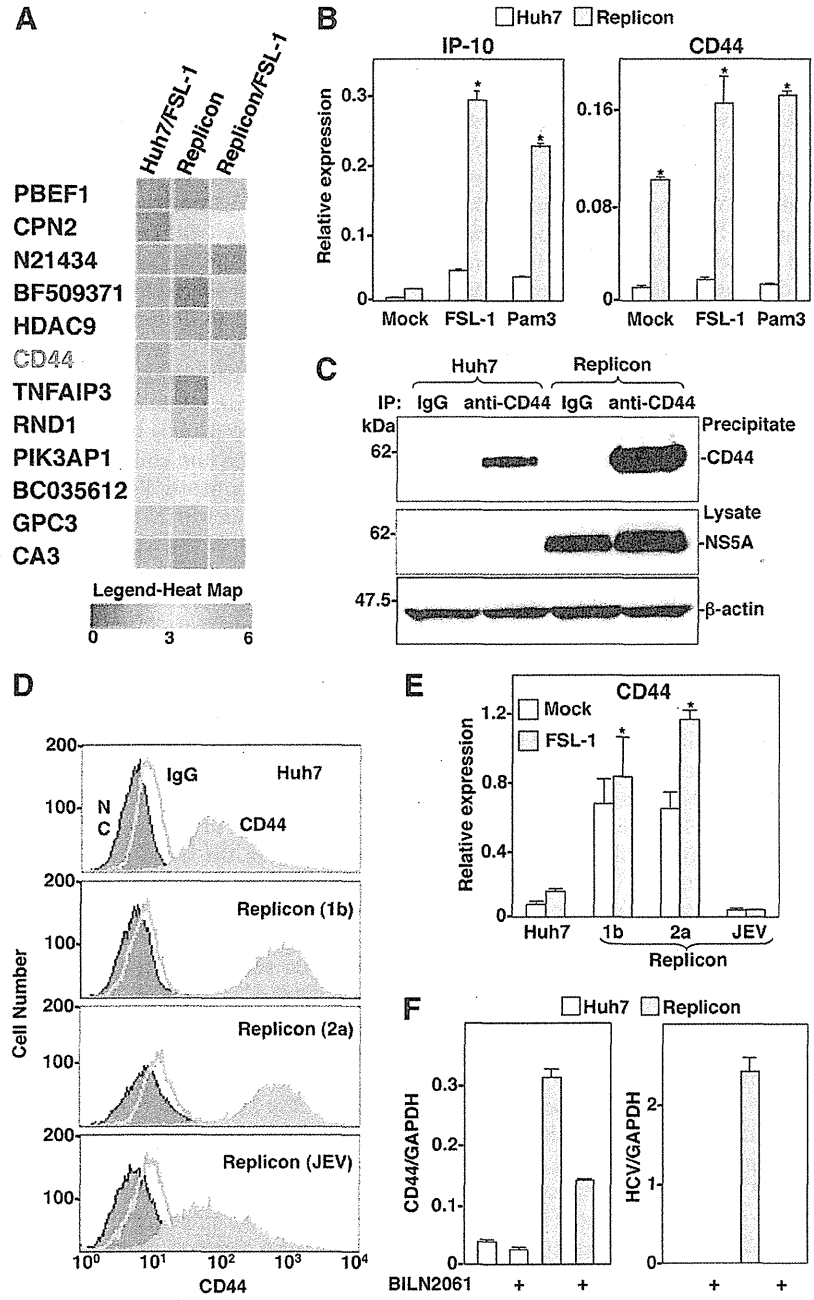

FIG 2 Expression of CD44 in HCV replicon-harboring cells is upregulated in accord with IP-10 production in response to TLR2 ligands. (A) Twelve genes in Huh7 and HCV replicon-harboring cells treated or not treated with FSL-1 were selected, and the resulting heat map is shown. PBEF1, pre-B-cell colony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

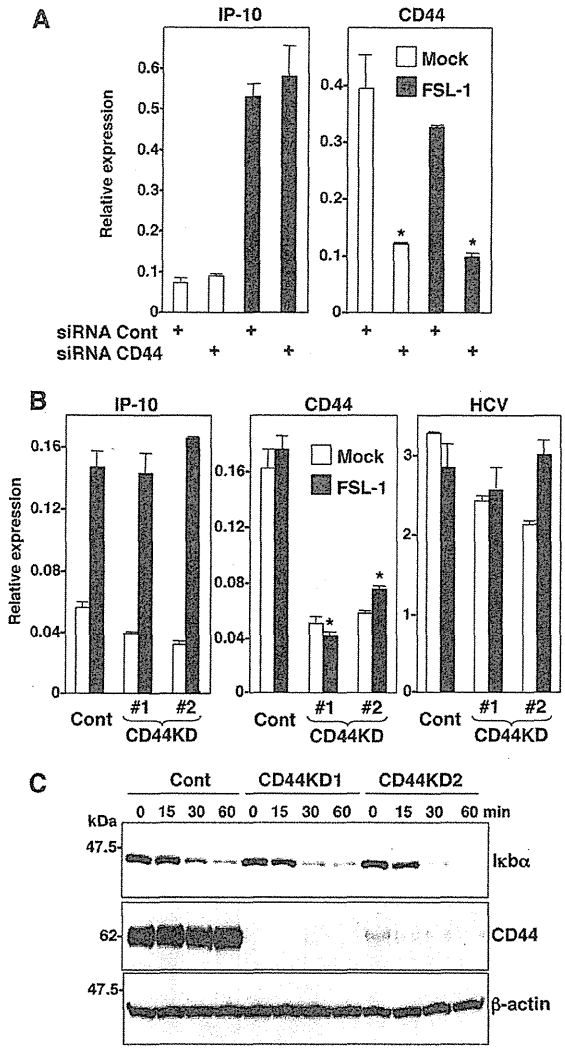

FIG 3 Effect of CD44 silencing on IP-10 production in replicon-harboring cells upon stimulation with TLR2 ligands derived from bacterial components. (A) HCV replicon-harboring cells were transfected with siRNA targeted to CD44 gene or control siRNA at a final concentration of 100 nM and stimulated with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

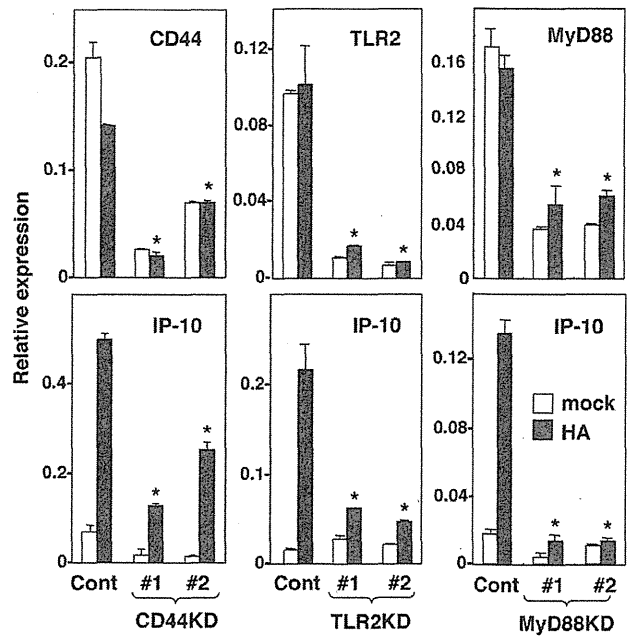

FIG 4 HA induces IP-10 production in human hepatoma cell lines through the TLR2-CD44-MyD88 axis. The stable knockdown cell clones based on the Huh7OK1 cells expressing siRNA targeted to the CD44, TLR2, or MyD88 gene (#1 and #2) or control siRNA (Cont) were stimulated with 500 μg/ml of HA. mRNA levels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

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

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

CD44 and TLR2 interact through their extracellular domains. To gain more insight into the IP-10 production by stimulation with HA through TLR2 and CD44, we determined the regions responsible for the interaction between CD44 and TLR2. The CD44 gene contains at least 20 exons, and various isoforms are generated through variable splicing of the internal 10 exons (Fig. 7A) (38). The wild-type and a mutant CD44 lacking the intracellular domain (CD44ΔTM; consists of amino acid residues from 1 to 223) but not a mutant missing the extracellular domain (CD44ΔEC; consists of amino acid residues from 223 to 361) were coprecipitated with TLR2 by immunoprecipitation analysis (Fig. 7B). A TLR2 mutant lacking the transmembrane region (TLR2ΔTM; consists of amino acid residues from 1 to 587) but not a mutant missing the extracellular domain (TLR2ΔEC; consists of amino acid residues from 588 to 784) exhibits a weak but substantial interaction with CD44 (Fig. 7C), indicating that CD44

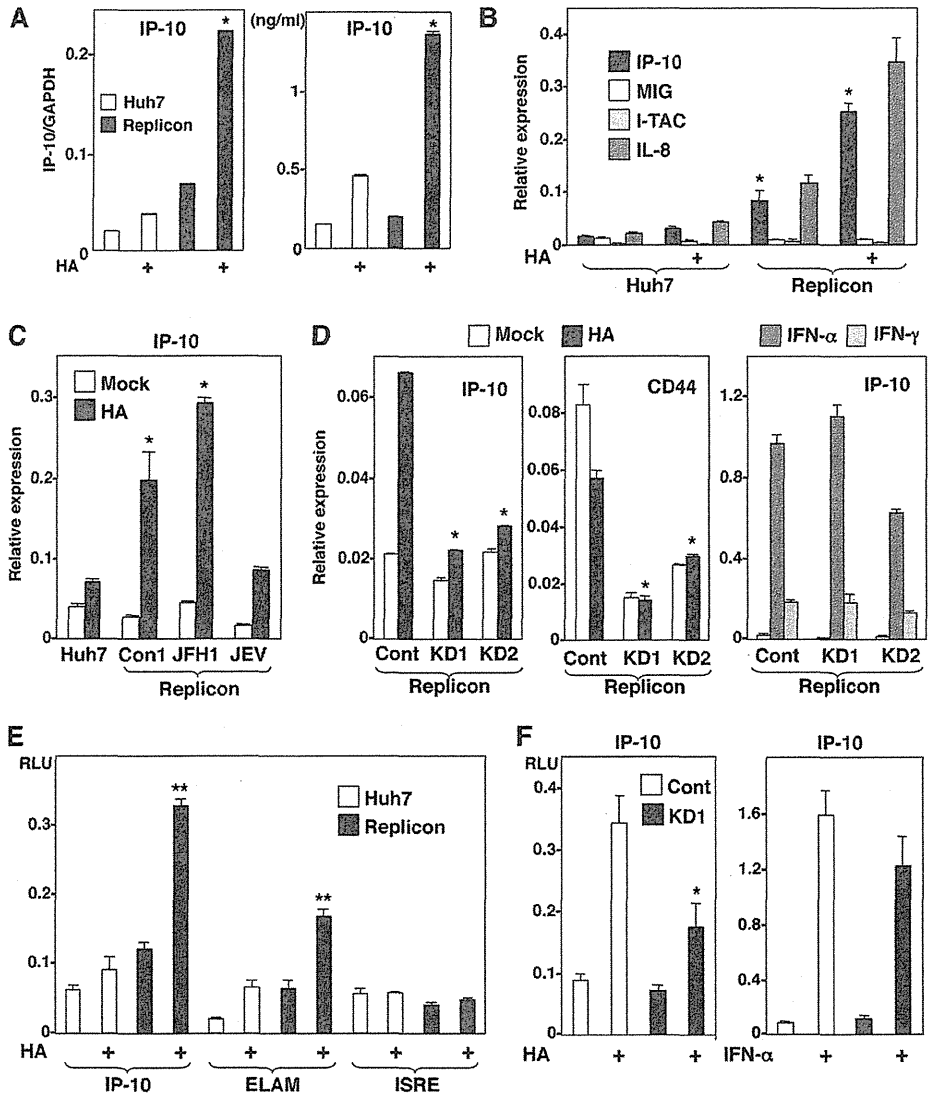

FIG 5 HA participates in IP-10 production in cells replicating HCV. (A) Huh7 and HCV replicon-harboring cells transfected with a plasmid encoding a FLAG-tagged TLR2 were stimulated with 500 μg/ml of HA, and IP-10 mRNA levels (left) and production of IP-10 in culture supernatants (right) 24 h after stimulation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

and TLR2 interact through their extracellular domains. Interestingly, the interaction between CD44 and TLR2 was enhanced by stimulation not only with HA but also PGN (Fig. 7D), although a TLR2 ligand (FSL-1) induces IP-10 production in cells replicating HCV through a CD44-independent pathway, as shown in Fig. 3. To further clarify the direct interaction between CD44 and TLR2, the extracellular domains of His-tagged CD44 (CD44ΔTM) and FLAG-tagged TLR2 (TLR2ΔTM) were expressed in insect cells. Purified samples were examined by Coomassie staining and immunoblotting (Fig. 7E). The CD44ΔTM applied in coats to the microplates exhibited binding to TLR2ΔTM but not to BSA in a dose-dependent manner (Fig. 7F, left). Furthermore, both PGN

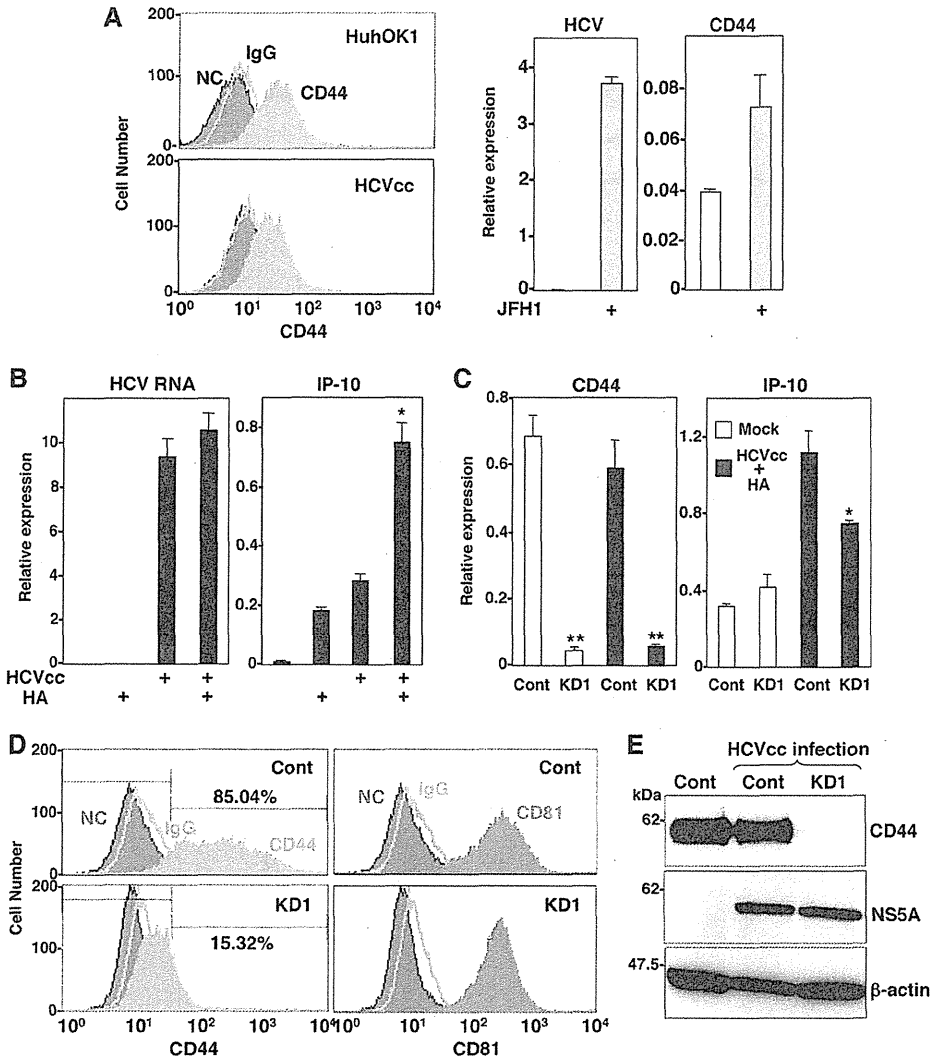

FIG 6 Enhancement of IP-10 production in HCVcc-infected cells upon stimulation with HA. (A) Left, cell surface expression of CD44 on Huh7OK1 cells infected with HCVcc at an MOI of 1 and incubated for 7 days was determined by using antibody that recognizes an ectodomain of CD44 (BU52 clones) and analyzed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

and HA also bound to CD44ΔTM in a dose-dependent manner, in contrast to a weak interaction with LPS (Fig. 7F, right). These results suggest that IP-10 is induced in cells replicating HCV upon stimulation with HA through an engagement of the extracellular domains of CD44 and TLR2.

DISCUSSION

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

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

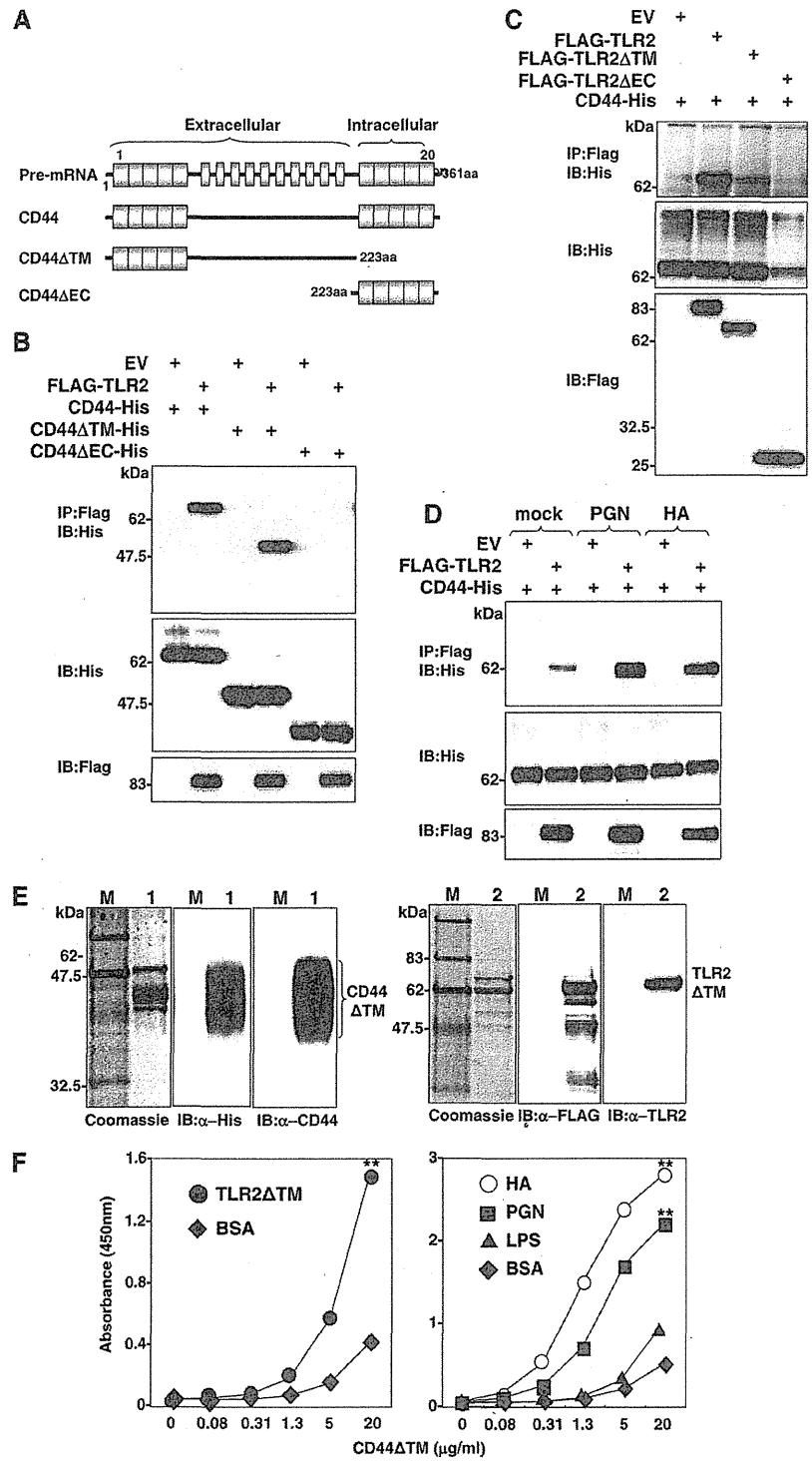

FIG 7 CD44 and TLR2 interact through their extracellular domains. (A) Structures of CD44 and its mutants used in this study. (B) FLAG-TLR2 was coexpressed with CD44-His, its mutants, or empty vector (EV) in 293T cells and immunoprecipitated with anti-FLAG antibody, and the precipitates were determined by immunoblotting (IB) with anti-His antibody. (C) CD44-His was coexpressed with FLAG-TLR2, its mutants, or empty vector in 293T cells and subjected to immunoprecipitation and immunoblotting using the appropriate antibodies. (D) Flag-TLR2 was coexpressed with CD44-His in 293T cells, stimulated with 20 μg/ml PGN or 500 μg/ml HA at 36 h posttransfection, and subjected to immunoprecipitation and immunoblotting using the appropriate antibodies at 24 h after stimulation. (E) Purified CD44ΔTM (lanes 1) and TLR2ΔTM (lanes 2) were examined by Coomassie staining and immunoblotting using antitag and specific antibodies. M denotes molecular mass markers. (F) Microtiter wells were coated with 20 μg/ml TLR2ΔTM (closed circles), 50 μg/ml HA (open circles), 40 μg/ml PGN (closed squares), 40 μg/ml LPS (closed triangles), or 50 μg/ml BSA (closed diamonds) at 4°C overnight and then incubated with the indicated concentrations of CD44ΔTM at room temperature for 1 h. The binding of CD44ΔTM was determined by measuring the absorbance at 450 nm. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$) versus the results for wells treated with BSA.

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

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

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

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

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

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

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REVIEW

Role of miR-122 and lipid metabolism in HCV infection

Takasuke Fukuhara · Yoshiharu Matsuura

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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.

T. Fukuhara · Y. Matsuura (✉)
Department of Molecular Virology,
Research Institute for Microbial Diseases, Osaka University,
3-1 Yamada-oka, Suita, Osaka 565-0871, Japan
e-mail: matsuura@biken.osaka-u.ac.jp

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Introduction

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

microRNA and virus infection

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

Liver-specific microRNA, miR-122

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

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

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

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

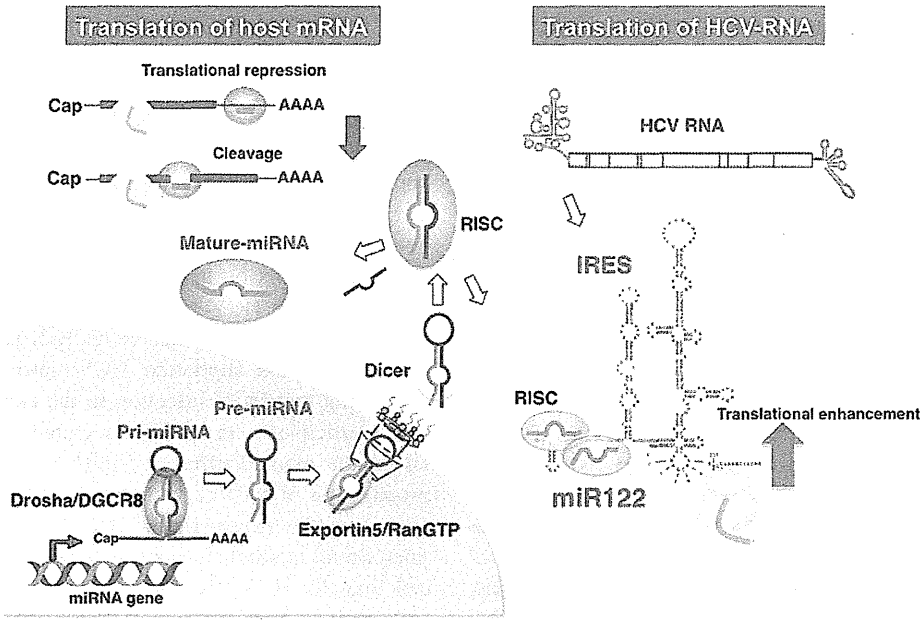

Fig. 1 miR-122 enhances the translation of hepatitis C virus (*HCV*) RNA. Primary miRNA (*pri-miRNA*) transcribed by RNA polymerase II in the nucleus is processed into precursor miRNA (*pre-miRNA*) by Drosha and DiGeorge syndrome critical region protein 8 (*DGCR8*). Pre-miRNA is exported into the cytoplasm 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, Ehrhadt 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,

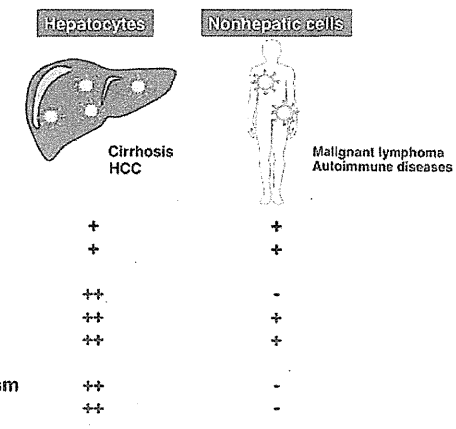

Fig. 2 HCV replication in hepatocytes and nonhepatic cells. Chronic HCV infection induces liver cirrhosis and hepatocellular carcinoma (*HCC*), and is also often associated with the development of extrahepatic manifestations (EHM) such as malignant lymphoma and autoimmune diseases. Not only hepatocytes but also nonhepatic cells express major HCV receptors, including CD81, SR-BI, 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.

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Exploitation of lipid components by viral and host proteins for hepatitis C virus infection

Kohji Moriishi[1]* *and Yashiharu Matsuura*[2]

[1] Department of Microbiology, Faculty of Medicine, University of Yamanashi, Chuo-shi, Yamanashi, Japan
[2] Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Suita-shi, Osaka, Japan

Edited by:
Yasuko Yokota, National Institute of Infectious Diseases, Japan

Reviewed by:
Glenn Randall, The University of Chicago, USA
Tetsuro Suzuki, Hamamatsu University School of Medicine, Japan

***Correspondence:**
Kohji Moriishi, Division of Medicine, Department of Microbiology, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, 1110 Shimokato, Chuo-shi, Yamanashi 409-3898, Japan.
e-mail: kmoriishi@yamanashi.ac.jp

Hepatitis C virus (HCV), which is a major causative agent of blood-borne hepatitis, has chronically infected about 170 million individuals worldwide and leads to chronic infection, resulting in development of steatosis, cirrhosis, and eventually hepatocellular carcinoma. Hepatocellular carcinoma associated with HCV infection is not only caused by chronic inflammation, but also by the biological activity of HCV proteins. HCV core protein is known as a main component of the viral nucleocapsid. It cooperates with host factors and possesses biological activity causing lipid alteration, oxidative stress, and progression of cell growth, while other viral proteins also interact with host proteins including molecular chaperones, membrane-anchoring proteins, and enzymes associated with lipid metabolism to maintain the efficiency of viral replication and production. HCV core protein is localized on the surface of lipid droplets in infected cells. However, the role of lipid droplets in HCV infection has not yet been elucidated. Several groups recently reported that other viral proteins also support viral infection by regulation of lipid droplets and core localization in infected cells. Furthermore, lipid components are required for modification of host factors and the intracellular membrane to maintain or up-regulate viral replication. In this review, we summarize the current status of knowledge regarding the exploitation of lipid components by viral and host proteins in HCV infection.

Keywords: HCV, hepatitis, lipid droplets, host factor

INTRODUCTION

Hepatitis C virus (HCV) is a major causative agent of chronic liver disease including steatosis, cirrhosis, and hepatocellular carcinoma. Epidemiological studies indicate that HCV is also associated with extrahepatic manifestations including type 2 diabetes mellitus, B-cell non-Hodgkin lymphoma, mixed cryoglobulinemia, and Sjögren's syndrome (Jacobson et al., 2010). It has been estimated that there are 170 million patients worldwide, of whom most are infected with HCV. Combination therapy with pegylated interferon (PEG-IFN) and ribavirin has been the standard treatment but it fails to cure ~50% of treated patients (Soriano et al., 2009).

Hepatitis C virus belongs to the genus *Hepacivirus* of the family Flaviviridae. The viral genome of HCV is characterized by a single positive strand RNA with a nucleotide length of 9.6 kb and it encodes a single polypeptide (**Figure 1**). This polyprotein is cleaved by host and viral proteases into structural and non-structural proteins (Harada et al., 1991; Hijikata et al., 1991; Grakoui et al., 1993a,b). Structural proteins, including the core protein and two envelope proteins, and the viroporin p7 are located within one-third of the N-terminal, while the remaining viral proteins are classified as non-structural proteins which form a replication complex with host factors (Grakoui et al., 1993c). HCV core protein is cleaved by signal peptide cleavage and then released from E1 (Santolini et al., 1994). After cleavage by signal peptidase (SP), the C-terminal transmembrane region of the core protein is further cleaved by signal peptide peptidase (SPP; Hussy et al., 1996; McLauchlan et al., 2002). The nucleocapsid, composed of matured core proteins and the viral genome, is surrounded by an envelope composed of host lipids and viral envelope proteins (Wakita et al., 2005). The life cycle of HCV is shown in **Figure 2**. The viral envelope proteins play a role in the binding to host receptors and membrane fusion for uncoating. Recently, several groups reported that the viral particle binds to a very low-density lipoprotein (VLDL), including apolipoprotein E (apoE), which is required for the binding step (Andre et al., 2002; Nielsen et al., 2006; Chang et al., 2007; Benga et al., 2010) as described below. The virus infects hepatocytes via entry factors known as receptors and co-receptors. The viral particle complex composed of the enveloped nucleocapsid and VLDL including apoE (Merz et al., 2011), is reported to bind to heparin sulfate (HS; Barth et al., 2003) and the low-density lipoprotein (LDL) receptor (LDLR; Agnello et al., 1999), although Albecka et al. (2012) recently reported that LDLR is required for optimal replication of the HCV genome rather than entry of the infectious viral particle. Other host factors may be involved in apoE-mediated entry. The HCV viral particle is transferred to the scavenger receptor class B type I (SR-BI; Scarselli et al., 2002; Bartosch et al., 2003) and CD81 (Pileri et al., 1998) through E2 binding and then enters cells with claudin-1 (CLDN1; Evans et al., 2007) and occludin (OCLN; Ploss et al., 2009) by endocytosis. The Niemann–Pick C1-like 1 cholesterol absorption receptor has recently been reported to be an HCV cell entry factor that is involved in the entry step between post-binding and

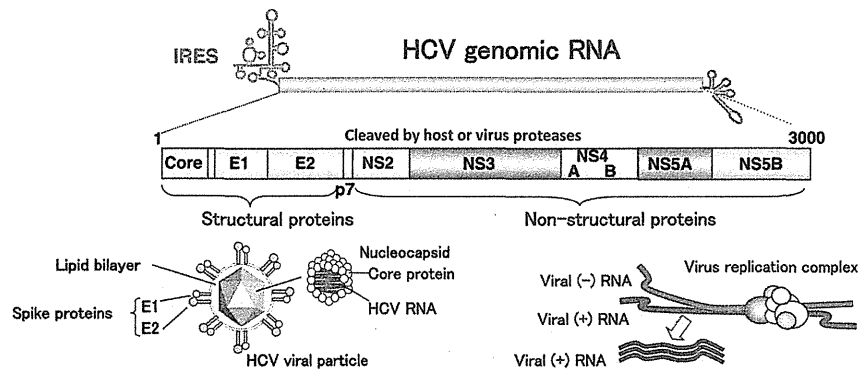

FIGURE 1 | Structure of HCV. HCV RNA encodes a polyprotein composed of about 3,000 amino acids. The core protein, and two envelope proteins are classified as structural protein, while NS2, NS3, NS4A, NS4B, NS5A, and NS5B are non-structural proteins.

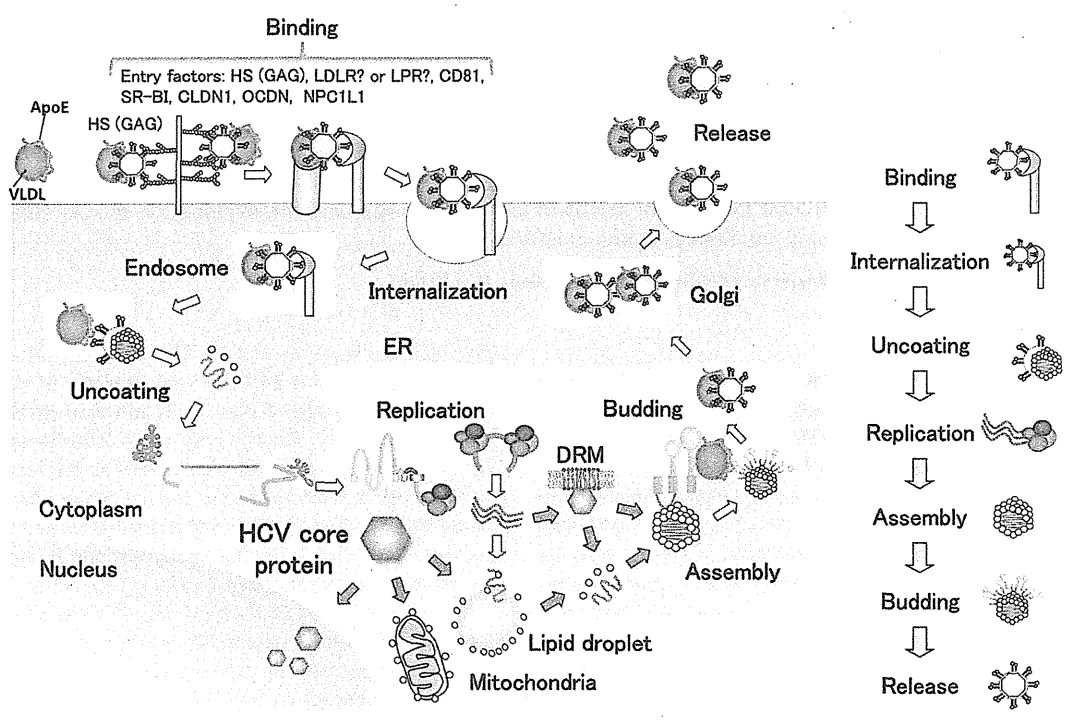

FIGURE 2 | The infection cycle of HCV. The HCV complex with VLDL binds to entry factors on the surface of hepatocytes and then enters cells by endocytosis. After uncoating, viral replication is carried out in the convoluted membrane structure called the membranous web. The viral nucleocapsid egresses into the lumen side of the ER and binds to VLDL. The HCV complex with VLDL is released from the infected cells.

pre-fusion (Sainz et al., 2012). The viral envelope fuses with the host plasma membrane in an endosome under a low pH condition (Takikawa et al., 2000; Hsu et al., 2003; Blanchard et al., 2006; Codran et al., 2006; Meertens et al., 2006; Tscherne et al., 2006). The capsid protein and viral genome are expected to be released into the cytoplasm of infected cells. The viral replication, assembly, and budding are summarized in **Figure 3** on the basis of current information. The viral genome is translated dependent on own internal ribosome entry site (Tsukiyama-Kohara et al., 1992) and transcribed by the translated and processed NS3 to NS5B (Lohmann et al., 1999). The viral protein NS4B induces a convoluted membrane structure (termed a membranous web) with host lipid components and proteins, in which the viral replication is carried out (Egger et al., 2002; Gosert et al., 2005; Ferraris et al., 2010). The newly synthesized viral positive stranded RNA genome is released from the membranous web and passes to the

core protein via NS5A (Masaki et al., 2008). The core protein is translocated on the surface of the lipid droplet or endoplasmic reticulum (ER) membrane for efficient formation of viral particles, and then encloses the synthesized viral genome to form a capsid near the membranous web (Miyanari et al., 2007; Boson et al., 2011). The capsids are enclosed by an endoplasmic membrane containing the viral envelope proteins E1 and E2 and are then released into ER lumen side, since intracellular envelope proteins are categorized as high-mannose type glycoproteins and the viral particle composed of core proteins and envelope proteins egresses into the lumen side of the intracellular compartment associated with lipid droplets (Miyanari et al., 2007; Vieyres et al., 2010). The viral particle is secreted through a host secretion pathway, although the mechanism by which HCV particles are secreted in infected cells remains poorly understood.

Although no effective vaccine for HCV has been developed, antiviral drugs targeting to the viral and host factors have been reported recently. The HCV replicon system was reported for a screening system based on cultured cells (Lohmann et al., 1999) and has been improved by modification of cell lines and marker genes and introduction of adaptive mutations in the region of the viral RNA genome for high efficiency of viral replication (Blight et al., 2000; Krieger et al., 2001; Lohmann et al., 2001; Ikeda et al., 2002; Pietschmann et al., 2002). The complete infectious cycle of HCV in cultured cells was established in a highly permissive cell line by using the genotype 2a strain JFH1 or its chimeric recombinant virus (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). A system based on the cultured cell line has been an exclusive drug-screening system for finding antiviral compounds that interfere with the process of viral RNA replication under intracellular conditions. NS3 forms a complex with cofactor NS4A (Failla et al., 1994, 1995; Koch et al., 1996). This interaction stabilizes NS3 and retains it on the ER where it acquires the ability of a protease against viral polyprotein (Wolk et al., 2000) and host protein IPS-1/MAVS (Foy et al., 2005; Loo et al., 2006), which is a molecule downstream of the RIG-I sensor molecule (Sumpter et al., 2005; Loo et al., 2008). NS3 serine protease activity is a target of the direct acting HCV antiviral drugs known as telaprevir and boceprevir, which are available clinically by combination therapy with PEG-INF and ribavirin (Hofmann and Zeuzem, 2011). The RNA helicase activity of NS3 and NS5B RNA-dependent RNA polymerase are also used for drug-screening in particular (Hicham Alaoui-Ismaili et al., 2000; Dhanak et al., 2002; Borowski et al., 2003; De Francesco et al., 2003; Boguszewska-Chachulska et al., 2004; Maga et al., 2005; Najda-Bernatowicz et al., 2010). Combination therapy using several compounds targeting host and viral factors may be able to completely eradicate the virus and suppress the pathogenicity induced by HCV infection.

Liver steatosis, which is characterized by accumulation of lipid droplets in hepatocytes, is significantly associated with the incidence of hepatocellular carcinoma in HCV-infected patients (Ohata et al., 2003). Severe liver steatosis has been frequently found in patients infected with the genotype 3a virus (Rubbia-Brandt et al., 2000; Adinolfi et al., 2001). Successful clearance of HCV reduces steatosis in genotype 3a patients, suggesting an association between genotype 3a and severe steatosis. Furthermore, HCV core protein derived from genotype 1 also induced liver steatosis in mouse and cultured cells (Barba et al., 1997; Moriya et al., 1997; Hope and McLauchlan, 2000). Lipid droplets containing triglycerides and cholesteryl ester are increased in cells expressing core protein and are surrounded by the core protein (Hope and McLauchlan, 2000). Non-structural proteins associate with the lipid droplets surrounded by HCV core proteins to supply the synthesized viral genome for viral assembly (Miyanari et al., 2007). Other lipid components are reported to be involved in formation of viral particles and the viral RNA replication as described below. This review mainly summarizes the viral and host factors that are associated with lipid metabolism with regard to HCV replication and pathogenicity.

THE ROLE OF VLDL IN HCV INFECTION

Hepatitis C virus replicates in a convoluted membrane structure as a membranous web (Egger et al., 2002; Gosert et al., 2005; Ferraris et al., 2010) and assembles in the area of the ER membrane-associated with lipid droplets surrounded by the core protein (Miyanari et al., 2007). The LDLR has also been proposed to function as one of entry factors described above for HCV entry, in which interaction between LDLR and HCV particles is facilitated though interaction of the virus with host lipoprotein components (Monazahian et al., 1999; Chang et al., 2007; Huang et al., 2007; Miyanari et al., 2007; Gastaminza et al., 2008). HCV RNA containing particles derived from infected human serum were fractionated in densities with a value of 1.03–1.25 g/ml (Thomssen et al., 1992, 1993). The HCV RNA particles of the fraction with a density of lower than 1.06 g/ml possessed infectivity against chimpanzees, while HCV RNA derived from fractions with a higher density showed poor infectious ability (Bradley et al., 1991; Hijikata et al., 1993). The infectious HCV particles form a LDL–virus complex in the sera of human patients (Andre et al., 2002). An LDL–virus complex was found in the fractions with very low to low buoyant densities (1.03–1.25 g/ml), which varied with the stage of infection (Pumeechockchai et al., 2002; Carabaich et al., 2005). HCV particles prepared from infected human serum forms a complex with lipoproteins designated as lipo-viro-particles (LVP; **Figure 3**; Andre et al., 2002; Nielsen et al., 2006). LVP includes triglycerides, HCV RNA, core protein, and apolipoproteins B and E (Andre et al., 2002), which are components of VLDLs and LDL (Brodsky et al., 2004).

Very low-density lipoprotein is formed with a hydrophobic particle composed of triglycerides and cholesteryl ester that is surrounded by a surface coat containing phospholipid, free cholesterol, and two dominant lipoproteins, apoB and apoE (review to see Havel, 2000). Both apoB and apoE were found in a low-density fraction of HCV RNA particles (Andre et al., 2002; Chang et al., 2007). HCV virions could also be precipitated with antibodies against apoB or apoE (Andre et al., 2002; Chang et al., 2007). ApoB and microsomal triglyceride transfer protein (MTP) are required for HCV assembly and production, since knockdown of apoB or a specific antibody to MTP could inhibit HCV production (Huang et al., 2007; Gastaminza et al., 2008). However, another report suggests that knockdown of apoB or antibodies to apoB exhibited no significant effect on HCV infectivity and production (Jiang and Luo, 2009). The monoclonal antibodies against apoE neutralized HCV infection in cultured cells (Chang et al., 2007; Jiang and Luo,

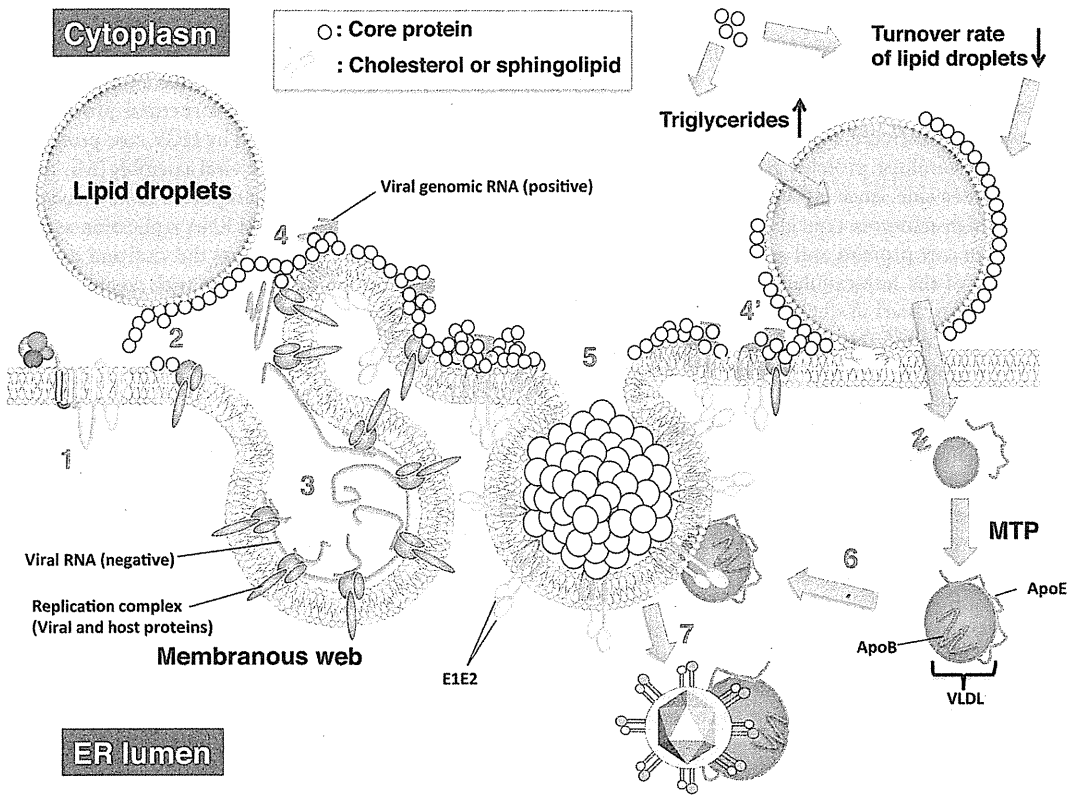

FIGURE 3 | Hepatitis C virus RNA replication and particle production on lipid droplets and the ER membrane. HCV RNA replication and viral particle production are thought to be carried out on the lipid droplets and ER membrane. The viral proteins take advantage of host lipid metabolism and intracellular compartments to produce viral components as described in the text. Each step is described below. (1) A newly synthesized or uncoated vial genomic RNA is translated to a viral polyprotein followed by a cleaving polyprotein. (2) Matured non-structural proteins form replication a complex with host factors. The core protein is translocated to lipid droplets by DGAT1. In cells infected with the highly infectious strain (e.g., Jc1), the core protein may be retained on ER membrane by cooperating with non-structural proteins. (3) NS4B is involved in the convoluted membrane structure dubbed the membranous web. Negative and positive strands of viral RNA are synthesized by the replication complex based on each other. (4) The positive strand of genomic RNA is enclosed with the core proteins, cooperating non-structural proteins. Envelope proteins, NS2, and p7 may determine recruitment of the core protein from lipid droplets to the ER membrane along microtubules. Lipid droplets hemi-fused with ER membrane (Wolins et al., 2006) may be involved in recruitment of core protein on the ER membrane (4'). (5) The core proteins enclose the viral RNA genome, and are then surrounded by envelopes. Non-structural proteins and other factors may support formation of nucleocapsid and budding. (6) VLDL binds to viral particles to form LVP before or after budding. (7) The viral particles egress into the ER lumen with VLDL including ApoE. The core protein upregulates synthesis or storage of triglycerides and cholesteryl ester, cooperating with host factors including DGAT1, and so on.

2009), while knockdown of apoE markedly reduced HCV infectivity and infectious viral production without affecting viral entry and replication (Chang et al., 2007; Berger et al., 2009; Jiang and Luo, 2009). Hishiki et al. (2010) suggested that the isoforms 3 and 4, but not 2, of apoE are critical for HCV infectivity dependent of affinity to LDLR. Furthermore, NS5A could interact with apoE in infected cells and colocalization of both proteins supports the notion of intracellular interaction in infected cells (Benga et al., 2010). The C-terminal alpha-helix region spanning from residue 205 to 280 was critical for NS5A–apoE interaction and viral production (Cun et al., 2010). ApoE included in LVP may directly bind to LDLR or LDLR-related proteins in hepatocytes (**Figure 2**), since apoE is a ligand for all members of the LDLR gene family (see review described by Herz et al., 2009). These results suggest that apoE is an essential host factor for HCV entry.

LOCALIZATION OF THE CORE PROTEIN ON BOTH ER AND LIPID DROPLETS IN INFECTED CELLS

Hepatitis C virus core protein is located at the N-terminus of the HCV polyprotein (**Figure 1**). The HCV core protein is cleaved from a precursor polyprotein by a SP, releasing it from an envelope E1 protein. Then, the C-terminal transmembrane region of the core protein is further processed by a SPP (McLauchlan et al., 2002). The intramembrane processing of the HCV core protein by SPP is critical for the production of infectious viral particles (Okamoto et al., 2008). The C-terminal end of the mature HCV core protein expressed in insect and human cell lines was determined to be Phe^{177} (Ogino et al., 2004; Okamoto et al., 2008). Randall et al. (2007) reported that the introduction of an siRNA targeted to SPP (called HM13) reduced the production of infectious HCV particles, suggesting that SPP is required for HCV

particle production. Our previous report (Okamoto et al., 2008) showed that the production of HCV in cells persistently infected with the JFH1 strain was impaired by treatment with an SPP inhibitor and that JFH1 viruses bearing SPP-resistant mutations in the core protein failed to propagate in a permissive cell line. These data suggest that intramembrane processing of HCV core protein by SPP is required for viral propagation. Matured core protein was found in a detergent-resistant membrane fraction, which was distinct from the classical lipid rafts (Matto et al., 2004). Our data also suggest that cleavage of HCV core protein by SPP is required for localization of HCV core protein in detergent-resistant membrane fractions including cholesterol and sphingolipid (**Figure 3**, step 4 and 5). Detergent-resistant membrane fractions may be derived from the membranous web where the viral replication complex synthesizes the viral RNA genome, since the replication complex is fractionated in lipid raft fractions including Vesicle-associated membrane protein-associated protein (VAP)-A, cholesterol, and sphingolipid (**Figure 3**, step 3; Shi et al., 2003; Aizaki et al., 2004; Gao et al., 2004; Sakamoto et al., 2005). Furthermore, an HCV core protein mutation resistant to SPP results in delayed localization of HCV core protein on lipid droplets and reduction of virus production (Targett-Adams et al., 2008). These reports suggest that cleavage of HCV core protein by SPP is required for its suitable intracellular localization for the viral assembly. Sequence analysis of the core protein suggests that high hydrophobicity is found in the region from amino acid residues 119 to 174, which is called domain 2 (Hope and McLauchlan, 2000). Domain 2 is critical for localization of the core protein on lipid droplets and shares common features with the core protein of GBV-B, but not of other viruses belonging to the Flaviviridae family (Hope et al., 2002). When three hydrophobic amino acids, Leu139, Val140, and Leu144, in the most hydrophobic peak in domain 2 were replaced with Ala to reduce hydrophobicity, the triple mutations in the core protein led to resistance to SPP cleavage, dislocation of the detergent-resistant membrane, and a reduction in virus production (Okamoto et al., 2004, 2008). Furthermore, comparative analysis between JFH1 and Jc1 suggest that binding strength of domain 2 of core for lipid droplets determines efficiency of virus assembly (Shavinskaya et al., 2007). These results suggest that hydrophobicity of domain 2 in the core protein is required for lipid droplet localization, SPP cleavage, and virus production.

Host lipid biogenesis is responsible for replication and assembly. HCV core protein contributes to the accumulation and production of host lipid components and is detected on the surface of lipid droplets (Hope and McLauchlan, 2000). The core protein is translocated into the lipid droplets near the replication complex and encloses newly synthesized viral RNA to form the nucleocapsid (**Figure 3**, step 2–4 or 4′), egresses into the lumen side of the ER, then is surrounded with host lipid components and viral envelope proteins (**Figure 3**, step 5; Miyanari et al., 2007). HCV core protein interacts with diacylglycerol acyl transferase 1 (DGAT1), which is required for the trafficking of core protein to lipid droplets (**Figure 3**, step 2; Herker et al., 2010). However, the translocation of the core protein to lipid droplets may not be required for efficient production of viral particles. The recombinant virus Jc1 exhibits a higher virus titer than the JFH1 strain (Lindenbach et al., 2006; Pietschmann et al., 2006). The core protein of the Jc1 strain is hardly detected on lipid droplets in infected cells and is mainly localized on ER membranes, together with envelope protein E2 (Miyanari et al., 2007; Shavinskaya et al., 2007; Boson et al., 2011). Expression of p7 increases the ER localization of core protein in the absence of envelope proteins (Boson et al., 2011). However, Miyanari et al. (2007) reported that the core protein of the Jc1 strain was mainly localized with envelope proteins on ER in cells transfected with a complete viral genome, but on lipid droplets in cells that were transfected with the viral genome lacking envelope protein genes. Expression of envelope proteins and p7 may determine intracellular localization of the core protein with regard to viral assembly (**Figure 3**, step 2 and 4 or 4′).

NS2 has been reported to be involved in the assembly process of HCV particles (Jones et al., 2007; Jirasko et al., 2008; Dentzer et al., 2009). NS2, which is composed of three transmembrane regions and a cytoplasmic domain in order after p7 (Lorenz et al., 2006), is known as the autoprotease of which C-terminal cytoplasmic domain is involved in *cis* cleavage at the NS2–NS3 junction (Santolini et al., 1995; Yamaga and Ou, 2002; Lorenz et al., 2006). Genetic interaction was implied between the N-terminal region of NS2 and the upstream structural proteins, since the first transmembrane of NS2 was identified as a genetic determinant for infectivity by construction of chimeric HCV with various genotypes (Pietschmann et al., 2006). Analyses by co-immunoprecipitation and imaging microscopy for interaction between NS2 and other viral proteins in cultured cells suggest that NS2 interacts with p7 and E2 on the ER-derived dotted structure closed to lipid droplets that are surrounded by HCV core protein (Popescu et al., 2011). NS2 also interacts with NS3/4A to recruit the core protein from lipid droplets to the cytoplasmic motile puncta along microtubules (Counihan et al., 2011). HCV p7 is a short hydrophobic protein composed of 63 amino acids and is encoded between the structural and non-structural proteins (Carrere-Kremer et al., 2002). The cytoplasmic loop of p7 is located between the N-terminal and C-terminal transmembrane regions (Carrere-Kremer et al., 2002). HCV p7 is known as a viroprotein that forms homooligomerize to be a ion channel, which is then involved in assembly and release of virus particle in infected cells by modulating pH equilibration in intracellular vesicles (Carrere-Kremer et al., 2002; Jones et al., 2007; Steinmann et al., 2007; Wozniak et al., 2010). Mutations of conserved amino acids required for ion channel activity impaired the production of infectious virus (Jones et al., 2007). However, recruitment of HCV core protein from lipid droplets to the ER assembly site was independent of the ion channel activity of HCV p7 (Boson et al., 2011). HCV p7 enhanced ER localization of the core protein without other viral proteins regardless of viral genotype, although compatibilities between two transmembrane regions of p7 and the first transmembrane domain of NS2 are responsible for ER localization of core protein and infection (Boson et al., 2011). The second transmembrane region of p7, rather than the first, is critical for compatibilities with NS2 regarding recruitment of core protein to the ER assembly site, although both transmembrane regions of p7 are important to sustain infectivity (Boson et al., 2011). These reports speculate that localization of the core protein on lipid droplets may contribute to suppression of virus production and maintenance of persistent HCV infection, while localization of the