CM did not induce MIP-1β expression significantly (Fig. 6E). We also observed only a marginal increase in MIP-1β mRNA level by solitary expression of each HCV protein in Huh7.5 cells by addition of LX2 CM (Fig. 6E).

Moreover, previous reports have suggested that ER stress, which is induced by HCV infection, leads to the generation of mature sterol regulatory element-binding protein 1 (SREBP-1) (36, 37) and that the mature SREBP-1c can bind to the C/EBPβ promoter (36, 38), leading to induction of C/EBPβ. Additionally, SREBP-1c promoter activity was upregulated in core-transgenic mice in a PA28γ-dependent manner (39). Indeed, we observed that endoplasmic reticulum (ER) stress-related genes and SREBP-1c expression were enhanced by HCV infection. However, induction of SREBP-1c is not involved in this process because HCV-infected cells that had SREBP-1c knocked down could still respond to HSC CM (data not shown).

Chronic inflammation is triggered by many events that also can increase the risk of developing cancer. For example, *Helicobacter pylori* is associated with gastric cancer, and inflammatory bowel disease is associated with colon cancer. In particular, IL-6 is one of the clinical targets for cancer-related inflammation (2). The level of IL-6 expression correlates with a rapid progression from hepatitis to hepatocellular carcinomas (40). The Ras and Jak/Stat pathway, which is downstream of the IL-6 receptor, is activated in hepatocellular carcinomas that have a poor prognosis (41). Additionally, IL-8 is a proinflammatory cytokine that specifically attracts and activates human neutrophils. In HCV patients, pegylated IFN-α-2a (PEG-IFN-α-2a) and ribavirin therapy decreased the neutrophil count in virologic responders compared to nonresponders (42). Enhancement of expression of these cytokines as well as MIP-1β, described here, suggests that the cross talk between HCV-infected hepatocytes and HSCs polarizes the cytokine profile toward a Th2-type immune response.

In conclusion, we have demonstrated that the cross talk between HSCs and HCV-infected hepatocytes results in the induction of inflammatory cytokines and chemokines, which promote the migration of CCR5-expressing cells in *in vitro* experiments. These results suggest that the induction of inflammatory cytokines and chemokines by HCV infection may recruit inflammatory cells such as cytotoxic T lymphocytes (CTL) and neutrophils to the liver, which induces liver cell injury leading to chronic hepatitis.

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## REFERENCES

1. **Bartosch B, Thimme R, Blum HE, Zoulim F.** 2009. Hepatitis C virus-induced hepatocarcinogenesis. J. Hepatol. **51**:810–820.
2. **Bowen DG, Walker CM.** 2005. Adaptive immune responses in acute and chronic hepatitis C virus infection. Nature **436**:946–952.
3. **Liaw YF, Lee CS, Tsai SL, Liaw BW, Chen TC, Sheen IS, Chu CM.** 1995. T-cell-mediated autologous hepatocytotoxicity in patients with chronic hepatitis C virus infection. Hepatology **22**:1368–1373.
4. **Shields PL, Morland CM, Salmon M, Qin S, Hubscher SG, Adams DH.** 1999. Chemokine and chemokine receptor interactions provide a mechanism for selective T cell recruitment to specific liver compartments within hepatitis C-infected liver. J. Immunol. **163**:6236–6243.
5. **Harvey CE, Post JJ, Palladinetti P, Freeman AJ, Ffrench RA, Kumar RK, Marinos G, Lloyd AR.** 2003. Expression of the chemokine IP-10 (CXCL10) by hepatocytes in chronic hepatitis C virus infection correlates with histological severity and lobular inflammation. J. Leukoc. Biol. **74**: 360–369.
6. **Napoli J, Bishop GA, McGuinness PH, Painter DM, McCaughan GW.** 1996. Progressive liver injury in chronic hepatitis C infection correlates with increased intrahepatic expression of Th1-associated cytokines. Hepatology **24**:759–765.
7. **Basu A, Meyer K, Lai KK, Saito K, Di Bisceglie AM, Grosso LE, Ray RB, Ray R.** 2006. Microarray analyses and molecular profiling of Stat3 signaling pathway induced by hepatitis C virus core protein in human hepatocytes. Virology **349**:347–358.
8. **Li K, Li NL, Wei D, Pfeffer SR, Fan M, Pfeffer LM.** 2012. Activation of chemokine and inflammatory cytokine response in hepatitis C virus-infected hepatocytes depends on Toll-like receptor 3 sensing of hepatitis C virus double-stranded RNA intermediates. Hepatology **55**:666–675.
9. **Wagoner J, Austin M, Green J, Imaizumi T, Casola A, Brasier A, Khabar KS, Wakita T, Gale M, Jr, Polyak SJ.** 2007. Regulation of CXCL-8 (interleukin-8) induction by double-stranded RNA signaling pathways during hepatitis C virus infection. J. Virol. **81**:309–318.
10. **Yu GY, He G, Li CY, Tang M, Grivennikov S, Tsai WT, Wu MS, Hsu CW, Tsai Y, Wang LH, Karin M.** 2012. Hepatic expression of HCV RNA-dependent RNA polymerase triggers innate immune signaling and cytokine production. Mol. Cell **48**:313–321.
11. **Friedman SL.** 2008. Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. Physiol. Rev. **88**:125–172.
12. **Friedman SL.** 2008. Mechanisms of hepatic fibrogenesis. Gastroenterology **134**:1655–1669.
13. **Hernandez-Gea V, Friedman SL.** 2011. Pathogenesis of liver fibrosis. Annu. Rev. Pathol. **6**:425–456.
14. **Coulouarn C, Corlu A, Glaise D, Guénon I, Thorgeirsson SS, Clément B.** 2012. Hepatocyte-stellate cell cross-talk in the liver engenders a permissive inflammatory microenvironment that drives progression in hepatocellular carcinoma. Cancer Res. **72**:2533–2542.
15. **Xu L, Hui AY, Albanis E, Arthur MJ, O'Byrne SM, Blaner WS, Mukherjee P, Friedman SL, Eng FJ.** 2005. Human hepatic stellate cell lines, LX-1 and LX-2: new tools for analysis of hepatic fibrosis. Gut **54**:142–151.
16. **Murakami K, Abe T, Miyazawa M, Yamaguchi M, Masuda T, Matsuura T, Nagamori S, Takeuchi K, Abe K, Kyogoku M.** 1995. Establishment of a new human cell line, LI90, exhibiting characteristics of hepatic Ito (fat-storing) cells. Lab. Invest. **72**:731–739.
17. **Zhang Z, Bryan JL, DeLassus E, Chang LW, Liao W, Sandell LJ.** 2010. CCAAT/enhancer-binding protein β and NF-κB mediate high level expression of chemokine genes CCL3 and CCL4 by human chondrocytes in response to IL-1β. J. Biol. Chem. **285**:33092–33103.
18. **Masaki T, Suzuki R, Murakami K, Aizaki H, Ishii K, Murayama A, Date T, Matsuura Y, Miyamura T, Wakita T, Suzuki T.** 2008. Interaction of hepatitis C virus nonstructural protein 5A with core protein is critical for the production of infectious virus particles. J. Virol. **82**:7964–7976.
19. **Freeman AJ, Pan Y, Harvey CE, Post JJ, Law MG, White PA, Rawlinson WD, Lloyd AR, Marinos G, Ffrench RA.** 2003. The presence of an intrahepatic cytotoxic T lymphocyte response is associated with low viral load in patients with chronic hepatitis C virus infection. J. Hepatol. **38**: 349–356.
20. **Zeremski M, Petrovic LM, Talal AH.** 2007. The role of chemokines as inflammatory mediators in chronic hepatitis C virus infection. J. Viral Hepat. **14**:675–687.
21. **Wald O, Weiss ID, Galun E, Peled A.** 2007. Chemokines in hepatitis C virus infection: pathogenesis, prognosis and therapeutics. Cytokine **39**: 50–62.
22. **Dolganiuc A, Oak S, Kodys K, Golenbock DT, Finberg RW, Kurt-Jones E, Szabo G.** 2004. Hepatitis C core and nonstructural 3 proteins trigger toll-like receptor 2-mediated pathways and inflammatory activation. Gastroenterology **127**:1513–1524.
23. **Abe T, Kaname Y, Hamamoto I, Tsuda Y, Wen X, Taguwa S, Moriishi**

K, Takeuchi O, Kawai T, Kanto T, Hayashi N, Akira S, Matsuura Y. 2007. Hepatitis C virus nonstructural protein 5A modulates the toll-like receptor-MyD88-dependent signaling pathway in macrophage cell lines. J. Virol. **81**:8953–8966.

24. **Kasprzak A, Zabel M, Biczysko W, Wysocki J, Adamek A, Spachacz R, Surdyk-Zasada J.** 2004. Expression of cytokines (TNF-alpha, IL-1alpha, and IL-2) in chronic hepatitis C: comparative hybridocytochemical and immunocytochemical study in children and adult patients. J. Histochem. Cytochem. **52**:29–38.
25. **Winwood PJ, Arthur MJ.** 1993. Kupffer cells: their activation and role in animal models of liver injury and human liver disease. Semin. Liver Dis. **13**:50–59.
26. **Wilkinson J, Radkowski M, Eschbacher JM, Laskus T.** 2010. Activation of brain macrophages/microglia cells in hepatitis C infection. Gut **59**: 1394–1400.
27. **Harrison JR, Kelly PL, Pilbeam CC.** 2000. Involvement of CCAAT enhancer binding protein transcription factors in the regulation of prostaglandin G/H synthase 2 expression by interleukin-1 in osteoblastic MC3T3-E1 cells. J. Bone Miner. Res. **15**:1138–1146.
28. **Akira S, Isshiki H, Sugita T, Tanabe O, Kinoshita S, Nishio Y, Nakajima T, Hirano T, Kishimoto T.** 1990. A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. EMBO. J. **9**:1897–1906.
29. **Nakajima T, Kinoshita S, Sasagawa T, Sasaki K, Naruto M, Kishimoto T, Akira S.** 1993. Phosphorylation at threonine-235 by a ras-dependent mitogen-activated protein kinase cascade is essential for transcription factor NF-IL6. Proc. Natl. Acad. Sci. U. S. A. **90**:2207–2211.
30. **Zhu S, Yoon K, Sterneck E, Johnson PF, Smart RC.** 2002. CCAAT/enhancer binding protein-beta is a mediator of keratinocyte survival and skin tumorigenesis involving oncogenic Ras signaling. Proc. Natl. Acad. Sci. U. S. A. **99**:207–212.
31. **Wegner M, Cao Z, Rosenfeld MG.** 1992. Calcium-regulated phosphorylation within the leucine zipper of C/EBP beta. Science **256**:370–373.
32. **Giltiay NV, Karakashian AA, Alimov AP, Ligthle S, Nikolova-Karakashian MN.** 2005. Ceramide- and ERK-dependent pathway for the activation of CCAAT/enhancer binding protein by interleukin-1beta in hepatocytes. J. Lipid Res. **46**:2497–2505.
33. **George A, Panda S, Kudmulwar D, Chhatbar SP, Nayak SC, Krishnan HH.** 2012. Hepatitis C virus NS5A binds to the mRNA cap-binding eukaryotic translation initiation 4F (eIF4F) complex and up-regulates host translation initiation machinery through eIF4E-binding protein 1 inactivation. J. Biol. Chem. **287**:5042–5058.
34. **Zhao LJ, Wang L, Ren H, Cao J, Li L, Ke JS, Qi ZT.** 2005. Hepatitis C virus E2 protein promotes human hepatoma cell proliferation through the MAPK/ERK signaling pathway via cellular receptors. Exp. Cell Res. **305**: 23–32.
35. **Qadri I, Choudhury M, Rahman SM, Knotts TA, Janssen RC, Schaack J, Iwahashi M, Puljak L, Simon FR, Kilic G, Fitz JG, Friedman JE.** 2012. Increased phosphoenolpyruvate carboxykinase gene expression and steatosis during hepatitis C virus subgenome replication: role of nonstructural component 5A and CCAAT/enhancer-binding protein β. J. Biol. Chem. **287**:37340–37351.
36. **Lei X, Zhang S, Barbour SE, Bohrer A, Ford EL, Koizumi A, Papa FR, Ramanadham S.** 2010. Spontaneous development of endoplasmic reticulum stress that can lead to diabetes mellitus is associated with higher calcium-independent phospholipase A2 expression: a role for regulation by SREBP-1. J. Biol. Chem. **285**:6693–6705.
37. **Joyce MA, Walters KA, Lamb SE, Yeh MM, Zhu LF, Kneteman N, Doyle JS, Katze MG, Tyrrell DL.** 2009. HCV induces oxidative and ER stress, and sensitizes infected cells to apoptosis in SCID/Alb-uPA mice. PLoS Pathog. **5**:e1000291. doi:10.1371/journal.ppat.1000291.
38. **Le Lay S, Lefrère I, Trautwein C, Dugail I, Krief S.** 2002. Insulin and sterol-regulatory element-binding protein-1c (SREBP-1C) regulation of gene expression in 3T3-L1 adipocytes. Identification of CCAAT/enhancer-binding protein beta as an SREBP-1C target. J. Biol. Chem. **277**: 35625–35634.
39. **Moriishi K, Mochizuki R, Moriya K, Miyamoto H, Mori Y, Abe T, Murata S, Tanaka K, Miyamura T, Suzuki T, Koike K, Matsuura Y.** 2007. Critical role of PA28gamma in hepatitis C virus-associated steatogenesis and hepatocarcinogenesis. Proc. Natl. Acad. Sci. U. S. A. **104**:1661–1666.
40. **Wong VW, Yu J, Cheng AS, Wong GL, Chan HY, Chu ES, Ng EK, Chan FK, Sung JJ, Chan HL.** 2009. High serum interleukin-6 level predicts future hepatocellular carcinoma development in patients with chronic hepatitis B. Int. J. Cancer **124**:2766–2770.
41. **Calvisi DF, Ladu S, Gorden A, Farina M, Conner EA, Lee JS, Factor VM, Thorgeirsson SS.** 2006. Ubiquitous activation of Ras and Jak/Stat pathways in human HCC. Gastroenterology **130**:1117–1128.
42. **Chung RT, Poordad FF, Hassanein T, Zhou X, Lentz E, Prabhakar A, Di Bisceglie AM.** 2010. Association of host pharmacodynamic effects with virologic response to pegylated interferon alfa-2a/ribavirin in chronic hepatitis C. Hepatology **52**:1906–1914.

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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.



## 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 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

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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′-GATCCCCCTCTAATGTGGAGTGCGACTTCAAGAGAGTCGCACTCCACATTAGAGTTTTTGGAAA-3′ (sense), 5′-AGCTTTTCCAAAAACTCTAATGTGGAGTGCGACTCTCTTGAAGTCGCACTCCACATTAGAGGGG-3′ (antisense). The oligonucleotides above were annealed and subcloned into the *Bgl*II-*Hind*III site, downstream from an RNA polymerase III promoter of pSUPER [15], to generate pSUPER-DDX5i. To construct pLV-DDX5i, the *Bam*HI-*Sal*I fragments of the pSUPER-DDX5i were subcloned into the *Bam*HI-*Sal*I 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 ($5 \times 10^4$ 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′-GAGGAGTTCCAGTTTCTGCG-3′ (forward), 5′-GCGCCTGGCAGATGGGGCAC-3′ (reverse); DDX5, 5′-ATGTCGGGTTATTCGAGTGA-3′ (forward), 5′-TTTCTCCCCAGGGTTTCCAA-3′ (reverse); INI1, 5′-ATGATGATGATGGCGCTGAG-3′ (forward), 5′-TCGGAACATACGGAGGTAGT-3′ (reverse); β-actin, 5′-TGACGGGGTCACCCACACTG-3′ (forward), 5′-AAGCTGTAGCCGCGCTCGGT-3′ (reverse); and HCV-JFH1, 5′-AGAGCCATAGTGGTCTGCGG-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 ($1 \times 10^3$ 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 ($1.5 \times 10^4$ 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 *Xba*I 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 ImmunoResearch, 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.

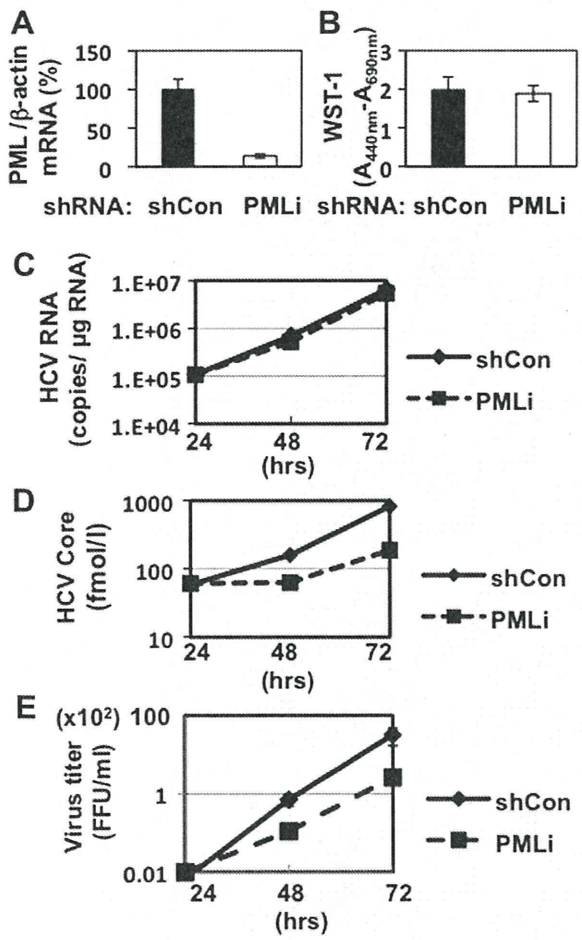


**Fig. 1.** PML is required for infectious HCV production. (A) Inhibition of PML mRNA expression by the shRNA-producing lentiviral vector. Real-time LightCycler RT-PCR for PML was performed as well as for β-actin mRNA. Each mRNA level was calculated relative to the level in RSc cells transduced with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

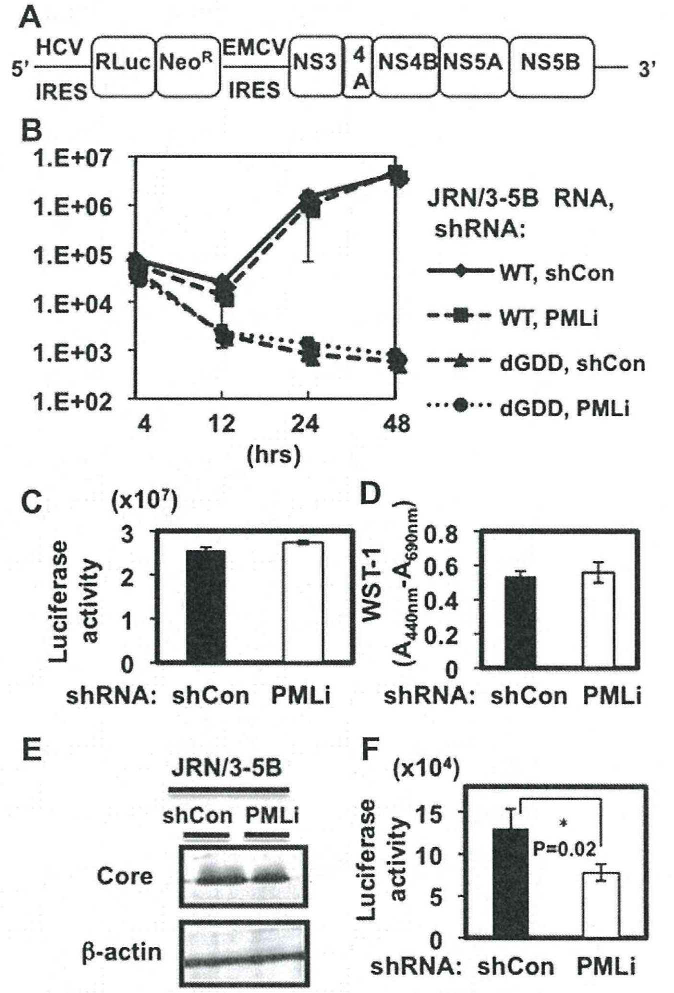


**Fig. 2.** PML is unrelated to the HCV RNA replication. Schematic gene organization of subgenomic JFH1 (JRN/3-5B) RNA encoding *Renilla* luciferase (RL) gene. *Renilla* luciferase gene (RLuc) is depicted as a box and is expressed as a fusion protein with Neo. (B) The transient replication of subgenomic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

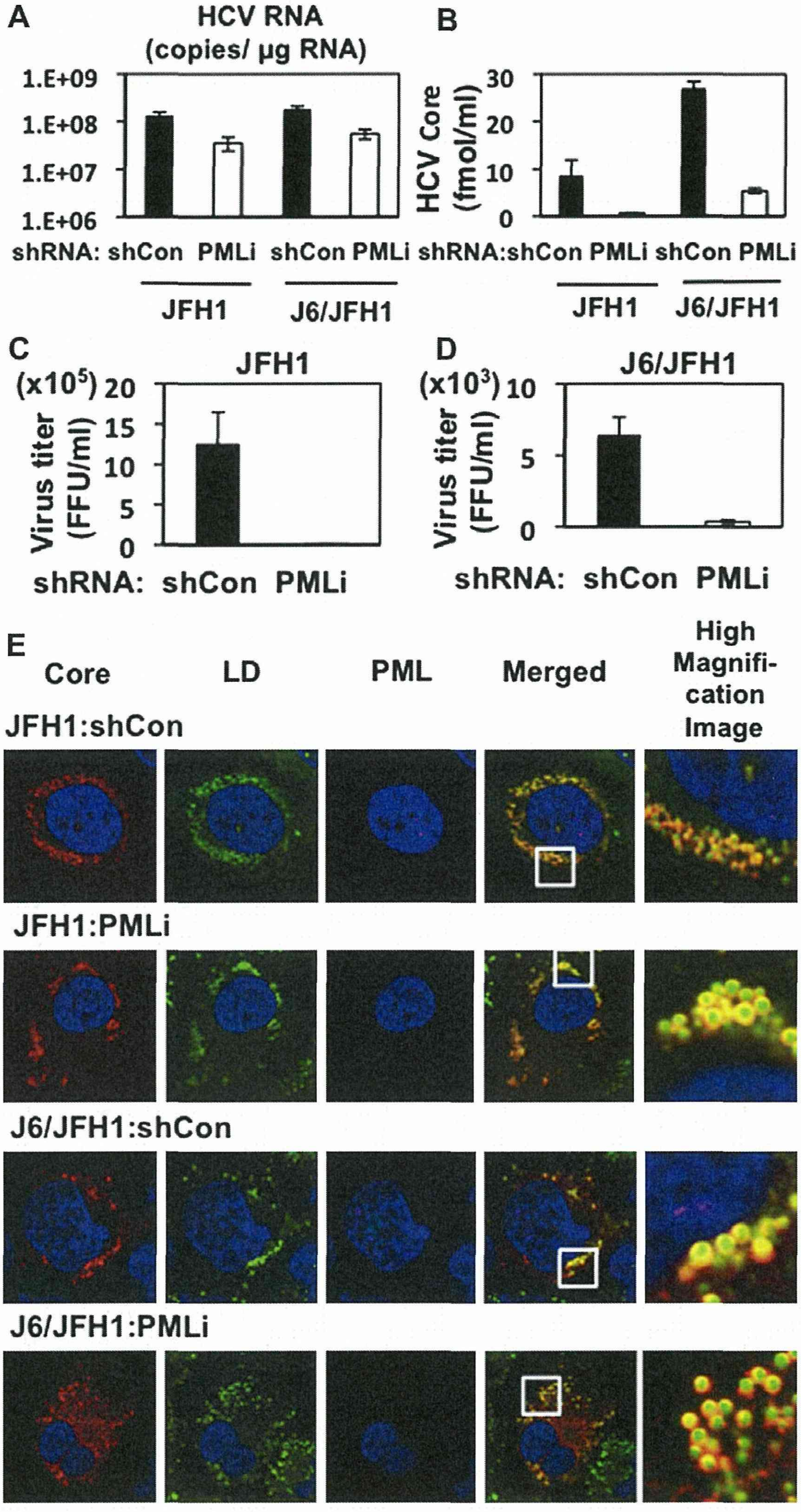


**Fig. 3.** PML is dispensable for the localization of HCV core to lipid droplet. (A) The levels of intracellular HCV RNA in PML knockdown or the control RSc cells 96 h after inoculation of HCV-JFH1 or HCV-J6/JFH1 were monitored by real-time LightCycler RT-PCR. Results from three independent experiments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

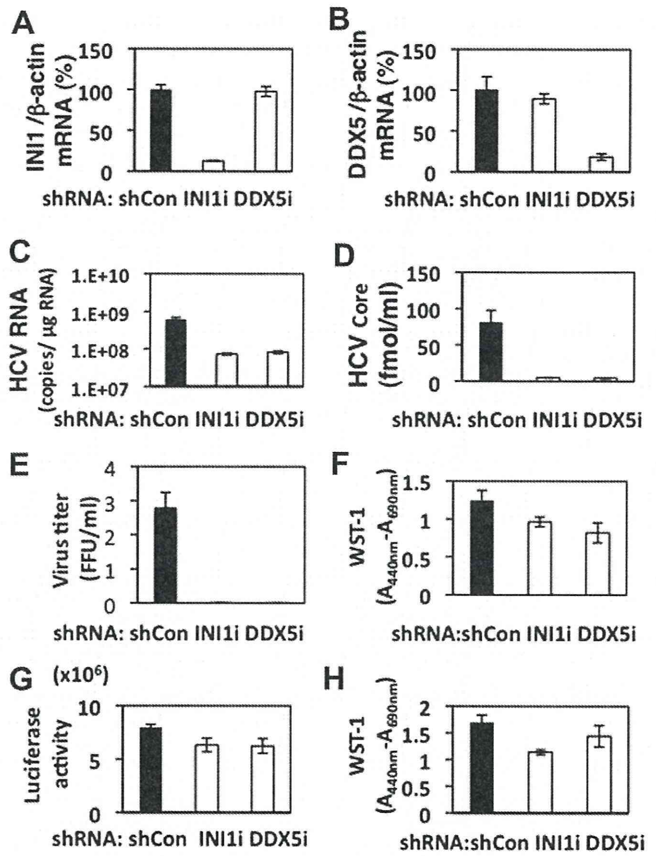


**Fig. 4.** INI1 and DDX5, PML-related proteins, are required for HCV production. (A, B) Inhibition of INI1 and DDX5 mRNA expressions by the shRNA-producing lentiviral vector. Real-time LightCycler RT-PCR for INI1 and DDX5 was performed as well as for β-actin mRNA in triplicate. Each mRNA level was calculated 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 ($\times 10^7$) 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.

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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## References

[1] N. Kato, Molecular virology of hepatitis C virus, Acta. Med. Okayama 55 (2001) 133–159.
[2] N. Kato, M. Hijikata, Y. Ootsuyama, et al., Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis, Proc. Natl. Acad. Sci. USA 87 (1990) 9524–9528.
[3] Y. Miyanari, K. Atsuzawa, N. Usuda, et al., The lipid droplet is an important organelle for hepatitis C virus production, Nat. Cell Biol. 9 (2007) 1089–1097.
[4] D.R. McGivern, S.M. Lemon, Tumor suppressors, chromosomal instability, and hepatitis C virus-associated liver cancer, Annu. Rev. Pathol.: Mech. Dis. 4 (2009) 399–415.
[5] K. Herzer, S. Weyer, P.H. Krammer, et al., Hepatitis C virus core protein inhibits tumor suppressor protein promyelocytic leukemia function in human hepatoma cells, Cancer Res. 65 (2005) 10830–10837.
[6] R.D. Everett, M.K. Chelbi-Alix, PML and PML nuclear bodies: implications in antiviral defence, Biochimie 89 (2007) 819–830.
[7] E.L. Reineke, H.Y. Kao, Targeting promyelocytic leukemia protein: a means to regulating PML nuclear bodies, Intl. J. Biol. Sci. 5 (2009) 366–376.
[8] M. Kuroki, Y. Ariumi, M. Ikeda, et al., Arsenic trioxide inhibits hepatitis C virus RNA replication through modulation of the glutathione redox system and oxidative stress, J. Virol. 83 (2009) 2338–2348.
[9] T. Wakita, T. Pietschmann, T. Kato, et al., Production of infectious hepatitis C virus in tissue culture from a cloned viral genome, Nat. Med. 11 (2005) 791–796.
[10] Y. Ariumi, M. Kuroki, K. Abe, et al., DDX3 DEAD-box RNA helicase is required for hepatitis C virus RNA replication, J. Virol. 81 (2007) 13922–13926.
[11] Y. Ariumi, M. Kuroki, H. Dansako, et al., The DNA damage sensors ataxia-telangiectasia mutated kinase and checkpoint kinase 2 are required for hepatitis C virus RNA replication, J. Virol. 82 (2008) 9639–9646.
[12] Y. Ariumi, M. Kuroki, Y. Kushima, et al., Hepatitis C virus hijacks P-body and stress granule components around lipid droplets, J. Virol. 85 (2011) 6882–6892.
[13] Y. Ariumi, M. Kuroki, M. Maki, et al., The ESCRT system is required for hepatitis C virus production, PLoS One 6 (2011) e14517.
[14] M. Ikeda, K. Abe, H. Dansako, et al., Efficient replication of a full-length hepatitis C virus genome, strain O, in cell culture, and development of a luciferase reporter system, Biochem. Biophys. Res. Co. 329 (2005) 1350–1359.
[15] T.P. Brummelkamp, R. Bernard, R. Agami, A system for stable expression of short interfering RNAs in mammalian cells, Science 296 (2002) 550–553.
[16] A.J. Bridge, S. Pebernard, A. Ducraux, et al., Induction of an interferon response by RNAi vectors in mammalian cells, Nat. Genet. 34 (2003) 263–264.
[17] Y. Ariumi, F. Serhan, P. Turelli, et al., The integrase interactor 1 (INI1) proteins facilitate Tat-mediated human immunodeficiency virus type 1 transcription, Retrovirology 3 (2006) 47.
[18] L. Naldini, U. Blömer, P. Gallay, et al., In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector, Science 272 (1996) 263–267.
[19] R. Zufferey, D. Nagy, R.J. Mandel, et al., Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo, Nat. Biotechnol. 15 (1997) 871–875.
[20] B.D. Lindenbach, M.J. Evans, A.J. Syder, et al., Complete replication of hepatitis C virus in cell culture, Science 309 (2005) 623–626.
[21] K. Ishii, K. Murakami, S.S. Hmwe, et al., Trans-encapsidation of hepatitis C virus subgenomic replicon RNA with viral structure proteins, Biochem. Biophys. Res. Co. 371 (2008) 446–450.
[22] E. Steinmann, C. Brohm, S. Kallis, et al., Efficient trans-encapsidation of hepatitis C virus RNAs into infectious virus-like particles, J. Virol. 82 (2008) 7034–7046.
[23] P. Turelli, V. Doucas, E. Craig, et al., Cytoplasmic recruitment of INI1 and PML on incoming HIV preintegration complexes: interference with early steps of viral replication, Mol. Cell 7 (2001) 1245–1254.
[24] G.J. Bates, S.M. Nicol, B.J. Wilson, et al., The DEAD box protein p68: a novel transcriptional coactivator of the p53 tumor suppressor, EMBO J. 24 (2005) 543–553.
[25] K. Watashi, M. Hijikata, A. Tagawa, et al., Modulation of retinoid signaling by a cytoplasmic viral protein via sequestration of Sp110b, a potent transcriptional corepressor of retinoic acid receptor, from the nucleus, Mol. Cell. Biol. 23 (2003) 7498–7509.
[26] K.S. Chang, J. Jiang, Z. Cai, et al., Human apolipoprotein E is required for infectivity and production of hepatitis C virus in cell culture, J. Virol. 81 (2007) 13783–13793.
[27] K. Moriishi, I. Shoji, Y. Mori, et al., Involvement of PA28γ in the propagation of hepatitis C virus, Hepatology 52 (2010) 411–420.
[28] L. Zannini, G. Buscemi, E. Fontanella, et al., REGγ/PA28γ proteasome activator interacts with PML and Chk2 and affects PML nuclear bodies number, Cell Cycle 8 (2009) 2399–2407.
[29] E. Yung, M. Sorin, A. Pal, et al., Inhibition of HIV-1 virion production by a transdominant mutant of integrase interactor 1, Nat. Med. 7 (2001) 920–926.
[30] C. Giorgi, K. Ito, H.K. Lin, et al., PML regulates apoptosis at endoplasmic reticulum by modulating calcium release, Science 330 (2010) 1247–1251.