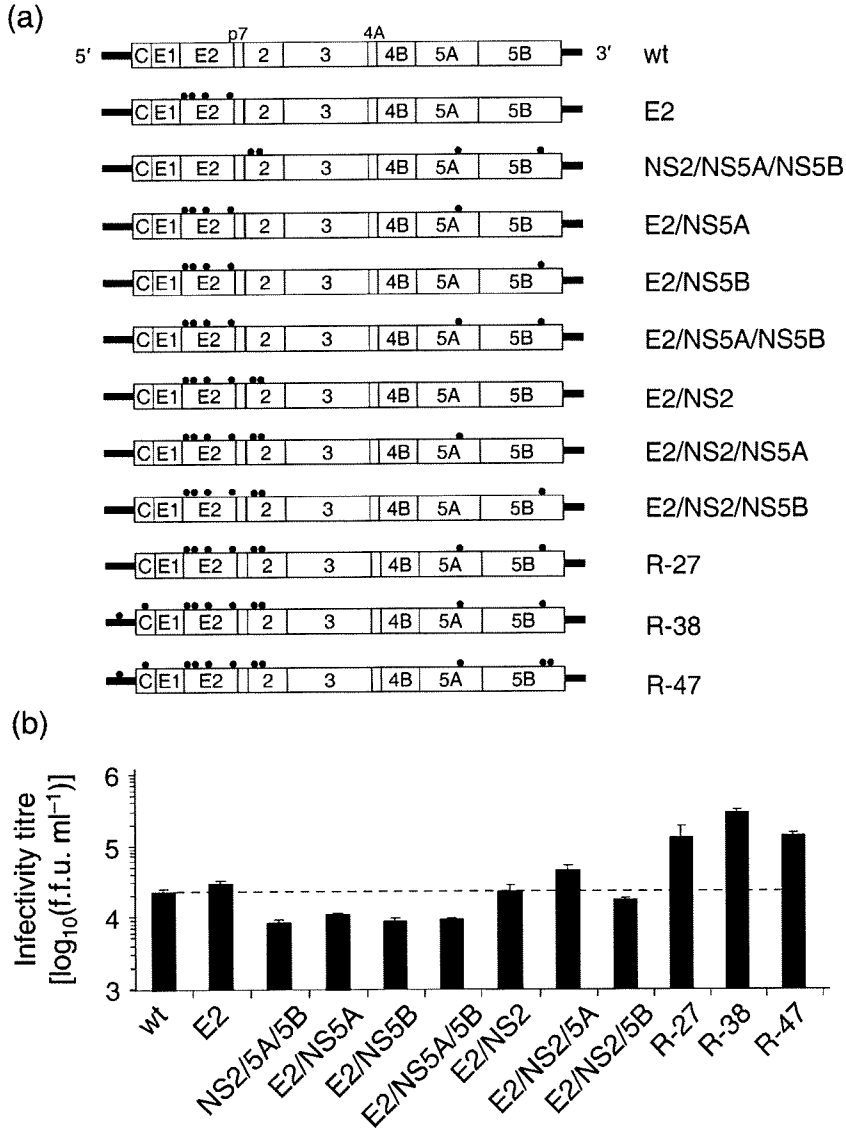

Fig. 3. Effects of combination of adaptive mutations on the production of infectious HCV. (a) Schematic representation of the wild-type (wt) and mutant chimeric HCV J6/JFH1 genomes. The HCV J6/JFH1 genomes with a combination of adaptive mutations at nt 146 (U to A) in the 5′-UTR and amino acid changes at K78E, T396A, T416A, N534H, A712V, Y852H, W879R, F2281L and M2876L are indicated by ●. (b) Recombinant mutant viruses with a combination of mutations were generated. Naïve Huh-7.5 cells were infected with each virus at an m.o.i. of 0.01 and cultured for 12 days. The ability of each mutant to release infectious virus particles was examined by titration assay. Infectivity titres reached maximal levels at 10 or 11 days post-infection and the maximal infectivity titres were plotted. Error bars represent SD for triplicate measurements.

We next examined the growth curves of the core protein levels by infecting cells with the recombinant viruses. The intracellular and extracellular core protein levels in cells infected with the P-1, R-27, R-38 and R-47 viruses were quantified. Huh-7.5 cells (1.2×10^5 cells per 12-well plate) were infected with these viruses at an m.o.i. of 0.2. The intracellular core protein levels in cells infected with the R-27, R-38 and R-47 viruses were 3- to 5-fold higher at day 1 post-infection than those in the P-1-infected cells. The intracellular core protein levels in the cells infected with the mutant viruses were 7- to 11-fold higher at day 3 post-infection than those in the P-1-infected cells (Fig. 5c). The extracellular core protein levels in the P-1-infected cells were comparable to the levels in cells infected with mutant viruses at day 1 post-infection. However, the extracellular core protein levels in cells infected with the R-27, R-38 and R-47 viruses increased more rapidly and reached 4.4- to 5.8-fold higher at day 3 post-infection than those in cells infected with the P-1 virus (Fig. 5d). Taken together, these data suggest that the adaptive mutants have advantages at the entry level, rather than the virus replication/translation level.

Table 2. Specific-infectivity titres of the recombinant adaptive mutant viruses

Virus	HCV RNA copies [$\log_{10}$(GE ml^{-1})]	Infectivity titre [$\log_{10}$(f.f.u. ml^{-1})]	Specific infectivity (f.f.u.: GE)
P-1	6.6±0.1	4.3±0.1	1:197
R-27	6.8±0.1	51±0.2	1:46
R-38	6.9±0	15.4±0.1	1:35
R-47	6.9±0.1	5.1±0.1	1:54

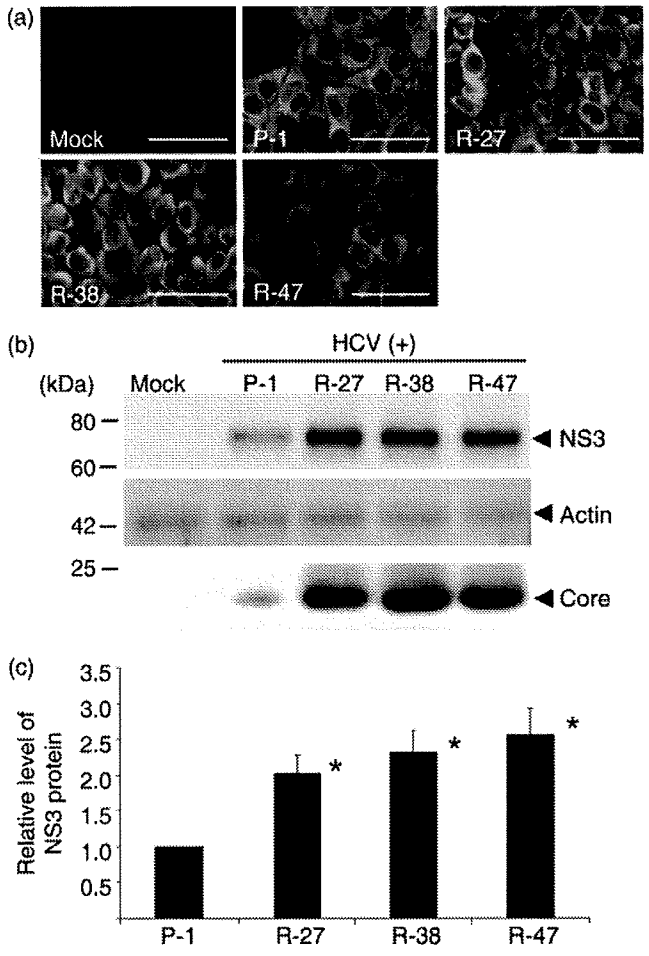

Fig. 4. Efficient expression of HCV proteins in Huh-7.5 cells infected with the adaptive mutants. Huh-7.5 cells (6×10^4 cells per 24-well plate) were infected with 200 µl P-1, R-27, R-38 or R-47 virus (6×10^4 f.f.u. ml^{-1}) at an m.o.i. of 0.2. (a) Cells were fixed 5 days post-infection and stained for immunofluorescence with anti-HCV-positive sera. Bars, 10 µm. (b) Immunoblot analysis of core and NS3 proteins in Huh-7.5 cells infected with R-27, R-38 and R-47 viruses. Data are representative of three independent experiments. (c) Quantification of the data shown in (b). Intensities of the gel bands were quantified by using the Scion Image for Windows program. The level of actin served as a loading control. Error bars represent SD for triplicate measurements. The difference between P-1 and the adaptive mutant (R-27, R-38 or R-47) was significant (*$P<0.05$ by Student's *t*-test).

Blocking of virus attachment and entry with anti-CD81 antibody

To determine whether the adapted mutant viruses have advantages at the entry level, we examined CD81-dependent entry into Huh-7.5 cells. Naïve Huh-7.5 cells were incubated with CD81-specific or non-specific antibody prior to inoculation. We scored infection by immunofluorescence at 24 h post-infection. As shown in Fig. 6(a), the anti-CD81 antibody inhibited the entry of the mutant viruses R-27, R-38 and R-47, as well as the wild-type virus, in a dose-dependent manner, suggesting that interaction between CD81 and HCV E2 glycoprotein is crucial for virus entry for all of these viruses. However, infections by the mutant viruses R-27, R-38 and R-47 were less dependent on CD81 than the wild-type virus. This result suggests that the mutations in the E2 glycoprotein confer an advantage to the mutant viruses at the entry level. We further analysed the mutant viruses to determine which mutation(s) is important for the advantage at the entry level. We infected Huh-7.5 cells with mutant viruses with a single point mutation in the E2 glycoprotein, such as T396A, T416A, N534H or A712V, or with all of the four mutations in E2. Blocking of virus entry with the anti-CD81 antibody was examined as shown in Fig. 6(b). Infection by the mutant virus N534H, as well as the mutant viruses E2, R-27, R-38 and R-47, was less dependent on CD81 than infection by the wild-type virus, whereas the other mutant viruses T396A, T416A and A712V showed a similar pattern to the wild type. These results indicate that the N534H mutation in the E2 region confers an advantage to the adaptive mutant viruses at the entry level.

DISCUSSION

In this study, we established an efficient HCV-production system by serial passaging of Huh-7.5 cells infected with the chimeric HCV J6/JFH1. Sequence analyses revealed that the adapted viruses possessed more than eight non-synonymous mutations in the genomes. Reverse-genetics analysis revealed that the recombinant viruses R-27, R-38 and R-47 exhibited higher expression of the HCV proteins than the wild-type virus. Moreover, we demonstrated that the N534H mutation in the E2 glycoprotein confers an advantage to the mutant viruses at the entry level.

The adaptive mutant viruses possessed four mutations (T396A, T416A, N534H and A712V) in E2. Two of these mutations (T416A and N534H) are in the regions that are involved in E2–CD81 binding and are, therefore, the possible target for neutralizing antibodies inhibiting E2–CD81 interactions (Helle & Dubuisson, 2008). The blocking of virus attachment and entry with CD81-specific antibody in this study revealed that the infections by the E2 R-27, R-38, R-47 and N534H mutants were less dependent on the CD81 molecule than that by the wild type J6/JFH1, suggesting that the N534H mutation gives the mutant viruses a selective advantage at the entry level. The N534H mutation is located in the sixth of 11 *N*-glycosylation sites, and is predicted to remove this *N*-glycosylation. The removal of *N*-glycosylation sites has been shown to have variable effects on CD81 binding and infectivity (Owsianka *et al.*, 2006; Roccasecca *et al.*, 2003). The glycans at positions 417, 532 and 645 (E2N1, E2N6 and E2N11) were shown to reduce the sensitivity of HCV pseudoparticles to antibody neutralization and to reduce the access of CD81 to its binding site on E2 (Goffard *et al.*, 2005). JFH-1 virus with the N534K mutation spread faster than the wild-type

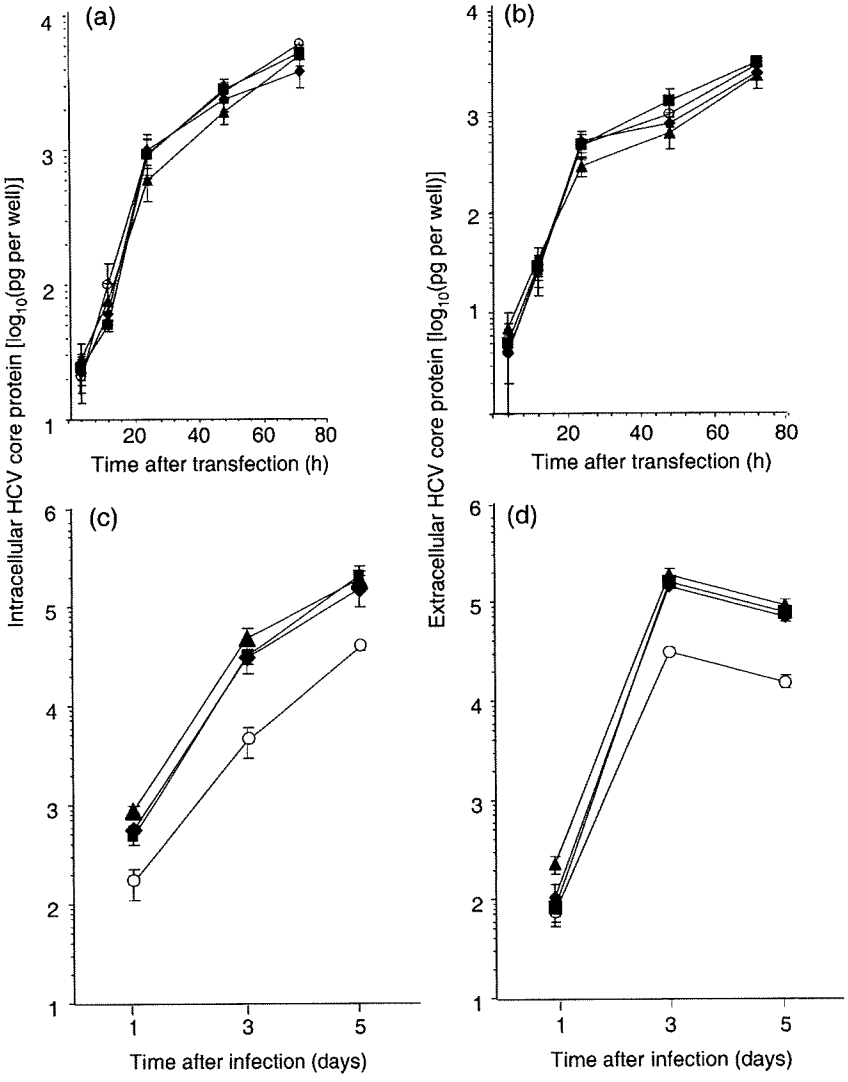

Fig. 5. Effects of adaptive mutations on the production of intracellular and extracellular core protein after transfection of *in vitro*-translated HCV RNAs or after infection of recombinant HCV. (a, b) After electroporation of 10 μg *in vitro*-translated HCV RNAs P-1 (○), R-27 (▲), R-38 (◆) and R-47 (■) into Huh-7.5 cells (5×10^6), the cells were divided into five sets, replated into a six-well plate and cultured. The cells and culture supernatants were harvested at the time points given. Intracellular (a) and extracellular (b) core protein levels were quantified by core protein-specific ELISA. (c, d) After Huh-7.5 cells (1.2×10^5 cells per 12-well plate) were infected with the P-1 (○), R-27 (▲), R-38 (◆) and R-47 (■) viruses at an m.o.i. of 0.2, the cells and culture supernatants were harvested at the time points given. Intracellular (c) and extracellular (d) core protein levels were quantified by core protein-specific ELISA.

JFH-1 virus after two successive amplifications in naïve cells, although the numbers of infectious viruses in the supernatant of transfected cells were initially low (Delgrange *et al.*, 2007). Our results in the growth curves of the viruses in the transfected cells and infected cells were consistent with their report. The CD81 inhibition assay in this study demonstrated clearly that the N534H mutation of the J6/JFH-1 virus confers a selective advantage for J6/JFH-1 at the entry level. To our knowledge, the present study is the first to prove that the mutation at site N534 gives infectious HCV a selective advantage at the entry level. These results raise two possibilities. One is that the N534H mutation in the E2 glycoprotein removes *N*-glycosylation and this mutant E2 glycoprotein possesses a higher affinity for the CD81 molecule, resulting in efficient entry to the cells. Another possibility is that the E2 glycoprotein with the N534H mutation gains higher affinity for other HCV receptors. Further investigation will be required to elucidate the mechanism of this adaptive mutation.

Our results showed that a combination of the mutations in E2, together with four additional mutations in NS2, NS5A and NS5B, resulted in higher infectivity of HCV, suggesting that the additional four mutations possess an advantage at different steps.

NS2 is a membrane-associated cysteine protease (Grakoui *et al.*, 1993; Hijikata *et al.*, 1993b; Lorenz *et al.*, 2006). The N terminus of NS2 consists of one or more transmembrane domains, whilst the C-terminal domain of NS2, together with the N-terminal one-third of NS3, forms the NS2–3 protease, an enzyme that catalyses a single cleavage at the NS2/NS3 boundary. The crystal structure of the C-terminal domain of NS2 has recently been determined and reveals a dimeric protease containing two composite active sites (Lorenz *et al.*, 2006). Jones *et al.* (2007) showed that NS2 and p7 are essential for HCV infectivity. The Y852 and W879 residues are located in the hydrophobic region of NS2. Although the exact topology of NS2 is disputed, the Y852H and W879R mutations would be predicted to lie

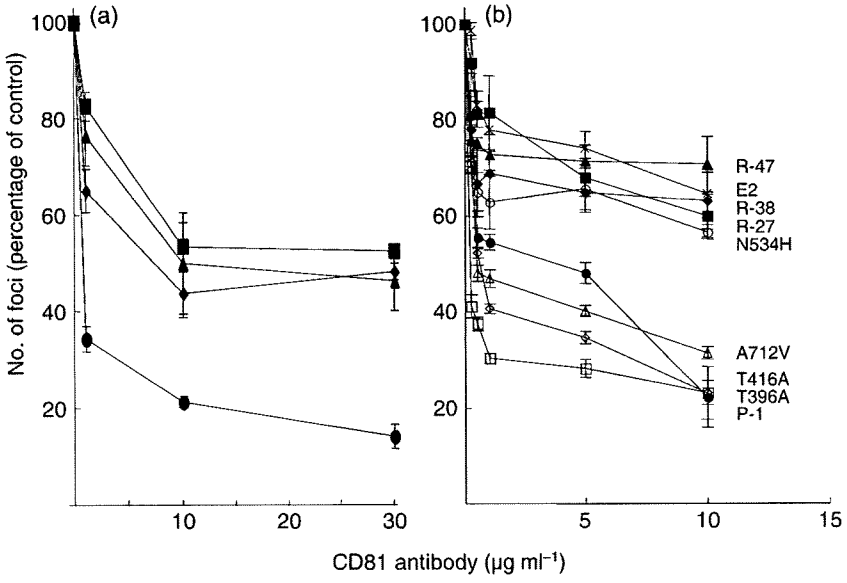

Fig. 6. Blocking of virus attachment and entry with anti-CD81 antibody. (a) Huh-7.5 cells (2×10^5 cells per six-well plate) were pretreated with 0, 1, 10 or 30 μg CD81 antibody (clone JS-81) ml^{-1} for 1 h and then infected with the wild-type (●, P-1) or recombinant mutant (■, R-27; ◆, R-38; ▲, R-47) viruses at an m.o.i. of 0.5. The cells were cultured for 24 h. The infection was monitored by HCV immunofluorescence and the numbers of HCV-positive foci were counted. Each result is expressed as a fraction of the number of foci observed in wells that received the control antibody instead of anti-CD81. Error bars represent SD for triplicate measurements. (b) Huh-7.5 cells (2×10^5 cells per six-well plate) were pretreated with 0, 0.25, 0.5, 1, 5 or 10 μg CD81 antibody ml^{-1} for 1 h and then infected with the wild-type (●, P-1) or recombinant (■, R-27; ◆, R-38; ▲, R-47; ×, E2; □, T396A; ◇, T416A; ○, N534H; △, A712V) viruses at an m.o.i. of 0.01. Blocking of virus entry with anti-CD81 antibody was examined. The infection was monitored by HCV immunofluorescence and the number of HCV-positive foci was counted.

within the second and third transmembrane domains, respectively (Yamaga & Ou, 2002). Murray *et al.* (2007) demonstrated that the A880P mutation increased infectious virus production significantly in the context of the J6/JFH1 genome, suggesting that the mutations in the transmembrane domain of NS2 play an important role in HCV infectivity. It is possible that the Y852H and W879R mutations in the transmembrane domain affect the topology and localization of NS2, and thereby HCV infectivity. Interestingly, NS2 has been found to interact with all other HCV NS proteins in *in vitro* pull-down assays, as well as cell-based colocalization and co-immunoprecipitation experiments (Dimitrova *et al.*, 2003; Hijikata *et al.*, 1993b), suggesting a role for NS2 as part of the replication complex.

Sequence analyses of HCV replicon cells revealed that highly adaptive mutations lie within the NS4B, NS5A and NS5B coding regions, with the majority clustering in NS5A. However, the mechanism underlying the replication enhancement is not known (Bartenschlager & Sparacio, 2007). The mutant viruses possessed an F2281L mutation that was located in domain II of NS5A. NS5A is an RNA-binding phosphoprotein composed of three domains that are separated by trypsin-sensitive low-complexity sequences (LCS I and LCS II) and an N-terminal amphipathic α-helix that anchors the protein stably to intracellular membranes (Brass *et al.*, 2002; Penin *et al.*, 2004; Tellinghuisen *et al.*, 2004). According to the X-ray crystal structure of domain I, it forms a dimer with a claw-like shape that can accommodate a single-stranded RNA molecule (Tellinghuisen *et al.*, 2005). Domain III of NS5A plays an important role in virus assembly and the production of infectious particles (Appel *et al.*, 2008; Masaki *et al.*, 2008; Tellinghuisen *et al.*, 2008). However, the role played by domain II of NS5A in the HCV replication cycle is unknown. Further examination will be required to clarify the effects of the F2281L mutation on the infectivity of the virus. Kaul *et al.* (2007) reported the V2941M mutation in NS5B in the context of the JFH1 genome. Lohmann *et al.* (2001) reported the R2884G mutation in the context of Con1-based replicon cells. Amino acid substitutions within NS5B may favour HCV replication and virus production in ways that remain to be determined.

Miyanari *et al.* (2007) proposed that HCV NS proteins and replication complexes are recruited to lipid droplet-associated membranes by the HCV core protein and that this recruitment is critical for producing infectious viruses. Cholesterol and sphingolipid associated with HCV particles are important for virion maturation and infectivity (Aizaki *et al.*, 2008). We speculate that the additional four mutations in NS2, NS5A and NS5B may confer an advantage in the maturation of virus particles or modification of virus envelopes with cholesterol and sphingolipid. Further investigation will be necessary to elucidate the mechanism of the adaptive mutations in NS2, NS5A and NS5B.

In conclusion, we have developed an efficient HCV-production system by passaging HCV J6/JFH1-infected Huh-7.5 cells. We have demonstrated that an efficient HCV-production system could be obtained by introducing adaptive mutations into the J6/JFH1 genome. The J6/JFH1-derived mutant viruses presented here would be a good tool for producing HCV particles with enhanced infectivity and for studying the molecular mechanism of HCV entry.

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REFERENCES

Aizaki, H., Morikawa, K., Fukasawa, M., Hara, H., Inoue, Y., Tani, H., Saito, K., Nishijima, M., Hanada, K. & other authors (2008). Critical role of virion-associated cholesterol and sphingolipid in hepatitis C virus infection. *J Virol* **82**, 5715–5724.

Appel, N., Zayas, M., Miller, S., Krijnse-Locker, J., Schaller, T., Friebe, P., Kallis, S., Engel, U. & Bartenschlager, R. (2008). Essential role of domain III of nonstructural protein 5A for hepatitis C virus infectious particle assembly. *PLoS Pathog* **4**, e1000035.

Bartenschlager, R. & Sparacio, S. (2007). Hepatitis C virus molecular clones and their replication capacity *in vivo* and in cell culture. *Virus Res* **127**, 195–207.

Bartosch, B., Vitelli, A., Granier, C., Goujon, C., Dubuisson, J., Pascale, S., Scarselli, E., Cortese, R., Nicosia, A. & Cosset, F. L. (2003). Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor. *J Biol Chem* **278**, 41624–41630.

Blight, K. J., Kolykhalov, A. A. & Rice, C. M. (2000). Efficient initiation of HCV RNA replication in cell culture. *Science* **290**, 1972–1974.

Blight, K. J., McKeating, J. A. & Rice, C. M. (2002). Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. *J Virol* **76**, 13001–13014.

Brass, V., Bieck, E., Montserret, R., Wolk, B., Hellings, J. A., Blum, H. E., Penin, F. & Moradpour, D. (2002). An amino-terminal amphipathic alpha-helix mediates membrane association of the hepatitis C virus nonstructural protein 5A. *J Biol Chem* **277**, 8130–8139.

Choo, Q. L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. W. & Houghton, M. (1989). Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* **244**, 359–362.

Choo, Q. L., Richman, K. H., Han, J. H., Berger, K., Lee, C., Dong, C., Gallegos, C., Coit, D., Medina-Selby, R. & other authors (1991). Genetic organization and diversity of the hepatitis C virus. *Proc Natl Acad Sci U S A* **88**, 2451–2455.

Delgrange, D., Pillez, A., Castelain, S., Cocquerel, L., Rouille, Y., Dubuisson, J., Wakita, T., Duverlie, G. & Wychowski, C. (2007). Robust production of infectious viral particles in Huh-7 cells by introducing mutations in hepatitis C virus structural proteins. *J Gen Virol* **88**, 2495–2503.

Dimitrova, M., Imbert, I., Kieny, M. P. & Schuster, C. (2003). Protein-protein interactions between hepatitis C virus nonstructural proteins. *J Virol* **77**, 5401–5414.

Evans, M. J., von Hahn, T., Tscherne, D. M., Syder, A. J., Panis, M., Wolk, B., Hatziioannou, T., McKeating, J. A., Bieniasz, P. D. & Rice, C. M. (2007). Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. *Nature* **446**, 801–805.

Goffard, A., Callens, N., Bartosch, B., Wychowski, C., Cosset, F. L., Montpellier, C. & Dubuisson, J. (2005). Role of N-linked glycans in the functions of hepatitis C virus envelope glycoproteins. *J Virol* **79**, 8400–8409.

Gottwein, J. M., Scheel, T. K., Hoegh, A. M., Lademann, J. B., Eugen-Olsen, J., Lisby, G. & Bukh, J. (2007). Robust hepatitis C genotype 3a cell culture releasing adapted intergenotypic 3a/2a (S52/JFH1) viruses. *Gastroenterology* **133**, 1614–1626.

Grakoui, A., McCourt, D. W., Wychowski, C., Feinstone, S. M. & Rice, C. M. (1993). A second hepatitis C virus-encoded proteinase. *Proc Natl Acad Sci U S A* **90**, 10583–10587.

Helle, F. & Dubuisson, J. (2008). Hepatitis C virus entry into host cells. *Cell Mol Life Sci* **65**, 100–112.

Hijikata, M., Mizushima, H., Akagi, T., Mori, S., Kakiuchi, N., Kato, N., Tanaka, T., Kimura, K. & Shimotohno, K. (1993a). Two distinct proteinase activities required for the processing of a putative nonstructural precursor protein of hepatitis C virus. *J Virol* **67**, 4665–4675.

Hijikata, M., Mizushima, H., Tanji, Y., Komoda, Y., Hirowatari, Y., Akagi, T., Kato, N., Kimura, K. & Shimotohno, K. (1993b). Proteolytic processing and membrane association of putative nonstructural proteins of hepatitis C virus. *Proc Natl Acad Sci U S A* **90**, 10773–10777.

Jones, C. T., Murray, C. L., Eastman, D. K., Tassello, J. & Rice, C. M. (2007). Hepatitis C virus p7 and NS2 proteins are essential for production of infectious virus. *J Virol* **81**, 8374–8383.

Kaul, A., Woerz, I., Meuleman, P., Leroux-Roels, G. & Bartenschlager, R. (2007). Cell culture adaptation of hepatitis C virus and *in vivo* viability of an adapted variant. *J Virol* **81**, 13168–13179.

Kuo, G., Choo, Q. L., Alter, H. J., Gitnick, G. L., Redeker, A. G., Purcell, R. H., Miyamura, T., Dienstag, J. L., Alter, M. J. & other authors (1989). An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. *Science* **244**, 362–364.

Lindenbach, B. D., Evans, M. J., Syder, A. J., Wolk, B., Tellinghuisen, T. L., Liu, C. C., Maruyama, T., Hynes, R. O., Burton, D. R. & other authors (2005). Complete replication of hepatitis C virus in cell culture. *Science* **309**, 623–626.

Lohmann, V., Korner, F., Koch, J., Herian, U., Theilmann, L. & Bartenschlager, R. (1999). Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* **285**, 110–113.

Lohmann, V., Korner, F., Dobierzewska, A. & Bartenschlager, R. (2001). Mutations in hepatitis C virus RNAs conferring cell culture adaptation. *J Virol* **75**, 1437–1449.

Lorenz, I. C., Marcotrigiano, J., Dentzer, T. G. & Rice, C. M. (2006). Structure of the catalytic domain of the hepatitis C virus NS2–3 protease. *Nature* **442**, 831–835.

Manns, M. P., Foster, G. R., Rockstroh, J. K., Zeuzem, S., Zoulim, F. & Houghton, M. (2007). The way forward in HCV treatment – finding the right path. *Nat Rev Drug Discov* **6**, 991–1000.

Masaki, T., Suzuki, R., Murakami, K., Aizaki, H., Ishii, K., Murayama, A., Date, T., Matsuura, Y., Miyamura, T., Wakita, T. & Suzuki, T. (2008). Interaction of hepatitis C virus nonstructural protein 5A with core

protein is critical for the production of infectious virus particles. *J Virol* **82**, 7964–7976.

McLauchlan, J., Lemberg, M. K., Hope, G. & Martoglio, B. (2002). Intramembrane proteolysis promotes trafficking of hepatitis C virus core protein to lipid droplets. *EMBO J* **21**, 3980–3988.

Miyanari, Y., Atsuzawa, K., Usuda, N., Watashi, K., Hishiki, T., Zayas, M., Bartenschlager, R., Wakita, T., Hijikata, M. & Shimotohno, K. (2007). The lipid droplet is an important organelle for hepatitis C virus production. *Nat Cell Biol* **9**, 1089–1097.

Muramatsu, S., Ishido, S., Fujita, T., Itoh, M. & Hotta, H. (1997). Nuclear localization of the NS3 protein of hepatitis C virus and factors affecting the localization. *J Virol* **71**, 4954–4961.

Murray, C. L., Jones, C. T., Tassello, J. & Rice, C. M. (2007). Alanine scanning of the hepatitis C virus core protein reveals numerous residues essential for production of infectious virus. *J Virol* **81**, 10220–10231.

Owsianka, A. M., Timms, J. M., Tarr, A. W., Brown, R. J., Hickling, T. P., Szwejk, A., Bienkowska-Szewczyk, K., Thomson, B. J., Patel, A. H. & Ball, J. K. (2006). Identification of conserved residues in the E2 envelope glycoprotein of the hepatitis C virus that are critical for CD81 binding. *J Virol* **80**, 8695–8704.

Penin, F., Brass, V., Appel, N., Ramboarina, S., Montserret, R., Ficheux, D., Blum, H. E., Bartenschlager, R. & Moradpour, D. (2004). Structure and function of the membrane anchor domain of hepatitis C virus nonstructural protein 5A. *J Biol Chem* **279**, 40835–40843.

Pietschmann, T., Lohmann, V., Kaul, A., Krieger, N., Rinck, G., Rutter, G., Strand, D. & Bartenschlager, R. (2002). Persistent and transient replication of full-length hepatitis C virus genomes in cell culture. *J Virol* **76**, 4008–4021.

Pileri, P., Uematsu, Y., Campagnoli, S., Galli, G., Falugi, F., Petracca, R., Weiner, A. J., Houghton, M., Rosa, D. & other authors (1998). Binding of hepatitis C virus to CD81. *Science* **282**, 938–941.

Poynard, T., Yuen, M. F., Ratziu, V. & Lai, C. L. (2003). Viral hepatitis C. *Lancet* **362**, 2095–2100.

Roccasecca, R., Ansuini, H., Vitelli, A., Meola, A., Scarselli, E., Acali, S., Pezzanera, M., Ercole, B. B., McKeating, J. & other authors (2003). Binding of the hepatitis C virus E2 glycoprotein to CD81 is strain specific and is modulated by a complex interplay between hypervariable regions 1 and 2. *J Virol* **77**, 1856–1867.

Russell, R. S., Meunier, J. C., Takikawa, S., Faulk, K., Engle, R. E., Bukh, J., Purcell, R. H. & Emerson, S. U. (2008). Advantages of a single-cycle production assay to study cell culture-adaptive mutations of hepatitis C virus. *Proc Natl Acad Sci U S A* **105**, 4370–4375.

Saito, I., Miyamura, T., Ohbayashi, A., Harada, H., Katayama, T., Kikuchi, S., Watanabe, Y., Koi, S., Onji, M. & other authors (1990). Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. *Proc Natl Acad Sci U S A* **87**, 6547–6549.

Scarselli, E., Ansuini, H., Cerino, R., Roccasecca, R. M., Acali, S., Filocamo, G., Traboni, C., Nicosia, A., Cortese, R. & Vitelli, A. (2002). The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. *EMBO J* **21**, 5017–5025.

Schaller, T., Appel, N., Koutsoudakis, G., Kallis, S., Lohmann, V., Pietschmann, T. & Bartenschlager, R. (2007). Analysis of hepatitis C virus superinfection exclusion by using novel fluorochrome gene-tagged viral genomes. *J Virol* **81**, 4591–4603.

Sumpter, R., Jr, Loo, Y. M., Foy, E., Li, K., Yoneyama, M., Fujita, T., Lemon, S. M. & Gale, M., Jr (2005). Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. *J Virol* **79**, 2689–2699.

Takigawa, Y., Nagano-Fujii, M., Deng, L., Hidajat, R., Tanaka, M., Mizuta, H. & Hotta, H. (2004). Suppression of hepatitis C virus replicon by RNA interference directed against the NS3 and NS5B regions of the viral genome. *Microbiol Immunol* **48**, 591–598.

Tellinghuisen, T. L., Marcotrigiano, J., Gorbalenya, A. E. & Rice, C. M. (2004). The NS5A protein of hepatitis C virus is a zinc metalloprotein. *J Biol Chem* **279**, 48576–48587.

Tellinghuisen, T. L., Marcotrigiano, J. & Rice, C. M. (2005). Structure of the zinc-binding domain of an essential component of the hepatitis C virus replicase. *Nature* **435**, 374–379.

Tellinghuisen, T. L., Foss, K. L., Treadaway, J. C. & Rice, C. M. (2008). Identification of residues required for RNA replication in domains II and III of the hepatitis C virus NS5A protein. *J Virol* **82**, 1073–1083.

Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H. G. & other authors (2005). Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* **11**, 791–796.

Yamaga, A. K. & Ou, J. H. (2002). Membrane topology of the hepatitis C virus NS2 protein. *J Biol Chem* **277**, 33228–33234.

Yi, M., Villanueva, R. A., Thomas, D. L., Wakita, T. & Lemon, S. M. (2006). Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells. *Proc Natl Acad Sci U S A* **103**, 2310–2315.

Yi, M., Ma, Y., Yates, J. & Lemon, S. M. (2007). Compensatory mutations in E1, p7, NS2, and NS3 enhance yields of cell culture-infectious intergenotypic chimeric hepatitis C virus. *J Virol* **81**, 629–638.

Zhong, J., Gastaminza, P., Cheng, G., Kapadia, S., Kato, T., Burton, D. R., Wieland, S. F., Uprichard, S. L., Wakita, T. & Chisari, F. V. (2005). Robust hepatitis C virus infection *in vitro*. *Proc Natl Acad Sci U S A* **102**, 9294–9299.

Zhong, J., Gastaminza, P., Chung, J., Stamataki, Z., Isogawa, M., Cheng, G., McKeating, J. A. & Chisari, F. V. (2006). Persistent hepatitis C virus infection *in vitro*: coevolution of virus and host. *J Virol* **80**, 11082–11093.

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HCV replication suppresses cellular glucose uptake through down-regulation of cell surface expression of glucose transporters☆

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See Editorial, pages 845–847

***Background/Aims*: Persistent infection with hepatitis C virus (HCV) causes extrahepatic diseases, including diabetes. We investigated the possible effect(s) of HCV replication on cellular glucose uptake and expression of the facilitative glucose transporter (GLUT) 2 and 1.**

***Methods*: We used Huh-7.5 cells harboring either an HCV subgenomic RNA replicon (SGR) or an HCV full-genomic RNA replicon (FGR), HCV-infected cells, and the respective cells treated with interferon (IFN). We also used liver tissue samples obtained from patients with or without HCV infection.**

***Results*: Glucose uptake and surface expression of GLUT2 and GLUT1 were suppressed in SGR, FGR and HCV-infected cells compared to the control cells. Expression levels of GLUT2 mRNA, but not GLUT1 mRNA, were lower in SGR, FGR and HCV-infected cells than in the control. Luciferase reporter assay demonstrated decreased GLUT2 promoter activities in SGR, FGR and HCV-infected cells. IFN treatment restored glucose uptake, GLUT2 surface expression, GLUT2 mRNA expression and GLUT2 promoter activities. Also, GLUT2 expression was reduced in hepatocytes of liver tissues obtained from HCV-infected patients.**

***Conclusions*: HCV replication down-regulates cell surface expression of GLUT2 partly at the transcriptional level, and possibly at the intracellular trafficking level as suggested for GLUT1, thereby lowering glucose uptake by hepatocytes.**
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***Keywords*: Diabetes mellitus; Down-regulation; Glucose uptake; GLUT1; GLUT2; Hepatitis C virus; Hepatocyte; Interferon; Replicon**

1. Introduction

Hepatitis C virus (HCV) is a small, enveloped RNA virus, which belongs to the genus *Hepacivirus* within the family *Flaviviridae*. The viral genome consists of single-stranded, positive-sense RNA of 9.6 kb that encodes a polyprotein of about 3000 amino acids. There are six major genotypes of HCV worldwide, with each genotype being further classified into a number of subtypes, such as HCV-1a and -1b [1,2]. The polyprotein is processed by host cellular and viral proteases to yield at least 10 structural and nonstructural (NS) proteins, such

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Abbreviations: FGR, full-genome RNA replicon; GLUT, glucose transporter; HBV, hepatitis B virus; HCV, hepatitis C virus; IFN, interferon; SGR, subgenomic RNA replicon.

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as core protein, envelope glycoproteins (E1 and E2), p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B [3,4].

HCV prevails in most parts of the world with an estimated number of about 170 million carriers and, hence, HCV infection is a major global healthcare problem [5]. Persistent infection with HCV causes not only liver diseases, including hepatitis, but also extrahepatic manifestations, such as type 2 diabetes [6–8]. While it has been known that liver cirrhosis impairs the glucose metabolism of the liver, there are some reports showing that HCV-infected patients over 40 years old have an increased risk for type 2 diabetes – three times higher than that for patients without HCV infection [9,10]. These reports imply the possibility that HCV infection directly predisposes the host towards type 2 diabetes. However, the precise mechanism(s) is poorly understood.

Glucose is transported into the cell via various isoforms of the facilitative glucose transporter (GLUT) that are present in most cells. Currently, a total of 14 isoforms have been identified in the GLUT family [11–13]. GLUT2 is expressed tissue-specifically in the liver, pancreatic β-cells, hypothalamic glial cells, retina and enterocytes [14]. On the other hand, GLUT1 is expressed at high levels in all fetal tissues and, in adults, it is widely expressed but most abundant in erythrocytes, endothelial cells of the blood–brain barrier, renal tubules of the kidney, and any kind of malignant cells including hepatocellular carcinoma [13].

In the present study, we demonstrated that HCV infection suppressed hepatocytic glucose uptake through down-regulation of surface expression of GLUT in a human hepatocellular carcinoma-derived cell line Huh-7.5. We also demonstrated that GLUT2 expression in hepatocytes of the liver tissues from HCV-infected patients was lower than in those from patients without HCV infection. We propose that HCV replication decreases glucose uptake and cell surface expression of GLUT, which would eventually lead to glucose metabolism disorder.

2. Materials and methods

2.1. Cell culture, HCV RNA replication, HCV infection and IFN treatment

A human hepatoma-derived cell line, Huh-7.5, which is highly permissive to HCV RNA replication [15], was kindly provided by Dr. C.M. Rice (The Rockefeller University, New York, NY, USA). The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum.

Huh-7.5 cells stably harboring an HCV-1b subgenomic RNA replicon (referred to as SGR cells, hereafter) were prepared as describe previously [16–18], using pFK5B/2884Gly (a kind gift from Dr. R. Bartenschlager, University of Heidelberg, Heidelberg, Germany). In SGR cells, the HCV subgenomic RNA replicon autonomously replicates to express NS3 to NS5B of HCV (Fig. 1). Cells harboring a full-length HCV-1b RNA replicon derived from pON/C-5B (referred to as FGR cells, hereafter) were described previously [19,20]. In FGR cells, the genome-size HCV RNA replicon autonomously replicates to express all the HCV proteins (the core protein, E1, E2, p7, NS2, NS3 to NS5B).

The pFL-J6/JFH1 plasmid that encodes the entire viral genome of a chimeric strain of HCV-2a, J6/JFH1 [21], was kindly provided by Dr. C.M. Rice. The HCV RNA genome was transcribed *in vitro* from pFL-J6/JFH1 and transfected to Huh-7.5 cells. The virus produced in the culture supernatant was used for infection experiments at multiplicities of infection of 1.0 and cultured for 5 days after virus infection.

In some experiments, SGR and FGR cells, as well as HCV-infected cells at 5 days after virus infection, were treated with 1000 IU/ml of IFN (Sigma, St. Louis, MI, USA) for 10 days to eliminate HCV replication.

2.2. Immunofluorescence

Cells were fixed with 3.7% paraformaldehyde and incubated with mouse monoclonal antibody against HCV NS5A (Chemicon International, Inc., Temecula, CA, USA) or HCV core (Abcam, Tokyo, Japan). The cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (MBL Co. Ltd., Nagoya, Japan), and observed under a fluorescent microscope (BX51; Olympus, Tokyo, Japan).

2.3. Immunoblotting

Cells were solubilized in lysis buffer as reported previously [22]. The cell lysates were electrophoresed subjected to 8% polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane (Millipore Corp., Billerica, MA, USA). The membranes were incubated with mouse monoclonal antibodies against HCV NS5A or NS3 (Chemicon), followed by incubation with peroxidase-conjugated goat anti-mouse IgG (MBL). The positive bands were visualized by using ECL detection system (GE Healthcare UK Ltd., Buckinghamshire, UK).

2.4. Uptake of 2-deoxy-D-glucose and thymidine

Cells cultured in 12-well plates were deprived of serum by incubation in serum-free medium for 12 h. The cells were then pre-incubated for 20 min in 450 μl of KRH (25 mM Hepes, 120 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 1.3 mM KH_2PO_4 and 0.1% BSA, pH 7.4). Glucose uptake assay was performed as describe previously [23]. In brief, glucose uptake was initiated by addition of 50 μl of reaction solution (KRH containing 0.5 mM, 0.25 μCi 2-deoxy-D-[1,2-^{3}H]glucose) to each well. As a negative control, 100 μM phloretin was added to reaction solution. After 10 min, transport was terminated by washing the cells with ice-cold KRH buffer containing 100 μM phloretin. The cells were solubilized by 0.1% sodium dodecyl sulfate, and the incorporated radioactivity was measured by liquid scintillation counter (LS6500; Beckman Coulter, Fullerton, CA). In some experiments, GLUT1 and GLUT2 were ectopically expressed by using the pCAGGS expression vector [24] and glucose uptake was measured as described above.

2.5. Flow cytometry

To examine cell surface expression of GLUT1 and GLUT2, cells harvested in PBS containing 0.2% EDTA were incubated with rabbit polyclonal antibodies against GLUT1 or GLUT2 (1:200; Alpha Diagnostic International, San Antonio, TX, USA) on ice for 1 h. After being washed, the cells were incubated with FITC-labeled goat anti-rabbit IgG (1:200; BD Pharmingen, Franklin Lakes, NJ, USA) on ice for another 1 h. Analysis was carried out using flow cytometer and a total of 10,000 live cell events were measured. Results were displayed graphically as overlaying histograms demonstrating the shift of the mean FITC staining value.

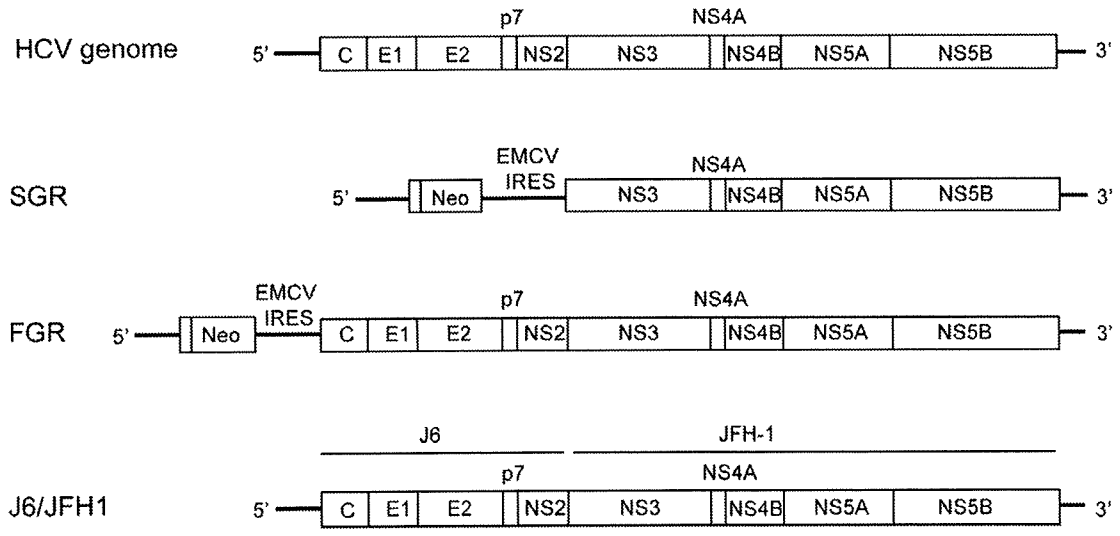

Fig. 1. The HCV genome and HCV RNA replicons. Schematic diagram of the HCV genome, SGR, FGR and the chimeric HCV J6/JFH1 genome are shown. EMCV IRES, encephalomyocarditis virus internal ribosome entry site; Neo, neomycin-resistance gene.

2.6. Real-time quantitative RT-PCR

Total cellular RNA was isolated using the TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA) and cDNA was generated using QuantiTect Reverse Transcription system (Qiagen, Valencia, CA, USA). Real-time quantitative PCR was performed on a SYBR *Premix Ex Taq* (Takara Bio, Kyoto, Japan) using SYBR green chemistry in ABI PRISM 7000 (Applied Biosystems, Foster, CA, USA). β-Glucuronidase was used as an internal control. The primers used are shown in Table 1.

2.7. Luciferase reporter assay

We constructed the human GLUT2 promoter-luciferase reporter gene (pGLUT2-1291Luc) by cloning a 1.6-kb genomic fragment that encompasses the human GLUT2 promoter (−1291 to +308) [14] into the pGL4 vector plasmid (Promega, Madison, WI, USA). pGLUT2-1291Luc thus contains a 1291-bp fragment of the human GLUT2 promoter upstream of the minimal promoter and the coding sequence of the *Photinus pyralis* (firefly) luciferase. pRL-CMV-*Renilla* (Promega) was used as an internal control. Cells were transfected with pGLUT2-1291Luc (1 μg) and pRL-CMV-*Renilla* (10 ng). After 24 h, a luciferase assay was performed by using Dual-luciferase reporter assay system (Promega). Firefly and *Renilla* luciferase activities were measured by Lumat LB 9501 (Berthold, Bad Wildbad, Germany). Firefly luciferase activity was normalized to *Renilla* luciferase activity for each sample.

2.8. Immunohistochemistry

Human adult liver autopsy materials and surgically removed liver tissues of patients with HCV- or HBV-associated hepatocellular carcinoma, and those with metastatic liver cancer were obtained with written informed consent. The tissues were fixed with 10% buffered formalin (pH 7.0), embedded in paraffin and sectioned at intervals of 4 μm. Immunohistochemical staining was performed with a DAKO ENVISION+ Kit (Dako, Glostrup, Denmark). In brief, fixed sections were treated with 3% hydrogen peroxide, and were autoclaved at 121 °C for 20 min. Then, the sections were incubated with a blocking solution and then with either anti-GLUT2 rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or normal rabbit IgG (Santa Cruz Biotechnology) as a control. The sections were incubated with horseradish peroxidase-labeled polymer-conjugated goat anti-rabbit IgG, followed by incubation in a chromogenic solution. The sections were then counterstained with hematoxylin and examined with a light microscope. GLUT2 expression levels were arbitrarily determined by two examiners, including a pathologist, in a blinded manner.

2.9. Statistical analysis

Results were expressed as mean ± SEM. Statistical significance was evaluated by ANOVA, and statistical significance was defined as $P < 0.05$.

Table 1
Sequences and positions of the primers used in this study.

Gene name (GenBank ID)	Primer	Position	PCR product (bp)
GLUT2	5′-TGGGCTGAGGAAGAGACTGT-3′	279–298	461
(J03810)	5′-AGAGACTGAAGGATGGCTCG-3′	739–720	
GLUT1	5′-TGAACCTGCTGGCCTTC-3′	437–453	399
(AK292791)	5′-GCAGCTTCTTTAGCACA-3′	835–819	
HCV NS5B	5′-ACCAAGCTCAAACTCACTCCA-3′	9191–9211	119
(AJ238799)	5′-AGCGGGGTCGGGCACGAGACA-3′	9309–9289	
β-glucuronidase	5′-ATCAAAAACGCAGAAAATACG-3′	1747–1767	238
(M15182)	5′-ACGCAGGTGGTATCAGTCTTG-3′	1984–1964	

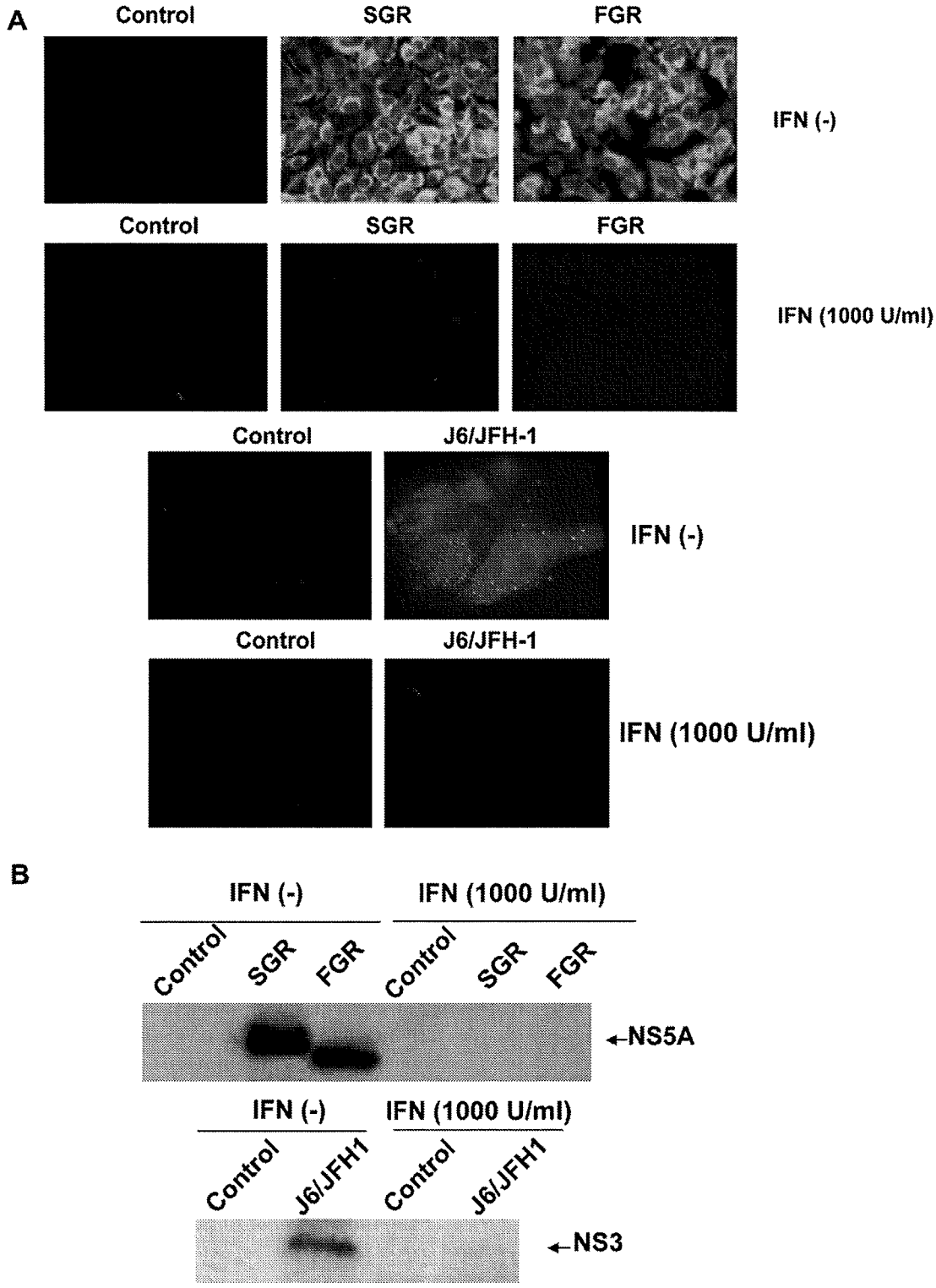

Fig. 2. Expression of HCV proteins in SGR, FGR, HCV-infected cells and the respective cells treated with IFN. (A) Cells were immunostained with anti-NS5A antibody (for SGR, FGR and the control cells) or anti-core antibody (for HCV-infected cells and the control). In parallel, cells were treated with IFN (1000 IU/ml) for 10 days to eliminate HCV replication before being subjected to immunostaining. (B) Cells were analyzed by immunoblotting with anti-NS5A antibody (upper panel) or anti-NS3 antibody (lower panel). In parallel, cells were treated with IFN (1,000 IU/ml) for 10 days to eliminate HCV replication before being subjected to immunoblotting.

3. Results

3.1. HCV protein expression in SGR, FGR, HCV-infected cells and those treated with IFN

Immunofluorescence analysis revealed that almost all the cells in SGR and FGR cultures, and >90% of the cells in the HCV J6/JFH1-infected culture were positive for HCV antigens (Fig. 2A). Western blot analysis also confirmed HCV protein expression in SGR, FGR and HCV-infected cells (Fig. 2B). In some experiments, HCV replication in SGR, FGR and HCV-infected cells was eliminated by IFN treatment for 10 days (Fig. 2A and B).

3.2. Selective suppression of cellular glucose uptake by HCV replication

2-Deoxygluose uptake levels in SGR, FGR and HCV-infected cells were significantly suppressed by about 50–60%, compared with the control Huh-7.5 cells (Fig. 3A and B). On the other hand, thymidine uptake, which was used as a control, did not significantly differ among all the cells tested (data not shown). Moreover, glucose uptake levels in SGR, FGR and HCV-infected cells were restored by IFN treatment (Fig. 3A and B). These results strongly suggest that cellular glucose uptake is selectively suppressed by HCV RNA replication.

3.3. Down-regulation of cell surface expression of GLUT2 and GLUT1 by HCV replication

GLUT2 is the principal glucose transporter of hepatocytes *in vivo* while GLUT1 is expressed in a wide variety of cultured cells. We therefore examined cell surface expression of GLUT2 and GLUT1 by flow cytometry analysis. As shown in Fig. 4A, cell surface expression of GLUT2 and GLUT1 was markedly down-regulated in SGR and FGR cells, compared with the control. On the other hand, cell surface expression of transferrin receptor was not significantly suppressed in SGR or FGR, compared with the control, with the result ensuring the specificity of the down-regulation of GLUT2 and GLUT1 cell surface expression in SGR and FGR (Fig. 4A). Moreover, treatment of SGR and FGR cells with IFN restored the surface expression of GLUT2 and GLUT1 (Fig. 4A). These results suggest that HCV RNA replication specifically mediates down-regulation of GLUT2 and GLUT1.

Down-regulation of GLUT2 surface expression was observed also in HCV-infected cells (Fig. 4B). On the other hand, down-regulation of GLUT1 surface expression was only marginal and, compared to that of GLUT2, less evidently observed in HCV-infected cells. As a control, cell surface expression of transferrin receptor did not differ at all between HCV-infected cells and the control. Again, treatment of HCV-infected cells with IFN restored surface expression of GLUT2 (Fig. 4B).

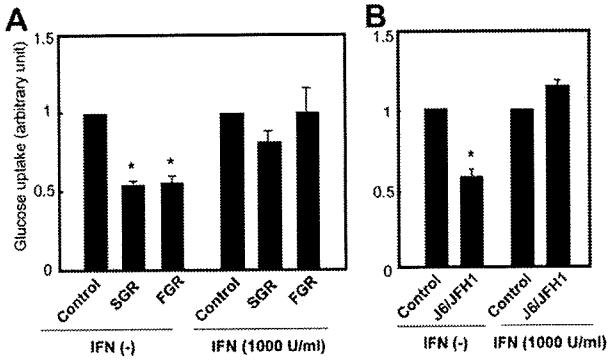

Fig. 3. Selective suppression of cellular glucose uptake by HCV replication. (A) Uptake of 2-deoxy-D-[1,2-^{3}H] glucose in SGR, FGR and HCV-negative control. In parallel, cells were treated with IFN (1000 IU/ml) for 10 days to eliminate HCV replication before being subjected to glucose uptake analysis. Data represent mean ± SEM of four independent experiments and the values for the control cells were arbitrarily expressed as 1.0. $^*P < 0.01$, compared with the control. (B) Uptake of 2-deoxy-D-[1,2-^{3}H] glucose in J6/JFH-1-infected cells and the uninfected control. In parallel, cells at 5 days after infection were treated with IFN (1000 IU/ml) for 10 days to eliminate HCV replication before being subjected to glucose uptake analysis.

3.4. Proteasomal degradation is not involved in the down-regulation of GLUT2 or GLUT1

Some viruses down-regulate cell surface molecules, such as immunoreceptors and intercellular adhesion molecules, through ubiquitination and proteasomal degradation of the target proteins [25]. To test this possibility, we treated SGR and FGR cells with lactacystin, a potent proteasome inhibitor. While lactacystin treatment enhanced cell surface expression of transferrin receptor, the same treatment did not increase cell surface expression of GLUT2 or GLUT1 in SGR or FGR cells (Fig. 5). This result suggested that down-regulation of cell surface expression of GLUT2 or GLUT1 in HCV-replicating cells was not due to increased degradation through the ubiquitin–proteasome system. The result rather implied the possible involvement of another mechanism(s), e.g., transcriptional suppression and/or impaired intracellular trafficking.

3.5. Transcriptional suppression of GLUT2, but not GLUT1, by HCV replication

To examine whether HCV RNA replication suppresses GLUT2 and GLUT1 expression at the transcriptional level, we measured mRNA expression levels by quantitative RT-PCR. The results obtained revealed that GLUT2 mRNA levels were reduced significantly in SGR, FGR and HCV-infected cells, compared to the control (Fig. 6A). It should be noted that the degree of GLUT2 mRNA suppression was greater in FGR

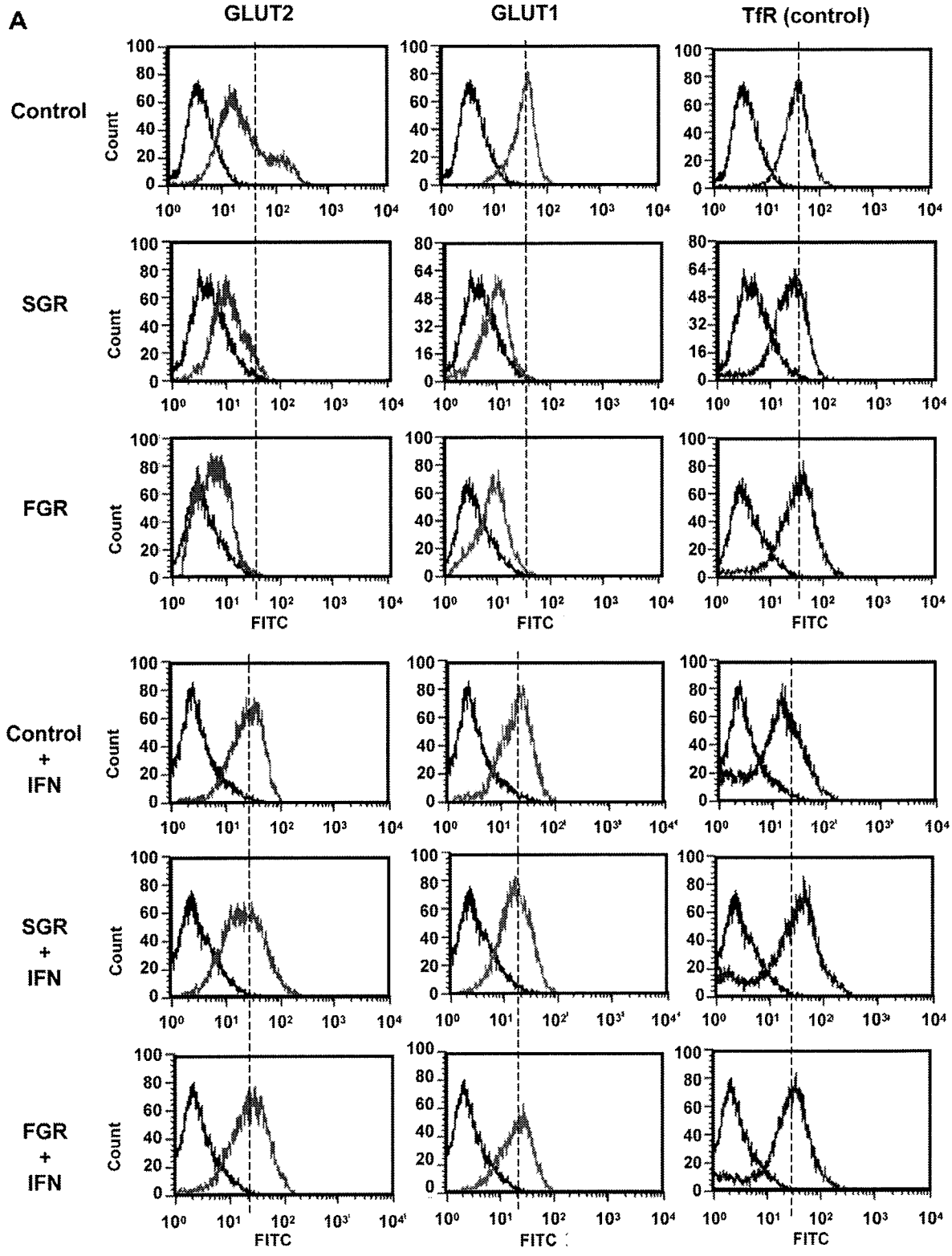

Fig. 4. Down-regulation of cell surface expressions of GLUT2 and GLUT1 by HCV replication. (A) SGR, FGR, the HCV-negative control cells were stained with specific antibodies, followed by FITC-conjugated second antibody (GLUT2, red line; GLUT1, green line) or stained with FITC-conjugated antibody alone (black line). Transferrin receptor (TfR) served as a control (blue line). In parallel, cells were treated with IFN (1000 IU/ml) for 10 days to eliminate HCV replication before being subjected to flow cytometry. (B) HCV-infected cells and the uninfected control were analyzed by flow cytometry as in (A). In parallel, cells at 5 days after infection were treated with IFN (1000 IU/ml) for 10 days to eliminate HCV replication before being subjected to flow cytometry analysis.

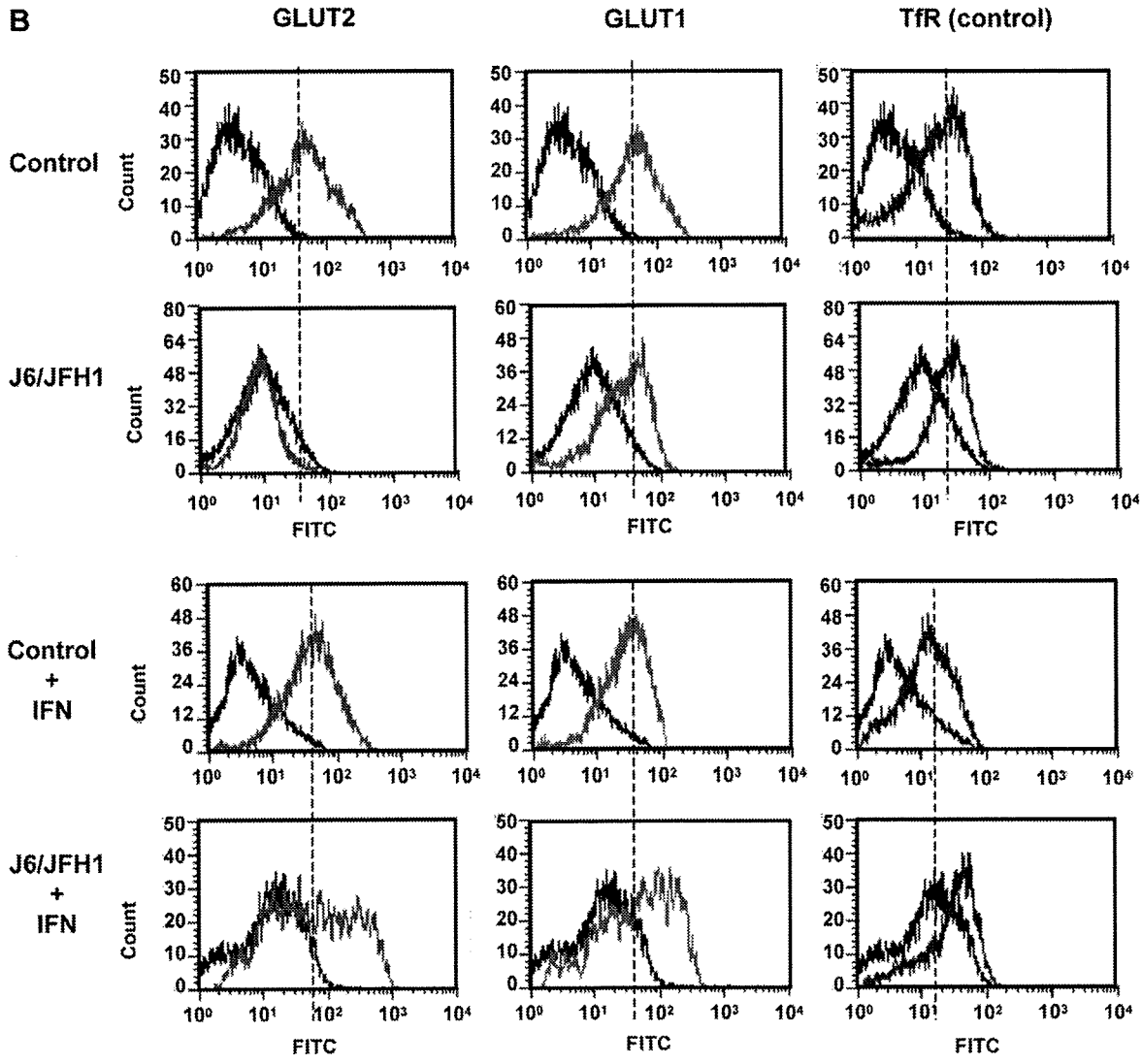

Fig. 4 (*continued*)

than in SGR cells. On the other hand, GLUT1 mRNA levels were not affected by HCV RNA replication (SGR and FGR) or HCV infection (Fig. 6B).

We also confirmed that GLUT2 mRNA expression levels in SGR, FGR and HCV-infected cells were restored by IFN treatment (Fig. 6A).

3.6. Suppression of GLUT2 promoter activity by HCV replication

Next, we performed luciferase reporter assay to examine the possible effect of HCV replication on GLUT2 promoter activities. The result obtained demonstrated that GLUT2 promoter activities were significantly suppressed in SGR, FGR and HCV-infected cells, compared to the control cells (Fig. 6C). Furthermore, GLUT2 promoter activities in SGR, FGR and HCV-infected cells were restored by IFN treatment. It is thus likely that HCV replication suppresses GLUT2 promoter activity, thereby decreasing GLUT2 mRNA levels.

3.7. Ectopically expressed GLUT1 or GLUT2 mediates increased glucose uptake in SGR, FGR and HCV-infected cells

We examined the possible effects of ectopically expressed GLUT1 and GLUT2 on glucose uptake in SGR, FGR and HCV-infected cells. Glucose uptake was significantly increased by ectopically expressed GLUT1 or GLUT2 in SGR, FGR and HCV-infected cells as well as in the control Huh-7.5 cells (Fig. 6D). It should be noted that, in this series of transient transfection experiments, only ca. 20% of the cells were ectopically overexpressing GLUT1 or GLUT2. These results collectively suggest the possibility that down-regulation

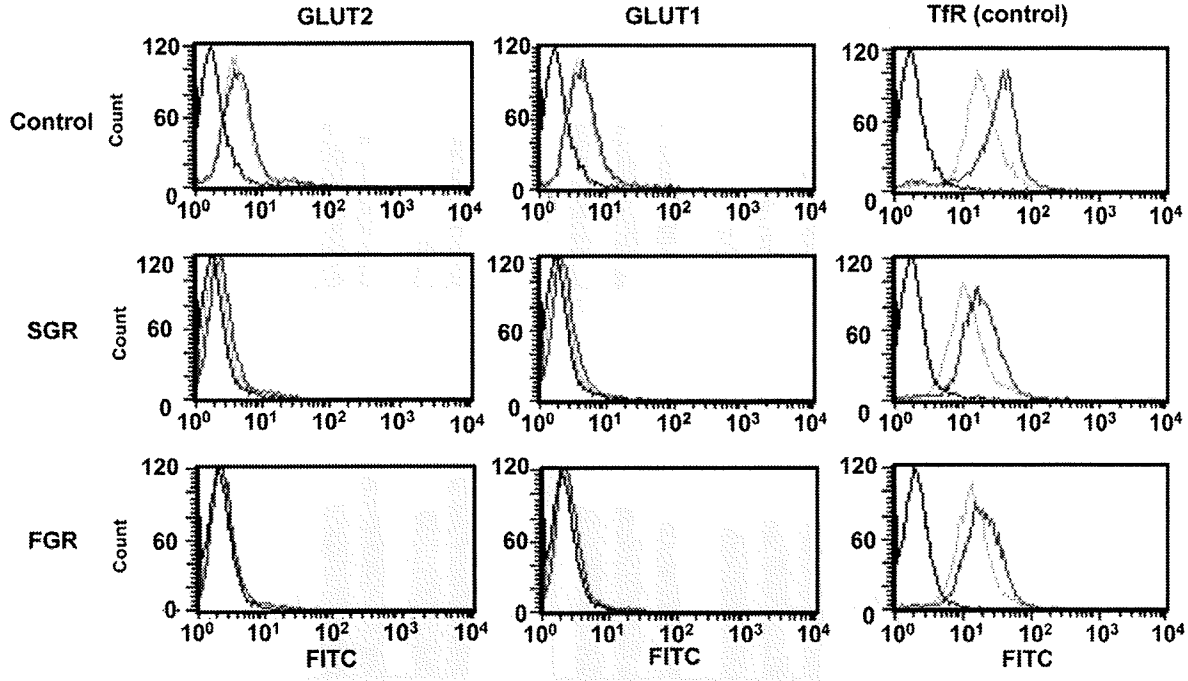

Fig. 5. Effects of lactacystin treatment on cell surface expression of GLUT2, GLUT1 and transferrin receptor (TfR). Cells were treated with lactacystin (10 μM) overnight to inhibit proteasomal degradation, and analyzed by flow cytometry. Cells treated with lactacystin are shown in red line and those left untreated in blue line. The negative controls stained with FITC-conjugated antibody alone are shown in black line.

of GLUT1 and GLUT2 expression is primarily involved in the decreased glucose uptake in SGR, FGR and HCV-infected cells.

3.8. Decreased GLUT2 expression in hepatocytes obtained from HCV-infected patients

GLUT2 is the principal glucose transporter expressed in hepatocytes *in vivo*. As shown in Fig. 7B, practically all hepatocytes obtained from patients without HCV infection showed positive staining for GLUT2, which was most evidently observed near the plasma membrane. On the other hand, hepatocytes obtained from HCV-infected patients showed markedly reduced GLUT2 staining in most, if not the entire, areas of the section, compared with the uninfected control (Fig. 7D). This heterogeneous staining pattern might reflect concomitant presence of areas comprising either virus-infected or uninfected hepatocytes in a tissue sample. Whereas all the sections obtained from 8 patients without HCV infection showed evenly positive staining for GLUT2, sections from 8 (89%) of 9 HCV-infected patients showed moderately to markedly reduced GLUT2 staining (Table 2). Reduced GLUT2 staining was observed also with hepatocytes in the liver tissues obtained from HBV-infected patients. However, the areas of reduced GLUT2 staining appeared to be more restricted in sections obtained from HBV-infected patients than in those from HCV-infected ones.

4. Discussion

HCV infection is known as an initiation and precipitating factor of type 2 diabetes [7–10,26,27]. Progression of liver fibrosis induced by persistent viral infection may induce diabetes [28]. Furthermore, it has been reported that the prevalence of diabetes is higher among patients with HCV-associated liver cirrhosis than in those with HBV-associated cirrhosis [7]. It is likely, therefore, that HCV infection itself is a risk factor of diabetes. Previous reports suggest that HCV infection directly causes insulin resistance that would cause the progression of diabetes [29–31]. However, the underlying mechanism(s) is not yet completely elucidated. In this study, we analyzed the effect of HCV infection on cellular glucose uptake and expression of glucose transporters.

We observed that glucose uptake was suppressed in cells harboring HCV RNA replicons (SGR and FGR) and those infected with HCV than in the control cells (Fig. 3). It has been reported that glucose disposal *in vivo* occurs through both insulin-dependent and insulin-independent mechanism [32]. We observed that treatment of SGR, FGR and the control Huh-7.5 cells with insulin (10^{-4} M to 10^{-9} M) increased glucose uptake by only about 50% from their basal levels (data not shown). Nevertheless, decreased glucose uptake by HCV-infected hepatocytes is a potential cause of hyperglycemia as the liver is a big organ accounting for 2% of the total body weight.

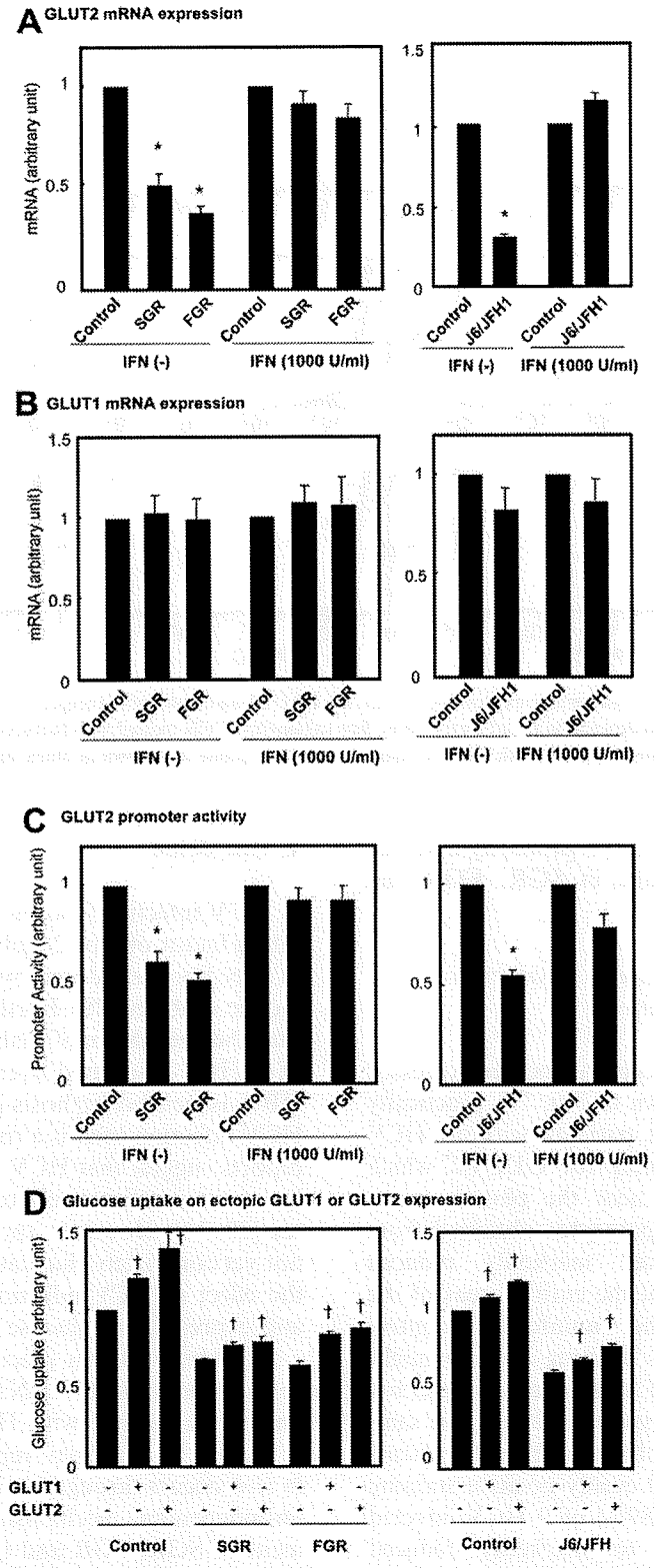

Fig. 6. Differential suppression of GLUT2 and GLUT1 mRNAs by HCV replication. (A and B) Quantitative RT-PCR analysis of mRNA for GLUT2 (A) and GLUT1 (B). mRNA expression levels of GLUT2 and GLUT1 in SGR, FGR and HCV-infected cells were determined and normalized with β-glucuronidase mRNA levels. In parallel, cells were treated with IFN (1000 IU/ml) for 10 days to eliminate HCV replication before being subjected to quantitative RT-PCR analysis. Data represent mean ± SEM of three independent experiments. $^{*}P < 0.01$, compared with the control. (C) GLUT2 promoter activities in SGR and FGR, HCV-infected cells were analyzed using luciferase reporter assay. In parallel, cells were treated with IFN (1000 IU/ml) for 10 days to eliminate HCV replication before being subjected to luciferase reporter assay. Data represent mean ± SEM of five independent experiments. $^{*}P < 0.01$, compared with the control. (D) Glucose uptake in cells ectopically expressing GLUT1 or GLUT2. Data represent mean ± SEM of two independent experiments. $^{\dagger}P < 0.01$, compared with mock transfected control.

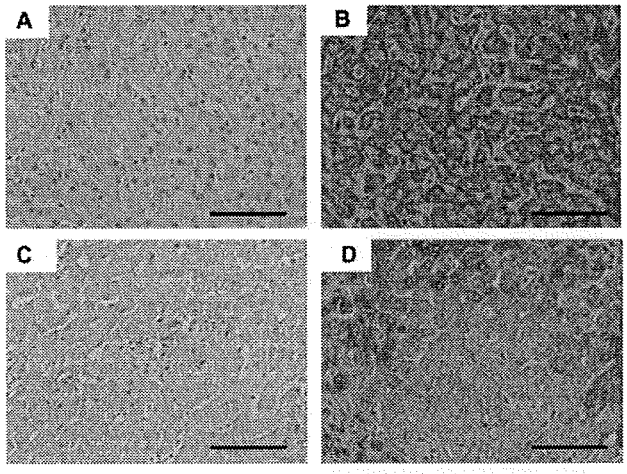

Fig. 7. Down-regulation of GLUT2 expression in HCV-infected human liver tissues *in vivo*. Normal human adult liver tissues (A and B) and HCV-infected, non-cancerous liver tissues (C and D) were fixed with formalin, sectioned and stained with normal rabbit IgG (A and C) or polyclonal anti-GLUT2 antibody (B and D). Scale bar = 100 μm.

Any proliferating cell requires energy sources, including glucose, and GLUTs play an important role in glucose uptake into the cell. In the liver, GLUT2 is the predominant glucose transporter, which regulates glucose metabolism by mediating a bidirectional transport, both entry and exit, of glucose into and from hepatocytes [13]. GLUT1, on the other hand, is known to be expressed in malignant cells including hepatocellular carcinoma [12,13] and a wide variety of cultured cells. In the present study we found that cell surface expression of GLUT2 and GLUT1 was markedly suppressed in SGR, FGR and HCV-infected cells compared to the control (Fig. 4A and B).

Table 2
Reduction of GLUT2 expression in hepatocytes of HCV-infected and HBV-infected human liver tissues.

Liver tissues	Sample No.	Reduction of GLUT2 expression
Uninfected	1	– *
	2	–
	3	–
	4	–
	5	–
	6	–
	7	–
	8	–
HCV-infected	9	1+ (Focal)[a]
	10	1+ (Focal)
	11	3+ (Diffuse)
	12	3+ (Diffuse)
	13	3+ (Diffuse)
	14	3+ (Focal)
	15	–
	16	2+ (Focal)
	17	3+ (Diffuse)
HBV-infected	18	–
	19	3+ (Diffuse)
	20	1+ (Focal)
	21	–
	22	2+ (Focal)
	23	1+ (Focal)
	24	2+ (Focal)

* -, no reduction; 1+, weak reduction; 2+, moderate reduction; 3+, strong reduction.

[a] Parentheses indicate either focal or diffuse appearance of the areas with reduced GLUT2 expression in each liver tissue sample.

GLUT2 expression is regulated at the transcriptional level, at least partly, by glucose [33]. It has been reported that hyperglycemia increases the GLUT2 mRNA and protein expression in an *in vivo* study [34]. Our present study demonstrated that GLUT2 mRNA expression was significantly suppressed in SGR, FGR and HCV-infected cells compared to the control (Fig. 6A). Consistent with this result, GLUT2 promoter activities, as measured by luciferase reporter assay, were suppressed in SGR, FGR and HCV-infected cells (Fig. 6C). In this connection, it was reported that GLUT2 promoter activities were up-regulated by sterol response element-binding protein (SREBP)-1c [35,36]. We confirmed in our study that GLUT2 promoter activities were up-regulated by over-expression of human SREBP-1c, and that the SREBP-1c-mediated GLUT2 promoter activities were suppressed significantly in SGR, FGR and HCV-infected cells (data not shown).

Unlike GLUT2 mRNA, GLUT1 mRNA was not suppressed by HCV RNA replication or HCV infection (Fig. 6B). Nevertheless, cell surface expression of GLUT1 was markedly down-regulated in SGR and FGR cells (Fig. 4A). As GLUT1 surface expression was not restored by treatment with lactacystin, a potent proteasome inhibitor (Fig. 5), it was unlikely that HCV-mediated suppression of GLUT1 surface expression was mediated through increased degradation by the ubiquitin-proteasome system. We assume that intracellular trafficking of GLUT1 (and possibly GLUT2 as well) is impaired by HCV RNA replication although we could not precisely prove it due mainly to the lack of an appropriate antibody that enables us to monitor GLUT1 trafficking. Further study is needed to elucidate the issue.

By means of immunohistochemical analysis, we confirmed that GLUT2 was strongly expressed in hepatocytes of the liver tissues obtained from all of 8 individuals without HCV infection (Fig. 7B and Table 2). More importantly, we demonstrated that GLUT2 expression was significantly down-regulated in hepatocytes obtained from 8 of 9 HCV-infected patients (Fig. 7D and Table 2). Interestingly, the areas where GLUT2 down-regulation was observed appeared to be scattered across the liver tissue sections. This may reflect the general observation that a group of hepatocytes in limited areas of the hepatic lobules, but not all the hepatocytes, are infected with HCV *in vivo*. By means of real-time quantitative PCR analysis, we found a tendency that levels of GLUT2 mRNA expression in liver tissues obtained from HCV-infected patients were lower than that obtained from uninfected controls although the dif-

ference was not statistically significant (data not shown). As stated above, not all the hepatocytes in the liver were infected with HCV and, therefore, the possible reduction of GLUT2 mRNA expression in HCV-infected hepatocytes might have been masked by the normal levels of expression in uninfected hepatocytes concomitantly present in the same tissue samples.

It should also be noted that GLUT2 staining was also reduced in hepatocytes obtained from HBV-infected patients, though to a lesser extent than that from HCV-infected ones (Table 2). We assume that inflammatory responses in the liver may trigger some intracellular event that leads to decreased GLUT2 expression in hepatocytes *in vivo*.

In conclusion, we have demonstrated for the first time that HCV replication inhibits cellular glucose uptake through down-regulation of cell surface expression of GLUT2 and possibly GLUT1. It is conceivable that the decreased glucose uptake by hepatocytes causes impaired glucose metabolism, leading eventually to the initiation and progression of diabetes mellitus during a prolonged period of HCV persistence.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2008.12.029.

References

[1] Simmonds P, Bukh J, Combet C, Deléage G, Enomoto N, Feinstone S, et al. Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. Hepatology 2005;42:962–973.

[2] Lu L, Li C, Fu Y, Thaikruea L, Thongswat S, Maneekarn N, et al. Complete genomes for hepatitis C virus subtypes 6f, 6i, 6j and 6m: viral genetic diversity among Thai blood donors and infected spouses. J Gen Virol 2007;88:1505–1518.

[3] Lindenbach BD, Rice CM. Unravelling hepatitis C virus replication from genome to function. Nature 2005;436:933–938.

[4] Appel N, Schaller T, Penin F, Bartenschlager R. From structure to function: new insights into hepatitis C virus RNA replication. J Biol Chem 2006;281:9833–9836.

[5] Shepard CW, Finelli L, Alter MJ. Global epidemiology of hepatitis C virus infection. Lancet Infect Dis 2005;5:558–567.

[6] Galossi A, Guarisco R, Bellis L, Puoti C. Extrahepatic manifestations of chronic HCV infection. J Gastrointestin Liver Dis 2007;16:65–73.

[7] Caronia S, Taylor K, Pagliaro L, Carr C, Palazzo U, Petrik J, et al. Further evidence for an association between non-insulin-dependent diabetes mellitus and chronic hepatitis C virus infection. Hepatology 1999;30:1059–1063.

[8] Mason AL, Lau JY, Hoang N, Qian K, Alexander GJ, Xu L, et al. Association of diabetes mellitus and chronic hepatitis C virus infection. Hepatology 1999;29:328–333.

[9] Mehta S, Levey JM, Bonkovsky HL. Extrahepatic manifestations of infection with hepatitis C virus. Clin Liver Dis 2001;5:979–1008.

[10] Mehta SH, Brancati FL, Sulkowski MS, Strathdee SA, Szklo M, Thomas DL. Prevalence of type 2 diabetes mellitus among persons with hepatitis C virus infection in the United States. Ann Intern Med 2000;133:592–599.

[11] Wu X, Freeze HH. GLUT14, a duplicon of GLUT3, is specifically expressed in testis as alternative spice forms. Genomics 2002;80:553–557.

[12] Macheda ML, Rogers S, Best JD. Molecular and cellular regulation of glucose transporter (GLUT) proteins in cancer. J Cell Physiol 2005;202:654–662.

[13] Godoy A, Ulloa V, Rodriguez F, Reinicke K, Yanez AJ, Garcia Mde L, et al. Differential subcellular distribution of glucose transporters GLUT1-6 and GLUT9 in human cancer: ultrastructural localization of GLUT1 and GLUT5 in breast tumor tissues. J Cell Physiol 2006;207:614–627.

[14] Ban N, Yamada Y, Someya Y, Miyawaki K, Ihara Y, Hosokawa M, et al. Hepatocyte nuclear factor-1α recruits the transcriptional co-activator p300 on the GLUT2 gene promoter. Diabetes 2002;51:1409–1418.

[15] Blight KJ, McKeating JA, Rice CM. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. J Virol 2002;76:13001–13014.

[16] Hidajat R, Nagano-Fujii M, Deng L, Tanaka M, Takigawa Y, Kitazawa S, et al. Hepatitis C virus NS3 protein interacts with ELKS-δ and ELKS-α, members of a novel protein family involved in intracellular transport and secretory pathways. J Gen Virol 2005;86:2197–2208.

[17] Nomura-Takigawa Y, Nagano-Fujii M, Deng L, Kitazawa S, Ishido S, Sada K, et al. Non-structural protein 4A of Hepatitis C virus accumulates on mitochondria and renders the cells prone to undergoing mitochondria-mediated apoptosis. J Gen Virol 2006;87:1935–1945.

[18] Inubushi S, Nagano-Fujii M, Kitayama K, Tanaka M, An C, Yokozaki H, et al. Hepatitis C virus NS5A protein interacts with and negatively regulates the non-receptor protein–tyrosine kinase Syk. J Gen Virol 2008;89:1231–1242.

[19] Ikeda M, Abe K, Dansako H, Nakamura T, Naka K, Kato N. Efficient replication of a full-length hepatitis C virus genome, strain O, in cell culture, and development of a luciferase reporter system. Biochem Biophys Res Commun 2005;329:1350–1359.

[20] Deng L, Nagano-Fujii M, Tanaka M, Nomura-Takigawa Y, Ikeda M, Kato N, et al. NS3 protein of Hepatitis C virus associates with the tumour suppressor p53 and inhibits its

function in an NS3 sequence-dependent manner. J Gen Virol 2006;87:1703–1713.
[21] Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, Liu CC, et al. Complete replication of hepatitis C virus in cell culture. Science 2005;309:623–626.
[22] Deng L, Adachi T, Kitayama K, Bungyoku Y, Kitazawa S, Ishido S, et al. Hepatitis C virus infection induces apoptosis through a Bax-triggered, mitochondrion-mediated, caspase 3-dependent pathway. J Virol 2008;82:10375–10385.
[23] Kanda H, Tamori Y, Shinoda H, Yoshikawa M, Sakaue M, Udagawa J, et al. Adipocytes from Munc18c-null mice show increased sensitivity to insulin-stimulated GLUT4 externalization. J Clin Invest 2005;115:291–301.
[24] Niwa H, Yamamura K, Miyazaki J. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 1991;108:193–199.
[25] Lehner PJ, Hoer S, Dodd R, Duncan LM. Downregulation of cell surface receptors by the K3 family of viral and cellular ubiquitin E3 ligase. Immunol Rev 2005;207:112–125.
[26] Mehta SH, Brancati FL, Strathdee SA, Pankow JS, Netski D, Coresh J, et al. Hepatitis C virus infection and incident type 2 diabetes. Hepatology 2003;38:50–56.
[27] Wang CS, Wang ST, Yao WJ, Chang TT, Chou P. Hepatitis C virus infection and the development of type 2 diabetes in a community-based longitudinal study. Am J Epidemiol 2007;166:196–203.
[28] Hui JM, Sud A, Farrell GC, Bandara P, Byth K, Kench JG, et al. Insulin resistance is associated with chronic hepatitis C virus infection and fibrosis progression. Gastroenterology 2003;125:1695–1704.
[29] Kawaguchi T, Yoshida T, Harada M, Hisamoto T, Nagao Y, Ide T, et al. Hepatitis C virus down-regulates insulin receptor substrates 1 and 2 through up-regulation of suppressor of cytokine signaling 3. Am J Pathol 2004;165:1499–1508.
[30] Miyamoto H, Moriishi K, Moriya K, Murata S, Tanaka K, Suzuki T, et al. Involvement of the PA28γ-dependent pathway in insulin resistance induced by hepatitis C virus core protein. J Virol 2007;81:1727–1735.
[31] Ader M, Ni TC, Bergman RN. Glucose effectiveness assessed under dynamic and steady state conditions. Comparability of uptake versus production components. J Clin Invest 1997;99:1187–1199.
[32] Banerjee S, Saito K, Ait-Goughoulte M, Meyer K, Ray RB, Ray R. Hepatitis C virus core protein upregulates serine phosphorylation of IRS-1 and impairs downstream Akt/PKB signaling pathway for insulin resistance. J Virol 2008;82:2606–2612.
[33] Im SS, Kim SY, Kim HI, Ahn YH. Transcriptional regulation of glucose sensors in pancreatic beta cells and liver. Curr Diabetes Rev 2006;2:11–18.
[34] Adachi T, Yasuda K, Okamoto Y, Shihara N, Oku A, Ueta K, et al. T-1095, a renal Na^+-glucose transporter inhibitor, improves hyperglycemia in streptozotocin-induced diabetic rats. Metabolism 2000;49:990–995.
[35] Im SS, Kang SY, Kim SY, Kim HI, Kim JW, Kim KS, et al. Glucose-stimulated upregulation of GLUT2 gene is mediated by sterol response element-binding protein-1c in the hepatocytes. Diabetes 2005;54:1684–1691.
[36] Kanayama T, Arito M, So K, Hachimura S, Inoue J, Sato R. Interaction between sterol regulatory element-binding proteins and liver receptor homolog-1 reciprocally suppresses their transcriptional activities. J Biol Chem 2007;282:10290–10298.

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Role of Oxysterol Binding Protein in Hepatitis C Virus infection▽†

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Hepatitis C virus (HCV) RNA genome replicates within the ribonucleoprotein (RNP) complex in the modified membranous structures extended from endoplasmic reticulum. A proteomic analysis of HCV RNP complexes revealed the association of oxysterol binding protein (OSBP) as one of the components of these complexes. OSBP interacted with the N-terminal domain I of the HCV NS5A protein and colocalized to the Golgi compartment with NS5A. An OSBP-specific short hairpin RNA that partially downregulated OSBP expression resulted in a decrease of the HCV particle release in culture supernatant with little effect on viral RNA replication. The pleckstrin homology (PH) domain located in the N-terminal region of OSBP targeted this protein to the Golgi apparatus. OSBP deletion mutation in the PH (ΔPH) domain failed to localize to the Golgi apparatus and inhibited the HCV particle release. These studies suggest a possible functional role of OSBP in the HCV maturation process.

Hepatitis C virus (HCV) infection is one of the leading causes of chronic hepatitis. HCV infection is associated with cirrhosis, steatosis, and hepatocellular carcinoma (33). The HCV RNA genome of ~9.6 kb is translated via an internal ribosome entry site element on the rough endoplasmic reticulum (ER) as a polyprotein precursor of about 3,010 amino acids that is co- and posttranslationally processed by cellular and viral proteases into mature structural and nonstructural (NS) proteins (33). HCV replicates within ribonucleoprotein (RNP) complexes associated with modified ER membranous structures (15). Recent work implicated lipid droplets that emanate from the ER as sites of RNA replication (28, 44). Almost all of the HCV NS proteins along with a variety of cellular factors are associated with the RNP complexes engaged in viral RNA replication (37). It is likely that these NS proteins not only participate in replication process but also are involved in the various steps of virion morphogenesis and assembly. Membrane-associated RNP complexes are generally composed of viral proteins, replicating RNA, host proteins, and altered cellular membranes (1). In this respect, a growing body of evidence implicates the functional role of NS5A in early steps of virion assembly and morphogenesis (3, 27, 45). NS5A is a phosphoprotein that migrates in sodium dodecyl sulfate gels as 56-kDa (basally phosphorylated) and 58-kDa (hyperphosphorylated) forms of proteins. The C-terminal domain III region of NS5A and the phosphorylated residue (Ser^{457}) are important for virion maturation (3, 27, 45). NS5A domain III contains the binding site for viral core protein, indicating the possible involvement of NS5A protein in virus assembly (27). NS5A anchors to the ER membrane by an N-terminal hydrophobic α-helix, and this attachment is needed for its key role(s) in viral replication (10). Studies suggest that phosphorylation of NS5A plays a functional role in viral replication (12). The hyperphosphorylated NS5A reduces its interaction with the human vesicle-associated membrane protein-associated protein A (VAP-A) (12). VAP-A binds both NS5A and NS5B (13, 17). These associations are important for RNA replication (13, 17).

HCV alters lipid homeostasis to benefit its infectious processes. Host lipids and their synthesis affect viral infectious process (21, 40, 51, 57). HCV RNA replication can be induced by saturated and monounsaturated fatty acids and inhibited by polyunsaturated fatty acids (18, 21). HCV gene expression induces lipogenesis by stimulating the activation of the sterol regulatory element binding proteins, the master regulators of lipid/fatty acid biosynthetic pathways (51). Reagents that interfere with host lipid biosynthetic pathways abrogate viral replication (21, 57). It has been suggested that HCV utilizes the very-low-density lipoprotein (VLDL) secretion pathway for its viral particle release (14, 19). These studies collectively suggest that host lipid metabolism plays a key role in the viral life cycle including replication, virion assembly, and secretion (56).

In the present study, we focus on the functional role of oxysterol binding protein (OSBP) that was identified by proteomic analysis as one of the host factors associated with the HCV RNP complexes. OSBP belongs to a family of the OSBP-related proteins. Originally discovered as a major cytosolic receptor for oxidized cholesterols, it undergoes translocation from the cytosolic/vesicular compartment to the Golgi apparatus upon ligand (hydroxycholesterol) binding (38). OSBP also binds to VAP-A via its FFAT motif (53). Golgi apparatus translocation of OSBP is regulated by the pleckstrin homology (PH) domain. This domain also harbors binding sites for phosphatidylinositol 4-phosphate (PI4P) and phosphatidylinositol 4,5-biphosphate ($PI4,5P_2$) (25). OSBP and OSBP-related pro-

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teins are implicated in cholesterol homeostasis, phospholipid metabolism, vesicular transport, and cell signaling (55). OSBP functions as sterol sensor that regulates the transport of ceramide from the ER to the Golgi apparatus for de novo synthesis of sphingomyelin by coordinated action with ceramide transport protein (CERT) (36). OSBP also functions as a scaffolding protein for two phosphatases (phosphatase 2A/HePTP) (49). This complex regulates the activity of extracellular signal-regulate kinase. This cytosolic 440-kDa complex disassembles by the addition of 25-hydroxycholesterol (25-HC) or depletion of cholesterol, both of which cause OSBP translocation to the Golgi compartment (49). Thus, in addition to its role in intracellular trafficking, OSBP appears to regulate cell signaling. We investigated the functional significance of OSBP association with HCV RNP complexes. RNA interference studies support a functional role of OSBP in virion morphogenesis and release process. The OSBP PH domain deletion mutant (ΔPH) failed to localize to the Golgi apparatus and caused an inhibition of the HCV particle release. Our work described herein also demonstrates that the association of OSBP with NS5A may also contribute to the overall HCV maturation process.

MATERIALS AND METHODS

Plasmids. The plasmids pJFH1, pSGR-JFH1, and pSGR-Luc-JFH1 were the generous gift of T. Wakita (22, 48). pSGR-JFH1-5A1ST, in which NS5A gene contains the One-STrEP tag (IBA, Göttingen, Germany), was generated by PCR-mediated mutagenesis using oligonucleotides described in Table S1 in the supplemental material. pFL-Luc-Jc1, an analogue to Luc-Jc1 (23), was constructed as described in Table S1 in the supplemental material. For constructing the human OSBP expression vector pFLAG-CMV-OSBP (where CMV is cytomegalovirus), OSBP1 cDNA was amplified from total RNA extracted from Huh7 cells by reverse transcription-PCR (RT-PCR), using oligonucleotides described in Table S1 in the supplemental material, and amplified product was digested with both HindIII and BamHI and cloned into the corresponding sites in the pFLAG-CMV2 vector (Sigma-Aldrich, St. Louis, MO). Expression vectors for mutant forms of OSBPs were constructed as described in Table S1 in the supplemental material. The VAP-A expression vector, pEF-FLAG-VAP-A, was kindly provided by Y. Matsuura (17). Generation of NS5A deletion mutants has been described previously (20). The NS5A derived from HCV JFH1 (genotype 2a) was cloned in the pEF1/Myc-His vector (Invitrogen, Carlsbad, CA) at the KpnI and XbaI sites by using a PCR-amplified fragment using primers described in Table S1 in the supplemental material. Lentiviral vectors, L-CMV-GFP-NheI (where GFP is green fluorescent protein), and packaging plasmids pMDL, pVSV-G (where VSV-G is vesicular stomatitis virus glycoprotein), and pREV were kindly provided by I. Verma (Salk Institute, La Jolla, CA) (46) and used for cloning short hairpin RNAs (shRNAs). The oligonucleotides used for constructing a scrambled shRNA (unrelated) and the OSBP-specific shRNA-1 and shRNA-2 are described in Table S1 in the supplemental material.

Cell culture. Human hepatoma cell lines Huh7 and Huh7.5.1 were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 mM minimal essential medium with nonessential amino acids (Invitrogen, Carlsbad, CA). The Huh7.5.1 cell line was a kind gift of F. Chisari (Scripps Institute, La Jolla, CA).

In vitro RNA transcription and RNA transfection. The plasmid encoding HCV (JFH1) was linearized by XbaI digestion, followed by mung bean nuclease treatment to blunt the XbaI-digested termini, and served as a template for RNA synthesis using a RiboMAX Large Scale RNA production system-T7 (Promega, Madison, WI). Synthesized RNAs were extracted by the acid guanidium thiocyanate phenol-chloroform (AGPC) method prior to transfection (7). Electroporation was used for RNA transfection. Subconfluent Huh7 or Huh7.5.1 cells were trypsinized and washed with ice-cold phosphate-buffered saline (PBS) and resuspended at 1×10^7 cells/ml in Cytomix buffer (47). Synthesized RNAs were mixed in 0.4 ml of cell suspension in a 4-mm gap electroporation cuvette (Genesee Scientific, San Diego, CA), pulsed at 260 V for 25 ms in a square wave mode of Gene Pulser Xcell electroporation system (Bio-Rad, Hercules, CA). For developing subgenomic replicon cell lines, Huh7 cells were transfected with in vitro synthesized replicon RNAs, incubated for 48 h in complete medium, and maintained in the presence of G418 (300 μg/ml). Stably expressing subgenomic replicon colonies were isolated after 3 weeks of growth in the presence of G418.

Proteomics analysis. Subgenomic replicon cell lines were established by transfecting SGR-JFH1 or SGR-JFH1-5A1ST RNA into Huh7 cells as described above, and isolated cell lines were designated SGR-JFH1 and SGR-JFH1-5A1ST, respectively. The NS5A protein complexes were purified by affinity chromatography from whole-cell lysates of the SGR-JFH1-5A1ST clone. Briefly, semiconfluent cell monolayers on 100-mm dishes were lysed with 4 ml of lysis buffer (0.2% deoxycholic acid, 0.25 M sucrose, 0.1 mM EDTA, and 3 mM Tris-HCl, pH 7.4) and incubated on ice for 20 min. The protein lysates were centrifuged at 25,000 × *g* for 30 min at 4°C. The cleared lysates were dialyzed against binding buffer (100 mM Tris-HCl, 150 mM NaCl, and 0.1% octylglucoside) and centrifuged again at 25,000 × *g* for 30 min at 4°C. The cleared lysates were loaded onto a StrepTactin-Sepharose column (1-ml bed volume; IBA) by filtering through 0.45-μm-pore-size polyethersulfone filter (Corning) and fractionated according to the manufacturer's instructions. The peak NS5A fraction from 30 preparations (12.5 μg) was collected and concentrated by acetone-methanol precipitation and subjected to multidimensional protein identification technology (MudPIT) analysis (5, 8, 9, 11, 26, 34, 39, 41). For the details of MudPIT analysis, see Materials and Methods in the supplemental material.

HCV infection and focus-forming unit assay. HCV JFH1 strain (48) was used for the production of HCV infectious viral particles. Huh7.5.1 cells were transfected with the in vitro synthesized RNA by electroporation. Ten days posttransfection cultured supernatants were collected. The infectious virion titers of collected supernatants were determined by a focus-forming-unit assay as described previously (58). HCV infection was performed at a multiplicity of infection (MOI) of 1.

Real-time RT-PCR. Total cellular RNAs were purified by the AGPC method (7). Viral RNAs were extracted from 100 μl of supernatant by the AGPC method. Five micrograms of *Saccharomyces cerevisiae* tRNA was added as a carrier (Sigma-Aldrich). HCV RNA was quantified on an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) as described previously (43). For the quantification of OSBP mRNA, 100 ng of total cellular RNA was subjected to cDNA synthesis using Improm II reverse transcriptase (Promega) oligo(dT) as a primer. Molecular copy number of OSBP and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was quantified by real-time PCR using SYBR premix Ex *Taq* (Takara Mirus Bio, Madison, WI) in an absolute quantification manner. The following set of primers was used for quantitative PCR: OSBP sense, 5′-AGAATACCCTTCGGACCCTCTC-3′; OSBP antisense, 5′-TCTTTTCATTGCTCTCAGCAGG-3′; GAPDH sense, 5′-GCCA TCAATGACCCCTTCATT-3′; and GAPDH antisense, 5′-TTGACGGTGCCA TGGAATTT-3′.

Western blotting analysis and immunoprecipitation. Huh7 cells or HCV-infected Huh7 cells were transfected with the indicated expression vectors by lipofection using TransIT-LT1 reagent (Mirus Bio, Madison, WI). Cells grown in a 35-mm dish were transiently transfected and incubated for 24 h, washed once with ice-cold PBS, and lysed on ice with 500 μl of lysis buffer (0.2% [wt/vol] deoxycholic acid, 20 mM Tris-HCl, 150 mM NaCl, 0.1 mM EDTA, 250 mM sucrose), supplemented with 1× Halt protease inhibitor single-use cocktail (Thermo Scientific, Rockford, IL). The cellular lysates were subjected to brief sonication, followed by centrifugation at 13,400 × *g* for 10 min. The clarified lysates were incubated with 5 μg of anti-FLAG M2 monoclonal antibody (Sigma-Aldrich) for 1 h. Five microliters of protein G-Sepharose 4 Fast Flow was added (GE Healthcare, Piscataway, NJ) for 2 h. Sepharose beads were collected by centrifugation and washed four times with 1 ml of lysis buffer. The immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Immunoblotting analysis was performed as described previously (52). The following antibodies were used for Western blotting analysis: mouse monoclonal anticore (Affinity Bioreagents, Golden, CO), goat polyclonal anti-OSBP (Novus biological, Littleton, CO), goat polyclonal anti-ApoB (Chemicon International, Temecula, CA), rabbit polyclonal anti-human albumin (MP Biomedicals, Solon, OH), rabbit polyclonal anti-adipose differentiation-related protein (Novus Biological), mouse monoclonal anti-fatty acid synthase (BD Bioscience), mouse monoclonal anti-NS3 (Virogen), and monoclonal antibody 9E10/A3 for NS5A (a generous gift from C. Rice).

Immunofluorescence microscopy. Infected and/or transfected cells were grown on glass coverslips, washed twice with PBS, and fixed in 3% paraformaldehyde in PBS supplemented with 2 mM $MgCl_2$ and 1.25 mM EGTA for 20 m at room temperature. Fixed cells were permeabilized and blocked in antibody-binding buffer (PBS, 0.2% [wt/vol] saponin, 0.2% [wt/vol] nonfat dry milk, 1% [wt/vol] bovine serum albumin, and 0.02% sodium azide) for 2 h at 4°C. Fixed cells were incubated in the presence of primary antibodies overnight. The primary antibodies used for immunofluorescence were mouse monoclonal 9E10/A3 for NS5A,