

**Figure 6. Immunization with rVV-N25 suppresses serum inflammatory cytokine levels.** (A) Daily cytokine levels in the serum of CN2-29<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup> mice during the week following immunization with LC16m8, rVV-CN2, rVV-N25, or rVV-CN5. Values represent means  $\pm$  SD (n = 3) and reflect the concentrations relative to those measured on day 0. The broken lines indicate the baseline data from wild-type mice. In all cases, n = 6 mice per group. (B) Liver sections from CN2-29<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup> and CN2-29<sup>(+/-)</sup>/MxCre<sup>(-/-)</sup> mice. (C) Histology activity index (HAI) scores of liver samples taken from CN2-29<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup>, or CN2-29<sup>(+/-)</sup>/MxCre<sup>(-/-)</sup> mice. (D) Liver sections from CN2-29<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup> mice in which TNF- $\alpha$  was neutralized and the IL-6 receptor was blocked. The scale bars indicate 50  $\mu$ m. (E) HAI scores of liver samples taken from CN2-29<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup> in which TNF- $\alpha$  was neutralized and the IL-6 receptor was blocked. Tg and non-Tg indicate CN2-29<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup> and CN2-29<sup>(+/-)</sup>/MxCre<sup>(-/-)</sup>, respectively. (F) Macrophages were the main producers of TNF- $\alpha$  and IL-6 in CN2-29<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup> mice following poly(I:C) injection. (G) Immunization with rVV-N25 reduced the number of macrophages in liver samples from CN2-29<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup> mice and suppressed TNF- $\alpha$  and IL-6 production from macrophages (Figure 6G). Significant relationships are indicated by a P-value. doi:10.1371/journal.pone.0051656.g006

hypothesis that inflammatory mediators played a key role in inducing hepatitis. Furthermore, to investigate whether TNF- $\alpha$  and IL-6 played particularly critical roles in the pathogenesis of chronic hepatitis in the transgenic mice, we neutralized TNF- $\alpha$  and blocked the IL-6 receptor in the livers of these mice. As expected, chronic hepatitis did not develop in these mice. (Figure 6D and E).

Next, to determine which cell population(s) produced TNF- $\alpha$ , IL-6, or both during continuous HCV expression in CN2-29<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup> mice, we isolated intrahepatic lymphocytes (IHLs) and labeled the macrophages (the F4/80<sup>+</sup> cells) with anti-TNF- $\alpha$  and anti-IL-6 antibodies using an intracellular cytokine detection method. Macrophages in CN2-29<sup>(+/-)</sup>/MxCre<sup>(-/-)</sup> mice produced small amounts of TNF- $\alpha$  and IL-6, while those in CN2-29<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup> mice produced much larger amounts of these cytokines (Figure 6F).

Finally, we evaluated whether rVV-N25 treatment affected the number of macrophages, cytokine production by macrophages, or both; specifically, we isolated IHLs from CN2-29<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup> mice 7 days after immunization with rVV-N25 or with LC16m8. The percentage of macrophages (CD11b<sup>+</sup>F4/80<sup>+</sup>) among IHLs and IL-6 production from these macrophages were significantly lower in rVV-N25-treated mice than in control mice (Figure 6G). Though the percentage of TNF- $\alpha$ -producing macrophages was not significantly different in rVV-N25-treated and control mice (P = 0.099), rVV-N25 treatment appeared to suppress these macrophages. These results demonstrated that rVV-N25 had a suppressive effect on activated macrophages, and they indicated that this suppression ameliorated the histological indicators of chronic hepatitis.

## Discussion

Various HCV transgenic mouse models have been developed and used to examine immune response to HCV expression and the effects of pathogenic HCV protein on hepatocytes [4,16,17]. However, these transgenic mice develop tolerance to the HCV protein; therefore, examining immune response to HCV protein has been difficult.

To overcome the problem of immune tolerance in mouse models of HCV expression, we developed an HCV model in mice that relies on conditional expression of the core, E1, E2, and NS2 proteins and the Cre/loxP switching system [5,6]; we showed that the injection of an Ad-Cre vector enhanced the frequency of HCV-specific activated CD8 T cells in the liver of these mice and caused liver injury. However, the Ad-Cre adenovirus vector alone causes acute hepatitis in wild-type mice. Nevertheless, the transgenic model was useful for evaluating interactions between the host immune system and viral protein (serum ALT level over 2,000 IU/L) [5]; HCV core protein levels were reduced and expression of this protein was transient (about 2 weeks). Therefore, this Ad-Cre-dependent model cannot be used to effectively investigate immune responses to chronic HCV hepatitis.

Here, we used poly (I:C)-induced expression of Cre recombinase to generate HCV transgenic mice in order to study the effect of HCV protein and confirmed that these mice developed chronic active hepatitis—including steatosis, lipid deposition, and hepatocellular carcinoma. These pathological findings in the transgenic mice were very similar to those in humans with chronic hepatitis C; therefore, this mouse model of HCV may be useful for analyzing the immune response to chronic hepatitis. However, experimental results obtained with this mouse model may not directly translate to clinical findings from patients with HCV infection because the expression of HCV proteins was not liver specific in these mice. Furthermore, poly(I:C) injection can activate innate immune responses and, consequently, might induce temporary liver injury [18]. Additionally, poly(I:C) injection has an adjuvant effect; specifically, it stimulates TLR3 signaling [19].

To evaluate whether poly(I:C) injection caused hepatitis in CN2-29<sup>(+/-)</sup>/MxCre<sup>(-/-)</sup> mice, we examined serum ALT levels and liver histology following poly(I:C) injection. We found that, following poly(I:C) injection, serum ALT levels in CN2-29<sup>(+/-)</sup>/MxCre<sup>(-/-)</sup> mice increased, reached a peak one day after injection, declined from day 1 to day 6, and were not elevated thereafter; this time-course indicated that poly(I:C) injection alone did not induce continuous liver injury (figure S6). Based on these findings, we believe that the effects of poly(I:C) injection in these mice did not confound our analysis of chronic hepatitis.

Immunization with rVV-N25 suppressed HCV protein levels in the liver, and this suppression was associated with ameliorated pathological chronic hepatitis findings (see Figure 3). Importantly, rVV-N25 treatment did not cause liver injury based on the serum ALT levels; therefore, this treatment was unlikely to have cytopathic effects on infected hepatocytes. These findings provided strong evidence that rVV-N25 treatment effectively halted the progression of chronic hepatitis. Immunization with plasmid DNA or with recombinant vaccinia virus can effectively induce cellular and humoral immune responses and exert a protective effect against challenge with HCV infection [20,21]. However, findings from these previous studies revealed HCV immunization of both uninfected, naive animals and immune-tolerant animals induced a HCV-specific immune response. In the model describe here; the animals were immune competent for HCV; therefore, our findings provided further important evidence that rVV-N25 was effective in the treatment of chronic hepatitis.

In addition, we demonstrated that rVV-N25 treatment in the absence of CD4 and CD8 T cells had no effect on HCV clearance. This important observation indicated that rVV-N25-induced HCV clearance was mediated by CD4 and CD8 T cells. Many studies have shown that spontaneous viral clearance during acute HCV infection is characterized by a vigorous, broadly reactive CD4 and CD8 T-cell response. [8,22] HCV clearance and hepatocellular cytotoxicity are both mediated by CD8 antigen-specific (cytotoxic T lymphocyte) CTLs [23]. Consistent with these observations, rVV-N25 treatment effectively induced the accumulation of NS2-specific CD8 T cells, which express high levels of

CD107a and IFN- $\gamma$  in the spleen. Notably, even with rVV-N25 immunization, the frequency of activated CD8 T cells was very low, and a minimum of 2-weeks incubation was required to distinguish the difference between rVV treatments. Even if a small population of specific CD8+ T cells played a relevant role in the reduction of core protein, it is difficult to assert that the only NS2-specific CD8+ T cells were important to this reduction. However, based on the results presented in Figure 4B, we are able to conclude that at least CD8+ and/or CD4+ T cells were important to the reduction in HCV core protein. Therefore, to elucidate the mechanism of HCV protein clearance, further investigation of not only the other T cell epitopes but also other immunocompetent cells is required.

Interestingly, rVV-N25 treatment—but not the rVV-CN2 or rVV-CN5 treatment—efficiently induced a HCV-specific activated CD8 T cells response; this difference in efficacy could have one or more possible causes. The HCV structural proteins (core, E1, and E2 proteins) in the rVV-CN construct may cause the difference; Saito et al. reported that injection with plasmid constructs encoding the core protein induced a specific CTL response in BALB/c mice [24]. Reportedly, CTL activity against core or envelope protein is completely absent from transgenic mice immunized with a plasmid encoding the HCV structural proteins, but core-specific CTL activity is present in transgenic mice that were immunized with a plasmid encoding the HCV core [21]. In contrast, when recombinant vaccinia virus expressing different regions of the HCV polyprotein were injected into BALB/c mice, only the HCV core protein markedly suppressed vaccinia-specific CTL responses [25]. Thus, the HCV core protein may have an immunomodulatory function [26]. Based on these reports and our results, we hypothesize that the causes underlying the effectiveness of rVV-N25 treatment were as follows: 1) this rVV construct included the core and envelope proteins and 2) the core protein had an immune-suppressive effect on CTL induction. Therefore, we suggest that exclusion of the core and envelope antigen as immunogen is one important factor in HCV vaccine design.

Interestingly, immunization with rVV-N25 rapidly suppressed the inflammatory response; however, immunization with either of the other rVVs did not (see Figure 6A). This result indicated that rVV-N25 may modulate inflammation via innate immunity, as well as via acquired immunity. Reportedly, Toll-like receptor (TLR)-dependent recognition pathways play a role in the recognition of poxviruses [27]. TLR2 and TLR9 have also been implicated in the recognition of the vaccinia virus [28,29]. These findings indicate that TLR on dendritic cells may modulate the immunosuppressive effect of rVV-N25 in our model of HCV infection; however, further examination of this hypothesis is required. The finding that pathological symptoms in the HCV transgenic mice were completely blocked by intravenous injection of TNF- $\alpha$  and IL-6 neutralizing antibodies indicated that the progression of chronic hepatitis depended on inflammatory cytokines in serum, rather than the HCV protein levels in hepatocytes. Lymphocytes, macrophages, hepatocytes, and adipocytes each produce TNF- $\alpha$  and IL-6 [30,31], and HCV-infected patients have elevated levels of TNF- $\alpha$  and IL-6 [32,33]. Both cytokines also contribute to the maintenance of hepato-steatosis in mice fed a high-fat diet [34], and production of TNF- $\alpha$  and IL-6 is elevated in obese mice due to the low grade inflammatory response that is caused by lipid accumulation [35]. These findings indicate that both cytokines are responsible for HCV-triggered hepato-steatosis, and anti-cytokine neutralization is a potential treatment for chronic hepatitis if antiviral therapy is not successful.

The reduction of macrophages in number might be due to the induction of apoptosis by vaccinia virus *in vitro* infection as

previously reported [36]. To understand the mechanisms responsible for the reduction of the number of macrophage, we performed another experiment to confirm whether the macrophages were infected with vaccinia virus inoculation. However, based on PCR analyses; vaccinia virus DNA was not present in liver tissue that contained macrophages (Figure S7). Furthermore, apoptosis of macrophages was not detected in liver samples (Data not shown). Based on these results, it is unlikely that the reduction in the number of macrophages was due to apoptosis induced by vaccinia virus infection. Although rVV-N25 reduced the number of macrophage, precise mechanism is still unknown. Further examination to elucidate the mechanism is required.

In conclusion, our findings demonstrated that rVV-N25 is a promising candidate for an HCV vaccine therapy. Additionally, the findings of this study indicate that rVV-N25 immunization can be used for prevention of HCV infection and as an antiviral therapy against ongoing HCV infection.

## Materials and Methods

### Ethics Statement

All animal care and experimental procedures were performed according to the guidelines established by the Tokyo Metropolitan Institute of Medical Science Subcommittee on Laboratory Animal Care; these guidelines conform to the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, Japan, 2006. All protocols were approved by the Committee on the Ethics of Animal Experiments of the Tokyo Metropolitan Institute of Medical Science (Permit Number: 11–078). All efforts were made to minimize the suffering of the animals.

### Animals

R6CN2 HCV cDNA (nt 294–3435) [37] and full genomic HCV cDNA (nt 1–9611) [38,39] were cloned from a blood sample taken from a patient (#R6) with chronic active hepatitis (Text S1). The infectious titer of this blood sample has been previously reported [40]. R6CN2HCV and R6CN5HCV transgenic mice were bred with Mx1-Cre transgenic mice (purchased from Jackson Laboratory) to produce R6CN2HCV-MxCre and R6CN5HCV-MxCre transgenic mice, which were designated CN2-29<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup> and RzCN5-15<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup> mice, respectively. Cre expression in the livers of these mice was induced by intraperitoneal injection of polyinosinic acid–polycytidylic acid [poly(I:C)] (GE Healthcare UK Ltd., Buckinghamshire, England); 300  $\mu$ L of a poly(I:C) solution (1 mg/mL in phosphate-buffered saline [PBS]) was injected three times at 48-h intervals. All animal care and experimental procedures were performed according to the guidelines established by the Tokyo Metropolitan Institute of Medical Science Subcommittee on Laboratory Animal Care.

### Histology and Immunohistochemical Staining

Tissue samples were fixed in 4% paraformaldehyde in PBS, embedded in paraffin, sectioned (4- $\mu$ m thickness), and stained with hematoxylin and eosin (H&E). Staining with periodic acid–Schiff stain, Azan stain, silver, or Oil-red-O was also performed to visualize glycogen degeneration, fibrillization, reticular fiber degeneration, or lipid degeneration, respectively.

For immunohistochemical staining, unfixed frozen liver sections were fixed in 4% paraformaldehyde for 10 min and then incubated with blocking buffer (1% bovine serum albumin in PBS) for 30 min at room temperature. Subsequently, the sections were incubated with biotinylated mouse anti-HCV core mono-

clonal antibody (5E3) for 2 h at room temperature. After being washed with PBS, the sections were incubated with streptavidin–Alexa Fluor 488 (Invitrogen). The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Fluorescence was observed using a confocal laser microscope (Laser scanning microscope 510, Carl Zeiss).

### Generation of rVVs

The pBR322-based plasmid vector pBMSF7C contained the ATI/p7.5 hybrid promoter within the hemagglutinin gene region of the vaccinia virus, which was reconstructed from the pSFJ1-10 plasmid and pBM vector [41,42]. Separate full-length cDNAs encoding either the HCV structural protein, nonstructural protein, or all HCV proteins were cloned from HCV R6 strain (genotype 1b) RNA by RT-PCR. Each cDNA was inserted into a separate pBMSF7C vector downstream of the pBMSF7C ATI/p7.5 hybrid promoter; the final designation of each recombinant plasmid was pBMSF7C-CN2, pBMSF7C-N25, or pBMSF7C-CN5 (Figure 2). They were then transfected into primary rabbit kidney cells infected with LC16m8 (multiplicity of infection = 10). The virus–cell mixture was harvested 24 h after the initial transfection by scrapping; the mixture was then frozen at  $-80^{\circ}\text{C}$  until use. The hemagglutinin-negative recombinant viruses were cloned as previously described [42] and named rVV-CN2, rVV-N25, or rVV-CN5. Insertion of the HCV protein genes into the LC16m8 genome was confirmed by direct PCR, and expression of each protein from the recombinant viruses was confirmed by western blot analysis. The titers of rVV-CN2, rVV-N25, and rVV-CN5 were determined using a standard plaque assay and RK13 cells.

### Statistical Analysis

Data are shown as mean  $\pm$  SD. Data were analyzed using the nonparametric Mann–Whitney or Kruskal–Wallis tests or ANOVA as appropriate; GraphPad Prism 5 for Macintosh (GraphPad) was used for all analyses.  $P$  values  $<0.05$  were considered statistically significant.

### Supporting Information

**Figure S1 HAI score of liver samples taken from CN2-29<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup> mice.**

(EPS)

**Figure S2 Lipid degeneration in samples of liver taken from CN2-29<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup> mice.**

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(EPS)

**Figure S3 HCV protein expression after infection of LC16m8, rVV-CN2, rVV-N25, or rVV-CN5 into HepG2 cells.**

(EPS)

**Figure S4 Effects of treatment with rVV-N25 in RzCN5-15<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup> mice.**

(EPS)

**Figure S5 Daily cytokine profiles of the serum from CN2-29<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup> mice during the week following inoculation with LC16m8, rVV-CN2, rVV-N25, or rVV-CN5.**

(EPS)

**Figure S6 The immune response following poly(I:C) injection in the acute phase.**

(EPS)

**Figure S7 Detection of vaccinia virus DNA in the skin, liver, and spleen after inoculation with attenuated vaccinia virus (Lister strain) or highly attenuated vaccinia virus (LC16m8 strain).**

(EPS)

**Table S1 Incidence of hepatocellular carcinoma in male and female transgenic mice at 360, 480, and 600 days after poly(I:C) injection.**

(EPS)

**Text S1 Supporting information including material and methods, and references.**

(DOCX)

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### Author Contributions

Performed the experiments: SS KK TC Y. Tobita TO FY Y. Tokunaga. Analyzed the data: SS KK TC MK. Contributed reagents/materials/analysis tools: KT-K TW TT MM K. Mizuno YH TH K. Matsushima. Wrote the paper: SS KK MK. Study concept and design: MK.

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Original article

# Selective estrogen receptor modulators inhibit hepatitis C virus infection at multiple steps of the virus life cycle

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## Abstract

We screened for hepatitis C virus (HCV) inhibitors using the JFH-1 viral culture system and found that selective estrogen receptor modulators (SERMs), such as tamoxifen, clomifene, raloxifene, and other estrogen receptor  $\alpha$  (ER $\alpha$ ) antagonists, inhibited HCV infection. Treatment with SERMs for the first 2 h and treatment 2–24 h after viral inoculation reduced the production of HCV RNA. Treating persistently JFH-1 infected cells with SERMs resulted in a preferential inhibition of extracellular HCV RNA compared to intracellular HCV RNA. When we treated two subgenomic replicon cells, which harbor HCV genome genotype 2a (JFH-1) or genotype 1b, SERMs reduced HCV genome copies and viral protein NS5A. SERMs inhibited the entry of HCV pseudo-particle (HCVpp) genotypes 1a, 1b, 2a, 2b and 4 but did not inhibit vesicular stomatitis virus (VSV) entry. Further experiment using HCVpp indicated that tamoxifen affected both viral binding to cell and post-binding events including endocytosis. Taken together, SERMs seemed to target multiple steps of HCV viral life cycle: attachment, entry, replication, and post replication events. SERMs may be potential candidates for the treatment of HCV infection.

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**Keywords:** HCV; Tamoxifen; SERM (Selective estrogen receptor modulator)

## 1. Introduction

Over 170 million people in the world are infected with the hepatitis C virus (HCV). Approximately 20% of infected patients develop cirrhosis and hepatocellular carcinoma after chronic HCV infection. No HCV vaccine is available yet, and the current standard of care, which consists of a combination of interferon (IFN) and ribavirin, is only effective for approximately 50% of infected patients, and many patients have serious side effects. Because of the urgent need for novel HCV therapeutics, research is being conducted to develop new

anti-HCV drugs. In addition to *in vitro* screening assays that target HCV-specific enzymes, other approaches that use replicon cells and the recently described Huh 7.5.1-JFH-1 (genotype 2a)-infection system have been developed [1]. The Huh 7.5.1-JFH-1-infection system is an excellent system to identify HCV inhibitors that interfere with individual steps of the HCV life cycle, such as viral attachment, entry, and release. This experimental system allows both viral and host components that are involved in HCV infection to be targeted. Although drugs that target the host components may be toxic, such drugs are unlikely to select for resistant viruses.

We screened chemicals using a cell-based screening system [2] and found that tamoxifen and other selective estrogen receptor modulators (SERMs) inhibited HCV infection. Tamoxifen has been successfully used for the treatment of breast cancer since it was found to be an ER antagonist over 30 years ago. Clomifene and raloxifene, which are compounds

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that are related to tamoxifen, have been developed and used for the treatment of breast cancer and for the treatment of anovulation and osteoporosis. Currently, these three SERMs and toremifene have been approved in Japan and the US, and next-generation SERMs are undergoing clinical evaluation.

Because tamoxifen exhibited the ability to inhibit HCV infection, we determined which SERMs could effectively inhibit HCV infection and be approved for clinical use. The first-generation SERMs—tamoxifen, clomifene, and raloxifene—were all effective against HCV as were other ER $\alpha$  antagonists. We examined whether SERMs could be utilized as new drugs for the treatment of HCV.

## 2. Materials and methods

### 2.1. Cells and virus

Human hepatoma cell line, Huh 7.5.1 cells and human embryonic kidney 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma–Aldrich Co. St. Louis, MO, USA) with 10% fetal bovine serum (FBS). HCV-JFH-1 (HCVcc) (genotype 2a) was the culture supernatant of infected Huh 7.5.1 cells as described previously [2]. A sub-genomic replicon cell line, clone #4-1, which harbors the genotype 2a (JFH-1) [3,4], and clone #5-15, that harbors the genotype 1b HCV genome [5], were also cultured in DMEM with FBS.

### 2.2. Chemicals

The SCADS inhibitor kit I was provided by the Screening Committee of Anticancer Drugs, supported by a Grant-in-Aid for Scientific Research on the Priority Area “Cancer” from The Ministry of Education, Culture, Sports, Science and Technology of Japan. Tamoxifen, diethylstilbestrol, triphenylethylene, 17 $\beta$ -estradiol, and brefeldin A were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Clomifene was purchased from LKT Laboratories, Inc. (St. Paul, MN, USA), and hydroxytamoxifen ((*z*)-4-hydroxytamoxifen) and raloxifene were purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). Chloroquine was purchased from WAKO (Osaka, Japan). Other chemicals were purchased from Tocris Bioscience (Bristol, UK).

### 2.3. Quantification of the viral titer in medium

Huh 7.5.1 cells were seeded in 96-well plates at a density of  $2 \times 10^4$  cells per well in a volume of 120  $\mu$ l. The next day, 15  $\mu$ l of media that contained the test compound and 15  $\mu$ l of the HCVcc virus stock solution at a moi of 0.01 were added to each well. After 5 days, 100  $\mu$ l of the culture supernatant was taken from each well, and viral RNA was extracted. Total RNA was also extracted from the cells. Quantitative real-time RT-PCR was then performed with One step SYBR PrimeScript RT-PCR Kit (Takara-Bio Co., Otsu, Japan) as described previously [2]. In the case of #4-1 replicon cell, as an internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured with primers 5'-CCACCCATGGCAAATTCC-3' and

5'-TGGGATTTCCATTGAT-3'. Cell growth was monitored using the MTT assay as described previously [6].

### 2.4. Western blotting

Western blotting was performed as previously described [2]. Briefly, cell lysates that contained equal quantities of protein were separated by SDS-PAGE, transferred onto PVDF membranes, and probed with antibodies against the core antigen (2H9), NS5A (Austral Biologicals, San Ramon, CA, USA), or GAPDH (Santa Cruz Biotech. Inc., Santa Cruz, USA). After incubation with horseradish peroxidase-conjugated secondary antibodies, the protein bands on the PVDF membranes were detected using an ECL system (GE Healthcare UK Ltd., Amersham Place, UK).

### 2.5. Production of and infection with pseudo-particles

HCV pseudo-particles (HCVpp) were generated using the following 3 plasmids: a Gag-Pol packaging construct (Gag-Pol 5349), a transfer vector construct (Luc 126), and a glycoprotein-expressing construct (HCV E1E2) (JFH-1, 2a). The generation of the pseudo-particles was performed according to the method described by Bartosch et al. [7]. To express the glycoproteins of other HCV genotypes, HCV E1E2 constructs of the genotypes 1a (H77), 1b (UKN1B 12.6), 2b (UKN2B 2.8), and 4 (UKN4 11.1) were generously provided by Dr. F. Cosset (INSERM, France) [8]. To produce VSVpp, a plasmid that coded the vesicular stomatitis virus (VSV) envelope, pCAG-VSV, was generously provided by Dr. Y. Matsuura (Osaka University, Japan). Gag-Pol 5349 (3.1  $\mu$ g), Luc 126 (3.1  $\mu$ g), and each of the individual glycoprotein-expression constructs (1.0  $\mu$ g) were co-transfected into 293T cells that were seeded on a 10-cm dish ( $2.5 \times 10^6$  cells) using TransIT-LT1 Transfection Reagent (21.6  $\mu$ l) (Mirus Bio LLC, Madison, WI, USA). The medium from the transfected cell cultures was harvested and used as the pseudo-particle stock. For the infection assay, Huh 7.5.1 cells were seeded onto a 48-well plate at a density of  $4 \times 10^4$  cells per well one day prior to infection. The medium was then removed, and the cells were subsequently infected with the pseudo-particles in the presence or absence of drug. The cells were then incubated for 3 h. The VSVpp preparation was diluted (1:600) to infect with similar RLU activity compared to the HCVpp. The supernatant was then removed, fresh culture medium was added to the cells, and the cells were incubated for an additional 3 days. The luciferase assays were performed using a luciferase assay system (Promega Co. Madison WI, USA). Anti-CD81 antibody (sc-23962) was purchased from Santa Cruz Biotech.

## 3. Results

### 3.1. Tamoxifen and estrogen receptor $\alpha$ antagonists inhibited HCV infection

Using quantitative RT-PCR, we screened the compounds in the SCADS inhibitor kit I. Drugs and HCVcc at a moi of 0.01

were added to Huh 7.5.1 cells. Five days later, the quantity of HCV RNA in the culture supernatant was measured using quantitative real-time RT-PCR [2]. We found that tamoxifen reduced the levels of JFH-1 RNA in the culture supernatant. We also examined the effects of other SERMs and agonists and antagonists of ER $\alpha$ . As shown in Fig. 1, tamoxifen, clomifene, and hydroxytamoxifen, which have a triphenylethylene backbone, exhibited intense inhibitory effects (EC<sub>50</sub>: approximately 0.1  $\mu$ M). Triphenylethylene showed reduced inhibitory activity (data not shown). Raloxifene also inhibited viral RNA production at a similar concentration. (EC<sub>50</sub>: approximately 0.1  $\mu$ M) (Fig. 1a). Tamoxifen and raloxifene display both ER $\alpha$  antagonist and agonist properties in a dose- and tissue-dependent manner [9]. In contrast, ICI 182,780 (fulvestrant), ZK164015, and MPP (methyl-piperidino-pyrazole) are exclusively antagonistic [10–12]. These ER $\alpha$  antagonists also showed inhibitory activity against JFH-1, but their EC<sub>50</sub> values were approximately 1  $\mu$ M (Fig. 1b). As the 50% toxic concentrations (TC<sub>50</sub>) for these compounds were observed to be greater than 10  $\mu$ M (Fig. 1a and b), these specific indexes are over 100. In contrast, the ER $\alpha$  agonists 17 $\beta$ -estradiol, diethylstilbestrol, and PPT (1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole) did not inhibit HCV (Fig. 1c). As expected, the SERMs that were observed to effectively inhibit HCV RNA production also reduced the core protein levels intracellularly (Fig. 1d).

### 3.2. SERMs inhibited more than one step of the JFH-1 life cycle

To determine which step of the JFH-1 life cycle was inhibited by the SERMs studied, we performed time-of-addition experiments. As described previously [2], JFH-1 appears to complete one infectious life cycle in approximately 48 h. Huh 7.5.1 cells were inoculated with JFH-1-containing medium (moi 0.1) with or without drug and were then incubated for 2 h. After the medium was removed, fresh medium with or without drug was added. The cells were then incubated for another 46 h. Treatment with 10  $\mu$ M tamoxifen for 48 h reduced the amount of viral RNA in the medium to 1.7% of levels observed in the control. Treatment with tamoxifen for the first 2 h after infection (0–2 h) reduced viral RNA to 2.3% of the levels observed in the control. The addition of tamoxifen to the fresh medium just after the removal of the virus (2–48 h) resulted in a reduction in the amount of viral RNA to 10.7% of the levels observed in the control. The addition of tamoxifen 24 h after viral inoculation (24–48 h) resulted in a decrease in the amount of viral RNA to 60% of the levels observed in the control (Fig. 2a). This result suggests that tamoxifen inhibits mainly viral entry and some steps during replication. 10  $\mu$ M of raloxifene exhibited a similar inhibitory pattern but less inhibited by the treatment after the entry step (Fig. 2b). A pure ER $\alpha$  antagonist, ICI 182,780 (30  $\mu$ M), also exhibited inhibition of both viral entry and the replication steps, but the inhibition of the entry step was not so marked (Fig. 2c).

To further investigate effect on HCV post replication, we infected HCV in the presence of the drugs for 72 h (moi 0.1)

and examined their effects on intracellular and extracellular HCV RNA levels. Brefeldin A, an inhibitor of protein transport [13], was used as a positive control of post replication inhibition. In this experimental setting, brefeldin A showed intracellular HCV RNA accumulation suggesting post replication inhibition (Fig. 2d). SERMs generally reduced HCV RNA in cell as well as HCV RNA in medium, although the extent of reduction was different (Fig. 2d). Lower concentration of SERMs reduced extracellular HCV RNA more robustly than intracellular HCV RNA. At a concentration of 0.1  $\mu$ M, tamoxifen exclusively inhibited HCV RNA in the culture supernatant but not intracellular HCV RNA levels, in a manner similar to that of brefeldin A (Fig. 2d). The results suggest that SERMs inhibit post replication step(s) such as assembly or release. Because low concentrations of tamoxifen failed to inhibit intracellular HCV RNA, SERMs potentially target post replication step(s) more efficiently than replication step. In this condition, higher concentrations (1 and 3  $\mu$ M) of tamoxifen seemed to inhibit intracellular HCV RNA rather than extracellular HCV RNA, although the reason is not clear.

To determine the effect of these drugs on chronic infection, we used pre-infected Huh 7.5.1 cells. We infected the cell with HCVcc at a moi of 0.01 and incubated for 3 days. Three days after infection, the drugs were added, and the cells were further incubated for 48 h. At the time of drug addition, the cells were persistently infected, and HCVcc was continuously produced and released into the culture supernatant, which is similar condition to chronic infection. HCV RNA was extracted from the culture supernatant and the cells after 48 h and measured copy number of HCV RNA. Both HCV RNA in the culture supernatant and that in the cell were reduced by treatment with the SERMs, but the intracellular HCV RNA levels were less reduced (Fig. 2e). This suggested that the SERMs caused preferential reduction in extracellular HCV RNA through interference with some post replication step(s), such as assembly or release. Brefeldin A accumulated intracellular HCV RNA, and reduced HCV RNA level in the culture supernatant (Fig. 2e).

These data suggested that the SERMs inhibit multiple steps in the HCV life cycle: entry, viral RNA replication and some post replication step(s).

### 3.3. SERMs inhibited copies and NS5A protein expression in replicon cells

To confirm the effect of these drugs on viral replication, we used two subgenomic replicon cells. The subgenomic replicon cells, derived from Huh7 cells, harbor HCV viral RNA that replicates autonomously, and they express viral proteins. We treated cells that harbored a subgenomic replicon (#4-1, genotype 2a) [3,4] with the SERMs for 48 h and measured the amount of cellular replicon RNA by quantitative RT-PCR. Treatment with 10  $\mu$ M of tamoxifen, raloxifene, or 3  $\mu$ M of clomifene, inhibited HCV RNA compare to GAPDH RNA, although statistical significance was shown in only the inhibition of 10  $\mu$ M of tamoxifen. ICI 182,780 did not show specific inhibition of HCV RNA (Fig. 3a).



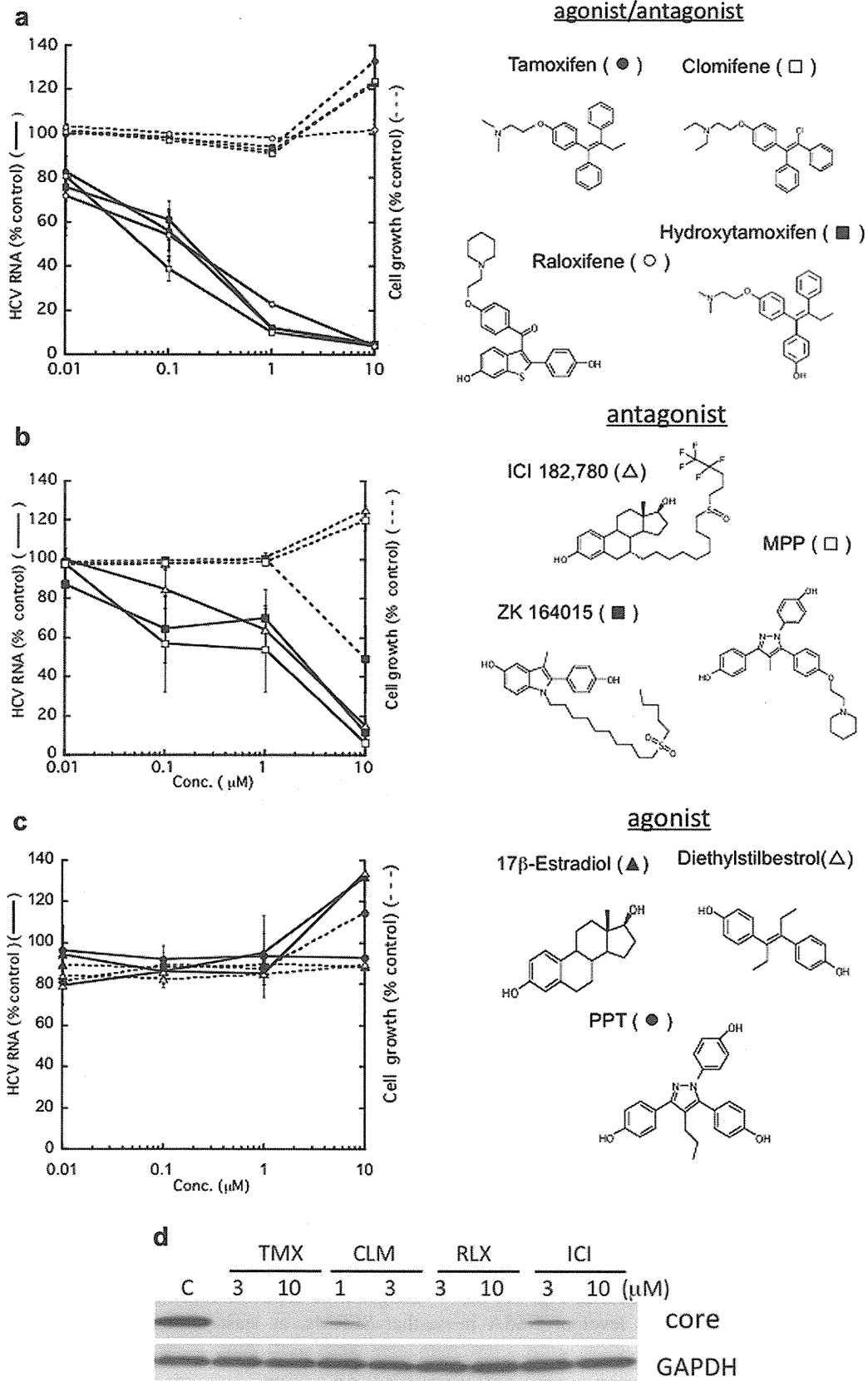


Fig. 1. Effects of SERMs on JFH-1 HCV RNA levels. a) Effects of tamoxifen, clomifene, and raloxifene. Huh 7.5.1 cells were infected with HCV JFH-1 (moi 0.01) in the presence of drugs and were incubated for 5 days. Drugs were added just before viral inoculation. HCV RNA in the medium was measured by tube-capture-RT-PCR [2]. Parallel cultures of cells without virus were analyzed using the MTT assay to detect the inhibition of cell growth due to drug exposure. Tamoxifen (closed circles), clomifene (open rectangles), hydroxytamoxifen (closed rectangles), and raloxifene (open circles). The percentages to control HCV RNA and

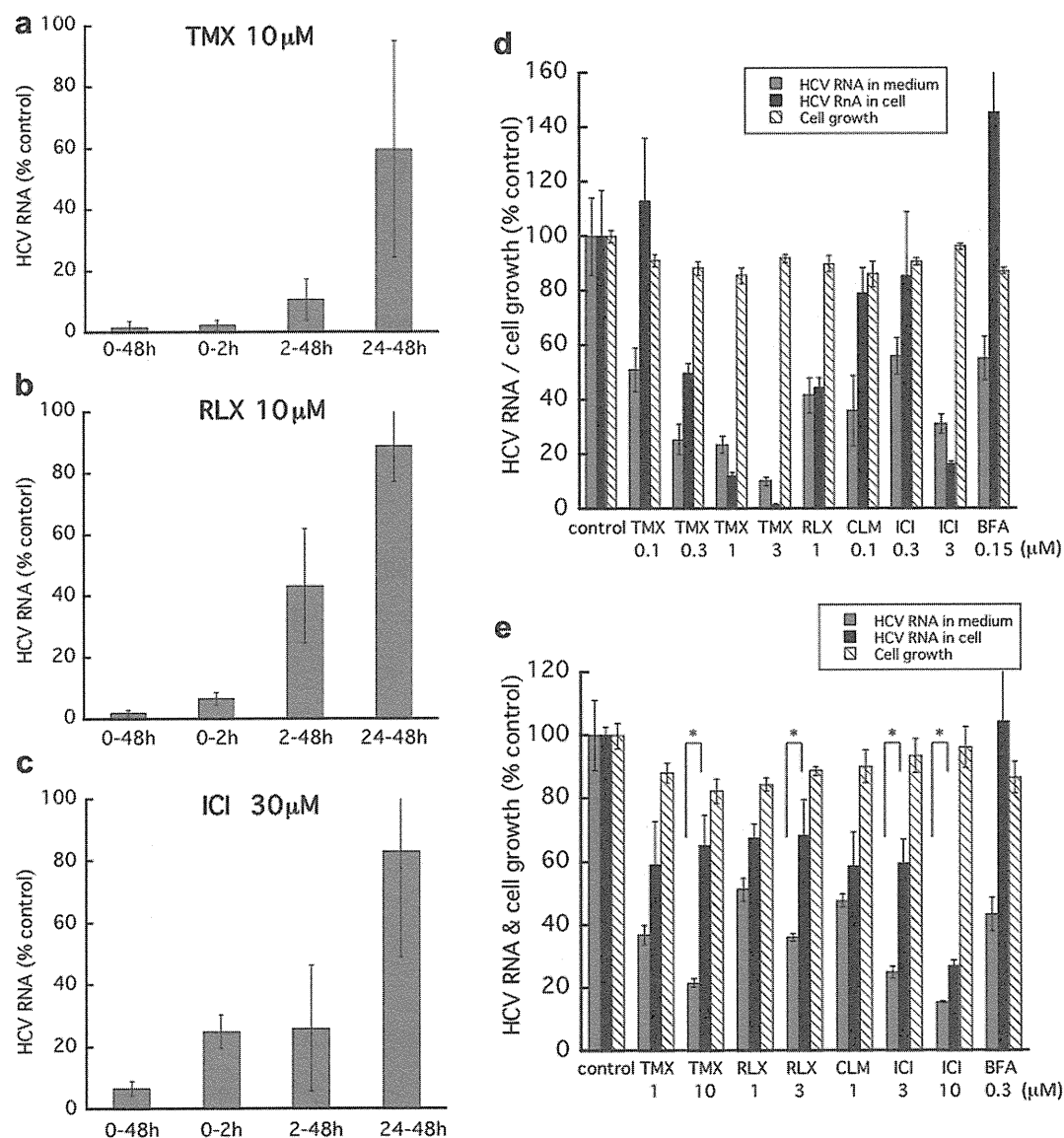


Fig. 2. Time-of-addition experiments (a–c) and the effect of SERMs on extracellular and intracellular HCV RNA in simultaneous infection (d) and in persistent infection (e). Huh 7.5.1 cells were treated with tamoxifen (TMX) (10 μM, a), raloxifene (RLX) (10 μM, b), or ICI 182,780 (ICI) (30 μM, c) during the following time periods: 0–2 h, 2–48 h, or 24–48 h after JFH-1 infection (moi 0.1). Forty-eight hours after infection, the culture supernatant was harvested, and HCV RNA was extracted and subjected to quantitative real-time RT-PCR to determine the number of copies of the JFH-1 genome. The data are the averages of three independent experiments and the standard deviation. d) Effect of treatment with SERMs for 3 days on extra- and intra-cellular HCV RNA levels. Huh 7.5.1 cells were infected with JFH-1 (moi 0.1) just after addition of the SERMs. Three days later, RNA was extracted from the cells and from the culture supernatant. The amount of HCV RNA was measured by quantitative real-time RT-PCR. Brefeldin A (BFA) was used as a positive control. e) Huh 7.5.1 cells were infected at a moi of 0.01, 3 days before addition of drugs. The infected cells were treated with SERMs for 48 h. RNA was subsequently extracted from the cells and the culture supernatant to determine the viral genome copy number. The results are presented as the percentage of control cells without drug. The data are the averages of triplicates and the error bars represent standard deviation. \**P*-value < 0.05. One representative experiment of two independent experiments is shown.

Next we treated this (#4-1, genotype 2a) and another replicon (#5-15, genotype 1b) [5] with the SERMs for 3 days and examined the effect of the compounds on the HCV NS5A protein levels by western blotting. As shown in Fig. 3b, the SERMs except ICI 182,780 reduced the level of NS5A in

accordance with the results in Fig. 3a. ICI 182,780 seemed to slightly reduce NS5A protein in #5-15 replicon cell. The SERMs did not reduce the protein levels of GAPDH in the subgenomic replicon cells (Fig. 3b). These results indicated that SERMs, at least tamoxifen, raloxifene and clomifene,

control cell growth are indicated by solid lines and dotted lines, respectively. b) Effect of the following ERα antagonists: ICI 182,780 (closed triangles), ZK164015 (closed rectangles), and MPP (open rectangles). c) Effect of the following ERα agonists: 17β-estradiol (closed triangles), diethylstilbestrol (open rectangles), and PPT (closed circles). The results are presented as percentages of the control cells that were not treated with drugs. Values are the averages of triplicates, and the error bars represent the standard deviation of the mean. One representative experiment of three independent experiments is shown. d) Huh 7.5.1 cells were infected (moi 0.01) in the presence of tamoxifen (TMX), clomifene (CLM), raloxifene (RLX), or ICI 182,780 (ICI) and incubated for 5 days. Cell lysates were blotted with anti-core and anti-GAPDH antibodies as described in the Section Materials and methods.

were effective not only against HCV genotype 2a but also HCV genotype 1b and that the compounds inhibited a HCV replication step. The growth of the replicon cells was suppressed by treatment with 10  $\mu\text{M}$  of clomifene. Clomifene at concentrations less than 10  $\mu\text{M}$  and tamoxifen, raloxifene and ICI 182,780 at 10  $\mu\text{M}$  concentration or lower did not inhibit cell growth (Fig. 3c).

### 3.4. SERMs inhibited entry of HCVpp but not VSVpp

To further examine the inhibition of early viral processes by the SERMs, we used infectious HCV pseudo-particles (HCVpp). Because HCVpp enter into cell dependent on HCV envelope protein but replicate dependent on retroviral system in the cell, we can exclude other effects of the drug except effect on HCV entry system. Pseudo-particles with the viral envelope glycoprotein mimic the entry of the parental virus, and this system has been used for investigation of HCV entry [7,8,18,20,21]. The infectious titer is determined by luciferase activity. We added tamoxifen to HCVpp- or VSVpp-

containing medium and incubated Huh 7.5.1 cells with this medium for 3 h. After washing the cells, fresh medium was added, and the cells were incubated for 3 days. Treatment with tamoxifen reduced the luciferase activity of the cells that were infected with HCVpp in a dose-dependent fashion. In contrast, the luciferase activity caused by VSVpp was not reduced by the same concentrations of tamoxifen (Fig. 4a). We also examined the effect of other SERMs, such as clomifene, raloxifene, ICI 182,780, ZK164015, and MPP, on HCVpp infection. All of these SERMs inhibited the luciferase activity caused by HCVpp but not the activity caused by VSVpp (Fig. 4b). ICI 182,780 showed a weaker effect compared to tamoxifen, clomifene and raloxifene. Next, we examined the effects of these drugs on various genotypes of HCVpp. Although the extent of inhibition was varied, the compounds inhibited all of the genotypes that were examined (Fig. 4c). At a concentration of 10  $\mu\text{M}$ , ICI 182,780 inhibited all of the genotypes of HCVpp other than genotype 2a. These results suggested that the SERMs inhibit entry of all genotypes of HCV.

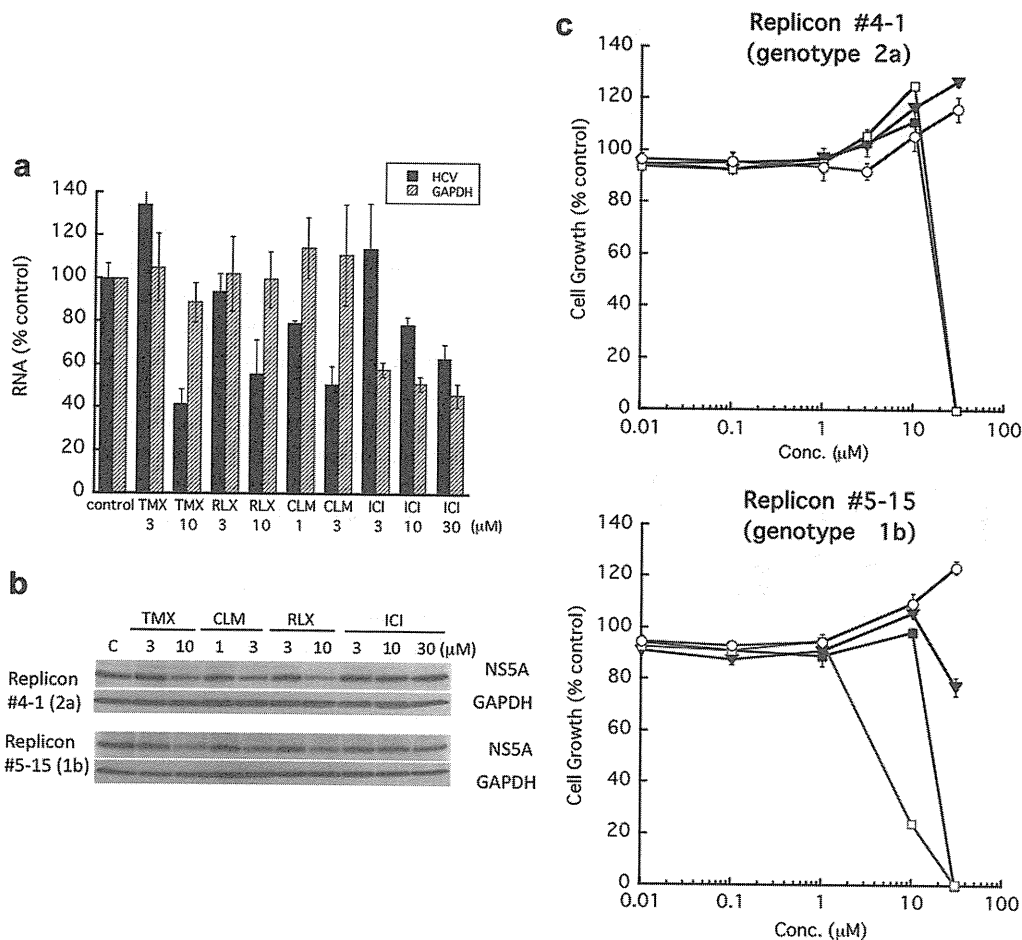


Fig. 3. The effect of SERMs on cells that harbored a subgenomic replicon. A subgenomic-replicon-harboring cell line clone #4-1 (genotype 2a) was treated with SERMs for 48 h. The total RNA was extracted from the cells, and amount of HCV RNA genome was measured. As an internal control, relative amount of GAPDH RNA was measured and indicated as percentage of control cells without drug (a). Another subgenomic-replicon-harboring cell line, clone #5-15 (genotype 1b) was treated with SERMs for 3 days. Cell lysates were subjected to western blotting with an anti-NS5A antibody or an anti-GAPDH antibody (b). Cells that were grown for 3 days in the presence of tamoxifen (closed rectangles), clomifene (open rectangles), raloxifene (closed triangles), or ICI 182,780 (open circles) were measured using the MTT assay. Cell growth is expressed as a percentage of control cells without drug (c). The values are the average of triplicate and the error bars represent the standard deviation of the mean. One representative experiment of two independent experiments is shown.

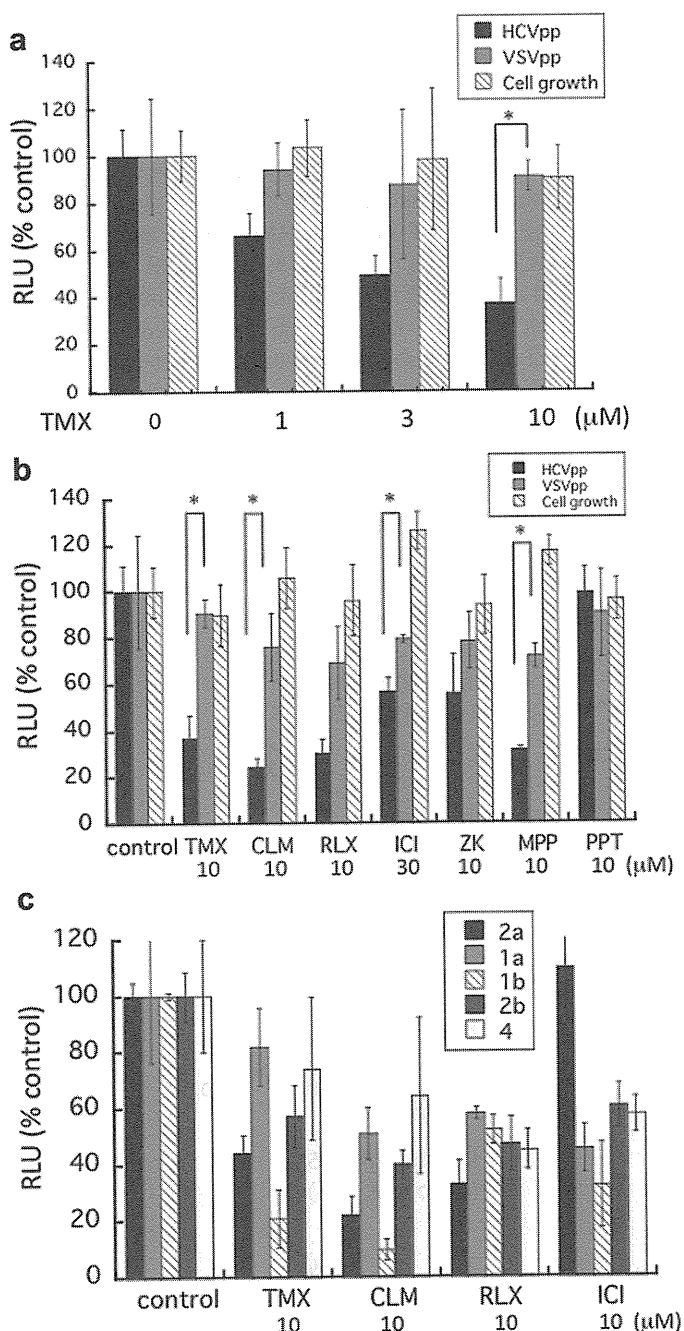


Fig. 4. Effect of SERMs on HCV pseudo-particle (HCVpp) infection. a) Huh 7.5.1 cells were incubated with pseudo-particles (HCVpp or VSVpp) in the presence or absence of tamoxifen for 3 h. The supernatants were removed, medium was added back to the cell cultures, and the cells were incubated for another 3 days. The VSVpp preparation was diluted 600 times so it was infected at similar RLU activity levels compared to HCVpp (approximately 5000 RLU). A parallel culture without pseudo-particles was analyzed using the MTT assay to evaluate the effect of the drugs on cell growth. b) Effects of various SERMs on HCVpp and VSVpp infection. c) Effects of SERMs on the various genotypes of HCVpp infection. The control luciferase activities were approximately 5000 RLU (genotype 2a), 3000 RLU (genotype 1a), 2400 RLU (genotype 1b), 3900 RLU (genotype 2b), and 860 RLU (genotype 4). The values are expressed as the percentage of control cells without drug. The data are the averages of three wells and the error bars are the standard deviation of the mean. \* $P$ -value < 0.05. One representative experiment of three independent experiments is shown.

### 3.5. Effect of tamoxifen on the attachment and entry steps

To better understand how tamoxifen blocks HCV entry, we performed an experiment to discriminate between the inhibition of HCV attachment to cells and the inhibition of post-binding entry events. HCV attaches to several cellular receptors via its E1 and E2 envelope proteins and enters via clathrin-mediated endocytosis [14–16]. We used HCVpp because infection with HCVpp is thought to simulate HCV entry [7,17,18] and the entry is independent of HCV replication. HCVpp binding to the cellular receptors was performed at 4 °C for 1.5 h. Under these conditions, HCVpp bind to the cells but entry is not efficient. The inoculum was removed, and fresh medium was added to the cells. The cells were subsequently incubated at 37 °C. In protocol I, the drug was administered during the binding step at 4 °C. After the shift to 37 °C, treatment with the drug was performed during first hour (protocol II) or after 1 h at 37 °C (protocol III) to distinguish between the inhibition of early and late post-binding events (Fig. 5a). The inoculum was removed after treatment, and fresh medium was added to the cells. We used chloroquine, a lysosome-tropic agent, as a control inhibitor for early entry (protocol II) [19]. We also used an anti-CD81 antibody that specifically inhibits HCV entry through the inhibition of the HCV cellular receptor protein CD81 at early entry [20,21]. As expected, chloroquine inhibited luciferase activity when the cells were treated during the early post-binding step (protocol II). This result suggested that endocytosis occurred primarily during the first post-binding period (protocol II). Anti-CD81 markedly inhibited luciferase activity during protocol II as reported [18,19]. Tamoxifen treatment did not result in clear differences between the protocols and the compound displayed similar activity regardless of the treatment period (Fig. 5b left). As a control, the same experiment was performed using VSVpp. Chloroquine inhibited the early entry step of VSVpp, but anti-CD81 and tamoxifen did not show any inhibition (Fig. 5b right).

Tamoxifen is a lipophilic weak base and inhibits acidification intracellularly [22]. Therefore, we examined whether the inhibition of the endocytosis of HCVpp by tamoxifen was dependent on its function as a weak base. Chloroquine is a weak base and inhibits endosome acidification. The pH sensitivity is considered a good indication of clathrin-dependent endocytosis. Previous reports have indicated that chloroquine inhibited HCVcc and HCVpp infection [14,19]. We adjusted the medium to pH 5.5 and incubated the cells in this acidic medium in the presence or absence of tamoxifen for 2 h post-binding. The acidification of the medium did not affect either the entry of HCVpp or the cell growth (Fig. 5c). Treatment with tamoxifen in the medium with a normal pH (pH 7.1) reduced HCVpp entry, and treatment with the drug in the acidic medium also reduced entry to a similar extent. In contrast, chloroquine treatment in regular medium reduced HCVpp entry, but entry was restored in the acidic medium (Fig. 5c). These results indicate that the inhibitory effect of tamoxifen was not dependent on the function of this compound as a base, unlike the effects of chloroquine.

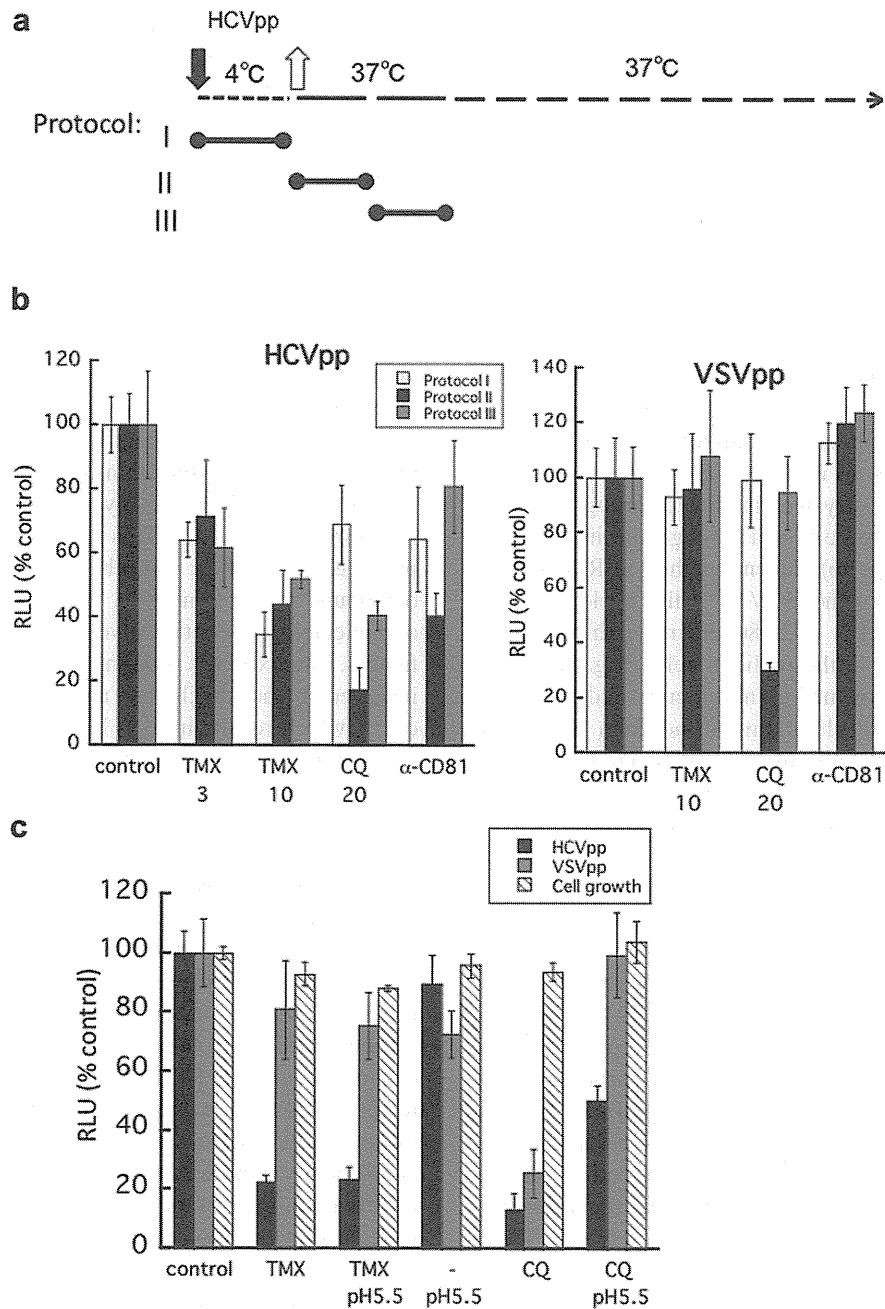


Fig. 5. Effect of tamoxifen on the attachment and endocytosis of HCVpp. a) Experimental design. HCVpp attachment to cells was performed at 4 °C for 1.5 h in the presence or absence of drug. Under these conditions, HCVpp bind to the cells but do not efficiently enter the cells. The inoculum was then removed, and fresh medium was added to the cells. The cells were subsequently incubated at 37 °C. The cells were treated with drug during the binding period at 4 °C (protocol I), during first hour after the shift to 37 °C (protocol II), or 1 h after the shift to 37 °C (protocol III). The drug-containing medium was removed for every treatment, and new medium was supplied to the cells. b) Effects of tamoxifen (TMX) (3 and 10  $\mu$ M), chloroquine (CQ) (20  $\mu$ M) and anti-CD81 antibody (20  $\mu$ g/ml) on HCVpp attachment (protocol I) and post-binding events (protocol II and III) (left). Effects of tamoxifen, chloroquine and anti-CD81 on VSVpp (right). c) Effects of exposure to low pH on the inhibition of HCVpp entry by tamoxifen and chloroquine. The cells were incubated with HCVpp at 4 °C for 1.5 h in the absence of drug. After removing the inoculum, regular (pH 7.1) or acidic medium that was adjusted with HCl to pH 5.5, either with or without drug (tamoxifen, 10  $\mu$ M, chloroquine, 20  $\mu$ M), was added to the cells. The cells were subsequently incubated at 37 °C. The drug-containing medium was removed after 2 h of incubation, and the cells were incubated for an additional 3 days with fresh, regular medium. The values are expressed as the percentage of control cells without drug. The data are the averages of three wells and the error bars represent the standard deviation of the mean. One representative experiment of three independent experiments is shown.

#### 4. Discussion

We screened for HCV inhibitors using the JFH-1-Huh 7.5.1 cell culture system and found that tamoxifen and ER $\alpha$  antagonists, but not ER $\alpha$  agonists, inhibited HCV JFH-1

infection. Although there are some reports about the HCV inhibitory effects of tamoxifen and other SERMs, we presented further information about the inhibitory effects of these substances. The time-of-addition experiments (Fig. 2a–c) suggested that these SERMs inhibit the entry and replication

steps in the HCV life cycle. These SERMs, except ICI 182,780, reduced level of HCV genome (genotype 2a) and NS5A (genotypes 1b and 2a) in the subgenomic replicon cells (Fig. 3), which supports the hypothesis that the inhibitory effect of the SERMs occurred during the replication steps. Further we observed that SERMs preferentially reduced extracellular HCV RNA compare to intracellular HCV RNA in the newly (Fig. 2d) and persistently infected cells (Fig. 2e). It suggests that the SERMs also target post replication step(s) in the viral life cycle, such as assembly and release. A low concentration of tamoxifen (0.1  $\mu\text{M}$ ) accumulated intracellular HCV RNA (Fig. 2d), which suggests that SERMs target post replication step(s) more efficiently than replication steps. Additionally, these compounds inhibited HCVpp infection (Fig. 4), which supports an inhibitory effect during the entry step. The inhibition of entry was mediated through the inhibition of viral binding to cells and through the inhibition of a post-binding event (Fig. 5b). Taken together, SERMs seemed to target multiple steps of the HCV viral life cycle.

Among the SERMs, ICI 182,780 did not exhibit distinct inhibition of replication in the replicon cells (Fig. 3a and b), although the compound seemed to inhibit entry and replication steps according to the result of time-of-addition experiment (Fig. 2c). The replicon cells are derived from Huh 7 cell. Although viral sensitivity to the SERMs might be dependent on the cell that HCV infect, it remains unclear whether ICI 182,780 inhibits replication step or not. However, the compound affected post replication step in a similar manner to other SERMs (Fig. 2d and e). It is unlikely that ICI 182,780 is an inhibitor with different mechanisms.

The inhibitory effect of tamoxifen on HCV replication has been previously reported by Watashi et al. [23]. They also demonstrated that ICI 182,780 inhibited HCV replication. This effect was discovered using a cell line that harbored a subgenomic replicon (genotype 1b). Additionally, these researchers reported that RNA interference-mediated knock-down of ER $\alpha$ , not ER $\beta$ , reduced HCV replication, but the reduction was not related to ERE-mediated transactivation activity. They suggested that ER $\alpha$  interacts with the HCV viral polymerase NS5B and that ER $\alpha$  promotes the participation of NS5B with the HCV replication complex. Using the Huh 7.5.1-JFH-1 screening system, Gastaminza et al. and Chockalingam et al. found that toremifene and raloxifene, respectively, function as HCV inhibitors. Gastaminza et al. [24] reported that toremifene inhibited HCV infection by inhibiting both the entry and release steps of the viral life cycle. Chockalingam et al. [25] determined that raloxifene inhibited the entry and replication steps, as we also observed. Our results are in accordance with these previous reports and other information about the inhibitory effects of SERMs.

Pseudo-particle experiments confirmed that SERMs affected the entry step of HCV viral life cycle (Fig. 4b), although the inhibitory effects were lower compare to those against HCVcc (Fig. 2a–c). The reason for the difference in sensitivity may account for some difference in the two entry systems. Otherwise, in the treatment with the drugs for the first 2 h of HCVcc infection, some amount of the drugs might enter

the cell and remain affecting the other steps. The SERMs affected not only genotype 2a but also other genotypes of HCVpp that were examined, suggests that these chemicals have effects on various genotypes of HCV. Although the SERMs appeared to inhibit multiple steps of the HCV life cycle, the primary target step in the viral life cycle might be the entry step. In the time-of-addition experiments, treatment with tamoxifen or raloxifene during the first 2 h was more effective than treatment during the subsequent 2–48 h (Fig. 2a). These SERMs are thought to primarily prevent viral entry and to inhibit post replication step and replication at higher concentrations.

As shown in Fig. 5, an experiment that could discriminate an effect on viral attachment from an effect at the post-binding processes indicated that tamoxifen inhibited both steps. The inhibition of endocytosis by tamoxifen was not rescued by exposure to a low pH. This suggests that the observed inhibition is the result of a mechanism that is independent of the compound's function as a base. HCV entry is a highly complicated process that involves numerous viral and cellular factors. Tamoxifen is thought to target multiple steps that are involved in the attachment and entry steps of the HCV life cycle, which results in high levels of inhibition.

At present, the mechanism of the entry inhibition by SERMs is not clear. It is possible that tamoxifen targets viral molecules, but we have no evidence to support this hypothesis. ER $\alpha$  might be a target molecule because all of the antagonists of ER $\alpha$  that were examined had an inhibitory effect. Watashi et al. indicated that ER $\alpha$  is involved in HCV replication [23]. ER $\alpha$  is thought to be present in the cytoplasm, which is where HCV replicates. However, it is doubtful that ER $\alpha$  is present on the cell surface where viral entry occurs. The addition of 17 $\beta$ -estradiol with tamoxifen did not prevent the inhibitory effect of tamoxifen in the HCVpp experiment (data not shown). This result suggests that tamoxifen does not compete with 17 $\beta$ -estradiol for the target molecules involved in HCV entry. Additionally, a pure ER $\alpha$  antagonist, ICI 182,780, was a less effective inhibitor of the entry step. Based on these results, it is thought that the molecule responsible for HCV entry that is targeted by SERMs is not ER $\alpha$ .

Tamoxifen has various targets other than ER $\alpha$ , such as P-glycoprotein (GPR30), calmodulin, and protein kinase C [26]. GPR30 (G protein-coupled receptor protein 30) is a membrane-associated estrogen receptor that is distinct from the classical ER [27]. Tamoxifen and ICI 182,780 are agonists of GPR30 [28]. We examined the effect of a specific GPR30 agonist, G-1, and a GPR30 antagonist, G-15, on HCVpp infection [29]. G-1 and G-15 did not inhibit HCVpp infection. Conversely, HCVpp infection was observed to increase upon addition of these compounds (data not shown). This result suggested that GPR30 is not involved in the inhibition of HCV entry.

We previously reported that a typical PKC inhibitor, bisindolylmaleimide I (BIM I), inhibited HCV replication [2]. BIM I (10  $\mu\text{M}$ ) inhibited both HCVpp and VSVpp infection in a similar manner by approximately 50% (data not shown). This suggests that BIM I has a different mechanism for the

inhibition of entry compared to tamoxifen. PKC is not thought to be involved in the HCV-specific inhibition of entry by SERMs.

There were few reports of HCV entry inhibitors until the development of the cell-culture JFH-1 infection system. It has recently been reported that fluphenazine, trifluoperazine and related chemicals exhibit a strong, dose-dependent inhibition of HCV entry without significantly affecting the entry of VSVpp [24,25]. These compounds are structurally similar to chlorpromazine, which is an inhibitor of the clathrin-coated pit formation that is required for HCV entry [14]. Interestingly, these compounds and the SERMs have a common structural characteristic: planar, multiple aromatic rings with a tertiary amine side chain. Tamoxifen, raloxifene and ER $\alpha$  antagonists all have this structure, but the ER $\alpha$  agonists do not have these structures. Fluphenazine and related chemicals may inhibit HCV entry through a mechanism that is similar to tamoxifen.

In summary, we observed a significant HCV inhibitory effect of various SERMs using the Huh 7.5.1 cell-JFH-1 infection system. Additionally, we demonstrated that SERMs could be useful for the treatment of HCV. Because it takes a great deal of time and money to develop a new drug from a novel chemical compound, it may be easier to use previously developed drugs that can be used for new applications. Tamoxifen, toremifene, and raloxifene are all drugs that have been in use for an extended period of time. In our present *in vitro* study, the effective concentrations for the HCV inhibitory effects of the SERMs were approximately 0.1–10  $\mu$ M. In the case of tamoxifen, 20 mg per day, administered for 8 weeks resulted in plasma concentrations of approximately 0.5  $\mu$ M. These concentrations could be sufficient to exert an anti-HCV effect. SERMs should be investigated to determine their efficacy for treating HCV clinically. Further examination of the mechanism of the entry inhibition mediated by SERMs would produce significant new data relevant to the understanding of HCV entry.

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## Replication of Hepatitis C Virus Genotype 3a in Cultured Cells

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**See Covering the Cover synopsis on page 1;  
see editorial on page 13.**

**Hepatitis C virus (HCV) genotype 3a is widespread worldwide, but no replication system exists for its study. We describe a subgenomic replicon system for HCV genotype 3a. We determined the consensus sequence of an HCV genome isolated from a patient, and constructed a subgenomic replicon using this clone. The replicon was transfected into HuH-7 cells and RNA replication was confirmed. We identified cell culture-adaptive mutations that increased colony formation multiple-fold. We have therefore established a genotype 3a replicon system that can be used to study this HCV genotype.**

*Keywords:* Virology; Experimental Model; HCVGT3; In Vitro Culture System.

Hepatitis C virus (HCV) infection leads to chronic infection and advanced liver diseases in most infected adults.<sup>1</sup> Of the 6 major HCV genotypes, genotypes 1 and 2 are the most prevalent in North America, Europe, and Japan,<sup>2,3</sup> and are the most highly studied. However, other genotypes display specific characteristics. For example, genotype 3a infection can result in hepatic steatosis<sup>4</sup> and telaprevir and boceprevir are less effective against genotype 3a.<sup>5</sup> Therefore, the pathogenesis and inhibitor sensitivity of all HCV genotypes should be studied. Although HCV subgenomic replicons are useful for understanding viral/host factors involved in HCV replication and inhibitor sensitivity, only HCV replicons for genotypes 1a, 1b, and 2a have been established.<sup>6–9</sup> Here, we report on the robust genotype 3a replication system.

An almost complete HCV genome was recovered from the serum of a patient with post-transplantation recurrent HCV infection. This serum exhibited higher infectivity than other tested sera toward primary human hepatocytes (Supplementary Figure 1A). The isolate, named S310, contained the following structural elements: a 5'UTR (nt 1-339), an open reading frame encoding 3021 aa (nt 340-9402), and a 3'UTR (nt 9403-9654). Only the last 44 nt of the X-region (nt 9611-9654) could not be recovered. Two major virus populations were found; S310/A contained Ala, Thr, Thr, and Ile, and S310/B

contained Thr, Ala, Ala, and Thr, at the 7<sup>th</sup>, 151<sup>st</sup>, 431<sup>st</sup>, and 472<sup>nd</sup> aa of the NS3 protein, respectively. S310 was clustered into genotype 3a by phylogenetic analysis (Supplementary Figure 1B). The complexity of the virus quasi-species in the serum was analyzed by sequencing the hypervariable region. Identical amino acid sequences in all 10 hypervariable region clones indicated a very low degree of diversity. The hypervariable region sequence of the JFH-1 strain also exhibited monoclonality,<sup>10</sup> which can be important for efficient replication in cultured cells.

Subgenomic replicons SGR-S310/A and SGR-S310/B were constructed and their replication efficiency was evaluated by G418-resistant colony-formation assay. After 3 weeks, a small number of colonies were visible for both replicons (Figure 1A). Because more colonies were observed in SGR-S310/A than in SGR-S310/B, we focused on SGR-S310/A (henceforth called SGR-S310). Ten cell colonies of SGR-S310 were isolated and analyzed for HCV replication. The mean RNA titer was  $9.1 \times 10^7 \pm 4.6 \times 10^7$  copies/ $\mu$ g total RNA (Figure 1B). HCV RNA (approximately 8 kb) was detected by Northern blotting (Supplementary Figure 2A). Viral proteins in the replicon cells were detected by immunofluorescence and Western blotting (Supplementary Figure 2B and 2C). To determine whether the G418 resistance of the cells was transmissible by cellular RNA transfection, we electroporated total cellular RNA isolated from 4 replicon clones into naïve HuH-7 cells. Multiple G418-resistant colonies appeared after transfection of the RNA isolated from the replicon clones (Supplementary Figure 3A), but not from the naïve HuH-7 cells. These results indicate that the replicon RNA in the parental colonies could replicate in naïve cells. Thus, the G418-resistant colonies that were isolated from cells electroporated with SGR-S310 synthetic RNA contained replicating viral RNA.

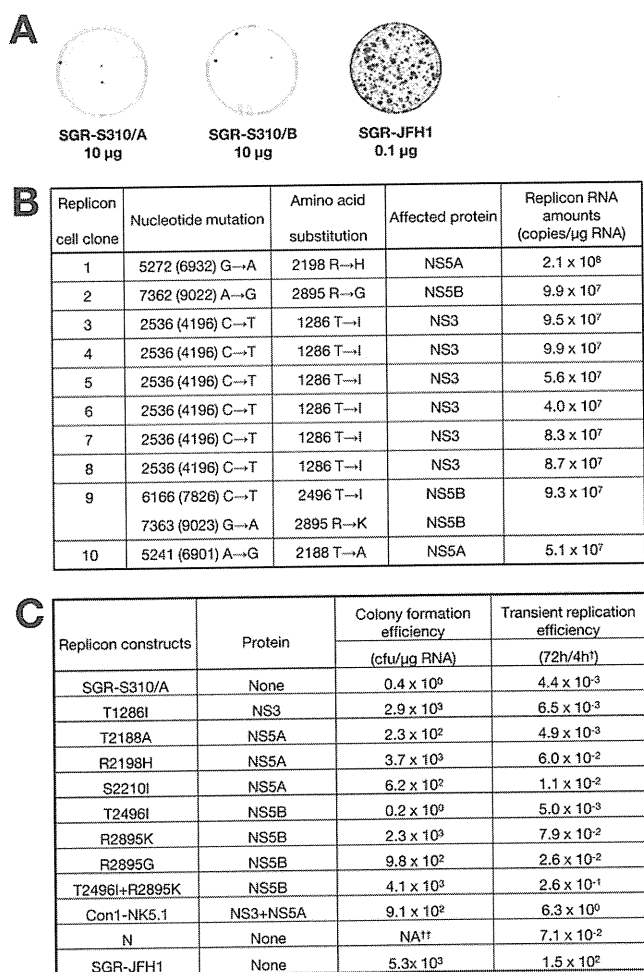
Replicating genomes have been shown to accumulate cell culture adaptive mutations, which increase their replication potential. To examine whether SGR-S310 acquired mutations, the complete HCV sequences from 10 replicon clones were sequenced. At least one nonsynonymous mutation was detected in the NS3-NS5B region of each replicon clone (Figure 1B). The following mutations were identified: T1286I in the NS3 helicase (6 of 10

*Abbreviation used in this paper:* HCV, hepatitis C virus.

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**Figure 1.** S310 subgenomic replicon analysis. (A) Three million HuH-7 cells were electroporated with 10 µg RNA from SGR-S310/A or SGR-S310/B or 0.1 µg RNA from SGR-JFH1. G418-selected colonies were fixed and stained after 3 weeks. (B) Non-synonymous mutations identified in the replicon genomes and HCV RNA titers in the replicon clones. Nucleotide positions within the S310 subgenomic replicon and within the full-length S310 genome (in parentheses) are given. (C) Replication potential of the adaptive mutants as determined by the colony-formation assay using Neo-replicons and by the transient replication assay using Fluc-replicons. <sup>†</sup>72 h/4 h, transient replication efficiency was determined as a ratio of luciferase activity in the transfected cells between 72 h and 4 h post transfection. <sup>††</sup>NA, not available.

clones); T2188A or R2198H in NS5A (2 clones); an R2895G substitution in NS5B (1 clone); and T2496I in NS5A plus R2895K in NS5B (1 clone). These mutations and the S2210I mutation (corresponding to S2204I in genotype 1 replicon)<sup>7,8</sup> were introduced, individually or in combination, into the parental SGR-S310 and the colony-formation efficiencies of the mutant replicons were tested. All mutations, except T2496I, increased the colony formation, indicating an adaptive phenotype (Figure 1C, Supplementary Figure 3B). Transient replication efficiency was also tested using firefly luciferase reporter replicons. SGR-S310/Luc did not replicate in Huh-7.5.1 cells, whereas the adaptive mutants displayed varying degrees of replication (Figure 1C, Supplementary Figure 3C). Adaptive mutations T2496I and R2895K, when combined to-

gether, most efficiently enhanced the colony formation as well as transient replication (Figure 1C). Interestingly, T1286I and R2895G found in our study correspond to the Con1 adaptive mutations T1280I and R2884G, respectively.<sup>11,12</sup> T2188A or R2198H in NS5A were identified in 2 replicon clones and are located close to S2210I. Indeed, S2210I also enhanced SGR-S310 replication, suggesting that this region might be important for HCV replication. S310 replicons with adaptive mutations were compared with genotype 1b (Con1 and N) and 2a (JFH-1) replicons. Colony-formation efficiencies of most S310 adaptive replicons were at levels comparable with Con1 and JFH-1 (Figure 1C, Supplementary Figure 3B). In contrast, S310 adaptive replicons replicated less efficiently than Con1-NK5.1 and JFH-1 replicons in transient replication assays. However, genotype 1b N replicon replicated at a level similar to some S310 adaptive replicons (Figure 1C, Supplementary Figure 3C). Future studies will dissect the detailed mechanisms that underlie the effects of these mutations.

Successful generation of a genotype 3a replicon provided a unique opportunity to compare the susceptibility of genotype 3a (SGR-S310), 1b (Con1<sup>13</sup>), and 2a (JFH-1/4-1<sup>13</sup>) replicons to HCV inhibitors. Interferon-alfa dose-dependently decreased the replication of all tested genotypes (Figure 2A), whereas a protease inhibitor, BILN-2061, was more effective against replicons from genotypes 1b and 2a than 3a (Figure 2B). The non-nucleoside polymerase inhibitor JTK-109 was more potent against genotype 1b and 3a (Figure 2C). However, the nucleoside polymerase inhibitor, PSI-6130, equally inhibited all genotypes (Figure 2D).

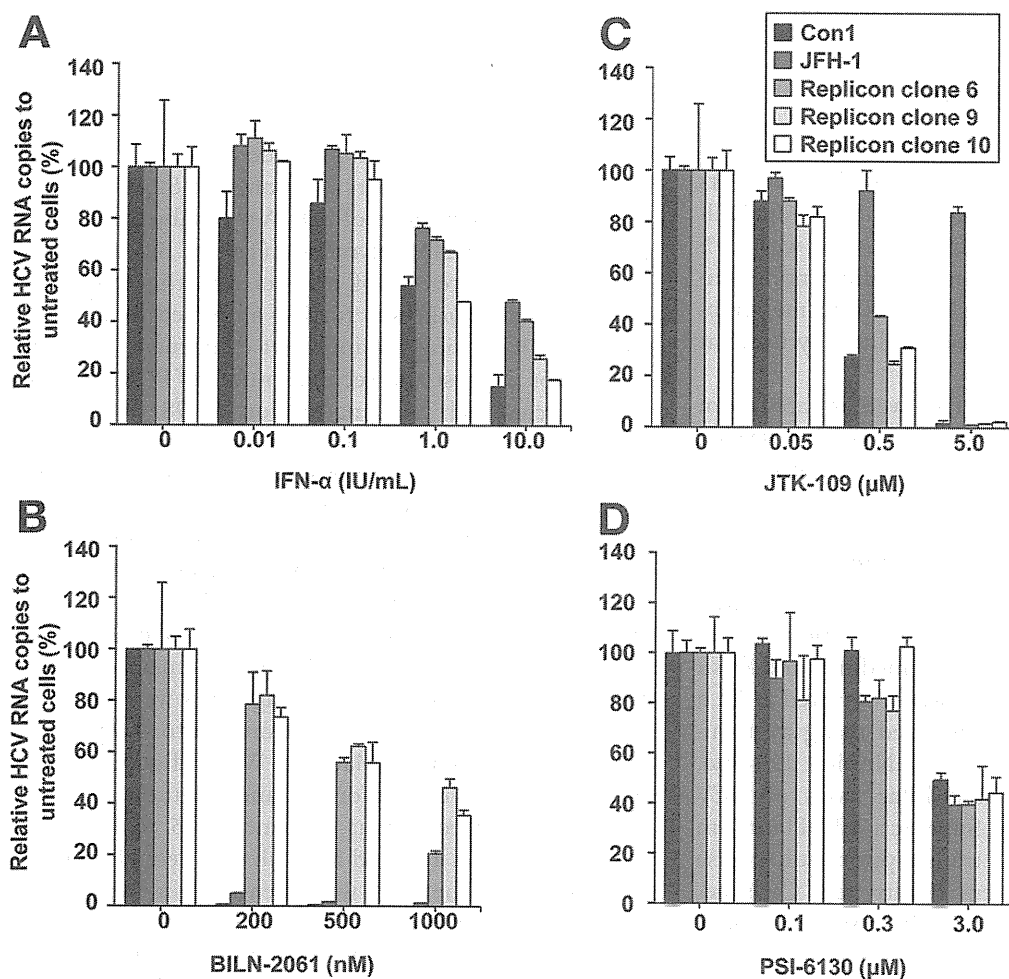
In conclusion, we established a subgenomic replicon for genotype 3a, which should be useful for understanding the specific characteristics of this genotype and for the screening of antiviral chemicals that are effective against this genotype. Construction of a full-length infectious S310 clone is in progress.

## Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at [www.gastrojournal.org](http://www.gastrojournal.org), and at <http://dx.doi.org/10.1053/j.gastro.2012.09.017>.

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**Figure 2.** Effects of antiviral inhibitors on the replication of S310 subgenomic replicons. Three cell clones (clone 6, 9 and 10) carrying genotype 3a S310 replicons and one cell clone each harboring genotype 1b Con1 and genotype 2a JFH-1 replicons were treated with the indicated concentrations of (A) interferon alpha, (B) HCV protease inhibitor BILN-2061, (C) the non-nucleoside polymerase inhibitor JTK-109, and (D) the nucleoside polymerase inhibitor PSI 6130 for 72 hours and replication levels were measured by quantifying intracellular HCV RNA. Results are means  $\pm$  standard deviations of 3 replicates.

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DDJ/EMBL/GenBank accession numbers: S310/A: AB691595, S310/B: AB691596, SGR-S310/A: AB691597, SGR-S310/B: AB691598, SGR-S310/Luc: AB691599.

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#### Conflicts of interest

The authors disclose no conflicts.

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## Supplementary Methods

### *Cell Culture*

The human hepatoma cell line HuH-7<sup>1</sup> and its derivative cell line Huh-7.5.1<sup>2</sup> were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, minimal essential medium nonessential amino acids, 100 U/mL penicillin, 100 µg/mL streptomycin, 10 mM HEPES, and 1 mM sodium pyruvate at 37°C in a 5% CO<sub>2</sub> incubator.

Primary human hepatocytes (PHH) were isolated from an encapsulated liver sample.<sup>3</sup> Isolated PHH were seeded in 12-well plates and cultured at 37°C in Lanford medium before infection.

### *PHH Infection With HCV-Positive Sera*

Three days post seeding, PHH were inoculated with HCV-positive sera. After 16 h of inoculation, monolayers were washed with William's E medium and fresh Lanford medium was added. Cells were harvested at 72 h post infection. Total RNA was isolated using a guanidinium isothiocyanate solution (RNable; Eurobio, Courtaboeuf, France) and intracellular levels of HCV RNA were quantified using the SuperScript III Platinum One-Step quantitative reverse transcription polymerase chain reaction (RT-PCR) system (Invitrogen, Carlsbad, CA) and a LightCycler480 real-time PCR system (Roche Diagnostics, Meylan, France).

### *HCV Genotype 3a Clone*

Clone S310 was isolated from a 71-year-old female patient suffering from post liver transplantation HCV recurrence. She was diagnosed with HCV genotype 3a infection at the age of 59 years and underwent liver transplantation 4 years later due to liver cirrhosis. HCV-RNA titer was  $2.8 \times 10^6$  copies/mL. Total RNA extracted from 100 µL serum using the acid-guanidinium isothiocyanate-phenol-chloroform method (Isogen-LS; Nippon Gene, Tokyo, Japan) was precipitated with isopropanol, washed with ethanol, and dissolved in 10 µL nuclease-free water. An aliquot of 4 µL was subjected to reverse transcription using random hexamers and Moloney murine leukemia virus reverse transcriptase (Superscript III; Invitrogen) at 42°C for 50 min and then at 50°C for 10 min.

### *Isolation of HCV*

The sequences of 4 isolates of genotype 3a (accession numbers AF046866, D28917,<sup>4</sup> X76918, and D17763<sup>5</sup>) that were obtained from the HCV database (<http://hcv.lanl.gov/content/sequence/HCV/ToolsOutline.html>) were aligned and PCR primers were designed based on the conserved sequences. These primers were used to amplify the complementary DNA (cDNA) of S310 into 9 overlapping fragments by nested PCR (nt 1–370, nt 127–1284, nt 1117–1997, nt 1704–3352, nt 3152–5080, nt

4869–6842, nt 6601–8129, nt 7988–9145, and nt 9082–9576; nucleotide numbers refer to the positions on S310, with nt 1 being the first nucleotide of the 5' UTR). The sequence of these primers is shown in Supplementary Table 1. Two microliters of cDNA was subjected to PCR using Pyrobest DNA polymerase (Takara Bio, Kyoto, Japan) and the outer set of primers, and this first-round PCR product (2 µL) was further amplified by a second round of PCR using the inner set of primers. PCR conditions for the first and second rounds of PCR consisted of 35 cycles each of denaturation at 98°C for 20 s, annealing at 55°C for 1 min, and extension at 72°C for 3 min. A fragment encompassing the 5' end of the viral genome (nt 1–370) was amplified by 5'RACE. Briefly, cDNA was synthesized with a 5' UTR primer (antisense), tailed with a dCTP homopolymer by using terminal deoxynucleotidyl transferase, and amplified by PCR (5' RACE System for Rapid Amplification of cDNA Ends; Invitrogen) using TaKaRa LA Taq polymerase (Takara Bio). The PCR products of all fragments were separated by agarose gel electrophoresis, cloned into the pGEM-T EASY vector (Promega, Madison, WI) and sequenced using the Big Dye Terminator Mix and an automated DNA sequencer. The consensus sequence of 5 to 9 isolated cDNA clones was adopted for each fragment. Two major populations of the virus were identified in the patient's serum that differed in 4 amino acids in the NS3 protein (aa 1039, 1183, 1463, and 1504), and these populations were designated as S310/A and S310/B (DDBJ/EMBL/GenBank accession number: AB691595 and AB691596, respectively). To assess the complexity of the HCV population in the patient's serum, the hypervariable region sequences of 10 clones were determined.

### *Computer Analysis*

A phylogenetic tree was constructed using the neighbor-joining method to examine the relationship between the polyprotein region of S310 and that of other HCV genotype 3a isolates available in the database. In order to analyze the diversity in each subgenomic region, the genetic distance was calculated between all possible pairs of genotype 3a isolates and between S310/A and other isolates using MacVector software (MacVector, Inc., Cary, NC). The ratios of these 2 values (mean genetic distance between S310/A and other isolates/mean genetic distance among all genotype 3a isolates) were compared.

### *Construction of Replicons*

Based on the consensus sequence of S310, we assembled pS310/A and pS310/B, which contained the full-length S310/A and S310/B cDNA, respectively, downstream of the T7 RNA polymerase promoter. Briefly the 9 amplicons described here were combined by overlapping PCR and ligated with pGEM-T EASY vectors to generate 6 plasmids (A through F) in such a way that each plasmid contained a unique restriction enzyme