Life cycle of the HCV reporter-assay system in Li23-derived cells

We investigated whether D7 cells produce infectious HCV. First, D7 cells were inoculated using the supernatant from RSc cells replicating JR/C5B with BX-2, and the supernatant was stored at 17 days after infection. Then, the supernatant derived from the D7 cells was used as an inoculum for reinfection of naïve D7 cells. RL activities were determined 2, 6, 10 and 14 days after reinfection (Fig. 6). RL activity was increased after reinfection in D7 cells and reached a plateau 10 days after reinfection. These data indicate that the JFH-1 reporter-assay system is also useful for monitoring the HCV life cycle in Li23-derived cell lines.

DISCUSSION

In this study, we developed an HCV production reporter-assay system using two distinct hepatoma cell lines, HuH-7 and Li23. Robust HCV RNA replication and virus production were achieved by the introduction of REMs into the structural region or the NS5B region. These REMs were obtained from JFH-1-infected long-term-cultured cells. The two REMs in NS5B (H2505Q and V2995L substitutions) derived from JFH-1 had different effects on replication of genotype 1b HCV-O RNA and genotype 2a JFH-1 RNA. Furthermore, the subcloned Li23-derived D7 cells produced by serial limiting dilution supported this HCV production reporter-assay system.

Several groups have reported JFH-1 reporter virus systems (Koutsoudakis et al., 2006; Marcello et al., 2006; Pietschmann et al., 2002; Wakita et al., 2005). However, robust reporter virus production was limited within the study using HuH-7-derived cells. Therefore, we attempted to developed a JFH-1 reporter virus assay system using our previously reported line of Li23 cells (Kato et al., 2009).
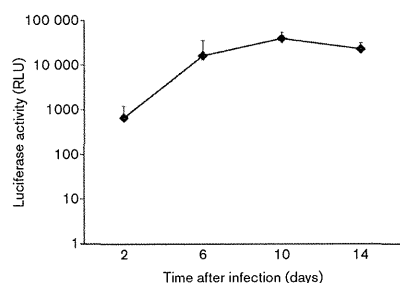

Fig. 6. HCV life cycle in Li23-derived D7 cells. D7 cells were inoculated with the supernatant from D7 cells after infection with JFH-1 with BX-2 mutants. RL activities were tested 2, 6, 10 and 14 days after infection.

The introduction of RL and EMCV-IRES genes into the HCV gene lengthened the genome of HCV by approximately 1.9 kb and led to a reduction in the efficiency of HCV RNA replication. To overcome this disadvantage, we adopted the following strategies: (i) introduce the REMs; (ii) select cloned Li23-derived cells with a highly permissive host condition by the serial limiting-dilution method. For the first purpose, we performed sequence analyses for HCV RNA from JFH-1-infected RSc cells. Mutations in the region from Core to NS2 or NS5B enhanced HCV RNA replication. However, combination of mutations from two different regions reduced HCV RNA replication (Fig. S2). The reason for this may be that these two mutation clusters were obtained from distinct RT-PCR-amplified clones and they were not necessarily located on the same viral genome. It has been reported that the combination of REMs exhibited an antagonistic effect on HCV RNA replication (Lohmann et al., 2001). For the second purpose, we selected highly permissive Li23-derived clonal cells by the limiting-dilution method. We obtained three Li23-derived subclonal cell lines, L8c15, C22 and D7, in order from parental Li23-derived ORL8c cells. The efficiency of infectivity was highest in D7 cells, followed in order by C22, L8c15 and Li23 cells. D7 cells were highly permissive for infection of HCV with NS5B mutations.

As shown in Fig. 3(a), the histidine at aa 2505 in JFH-1 was conserved in the replication-competent O, 1B-4 and KAH5 strains at the corresponding position, aa 2482. The valine at aa 2995 in JFH-1 was alanine in strains O, 1B-4 and KAH5 at the corresponding position, aa 2972. The REMs in genotype 1b HCV were usually obtained by selection with neomycin after HCV RNA electroporation. Pietschmann et al. (2009) reported that REMs impaired infectious virus production. Most REMs are located in the NS3 and NS5A regions (Abe et al., 2007; Blight et al., 2002; Lohmann et al., 2001; Pietschmann et al., 2002). NS5A is a key molecule for virus production, and REMs affect the phosphorylation status of NS5A and the interaction with Core (Kato et al., 2008; Masaki et al., 2008; Tellinghuisen et al., 2008). In contrast, our REMs in NS5B were obtained in JFH-1-infected long-term cell culture without drug selection. Taking this information into account, we considered that H2505Q in NS5B might not interfere with genotype 1b virus production. We attempted to apply this REM from genotype 2a to genotype 1b and found that H2505Q enhanced replication of the genotype 1b HCV-O replicon. We are currently investigating whether our NS5B REM could enhance genotype 1b HCV production. As for the substitution at aa 2995 in JFH-1 (aa 2972 in genotype 1b), we should be careful in interpretation, because the backgrounds at this position are different between genotypes 2 and 1. Analysis of an HCV database (http://s2as02.genes. nig.ac.jp/) revealed that the consensus amino acids at position 2995 in genotype 2 and at 2972 in genotype 1 were valine and alanine, respectively. Furthermore, alanine and valine are not found at position 2995 in genotype 2 or at 2972 in genotype 1, respectively. These observations

indicate that amino acid substitution between alanine and valine at these positions may be lethal for HCV of both genotypes. The amino acid at position 2995 in genotype 2 (2972 in genotype 1) is just upstream of a cis-acting replication element in NS5B. Therefore, the nucleotide at this position may affect the HCV RNA replication. To clarify this issue, further study will be needed.

A comparative study using HuH-7- and Li23-based JFH-1 reporter-assay systems would be expected to reveal new information on virus entry and release steps, because the backgrounds of these cells are different. Our recent study of these cells revealed the difference in sensitivities to anti-HCV reagents including ribavirin and methotrexate (Mori et al., 2011; Ueda et al., 2011). Furthermore, the IL28B genotype was different between HuH-7 and Li23 cells. The IL28B genotype (rs8099917) of HuH-7 cells renders them resistant to pegylated IFN and ribavirin, and Li23 cells are sensitive to pegylated IFN and ribavirin (M. Ikeda and N. Kato, unpublished data).

Recently, it was reported that stable expression of miR122 enhanced JFH-1 HCV production in Hep3B and HepG2 (Kambara et al., 2012; Narbus et al., 2011). It is noteworthy that the expression of miR122 in Li23-derived cells was almost the same as that in HuH-7 cells (Fig. S3). High-level expression of miR122 in Li23 cells may be one of the reasons that Li23 cells can support HCV production as robust as that in HuH-7 cells among the hepatocyte-derived cell lines. Interestingly, the expression levels of miR122 are higher in ORL8c, L8c15 and D7 cells, but not in C22 cells, than those in parental Li23 cells (Fig. S3). This result suggests that the expression level of miR122 may partly contribute to the fitness of HCV replication and production.

So far, we have only little information regarding the mechanism by which subclonal cells support HCV replication and production more efficiently than the parental cells. In this study, we found that the expression levels of CLDN1 and NPC1L1 in Li23-derived subclonal cells were higher than those in the parental Li23 cells. These results suggest that a high expression level of these entry factors in Li23-derived subclonal cells may contribute to enhanced virus entry. In the course of the experiment to determine the expression levels of NPC1L1 in HuH-7- and Li23-derived cell lines, we found that RSc cells expressed a very low level of NPC1L1 compared with the parental HuH-7 cells. Possible mechanisms for this are: (i) very low-level expression of NPC1L1 is sufficient for HCV entry; (ii) an unknown entry factor compensates for NPC1L1 in the entry step in RSc cells. Further study will be needed to clarify this issue.

In summary, we have developed JHF-1 reporter-assay systems using HuH-7-derived RSc and Li23-derived D7 cells. Expression levels of CLDN1 and NP1C1L were higher than those in the parental Li23 cells. We found different effects of REMs (V2995L and H2505Q) in NS5B on virus RNA replication in genotype 2a and 1b HCV strains. These findings will become useful tools for the study of the life cycle of HCV.

METHODS

HCV infection and determination of f.f.u. To determine f.f.u. ml^{-1}, 6×10^3 cells were plated onto a 96-well plate 24 h before infection. The supernatant of HCV RNA-replicating cells was diluted serially and was used as an inoculum. Forty-eight hours after infection, the cells were fixed and Core was stained with anti-Core antibody and HRP-conjugated mouse anti-IgG antibody. Then, the expression of Core was visualized with a DAB substrate kit (DAKO). Culture supernatants and cells were collected for quantification of Core by ELISA (Mitsubishi Kagaku Bio-Clinical Laboratories).

Quantitative RT-PCR analysis. Quantitative RT-PCR analysis for HCV receptors was performed using real-time LightCycle PCR (Roche Diagnostics) as described previously (Ikeda *et al.*, 2005). The primer pairs for CD81, SR-BI, CLDN1 and OCLN were reported previously (Nakamuta *et al.*, 2011). The primer pair NPC1L1 (5′-AGATCTTCTTCTTCCGCCTCCA-3′) and NPC1L1R (5′-TGCCAG-AGCCGGGTTAAC-3′) was used for NPC1L1.

Statistical analysis. Luciferase activities were compared statistically between the various treatment groups using Student's *t*-test. *P*-values of <0.05 were considered statistically significant. The mean±SD was determined from at least three independent experiments.

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PML tumor suppressor protein is required for HCV production

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ABSTRACT

PML tumor suppressor protein, which forms discrete nuclear structures termed PML-nuclear bodies, has been associated with several cellular functions, including cell proliferation, apoptosis and antiviral defense. Recently, it was reported that the HCV core protein colocalizes with PML in PML-NBs and abrogates the PML function through interaction with PML. However, role(s) of PML in HCV life cycle is unknown. To test whether or not PML affects HCV life cycle, we examined the level of secreted HCV core and the infectivity of HCV in the culture supernatants as well as the level of HCV RNA in HuH-7-derived RSc cells, in which HCV-JFH1 can infect and efficiently replicate, stably expressing short hairpin RNA targeted to PML. In this context, the level of secreted HCV core and the infectivity in the supernatants from PML knockdown cells was remarkably reduced, whereas the level of HCV RNA in the PML knockdown cells was not significantly affected in spite of very effective knockdown of PML. In fact, we showed that PML is unrelated to HCV RNA replication using the subgenomic HCV-JFH1 replicon RNA, JRN/3-5B. Furthermore, the infectivity of HCV-like particle in the culture supernatants was significantly reduced in PML knockdown JRN/3-5B cells expressing core to NS2 coding region of HCV-JFH1 genome using the trans-packaging system. Finally, we also demonstrated that INI1 and DDX5, the PML-related proteins, are involved in HCV production. Taken together, these findings suggest that PML is required for HCV production.

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1. Introduction

Hepatitis C virus (HCV) is the causative agent of chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma. HCV is an enveloped virus with a positive single-stranded 9.6 kb RNA genome, which encodes a large polyprotein precursor of approximately 3000 amino acid residues. This polyprotein is cleaved by a combination of the host and viral proteases into at least 10 proteins in the following order: core, envelope 1 (E1), E2, p7, non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B [1,2]. HCV core protein forms a viral capsid and is essential for infectious virion production. The core protein is targeted to lipid droplets. Recently, lipid droplets have been found to be involved in an important cytoplasmic organelle for HCV production [3].

In addition, HCV core has been reported to facilitate cellular transformation as well as development of hepatocellular

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carcinoma in HCV core-transgenic mice [4]. Interactions of core with tumor suppressor proteins such as p53 and DDX3 may lead to enhanced cellular proliferation [4]. Indeed, HCV core interacts with promyelocytic leukemia (PML) protein and inhibits the PML tumor suppressor pathway through interfering with the PML-mediated apoptosis-inducing function [5]. PML forms discrete nuclear structures termed PML-nuclear bodies (PML-NBs) and associates with several cellular functions, including cell proliferation, apoptosis and antiviral defense [6,7]. In acute promyelocytic leukemia (APL) patient, the PML gene is fused with the retinoic acid receptor-α (RARα) gene, thus resulting in expression of an oncogenic PML-RARα fusion protein [6,7]. Conversely, treatment of APL patient with arsenic trioxide leads to reformation of PML-NBs and results in disease remission [6,7], indicating that PML is a target of arsenic trioxide. Interestingly, we have recently demonstrated that arsenic trioxide strongly inhibited HCV infection and HCV RNA replication without cell toxicity [8]. However, the role of PML in HCV life cycle yet remains unclear. To investigate the possible involvement of PML in HCV life cycle, we examined the accumulation of HCV RNA as well as the release of HCV core into culture

supernatants from cells rendered defective for PML by RNA interference. The results provide evidence that PML is required for HCV production.

2. Materials and methods

2.1. Cell culture

293FT cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS). The three HuH-7-derived cell lines: RSc cured cells that cell culture-generated HCV-JFH1 (JFH1 strain of genotype 2a) [9] could infect and effectively replicate [10–13], OR6c cells is cured cells of OR6 cells harboring the genome-length HCV-O RNA with luciferase as a reporter [14] or OR6c JRN/3-5B cells harboring the subgenome HCV-JFH1 RNA with luciferase as a reporter were cultured in DMEM with 10% FBS as described previously [13].

2.2. RNA interference

Oligonucleotides with the following sense and antisense sequences were used for the cloning of short hairpin RNA (shRNA)-encoding sequences targeted to DDX5 in a lentiviral vector: 5′-GATCCCCCTCTAATGTGGAGTGCGACTTCAAGAGAGTCGCACTCCACA TTAGAGTTTTTGGAAA-3′ (sense), 5′-AGCTTTTTCCAAAAACTCTAATGT GGAGTGCGACTCTCTTGAAGTCGCACTCCACATTAGAGGGG-3′ (antisense). The oligonucleotides above were annealed and subcloned into the BglII-HindIII site, downstream from an RNA polymerase III promoter of pSUPER [15], to generate pSUPER-DDX5i. To construct pLV-DDX5i, the BamHI-SalI fragments of the pSUPER-DDX5i were subcloned into the BamHI-SalI site of pRDI292, an HIV-1-derived self-inactivating lentiviral vector containing a puromycin resistance marker allowing for the selection of transduced cells [16]. We previously described pLV-PMLi [8] and pLV-INI1i [17], respectively.

2.3. Lentiviral vector production

The vesicular stomatitis virus (VSV)-G-pseudotyped HIV-1-based vector system has been described previously [18,19]. The lentiviral vector particles were produced by transient transfection of the second-generation packaging construct pCMV-ΔR8.91 [18,19] and the VSV-G-envelope-expressing plasmid pMDG2 as well as pLV-PMLi into 293FT cells with FuGene6 (Roche Diagnostics, Mannheim, Germany).

2.4. HCV infection experiments

The supernatants was collected from cell culture-generated HCV-JFH1-infected RSc cells at 5 days post-infection and stored at −80 °C after filtering through a 0.45 μm filter (Kurabo, Osaka, Japan) until use. For infection experiments with HCV-JFH1 virus or J6/JFH1 [20], RSc cells (5 × 10^4 cells/well) were plated onto 6-well plates and cultured for 24 h (hrs). We then infected the cells at a multiplicity of infection (MOI) of 0.05. The culture supernatants were collected at the indicated time post-infection and the levels of the core protein were determined by enzyme-linked immunosorbent assay (Mitsubishi Kagaku Bio-Clinical Laboratories, Tokyo, Japan). Total RNA was isolated from the infected cellular lysates using RNeasy mini kit (Qiagen, Hilden, Germany) for quantitative RT-PCR analysis of intracellular HCV RNA. The infectivity of HCV-JFH1 in the culture supernatants was determined by a focus-forming assay at 48 h post-infection.

2.5. Quantitative RT-PCR analysis

The quantitative RT-PCR analysis for HCV RNA was performed by real-time LightCycler PCR (Roche) as described previously [14]. We used the following forward and reverse primer sets for the real-time LightCycler PCR: PML, 5′-GAGGAGTTCCAGTTTCT GCG-3′ (forward), 5′-GCGCCTGGCAGATGGGCAC-3′ (reverse); DDX5, 5′-ATGTCGGGTTATTCGAGTGA-3′ (forward), 5′-TTTCTCC CCAGGGTTTCCAA-3′ (reverse); INI1, 5′-ATGATGATGATGGCGCTG AG-3′ (forward), 5′-TCGGAACATACGGAGGTAGT-3′ (reverse); β-actin, 5′-TGACGGGGTCACCCACACTG-3′ (forward), 5′-AAGCTGTAG CCGCGCTCGGT-3′ (reverse); and HCV-JFH1, 5′-AGAGCCATAGTGGT CTGCGG-3′ (forward), 5′-CTTTCGCAACCCAACGCTAC-3′ (reverse).

2.6. Western blot analysis

Cells were lysed in buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 4 mM EDTA, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride. Supernatants from these lysates were subjected to SDS–polyacrylamide gel electrophoresis, followed by immunoblot analysis using anti-HCV core (CP-9 and CP-11; Institute of Immunology, Tokyo, Japan) or anti-β-actin antibody (Sigma).

2.7. WST-1 assay

RSc or OR6c JRN/3-5B cells (1 × 10^3 cells/well) were plated onto 96-well plates and cultured. The cells were subjected to the WST-1 cell proliferation assay (Takara Bio, Otsu, Japan) according to the manufacturer's protocol. The absorbance was read using a microplate reader at 440 nm with a reference wavelength of 690 nm.

2.8. Renilla luciferase (RL) assay

OR6c JRN/3-5B cells (1.5 × 10^4 cells/well) were plated onto 24-well plates and cultured for 72 h, then, subjected to the RL assay according to the manufacturer's instructions (Promega, Madison, WI, USA). A lumat LB9507 luminometer (Berthold, Bad Wildbad, Germany) was used to detect RL activity.

2.9. RNA synthesis and transfection

Plasmid pJRN/3-5B was linearized by digestion with XbaI and was used for RNA synthesis with T7 MEGAscript (Ambion) as previously described [13]. In vitro transcribed RNA was transfected into OR6c cells by electroporation as described previously [14].

2.10. Immunofluorescence and confocal microscopic analysis

Cells were fixed in 3.6% formaldehyde in phosphate-buffered saline (PBS), permeabilized in 0.1% Nonidet P-40 in PBS at room temperature, and incubated with anti-PML antibody (PM001, MBL) and anti-HCV core at a 1:300 dilution in PBS containing 3% bovine serum albumin (BSA) at 37 °C for 30 min. They were then stained with anti-Cy3-conjugated anti-mouse antibody (Jackson Immuno-Research, West Grove, PA) or Alexa Fluor 647-conjugated anti-rabbit antibody (Molecular Probes, Invitrogen) at a 1:300 dilution in PBS containing BSA at 37 °C for 30 min. Lipid droplets and nuclei were stained with BODIPY 493/503 (Molecular Probes, Invitrogen) and DAPI (4′,6′-diamidino-2-phenylindole), respectively. Following extensive washing in PBS, the cells were mounted on slides using a mounting media of SlowFade Gold antifade reagent (Invitrogen) added to reduce fading. Samples were viewed under a confocal laser-scanning microscope (FV1000; Olympus, Tokyo, Japan).

3. Results

3.1. PML is involved in the propagation of HCV

To investigate the potential role(s) of PML in HCV life cycle, we first used lentiviral vector-mediated RNA interference to stably knockdown PML in HuH-7-derived RSc cured cells that HCV-JFH1 [9] could infect and effectively replicate [10–13]. Real-time RT-PCR analysis for PML demonstrated a very effective knockdown of PML in RSc cells transduced with lentiviral vector expressing shRNA targeted to PML (Fig. 1A). To test the cell toxicity of shRNA, we examined WST-1 assay. In spite of very effective knockdown of PML, we demonstrated that the shRNA targeted to PML did not affect the cell viabilities (Fig. 1B). We next examined the level of secreted HCV core and the infectivity of HCV in the culture supernatants as well as the level of HCV RNA in PML knockdown RSc cells 24, 48, or 72 h after HCV-JFH1 infection at an MOI of 0.05. The results showed that the level of HCV RNA in PML knockdown cells was not affected until 72 h post-infection (Fig. 1C), while the release of HCV core protein into the culture supernatants

was significantly suppressed in PML knockdown cells at 48 or 72 h post-infection (Fig. 1D). Consistent with this finding, the infectivity of HCV in the culture supernatants was also significantly suppressed in the PML knockdown cells at 48 or 72 h post-infection (Fig. 1E). We also obtained similar results using siRNA specific for human PML (siGENOME SMRT pool M-006547-01-0005, Dharmacon, Thermo Fisher Scientific, Waltham, MA) (data not shown). These results suggested that PML is associated with propagation of HCV.
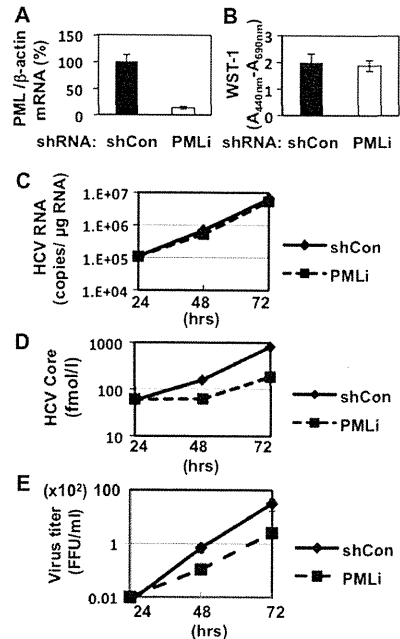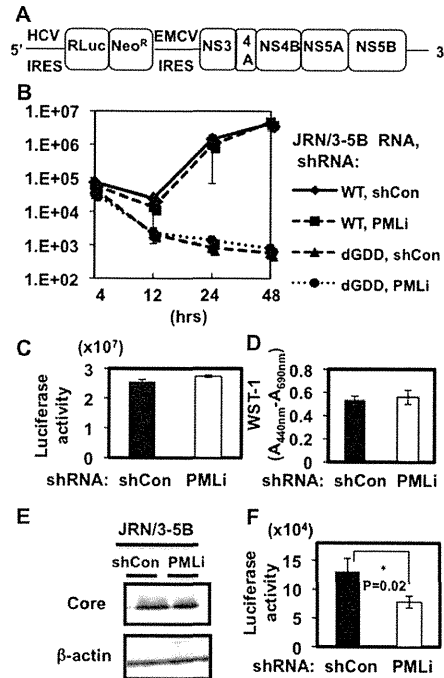

Fig. 1. PML is required for infectious HCV production. (A) Inhibition of PML mRNA expression by the shRNA-producing lentiviral vector. Real-time LightCycler RT-PCR for PML was performed as well as for β-actin mRNA. Each mRNA level was calculated relative to the level in RSc cells transduced with a control lentiviral vector (shCon) which was assigned as 100%. (B) WST-1 assay of the PML knockdown (PMLi) or the control (shCon) RSc cells. (C) The levels of intracellular genome-length HCV-JFH1 RNA in the PML knockdown or the control cells at 24, 48 or 72 h post-infection at an MOI of 0.05 were monitored by real-time LightCycler RT-PCR. (D) The levels of HCV core in the culture supernatants from the PML knockdown or the control RSc cells 24, 48 or 72 h after inoculation of HCV-JFH1 were determined by ELISA. (E) The infectivity of HCV in the culture supernatants was determined by a focus-forming assay at 48 h post-infection. All experiments were done in triplicate.

Fig. 2. PML is unrelated to the HCV RNA replication. Schematic gene organization of subgenomic JFH1 (JRN/3-5B) RNA encoding Renilla luciferase (RL) gene. Renilla luciferase gene (RLuc) is depicted as a box and is expressed as a fusion protein with Neo. (B) The transient replication of subgenomic HCV-JFH1 replicon in the PML knockdown (PMLi) or the control OR6c cells (shCon) after electroporation of in vitro transcribed JRN/3-5B RNA (10 μg) was monitored by RL assay at the indicated time. The results of Renilla luciferase activity are shown. dGDD indicates the deletion of the GDD motif in the NS5B polymerase, and the subgenomic HCV replicon with the deletion of GDD was used as a negative control. (C) The level of HCV RNA replication in PML knockdown (PMLi) or the control (shCon) OR6c JRN/3-5B cells was monitored by RL assay. The results shown are means from three independent experiments. (D) WST-1 assay of the PML knockdown or the control JRN/3-5B cells. (E) The level of HCV core protein in OR6c JRN/3-5B cells by expression of HCV core to NS2 coding region of HCV-JFH1 using mouse retroviral vector. pCX4bsr-JFH1-myc-C-NS2 and pMDG2 were cotransfected into Plat-E cells, mouse retroviral packaging cells. Mouse retroviral vector was obtained from their culture supernatants and transduced into OR6c JRN/3-5B PML knockdown or the control cells. The results of Western blot analysis of cellular lysates with anti-HCV core or an anti β-actin antibody are shown. (F) The level of HCV RNA replication in RSc cells 72 h after inoculation of HCV-like particles produced using trans-packaging system was monitored by RL assay. Asterisk indicates significant difference compared to the control. *P = 0.02.
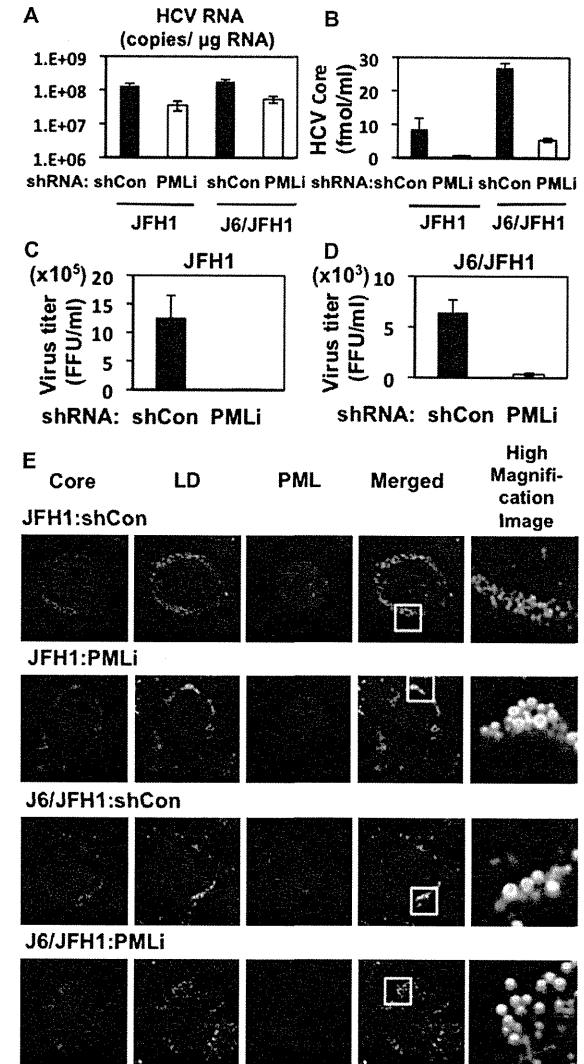
Fig. 3. PML is dispensable for the localization of HCV core to lipid droplet. (A) The levels of intracellular HCV RNA in PML knockdown or the control RSc cells 96 h after inoculation of HCV-JFH1 or HCV-J6/JFH1 were monitored by real-time LightCycler RT-PCR. Results from three independent experiments are shown (A–C). (B) The levels of HCV core in the culture supernatants from the PML knockdown RSc cells at 96 h post-infection were determined by ELISA. (C, D) The infectivity of HCV in the culture supernatants was determined by a focus-forming assay at 48 h post-infection. (E) HCV core localizes to lipid droplet (LD) in the PML knockdown (PMLi) or the control (shCon) cells after infection with either HCV-JFH1 or HCV-J6/JFH1. Cells were fixed 72 h post-infection and were then examined by confocal laser scanning microscopy.

3.2. PML is unrelated to HCV RNA replication

To examine whether or not PML is involved in HCV RNA replication, we used the subgenomic replicon RNA of HCV-JFH1, JRN/

3-5B, encoding Renilla luciferase gene for monitoring the HCV RNA replication (Fig. 2A). In vitro transcribed JRN/3-5B RNA was transfected into the PML knockdown OR6c cells by electroporation and we examined the luciferase activity. Consequently, the

luciferase activity in the PML knockdown cells was similar to that of the control cells (Fig. 2B), indicating that shRNA targeted to PML could not affect the transient HCV RNA replication. As well, the level of HCV RNA in PML knockdown HuH-7-derived OR6c JRN/3-5B cells harboring the subgenomic replicon RNA of HCV-JFH1 and the cell growth was not affected (Fig. 2C and D), suggesting that PML is unrelated to the HCV RNA replication. To further confirm whether or not PML is involved in HCV production, we used trans-packaging system [21,22], that HCV subgenomic replicon was efficiently encapsidated into infectious virus-like particles by expression of HCV core to NS2 coding region. In fact, infectious HCV-like particles were produced and released into the culture medium from PML knockdown JRN/3-5B cells stably expressing core to NS2 coding region of HCV-JFH1 genome by mouse retroviral vector (Fig. 2E). We could monitor the HCV RNA replication by Renilla luciferase assay in target naïve RSc cells after the inoculation of infectious HCV-like particles. Consequently, the release of infectious HCV-like particles into the culture supernatants was significantly suppressed in PML knockdown cells at 72 h post-infection (Fig. 2F). Thus, we conclude that PML is associated with HCV production.

3.3. PML is required for the late step in the HCV-JFH1 life cycle

To avoid the possibility of specific finding when we only used HCV-JFH1, we examined another strain of HCV-J6JFH1 [20]. For this, we analyzed the level of HCV core and the infectivity in the culture supernatant as well as the level of HCV RNA in the PML knockdown RSc cells 96 h after inoculation of HCV-J6/JFH1. In this context, the level of HCV RNA in PML knockdown cells was only somewhat decreased (Fig. 3A), while the level of core and the infectivity in the culture supernatants was remarkably reduced (Fig. 3B–D), indicating that PML is required for infectious HCV-J6/JFH1 production as well as HCV-JFH1.

Since lipid droplets have been shown to be involved in an important cytoplasmic organelle for HCV production [3], we performed immunofluorescence and confocal microscopic analyses to determine whether or not HCV core misses localization into lipid droplets in the PML knockdown cells. We found that the core protein was targeted into lipid droplets even in PML knockdown RSc cells as well as in the control RSc cells after infection with either HCV-JFH1 or HCV-J6/JFH1 (Fig. 3E). This suggests that PML plays a role in the late step after the core is targeted into lipid droplet in the HCV life cycle. Importantly, HCV did not disrupt the formation of PML-NBs in response to HCV infection (Fig. 3E) unlike HIV-1 and other DNA viruses [6,7,23].

3.4. INI1 and DDX5, PML-related proteins, are involved in HCV production

Finally, we established the INI1 or DDX5, PML-related protein [23,24] knockdown RSc or OR6c JRN/3-5B cells by lentiviral vector expressing shRNA target to INI1 [17] or DDX5 to examine potential role of INI1 and DDX5 in HCV life cycle. Consequently, we found that the release of HCV core or the infectivity of HCV into the culture supernatants was significantly suppressed in the INI1 or DDX5 knockdown RSc cells 96 h after HCV-JFH1 infection, while the RNA replication in the knockdown cells was only somewhat decreased in spite of the very effective knockdown of INI1 or DDX5 mRNA without growth inhibition (Fig. 4A–F), suggesting that INI1 and DDX5 are involved in HCV life cycle. To confirm whether or not these proteins are involved in HCV RNA replication, we examined the luciferase assay in the INI1 or DDX5 knockdown OR6c JRN/3-5B cells. In this context, the shRNA target to INI1 or DDX5 did not affect the luciferase activity and the cell growth in these
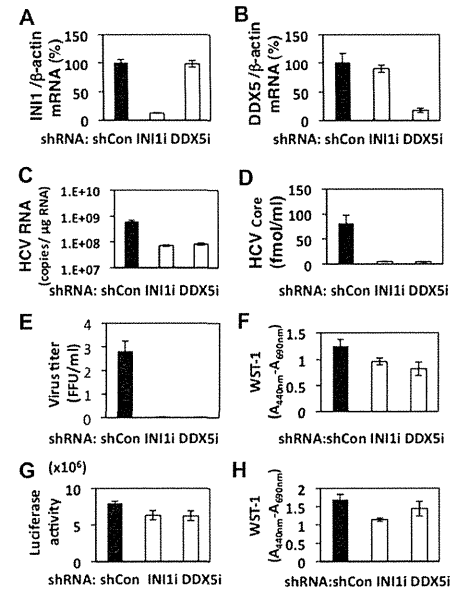
knockdown cells (Fig. 4G and H), suggesting that both INI1 and DDX5 are required for HCV production like PML.

4. Discussion

So far, the PML tumor suppressor protein, which forms PML-NBs, has been implicated in host antiviral defenses [6,7]. In fact, PML is induced by interferon after viral infection and suppresses some viral replication [6,7]. In contrast, PML-NBs are often disrupted or sequestered in the cytoplasm by infection with several DNA or RNA viruses to protect from the antiviral function of PML [6,7,23]. In case of HCV, Herzer et al. recently reported that the HCV core protein colocalizes with PML in PML-NBs and abrogates the PML function through interaction with PML isoform IV by over-expression studies [5]. However, we did not observe such colocalization of HCV core with PML and HCV did not affect the formation of PML-NBs in response to HCV-JFH1 infection (Fig. 3E). Interestingly, Watashi et al., previously demonstrated the HCV core modulates the retinoid signaling pathway through sequestration of

Sp110b, PML-related potent transcriptional corepressor of retinoic acid receptor, in the cytoplasm from nucleus [25].

In contrast, we have demonstrated that PML is required for infectious HCV production (Fig. 1). However, the molecular mechanism(s) how PML regulates HCV production yet remains unclear. At least, PML seems to be unrelated to the HCV RNA replication (Fig. 2). In this regard, several host factors including apolipoprotein E, components of ESCRT system, and PA28γ have been implicated in infectious HCV production [13,26,27]. Indeed, PA28γ, a proteasome activator, interacts with HCV core and affects nuclear retention and stability of the core protein. Importantly, PA28γ participates in the propagation of infectious HCV by regulation of degradation of the core protein [27]. Intriguingly, Zannini reported that PA28γ interacts with PML and Chk2 and affects PML-NBs number [28]. Accordingly, we demonstrated that ATM and Chk2, which phosphorylates PML and regulates the PML function, are involved in HCV life cycle [11]. In addition, other PML-related proteins such as INI1 and DDX5 seem to be involved in HCV production (Fig. 4). Indeed, INI1, also known as hSNF5, is incorporated into HIV-1 virion and is required for efficient HIV-1 production [29]. On the other hand, cytoplasmic PML may be involved in HCV production, since endoplasmic reticulum (ER) and lipid droplets are important cytoplasmic organelle for the HCV life cycle. In this regard, Giorgi et al. recently reported that cytoplasmic PML specifically enriches at ER [30], suggesting that cytoplasmic PML may be associated with HCV production. Altogether, the PML pathway seems to be involved in infectious HCV production.

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