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Short
Communication

Production of single-round infectious chimeric flaviviruses with DNA-based Japanese encephalitis virus replicon

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A method for rapid production of single-round infectious particles (SRIPs) of flavivirus would be useful for viral mutagenesis studies. Here, we established a DNA-based production system for SRIPs of flavivirus. We constructed a Japanese encephalitis virus (JEV) subgenomic replicon plasmid, which lacked the C-prM-E (capsid–pre-membrane–envelope) coding region, under the control of the cytomegalovirus promoter. When the JEV replicon plasmid was transiently co-transfected with a JEV C-prM-E expression plasmid into 293T cells, SRIPs were produced, indicating successful *trans*-complementation with JEV structural proteins. Equivalent production levels were observed when C and prM–E proteins were provided separately. Furthermore, dengue types 1–4, West Nile, yellow fever or tick-borne encephalitis virus prM–E proteins could be utilized for production of chimeric flavivirus SRIPs, although the production was less efficient for dengue and yellow fever viruses. These results indicated that our plasmid-based system is suitable for investigating the life cycles of flaviviruses, diagnostic applications and development of safer vaccine candidates.

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Japanese encephalitis virus (JEV) is the leading cause of viral encephalitis with severe mortality in eastern and south-eastern Asia, and is estimated to be responsible for 67 900 cases annually, mostly in children (Campbell *et al.*, 2011). The virus is transmitted by *Culex* mosquito vectors between pigs and/or wild birds, and humans and horses are thought to be dead-end hosts. JEV is a member of the genus *Flavivirus* within the family *Flaviviridae*, which includes dengue virus (DENV), West Nile virus (WNV), yellow fever virus (YFV) and tick-borne encephalitis virus (TBEV). JEV is an enveloped single-stranded positive-sense RNA virus with an 11 kb genome that is translated as a single large polyprotein. The polyprotein is co-translationally cleaved by host and viral proteases into three structural proteins – capsid (C), pre-membrane (prM) and envelope

(E) – and seven non-structural (NS) proteins (Sumiyoshi *et al.*, 1987).

For several flaviviruses, subgenomic replicons, which lack structural protein genes but can replicate in cells, have been constructed (Khromykh & Westaway, 1997; Pang *et al.*, 2001; Shi *et al.*, 2002). In addition, the expression of viral structural proteins in cells harbouring replicon RNA has been shown to produce single-round infectious particles (SRIPs), which are infectious, but progeny viruses cannot be spread from the infected cells, as the packaged genome lacks structural protein genes (Gehrke *et al.*, 2003; Jones *et al.*, 2005; Khromykh *et al.*, 1998; Ng *et al.*, 2007; Scholle *et al.*, 2004; Yun *et al.*, 2009). Furthermore, *trans*-packaging of replicons by the prM–E proteins from heterologous flaviviruses have been reported (Ansarah-Sobrinho *et al.*, 2008; Yoshii *et al.*, 2008).

A method for rapidly producing SRIPs of flaviviruses would be useful for viral mutagenesis studies, diagnostic applications and the production of vaccines with reduced

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One supplementary figure is available with the online version of this paper.

risk of infection. In this study, to establish a DNA-based production system for SRIPs, we constructed a JEV subgenomic replicon plasmid, which lacked the C-prM-E coding region, under the control of the cytomegalovirus (CMV) promoter. As DNA-based replicons can be transfected directly into eukaryotic cells without *in vitro* transcription, SRIPs can be rapidly produced by co-transfection with structural protein-expression plasmids.

In order to generate a subgenomic replicon from the JEV Nakayama strain (McAda *et al.*, 1987), viral RNA was extracted from infected Vero cells, reverse transcribed into cDNA and amplified in individual dsDNA fragments containing T7 RNA polymerase promoter and hepatitis delta virus ribozyme (HDV-RZ) as shown in Fig. 1(a). For deletion of the C-prM-E region, a synthetic antisense oligonucleotide was used to add a *Bsp*TI site at codons 17–18 of the C-coding region following the cyclization sequence, and a sense oligonucleotide was designed that added the *Bsp*TI site before the C-terminal transmembrane domain, which consists of 30 aa of the E protein coding sequence, in order to permit ligation of C to NS1. The five individual fragments required to produce a replicon-length cDNA were readily assembled into the low-copy-number plasmid pACYC177, designated pJEVrep#97. Replication of *in vitro*-transcribed RNAs derived from the plasmid was confirmed in RNA-transfected cells (data not shown). Next, to construct a DNA-based replicon plasmid, the T7 RNA polymerase promoter was replaced with the CMV promoter, and the simian virus 40 polyadenylation signal was inserted downstream of the HDV-RZ; the resulting plasmid was designated pCMV-JErep (Fig. 1a). pCMV-JErep-fs, which contains a frameshift mutation through a 4 nt insertion upstream of the GDD motif of RNA-dependent RNA polymerase in NS5, was also constructed as a negative control with no replication activity. To characterize the replication activity of the plasmid-derived replicon, 293T cells were transfected with plasmids as described previously (Suzuki *et al.*, 2013). Indirect immunofluorescence with an anti-dsRNA antibody showed positive staining in the cytoplasm of cells transfected with pCMV-JErep plasmid or infected with JEV Nakayama strain, whereas no signal was detected in the cells transfected with pCMV-JErep-fs, indicating the ability of viral RNAs transcribed intracellularly from the plasmid pCMV-JErep to replicate in cells (Fig. 1b). It should be noted that NS1 protein was detected in the cytoplasm of cells transfected with both pCMV-JErep and pCMV-JErep-fs.

We also constructed expression plasmids for JEV C-E, mature C consisting of 105 aa, and prM-E, which we designated pCAG-JECE, pCAG-JEC and pCAG-JEprME, respectively (Fig. 2a). To reduce sequence homology and intergenomic recombination potential with the truncated C and E genes in the subgenomic replicon, 21 nt mutations were incorporated into the 5' region of the C gene and 3' region of the E gene. These changes also include two nucleotides in the conserved 5' cyclization sequence (CS) (Hahn *et al.*, 1987; Khromykh *et al.*, 2001), producing a

sequence that was non-complementary to the 3' CS of the replicon genome, thereby preventing replication of a recombinant genome. To produce SRIPs of JEV, 293T cells were transfected with a mixture of two (pCMV-JErep and pCAG-JECE) or three (pCMV-JErep, pCAG-JEC and pCAG-JEprME) plasmids. The infectivity of SRIPs was determined by inoculating the culture supernatant of transfected cells into Vero cells, followed by immunostaining with anti-NS1 antibody. 293T cells produced a titre of 6.9×10^5 IU ml⁻¹ (Fig. 2b) or 7.9×10^5 IU ml⁻¹ (Fig. 2c) 3 days after transfection with two or three plasmids, respectively. In contrast, no infectious particles were detected in the supernatant when one of the two or three plasmids was omitted or the replicon containing a frameshift mutation was introduced. The production levels of SRIPs from cells transfected with two or three plasmids were similar, as shown in Fig. 2(d).

In order to confirm that the SRIPs have only single-round infectivity potential, Vero cells were inoculated with medium harvested from 293T cells transfected with replicon and structural protein plasmids, and were examined for antigen-positive cells. SRIPs were demonstrated to be infectious in the first round (Fig. 3a). However, no antigen-positive cells were observed in a second round, in which the supernatants of the cells infected with SRIPs were transferred to naive Vero cells (Fig. 3a). As a control, supernatant from JEV-infected cells produced antigen-positive cells in second-round infection.

We then evaluated whether the SRIPs could be used in neutralization tests instead of infectious live virus by using anti-JEV sera raised in rabbits as a representative antibody. Serial fourfold dilutions of serum were mixed with aliquots of SRIPs or virus of equivalent infectivity. The virus-antibody mixture was incubated for 1 h at room temperature, followed by titration for infectivity on Vero cell monolayers in a 96-well plate. The neutralizing activity of each antibody dilution was expressed as a percentage of the infectivity obtained with the control, which was tested in the absence of any serum. Infection with SRIPs and JEV Nakayama strain were similarly neutralized by anti-JEV antibody in a dose-dependent manner, although normal serum did not affect infection with SRIPs and JEV (Fig. 3b).

Next, to examine whether SRIPs derived from other flaviviruses could be generated using our plasmid-based method, we used prM-E expression plasmids for the following viruses: DENV1, Mochizuki strain; DENV2, New Guinea C strain; DENV3, H87 strain; DENV4, H241 strain (Konishi *et al.*, 2006); WNV, NY99-6922 strain (Ishikawa *et al.*, 2007); YFV, 17D strain; and TBEV, Oshima 5-10 strain (Yoshii *et al.*, 2003). Detection of each E protein in cells transfected with prM-E expression plasmids by immunofluorescence revealed indistinguishable efficiency of transfection as shown in Fig. S1 (available in JGV Online). Efficient production of chimaeric flavivirus SRIPs by co-transfection with JEV C and JEV replicons was achieved for

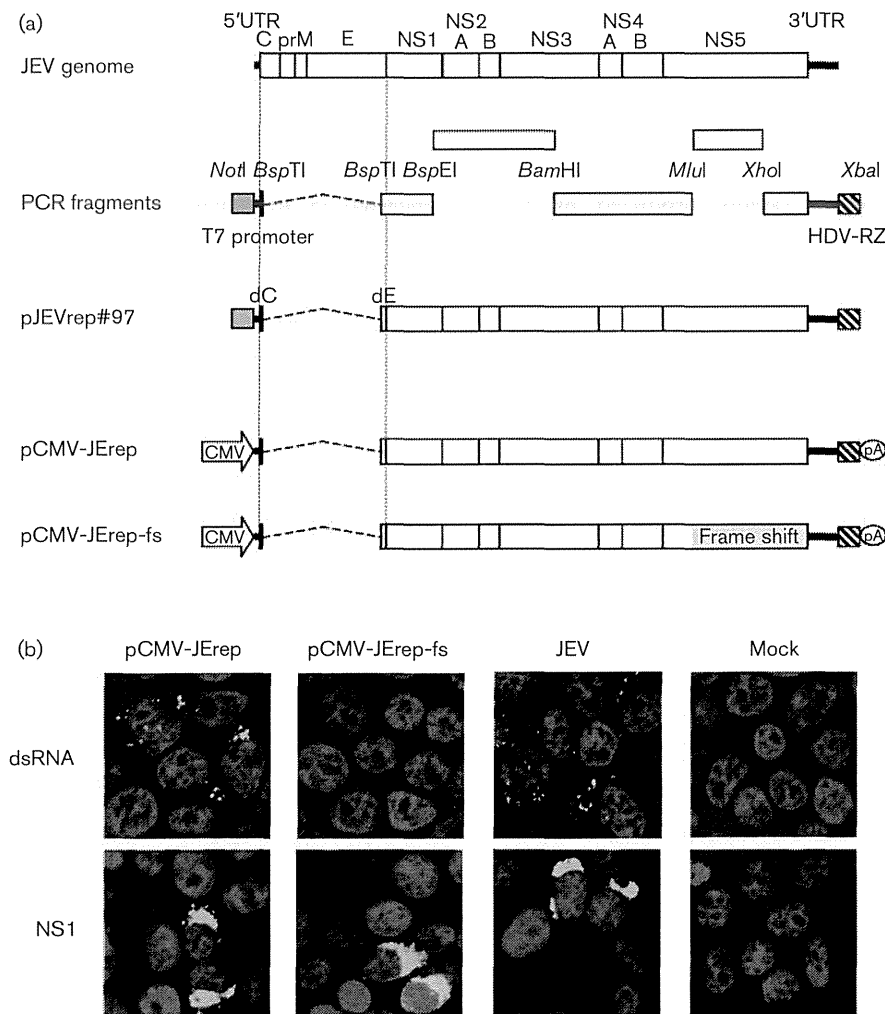


Fig. 1. (a) Schematic representation of the position of the JEV genome showing restriction enzymes sites (*NotI*, *BspTI*, *BspEI*, *BamHI*, *MluI*, *XhoI* and *XbaI*), fragments used to assemble for replicon construction, position of the T7 promoter, CMV promoter (CMV), HDV-RZ and polyadenylation signal (pA). (b) 293T cells were transfected with the indicated plasmids or were infected or mock-infected with JEV. Two days post-transfection or post-infection, cells were fixed and permeabilized as described previously (Suzuki *et al.*, 2013). Samples were then incubated with anti-dsRNA antibody (J2; English & Scientific Consulting) or anti-NS1 antibody (2D5; Konishi *et al.*, 2004). Green signals were obtained with Alexa-Fluor-488-labelled goat anti-mouse IgG secondary antibody (Invitrogen). Cell nuclei were counterstained with DAPI.

WNV and TBEV, although production of SRIPs was less efficient for DENV1-4 and YFV (Fig. 3c).

It is curious that TBEV prM-E protein can be utilized efficiently for assembly of SRIPs in combination with the JEV C protein and replicon RNA producing equivalent titres to JEV and WNV, as TBEV is a tick-borne virus and is classified as a distinct serogroup from JEV. In contrast, production of DENV- and YFV-SRIPs was less efficient. The low infectious titre of SRIPs containing at least dengue prM-E may be explained by the low specific infectivity of particles encapsidated in DENV envelope protein (van der Schaar *et al.*, 2007; Winkelmann *et al.*, 2011), although we were unable to exclude the possibility that the viral assembly and/or secretion with dengue prM-E is not

efficient (Chang *et al.*, 2003; Hsieh *et al.*, 2008). Adaptive mutations in structural and NS proteins could possibly enhance the production of infectious particles by improving the specific infectivity of the resulting particles (Winkelmann *et al.*, 2011). In addition, it has been reported that a chimaeric WNV genome with DENV2 prM-E genes but lacking the C gene replicates much better in DENV2-C-expressing cells than in WNV-C-expressing cells (Suzuki *et al.*, 2009), thus suggesting that the combination of homologous C protein and prM-E proteins improves the production of viral particles. Therefore, it is possible to obtain a better yield of dengue SRIPs by using DENV C protein instead of JEV C protein. Such DENV-SRIPs can be useful for studying infection-enhancing and neutralizing antibody activities.

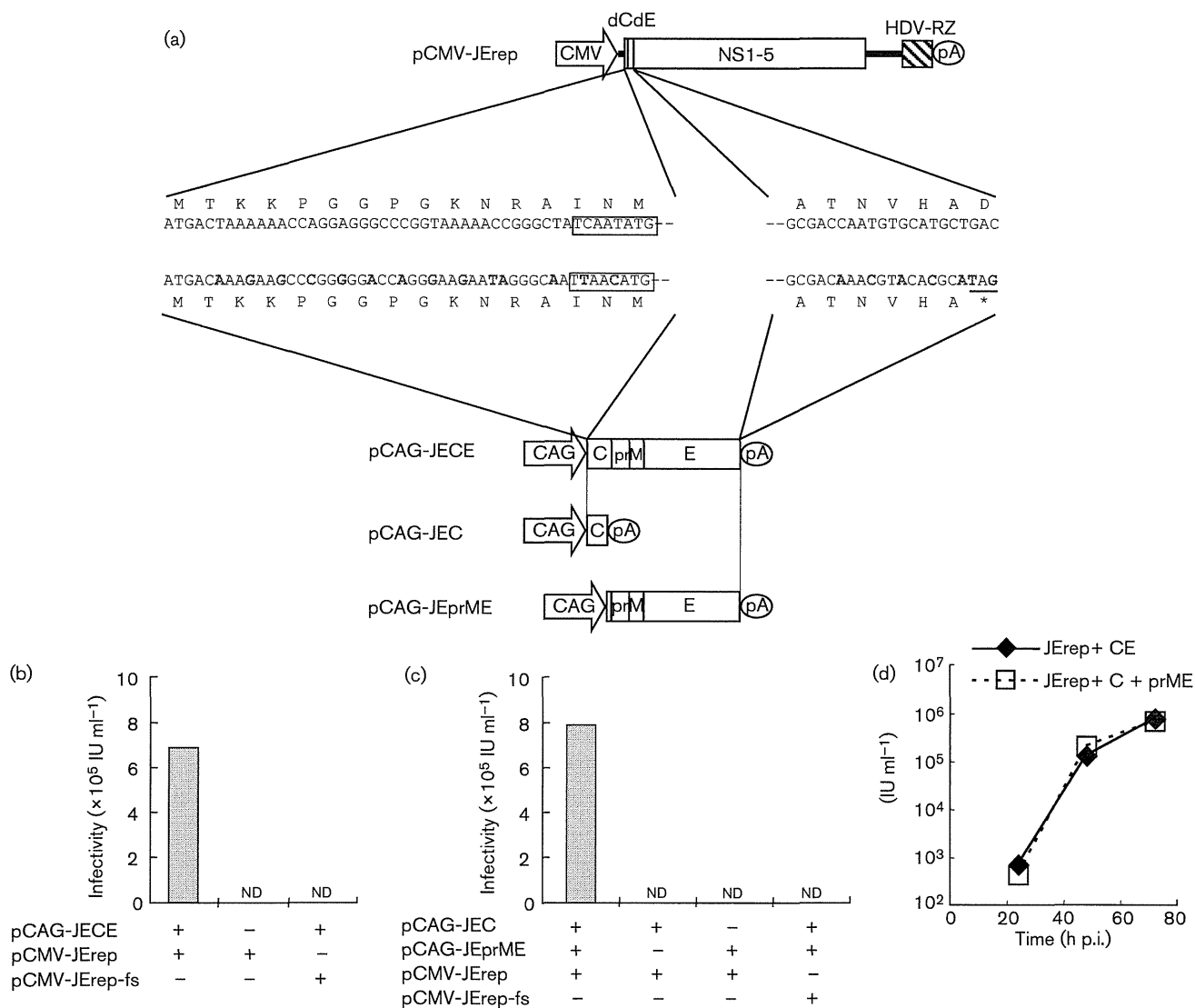


Fig. 2. Schematic representation of JEV replicon and structural protein-expression plasmids. (a) Top: JEV subgenomic replicon with deletion of structural proteins. This replicon contains a partial C and E gene. Bottom: JEV structural protein-expression plasmids showing the region of overlap with JEV replicon. Boxes indicate the 8 nt in the 5' CS that are 100 % conserved among all mosquito-borne flaviviruses. The JEV C-E, C and prM-E coding sequences harbour silent mutations designed to prevent homologous recombinations that included two changes to the CS sequence, which must be 100 % complementary to the 3' CS of JEV in order to permit genome replication. The termination codon is underlined. Nucleotide substitutions are shown in red. (b, c) Titres of JEV-SRIPs produced by transfection of 293T cells with replicon plasmid and structural protein-expression plasmids. Dilutions of supernatant collected at 3 days post-transfection were used to inoculate monolayers of Vero cells. Cells were fixed at 2 days post-infection and stained with anti-NS1 antibody. Stained cells were then counted to determine the titres (IU ml⁻¹) produced by transfections. ND, Not detected. (d) Time course of JEV SRIP production from transfected cells. At each time point, medium was removed and frozen for subsequent titration and fresh medium was added. JEV SRIP titres were determined by assaying infectivity in Vero cells.

The plasmid-based production system offers an advantage for vaccine production in terms of stability and safety, as this method is able to reduce the chance of mutations in the structural protein region, as well as the risk of infection when compared with live virus production. In addition, our replicon plasmids have the potential for application to DNA-based vaccines, as described

previously (Cao *et al.*, 2011; Chang *et al.*, 2008; Huang *et al.*, 2012).

In conclusion, we established a DNA-based production system for SRIPs of flaviviruses. This system has potential value as a basic research and diagnostic tool, and could be used to enhance the safety of neutralization assay, as well as vaccine production.

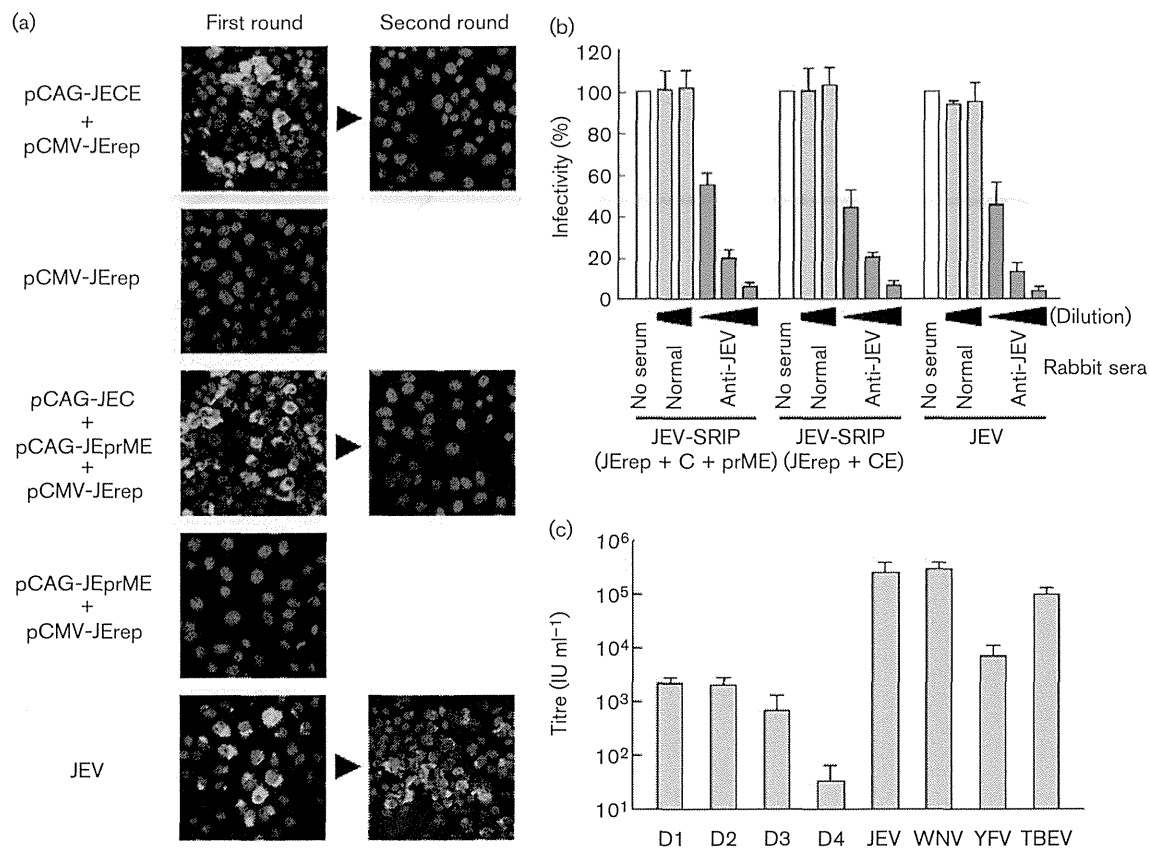


Fig. 3. Vero cells were inoculated with supernatant of 293T cells transfected with the indicated plasmids or infected with JEV. Two days post-inoculation, culture supernatants were collected, and cells were fixed and stained with NS1 antibodies (first round). Naive Vero cells were reinfected with culture supernatants from the first round. Two days post-inoculation, cells were fixed and stained with NS1 antibodies (second round). Cell nuclei were counterstained with DAPI. (b) JEV SRIP inoculum was incubated with serially diluted (1:2000, 1:8000 and 1:32000) rabbit normal serum or anti-JEV serum for 1 h at room temperature, followed by inoculation onto Vero cells. Cells were immunostained with anti-NS1 antibody at 2 days post-infection, and antigen-positive cells were counted and used to calculate a titre based on f.f.u. ml⁻¹ for spreading infections or IU ml⁻¹ for non-spreading infections. Data for each condition are means of values obtained from three independent experiments with error bars showing SD. The value for controls without serum (no serum) was set at 100%. (c) Infectious titres of flavivirus SRIPs, including dengue types 1–4 (D1–4), produced by transfection of 293T cells with pCMV-JErep, pCAG-JEC and flavivirus prM-E expression plasmids. Dilution of supernatant collected at 3 days post-transfection was used to inoculate monolayers of Vero cells. Cells were fixed at 2 days post-infection and stained with anti-JEV NS1 antibody, and stained cells were counted in order to determine titres.

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Alternative endocytosis pathway for productive entry of hepatitis C virus

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Previous studies have shown that hepatitis C virus (HCV) enters human hepatic cells through interaction with a series of cellular receptors, followed by clathrin-mediated, pH-dependent endocytosis. Here, we investigated the mechanisms of HCV entry into multiple HCV-permissive human hepatocyte-derived cells using trans-complemented HCV particles (HCVtcp). Knockdown of CD81 and claudin-1, or treatment with bafilomycin A1, reduced infection in Huh-7 and Huh7.5.1 cells, suggesting that HCV entered both cell types via receptor-mediated, pH-dependent endocytosis. Interestingly, knockdown of the clathrin heavy chain or dynamin-2 (Dyn2), as well as expression of the dominant-negative form of Dyn2, reduced infection of Huh-7 cells with HCVtcp, whereas infectious entry of HCVtcp into Huh7.5.1 cells was not impaired. Infection of Huh7.5.1 cells with culture-derived HCV (HCVcc) via a clathrin-independent pathway was also observed. Knockdown of caveolin-1, ADP-ribosylation factor 6 (Arf6), flotillin, p21-activated kinase 1 (PAK1) and the PAK1 effector C-terminal binding protein 1 of E1A had no inhibitory effects on HCVtcp infection into Huh7.5.1 cells, thus suggesting that the infectious entry pathway of HCV into Huh7.5.1 cells was not caveolae-mediated, or Arf6- and flotillin-mediated endocytosis and macropinocytosis, but rather may have occurred via an undefined endocytic pathway. Further analysis revealed that HCV entry was clathrin- and dynamin-dependent in ORL8c and HepCD81/miR122 cells, but productive entry of HCV was clathrin- and dynamin-independent in Hep3B/miR122 cells. Collectively, these data indicated that HCV entered different target cells through different entry routes.

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INTRODUCTION

Over 170 million people worldwide are chronically infected with hepatitis C virus (HCV), and are at risk of developing chronic hepatitis, cirrhosis and hepatocellular carcinoma (Hoofnagle, 2002). HCV is an enveloped virus belonging to the family *Flaviviridae*. Its genome is an uncapped 9.6 kb positive-stranded RNA consisting of the 5'-UTR, an ORF encoding viral proteins and the 3'-UTR (Suzuki *et al.*, 2007). A precursor polyprotein is further processed into structural proteins (core, E1, and E2), followed by p7 and non-structural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B), by cellular and viral proteases.

Host–virus interactions are required during the initial steps of viral infection. Viruses enter the cells by various pathways, such as receptor-mediated endocytosis followed by pH-dependent or -independent fusion from endocytic compartments, or pH-independent fusion at the plasma membrane coupled with receptor-mediated signalling and coordinated disassembly of the actin cortex (Grove & Marsh, 2011). It was reported previously that CD81 (Bartosch *et al.*, 2003; McKeating *et al.*, 2004; Pileri *et al.*, 1998), scavenger receptor class B type I (SR-BI) (Bartosch *et al.*, 2003; Scarselli *et al.*, 2002), claudin-1 (Evans *et al.*, 2007; Liu *et al.*, 2009) and occludin (Benedicto *et al.*, 2009; Liu *et al.*, 2009; Ploss *et al.*, 2009) are critical molecules for HCV entry into cells. Recently, epidermal growth factor receptor and ephrin receptor type A2 were also identified as host cofactors for HCV entry, possibly by modulating interactions between CD81 and claudin-1 (Lupberger *et al.*,

Two supplementary figures are available with the online version of this paper.

2011). In addition, Niemann–Pick C1-like 1 (NPC1L1) cholesterol absorption receptor has been shown to play a role in HCV entry, probably at the fusion step (Sainz *et al.*, 2012).

Following receptor binding, HCV has been reported to enter cultured cells via clathrin-mediated endocytosis, the most common and best-characterized mode of endocytosis, following membrane fusion in early endosomes (Blanchard *et al.*, 2006; Codran *et al.*, 2006; Coller *et al.*, 2009; Meertens *et al.*, 2006; Trotard *et al.*, 2009) using retrovirus-based HCV pseudoparticles (HCVpp) and cell culture-produced HCV (HCVcc). Early steps in 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. However, as HCVpp are generated in non-hepatic cells such as human embryo kidney 293T cells, it is likely that the cell-derived component(s) of HCVpp differ from those of HCVcc.

In the present study, we readdressed the HCV endocytosis pathway using trans-complemented HCV particles (HCVtcp) (Suzuki *et al.*, 2012), of which the packaged genome is a subgenomic replicon. HCVtcp, generated in Huh-7 or its derivative cell lines with two plasmids, are infectious, but support only single-round infection, thereby allowing us to examine infectious viral entry without the influence of reinfection. In addition, HCVtcp is useful for quantifying productive infection by measuring luciferase activity. Furthermore, it has been shown that the HCVtcp system is more relevant as a model of HCV infection than HCVpp (Suzuki *et al.*, 2012). Our results demonstrated conclusively that, in addition to the clathrin-mediated endocytosis pathway, HCV was capable of utilizing the clathrin- and dynamin-independent pathways for infectious entry of HCV into human liver-derived cells.

RESULTS

HCV entry depends on receptor-mediated, pH-dependent endocytosis

HCV has been shown to enter permissive cells through clathrin-mediated endocytosis and low pH-dependent fusion with endosomes mostly using HCVpp (Codran *et al.*, 2006; Meertens *et al.*, 2006; Trotard *et al.*, 2009), although some researchers have used HCVcc with limited cell lines (Blanchard *et al.*, 2006; Coller *et al.*, 2009). However, several distinct characteristics between HCVpp and HCVcc have recently been revealed with regard to morphogenesis and entry steps (Helle *et al.*, 2010; Sainz *et al.*, 2012; Suzuki *et al.*, 2012; Vieyres *et al.*, 2010). Therefore, in this study, we used HCVtcp, which exhibit similar characteristics to HCVcc when compared with HCVpp and support single-round infection (Suzuki *et al.*, 2012).

Initially, to determine whether receptor candidates such as CD81, claudin-1, occludin and SR-BI are essential for HCV

entry into Huh-7 and Huh7.5.1 cells, we examined the knockdown effect of these molecules on HCVtcp infection. Knockdown of these receptors was confirmed by immunoblotting (Fig. 1a) and FACS analysis (Fig. 1b). It should be noted that the luciferase activity in Huh7.5.1 was approximately four times higher than that in Huh-7 cells when the same amount of inoculum was used for infection (Fig. S1, available in the online Supplementary Material), and knockdown did not affect cell viability (data not shown). Knockdown of CD81 and claudin-1 significantly reduced the infection of Huh-7 and Huh7.5.1 cells with HCVtcp derived from genotype 2a (Fig. 1c). Knockdown of occludin led to a moderate reduction in infection; however, only a marginal effect was observed in SR-BI knockdown in both Huh-7 and Huh7.5.1 cells (Fig. 1c), possibly due to the reduced requirement for SR-BI during virus entry by adaptive mutation in E2 (Grove *et al.*, 2008).

Next, to examine whether HCV entry was pH-dependent, Huh-7 and Huh7.5.1 cells were pretreated with bafilomycin A1, an inhibitor of vacuolar H⁺-ATPases that impairs vesicle acidification, and then infected with HCVtcp. At 72 h post-infection, luciferase activity and cell viability were determined. Bafilomycin A1 inhibited HCVtcp infection in a dose-dependent manner without affecting cell viability in both Huh-7 and Huh7.5.1 cells (Fig. 2a, b). We also confirmed that treatment with bafilomycin A1 after HCVtcp infection had a minor effect on luciferase activity (Fig. 2c). These results indicated that the infectious route of HCVtcp into Huh-7 and Huh7.5.1 cells is receptor-mediated and involves pH-dependent endocytosis.

Knockdown of clathrin heavy chain (CHC) or dynamin-2 (Dyn2) reduces HCVtcp infection in Huh-7 cells, but not in Huh7.5.1 cells

Among the known pathways of pH-dependent viral endocytosis, clathrin-mediated dynamin-dependent endocytosis is a major endocytosis pathway. Chlorpromazine, an inhibitor of clathrin-dependent endocytosis, has been commonly used to study clathrin-mediated endocytosis; however, it exerts multiple side-effects on cell function as it targets numerous receptors and intracellular enzymes, and alters plasma membrane characteristics (Sieczkarski & Whittaker, 2002a). Therefore, we examined the HCV endocytosis pathway by knockdown of specific molecules required for the endocytosis pathway. CHC, a major structural protein in clathrin-coated vesicles, and Dyn2, a GTPase essential for clathrin-coated-pit scission from the plasma membrane, play important roles in the clathrin-mediated pathway. Another well-studied model of viral entry is caveolin-mediated endocytosis. The role of dynamin in both clathrin-mediated endocytosis and caveolae-dependent endocytosis has been established (Marsh & Helenius, 2006; Miaczynska & Stenmark, 2008). To examine the endocytosis pathways of HCV, small interfering RNAs (siRNAs) for CHC, Dyn2 and caveolin-1 (Cav1), or scrambled control siRNA, were transfected into Huh-7 or

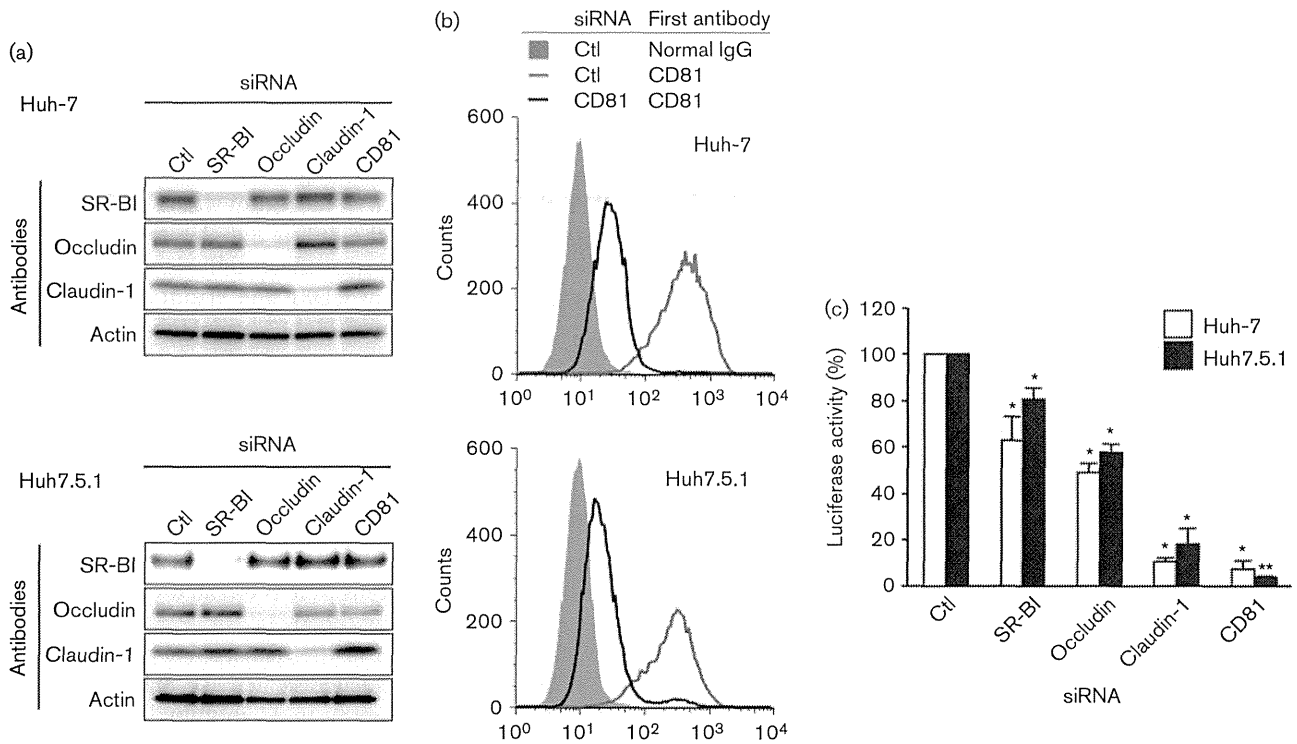


Fig. 1. Knockdown effect of receptor candidate molecules on HCV infection. (a) Huh-7 or Huh7.5.1 cells were transfected with the indicated small interfering RNAs (siRNA), harvested at 48 h post-transfection and the specific knockdown of each protein was verified by immunoblotting. (b) Huh-7 or Huh7.5.1 cells were transfected with CD81 or control siRNAs, harvested at 48 h post-transfection and the cell surface expression of CD81 was verified by FACS analysis. (c) Cells transfected with siRNA were infected with the same amount of HCVtcp at 48 h post-transfection. Firefly luciferase activity in the cells was determined at 72 h post-infection and is expressed relative to the activity with control siRNA transfection. The value for control (Ctl) siRNA was set at 100 %. Data represent the mean \pm sd. Statistical differences between controls and each siRNA were evaluated using Student's *t*-test. * $P < 0.05$, ** $P < 0.001$ versus control.

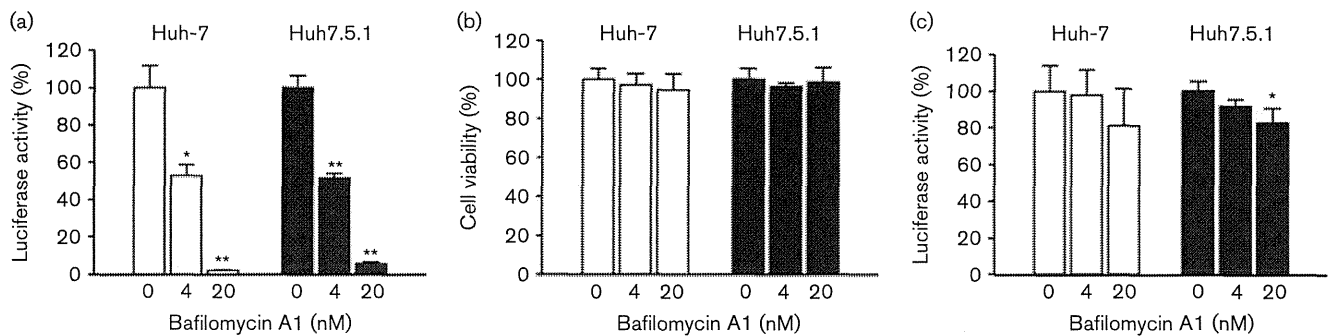


Fig. 2. Role of endosomal low pH in HCV infection. Cells were treated with bafilomycin A1 for 1 h at the indicated concentrations and infected with HCVtcp. (a, b) Luciferase activity (a) and cell viability (b) were determined at 72 h post-infection, and expressed relative to amounts observed in controls. (c) Cells were treated with bafilomycin A1 for 1 h at the indicated concentrations 48 h after HCVtcp infection. Luciferase activity was determined at 10 h post-treatment and expressed relative to amounts observed in controls. Data represent the mean \pm sd. Statistical differences between controls and indicated concentrations were evaluated using Student's *t*-test. * $P < 0.05$, ** $P < 0.001$ versus control.

Huh7.5.1 cells, followed by infection with HCVtcp. Expression of CHC, Dyn2 and Cav1 was downregulated by transfection of specific siRNAs (Fig. 3a, b), whereas expression of SR-BI, occludin, claudin-1 and CD81 was not reduced (Figs 3a and S2). As indicated in Fig. 3(c), luciferase activity from HCVtcp was significantly reduced by knockdown of CHC and Dyn2 in Huh-7 cells, but not in Huh7.5.1 cells. Knockdown of Cav1 showed no inhibitory effects on HCVtcp entry into either cell line. Dynamin-independent entry in Huh7.5.1 cells was also observed using HCVtcp derived from genotype 1b (data not shown). Knockdown of CHC or Dyn2 also reduced entry of HCVcc in Huh-7 cells, but had no inhibitory effects in Huh7.5.1 (Fig. 3d). To rule out the possibility of effects on CHC and Dyn2 knockdown on viral RNA replication, HCVtcp were also

inoculated before siRNA transfection. Luciferase activity was not affected by knockdown of CHC or Dyn2 in either cell line, whereas marked inhibition was observed for phosphatidylinositol 4-kinase (PI4K) (Fig. 3e). These data suggested that HCV entry was clathrin-mediated and dynamin-dependent in Huh-7 cells, but productive entry of HCV was clathrin- and dynamin-independent in Huh7.5.1 cells.

Expression of the dominant-negative form of Dyn2 reduces HCV infection in Huh-7 cells, but not in Huh7.5.1 cells

We also examined the role of dynamin in infectious entry of HCV into Huh-7 and Huh7.5.1 cells by overexpression of the dominant-negative form of Dyn2 (Dyn-K44A), which

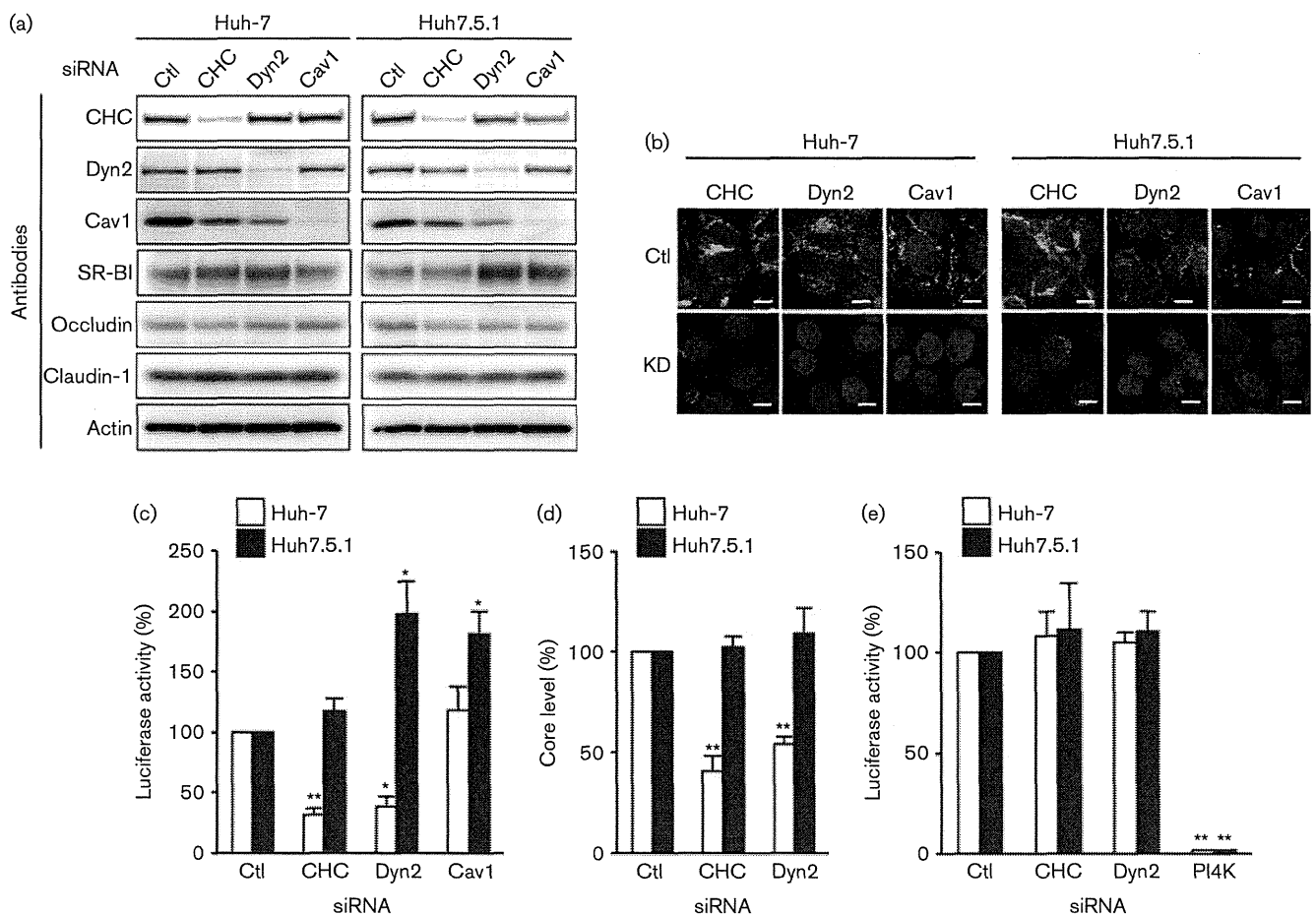


Fig. 3. Effects of CHC, Dyn2 and Cav1 knockdown on HCV infection. (a, b) Huh-7 cells or Huh7.5.1 cells were transfected with the indicated siRNAs and the specific knockdown (KD) of each protein was verified by immunoblotting (a) or immunostaining (b) at 48 h post-transfection. Bar, 50 μm. (c) Cells were transfected with the indicated siRNAs, followed by infection with HCVtcp at 48 h post-transfection. Firefly luciferase activity in the cells was subsequently determined at 3 days post-infection. The value for control (Ctl) siRNA was set at 100%. Data represent the mean ± SD. (d) Cells were transfected with siRNA, followed by infection with HCVcc at 48 h post-transfection. Intracellular core levels were quantified at 24 h post-infection. The value for control siRNA was set at 100%. Data represent the mean ± SD. (e) Cells were infected with HCVtcp, followed by transfection with the indicated siRNAs. Luciferase activity in the cells was subsequently determined at 2 days post-transfection. The value for control siRNA was set at 100%. Data represent the mean ± SD. Statistical differences between controls and each siRNA were evaluated using Student's *t*-test. **P* < 0.05, ***P* < 0.001 versus control.

has been shown to effectively block clathrin-dependent and caveolar endocytosis (Damke *et al.*, 1995). Expression of haemagglutinin (HA)-tagged Dyn-K44A reduced the number of HCV-infected Huh-7 cells, but not Huh7.5.1 cells, as compared with WT HA-tagged Dyn2 (Dyn-WT), as shown in Fig. 4(a, b). Interestingly, internalization of transferrin, which is known to be mediated by clathrin-dependent endocytosis, was reduced in both Huh-7 and Huh7.5.1 cells expressing Dyn-K44A, whereas cells expressing Dyn-WT showed efficient endocytosis of transferrin (Fig. 4c, d). Collectively, these results suggested that dynamin participated in the internalization of HCV in Huh-7 cells, but was

not absolutely required in Huh7.5.1 cells, although transferrin was taken up via dynamin-dependent endocytosis in both Huh-7 and Huh7.5.1 cells.

Flotillin-1 or the GTPase regulator associated with focal adhesion kinase 1 (GRAF1) play no major role during HCV infection of Huh7.5.1 cells

In order to dissect the major endocytosis pathways of HCVtcp in Huh7.5.1 cells, we investigated the role of alternative routes of HCV entry by siRNA knockdown. We silenced essential factors for the clathrin- or dynamin-independent pathways

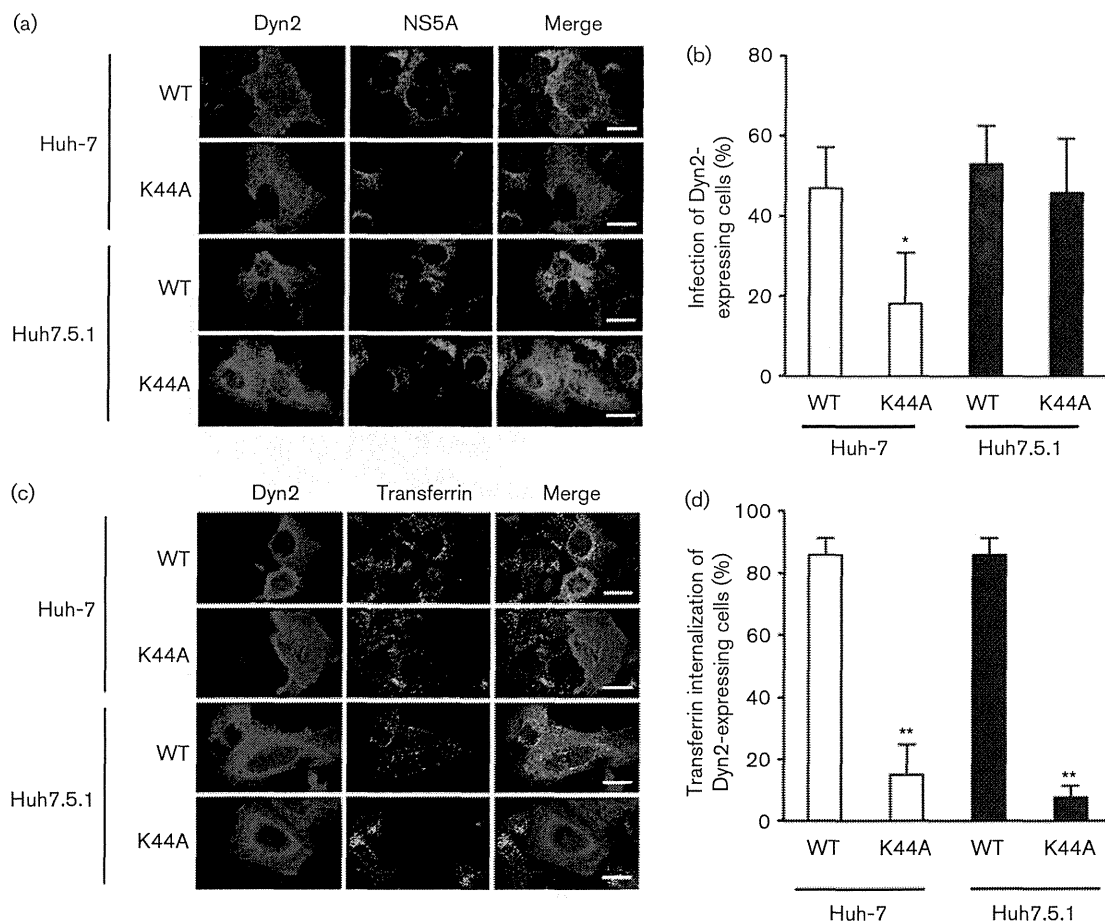


Fig. 4. Dynamin participates in the internalization of HCV in Huh-7 cells, but not in Huh7.5.1 cells. (a) Cells were transfected with HA-tagged WT Dyn2 (Dyn-WT) or dominant-negative Dyn2 (Dyn-K44A) expression plasmids. At 2 days post-transfection, cells were infected with HCVtcp, which possessed a subgenomic replicon without the luciferase gene. After 3 days, cells were fixed and HA-Dyn2 or HCV NS5A stained with anti-HA or anti-NS5A antibodies, respectively. Cell nuclei were counterstained with DAPI. Bar, 100 μ m. (b) Data were quantified as the population of HCVtcp-infected cells among HA-positive cells. At least 20 HA-positive cells were evaluated in triplicate experiments. Data represent the mean \pm SD. (c) Cells were transfected with HA-tagged Dyn-WT or Dyn-K44A expression plasmids. At 2 days post-transfection, cells were incubated with Alexa Fluor-488 labelled transferrin at 37 $^{\circ}$ C in a 5% CO₂ incubator. After 30 min of incubation, cells were washed, fixed and stained with anti-HA antibodies. Cell nuclei were counterstained with DAPI. Bar, 100 μ m. (d) Data were quantified as the population of transferrin-internalized cells among HA-positive cells. At least 20 HA-positive cells were evaluated in triplicate experiments. Data represent the mean \pm SD. Statistical differences between Dyn-WT and Dyn-K44A were evaluated using Student's *t*-test. * P <0.05, ** P <0.001 versus Dyn-WT.

including flotillin-dependent endocytosis, ADP-ribosylation factor 6 (Arf6)-dependent endocytosis, clathrin-independent carrier/glycosylphosphatidylinositol-enriched early endosomal compartment (CLIC/GEEC) endocytic pathway and macropinocytosis in Huh7.5.1 cells. Flotillin-1 and Arf6 are indispensable components of the flotillin and Arf6 pathways, respectively. Knockdown of flotillin-1 or Arf6 had no inhibitory effects on HCVtcp infection in Huh7.5.1 cells (Fig. 5a). The CLIC/GEEC endocytic pathway has recently become better defined and is regulated by the GTPase regulator associated with focal adhesion kinase-1 (GRAF1). However, GRAF1 was not detected in Huh-7 or Huh7.5.1 cells (Fig. 5b); thus, it is unlikely that the CLIC/GEEC pathway was involved in HCV entry in Huh7.5.1 cells. In addition, knockdown of p21-activated kinase 1 (PAK1) and the PAK1 effector C-terminal binding protein 1 of E1A (CtBP1), which play important regulatory roles in the process of macropinocytosis, did not inhibit HCVtcp infection in Huh7.5.1 cells (Fig. 5c). Taken together, these results suggested that the entry of HCVtcp into Huh7.5.1 cells was not mediated mainly by flotillin-dependent endocytosis,

Arf6-dependent endocytosis, the CLIC/GEEC endocytic pathway and macropinocytosis.

Clathrin-dependent and -independent pathways for HCV entry in other hepatic cells

We further examined the endocytosis pathways for HCV in non-Huh-7-related human liver-derived cell lines. Three HCVcc permissive hepatocellular carcinoma cell lines, Li23-derived ORL8c (Kato *et al.*, 2009), HepCD81/miR122 cells (HepG2/CD81 cells overexpressing miR122) and Hep3B/miR122 (Kambara *et al.*, 2012), were transfected with siRNA for CHC, Dyn2 or claudin-1, followed by infection with HCVtcp. Immunoblotting was performed in order to confirm knockdown of target proteins (Fig. 6a). Although knockdown of CHC or Dyn2 expression inhibited HCVtcp infection of ORL8c and HepCD81/miR122 cells, HCVtcp infection of Hep3B/miR122 cells was not affected (Fig. 6b), thus suggesting that productive entry of HCV is clathrin- and dynamin-independent in Hep3B/miR122 cells.

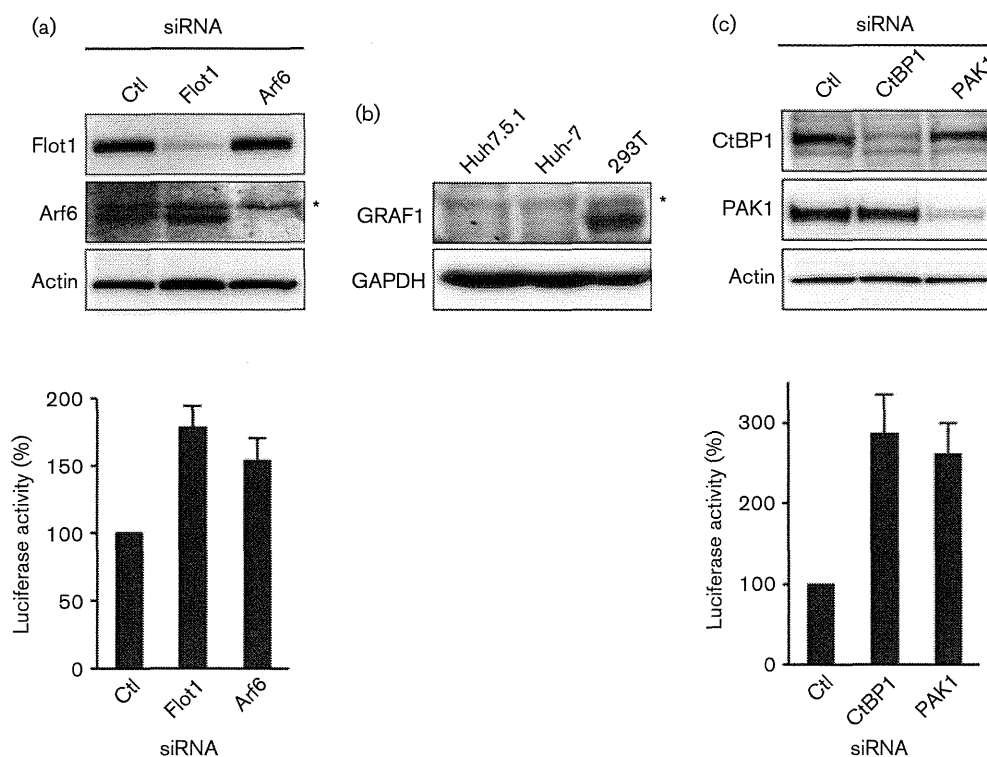


Fig. 5. Role of an alternative endocytosis pathway of HCV in Huh7.5.1 cells. (a) Huh7.5.1 cells were transfected with flotillin-1 (Flot1) or Arf6 siRNAs and specific knockdown of each protein was verified by immunoblotting (upper). Non-specific bands are marked with an asterisk. Cells transfected with siRNA were infected with HCVtcp. Luciferase activity (lower) was determined at 72 h post-infection and expressed relative to the amount observed in control (Ctl) siRNA transfection. Data represent the mean \pm SD. (b) Expression of GRAF1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in Huh7.5.1, Huh-7 and 293T cells was analysed by immunoblotting. Non-specific bands are marked with an asterisk. (c) Huh7.5.1 cells were transfected with CtBP1 or PAK1 siRNA and specific knockdown of each protein was verified by immunoblotting (upper). Cells transfected with siRNA were infected with the HCVtcp. Luciferase activity (lower) was determined at 72 h post-infection and expressed relative to the amount observed in control (Ctl) siRNA transfection. Data represent the mean \pm SD.

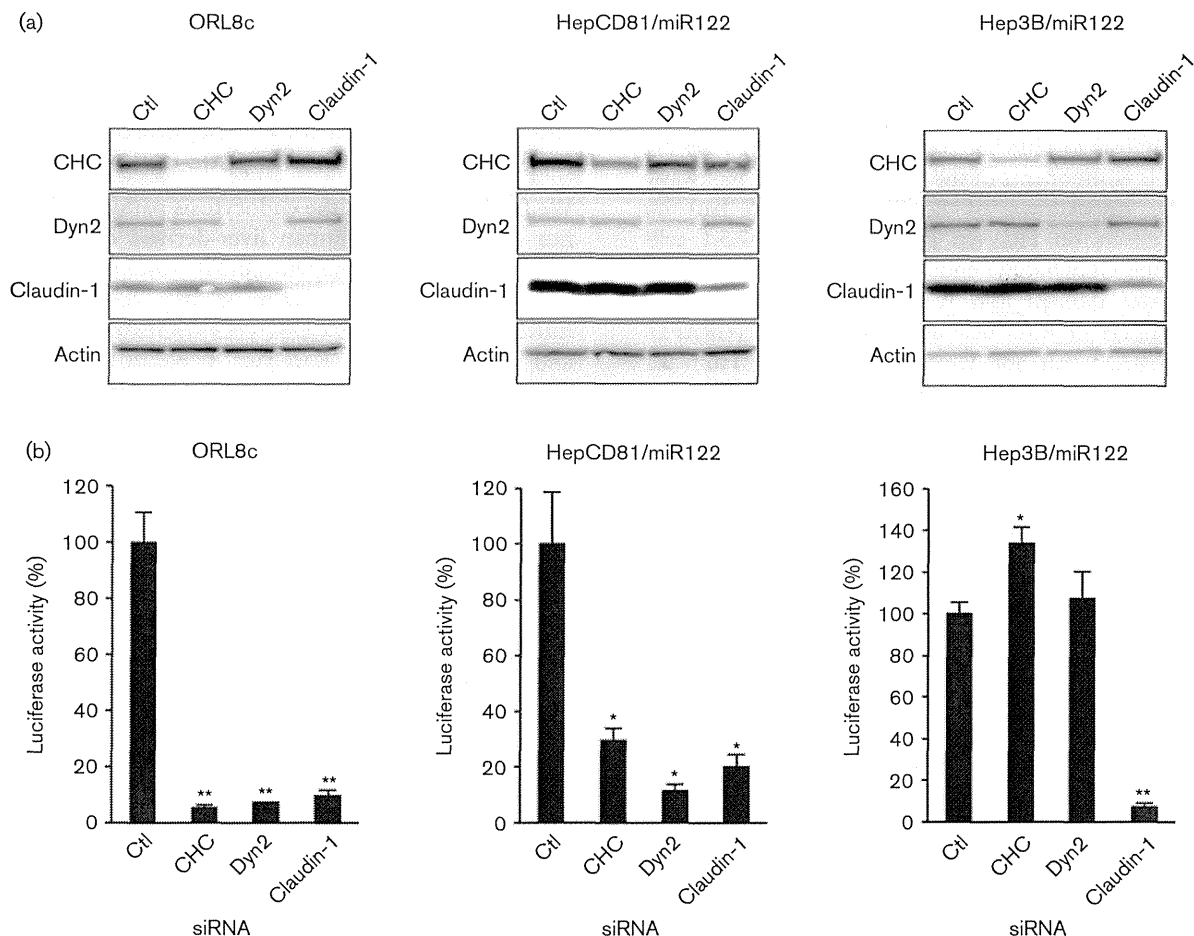


Fig. 6. Clathrin-dependent and -independent pathway of HCV entry in other HCV-permissive cells. The indicated cells were transfected with the indicated siRNAs and then infected with HCV_{top} at 48 h post-transfection. (a) Specific knockdown of each protein was verified by immunoblotting. (b) Luciferase activity was determined at 72 h post-infection and expressed relative to the amount observed in the control (Ctl) siRNA transfection. Data represent the mean \pm SD. Statistical differences between controls and each siRNA were evaluated using Student's *t*-test. * $P < 0.05$, ** $P < 0.001$ versus control.

In summary, we identified an alternative clathrin- and dynamin-independent entry pathway for HCV in at least two independent cell lines, Huh7.5.1 and Hep3B/miR122 cells, in addition to the previously reported clathrin- and dynamin-dependent pathway. These findings provided clues for understanding the molecular mechanisms of the endocytosis pathway for HCV infection.

DISCUSSION

Many viruses have been shown to utilize a number of different endocytic pathways to productively infect their hosts. Clathrin-dependent endocytosis would appear to be the most commonly used, but it is increasingly clear that a number of clathrin-independent endocytosis pathways are also used by several different viruses (Mercer *et al.*, 2010). In the case of HCV, it has been reported that viral entry is mediated by clathrin-dependent endocytosis (Blanchard

et al., 2006; Codran *et al.*, 2006; Coller *et al.*, 2009; Meertens *et al.*, 2006; Trotard *et al.*, 2009). In these papers, HCVpp was used at least in part for analysis of HCV entry pathway. However, recent reports have revealed several different characteristics between HCVpp and HCVcc.

Viral entry has been addressed primarily by pharmacologic inhibitor studies, immunofluorescence and electron microscopy, by transfection with dominant-negative constructs, and more recently by siRNA knockdown. Analysis of endocytosis pathways using pharmacological inhibitors has raised concerns about specificity. For example, chlorpromazine, an inhibitor of clathrin-mediated endocytosis, has been shown to exert multiple side-effects on cell function as it targets numerous receptors and intracellular enzymes, and alters plasma membrane characteristics (Sieczkarski & Whittaker, 2002a). Methods for elucidating the viral endocytosis pathway by co-localization of virus particles with host factor also have limitations. Electron and

fluorescence microscopy, which require a high particle number, do not allow the differentiation of infectious and non-infectious particles. Infectious particles of HCV in the supernatant of infected cells appeared to represent only a small portion of secreted virus particles (Akazawa *et al.*, 2008) and it is unclear whether the viral particles observed by microscopy could lead to productive infection. Therefore, we utilized HCVtcp, which is useful for determining productive entry of the virus without reinfection, and a combination of siRNA knockdown and dominant-negative mutants for analysis of the productive route of infection. Although HCVcc is also utilized in analysis of productive entry, it cannot completely exclude the effects of reinfection by virus produced by infected cells. Reduction of HCVcc infection by knockdown of CHC and Dyn2 was moderate when compared with that of HCVtcp (Fig. 3c, d), thus suggesting slight effects due to reinfection in HCVcc.

The data presented here demonstrate for the first time to our knowledge that HCV is able to enter cells via dynamin-independent endocytosis in addition to the previously described classical clathrin- and dynamin-dependent pathway. First, knockdown of CHC and Dyn2 had no inhibitory effects on HCVtcp and HCVcc entry into Huh7.5.1 cells. Second, overexpression of dominant-negative Dyn2 had no inhibitory effects on HCVtcp in Huh7.5.1 cells. Finally, in addition to Huh7.5.1 cells, Hep3B/miR122 cells were also shown to be infected with HCV via clathrin- and dynamin-independent pathways. We further investigated the role of alternative minor routes of HCV entry into Huh7.5.1 cells; however, the productive endocytosis pathway could not be defined. It should be noted that inhibition of alternative endocytosis routes by siRNA led to an increase of luciferase activity (Figs 3c and 5a, c). This could be explained by the inhibition of a particular endocytosis pathway resulting in a compensatory increase in alternative endocytosis pathways (Damke *et al.*, 1995).

Although we confirmed an alternative endocytosis pathway for the productive entry of HCV, it is not clear why and how the two independent endocytosis pathways operate in different cell lines. SV40 can enter cells via caveolae-dependent (Norkin *et al.*, 2002; Pelkmans *et al.*, 2001) and -independent (Damm *et al.*, 2005) pathways. Influenza virus enters cells via clathrin-mediated endocytosis (Matlin *et al.*, 1981) in addition to non-clathrin-mediated, non-caveola-mediated internalization pathways (Sieczkarski & Whittaker, 2002b). Entry of dengue virus type 2 is clathrin-dependent in HeLa and C6/36 cells (Acosta *et al.*, 2008; Mosso *et al.*, 2008; van der Schaar *et al.*, 2008), and is clathrin-independent in Vero cells (Acosta *et al.*, 2009). Different receptor usage may determine the consequential route of entry. However, we did not observe any differences between Huh-7 and Huh7.5.1 cells in terms of knockdown effects of receptor candidate molecules on HCV infection, as shown in Fig. 1(c), although we cannot exclude the possibility that other undefined receptors are associated with viral entry. Huh7.5.1 cells were established by

elimination of the HCV genome from replicon cells derived from Huh-7 cells (Blight *et al.*, 2002; Zhong *et al.*, 2005) and they exhibit more potent replication of HCV than the original Huh-7 cells. Further study showed that the increased permissiveness of cured cells results from a mutation in the retinoic acid-inducible gene I (Sumpter *et al.*, 2005), which impairs IFN signalling. In addition, it has been shown that cured cell lines express higher levels of miR122 than parental cells participating in the efficient propagation of HCVcc (Kambara *et al.*, 2012). As it is unclear whether these changes are the reason for a distinct endocytosis pathway, it will be of interest to explore these associations in further studies.

In conclusion, we confirmed an alternative clathrin-independent endocytosis pathway in HCV-permissive human hepatic-derived cells, in addition to the previously reported clathrin-dependent endocytosis pathway. This paper highlights the fact that clathrin- and dynamin-mediated endocytosis is the main route of HCV entry for Huh-7, HepCD81/miR122 and ORL8c cells, whilst clathrin and dynamin do not play a major role during the productive route of HCV infection in Huh7.5.1 and Hep3B/miR122 cells. Taken together, these studies suggest that different cell entry pathways for HCV infection may be utilized in different cell types, although further studies are necessary in order to understand this phenomenon.

METHODS

Cells. The human hepatocellular carcinoma cell lines Huh-7, Huh7.5.1, Hep3B/miR122 and HepG2/CD81, which overexpressed miR122 (Kambara *et al.*, 2012), were maintained in Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemical Industries) containing non-essential amino acids, penicillin (100 U ml⁻¹), streptomycin (100 µg ml⁻¹) and 10 % FBS. Li23-derived ORL8c cells (Kato *et al.*, 2009) were maintained in F12 medium and DMEM (1 : 1, v/v) supplemented with 1 % FBS, epidermal growth factor (50 ng ml⁻¹), insulin (10 µg ml⁻¹), hydrocortisone (0.36 µg ml⁻¹), transferrin (5 µg ml⁻¹), linoleic acid (5 µg ml⁻¹), selenium (20 ng ml⁻¹), prolactin (10 ng ml⁻¹), gentamicin (10 µg ml⁻¹), kanamycin monosulfate (0.2 mg ml⁻¹) and fungizone (0.5 µg ml⁻¹). All cell lines were cultured at 37 °C in a 5 % CO₂ incubator.

Preparation of viruses. HCVtcp and HCVcc derived from JFH-1 with adaptive mutations in E2 (N417S), p7 (N765D) and NS2 (Q1012R) were generated as described previously (Suzuki *et al.*, 2012). For HepCD81/miR122 and ORL8c cells, HCVtcp containing the *Gaussia* luciferase (GLuc) reporter gene were used. To do this, plasmid pHH/SGR-JFH1/GLuc/NS3m carrying the bicistronic sub-genomic HCV replicon containing the GLuc reporter gene and the NS3 adaptive mutation was constructed by replacement of the firefly luciferase (FLuc) gene of pHH/SGR-Luc containing the NS3 mutation (N1586D) (Suzuki *et al.*, 2012) with the GLuc gene of pCMV-GLuc (NEB).

Plasmids. HA-tagged Dyn2, a dominant-negative Dyn2 (K44A) in which Lys44 was replaced with Ala, was cloned into pcDNA3.1 as described previously (Kataoka *et al.*, 2012).

Gene silencing by siRNA. siRNAs were purchased from Sigma-Aldrich and were introduced into the cells at a final concentration of

30 nM using Lipofectamine RNAiMAX (Invitrogen) in accordance with the manufacturer's instructions. Target sequences of the siRNAs were: occludin (5'-GCAAGAUCACUAUGAGACA-3'), SR-BI (5'-GAGCUUUGGCCUUGGUCUA-3'), CD81 (5'-CUGUGAUCUAUGAUUCUUGCA-3'), CHC (5'-CUAGCUUUGCACAGUUUAA-3'), Dyn2 (5'-CCCUCAAGGAGGCGCUCAA-3'), Cav1 (5'-CCCUAAAACACCUCAACGAU-3'), flotillin-1 (5'-CCUAUGACAUCGAGGUCAA-3'), Arf6 (5'-CAGUUCUUGGUAAGUCCU-3'), CtBP1 (5'-GACUCGACGCGUGGCCACA-3') and PAK1 (5'-GCAUCAAUUCCUGAAGAUU-3'). Target sequences of the siRNAs for claudin-1, PI4K and scrambled negative control were as described previously (Suzuki *et al.*, 2013).

Immunoblotting. Cells were washed with PBS and incubated with passive lysis buffer (Promega). Lysates were sonicated for 10 min and added to the same volume of 2 × SDS-PAGE sample buffer. Protein samples were boiled for 10 min, separated by SDS-PAGE and then transferred to PVDF membranes (Merck Millipore). After blocking, membranes were probed with primary antibodies, followed by incubation with peroxidase-conjugated secondary antibody. Antigen-antibody complexes were visualized using an enhanced chemiluminescence detection system (SuperSignal West Pico Chemiluminescent Substrate; Thermo Scientific) in accordance with the manufacturer's protocols.

Flow cytometry. Cultured cells detached by treatment with trypsin were incubated with anti-CD81 antibody or anti-mouse IgG antibody for 1 h at 4 °C. After being washed with PBS containing 0.1 % BSA, cells were incubated with an Alexa Fluor 488-conjugated anti-mouse secondary antibody (Invitrogen) for 1 h at 4 °C, washed repeatedly and resuspended in PBS. Analyses were performed using a FACSCalibur system (Becton Dickinson).

Reagents and antibodies. Bafilomycin A1 was obtained from Wako Pure Chemical Industries. Alexa Fluor 488-conjugated transferrin was obtained from Invitrogen. For immunoblotting, anti-SR-BI (NB400-104; Novus Biologicals), anti-occludin (71-1500; Invitrogen), anti-claudin-1 (51-9000; Invitrogen), anti-Dyn2 (ab3457; Abcam), anti-Cav1 (N-20; Santa Cruz Biotechnology), anti-flotillin (H-104; Santa Cruz Biotechnology), anti-Arf6 (ab77581; Abcam) and anti-PAK1 (2602; Cell Signaling Technology) rabbit polyclonal antibodies; anti-CD81 (JS-81; BD Biosciences), anti-β-actin (AC-15; Sigma-Aldrich), anti-CHC (23; BD Biosciences), anti-GRAF1 (SAB1400439; Sigma-Aldrich) and anti-glyceraldehyde 3-phosphate dehydrogenase (6C5; Merck Millipore) mouse mAb; and anti-CtBP1 goat polyclonal antibody (C-17; Santa Cruz Biotechnology) were used. For immunofluorescence staining, anti-CHC mAb (X22) and anti-HA rat polyclonal antibody (3F10) were obtained from Thermo Scientific and Roche Applied Science, respectively. Anti-NS5A antibody was a rabbit polyclonal antibody against synthetic peptides. Alexa Fluor 488- or 555-labelled secondary antibodies were obtained from Invitrogen.

DNA transfection. Cell monolayers were transfected with plasmid DNA using TransIT-LT1 transfection reagent (Mirus) in accordance with the manufacturer's instructions.

Treatment of cells with bafilomycin A1 and cell viability. Cells were preincubated with various concentrations of bafilomycin A1 for 60 min at 37 °C. Preincubated cells were then infected with HCVtcp. Cells treated with 0.1 % DMSO were used as controls. Cell viability was analysed by the Cell Titre-Glo Luminescent Cell Viability Assay (Promega).

Uptake of transferrin. Cells were grown on glass coverslips. After cells were transfected with HA-tagged Dyn2 expression plasmids, Alexa Fluor 488-conjugated transferrin at 20 µg ml⁻¹ was added and incubated for 30 min. Cells were washed with PBS and fixed in 4 % paraformaldehyde.

Immunofluorescence analysis. Huh7.5.1 and Huh-7 cells were fixed with 4 % paraformaldehyde in PBS for 30 min, and were then blocked and permeabilized with 0.3 % Triton X-100 in a non-fat milk solution (Block Ace; Snow Brand Milk Products) for 60 min at room temperature. Samples were then incubated with anti-CHC, anti-Dyn2, anti-Cav1, anti-NS5A or anti-HA for 60 min at room temperature, washed three times with PBS, and then incubated with secondary antibodies for 60 min at room temperature. Finally, samples were washed three times with PBS, rinsed briefly in double-distilled H₂O and mounted with DAPI mounting medium. The signal was analysed using a Leica TCS SPE confocal microscope.

Luciferase assay. For quantification of FLuc activity in HCVtcp-infected cells, cells were lysed with passive lysis buffer (Promega) at 72 h post-infection. FLuc activity of the cells was determined using a luciferase assay system (Promega). For quantification of GLuc activity in supernatants of HCVtcp-infected cells, the *Renilla* Luciferase Assay System (Promega) was used. All luciferase assays were performed at least in triplicate.

Quantification of HCV core protein. HCV core protein was quantified using a highly sensitive enzyme immunoassay (Lumipulse G1200; Fujirebio) in accordance with the manufacturer's instructions.

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Development of Hepatitis C Virus Genotype 3a Cell Culture System

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Hepatitis C virus (HCV) genotype 3a infection poses a serious health problem worldwide. A significant association has been reported between HCV genotype 3a infections and hepatic steatosis. Nevertheless, virological characterization of genotype 3a HCV is delayed due to the lack of appropriate virus cell culture systems. In the present study we established the first infectious genotype 3a HCV system by introducing adaptive mutations into the S310 strain. HCV core proteins had different locations in JFH-1 and S310 virus-infected cells. Furthermore, the lipid content in S310 virus-infected cells was higher than Huh7.5.1 cells and JFH-1 virus-infected cells as determined by the lipid droplet staining area. **Conclusion:** We believe that this genotype 3a infectious cell culture system will be a useful experimental model for studying genotype 3a viral life cycles, molecular mechanisms of pathogenesis, and genotype 3a-specific antiviral drug development. (HEPATOLOGY 2014;00:000-000)

About 170 million people worldwide are infected with hepatitis C virus (HCV), which causes chronic liver disease at a high rate, leading to complications including endstage liver disease, liver cirrhosis, and hepatocellular carcinoma.¹ HCV is classified into seven major genotypes.² Genotype 1b is the most prevalent HCV genotype in Asian countries, followed by genotype 3a. Genotype 3a infections are more prevalent in South Asian countries with large populations.^{3,4} A high incidence of hepatic steatosis is associated with genotype 3a infection.⁵⁻⁷ Interferon and ribavirin combination therapy is not satisfactory in genotype 3a-infected patients, although it is more effective than in genotype 1b-infected patients.⁵ The recently developed protease inhibitors telaprevir and boceprevir are also less effective against genotype 3a infection.⁸ New antiviral drug development against genotype 3a HCV is necessary to improve treatment efficiency in genotype 3a-infected patients.

HCV subgenomic replicon systems are useful tools for the study of viral replication mechanisms and anti-

viral drug development. Recently, genotype 3a replicon systems were established.^{9,10} The S310 replicon with adaptive mutations replicated efficiently in cell culture. Genotype 3a infections have a different pathogenesis as compared to other genotype infections (for example, steatosis). Previous studies demonstrated that cells expressing genotype 3a core protein had increased lipid accumulation.¹¹⁻¹³ Therefore, an efficient infectious viral system recapitulating the full life cycle is now essential to determine the precise pathogenesis of genotype 3a infection.

In the present study we established an infectious genotype 3a HCV cell culture system by using S310 strains. The full-length S310 clones replicated efficiently and produced infectious viral particles. There were different HCV core localization patterns between genotype 3a S310- and genotype 2a JFH-1-infected cells. Interestingly, the lipid content in S310 virus-infected cells was higher than Huh7.5.1 cells and JFH-1 virus-infected cells as determined by lipid droplet staining area. This cell culture system will be very

Abbreviations: DMEM, Dulbecco's Modified Eagle Medium; HCV, hepatitis C virus; IgG, immunoglobulin G; LD, lipid droplet; MOI, multiplicity of infection; RT-PCR, reverse-transcriptase polymerase chain reaction; SGR, subgenomic replicon.

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useful for the study of viral life cycles, molecular mechanisms of pathogenesis, and specific antiviral drug development for genotype 3a HCV.

Materials and Methods

Details of the procedures are described in the Supporting Information.

Cell Culture. HuH-7 cells (obtained from Dr. Francis V. Chisari, Scripps Research Institute California) and a derivative cell line, Huh7.5.1 cells (obtained from Dr. Francis V. Chisari) were cultured as described in the Supporting Information.

HCV Plasmid Construction. Plasmids used in the analysis were constructed based on pS310 (S310/A, DDBJ/EMBL/GenBank accession number: AB691595).⁹ We constructed the full-length adapted pS310 by introducing the adaptive mutations from the SGR-S310 replicon assay. pJ6/JFH1 was previously obtained from pJFH1 by replacement with the 5' untranslated region (UTR) to p7 region (EcoRI-BclI) of the J6CF strain (a kind gift from Dr. Jens Bukh).¹⁴ pS310/JFH1 was obtained from pJFH1 by replacement with the core to NS2 C3 junction of the pS310 strain.¹⁵ pS310/JFH1 thus included nucleotide (nt) 1 to 2892 (amino acid [aa] 1 to 851) of S310 and nt 2888 to 9678 (aa 850 to 3033) of JFH-1 (accession number: AB047639).

RNA Synthesis and Transfection. Full-length HCV RNA was synthesized from pS310, pJFH1, pJ6/JFH1, pS310/JFH1 and the derivatives of pS310 constructs with adaptive mutations. The synthesized HCV RNA (10 μ g) was transfected into Huh7.5.1 cells by electroporation, as described previously.¹⁶⁻¹⁸

Quantification of HCV Core Protein and RNA. The concentrations of HCV core protein in the culture media and cell lysates were measured by a chemiluminescent enzyme immunoassay (Lumipulse II HCV core assay, Ortho Clinical Diagnostics, Tokyo, Japan).¹⁹ Real-time quantitative reverse-transcription polymerase chain reaction (RT-PCR) was performed to determine the copy numbers of HCV RNA as described previously.²⁰

RT-PCR and Sequencing Analysis. Total RNA was extracted and purified from the culture medium or cell pellet of the HCV RNA-transfected cells. HCV

cDNA was synthesized and amplified by RT-PCR as described previously.²¹⁻²³ The sequence of each amplified DNA was determined directly by using specific primers.

Determination of Infectivity. Infectivity of HCV was quantified by counting the infected foci by using fluorescence microscopy (Olympus, Tokyo, Japan), and the infectivity was expressed as the number of focus-forming units per milliliter (ffu/mL).^{16,24}

Immunofluorescence Analysis and Lipid Content Quantification. Transfected or infected cells were fixed and then stained with anti-core monoclonal antibody (2H9), DAPI, and BODIPY to determine the sublocalization of core protein, nucleus, and lipid droplets (LDs), respectively. BioZero (Keyence, Tokyo, Japan) or Leica TSE SPE confocal fluorescence microscopy (Leica Microsystems, Wetzlar, Germany) were used for the observation. The LD content of cells was quantified by MetaMorph (Leica MM AF Software) analysis.²⁵

Western Blot Analysis. Cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF membrane. HCV proteins (core, E2, and NS3) and host proteins were detected by specific antibodies.

Sucrose Density Gradient Analysis. Culture medium derived from the transfected cells was analyzed by sucrose gradient ultracentrifugation analysis.

Anti-HCV Drug Treatment. HCV-infected cells were tested with various concentrations of interferon α (IFN α , MSD K.K., Tokyo, Japan), the NS3 protease inhibitor VX-950 (Selleck Chemicals, Houston, TX), the NS5A inhibitor BMS-790052 (Selleck Chemicals), an immunosuppressant cyclosporin A (CsA, Sigma, St. Louis, MO) or the NS5B polymerase inhibitors JTK-109 (Japan Tobacco, Osaka, Japan) and PSI-6130 (Pharmasset, Princeton, NJ). After a 72-hour incubation the culture media were harvested and HCV core protein levels were quantified.

Statistical Analysis. Results were obtained from at least three independent experiments. Data are expressed as the mean \pm SD. Statistical analysis was performed using Welch's t-test. $P < 0.05$ was considered statistically significant.

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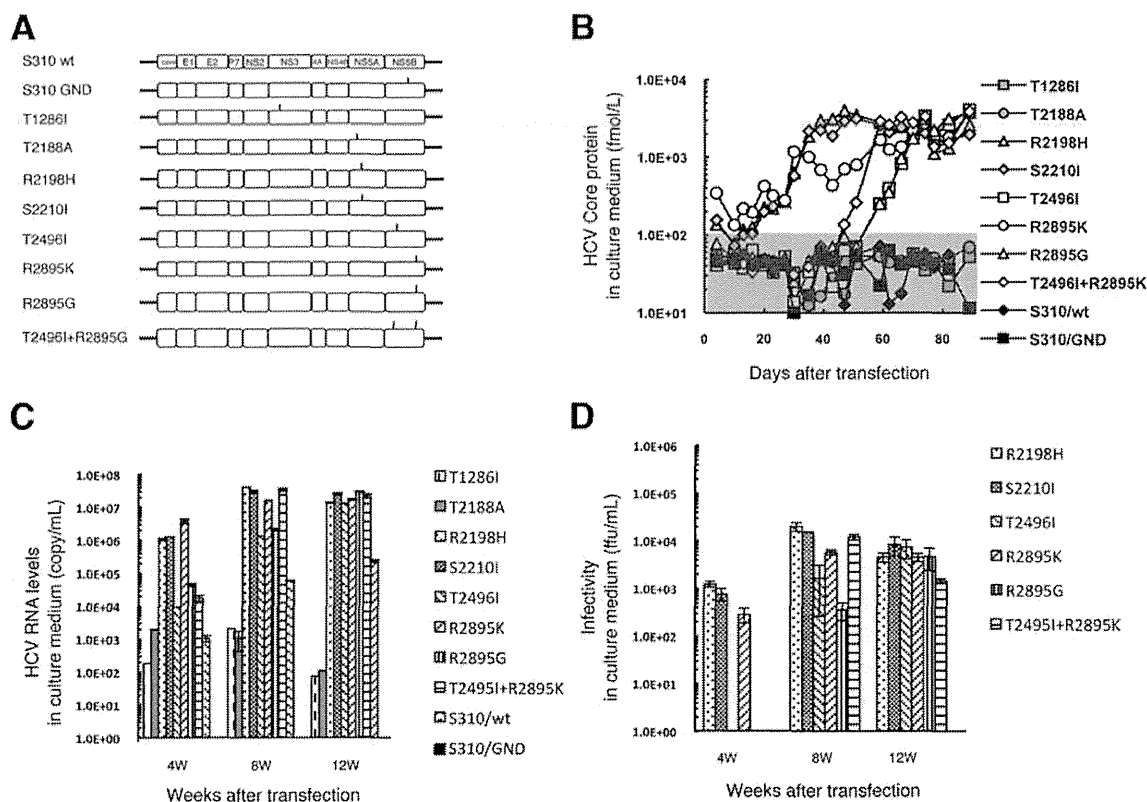


Fig. 1. Structure of full-length adapted S310 constructs and long-term culture of full-length adapted S310 clones. (A) Each mutation from subgenomic replicon clones was introduced into full-length wild-type S310. The position of each mutation is indicated by vertical lines. (B) Huh7.5.1 cells were transfected with the transcribed RNA from each construct. The cells were passaged every 3-5 days and HCV core protein levels in the culture medium at each passage were determined. Gray zone (<100 fmol/L) indicates the value below the detection limit, which was determined by mean \pm 3 SD of the detection values of control culture media. (C) RNAs in the culture medium were isolated. Copy numbers of HCV RNA were determined by real-time detection RT-PCR. The culture medium at 4, 8, and 12 weeks after transfection was used. (D) Infectious titers in the culture medium were determined by focus formation assay. (E) Each passaged cell was seeded onto a slide glass. The cells were fixed, probed with the core specific antibody (green) or DAPI for nucleus staining (blue), and examined by confocal microscopy. The cells at 4, 8, and 12 weeks after transfection are shown. (F) Western blot analysis. Cell lysates were prepared from S310 clones (1; R2198H, 2; S2210I, 3; T2496I, 4; R2895K, 5; R2895G, 6; T2496I+R2895K), Huh7.5.1 cells (7), JFH-1-infected cells (8), and J6/JFH1-infected cells (9). Protein (20 μ g) was separated by 12.5% SDS-PAGE, and each protein was detected by core, E2, NS3, beta actin, and GAPDH antibody. Arrows indicate the position of each protein. All assays were performed in triplicate, and data are presented as means \pm standard deviation.

Results

Full-Length Constructs With Adaptive Mutations. In our previous study, several adaptive mutations were found in the genotype 3a S310 subgenomic replicon assay.⁹ Those mutations were T1286I (NS3), T2188A (NS5A), R2198H (NS5A), S2210I (NS5A), T2496I (NS5B), R2895K (NS5B), R2895G (NS5B), and T2496I (NS5B) + R2895G (NS5B). We introduced these mutations into full-length S310 wild-type constructs (Fig. 1A).

Full-Length HCV Replication and Viral Production in Long-Term Culture. Viral RNA was synthesized from the full-length S310 wild- and mutant-type constructs and transfected into Huh7.5.1 cells. To examine whether these S310 constructs with adaptive

mutations could continuously produce infectious virus, transfected cells were serially passaged and secreted HCV core protein levels in the culture medium were monitored (Fig. 1B). After RNA transfection, the HCV core protein levels of three mutant-type S310 constructs (R2198H, S2210I, and R2895K) continuously increased, and finally they plateaued at \sim 2,000-4,000 fmol/L. Interestingly, from 6-8 weeks after transfection the HCV core protein levels of the other three clones with different adaptive mutations (T2496I, R2895G, and T2496I + R2896K) increased rapidly, and their core protein levels also reached the same levels as the former three clones (R2198H, R2210I, and R2895K). Two other mutant-type (T1286I, T2188A) and wild-type S310 constructs as well as replication-incompetent mutant S310/GND