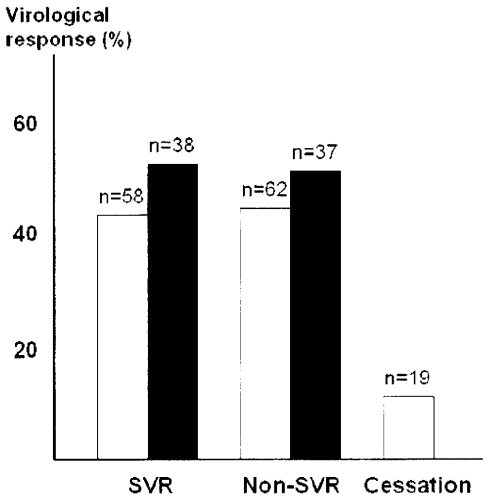

Fig. 2. Virological response in patients treated with peginterferon plus ribavirin for 48 weeks. The results are shown for all 139 subjects (open bars) and for 75 cases with good adherence of >80% of the scheduled dosages (closed bars). SVR, sustained virological response.

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

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)

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[a]
Sex (men/women)	18/10	21/19	NS[b]
Pre-treatment HCV RNA (KIU/ml)	1,635 ± 930	2,087 ± 1,422	NS[a]
Alanine aminotransferase level (U/L)	80 ± 62	71 ± 47	NS[a]
Stage of liver fibrosis			
F1 or F2/F3 or F4	19/9	28/12	NS[b]
Drug adherence dosage (%)			
Pegylated interferon	97.7 ± 5.2	95.2 ± 7.3	NS[a]
Ribavirin	96.8 ± 6.4	95.3 ± 7.7	NS[a]

NS, not significant.
[a]t-test.
[b]χ^2 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 ± 0.94 vs. 6.25 ± 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.
*$P < 0.05$.
**$P = 0.08$; χ^2 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[a]
Non-SVR (n = 10)	3 (30)	7 (70)	

SVR, sustained virological response.
[a]χ^2 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.I3 ± 0.71	NS[a]
Non-SVR (n = 35)	6.33 ± 0.59	6.32 ± 0.55	NS[a]
Total (n = 68)	5.98 ± 0.94	6.25 ± 0.62	NS[a]

SVR, sustained virological response. NS, not significant.
[a]*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 NS3polymorphisms with clinical outcome.

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ORIGINAL ARTICLE

17β-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 17β-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_2, but not P_4, significantly inhibited production of the HCV virion without inhibiting HCV RNA replication or HCV protein synthesis. E_2–mediated inhibition of HCV virion production was abolished by a nuclear estrogen receptor (ER) antagonist ICI182780. Moreover, treatment with the ERα-selective agonist 4, 4′, 4″- (4-propyl-[1H]-pyrazole-1, 3, 5-triyl)trisphenol (PPT), but not with the ERβ-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_2, inhibits the production of HCV infectious particles in an ERα–dependent manner.

Key words 17β-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_2, 17β-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_4, 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_2, 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; ERα, ERβ 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_2 and P_4, 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 μg/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 (2×10^5 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_2 and P_4 were purchased from Sigma–Aldrich (St Louis, MO, USA). ICI182780 (an antagonist of ERα and ERβ), PPT (an ERα-selective agonist) (20) and DPN (an ERβ-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_2 and P_4 used in this study were 0.4 μM and 3 μM, respectively, which correspond to the estimated highest concentrations in the sera of pregnant women. ICI182780 was used at a concentration of 1 μM, PPT and DPN at 0.1, 1 and 10 μM, and G-1 at 0.1 and 1 μM. As G-1 has been reported to lose its GPR30-binding specificity at concentrations over 1 μM, a concentration of 10 μM 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 (2.0×10^4 cells/well) were inoculated with HCV and treated with E_2, P_4 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′-ACCAAGCTCAAACTCACTCCA-3′ and 5′-AGCGGGGTCGGGCAC GAGACA-3′ (23);

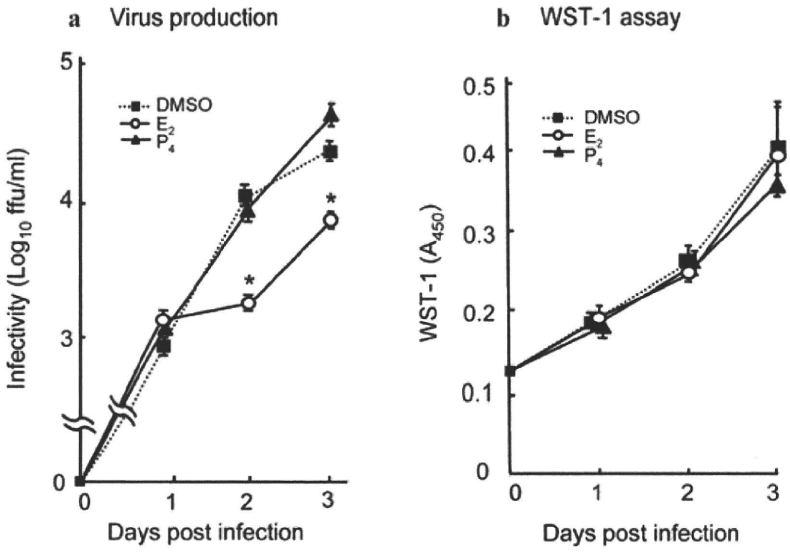

Fig. 1. Effects of E_2 and P_4 on HCV virion production and cell growth. (a) HCV virion production. Huh-7.5 cells were inoculated with HCV at a multiplicity of infection of 1.0, incubated for 2 hr, and cultured for 0, 1, 2 and 3 days after virus infection. The HCV-infected cells were treated with E_2 (0.4 μM), P_4 (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_2, P_4 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_2 inhibits HCV virion production, but not HCV RNA replication or HCV protein synthesis

We first examined the effect of E_2 or P_4 treatment on HCV virion production. At 2 hr after virus inoculation, the HCV-infected Huh-7.5 cells were treated with E_2 (0.4 μM) or P_4 (3 μM) for 3 days. Culture supernatants were collected every day and titrated for viral infectivity. As shown in Figure 1a, E_2 treatment significantly suppressed HCV virion production at 2 and 3 days postinfection, whereas treatment with P_4 did not. The same treatment (E_2 or P_4) did not exert significant cytotoxicity (Fig. 1b). Next, we examined the effect of E_2 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_2 or P_4 (Fig. 2a–c). Moreover, treatment of the cells with E2 either prior to, or during, virus inoculation did not significantly inhibit HCV virion production (Fig. 3a). These results collectively suggest that E_2 inhibits HCV virion production, but not at the level of virus entry, RNA replication or protein synthesis. We also observed that E_2 –mediated inhibition of HCV virion production occurs in a dose-dependent manner (Fig. 3b).

A nuclear estrogen receptor antagonist, ICI182780, abolishes E_2-mediated inhibition of HCV virion production

We hypothesized that E_2 signaling through nuclear ER (ERα and ERβ) was involved in the E_2–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_2–mediated inhibition of HCV virion production (Fig. 4).

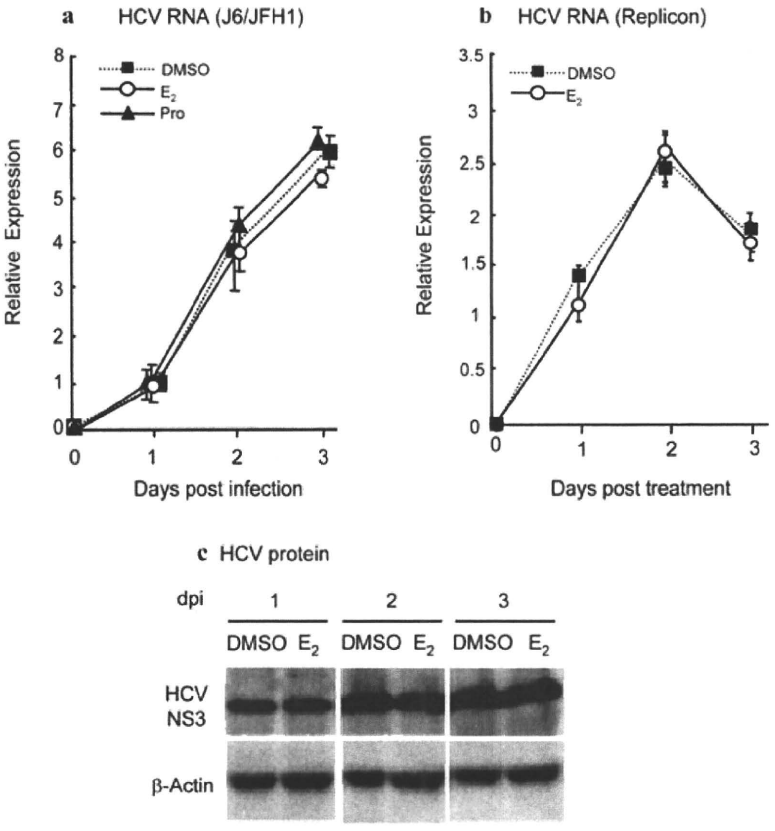

Fig. 2. Effects of E_2 and P_4 on HCV RNA replication and HCV protein synthesis. (a) HCV RNA replication. Huh-7.5 cells were inoculated with HCV at a multiplicity of infection of 1.0, incubated for 2 hr, and cultured for 0, 1, 2 and 3 days after virus infection. The HCV-infected cells were treated with E_2 (0.4 μM) or DMSO (control) from 2 hr to sampling time (days 1, 2 and 3). HCV RNA replication levels were determined by real-time quantitative RT-PCR and normalized with β-actin mRNA levels. Data are shown as mean ± SEM. (b) Huh-7.5 cells harboring a full-genomic HCV RNA replicon (23) were treated with E_2 (0.4 μM) or DMSO, and HCV RNA replication levels determined as in (a). (c) HCV protein synthesis. HCV-infected cells were treated with E2 or DMSO as in (a) and the amount of HCV protein synthesis determined by immunoblot analysis using anti-NS3 antibody. The degree of β-actin expression as determined by anti-β-actin antibody served as a control. dpi, days postinfection.

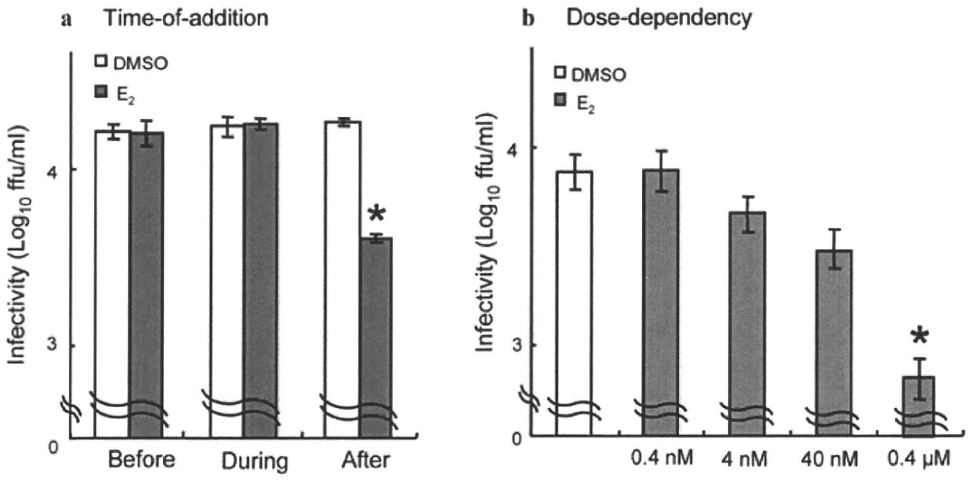

Fig. 3. Kinetic analysis of E_2–mediated inhibition of HCV virion production. (a) Time-of-addition experiment. Huh-7.5 cells were inoculated with HCV at a multiplicity of infection of 1.0, incubated for 2 hr, and cultured up to 2 days after virus infection. Treatment of the cells with E_2 (0.4 μM) was performed before or during virus inoculation for 2 hr, or after virus inoculation until sampling time (day 2). The culture supernatants of HCV-infected cells were assayed for viral infectivity. Data are shown as mean ± SEM. *$P < 0.05$, compared with DMSO control. (b) Dose–dependency experiment. Huh-7.5 cells were inoculated with HCV as in (a). The HCV-infected cells were treated with various concentrations of E_2 (0.4 nM to 0.4 μM]) from 2 hr postinfection to sampling time (day 2). The culture supernatants of HCV-infected cells were assayed for viral infectivity. Data are shown as mean ± SEM. *$P < 0.05$, compared with DMSO control.

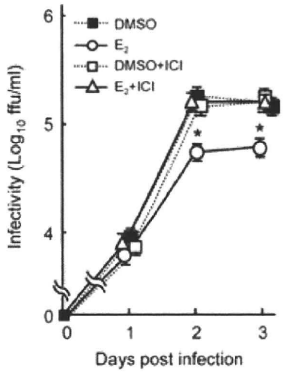

Fig. 4. Effects of ER antagonist, ICI182780, on HCV virion production. Huh-7.5 cells were inoculated with HCV at a multiplicity of infection of 1.0, incubated for 2 hr, and cultured for 0, 1, 2 and 3 days after virus infection. The HCV-infected cells were treated with E_2 (0.4 μM) and/or ICI182780 (1 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. *$P < 0.05$, compared with DMSO control.

Estrogen receptor-α-selective agonist 4, 4′, 4″- (4-propyl-[1H]-pyrazole-1, 3, 5-triyl) trisphenol inhibits HCV virion production

To determine which estrogen receptor(s) is/are involved in the E_2–mediated down-regulation of HCV virion production, we used receptor-specific agonists, such as PPT (an ERα-selective agonist) (20), DPN (an ERβ-selective agonist) (21) and G-1 (a GPR30-selective agonist) (22). Treatment of cells with PPT (10 μM), but not with DPN (10 μM) or G-1 (1 μM), significantly inhibited HCV virion production (Fig. 5). PPT treatment at a concentration of 1 μM also brought about a weak, but significant, inhibition of HCV virion production at 2 days postinfection. On the other hand, PPT did not mediate significant cytotoxicity at the concentrations tested (data not shown).

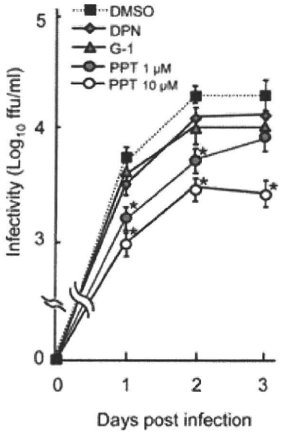

Fig. 5. Effects of ER-specific agonists on HCV virion production. Huh-7.5 cells were inoculated with HCV at a multiplicity of infection of 1.0, incubated for 2 hr, and cultured for 0, 1, 2 and 3 days after virus infection. The HCV-infected cells were treated with PPT (ERα-selective agonist; 1 and 10 μM), DPN (ERβ-selective agonist; 10 μM) or G-1 (GPR30-selective agonist; 1 μM) from 2 hr postinfection to sampling time (days 1, 2 and 3). The culture supernatants of HCV-infected cells were assayed for viral infectivity. Data are shown as mean ± SEM. *$P < 0.05$, compared with DMSO control.

DISCUSSION

We have demonstrated in the present study that treatment of Huh-7.5 cells with E_2 inhibits HCV virion production, but not HCV RNA replication or HCV protein synthesis (Figs 1 and 2). Treatment of the cells with E_2 either prior to, or during, virus inoculation did not significantly suppress HCV virion production (Fig. 3a). These results collectively suggest that E_2 inhibits HCV infection at the virion assembly/secretion level, but not at the level of virus attachment/entry, virus RNA replication or virus protein synthesis. E_2 has been reported to possess antioxidant and anti-apoptotic activities in fibrotic liver and cultured hepatocytes (24, 25). It should be noted, however, that E_2 did not exert anti-apoptotic or cytotoxic (pro-apoptotic) effect under our experimental conditions (Fig. 1b). In contrast to E_2, another female hormone, P_4, did not significantly affect HCV virion production (Fig. 1a).

E_2-mediated inhibition of HCV virion production was abolished by a nuclear ER (ERα and ERβ) antagonist, ICI182780 (Fig. 4), this result suggesting that suppression of HCV virion production may be induced by ER signal transduction. Three types of ER have been reported so far; ERα, ERβ and GPR30 (11–15). To determine which ER is involved in the suppression of HCV virion production, we used ER-specific agonists, PPT (for ERα) (20), DPN (for ERβ) (21) and G-1 (for GPR30) (22). We found that PPT, but not DPN or G-1, inhibits the production of HCV infectious particles (Fig. 5), suggesting that ERα plays an important role in the inhibition of HCV virion production. It has been reported that, in hepatocytes, ERα constitutes a minor proportion of the total ER, and that an estrogen-mediated anti-apoptotic effect is mediated principally through ERβ (26). However, the importance of ERα–mediated signal transduction should not be ignored. The rationale for this assertion is that ERα is known to be involved in lipid metabolism (27), that certain lipid metabolism disorder(s) possibly result(s) in abnormal accumulation of lipid droplets, and that such an accumulation is required for HCV virion maturation in virus-infected cells (27), that certain lipid metabolism disorder(s) possibly result(s) in abnormal accumulation of

lipid droplets, and that such an accumulation is required for HCV virion maturation in virus-infected cells (28). Also, we should not yet exclude the possible importance of ERβ and GPR30, because they may not be expressed at a sufficient level in the Huh7.5 cell line maintained in our laboratory.

Other relevant observations are that ERα interacts with HCV NS5B, the viral RNA polymerase, and promotes association of NS5B with the replication complex in human hepatoma-derived Huh-7 cells, and that tamoxifen, a competitive inhibitor of estrogens, suppresses the ERα–mediated association of NS5B with the replication complex, thereby inhibiting HCV RNA replication (29). Similarly, E_2 binding to ERα may abrogate its interaction with NS5B. However, in our experiments we did not observe E_2–mediated inhibition of HCV RNA replication (Fig. 2a,b). We therefore assume that E_2 inhibits HCV virion production through a mechanism other than E_2–ERα–NS5B interactions. Further study is needed to elucidate this issue.

In conclusion, the most potent physiological estrogen, E_2, inhibits production of HCV infectious particles in Huh-7.5 cell cultures in an ERα–dependent manner. This may explain, at least in part, why the incidence of HCV-associated liver disease is lower in premenopausal women than in postmenopausal women and men.

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Short Communication

Gene expression profile of Li23, a new human hepatoma cell line that enables robust hepatitis C virus replication: Comparison with HuH-7 and other hepatic cell lines

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Aim: Human hepatoma cell line HuH-7-derived cells are currently the only cell culture system used for robust hepatitis C virus (HCV) replication. We recently found a new human hepatoma cell line, Li23, that enables robust HCV replication. Although both cell lines had similar liver-specific expression profiles, the overall profile of Li23 seemed to differ considerably from that of HuH-7. To understand this difference, the expression profile of Li23 cells was further characterized by a comparison with that of HuH-7 cells.

Methods: cDNA microarray analysis using Li23 and HuH-7 cells was performed. Li23-derived ORL8c cells and HuH-7-derived RSc cells, in which HCV could infect and efficiently replicate, were also used for the microarray analysis. For the comparative analysis by reverse transcription polymerase chain reaction (RT–PCR), human hepatoma cell lines (HuH-6, HepG2, HLE, HLF and PLC/PRF/5) and immortalized hepatocyte cell line (PH5CH8) were also used.

Results: Microarray analysis of Li23 versus HuH-7 cells selected 80 probes to represent highly expressed genes that have ratios of more than 30 (Li23/HuH-7) or 20 (HuH-7/Li23). Among them, 17 known genes were picked up for further analysis. The expression levels of most of these genes in Li23 and HuH-7 cells were retained in ORL8c and RSc cells, respectively. Comparative analysis by RT–PCR using several other hepatic cell lines resulted in the classification of 17 genes into three types, and identified three genes showing Li23-specific expression profiles.

Conclusion: Li23 is a new hepatoma cell line whose expression profile is distinct from those of frequently used hepatic cell lines.

Key words: hepatitis C virus, hepatoma cell line, HuH-7, Li23, microarray

INTRODUCTION

HUH-7, A HUMAN hepatoma cell line,[1] is frequently used in the research of hepatitis C virus (HCV), since an HCV replicon system enabling HCV subgenomic RNA replication was developed using HuH-7 cells.[2] Even with the use of an efficient HCV production system developed in 2005,[3] HuH-7-derived cells are still used as the only cell line for persistent HCV production systems.

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We previously developed HCV replicon systems[4,5] and an HCV production system[6] using HuH-7-derived cells. Furthermore, we recently found a new human hepatoma cell line, Li23, that enables robust HCV RNA replication and persistent HCV production.[7] In that study, using microarray analysis, we excluded the possibility that the obtained Li23-derived cells were derived from contamination of HuH-7-derived cells used for HCV replication.[7] In addition, we noticed that the gene expression profile of Li23 cells seemed considerably different from that of HuH-7 cells. Therefore, we assumed that the Li23 cell line possesses a unique expression profile among widely used human hepatoma cell lines. To evaluate this assumption, we further characterized the expression profile of Li23 cells by comparing it with those of other human hepatoma cell lines, including HuH-7,[1] HuH-6,[8] HepG2,[9] HLE,[10] HLF[10] and PLC/PRF/5.[11] Human immortalized hepatocyte cell line

PH5CH8[12] was also used for the comparison. Here, we show that the Li23 cell line possesses a distinct expression profile among hepatic cell lines.

METHODS

Cell culture

HUH-7, HUH-6, HEPG2, HLE, HLF and PLC/PRF/5 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Li23 and PH5CH8 cells were maintained as described previously.[7] Cured cells (Li23-derived ORL8c and HuH-7-derived RSc), from which the HCV RNA had been eliminated by interferon (IFN) treatment, were also maintained as described previously.[7]

cDNA microarray analysis

Li23, ORL8c, HuH-7 and RSc cells (1×10^6 each) were plated onto 10-cm diameter dishes and cultured for 2 days. Total RNA from these cells were prepared using the RNeasy extraction kit (QIAGEN, Hilden, Germany). cDNA microarray analysis was performed according to the methods described previously.[7] Differentially expressed genes were selected by comparing the arrays from Li23 and HuH-7 cells. The selected genes were further compared with the array from ORL8c or RSc cells.

Reverse transcription polymerase chain reaction

Reverse transcription polymerase chain reaction (RT-PCR) was performed to detect cellular mRNA as described previously.[13] Briefly, total RNA (2 μg) was reverse-transcribed with M-MLV reverse transcriptase (Invitrogen, San Diego, CA, USA) using an oligo dT primer (Invitrogen) according to the manufacturer's protocol. One-tenth of the synthesized cDNA was used for PCR. The primers arranged for this study are listed in Table 1. In addition, we used primer sets for New York esophageal squamous cell carcinoma 1 (NY-ESO-1), β-defensin-1 (DEFB1), lectin, galactoside-binding, soluble 3 (LGALS3)/Galectin-3, melanoma-specific antigen family A6 (MAGEA6), UDP glycosyltransferase 2 family polypeptide B4 (UGT2B4), transmembrane 4 superfamily member 3 (TM4SF3), insulin-like growth factor binding protein 2 (IGFBP2), arylacetamide deacetylase (AADAC), albumin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as described previously.[7]

RESULTS

Genes showing pronounced differences in gene expression between Li23- and HuH-7-derived cells

WE RECENTLY ESTABLISHED several Li23-derived cell lines showing robust HCV RNA replication.[7] In convenient microarray analysis using these cell lines, we noticed that the gene expression profile of Li23 cells differed considerably from that of HuH-7 cells, and that several genes, including cancer antigens such as NY-ESO-1 and MAGEA6, were highly expressed in Li23 cells but were not expressed in HuH-7 cells.[7] However, it

Table 1 Primers used for reverse transcription polymerase chain reaction analysis

Gene (accession no.)	Direction	Nucleotide sequence (5′–3′)	Products (bp)
Cancer antigen 45, A5	Forward	TGGAGATGACCTAGAATGCAG	218
(CT45A5); NM_001007551	Reverse	CTCGTCTCATACATCTTGCTG	
Four-and-a-half LIM domain 1	Forward	GGAATCACTTACCAGGATCAG	243
(FHL1; NM_001449)	Reverse	TTTGCAGTGGAAGCAGTAGTC	
Thymosin β4, X-linked	Forward	ACCAGACTTCGCTCGTACTC	179
(TMSB4X; NM_021109)	Reverse	TCGCCTGCTTGCTTCTCCTG	
Lectin, galactoside-binding, soluble 1	Forward	CAACACCATCGTGTGCAACAG	253
(LGALS1; NM_002305)	Reverse	CAGCTGCCATGTAGTTGATGG	
Interferon-induced transmembrane protein 2	Forward	CCTCTTCATGAACACCTGCTG	184
(IFITM2; NM_006435)	Reverse	CACTGGGATGATGATGAGCAG	
Apolipoproteins A1	Forward	ACTGTGTACGTGGATGTGCTC	273
(APOA1; X02162)	Reverse	CTTCTTCTGGAAGTCGTCCAG	
α-2-HS-glycoprotein	Forward	AACCGAACTGCGATGATCCAG	248
(AHSG; NM_001622)	Reverse	TTCGACAGCATGCTCCTTCAG	
Gap junction protein-α 1	Forward	CATCTTCATGCTGCTGCTGTC	253
(GJA1; NM_000165)	Reverse	GTTTCTGTGGCCACTAACCAG	

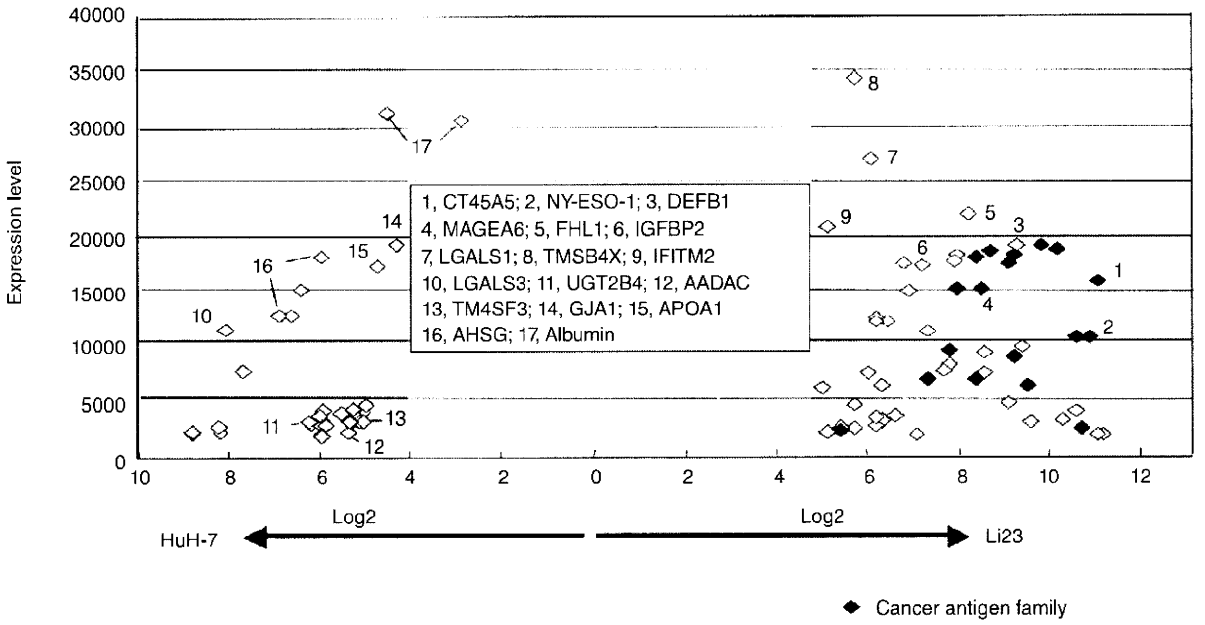

Figure 1 Genes showing pronounced differences in gene expression between Li23 and HuH-7 cells. The probes showing expression levels of more than 2000 and ratios of more than 30 (Li23/HuH-7) or 20 (HuH-7/Li23) are presented.

is unclear whether the expression profiles of these genes are characteristics of Li23 cells.

To clarify this point, comprehensive microarray analysis using Li23 and HuH-7 cells was performed. This revealed 4119 and 3570 probes whose expression levels were upregulated and downregulated at ratios of more than 2 and less than 0.5 in Li23 versus HuH-7 cells, respectively. From among these probes, we selected those showing ratios of more than 30 (Li23/HuH-7) and 20 (HuH-7/Li23), and further selected the probes showing expression levels of more than 2000 (actual value of measurement). By these selections, 80 probes were assigned (Fig. 1). The most distinguishing characteristic of the comparison is that the cancer antigen family (18 probes) was highly expressed in Li23 cells but was not highly expressed in HuH-7 cells (Fig. 1). From these probes, 14 known genes showing expression levels above 10 000 (#1–10 and #14–17 in Fig. 1) and three additional known genes (#11–13 in Fig. 1) were chosen as representative genes for further analysis.

Regarding the total of 17 genes, the expression levels in Li23 versus ORL8c or HuH-7 versus RSc were compared. The expression levels of most of the 17 genes were maintained between Li23 and ORL8c cells or between HuH-7 and RSc cells (Table 2). These results indicate that ORL8c and RSc cells retained the characteristics of parent Li23 and HuH-7 cells, respectively. However, it was notable that the expression levels of apolipoprotein A1 (APOA1), α-2-HS-glycoprotein (AHSG), and albumin were significantly higher in ORL8c cells than in Li23 cells, suggesting that ORL8c is selected as a specific clone from Li23 cell populations.

Expression profiles of representative genes whose expression levels showed drastic differences between Li23 and HuH-7 cells among human hepatic cell lines

Regarding the 17 genes selected above, we performed comparative analyses by RT-PCR using Li23, HuH-7, HuH-6, HepG2, HLE, HLF, PLC/PRF/5 and PH5CH8 cells in order to clarify whether or not these genes exhibit Li23-specific expression profiles. The results of the RT-PCR performed after optimization of PCR conditions in each gene resulted in the classification of the 17 genes into three types (A, B and C in Fig. 2). NY-ESO-1 and DEFB1 (high expression in Li23 only), and LGALS3/Galectin-3 (no expression in Li23 only) belonged to type A, which showed a Li23-specific feature. Type B showed that the expression levels in Li23, HLE, HLF, PLC/PRF/5 and/or PH5CH8 cells were greatly higher or lower than those in HuH-7, HuH-6 and HepG2 cells. Type B consisted of cancer antigen 45, A5

Table 2 Representative genes showing pronounced differences in gene expression between Li23 and HuH-7 cells

Gene	Accession no.	Li23	Li23-derived ORL8c	HuH-7	HuH-7-derived RSc
Cancer antigen 45, A5 (CT45A5)	NM_001007551	15 857†	10 508	8	23
Cancer testis antigen 1A (NY-ESO-1/CTAG1A)	U87459	9 005	5 503	5	8
β-Defensin-1 (DEFB1)	U73945	18 311	8 326	31	7
Melanoma-specific antigen family A6 (MAGEA6)	U10691	15 168	17 050	42	35
Four-and-a-half LIM domain 1 (FHL1)	NM_001449	21 851	13 428	77	79
Insulin-like growth factor binding protein 2 (IGFBP2)	NM_000597	17 429	8 931	117	13
Lectin, galactoside-binding, soluble 1 (LGALS1)	NM_002305	26 694	27 098	379	11
Thymosin β4, X-linked (TMSB4X)	NM_021109	34 273	26 199	648	307
IFN-induced transmembrane protein 2 (IFITM2)	NM_006435	20 762	9 645	595	637
Lectin, galactoside-binding, soluble 3 (LGALS3/Galectin 3)	BC001120	41	70	10 973	6 020
UDP glycosyltransferase 2 family polypeptide B4 (UGT2B4)	NM_021139	40	57	2 863	7 546
Arylacetamide deacetylase (AADAC)	NM_001086	57	73	2 282	4 746
Transmembrane 4 superfamily member 3 (TM4SF3)	NM_004616	95	51	3 220	1 265
Gap junction protein-α 43 KDa (GJA1)	NM_000165	951	2	19 090	19 485
Apolipoprotein A1 (APOA1)	X02162	673	7 230	16 920	15 202
α-2-HS-glycoprotein (AHSG)	NM_001622	308	6 373	18 436	26 000
Albumin	AF116645	4 304	30 111	30 234	33 140
	D16931	1 387	23 615	30 668	39 144

†Signal intensity in human genome U133 Plus 2.0 array.

(CT45A5), MAGEA6, four-and-a-half LIM domains 1 (FHL1), Thymosin B4, X-linked (TMSB4X), lectin, galactoside-binding, soluble 1 (LGALS1) and IFN-induced transmembrane protein 2 (IFITM2) – all of which were highly expressed in Li23 cells – and APOA1, AHSG and UGT2B4, which were highly expressed in HuH-7 cells. The remaining five genes were assigned to type C and showed more complex expression profiles (Fig. 2). For instance, Gap junction protein-α 43 kDa (GJA1) expression was observed in HuH-7, HLE, HLF, PLC/PRF/5 and PH5CH8 cell lines, but not in Li23, HuH-6 or HepG2 cell lines. In addition, IGFBP2 expression was observed in Li23, HuH-6 and PH5CH8 cell lines, but not in the other cell lines. Together, these results indicate that the Li23 cell line possesses a distinct expression profile among frequently used hepatic cell lines.

DISCUSSION

IN THIS STUDY, we assigned 17 known genes that showed drastic differences between Li23 and HuH-7 cells, and classified the expression profiles of these genes into at least three types among frequently used hepatic cell lines. Three genes (NY-ESO-1, DEFB1 and LGALS3/Galectin-3) were identified as the representative showing Li23-specific expression.

NY-ESO-1 is a well-characterized cancer-testis antigen (CTAG) that appears to be the most immunogenic CTAG known to date.[14] NY-ESO-1 is expressed in malignant tumors such as melanoma, lung carcinoma and bladder cancer, which are called "CTAG-rich" tumor types, but are expressed solely in the testis among normal adult tissues.[15] Because a spontaneous immune response to NY-ESO-1 is frequently observed in patients with malignant tumors including hepatocellular carcinoma,[16] cancer vaccine trials based on NY-ESO-1 are currently underway.[15] However, the biological role of NY-ESO-1 in both tumors and testis remains poorly understood. Accordingly, the Li23 cell line may be useful for the study of the biological role of NY-ESO-1.

Human defensins, which are small cationic peptides produced by neutrophils and epithelial cells, form two genetically distinct subfamilies, α-defensin and β-defensin. DEFB1, identified in this study, is one of six members belonging to β-defensins and appears to be involved in the antimicrobial defense of the epithelia of surfaces.[16,17] Although α-defensins consisting of six members are known to be expressed in a variety of tumors, DEFB1 is downregulated in some tumor types in which it could behave as a tumor suppressor protein.[18] Our study revealed that except DEFB1 in Li23 cells, no α- or β-defensin members were expressed in the

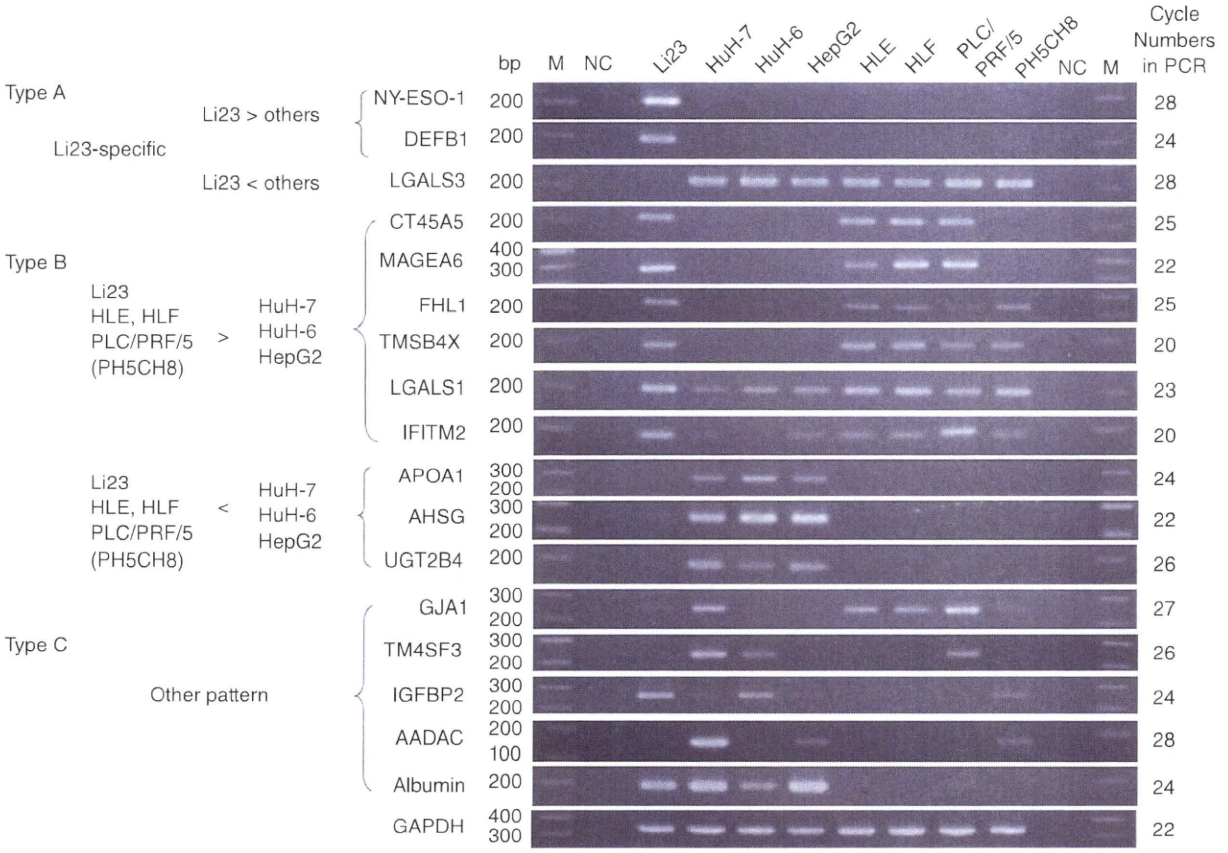

Figure 2 Expression profiles of representative genes, whose expression levels showed drastic differences between Li23 and HuH-7 cells, among human hepatic cell lines. Reverse transcription polymerase chain reaction (RT–PCR) analysis was performed as described in Methods. PCR products were detected by staining with ethidium bromide after separation by electrophoresis on 3% agarose gels.

hepatic cell lines tested in this study (data not shown). Because the molecular mechanism underlying DEFB1 expression or its role in oncogenesis remains to be clarified, Li23 cells may be useful for a study like that.

LGALS3/Galectin-3 is the most studied member of the galectin family, which is characterized by specific binding of β-galactosides through the carbohydrate-recognition domain.[19] LGALS3/Galectin-3 is ubiquitously expressed in numerous cell and tissue types; it is located in both nuclei and cytoplasm, and is secreted through a non-classical pathway. To date, LGALS3/Galectin-3 was found to be involved in many regulations including development, immune reaction, tumorigenesis, and tumor growth and metastasis.[19,20] Indeed, the overexpression of LGALS3/Galectin-3 in cirrhotic and hepatocellular carcinoma has also been reported.[21] In such situations, the absence of LGALS3/Galectin-3 expression in the Li23 cell line is a unique feature among hepatic cell lines, which show high expression levels. Accordingly, the Li23 cell line might be useful as a LGALS3/Galectin-3-null cell line for various studies including those on tumor growth and metastasis.

Although we identified Li23-specific genes showing distinct expression levels among hepatic cell lines examined, microarray analysis revealed that the expression profiles of Li23 and HuH-7 cells, both of which possess an environment for robust HCV replication, differed considerably. Accordingly, such differences may affect the properties or multiplications of HCV, such as susceptibility to anti-HCV reagents, the mutation rate of the HCV genome and the efficiency of HCV replication. Further comparative analysis using Li23 and HuH-7 cells will help to resolve these uncertain subjects.

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The ESCRT System Is Required for Hepatitis C Virus Production

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Abstract

Background: Recently, lipid droplets have been found to be involved in an important cytoplasmic organelle for hepatitis C virus (HCV) production. However, the mechanisms of HCV assembly, budding, and release remain poorly understood. Retroviruses and some other enveloped viruses require an endosomal sorting complex required for transport (ESCRT) components and their associated proteins for their budding process.

Methodology/Principal Findings: To determine whether or not the ESCRT system is needed for HCV production, we examined the infectivity of HCV or the Core levels in culture supernatants as well as HCV RNA levels in HuH-7-derived RSc cells, in which HCV-JFH1 can infect and efficiently replicate, expressing short hairpin RNA or siRNA targeted to tumor susceptibility gene 101 (TSG101), apoptosis-linked gene 2 interacting protein X (Alix), Vps4B, charged multivesicular body protein 4b (CHMP4b), or Brox, all of which are components of the ESCRT system. We found that the infectivity of HCV in the supernatants was significantly suppressed in these knockdown cells. Consequently, the release of the HCV Core into the culture supernatants was significantly suppressed in these knockdown cells after HCV-JFH1 infection, while the intracellular infectivity and the RNA replication of HCV-JFH1 were not significantly affected. Furthermore, the HCV Core mostly colocalized with CHMP4b, a component of ESCRT-III. In this context, HCV Core could bind to CHMP4b. Nevertheless, we failed to find the conserved viral late domain motif, which is required for interaction with the ESCRT component, in the HCV-JFH1 Core, suggesting that HCV Core has a novel motif required for HCV production.

Conclusions/Significance: These results suggest that the ESCRT system is required for infectious HCV production.

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Introduction

Hepatitis C virus (HCV) is a causative agent of chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma. HCV is an enveloped virus with a positive single stranded 9.6 kb RNA genome, which encodes a large polyprotein precursor of approximately 3,000 amino acid residues. This polyprotein is cleaved by a combination of the host and viral proteases into at least 10 proteins in the following order: Core, envelope 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B [1]. HCV Core, a highly basic RNA-binding protein, forms a viral capsid and is targeted to lipid droplets [2–6]. The Core is essential for infectious virion production [7]. NS5A, a membrane-associated RNA-binding phosphoprotein, is also involved in the assembly and maturation of infectious HCV particles [8,9]. Intriguingly, NS5A is a key regulator of virion production through the phosphorylation by casein kinase II [9]. Recently, lipid droplets have been found to be involved in an important cytoplasmic organelle for HCV production [4]. Indeed, NS5A is known to colocalize with the Core on lipid droplets [5], and the interaction between NS5A and the Core is critical for the production of infectious HCV particles [3]. However, the host factor involved in HCV assembly, budding, and release remains poorly understood.

Budding is an essential step in the life cycle of enveloped viruses. Endosomal sorting complex required for transport (ESCRT) components and associated factors, such as tumor susceptibility gene 101 (TSG101, a component of ESCRT-I), charged multivesicular body protein 4b (CHMP4b, a component of ESCRT-III), and apoptosis-linked gene 2 interacting protein X (ALIX, a TSG101- and CHMP4b-binding protein), have been found to be involved in membrane remodeling events that accompany endosomal protein sorting, cytokinesis, and the budding of several enveloped viruses, such as human immunodeficiency virus type 1 (HIV-1) [10–12]. The ESCRT complexes I, II, and III are sequentially, or perhaps concentrically recruited to the endosomal membrane to sequester

cargo proteins and drive vesicularization into the endosome. Finally, ESCRT-III recruits Vps4 (two isoforms, Vps4A and Vps4B), a member of the AAA-family of ATPase that disassembles and thereby terminates and recycles the ESCRT machinery.

Since HCV is also an enveloped RNA virus, we hypothesized that the ESCRT system might be required for HCV production. To test this hypothesis, we examined the release of HCV Core into culture supernatants from cells rendered defective for ESCRT components by RNA interference. The results provide evidence that the ESCRT system is required for HCV production.

Materials and Methods

Cell Culture

293FT cells (Invitrogen, Carlsbad, CA) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS). The HuH-7-derived cell line, RSc cured cells that cell culture-generated HCV-JFH1 (JFH1 strain of genotype 2a) [13] could infect and effectively replicate [14–16] and OR6c and OR6 cells harboring the genome-length HCV-O RNA with luciferase as a reporter were cultured in DMEM with 10% FBS as described previously [17,18].

Plasmid Construction

To construct pcDNA3-FLAG-Alix, a DNA fragment encoding Alix was amplified from total RNAs derived from RSc cells by RT-PCR using the follwing pairs of primers: Forward 5′-CGGG-ATCCAAGATGGCGACATTCATCTCGGT-3′ and reverse 5′-CCGGCGGCCGCTTACTGCTGTGGATAGTAAG-3′. The obtained DNA fragment was subcloned into *Bam*HI-*Not*I of pcDNA3-FLAG vector [19], and the nucleotide sequences were determined by Big Dye termination cycle sequencing using an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA). The plasmid of pJRN/3-5B was based on pJFH1 [13] and was constructed as previously described [20].

RNA synthesis, RNA transfection, and Selection of G418-resistant cells

Plasmid pJRN/3-5B were linearized by *Xba*I and used for the RNA synthesis with the T7 MEGAScript kit (Ambion, Austin, TX). *In vitro* transcribed RNA was transfected into OR6c cells by electroporation [17,18]. The transfected cells were selected in culture medium containing G418 (0.3 mg/ml) for 3 weeks. We referred to them as OR6c/JRN 3-5B cells.

Immunofluorescence and Confocal Microscopic Analysis

Cells were fixed in 3.6% formaldehyde in phosphate-buffered saline (PBS) and permeabilized in 0.1% Nonidet P-40 (NP-40) in PBS at room temperature as previously described [21]. Cells were incubated with anti-HCV Core antibody (CP-9 and CP-11 mixture; Institute of Immunology, Tokyo, Japan), anti-Myc-Tag antibody (PL14; Medical & Biological Laboratories, MBL, Nagoya, Japan), anti-Alix antibody (Covalab, Villeurbanne, France), and/or anti-FLAG polyclonal antibody (Sigma, St. Louis, MO) at a 1:300 dilution in PBS containing 3% bovine serum albumin (BSA) at 37°C for 30 min. Cells were then stained with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA) or anti-Cy3-conjugated anti-mouse antibody (Jackson ImmunoResearch) at a 1:300 dilution in PBS containing BSA at 37°C for 30 min. Lipid droplets and nuclei were stained with BODIPY 493/503 (Molecular Probes, Invitrogen) and DAPI (4′,6′-diamidino-2-phenylindole), respectively. Following extensive washing in PBS, cells were mounted on slides using a mounting media of 90% glycerin/10% PBS with 0.01% *p*-phenylenediamine added to reduce fading. Samples were viewed under a confocal laser-scanning microscope (LSM510; Zeiss, Jena, Germany).

RNA Interference

The following siRNAs were used: human TSG101 (siGENOME SMARTpool M-003549-01-0005 and 5′-CCUCCAGU-CUUCUCUCGUCUU-3′ sense, 5′-GACGAGAGAAGACUG-GAGGUU-3′ antisense), human Alix/PDCD6IP (siGENOME SMARTpool M-004233-02-0005), human Vps4B (siGENOME SMARTpool M-013119-02-0005), human CHMP4b (siGENOME SMARTpool M-018075-00-0005), and siGENOME Non-Targeting siRNA Pool#1 (D-001206-13-05) (Dharmacon, Thermo Fisher Scientific, Waltham, MA) as a control. siRNAs (50 nM final concentration) were transiently transfected into either RSc cells [14–16] or OR6 cells [17,18] using Oligofectamine (Invitrogen) according to the manufacturer's instructions. Oligonucleotides with the following sense and antisense sequences were used for the cloning of short hairpin (sh) RNA-encoding sequences against TSG101, Alix, Vps4B, or CHMP4b in lentiviral vector: TSG101i, 5′-GATCCCC GGAGGAAATGGATCGTGCCTT-CAAGAGAGGCACGATCCATTTCCTCCTTTTTGGAAA-3′ (sense), 5′-AGCTTTTCCAAAAAGGAGGAAATGGATCGTG-CCTCTCTTGAAGGCACGATCCATTTCCTCCGGG-3′ (antisense); Alixi, 5′-GATCCCC GGAGGTGTTCCCTGTCTTG-TTCAAGAGACAAGACAGGGAACACCTCCTTTTTGGAA-A-3′ (sense), 5′-AGCTTTTCCAAAAAGGAGGTGTTCCCTG-TCTTGTCTCTTGAACAAGACAGGGAACACCTCCGGG-3′ (antisense); Vps4Bi, 5′-GATCCCC GGAGAATCTGATGATC-CTGTTCAAGAGACAGGATCATCAGATTCTCCTTTTTG-GAAA-3′ (sense), 5′-AGCTTTTCCAAAAAGGAGAATCT-GATGATCCTGTCTCTTGAACAGGATCATCAGATTCTC-CGGG-3′ (antisense); CHMP4bi, 5′-GATCCCC GAGGAG-GACGACGACATGATTCAAGAGATCATGTCGTCGTCC-TCCTCTTTTTGGAAA-3′ (sense), 5′-AGCTTTTCCAAAAA-GAGGAGGACGACGACATGATCTCTTGAATCATGTCG-TCGTCCTCCTCGGG-3′ (antisense); Broxi, 5′-GATCCCCG-GATGACAGTACTAAACCCTTCAAGAGAGGGTTTAGTA-CTGTCATCCTTTTTGGAAA-3′ (sense), 5′-AGCTTTTC-CAAAAAGGATGACAGTACTAAACCCTCTCTTGAAGGG-TTTAGTACTGTCATCCGGG-3′ (antisense). The oligonucleotides above were annealed and subcloned into the *Bgl*II-*Hin*dIII site, downstream from an RNA polymerase III promoter of pSUPER [22], to generate pSUPER-TSG101i, pSUPER-Alixi, pSUPER-Vps4Bi, and pSUPER-CHMP4bi, respectively. To construct pLV-TSG101i, pLV-Alixi, pLV-Vps4Bi, and pLV-CHMP4bi, the *Bam*HI-*Sal*I fragments of the corresponding pSUPER plasmids were subcloned into the *Bam*HI-*Sal*I site of pRDI292 [23], an HIV-1-derived self-inactivating lentiviral vector containing a puromycin resistant marker allowing for the selection of transduced cells, respectively.

Lentiviral Vector Production

The vesicular stomatitis virus (VSV)-G-pseudotyped HIV-1-based vector system has been described previously [24–26]. The lentiviral vector particles were produced by transient transfection of the second-generation packaging construct pCMV-ΔR8.91 [24–26] and the VSV-G-envelope-expressing plasmid pMDG2 as well as pRDI292 into 293FT cells with FuGene6 (Roche Diagnostics, Basel, Switzerland).

HCV Infection Experiments

The supernatants was collected from cell culture-generated HCV-JFH1 [13]-infected RSc cells [14–16] at 5 days post-

infection and stored at −80°C after filtering through a 0.45 μm filter (Kurabo, Osaka, Japan) until use. For infection experiments with HCV-JFH1 virus, RSc cells (1×10^5 cells/well) were plated onto 6-well plates and cultured for 24 hours (hrs). Then, we infected the cells with 50 μl (equivalent to a multiplicity of infection [MOI] of 0.1) of inoculum. The culture supernatants were collected and the levels of HCV Core were determined by enzyme-linked immunosorbent assay (ELISA) (Mitsubishi Kagaku Bio-Clinical Laboratories, Tokyo, Japan). Total RNA was isolated from the infected cellular lysates using RNeasy mini kit (Qiagen, Hilden, Germany) for quantitative RT-PCR analysis of intracellular HCV RNA. The infectivity of HCV in the culture supernatants was determined by a focus-forming assay at 48 hrs post-infection. The HCV infected cells were detected using anti-HCV Core antibody (CP-9 and CP-11). Intracellular HCV infectivity was determined by a focus-forming assay at 48 hrs post-inoculation of lysates by repeated freeze and thaw cycles (three times).

Quantitative RT-PCR Analysis

The quantitative RT-PCR analysis for HCV RNA was performed by real-time LightCycler PCR (Roche) as described previously [17,18]. We used the following forward and reverse primer sets for the real-time LightCycler PCR: TSG101, 5′-ATGGCGGTGTCGGAGAGCCA-3′ (forward), 5′-AACAGGTTTGAGATCTTTGT-3′ (reverse); Alix, 5′-ATGGCGACATTCATCTCGGT-3′ (forward), 5′-TACTGGGCCTGCTCTTCCCC-3′ (reverse); Vps4B, 5′-ATGTCATCCACTTCGCCCAA-3′ (forward), 5′-ATACTGCACAGCATGCTGAT-3′ (reverse); CHMP4b, 5′-ATGTCGGTGTTCGGGAAGCT-3′ (forward), 5′-ATCTCTTCCGTGTCCCGCAG-3′ (reverse); Brox, 5′-ATGACCCATTGGTTTCATAG-3′ (forward), 5′-CCTGGATGACCTCAAGTCAT-3′ (reverse); β-actin, 5′-TGACGGGGTCACCCACACTG-3′ (forward), 5′-AAGCTGTAGCCGCGCTCGGT-3′ (reverse); and HCV-JFH1, 5′-AGAGCCATAGTGGTCTGCGG-3′ (forward), 5′-CTTTCGCAACCCAACGCTAC-3′ (reverse).

MTT Assay

Cells (5×10^3 cells/well) were plated onto 96-well plates and cultured for 24, 48 or 72 hrs, then, subjected to the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromid (MTT) assay according to the manufacturer's instructions (Cell proliferation kit I, Roche). The absorbance was read using a microplate reader (Multiskan FC, Thermo Fisher Scientific) at 550 nm with a reference wavelength of 690 nm.

Renilla Luciferase (RL) Assay

OR6 cells (1.5×10^4 cells/well) [17,18] were plated onto 24-well plates and cultured for 24 hrs. The cells were transfected with siRNAs (50 nM) using Oligofectamine and incubated for 72 hrs, then, subjected to the RL assay according to the manufacturer's instructions (Promega, Madison, WI). A lumat LB9507 luminometer (Berthold, Bad Wildbad, Germany) was used to detect RL activity.

Western Blot Analysis

Cells (2×10^5 cells/well) were plated onto 6-well plates and cultured for 24 or 48 hrs. Cells were lysed in buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 4 mM EDTA, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF). Supernatants from these lysates were subjected to SDS-polyacrylamide gel electrophoresis, followed by immunoblot analysis using anti-TSG101 antibody (BD Transduction Laboratories, San Jose, CA), anti-Alix antibody, anti-Vps4B antibody (Abnova, Taipei, Taiwan) (A302-078A; Bethyl Laboratories, Montgomery, TX), anti-CHMP4B antibody (sc-82557; Santa Cruz Biotechnology, Santa Cruz, CA), anti-HCV Core antibody, anti-β-actin antibody (Sigma), anti-Myc-Tag antibody, anti-FLAG antibody (M2; Sigma), anti-Chk2 antibody (DCS-273; MBL), anti-heat shock protein (HSP) 70 antibody (BD), Living Colors A.v. monoclonal antibody (JL-8; Clontech, Mountain View, CA), anti-HCV NS5A monoclonal antibody (no. 8926; a generous gift from A Takamizawa, The Research Foundation for Microbial Diseases of Osaka University, Japan), or anti-HCV NS5A polyclonal antibody (a generous gift from K Shimotohno, Chiba Institute of Technology, Chiba, Japan).

Immunoprecipitation Analysis

Cells were lysed in buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 1 mM PMSF, and protease inhibitor cocktail containing 104 μM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 80 nM aprotinin, 2.1 μM leupeptin, 3.6 μM bestatin, 1.5 μM pepstatin A, and 1.4 μM E-64 (Sigma). Lysates were pre-cleaned with 30 μl of protein-G-Sepharose (GE Healthcare Bio-Sciences). Pre-cleaned supernatants were incubated with 5 μl of Living Colors A.v. monoclonal antibody or anti-FLAG antibody at 4°C for 1 hr. Following absorption of the precipitates on 30 μl of protein-G-Sepharose resin for 1 hr, the resin was washed four times with 700 μl lysis buffer. Proteins were eluted by boiling the resin for 5 min in 1× Laemmli sample buffer. The proteins were then subjected to SDS-PAGE, followed by immunoblotting analysis using either anti-FLAG antibody, Living Colors A.v. monoclonal antibody or anti-HCV Core antibody.

Statistical Analysis

Statistical comparison of the infectivity of HCV in the culture supernatants between the knockdown cells and the control cells was performed using the Student's *t*-test. *P* values of less than 0.05 were considered statistically significant. All error bars indicate standard deviation.

Results

The ESCRT system is required for HCV production

To investigate the potential role(s) of the ESCRT system in the HCV life cycle, we first used lentiviral vector-mediated RNA interference to stably knockdown the ESCRT components, including TSG101, Alix, Vps4B, or CHMP4b in HuH-7-derived RSc cured cells that cell-culture-generated HCVcc (HCV-JFH1, genotype 2a) [13] could infect and effectively replicate [14–16]. We used puromycin-resistant pooled cells 10 days after the lentiviral transduction in all experiments. Western blot and real-time LightCycler RT-PCR analyses for TSG101, Alix, Vps4B, or CHMP4b demonstrated a very effective knockdown of each ESCRT component in RSc cells transduced with lentiviral vectors expressing the corresponding shRNAs (Fig. 1A–E). Importantly, we noticed that the depletion of ESCRT components did not affect the levels of several cellular proteins, including HSP70, Chk2, and b-actin (Fig. 1A). To test the cell toxicity of each shRNA, we examined colorimetric MTT assay. In this context, we demonstrated that the shRNAs did not affect the cell viabilities (Fig. 1F). We next examined the levels of HCV Core and the infectivity of HCV in the culture supernatants as well as the level of HCV RNA in the TSG101, Alix, Vps4B, or CHMP4b stable knockdown RSc cells 97 h after HCV-JFH1 infection at an MOI of 0.1. The results showed that the release of HCV Core into the culture supernatants