**Original Article**

**Isolation and Characterization of an Huh.7.5.1-Derived Cell Clone Highly Permissive to Hepatitis C Virus**

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**SUMMARY:** An efficient cell culture and infection system for hepatitis C virus (HCV) facilitates analyses of the complete virus life cycle. Human hepatic Huh7.5.1 cells and an HCV-JFH1 strain have been widely employed in infection experiments. In the present study, cultured Huh7.5.1 cells exhibited heterogeneous phenotypes of HCV infection. Using single-cell cloning of Huh7.5.1 cells, we isolated a clone highly permissive to HCV (Huh7.5.1-8) and a CD81-defective clone nonpermissive to HCV (Huh7.5.1-5). Expression of CD81 in Huh7.5.1-5 cells restored permissiveness to HCV, indicating that CD81 is essential for HCV infection and a defect in CD81 causes nonpermissiveness to HCV in Huh7.5.1-5 cells. Huh7.5.1-8 cells had approximately 10-fold higher HCV replication rates, with cellular HCV RNA copy numbers of >10^9 copies/μg of cellular RNA and viral titers of >10^6 infectious units/ml of culture supernatant. Permissiveness of Huh7.5.1-8 cells to HCV infection was phenotypically very stable because there was no difference in permissiveness after more than 100 passages (1-year culture). This efficient cell culture system for HCV using Huh7.5.1-8 cell provides a powerful tool for studying the HCV life cycle and constructing antiviral strategies.

**INTRODUCTION**

Hepatitis C virus (HCV) is an enveloped, positivesense, single-stranded RNA virus belonging to the Flaviviridae family (1). Fifteen million people are infected with HCV worldwide (2), and HCV is a major causative agent of severe liver diseases such as cirrhosis and hepatocellular carcinoma. Therefore, HCV infection is recognized as a major global healthcare burden (3). Although recent HCV therapy with direct-acting antivirals agents that directly target HCV enzymes has remarkably improved treatment outcomes, drug-resistant viruses have emerged during these treatments (4,5), warranting the development of alternative antivirals that target host machinery involved in HCV infection.

Decades after the discovery of HCV in 1989, a robust cell culture system that allowed completion of the HCV life cycle was developed using an HCV-JFH1 strain in 2005 (6–8). In vitro infection systems, only Huh7-derived cell lines, which were originally isolated from human hepatocellular carcinoma, have been found to be highly permissive to HCV (9,10). Accordingly, Huh7 cells have been frequently used in studies of HCV infection (10). In addition, Blight et al. established an Huh7.5 cell line highly permissive to HCV replicons (11), and the subsequent Huh7.5.1 cell line was generated from a cured HCV replicon cell line as a cell clone with a higher degree of permissiveness to HCV infection. These cells show viral titers of 10^9–10^10 infectious units/ml of culture supernatant in the HCV-JFH1 infection system, and the virus can be serially passaged without loss of infectivity (8). Very recent studies have also shown other hepatic and nonhepatic cell lines that endogenously express higher levels of miR-122 and support HCV infection (12–15), although infection levels in these models are similar to or lower than those in Huh7-derived cells.

In the present study, cultured Huh7.5.1 cells had heterogeneous permissiveness to HCV infection. Thus, we isolated Huh7.5.1-derived cell clones using single-cell cloning and identified clones with higher and lower permissiveness to HCV infection. These established cell clones will contribute to further elucidation of the HCV life cycle and the development of novel antivirals and vaccines.

**MATERIALS AND METHODS**

**Cell culture:** Human hepatoma Huh-7.5.1 cells (8) were kindly provided by Dr. Francis V. Chisari (The Scripps Research Institute, La Jolla, CA, USA). Huh-7.5.1 cells and Huh-7.5.1-derived cell clones were maintained at 37°C in an atmosphere of 5% CO2 in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (FBS), 0.1 mM nonessential amino acids, 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate (normal medium). Cells were usually passaged every 3–5 days at a density of >5 × 10^5 cells/10-cm dish.

**Single-cell cloning:** Huh7.5.1 cells were diluted in...
normal medium and were seeded into 10-cm dishes at 1,000 cells/dish. Cells were cultured until visible colonies formed, and colonies were isolated and re-cloned in 96-well plates using the limiting dilution method.

**Antibodies and plasmids:** Rabbit monoclonal antibodies (mAbs) against scavenger receptor class B type I (SRBI; clone EP1556Y) and mouse mAbs against glyceraldehyde 3-phosphate dehydrogenase (GAPDH; clone 6C5) were purchased from Abcam, Plc. (Cambridge, UK). Rabbit anti-claudin-1 polyclonal antibodies and mouse anti-occludin mAbs (clone OC-3F10) were purchased from Life Technologies (Carlsbad, CA, USA). Mouse anti-CD81 mAbs (clone JS-81) were purchased from BD Bioscience Pharmingen (San Jose, CA, USA). Mouse mAbs against the low-density lipoprotein receptor (LDLR; clone 15C8) were purchased from EMD Millipore (Billerica, MA, USA). Mouse mAbs against the HCV core protein (clone 2H9) were described previously (6). The human CD81 expression vector pcDNA3.1-hCD81 was generously provided by Dr. Y. Matsuura (Osaka University, Osaka, Japan).

**Infection of cells with HCV:** Huh7.5.1-8 cells were cultured with HCV-JFH1 (genotype 2a; HCVcc; 6). In brief, cells were incubated with HCVcc diluted in normal medium for 2 h at 37°C as described previously (16). Viral titers were then determined using fluorescent focus assay as described by Kato et al. (17), except for the use of Huh7.5.1-8 cells, as detailed below.

**Immunofluorescence analysis:** Immunofluorescence staining with mAbs against the HCV core protein was performed as described previously (16). In brief, cells were cultured on collagen-coated glass cover slips (Asahi Glass Co., Ltd., Tokyo, Japan) in 24-well plates and were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 30 min. After washing with PBS containing 30 mM glycine, cells were permeabilized with PBS containing 0.1% Triton X-100 for 10 min, blocked with 5% (w/v) skim milk in PBS for 30 min, incubated with a 1:500 dilution of mouse mAbs against the anti-HCV core protein (2H9), and then incubated with 1:500 dilutions of AlexaFluor488-conjugated goat anti-mouse IgG (Life Technologies) and 4',6'-diamidino-2-phenylindole (DAPI, 10 mg/ml, Life Technologies). Stained cells were observed under a fluorescence microscope (Biozero BZ-8100; Keyence Corp., Osaka, Japan).

**Quantification of HCV RNA:** Total RNA from culture supernatants and cultured cells was extracted and purified using the Viral Nucleic Acid Extraction Kit I and a Blood/Cultured Cell Total RNA mini Kit (FAVORGEN Biotech Corp., Pingtung City, Taiwan), respectively. Viral RNA contents were determined using one-step quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) with RNA-direct™ Realtime PCR Master Mix (Toyobo Co. Ltd., Osaka, Japan), the specific primers 5'-ACGGGCTTTAATTGCAACAGG-3' and 5'-ACGGGTGATGCAGGAGACAACAGCAGGCAACACG-3', and the Taqman probe [6-FAM]-AGCAAGAATAGAAAAGGGGAACCGGTTAGC-TAMRA-6-FAM]. PCR analyses were performed using the LightCycler® Real-Time PCR System (F. Hoffmann-La Roche AG Konzern-Hauptzitz, Basel, Switzerland) at 90°C for 1 min, 61°C for 20 min, and 95°C for 30 s, followed by 45 cycles of 95°C for 0 s and 61°C for 45 s.

**Replication activity assays with subgenomic replicons:** The subgenomic replicon plasmids pSGR-JFH1/Luc and pSGR-JFH1/Luc-GND (18) carried the luc gene and were based on the HCV-JFH1 sequence. The latter contained a GDD-to-GN mutation in NS5B that abolishes RNA polymerase activity, and it was linearized and transcribed in vitro into RNA using T7 RNA polymerase. Replicon RNA was then purified as described previously (18) using the AmpliScribe™ T7 High Yield Transcription Kit (Epitect Biotechnologies, Madison, WI, USA). Cells were seeded in 24-well plates at 1 × 10^4 cells and were cultured for 1 day, and replicon RNA (2 μg each) from pSGR-JFH1/Luc or pSGR-JFH1/Luc-GND was transfected into cells using DMRIE-C Transfection Reagent (Life Technologies). After culture for 4 h, the culture medium was discarded and replaced with fresh normal medium, and cells were incubated for 1–4 days. Subsequently, the cells were lysed in 100 μl of Cell Culture Lysis Reagent (Promega Corp., Madison, WI, USA) and 5-μl aliquots were mixed with 25 μl of Luciferase assay substrate (PicaGene, Toyo Ink Mfg. Co., Ltd., Tokyo, Japan) for measuring the luciferase activity using a Luminescencer-PSN luminometer (ATTO Co., Ltd., Tokyo, Japan).

**Infection of cells with HCV pseudo-particles:** HCV pseudo-particles (HCVpp) were generated as described previously (19). In brief, a Gag-Pol packaging vector (Gag-Pol 5349), a reporter vector (Luc 126), and an envelope glycoprotein (HCV E1 and E2)-expressing vector (JFH1, genotype 2a; TH, genotype 1b (20)) were transfected into HEK293T cells. After culture, medium from transfected cells was collected and used as the HCVpp stock.

Prior to infection, cells were seeded at a density of 5 × 10^5 cells/well in 48-well plates, cultured for 1 day, and then incubated with HCVpp for 6 h at 37°C. After washing 3 times with normal medium, cells were cultured in normal medium for an additional 2 days at 37°C. Luciferase activity in cell lysates was then measured as described above in the “Replication activity assays with subgenomic replicons” section.

**Flow cytometry analyses:** Cells were detached using PBS containing 1 mM ethylenediaminetetraacetic acid and then treated with anti-CR81 or anti-SRBI antibodies at a dilution of 1:500 in PBS containing 2% FBS for 30 min at 4°C. After washing with PBS, the cells were incubated with AlexaFluor 488 goat anti-mouse or anti-rabbit IgG (Life Technologies) and 4',6'-diamidino-2-phenylindole (DAPI, 10 mg/ml, Life Technologies). Stained cells were observed under a fluorescence microscope (Biozero BZ-8100; Keyence Corp., Osaka, Japan).

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**Immunoblot analysis:** Immunoblotting was performed as described previously (16). In brief, cell lysates containing equal amounts of protein were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoro-
ride membranes and probed with the following antibodies: anti-SRBI (dilution, 1:1,000), anti-GAPDH (dilution, 1:20,000), anti-claudin-1 (dilution, 1:2,000), anti-occludin (dilution, 1:1,000), horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA), and HRP-conjugated goat anti-mouse IgG
A Highly Permissive Cell Line for HCV Infection

Huh7.5.1 cells exhibited heterogeneous HCV infection phenotypes: Cultured Huh7.5.1 cells were infected with varying copy numbers of HCVcc (1.14 × 10^7 and 1.14 × 10^8 copies/ml), and ratios of cells with visible staining of the HCV core protein were similar (38% and 35%, respectively; Fig. 1). However, HCV core protein staining varied between infected cells (Fig. 1), indicating phenotypic heterogeneity of HCV infection among these Huh7.5.1 cells.

Isolation of a highly HCV permissive Huh7.5.1-derived cell clone: To establish an Huh7.5.1 cell clone highly permissive to HCV, we isolated Huh7.5.1-derived cell clones using single-cell cloning as described in the Materials and Methods. Cloned cells were then infected with HCVcc, and cell lysates were examined using immunoblotting analyses with an anti-HCV core protein mAb. Cell clones exhibited varying expression levels of the HCV core protein, producing variable HCV permissiveness among clones (Fig. 2), as indicated in Fig. 1. Subsequently, a highly permissive clone was designated Huh7.5.1-8, and higher HCV growth rates were shown (Fig. 2, lane h). Infection of Huh7.5.1-8 cells with HCVcc at 1.14 × 10^7 copies/ml produced intense anti-HCV core protein staining in all cells (Fig. 3B). However, no morphological differences were observed.

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served between Huh7.5.1 and Huh7.5.1-8 cells.

**Characterization of Huh7.5.1-8 cells:** In time course comparisons of HCV production in parental Huh7.5.1 cells and cloned Huh7.5.1-8 cells, HCV RNA contents were significantly higher in Huh7.5.1-8 cells and culture supernatants than in Huh7.5.1 cells and supernatants at 2, 4, and 6 days post-infection (dpi; Figs. 4A and 4B). Examination of infectivity titers in culture supernatants from cell strains at 4 dpi showed $4.56 \times 10^6 \pm 2.61 \times 10^4$ focus-forming units (ffu)/ml of culture supernatant from Huh7.5.1-8 cells, which was more than 10-fold higher than that of Huh7.5.1 cells (Table 1). However, numbers of ffu per $10^5$ HCV RNA copies did not differ between Huh7.5.1 and Huh7.5.1-8 cells ($105 \pm 6$ vs. $69 \pm 4$; Table 1), indicating that both cells produced viral particles with comparable infectivity.

To identify steps in the HCV life cycle that were enhanced in Huh7.5.1-8 cells, transient-replication assays were performed using a subgenomic replicon carrying the luciferase reporter gene SGR-JFH1/Luc-wt (21). As shown in Fig. 5, luciferase activity in replicon-transfected Huh7.5.1-8 cells progressively increased for 2 days and then reached a plateau, whereas that in the replicon-

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**Table 1. Infectivity titers in culture medium of Huh7.5.1 and Huh7.5.1-8 cells infected with HCVcc**

<table>
<thead>
<tr>
<th>Cell</th>
<th>Infectivity</th>
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<tr>
<td></td>
<td>ffu/ml</td>
</tr>
<tr>
<td>Huh7.5.1 cells</td>
<td>$(4.06 \pm 0.22) \times 10^4$</td>
</tr>
<tr>
<td>Huh7.5.1-8 cells</td>
<td>$(4.56 \pm 0.26) \times 10^6$</td>
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<tr>
<td></td>
<td>ffu/10^5 HCV RNA copies</td>
</tr>
<tr>
<td>Huh7.5.1 cells</td>
<td>$105 \pm 32$</td>
</tr>
<tr>
<td>Huh7.5.1-8 cells</td>
<td>$69 \pm 5$</td>
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$n = 3.$
transfected Huh7.5.1 cells gradually increased and reached a plateau at day 3. In subsequent experiments, the replication-incompetent mutant of the replicon, SGR-JFH1/Luc-GND (21) showed similar background levels of luciferase activity in both cell strains, confirming that observed increases in activity were dependent on viral RNA replication. These results indicate more than 10-fold greater HCV RNA replication activity in Huh7.5.1-8 cells than in parental Huh7.5.1 cells.

In further experiments, HCV entry activity was determined in these cells using HCVpp, which carries a luciferase reporter gene (19). At 2 dpi, luciferase activity of Huh7.5.1 cells infected with HCVpp (genotype 2a of 1b) was approximately half of that of Huh7.5.1-8 cells (Fig. 6A), suggesting the presence of cell subpopulations with relatively lower HCV entry activity among Huh7.5.1 cells. HCV entry into host cells is mediated by multiple host factors, including CD81, SRβ1, claudin-1, occludin, and LDLR (22–25). Immunoblot analyses revealed no differences in protein levels of SRβ1, claudin-1, occludin, or LDLR between Huh7.5.1 and Huh7.5.1-8 cells, whereas lower CD81 protein expres-

Fig. 6. A subpopulation of Huh7.5.1 cells was CD81-defective. (A) Huh7.5.1, Huh7.5.1-8, and Huh7.5.1-5 cells were infected with HCVpp for 6 h: genotype 1b (white) and genotype 2a (black). At 2 dpi, the luciferase activities of cell lysates were measured using a luminometer. Data are presented as means ± SD (n = 3). (B) Huh7.5.1 cells, Huh7.5.1-8 cells, and Huh7.5.1-5 cells were lysed, and an equal portion of each cell lysate (11 μg) was subjected to immunoblotting for CD81, SRβ1, claudin-1, occludin, LDLR, and GAPDH proteins, as described in the Materials and Methods. The relative amounts of CD81/GAPDH, SRβ1/GAPDH, Claudin-1/GAPDH, Occludin/GAPDH, and LDLR/GAPDH in these cells were shown. (C) Huh7.5.1, Huh7.5.1-8, and Huh7.5.1-5 cells were stained with mAb against the extracellular domain of CD81 and analyzed by flow cytometry, as described in the Materials and Methods.
Higher HCV permissiveness of Huh7.5.1-8 cells was reflected by higher replication activity of HCV (Fig. 5) in these cells. In addition, sustained expression of HCV infection factors, particularly CD81, resulted in complete viral replication in Huh7.5.1-8 cells (Fig. 7). Huh7.5.1-8 cells were originally derived from a human hepatocellular carcinoma, their genomes may be unstable. Accordingly, gene expression levels varied between parental Huh7.5.1 cells and cloned cells (Table 2 and Fig. 7). Following single-cell cloning, we isolated an Huh7.5.1-8 cell clone, another clone that was not permissive to HCV infection (Huh7.5.1-5) was isolated (Fig. 2, lane e). Huh7.5.1-5 cells also resisted entry of HCVpp (Fig. 6A) and were deficient in CD81 (Figs. 6B and 6C). In addition, HCV infectability was restored after the expression of human CD81 in Huh7.5.1-5 cells (Fig. 8). In agreement with previous studies (22,26), the present data indicate that CD81 is essential for HCV infection in hepatocytes (22,26).

**DISCUSSION**

Under the present culture conditions, Huh7.5.1 cells showed heterogeneous phenotypes for HCV infection, as shown in previous studies of phenotypically distinct Huh7 cell lines (11,27–33). Because Huh7-derived cells were originally derived from a human hepatocellular carcinoma, their genomes may be unstable. Accordingly, gene expression levels varied between parental Huh7.5.1 cells and cloned cells (Table 2 and Fig. 7). Following single-cell cloning, we isolated an Huh7.5.1-8 cell clone highly permissive to HCV infection, with cellular HCV RNAs of >10^6 copies/μg of total RNA (13-fold higher) and viral titers of >4×10^6 infectious units/ml of culture supernatant (11-fold higher). In addition, the HCV infection phenotype was very stable in Huh7.5.1-8 cells, indicating their utility in studies of reproductive HCV infection.

Huh7.5.1-8 cells, including neurotensin (NTS), anterior gradient 2 (AGR2), group-specific component (GC), alpha-fetoprotein (AFP), albumin (ALB), ribosomal protein L39 (RPL39), and sec61 gamma subunit (SEC61G). However, no genes were expressed at lower levels in Huh7.5.1-8 cells than in Huh7.5.1 (Table 2). In subsequent real-time qRT-PCR experiments, differences in mRNA expression reflected those identified in RNAseq analyses, and all, except RPL39, upregulated, suggesting an involvement in enhanced viral replication in Huh7.5.1-8 cells (Fig. 7). Huh7.5.1-8 cell clones were CD81 deficient and HCV nonpermissive: In addition to the Huh7.5.1-8 cell clone, another clone that was not permissive to HCV infection (Huh7.5.1-5) was isolated (Fig. 2, lane e). Huh7.5.1-5 cells also resisted entry of HCVpp (Fig. 6A) and were deficient in CD81 (Figs. 6B and 6C). In addition, HCV infectability was restored after the expression of human CD81 in Huh7.5.1-5 cells (Fig. 8). In agreement with previous studies (22,26), the present data indicate that CD81 is essential for HCV infection in hepatocytes (22,26).
ing studies and may facilitate detailed investigations of HCV replication activity in Huh7.5.1-8 cells remain to be explored. Immunoblotting, and qRT-PCR analyses of Huh7.5.1-8 cells were easily detected using immunohistochemical, immunoblotting, and qRT-PCR analyses of Huh7.5.1-8 cells, and the protein SEC61G is required for tumor cell survival (40). In addition, AGR2 acts as a p53 tumor suppressor inhibitor (41). Thus, higher expression levels of these genes may facilitate the growth of Huh7.5.1-8 cells. Because HCV replication is known to vary between stages of cell growth (42–44), further investigations of the 6 genes identified in the present study are required to determine their roles in the HCV life cycle, including RNA replication.

In a previous study, Akazawa et al. isolated several CD81-deficient Huh7 clones (26). Thus, we established a CD81-deficient Huh7.5.1-5 cell clone from the present study with varying HCV permissiveness, including a highly HCV-permissive Huh7.5.1-8 cell clone with higher replication activity and an HCV-nonpermissive Huh7.5.1-5 cell clone deficient in CD81. HCV infection was easily detected using immunohistochemical, immunoblotting, and qRT-PCR analyses of Huh7.5.1-8 cells. Although the mechanisms underlying increased HCV replication activity in Huh7.5.1-8 cells remain unknown, these isolated cell clones provide a model of HCV infection that is widely applicable to drug screening studies and may facilitate detailed investigations of the HCV life cycle.

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Conflict of interest None to declare.

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