

## Rab13 Is Involved in the Entry Step of Hepatitis C Virus Infection

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Membrane transport probably participates in the lifecycle of hepatitis C virus (HCV). Rab proteins are essential host factors for HCV RNA replication, but these proteins' roles in other steps of the HCV lifecycle are not clear. The tight junction (TJ) plays a key role in HCV infection. Rab13 regulates the endocytic recycling of the TJ-associated proteins. Here we investigated whether Rab13 is involved in the HCV entry step. We used HuH-7-derived RSc cells and Li23-derived D7 cells. To evaluate the effect of Rab13 in HCV infection, we transfected the cells with siRNA targeting Rab13 before HCV infection. The down-regulation of Rab13 inhibited HCV infection. The D7 cells had showed a greater inhibitory effect against HCV infection compared to that in the RSc cells by Rab13 knockdown. Next, to evaluate the effect of Rab13 after infection, we inoculated the cells with HCV before transfection of the siRNA. The down-regulation of Rab13 did not show any effects after HCV infection. We further examined whether Rab13 would influence HCV RNA replication by using HCV replicon-harboring cells. The results revealed that Rab13 did not affect the step of HCV RNA replication. These results suggest that Rab13 plays an important role in the step of HCV entry.

**Key words:** hepatitis C virus, Rab13, occludin, claudin 1

**H**epatitis C virus (HCV) causes chronic hepatitis and can progress to fatal liver cirrhosis and hepatocellular carcinoma. The exclusion of the virus is thus a strategy for preventing the progress of the disease after HCV infection. HCV belongs to the *Flaviviridae* family. The genome of HCV is a single-stranded 9.6-kb RNA. The genome encodes a single polyprotein of 3,000 amino acids. The host and viral protease cleave the polyprotein into 10 mature proteins:

Core, envelope 1 (E1), E2, p7, nonstructural 2 (NS2), NS3, NS4A, NS4B, NS5A and NS5B [1–3].

The HCV entry into cells consists of several steps [4]. The first attachment of HCV particles onto the cell surface require host factors, such as glycosaminoglycans and low-density lipoprotein receptor [5, 6]. Then, HCV E2 binds to the cell surface receptors: scavenger receptor class B type I (SR-BI) and CD81 [7–10]. CD81 and SR-BI act as the binding receptors for HCV infection. CD81-HCV complexes then inter-

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act with claudin1 (CLDN1) and occludin (OCLN). CLDN1 and OCLN are the main tight junction (TJ) proteins and act as the entry receptors for HCV infection. After interaction with these TJ proteins, HCV complexes are internalized via endocytosis.

We reported that statins, which are HMG-CoA reductase inhibitors, suppressed HCV RNA replication via inhibition of the biosynthesis of geranylgeranyl pyrophosphate (GGPP) [11]. Rabs need modification with GGPP for their activation by geranylgeranyltransferase II. We have therefore tried to clarify the effect of Rabs in each step of the HCV lifecycle.

The Rab proteins belong to the Ras superfamily of small GTPases. The Rab family consists of more than 60 subtypes. As membrane transport plays a significant role in various steps in the HCV lifecycle, the Rab proteins seem to be essential host factors for HCV infection, replication, and assembly. For example, Rab5 and Rab7 are important for HCV RNA replication, and Rab18 is required for viral assembly [12–15].

However, there is apparently no prior study about Rabs regarding the entry step of HCV. CLDN1 and OCLN are the entry receptors for HCV infection, and the membrane trafficking of them is regulated by Rab13 [16, 17]. The Rab13 translocates the TJ proteins to cytoplasmic vesicles and recycles them to the TJ.

The genotype 2a JFH-1 strain produces robust infectious HCV in cell culture [18]. To clarify the role of Rab13 in the HCV lifecycle, we used our developed HCV-JFH-1 reporter system [19]. Here, we investigated whether Rab13 is essential in the HCV entry step.

## Materials and Methods

**Cell culture and HCV RNAs.** RSc is a clonal cell line from the human hepatoma cell line HuH-7. We cultured RSc cells in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Life Technologies) [19]. D7 is a clonal cell line from the human hepatoma cell line Li23 [19]. We maintained D7 cells in F12 medium (Life Technologies) and DMEM (1 : 1 in volume) supplemented with 1% FBS and epidermal growth factor (50 ng/ml; Pepro

Tech, Rocky Hill, NJ). The subgenomic HCV-JFH-1 replicon contains Renilla luciferase (*RL*) and neomycin phosphotransferase genes in the first cistron and NS3 to NS5B region of HCV-JFH-1 in the second cistron [20]. The subgenomic HCV-JFH-1 replicon-harboring cells were cultured in the above medium supplemented with G418 (0.3 mg/ml; Geneticin, Invitrogen, Carlsbad, CA, USA).

**siRNA.** We introduced small interfering RNAs (siRNAs) targeting Rab13 (M-008389-00, Thermo Fisher Scientific, Wilmington, DE, USA) or control siRNAs (D-001206-13-20, Thermo Fisher Scientific) into RSc and D7 cells by using Lipofectamine RNAiMAX transfection reagent according to the manufacturer's protocol (Life Technologies).

**RL assay.** The cells ( $2 \times 10^4$  cells) were plated onto 24-well plates and cultured for 48 h after transfection or infection. The cells were harvested with Renilla lysis reagent (Promega, Madison, WI, USA) and subjected to the RL assay.

**WST-1 cell proliferation assay.** For the WST-1 assay, the cells ( $4 \times 10^3$  cells) were plated onto a 96-well plate at 24 h before transfection or infection. The cells were subjected to a WST-1 cell proliferation assay at 48 h after transfection or infection, (Takara Bio, Otsu, Japan).

**Western blot analysis.** The cells ( $1 \times 10^5$  cells) were plated onto 6-well plates and cultured for 48 h after transfection or infection. Preparation of the cell lysates, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting were then performed as described previously [21]. The antibodies for detection were Core (CP11; Institute of Immunology, Tokyo), NS5A (Dr. T. Wakita, National Institute of Infectious Diseases, Tokyo), Rab13 (Atlas Antibodies, Stockholm, Sweden) and  $\beta$ -actin (AC-15; Sigma, St. Louis, MO, USA). We performed an enhanced chemiluminescence assay (Perkin Elmer Life Science, Wellesley, MA, USA) to detect immunocomplexes on the membranes.

**HCV infection.** RSc or D7 cells ( $2 \times 10^4$  cells) were plated onto a 24-well plate 24 h before transfection or infection. To evaluate the effect of Rab13 in infection, we first transfected the cells with siRNA and cultured them for 24 h. The cells were then inoculated with supernatant from RSc cells containing reporter HCV-JFH-1 (JR/C5B/BX-2) at a multiplicity of infection (MOI) of 0.2 [19]. After infection, the

cells were cultured for 48h and subjected to an RL assay as described previously [19]. The JR/C5B/BX-2 contains the *RL* gene in the first cistron following the encephalomyocarditis virus-internal ribosomal entry site gene and the open reading frame (ORF) of HCV-JFH-1 in the second cistron. To evaluate the effect of Rab13 after infection, we inoculated the cells with reporter HCV-JFH-1 at the MOI of 0.2 and cultured the cells for 24h. The cells were then transfected with siRNA and cultured for 48h, and subjected to the RL assay.

**Immunofluorescence staining.** D7 cells ( $2 \times 10^4$  cells) were plated onto a microscope slide and cultured for 48h after infection. The cells were then washed in phosphate-buffered saline (PBS) and fixed using 3% paraformaldehyde in PBS for 15min at room temperature (RT). The cells were washed 3 times with PBS and permeabilized with 0.25% Triton X-100 for 10min at RT. Next, the cells were washed 3 times with PBS and incubated in 1% bovine serum albumin (BSA) for 1h. The cells were incubated with primary antibodies in 1% BSA overnight at 4°C. The primary antibodies used were anti-Core and Rab13 antibodies (1 : 150).

The cells were then washed 3 times with PBS and incubated with secondary antibodies for 30min at RT. Alexa fluor 488-conjugated goat anti-rabbit antibody and Alexa fluor 564-conjugated goat anti-mouse antibody were used as the secondary antibodies. The cells were then stained with DAPI for 5min at RT. DAPI was used for visualization of the nucleus.

**Statistical analysis.** Data represent the mean  $\pm$  standard error obtained from at least triplicate experiments. *P*-values were determined by Student's *t*-test. Significance was accepted at  $*p < 0.01$ .

## Results

**Rab13 is involved in HCV infection.** Rab13 regulates the endocytic recycling of the TJ-associated proteins CLDN1 and OCLN. As CLDN1 and OCLN play key roles in HCV infection, we examined whether Rab13 is required for HCV infection. To do this, we used an HCV-JFH-1 reporter assay system [19] and 2 types of liver cells. HuH-7-derived RSc cells and Li23-derived D7 cells are highly permissive cell lines for HCV infection [19]. To evaluate the effect of Rab13 in HCV infection, we transfected RSc and D7

cells with siRNA targeting Rab13 before HCV infection (Fig. 1A). The cells were inoculated with reporter HCV-JFH-1, and we monitored the infection by measuring the RL activity at 48h after infection. The downregulation of Rab13 exhibited a weak inhibition of HCV infection in the RSc cells (Fig. 1B) and significant inhibition in the D7 cells (Fig. 1C). The downregulation of Rab13 did not exhibit cytotoxicity to the RSc or D7 cells until 2 nM of siRNA was used (Fig. 1B, C).

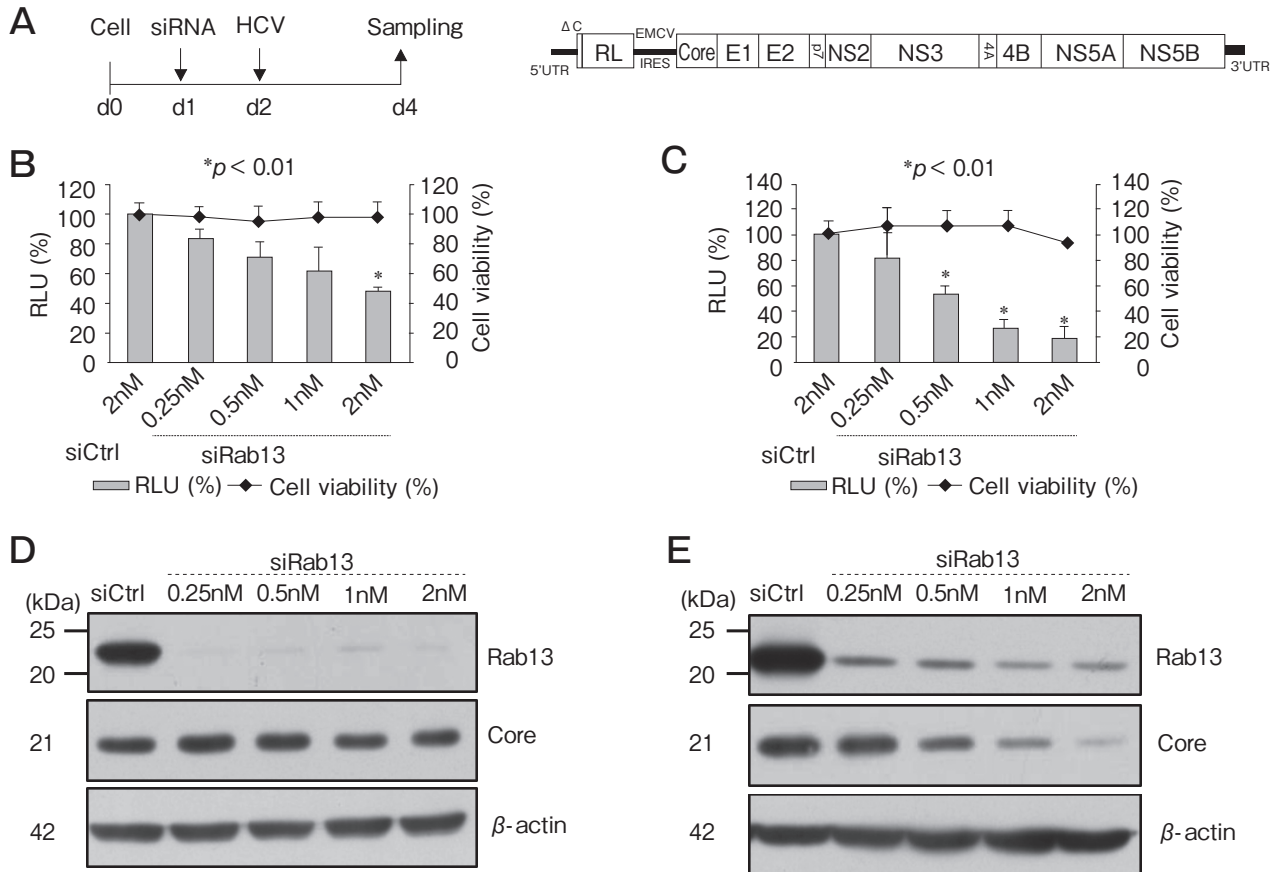
The knockdown of Rab13 after HCV infection exhibited a marginal reduction of the Core in the RSc cells (Fig. 1D). In the D7 cells, the knockdown of Rab13 significantly reduced the Core in a dose-dependent manner (Fig. 1E). The inhibitory effect on HCV infection by Rab13 suppression was stronger in the D7 cells than in the RSc cells. These results suggest that Rab13 is associated with the HCV infection.

**The suppression of Rab13 after HCV infection had no effect.**

We examined the effect of the Rab13 after infection. The cells were inoculated with reporter HCV-JFH-1 for 24h. After infection, the cells were transfected with siRNA and cultured for 48h. The effect of Rab13 knockdown at post-infection was assessed using the RL assay (Fig. 2A). The knockdown of Rab13 did not inhibit HCV proliferation in the RSc cells and exhibited marginal inhibition in the D7 cells at 2 nM of siRNA (Fig. 2B, C). The downregulation of Rab13 did not exhibit cytotoxicity to the RSc and D7 cells until 2 nM siRNA was used (Fig. 2B, C). There was no reduction of Core protein expression in the RSc or D7 cells until 2 nM of siRNA was used (Fig. 2D, E). These results suggest that Rab13 does not play a significant role in the HCV lifecycle after the viral entry step.

**The suppression of Rab13 does not inhibit the replication of subgenomic HCV replicon.**

We further examined whether Rab13 would influence HCV RNA replication by using HCV-JFH-1 replicon-harboring cells. In this replicon system, subgenomic HCV RNA could replicate but could not produce infectious virus. The cells were transfected with siRNA and cultured for 48h (Fig. 3A). The downregulation of Rab13 did not inhibit HCV RNA replication in the RSc or D7 cells (Fig. 3B, C). The lower concentration of siRNA exhibited enhancement of HCV RNA replication in both RSc and D7 cells. The knockdown of Rab13 did not decrease the NS5A pro-

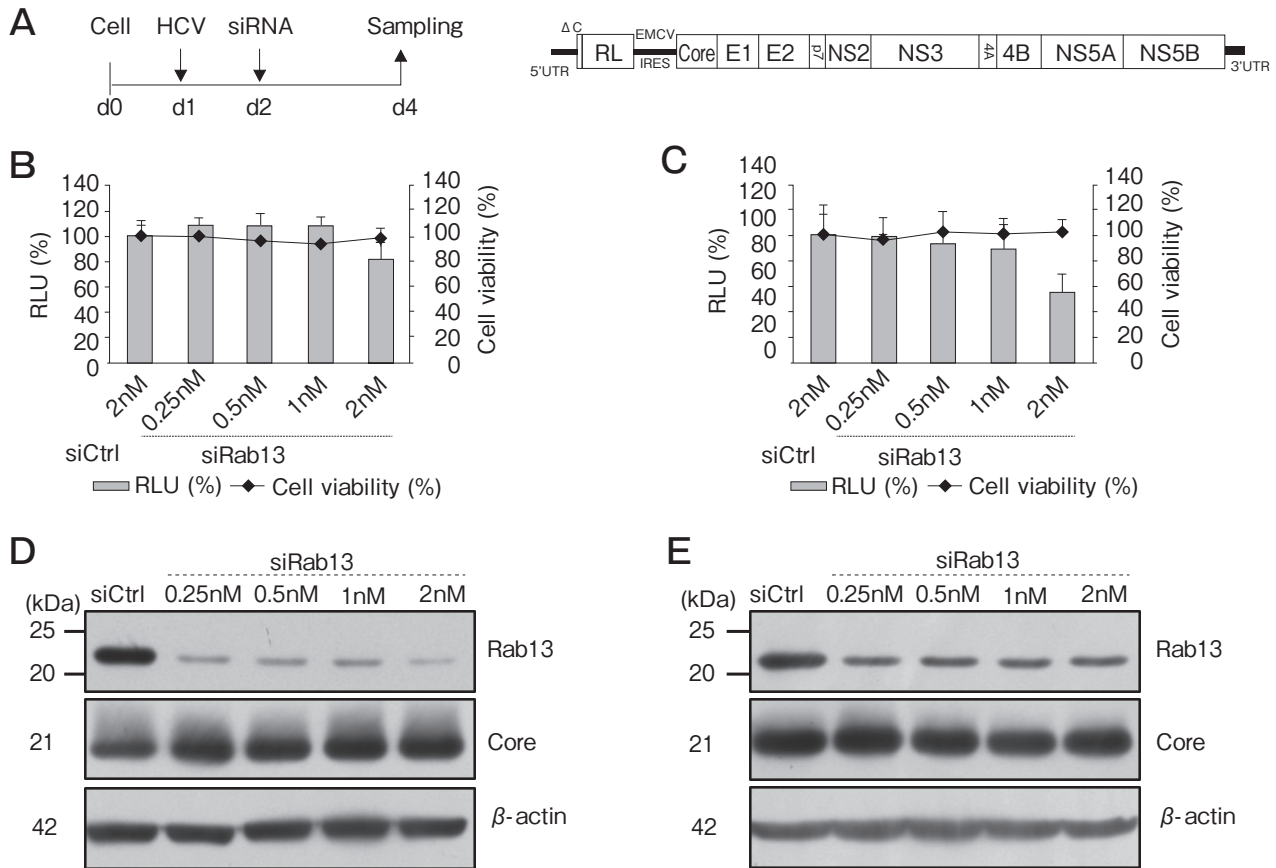


**Fig. 1** Rab13 is involved in HCV infection. **(A)** The experimental procedure used to identify the effect of Rab13 in the HCV entry step and the schematic gene organization of reporter HCV-JFH-1. The reporter HCV-JFH-1 contains the *RL* gene in the first cistron following the encephalomyocarditis virus-internal ribosomal entry site gene and the ORF of HCV-JFH-1 in the second cistron.  $\Delta C$ : 12 N-terminal amino acid residues of the Core. **(B)** RSC cells were transfected with siRNA (0.25, 0.5, 1, 2nM) 24h before HCV infection. The reporter HCV-JFH-1 was used as an inoculum after the removal of siRNA. The cells were infected with the reporter HCV-JFH-1 and cultured for 48h. The inhibition of HCV infection was assessed by relative RL activity (relative light units: RLU). The relative RL activity is shown as a percentage of the control value. The cell viability was determined by WST-1 assay. Bars: mean  $\pm$  standard deviation (SD) of triplicate data points. **(C)** D7 cells were transfected with siRNA (0.25, 0.5, 1, 2nM) 24h before HCV infection. The reporter HCV-JFH-1 was used as an inoculum after the removal of siRNA. The cells were infected with the reporter HCV-JFH-1 and cultured for 48h. The inhibition of HCV infection was assessed by RL activity. The cell viability was determined by WST-1 assay. **(D)** RSC cells were transfected with siRNA (0.25, 0.5, 1, 2nM) at 24h before HCV infection. The authentic HCV-JFH-1 in the supernatant was used as an inoculum. The cells were infected with HCV and cultured for 48h. The expressions of Rab13 and Core were analyzed by immunoblotting using anti-Rab13 and anti-Core antibodies.  $\beta$ -actin was used as a control for the amount of protein loaded per lane. **(E)** D7 cells were transfected with siRNA (0.25, 0.5, 1, 2nM) at 24h before HCV infection. The authentic HCV-JFH-1 in the supernatant was used as an inoculum. The cells were infected with HCV and cultured for 48h. The expressions of Rab13 and Core were analyzed by immunoblotting using anti-Rab13 and anti-Core antibodies.  $\beta$ -actin was used as a control for the amount of protein loaded per lane.

tein expression (Fig. 3D, E). These results revealed that Rab13 did not inhibit the replication of subgenomic HCV RNA.

**The downregulation of Rab13 inhibited HCV infection.** We observed that Rab13 is required for HCV entry in D7 cells, so we further examined whether Rab13 is required for HCV entry in D7 cells

by confocal microscopy. The D7 cells were transfected with siRNA and infected with HCV-JFH-1 for 48h. The Rab13 and Core were observed in siCtrl-treated cells (Fig. 4, middle panels). In contrast, the siRab13-treated cells decreased the Core (Fig. 4, lower panels). These results indicate that Rab13 is associated with the HCV infection step.



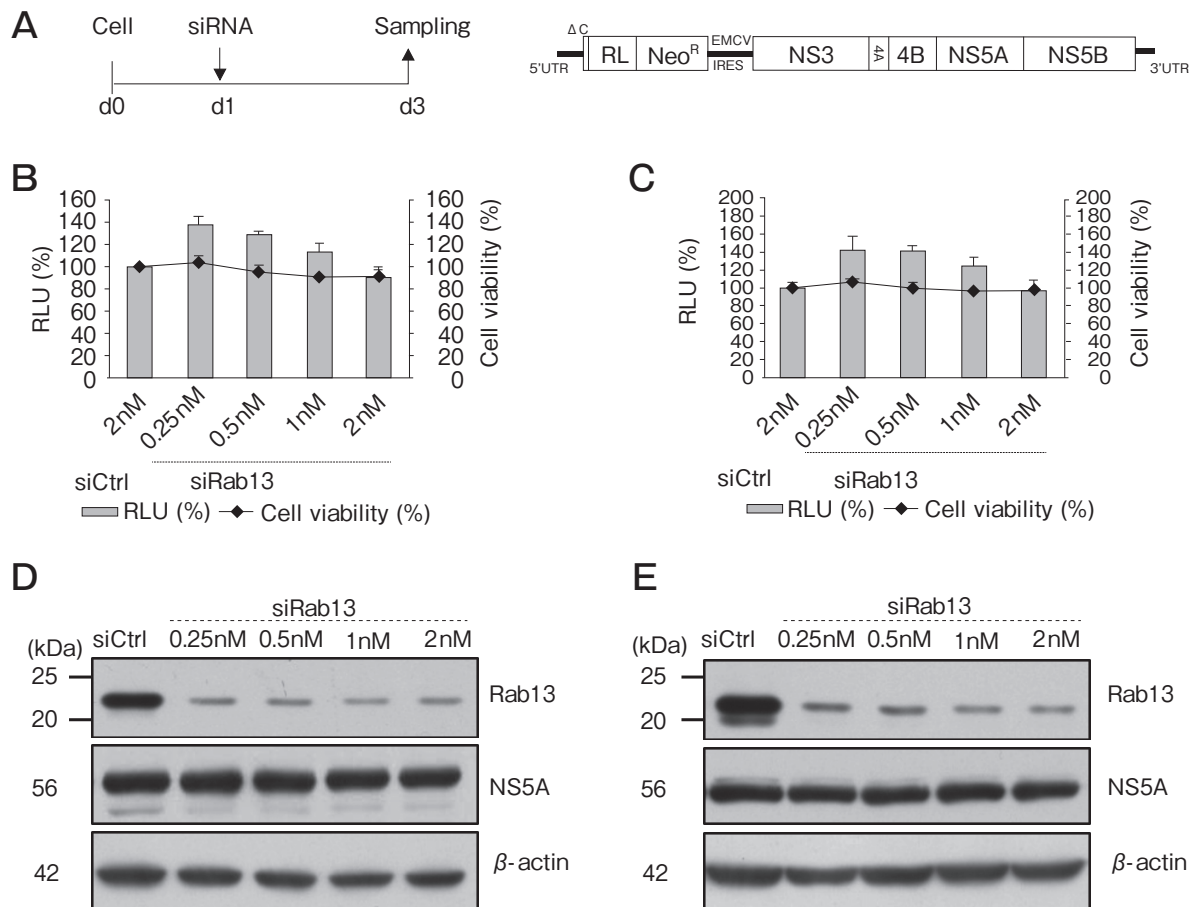
**Fig. 2** The suppression of Rab13 after HCV infection had no effect. **(A)** The experimental procedure for determining the effect of Rab13 in the HCV entry step and the gene organization of reporter HCV-JFH-1. **(B)** Rsc cells were infected with the reporter HCV-JFH-1 before the transfection of siRNA, and 24h after infection, the cells were transfected with siRNA (0.25, 0.5, 1, 2nM) and cultured for 48h. The inhibitory effect on HCV proliferation after infection was assessed by relative RL activity, which is shown as a percentage of the control. The cell viability was determined by WST-1 assay. Bars: the mean  $\pm$  SD of triplicate data points. **(C)** D7 cells were infected with the reporter HCV-JFH-1 before the transfection of siRNA and then cultured for 24h. After infection, the cells were transfected with siRNA (0.25, 0.5, 1, 2nM) and cultured for 48h. The inhibitory effect on HCV proliferation after infection was assessed by relative RL activity. The cell viability was determined by WST-1 assay. **(D)** Rsc cells were infected with authentic HCV-JFH-1 and cultured for 24h. After infection, the cells were transfected with siRNA (0.25, 0.5, 1, 2nM) and cultured for 48h. The expressions of Rab13 and Core were analyzed by immunoblotting using anti-Rab13 and anti-Core antibodies.  $\beta$ -actin was used as a control for the amount of protein loaded per lane. **(E)** D7 cells were infected with authentic HCV-JFH-1 virion and cultured for 24h. After infection, the cells were transfected with siRNA (0.25, 0.5, 1, 2nM) and cultured for 48h. The expressions of Rab13 and Core were analyzed by immunoblotting using anti-Rab13 and anti-Core antibodies.  $\beta$ -actin was used as a control for the amount of protein loaded per lane.

### Discussion

The results of the present study demonstrated that Rab13 is required for the HCV entry step in the HCV lifecycle. In particular, the knockdown of Rab13 significantly inhibited HCV infection and reduced the Core in D7 cells. Rab13 did not support the other steps.

It is likely that membrane transport participates in

the lifecycle of HCV. It was reported that Rab proteins are essential host factors for the HCV lifecycle, and that Rab proteins are important for HCV RNA replication and viral assembly [12–15]. However, we were unable to find any prior study of Rab regarding the HCV entry step. We focused here on Rab13, which regulates TJ assembly and the membrane traffic [16, 17]. The TJ plays a key role in HCV's entry into cells. Rab13 regulates the endocytic recycling of

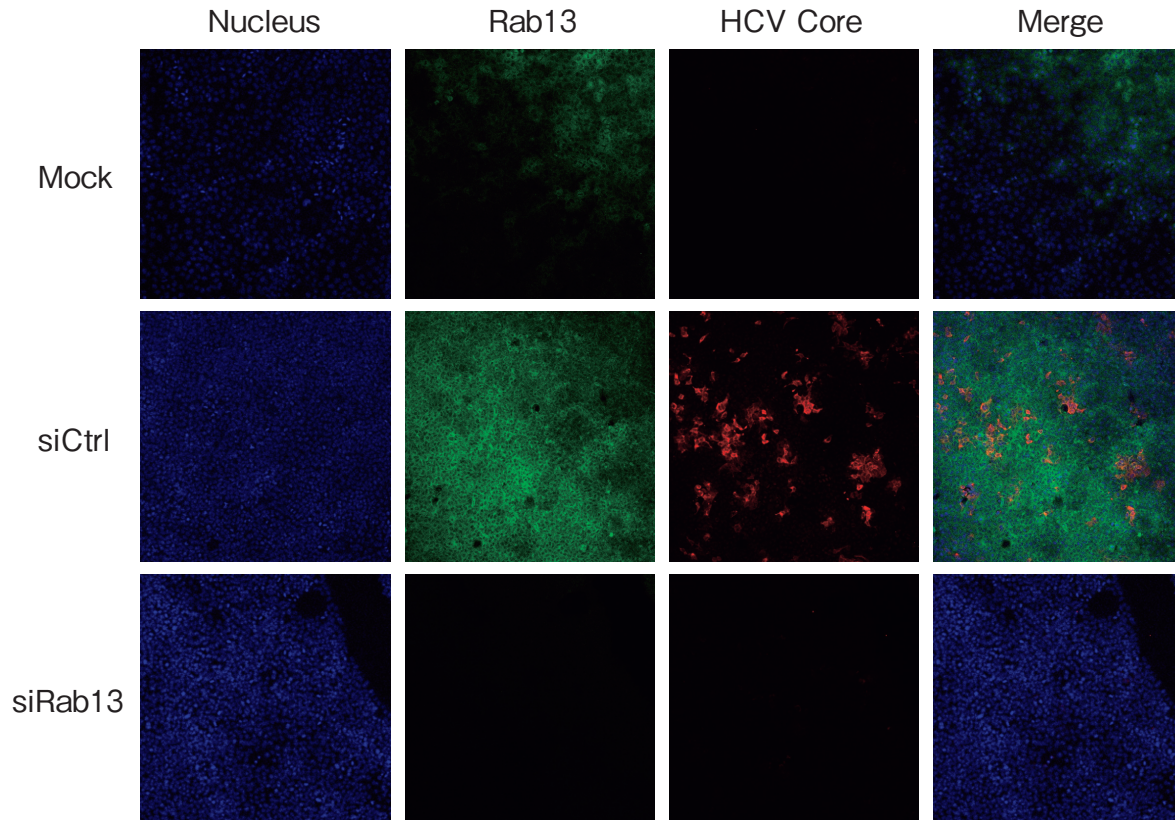


**Fig. 3** The suppression of Rab13 does not inhibit the replication of subgenomic HCV replicon. **(A)** The experimental procedure used to determine the effect of Rab13 on HCV RNA replication. The gene organization of subgenomic HCV replicon is described in the Materials and Methods section. **(B)** Subgenomic HCV-JFH-1 RNA (JRN/35B) replicating RSc cells were transfected with siRNA (0.25, 0.5, 1, 2nM) for 48h. The relative RL activity for HCV RNA replication is expressed as a percentage of the control. Bars: mean  $\pm$  SD of triplicate data points. **(C)** Subgenomic HCV-JFH-1 RNA (JRN/35B) replicating D7 cells were transfected with siRNA (0.25, 0.5, 1, 2nM) and cultured for 48h. The relative RL activity for HCV RNA replication is expressed as a percentage of control. **(D)** Subgenomic HCV-JFH-1 RNA (JRN/35B) replicating RSc cells were transfected with siRNA (0.25, 0.5, 1, 2nM) for 48h. The expressions of Rab13 and NS5A were analyzed by immunoblotting using anti-Rab13 and anti-NS5A antibodies.  $\beta$ -actin was used as a control for the amount of protein loaded per lane. **(E)** Subgenomic HCV-JFH-1 RNA (JRN/35B) replicating D7 cells were transfected with siRNA (0.25, 0.5, 1, 2nM) and cultured for 48h. The expressions of Rab13 and NS5A were analyzed by immunoblotting using anti-Rab13 and anti-NS5A antibodies.  $\beta$ -actin was used as a control for the amount of protein loaded per lane.

TJ-associated proteins, CLDN1 and OCLN, which are HCV entry receptors.

We first investigated whether the downregulation of Rab13 would inhibit HCV infection by using 2 distinct hepatoma cell lines, HuH-7 and Li23. The results indicated that the downregulation of Rab13 by siRNA inhibited HCV infection (Figs. 1, 4). Rab13 may be required for HCV infection, but it was not essential for HCV RNA replication (Figs. 2, 3).

The siRab13 treatment in the Li23-derived D7 cells had a greater inhibitory effect against HCV infection compared to that in the HuH-7-derived RSc cells. We reported differential gene expressions between Li23 and HuH-7 cells [22]. For example, the expression of Rab escort proteins (REPs) is higher in Li23 cells than in HuH-7 cells. REPs are one of the host components for the geranylgeranylation of Rabs. The REP consists of REP-1 and REP-2, and interestingly,



**Fig. 4** Rab13 is required for HCV infection. D7 cells were transfected with siCtrl (middle panels) or siRab13 (lower panels) (100 nM) at 24 h before HCV infection. The cells were mock-infected (upper panels) or infected with authentic HCV-JFH-1 at a MOI of 2 (middle and lower panels) and cultured for 48 h. The cells were then stained with HCV Core (red) and Rab13 (green) antibody and observed by confocal microscopy.

REP-1 is expressed in the Li23 cell line but not in the HuH-7 cell line (unpublished data). The lack of the REP-1 may affect the different inhibitory effects against HCV infection with the siRab13 treatment between the 2 cell lines.

We reported that the OCLN expression in Li23-derived D7 cells is two times higher than in HuH-7-derived RSc cells and that the CLDN1 expression in HuH-7-derived RSc cells is 1.5 times higher than in Li23-derived D7 cells [19]. The different expression balance may affect the HCV infection via Rab13. The different expressions of unknown host factors regarding Rab modification and HCV proliferation may have influenced the results of the present experiments with siRab13. To clarify this issue, further studies are needed.

Recent studies show that HCV entry requires several host factors. The TJ-associated proteins

CLDN1 and OCLN go and return between the TJs and the cytoplasm. These proteins do not interact with the HCV particles. CLDN1 and OCLN play roles in the post-attachment step by interacting with HCV E2-CD81 [23, 24]. The inhibition of HCV infection by the downregulation of Rab13 is thus not due to the direct interaction with the HCV particles.

It was recently reported that protein kinase A (PKA) signaling was required for HCV entry. PKA signaling promoted the CD81-CLDN1 interaction in TJ. Inhibition of PKA activity induced a reorganization of CLDN1 from TJs to the cytoplasm [25]. In another study, Rab13 interacted with PKA and was implicated in TJ assembly [26]. The active-form Rab13-GTP bound to PKA and inhibited phosphorylation, resulting in the localization of CLDN1 in the cytoplasm, whereas the inactive-form Rab13-GDP did not bind to PKA, resulting in recycling CLDN1 to the

TJ. Collectively, these results raise the possibility that the inhibition of HCV infection by the down-regulation of Rab13 is related to PKA signaling.

In conclusion, our present findings demonstrate that Rab13 is involved in the HCV entry step.

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