

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).  $\beta$ -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  $\mu$ g) 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

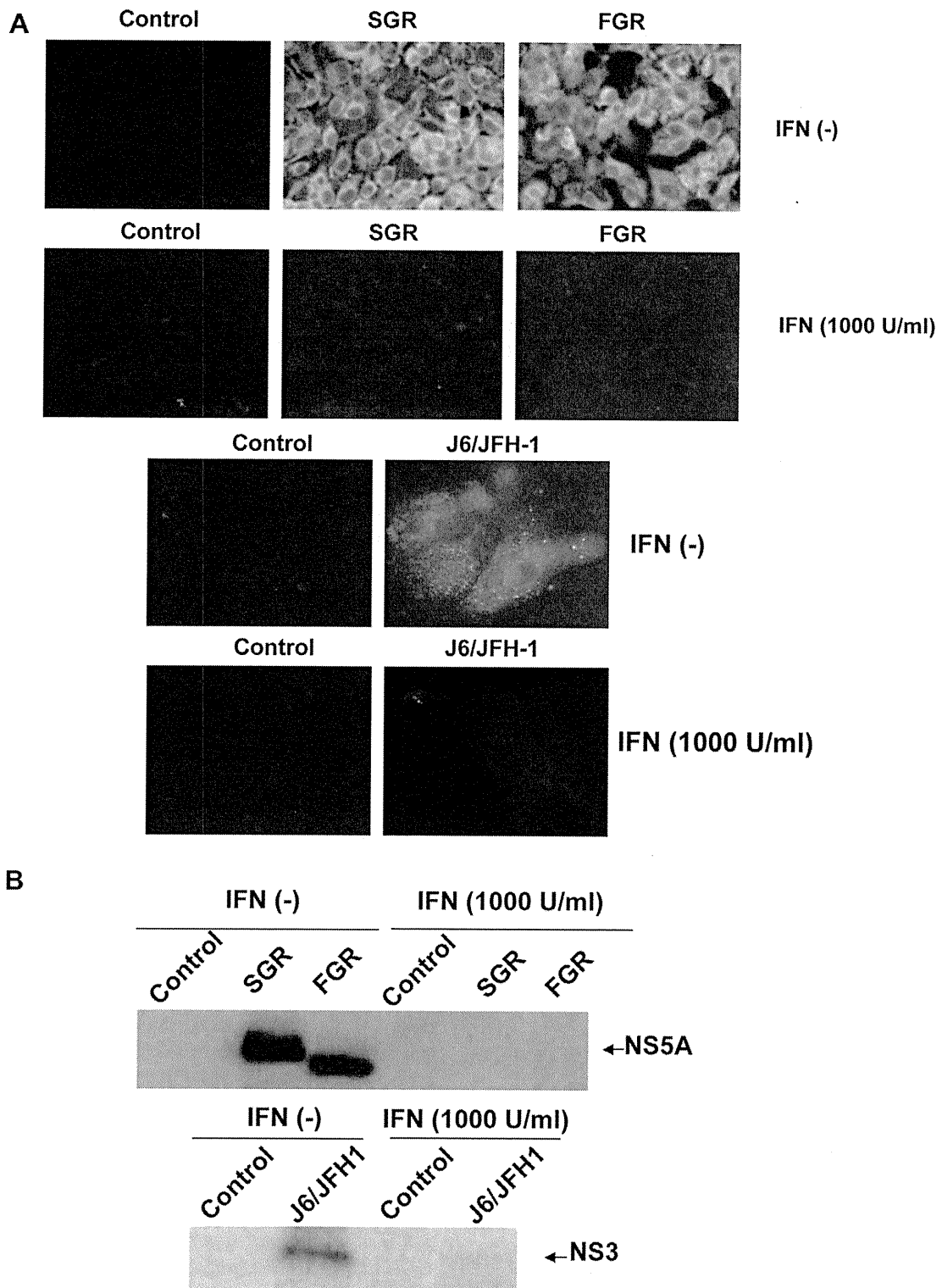
Human adult liver autopsy materials and surgically removed liver tissues of patients with HCV- or HBV-associated hepatocellular carcinoma, and those with metastatic liver cancer were obtained with written informed consent. The tissues were fixed with 10% buffered formalin (pH 7.0), embedded in paraffin and sectioned at intervals of 4  $\mu$ m. 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  $^{\circ}$ 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  $\pm$  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 (J03810)	5'-TGGGCTGAGGAAGAGACTGT-3'	279–298	461
	5'-AGAGACTGAAGGATGGCTCG-3'	739–720	
GLUT1 (AK292791)	5'-TGAACCTGCTGGCCTTC-3'	437–453	399
	5'-GCAGTCTCTTTAGCACA-3'	835–819	
HCV NS5B (AJ238799)	5'-ACCAAGCTCAAACCTCACTCCA-3'	9191–9211	119
	5'-AGCGGGGTCGGGCACGAGACA-3'	9309–9289	
$\beta$ -glucuronidase (M15182)	5'-ATCAAAAACGCAGAAAATACG-3'	1747–1767	238
	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-Deoxyglucose 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 vari-

ety 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).

#### 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

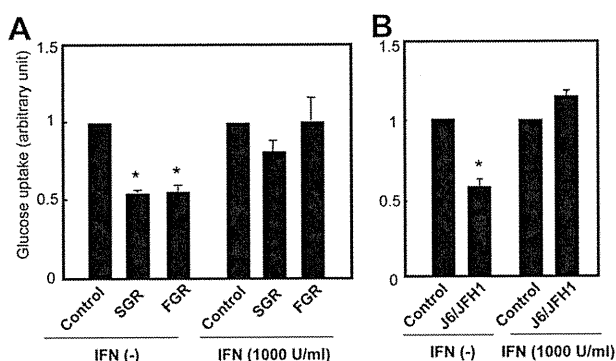


Fig. 3. Selective suppression of cellular glucose uptake by HCV replication. (A) Uptake of 2-deoxy-D-[1,2-<sup>3</sup>H] glucose in SGR, FGR and HCV-negative control. In parallel, cells were treated with IFN (1000 IU/ml) for 10 days to eliminate HCV replication before being subjected to glucose uptake analysis. Data represent mean  $\pm$  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-<sup>3</sup>H] glucose in J6/JFH1-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.

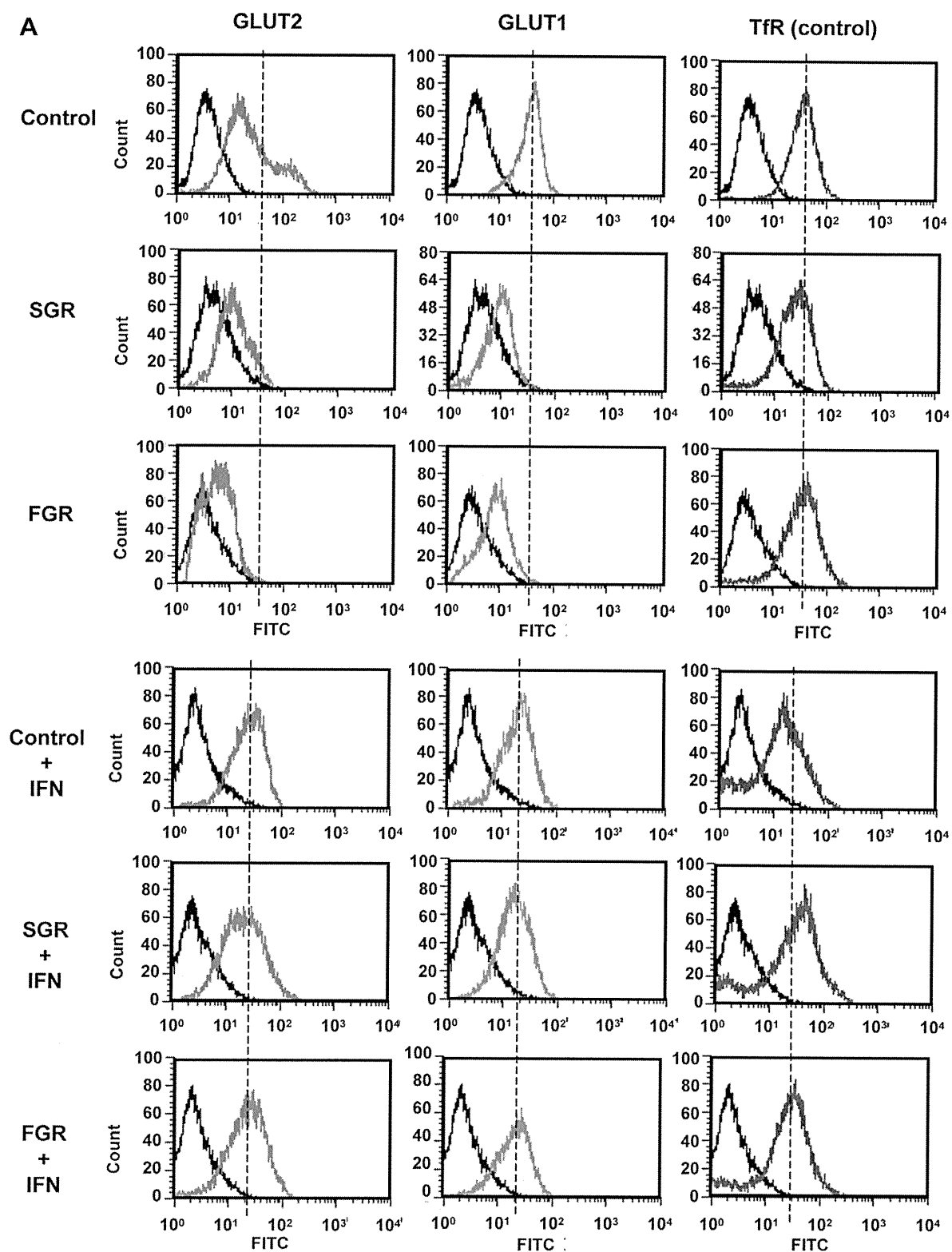


Fig. 4. Down-regulation of cell surface expressions of GLUT2 and GLUT1 by HCV replication. (A) SGR, FGR, the HCV-negative control cells were stained with specific antibodies, followed by FITC-conjugated second antibody (GLUT2, red line; GLUT1, green line) or stained with FITC-conjugated antibody alone (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.

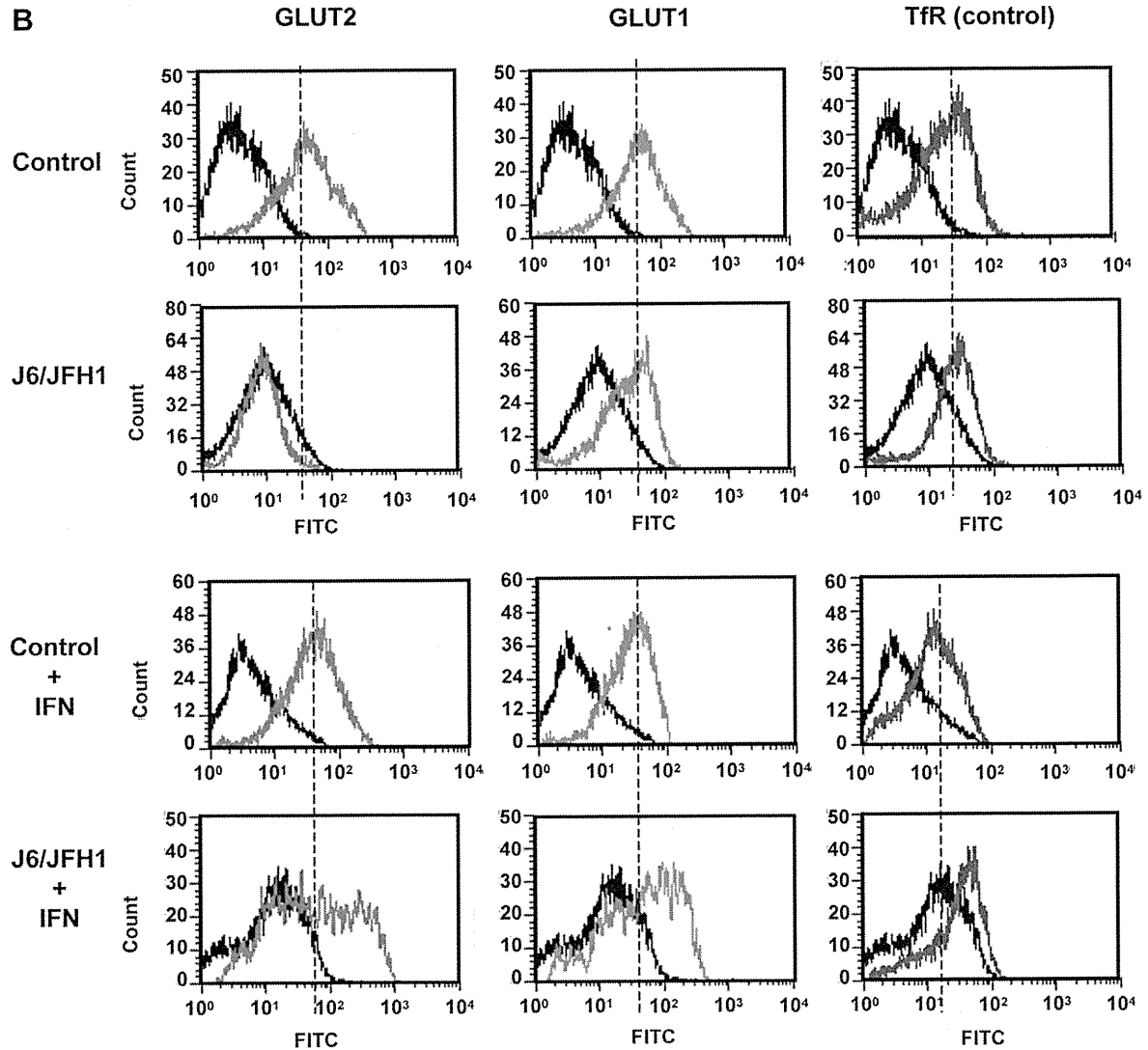


Fig. 4 (continued)

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

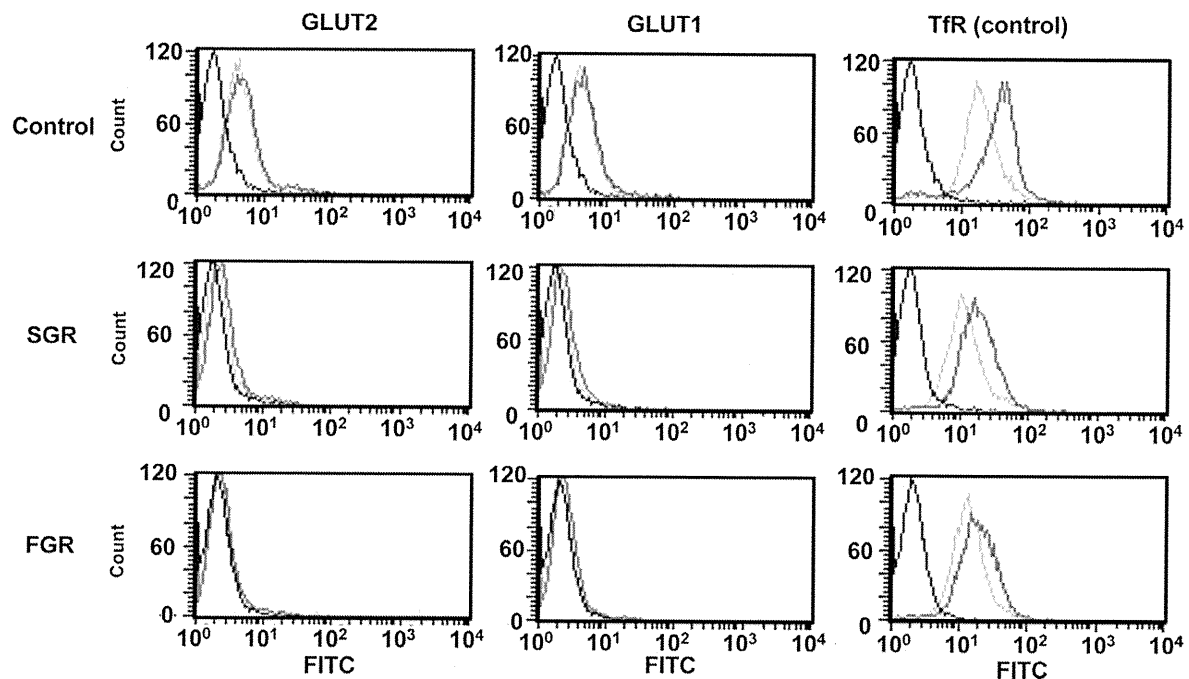


Fig. 5. Effects of lactacystin treatment on cell surface expression of GLUT2, GLUT1 and transferrin receptor (TfR). Cells were treated with lactacystin (10  $\mu$ M) overnight to inhibit proteasomal degradation, and analyzed by flow cytometry. Cells treated with lactacystin are shown in red line and 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.

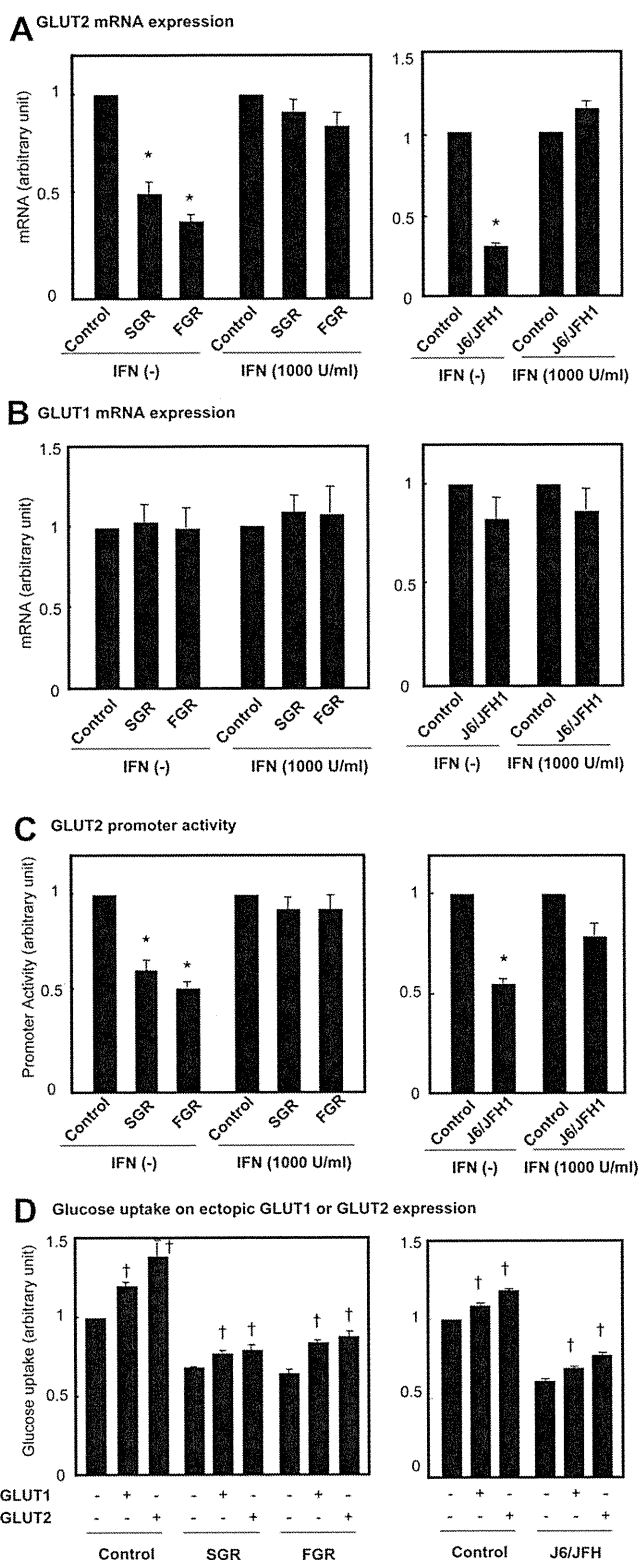
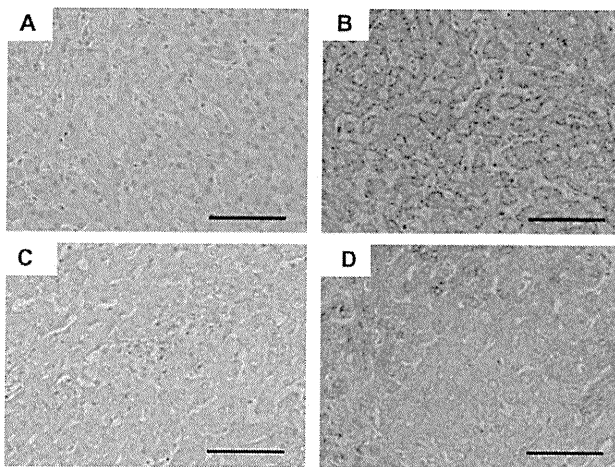


Fig. 6. Differential suppression of GLUT2 and GLUT1 mRNAs by HCV replication. (A and B) Quantitative RT-PCR analysis of mRNA for GLUT2 (A) and GLUT1 (B). mRNA expression levels of GLUT2 and GLUT1 in SGR, FGR and HCV-infected cells were determined and normalized with  $\beta$ -glucuronidase mRNA levels. 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  $\pm$  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  $\pm$  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  $\pm$  SEM of two independent experiments. † $P < 0.01$ , 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  $\mu$ m.

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

**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) <sup>a</sup>
	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.

<sup>a</sup> Parentheses indicate either focal or diffuse appearance of the areas with reduced GLUT2 expression in each liver tissue sample.

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).

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 Gastrointest 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 splice 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-1 $\alpha$  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- $\delta$  and ELKS- $\alpha$ , 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 PA28 $\gamma$ -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<sup>+</sup>-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.

# Secondary Structure of the Amino-Terminal Region of HCV NS3 and Virological Response to Pegylated Interferon Plus Ribavirin Therapy for Chronic Hepatitis C

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The aim of the study was to identify a predictive marker for the virological response in hepatitis C virus 1b (HCV-1b)-infected patients treated with pegylated interferon plus ribavirin therapy. A total of 139 patients with chronic hepatitis C who received therapy for 48 weeks were enrolled. The secondary structure of the 120 residues of the amino-terminal HCV-1b non-structural region 3 (NS3) deduced from the amino acid sequence was classified into two major groups: A and B. The association between HCV NS3 protein polymorphism and virological response was analyzed in patients infected with group A ( $n = 28$ ) and B ( $n = 40$ ) isolates who had good adherence to both pegylated interferon and ribavirin administration ( $>95\%$  of the scheduled dosage) for 48 weeks. A sustained virological response (SVR) representing successful HCV eradication occurred in 33 (49%) in the 68 patients. Of the 28 patients infected with the group A isolate, 18 (64%) were SVR, whereas of the 40 patients infected with the group B isolate 15 (38%) were SVR. The proportion of virological responses differed significantly between the two groups ( $P < 0.05$ ). These results suggest that polymorphism in the secondary structure of the HCV-1b NS3 amino-terminal region influences the virological response to pegylated interferon plus ribavirin therapy, and that virus grouping based on this polymorphism can contribute to prediction of the outcome of this therapy. *J. Med. Virol.* 82:1364–1370, 2010. © 2010 Wiley-Liss, Inc.

**KEY WORDS:** hepatitis C; interferon; ribavirin; interaction; polymorphism

## INTRODUCTION

Hepatitis C virus (HCV) is the major pathogen that causes chronic liver diseases with a risk of progression to cirrhosis and hepatocellular carcinoma. Currently, the standard treatment for chronic hepatitis C is antiviral therapy using pegylated interferon (Peg-IFN) plus ribavirin (RBV), and this approach is most effective for eradication of HCV viremia. However, even with the widely used treatment regimen of 48 weeks, the rate of sustained virological response (SVR), which indicates eradication of viremia, is still approximately 50% for patients infected with the therapy-resistant HCV genotype 1b (HCV-1b) with a high viral load [Manns et al., 2001; Bruno et al., 2004; Hadziyannis et al., 2004]. It would be useful to predict the virological response to this therapy and to identify patients who would obtain beneficial therapeutic effects before treatment, in order to avoid any serious side effect and to eliminate those who would not be helped by the treatment. In the future it will be important to establish a protocol of tailor-made medicine for chronic hepatitis C.

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Both the HCV genotype and pre-treatment viral load are major viral factors that influence the response to IFN-based antiviral therapy, but IFN resistance is also partly due to variation of the amino acid sequence encoded by HCV itself. Enomoto et al. [1996] proposed that variation of 40 amino acids within the NS5A region (aa 2,209–2,248), which is referred to as the IFN sensitivity-determining region (ISDR), is well correlated with IFN responsiveness. ISDR and its adjacent sequence bind and inhibit the enzymatic activity of a double-stranded RNA-activated protein kinase (PKR), which can have an antiviral effect, and therefore the combined region is referred to as the PKR-binding domain (PKR-BD) [Gale et al., 1997, 1998]. A correlation between sequence variation in the PKR-BD and IFN responsiveness has been reported [Nousbaum et al., 2000], and some reports show a correlation between IFN responsiveness and the sequence diversity of variable region 3 (V3) (aa 2,356–2,379) or surrounding regions near the carboxy terminus of NS5A [Murphy et al., 2002; Sarrazin et al., 2002; Puig-Basagoiti et al., 2005]. A high degree of amino acid substitution in the V3 and pre-V3 regions (aa 2,334–2,355) of NS5A, which is referred to as the IFN/RBV resistance-determining region (IRRDR) (aa 2,334–2,379), has been associated with SVR in Peg-IFN/RBV combination therapy for patients infected with HCV-1b [El-Shamy et al., 2007, 2008]. In addition to these findings in non-structural proteins of the virus, amino acid substitution in a structural region of HCV has been reported to be a predictive viral marker for the virological response to PegIFN/RBV therapy. Amino acid polymorphisms in the HCV core region (Arg70 vs. Gln70 and Leu91 vs. Met91) correlate with virological outcome and on-treatment viral kinetics in Peg-IFN/RBV therapy [Akuta et al., 2006, 2007], and a double wild-type HCV core (Arg70 and Leu91) may be a significant predictor of SVR in Peg-IFN/RBV therapy [Akuta et al., 2007].

Interactions between viral and host proteins in infected cells may influence therapeutic effects and the natural history of infection, since the HCV NS3 region has a significant effect on immunity. The amino-terminal part of this region encodes a serine protease, for which the minimum activity has been mapped to a region between aa 1,059 and 1,204 [Yamada et al., 1998]. The serine protease inactivates Cardif, a caspase recruitment domain (CARD)-containing adaptor protein that interacts with the RNA helicase retinoic acid inducible gene 1 (RIG-1)-dependent antiviral pathway in infected cells [Foy et al., 2003; Meylan et al., 2005; Evans and Seeger, 2006]. This action inhibits phosphorylation and subsequent heterodimerization of interferon regulatory factor-3 (IRF-3), which is essential for activation of IFN signaling through translocation of IRF-3 heterodimers into the nucleus, and eventually blocks IFN-beta production. In addition, inactivation of IRF-3 is postulated to influence the therapeutic effect of IFN-based antiviral therapy, because the IRF-3 heterodimer translocates into the nucleus to bind to the IFN-stimulated response element that produces

many antiviral proteins, including 2',5'-oligoadenylate synthetase and PKR [Nakaya et al., 2001; Grandvaux et al., 2002]. Collectively, these findings suggest that polymorphisms in HCV NS3 structure deduced from sequence variation may influence IFN-related signaling and the antiviral effect of IFN-based anti-HCV therapy.

We have focused on polymorphisms in the secondary structure of the viral polyprotein that interacts with host proteins involved in immunity, with the aim of identification of predictive viral markers for the response to Peg-IFN/RBV therapy. In this study, we examined the potential correlation between polymorphisms in the secondary structure of the HCV NS3 amino-terminal region and virological responses to Peg-IFN/RBV therapy in patients infected with HCV-1b with a high viral load.

## PATIENTS AND METHODS

### Patients and Treatment Regimen With Peg-IFN Plus Ribavirin

A total of 139 consecutive patients diagnosed with chronic hepatitis C were enrolled in the study from December 2004 to March 2007. These patients included 81 men and 58 women, and were aged from 31 to 75 years old (mean  $\pm$  SD, 56.8  $\pm$  8.7 years old). All patients were infected with HCV-1b with a high viral load of over 100 KIU/ml, and all received Peg-IFN/RBV therapy. Patients with alcoholic liver injury, autoimmune liver disease, and those who had symptoms of decompensated cirrhosis including ascites were excluded. Briefly, all patients were treated with a combination of Peg-IFN-alpha 2b (Pegintron<sup>®</sup>; Schering-Plough, Kenilworth, NJ) and RBV (Rebetol<sup>®</sup>; Schering-Plough) for 48 weeks. Peg-IFN was administered subcutaneously once a week and RBV was given orally twice a day for the total dose. The dosages were determined on the basis of body weight according to the Japanese standard prescription information supplied by the Japanese Ministry of Health, Labour and Welfare, and there was a limit for calculating the optimized dose: patients with body weights of 35–45, 46–60, 61–75, and 76–90 kg were given Peg-IFN at doses of 60, 80, 100, and 120  $\mu$ g, respectively, and those with body weights of <60, 60–80, and >80 kg were given RBV at doses of 600, 800, and 1,000 mg, respectively. The dose of Peg-IFN or RBV was reduced according to the Japanese standard criteria based on the white blood cell count, neutrophil count, hemoglobin concentration and platelet count [Hiramatsu et al., 2008].

### Virological Tests and Response to Peg-IFN Plus Ribavirin

Virological responses were evaluated at 12 weeks after the start of treatment with an early depletion of viremia referred to as an early virological response (EVR), at the end of treatment with depletion of viremia referred to as an end of treatment virological response (ETR), and at 24 weeks after completion of treatment,

with a clinical outcome of a sustained virological response (SVR) representing successful HCV eradication. All patients were negative for hepatitis B surface antigen. Quantification of serum HCV RNA was performed using an RT-PCR-based commercial kit (Amplicor HCV monitor test, ver. 2.0, Roche Diagnostics, Tokyo, Japan). This Amplicor HCV RNA assay has a lower limit of detection of 50 IU/ml. SVR was determined by monitoring negativity for HCV RNA monthly for 6 months. The real-time PCR assay kit (COBAS TaqMan HCV Auto, Roche Diagnostics) for more precise quantitation of HCV viremia has recently become available and pre-treatment viral titers were re-evaluated using preserved serum samples. This real-time PCR assay has a lower limit of detection of 15 IU/ml. The study protocol was approved by the Ethics Committee of Yamagata University Hospital. Informed consent was obtained from all patients.

#### PCR Amplification of the Amino-Terminal Region of NS3

RNA was extracted from 50  $\mu$ l of serum using an RNeasy Mini kit (Qiagen, Tokyo, Japan). To amplify the region of the HCV genome encoding the amino-terminal region of NS3 (1,027–1,206), a one-step PCR was performed in a tube using the Superscript One-Step RT-PCR kit with Platinum Taq (Gibco-BRL, Tokyo, Japan) and an outer set of primers: NS3-F1 (sense primer; 5'-ACA CCG CGG CGT GTG GGG ACA T-3'; nucleotides 3,295–3,316) and NS3-AS2 (antisense primer; 5'-GCT CTT GCC GCT GCC AGT GGG A-3'; nucleotides 4,040–4,019), as reported previously [Ogata et al., 2002a, 2003]. PCR was initially performed at 45°C for 30 min at RT and then at 94°C for 2 min, followed by the first-round PCR for forty 3-min cycles at 94°, 55°, and 72°C for 1 min each. The second-round PCR was performed with *Pfu* DNA polymerase (Promega, Tokyo, Japan) and an inner set of primers: NS3-F3 (sense primer; 5'-CAG GGG TGG CGG CTC CTT-3'; nucleotides 3,390–3,407) and NS3-AS1 (antisense primer; 5'-GCC ACT TGG AAT GTT TGC GGT A-3'; nucleotides 4,006–3,985). The second-round PCR was performed for 35 cycles, with each cycle consisting of 1 min at 94°C, 1.5 min at 55°C, and 3 min at 72°C. This method allowed amplification of the corresponding portion of the HCV genome from HCV-1b RNA-positive samples. The amplified fragments were purified with a QIAquick PCR purification kit (Qiagen) and directly sequenced (without being subcloned) in both directions using a dRhodamine Terminator Cycle Sequencing Ready Reaction kit and an ABI 377 sequencer (Applied Biosystems, Tokyo, Japan).

#### Classification of the Secondary Structure of the HCV-1b NS3 Amino-Terminal Region

The secondary structure of the amino-terminal region of HCV NS3 was predicted by computer-assisted Robson analysis [Garnier et al., 1978] with Genetyx-Mac software (ver.10.1; Software Development Co., Tokyo,

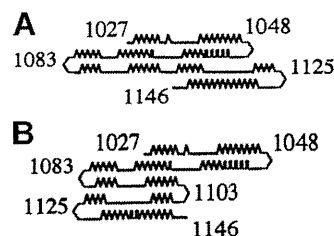


Fig. 1. Secondary structure of the 120 amino-terminal residues of HCV-1b nonstructural 3 (NS3) region classified into two major groups: A and B. The looped, zigzag, straight, and bent lines represent  $\alpha$ -helix,  $\beta$ -sheet, coil, and turn structures, respectively. The numbers indicate amino acid positions. A: Group A, (B) Group B.

Japan). Previously, the full-length secondary structure of the HCV-1b NS3 region was analyzed, and this showed that the secondary structure deduced from the carboxy-terminal 60 residues was well conserved in terms of linear structure, without any turn structure [Ogata et al., 2002a]. We have shown that the secondary structure of the 120 residues in the amino-terminal region of HCV-1b NS3 can be classified into two major groups: A and B (Fig. 1) [Ogata et al., 2002a, 2003]. Briefly, the criteria for this classification are as follows: in group A isolates, the carboxy-terminal 20 residues (aa 1,125–1,146) are oriented leftward relative to a domain composed of the remaining amino-terminal region; whereas in group B isolates, the same 20 residues are oriented rightward relative to the rest of the amino-terminal domain.

#### Analysis of Amino Acid Substitutions in the Core Region

To amplify a region of the HCV genome encoding the core region including positions 70 and 91, reverse transcription and the first-round PCR were performed in a tube by the Superscript One-Step RT-PCR kit with Platinum *Taq* (Gibco-BRL) and an outer set of primers, followed by second-round PCR with an inner set of primers in accordance with procedures reported previously [Ogata et al., 2002b]. The sequences of the amplified fragments were determined by direct sequencing.

#### Statistical Analysis

Data were analyzed by a  $\chi^2$  test for independence with a two-by-two contingency table and a Student *t*-test. A *P*-value <0.05 was considered significant.

## RESULTS

#### Virological Response and Adherence to the Peg-IFN Plus Ribavirin Regimen

Rates of virological responses in patients treated with PegIFN/RBV combination therapy for 48 weeks are shown in Figure 2. Of the 139 patients enrolled in the study, SVR, non-SVR and cessation of therapy occurred in 58 (42%), 62 (45%), and 19 (14%), respectively. Serious

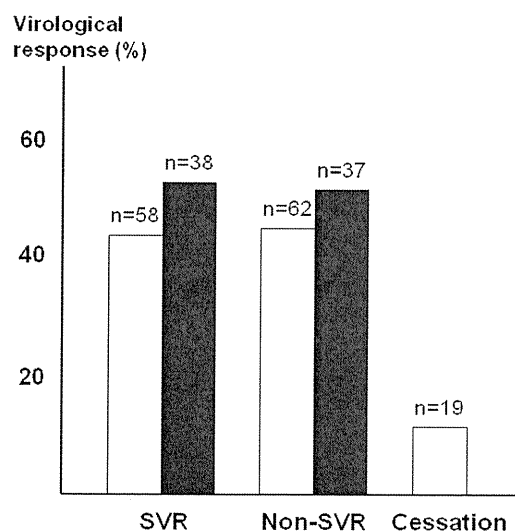


Fig. 2. Virological response in patients treated with peginterferon plus ribavirin for 48 weeks. The results are shown for all 139 subjects (open bars) and for 75 cases with good adherence of >80% of the scheduled dosages (closed bars). SVR, sustained virological response.

adverse events that necessitated discontinuation of this therapy were depression in one patient, thyroid function disorder in 2, general itching in 2, infection in 2, anorexia in 2, occurrence of hepatocellular carcinoma in 2, and a decreased neutrophil count in 2. Six patients also terminated this therapy at their own request. Of the 139 patients, 75 (54%) received >80% of the scheduled dosage of Peg-IFN and RBV designated before treatment, and of these 75 cases SVR and non-SVR occurred in 38 (51%) and 37 (49%), respectively.

#### Prevalence of Types of Secondary Structure of the Amino-Terminal Region of HCV NS3

The prevalence of the types of secondary structure of HCV NS3 in the 139 subjects is shown in Table I. Among these subjects, 43 (31%), 70 (50%), and 26 (19%) were classified into groups A, B, and others, including 3 of mixed type (A plus B) and 23 of non-A, non-B type. Of the 75 cases with good adherence to administration of >80% of the scheduled dosage, 28 (37%), 40 (53%) and 7 (9%) were classified into groups A, B, and others. The amino acid data of group A and B in the cases with good adherence to administration are available in the DDBJ/EMBL/GenBank databases with the accession numbers AB548070–AB548137. Our analysis revealed no specific correlations between amino acid sequences

TABLE I. Prevalence of the HCV NS3 Secondary Structure Type

	Group A (%)	Group B (%)	Others (%)
Enrolled cases (n = 139)	43 (31)	70 (50)	26 (19)
Adherent cases (n = 75)	28 (37)	40 (53)	7 (9)

and the secondary structure deduced by the Robson method, as we have reported previously [Ogata et al., 2003].

#### Characteristics of Adherent Patients Based on Different HCV NS3 Structure Types

The virological responses to Peg-IFN/RBV combination therapy for patients infected with group A and B isolates were assessed in the 68 subjects with good adherence to the scheduled dosage of Peg-IFN and RBV. The characteristics of patients infected with group A and B isolates are shown in Table II. Age, gender, pre-treatment level of serum HCV RNA and ALT, and frequency of fibrosis stage did not differ significantly between the two groups. Peg-IFN/RBV combination therapy was completed in all the patients, and the total administered dosages of Peg-IFN and RBV was >95% of the scheduled dosage in both groups.

#### Relationship Between Virological Responses and Polymorphisms in the HCV NS3 Amino-Terminal Region

In the 68 patients who received >95% of the scheduled doses of Peg-IFN and RBV for 48 weeks, SVR and non-SVR occurred in 33 (49%) and 35 (51%), respectively. The EVR, ETR, and SVR rates in patients infected with group A and B isolates are shown in Table III. There was a significant difference in the rates of EVR between subjects infected with group A and B isolates: EVR was achieved in 19 of 28 (68%) patients with group A infection, compared to 17 of 40 (43%) with group B infection ( $P < 0.05$ ). The final outcome also differed significantly between subjects infected with group A and B isolates: SVR was achieved in 18 of 28 (64%) patients with group A infection, compared to 15 of 40 (38%) with group B infection ( $P < 0.05$ ).

#### Polymorphisms in Core Amino Acids 70/91 and in the HCV NS3 Secondary Structure

The wild-type core sequence (Arg70, Leu91) has been associated with SVR in Peg-IFN/RBV combination therapy, while the non-double wild-type containing one or two substitutions at positions 70 and/or 91 was associated with non-SVR [Akuta et al., 2007]. Therefore, we examined substitutions at positions 70 and 91 in the HCV core region in pre-treatment serum samples of 44 cases that were available for testing. The double wild-type 70/91 sequence was found in 22 of the 44 cases (50%), of which 12 were SVR and 10 were non-SVR. Combination analysis of polymorphisms of the HCV core 70/91 positions and the NS3 amino-terminal region showed that 10 (83%) of the 12 SVR cases and only 3 (30%) of the 10 non-SVR cases with the double wild-type core had a group A polymorphism in HCV NS3 (Table IV). Thus, combination analysis of the core and NS3 regions may improve prediction of the outcome of Peg-IFN/RBV therapy.

TABLE II. Characteristics of Adherent Patients Infected With HCV Group A and B Isolates

	Group A (n = 28)	Group B (n = 40)	P
Age (years)	55.5 ± 9.5	55.5 ± 8.9	NS <sup>a</sup>
Sex (men/women)	18/10	21/19	NS <sup>b</sup>
Pre-treatment HCV RNA (KIU/ml)	1,635 ± 930	2,087 ± 1,422	NS <sup>a</sup>
Alanine aminotransferase level (U/L)	80 ± 62	71 ± 47	NS <sup>a</sup>
Stage of liver fibrosis			
F1 or F2/F3 or F4	19/9	28/12	NS <sup>b</sup>
Drug adherence dosage (%)			
Pegylated interferon	97.7 ± 5.2	95.2 ± 7.3	NS <sup>a</sup>
Ribavirin	96.8 ± 6.4	95.3 ± 7.7	NS <sup>a</sup>

NS, not significant.

<sup>a</sup>t-test.<sup>b</sup>χ<sup>2</sup> test.

### Re-Evaluation of Pre-Treatment HCV Viremia Status Using Real-Time PCR

Since the viral titer before treatment is a major predictive marker of the outcome of Peg-IFN/RBV therapy, we re-evaluated the pre-treatment viral titers more precisely using preserved serum samples taken within 1 month before treatment, using a real-time PCR assay. The pre-treatment viral titers did not differ significantly between sera with group A and B isolates ( $5.98 \pm 0.94$  vs.  $6.25 \pm 0.62$  logIU/ml) (Table V). The secondary structure polymorphisms of HCV NS3 were independent of the pre-treatment viral titers.

### DISCUSSION

Antiviral therapy with Peg-IFN/RBV for 48 weeks fails to eradicate HCV in about half of patients infected with a high titer of HCV genotype 1b, and the severe adverse events and high costs associated with this therapy require outcome prediction to allow targeted treatment for chronic hepatitis C. The pre-treatment viral titer, viral factors that influence the virological response to IFN-based anti-HCV therapy have been widely investigated. Viral kinetics showing prompt seronegativity after the start of treatment is a critical factor for achieving SVR, and thus the possible correlation between an early virological response and genetic sequence variation of the HCV has been studied. In particular, amino acid substitutions in the HCV core region at positions 70 and 91 or multiple mutations detected in the IRRDR of the HCV NS5A region are useful markers for predicting EVR and subsequent SVR.

TABLE III. Virological Responses in Subjects With Different Polymorphisms in the Secondary Structure of HCV NS3

	EVR*	ETR**	SVR*
Group A (n = 28)	19 (68%)	23 (82%)	18 (64%)
Group B (n = 40)	17 (43%)	25 (63%)	15 (38%)

EVR: early virological response at 12 weeks after the start of treatment.

ETR: virological response at the end of treatment.

SVR: sustained virological response 24 weeks after completion of treatment.

\*P &lt; 0.05.

\*\*P = 0.08; χ<sup>2</sup> test.

To date, the influence of several single amino acid substitutions and accumulation of these changes in the viral genome on the effect of IFN-based anti-HCV therapy has been examined. Since interactions between host and viral proteins in infected cells may influence the therapeutic effect of an antiviral agent, we focused on the association of structural polymorphism of a viral protein with the effect of Peg-IFN/RBV combination therapy in this study. Our results suggest that polymorphism analysis of secondary structure deduced from sequence variations in the HCV NS3 amino-terminal region can be used to predict viral responses to this therapy.

Amino acid sequences of the HCV NS3 amino-terminal region, which encodes a serine protease, vary greatly among HCV isolates. Interactions between HCV NS3 and host proteins may influence both oncogenesis and immunity, and thus elucidation of the biological significance of these interactions could result in a new prognostic marker for HCC or a predictive marker for anti-HCV therapy. First, HCV NS3 interacts with the p53 tumor suppressor to suppress p53-dependent apoptosis or p21 transcriptional activity [Ishido and Hotta, 1998; Kwun et al., 2001; Deng et al., 2006]. Transfection of a plasmid expressing the amino-terminal portion of HCV NS3 induces cell transformation in vitro, and transplanted cells proliferate with sarcoma-like features in vivo [Sakamuro et al., 1995]. These findings suggest that NS3 may be involved in the oncogenic pathway in HCV infection. We have shown that the secondary structure of the 120-residue amino-terminal region of NS3 (1,027–1,146) is classifiable into two major groups: A and B. This region encodes a serine protease and also includes p53-binding sites. Our

TABLE IV. Treatment Outcome of Cases With a Double Wild-Type Core Region and Different HCV NS3 Structural Polymorphism

	Group A (%)	Group B (%)	P
SVR (n = 12)	10 (83)	2 (17)	0.02 <sup>a</sup>
Non-SVR (n = 10)	3 (30)	7 (70)	

SVR, sustained virological response.

<sup>a</sup>χ<sup>2</sup> test.



TABLE V. Pre-Treatment HCV RNA Levels Measured by Real-Time PCR for Subjects With Different HCV NS3 Structural Polymorphism

	Group A	Group B	P
SVR (n = 33)	5.78 ± 1.05	6.13 ± 0.71	NS <sup>a</sup>
Non-SVR (n = 35)	6.33 ± 0.59	6.32 ± 0.55	NS <sup>a</sup>
Total (n = 68)	5.98 ± 0.94	6.25 ± 0.62	NS <sup>a</sup>

SVR, sustained virological response. NS, not significant.  
<sup>a</sup>t test.

previous cross-sectional studies revealed that the prevalence of group B infection is significantly higher in HCC cases than in non-HCC cases [Ogata et al., 2003], and that the group B infection is an independent risk factor for development of HCC in patients with chronic HCV infection [Nishise et al., 2007]. Second, NS3 interacts with host proteins associated with IFN signaling and thus influences cellular immunity. Since the serine protease encoded by the amino-terminal region of NS3 inhibits the IFN-signaling pathway, polymorphism of this region is likely to influence the effect of Peg-IFN/RBV combination therapy.

Several factors associated with the virological response to this therapy are well known, with adherence to both IFN and RBV strongly influencing outcome [Pearlman, 2004; Arase et al., 2005; Yamada et al., 2008]. In this study, we analyzed 75 cases in which >80% of the scheduled dosage of both drugs was administered. Of these cases, 28 (37%) and 40 (53%) were infected with group A and B isolates, respectively, which were similar rates to those for the 139 cases in the overall study. Age, gender, viral load before treatment, ALT level, proportion of fibrosis stage and adherence to Peg-IFN and RBV did not differ between the group A and B cases. However, the frequencies of SVR and EVR were significantly higher in group A, and those for non-EVR and non-SVR were significantly higher in group B. The results suggest that infection with the group B isolate, which correlates with a higher rate of HCC, is resistant to Peg-IFN/RBV therapy. The pre-treatment viremia status in the 68 cases with group A or B isolates showed no significant differences between the two groups of patients. Therefore, these results suggest that the secondary structure of the HCV NS3 amino-terminal region may be useful for prediction of the outcome of Peg-IFN/RBV combination therapy. In this initial study setting, the relationship of these polymorphisms to the frequency of rapid viral response at 4 weeks after the start of treatment was not evaluated. It will be important to assess this relationship in a future study.

The polymorphism in HCV core region (Arg70/Leu91) is a useful predictive marker for virological responses in Peg-IFN/RBV therapy [Akuta et al., 2007]. Interestingly, a combined analysis of polymorphisms of the core region (which encodes a structural protein) and HCV NS3 (a nonstructural protein) improved the prediction rate. Therefore, analysis of NS3 polymorphism in combination with the core structural polymorphism

appears to improve prediction of the outcome of Peg-IFN/RBV therapy. A larger, multi-center prospective study would be necessary to validate the present results. In conclusion, the results of this study suggest that secondary structure polymorphism in the amino-terminal region of HCV NS3 is a useful predictive marker of the effect of Peg-IFN/RBV combination therapy for chronic hepatitis C. Although the present findings are clinically important, and will be helpful for predicting the outcome of Peg-IFN/RBV therapy, further in vitro studies will be needed to elucidate the molecular mechanism underlying the association of HCV NS3 polymorphisms with clinical outcome.

## REFERENCES

- Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, Kobayashi M, Saitoh S, Watahiki S, Sato J, Kobayashi M, Arase Y, Ikeda K, Kumada H. 2006. Predictive factors of virological non-response to interferon-ribavirin combination therapy for patients infected with hepatitis C virus of genotype 1b and high viral load. *J Med Virol* 78:83–90.
- Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Kobayashi M, Arase Y, Ikeda K, Kumada H. 2007. Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: Amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. *J Hepatol* 46:403–410.
- Arase Y, Ikeda K, Tsubota A, Suzuki F, Suzuki Y, Saitoh S, Kobayashi M, Akuta N, Someya T, Hosaka T, Sezaki H, Kobayashi M, Kumada H. 2005. Significance of serum ribavirin concentration in combination therapy of interferon and ribavirin for chronic hepatitis C. *Intervirology* 48:138–144.
- Bruno S, Cammà C, Di Marco V, Rumi M, Vinci M, Camozzi M, Rebusci C, Di Bona D, Colombo M, Craxi A, Mondelli MU, Pinzello G. 2004. Peginterferon alfa-2b plus ribavirin for naïve patients with genotype 1 chronic hepatitis C: A randomized controlled trial. *J Hepatol* 41:474–481.
- Deng L, Nagano-Fujii M, Tanaka M, Nomura-Takigawa Y, Ikeda M, Kato N, Sada K, Hotta H. 2006. NS3 protein of hepatitis C virus associated with the tumor suppressor p53 and inhibits its function in an NS3 sequence-dependent manner. *J Gen Virol* 87:1703–1713.
- El-Shamy A, Sasayama M, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, Hotta H. 2007. Prediction of efficient virological response to pegylated interferon/ribavirin combination therapy by NS5A sequences of hepatitis C virus and anti-NS5A antibodies in pre-treatment sera. *Microbiol Immunol* 51:471–482.
- El-Shamy A, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, Hotta H. 2008. Sequence variation in hepatitis C virus nonstructural protein 5A predicts clinical outcome of pegylated interferon/ribavirin combination therapy. *Hepatology* 48:38–47.
- Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Ogura Y, Izumi N, Marumo F, Sato C. 1996. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *N Engl J Med* 334:77–81.
- Evans JD, Seeger C. 2006. Cardif: A protein central to innate immunity is inactivated by the HCV NS3 serine protease. *Hepatology* 43:615–617.
- Foy E, Li K, Wang C, Sumpter R, Jr., Ikeda M, Lemon SM, Gale M, Jr. 2003. Regulation of interferon regulatory factor-3 by the hepatitis C virus serine protease. *Science* 300:1145–1148.
- Gale MJ, Jr., Korth MJ, Tang NM, Tan SL, Hopkins DA, Dever TE, Polyak SJ, Gretch DR, Katze MG. 1997. Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. *Virology* 230:217–227.
- Gale MJ, Jr., Korth MJ, Katze MG. 1998. Repression of the PKR protein kinase by the hepatitis C virus NS5A protein: A potential mechanism of interferon resistance. *Clin Diagn Virol* 10:157–162.
- Garnier J, Osguthorpe DJ, Robson B. 1978. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. *J Mol Biol* 120:97–120.



- Grandvaux N, Servant MJ, tenOever B, Sen GC, Balachandran S, Barber GN, Lin R, Hiscott J. 2002. Transcriptional profiling of interferon regulatory factor 3 target genes: Direct involvement in the regulation of interferon-stimulated genes. *J Virol* 76:5532–5539.
- Hadziyannis SJ, Sette H, Jr., Morgan TR, Balan V, Diago M, Marcellin P, Ramadori G, Bodenheimer H, Jr., Bernstein D, Rizzetto M, Zeuzem S, Pockros PJ, Lin A, Ackrill AM. 2004. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: A randomized study of treatment duration and ribavirin dose. *Ann Intern Med* 140:346–355.
- Hiramatsu N, Kurashige N, Oze T, Takehara T, Tamura S, Kasahara A, Oshita M, Katayama K, Yoshihara H, Imai Y, Kato M, Kawata S, Tsubouchi H, Kumada H, Okanou T, Kakumu S, Hayashi N. 2008. Early decline of hemoglobin can predict progression of hemolytic anemia during pegylated interferon and ribavirin combination therapy in patients with chronic hepatitis C. *Hepatol Res* 38:52–59.
- Ishido S, Hotta H. 1998. Complex formation of the nonstructural protein 3 of hepatitis C virus with the p53 tumor suppressor. *FEBS Lett* 438:258–262.
- Kwon HJ, Jung EY, Ahn JY, Lee MN, Jang KL. 2001. p53-dependent transcriptional repression of p21(waf1) by hepatitis C virus NS3. *J Gen Virol* 82:2235–2241.
- Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK. 2001. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: A randomized trial. *Lancet* 358:958–965.
- Meylan E, Curran J, Hofmann K, Moradpour D, Binder M, Bartenschlager R, Tschopp J. 2005. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* 437:1167–1172.
- Murphy MD, Rosen HR, Marousek GI, Chou S. 2002. Analysis of sequence configurations of the ISDR, PKR-binding domain, and V3 region as predictors of response to induction interferon-alpha and ribavirin therapy in chronic hepatitis C infection. *Dig Dis Sci* 47:1195–1205.
- Nakaya T, Sato M, Hata N, Asagiri M, Suemori H, Noguchi S, Tanaka N, Taniguchi T. 2001. Gene induction pathways mediated by distinct IRFs during viral infection. *Biochem Biophys Res Commun* 283:1150–1156.
- Nishise Y, Saito T, Sugahara K, Ito JI, Saito K, Togashi H, Nagano-Fujii M, Hotta H, Kawata S. 2007. Risk of hepatocellular carcinoma and secondary structure of hepatitis C virus (HCV) NS3 protein amino-terminus, in patients infected with HCV subtype 1b. *J Infect Dis* 196:1006–1009.
- Nousbaum J, Polyak SJ, Ray SC, Sullivan DG, Larson AM, Carithers RL, Jr., Gretch DR. 2000. Prospective characterization of full-length hepatitis C virus NS5A quasispecies during induction and combination antiviral therapy. *J Virol* 74:9028–9038.
- Ogata S, Ku Y, Yoon S, Makino S, Nagano-Fujii M, Hotta H. 2002a. Correlation between secondary structure of an amino-terminal portion of the nonstructural protein 3 (NS3) of hepatitis C virus and development of hepatocellular carcinoma. *Microbiol Immunol* 46:549–554.
- Ogata S, Nagano-Fujii M, Ku Y, Yoon S, Hotta H. 2002b. Comparative sequence analysis of the core protein and its frameshift product, the F protein, of hepatitis C virus subtype 1b strains obtained from patients with and without hepatocellular carcinoma. *J Clin Microbiol* 40:3625–3630.
- Ogata S, Florese RH, Nagano-Fujii M, Hidajat R, Deng L, Ku Y, Yoon S, Saito T, Kawata S, Hotta H. 2003. Identification of hepatitis C virus (HCV) subtype 1b strains that are highly, or only weakly, associated with hepatocellular carcinoma on the basis of the secondary structure of an amino-terminal portion of the HCV NS3 protein. *J Clin Microbiol* 41:2835–2841.
- Pearlman BL. 2004. Hepatitis C treatment update. *Am J Med* 117:344–352.
- Puig-Basagoiti F, Forns X, Furci I, Ampurdanés S, Giménez-Barcons M, Franco S, Sánchez-Tapias JM, Saiz JC. 2005. Dynamics of hepatitis C virus NS5A quasispecies during interferon and ribavirin therapy in responder and non-responder patients with genotype 1b chronic hepatitis C. *J Gen Virol* 86:1067–1075.
- Sakamuro D, Furukawa T, Takegami T. 1995. Hepatitis C virus nonstructural protein NS3 transforms NIH 3T3 cells. *J Virol* 69:3893–3896.
- Sarrazin C, Herrmann E, Bruch K, Zeuzem S. 2002. Hepatitis C virus nonstructural 5A protein and interferon resistance: A new model for testing the reliability of mutational analyses. *J Virol* 76:11079–11090.
- Yamada K, Mori A, Seki M, Kimura J, Yuasa S, Matsuura Y, Miyamura T. 1998. Critical point mutations for hepatitis C virus NS3 proteinase. *Virology* 246:104–112.
- Yamada G, Iino S, Okuno T, Omata M, Kiyosawa K, Kumada H, Hayashi N, Sakai T. 2008. Virological response in patients with hepatitis C virus genotype 1b and a high viral load: Impact of peginterferon-alpha-2a plus ribavirin dose reductions and host-related factors. *Clin Drug Investig* 28:9–16.

ORIGINAL ARTICLE

## 17 $\beta$ -estradiol inhibits the production of infectious particles of hepatitis C virus

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### ABSTRACT

Persistent infection with hepatitis C virus causes serious liver diseases, such as chronic hepatitis, hepatic cirrhosis and hepatocellular carcinoma. The male gender is one of the critical factors in progression of hepatic fibrosis due to chronic HCV infection; thus female hormones may play a role in delaying the progression of hepatic fibrosis. It has also been reported that women are more likely than men to clear HCV in the acute phase of infection. These observations lead the present authors to the question: do female hormones inhibit HCV infection? In this study using HCV J6/JFH1 and Huh-7.5 cells, the possible inhibitory effect(s) of female hormones such as 17 $\beta$ -estradiol (the most potent physiological estrogen) and progesterone on HCV RNA replication, HCV protein synthesis and production of HCV infectious particles (virions) were analyzed. It was found that E<sub>2</sub>, but not P<sub>4</sub>, significantly inhibited production of the HCV virion without inhibiting HCV RNA replication or HCV protein synthesis. E<sub>2</sub>-mediated inhibition of HCV virion production was abolished by a nuclear estrogen receptor (ER) antagonist ICI182780. Moreover, treatment with the ER $\alpha$ -selective agonist 4, 4', 4''-(4-propyl-[1H]-pyrazole-1, 3, 5-triyl)trisphenol (PPT), but not with the ER $\beta$ -selective agonist 2, 3-bis(4-hydroxyphenyl)-propionitrile (DPN) or the G protein-coupled receptor 30 (GPR30)-selective agonist 1-(4-[6-bromobenzo 1, 3 dioxol-5-yl]-3a, 4, 5, 9b-tetrahydro-3H-cyclopenta [c] quinolin-8-yl)-ethanone (G-1), significantly inhibited HCV virion production. Taken together, the present results suggest that the most potent physiological estrogen, E<sub>2</sub>, inhibits the production of HCV infectious particles in an ER $\alpha$ -dependent manner.

**Key words** 17 $\beta$ -estradiol, estrogen receptor, hepatitis C virus, sex difference.

HCV, an enveloped RNA virus which belongs to the genus *Hepacivirus* within the family *Flaviviridae*, prevails in most parts of the world with an estimated number of about 170 million carriers; hence HCV infection is a major global health-care problem (1). Persistent infection with HCV causes serious liver diseases, such as chronic hepatitis, hepatic cirrhosis and hepatocellular carcinoma

(2, 3). In the USA, the prevalence of anti-HCV antibodies is twice as high in men as in women (4). The male gender is thought to be one of the critical factors in progression of hepatic fibrosis in chronic HCV infection (5, 6). It has also been reported that progression of hepatic fibrosis is faster in postmenopausal than in premenopausal women, and that hormone replacement therapy with estrogen and

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**List of Abbreviations:** DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DPN, 2, 3-bis(4-hydroxyphenyl)-propionitrile; E<sub>2</sub>, 17 $\beta$ -estradiol; ER, estrogen receptor; G-1, 1-(4-[6-bromobenzo 1, 3 dioxol-5-yl]-3a, 4, 5, 9b-tetrahydro-3H-cyclopenta [c] quinolin-8-yl)-ethanone; GPR30, G protein-coupled receptor 30; HCV, hepatitis C virus; P<sub>4</sub>, progesterone; PPT, 4, 4', 4''-(4-propyl-[1H]-pyrazole-1, 3, 5-triyl)trisphenol; SEM, standard error of the mean.

progesterone significantly delays progression of hepatic fibrosis in postmenopausal women (6, 7). This potential innate resistance of premenopausal women to hepatic fibrosis may be attributed to female hormones, such as estrogens and progesterone. In fact, E<sub>2</sub>, the most potent physiological estrogen, has been reported to suppress the progression of liver fibrosis and hepatocarcinogenesis (8, 9). Moreover, women are more likely than men to clear HCV in the acute phase of infection, even within a few months after infection (10). These observations imply the possibility that female hormones inhibit HCV infection, either at the level(s) of virus attachment/entry, virus RNA replication, virus protein synthesis or production of infectious virus particles (virions).

Estrogens utilize three kinds of ER; ER $\alpha$ , ER $\beta$  and GPR30 (11–15). Specific agonists and antagonists of ER are available and widely used to examine the roles of estrogens. In the present study, we examined the possible effects of female hormones, especially E<sub>2</sub> and P<sub>4</sub>, on HCV RNA replication, protein synthesis and virion production in cultured cells.

## MATERIALS AND METHODS

### Cell culture and virus infection

A human hepatoma-derived cell line, Huh-7.5, which is highly permissive to HCV RNA replication (16), was kindly provided by Dr. C. M. Rice (The Rockefeller University, New York, NY, USA). The cells were maintained in phenol red-free DMEM (Sigma–Aldrich, St Louis, MO, USA) supplemented with 10% heat-inactivated and charcoal-stripped FBS (Israel Beit Haemek, Haemek, Israel), 0.1 mM non-essential amino acids (Invitrogen, Carlsbad, CA, USA), 100 IU/mL penicillin and 100  $\mu$ g/mL streptomycin (Invitrogen).

The pFL-J6/JFH1 plasmid that encodes the entire viral genome of a chimeric strain of HCV-2a, J6/JFH1 (17) was kindly provided by Dr. C. M. Rice. A cell culture-adapted mutant derived from J6/JFH1 (P-47 strain) (18, 19) was used for infection experiments. The virus was inoculated into Huh-7.5 cells at a multiplicity of infection of 1.0 and incubated for 2 hr. After the residual virus had been removed by washing, the cells were cultured in the presence or absence of female hormones, and agonists and an antagonist of estrogen receptors (see below). Culture supernatants were collected at 0, 1, 2 and 3 days postinfection and virus titers were determined, as described below.

### Virus titration

Culture supernatants containing HCV were serially diluted 10-fold in DMEM and inoculated into Huh-7.5 cells

( $2 \times 10^5$  cells per well in a 24-well plate). After incubation at 37°C for 6 hr, the cells were fed with fresh DMEM. At 24 hr postinfection, the cells were fixed with ice-cold methanol, blocked with 5% goat serum in PBS and subjected to immunofluorescence analysis using mouse monoclonal antibody against the HCV core protein (2H9) and Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L, Molecular Probes, Eugene, OR, USA). Hoechst 33342 (Molecular Probes) was used for counterstaining of the nuclei. HCV-positive foci were counted under a fluorescent microscope (BX51; Olympus, Tokyo, Japan) and virus titers were expressed as focus-forming units per ml, as reported previously (18, 19).

### Chemicals

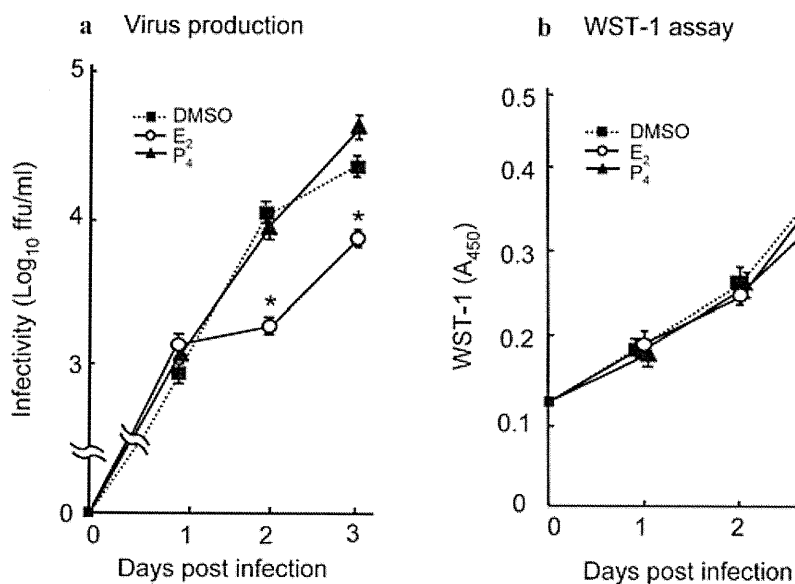
E<sub>2</sub> and P<sub>4</sub> were purchased from Sigma–Aldrich (St Louis, MO, USA). ICI182780 (an antagonist of ER $\alpha$  and ER $\beta$ ), PPT (an ER $\alpha$ -selective agonist) (20) and DPN (an ER $\beta$ -selective agonist) (21) were purchased from Tocris Bioscience (Bristol, UK). G-1 (a GPR30-selective agonist) (22) was purchased from Calbiochem (Darmstadt, Germany). DMSO, which was used as a solvent, was obtained from Wako Pure Chemical Industries (Osaka, Japan). The concentrations of E<sub>2</sub> and P<sub>4</sub> used in this study were 0.4  $\mu$ M and 3  $\mu$ M, respectively, which correspond to the estimated highest concentrations in the sera of pregnant women. ICI182780 was used at a concentration of 1  $\mu$ M, PPT and DPN at 0.1, 1 and 10  $\mu$ M, and G-1 at 0.1 and 1  $\mu$ M. As G-1 has been reported to lose its GPR30-binding specificity at concentrations over 1  $\mu$ M, a concentration of 10  $\mu$ M for G-1 was not tested. The final concentration of DMSO as a control never exceeded 0.01%.

### Cell viability assay

Cells plated on 96-well microtiter plates ( $2.0 \times 10^4$  cells/well) were inoculated with HCV and treated with E<sub>2</sub>, P<sub>4</sub> or DMSO. The cell viability in each well was determined by WST-1 assay (Roche Diagnostics, Mannheim, Germany) until 3 days postinfection.

### Real-time quantitative RT-PCR

Total cellular RNA was isolated using the RNAiso reagent (Takara Bio, Kyoto, Japan) and cDNA was generated using the QuantiTect Reverse Transcription system (Qiagen, Valencia, CA, USA). Real-time quantitative PCR was performed on a SYBR *Premix Ex Taq* (Takara Bio) using SYBR green chemistry in ABI PRISM 7000 (Applied Biosystems, Foster, CA, USA). Primer sets used in this study are shown below: HCV NS5B, 5'-ACCAAGCTCAAACCTCACTCCA-3' and 5'-AGCGGGTTCGGGCAC GAGACA-3' (23);



**Fig. 1. Effects of E<sub>2</sub> and P<sub>4</sub> on HCV virion production and cell growth.** (a) HCV virion production. Huh-7.5 cells were inoculated with HCV at a multiplicity of infection of 1.0, incubated for 2 hr, and cultured for 0, 1, 2 and 3 days after virus infection. The HCV-infected cells were treated with E<sub>2</sub> (0.4 μM), P<sub>4</sub> (3 μM) or DMSO (control) from 2 hr postinfection to sampling time (days 1, 2 and 3). The culture supernatants of HCV-infected cells were assayed for virus infectivity. Data are shown as mean ± SEM. (b) Cell growth. HCV-infected cells were treated with E<sub>2</sub>, P<sub>4</sub> or DMSO (control) from 2 hr to 3 days postinfection. Cell growth in each culture was determined by WST-1 assay. Data are shown as mean ± SEM.

β-actin, 5'-GCGGGAAATCGTGCGTGACATT-3' and 5'-GATGGAGTTGAAGGTAGTTTCGTG-3'.

### Immunoblotting

Cells were solubilized in lysis buffer as reported previously (18, 19). The cell lysates were subjected to 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membranes were incubated with mouse monoclonal antibodies against HCV NS3 (Chemicon International, Temecula, CA, USA), followed by incubation with peroxidase-conjugated goat anti-mouse IgG (Medical & Biological Laboratories Co. Ltd., Nagoya, Japan). The positive bands were visualized by using ECL detection system (GE Healthcare UK, Buckinghamshire, UK).

### Statistical analysis

Results were expressed as mean ± SEM. Statistical significance was evaluated by one-way analyses of variances.

## RESULTS

### E<sub>2</sub> inhibits HCV virion production, but not HCV RNA replication or HCV protein synthesis

We first examined the effect of E<sub>2</sub> or P<sub>4</sub> treatment on HCV virion production. At 2 hr after virus inoculation, the HCV-infected Huh-7.5 cells were treated with E<sub>2</sub> (0.4 μM)

or P<sub>4</sub> (3 μM) for 3 days. Culture supernatants were collected every day and titrated for viral infectivity. As shown in Figure 1a, E<sub>2</sub> treatment significantly suppressed HCV virion production at 2 and 3 days postinfection, whereas treatment with P<sub>4</sub> did not. The same treatment (E<sub>2</sub> or P<sub>4</sub>) did not exert significant cytotoxicity (Fig. 1b). Next, we examined the effect of E<sub>2</sub> on HCV RNA replication and HCV protein synthesis under the same experimental conditions. We found that HCV RNA replication and HCV protein synthesis in both HCV-infected cells and HCV RNA replicon-harboring cells (23) were all unaffected by treatment with E<sub>2</sub> or P<sub>4</sub> (Fig. 2a–c). Moreover, treatment of the cells with E<sub>2</sub> either prior to, or during, virus inoculation did not significantly inhibit HCV virion production (Fig. 3a). These results collectively suggest that E<sub>2</sub> inhibits HCV virion production, but not at the level of virus entry, RNA replication or protein synthesis. We also observed that E<sub>2</sub>-mediated inhibition of HCV virion production occurs in a dose-dependent manner (Fig. 3b).

### A nuclear estrogen receptor antagonist, ICI182780, abolishes E<sub>2</sub>-mediated inhibition of HCV virion production

We hypothesized that E<sub>2</sub> signaling through nuclear ER (ERα and ERβ) was involved in the E<sub>2</sub>-mediated inhibition of HCV virion production. To test this possibility, we used ICI182780 (1 μM), an antagonist of ERα and ERβ. The results clearly demonstrated that treatment of cells with ICI182780 abolished E<sub>2</sub>-mediated inhibition of HCV virion production (Fig. 4).