mRNAs in response to O cell RNA and polyI:C (Fig. 2C, 2D). IPS-1 KO severely reduced IFN-λ2/3 mRNA expression in BM-DCs and BM-Mfs in response to O cell RNA (Fig. 2C). These results indicated that IPS-1 in BM-DCs and BM-Mfs plays a crucial role in IFN-λ2/3 mRNA expression in response to cytoplasmic HCV RNA.

Mice have $CD4^+$, $CD8^+$, and DN DCs. Thus, we next examined the IFN-β and -λ2/3 mRNA expression in these mouse DC subsets. As seen with BM-DCs, the mouse DCs expressed IFN-β and -λ2/3 mRNA in response to polyI:C but not O cell RNA in the culture medium, whereas stimulation with polyI:C or O cell RNA by transfection strongly induced their expression (Fig. 3A, 3B). Interestingly, $CD8^+$ DCs highly expressed IFN-λ2/3 mRNA in response to stimulation with polyI:C or O cell RNA by transfection compared with $CD4^+$ and DN DCs (Fig. 3A, 3B), and IPS-1 KO but not TICAM-1 KO severely reduced IFN-λ2/3 expression in $CD8^+$ DCs in response to O cell RNA transfection (Fig. 3C, 3D). This indicated that IPS-1 was essential for IFN-λ2/3 mRNA expression in $CD8^+$ DCs in response to cytoplasmic HCV RNA.

It was recently reported that exosomes mediate cell-to-cell transfer of HCV RNA from infected cells to cocultured DCs (27). We examined the production of IFN-β and -λ2/3 by $CD8^+$ DCs that were cocultured with O cells and Oc cells. Coculture with O cells but not Oc cells induced IFN-β and -λ2/3 production by $CD8^+$ DCs (Fig. 4A, 4B). Interestingly, TICAM-1 KO abolished IFN-λ2/3 mRNA expression and protein production, whereas IPS-1 KO failed to reduce IFN-λ2/3 mRNA expression and protein production in $CD8^+$ DCs (Fig. 4C, 4D). This suggested that TICAM-1 but not IPS-1 was essential for IFN-λ2/3 production by $CD8^+$ DCs when cocultured with hepatocytes with HCV replicons.

Type III IFN increases RIG-I expression in $CD8^+$ DC

The receptor for type III IFN consists of IL-10RB and IL-28Rα subunits (8). DN and $CD4^+$ DCs and NK cells did not express IL-28Rα mRNA, whereas $CD8^+$ DCs expressed both IL-10RB and IL-28Rα mRNAs (Fig. 5A). Thus, we investigated the effects of IFN-λ on DC function.

First, we examined DC cell surface markers. Unlike IFN-α, IFN-λ3 hardly increased CD40, 80, and 86 surface marker expressions on $CD8^+$ DCs (Fig. 5B). Second, we examined the effects of IFN-λ3 on cross-priming because $CD8^+$ DCs have high cross-priming capability. OVA, IFN-α, and/or IFN-λ3 were i.p. injected into mice according to the indicated schedules (Fig. 5C). Seven days after injection, OVA (SL8)-specific $CD8^+$ T cells in spleens were quantified by tetramer staining. For a positive control, OVA and polyI:C were i.p. injected into mice. The results showed that IFN-λ3 failed to increase OVA-specific $CD8^+$ T cells in the spleens and suggested that IFN-λ3 failed to promote cross-priming at least in our experimental condition (Fig. 5C).

Third, we examined NK cell activation by DCs. NK cells and DCs were isolated from mouse spleens and were cocultured for 24 h in the presence of IFN-α, λ3, or polyI:C. Although IFN-γ production was increased by IFN-α stimulation, IFN-λ3 failed to increase IFN-γ production (Fig. 5D). Next, we investigated a cell surface marker for NK cells when cocultured with DCs. The expression of CD69, a NK cell activation marker, was not increased by IFN-λ3 stimulation (Fig. 5E). These results indicated that, unlike IFN-α, IFN-λ3 failed to enhance the activation of NK cells by DCs.

Fourth, we investigated the expression of antiviral genes in $CD8^+$ DCs in response to IFN-λ3 stimulation. Interestingly, IFN-λ3 stimulation increased RIG-I and Mx1 but not TLR3 mRNA expression in $CD8^+$ DCs (Fig. 6A). In addition, pretreatment with IFN-λ3 augmented IFN-λ2/3 mRNA expression in $CD8^+$ DCs in response to HCV RNA (Fig. 6B). Taken together, type III IFN induced RIG-I and antiviral protein expression but failed to promote DC-mediated NK cell activation and cross-priming.

Hepatocytes express type III IFN receptors. Thus, we examined the effects of IFN-λ on mouse hepatocytes. As with IFN-α, IFN-λ3 stimulation induced both TLR3 and RIG-I mRNA expression in mouse hepatocyte (Fig. 6C). Antiviral nucleases, ISG20 and RNaseL, and an IFN-inducible gene, Mx1, were induced by IFN-λ3 or IFN-α treatment (Fig. 6C). Pretreating mouse hepatocytes with IFN-λ3 enhanced IFN-β and -λ2/3 mRNA expression in response to stimulation with HCV RNA by transfection (Fig. 6D). These results indicated that IFN-λ3 induced cytoplasmic antivirus protein expression in mouse hepatocytes. We confirmed that IFN-λ3 treatment significantly reduced HCV RNA levels in O cells with HCV replicons (Fig. 6E). A previous study also reported that IFN-λ inhibits HCV replication (13).

Discussion

Previous studies have established the importance of the TLR3 pathway for type III IFN production in response to polyI:C (15) or HCV (17). In this study, we established the importance of IPS-1–dependent pathway for type III IFN production in response to cytoplasmic HCV RNA in vivo and in vitro using a mouse model. These data indicated that there are at least two main pathways for type III IFN production in vivo, as follows: one is TICAM-1 dependent, and the other is IPS-1 dependent.

We revealed that IFN-λ was efficiently produced by $CD8^+$ DCs, the mouse counterpart of human $BDCA3^+$ DCs, in response to

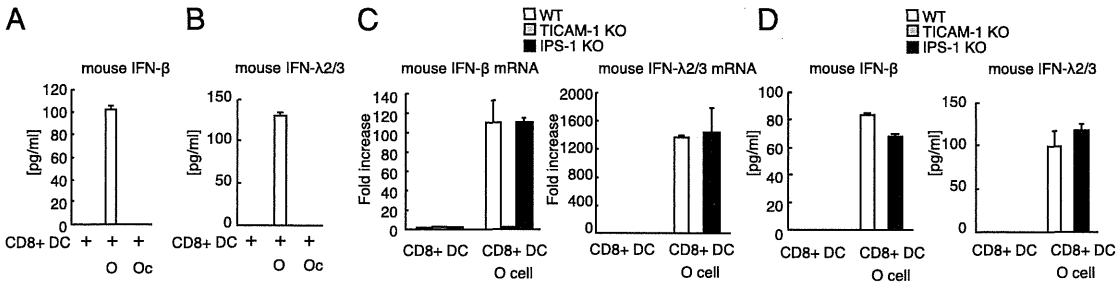

FIGURE 4. IFN-β and –λ production by $CD8^+$ DCs cocultured with hepatocytes with HCV replicons. (**A** and **B**) $CD8^+$ DCs isolated from wild-type spleens were cocultured with O cells (with HCV replicons) or Oc cells (without HCV replicons). After 24 h of coculture, IFN-β (A) and –λ2/3 (B) concentrations in culture medium were determined by ELISA. (**C**) $CD8^+$ DCs isolated from wild-type, TICAM-1 KO, or IPS-1 KO spleens were cocultured with O cells with HCV replicons for six hours, and then IFN-β and –λ2/3 mRNA expression was determined by RT-qPCR. (**D**) $CD8^+$ DCs isolated from wild-type, TICAM-1 KO, or IPS-1 KO spleens were cocultured with O cells with HCV replicons. IFN-β and –λ2/3 concentrations in culture medium were determined by ELISA.

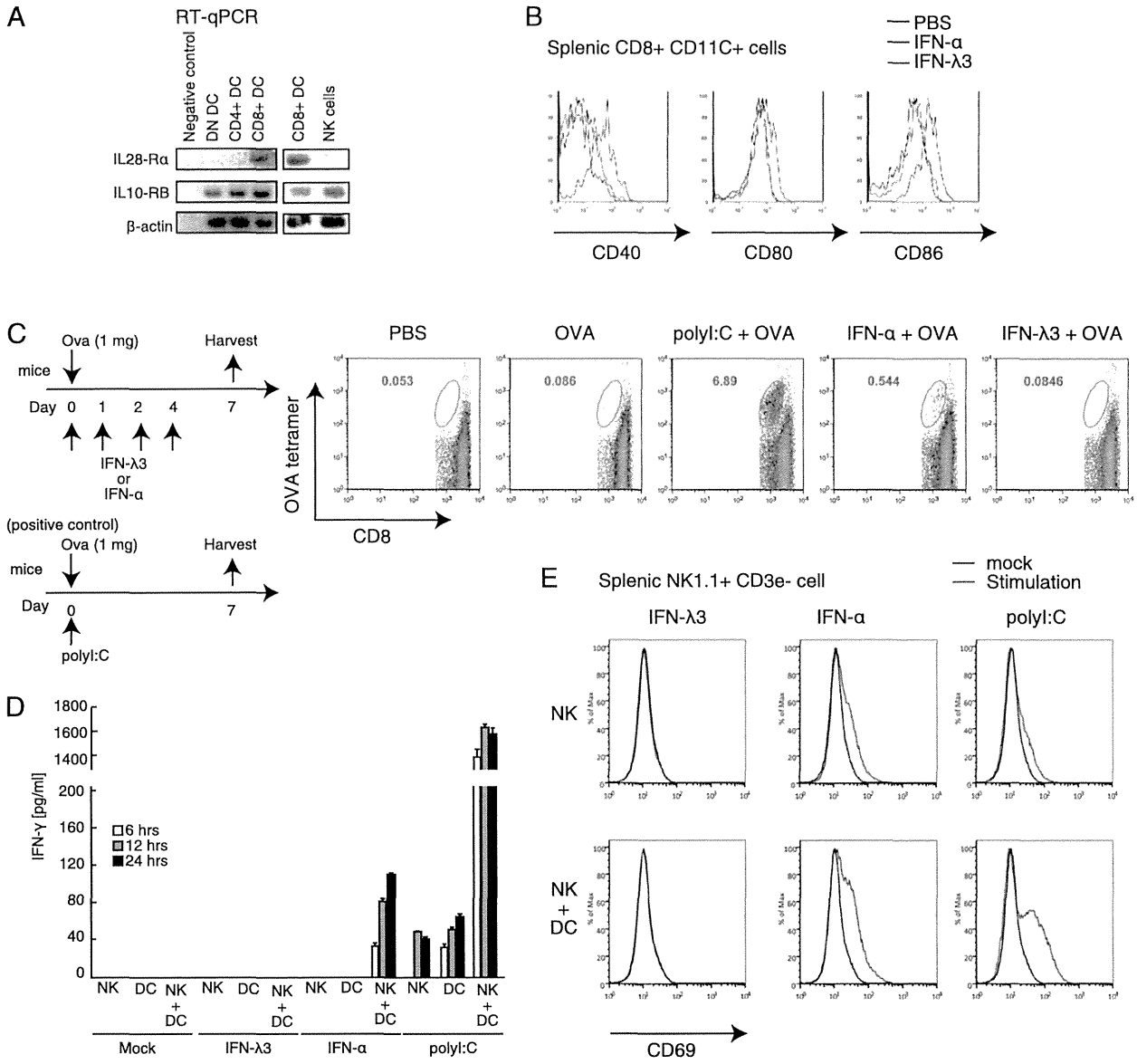

FIGURE 5. IFN-λ effects on DC functions. (**A**) DN, $CD4^+$, $CD8^+$ DCs, and NK cells were isolated from wild-type mouse spleens. IL-28Rα and IL-10RB mRNA were determined by RT-PCR. (**B**) A total of 0.5 μg IFN-λ3 or 1×10^5 IU IFN-α was i.p. injected into mice. Six hours after injection, spleen $CD8^+$ DCs were isolated, and cell surface expressions of CD40, 80, and 86 were determined by FACS analysis. (**C**) OVA and IFN-λ or IFN-α were i.p. injected into mice on day 0, and then IFN-λ or IFN-α was injected into mice on days 1, 2, and 4. Spleens were excised on day 7, and OVA (SL8)-specific $CD8^+$ T cells were determined by a tetramer assay. For a negative control, PBS in place of IFN was injected on days 0, 1, 2, and 4. For a positive control, polyI:C and OVA were injected into mice on day 0. (**D**) NK cells and $CD11c^+$ DCs were isolated from mouse spleens and then stimulated with 1000 U/ml IFN-α, 100 ng/ml IFN-λ3, or 100 μg/ml polyI:C. IFN-γ concentrations in the culture medium at the indicated times were determined by ELISA. (**E**) NK cells were isolated from mouse spleens and then cultured with or without spleen $CD11c^+$ DCs. Cells were stimulated with 1000 U/ml IFN-α, 100 ng/ml IFN-λ3, or 20 μg polyI:C. CD69 expression on NK cells was determined by FACS analysis.

cytoplasmic HCV RNA. Moreover, our data showed that IFN-λ stimulation increased the mRNA expression of RIG-I but not that of TLR3 in $CD8^+$ DCs, and $CD8^+$ DCs required IPS-1 to produce IFN-λ in response to stimulation with cytoplasmic HCV RNA. Furthermore, IFN-λ enhanced the mRNA expression of IFN-λ itself in $CD8^+$ DCs, which suggested a positive feedback loop for IFN-λ mRNA expression in $CD8^+$ DCs. IFN-λ failed to promote DC-mediated NK activation or cross-priming at least in our experimental conditions, whereas antiviral proteins, such as ISG20 and RNaseL, were efficiently induced by IFN-λ stimulation in hepatocytes and $CD8^+$ DCs. These results established a novel role of IPS-1 in innate immune response against HCV via IFN-λ production. IFN-λ pretreatment markedly increased IFN-β mRNA expression in response to HCV RNAs in mouse hepatocyte but not in $CD8^+$ DCs (Fig. 6B, 6D). Although the underlying mechanism is unclear, it is possible that there is a cell-type–specific role of IFN-λ.

It was recently reported that $BDCA3^+$ DCs require TLR3 for type III IFN production in response to cell-cultured HCV (17). They used a HCV 2a JFH1 strain that cannot infect human DCs in vitro (5). We also showed that the TLR3 adaptor TICAM-1 was essential for type III IFN production by $CD8^+$ DCs when cocultured with O cells with HCV replicons. Thus, TLR3 appears to be essential for type III IFN production by DCs that are not infected with HCV. It

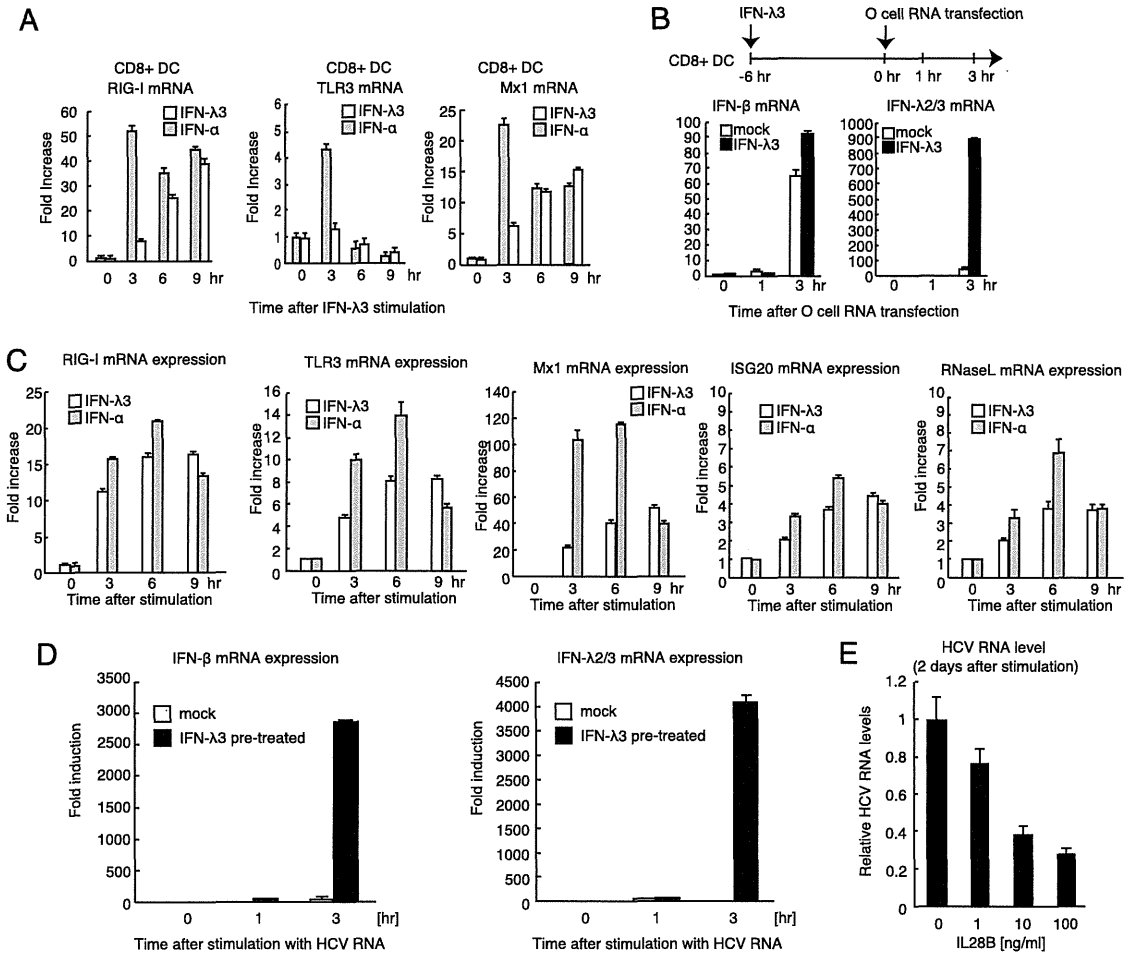

FIGURE 6. Antiviral responses induced by IFN-λ. (**A**) Mouse spleen CD8$^+$ DCs were stimulated with 100 ng/ml IFN-λ3 or 1000 IU/ml IFN-α, after which RIG-I, TLR3, and Mx1 mRNA levels were determined by quantitative RT-PCR. (**B**) Mouse spleen CD8$^+$ DCs were treated with 100 ng/ml IFN-λ3 for 6 h. O cell RNA was transfected into CD8$^+$ DCs, and IFN-β and -λ2/3 mRNA levels were determined by quantitative RT-PCR at the indicated times. (**C**) Mouse hepatocyte cell line cells were stimulated with 1000 U/ml IFN-α or 100 ng/ml IFN-λ3. RIG-I, TLR3, Mx1, ISG20, and RNaseL mRNA levels were determined by quantitative RT-PCR. (**D**) Mouse hepatocyte cell line cells were treated with 100 ng/ml IFN-λ3 for 6 h, and then O cell RNA was transfected into these cells. IFN-β and -λ2/3 mRNA levels were measured by quantitative RT-PCR at the indicated times. (**E**) O cells that contain HCV 1b full-length replicons were treated with human IL-28B at indicated concentration for 2 d. HCV RNA levels were determined by quantitative RT-PCR. HCV RNA levels were normalized to GAPDH mRNA expression.

has been shown that exosomes are internalized efficiently by DCs and sorted into early endosomes, where TLR3 is localized (28, 29). Unlike the transfected HCV RNA, exosome-enclosed HCV RNA might be efficiently sorted and released within early endosomes of CD8$^+$ DC, where TLR3 is localized, leading to TLR3-dependent IFN-λ2/3 production. Although HCV JFH1 infection particles fail to infect DCs in vitro, previous studies indicated that HCV infects DCs in chronically infected patients (23, 24, 30). In human patient DCs and hepatocytes infected with HCV, the IPS-1 pathway could play a pivotal role in type III IFN production.

Knockout of TICAM-1 failed to reduce IFN-λ2/3 mRNA expression in mouse liver after HCV RNA hydrodynamic injection, whereas knockout of TICAM-1 abolished IFN-λ2/3 levels in sera after HCV RNA hydrodynamic injection (Fig. 1B, 1D). Considering that there is a positive feedback loop for IFN-λ production, it is possible that TICAM-1 and IPS-1 pathways augment IFN-λ production each other in vivo; however, we do not exclude a possibility that TICAM-1 is involved in posttranscriptional step of IFN-λ production.

HCV NS3-4A protease cleaves IPS-1 to suppress host innate immune responses (31, 32). However, it is notable that a mutation within the *RIG-I* gene in HuH7.5 cells increases cellular permissiveness to HCV infection (33). This indicates that the RIG-I pathway is functional at least during the early phase of HCV infection before NS3-4A cleaves IPS-1. Thus, we propose that IPS-1 is important for type III IFN production during the early phase of HCV infection.

IFN-α augmented DC-mediated NK cell activation and cross-priming, whereas IFN-λ failed to augment DC-mediated NK cell activation and cross-priming in our experimental conditions. However, as seen with IFN-α, IFN-λ could induce RNaseL and ISG20 mRNA expression. These data indicated that IFN-λ induces cytoplasmic antiviral proteins to eliminate infected virus. A previous study showed that IPS-1 is required for initial antiviral response but dispensable for the protective adaptive immune response to influenza A virus (34). Thus, it is expected that IPS-1–mediated IFN-λ production would be required for initial antiviral response to HCV infection.

In summary, our results provide insights into type III IFN production mechanism in response to HCV RNA in vivo and identify IPS-1 as a molecule crucial for producing type III IFN from hepatocyte and CD8$^+$ DCs in response to cytoplasmic HCV RNA.

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Disclosures

The authors have no financial conflicts of interest.

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Anti-HCV activity of the Chinese medicinal fungus *Cordyceps militaris*

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ABSTRACT

Persistent hepatitis C virus (HCV) infection causes chronic liver diseases and is a global health problem. Although the sustained virologic response rate in the treatment of genotype 1 using new triple therapy (pegylated-interferon, ribavirin, and telaprevir/boceprevir) has been improved by more than 70%, several severe side effects such as skin rash/ageusia and advanced anemia have become a problem. Under these circumstances, a new type of anti-HCV oral drug with few side effects is needed. Our recently developed HCV drug assay systems, including the HuH-7 cell line-derived OR6 and AH1R, and the Li23 cell line-derived ORL8 and ORL11, allow genome-length HCV RNAs (several strains of genotype 1b) encoding renilla luciferase to replicate efficiently. Using these systems as anti-HCV candidates, we have identified numerous existing medicines that can be used against HCV with few side effects, such as statins and teprenon. To obtain additional anti-HCV candidates, we evaluated a number of oral health supplements, and found that the capsule but not the liquid form of *Cordyceps militaris* (CM) (*Ascomycotinanorth, North Chinese caterpillar fungus*), which is used as a Chinese herbal medicine, exhibited moderate anti-HCV activity. In combination with interferon-α or ribavirin, CM exhibited an additive inhibitory effect. Among the main components of CM, cordycepin, but not ergosterol, contributed to the anti-HCV activity of CM. In consideration of all these results, we suggest that CM would be useful as an oral anti-HCV agent in combination with interferon-α and/or ribavirin.

1. Introduction

Hepatitis C virus (HCV) infection frequently causes chronic hepatitis, which often leads to liver cirrhosis and hepatocellular carcinoma. Since approximately 170 million people are infected with HCV worldwide, HCV infection is a serious global health problem [1]. HCV is an enveloped virus with a positive single-stranded RNA of the *Flaviviridae* family. The HCV genome encodes a large polyprotein precursor of approximately 3000 amino acids, which is cleaved into 10 proteins in the following order: Core, envelope 1 (E1), E2, p7, non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B [2,3].

Recently, a new therapy for hepatitis C (genotype 1) with a combination of pegylated-interferon (PEG-IFN), ribavirin (RBV), and telaprevir/boceprevir (inhibitor of HCV NS3-4A protease) has been started as a global standard therapy [4]. Although the sustained virological response (SVR) in this therapy has improved approximately 70–80% [5], this therapy has several problems, such as severe side effects (skin rash, ageusia, advanced anemia, etc.), emergence of resistant viruses, and high treatment cost [5,6].

Although cells derived from the human hepatoma cell line HuH-7 have been used as the preferred culture system for the study of HCV life cycles and for the development of anti-HCV drugs [7], we previously found a new human hepatoma cell line, Li23, that enables reproducibility of the HCV life cycle [8]. Using the Li23 cell line, we developed Li23-derived drug assay systems (ORL8 and ORL11) in which a genome-length HCV RNA (the O strain of genotype 1b derived from an HCV-positive healthy carrier) encoding renilla luciferase (RL) replicates efficiently [8], based on a method previously reported in the development of a HuH-7-derived drug assay system (OR6) [9]. Since we demonstrated that the gene expression profile of Li23 cells was distinct from that in HuH-7 cells [10], and that the anti-HCV targets in Li23-derived cells (ORL8 and ORL11) were distinct from those in HuH-7-derived cells (OR6 and AH1R, which was developed using the AH1 strain of genotype 1b) [11–14], we considered that we might find a new type of anti-HCV agent by conducting a search using these two kinds of cell-based HCV RNA-replication assay systems. Indeed, we recently found that the preclinical antimalarial drugs N-89 and N-251 [15,16] exhibited potent anti-HCV activities [17].

Here, we report the further discovery that an oral health supplement used as a Chinese herbal medicine, *Cordyceps militaris*

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(CM) (*Ascomycotinanorth, North Chinese caterpillar fungus*), exhibited moderate anti-HCV activity.

2. Materials and methods

2.1. Cell cultures

HuH-7-derived OR6 [9] and AH1R [12] cells harboring genome-length HCV RNA encoding RL and HuH-7-derived polyclonal sOR [18] cells harboring subgenomic HCV replicon RNA encoding RL were cultured in the medium used for HuH-7 cells in the presence of G418 (0.3 mg/ml; Geneticin, Invitrogen, Carlsbad, CA) as described previously [17]. Li23-derived ORL8 [8] cells harboring genome-length HCV RNA encoding RL and Li23-derived polyclonal sORL8 [8] cells harboring subgenomic HCV replicon RNA encoding RL were also cultured in the medium used for Li23 cells in the presence of G418 (0.3 mg/ml) as described previously [8].

2.2. Reagents

The capsule and liquid forms of CM were purchased from CAITAC (Okayama, Japan). RBV was kindly provided by Yamasa (Chiba, Japan). Human IFN-α and vitamin E (VE) were purchased from Sigma-Aldrich (St. Louis, MO). Cordycepin was purchased from Wako (Osaka, Japan). Ergosterol and cyclosporine A (CsA) were purchased from Tokyo Chemical Industry (Tokyo, Japan).

2.3. RL assay

The RL assay was performed as described previously [8,14]. Briefly, the cells were plated onto 24-well plates (2×10^4 cells per well) in triplicate and then treated with each reagent at several concentrations for 72 h. After treatment, the cells were subjected to luciferase assay using an RL assay system (Promega, Madison, WI). From the assay results, the 50% effective concentration (EC_{50}) of each reagent was determined.

2.4. WST-1 cell proliferation assay

The WST-1 cell proliferation assay was performed as described previously [14]. Briefly, the cells were plated onto 96-well plates (1×10^3 cells per well) in triplicate and then treated with each reagent at several concentrations for 72 h. After treatment, the cells were subjected to the WST-1 cell proliferation assay (Takara Bio, Otsu, Japan) according to the manufacturer's protocol. From the assay results, the 50% cytotoxic concentration (CC_{50}) of each reagent was determined.

2.5. Western blot analysis

The preparation of cell lysates, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and immunoblotting analysis were performed as described previously [19]. The antibodies used in this study were those against HCV Core (CP11; Institute of Immunology, Tokyo, Japan), NS5B (a generous gift from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science, Japan), and β-actin (AC-15; Sigma–Aldrich) as the control for the amount of protein loaded per lane.

2.6. Selective index (SI)

The SI value of each reagent was determined by dividing the CC_{50} value by the EC_{50} value.

2.7. Statistical analysis

Determination of the significance of differences among groups was assessed using the Student's *t*-test. Values of $P < 0.05$ were considered significant.

3. Results

3.1. The capsule form of CM, used as an oral health supplement, showed anti-HCV activity in both HuH-7- and Li23-derived HCV RNA-replicating cells

During the course of evaluating various oral health supplements for their anti-HCV activities using our previously developed HuH-7- and Li23-derived HCV assay systems, there was an opportunity to evaluate CM known as one of the Chinese herbal medicine. We first evaluated the anti-HCV activities of the capsule and liquid forms of CM using HuH-7-derived OR6 and AH1R assay systems and an Li23-derived ORL8 assay system, all of which enable monitoring of the replication of genome-length HCV RNA. The results revealed that the capsule form but not the liquid form of CM possessed moderate anti-HCV activities in all assay systems (Fig. 1A and B). The EC_{50} and SI values of the capsule form of CM were calculated in each assay system (EC_{50} 62 μg/ml, SI 1.9 in the OR6 assay; EC_{50} 54 μg/ml, SI >5.6 in the ORL8 assay; EC_{50} 31 μg/ml, SI 5.2 in the AH1R assay) (Table 1). The anti-HCV activities of the capsule form of CM found in the OR6, ORL8, and AH1R assays were confirmed by Western blot analysis of the HCV Core and NS5B (Fig. 1C). We next examined the activities of the capsule form of CM using HuH-7-derived polyclonal sOR and Li23-derived polyclonal sORL8 assay systems that enable monitoring of the replication of HCV subgenomic replicon RNA. These assays also showed that the capsule form of CM possessed anti-HCV activity with EC_{50} values less than those in the OR6 and ORL8 assays (Supplementary Fig. S1 and Table 1). Taken together, these results indicate that the anti-HCV activity of CM is not dependent on the specific cloned cell line, HCV strain, or HCV structural proteins.

3.2. Additive effect of the anti-HCV activities of CM in combination with IFN-α or RBV

To determine the intake effect of CM in the current HCV treatment, we examined the anti-HCV activity of the capsule form of CM in combination with IFN-α or RBV using an Li23-derived ORL8 assay system. The results revealed that the anti-HCV effects of CM plus IFN-α or RBV were additive (Fig. 2A and B). Although we observed that the anti-HCV activities of CM in combination with 4 IU/ml of IFN-α or 25 μM of RBV were greater than the expected sum of the constituent activities, these differences were not statistically significant (Fig. 2A and B). Therefore, these results suggest that the anti-HCV effects of CM do not interfere with those of IFN-α and RBV, and in fact may even augment them.

3.3. Cordycepin, but not ergsterol, is responsible for the anti-HCV activity of CM

We next examined which component of CM is responsible for the anti-HCV activity. The Japanese Food Research Laboratories (Tokyo, Japan) have reported that the main components of CM are as follows: β-glucan 8.40 g, cordycepin 4.95 g, mannitol 4.52 g, ergosterol 0.75 g, superoxide dismutase 860,000 U, copper 2.13 mg, zinc 17.1 mg, and selenium 80 μg per 100 g of CM (http://www.caitac.co.jp/matsubaratouchukasou/syouhin.html). From this information, we speculated that cordycepin or ergosterol might have anti-HCV activity (Fig. 3A). Thus, we evaluated

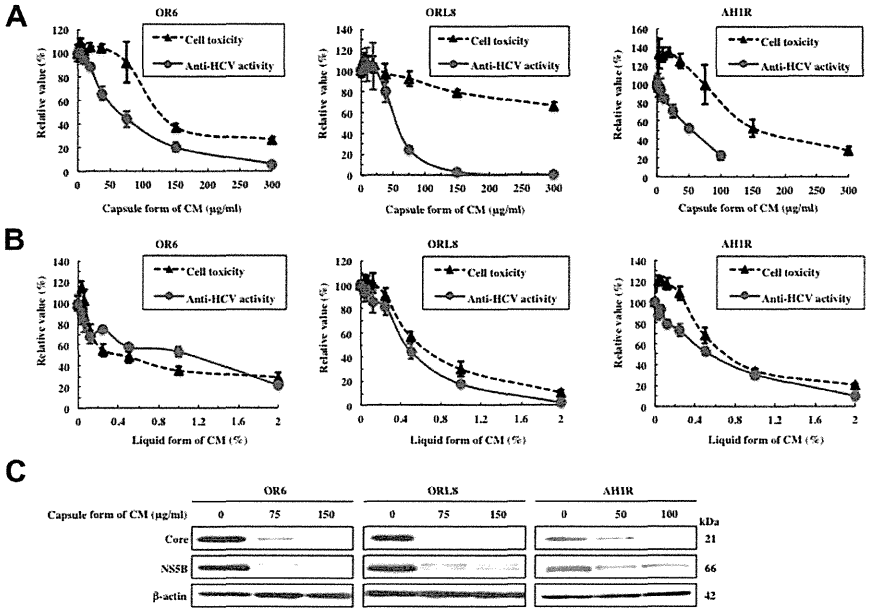

Fig. 1. Anti-HCV activities of the capsule form of CM detected in the OR6, ORL8, and AH1R assays. (A) Effects of the capsule form of CM on genome-length HCV RNA replication. OR6, ORL8, and AH1R cells were treated with the capsule form of CM for 72 h, followed by RL assay (red circles) and WST-1 assay (black triangles). The relative value (%) calculated at each point, when the level in non-treated cells was assigned as 100%, is presented here. Data are expressed as the means ± standard deviation of triplicate assays. (B) The liquid form of CM did not inhibit the genome-length HCV RNA replication. The RL and WST-1 assay were performed as described in (A). (C) Western blot analysis of the cells treated with the capsule form of CM. HCV Core and NS5B were detected using anti-core and anti-NS5B antibodies. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Anti-HCV activities of 4 reagents evaluated in this study.

Assay system	Genome-length HCV RNA									HCV subgenomic replicon					
Cell origin	OR6 HuH-7			ORL8 Li23			AH1R HuH-7			sOR HuH-7			sORL8 Li23		
HCV strain	O			O			AH1			O			O		
Compound (concentration)	EC_{50}	CC_{50}	SI	EC_{50}	CC_{50}	SI	EC_{50}	CC_{50}	SI	EC_{50}	CC_{50}	SI	EC_{50}	CC_{50}	SI
Capsule form of CM (μg/ml)	62	120	1.9	54	>300	>5.6	31	160	5.2	12	45	3.8	30	120	4.0
Liquid form of CM (%)	1.1	0.44	0.40	0.54	0.70	1.3	0.45	0.59	1.3	ND			ND		
Cordycepin (μg/ml)	2.6	3.5	1.3	3.8	3.6	0.95	0.58	1.9	3.3	1.7	3.0	1.8	21	19	0.90
Ergosterol (μg/ml)	>4.0	>4.0	<1.0	>4.0	>4.0	<1.0	>4.0	>4.0	<1.0	>4.0	>4.0	<1.0	>4.0	>4.0	<1.0

ND, not determined.

cordycepin and ergosterol using HuH-7-derived OR6 and AH1R assay systems. The results of both assays revealed that cordycepin, but not ergosterol, possessed anti-HCV activity (Fig. 3B and C). The EC_{50} and SI values of cordycepin were calculated in each assay (EC_{50} 2.6 μg/ml, SI 1.3 in the OR6 assay; EC_{50} 0.58 μg/ml, SI 3.3 in the AH1R assay) (Table 1). If all of the anti-HCV activity of CM was attributable to cordycepin (4.95% of content), the EC_{50} values, 62 and 31 μg/ml of CM, obtained by OR6 assay and AH1R assay would correspond to 3.0 and 1.6 μg/ml of cordycepin, respectively. Therefore, these results suggest that cordycepin is an integral component for the anti-HCV activity of CM. However, we were not able to confirm the anti-HCV activity of cordycepin in the Li23-derived ORL8 or sORL8 assay, although we did detect anti-HCV activity of cordycepin in the HuH-7-derived sOR assay (Supplementary Fig. S2 and Table 1). Ergosterol did not exhibit any anti-HCV activities in these assays (Supplementary Fig. S3 and Table 1). Taken together, these results suggest that cordycepin is a responsible compound for the anti-HCV activity of CM, although the anti-HCV activity of cordycepin may depend on the cell strain used in the assay, unlike the anti-HCV activity of CM.

4. Discussion

In the present study, using cell-based HCV RNA-replication assay systems, we found that CM, an oral health supplement, possessed moderate anti-HCV activity, and showed an additive inhibitory effect in combination with IFN-α or RBV. Furthermore, we identified cordycepin as a responsible component for the anti-HCV activity of CM.

It is interesting that the liquid form did not show any anti-HCV activity, while the capsule form did. Because cordycepin is probably present in both CM formulations, cordycepin may be unstable in the liquid preparation, or compounds that inhibit the anti-HCV activity of cordycepin may also be present in the liquid formulation. Therefore, the anti-HCV activity of CM may depend on the formulation method.

The molecular mechanism underlying the anti-HCV activity of CM is also interesting. Since cordycepin was found to be a responsible component for the anti-HCV activity of CM and cordycepin is known to be an analog of nucleoside, we can estimate that cordycepin inhibits the RNA-dependent RNA polymerase (NS5B) of HCV. Previously, we reported that anti-HCV agents could be

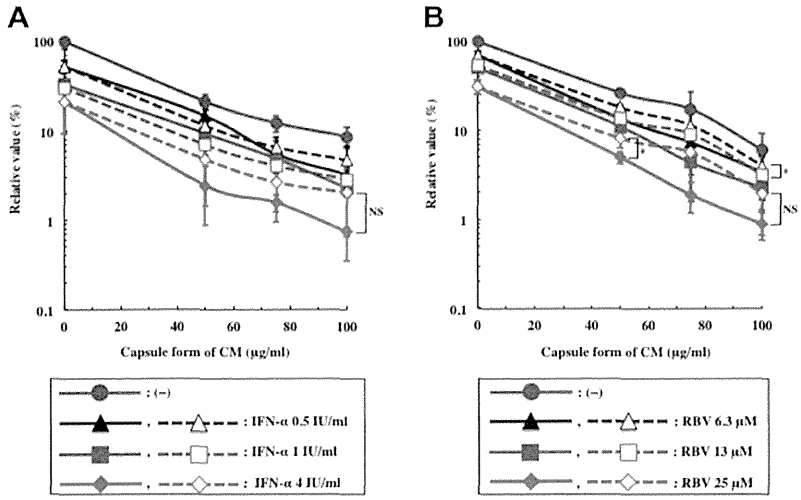

Fig. 2. Additive inhibitory effects of the capsule form of CM when used in combination with IFN-α or RBV on HCV RNA replication in ORL8 cells. Open symbols in the broken lines show the values expected as an additive anti-HCV effect and closed symbols in the solid lines show the values obtained by the ORL8 assay. ORL8 cells were treated with the capsule form of CM in combination with IFN-α (A) or RBV (B) for 72 h and subjected to RL assay. $^{*}P < 0.05$; NS, not significant.

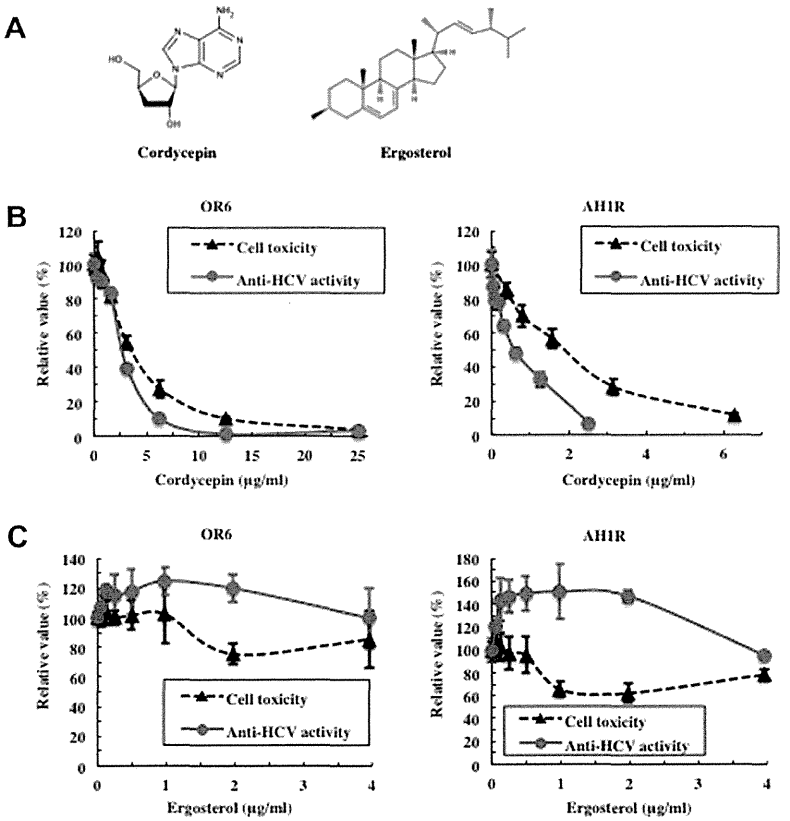

Fig. 3. Cordycepin is a responsible compound for anti-HCV activity of CM. (A) Structures of cordycepin and ergosterol. (B) Effect of cordycepin on genome-length HCV RNA replication. The RL and WST-1 assays using OR6 and AH1R cells were performed as described in Fig. 1A. (C) Ergosterol did not inhibit the genome-length HCV RNA replication. The RL and WST-1 assays using OR6 and AH1R cells were performed as described in Fig. 1A.

classified into two types: those whose anti-HCV activity is canceled by the antioxidant VE, and those whose activity is not canceled by VE [20]. To date, we have reported that CsA, N-251 (preclinical antimalarial drug), β-carotene, vitamin D2, and linoleic acid belong to the former group, and IFN-α, IFN-β, RBV, and statins belong to the latter [11,17,20]. We currently speculate that the oxidative stress induced by the former anti-HCV agents causes the anti-HCV activity via activation of the extracellular signal-regulated kinase signaling pathway [21]. Therefore, using the ORL8 assay system, we evaluated which group CM belonged to, and determined that the anti-HCV activity of CM was not canceled by VE, whereas the anti-HCV activity of CsA was completely cancelled by VE (Supplementary Fig. S4). These results suggest that the induction of oxidative stress is not associated with the anti-HCV

activity of CM, and support our initial estimation that cordycepin is a responsible component for the anti-HCV activity of CM and directly inhibits the NS5B polymerase.

In this study, we identified cordycepin as a responsible component of CM for the anti-HCV activity, because the EC_{50} value of cordycepin was comparable to the concentration calculated from the content of the capsule form of CM. However, the cell toxicity of cordycepin was stronger than that of the capsule form of CM. For example, in AH1R cells, the CC_{50} value of cordycepin was 1.9 μg/ml, whereas the value of the capsule type of CM was 160 μg/ml (equivalent to 7.9 μg/ml of cordycepin) (Table 1). Cordycepin is a promising preclinical drug that exhibits anti-tumor activities both *in vitro* and *in vivo* [22]. Since the cell lines that we established and applied to the HCV assay (OR6, ORL8, AH1R, etc.) were derived from HuH-7 or Li23 hepatoma cells, the obtained low CC_{50} values of cordycepin would be reasonable. Therefore, the high CC_{50} values obtained for the capsule form of CM are notable, and would seem to suggest that CM in the capsule form contains certain components that reduce the cytotoxicity of cordycepin. For this reason, we anticipate that the capsule form of CM will be useful as an oral supplement for the treatment of HCV with a minimal side effect profile.

In conclusion, we found that capsule form of CM, which is used as an oral health supplement, exhibited a moderate inhibitory effect on HCV RNA replication. This agent would therefore be useful as an additional component in an existing therapeutic regimen using HCV-specific inhibitors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.03.150.

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Rab18 is required for viral assembly of hepatitis C virus through trafficking of the core protein to lipid droplets

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ABSTRACT

During persistent infection of HCV, the HCV core protein (HCV-JFH-1 strain of genotype 2a) is recruited to lipid droplets (LDs) for viral assembly, but the mechanism of recruitment of the HCV core protein is uncertain. Here, we demonstrated that one of the Ras-related small GTPases, Rab18, was required for trafficking of the core protein around LDs. The knockdown of Rab18 reduced intracellular and extracellular viral infectivity, but not intracellular viral replication in HCV-JFH-1-infected RSc cells (an HuH-7-derived cell line). Exogenous expression of Rab18 increased extracellular viral infectivity almost two-fold. Furthermore, Rab18 was co-localized with the core protein in HCV-JFH-1-infected RSc cells, and the knockdown of Rab18 blocked recruitment of the HCV-JFH-1 core protein to LDs. These results suggest that Rab18 has an important role in viral assembly through the trafficking of the core protein to LDs.

Introduction

Hepatitis C virus (HCV) is an enveloped positive single-stranded RNA virus belonging to the *Flaviviridae* family (Choo et al., 1989). The HCV genome encodes a large polyprotein precursor of approximately 3000 amino acid (aa) residues, which is cleaved co- and post-translationally into at least ten proteins in the following order: core, envelope 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (Kato, 2001). Persistent HCV infection in the liver causes chronic hepatitis, and then highly progresses to liver cirrhosis and hepatocellular carcinoma (Ohkoshi et al., 1990). Therefore, the elimination of HCV RNA by the anti-HCV reagents such as interferon is necessary to block the progression of liver diseases such as liver cirrhosis and hepatocellular carcinoma. To date, the HCV-JFH-1 strain (genotype 2a) has mainly been used to study the complete life cycle of HCV worldwide. In HCV-JFH-1-infected human hepatoma HuH-7 cells, viral replication intermediate, double-stranded RNA, is detected adjacent to the membranes of the endoplasmic reticulum (ER) (Targett-Adams et al., 2008). In addition, the HCV replication complex is formed on a detergent-resistant membrane (Shi et al., 2003). These results suggest that HCV RNA replication occurs on lipid rafts and the membranous web at the cytosolic side of the ER. Following viral replication, the HCV core protein matures through the translation of a large polyprotein precursor from HCV RNA and then the processing by signal peptide peptidase. The matured HCV-JFH-1 core protein has been shown to be trafficked to lipid droplets (LDs) for viral assembly (Miyanari et al., 2007).

LDs are important organelles for lipid metabolism. LDs are covered by a phospholipid monolayer, and accumulate excessive neutral lipids such as triglycerides. A proteomics analysis revealed that a number of host factors are associated with LDs (Brasaemle et al., 2004). These host factors are required for acquisition, storage, lipolysis, transport and/or release of lipids, respectively. The first of these LD-associated factors to be identified were members of the PAT family of proteins, including perilipin (PLIN), ADRP (adipose differentiation-related protein; also named adipophilin or PLIN2), and TIP47 (also named PLIN3). PLIN is expressed only in adipocytes and steroidogenic cells, whereas ADRP and TIP47 are expressed in various cell types. ADRP and TIP47 have similar sequences and three-dimensional structures (Hickenbottom et al., 2004), but their intracellular distributions in HuH-7 cells are different (Ohsaki et al., 2006). ADRP localizes exclusively to the surface of LDs in HuH-7 cells, whereas only some of total TIP47 localizes to the LD surface in this cell line. During viral assembly, the core protein is trafficked to ADRP on LDs (Counihan et al., 2011). ADRP is displaced from the surface of LDs to the cytoplasm by the core protein, and then subjected to degradation (Boulant et al., 2008). These displacements

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0042-6822/

of ADRP also cause the redistribution of LDs around the nucleus (Boulant et al., 2008). These observations imply that the core protein may increase the probability of an interaction between the sites of viral replication at the ER and viral assembly at the LDs. However, the precise mechanism of intracellular trafficking of the core protein to ADRP on LDs is still uncertain.

A family of Ras-related small GTPases plays an important role in the membrane trafficking between organelles such as the ER, Golgi, early/late endosomes, LDs, and so on (Hutagalung and Novick, 2011). One of the Ras-related small GTPases, Rab18, is required for membrane trafficking between the ER and Golgi (Dejgaard et al., 2008). On the other hand, Rab18 is an LD-associated protein, and the ectopic expression of Rab18 induces the close apposition of LDs to ER membranes through the reduction of ADRP (Ozeki et al., 2005). These observations imply that Rab18 may be required for membrane trafficking through the redistribution of LDs around the ER. Recently, Salloum et al. reported that Rab18 bound HCV-JFH-1 NS5A and may have promoted the interaction between sites of viral replication and LDs in HCV-Jc1-infected Huh7.5.1 cells (Salloum et al., 2013). However, HCV-Jc1 is an intragenotypic recombinant encoding core to NS2 from the HCV-J6 strain (genotype 2a) in the context of HCV-JFH-1, and does not exist in nature. In addition, although HCV-Jc1 was shown to be more robust in the release of viral particles than HCV-JFH-1, the HCV-J6 core protein did not associate with LDs (Shavinskaya et al., 2007). On the other hand, the HCV-JFH-1 core protein does associate with LDs, and LD-associated core proteins recruit HCV NS protein from the ER to LDs (Miyanari et al., 2007). These results imply that HCV-Jc1 may release viral particles via an intracellular organelle distinct from the LDs. Therefore, we hypothesized that Rab18 first trafficked the HCV-JFH-1 core protein and subsequently NS5A to LD. To prove this hypothesis, we examined the association of the HCV-JFH-1 core protein with LDs and the levels of viral assembly in Rab18-knockdown cells.

Here, we show that Rab18 is required for trafficking of the HCV-JFH-1 core protein to LDs and the subsequent assembly of HCV. Rab18 may be involved in the maturation of viral particles through membrane trafficking of the HCV-JFH-1 core protein from the sites of viral replication at the ER to viral assembly at the LDs in human hepatocytes.

Results

RSc cells show higher viral productivity than Huh7.5 cells

To date, human hepatoma HuH-7 cells have mainly been used to study the complete life cycle of HCV in studies worldwide. One of the sublines of HuH-7 cells, Huh7.5, is used in many laboratories for its high susceptibility to infection with the HCV-JFH-1 strain (genotype 2a). On the other hand, we have previously established several types of HCV RNA-replicating cells (genotype 1b, O strain), such as sO cells (Kato et al., 2003, sub-genomic HCV RNA), O cells (Ikeda et al., 2005, genome-length HCV RNA), and OR6 cells (Ikeda et al., 2006, genome-length HCV RNA-encoding renilla luciferase) derived from HuH-7 cells, and their "cured" cells (sOc (Kato et al., 2003), Oc (Ikeda et al., 2005), OR6c (Ikeda et al., 2006), respectively) by the elimination of HCV RNA (Fig. 1A). The cured cell lines have been reported to increase the permissiveness of HCV (Blight et al., 2002). We also previously reported that Oc cells showed higher permissiveness of HCV than sOc cells (Abe et al., 2007). RSc cells are one of our cured cell lines derive from OR6c cells, and have mainly been used to study the complete life cycle of HCV in our laboratory (Ariumi et al., 2007, 2008; Kato et al., 2009). However, we have no information on viral susceptibility of our cured cell lines to HCV-JFH-1 infection. To identify which of our cured cell lines would be most useful for the infection with HCV-JFH-1, we first compared the amounts of LDs by two methods, i.e., a confocal microscope and flow cytometry. The LDs were stained with BODIPY493/503 and observed under a confocal microscope. In our cured cells (sOc, Oc, OR6c, and RSc cells), the numbers rather than the sizes of LDs have increased compared to HuH-7 cells (Fig. 1B). In addition, the mean fluorescence intensity of BODIPY493/503-stained cells has increased by the enhancements of the numbers of LDs (Fig. 1C). These qualitative and quantitative analyses revealed that our cured cells (sOc, Oc, OR6c, and RSc cells) formed higher levels of LDs than HuH-7 and Huh7.5 cells (Fig. 1B and C). Interestingly, irrespective of the quantitative difference of LDs, the levels of viral replication at 72 h after the viral inoculation of HCV-JFH-1 were comparable between each of our cured cell lines and Huh7.5 cells, but not between each of the cured cell lines and HuH-7 cells (Fig. 1D). Moreover, the time-course analysis showed that the capacities of HCV RNA replication were almost comparable between RSc and Huh7.5 cells (Fig. 1E). These results suggest that the levels of HCV RNA replication do not depend on the amount of LDs. Next, to compare the levels of viral assembly and viral productivity between RSc and Huh7.5 cells, we examined the infectivity of the cell lysates (intracellular infectivity) and the supernatants (extracellular infectivity) derived from both lines of HCV-JFH-1-infected cells. The intracellular and extracellular infectivities of HCV-JFH-1-infected RSc cells were significantly higher than those of HCV-JFH-1-infected Huh7.5 cells (Fig. 1F). These results suggest that RSc cells possess higher viral productivity in response to infection with HCV-JFH-1 than Huh7.5 cells.

Rab18 is required for viral production, but not viral RNA replication

As the first step of viral assembly, the HCV-JFH-1 core protein displaces ADRP from the surface of LDs to the cytoplasm (Boulant et al., 2008; Counihan et al., 2011). In the present study, we tried to clarify how the core protein is trafficked to LDs by using RSc and Huh7.5 cells. It has been reported that Rab18, one of the Ras-related small GTPase family members, induces the close apposition of LDs to ER membranes through the reduction of ADRP (Ozeki et al., 2005). Based on these previous findings, we hypothesized that Rab18 is required for trafficking of the HCV-JFH-1 core protein to LDs. To prove this hypothesis, we first examined the expression levels of Rab18 in RSc and Huh7.5 cells. The expression levels of Rab18 were almost comparable between RSc cells and Huh7.5 cells at both the transcript (Fig. 2A) and protein levels (Fig. 2B). Two other members of the Ras-related small GTPases, Rab5 and Rab7, were also present at almost the same levels in RSc and Huh7.5 cells. We next examined the effect of the knockdown of Rab18 against HCV replication in RSc cells. The knockdown of Rab18 (Fig. 2C) had no effect on the RNA replication step (Fig. 2D). Rab18-knockdown Huh7.5 cells and genome-length HCV RNA-replicating O cells (Kato et al., 2009) also showed similar results (Fig. 2E and F, Supplemental Fig. S1A and B). However, we found that the knockdown of Rab18 caused a significant decrease in viral productivity in both RSc and Huh7.5 cells (Fig. 2G). In addition, the knockdown of ADRP (Supplemental Fig. S2A) also decreased viral productivity rather than HCV RNA replication (Supplemental Fig. S2B). Furthermore, we demonstrated that the overexpression of Rab18 (Fig. 2H) recovered the viral productivity (Fig. 2I) rather than viral RNA replication (Fig. 2J). From these results, we conclude that Rab18 is required for viral production of HCV.

Rab18 is required for viral assembly through the trafficking of the HCV-JFH-1 core protein to LDs

To clarify whether Rab18 is required for the viral assembly step, we first examined the localization of Rab18 and the HCV core protein in HCV-JFH-1-infected cells. The results revealed that Rab18

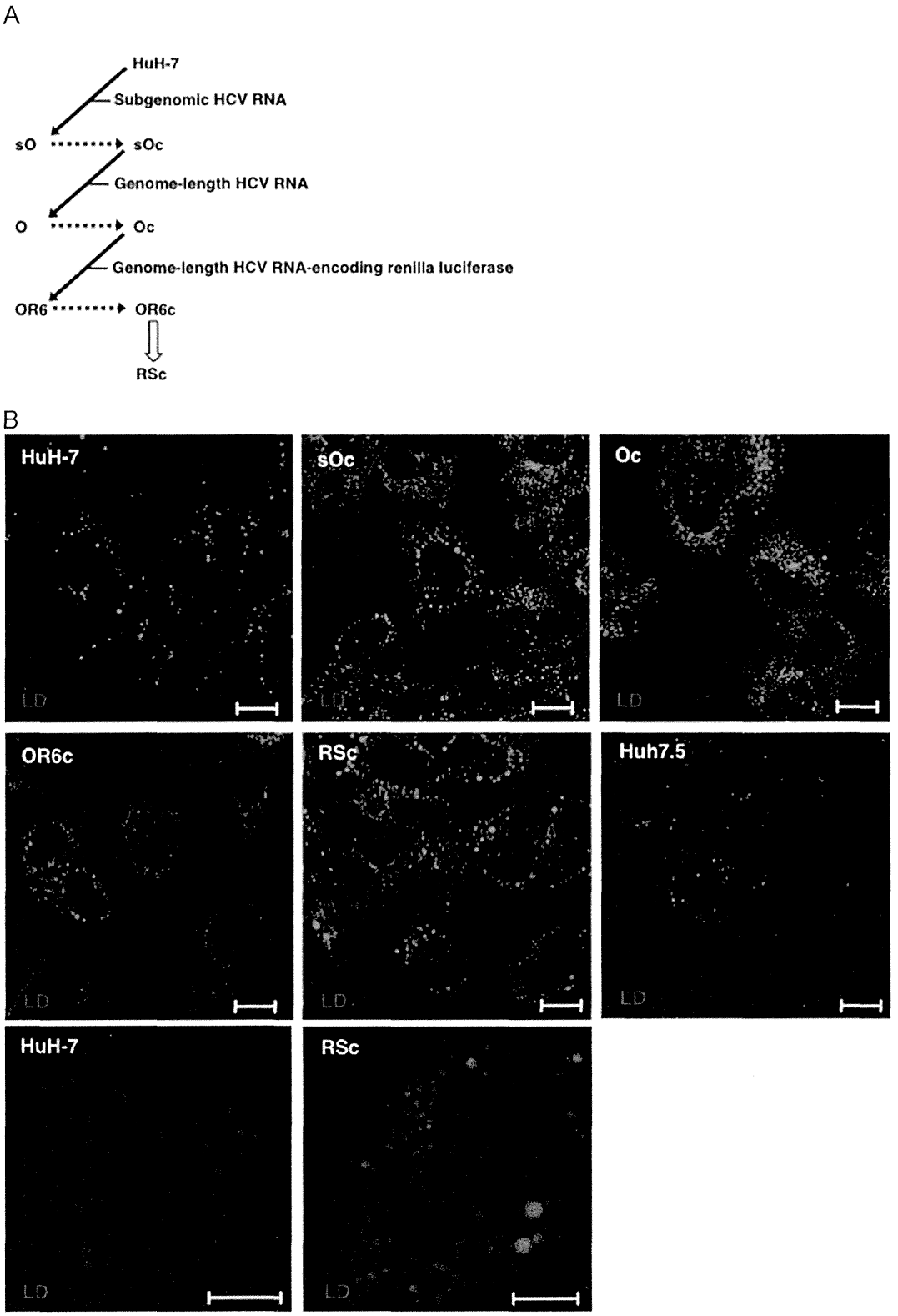

Fig. 1. RSc cells possess higher viral productivity in response to the infection with HCV-JFH-1 than Huh7.5 cells. (A) Outline for the establishment of our HCV RNA-replicating cells and their "cured" cells. Our HCV RNA-replicating cells (sO, O, and OR6 cells) are independently established by the transfection of HCV RNA into HuH-7 or cured cells (sOc or Oc cells) as previously reported (Ikeda et al., 2005, 2006; Kato et al., 2003). To prepare the cured cells, HCV RNA was eliminated from HCV RNA-replicating cells by the interferon treatment. RSc cells are one of the sublines of OR6c cells. The arrows with solid and dashed lines show the transfection of HCV RNA and the interferon treatment, respectively. (B) Visualization of LD under a confocal microscope. The panels show the fluorescence of LD by staining with BODIPY493/503. Bars, 20 μm. (C) Measurement of the mean fluorescence intensity of BODIPY493/503-stained cells by a flow cytometer. These levels were calculated relative to the level in HuH-7 cells, which was set at 1. (D) Quantitative RT-PCR analysis of HCV RNA in our cured cells 72 h after infection with HCV-JFH-1. Total RNA extracted from the cells was subjected to quantitative RT-PCR analysis. The experiments were performed in at least triplicate. (E) Time-course analysis of HCV RNA in RSc and Huh7.5 cells after infection with HCV-JFH-1. Total RNA was extracted from HCV-JFH-1-infected cells at each time point. (F) Quantitative RT-PCR analysis of HCV RNA in Huh7.5 cells 72 h after infection with intracellular (left panel) and extracellular (right panel) HCV-JFH-1. As intracellular or extracellular HCV-JFH-1, the lysate or the supernatant was recovered from RSc cells (designated J-RSc in the figure) and Huh7.5 cells (designated J-Huh7.5) 24 h after infection with HCV-JFH-1.

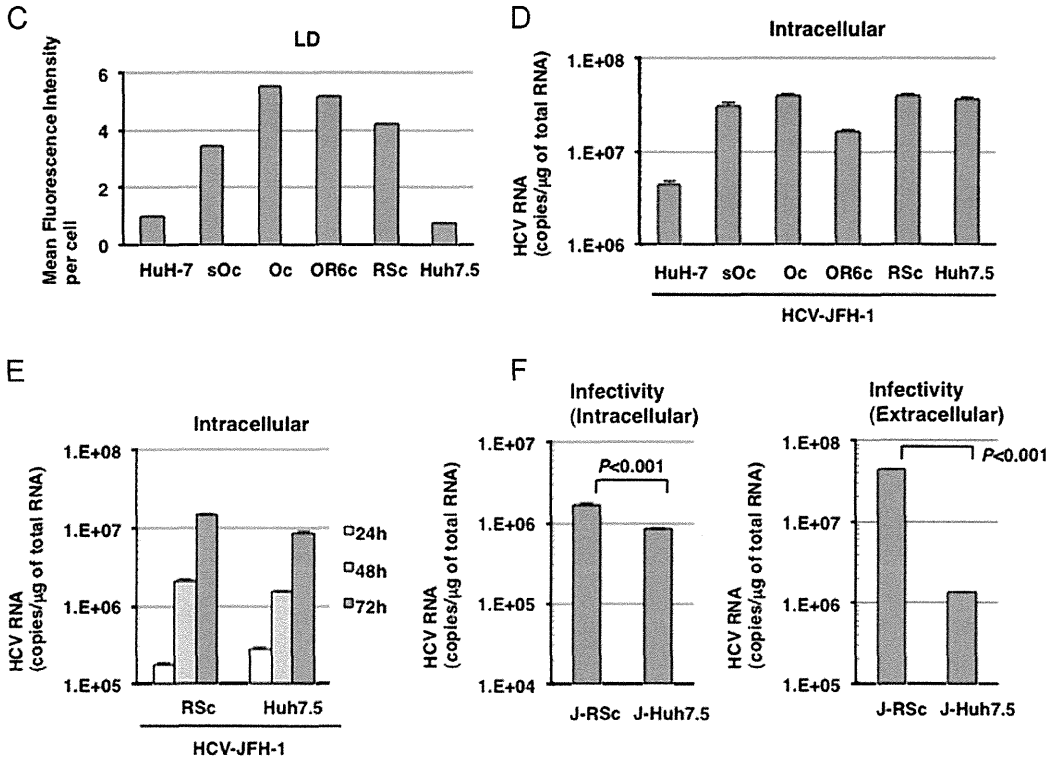

Fig. 1. (*continued*)

was co-localized with the core protein in HCV-JFH-1-infected RSc and Huh7.5 cells (Fig. 3A). We previously reported that the core protein was not recruited to LDs in O cells, from which no infectious virus was produced (data not shown). In fact, in contrast to HCV-JFH-1-infected RSc and Huh7.5 cells, co-localization of Rab18 and the core protein was not observed in O cells (Fig. 3B), although the expression of Rab18 were almost comparable levels among these cell lines (Supplemental Fig. S1C). We next examined the infectivity of intracellular viral particles in HCV-JFH-1-infected RSc siRab18 cells. The results revealed that the knockdown of Rab18 inhibited 90% of the production of intracellular viral particles (Fig. 3C) and the recruitment of the core protein to LDs (Fig. 3D). However, the knockdown of Rab4 (early endosome marker), other Ras-related small GTPase family member, inhibited only 40% of the production of intracellular viral particles (Supplemental Fig. S3C). These results suggest that Rab18 is particularly required for viral assembly through the trafficking of the core protein to LDs. Rab18 may be involved in the maturation of viral particles through membrane trafficking of the core protein from the ER to LDs.

Discussion

Previous proteomics analysis showed that a number of host factors were associated with LDs (Brasaemle et al., 2004). These LD-associated proteins may be required for the life cycle of HCV as well as the metabolism of lipids. During viral production, the HCV core protein is recruited to LDs in HCV-JFH-1-infected cells (Miyanari et al., 2007). In the present study, we suggest that one of the LD-associated proteins, Rab18, is required for trafficking of the HCV core protein to LDs and subsequent viral assembly.

The HCV core protein consists of three domains (domains 1, 2, and 3). Domain 2 (aa 118–173) contains two proline residues at aa positions 138 and 143, and a YATG sequence ranging from aa positions 164–167 that is essential for the association with LDs (Hope et al., 2002). These are conserved in both the JFH-1 strain (genotype 2a) and O strain (genotype 1b). However, the core protein was associated with LDs in HCV-JFH-1-infected RSc cells (Fig. 3D), but not in genome-length HCV RNA-replicating O cells (Kato et al., 2009). Matto et al. found that there were two morphologically distinct populations of the core protein (the ring-like and the dot-like pattern) in genome-length HCV RNA (genotype 1b)-replicating cells (Matto et al., 2004). The ring-like core protein was associated with LDs, and the dot-like core protein was associated with the detergent-resistant membranes and the lipid rafts essential for viral replication (Matto et al., 2004). We previously demonstrated that the core protein of the O strain showed a dot-like pattern (Kato et al., 2009), and that infectious virus was not produced from O cells (data not shown). In contrast to the core protein of the JFH-1 strain, the core protein of the O strain was not trafficked to LDs, and may have remained at the detergent-resistant membranes and the lipid rafts.

We also demonstrated that the core protein was co-localized with Rab18 in HCV-JFH-1-infected RSc cells, but not in O cells (Fig. 3B). In addition, we demonstrated that the knockdown of Rab18 did not inhibit HCV RNA replication in O cells as well as HCV-JFH-1-infected RSc cells (Supplemental Figs. S1B and S3B). However, in contrast to our results, Salloum et al. have previously observed that the knockdown of Rab18 inhibited HCV RNA replication in OR6 cells (Salloum et al., 2013). From these results, we speculate the clonality of HuH-7 cells as one of causes of this discrepancy. On the other hand, it also remains the possibility that the induction of IFN-β by shRNA reduced HCV RNA replication. Kenworthy et al. has previously reported that the introduction of shRNA by lentiviral vector may induce IFN-β (Kenworthy et al., 2009). Rab18 knockdown cells in the Salloum's paper were generated by the introduction of shRNA using lentiviral vector. In addition, Rab18 overexpression did not enhance HCV RNA replication in OR6 cells (Salloum et al., 2013). Another proteomic analysis suggested that Rab18 is upregulated in the lipid raft fraction of

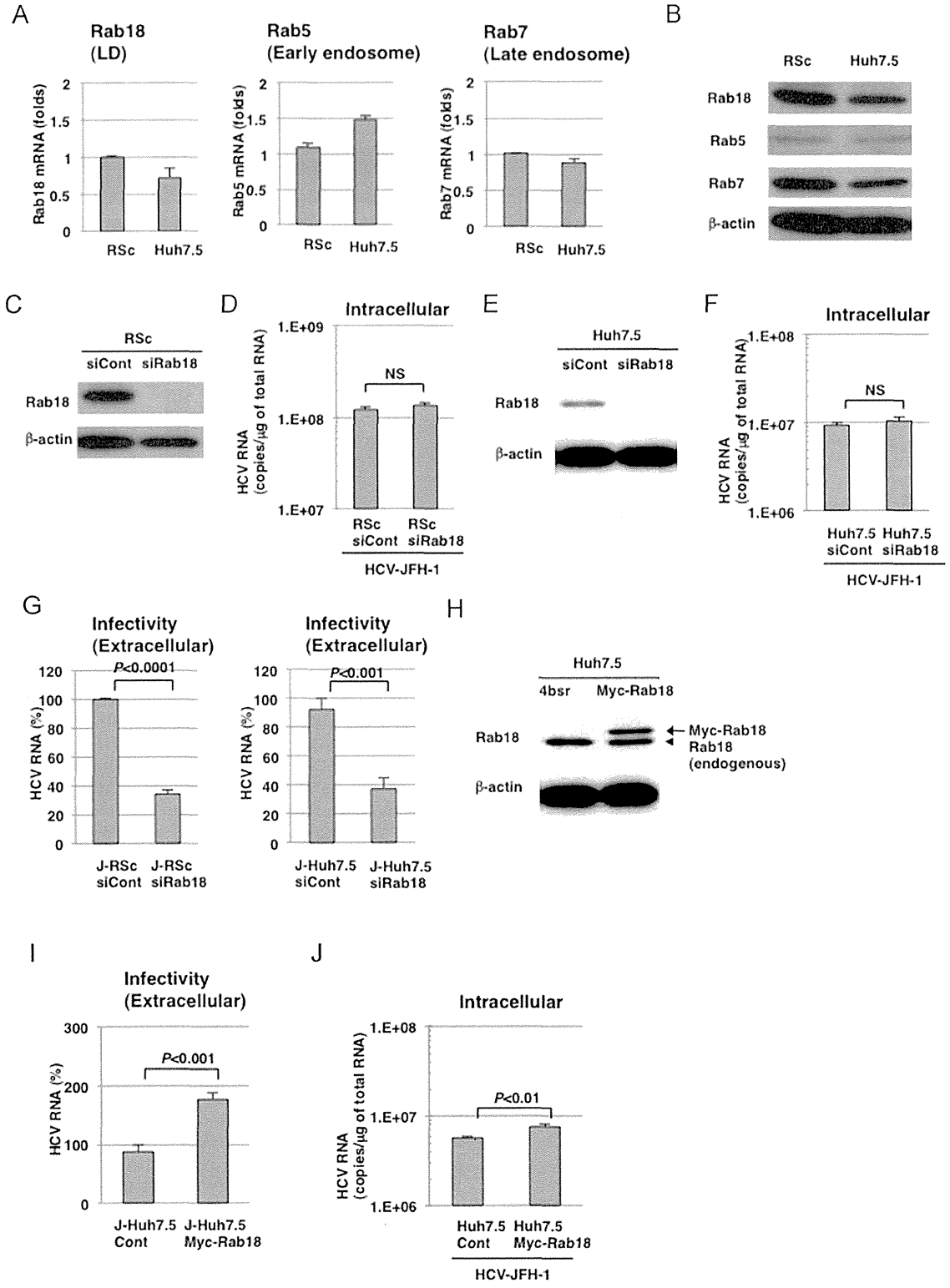

Fig. 2. Rab18 is required for viral production, but not viral RNA replication. (A) Quantitative RT-PCR analysis of Rab18, Rab5, and Rab7 mRNA in RSc and Huh7.5 cells. These levels were calculated relative to the level in RSc cells, which was set at 1. (B) Western blot analysis of Rab18, Rab5, and Rab7 in RSc and Huh7.5 cells. β-actin was included as a loading control. (C) Western blot analysis of Rab18 expression in RSc cells transfected with Rab18-specific (designated RSc siRab18 in the figure) or control (designated RSc siCont) siRNA. Cell lysates were prepared from RSc cells 120 h after transfection with Rab18-specific or control siRNA. (D) Quantitative RT-PCR analysis of HCV RNA in RSc siRab18 cells 24 h after infection with HCV-JFH-1. Transfection was performed 96 h before infection with HCV-JFH-1. NS: no significance. (E) Western blot analysis of Rab18 expression in Huh7.5 cells transfected with Rab18-specific (designated Huh7.5 siRab18) or control (designated Huh7.5 siCont) siRNA. (F) Quantitative RT-PCR analysis of HCV RNA in Huh7.5 siRab18 and siCont cells 24 h after infection with HCV-JFH-1. (G) Quantitative RT-PCR analysis of HCV RNA in Huh7.5 cells 72 h after infection with extracellular HCV-JFH-1. (Left panel) As extracellular HCV-JFH-1, the supernatant was recovered from RSc siCont cells (designated J-RSc siCont) or RSc siRab18 cells (designated J-RSc siRab18) 24 h after infection with HCV-JFH-1. (Right panel) The supernatant was also recovered from Huh7.5 siRab18 cells (designated J-Huh7.5 siRab18) and Huh7.5 siCont cells (designated J-Huh7.5 siCont) 24 h after infection with HCV-JFH-1. (H) Western blot analysis of Rab18 expression in Huh7.5 cells stably expressing Myc-tagged Rab18 (designated Huh7.5 Myc-Rab18). The arrow and arrowhead indicate exogenous (Myc-tagged) and endogenous Rab18, respectively. (I) Quantitative RT-PCR analysis of HCV RNA in Huh7.5 cells 72 h after infection with extracellular HCV-JFH-1. As extracellular HCV-JFH-1, the supernatant was recovered from Huh7.5 Cont (designated J-Huh7.5 Cont) or Huh7.5 Myc-Rab18 (designated J-Huh7.5 Myc-Rab18) 24 h after infection with HCV-JFH-1. (J) Quantitative RT-PCR analysis of HCV RNA in Huh7.5 Myc-Rab18 cells 24 h after infection with HCV-JFH-1.

genome-length HCV RNA (genotype 1b)-replicating cells (Mannova et al., 2006). These results may suggest that Rab18 also remained at the detergent-resistant membranes and the lipid rafts in the genome-length HCV RNA (genotype 1b)-replicating cells such as O cells. In addition, the gene silencing of Rab18 (Rab18-knockdown JFH-1-infected RSc cells) blocked localization of the HCV-JFH-1 core protein to LDs (Fig. 3D). Interestingly, the morphology of the population of HCV-JFH-1 core proteins was changed from a ring-like pattern to a dot-like pattern by the gene silencing of Rab18 (Fig. 3D). The HCV replication complex is formed on detergent-resistant membranes of the ER lumen (Shi et al., 2003). The ectopic expression of Rab18 induces the close apposition of LD to ER membranes through the reduction of ADRP (Ozeki et al., 2005). Rab18 may be one of the key host factors for the switch from the viral replication step to the viral assembly step through the close apposition of the detergent-resistant membranes to LDs. Rab18 may be an important target for the development of more effective anti-HCV reagents.

Materials and methods

Cell culture, reagents, and plasmids

Human hepatoma Huh7.5 cells were provided by Apath LLC (Brooklyn, NY). Huh7.5 cells, HuH-7 cells, and our established HuH-7-derived cells (sOC, Oc, OR6c, and RSc cells) were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum. Blasticidin (2 μg/ml) was used for the selection of Huh7.5 cells exogenously expressing Myc-Rab18. G418 (0.3 mg/ml) was also used for the selection of genome-length HCV RNA-replicating O cells.

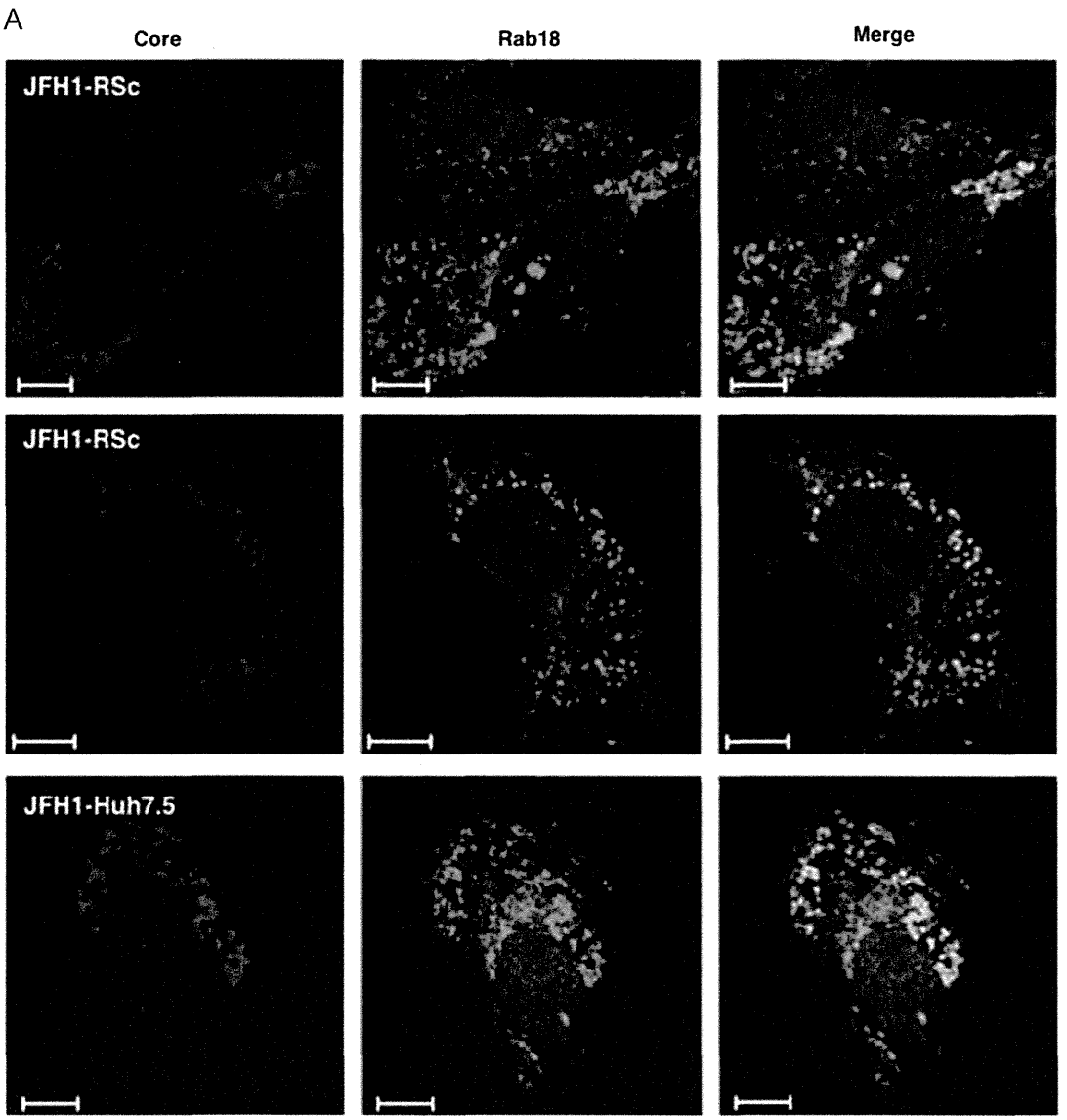

Fig. 3. Rab18 is required for viral assembly through the trafficking of HCV-JFH-1 core protein on LDs. (A) Visualization of the HCV-JFH-1 core protein (red) and Rab18 (green) under a confocal microscope. The panels show RSc or Huh7.5 cells 72 h after infection with HCV-JFH-1. Bars, 10 μm. (B) Visualization of the core protein (red) and Rab18 (green) in O cells. Bars, 10 μm. (C) Quantitative RT-PCR analysis of HCV RNA in Huh7.5 cells 72 h after infection with intracellular HCV-JFH-1. As intracellular HCV-JFH-1, the lysate was prepared from RSc siRab18 cells (designated J-RSc siRab18) and RSc siCont cells (designated J-RSc siCont) 24 h after infection with HCV-JFH-1. (D) Visualization of the HCV-JFH-1 core protein (red) and LD (green) under a confocal microscope. The panels show RSc siRab18 and siCont cells 72 h after infection with HCV-JFH-1. Bars, 10 μm.

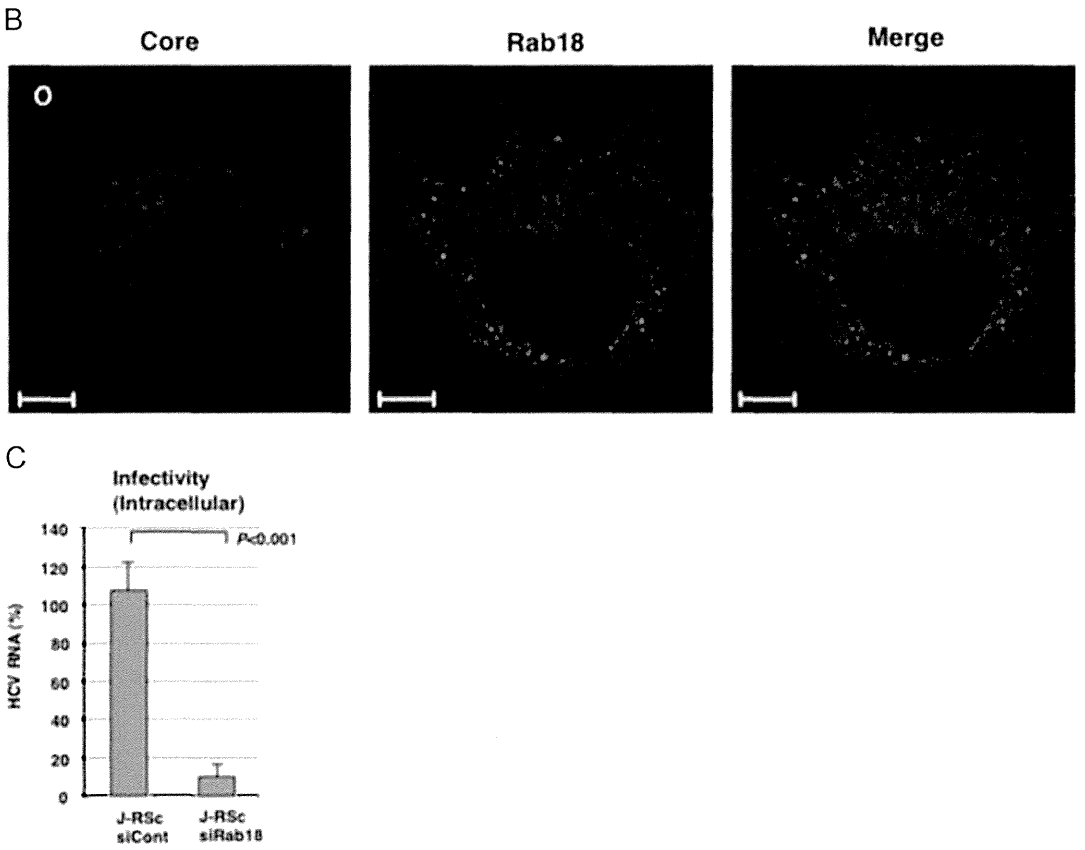

Fig. 3. (*continued*)

Immunofluorescence analysis

The LDs were stained with BODIPY493/503 (Invitrogen), and then photographed under a confocal microscope. Anti-Core antibody (CP11; Institute of Immunology, Tokyo, Japan) and anti-Rab18 antibody (Sigma, St. Louis, MO) were used as the primary antibodies. AlexaFluor 488-conjugated goat anti-rabbit antibody and AlexaFluor 594-conjugated goat anti-mouse antibody (Invitrogen) were used as the secondary antibodies. The intracellular localizations of HCV core protein and Rab18 were visualized and photographed under a confocal microscope as previously reported (Dansako et al., 2008). 4′-6-diamino-2-phenylindole (DAPI; Sigma) was used for visualization of the nucleus.

Flow cytometric analysis

The LDs were stained with BODIPY493/503 and then their mean fluorescence intensity was measured by a flow cytometer. The levels of LDs were calculated relative to the level in HuH-7 cells, which was set at 1.

Infection with HCV-JFH-1

The cells were infected with HCV-JFH1 (genotype 2a) for the appropriate time at a multiplicity of infection (MOI) of 1, and then the samples were prepared for the Western blot analysis, immunofluorescence analysis, and quantitative reverse transcription (RT)-PCR. Cell lysates and supernatants were prepared from the HCV-JFH-1-infected cells to monitor intracellular and extracellular infectivity. Intracellular HCV-JFH-1 was prepared from HCV-JFH-1-infected cells by repeated freeze-thaw cycles.

Generation of Rab18-knockdown cells

Small interfering RNAs (siRNAs) targeting Rab18 (Thermo Scientific; M-010824-00-0005) were prepared to generate Rab18-knockdown cells. siRNAs targeting Rab18 or non-targeting siRNAs (Thermo Scientific; D-001206-13-20) were introduced into RSc or Huh7.5 cells by DharmaFECT transfection reagent (Thermo Fisher Scientific, Waltham, MA). After transfection for the appropriate amount of time, Rab18-knockdown cells were infected with HCV-JFH-1.

Quantitative RT-PCR analysis

Total cellular RNA was isolated from HCV-JFH-1-infected cells by using an RNeasy mini kit (Qiagen, Hilden, Germany). RT was performed as previously described (Dansako et al., 2009). A SYBR Premix Ex Taq kit (TaKaRa Bio, Otsu, Japan) was used to measure the RNA levels of Rab18, Rab5, Rab7, GAPDH, or HCV. We used the following forward and reverse primer sets for quantitative PCR: for Rab18, 5′-GCGGAACGGGGTCAGGATGG-3′ (forward) and 5′-AAGAG CAGGCTGGACTTGCCC-3′ (reverse); for Rab5, 5′-GCTTGCTGCGGTCT CAGGTTTCT-3′ (forward) and 5′-TGGCCCGTTGGGTCTTGTTGC-3′ (reverse); for Rab7, 5′-CTCATCCAGGCCAGTCCCCGA-3′ (forward) and 5′–CCCGCTTTGTGGCCACTTGTC-3′ (reverse); for HCV and GAPDH, the primer sets are given in Dansako et al., 2013 and Dansako et al., 2003, respectively. The levels of Rab18, Rab5, Rab7, and HCV were normalized to the levels of GAPDH mRNA. The mRNA levels of Rab18, Rab5, and Rab7 in Huh7.5 cells were calculated relative to the level in RSc cells, which was set at 1. In vitro-transcribed HCV-JFH-1 RNA was used as the standard to calculate the amount of HCV RNA in HCV-JFH-1-infected cells. Data are the means ± SD from three independent experiments.

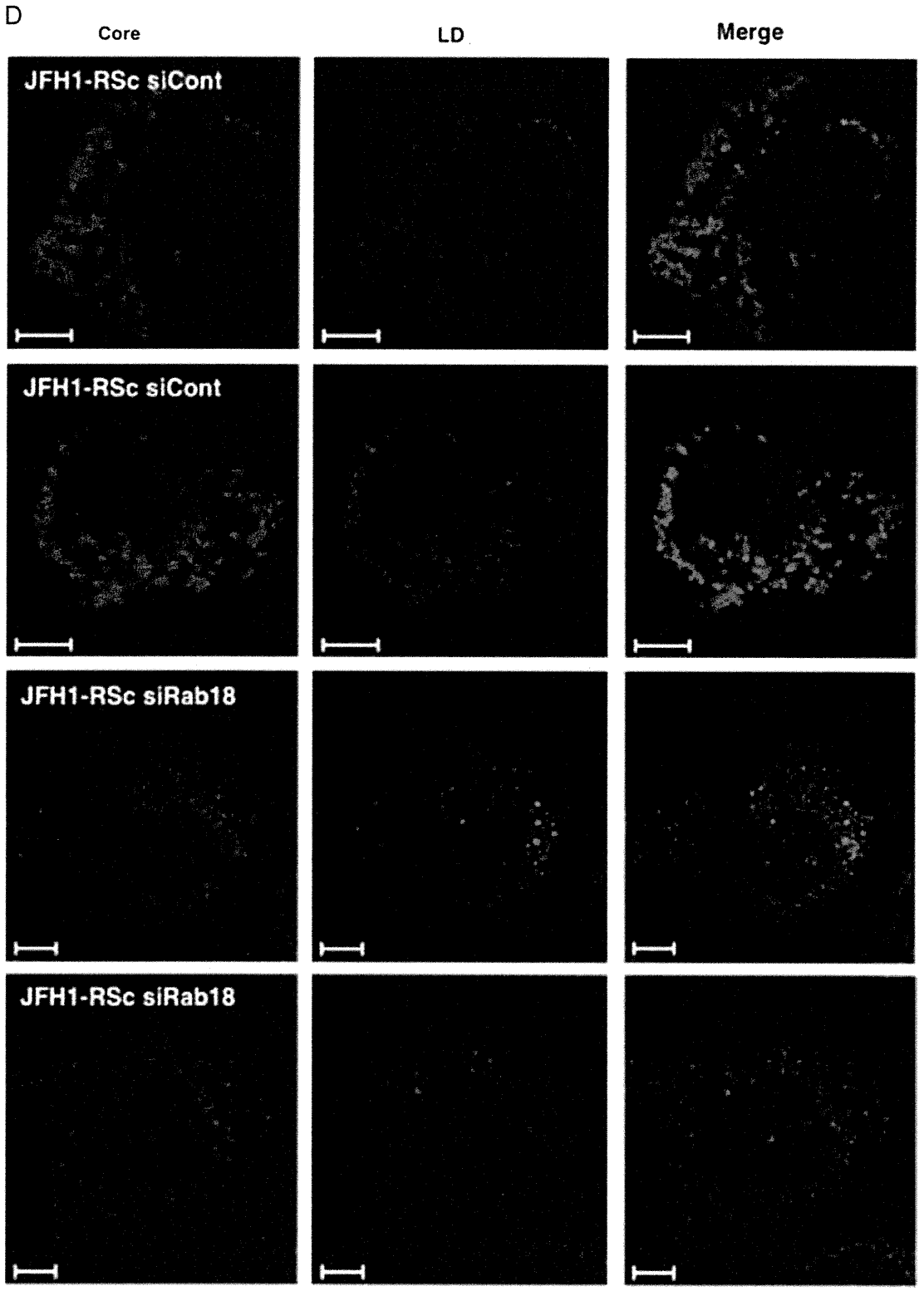

Fig. 3. (*continued*)

Western blot analysis

Preparation of cell lysates, and SDS-polyacrylamide gel electrophoresis were performed as previously described (Dansako et al., 2005). Gel was transferred to an Immobilon PVDF membrane (Millipore, Billerica, MA) by using a semi-dry transfer system: Horize BLOT 2MR (ATTO, Tokyo, Japan). Anti-Core (CP11; Institute of Immunology Co.), anti-Myc (PL14; Medical & Biological Laboratories, Nagoya, Japan), anti-Rab18 (Sigma), anti-Rab5 (S-19; Santa Cruz Biotechnology. Santa Cruz, CA), anti-Rab7 (Sigma),

and anti-β-actin antibody (AC-15; Sigma) were used in this study as primary antibodies. HRP-conjugated anti-mouse-IgG or anti-rabbit-IgG was used in this study as a secondary antibody (Cell Signaling Technology, Beverly, MA). Immunocomplexes were detected as previously described (Dansako et al., 2007).

Statistical analysis

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

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.05.017.

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GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Hepatitis C Virus Core Protein Suppresses Mitophagy by Interacting with Parkin in the Context of Mitochondrial Depolarization

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Hepatitis C virus (HCV) causes mitochondrial injury and oxidative stress, and impaired mitochondria are selectively eliminated through autophagy-dependent degradation (mitophagy). We investigated whether HCV affects mitophagy in terms of mitochondrial quality control. The effect of HCV on mitophagy was examined using HCV-Japanese fulminant hepatitis-1–infected cells and the uncoupling reagent carbonyl cyanide *m*-chlorophenylhydrazone as a mitophagy inducer. In addition, liver cells from transgenic mice expressing the HCV polyprotein and human hepatocyte chimeric mice were examined for mitophagy. Translocation of the E3 ubiquitin ligase Parkin to the mitochondria was impaired without a reduction of pentaerythritol tetranitrate–induced kinase 1 activity in the presence of HCV infection both *in vitro* and *in vivo*. Coimmunoprecipitation assays revealed that Parkin associated with the HCV core protein. Furthermore, a Yeast Two-Hybrid assay identified a specific interaction between the HCV core protein and an N-terminal Parkin fragment. Silencing Parkin suppressed HCV core protein expression, suggesting a functional role for the interaction between the HCV core protein and Parkin in HCV propagation. The suppressed Parkin translocation to the mitochondria inhibited mitochondrial ubiquitination, decreased the number of mitochondria sequestered in isolation membranes, and reduced autophagic degradation activity. Through a direct interaction with Parkin, the HCV core protein suppressed mitophagy by inhibiting Parkin translocation to the mitochondria. This inhibition may amplify and sustain HCV-induced mitochondrial injury. *(Am J Pathol 2014, 184: 3026–3039; http://dx.doi.org/10.1016/j.ajpath.2014.07.024)*

Oxidative stress is present in chronic hepatitis C to a greater degree than in other inflammatory liver diseases.[1,2] The hepatitis C virus (HCV) core protein induces the production of reactive oxygen species (ROS)[3,4] through mitochondrial electron transport inhibition.[5] Because the mitochondria are targets for ROS and ROS generators, HCV-induced ROS have the potential to injure mitochondria. In addition, hepatocellular mitochondrial alterations have been observed in patients with chronic hepatitis C.[6] We previously identified a ROS-associated iron metabolic disorder[7] and demonstrated that transgenic mice expressing the HCV polyprotein develop hepatocarcinogenesis related to mitochondrial injury induced by HCV and iron overload.[8] Therefore, impaired mitochondrial function may play a critical role in the development of hepatocellular carcinoma (HCC) in patients with chronic HCV infection. Conversely, the affected mitochondria are selectively eliminated through the autophagy-dependent degradation of mitochondria (referred to as mitophagy) in both physiological and pathological settings to maintain the mitochondrial quality.[9,10] On the

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basis of these observations, we hypothesized that HCV may suppress mitophagy, which could lead to the sustained presence of affected mitochondria, increased ROS production, and the development of HCC.

Mitochondrial membrane depolarization precedes mitophagy induction,[11] which is selectively controlled by a variety of proteins in mammalian cells, including pentaerythritol tetranitrate–induced kinase 1 (PINK1) and the E3 ubiquitin ligase Parkin.[12–19] PINK1 facilitates Parkin targeting of the depolarized mitochondria.[12–15] Although Parkin ubiquitinates a broad range of mitochondrial outer membrane proteins,[14,17–19] it remains unclear how Parkin enables the damaged mitochondria to be recognized by the autophagosome. Structures containing autophagy-related protein 9A and the uncoordinated family member-51–like kinase 1 complex independently target depolarized mitochondria at the initial stages of Parkin-mediated mitophagy, whereas the autophagosomal microtubule-associated protein light chain 3 (LC3) is critical for efficient incorporation of damaged mitochondria into the autophagosome at a later stage.[20] LC3-I undergoes post-translational modification by phosphatidylethanolamine to become LC3-II, and LC3-II insertion into the autophagosomal membrane is a key step in autophagosome formation. In addition, the autophagic adaptor p62 is recruited to mitochondrial clusters and is essential for mitochondrial clearance,[13] although it remains controversial as to whether p62 is essential for mitochondrial recognition by the autophagosome[13] or rather is important for perinuclear clustering of depolarized mitochondria.[19,21] Our aim was to examine whether HCV suppresses mitophagy. We found that HCV core protein inhibits the translocation of Parkin to affected mitochondria by interacting with Parkin and subsequently suppressing mitophagy. These results imply that mitochondria affected by HCV core protein are unlikely to be eliminated, which may intensify oxidative stress and increase the risk of hepatocarcinogenesis.

Materials and Methods

Cell Culture, HCV Infection Experiments, and Mitochondrial Depolarization

HCV-Japanese fulminant hepatitis-1 (JFH1)–infected Huh7 cells have previously been described in detail.[22] The supernatants were collected from cell culture–generated JFH1-Huh7 cells at 21 days after infection and stored until use at −80°C after filtering through a 0.45-μm filter. For infection experiments with the HCV-JFH1 virus, 1×10^5 Huh7 cells per well were plated onto 6-well plates and cultured for 24 hours. Then, we infected the cells with 50 μL (equivalent to a multiplicity of infection of 0.1) of inoculum. The culture supernatants were collected, and the levels of the HCV core were determined using an enzyme-linked immunosorbent assay (ELISA; Mitsubishi Kagaku Bio-Clinical Laboratories, Tokyo, Japan). Total RNA was isolated from the infected cellular lysates using an RNeasy mini kit (Qiagen, Hilden, Germany) for quantitative RT-PCR analysis of the intracellular HCV RNA. The HCV infectivity in the culture supernatants was determined by a focus-forming assay at 48 hours after infection. The HCV-infected cells were detected using an anti-HCV core antibody (CP-9 and CP-11, Institute of Immunology, Ltd, Tokyo, Japan). Intracellular HCV infectivity was determined using a focus-forming assay at 48 hours after inoculation of the lysates by repeated freeze-and-thaw cycles (three times).

To depolarize the mitochondria, the cells were treated with 10 μmol/L carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; Sigma-Aldrich, St. Louis, MO) for 1 to 2 hours or 1 μmol/L valinomycin (Sigma-Aldrich) for 3 hours; CCCP represses ATP synthesis through the loss of the H^+ gradient without affecting mitochondrial electron transport, which is known to induce mitochondrial fragmentation.[13]

Animals

The pAlbSVPA-HCV vector, which contains the full-length polyprotein-coding region under the control of the murine albumin promoter/enhancer, has previously been described in detail.[23,24] Of the four transgenic lineages with evidence of RNA transcription of the full-length HCV-N open reading frame (FL-N), the FL-N/35 lineage proved capable of breeding large numbers.[24] Urokinase-type plasminogen activator–transgenic severe combined immunodeficiency mice were generated, and human hepatocytes were transplanted to generate chimeric mice.[25] The chimeric mice were injected with genotype *1b* HCV-positive human serum samples, as described previously.[26] The mouse livers were extracted 12 weeks after the infection, when the serum HCV RNA titers had increased over baseline levels. Male FL-N/35 transgenic mice, age-matched C57BL/6 mice (control), and chimeric mice with and without HCV infection were fed, maintained, and then euthanized by i.p. injection of 10% nembutal sodium, according to the guidelines approved by the Institutional Animal Care and Use Committee. The study protocol for obtaining human serum samples conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Review Committee.

Measurement of HCV RNA and Human Albumin in the Serum of Chimeric Mice

HCV RNA[26] and human albumin[25] were quantified as described previously. Human albumin levels in the serum of chimeric mice were determined using the Human Albumin ELISA Quantification kit (Bethyl Laboratories Inc., Montgomery, TX).

Measurement of Mitochondrial Membrane Potential

The mitochondrial membrane potential ($\Delta\Psi$) was measured using a Cell Meter JC-10 Mitochondrial Membrane Potential Assay kit (AAT Bioquest, Inc., Sunnyvale, CA), according to the manufacturer's instructions. The fluorescent intensities