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A single point mutation in E2 enhances hepatitis C virus infectivity and alters lipoprotein association of viral particles

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ABSTRACT

Hepatitis C virus (HCV) infection is a major worldwide health problem. Our previous results showed that HCV evolved to gain the enhanced infectivity and altered buoyant density distribution during persistent infections *in vitro*. Here we showed that a point mutation I414T in HCV E2 was mainly responsible for these phenotypic changes. While the I414T mutation had no significant effect on HCV RNA replication and viral entry, it enhanced the production of infectious viral particles and decreased the dependency of viral entry on the levels of HCV receptors. Furthermore, we showed that the I414T mutation reduced the association of viral particles with low-density lipoprotein or very low-density lipoproteins during the virus secretion process, and the infection of the delipidated virus was more sensitive to the blockade by an anti-E2 neutralizing antibody and recombinant CD81 proteins. Our results provided more insights into understanding the roles of lipoprotein associations in HCV life cycle.

Introduction

Hepatitis C virus (HCV), the causative agent of acute and chronic hepatitis C, is a positive-strand RNA virus that belongs to the *Flaviviridae* family. More than 170 million people have been infected with HCV worldwide, and many of them are subject to high risk of developing liver cirrhosis and hepatocellular carcinoma (Lauer and Walker, 2001). The HCV genome encodes a single polyprotein that is processed by a combination of host and viral peptidases into at least 10 structural and nonstructural proteins. The structure proteins include core, E1 and E2 that form virus particles. The non-structure proteins include P7, NS2, NS3, NS4A, NS4B, NS5A and NS5B (Bartenschlager and Lohmann, 2000). The HCV envelope is formed by the two heavily N-glycosylated type I transmembrane proteins E1 and E2, which are present on the surface of viral particles as heterodimers anchored to a double-layer lipid membrane (Op De Beeck et al., 2001).

Several putative HCV receptors/factors have been identified so far. The tetraspanin molecule CD81 and the scavenger receptor class B type I (SR-BI) were both identified as HCV receptors based on their interactions with recombinant soluble E2 protein (Pileri et al., 1998; Scarselli et al., 2002), and further confirmed in the studies of infection of HCV pseudotyped retroviral particles (HCVpp) (Bartosch et al., 2003; Grove et al., 2007; Kapadia et al., 2007) and the recently established *in vitro* infectious HCV system (HCVcc) (Kapadia et al., 2007; Koutsoudakis et al., 2006, 2007; Tscherne et al., 2006; Voisset et al., 2005). In addition, recent data support a role for claudin-1 (CLDN1) and occludin, components of the tight junctions, in a late stage of HCV entry, presumably after the initial virus attachment and binding to CD81 and SR-B1 (Evans et al., 2007; Liu et al., 2009; Ploss et al., 2009). Other identified entry factors, including the low-density lipoprotein (LDL) receptor (LDLR) (Agnello et al., 1999), glycosaminoglycan (Barth et al., 2003), and asialoglycoprotein receptor (Saunier et al., 2003), seem to act as nonspecific attachment factors to mainly allow viral particles to concentrate on the cell surface.

HCV particles found *in vivo* and *in vitro* are heterogeneous in density and size. HCV has long been known to associate with LDL and very low-density lipoprotein (VLDL) circulating in patient blood (Thomssen et al., 1992). HCV E1/E2 envelope proteins have been found to bind to LDL, VLDL and high-density lipoprotein (HDL) (Monazahian et al., 2000). Accumulating evidence suggests the association between HCV and LDL/VLDL is important for virus entry and virus egress (Chang et al., 2007; Gastaminza et al., 2008; Huang et al., 2007; Meunier et al., 2008; Molina et al., 2007; Nahmias et al., 2008), and HCVcc entry can be blocked by antibodies against apolipoprotein C1 (Dreux et al., 2007; Meunier et al., 2005, 2008) or apolipoprotein E (Chang et al., 2007).

Our previous studies showed that HCV infection could be persistent in cell culture for up to 6 months during which the virus evolved to gain the enhanced infectivity and altered buoyant density distribution (Zhong et al., 2006). Here we reported that a point mutation I414T in E2 was responsible for the changes of viral

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infectivity and buoyant density distribution. While the I414T mutation had no significant effect on HCV RNA replication and viral entry, it promoted the production of infectious viral particles and reduced the dependency of viral entry on the levels of HCV receptors. Furthermore, we showed that the I414T mutation reduced the association of viral particles with LDL/VLDL during the virus secretion process, and the infection of this less lipidated virus was more sensitive to the blockade by an anti-E2 neutralizing antibody and recombinant CD81 proteins, but less sensitive to anti-apolipoprotein antibodies. Our results provided more insights into the understanding of the roles of lipoprotein associations in HCV life cycle.

Results

I414T accelerated JFH-1 infection kinetics and increased viral titers

In our previous studies, we established persistent HCV infection in cell culture for up to 6 months. During the persistent infection, the viral infectivity was greatly enhanced (Zhong et al., 2006). Our sequencing analysis revealed that three point mutations in E2, V388P, I414T and L644I, were selected during a persistent HCV infection (Exp 2 in (Zhong et al., 2006)). To examine the effect of this mutation on HCV infection, we engineered each individual mutation to JFH-1 and obtained recombinant mutant viruses as described in Materials and methods. Next we compared the ability of the wild-type and mutant viruses in expanding in naïve cells. Naïve Huh-7.5.1 cells were inoculated with V388P, I414T, L644I or wild-type viruses at a multiplicity of infection (MOI) of 0.005, and the supernatant infectivity titers at the indicated time points after the inoculation were determined by the titration assay. As shown in Fig. 1A, V388P and L644I did not accelerate HCV expansion kinetics, while the I414T mutant virus expanded faster than wild-type JFH-1, and the peak titers of I414T were about 5-fold higher than that of wild type. We also determined the intracellular HCV RNA levels at the indicated time points after the inoculation of wild-type and I414T viruses by quantitative RT-PCR. Consistently, I414T virus expanded faster than the wild-type JFH-1 (Fig. 1B). Furthermore, immunofluorescence staining of HCV E2 proteins in the infected cells at day 5 post-infection showed that I414T virus expanded to about 70% of total cell population while wild type expanded to only 20% (Fig. 1C). These results demonstrated that the I414T mutation enhanced JFH-1 infectivity.

I414T did not enhance viral entry

HCV glycoprotein E2 interacts with cellular receptors CD81 and SR-B1 that mediate HCV entry (Op De Beeck et al., 2001). It is possible that the I414T mutation in E2 could improve JFH-1 infectivity by enhancing HCV entry. To test this hypothesis, we first analyzed the entry of wild-type and I414T JFH-1 infection in Huh-7.5.1 cell. The same infectious units of wild-type and I414T viruses were incubated with Huh-7.5.1 cells. At the indicated time points, the viral inocula were removed and the cells were supplemented with fresh complete medium. The number of viruses that had entered the cells and the number of remaining viruses in the viral inocula at each time point after inoculation were determined, respectively. As shown in Figs. 2A and B, no significant difference was observed between wild type and I414T, suggesting that the I414T mutation did not confer an advantage at the level of HCV entry. To further confirm this, we compared the

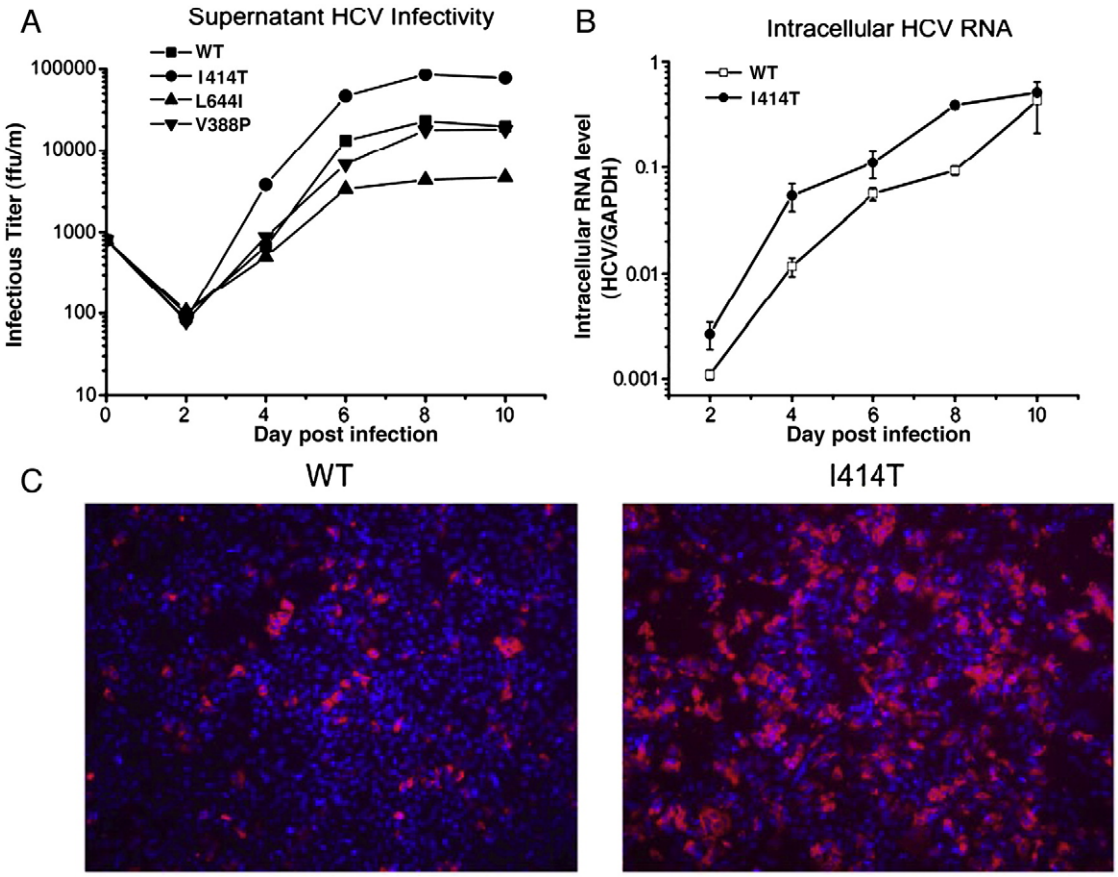

Fig. 1. The I414T mutation enhanced JFH-1 infectivity. Huh-7.5.1 cells were infected with wild-type or E2mutant JFH-1 viruses at an MOI of 0.005. (A) Cell culture supernatants were collected at the indicated days and analyzed for the infectivity titers by a titration assay. (B) Intracellular HCV RNA levels were determined by quantitative RT-PCR and normalized to cellular GAPDH levels. The error bar represented triplicates of each time point. (C) Immunofluorescence staining of HCV E2 proteins in the infected cells at day 5 post-infection.

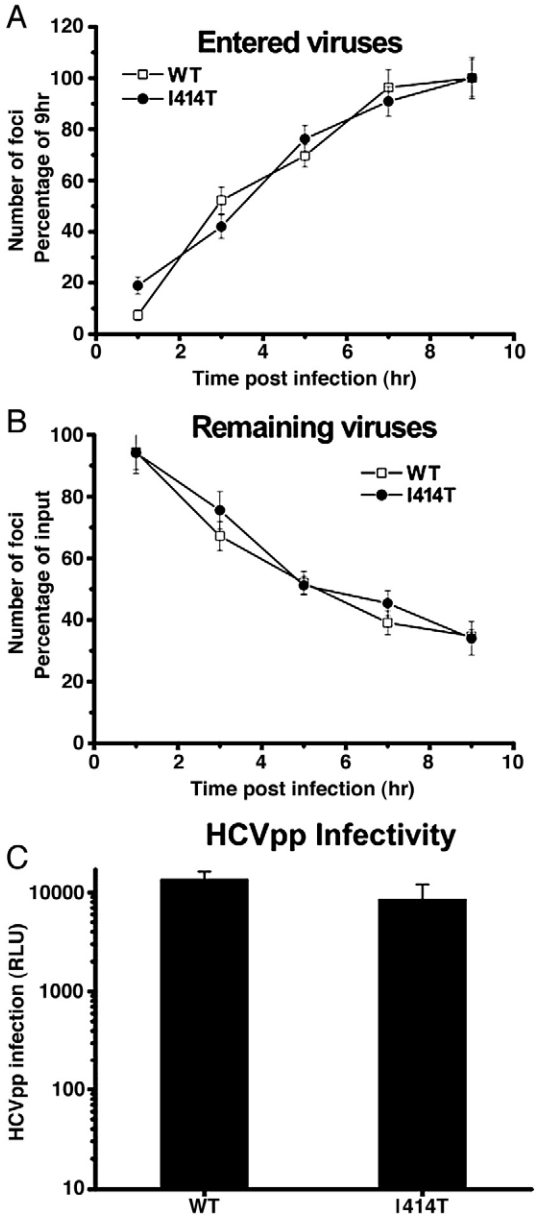

Fig. 2. I414T did not enhance JFH-1 entry. (A and B) Infection of wild-type and I414T viruses in Huh-7.5.1 cells. Fifty infectious units of wild-type and I414T JFH-1 were inoculated to Huh-7.5.1 cells and withdrawn at the indicated time points. (A) The number of viruses that had entered the cells was quantified by immunofluorescence staining at 72 h post-infection. The data were expressed as the percentage of HCV-positive focus number to that at 9 h post-infection (30 ffu). The error bar represented 12 replicates. (B) The number of remaining viruses in the withdrawn viral inocula was determined by a titration assay. The data were expressed as the percentage of total input (50 ffu). The error bar represented four replicates. (C) The infection of Huh-7.5.1 cells with HCVpp bearing the wild-type or I414T mutant E2 proteins. HCVpp produced from transfection of HEK293T cells were normalized according to their HIV p24 contents, and then were used to infect Huh-7.5.1 cells for 3 days. The lysate of infected cells were assayed for the luciferase activities. The error bar represented triplicates.

infection of pseudotyped HCV particles (HCVpp) bearing the wild-type or I414T mutant E2 proteins (Fig. 2C). No difference was detected between wild type and I414T either.

Next we analyzed the binding of soluble E2 protein (sE2) containing the I414T mutation to the HCV receptors CD81 or SRB1. The recombinant his-tagged sE2 proteins were prepared as described in Materials and methods, and quantified by western blot using an anti-his antibody. The normalized wild-type and I414T mutant sE2 proteins were incubated with CHO cells stably expressing human CD81 or SRB1, and the binding of sE2 to CHO-CD81 or CHO-SRB1 cells was detected by FACS. As shown in Fig. 3, the I414T mutation did not appear to enhance sE2 interaction with human CD81 or SRB1. Altogether, these data suggest that the I414T mutation did not facilitate HCV entry.

I414T enhanced production of infectious viral particles

To determine whether I414T has any effects on virus production, we engineered the I414T mutation to the JFH-1 full-length genomic replicon that is bi-cistronic and harbors a neomycin-resistant marker (Fig. 4A). It has been shown that the cells stably replicating the JFH-1 full-length genomic replicon RNA were able to produce infectious viruses (Akazawa et al., 2008), therefore to eliminate the effect of re-infection on the quantification of virus production, we transfected the JFH-1 full-length genomic replicon RNA into R3 cell line, a CD81-negative Huh-7 cell that does not support JFH-1 infection (Zhong et al., 2006). After the G418 selection, the survival colonies were pooled, expanded and analyzed for HCV RNA levels in the cells and infectivity titers in the culture supernatants. As shown in Fig. 4B, the intracellular HCV RNA levels of the I414T replicon cells were comparable with that of wild type, but the infectivity titers in the supernatants of the I414T replicon cells were about 4 times higher than that of wild type (Fig. 4B). To further distinguish whether the increase of infectivity titers was due to the increase of general virus production or the increase of proportion of infectious viral particles among the total supernatant encapsidated HCV RNA, we measured the HCV RNA levels in the culture supernatants. As shown in Fig. 4B, the total HCV RNA levels in the I414T replicon cell supernatants were similar to those in the wild-type replicon cell supernatants, suggesting that I414T only increased the proportion of infectious particles among the total viral particles in the supernatants.

The enhanced virus production could result from the improved viral assembly or from the improved virus secretion. To investigate at which stage I414T improved virus production, we compared intracellular and extracellular HCV infectivity titers of the wild-type and mutant viruses. The intracellular viruses were prepared as previously described (Gastaminza et al., 2008). As shown in Fig. 4C, while the levels of intracellular viral infectivity were comparable, the I414T extracellular infectivity was about four-fold higher than wild type. The ratio of extracellular and intracellular viral infectivity was 188:1 for I414T and 56:1 for wild type. These data suggest I414T enhanced virus production by improving virus secretion.

I414T changed the buoyant density of JFH-1 virus

To compare biophysical properties of the viral particles, we analyzed the buoyant densities of wild-type and I414T viruses collected from culture supernatants in a 20–60% sucrose gradient ultracentrifugation. As expected, the infectivity of both wild-type and I414T viruses was distributed over a broad range of buoyant density. However, the infectivity in the lowest-density fraction of I414T was significantly reduced compared with that of wild type (Fig. 5A). Analysis of HCV RNA content of each fraction showed that the mutation caused the similar density change (Fig. 5B). It has been shown by a number of laboratories that matured HCV particles are associated with LDL or VLDL, rendering the heterogeneous buoyant density of viral particles, and LDL/VLDL is added to intracellular infectious HCV particles as the viruses exit host cells (Gastaminza et al., 2008). The lack of infectivity at the lowest density fraction may be indicative of less LDL/VLDL association in I414T viral particles during virus secretion. To test this hypothesis, we analyzed the buoyant density of intracellular wild-type and I414T mutant viral particles. As shown in Fig. 5C, both intracellular viral particles had a peak infectivity titer at the density of around 1.15 g/l, with no significant difference between wild type and I414T. These data

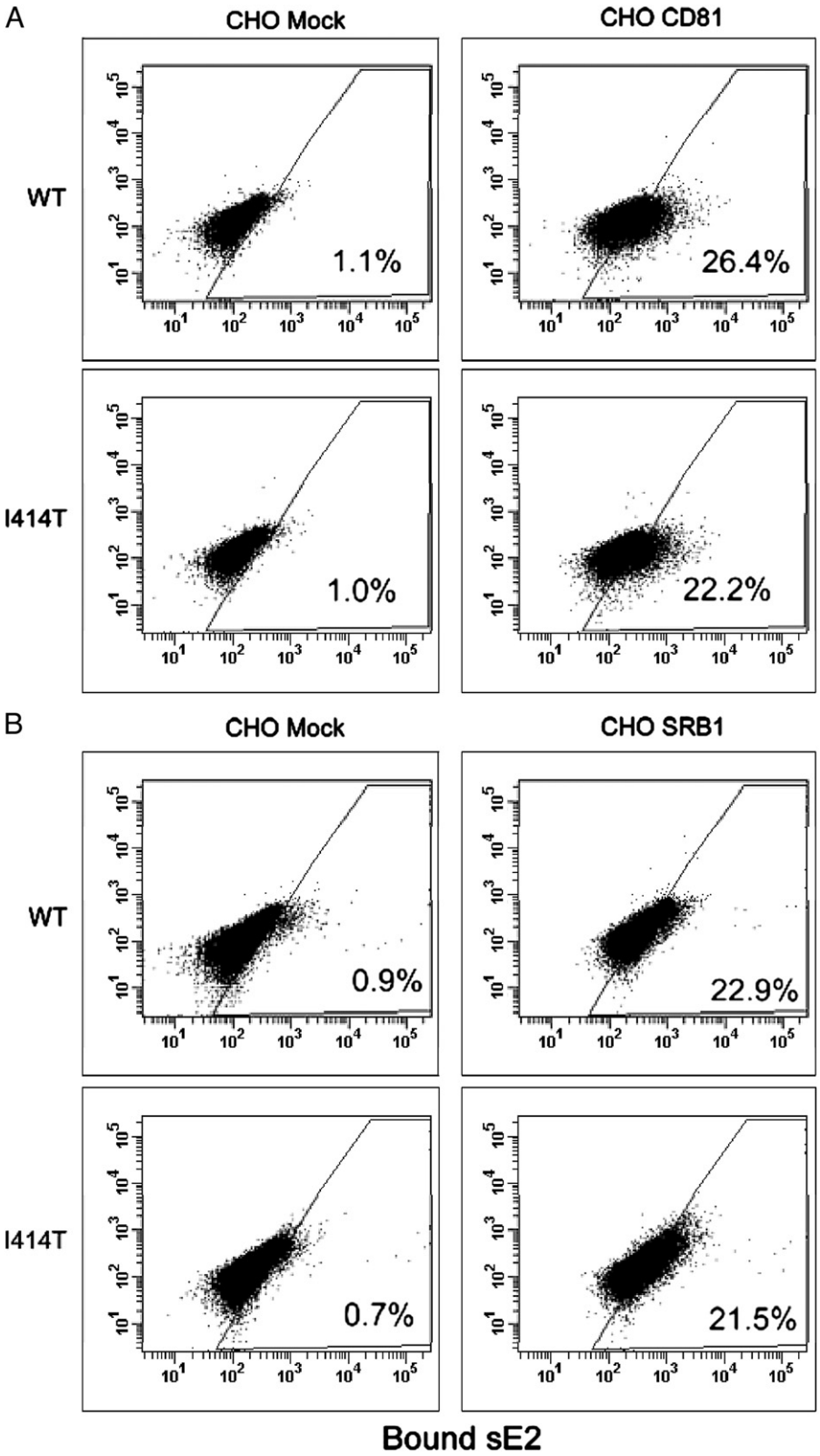

Fig. 3. I414T did not enhance the binding of E2 to human CD81 or SRB1. The his-tagged wide-type or I414T soluble E2 proteins were incubated with CHO cells stably expressing (A) human SRB1 or (B) human CD81 on the cell surface. Naïve CHO cells were used as mock control. The binding efficiency was detected by FACS analysis using a mouse monoclonal anti-his tag antibody, followed by a goat-anti-mouse secondary antibody labeled with Alexa555. The number in the panel shows the percentage of sE2 binding cells.

suggested that the difference of buoyant density distribution between the wild-type and I414T mutant extracellular viral particles may occur during the process of viral particle secretion, likely due to the different VLDL/LDL association.

The entry of I414T was less dependent upon the levels of host cell receptors

I414T mutation was selected from a persistent HCV infection during which host cells evolved to become less permissive for JFH-1

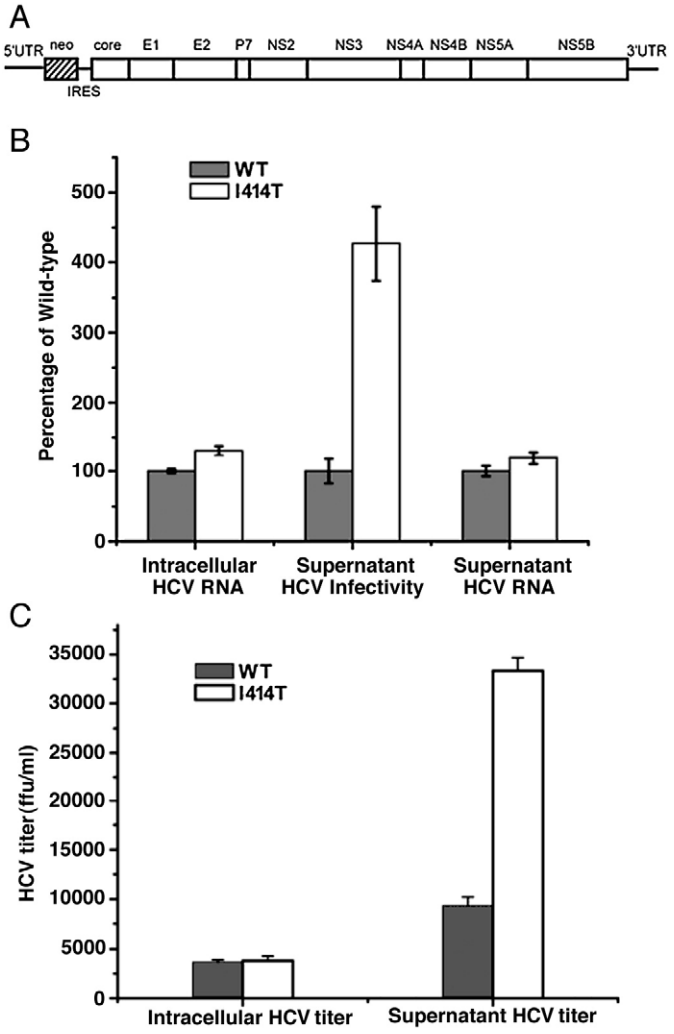

Fig. 4. The I414T mutation enhanced the production of infectious viral particles. (A) The structure of JFH-1 full-length genomic replicon. (B) The intracellular and supernatant HCV RNA levels and supernatant viral infectivity of R3 cells (CD81-negative subclone of Huh7) that stably expressed the wild-type or I414T JFH-1 full-length genomic replicon RNA. HCV RNA levels were analyzed by quantitative RT-PCR and normalized to the internal GAPDH control. The error bar represented two replicates. The supernatant infectivity titers were determined by a titration assay and normalized to the number of cells from which the viral supernatants were collected. HCV RNA levels were analyzed by quantitative RT-PCR. The error bar represented three replicates. All data were expressed as the percentage of wild type. The wild-type intracellular HCV RNA levels, supernatant infectivity and supernatant HCV RNA levels were 2.6×10^7 copies/g total RNA, 180 ffu/ml and 5.8×10^5 copies/ml respectively. (C) The intracellular and supernatant HCV infectivity of Huh-7 cells that were persistently infected by wild-type or I414T mutant viruses. Intracellular and supernatant viruses were prepared as described in Materials and methods. Infectivity was detected by titration assay.

infection by reducing the CD81 levels on the cell surface (Zhong et al., 2006). It was conceivable to speculate that the I414T mutation may confer an advantage at the step of viral entry when the CD81 levels on the host cell surface are reduced. To test this hypothesis, we compared the entry of wild-type and I414T JFH-1 viruses in the Huh-7.5.1 cells that were treated with a serial dose of anti-CD81 monoclonal antibodies. Interestingly, as shown in Fig. 6A, the infection of I414T was less sensitive to the anti-CD81 antibody blockade than that of wild type. Next we compared the entry of wild-type and I414T JFH-1 viruses when SR-B1 on the host cell surface was blocked by anti-SR-B1 sera. In consistent with the CD81 blockade, the blockade of SR-B1 on the host cell surface inhibited wild type more efficiently than I414T (Fig. 6B). Claudin-1 was recently identified as an important HCV entry factor. Therefore, we also compared the infection of wild-type and I414T JFH-1 viruses in the Huh-7.5.1 cells in which the claudin-1 expression levels were reduced by a claudin-1 specific shRNA. The western blot analysis showed that the claudin-1 specific shRNA decreased the expression of claudin-1 in a dose-dependent manner (data not shown). Importantly, as shown in Fig. 6C, the infection of I414T was less sensitive to the reduction of claudin-1 levels than that of wild type. Altogether, these data indicated that the infection of I414T virus was less dependent upon the levels of viral receptors/co-receptors on the host cell surface.

I414T was more sensitive to an anti-E2 neutralizing antibody and CD81-LEL treatment

Our foregoing results suggested the I414T mutation in E2 reduced the association of viral particles with LDL/VLDL, which may lead to more exposure of viral envelope glycoproteins on viral particles. To test this hypothesis, we first determined the sensitivity of wild type or I414T infection to a previously reported human anti-E2 monoclonal antibody (Law et al., 2008). As shown in the Fig. 7A, the anti-E2 antibody inhibited

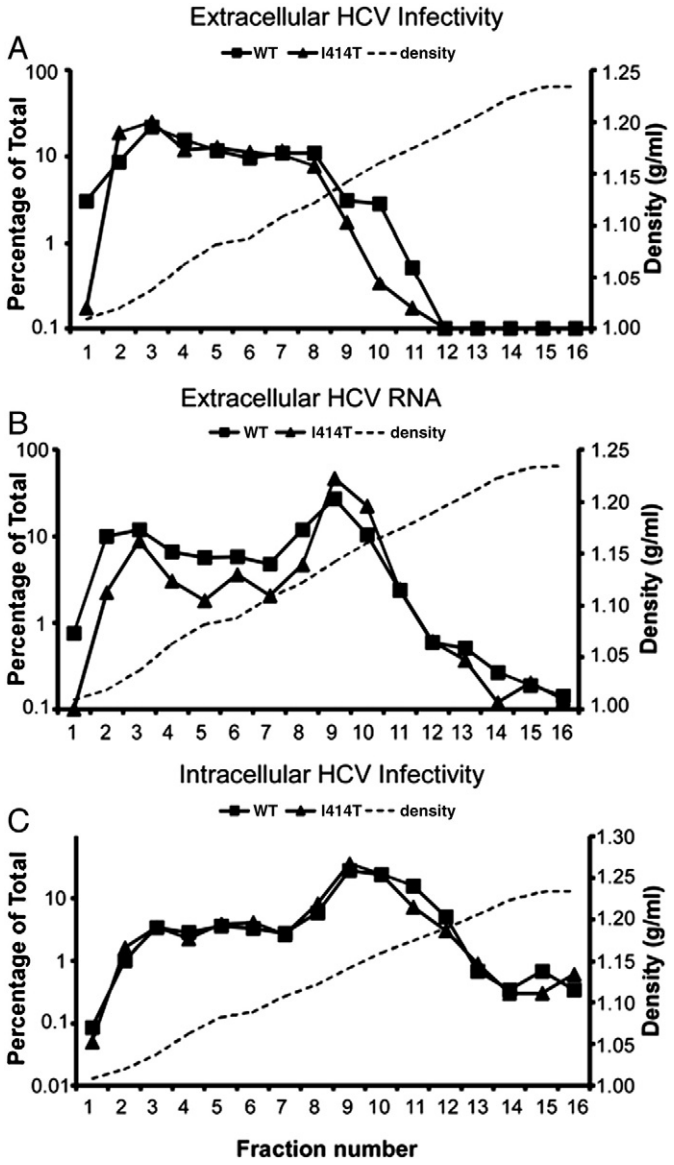

Fig. 5. I414T changed the buoyant density of JFH 1 virus. The extracellular (A and B) and intracellular (C) viral particles of wild-type and I414T JFH-1 were analyzed in a 20-60% sucrose gradient. The infectivity titers (A and C) and HCV RNA levels (B) of each fraction were determined by titration assay and quantitative RT-PCR respectively. The data were expressed as the percentage of the total titers of all density fractions.

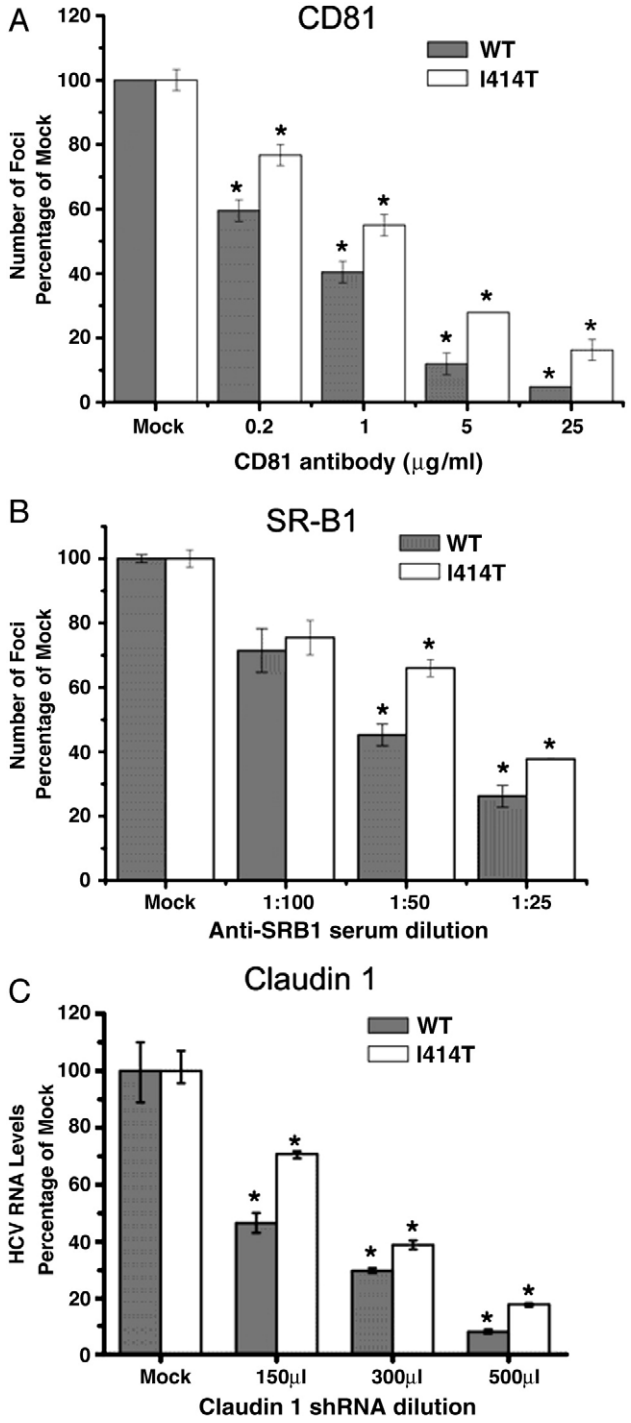

Fig. 6. The I414T virus infection was less dependent on the availability of host receptors. Huh-7.5.1 cells were pre-incubated with (A) anti-CD81 monoclonal antibody (clone 5A6) or (B) Rat anti-SR-B1 serum at the indicated doses at 37 °C for 1 h, and then infected with the same infectious units of wild-type or I414T JFH-1. The infection was analyzed with immunofluorescence staining, and the data were expressed as the percentage of infection in the mock treated Huh-7.5.1 cells. (C) Huh-7.5.1 cells were transduced with lentiviruses expressing claudin-1 specific shRNA, followed by the infection with wild-type or I414T mutant JFH-1. The intracellular HCV RNA levels were determined by quantitative RT-PCR. The data were expressed as the percentage of infection in the mock treated Huh-7.5.1 cells. The error bar represented three replicates (*$P<0.05$).

the infection of I414T much more efficiently than that of wild type. Consistently, the infection of I414T was more sensitive to recombinant large extracellular loop of CD81, a binding site of HCV E2 (Fig. 7B). Interestingly, however, the anti-E2 monoclonal antibody blocked the infection of wild-type and I414T mutant HCVpp at a similar efficiency (Fig. 7C), presumably because HCVpp may lack lipoprotein association during HCVpp genesis in HEK293T cells (Burlone and Budkowska, 2009). Altogether, these data indicated that HCV envelope glycoproteins on the I414T viral particles were more accessible for the neutralizing antibody or receptor binding.

The LDL/VLDL association determined the sensitivity of JFH-1 to the anti-E2 neutralizing antibody

To further confirm that I414T mutation increased the sensitivity of JFH-1 to the anti-E2 neutralizing antibody by reducing the LDL-VLDL association of viral particles, we used the anti-E2 antibody to neutralize the infection of intracellular wild-type or I414T viruses which share the similar buoyant density due to the lack of LDL/VLDL association. As shown in Fig. 8A, although the anti-E2 antibody inhibited the infection of extracellular I414T virus more efficiently than that of extracellular wild-type virus, no significant difference in the blocking efficiency was observed between the intracellular wild type and I414T. In addition, the intracellular viruses that lacked the LDL/VLDL association was apparently more sensitive to the anti-E2 neutralizing antibody than the extracellular viruses, providing additional evidence that the LDL/VLDL association reduces the sensitivity of HCV to the anti-E2 neutralization.

It has been reported that HCV viral particles were associated with apoE and apoC, and the antibodies against these apolipoproteins were able to neutralize HCV infection (Chang et al., 2007; Meunier et al., 2008). Therefore, we compared the sensitivity of wild type and I414T to anti-apoE or anti-apoC antibodies. As shown in Figs. 8B and C, I414T infection was less sensitive to the apolipoprotein blockade, suggesting that the mutant virus may have less apolipoprotein contents. Furthermore, we compared the sensitivity of extracellular and intracellular wild-type JFH-1 to the apolipoprotein blockade. As shown in Fig. 8D, similar to the I414T extracellular viruses, the infection of the intracellular wild-type viruses was less sensitive to the ApoE blockade. Altogether, these data strongly suggested that the virus–lipoprotein association could determine the interactions between the viral glycoproteins and their receptors or neutralizing antibodies.

Discussion

It has been reported by many groups that the infectivity of HCV could be enhanced during a long-term cell culture. Many mutations have been identified across the HCV genome that are responsible for the enhancement of infectivity (Delgrange et al., 2007; Kaul et al., 2007; Russell et al., 2008; Zhong et al., 2006). In our studies we found that the mutation of isoleucine to threonine at amino acid 414 in E2 led to more rapid viral expansion kinetics, higher infectious titers, increased specific infectivity and buoyant density changes. The HCV RNA levels in the wild-type and I414T mutant full-length genomic replicon cells or in the cell culture supernatants were similar, suggesting this mutation did not enhance HCV RNA replication in the cells or general virus particles secretion. Nevertheless, the I414T culture supernatants possessed about 4 times more infectious viral particles than wild type, indicating this mutation increased the proportion of infectious viral particles among total encapsulated HCV RNA population. Given the fact that HCV E2 glycoprotein is important for HCV entry, this mutation could give virus an advantage at the stage of virus entry. However, our results indicated that this mutation did not accelerate HCVcc or HCVpp entry in Huh-7.5.1 cells. Instead, interestingly, this mutation did confer an advantage at the virus entry when the levels of host receptors (CD81, SR-B1 or Claudin-1) were reduced. These results should make sense because the mutation was selected from a persistent HCV infection cell culture during which host cells became less permissive for HCV infection by reducing the CD81 levels on the cell surface. The I414T mutation that reduced the

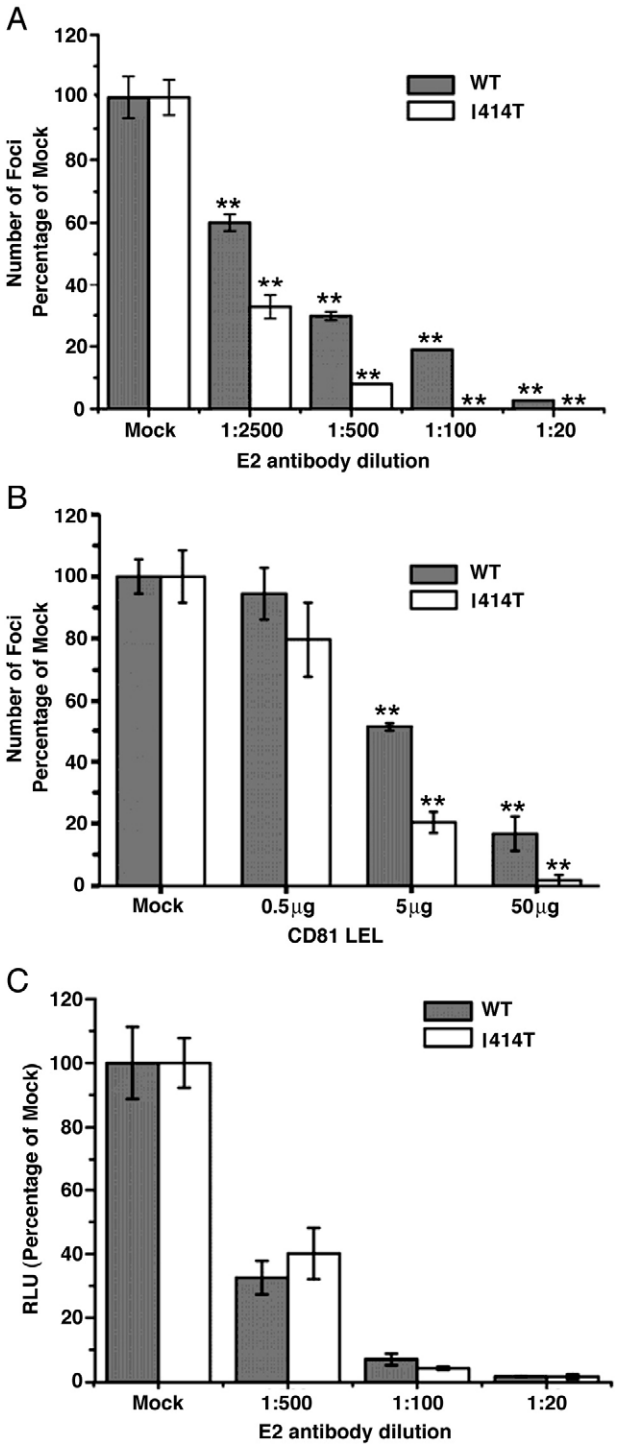

Fig. 7. I414T was more sensitive to an anti-E2 neutralizing antibody and CD81-LEL treatment. The wild-type or I414T mutant viruses were pre-incubated with a serial dose of (A) an anti-E2 neutralizing antibody or (B) CD81 LEL for 1 h at 37 °C, and then used to infect Huh-7.5.1 cells. The infection was analyzed at 72 h post-infection by immunofluorescence staining. (C) The wild-type or I414T mutant HCVpp were blockaded by the anti-E2 neutralizing antibody and HCVpp infection was determined by luciferase reporter assay. The data were expressed as the percentage of infection in the mock treated virus. The error bar represented three replicates (**$P<0.01$).

dependency of HCV entry on the levels of HCV receptors should benefit viral survival during the persistent infection.

HCV is believed to closely associate with host derived LDL/VLDL and exist in patient blood and in cell culture supernatants in a form of lipoprotein–virus complex. The change of buoyant density of the I414T mutant virus may be an indicative of an altered interaction of viral particles with lipoproteins. We hypothesized that the lack of infectivity in the lowest density fraction of the I414T mutant virus resulted from the reduction of association with LDL/VLDL during the virus secretion process. This hypothesis was supported by the observation that the delipidated intracellular viral particles of I414T shared a similar buoyant density distribution with that of wild type. Furthermore, our results showed that the infection of I414T mutant virus was much more sensitive to blockade by an anti-E2 neutralizing antibody or the recombinant CD81-LEL than that of the wild-type virus, while this difference was not observed in the infection of the delipidated intracellular HCVcc or HCVpp. Our interpretation for this observation was that the reduction of virus–lipoprotein association leads to more exposure of envelope glycoproteins on the surface of viral particles, making them more accessible for the neutralizing antibody or cell receptors. We also found that the infection of I414T virus was less sensitive to the blockade by the anti-ApoC1 or anti-ApoE antibody, supporting the notion that there were less apolipoprotein contents in the mutant viral particles. Recently Grove et al. (2008) reported another point mutation G451R in E2 that arose during the persistent infection *in vitro*. Although some differences exist between these two mutant viruses, such as the CD81 binding affinity and the dependency of virus infection on HCV receptors, both studies established a strong relationship between viral infectivity and virus–lipoprotein association. An important remaining question is how these mutations in E2 alter the lipoprotein association during the virus secretion process. Obviously more investigation will be needed to address this question.

Materials and methods

Cell culture, in vitro transcription, and HCV RNA transfection and molecular cloning

The cell culture condition and protocols for *in vitro* transcription and HCV RNA electroporation have been described previously (Zhong et al., 2005). Individual viral mutations were introduced into the pJFH-1 plasmid (Kato et al., 2003) by two-step recombinant PCR using primers containing the mutation, followed by restriction digestion and ligation. To make the HCV E1-E2 expression constructs for HCVpp genesis, the wild-type or I414T mutant E1 and E2 sequences were amplified by PCR and inserted into the *Bam*HI and *Not*I sites of pCDNA3.1. To make CD81 and SRB1 expression constructs, human CD81 and SRB1 cDNAs were amplified by PCR and inserted into the *Hin*dIII and *Not*I sites of pLEGFP-N1. To make the soluble E2 expression constructs, the C-terminal truncated wild-type or I414T mutant E2 sequences (384–687) were added a histidine tag at the C-terminal by PCR using the primers -GGAGCAGTCTTCGTTTCGCCCGGCACCAC-CACCGTTGGAGGC- and -CACAGCAGATCTTTA*GTGGTGGTGGTGGT-GGTG*GGGTAAGTCTGAGTAGGTGCAGGG-(C-terminal histidine tag is italicized). The tPA fragment was amplified using the sense primer -CATGGGTCTTTTCTGCAGTCACCGTCCTTAGAT- and the antisense primer -GCCTCCAACGGTGGTGGTGCCGGGCGAAACGAAGACTGCTCC-. The E2 fragments were then fused with the tPA fragment by PCR, and inserted into the *Bgl*II and *Pst*I sites of pVIJns vector (Scarselli et al., 2002). All constructs were verified by DNA sequencing.

RNA analysis, indirect immunofluorescence, titration analysis, HCV infection kinetics assay and sedimentation equilibrium gradient analysis

These analyses were performed as previously described (Zhong et al., 2005).

Intracellular particle preparation

Twenty million Huh-7.5.1 cells infected with wild-type or I414T mutant viruses were collected by trypsinization and resuspended in

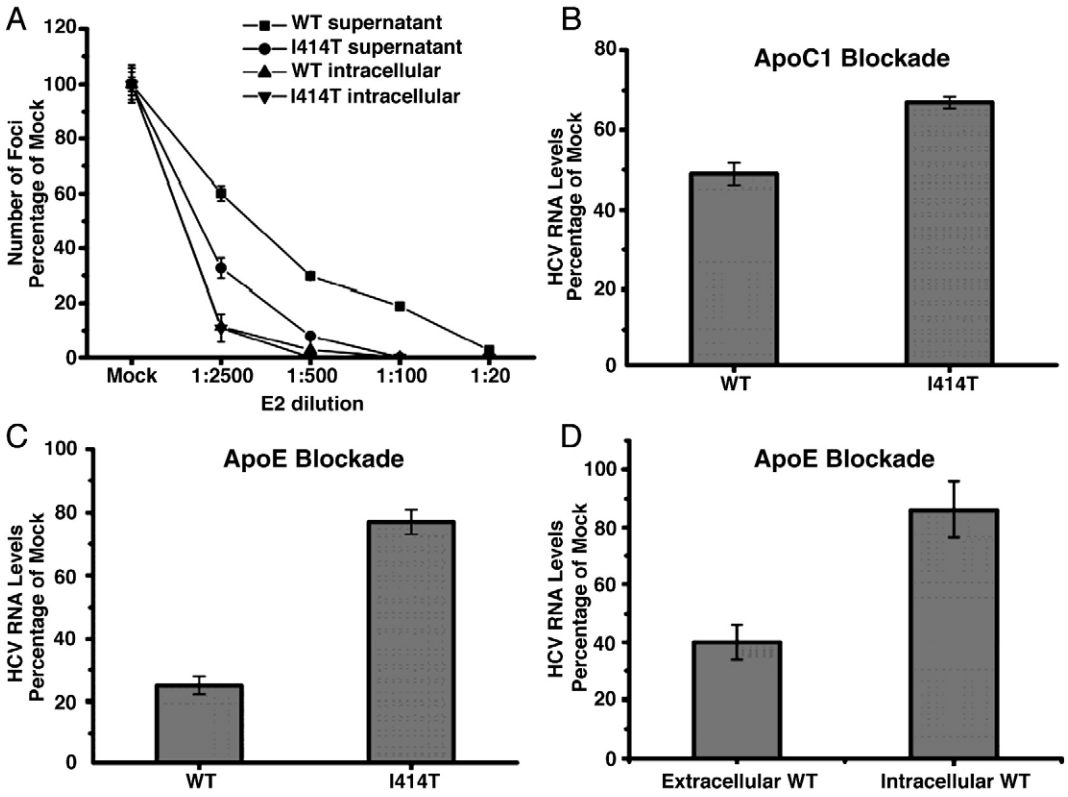

Fig. 8. LDL-VLDL association altered the sensitivity of HCV infection to the E2 neutralization. (A) The wild-type or I414T extracellular or intracellular viruses were assayed for their sensitivities to the neutralization by an anti-E2 monoclonal antibody. (B and C) The wild-type or I414T extracellular viruses were assayed for their sensitivities to an anti-ApoC1 antibody (B) or anti-ApoE antibody (C). (D) The extracellular or intracellular wild-type JFH-1 viruses were assayed for their sensitivities to the anti-ApoE antibody. In all cases, the viruses were pre-incubated with the antibodies for 1 h at 37 °C, and then inoculated to Huh-7.5.1 cells for 4 h. The intracellular RNA levels at 72 h post-infection were analyzed by quantitative RT-PCR. The data were expressed as the percentage of the mock treatment control. The error bar represented three replicates.

2 ml of complete medium. The cells were lysed by four freeze–thaw cycles between −80 °C isopropanol and a 37 °C water bath. Cell debris was removed by centrifugation twice at 4000 rpm for 5 min. The supernatant was collected and used for the infection.

Virus entry assay

Fifty focus-forming units of wild-type or I414T viruses were inoculated to 1×10^4 Huh-7.5.1 cells. The inocula were removed at the indicated time points and transferred to 1×10^4 naïve Huh-7.5.1 cells in a 96-well to quantify the number of viruses that have not entered the cells. In the meantime, the inoculated cells were washed three times with PBS and cultured in fresh complete medium. The cells were fixed at 72 h post-infection and analyzed by indirect immunofluorescence staining as previously described (Zhong et al., 2005).

JFH-1 replicon RNA transfection

Ten micrograms of *in vitro*-transcribed full-length JFH-1 replicon RNA containing a neo marker (Date et al., 2007) was electroporated into 4×10^6 R3 cells, CD81 negative subclone of Huh7 cells (Zhong et al., 2006). After electroporation, one-third of the cells were plated in a 10-cm dish. G418 (500 μg/ml) was added 1 day after transfection and was refreshed every 3 to 4 days. G418-resistant colonies were pooled to expand or stained with crystal violet 3 weeks after transfection.

Blockade of HCV infection by an anti-CD81 antibody and anti-SR-B1 serum

Ten thousand of Huh-7.5.1 cells in a 96-well plate were pretreated with 0, 0.1, 0.25, 1, 2.5, and 25 μg/ml of CD81 antibody (clone 5A6; Santa Cruz Biotechnology, Santa Cruz, CA) or 1:25, 1:50, 1:100 diluted anti SR-B1 rat serum (Zeisel et al., 2007) for 1 h and then infected with 50 focus-forming units of the wild-type or I414T mutant viruses for 5 h. The viruses were removed, and the cells were washed with PBS and then supplemented with complete medium. The efficiency of the infection was determined 3 days later by counting the number of HCV-positive foci by immunofluorescence as described above.

Blockade of HCV infection by CD81-LEL, anti-E2, anti-ApoC1 or anti-ApoE antibodies

Fifty focus-forming units of the wild-type or I414T mutant viruses were pre-incubated with various amount of purified large extracellular loop (LEL) of CD81 (Drummer, Wilson, and Poumbourios, 2002), anti-E2 (Law et al., 2008), anti-ApoC1 (Biodesign, Saco, ME) or anti-ApoE (Merck, Whitehouse Station, NJ) antibodies at 37 °C for 1 h, and then added to 1×10^4 Huh-7.5.1 cells for 8 h. The viruses were removed, and the cells were washed with PBS for three times and then supplemented with complete medium. The efficiency of the infection was determined 3 days later by quantitative RT-PCR analysis or by immunofluorescence staining as described above.

HCVpp genesis and infection

HCV pseudotyped retroviral particles (HCVpp) were generated as previously described (Hsu et al., 2003). Briefly 293T cells were co-transfected with the envelope-deficient HIV genome pNL4-3.Luc.R-.E- and a plasmid expressing wild-type or I414T mutant glycoproteins. The medium was replaced with complete medium after 18 h. Supernatants were collected after 72 h and clarified by centrifugation. For infection experiments, HCVpp supernatants were first normalized according to their HIV p24 levels by western blot, and then were used to infect 1×10^4 Huh-7.5.1 cells for 72 h. The infected cells were lysed

with 20 μl of cell culture lysis reagent (Promega, Madison, WI), and infection was measured by quantifying the expression of the luciferase reporter using 50 μl of luciferase substrate (Promega) and a Centro LB960 luminometer (Berthold Technologies, Bad Wildbad, Germany). The neutralization of HCVpp infection was carried out by pre-incubating 150 μl of wild-type or I414T mutant HCVpp with a serial dilution of anti-E2 antibodies (Law et al., 2008) at 37 °C for 1 h, and then added to 3×10^4 Huh-7.5.1 cells for 72 h. The efficiency of the infection was determined by the luciferase assay as described above.

Soluble E2 protein binding assay

HEK293T cells were transfected with 10 μg of plasmids expressing His-tagged HCV E2 proteins by the calcium phosphate method. Culture supernatants were harvested 72 h after transfection, concentrated 60 times using centrifugal filter devices (Amicon® Ultra-15, 10000 MWCO). The lentiviral vector pLEGFP-N1 (Invitrogen) containing human CD81 or SR-B1 was co-transfected with the plasmids expressing HIV gal-pol protein and VSV glycoprotein into HEK293T cells. The supernatants collected at 72 h post-transfection were used to transduce CHO cells. The expression of the receptors on the cell surface was confirmed by FACS using a mouse anti-hCD81 monoclonal antibody (clone 5A6, Santa Cruz Biotechnology) or Rabbit anti-hSRB1 antibody (NB400-104, Novus, Littleton, CO). The CD81-positive cells were sorted out, and then expanded for the E2 binding assay. To analyze the binding of sE2 to the receptors, 150 μl of concentrated sE2 culture supernatants were incubated with parental CHO cells or with CHO cells expressing SR-BI or CD81 at 37 °C for 1 h. After washing with PBS, the cells were incubated with 800 ng of anti-His mouse monoclonal antibody (Sigma, St. Louis, MO) on ice for 30 min, and then with 50 ng of Alexa555-conjugated goat-anti-mouse IgG (Invitrogen), followed by FACS analysis on a FACS Calibur flow cytometer (BD Biosciences, San Diego, CA).

RNA interference

Vector pSQR expressing shRNAs targeting the CLDN1 reference sequence (Sigma) was transfected into HEK293T cells together with two packaging plasmids encoding VSV glycoprotein and GAG-pol (Rubinson et al., 2003). The resulting supernatants were collected 72 h after transfection, clarified by centrifugation and used to infect Huh-7.5.1 cells. The expression levels of CLDN1 in infected cells were determined by western blot using a mouse anti-CLDN1 monoclonal (Zymed Laboratories, San Francisco, CA).

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