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Genetic Characterization of Hepatitis C Virus in Long-Term RNA Replication Using Li23 Cell Culture Systems

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Abstract

Background: The most distinguishing genetic feature of hepatitis C virus (HCV) is its remarkable diversity and variation. To understand this feature, we previously performed genetic analysis of HCV in the long-term culture of human hepatoma HuH-7-derived HCV RNA-replicating cell lines. On the other hand, we newly established HCV RNA-replicating cell lines using human hepatoma Li23 cells, which were distinct from HuH-7 cells.

Methodology/Principal Findings: Li23-derived HCV RNA-replicating cells were cultured for 4 years. We performed genetic analysis of HCVs recovered from these cells at 0, 2, and 4 years in culture. Most analysis was performed in two separate parts: one part covered from the 5′-terminus to NS2, which is mostly nonessential for RNA replication, and the other part covered from NS3 to NS5B, which is essential for RNA replication. Genetic mutations in both regions accumulated in a time-dependent manner, and the mutation rates in the 5′-terminus-NS2 and NS3-NS5B regions were $4.0–9.0 \times 10^{-3}$ and $2.7–4.0 \times 10^{-3}$ base substitutions/site/year, respectively. These results suggest that the variation in the NS3-NS5B regions is affected by the pressure of RNA replication. Several in-frame deletions (3–105 nucleotides) were detected in the structural regions of HCV RNAs obtained from 2-year or 4-year cultured cells. Phylogenetic tree analyses clearly showed that the genetic diversity of HCV was expanded in a time-dependent manner. The GC content of HCV RNA was significantly increased in a time-dependent manner, as previously observed in HuH-7-derived cell systems. This phenomenon was partially due to the alterations in codon usages for codon optimization in human cells. Furthermore, we demonstrated that these long-term cultured cells were useful as a source for the selection of HCV clones showing resistance to anti-HCV agents.

Conclusions/Significance: Long-term cultured HCV RNA-replicating cells are useful for the analysis of evolutionary dynamics and variations of HCV and for drug-resistance analysis.

Introduction

Hepatitis C virus (HCV) infection frequently causes chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma. Such persistent infection has now become a serious health problem, with more than 170 million people worldwide infected with HCV [1]. HCV is an enveloped positive single-stranded RNA (9.6 kb) virus belonging to the *Flaviviridae* family, and the HCV genome encodes a large polyprotein precursor of approximately 3000 amino acid (aa) residues. This polyprotein is cleaved by a combination of host and viral proteases into at least 10 proteins in the following order: core, envelope 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B [2,3].

The initial development of a cell culture-based replicon system [4] and a genome-length HCV RNA-replicating system [5] using genotype 1b strains led to rapid progress in investigations into the mechanisms underlying HCV replication [6,7]. HCV replicon RNA (approximately 8 kb) is a selectable, bicistronic HCV RNA with the first cistron, the neomycin phosphotransferase (Neo^R) gene, being translated under control of the HCV internal ribosome entry site (IRES) and the second cistron, the NS3-NS5B regions, being translated under control of the encephalomyocarditis virus (EMCV) IRES. Genome-length HCV RNA (approximately 11 kb) possesses the Core-NS5B regions in substitution for the NS3-5B regions of the replicon in addition to the replicon structure. It was reported that infectious HCV particles are not produced in genome-length HCV RNA-replicating cell systems using genotype 1b strains [6,8]. However, in 2005, an efficient virus production system using the JFH-1 strain of genotype 2a was developed using HuH-7-derived cells [9]. Since then, this infectious HCV system became a powerful tool to study the full viral life cycle [10].

The most distinguishing feature of the HCV RNA is its remarkable diversity and variation. To date, six major HCV genotypes, each having a large number of subtypes, have been found to show more than a 20% difference at the nucleotide level compared with any other genotypes [11,12]. An approximately 5–8% difference at the nucleotide level has been observed within a single genotype [3]. Furthermore, an approximately 1% difference at the nucleotide level is also observed among HCV genomes in an individual [13]. Although genetic analyses of HCV using *in vivo* specimens have estimated that the genetic mutation rate of HCV is $1.4–1.9 \times 10^{-3}$ base substitutions/site/year [14–16], the potential variability of HCV is not clear due to the selective pressure of immune system functions *in vivo* [17,18].

To define the actual genetic mutation frequency of HCV, we previously performed genetic analysis of HCV [19,20] using human hepatoma HuH-7 cell culture-based HCV replicon systems or genome-length HCV RNA-replication systems. In studies using the 1B-1 or O strain of genotype 1b, the accumulation of genetic mutations (mutation rate is 3.0–4.8×10^{-3} base substitutions/site/year), the enlargement of genetic diversity, and an increase in GC contents of HCV RNA were observed in a time-dependent manner during a 2-year cell culture [19,20]. These results suggest that the long-term culture of HCV RNA-replicating cells is useful for understanding the evolutionary dynamics and variations of HCV. However, HuH-7-derived cells are the only cell culture system used thus far for robust HCV replication [6,7]. Therefore, it remains unclear whether our results obtained from HuH-7-derived HCV RNA-replicating cell culture systems reflect the general features of HCV's genetic diversity and variation. On the other hand, in 2009 we established four new human hepatoma Li23 cell-derived genome-length HCV RNA (O strain of genotype 1b; GenBank accession no. AB191333)-replicating cell lines, OL (polyclonal; a mixture of approximately 200 clones), OL8 (monoclonal), OL11 (monoclonal), and OL14 (monoclonal) [21], and have been culturing them for more than 4 years. Since we demonstrated that the gene expression profile of Li23 cells was distinct from those in HuH-7 cells [22], and that anti-HCV targets in Li23-derived cells were distinct from those in HuH-7-derived cells [23–25], we expected to find distinct HCV variability and diversity from those observed previously in HuH-7-derived cells. To clarify this point, we carried out comprehensive genetic analysis of HCVs obtained from 0-year, 2-year, and 4-year cultures of OL, OL8, OL11, and OL14 cells, and compared them with the original ON/C-5B/QR,KE,SR RNA [21].

Here, we report the evolutionary HCV dynamics occurring in the long-term replication of genome-length HCV RNAs using Li23-derived cell culture systems.

Materials and Methods

Cell Cultures

The human hepatoma Li23 cell line, which was established and characterized in 2009, consists of human hepatoma cells from a Japanese male (age 56) [21]. The Li23 cells were cultured in modified medium for human immortalized hepatocytes, as described previously [21,26]. Genome-length HCV RNA-replicating cells (Li23-derived OL, OL8, OL11, and OL14 cells) were cultured in the medium for the Li23 cells in the presence of 0.3 mg/ml of G418 (Geneticin, Invitrogen, Carlsbad, CA). These cells were passaged every 7 days for 4 years. HCV RNA-replicating cells possess the G418-resistant phenotype, because Neo^R as a selective marker was produced by the efficient replication of HCV RNA. Therefore, when HCV RNA is excluded from the cells or when its level decreases, the cells are killed in the presence of G418. In this study, OL, OL8, OL11, and OL14 cells were renamed OL(0Y), OL8(0Y), OL11(0Y), and OL14(0Y) cells, respectively, to specify the time at which the cells were established. These "0Y" cells of passage number 3 were used in this study. Two-year cultures of OL(0Y), OL8(0Y), OL11(0Y), and OL14(0Y) cells were designated OL(2Y), OL8(2Y), OL11(2Y), and OL14(2Y) cells, respectively. Four-year cultures of OL(0Y), OL8(0Y), OL11(0Y), and OL14(0Y) cells were designated OL(4Y), OL8(4Y), OL11(4Y), and OL14(4Y) cells, respectively.

Quantification of HCV RNA

Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis for HCV RNA was performed using a real-time LightCycler PCR (Roche Diagnostics, Basel, Switzerland) as described previously [21,27]. Experiments were done in triplicate.

Western Blot Analysis

The preparation of cell lysates, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblotting analysis with a PVDF membrane was performed as described previously [28]. The antibodies used to examine the expression levels of HCV proteins were those against NS4A (a generous gift from Dr. A. Takamizawa, Research Foundation for Microbial Diseases, Osaka University) and NS5B (a generous gift from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science, Japan). Anti-β-actin antibody (AC-15; Sigma, St. Louis, MO) was also used to detect β-actin as an internal control. Immunocomplexes on the membranes were detected by enhanced chemiluminescence assay (Western Lightning Plus-ECL; Perkin-Elmer Life Sciences, Boston, MA).

RT-PCR and Sequencing

To amplify genome-length HCV RNA, RT-PCR was performed separately in two fragments as described previously [21,27]. Briefly, one fragment covered from the 5′-terminus to NS3, with a final product of approximately 5.1 kb, and the other fragment covered from NS2 to NS5B, with a final product of approximately 6.1 kb. These fragments overlapped at the NS2 and NS3 regions and were used for sequence analysis of the HCV open reading frame (ORF) after cloning into pBR322MC [28]. SuperScript II (Invitrogen, Carlsbad, CA) and KOD-plus DNA polymerase (Toyobo, Osaka, Japan) were used for RT and PCR, respectively. Plasmid inserts were sequenced in both the sense and antisense directions using Big Dye terminator cycle sequencing on an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA). The nucleotide sequences of each of 10 (OL cell series) or 3 (OL8, OL11, and OL14 cell series) independent clones obtained were determined.

Molecular Evolutionary Analysis

Nucleotide and deduced aa sequences of the clones obtained by RT-PCRs were analyzed by neighbor-joining analysis using the program GENETYX-MAC (Software Development, Tokyo, Japan).

Antiviral Assay

To monitor the anti-HCV activity of telaprevir, genome-length HCV RNA-replicating cells were plated onto 6-well plates (2×10^5 cells for OL(0Y) cells or 8×10^4 for OL(4Y), OL8(4Y), OL11(4Y), or OL14(4Y) cells per well). After 24 hrs in culture, the cells were treated with telaprevir (a generous gift from Dr. T. Furihata, Chiba University, Japan) at 0.2 μM or 0.4 μM for 3 days. After
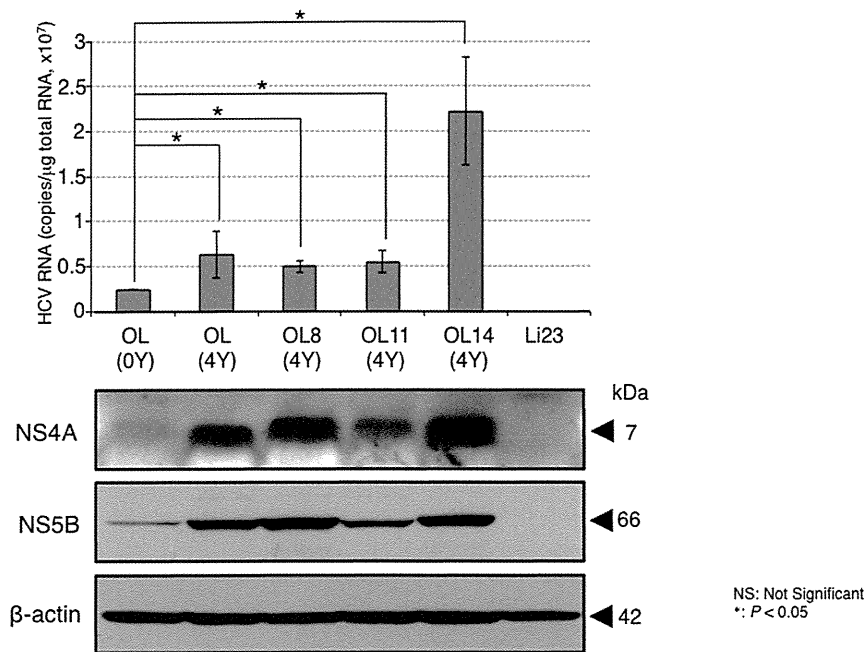
Figure 1. Characterization of genome-length HCV RNA-replicating cells after 4 years in culture. (A) Quantitative analysis of intracellular genome-length HCV RNA. The total RNAs from OL(4Y), OL8(4Y), OL11(4Y), and OL14(4Y) cells used were analyzed. The levels of intracellular genome-length HCV RNA were quantified by LightCycler PCR. OL(0Y) and Li23 cells were used as a positive and a negative control, respectively. (B) Western blot analysis. The cellular lysates from the cells used for RT-PCR analysis were also used for comparison. NS4A and NS5B were detected by Western blot analysis. β-actin was used as a control for the amount of protein loaded per lane.
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treatment, the cells were subjected to quantitative RT-PCR analysis for HCV RNA.

Statistical Analysis

The significance of differences among groups was assessed using Student's *t*-test. *P*<0.05 was considered significant.

Results

Efficient replication of genome-length HCV RNA is maintained in long-term cell culture

To prepare the specimens for the genetic analysis of HCV, genome-length HCV RNA-replicating OL(0Y), OL8(0Y), OL11(0Y), and OL14(0Y) cells were cultured for 4 years. Since we previously demonstrated that the levels of HCV RNAs increased in all cases after 2 years of constitutive HCV RNA replication [26], in the present study we examined the levels of intracellular HCV RNAs after the cell culture of 4 years by quantitative RT-PCR. The results revealed that the levels of HCV RNAs in all cases were significantly higher than that of OL(0Y) cells (Fig. 1). Western blot analysis for HCV NS4A and NS5B also showed that the expression levels in all cases were higher than that of OL(0Y) cells. However, the present results were matched with previous findings regarding a 2 year-culture [26], revealing that the levels of HCV RNAs of OL8(4Y) and OL11(4Y) cells become lower than those of OL8(0Y) or (2Y) and OL11(0Y) or (2Y) cells, respectively. Unlike the results for the OL8 or OL11 series, the levels of HCV RNAs of OL(4Y) or OL14(4Y) cells were each maintained throughout cultures of 2 years and 4 years. Overall, we showed that the HCV RNA levels in all cases were more than 5×10⁶ copies/μg of total RNA, indicating that efficient HCV RNA replication occurred during those 4 years.

We next examined whether infectious HCV particles are produced from genome-length HCV RNA-replicating cells after 4 years of culture, although it has been reported that infectious particles were not produced in genome-length HCV RNA-replicating cell systems [6,8]. To clarify this point, we performed infection experiments to HCV (JFH-1) susceptible HuH-7-derived RSc and Li23-derived ORL8 cells [21] using the supernatant of OL(0Y), OL(4Y), OL8(4Y), OL11(4Y), or OL14(4Y) cells as an inoculum. At 7 days and 8 days post-infection, we quantified the Core in the supernatants by enzyme-linked immunosorbent assay and HCV RNA in the cells by quantitative RT-PCR. The results (Fig. S1) showed that both Core and HCV RNA were not detected in our long-term cultured cells, suggesting that the cells produced no infectious virus particles over time.

Genetic variations of genome-length HCV RNAs during long-term cell culture

To clarify the genetic variations of HCVs during the period of cell culture, we carried out sequence analysis of genome-length HCV RNAs obtained from OL(2Y), OL(4Y), OL8(2Y), OL8(4Y), OL11(2Y), OL11(4Y), OL14(2Y), and OL14(4Y) cells. The determined nucleotide sequences of genome-length HCV RNAs were compared with those of the original ON/C-5B/QR,KE,SR RNA [21] used for the establishment of the OL(0Y), OL8(0Y), OL11(0Y), and OL14(0Y) cell lines. To compare the nucleotide sequences, the data on genome-length HCV RNAs from OL(0Y), OL8(0Y), OL11(0Y), and OL14(0Y) cells were also used [21]. Most of the sequence analysis was performed in two separate parts: one part covers from the 5′-terminus to NS2, which is mostly nonessential for RNA replication, and the other part covers from NS3 to NS5B, which is essential for RNA replication. The results revealed that the numbers of base substitutions in both regions
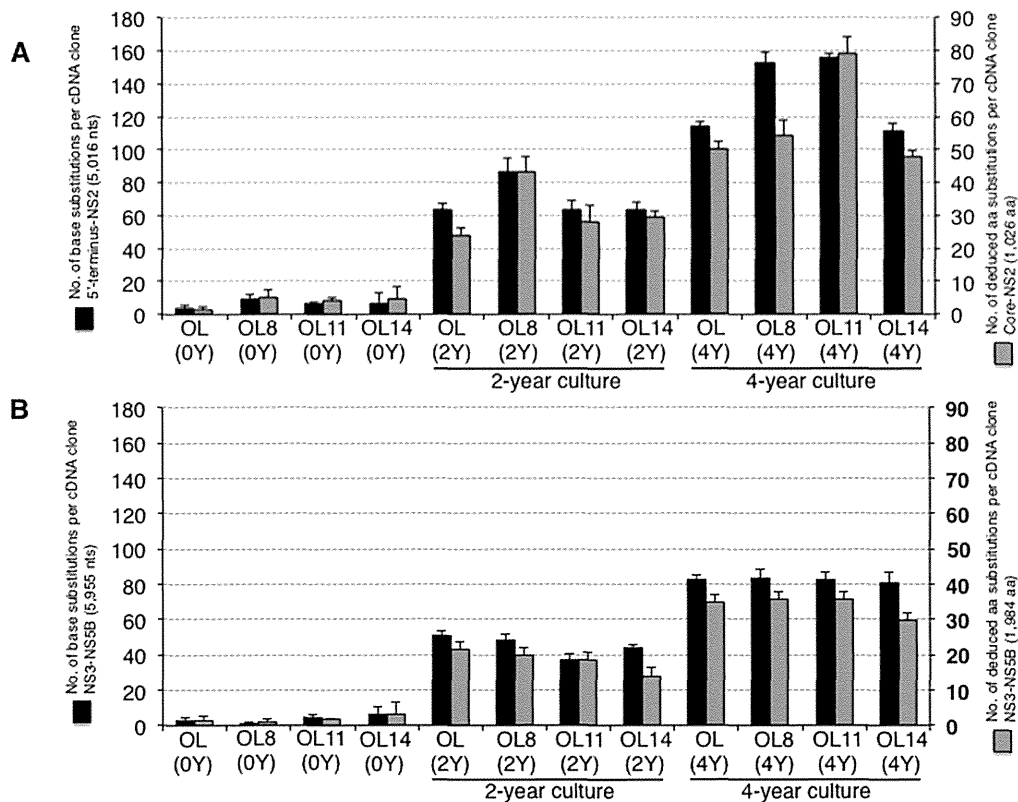
Figure 2. Genetic variations occurring in long-term replication of genome-length HCV RNAs. (A) Genetic variations in the 5'-terminus-NS2 regions. The left vertical line indicates the mean numbers of base substitutions detected per cDNA clone, by comparison with ON/C-5B/QR,KE,SR RNA [21]. The right vertical line indicates the mean numbers of aa substitutions in the Core-NS2 regions deduced per cDNA clone, by comparison with the original aa sequences deduced from ON/C-5B/QR, KE, SR RNA [21]. (B) Genetic variations in the NS3-NS5B regions. The mean numbers of base substitutions and aa substitutions are indicated as shown in (A).
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increased in a time-dependent manner (Fig. 2A and 2B). The numbers of deduced aa substitutions in HCV ORFs correlated well with the numbers of base substitutions of genome-length HCV RNAs (Fig. 2A and 2B). These base substitutions were considered mutations that occurred during the intracellular replication of genome-length HCV RNA. Based on the results after 2 or 4 years in culture, we calculated the apparent mutation rates of genome-length HCV RNAs in these cell lines. For this analysis, genome-length HCV RNA was divided into three parts: the 5'-terminus-EMCV IRES regions (partly essential for RNA replication), the Core-NS2 regions (nonessential for RNA replication), and the NS3-NS5B regions (essential for RNA replication). The results revealed that the mutation rates (base substitutions/site/year) in the three distinct regions calculated from the data of the 2-year culture were about the same as the mutation rates calculated from the data of the 4-year culture (Fig. 3). These results suggest that genetic variations of HCV have occurred at the same speed for four years in Li23-derived genome-length HCV RNA replicating cells. Furthermore, we noticed that the mutation rates in the NS3-NS5B regions $(2.7-4.0 \times 10^{-3})$ were lower than those in the 5'-terminus-EMCV IRES regions $(4.1-6.9 \times 10^{-3})$ and the Core-NS2 regions $(5.3-9.1 \times 10^{-3})$ (Fig. 3). Moreover, we examined the numbers of synonymous (dS) and nonsynonymous (dN) mutations with transition (Ts) or transversion (Tv) in two divided regions (Core-NS2 and NS3-NS5B). The results are summarized in Table 1. The dN/dS ratio in the Core-NS2 and NS3-NS5B regions were 1.55 to 3.00 and 0.45 to 1.06, respectively. These values imply the positive selection in Core-NS2 regions and the

purifying (stabilizing) selection in NS3-NS5B regions except OL11(2Y) and OL8(4Y) cells. Since the dN/dS ratios in NS3-NS5B regions of OL11(2Y) and OL8(4Y) cells were 1.06 and 1.03, respectively, we can estimate that neutral selection acted in these cells. In addition, the Ts/Tv ratios in the Core-NS2 and NS3-5B regions were 3.50 to 7.21 and 3.58 to 10.08, respectively. These results showed a tendency similar to that found in a previous study [20] using HuH-7-derived genome-length HCV RNA-replicating cells, suggesting that the NS3-NS5B regions, which are essential for RNA replication, are evolutionarily limited. Together these results indicate that HCV can mutate at the same level in both HuH-7-derived cells and Li23-derived cells.

Characterization of aa substitutions in HCV ORFs during long-term cell culture

We next characterized aa substitutions in HCV ORFs that occurred during 4 years in culture of OL(0Y), OL8(0Y), OL11(0Y), and OL14(0Y) cells. The conserved aa substitutions (mutated in all 10 clones sequenced in the cases of OL(2Y) or OL(4Y) cells and mutated in all 3 clones sequenced in the cases of OL8(2Y), OL8(4Y), OL11(2Y), OL11(4Y), OL14(2Y), or OL14(4Y) cells) are summarized in Table 2 (Core-p7 regions) and Table 3 (NS2-NS5B regions). Among the many aa substitutions, only 19 were the same as those detected in the 2-year culture of one of five kinds of HuH-7-derived genome-length HCV RNA (O strain of genotype 1b)-replicating cell lines [20] (Tables 2 and 3). In addition, 17 aa were substituted to the type of
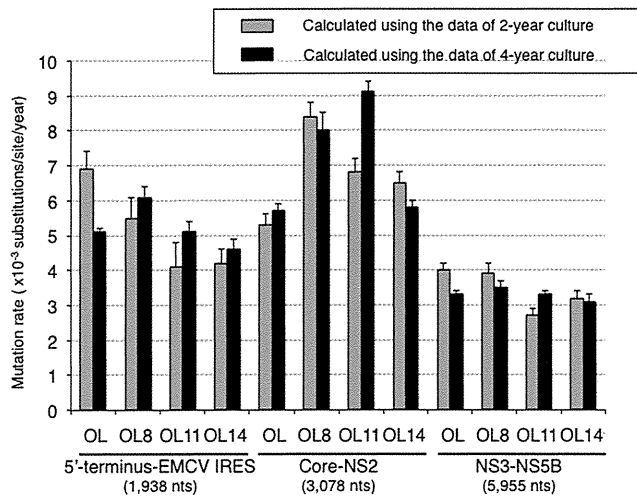
Figure 3. Mutation rates of genome-length HCV RNAs in long-term cell culture. The mutation rates of three regions (5'-terminus-EMCV-IRES, Core-NS2, and NS3-NS5B) of genome-length HCV RNAs (OL, OL8, OL11, and OL14) were calculated using the sequence data obtained from 2- or 4-year cell culture. The vertical line indicates the means of the mutation rates calculated using the nucleotide sequences of 10 clones (OL) or 3 clones (OL8, OL11, or OL14) of genome-length HCV RNAs, by comparison with the original sequence (ON/C-5B/QR,KE,SR RNA) [21].
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JFH-1 strain (genotype 2a: accession number AB237837) (Tables 2 and 3). We noticed that 12 aa substitutions were commonly detected in at least two different cell lines (Tables 2 and 3). The remaining 338 conserved aa substitutions were independently caused in each of the Li23-derived genome-length HCV RNA-replicating cell lines (Tables 2 and 3). However, from these results, we cannot conclude it whether genetic variations of HCV occur in a cell-line-specific manner or in a random manner.

Genetic deletions were characterized in the first half of genome-length HCV RNAs during long-term cell culture

Recently, Pacini et al. demonstrated that naturally occurring HCV subgenomic RNAs, mostly lacking the E1 or E2 region, were capable of autonomous replication and could be packaged and secreted in viral particles [29]. In the present cell-based study also, we detected several conserved deletions within genome-length HCV RNAs, although a previous study using HuH-7-derived cell lines did not reveal any conserved deletions [20]. As shown in Figure 4, all deletions were located in the first half of genome-length HCV RNA. In OL8(2Y) and OL8(4Y) cells, a conserved 51 nucleotides (nts) deletion in frame was detected, resulting in a 17 aa deletion (aa 686–702 in the E2). In OL14(2Y) and OL14(4Y) cells also, two kinds of conserved 3 nts deletion in frame were detected, resulting in a 1 aa deletion in each (aa 414 in the E2 and aa 847 in the NS2). Furthermore, a conserved 105 nts deletion in frame was observed in OL14(4Y) cells, resulting in a 35 aa deletion (aa 725–746 in the E2 and aa 747–759 in the NS2). In addition, 26 nts (nt 1248–1273) located between the Neo^R gene and IRES was conservatively deleted in OL11(2Y) and OL11(4Y) cells. These results suggest that nonessential regions for RNA replication are deleted during long-term culture of Li23-derived cells. However, such deletion was not caused in the OL cell series.

Genetic diversity of genome-length HCV RNA arising during long-term cell culture

Based on the sequence data of all clones obtained after 0-year, 2-year, and 4-year culture, we examined the genetic diversities of genome-length HCV RNAs by the construction of phylogenetic trees. The results revealed that the genetic diversities of genome-length HCV RNAs were clearly expanded at both the nucleotide (Fig. 5) and aa (Fig. S2) sequence levels in the 5'-terminus-NS2 regions and the NS3-NS5B regions, and that the 10 clones derived from OL cell series and 3 clones derived from each other cell series were clustered and located at similar genetic distances from the origin (ON/C-2 or O/3-5B/QR,KE,SR for the nucleotide sequence level and O/C-2 or O/3-5B/QR,KE,SR for the aa sequence level [21]) (Fig. 5 and Fig. S2).

We next compared the nucleotide sequences among 10 independent OL(4Y) clones obtained after 4-year cell culture. In

Table 1. Base substitutions occurring in genome-length HCV RNAs during long-term cell culture.

Full-length HCV RNA series	Ts				Tv				dN/dS		Ts/Tv	
	dN		dS		dN		dS					
	C-NS2	NS3-5B	C-NS2	NS3-5B	C-NS2	NS3-5B	C-NS2	NS3-5B	C-NS2	NS3-5B	C-NS2	NS3-5B
OL(2Y)	21.2±1.4	11.5±1.4	9.1±1.5	29.3±2.0	3.1±1.4	9.0±1.5	1.1±0.3	0.5±0.5	2.38	0.69	7.21	4.29
OL8(2Y)	34.3±4.9	14.3±1.2	12.3±2.1	23.7±.25	7.7±0.6	5.7±1.2	1.7±0.6	4.3±0.6	3.00	0.71	5.00	3.80
OL11(2Y)	23.3±4.0	13.0±1.0	17.0±3.6	16.0±4.0	6.0±3.6	6.0±1.7	0.7±0.6	2.0±0	1.66	1.06	6.05	3.63
OL14(2Y)	18.7±1.5	11.0±1.0	16.3±2.1	29.3±4.7	8.7±0.6	2.7±2.9	1.3±0.6	1.3±1.5	1.55	0.45	3.50	10.08
OL(4Y)	47.4±3.2	22.1±1.7	16.4±2.0	45.1±2.5	5.1±0.9	13.1±1.2	4.0±0.5	2.3±0.5	2.57	0.74	7.01	4.36
OL8(4Y)	56.7±4.2	35.7±1.2	29.7±2.5	38.3±2.3	14.3±0.6	12.3±0.6	1.3±0.6	8.3±0.6	2.29	1.03	5.51	3.58
OL11(4Y)	66.7±4.9	26.3±0.6	30.0±5.6	42.0±3.6	16.3±2.9	6.7±1.5	4.3±0.6	6.7±3.2	2.42	0.68	4.68	5.13
OL14(4Y)	34.3±1.5	23.7±1.2	27.3±3.5	47.3±2.9	10.3±1.2	5.0±0	1.3±0.6	3.7±1.5	1.56	0.56	5.29	8.19

Base substitutions were counted by comparison with the sequence of genome-length HCV RNA (ON/C-5B/QR,KE,SE [20]).
Average numbers of base substitutions per cDNA clone are shown.
Ts: Transition; Tv: Transversion; dN: Nonsynonymous; dS: Synonymous.
doi:10.1371/journal.pone.0091156.t001

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Table 2. Conservative aa substitutions occurring during long-term replication of genome-length HCV RNAs (I).

Region	OL		OL8		OL11		OL14	
Core (1~191)	V46A	**T52A**	**K10R**[a,b]	T11S	(I30T)	**S53P**	**K10R**[a,b]	**K12N**[b]
	L133F[b]	**G146R**	**Q20R**	V31A	T125S	**L133S**	**K23M**	E54G
	N163D	**L185S**	W76R	E89V[b]	M134T	**L139P**[b]	**S56P**	I65V
			L91P	N118I	A150T	**N163T**[a,b]	A180V	
			E159V	N163T[a,b]				
			P170A					
E1 (192~383)	**Y201H**	Y214C	C207Y	**V230A**	D206G	V240L	V203I	**C226R**[b]
	D218T	**L246P**	C281Y	**V284A**	A241T	S251G[a]	S251G[a]	**Y276H**
	F271S	I287N	**L286P**	F293L	S283P	**V284G**	L308S	**A343V**[c]
	Y298H[c]	C306S	S294L	**V313A**[b]	C304R	**M318V**[a]	**A380S**	
	W320R	**L332P**	(M318V)[a]	M323L	V365A	L377F		
	L359F		**T329A**	**L338F**				
			Q342R	V344G				
			A351P	**S363P**				
			W368R	F378L				
E2 (384~746)	R386C	I414T	**R386H**	N395D	S408P	R424G	I411V	**I414Δ**
	S450P	M456T	K410E	**N417D**	**L427P**	(G436E)	**S419R**	I422T
	E464A[c]	N532G	**N428D**	(I462V)	F447L	(S449P)	R483G	D520G
	N556S	K596E	I462A	**D481E**	S449L	**F465L**	K562E	T563M
	R614G	**M631T**	**Y507H**[b]	**G523S**	(Q467H)	V514G	C564W	**T680S**[c]
	E650G	L692P	**L537P**[b]	**N548S**[c]	E533G	C569R	**D698G**	V699A
	V710A	L721P[a]	**T561S**	E591G	**N577T**[c]	(L603M)	Δ725-746	
			S668P	I674T[b]	V609I	D610G		
			Δ686-702	**V709A**	Y611C	W616R		
			W736R		N623S	S663G		
					F679L	V710I		
					V712A	L721P[a]		
					V731A			
p7 (747~809)	E749K	**G764S**	**S767P**	(L797I)	N750D	L766F	(L748P)	Δ747-759
	L769P				L799P		**F771L**	**I778V**

[a]Conservative aa substitutions detected in at least two of four cell line series.
[b]Conservative aa substitutions detected in HuH-7-derived cell line series (O, OA, OB, OD, or OE) used in the previous study [20].
[c]Conservative aa substitutions that became the same aa as the JFH-1 strain.
Conservative aa substitutions detected after 2-year and 4-year cultures are shown by bold letters.
Conservative aa substitutions detected only after 2-year culture are shown within parentheses.
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the 5′-terminus-NS2 regions and the NS3-NS5B regions derived from OL(4Y) cells, 0.38–1.28% and 0.22–0.56% differences in nucleotide sequences were observed, respectively. These results suggest that the quasispecies nature of genome-length HCV RNA was acquired steadily over long-term intracellular RNA replication.

Classification of mutations occurred in genome-length HCV RNAs during long-term cell culture

We next examined the mutation patterns occurring in genome-length HCV RNAs. The results revealed that U to C and A to G transition mutations were the most and second-most frequent mutations in total, although three cases (OL8(2Y), OL8(4Y), and OL14(4Y)) showed the opposite result (Table 4). High frequencies of U to C and A to G mutations were also observed in a previous study using HuH-7-derived HCV replicon- or genome-length HCV RNA-replicating cell lines [19,20]. The rarest mutation was C to G transversion in 2-year and 4-year cultures (Table 4). This result was the same as in a previous report using HuH-7-derived cell systems [20]. Since the frequency of U to C and A to G mutations was two or three times higher than that of C to U and G to A mutations, the GC content of HCV RNA increased significantly in a time-dependent manner in both the 5′-terminus-NS2 regions (Fig. 6A) and the NS3-NS5B regions (Fig. 6B). The increase in GC content of HCV RNA was observed in all Li23-derived cells after 2-year or 4-year culture. In the 5′-terminus-NS2 regions of HCV RNA, a remarkable (more than 1%) increase in GC content was found after the 4-year culture of all the cells except OL14(0Y) cells (Fig. 6A).

The time-dependent increase in the GC content of the HCV RNA may gradually change to an energetically stable form during

Table 3. Conservative aa substitutions occurring during long-term replication of genome-length HCV RNAs (II).

Region	OL		OL8		OL11		OL14	
NS2	Y835H	F886L[b]	**M814I**	**I824V**	W845R	**V853A**	F823S	**W844R**
(810~1026)	**L892S**		L849F	**R852G**	D871G	**T877A**	**Q847H**	**Y848Δ**
			A855T	**Q903R**[a,c]	I885T	(P898L)	Q903R[a,c]	I983T
			K927R	E1019G	Q903R[a,c]	V913A		
					L924S			
NS3	V1081A	**E1202A**	**P1122S**	V1415I	**S1173L**	M1205V	M1268V	P1290H
(1027~1657)					**T1280A**	(I1412V)	D1581G	R1596K
					F1501Y[c]	Q1606R	A1647T[c]	
					F1644L			
NS4A	Q1703R							
(1658~1711)								
NS4B	**S1827T**[c]	**V1880A**[b]			I1769V	**Q1804R**	A1743V	S1827A
(1712~1972)	**P1908L**	L1956M			Q1955R		V1906A	
NS5A	**L2003F**	H2057R	**R1978K**	D1979E	K2050R	**F2099Y**[c]	L2125V[c]	**D2220G**[a]
(1973~2419)	S2246P	I2252S	K1998R	S2079Y	**T2217I**	I2274V	F2281L[a,c]	**D2292E**[a,b]
	T2278A	**F2281L**[a,c]	K2212R[c]	**D2220G**[a]	K2277R	S2283P[a]	F2352L	S2355T
	S2283P[a]	**D2292E**[a,b]	**E2263G**	**E2265V**	K2320R[a]	**T2336S**[c]	S2373P	D2374N
	K2320R[a]	S2338P	V2270A	K2280D	T2351A	**F2352S**	A2382V	G2396R
	S2355P	P2369H	**Y2293H**	D2305N	**W2405R**[a,b]		**S2401N**	W2405R[a,b]
	S2384P	**M2388T**	S2342P[b]	L2347R			**C2418R**[b]	
	G2403R	**S2409R**	F2352V	T2364A				
			D2377G[b]	**S2380T**				
			D2381G	S2387P[b]				
			W2405R[a,b]	S2406A				
			E2410K					
NS5B	K2470R	**D2771N**[c]	S2975G[c]	**I3004V**	K2493R	T2549A	**A2444T**	**H2539R**
(2420~3010)	L2853I	Q2933R			K2689R	Q2728R	V2918I	
	V3000A							

[a]Conservative aa substitutions detected in at least two of four cell line series.
[b]Conservative aa substitutions detected in HuH-7-derived cell line series (O, OA, OB, OD, or OE) used in the previous study [20].
[c]Conservative aa substitutions that became the same aa as JFH-1strain.
Conservative aa substitutions detected after 2-year and 4-year cultures are shown by bold letters.
Conservative aa substitutions detected only after 2-year culture are shown in parentheses.
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RNA replication. We assumed that the increase in GC content is due to an increase in G- and C-ending codons, except for AGG and UUG codons, for efficient expression in human cells, so-called codon optimization [30], and we examined this possibility. The results in the NS3-NS5B regions revealed the time-dependent increase of G- and C-ending codons, except for AGG and UUG codons, in all four cell series (Table 5). However, this phenomenon was not remarkable in the Core-NS2 regions (Table 5). These results suggest that codon usage in the NS3-NS5B regions adapts to efficient translation in the human cells in a time-dependent manner. Further long-term cell cultures will clarify this point.

Usefulness of long-term cultured genome-length HCV RNA-replicating cells as a source of resistant HCV for anti-HCV agents

As described above, we demonstrated that genetic mutations and the diversity of HCV RNA expanded during long-term culture of genome-length HCV RNA-replicating cells. From these

results, we assumed that these HCV populations that mimic the state of long-term persistent infection become the source of resistant HCV for anti-HCV agents. To clarify this point, we examined the effect of telaprevir, an inhibitor of HCV NS3-4A protease, which is the first directly acting antiviral reagent to be used for the treatment of HCV genotype 1, using 4-year cultured cell lines [31]. To know the effective concentration area, we first evaluated the anti-HCV activity of telaprevir using our previously developed HCV reporter assay systems (HuH-7-derived OR6 [27] and Li23-derived ORL8 [21]). The results revealed that 50% effective concentration (EC_{50}) values were 0.17 µM and 0.14 µM in the OR6 and ORL8 assay systems, respectively, indicating that telaprevir exhibited strong anti-HCV activities in our HCV cell culture systems (data not shown). In reference to these EC_{50} values, we next examined the anti-HCV activity of telaprevir at 0.2 and 0.4 µM for 3 days on OL(4Y), OL8(4Y), OL11(4Y), and OL14(4Y) cells. OL(0Y) cells were also used as a control. Telaprevir at 0.2 and 0.4 µM inhibited approximately 60% and
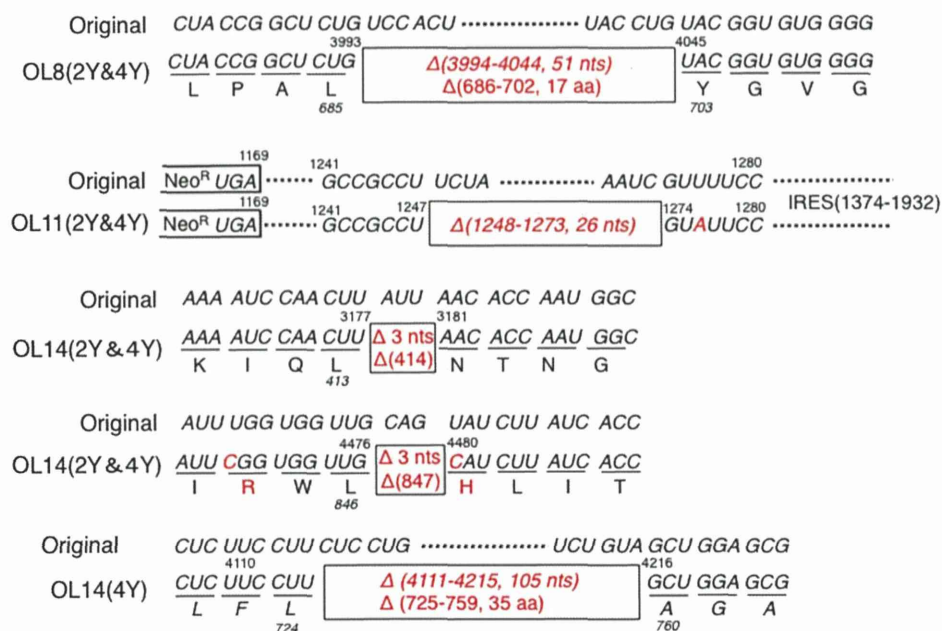
Figure 4. Genetic deletions occurred in the first half of genome-length HCV RNAs during the long-term cell culture. The conservative deleted portions in the genome-length HCV RNAs derived from OL8(2Y), OL8(4Y), OL11(2Y), OL11(4Y), OL14(2Y), or OL14(4Y) cells were shown by boxes. The original sequence was from ON/C-5B/QR,KE,SR RNA [21].
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80%, respectively, of HCV RNA replication on OL(0Y) cells, as expected from the results of the reporter assay, and that the anti-HCV activities of telaprevir on OL(4Y), OL11(4Y), and OL14(4Y) cells were similar to that on OL(0Y) cells (Fig. 7A). Unexpectedly, however, HCV RNA replication on OL8(4Y) cells was highly sensitive to telaprevir. Approximately 97% of HCV RNA replication was inhibited by 0.2 μM of telaprevir (Fig. 7A). These results suggest that HCV mutations that occur during long-term cell culture do not control the anti-HCV activity of telaprevir. Next we examined the possibility that long-term cultured cells can become the source of telaprevir-resistant HCV. First, OL(0Y) and OL(4Y) cells were treated with or without 0.4 μM of telaprevir (3 times at 6-day intervals) and 0.8 μM of telaprevir (3 times at 6-day intervals) in the presence of G418. The growth of the cells treated with telaprevir first slowed but then recovered. In this stage, we checked the anti-HCV activity of telaprevir at 0.2 μM for 3 days on telaprevir-treated OL(0Y) and OL(4Y) cells (designated OL(0Y)T and OL(4Y)T cells, respectively) with untreated OL(0Y) and OL(4Y) cells. The results clearly indicated that OL(0Y)T and OL(4Y)T cells completely converted telaprevir-sensitive phenotypes into telaprevir-resistant phenotypes (Fig. 7B). It is noteworthy that telaprevir-resistant OL(4Y)T cells were provided without a decrease in the level of HCV RNA replication. These results suggest that long-term cultured OL(4Y) cells may easily convert the phenotypes against anti-HCV drugs such as telaprevir.

Discussion

In the present study, using Li23-derived cells unlike HuH-7, we characterized the genetic evolution and dynamics of HCV in the long-term culture of four kinds of genome-length HCV RNA-replicating cells, and demonstrated that genetic mutations of HCV accumulated and the genetic diversity of HCV expanded in a time-dependent manner. The GC content of HCV RNA was also significantly increased in a time-dependent manner. These

phenomena, including the increased mutation rates, were consistent with those observed in the previous study using HuH-7-derived cell culture systems [19,20]. However, we detected several in-frame deletions in the structural regions, suggesting that the environment maintaining RNA genomic stability differs between Li23 and HuH-7 cells. Furthermore, we observed for the first time that GC content in nonstructural regions increased for codon optimization in human cells. Moreover, we demonstrated that the long-term cultured genome-length HCV RNA-replicating cells were useful as a library source for the isolation or characterization of resistant HCVs against anti-HCV agents.

Using Li23-derived cell culture systems, we observed that the mutation rates of HCV RNAs were $4.0–9.0 \times 10^{-3}$ and $2.7–4.0 \times 10^{-3}$ base substitutions/site/year in 5′-terminus-NS2 regions and NS3-NS5B regions, respectively. These values were 2.1–6.4 times and 1.4–2.9 times higher than those ($1.4–1.9 \times 10^{-3}$ base substitutions/site/year) previously obtained in chimpanzees [15,16] and in a patient [14] with chronic hepatitis C. Since we previously found that the mutation rates of genome-length HCV RNAs were $4.4–7.4 \times 10^{-3}$ and $2.5–3.7 \times 10^{-3}$ base/substitutions/site/year in 5′-terminus-NS2 regions and NS3-NS5B regions, respectively, using HuH-7-derived cell culture systems [21], most of the mutation rates were proved not to change, regardless of the cell type. Since the selective pressures of the humoral immune responses [17] targeting the envelope proteins and cellular immune responses [18] targeting all HCV proteins function *in vivo*, the mutation rates obtained using the cell culture systems without the immunological pressure would be reasonable values as a potential mutation rate of HCV in RNA replication.

Thus far, many studies using the HCV replicon system, including the whole-virus system of JFH-1 strain HCV, have clarified the aa positions that are essential for the efficient HCV reproduction [32–34]. On the basis of those reports, we made lists of functional aas in HCV genotype 1 (partly genotype 2a) (Tables S1 and S2) and then checked whether the position of each
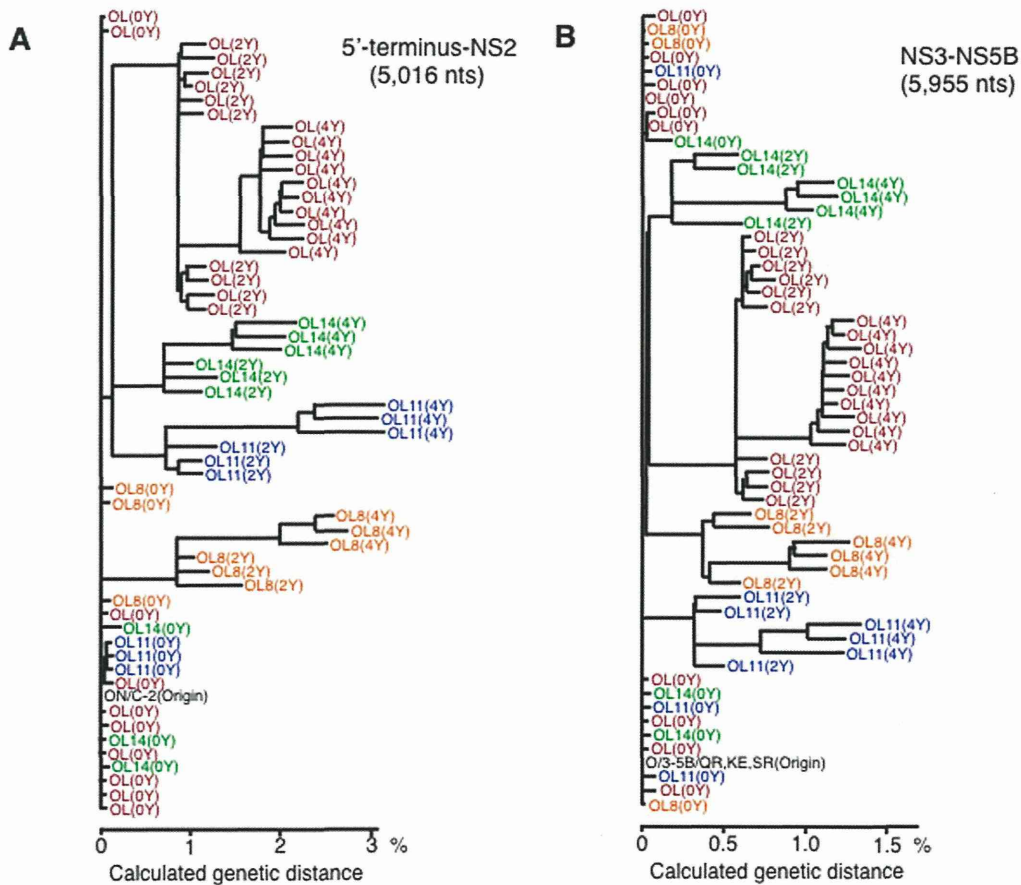
Figure 5. Phylogenetic trees of genome-length HCV RNA populations obtained in long-term cell culture. The phylogenetic trees are depicted on the basis of nucleotide sequences of all cDNA clones obtained by 0-year, 2-year, and 4-year cultures of OL, OL8, OL11, and OL14 cells. (A) The 5'-terminus-NS2 regions of genome-length HCV RNA. ON/C-2 indicates the original sequences of the 5'-terminus-NS2 regions of ON/C-5B/QR,KE,SR RNA [21]. (B) The NS3-NS5B regions of genome-length HCV RNA. O/3-5B/QR,KE,SR indicates the original sequences of the NS3-NS5B regions of ON/C-5B/QR,KE,SR RNA [21].
doi:10.1371/journal.pone.0091156.g005

functional aa was the same as the position of the aa substitution detected in this study. This investigation revealed that most of the functional aas were conserved during the 4-year culture of genome-length HCV RNA-replicating cells, suggesting that the basic HCV RNA replication mechanism does not change during long-term cell culture. However, as we observed several aa substitutions in the Core from OL11 series, the function of the Core may be lost in long-term-cultured OL11 cells, although the Core is not essential for RNA replication.

Although our report is the only one to conduct genetic variation and diversity analyses of HCV during the long-term HCV RNA replication of genotype 1b in cell culture, several similar reports use long-term HCV RNA (JFH-1 strain of genotype 2a)-replicating HuH-7-derived cells [35–41]. In those studies, many adaptive mutations were found as the result of long-term persistent HCV reproduction. Although it is a bit complicated to decide the corresponding aa positions exactly, as the O strain and JFH-1 strain belong to different genotypes, we examined whether the substituted aas detected in this study were found in those adaptive mutations obtained from reports using the JFH-1 strain. We noticed that only I414T substituted between 2- and 4-year cultures of OL cells was the same aa substitution as the JFH-1 strain (Table S3). It is unlikely that this substitution functions as an adaptive mutation for RNA replication because the HCV RNA level

decreased between 2- and 4-year cultures (Fig. 1 and [26]). It is also unlikely that this substitution increases virus production because virus particles were not produced from the cells cultured for 2 or 4 years (Fig. S1). However, we can exclude the possibility that other aa substitutions detected at the corresponding positions to the JFH-1 strain are adaptive mutations.

In our previous study using HuH-7-derived cell culture systems, we noticed that none of the aa substitutions were detected in the N-terminal half (242 aa of aa 1976 to 2217) of the NS5A after 2-year cultures, suggesting that this region would be the most critical for maintaining RNA replication. However, we detected many aa substitutions in this region in all Li23-derived cell lines after 2-year or 4-year cultures (Table 3). These were the following aa substitutions: L2003F and H2057R in OL series; R1978K, D1979E, K1998R, S2079Y, and K2212R in OL8 series; K2050R, F2099Y, and T2217I in OL11 series; L2125V in OL14 series. These results suggest that the N-terminal half of NS5A also possesses further variability to allow a better environment for HCV RNA reproduction. Another interesting feature we noticed is that several aa substitutions were spontaneously detected in the interferon (IFN) sensitivity determining region (ISDR) [42] (aa 2209–2248) and in the IFN/Ribavirin (RBV) resistance-determining region (IRRDR) [43] (aa 2334–2379) of NS5A in the cells without IFN or RBV treatment. In
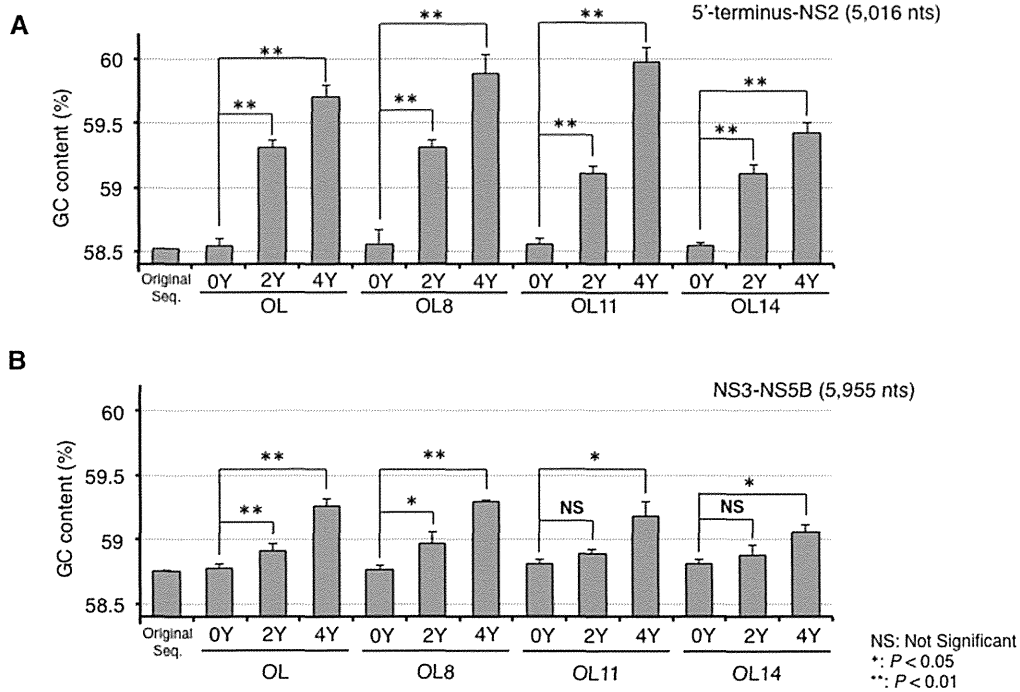
Figure 6. Increased GC content of genome-length HCV RNAs occurring in long-term RNA replication. The GC content of cDNA clones obtained by 0-year, 2-year, and 4-year culture of OL, OL8, OL11, and OL14 cells was calculated. The values indicate the means of 10 clones (OL) or 3 clones (OL8, OL11, or OL14). (A) The 5′-terminus-NS2 regions. (B) The NS3-NS5B regions.
doi:10.1371/journal.pone.0091156.g006

Table 4. Base substitution patterns occurred in genome-length HCV RNAs during the long-term cell culture.

Base substitution pattern	Average numbers of base substitutions per cDNA clone								Sum		HuH-7-derived
	OL	OL	OL8	OL8	OL11	OL11	OL14	OL14	OL~OL14	OL~OL14	O, OA, OB, OD&OE
	(2Y)	(4Y)	(2Y)	(4Y)	(2Y)	(4Y)	(2Y)	(4Y)	(2Y)	(4Y)	(2Y)*
Transition											
U →C	46.0	79.9	38.7	69.3	31.0	74.7	32.7	51.0	37.1±6.8	68.7±12.6	32.1±3.5
A →G	25.0	39.4	39.3	77.0	26.0	71.3	29.3	57.7	29.9±6.5	61.4±16.7	30.5±6.2
C →U	13.3	22.7	14.7	27.0	15.3	32.7	16.3	29.7	14.9±1.3	28.0±4.2	11.3±2.2
G →A	8.7	15.5	10.7	20.0	10.3	19.0	11.7	24.3	10.4±1.3	19.7±3.6	10.5±4.0
Transversion											
C →A	6.1	9.1	9.0	9.7	1.3	6.3	4.0	3.3	5.1±3.3	7.1±2.9	1.7±1.1
U →G	2.2	6.5	1.0	6.0	2.7	7.0	1.0	6.7	1.7±0.9	6.6±0.4	2.5±1.3
A →U	1.4	1.8	4.7	13.0	2.3	8.0	2.7	2.7	2.8±1.4	6.4±5.2	2.2±1.4
U →A	1.8	3.5	3.3	4.3	5.7	10.0	1.7	5.7	3.1±1.9	5.9±2.9	2.8±1.3
A →C	3.9	5.7	3.0	3.7	1.0	4.7	3.0	4.3	2.7±1.2	4.6±0.8	3.9±0.8
G →U	1.2	2.2	1.3	2.3	1.3	4.3	3.3	3.3	1.8±1.0	3.0±1.0	1.9±0.6
G →C	3.3	4.1	1.0	1.7	1.3	2.3	1.0	1.0	1.7±1.1	2.3±1.3	2.4±1.6
C →G	0.2	3.4	1.0	1.3	1.0	0.0	0.7	2.0	0.7±0.4	1.7±1.4	1.5±1.3

Base substitutions were counted by the comparison with the sequence of genome-length HCV RNA (ON/C-5B/QR,KE,SR [20]).
*Data from the previous study [20].
doi:10.1371/journal.pone.0091156.t004

Table 5. Contribution degrees of the G- and C-ending codons except AGG and UUG codons in the GC content increase during 2-year or 4-year cell cultures.

C-NS2

	OL	OL8	OL11	OL14
2Y culture	9.3*/24.0** (39%)	7.3/27.7 (26%)	4.3/20.6 (21%)	3.0/17.4 (17%)
4Y culture	9.8/38.1 (26%)	6.7/49.8 (13%)	17.7/54.7 (32%)	5.0/24.3 (21%)

NS3-5B

	OL	OL8	OL11	OL14
2Y culture	2.1/9.0 (23%)	4.0/12.7 (31%)	0/7.9 (0%)	3.3/6.7 (49%)
4Y culture	12.5/29.9 (42%)	13.7/32.0 (43%)	6.7/25.8 (24%)	16.0/18.0 (89%)

*The increased numbers of G- and C-ending codons except AGG and UUG codons per cDNA clone.
**The increased numbers of G and C per cDNA clone.
doi:10.1371/journal.pone.0091156.t005

ISDR, K2212R (OL8 series), T2217I (OL11 series), D2220G (OL8 and OL14 series), and S2246P (OL series) were detected. Furthermore, in IRRDR, T2336S (OL11 series), S2338P (OL series), S2342P (OL8 series), L2347R (OL8 series), T2351A (OL11 series), F2352V (OL8 series), F2352S (OL11 series), F2352L (OL14 series), S2355P (OL series), S2355T (OL14 series), T2364A (OL8 series), P2369H (OL series), S2373P (OL14 series), D2374N (OL14 series), and D2377G (OL8 series) were detected (Table 3). These aa substitutions except for D2220G also appeared in a seemingly random manner, although aa 2352 and 2355 were hot spots for aa substitutions in the Li23-derived cell culture system but not in the HuH-7-derived cell culture system [20]. These results suggest that the sensitivity to IFN or RBV might change during long-term cell culture, although it has not yet been proved that variations in ISDR or IRRDR may change the sensitivity to IFN or RBV.

When we explored this possibility, we newly noticed that L2003F (L31F in NS5A) was detected as a conservative aa in
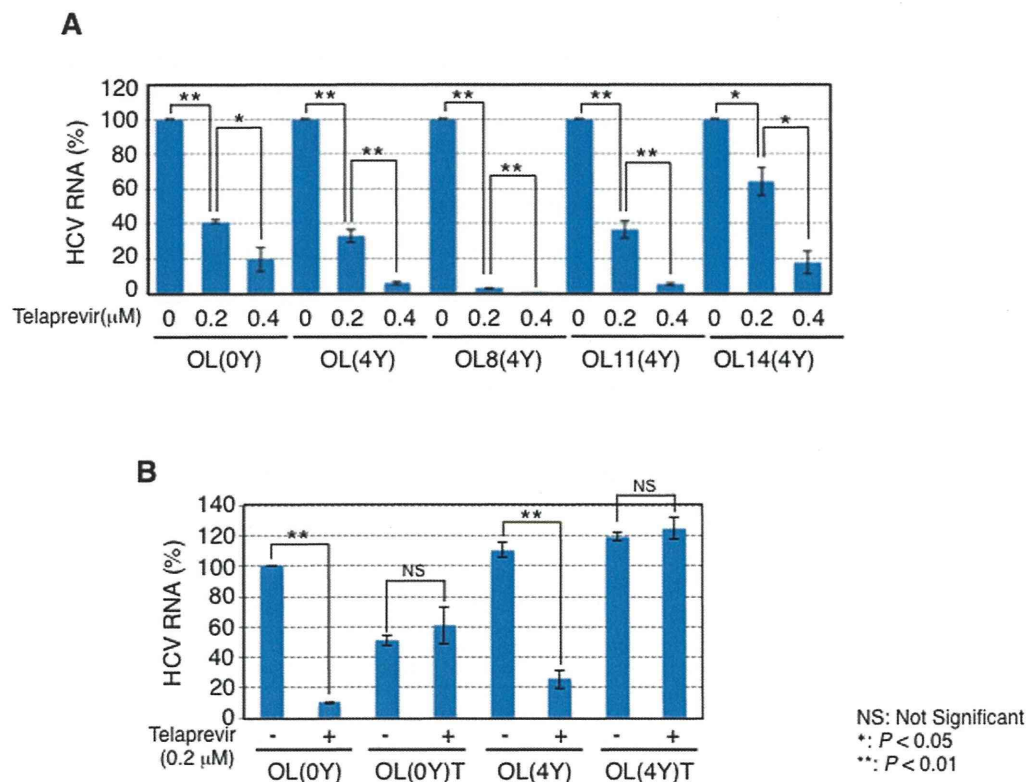

Figure 7. Sensitivity to telaprevir of the 4-year cultured genome-length HCV RNA-replicating cells. (A) Telaprevir sensitivities on genome-length HCV RNA replication in OL(4Y), OL8(4Y), OL11(4Y), and OL14(4Y) cells. OL(0Y) cells were used as a control. The cells were treated with telaprevir for 72 h, and then the levels of intracellular genome-length HCV RNA were quantified by LightCycler PCR. (B) Telaprevir-treated OL(0Y) and OL(4Y) cells (designated as OL(0Y)T and OL(4Y)T, respectively) became telaprevir-resistant easily. Telaprevir treatment and quantitative RT-PCR were preformed as shown in (A).
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OL(2Y) and OL(4Y) cells. F in aa 2003 has been reported as an aa showing low-level resistance to daclatasvir (BMS-790052), an NS5A inhibitor that will soon serve as a clinical cure [44]. Furthermore, V1081A (V55A in NS3) was also detected as a conservative aa in OL(4Y) cells. A in aa 1081 has been reported as an aa showing low-level resistance to boceprevir, an NS3-4A serine protease inhibitor that was approved as a new direct-acting antiviral drug [45]. These facts indicate that clones resistant to anti-HCV agents emerge naturally without treatment. Since V1081A and L2003F were detected in all HCV clones derived from OL(4Y) cells, these aa substitutions may possess some advantage for cell proliferation. Furthermore, as a minor population, a larger number of resistant HCV clones may emerge from such a long-term cell culture. Although neither daclatasvir nor boceprevir was available in this study, we demonstrated that telaprevir-treated OL(4Y) cells completely and easily converted a telaprevir-sensitive phenotype into a telaprevir-resistant phenotype without a decrease in the level of HCV RNA replication, suggesting that telaprevir-resistant HCV clones rapidly became dominant populations in the telaprevir-treated OL(4Y) cells.

As well as V1081A and L2003F, we noticed for the first time that D2292E (D320E in NS5A) appeared in OL(2Y), OL(4Y), OL14(2Y), and OL14(4Y) cells as a conservative aa substitution, although our previous study using HuH-7-derived cells detected D2292E as a conservative aa substitution after 2-year cultures of genome-length HCV RNA-replicating OB and OE cells [20]. It has been reported that D2292E is an aa substitution that causes resistance to cyclosporine (CsA) and other cyclophilin inhibitors, including NIM811 and DEB025 [46,47]. These facts also indicate that the HCV species possessing D2292E substitution can become the main species naturally in cultured cells without CsA or other treatments.

This study demonstrated that a single genome-length HCV RNA could exhibit a remarkable diversity after 4 years in cell culture with RNA replication. Our results, together with previous results, suggest that such diversity of HCV obtained by long-term cell culture may be useful not only for understanding the genetic variations and diversity of HCV but also for the examination of the resistant spectrum of anti-HCV agents.

Supporting Information

Figure S1 No infectious virus production from long-term cultured genome-length HCV RNA-replicating cells. HCV infection to RSc (1×10^4) and ORL8c (5×10^3) cells was performed using the supernatant (each 1 ml after filtering through a 0.20-μm filter [Kurabo, Osaka, Japan]) of OL(0Y), OL(4Y), OL8(4Y), OL11(4Y), or OL14(4Y) cells as an inoculum,

as described previously [23]. As a positive control, HCV JFH-1 virus was used for the infection at a multiplicity of infection of 0.1 or 1.0. At 7 days and 8 days, (A) the levels of Core in the supernatant after filtering through a 0.20-μm filter were quantified by enzyme-linked immunosorbent assay (Mitsubishi Kagaku Bio-Clinical Laboratories, Tokyo, Japan) and (B) the levels of intracellular HCV RNA were quantified by LightCycler PCR, as described previously [21,27].
(TIF)

Figure S2 Phylogenetic trees of deduced aa in ORF of genome-length HCV RNA populations obtained in long-term cell culture. The phylogenetic trees are depicted on the basis of aa sequences deduced from all cDNA clones obtained by 0-year, 2-year, and 4-year cultures of OL, OL8, OL11, and OL14 cells. (A) The Core-NS2 regions in ORF of genome-length HCV RNA. O/C-2 indicates the original aa sequences of the Core-NS2 regions in ORF of ON/C-5B/QR,KE,SR RNA [21]. (B) The NS3-NS5B regions in ORF of genome-length HCV RNA. O/3-5B/QR,KE,SR indicates the original aa sequences of the NS3-NS5B regions in ORF of ON/C-5B/QR,KE,SR RNA [21].
(TIF)

Table S1 Comparative list of functional aas in HCV genotype 1 and aa substitutions detected in this study (I).
(DOC)

Table S2 Comparative list of functional aas in HCV genotype 1 and aa substitutions detected in this study (II).
(DOC)

Table S3 Hereditary aa substitutions detected in persistent HCV JFH-1 (genotype 2a) infection; comparison with aa substitutions detected in this study.
(DOC)

Acknowledgments

Author Contributions

Conceived and designed the experiments: NK. Performed the experiments: NK HS YU HD. Analyzed the data: NK HS YU HD. Contributed reagents/materials/analysis tools: KM SS HD MI. Wrote the paper: NK.

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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.

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.*,

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 nonhepatic 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
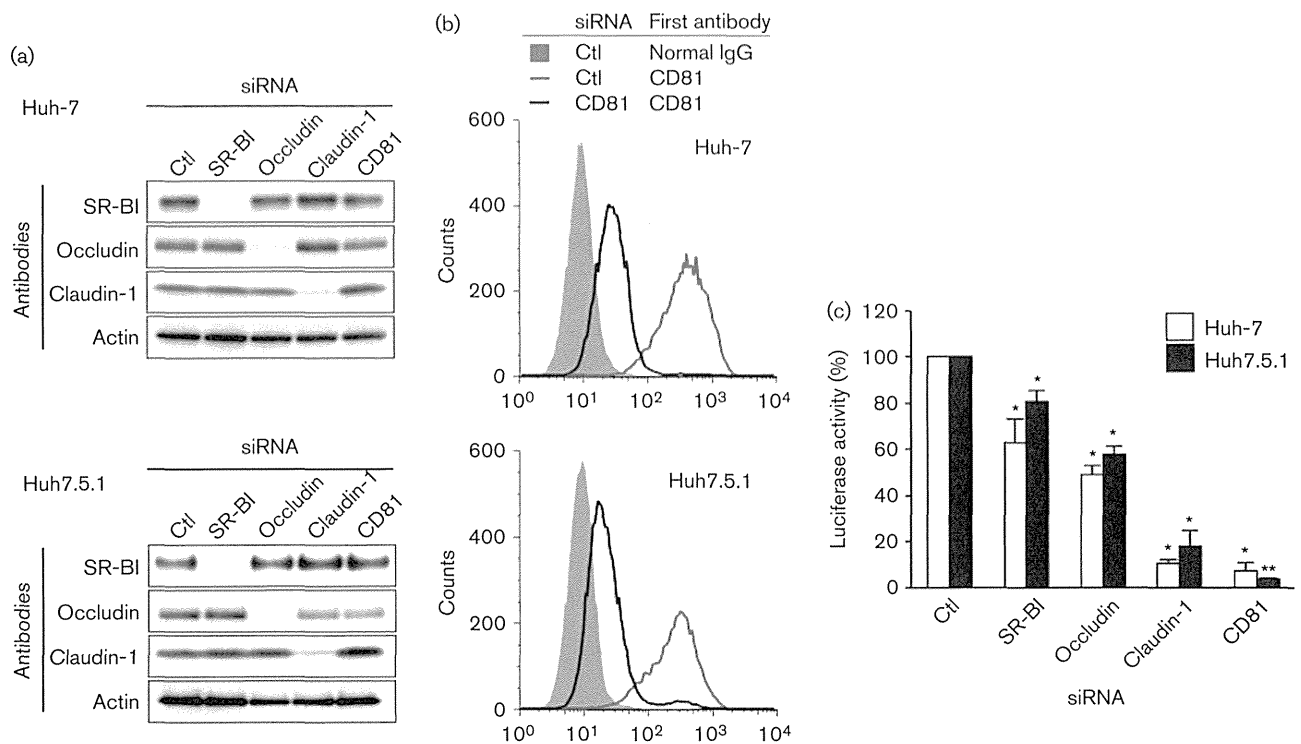
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Fig. 1. Knockdown effect of receptor candidate molecules on HCV infection. (a) Huh-7 or Huh7.5.1 cells were transfected with the indicated small interfering RNAs (siRNA), harvested at 48 h post-transfection and the specific knockdown of each protein 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 ± SD. Statistical differences between controls and each siRNA were evaluated using Student's t-test. *P<0.05, **P<0.001 versus control.
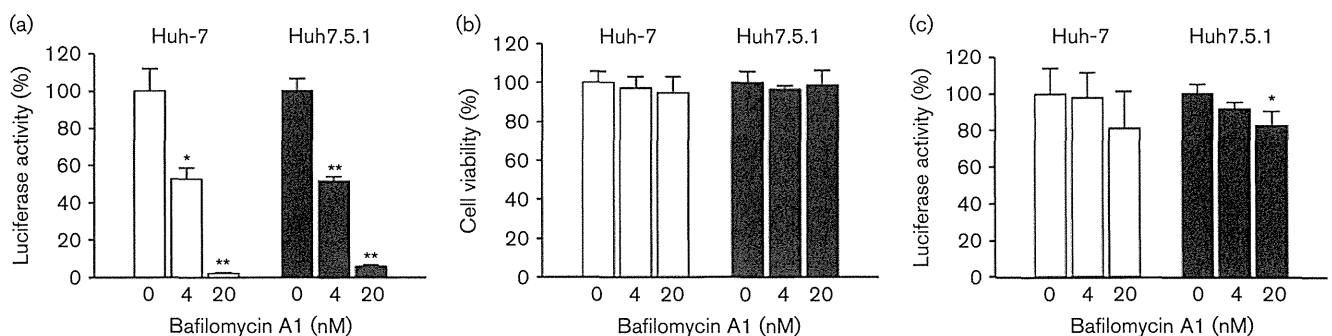

Fig. 2. Role of endosomal low pH in HCV infection. Cells were treated with bafilomycin A1 for 1 h at the indicated concentrations and infected with HCVtcp. (a, b) Luciferase activity (a) and cell viability (b) were determined at 72 h post-infection, and expressed relative to amounts observed in controls. (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 ± 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 phospha-tidylinositol 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
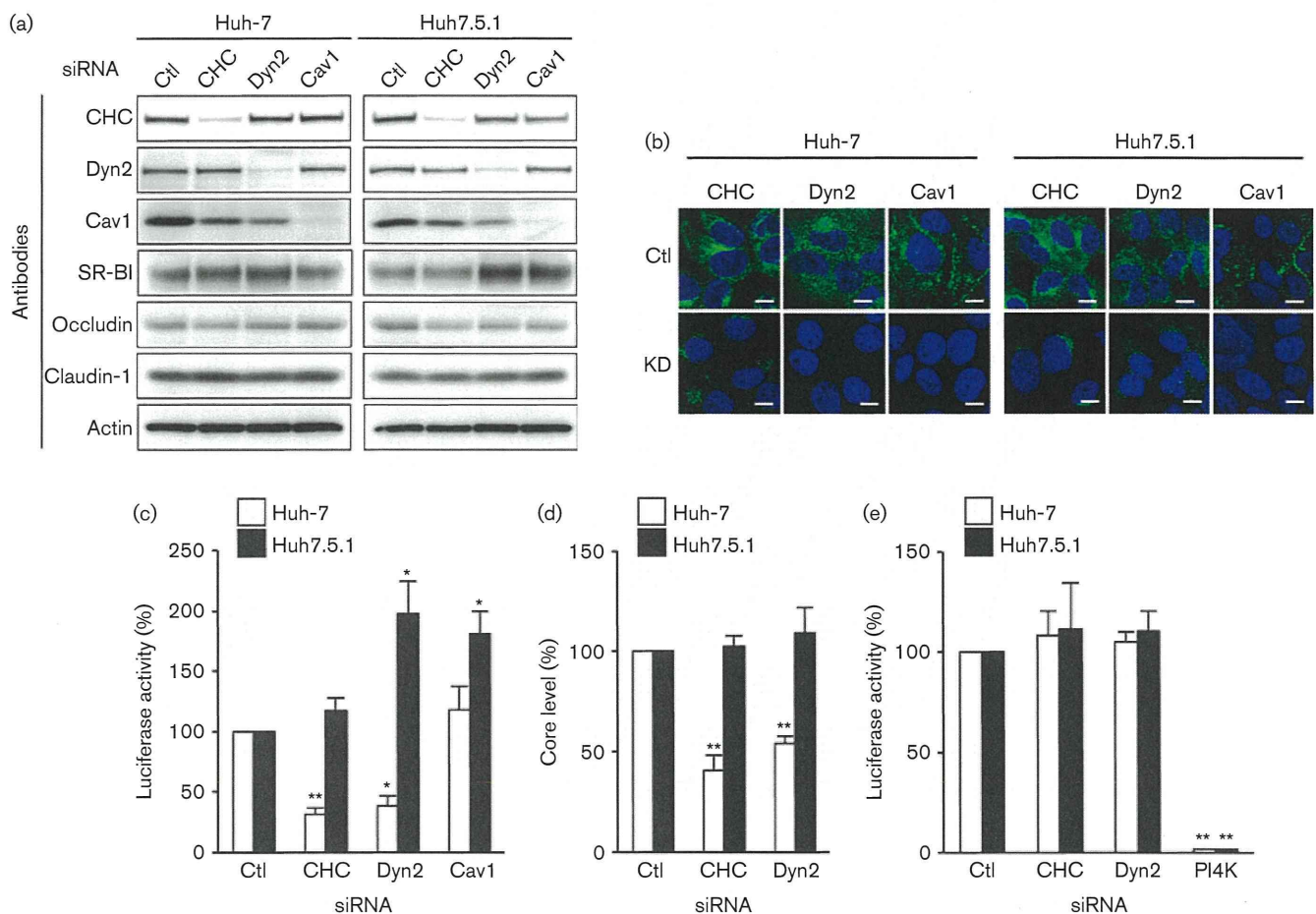

Fig. 3. Effects of CHC, Dyn2 and Cav1 knockdown on HCV infection. (a, b) Huh-7 cells or Huh7.5.1 cells were transfected with the indicated siRNAs and the specific knockdown (KD) of each protein was verified by immunoblotting (a) or immunostaining (b) at 48 h post-transfection. Bar, 50 µm. (c) Cells were 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
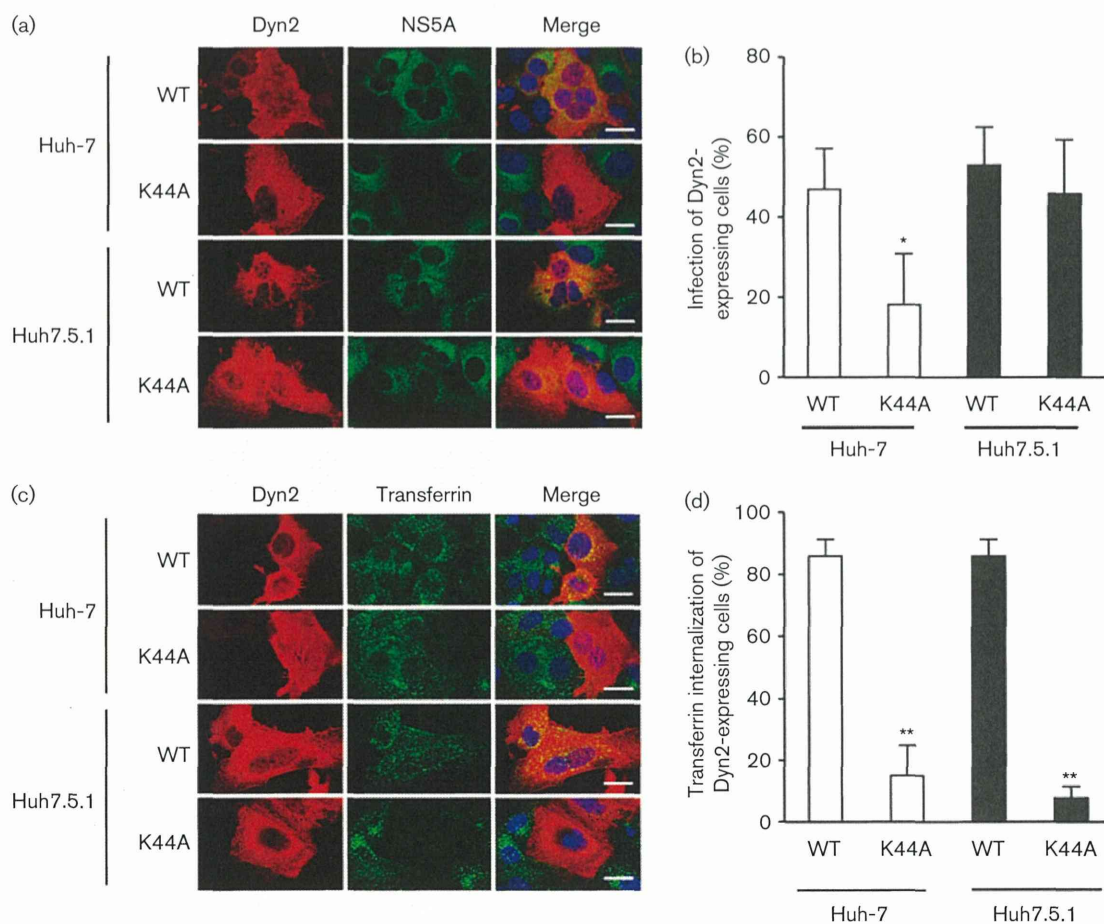

Fig. 4. Dynamin participates in the internalization of HCV in Huh-7 cells, but not in Huh7.5.1 cells. (a) Cells were transfected with HA-tagged WT Dyn2 (Dyn-WT) or dominant-negative Dyn2 (Dyn-K44A) expression plasmids. At 2 days post-transfection, cells were infected with HCVtcp, which possessed a subgenomic 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 μm. (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 ± 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 °C in a 5 % CO_2 incubator. After 30 min of incubation, cells were washed, fixed and stained with anti-HA antibodies. Cell nuclei were counterstained with DAPI. Bar, 100 μm. (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 ± 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.
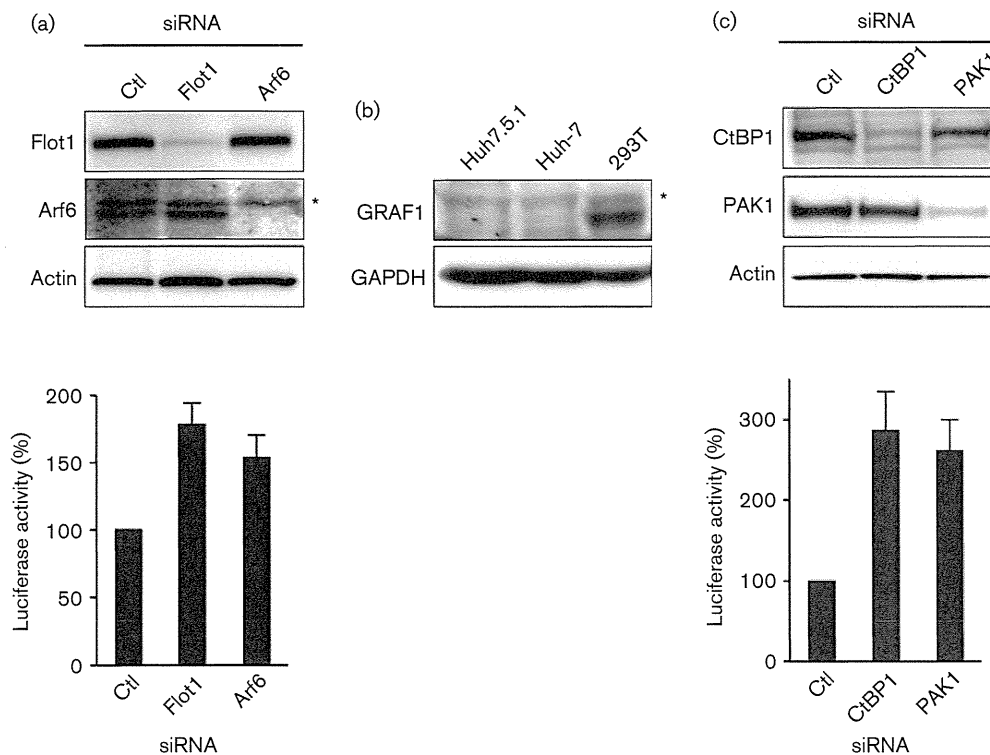

Fig. 5. Role of an alternative endocytosis pathway of HCV in Huh7.5.1 cells. (a) Huh7.5.1 cells were transfected with flotillin-1 (Flot1) or Arf6 siRNAs and specific knockdown of each protein was verified by immunoblotting (upper). Non-specific bands are marked with an asterisk. Cells transfected with 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 ± 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 ± SD.