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# A Subclone of HuH-7 with Enhanced Intracellular Hepatitis C Virus Production and Evasion of Virus Related-Cell Cycle Arrest

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## Abstract

Hepatitis C virus (HCV) cell culture system with JFH-1 strain and HuH-7 cells enabled us to produce infectious HCV particles *in vitro*, and such system is useful to explore the anti-HCV compounds and to develop the vaccine against HCV. In the present study, we describe the derivation of a cell line that permits improved production of HCV particles. Specifically, we characterized several subclones that were isolated from the original HuH-7 cell line by limiting dilution. These HuH-7 subclones displayed a notable range of HCV production levels following transfection by full-genome JFH-1 RNA. Among these subclones, HuH-7T1 produced HCV more efficiently than other subclones and Huh-7.5.1 that is known to be highly permissive for HCV replication. Upon transfection with full-genome RNA, HCV production was increased ten-fold in HuH-7T1 compared to Huh-7.5.1. This increase in viral production correlated with increased efficiency of intracellular infectious virus production. Furthermore, HCV replication did not induce cell cycle arrest in HuH-7T1, whereas it did in Huh-7.5.1. Consequently, the use of HuH-7T1 as host cells could provide increased population of HCV-positive cells and elevated viral titer. In conclusion, we isolated a HuH-7 subclone, HuH-7T1, that supports efficient HCV production. High efficiency of intracellular infectious virus production and evasion of cell cycle arrest were important for this phenotype. We expect that the use of this cell line will facilitate analysis of the underlying mechanisms for HCV particle assembly and the cell cycle arrest caused by HCV.

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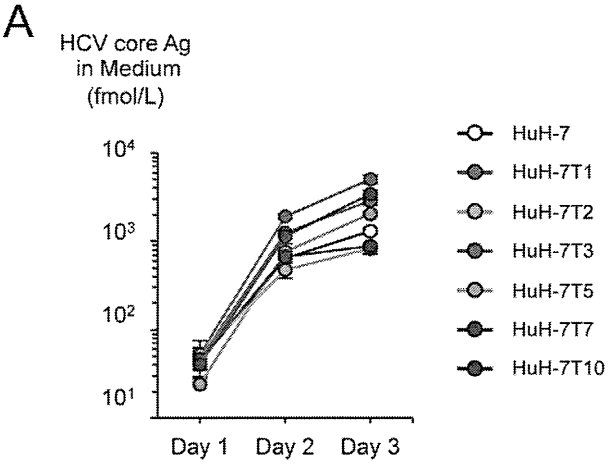
## Introduction

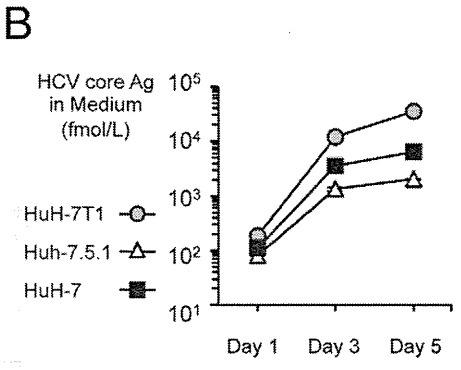
Hepatitis C virus (HCV) is a major cause of chronic liver disease [1,2]. Currently, approximately 200 million people are infected with HCV worldwide and are at continued risk of developing chronic liver diseases such as chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [3,4]. Historically, the lack of a cell culture system capable of producing virus particles hampered progress in the field of HCV research. Subsequently, a robust HCV cell culture system was developed using HCV JFH-1 strain that had been cloned from a fulminant hepatitis patient [5,6,7]. JFH-1 was the first HCV strain that could replicate and produce HCV particles autonomously *in vitro*, thereby facilitating investigation of the entire life cycle of the virus. This HCV cell culture system employed HuH-7 cell line, which was established from a hepatocellular carcinoma [5,8], as a host. Since the HCV replicon system enabling HCV subgenomic RNA replication was originally developed using HuH-7 [9], this cell line has been used in the research field of HCV most frequently. However, HuH-7 is known to be heterogeneous. Notably, Saintz et al. reported that HuH-7 cell lines obtained from various laboratories exhibit distinct morphological, cell growth, and HCV susceptibility properties [10]. We also found that single-cell cloning of HuH-7 maintained in our laboratory yielded multiple subclones that exhibited different characteristics of HCV infection and replication [11]. In the present study, we derived cell lines from original HuH-7 obtained from the cell bank and screened to identify a cell line with improved production of infectious HCV particles. As we report here, we obtained one such clone (HuH-7T1) and performed an initial characterization of the HCV life cycle in this host.

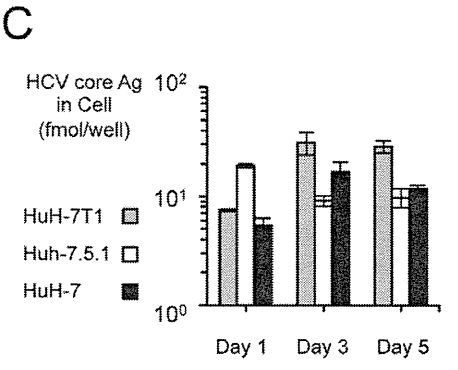
## Materials and Methods

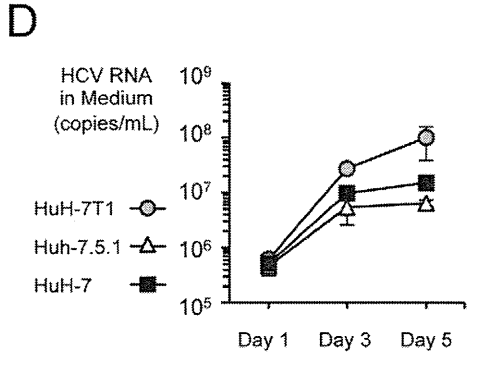
### Cell culture

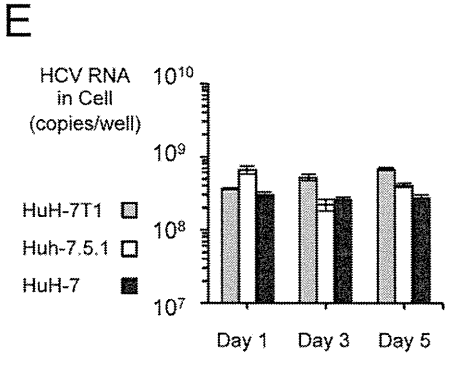
The original HuH-7 cell line (catalog number; JCRB0403) was purchased from Health Science Research Resources Bank (Osaka, Japan). The cured cell line, Huh-7.5.1, was a kind gift from Dr. Francis V. Chisari (Scripps Research Institute, La Jolla, CA) [6]. These cell lines were cultured at 37°C in a 5% $CO_2$ environment using Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum.

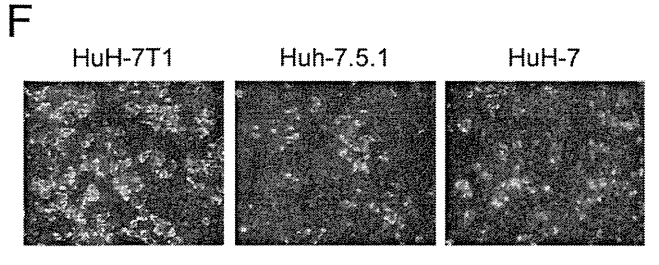

A
HCV core Ag
in Medium
(fmol/L)
10^4
10^3
10^2
10^1
Day 1
Day 2
Day 3
HuH-7
HuH-7T1
HuH-7T2
HuH-7T3
HuH-7T5
HuH-7T7
HuH-7T10


B
HCV core Ag
in Medium
(fmol/L)
10^5
10^4
10^3
10^2
10^1
HuH-7T1
Huh-7.5.1
HuH-7
Day 1
Day 3
Day 5


C
HCV core Ag
in Cell
(fmol/well)
10^2
10^1
10^0
HuH-7T1
Huh-7.5.1
HuH-7
Day 1
Day 3
Day 5


D
HCV RNA
in Medium
(copies/mL)
10^9
10^8
10^7
10^6
10^5
HuH-7T1
Huh-7.5.1
HuH-7
Day 1
Day 3
Day 5


E
HCV RNA
in Cell
(copies/well)
10^10
10^9
10^8
10^7
HuH-7T1
Huh-7.5.1
HuH-7
Day 1
Day 3
Day 5


F
HuH-7T1
Huh-7.5.1
HuH-7

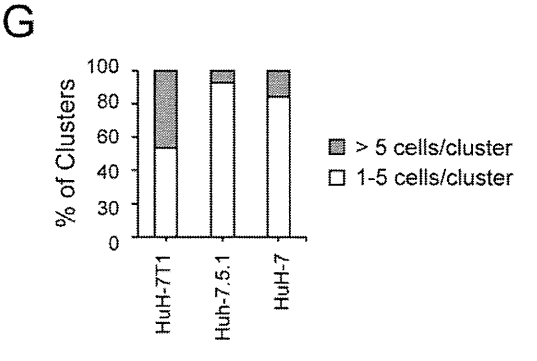

G
% of Clusters
100
80
60
40
30
0
HuH-7T1
Huh-7.5.1
HuH-7
> 5 cells/cluster
1-5 cells/cluster

**Figure 1. HCV production in HuH-7 subclones.** (A) Two micrograms of JFH-1 RNA were electroporated into the HuH-7 subclones. Culture medium was harvested at Days 1, 3, and 5, and HCV core protein levels in the culture medium were measured. Assays were performed three times independently, and data are presented as mean ± standard deviation. (B–D) Comparison of HCV production among HuH-7T1, Huh-7.5.1 and HuH-7. HCV core protein (B and C) and HCV RNA (D and E) levels in cells and culture medium were measured. Assays were performed three times independently, and data are presented as mean ± standard deviation. (F) HCV-positive cells at Day 3 post-transfection were visualized with anti-core antibody (green); nuclei were visualized with DAPI (blue). (G) The number of HCV positive cells within a cluster were counted and classified into 2 groups (>5 cells/cluster and 1–5/cluster). More than 100 foci were counted. The percentages of each group are shown.
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## Single cell cloning by limiting dilution

The original HuH-7 cell line was diluted with medium at 1 cell/mL and seeded at 100 μL/well in 96-well plates. Six subclones were obtained and resulting subclones were expanded and stored at −80°C pending further characterization. The characteristics of obtained subclones were maintained after passages over several months.

## HCV constructs and RNA transfection

pJFH1 is a full-length JFH-1 clone whose construction was reported previously [5]. pSGR-JFH1-Luc (a JFH-1 subgenomic replicon construct containing a firefly luciferase-encoding reporter gene) and pSGR-JFH1/GND-Luc (a replication-defective mutant construct) also were described previously [12]. pH77S.2, a full-length H77S.2 construct, was a kind gift from Dr. Stanley M Lemon (University of North Carolina at Chapel Hill, Chapel Hill, NC). This construct is a derivative of strain H77S (genotype 1a) harboring an additional mutation, and produces infectious virus in cultured cells after full-genome RNA transfection [13]. RNA synthesis and transfection were performed as described previously [14,15].

## Quantification of HCV core protein and RNA

The concentration of HCV core protein in the culture medium and cell lysate was measured using a chemiluminescent enzyme immunoassay (Lumipulse Ortho HCV antigen, Fujirebio, Tokyo, Japan) in accordance with the manufacturer's instructions. The concentration of HCV RNA was measured as described previously [16].

## Determination of infectivity titers

To determine the intracellular infectivity of the HCV RNA-transfected cells, a cell lysate of HCV RNA-transfected cells cultured in a 10 cm dish was generated by subjecting the cells to four freeze-thaw cycles. The culture supernatant and cell lysate were serially diluted and inoculated into naive Huh-7.5.1 seeded at $1\times10^4$ cells/well in poly-D-lysine-coated 96-well plates (BD, Franklin Lakes, NJ), and the inoculated plates were incubated for another 3 days at 37°C. The cells were then fixed with methanol, and the infected foci were visualized by staining with anti-core antibody (clone 2H9 [5,8] for JFH-1 and c7-50 (Abcam, Cambridge, MA) for H77S.2) and Alexa Fluor 488 Goat Anti-mouse IgG (Invitrogen, Carlsbad, CA). The infectivity titer was quantified by counting the stained foci and expressing the value as the number of focus-forming units (FFU).

**Table 1.** Infectivity titers in culture medium and cells of HuH-7T1 and Huh-7.5.1 transfected with JFH-1 RNA.

| Cell Line | Infectivity | | Secretion Rate |
|---|---|---|---|
| | Medium (FFU/dish) | Cells (FFU/dish) | |
| HuH-7T1 | $2.23\times10^6\pm3.15\times10^5$ * | $1.11\times10^4\pm1.15\times10^3$ * | $2.00\times10^2\pm1.98\times10^1$ * |
| Huh-7.5.1 | $9.92\times10^4\pm2.98\times10^4$ | $1.34\times10^2\pm1.42\times10^1$ | $7.30\times10^2\pm1.40\times10^2$ |

*$P<0.05$ as compared with Huh-7.5.1.
doi:10.1371/journal.pone.0052697.t001

## Flow cytometric analysis

For cell cycle distribution analyses, cells were labeled with 5-ethynyl-2′-deoxyuridine (EdU) for 4 h prior to harvest. The harvested cells were fixed in 4% paraformaldehyde, permeabilized, and stained with anti-nonstructural (NS) 5A antibody (clone KS0265-1; raised by immunization with JFH-1 NS5A) and Alexa Fluor 647 Goat Anti-mouse IgG (Invitrogen). Incorporated EdU was stained with Alexa Fluor 488 azide by using the Click-iT EdU flow cytometry kit (Invitrogen) according to the manufacturer's instructions. Following treatment with RNase A, 7-aminoactinomycin D (7-AAD) was added. Samples were analyzed using a FACS Calibur flow cytometer. The population of cells in G0/G1, S, or G2/M phases of the cell cycle was determined using FlowJo software (Tree Star, Inc., Ashland, OR).

## Immunostaining

Infected cells were cultured on glass cover slips in a 12-well plate. Cells were fixed in 4% paraformaldehyde and permeabilized. After blocking, HCV-positive cells were visualized by staining with anti-core antibody (clone 2H9) and Alexa Fluor 488 Goat Anti-mouse IgG, and nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI).

## Virus entry assay

HCV pseudo type virus (HCVpp) harboring the JFH-1 E1 and E2 glycoprotein was prepared as described previously [11]. Target

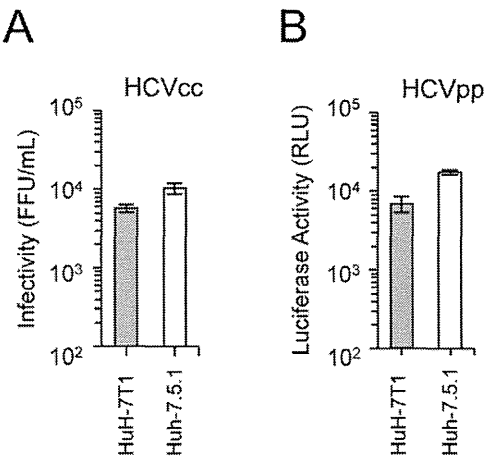


**Figure 2. Comparison of infection in HuH-7T1 and Huh-7.5.1.** (A) Infection of HCVcc into HuH-7T1 and Huh-7.5.1. The cells were fixed 3 days after infection and infected foci were counted. (B) Infection of HCVpp into HuH-7T1 and Huh-7.5.1. The cells were harvested 3 days after infection, and the luciferase activity in the cell lysate was measured.
doi:10.1371/journal.pone.0052697.g002

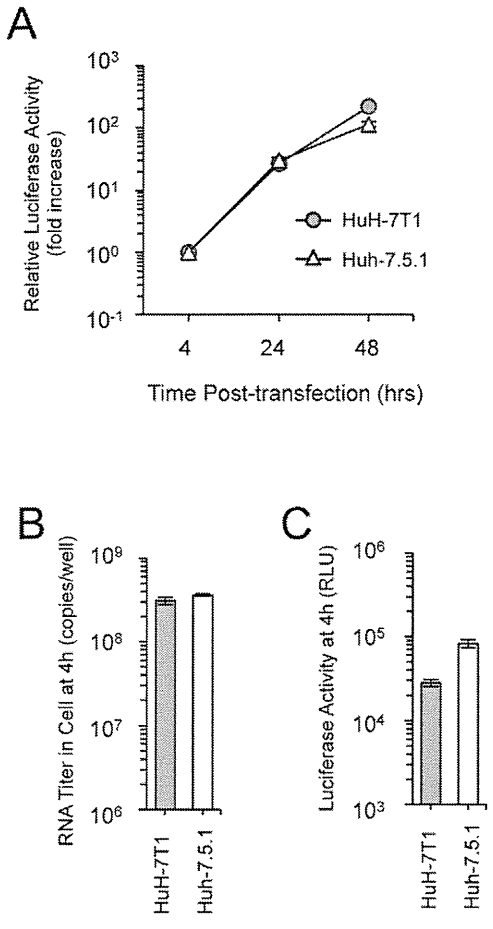


**Figure 3. Comparison of replication in HuH-7T1 and Huh-7.5.1.** (A) Five micrograms of JFH-1 subgenomic replicon RNA was electroporated into HuH-7T1 and Huh-7.5.1. The cells were harvested at indicated time points. The luciferase activity in the cell lysates was normalized to the data at 4 h after transfection; values are expressed as fold increases. (B and C) Comparison of transfection and translation efficiencies. Five micrograms of JFH-1/GND-Luc RNA was transfected into HuH-7T1 and Huh-7.5.1. The cells were harvested at 4 h after transfection, and the amount of transfected RNA in cells (B) and luciferase activity in the cell lysates (C) were measured.
doi:10.1371/journal.pone.0052697.g003

cells were seeded into 48-well plates at a density of $2\times10^4$ cells/well. On the following day, a 100-μL aliquot of each diluted supernatant containing HCVpp was added to each well and incubated for 3 h. The supernatants were replaced with fresh medium, and the cells were incubated for 72 h at 37°C. Cells were lysed with Passive Lysis Buffer (Promega, Madison, WI). Luciferase activities were quantified using a luciferase assay system (Promega). Assays were performed in triplicate; data are presented as mean ± standard deviation.

Cell culture-generated HCV JFH-1 virus (HCVcc) was prepared as follows: culture medium from JFH-1 RNA-transfected cells was collected and 40-times concentrated using Amicon Ultra-15 filter units (100-kDa cutoff; Millipore, Bedford, MA) and stored at −80°C until use. HCVcc was inoculated into target cells, and infectivity titer was determined as described.

### Luciferase assay

Luciferase activity of subgenomic reporter replicon RNA-transfected cell lysate was measured as described previously [14,15].

### Statistical analysis

Significant differences were evaluated using the Student's t-test. $P<0.05$ was considered significant.

## Results

### Isolation of HuH-7 subclones with improved HCV production

To obtain cell lines with improved HCV production potential, we used limiting dilution to establish six subclones (HuH-7T1, HuH-7T2, HuH-7T3, HuH-7T5, HuH-7T7, and HuH-7T10) from the original HuH-7 purchased from the cell bank. We transfected JFH-1 RNA into each of these subclones and measured the level of core protein in the culture medium. These subclones displayed a range of core protein production levels. (Fig. 1A). Compared to the original HuH-7, four (HuH-7T1, HuH-7T3, HuH-7T5 and HuH-7T10) and two (HuH-7T2 and HuH-7T7) subclones produced higher or lower amounts of HCV core protein, respectively. Among these subclones, we chose HuH-7T1 for further characterization because this subclone produced HCV core protein at the highest level (Fig. 1A). Then, we compared core protein production of HuH-7T1 with Huh-7.5.1, a cell line reported to be highly permissive for HCV replication [6]. After JFH-1 RNA transfection, HCV core protein level in the culture medium of HuH-7T1 was 17.6-fold higher than that seen with Huh-7.5.1 (Fig. 1B). HCV core protein levels in cell lysate of HuH-7T1 were lower at Day 1, but higher at Days 3 and 5 after transfection, compared to Huh-7.5.1 (Fig. 1C). HCV RNA levels in the culture medium and cell lysates of these cells showed similar tendencies (Fig. 1D and 1E). The infectivity titer in culture medium of HuH-7T1 at Day 5 was 22.5-fold higher than that of Huh-7.5.1 (Table 1), indicating that HuH-7T1 supported production of infectious HCV particles to levels higher than those seen in Huh-7.5.1. The number of HCV-positive cells of HuH-7T1 at Day 5 also was higher than that seen with Huh-7.5.1 (Fig. 1F). The percentage of HCV positive cell clusters consisting of more than 5 cells was higher in HuH-7T1 than in Huh-7.5.1 (Fig. 1G). We also assessed if HuH-7T1 produced higher amount of core protein after infection of HCVcc. HuH-7T1 produced higher amount of HCV core protein than Huh-7.5.1 after JFH-1 virus infection at the same multiplicity of infection (Fig. S1A), and HCV core protein levels in cell lysate of HuH-7T1 were also higher than that of Huh-7.5.1 (Fig. S1B). These data indicated that HuH-7T1 produced infectious HCV particles more efficiently than Huh-7.5.1 after JFH-1 RNA transfection and JFH-1 virus infection.

The original HuH-7 could produce higher amount of HCV core protein than Huh-7.5.1 after JFH-1 RNA transfection (Fig. 1B). However, in the experiment of HCVcc infection, HuH-7 produced lower amount of HCV core protein than Huh-7.5.1 in culture medium (Fig. S1A) and in cell lysate (Fig. S1B).

### Analysis of HCV life cycle in HuH-7T1

To clarify the underlying mechanism of the enhanced virus production in HuH-7T1, we assessed the efficiencies of each step in the HCV life cycle. The viral infection step was assessed by using HCVcc and HCVpp. The HCVcc system uses cell culture-generated HCV and detects steps from viral attachment through replication. On the other hand, the HCVpp system uses the

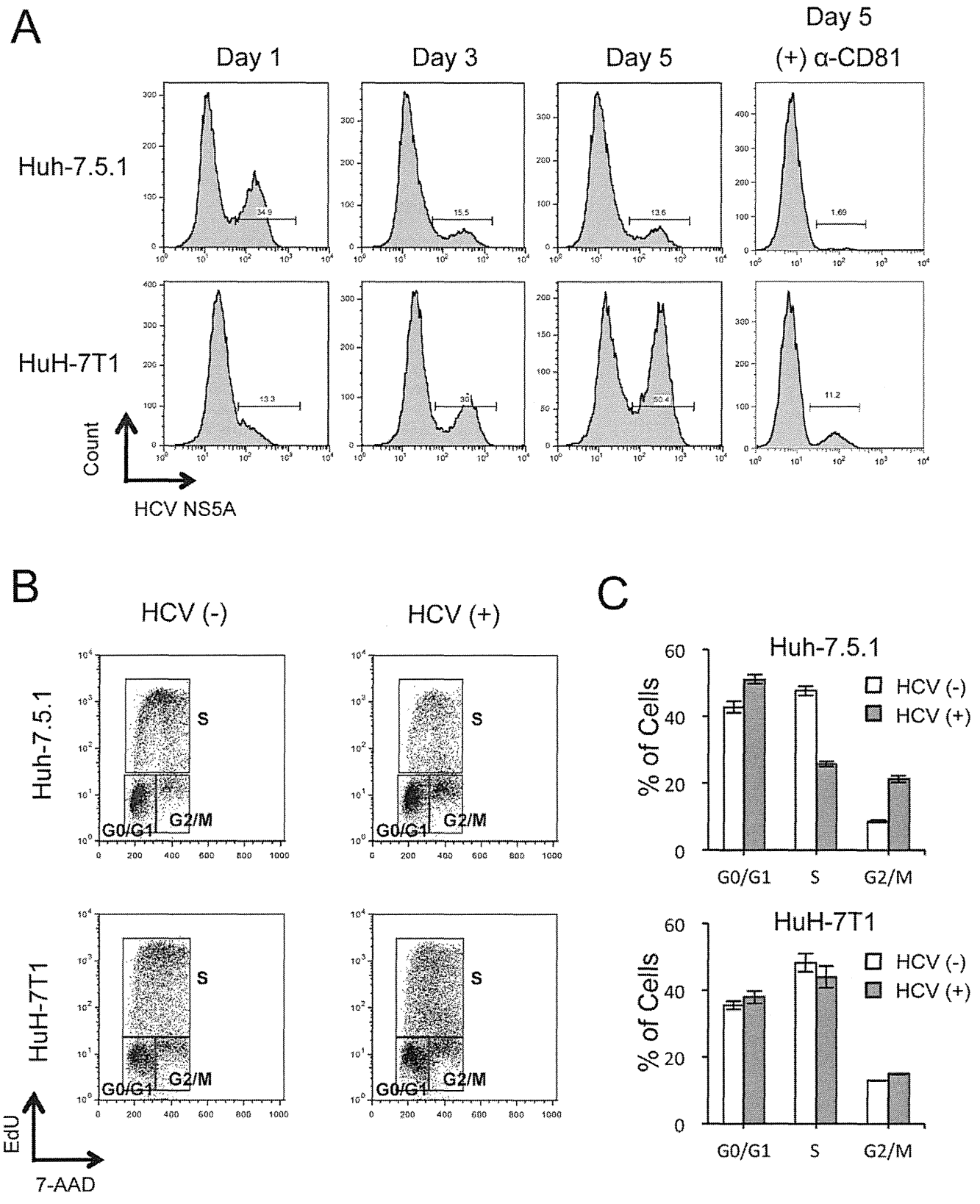


**Figure 4. Effects of HCV replication on cell proliferation of Huh-7.5.1 and HuH-7T1.** (A) Population of HCV-positive cells after JFH-1 RNA transfection. Two micrograms of JFH-1 RNA was electroporated into Huh-7.5.1 and HuH-7T1 and cultured with or without 10 mg/mL of anti-CD81 antibody (clone JS-81, BD). Cells were harvested at Days 1, 3, and 5. After fixing, cells were stained with anti-NS5A antibody and analyzed by flow cytometry. (B, C) Cell cycle distribution of HCV-positive and -negative cells after JFH-1 RNA transfection. Two micrograms of JFH-1 RNA was electroporated into Huh-7.5.1 and HuH-7T1. Cells were pulse-labeled with EdU and analyzed for cell cycle distribution. The percentages of cells in G0/

G1, S, and G2/M phases of the cell cycle were calculated by gating with FlowJo software. (B) Representative cell cycle distributions of HCV-negative and -positive cells. (C) Percentages of cells in each phase of the cell cycle for HCV-negative and -positive populations. Assays were performed three times independently; data are presented as mean ± standard deviation.
doi:10.1371/journal.pone.0052697.g004

retroviral particles harboring the HCV envelope protein and a luciferase reporter gene, and measures infection efficiency in the absence of HCV replication [11]. The infectivity titer of HCVcc in HuH-7T1 was 33.0%±8.1% of that in Huh-7.5.1 (Fig. 2A). To evaluate the infection efficiency of HCVpp, cellular luciferase activity was measured after HCVpp infection. The luciferase activity in HuH-7T1 was 39.5%±9.0% of that in Huh-7.5.1 (Fig. 2B). As there were differences in infection efficiencies of HCVcc and HCVpp between these cell lines, we analyzed cell-surface expression of the HCV receptor, CD81, using flow cytometry. The population of CD81-expressing cells was slightly lower in HuH-7T1 than in Huh-7.5.1, and HuH-7T1 showed a broad peak of CD81 expression, indicating that CD81 expression level in each cell varied (Fig. S2). Taken together, these results indicated that the susceptibility for HCV infection in HuH-7T1 was lower than in Huh-7.5.1. This distinction presumably reflected the reduced population of CD81-expressing cells, implying that this step was not responsible for the enhanced virus production in HuH-7T1.

We assessed RNA replication efficiency by transfection with a subgenomic JFH-1 replicon RNA that harbored a luciferase-encoding gene. Subgenomic replicon assay revealed that RNA replication in HuH-7T1 demonstrated similar kinetics to that seen in Huh-7.5.1 when compared with the fold-increase value over 4 h of each cells (Fig. 3A), but the absolute luciferase activities of HuH-7T1 were lower than that of Huh-7.5.1 at all time points tested (Fig. S3). We then compared RNA transfection efficiency by measuring the RNA titers of the transfected replication-defective subgenomic replicon RNA (SGR-JFH1/GND-Luc) in the cells. The amount of replicon RNA in the two cell lines was same level at 4 h after transfection (Fig. 3B). However, the luciferase activity in HuH-7T1 was 2.9-times lower than that of Huh-7.5.1 at 4 h after transfection (Fig. 3C). Thus, translation efficiency of HCV genome was lower in HuH-7T1 than in Huh-7.5.1. Taken together, neither the translation or replication step was responsible for enhanced virus production in HuH-7T1.

To assess the efficiencies of intracellular infectious virus production and secretion, we compared infectivity titers in cells and medium of JFH-1 RNA-transfected HuH-7T1 and Huh-7.5.1. At Day 5 after transfection, the intracellular infectivity of HuH-7T1 was 83-fold higher than that of Huh-7.5.1 (Table 1). However, the core protein level of the cells of HCV RNA-transfected HuH-7T1 at Day 5 was only 2.9-fold higher than that of Huh-7.5.1 (Fig. 1B), indicating that infectious HCV particles were assembled more efficiently in HuH-7T1 than in Huh-7.5.1. Virus secretion efficiencies also were assessed by comparing the ratio of infectivity titers in cells and supernatants, and were 3.7-fold lower in HuH-7T1 compared to Huh-7.5.1 (Table 1). Taken together, these results indicated that the efficiency of intracellular infectious virus production was significantly higher in HuH-7T1 than that in Huh-7.5.1, whereas virus secretion efficiency was slightly lower in HuH-7T1 than that in Huh-7.5.1. Therefore, the enhanced intracellular infectious virus production was considered to be responsible for the advantage of HuH-7T1.

## Gene expression analysis

To identify the host factors regarding the concerned properties of HuH-7T1, we measured gene expression levels for genes that encode cellular factors reported to be involved in the HCV life cycle. Among 37 host factor-encoding transcripts tested, none except for miR-122 showed more than 2-fold higher or lower expression levels in HuH-7T1 compared to Huh-7.5.1 (Fig. S4). The gene expression level of miR-122 was approximately 5-times lower in HuH-7T1 than in Huh-7.5.1.

## Cell cycle analysis of HuH-7T1 and Huh-7.5.1

Although we found that intracellular infectious HCV particles produced more efficiently in HuH-7T1 than in Huh-7.5.1, we thought that there were other possible steps associated with the efficient virus production of HuH-7T1. Because, when HCV RNA is transfected, HuH-7T1 forms the larger HCV positive cell clusters than Huh-7.5.1 (Fig. 1G), although viral entry is less

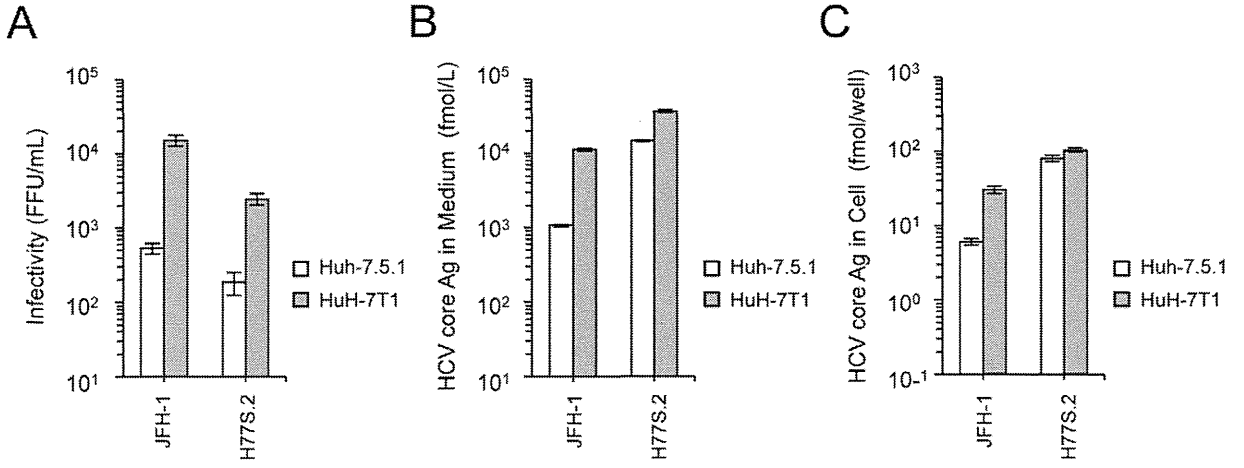


**Figure 5. Infectious virus production of H77S.2 in HuH-7T1 and Huh-7.5.1.** Two micrograms of JFH-1 RNA or 10 micrograms of H77S.2 RNA were electroporated into Huh-7.5.1 and HuH-7T1. Culture medium and cells were harvested at Day 3, and infectivity titer and HCV core level were determined.
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efficient in HuH-7T1 as compared with Huh-7.5.1. To determine other advantages of HuH-7T1, we used flow cytometry to monitor the population of the HCV-positive cells after RNA transfection. At Day 1, the population of HCV-positive cells was higher in Huh-7.5.1 (34.9%) than in HuH-7T1 (13.3%) (Fig. 4A). However, the population of HCV-positive cells in HuH-7T1 increased from Day 1 to Day 5, while that in Huh-7.5.1 decreased over the same interval. When we added anti-CD81 antibody to the medium to exclude the effect of re-infection of the progeny virus, we found that the population of HCV-positive cells in HuH-7T1 did not change from Day 1 to Day 5, while that in Huh-7.5.1 decreased more severely. From these data, we hypothesized that proliferation of HCV-positive cells differed between these cell lines. To clarify this point, we compared the cell cycle distribution of HCV-positive and -negative cells after JFH-1 RNA transfection (Fig. 4B). In Huh-7.5.1, the fraction of cells in S phase was lower among HCV-positive cells than among HCV-negative cells (25.7%±0.8% vs 47.6%±1.5%, respectively; $P<0.05$; Fig. 5C); conversely, the fraction of cells in G0/G1 and G2/M phases was higher among HCV-positive cells compared to HCV-negative cells (51.0%±1.4% vs. 42.8%±1.7%, 21.2%±1.1% vs. 8.6%±0.4%, respectively; $P<0.05$, Fig. 4C), indicating that cell proliferation was suppressed by HCV replication in Huh-7.5.1. By contrast, in HuH-7T1, the fraction of cells in S phase was not significantly different for HCV-positive and -negative cells (Fig. 4C). Thus, unlike Huh-7.5.1, HuH-7T1 evaded the cell cycle arrest associated with HCV replication.

We also analyzed HCV-related apoptosis by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay and found that apoptosis was observed in a limited number of HCV-positive cells (Fig. S5) as we reported previously [17].

### Comparisons of virus production level of H77S.2 (genotype 1a) between HuH-7T1 and Huh-7.5.1

To test whether HuH-7T1 could enhance viral production of HCV strains other than JFH-1, we transfected H77S.2 RNA into HuH-7T1 and Huh-7.5.1 and compared the infectious virus production. As seen with JFH-1 RNA transfection, H77S.2 RNA transfection of HuH-7T1 resulted in increased (13.1-fold) levels of infectious virus and increased (2.5-fold) level of HCV core in medium compared to Huh-7.5.1 (Fig. 5A and 5B), although intracellular HCV core was slightly higher in HuH-7T1 than in Huh-7.5.1 (Fig. 5C).

## Discussion

Increased efficiency of virus production can be achieved by viral adaptations associated with enhancement of steps in the viral life cycle. A number of adaptive mutations that could enhance viral genome replication or viral particle assembly has been reported, although the effects of some of these mutations were strain specific [18,19], and none of these has been reported to be applicable to multiple strains and genotypes. Therefore, to obtain the efficient virus production with multiple HCV strains, several cell lines permissive for HCV have been established [6,20,21,22,23]. Generally, they were generated by curing replicon cells in which HCV subgenomic replicon replicated efficiently. As a result, these cured cells support primarily the HCV RNA replication and it is not sufficient to obtain large amounts of virus. The Huh-7.5.1 strain is an example of such a cured cell, and is known to have a loss-of-function mutation in the gene encoding RIG-I, thereby impairing a part of innate immune system and permitting increased HCV replication [20,24]. In the present study, we used another strategy to obtain the cell line for efficient HCV production, namely the use of limiting dilution to isolate a cell line with the desired properties. Our resulting cell line (designated HuH-7T1) produced infectious virus more efficiently than Huh-7.5.1, while supporting a more rapid increase of HCV infected cells.

To identify the affected steps of the viral life cycle in HuH-7T1, we systematically used various assays to investigate the steps of viral infection, translation, replication, infectious viral particle production, and secretion. The HCV infection step was assessed by two assays, using HCVcc and HCVpp. Both assays indicated that the HCV infection efficiency was lower in HuH-7T1 than in Huh-7.5.1. It has been reported that the susceptibility for HCV infection was associated with CD81 expression levels [11,25]. We observed that the population of CD81-expressing cells was lower in HuH-7T1 than in Huh-7.5.1. Therefore, the lower infection efficiency of HuH-7T1 was probably due to the reduced number of CD81-expressing cells. We found that the efficiency of genome translation was lower, but the efficiency of replication was similar in HuH-7T1 compared with Huh-7.5.1. By the gene expression analysis, miR-122 was detected as less expressed in HuH-7T1, and it may be responsible for the lower translation efficiency of HuH-7T1. In contrast, the efficiency of intracellular infectious viral particle production was substantially higher in HuH-7T1 than in Huh-7.5.1. We measured the expression levels of genes encoding host factors involved in viral particle assembly, but did not identify any responsible genes for HuH-7T1 phenotype. A comprehensive microarray analysis would be needed to determine the responsible host factors. We also found that virus secretion efficiency was lower in HuH-7T1 than in Huh-7.5.1. Nevertheless, virus production in HuH-7T1 was significantly higher than that in Huh-7.5.1, suggesting that the enhancement of intracellular viral particle production efficiency in HuH-7T1 was sufficient to overcome other disadvantages compared to Huh-7.5.1.

Immunostaining analysis clearly indicated that the number of HCV-positive cells at Day 5 after RNA transfection was larger for HuH-7T1 than for Huh-7.5.1, and the percentage of HCV positive cell clusters consisting of more than 5 cells was higher in HuH-7T1 than in Huh-7.5.1. These effects may not be fully explained by the difference in intracellular viral particle production efficiency. Thus, we focused on the cell proliferation of HCV-replicating cells in HuH-7T1 and Huh-7.5.1. Flow cytometry analysis revealed that the HCV-positive cell population increased in HuH-7T1 from Day 1 to Day 5, in contrast to the decrease seen in Huh-7.5.1 cells during the same interval. A detailed analysis of the cell cycle populations revealed that the ratio of S-phase cells was reduced by HCV replication in Huh-7.5.1, but not in HuH-7T1. Thus, cell proliferation was suppressed by HCV replication in Huh-7.5.1, but not in HuH-7T1. The time-dependent reduction of the HCV-positive cell population observed in Huh-7.5.1 probably resulted from decreased proliferation activity of HCV-replicating cells relative to HCV-negative cells in spite of the efficient re-infection of the progeny virus. In the case of HuH-7T1, the HCV-positive cells could proliferate as like as the HCV-negative cells, and as a result, the HCV-positive cell population was increased by the re-infection of the progeny virus allowing production of large amounts of viruses.

Cell cycle arrest associated with HCV replication in cell culture has been reported previously. Walters et al. observed S-phase reduction in Huh7.5 cells infected with J6/JFH-1 chimeric viruses, but could not identify the factor(s) responsible for the delay in cell cycle progression [26,27]. Another group also reported an increase in G2/M phase and reduction in S phase in Huh7.5 cells following transfection of JFH-1 and its chimeric viral RNA, and suggested

that the degree of cell cycle arrest was related to the intracellular level of viral protein [26,27]. Additionally, there are numerous papers reporting the relationship between cell cycle arrest and individual HCV proteins such as core [28,29,30], NS2 [31] and NS5B [32,33,34]. However, effects of these HCV proteins on cell cycle remain controversial, and the mechanisms of cell cycle arrest caused by HCV replication remain unclear. Since HuH-7T1 are resistant to cell cycle arrest by HCV replication while Huh-7.5.1 are sensitive, these cell lines should help to clarify the mechanism of cell cycle arrest while facilitating the identification of host and viral factors involved therein.

The improved viral production in the HuH-7T1 was observed also with another HCV strain, H77S.2. This viral strain is a derivative of H77S [35], which is genotype 1a and produces infectious virus in cultured cells following full-genome RNA transfection [13]. Although the H77S.2 strain could replicate, and secreted HCV core protein more efficiently than JFH-1 in Huh-7.5.1, infectious virus production was less efficient as compared with JFH-1 and infectivity in the medium of H77S.2 RNA-transfected Huh-7.5.1 was at a detectable level. These data implied that H77S.2 mainly secreted unassembled HCV core proteins or noninfectious virus particles. In HuH-7T1, the infectious virus production of H77S.2 was enhanced about ten times, and HCV core level in the medium was enhanced about three times, indicating that HuH-7T1 enhanced infectious virus production. These data also indicated that large amounts of infectious viruses could also be obtained with other HCV strains in HuH-7T1.

In conclusion, we isolated a HuH-7 subclone, HuH-7T1, that displays improved ability to produce infectious HCV virus particles. Enhanced intracellular infectious virus production and evasion of cell cycle arrest were important for the increased efficiency of viral production. This cell line is expected to facilitate HCV research both by providing increased amounts of HCV particles and by permitting the identification of cellular factors involved in viral particle production.

## Supporting Information

**Figure S1 Kinetics of JFH-1 virus infection on HuH-7T1, huh-7.5.1 and HuH-7.** Target cells were seeded into 12-well plates at a density of $2\times10^5$ cells/well. On the following day, the cells were infected with JFH-1 virus at a multiplicity of infection of 0.1 and incubated for 72 h at 37°C. Culture medium and cells were harvested at Days 1, 3, and 5, and HCV core protein levels in the culture medium and in the cells were measured. Assays were performed three times independently, and data are presented as mean ± standard deviation.
(TIF)

**Figure S2 Expression levels of CD81 in HuH-7T1 and Huh-7.5.1.** Analysis of CD81 expression on cell surface of HuH-7T1 and Huh-7.5.1 by flow cytometry. The assays were performed three times independently; representative data are shown. Percentages of CD81-positive cells are shown above the histogram.
(TIF)

**Figure S3 Comparison of absolute luciferase activity in HuH-7T1 and Huh-7.5.1.** Absolute measurement data of luciferase activity at Fig. 3A was plotted.
(TIF)

**Figure S4 Expression levels of genes associated with HCV life cycle in HuH-7T1 and Huh-7.5.1.** Total cellular RNA was extracted from Huh-7.5.1 and HuH-7T1, and cDNA was synthesized using Superscript III reverse transcriptase (except for miR-122) or TaqMan MicroRNA RT Kit (miR-122). Quantitative PCR was performed using gene-specific primer and probe sets. Data are expressed as a fold-difference of expression compared to that in Huh-7.5.1. Dashed lines indicate 2-fold higher or lower expression levels compared to Huh-7.5.1.
(TIF)

**Figure S5 Apoptosis assay of JFH-1 RNA-transfected cells.** Two micrograms of JFH-1 RNA was electroporated into Huh-7.5.1 and HuH-7T1. Cells were harvested at Day 3 and fixed in 4% paraformaldehyde, permeabilized, and stained with anti-NS5A antibody (clone KS0265-1) and Alexa Fluor 647 Goat Anti-mouse IgG (Invitrogen). Apoptosis was detected by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL). Samples were analyzed using a FACS Calibur flow cytometer.
(TIF)

**Text S1 Supporting Materials and Methods.**
(DOC)

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## Author Contributions

Conceived and designed the experiments: AM TW SM TK. Performed the experiments: AM NS SY MIS TM SK TK. Analyzed the data: AM TK. Contributed reagents/materials/analysis tools: SY MIS SM. Wrote the paper: AM SM TK.

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# *Trans*-complemented hepatitis C virus particles as a versatile tool for study of virus assembly and infection

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ABSTRACT

In this study, we compared the entry processes of *trans*-complemented hepatitis C virus particles (HCVtcp), cell culture-produced HCV (HCVcc) and HCV pseudoparticles (HCVpp). Anti-CD81 antibody reduced the entry of HCVtcp and HCVcc to almost background levels, and that of HCVpp by approximately 50%. Apolipoprotein E-dependent infection was observed with HCVtcp and HCVcc, but not with HCVpp, suggesting that the HCVtcp system is more relevant as a model of HCV infection than HCVpp. We improved the productivity of HCVtcp by introducing adapted mutations and by deleting sequences not required for replication from the subgenomic replicon construct. Furthermore, blind passage of the HCVtcp in packaging cells resulted in a novel mutation in the NS3 region, N1586D, which contributed to assembly of infectious virus. These results demonstrate that our plasmid-based system for efficient production of HCVtcp is beneficial for studying HCV life cycles, particularly in viral assembly and infection.



## Introduction

Over 170 million people worldwide are chronically infected with hepatitis C virus (HCV), and are at risk of developing chronic liver diseases (Hoofnagle, 2002). HCV is an enveloped virus of the family *Flaviviridae*, and its genome is a positive-strand RNA consisting of the 5′-untranslated region (UTR), an open reading frame encoding viral proteins (core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) and the 3′-UTR (Suzuki et al., 2007).

Host–virus interactions are required during the initial steps of viral infection. It was previously reported that CD81 (Bartosch et al., 2003a, b; McKeating et al., 2004; Pileri et al., 1998), scavenger receptor class B type I (Bartosch et al., 2003a, b; Scarselli et al., 2002), claudin-1 (Evans et al., 2007; Liu et al., 2009) and occludin (Benedicto et al., 2009; Evans et al., 2007; Liu et al., 2009; Ploss et al., 2009) are critical molecules for HCV entry into cells. CD81 interacts with HCV E2 via a second extracellular loop (Bartosch et al., 2003a, b; Hsu et al., 2003) and its role in the internalization process was confirmed (Cormier et al., 2004; Flint et al., 2006). It has also been shown that infectious HCV particles produced in cell cultures (HCVcc) exist as apolipoprotein E (ApoE)-enriched lipoprotein particles (Chang et al., 2007) and that ApoE is important for HCV infectivity (Owen et al., 2009).

Investigation of HCV had been hampered by difficulties in amplifying the virus in vitro before development of robust cell culture systems based on JFH-1 isolates (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). Retrovirus-based HCV pseudoparticles (HCVpp), in which cell entry is dependent on HCV glycoproteins, have been used to study virus entry (Bartosch et al., 2003a; Hsu et al., 2003). Vesicular stomatitis virus (VSV)-based pseudotypic viruses bearing HCV E1 and E2 and replication-competent recombinant VSV encoding HCV envelopes have also been available as surrogate models for studies of HCV infection (Mazumdar et al., 2011; Tani et al., 2007).

It was recently shown that HCV subgenomic replicons can be packaged when structural proteins are supplied in *trans* (Adair et al., 2009; Ishii et al., 2008; Masaki et al., 2010; Steinmann et al., 2008). These *trans*-complemented HCV particles (HCVtcp) are infectious, but support only single-round infection and are unable to spread. Establishment of flexible systems to efficiently produce HCVtcp should contribute to studying HCV assembly, in particular encapsidation of the viral genome, and entry to cells with less stringent biosafety and biosecurity measures. Although single-round infection can be achieved by using the HCVcc system with receptor knock-out

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cells, the single-round HCVcc system is not suitable for studying virus entry. We previously described plasmid-based production of HCVcc and HCVtcp (Masaki et al., 2010). Here, we demonstrated that HCVtcp production can be enhanced by introducing the previously reported cell-culture adaptive mutations and by deleting sequences not essential for replication in the subgenomic replicon construct. By providing genotype 1b-derived core-to-p7 in addition to intragenotypic viral proteins, chimeric HCVtcp were generated. Furthermore, blind passage of HCVtcp in the packaging cells resulted in the identification of a novel cell culture-adaptive mutation in NS3 that enables us to establish the efficient production of HCVtcp with structural proteins from various strains. Taken together, our system for producing single-cycle infectious HCV particles should be useful in the study of entry and assembly steps of the HCV life cycles. This technology may also have potential to be the basis for the safer vaccine development.

## Results

### *Enhancement of HCVtcp production by adaptive mutations in E2, p7 and NS2 and by deleting sequences not essential for replication from replicon construct*

In our HCVtcp system, the RNA polymerase I (Pol I)-driven replicon plasmid, which carries a dicistronic subgenomic luciferase reporter replicon of JFH-1 strain with a Pol I promoter and terminator (pHH/SGR-Luc), as well as a plasmid containing core-NS2 cDNA under the CAG promoter (pCAGC-NS2) were used (Masaki et al., 2010). In an effort to improve the yield of HCVtcp production, cell culture-adaptive mutations in E2 (N417S), p7 (N765D) and NS2 (Q1012R) which were previously selected from serial passage of HCVcc (Russell et al., 2008) were introduced into the core-NS2 expression plasmid (Fig. 1A) (residues are numbered

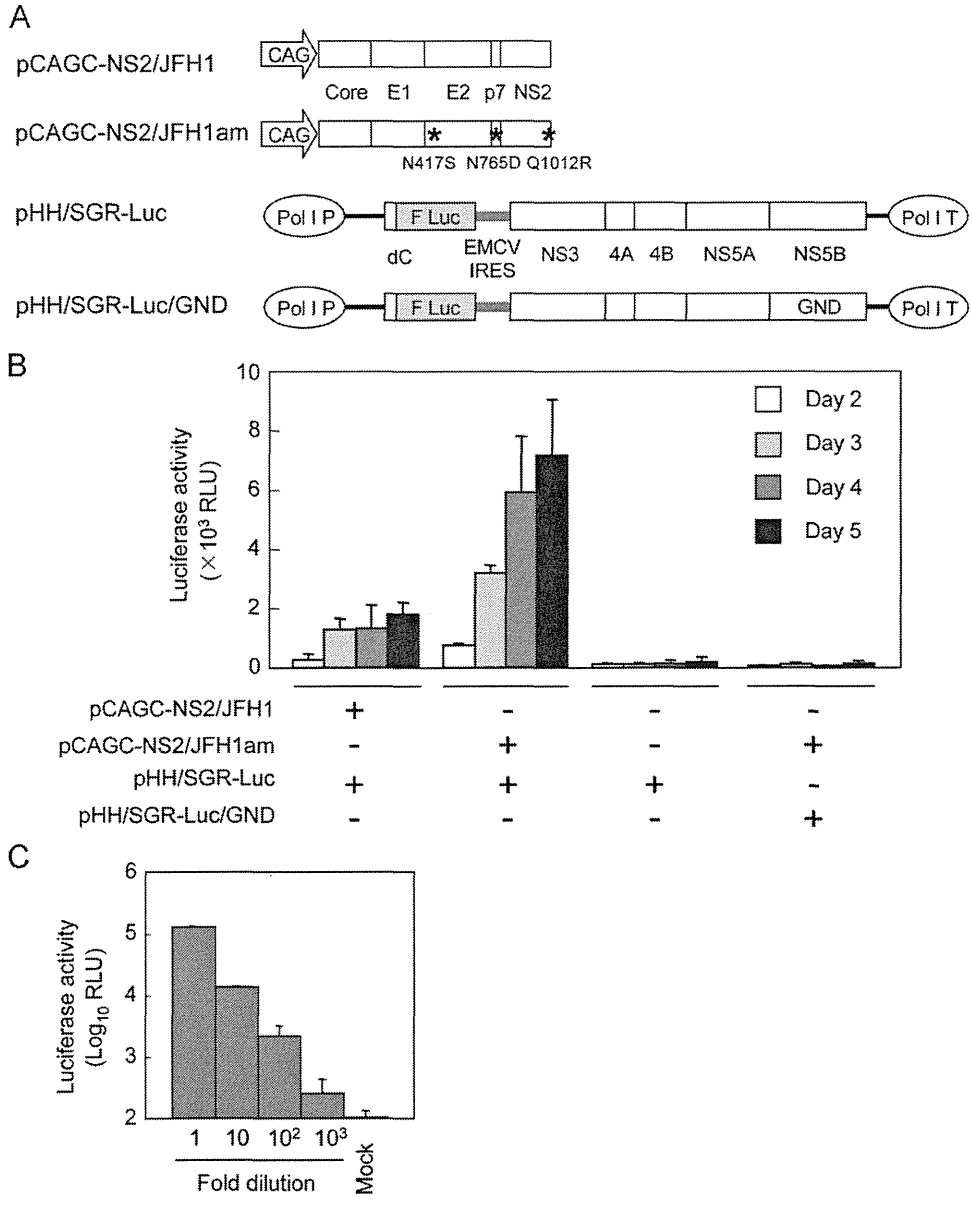


**Fig. 1.** HCVtcp production by two-plasmid transfection. (A) Schematic representation of plasmids is shown. HCV polyproteins derived from JFH-1 are indicated by white boxes. HCV UTRs are indicated by bold lines. The internal ribosomal entry site from encephalomyocarditis virus (EMCV IRES) is denoted as gray lines. Adaptive mutations are indicated as asterisks. F Luc: firefly luciferase gene; CAG: CAG promoter; Pol I P: RNA polymerase I promoter; Pol I T: RNA polymerase I terminator; GND: replication-deficient GND mutation. (B) Luciferase activity in Huh7.5.1 cells inoculated with supernatant from cells transfected with indicated plasmids at the indicated time points. Data are averages of triplicate values with error bars showing standard deviations. (C) Luciferase activity in cells inoculated with serially diluted HCVtcp.

according to positions within the JFH-1 polyprotein). Supernatants of cells transfected with plasmids (Fig. 1A) were collected and were used to infect Huh7.5.1 cells, which were analyzed by luciferase assay. Introduction of adaptive mutations (pCAGC-NS2/JFH1am) resulted in more than 4-fold higher production of HCVtcp at 5 day post-transfection, as compared to wild-type (WT) (pCAGC-NS2/JFH1) (Fig. 1B), indicating that the adaptive mutations contribute to enhancing HCVtcp production. To confirm that luciferase activity levels in HCVtcp-infected cells are correlated with the number of infectious particles, Huh7.5.1 cells were inoculated with serial dilutions of HCVtcp. Luciferase activity was well correlated with viral load (Fig. 1C), indicating that luciferase assay in HCVtcp-infected cells can be used to quantify HCV infection.

In order to further explore the efficient production of HCVtcp, we generated replicon constructs that lack the luciferase gene or include the partial coding sequences for structural proteins instead of reporter (Fig. 2A). Replication of each replicon in plasmid-transfected cells was then assessed by Western blotting (Fig. 2B). Among the constructs tested, NS5B levels were lowest in cells expressing pHH/SGR-Luc. NS5B levels in cells replicating other replicons appeared to be comparable. Cells were infected with supernatants of cells transfected with each replicon plasmid, along with pCAGC-NS2/JFH1am, followed by infectious unit assay (Fig. 2C). The highest production of HCVtcp was obtained from cells transfected with pHH/SGR, where the luciferase sequence was deleted from pHH/SGR-Luc, thus suggesting that deletion of the sequence not essential for RNA replication in the replicon may contribute to enhancing HCVtcp production.

*Production of chimeric HCVtcp by providing heterologous core-p7*

In order to elucidate whether *trans*-encapsidation of JFH-1 replicon can be achieved by providing core-p7 from other HCV strains, core-NS2 plasmids were constructed (Fig. 3A). In these plasmids, core through the N-terminal 33 aa of NS2, which contains transmembrane domain 1 of NS2, was derived from either H77c (genotype 1a), THpa (genotype 1b), Con1 (genotype 1b) or J6 (genotype 2a) strain. Residual NS2 was derived from JFH-1, as described previously (Pietschmann et al., 2006). HCVtcp was efficiently produced by core-p7 of J6 and THpa strains, but its production was less efficient in the case of Con1 strain. *Trans*-packaging was not detectable when core-p7 of H77c strain was used (Fig. 3C). Among HCV strains tested, difference in luciferase activity levels in HCVtcp-infected cells (Fig. 3C) were in agreement with that in the viral RNA levels in the culture supernatants of the transfected cells (Fig. 3B). Although the efficacy of *trans*-complementation was variable among strains, chimeric HCVtcp can be generated by providing genotype 1b-derived core-p7 in addition to intragenotypic viral proteins, and was used in subsequent studies.

*ApoE- and CD81-dependent infection by HCVtcp*

There is accumulating evidence that apolipoproteins, particularly ApoE, contribute to HCV production and infectivity (Chang et al., 2007; Owen et al., 2009). To determine whether ApoE is involved in infection of target cells by HCVtcp, we infected cells in the presence of increasing concentrations of anti-ApoE antibody.

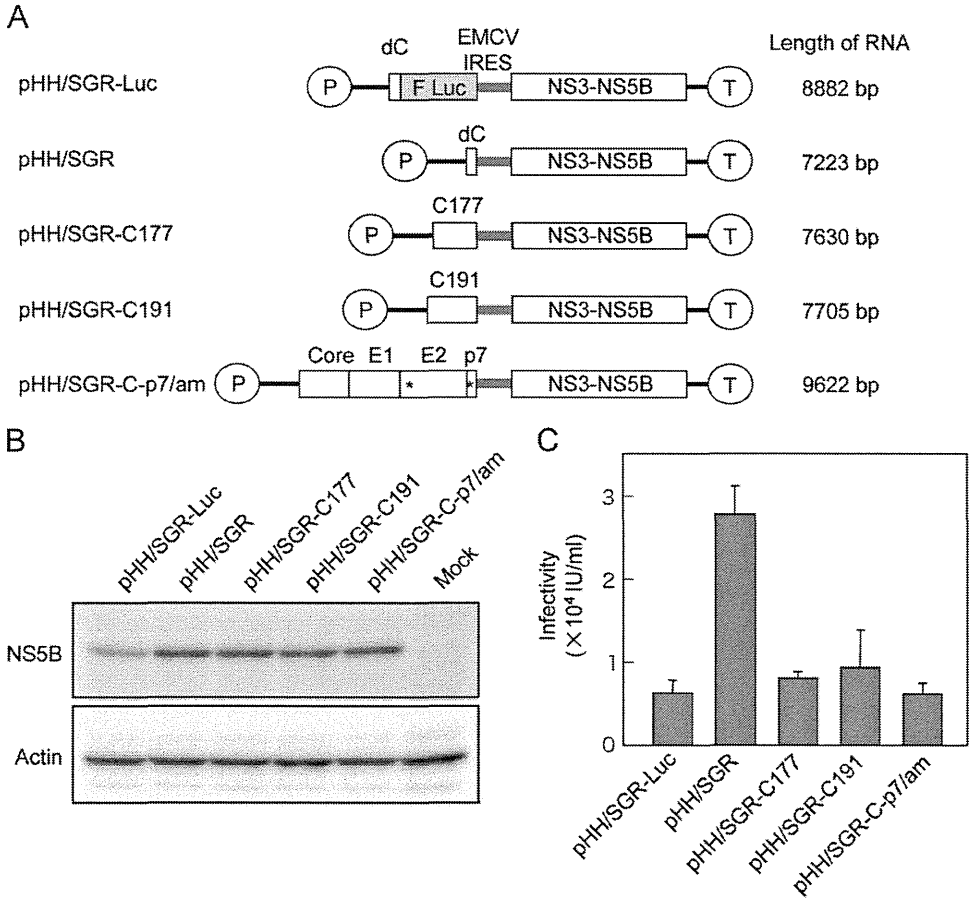


**Fig. 2.** Production of HCVtcp with different replicon constructs. (A) Schematic representation of plasmids used for production of HCVtcp. Deduced length of transcribed RNA from each construct is shown on the right. HCV polyproteins from JFH-1 strain are indicated by open boxes. HCV UTRs are indicated by bold lines. The EMCV IRES is denoted by gray bars. Adaptive mutations are indicated by asterisks. F Luc: firefly luciferase gene; P: RNA polymerase I promoter; T: RNA polymerase I terminator. (B) Detection of NS5B and actin in Huh7.5.1 cells transfected with indicated plasmids at 4 day post-transfection. (C) Infectivity of culture supernatants from cells transfected with indicated replicon plasmids along with pCAGC-NS2/JFH1am at 4 day post-transfection.

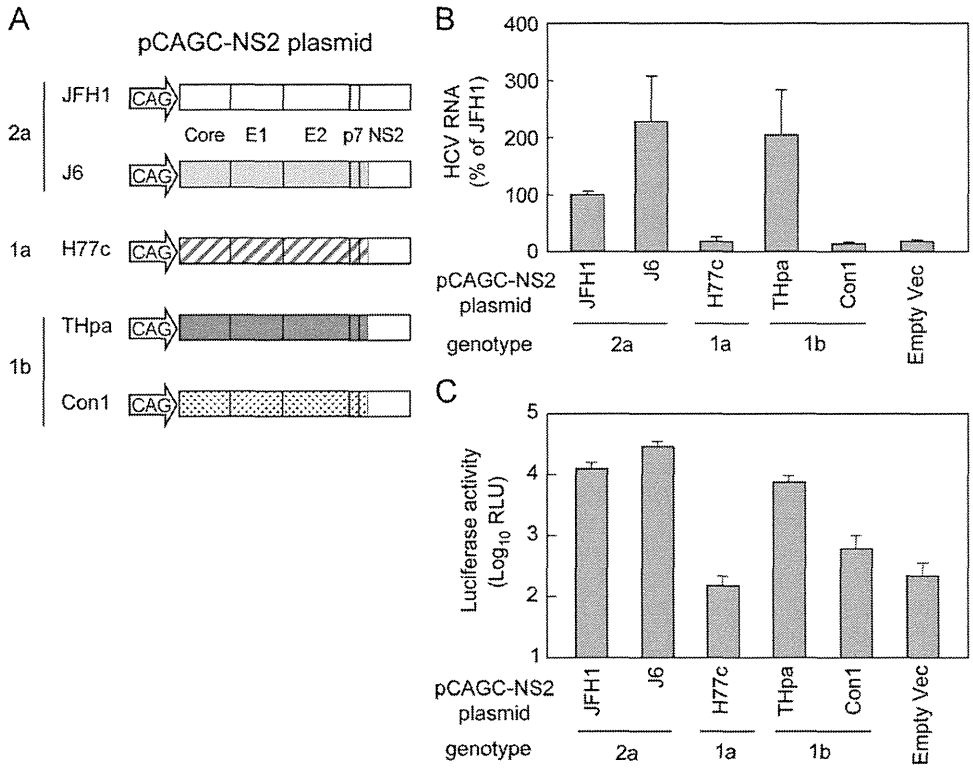


**Fig. 3.** HCVtcp production with structural proteins from various strains. (A) Schematic representation of plasmids used. HCV polyproteins of JFH-1, J6, H77c, THpa and Con1 strain are shown in the open box, bright gray box, box with diagonal lines, dark gray box and dotted box, respectively. (B) Relative levels of HCV RNA in the supernatant from cells transfected with indicated plasmids along with pHH/SGR-Luc. (C) Luciferase activity in cells inoculated with supernatant from cells transfected with indicated plasmids along with pHH/SGR-Luc at 4 day post-transfection.

pCAGC-NS2/THpa and pCAGC-NS2/JFH1am were used as core-NS2 plasmids for HCVtcp production carrying core-p7 derived from genotypes 1b and 2a (HCVtcp-1b and HCVtcp-2a, respectively). HCVpp derived from JFH-1 and VSVpp were generated and used for comparison. Infection with HCVtcp-1b or HCVtcp-2a was blocked by anti-ApoE antibody in a dose-dependent manner. In contrast, anti-ApoE antibody did not affect infection with HCVpp and VSVpp (Fig. 4A).

The CD81 dependence of infection was also compared between HCVtcp and HCVpp (Fig. 4B). Anti-CD81 antibody inhibited the entry of HCVtcp-1b, HCVtcp-2a, and HCVpp in a dose-dependent manner. The antibody had no effect on VSVpp infection. HCVtcp infection appears to be more sensitive to anti-CD81 antibody when compared with HCVpp infection; more than 60% inhibition was observed at 0.08 μg/mL anti-CD81 antibody for HCVtcp-1b and HCVtcp-2a, whereas approximately 50% inhibition was observed for HCVpp at 2 μg/mL antibody. Neutralization of HCVcc by anti-ApoE and anti-CD81 antibodies was also determined. Antibodies blocked HCVcc infection (Fig. 4C and D), as observed with HCVtcp. These results suggest that ApoE, as well as CD81, play an important role in HCVtcp infection. Thus, HCVtcp may be more useful for evaluating the HCV entry process than HCVpp.

*Identification of novel culture-adaptive mutation in NS3 by serial passage of HCVtcp in packaging cells*

The HCVtcp system was further applied to analyses of genetic changes during serial passages in target cells. As an initial attempt, supernatants of cells co-transfected with pCAGC-NS2/JFH1am and pHH/SGR were inoculated into Huh7.5.1 cells transiently transfected with pCAGC-NS2/JFH1am. However, infectious titer was lost after repeated inoculation, likely due to low HCVtcp titers and low efficiency of plasmid transduction (data not shown). To overcome this, we utilized recombinant adenovirus vectors (rAdVs) to provide core-NS2. As we were not able to obtain rAdV directly expressing core-NS2, conditional transgene expression based on a Cre-loxP strategy was employed (Kanegae et al., 1995). We constructed an rAdV containing core-NS2 gene downstream of a stuffer DNA flanked by a pair of loxP sites (AxCALNLH-CNS2). When cells were doubly infected with AxCALNLH-CNS2 and the Cre-expressing rAdV, AxCANCre (Kanegae et al., 1995), the Cre-mediated excisional deletion removed the stuffer DNA, resulting in core-NS2 expression under control of the CAG promoter (Fig. 5A). As expected, tightly regulated production of HCVtcp was observed. The cells infected with AxCANCre and AxCALNLH-CNS2 along with transduction of pHH/SGR-Luc produced HCVtcp at high levels. Production of HCVtcp was undetectable when either AxCANCre or AxCALNLH-CNS2 was not infected (Fig. 5B). The Cre-mediated rAdV expression system appears to have yielded considerably higher production of HCVtcp when compared with the settings for plasmid co-transfection.

Supernatants from cells in which core-NS2 was expressed using rAdVs and the subgenomic RNA derived from pHH/SGR replicated were inoculated into cells infected with AxCALNLH-CNS2 and AxCANCre (Fig. 6A). Blind passage was performed by sequentially transferring culture supernatants to cells infected with the above rAdVs. The two independent 10 blind passages (p10) showed virus titers of $>1\times10^6$ IU/mL, which were markedly higher than those of the passage 0 (p0) stock cultures ($4\times10^4$ IU/mL). Side-by-side infection analysis revealed that the HCVtcp p10 #1 achieved a virus titer approximately 36 times higher than that of HCVtcp p0 on the packaging cells at 6 day post-infection (Fig. 6B). Sequencing of the entire replicon in the supernatants at p10 in two independent experiments revealed

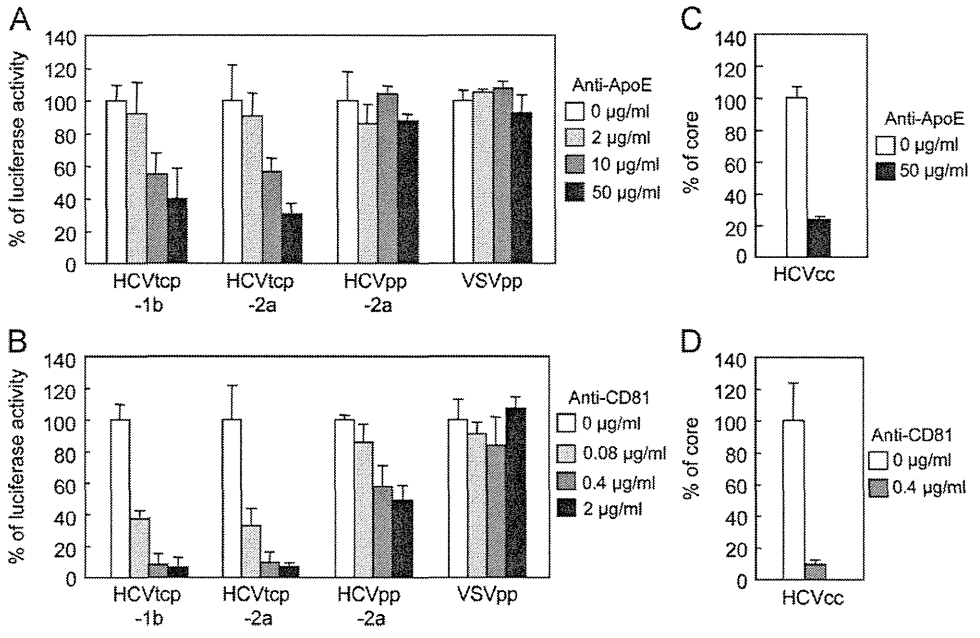


**Fig. 4.** Effects of anti-ApoE and anti-CD81 antibodies on HCV entry. (A) Aliquots of virus sample were incubated with increasing concentrations of anti-ApoE antibodies for 1 h and were then added to Huh7.5.1 cells. Luciferase activity was determined at 72 h post-infection and is expressed relative to activity without antibodies (white bar). (B) Huh7.5.1 cells were preincubated for 1 h with increasing concentrations of anti-CD81 antibodies, followed by inoculating virus samples. Luciferase activity was determined and expressed as shown in (A). (C) Aliquots of HCVcc were incubated with anti-ApoE antibodies for 1 h and were then added to Huh7.5.1 cells at an MOI of 0.05. Intracellular core levels were quantitated at 24 h post-infection and are expressed relative to levels without antibodies (white bar). (D) Huh7.5.1 cells were preincubated for 1 h with anti-CD81 antibodies. HCVcc infection and measurement of core proteins were performed as indicated in (C).

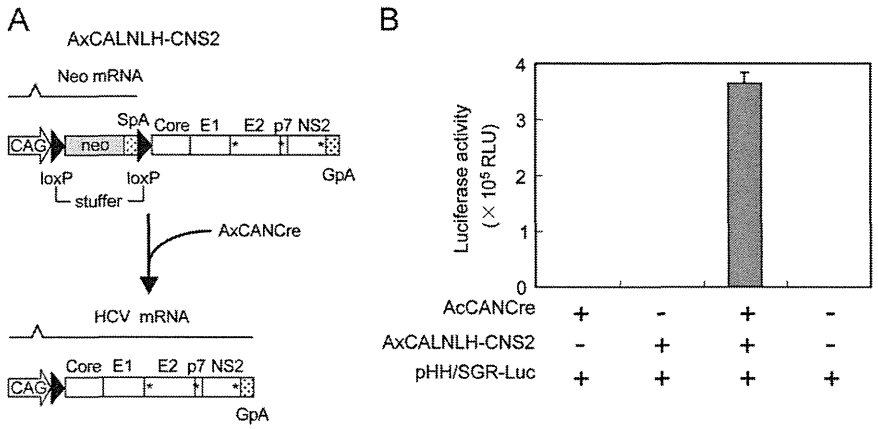


**Fig. 5.** Transgene activation mediated by rAdVs expressing Cre recombinase under control of CAG promoter. (A) Cre recobminase expressed by AxCANCre recognizes a pair of its target sequences loxP in AxCALNLH-CNS2, and removes the stuffer region resulting in expression of HCV core-NS2 polyprotein by CAG promoter. CAG: CAG promoter; SpA: SV40 early polyA signal; GpA: rabbit b-globin poly(A) signal. (B) Luciferase activity in Huh7.5.1 cells inoculated with 4-day post-transfection culture supernatant from cells transfected with pHH/SGR-Luc, and then infected with indicated rAdVs.

that both passaged HCVtcp had an identical nonsynonymous mutation in the NS3 region (N1586D) (Fig. 6C).

In order to examine the role of NS3 mutation identified on HCV RNA replication and on HCVtcp production, the N1586D mutation was introduced into pHH/SGR-Luc. Luciferase activities of the N1586D-mutated replicon were apparently lower than those of the WT-replicon, thus suggesting that the NS3 mutation reduced viral RNA replication (Fig. 7A). HCV RNA levels in the supernatants of cells transfected with WT- or mutant replicon plasmid along with pCAGC-NS2/JFH1am and luciferase activity in cells inoculated with supernatants from the transfected cells were then determined (Fig. 7B). The viral RNA level secreted from cells replicating the N1586D-mutated replicon was lower than that from cells replicating WT replicon (Fig. 7B, left). By contrast, a significantly higher infectivity of HCVtcp produced from the mutant replicon-cells was observed, as compared to WT replicon-cells (Fig. 7B, right), suggesting that the adaptive mutation increased the specific infectivity (almost 9-fold) of the virus particles. To further determine whether the N1586D mutation affects infectious viral assembly and/or virus release, we used the CD81-negative Huh-7 subclone, Huh7-25 (Akazawa et al., 2007), which may produce infectious particles, but is not susceptible to HCV entry due to a lack of CD81 expression, therefore allowing us to examine viral assembly and release without the influence of reinfection by produced HCVtcp. Measurement of intracellular and extracellular HCVtcp indicated that Huh7-25 cells replicating the N1586D-mutated replicon produced more infectious virus than WT in both supernatants and cell lysates (Fig. 7C). Thus, it can be concluded that the N1586D mutation contributes to enhanced infectious viral assembly, not RNA replication. We could not exclude the possibility that N1586D mutation affects virus release, since the mutation enhanced extracellular virus titers more than did the intracellular titer.

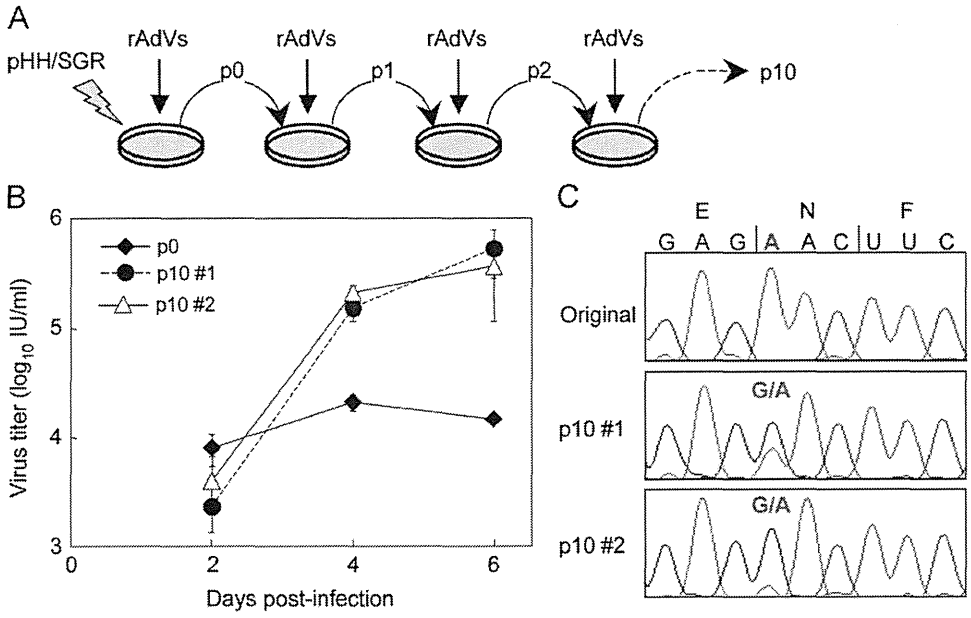


**Fig. 6.** Genotypic changes in HCVtcp following blind passage. (A) Experimental procedure for blind passage of HCVtcp. Huh7.5.1 cells were transfected with pHH/SGR and were doubly infected with AxCANCre and AxCALNLH-CNS2. Culture fluids were collected and were inoculated into cells infected with AxCANCre and AxCALNLH-CNS2. These procedures were repeated 10 times with two independent samples (#1 and #2). (B) Growth curves of HCVtcp p0 and p10 on Huh7.5.1 cells expressing core-NS2. Cells were infected with HCVtcp at an MOI of 0.05, and medium was collected at the indicated time points and subjected to titration. (C) Nucleotide sequences of original and blind-passaged replicons from HCVtcp. Nucleotides of mutated position are shown in red and bold.

The impact of the N1586D mutation on production of intra- and intergenotypic HCVtcp chimeras was also investigated. The N1586D mutation in the replicon enhanced the production of chimeric HCVtcp by providing core-p7 from all strains examined, although not statistically significant in THpa, and Con1 strains (Fig. 7D). Finally, to determine whether the N1586D mutation was responsible for enhancing HCVcc production, this mutation was introduced into pHHJFH1, which carries the full-length wild-type JFH-1 cDNA (Masaki et al., 2010), yielding pHHJFH1N1586D. The virus titer obtained from cells transfected with the pHHJFH1N1586D was significantly higher than that of WT (Fig. 7E), thus demonstrating that the N1586D mutation enhances yields of HCVcc, in addition to HCVtcp.

## Discussion

Single-round infectious viral particles generated by *trans*-packaging systems are considered to be valuable tools for studying virus life cycles, particularly the steps related to entry into target cells, assembly and release of infectious particles. However, limited HCV strains have been applied for the efficient production of HCVtcp to date. In this study, we improved the HCVtcp system in order to enhance the productivity of infectious particles. Production of chimeric HCVtcp by providing genotype 1b-derived core-p7, in addition to intragenotypic viral proteins, was also confirmed. Furthermore, we exploited the system to investigate genetic changes during serial passage of target cells and identified a novel cell culture-adaptive mutation in NS3, which also contributes to enhance the productivity of HCVtcp.

HCVpp (Bartosch et al., 2003a; Hsu et al., 2003) has proven to be a valuable surrogate system by which the study of viral and cellular determinants of the viral entry pathway is possible. Early steps of HCV infection, including the role of HCV glycoprotein heterodimers, receptor binding, internalization and pH-dependent endosomal fusion, have been at least in part mimicked by HCVpp (Lavie et al., 2007). However, as HCVpp is generated in non-hepatic cells such as the human embryo kidney cells 293T, it is likely that the cell-derived component(s) of HCVpp differ from those of HCVcc. Hepatocytes play a role in maintaining lipid homeostasis in the body by assembling and secreting lipoproteins, including VLDL. It is highly likely that HCV exploits lipid synthesis pathways, as there is a tight link between virion formation and VLDL synthesis. Down-regulation of ApoE considerably reduces HCV production (Benga et al., 2010; Chang et al., 2007; Hishiki et al., 2010; Jiang and Luo, 2009; Owen et al., 2009). Infectivity of HCVcc is also neutralized by anti-ApoE antibodies (Chang et al., 2007). These data suggest that ApoE is important for HCV infectivity. Furthermore, Niemann-Pick C1-like 1 (NPC1L1), involving cholesterol uptake receptor, was recently identified as a host factor for HCV entry (Sainz et al., 2012). Knockdown of NPC1L1 had no effect on the entry of HCVpp whereas HCVcc entry was impaired, possibly due to different cholesterol content of these particles. Here, we found that the anti-ApoE antibody neutralized infection by HCVtcp and HCVcc, but not by HCVpp (Fig. 4A and C), thus suggesting that biogenesis and/or secretion pathways of VLDL are involved in HCVtcp similarly to HCVcc, but not in HCVpp.

We also observed that infectivity of HCVtcp and HCVcc is more efficiently neutralized by the anti-CD81 antibody, as compared to that of HCVpp (Fig. 4B and D). It has recently been reported that E2 of HCVcc contained both high-mannose-type and complex-type glycans, whereas most of the glycans on HCVpp-associated E2 were complex-type, which is matured by Golgi enzymes (Vieyres et al., 2010). Mutational analysis of the N-linked glycosylation sites in E1/E2 demonstrated that several glycans on E2 may affect the sensitivity of HCVpp against antibody neutralization, as well as access of CD81 to its binding site on E2 (Helle et al., 2010). The differences in sensitivity between HCVtcp and HCVpp to neutralization by anti-CD81 antibody observed here may be due to differences in carbohydrate composition of HCV glycoproteins during expression and processing of E1/E2 in cells and morphogenesis of HCVtcp and HCVpp.

By analyzing the various replicons for *trans*-packaging, we observed the highest production of HCVtcp with replicons from pHH/SGR, which lacked sequences not essential for RNA

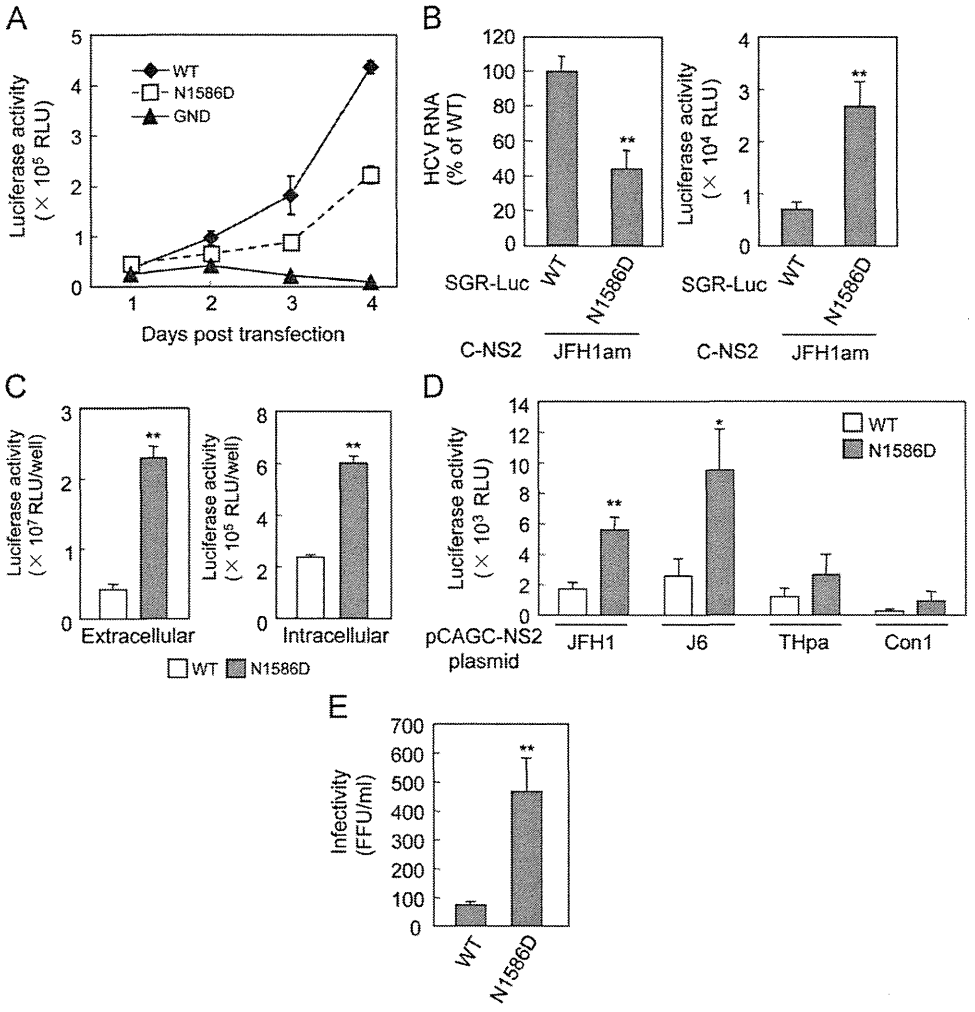


**Fig. 7.** Effects of N1586D mutation on RNA replication and production of HCVtcp or HCVcc. (A) RNA replication of replicons in cells transfected with pHH/SGR-Luc (WT) or N1586D mutant. Luciferase activities at 1 to 4 day post-transfection were determined. (B) Relative levels of HCV RNA in the supernatants from cells transfected with pHH/SGR-Luc (WT) or N1586D mutant plasmid along with pCAGC-NS2/JFH1am were shown in the left panel. Luciferase activities in cells inoculated with supernatants from cells transfected with indicated plasmids at 4 day post-transfection were shown in the right panel. (C) Luciferase activity in cells inoculated with supernatant and cell lysates from Huh7-25 cells transfected with pHH/SGR-Luc (WT) or N1586D mutant plasmid along with pCAGC-NS2/JFH1am at 5 day post-transfection. (D) Luciferase activity in cells inoculated with culture supernatant from cells transfected with pHH/SGR-Luc (WT) or N1586D mutant plasmid along with indicated core-NS2 plasmids at 4 day post-transfection. (E) Infectivity of supernatant from cells transfected with pHH/JFH1 (WT) or its derivative plasmid containing N1586D mutation at 6 day post-transfection. Statistical differences between WT and N1586D were evaluated using Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.005$ vs. WT.

replication, while less efficient productivity was observed from pHH/SGR-Luc, pHH/SGR-C177, pHH/SGR-C191 and pHH/SGR-C-p7/am (Fig. 2C). Differences in the replication efficiency of the replicon do not appear to be a major determinant for HCVtcp productivity, at least in the present settings, as all replicon constructs except pHH/SGR-Luc replicated at similar levels, as confirmed by Western blotting (Fig. 2B). Although the shorter viral genome sequence may offer advantages over the longer sequence, further investigation is required in order to understand the molecular mechanisms underlying viral genome packaging. By comparing pHH/SGR vs. pHH/SGR-C177, pHH/SGR-C191 and pHH/SGR-C-p7/am, it is likely that the expression of the structural protein in *cis* does not increase HCVtcp production when sufficient amounts of structural proteins are supplied in *trans*.

Blind passage of HCVtcp in packaging cells infected with rAdVs providing core-NS2 enabled us to identify a novel culture-adaptive mutation in NS3. The N-terminal third of NS3 forms a serine protease, together with NS4A, and its C-terminal two-thirds exhibits RNA helicase and RNA-stimulated NTPase activities. In addition, similarly to flaviviruses (Kummerer and Rice, 2002; Liu et al., 2002), it is now apparent that HCV NS3 is also involved in viral morphogenesis (Han et al., 2009; Ma et al., 2008), although its precise role and underlying molecular mechanism(s) have not fully been elucidated. Two cell-culture adaptive NS3 mutations which are involved in HCV assembly have been identified. The Q1251L mutation in helicase subdomain 1 resulted in approximately 30-fold higher production of HCV without affecting NS3 enzymatic activities (Ma et al., 2008). The M1290K adaptive mutation was also located in subdomain 1 of the NS3 helicase (Han et al., 2009). The N1586D mutation identified here was located in subdomain 3 of helicase. Analogous to Q1251L and M1290K, the N1586D mutation enhanced the infectious viral assembly by increasing specific infectivity without affecting the efficiency of viral RNA replication. Considering the possibility that NS3 plays a role in linking between the viral replicase and assembly sites (Jones et al., 2011), it is likely that NS3 helicase is one of the determinants for interaction with the structural proteins. Our results, together with earlier studies, suggest that chimeric and defective mutations as well as supplying the viral components in *trans*, function as selective pressures in virion assembly.

In summary, we have established a plasmid-based reverse genetics for efficient production of HCVtcp with structural

proteins from various strains. Single-round infectious HCVtcp can complement the HCVcc and HCVpp systems as a valuable tool for the study of HCV life cycles.

## Materials and methods

### *Cells*

Huh7 derivative cell line Huh7.5.1 and Huh7-25 were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with nonessential amino acids, 100 U of penicillin/mL, 100 μg of streptomycin/mL, and 10% fetal bovine serum at 37 °C in a 5% $CO_2$ incubator.

### *Plasmids*

Plasmids pHHJFH1, pHH/SGR-Luc, pHH/SGR-Luc/GND and pCAG/C-NS2 were as described previously (Masaki et al., 2010). In this study, plasmid pCAG/C-NS2 was designated as pCAGC-NS2/JFH. The plasmid pCAGC-NS2/JFHam having adaptive mutations in E2 (N417S), p7 (N765D), and NS2 (Q1012R) in pCAGC-NS2/JFH was constructed by oligonucleotide-directed mutagenesis. These mutations were also introduced in pHHJFH1, resulting in pHHJFH1am. To generate core-NS2 expression plasmids with different strains of HCV, the cDNA coding core to the first transmembrane region of NS2 (33 amino acids) in pCAGC-NS2/JFH was replaced with the corresponding sequence of the J6 (Lindenbach et al., 2005), H77c (Yanagi et al., 1997), THpa (Shirakura et al., personal communication) and Con1 (Koch and Bartenschlager, 1999) strains. The THpa sequence contained the P to A mutation at 328 aa at E1 in the original TH strain. To generate pHH/SGR, pHH/SGR-Luc was digested with MluI and PmeI, followed by Klenow enzyme treatment and self-ligation to delete the luciferase coding sequence. To generate pHH/SGR-C177, pHH/SGR-C191 and pHH/SGR-C-p7/am, cDNA coding the partial core and luciferase in pHH/SGR-Luc were replaced with coding sequences for mature core (177aa), full-length core (191aa) or core-p7 polyprotein containing adaptive mutations in E2 and p7, respectively. The selected NS3 mutation (N1586D) was introduced into pHH/SGR-Luc and pHHJFH1 by oligonucleotide-directed mutagenesis.

### *Generation of viruses*

HCVcc and HCVtcp were generated as described previously (Masaki et al., 2010). For the production of HCVpp-2a, plasmid pcDNAdeltaC-E1-E2(JFH1)am having adaptive mutations in E2 (N417S) in pcDNAdeltaC-E1-E2(JFH1) (Akazawa et al., 2007) was constructed by oligonucleotide-directed mutagenesis. Murine leukemia virus pseudotypes with VSV G glycoprotein expressing luciferase reporter (VSVpp) were generated in accordance with previously described methods (Akazawa et al., 2007; Bartosch et al., 2003a).

### *Luciferase assay*

Huh7.5.1 cells were seeded onto a 24-well plate at a density of $3 \times 10^4$ cells/well 24 h prior to inoculation with reporter viruses. Cells were incubated for 72 h, followed by lysis with 100 μL of lysis buffer. Luciferase activity of the cells was determined using a luciferase assay system (Promega, Madison, WI). All luciferase assays were performed in triplicate.

### *Quantification of HCV infectivity and HCV RNA*

To determine the titers of HCVtcp and HCVcc, Huh7.5.1 cell monolayers prepared in multi-well plates were incubated with dilutions of samples and then replaced with media containing 10% FBS and 0.8% carboxymethyl cellulose. Following incubation for 72 h, monolayers were fixed and immunostained with rabbit polyclonal anti-NS5A antibody, followed by Alexa Fluor 488-conjugated anti-rabbit secondary antibody (Invitrogen), and stained foci or individual cells were counted and used to calculate a titer of focus-forming units (FFU)/mL for spreading infections or infectious units (IU)/mL for non-spreading infections. For intracellular infectivity, the cell pellet was resuspended in culture media, and cells were lysed by four freeze–thaw cycles. Cell debris was pelleted by centrifugation for 5 min at 4000 rpm. Supernatant was collected and used for titration. To determine the amount of HCV RNA in culture supernatants, RNA was extracted from 140 μL of culture medium by QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA) and treated with DNase (TURBO DNase; Ambion, Austin, TX) at 37 °C for 1 h. Extracted RNA was further purified by using an RNeasy Mini Kit, which includes RNase-free DNase digestion (QIAGEN). Copy numbers of HCV RNA were determined by real-time quantitative reverse transcription-PCR as described previously (Wakita et al., 2005).

### *Antibodies*

Mouse monoclonal antibodies against actin (AC-15) and CD81 (JS-81) were obtained from Sigma (St. Louis, MO) and BD Biosciences (Franklin Lakes, NJ), respectively. Goat polyclonal antibody to ApoE (LV1479433) was obtained from Millipore (Tokyo, Japan). Anti-NS5A and anti-NS5B antibodies were rabbit polyclonal antibody against synthetic peptides.

### *Neutralization assay*

For neutralization experiments with anti-CD81 antibody, Huh7.5.1 cells were incubated with dilutions of anti-CD81 antibody for 1 h at 37 °C. Cells were then infected with viruses for 5 h at 37 °C. For neutralization experiments with anti-ApoE antibody, viruses were incubated with various concentrations of anti-ApoE antibody at room temperature for 1 h and cells were infected with viruses for 5 h at 37 °C. Following infection, supernatant was removed and cells were incubated with culture medium, and luciferase activity was determined at 3 day post-infection for HCVtcp and pseudotyped viruses. For neutralization experiments with HCVcc generated with pHHJFH1am, a multiplicity of infection (MOI) of 0.05 was used for inoculation, and intracellular core protein levels were monitored by ELISA (Ortho Clinical Diagnostics) at 24 h post-infection.

### *Immunoblotting*

Transfected cells were washed with PBS and incubated with lysis buffer (50 mM Tris–HCl, pH 7.4, 300 mM NaCl, 1% triton X-100). Lysates were then sonicated for 5 min and were added to the same volume of SDS sample buffer. Protein samples were boiled for 10 min, separated by SDS-PAGE, and transferred to PVDF membrane. After blocking, membranes were probed with first antibodies, followed by incubation with peroxidase-conjugated secondary antibody. Antigen–antibody complexes were visualized using an enhanced chemiluminescence detection system (Super Signal West Pico Chemiluminescent Substrate; PIERCE, Rockford, IL), in accordance with the manufacturer's protocols.

*Generation of recombinant adenoviruses*

rAdV, AxCANCre, expressing Cre recombinase tagged with nuclear localization signal under CAG promoter was prepared as described previously (Baba et al., 2005). The target rAdV AxCALNLH-CNS2 expressing HCV core-NS2 polyprotein with adaptive mutations in E2, p7 and NS2 was generated as follows. Cosmid pAxCALNLwit2 is identical to pAxCALNLw (Sato et al., 1998), except that both the terminal sequences of the rAdV genome are derived from pAxCAwit2 (Fukuda et al., 2006). The core-NS2 fragment obtained from pCAGC-NS2/JFH1am by StuI-EcoRI digestion and subsequent Klenow treatment was inserted into the SwaI site of pAxCALNLwit2. The resultant cosmid pAxCALNLH-CN2it2 was digested with PacI and transfected into 293 cells to generate rAdV AxCALNLH-CNS2.

*Preparation of packaging cells for HCVtcp*

Huh7.5.1 cells were coinfected with AxCANCre at an MOI of 1 and AxCALNLH-CNS2 at an MOI of 3 for expression of JFH-1 core-NS2 polyprotein containing the adaptive mutations in E2, p7 and NS2.

*RNA preparation, RT-PCR and sequencing*

Total cellular RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA), and subjected to reverse transcription with random hexamer and Superscript III reverse transcriptase (Invitrogen). Three fragments of HCV cDNAs that cover the entire HCV subgenomic replicon genome, were amplified by nested PCR with TaKaRa Ex Taq polymerase (Takara, Shiga, Japan). Amplified products were separated by agarose gel electrophoresis, and were used for direct DNA sequencing.

## Acknowledgments

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# 25-Hydroxyvitamin $D_3$ Suppresses Hepatitis C Virus Production

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**Because the current interferon (IFN)-based treatment for hepatitis C virus (HCV) infection has a therapeutic limitation and side effects, a more efficient therapeutic strategy is desired. Recent studies show that supplementation of vitamin D significantly improves sustained viral response via IFN-based therapy. However, mechanisms and an active molecular form of vitamin D for its anti-HCV effects have not been fully clarified. To address these questions, we infected HuH-7 cells with cell culture-generated HCV in the presence or absence of vitamin $D_3$ or its metabolites. To our surprise, 25-hydroxyvitamin $D_3$ [25(OH)$D_3$], but not vitamin $D_3$ or 1,25-dihydroxyvitamin $D_3$, reduced the extra- and intracellular levels of HCV core antigen in a concentration-dependent manner. Single-cycle virus production assay with a CD81-negative cell line reveals that the inhibitory effect of 25(OH)$D_3$ is at the level of infectious virus assembly but not entry or replication. Long-term 25(OH)$D_3$ treatment generates a HCV mutant with acquired resistance to 25(OH)$D_3$, and this mutation resulting in a N1279Y substitution in the nonstructural region 3 helicase domain is responsible for the resistance.** ***Conclusion:*** **25(OH)$D_3$ is a novel anti-HCV agent that targets an infectious viral particle assembly step. This finding provides insight into the improved efficacy of anti-HCV treatment via the combination of vitamin $D_3$ and IFN. Our results also suggest that 25(OH)$D_3$, not vitamin $D_3$, is a better therapeutic option in patients with hepatic dysfunction and reduced enzymatic activity for generation of 25(OH)$D_3$.** (HEPATOLOGY 2012;56:1231-1239)

Hepatitis C virus (HCV) infection affects about 200 million people worldwide.[1,2] The majority of HCV-infected patients fail to clear the virus and develop chronic liver diseases, including cirrhosis and hepatocellular carcinoma. Standard treatment for chronic hepatitis C is currently based on a combination of pegylated interferon (IFN) and ribavirin.[2] However, the therapy is accompanied by substantial side effects and is only effective in about half of patients.[3,4] Thus, it is critical to provide a new therapeutic modality against chronic hepatitis C. Recently, vitamin D supplementation has been shown to improve the efficacy of combination therapy with IFN and ribavirin.[5,6] However, mechanisms of this effect have not yet been fully elucidated.

Vitamin D absorbed in the intestine from diet or synthesized in the skin is converted to 25-hydroxyvitamin D [25(OH)D] in the liver. Released 25(OH)D is bound to α-globulin and transported to proximal tubules of the kidney,[7] where 25(OH)D is hydroxylated either by 25(OH)D-1 alpha-hydroxylase to generate the active form, 1,25-dihydroxyvitamin D [1,25$(OH)_2$D], or by 25(OH)D-24-hydroxylase to form the biologically inactive form, 24,25-dihydroxyvitamin D [24,25$(OH)_2$D]. 1,25$(OH)_2$D is a key hormone for calcium and bone homeostasis, and its production is tightly regulated by plasma levels of calcium and phosphorus and parathyroid hormone, In addition, vitamin D has nonskeletal actions, and vitamin D deficiency is associated with many diseases including cancer, autoimmune disorder, cardiovascular disease, insulin resistance, and infectious diseases.[8-13] Thus it is not surprising that vitamin D status also

*Abbreviations: 1,25$(OH)_2$D, 1,25-dihydroxyvitamin D; 24,25$(OH)_2$D, 24,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D; Ag, antigen; DMEM, Dulbecco's modified Eagle's medium; HCV, hepatitis C virus; HCVcc, cell culture-generated HCV JFH-1 virus; HCVpp, HCV pseudoparticles; IFN, interferon; ISG, IFN-stimulated gene; JFH-1/wt, wild-type JFH-1; MLV, murine leukemia virus; NS3, nonstructural region 3; PCR, polymerase chain reaction; WST-8, water-soluble tetrazolium salt 2-(2-methoxy-4- nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt.*

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