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.

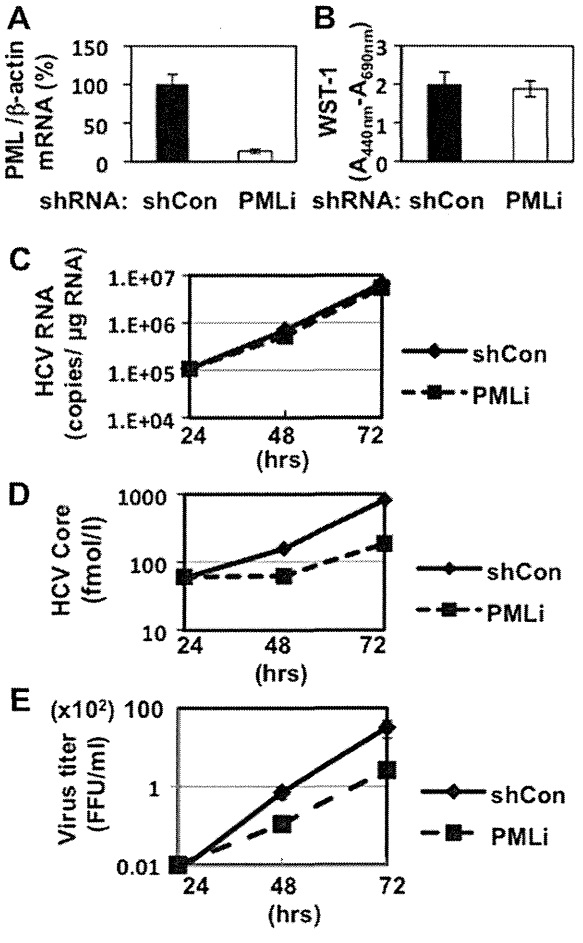

Fig. 1. PML is required for infectious HCV production. (A) Inhibition of PML mRNA expression by the shRNA-producing lentiviral vector. Real-time LightCycler RT-PCR for PML was performed as well as for β-actin mRNA. Each mRNA level was calculated relative to the level in RSc cells transduced with a control 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.

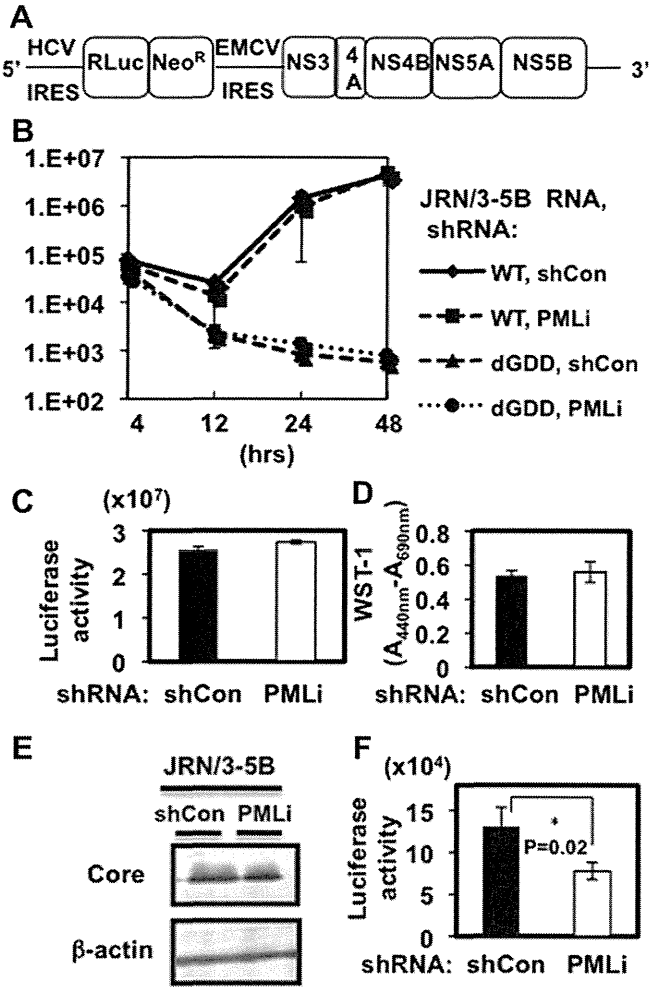

Fig. 2. PML is unrelated to the HCV RNA replication. Schematic gene organization of subgenomic JFH1 (JRN/3-5B) RNA encoding *Renilla* luciferase (RL) gene. *Renilla* luciferase gene (RLuc) is depicted as a box and is expressed as a fusion protein with Neo. (B) The transient replication of subgenomic 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. *$P = 0.02$.

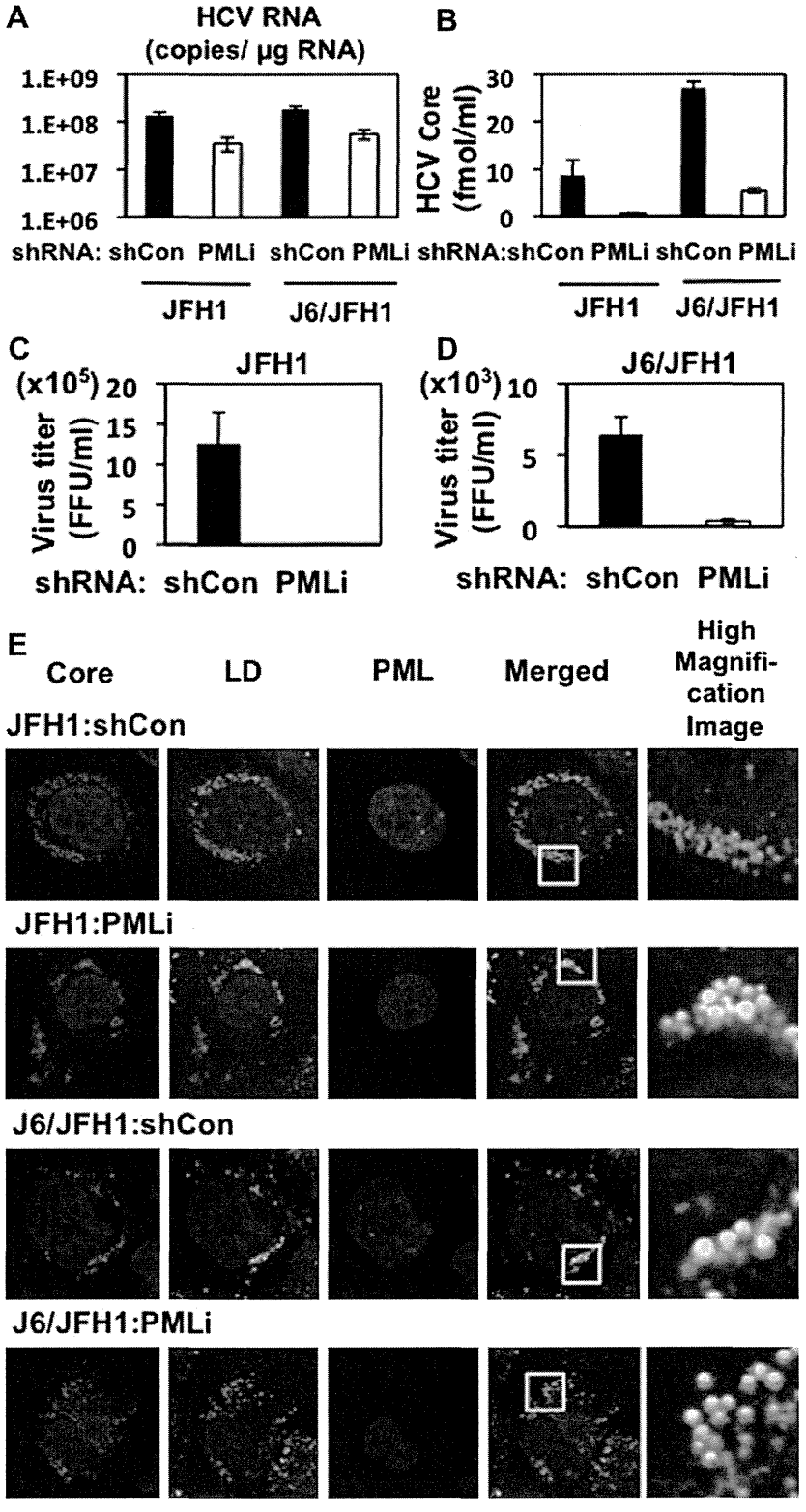

Fig. 3. PML is dispensable for the localization of HCV core to lipid droplet. (A) The levels of intracellular HCV RNA in PML knockdown or the control RSc cells 96 h after inoculation of HCV-JFH1 or HCV-J6/JFH1 were monitored by real-time LightCycler RT-PCR. Results from three independent experiments 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.

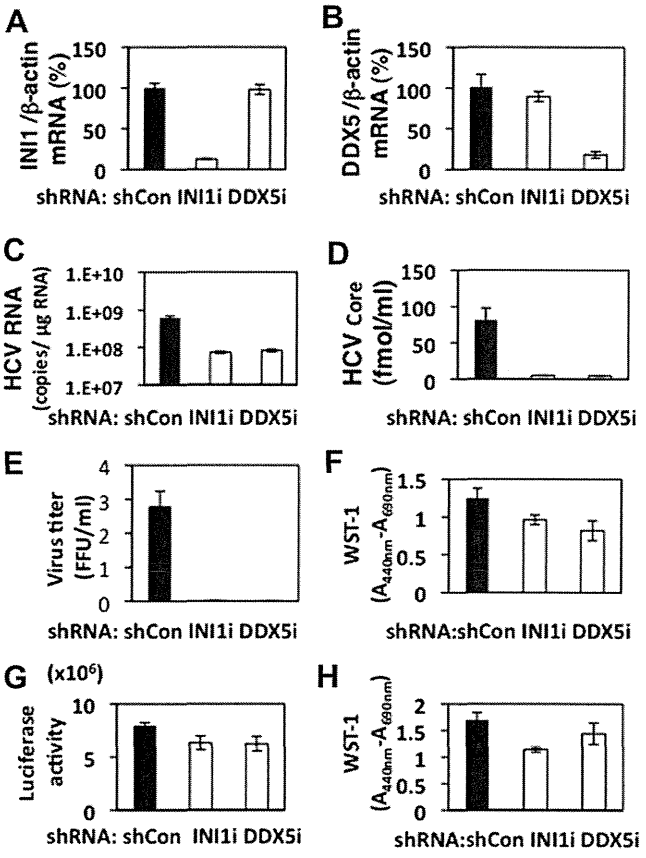

Fig. 4. INI1 and DDX5, PML-related proteins, are required for HCV production. (A, B) Inhibition of INI1 and DDX5 mRNA expressions by the shRNA-producing lentiviral vector. Real-time LightCycler RT-PCR for INI1 and DDX5 was performed as well as for β-actin mRNA in triplicate. Each mRNA level was calculated relative to the level in RSc cells transduced with a control lentiviral vector (Con) which was assigned as 100%. (C) The levels of intracellular genome-length HCV-JFH1 RNA in each knockdown cells at 96 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 INI1 (INI1i) or DDX5 knockdown (DDX5i) RSc cells 96 h after inoculation of HCV-JFH1 were determined by ELISA. (E) The infectivity of HCV-JFH1 in the culture supernatants was determined by a focus-forming assay at 48 h post-infection. Virus titer is shown as ($x10^7$) FFU/ml. (F) WST-1 assay of each knockdown RSc cells at 96 h post-infection. (G) The HCV RNA replication level in INI1 and DDX5 knockdown OR6c JRN/3-5B cells was monitored by RL assay. (H) WST-1 assay of each knockdown OR6c JRN/3-5B cells. All results shown are means from three independent experiments.

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.

Acknowledgments

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