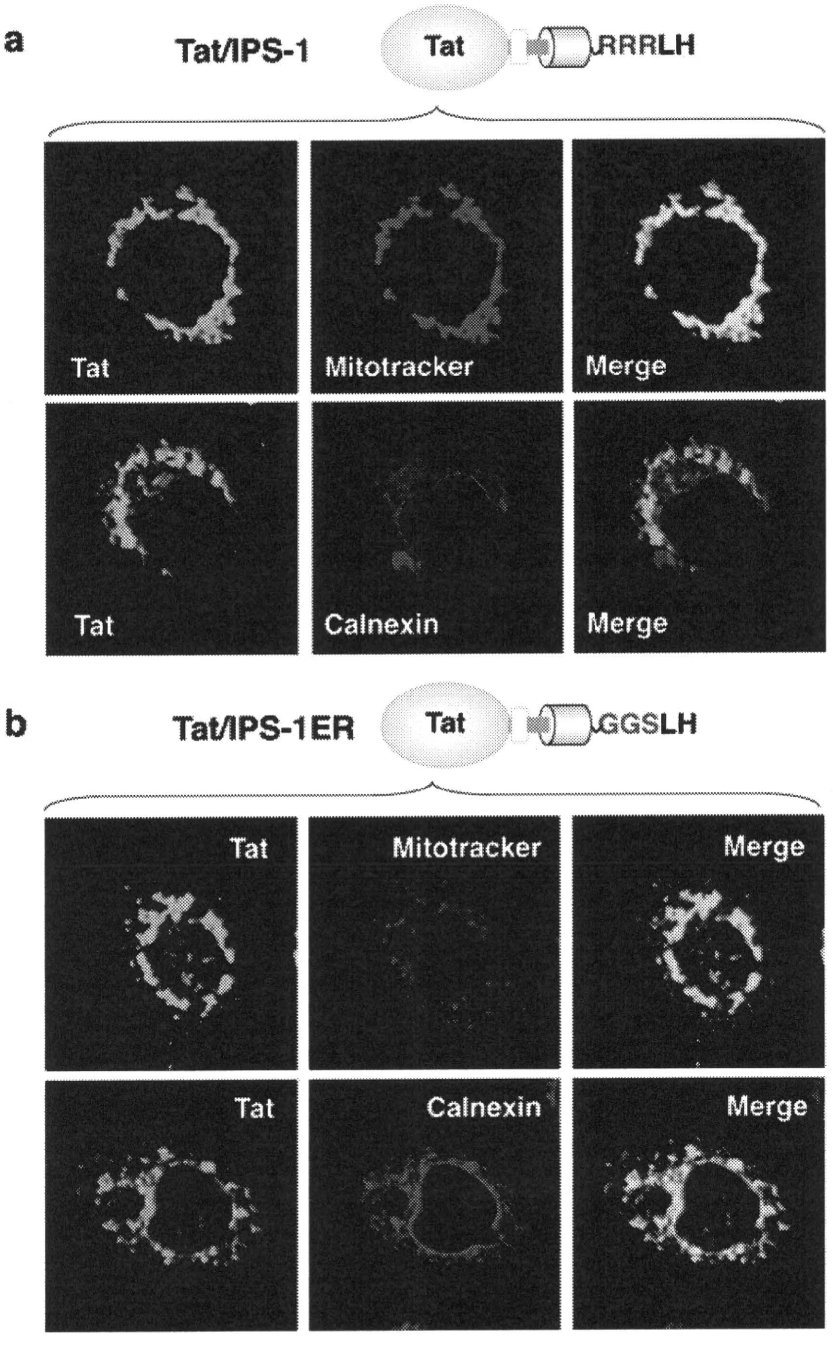

Fig. 2. Subcellular localization of the chimeric transcription factors. Huh7OK1 cells were transfected with expressing plasmids encoding (a) Tat/IPS-1 or (b) Tat/IPS-1ER. The subcellular localization of the Tat (green), mitochondrial and ER markers (Mitotracker and Calnexin, red), respectively, was observed by immunofluorescence assay.

Subcellular localization of the chimeric transcription factors

To determine the intracellular localization of Tat/IPS-1, Huh7OK1 cells were transfected with pCAG-Tat/IPS-1 and examined by immunofluorescent assay (Fig. 2a). Although Tat/IPS-1 was co-localized with the mitochondrial marker Mitotracker as we expected, expression of Tat/IPS-1 induced severe aggregation of mitochondria and cell death. Accumulation of artificial proteins on the mitochondria membrane may induce mitochondrial dysfunction that leads to cell death. Horie *et al.* have reported that the C-terminal net positive charge of C-tail-anchored proteins, such as Tom5, is crucial for mitochondrial targeting, and that mutants reducing the net charge are distributed throughout the intracellular membrane (32). Four out of

five C-terminal residues of IPS-1 (RRRLH) are positively charged (Fig. 1a). To modify the intracellular localization of Tat/IPS-1, we constructed an expression plasmid pCAG-Tat/IPS-1ER encoding Tat/IPS-1 possessing substitution of three arginine residues in the C-terminal five residues with non-charged amino acid glycine residues (GGGLH) (Fig. 1a) and examined the intracellular localization (Fig. 2b). Tat/IPS-1ER was co-localized with an ER marker Calnexin but not with Mitotracker and neither mitochondrial accumulation nor cell death was observed in the cells expressing Tat/IPS-1ER. These results indicate that Tat/IPS-1ER resides in the ER membrane and, unlike Tat/IPS-1, exhibits no severe cell toxicity. Therefore, we prepared Gal4-TBP/IPS-1ER in a similar way and used these ER-localized transcription factors in the following experiments.

Activation of chimeric transcription factors by HCV NS3/4A protease and establishment of indicator cell lines for HCV

To confirm that the chimeric transcription factors had been cleaved by NS3/4A protease, 293T cells were transfected with pCAG-Tat/IPS-1ER and/or pBIND-Gal4-TBP/IPS-1ER together with a plasmid encoding HCV NS3/4A protease or its defective mutant NS3/4A S139A (Fig. 3a). The cleaved products of Tat/IPS-1ER and Gal4-TBP/IPS-1ER were detected by anti-myc and anti-Gal4 DB antibodies, respectively, in cells co-expressing with NS3/4A but not with a S139A mutant. Immunofluorescent analyses revealed clear nuclear localization of the transcription factors cleaved off from the ER by expression of NS3/4A protease (Fig. 3b). Next, to examine whether or not reporter gene expression under the control of GAL4UAS and the HIV-1 LTR tandem promoter was dependent on the cleavage of transcription factors by HCV NS3/4A protease, Huh7OK1 cells were transfected with the plasmids encoding the transcription factors, pBIND-Gal4-TBP/IPS-1ER and pCAG-Tat/IPS-1ER, together with pGL4.31LTR-Luc, and luciferase activity was determined (Fig. 3c). No significant reporter gene activation was observed in cells co-transfected with pGL4.31LTR-Luc and pCAG-Tat/IPS-1ER irrespective of the expression of NS3/4A protease, and only a two-fold increase in luciferase was observed in cells co-transfected with pGL4.31LTR-Luc and pBIND-Gal4-TBP/IPS-1ER and then expressed with NS3/4A protease but not with NS3/4A S139A. Gal4-TBP and Tat have previously been shown to cooperatively accelerate initiation and elongation of the polymerase reaction (33). Synergistic enhancement (a more than forty-fold increase) of luciferase expression with a low background was achieved by expression of NS3/4A, but not by expression of NS3/4A S139A, in cells co-transfected with pGL4.31LTR-Luc, pCAG-Tat/IPS-1ER and pBIND-Gal4-TBP/IPS-1ER. In addition, specific induction of the reporter gene was detected in Huh9–13 cells harboring the subgenomic replicon of a genotype 1b Con1 strain (Fig. 3d). These results clearly indicate that the chimeric transcriptional factors, Tat/IPS-1ER and Gal4-TBP-1ER, were released from the ER membrane by HCV NS3/4A protease, and synergistically activated reporter gene expression under the control of GAL4UAS and the HIV-1 LTR tandem promoter.

To establish indicator cell lines for HCV, Huh7OK1 cells transfected with pIRES-TG encoding the ER-localized transcription factors (Tat/IPS-1ER and Gal4-TBP/IPS-1ER; Fig. 1a) were further transfected with pGL4.31LTR encoding reporter genes under the control of GAL4UAS and the HIV-1 LTR tandem promoter (Fig. 1b), and two indicator cell lines capable of detecting HCV propagation (Huh7OK1/TG-Luc and Huh7OK1/TG-LNGFR) were established (Fig. 1c).

Specific reporter gene activation upon infection with JFH-1 virus

To determine the specificity of reporter gene expression in the indicator cell lines in response to HCV infection, the Huh7OK1/TG-Luc cell line stably expressing Tat/IPS-1ER and Gal4-TBP/IPS-1ER transcription factors and carrying the luciferase gene under the control of the GAL4UAS and HIV-1 LTR promoter was infected with JFH-1 virus at an MOI of 0.0001 to 1.0, and the luciferase activity and intracellular viral RNA levels were determined at 96-hr post-infection. Luciferase activity exhibited a good correlation with HCV RNA in the range of 10^3 to 10^5 luciferase units ($R^2 = 0.98704$) (Fig. 4a). To confirm the specificity of reporter gene expression of the indicator cell line, Huh7OK1/TG-Luc cells were infected with JFH-1 virus, HCVrv (29), or JEV (a member of the family *Flaviviridae)* and the luciferase activities determined (Fig. 4b). Dose-dependent induction of luciferase was observed in cells infected with JFH-1 virus, but not in those with infected with HCVrv or JEV, suggesting specific detection of HCV replication by the indicator cell line. To further examine specific induction of the reporter gene upon infection with HCV, we assessed the effects of inhibitors of HCV entry or RNA replication on reporter gene expression (Fig. 4c). Luciferase activity was reduced in a dose-dependent manner by treatment with anti-CD81 antibody (34), but not with the isotype control, consistent with a previous observation (5). Its activity was also suppressed in a dose-dependent manner by treatment with Mβ-CD (35), IFN- α (36) and sera from hepatitis C patients, but not by sera from healthy donors. These results indicate that the Huh7OK1/TG-Luc cell line can be used as a reliable and convenient tool to

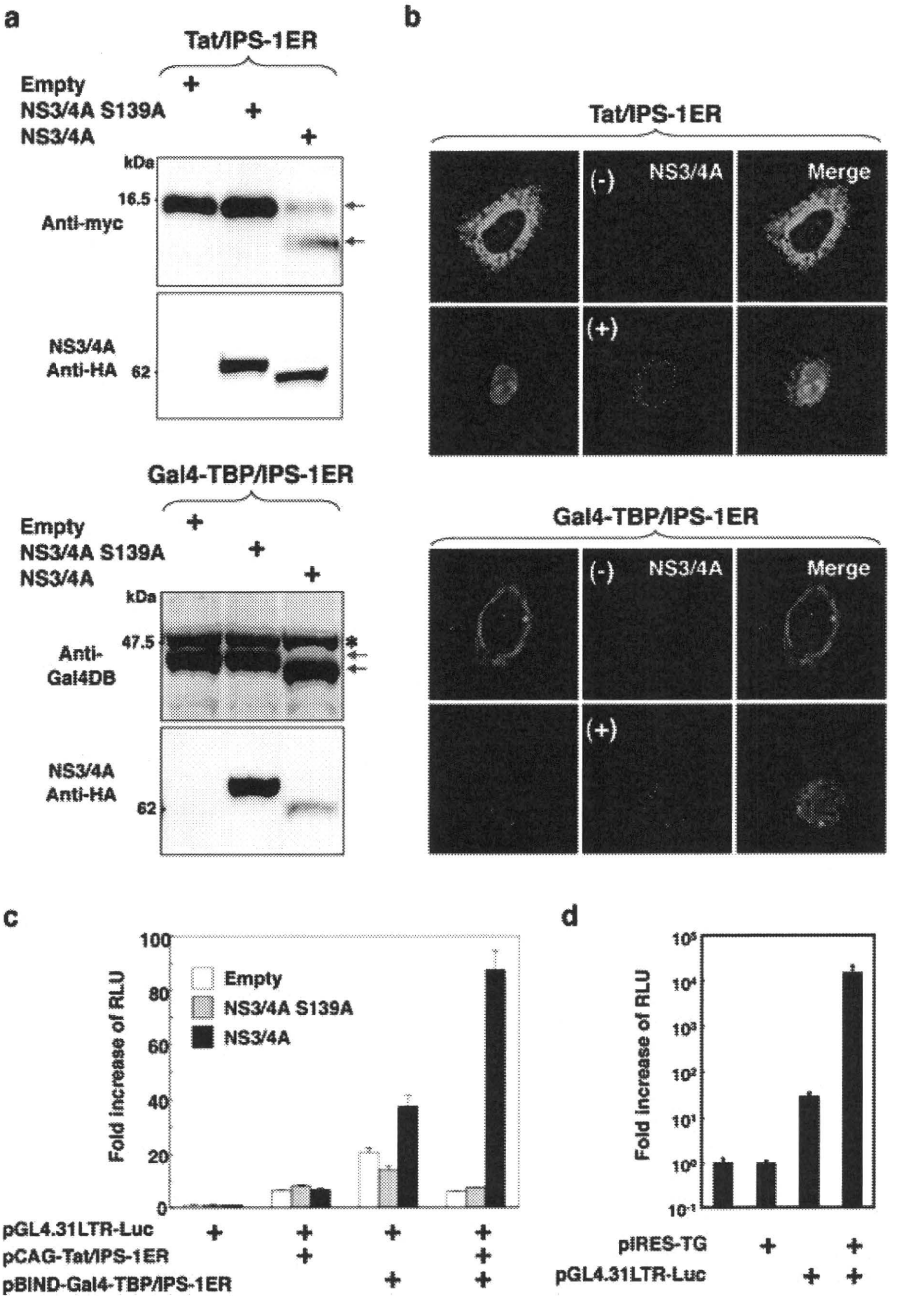

Fig. 3. Activation of the chimeric transcription factors by HCV NS3/4A protease. (**a**) Immunoblotting analyses of 293T cells expressing Tat/IPS-1ER or Gal4-TBP/IPS-1ER in the presence of the active or inactive form of HCV NS3/4A proteases. Tat/IPS-1ER, Gal4-TBP/IPS-1ER and HA-tagged NS3/4A were detected with anti-myc, anti-Gal4 DB and anti-HA antibody, respectively. *, non-specific bands recognized by anti-Gal4 DB antibody; blue arrows, processed transcription factors; red arrows, unprocessed transcription factors. (**b**) Nuclear localization of the chimeric transcription factors after processing by NS3/4A protease. Huh7OK1 cells were transfected with the expression plasmids of Tat/IPS-1ER (upper half of panels) or Gal4-TBP/IPS-1ER (lower half of panels) together. Cells were fixed and stained with appropriate antibodies at 48-hr post-transfection. (**c**) Huh7OK1 cells were transfected with pGL4.31LTR-Luc, pBIND-Gal4-TBP/IPS-1ER and pCAG-Tat/IPS-1ER in various combinations, together with pGL4.31LTR-Luc and pTK-Rluc, and luciferase activity was determined by a dual luciferase assay at 48-hr post-transfection. The relative luciferase activity was determined after standardization with the expression of the *Renilla* luciferase. The value of the cells transfected with the empty vectors was defined as 1. +, with a plasmid for NS3/4A protease; −, without a plasmid for NS3/4A protease. (**d**) Huh 9–13 cells harboring a subgenomic replicon derived from a genotype 1b Con1 strain were transfected with pIRES-TG, pGL4.31LTR-Luc as indicated combination, together with pTK-Rluc. The relative luciferase activity was determined after standardization with the expression of *Renilla* luciferase. The value of the cells transfected with the empty vectors was defined as 1.

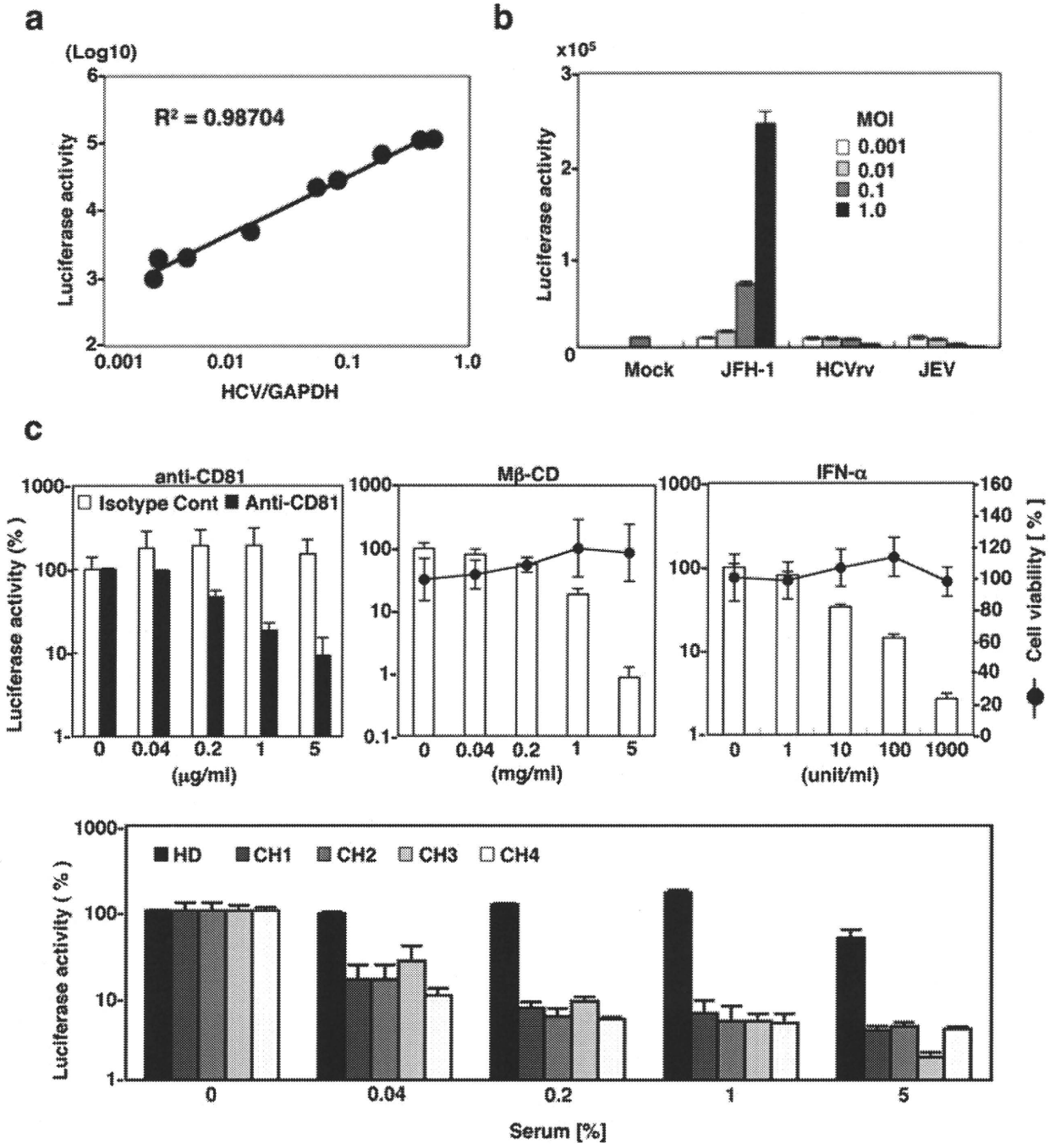

Fig. 4. Luciferase expression in the Huh7OK1/TG-Luc cell line upon infection with JFH-1 virus. (**a**) Huh7OK1/TG-Luc cells were infected with JFH-1 virus at an MOI 0.0001 to 1.0, and luciferase activity and intracellular viral RNA levels were determined at 96-hr post-infection. (**b**) Huh7OK1/TG-Luc cells were inoculated with JFH-1 virus, HCVrv or JEV at an MOI of 0.001 to 1, and luciferase activities were determined at 48-hr post-infection. (**c**) For neutralization by anti-CD81 antibody, Huh7OK1/TG-Luc cells were pre-treated with anti-CD81 monoclonal antibody or with an isotype control IgG for 1 hr before infection (upper left panel), and luciferase activity was determined at 3 days of incubation. The cells were also pre-treated with Mβ-CD (upper middle panel) for 1hr before infection. The cells were also incubated with JFH-1 virus together with IFN-α (upper right panel) or HCV patient sera (lower panel) at the indicated concentrations, and luciferase activity was determined at 4 days of incubation. The value of luciferase activity in untreated cells was defined as 100%. Cell viability was determined by CellTiter 96 AQueous One Solution Cell Proliferation Assay. HD, serum from healthy donor; CH1, CH2, CH3 or CH4, serum from each chronic hepatitis C patient.

detect and quantify the propagation of HCV based on luciferase expression.

Establishment of an indicator cell line capable of expressing LNGFR upon infection with HCV

To develop an indicator line capable of selectively collecting HCV-infected cells based on expression of cell surface molecules, we established an Huh7OK1/TG-LNGFR cell line possessing the LNGFR gene under the control of the GAL4UAS and HIV-1 LTR tandem promoter. LNGFR is mainly expressed in the cells of nervous systems, but not in hepatocytes, and immunomagnetic cell sorting systems have been proven to be effective for separating LNGFR-positive cells (37, 38). Expression of LNGFR was induced in Huh7OK1/TG-LNGFR cells upon infection with JFH-1 virus, whereas no expression of LNGFR was detected in uninfected Huh7OK1/TG-LNGFR cells or Huh7OK1/TG-Luc cells infected with JFH-1 virus (Fig. 5a), indicating that Huh7OK1/TG-LNGFR cells can specifically induce expression of LNGFR upon infection with HCV. Next, to demonstrate the ability of the system to isolate HCV-infected cells, Huh7OK1/TG-LNGFR cells were infected with JFH-1 virus at an MOI of 0.1 and sorted by anti-LNGFR antibody-coated magnetic beads at 4 days post-infection. The proportions of cells exhibiting positivity for only NS5A or only LNGFR, or double-positivity for both proteins before sorting were determined to be 14.17%, 5.99% and 3.20% of the total cell population, respectively (Fig. 5b middle panel). The reason for not all HCV-infected cells inducing expression of LNGFR remains unclear at this stage, but it might be because of either a difference in the sensitivity of the antibodies used for detection of NS5A and LNGFR or the presence of an unknown inhibitory mechanism of LNGFR expression on the cell surface. After sorting, the proportion of NS5A and LNGFR double-positive cells was increased to up to 48.05% of the recovered cells inoculated with JFH-1 virus (Fig. 5b right panel). These results suggest that the indicator cell system capable of expressing LNGFR upon infection with HCV is effective for concentrating HCV-infected cells.

Application of Huh7OK1/TG-Luc cells for screening of anti-HCV compounds

To determine the ability of the indicator cell lines to screen anti-HCV compounds, approximately 1300 chemical compounds were obtained from the library of the Chemical Biology Research Initiative at the University of Tokyo and screened by using Huh7OK1/TG-Luc cells. Cells seeded in 96-well plates (5000 cells/well) were incubated with culture medium containing JFH-1 virus at an MOI of 1.0 and various concentrations of each compound, and luciferase activities were determined after 4 days of incubation. Two of the compounds we examined, fiduxosin hydrochloride and flunarizine dihydrochloride, exhibited significant reduction of luciferase expression without severe cell toxicity (Fig. 6a). The antiviral activities of these compounds were confirmed by the reduction of HCV RNA in the cells in a dose-dependent manner (Fig. 6b). Although fiduxosin and flunarizine are known to be antagonists of alpha-1 adrenoceptor (39) and T-type calcium ion channels (40), respectively, the mechanisms by which they inhibit HCV propagation are not known. These results indicate that the Huh7OK1/TG-Luc cell line is applicable for a reliable cell-based assay for high-throughput screening of anti-HCV drugs.

DISCUSSION

The establishment of indicator cell lines based on MAGI assays (30) has made a great contribution to our understanding of HIV-1 life cycles and the development of anti-HIV-1 reagents (41, 42). In this study, we have established indicator cell lines for HCV infection in the same manner using a MAGI assay. To establish indicator cell lines for HCV, HCV NS3/4A-mediated trans-activation of reporter gene systems based on chimeric transcription factors containing the cleavage sequence of the NS3/4A protease derived from HCV polyprotein have been reported (19–21). Breiman *et al.* (21) reported construction of a chimeric transcription factor consisting of a fusion protein of the Gal4 DB domain with the VP16 trans-activator protein of herpes simplex virus (Gal4VP16), NS3/4A protease-cleavage sequences between HCV NS5A and NS5B proteins and a portion of the ER-resident protein PERK for anchoring at the ER membrane. Upon infection with HCV, the Gal4VP16 was cleaved off by the NS3/4A protease and activated the transcription of reporter genes. NS3/4A protease-cleavage sites from NS4A to NS5B junctions were inserted between green fluorescent protein and SEAP, and HCV infection was quantified by the SEAP activity secreted into culture supernatants (19, 20). However, in the previous studies these chimeric constructs containing NS3/4A protease-cleavage sites derived from HCV proteins exhibited only partial cleavage upon co-expression of NS3/4A protease, suggesting that processing of the HCV sequences in the fusion proteins is not efficiently recognized by HCV protease.

To overcome this obstacle, we utilized the C-terminal portion of the mitochondria-resident IPS-1 as a transmembrane anchor of the chimeric transcriptional factors. It has been reported that cleavages of IPS-1 are observed in cell cultures by expression of NS3/4A derived from several HCV strains of different genotypes (43) and also in the liver tissue of patients with chronic hepatitis C

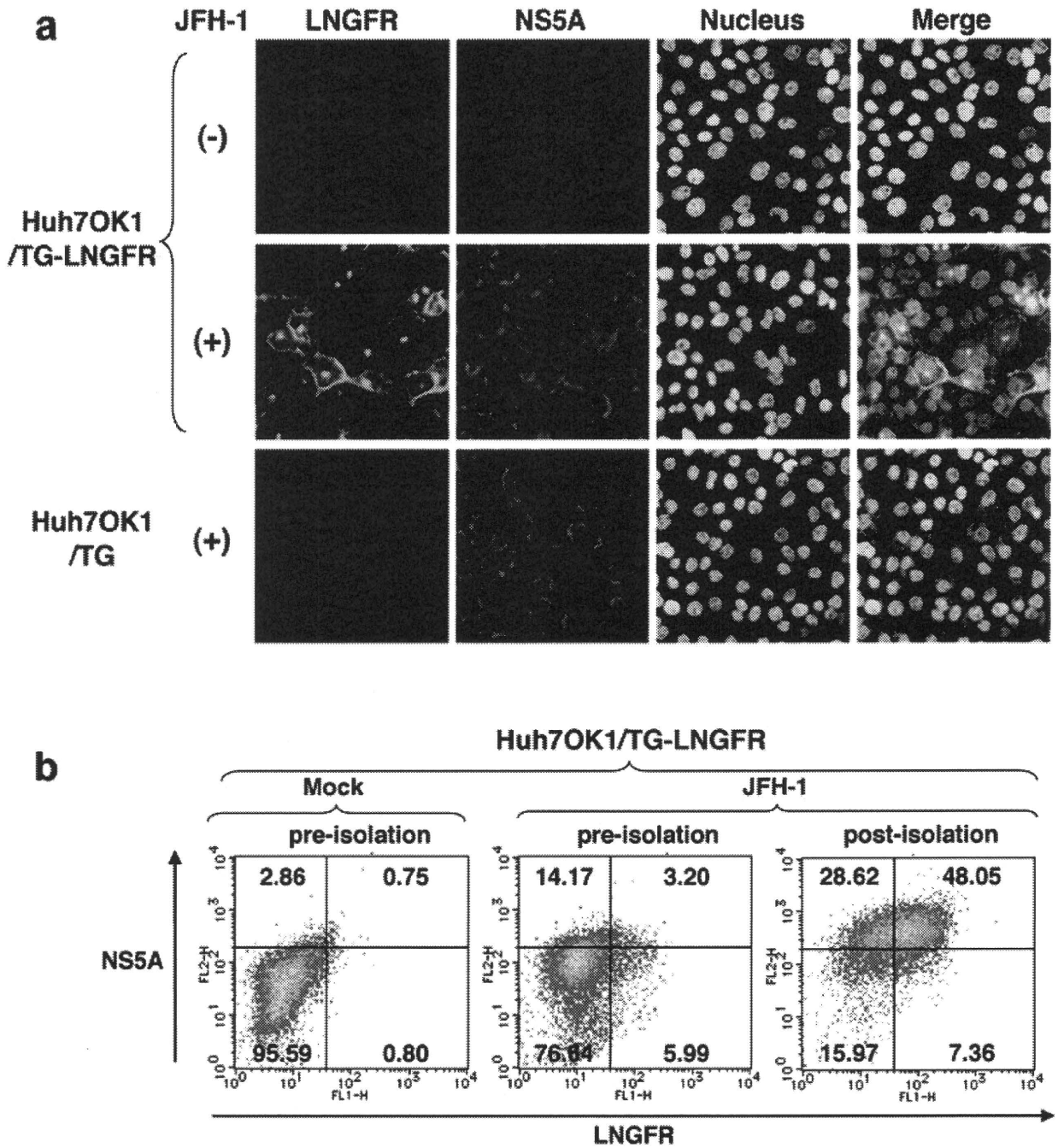

Fig. 5. Isolation of HCV-infected cells expressing LNGFR by immunomagnetic cell sorting. (**a**) Huh7OK1/TG-LNGFR cells or Huh7OK1/TG cells were infected with JFH-1 virus at an MOI of 0.1, and the expressions of LNGFR and of HCV NS5A were determined by immunofluorescence assay using anti-LNGFR and anti-NS5A antibodies, respectively. Nuclei were stained by TO-PRO-3 iodide (cyan). (**b**) Huh7OK1/TG-LNGFR cells were inoculated with JFH-1 virus at an MOI of 0.1 and subjected to MACS separation using anti-LNGFR magnetic beads at 4 days post-infection. LNGFR- and NS5A-positive cells were analyzed by flow cytometry before and after isolation by MACS. Green, expression of LNGFR; red, expression of HCV NS5A.

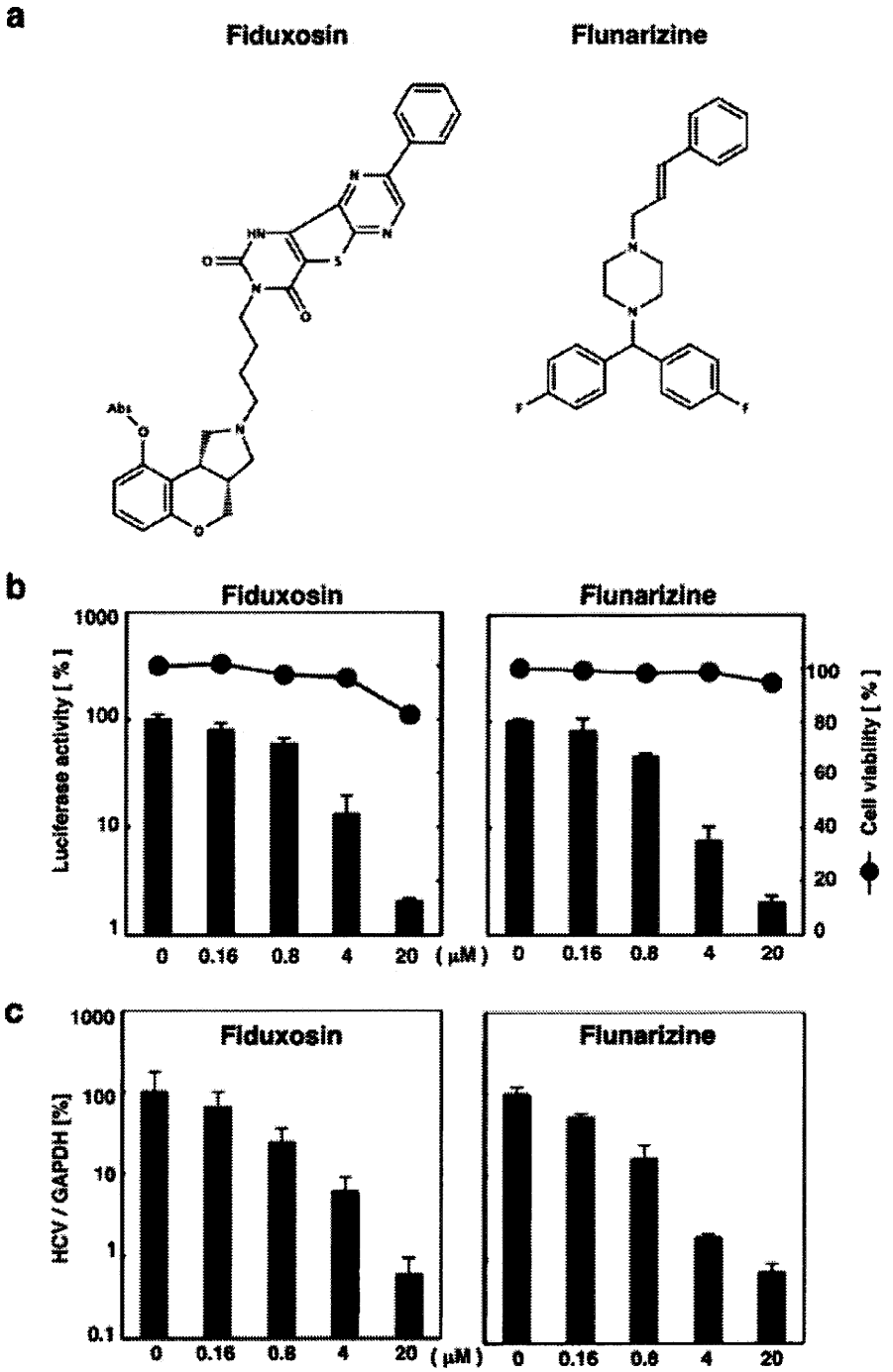

Fig. 6. Application of Huh7OK1/TG-Luc cells for screening of anti-HCV compounds. (**a**) Chemical structures of fiduxosin hydrochloride and flunarizine dihydrochloride. (**b**) Huh7OK1/TG-Luc cells were incubated with JFH-1 virus at an MOI of 1.0 together with each compound at a concentration of 0, 0.16, 0.8, 4 or 20 μM, and the cell viability and luciferase activity were determined at 4 days post-inoculation. The values of cell viability and luciferase activity in untreated cells were defined as 100%. (**c**) Total RNA was prepared from the cells and HCV RNA and GAPDH mRNA were measured by real-time quantitative PCR. The relative value of HCV RNA was determined by normalization of GAPDH mRNA. The value of HCV RNA/GAPDH RNA in untreated cells was defined as 100%.

infection (43), suggesting that chimeric constructs fused with the C-terminal region of IPS-1 can be cleaved by NS3/4A protease in a broad range of HCV genotypes. Furthermore, to avoid induction of mitochondrial dysfunction and cell death, we modified the C-terminal residues of IPS-1of the chimeric transcription factors to achieve localization on the ER membrane. HCV NS3/4A is an ER-membrane associated protease, and subcellular localization and distance of the cleavage site of the substrates from the membrane could be crucial for efficient processing. Judging from the processing of the Tat/IPS-1ER and Gal4TBP/IPS-1ER proteins by co-expression of NS3/4A protease, the ER-anchored C-terminal domain of IPS-1 was efficiently cleaved by NS3/4A protease.

One of the difficulties in development of indicator cells is a low S/N ratio. When we examined the combination of Tat/IPS-1ER and an HIV-1 LTR promoter, a high background of luciferase expression was observed, probably due to spontaneous release of an undetectable population of the transcription factor from the ER and contingent activation of the target promoter (data not shown). A previous report has indicated that Gal4-TBP and Tat proteins accelerate transcription at different steps (33). Gal4-TBP and Tat accelerate recruitment of RNA polymerase II holoenzyme to initiate transcription, and of P-TEFb to enhance elongation of the polymerase reaction, respectively. As we expected, synergistic enhancement of luciferase expression with a high S/N ratio was achieved by the combination of the two chimeric transcription factors and the GAL4UAS and HIV-1 LTR tandem promoter. Furthermore, a close correlation between expression and viral RNA replication upon HCV infection was observed in Huh7OK1/TG-Luc cells. These results indicate that the Huh7OK1/TG-Luc cell line is useful for quantifying HCV replication with a high sensitivity and specificity and applicable for high-throughput screening of anti-HCV compounds. Among the 1300 compounds screened here, we identified fiduxosin hydrochloride and flunarizine dihydrochloride as anti-HCV agents. Fiduxosin is known as an alpha1-adrenoceptor antagonist (39). Previous screening of a whole-genome siRNA library has shown that knockdown of alpha-1 adrenoceptor A, B, or D expression significantly reduces the replication of the genotype 1b subgenomic replicon (44), suggesting that alpha-1 adrenoceptors participate in the replication of HCV. Flunarizine is an antagonist of T-type calcium ion channels (40) and has been shown to induce expression of HO-1 (which is an antioxidant defense and key cytoprotective enzyme) through PI3K/Nrf2 signaling in an auditory cell line (45). It has been reported that overexpression of HO-1 suppresses HCV replication in genotype 1b replicon cells (46). Although further studies are required, it is reasonable to speculate that HO-1induced by treatment with flunarizine participates in the inhibition of HCV replication.

Another advantage of the indicator system is its flexibility in the choice of reporter genes. For easy separation and concentration of HCV-infected cells by immunomagnetic sorting, we established an Huh7OK1/TG-LNGFR cell line and demonstrated that a fifteen-fold concentration of HCV-infected cells could be achieved with a single sorting step. In addition, it would be possible to use this system in research on various cell lines other than Huh7 derivatives and primary hepatocytes in order to isolate novel HCV strains that exhibit no susceptibility to Huh7-derived cell lines. Although a few HCV strains for productive infection in cultured cells have been reported (3, 6, 47), no reliable and robust cell culture system for propagation of serum-derived HCV has thus far been established. We have inoculated sera from the chronic and window periods of patients with hepatitis C, but so far no significant reporter gene expression has been obtained in the indicator cell lines described in this study (data not shown), indicating that more factors are required for efficient propagation of serum-derived, naturally occurring HCV.

While it has been reported that HCV can spread through cell-to-cell pathways even in the presence of neutralizing antibodies (48–51), the precise mechanisms are not known. Although human CD81 and Claudin-1 are known to be major receptor candidates for HCV entry, cell-to-cell transmission between hepatoma cells is dependent on the expression of Claudin-1 but not of human CD81 (49, 50). In addition, peripheral blood B cells, in which JFH-1 virus cannot replicate, are able to transfer JFH-1 virus to hepatoma cells through SR-BI-, DC-SIGN- and L-SIGN-dependent pathways (51). The indicator cell lines established in this study could be used as recipient cells to investigate cell-to-cell transmission.

In conclusion, we have constructed chimeric transcription factors which are specifically and efficiently cleaved by HCV NS3/4A cleavage and established indicator cell lines capable of monitoring infection with JFH-1 virus based on reporter gene activation through cleavage of the transcription factors by HCV protease. By introducing the present system into various cell lines and modifying the reporter gene, it might be possible to establish a cell culture system capable of propagating serum-derived HCV.

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Involvement of PA28γ in the Propagation of Hepatitis C Virus

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We have reported previously that the proteasome activator PA28γ participates not only in degradation of hepatitis C virus (HCV) core protein in the nucleus but also in the pathogenesis in transgenic mice expressing HCV core protein. However, the biological significance of PA28γ in the propagation of HCV has not been clarified. PA28γ is an activator of proteasome responsible for ubiquitin-independent degradation of substrates in the nucleus. In the present study, knockdown of PA28γ in cells preinfection or postinfection with the JFH-1 strain of HCV impaired viral particle production but exhibited no effect on viral RNA replication. The particle production of HCV in PA28γ knockdown cells was restored by the expression of an small interfering RNA (siRNA)-resistant PA28γ. Although viral proteins were detected in the cytoplasm of cells infected with HCV, suppression of PA28γ expression induced accumulation of HCV core protein in the nucleus. HCV core protein was also degraded in the cytoplasm after ubiquitination by an E3 ubiquitin ligase, E6AP. Knockdown of PA28γ enhanced ubiquitination of core protein and impaired virus production, whereas that of E6AP reduced ubiquitination of core protein and enhanced virus production. Furthermore, virus production in the PA28γ knockdown cells was restored through knockdown of E6AP or expression of the siRNA-resistant wild-type but not mutant PA28γ incapable of activating proteasome activity. *Conclusion:* Our results suggest that PA28γ participates not only in the pathogenesis but also in the propagation of HCV by regulating the degradation of the core protein in both a ubiquitin-dependent and ubiquitin-independent manner. (HEPATOLOGY 2010;52:411-420)

Over 170 million individuals worldwide are infected with hepatitis C virus (HCV), which is a major etiological agent of liver diseases, including hepatic steatosis, cirrhosis, and hepatocellular carcinoma (HCC).[1] HCV is classified into the genus Hepacivirus of the *Flaviviridae* family and has a positive, single-strand RNA genome that encodes a single polyprotein consisting of about 3,000 amino acids.[2] The N-terminal one-third of the polyprotein is occupied by the structural proteins, and the remaining portion consists of nonstructural proteins involved in viral replication and assembly. Host and viral proteases cleave the appropriate sites of the polyprotein, resulting in generation of at least 10 viral proteins. The capsid (core), E1 and E2 proteins, and p7 are cleaved off by signal peptidase from the polyprotein. Furthermore, the C-terminal signal sequence of the core protein is processed by signal peptide peptidase.[3] Our recent data indicate that signal peptide peptidase cleaves the polyprotein between Phe[177] and Leu[178] in the signal sequence, and this processing is required for HCV propagation.[4] The mature core proteins make nucleocapsid with viral RNA, and HCV particles bud into the lumen of the endoplasmic reticulum bearing E1 and E2 glycoproteins on the host lipid components, and are released from the host cells.

Several reports suggest that HCV core protein plays an important role in the development of various outcomes of liver failure, including steatosis and HCC.[5,6]

Abbreviations: HA, hemagglutinin; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; JEV, Japanese encephalitis virus; moi, multiplicity of infection; shRNA, short hairpin RNA; siRNA, small interfering RNA.

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We have reported previously that HCV core protein specifically interacts with a proteasome activator PA28γ/REGγ in the nucleus and is digested by a PA28γ-dependent proteasome activity.[7] *In vivo* experiments in a mouse model suggest that PA28γ plays a critical role in the pathogenesis induced by HCV core protein.[8,9] PA28γ forms a homoheptamer in the nucleus and enhances the proteasome-mediated cleavage after basic amino acid residues, whereas PA28α and PA28β exhibit 41% and 34% homology to PA28γ, respectively, and form a heteroheptamer in the cytoplasm to activate cleavage after hydrophobic, acidic, or basic amino acid residues.[10] Recently, several groups reported that PA28γ interacts with steroid receptor coactivator-3 and cell cycle suppressors such as $p21^{WAF1/CIP1}$, $p16^{INK4A}$, and $p19^{ARF}$, and enhances the degradation of these proteins in a ubiquitin- and adenosine triphosphate–independent manner.[11-13] Furthermore, other mechanisms of ubiquitin-independent degradation have been considered for cell cycle regulation, summarized in the review of Jariel -Encontre et al.[14] However, the precise physiological functions of PA28γ are largely unknown *in vivo*, because PA28γ-knockout mice exhibit only mild growth retardation and live approximately as long as their control littermates.[15,16]

HCV core protein is degraded in a PA28γ-dependent and ubiquitin-independent manner in the nucleus,[7,17] while E6AP is also involved in the degradation of the core protein in a ubiquitin-dependent manner.[17,18] E6AP is a member of E3 ligases, which catalyze ubiquitin ligation of host and foreign proteins. Knockdown of E6AP suppressed degradation of HCV core protein and enhanced the release of infectious particles, suggesting that E6AP negatively regulates HCV propagation.[18] However, the role of PA28γ in the propagation of HCV has not yet been characterized. In this study, we examined the biological significance of PA28γ in the propagation of HCV.

Materials and Methods

Transfection, Immunoblotting, and RNA Interference. Plasmid DNA was transfected into Huh7OK1 cells by way of liposome-mediated transfection using Lipofectamine LTX with Plus reagent (Invitrogen, Carlsbad, CA). Expression of HCV core protein was determined by way of enzyme-linked immunosorbent assay as described.[19] Immunoblotting was performed as described.[8] The small interfering RNAs (siRNAs) targeted to the PA28γ gene were purchased from Ambion (Austin, TX) and were introduced into the cell lines using Lipofectamine RNAiMax (Invitrogen). siRNAs with the Ambion siRNA ID numbers 138669 and 138670 were designated as siPA28γ1 and siPA28γ2, respectively. Antibodies and plasmids are described in the Supporting Information.

Cell Lines and Virus Infection. All cell lines were cultured at 37°C under the conditions of humidified atmosphere and 5% CO_2. The human hepatoma cell line Huh7OK1 and derivative cell lines were maintained in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) supplemented with nonessential amino acids, sodium pyruvate, and 10% fetal bovine serum. The Huh7-derived cell line harboring a subgenomic or a full-length HCV replicon RNA[20] was maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, nonessential amino acids, sodium pyruvate, and 1 mg/mL G418 (Nakarai Tesque, Kyoto, Japan). Huh7OK1 cells were transfected with pSilencer-shPA28γ4 or a control plasmid, pSilencer 2.1 U6 hygro negative control (Ambion), and drug-resistant clones were selected by treatment with hygromycin (Wako, Tokyo, Japan) at a final concentration of 100 μg/mL. Huh7OK1 cells transfected with the control plasmid were selected with puromycin and designated as shCntrl, whereas those transfected with pSilencer-shPA28γ4 were established by limited dilution,[8] and two of the resulting cell lines were designated as KD5 and KD7. Plasmids encoding wild-type or mutant PA28γ complementary DNAs resistant to siRNA against PA28γ were prepared by using the silent mutations as reported.[8] These plasmids were transfected into Huh7OK1 cells and cultivated in medium containing 0.1 μg/mL of puromycin for 2 days. The surviving cells were used for virus infection. The shCtrl and KD5 cells were transformed with pSilencer shE6AP or pSilencer 3.1 H1 puro negative control (Ambion) and treated with 0.1 μg/mL of puromycin for 2 days. The surviving cells were infected with JFH-1 virus at a multiplicity of infection (moi) of 0.05. The viral RNA derived from the plasmid pJFH1 was transcribed and introduced into Huh7OK1 cells according to the method of Wakita et al.[21] The infectivity of JFH1 strain was determined using a focus-forming assay[21] and is expressed in focus-forming units. The Huh7 cell line harboring subgenomic replicon RNA of the Con1 or JFH1 strain was prepared according to the method of Pietschmann et al.[22] The infectivity of the Japanese encephalitis virus (JEV) was determined by an immunostaining focus assay as described[23] and is expressed in focus-forming units. Colony formation and replication assays, quantitative

reverse-transcription polymerase chain reaction, and estimation of cell growth was performed as described in the Supporting Information.

Immunofluorescent Staining. Huh7OK1-derived cells were seeded at 0.5×10^4 cells/well in an eight-well chamber slide, infected with JFH-1 virus at an moi of 0.3 after incubation at 37°C for 24 hours, stained with Bodipy 558/568 C_{12} according to the method of Targett-Adams et al.[24] at 4 days postinfection, and then fixed at 4°C for 30 mintues with 4% paraformaldehyde in phosphate-buffered saline. After treatment of cells with 1 μg/mL of RNase A, nuclei were stained with 50 μM Heachest 33258. The fixed cells were permeabilized with 20 mM Tris-HCl containing 1% Nonidet P-40 and 135 mM NaCl at room temperature for 5 minutes, reacted with rabbit anti-core or anti-NS5A antibody followed by Alexa Fluor 488-goat antibody to rabbit immunoglobulin G, washed three times with phosphate-buffered saline, and observed with a FluoView FV1000 laser scanning confocal microscope (Olympus, Tokyo, Japan). The percentage of the area occupied by the core protein in nucleus and cytoplasm was calculated using Image-Pro software (Media Cybernetics). The percentage of the nuclear core protein to the total core protein was examined randomly in 10 fields of every three wells. The percentage of the nuclear NS5A to total NS5A was estimated by the same method as the ratio of the core protein.

Results

Transient Knockdown of PA28γ Prior to or After Infection With HCV Reduces Particle Production. We reported previously that Huh7OK1 cells are as permissive to JFH-1 virus infection as Huh7.5.1 cells.[25] The Huh-7OK1 cell line retained the ability to produce type I IFNs through the RIG-I–dependent signaling pathway upon infection with RNA viruses and exhibited a cell surface expression level of human CD81 comparable to that of the parental cell line. However, the mechanism through which the Huh7 OK1 cell line exhibits highly permissive to JFH-1 virus infection has not been clarified yet. Two siRNAs were used to knock down PA28γ, but only one, siPA28γ1, was used because the other had off-target effects (Supporting Fig. 1). To examine the effect of PA28γ on the propagation of HCV, siPA28γ1 was introduced into Huh7OK1 cells 24 hours before infection. The levels of viral RNA, core protein, and infectious viral titer were determined at 48 and 96 hours postinfection. Viral RNA in the culture supernatant and cells was clearly reduced by the knockdown of PA28γ at 48 and 96 hours postinfection, respectively (Fig. 1A), whereas a significant reduction of core protein expression was detected at 96 hours but not at 48

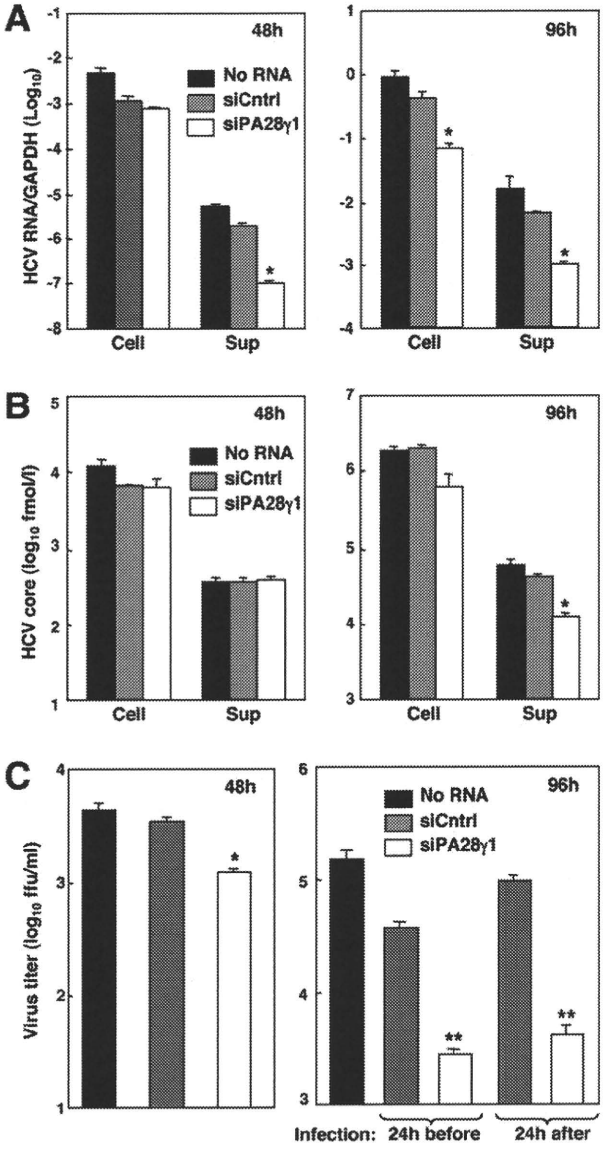

Fig. 1. Transient knockdown of PA28γ before or after infection with HCV reduces particle production. (A) Huh7OK1 cells transfected with a control siRNA (siCntrl) or PA28γ siRNA1 were infected with JFH-1 virus at 24 hours posttransfection and then harvested at 48 hours (left panel) and 96 hours postinfection (right panel). The quantity of HCV RNA in cells and supernatants was determined by way of quantitative reverse-transcription polymerase chain reaction. (B) The expression of HCV core protein in cells and supernatants at 48 hours (left panel) and 96 hours (right panel) postinfection was determined by ELISA. (C) Huh7OK1 cells that were transfected with siCntrl or PA28γ siRNA1 were infected with JFH-1 virus at 24 hours posttransfection. The infectivity of the virus in the culture supernatant was determined by a focus-forming assay at 48 hours postinfection (left panel). Those transfected with the siRNAs at 24 hours before and after infection with JFH-1 virus were determined similarly at 96 hours postinfection (right panel). $*P < 0.05$, $**P < 0.01$ versus control siRNA-transfected cells. Data are representative of three independent experiments.

hours postinfection (Fig. 1B). Infectious viral titer in the culture supernatant was significantly reduced at 48 and 96 hours postinfection by the PA28γ knockdown (Fig. 1C), consistent with the suppression of the viral RNA in the supernatant. Furthermore, a comparable suppression of the production of infectious particles in the supernatant was also achieved by introducing siPA28γ1 into cells even at 24 hours postinfection (Fig. 1C, right panel). These results suggest that PA28γ participates in the regulation of HCV propagation in postentry steps.

Stable Knockdown of PA28γ Impairs Viral Propagation. To establish the PA28γ knockdown cell lines, Huh7OK1 cells were transfected with a plasmid encoding a short hairpin RNA (shRNA) targeted to PA28γ and selected with hygromycin, resulting in two clones—KD5 and KD7—that exhibited a clear reduction of PA28γ expression (Fig. 2A). Although the suppression of PA28γ expression in KD7 cells was slightly more efficient than that in KD5 cells, the growth of KD7 cells was impaired (Fig. 2B). Viral production in the culture supernatants in cells infected with the JFH-1 virus was significantly impaired in PA28γ knockdown KD5 cells compared with control cells (Fig. 2C). The viral RNA and core protein in the supernatant were also reduced in KD5 cells (Fig. 2D). Expression of siRNA-resistant PA28γ in PA28γ knockdown KD5 and KD7 cells recovered virus production in the supernatant to a level similar to that in the control cells transfected with an empty vector, and overexpression of siRNA-resistant PA28γ in control cells slightly enhanced virus production (Fig. 2E). Our previous data suggest that capsid protein of JEV does not bind to PA28γ.[7] To examine whether PA28γ regulates JEV propagation, KD5 and shCntrl cells were infected with JEV at an moi of 0.5. The infectivity of JEV in KD5 cells was similar to that in shCntrl cells (Fig. 2F), suggesting that PA28γ does not participate in the virus production pathway of JEV. These results further support the notion that PA28γ participates in HCV propagation.

Knockdown of PA28γ Exhibits No Effect on Viral RNA Replication. Although knockdown of PA28γ resulted in the suppression of viral particle and RNA production in the culture supernatant at 48 hours postinfection with JHF-1 virus, viral RNA in the cells was not reduced (Fig. 1), suggesting that PA28γ does not participate in viral replication. To gain more insight on this point, we examined the effect of PA28γ knockdown on RNA replication in replicon cells. Transient knockdown of PA28γ through introduction of siPA28γ into the subgenomic HCV replicon cells derived from the Con1 or JFH-1 strain induced no significant reduction of HCV RNA (Fig. 3A). Furthermore, luciferase activities in the stable PA28γ

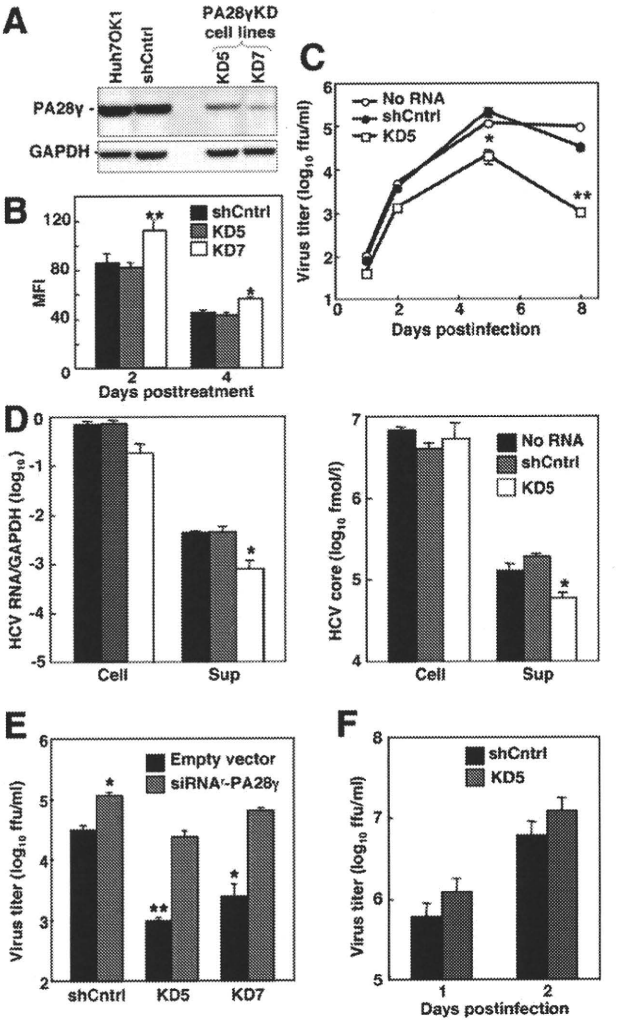

Fig. 2. Establishment of PA28γ knockdown cell lines and propagation of HCV. (A) Huh7OK1 cells were transfected with pSilencer shPA28γ or control plasmid and selected by hygromycin at 48 hours posttransfection. Two PA28γ knockdown cell lines (KD5 and KD7) and one control cell line (shCntrl) were established, and PA28γ knockdown was confirmed by way of immunoblotting. (B) Growth of the cell lines was determined by staining with carboxyfluorescein succinimidyl ester. (C,D) KD5 and shCntrl cell lines were infected with the JFH-1virus at an moi of 0.05. The infectious virus titers in the culture supernatants (C) was determined by way of focus-forming assay. The virus RNA (D, left panel) and the core protein (D, right panel) in both cell and the supernatant were determined at 5 days postinfection by way of ELISA and quantitative reverse-transcription polymerase chain reaction, respectively. (E) The plasmid encoding an siRNA-resistant PA28γ or empty vector was transfected into the cell lines, seeded at 5×10^4 cells into a six-well plate after cultivation in the presence of puromycin for 2 days, and infected with JFH-1 virus at an moi of 0.05. The viral titers were determined at 5 days postinfection. $^*P < 0.05$, $^{**}P < 0.01$ versus shCntrl cells transfected with an empty vector. (F). KD5 and shCntrl cell lines were infected with the JEV virus at an moi of 0.5. The infectivity of JEV in the supernatant was determined at 1 and 2 days postinfection. Data are representative of three independent experiments.

knockdown cell line KD5 and the control cell line transfected with the subgenomic replicon RNA (WT) were gradually increased until 4 days posttransfection, whereas luciferase activities in the same two cell lines transfected with the polymerase-dead replicon RNA (GND) were decreased in a time-dependent manner (Fig. 3B). Next, to explore the effect of PA28γ knockdown on the viral replication over a longer period, replicon RNA encoding the neomycin-resistance gene was transfected into the cell lines for a colony formation assay. The numbers of colonies in the KD5 cell line after 4 weeks of selection with G418 were similar to those in the control cell line (Fig. 3C). To further clarify the roles of PA28γ on the postreplication steps, *in vitro* transcribed full-length viral RNA was transfected into Huh7OK1 cells, and siPA28γ1 was then introduced into the cells at 24 hours posttransfection of viral RNA. Intracellular core protein was increased in a time-dependent manner, but no significant difference was observed between cells transfected with control siRNA and those transfected with siPA28γ1 (Fig. 3D, left panel). However, infectious virus titers in the supernatant were significantly decreased by the transient and stable knockdown of PA28γ compared with control cells (Fig. 3D, middle and right panels). Furthermore, PA28γ did not contribute to the virus production of JEV (Fig. 2F), suggesting that the general sorting pathway of the flavivirus is functional under

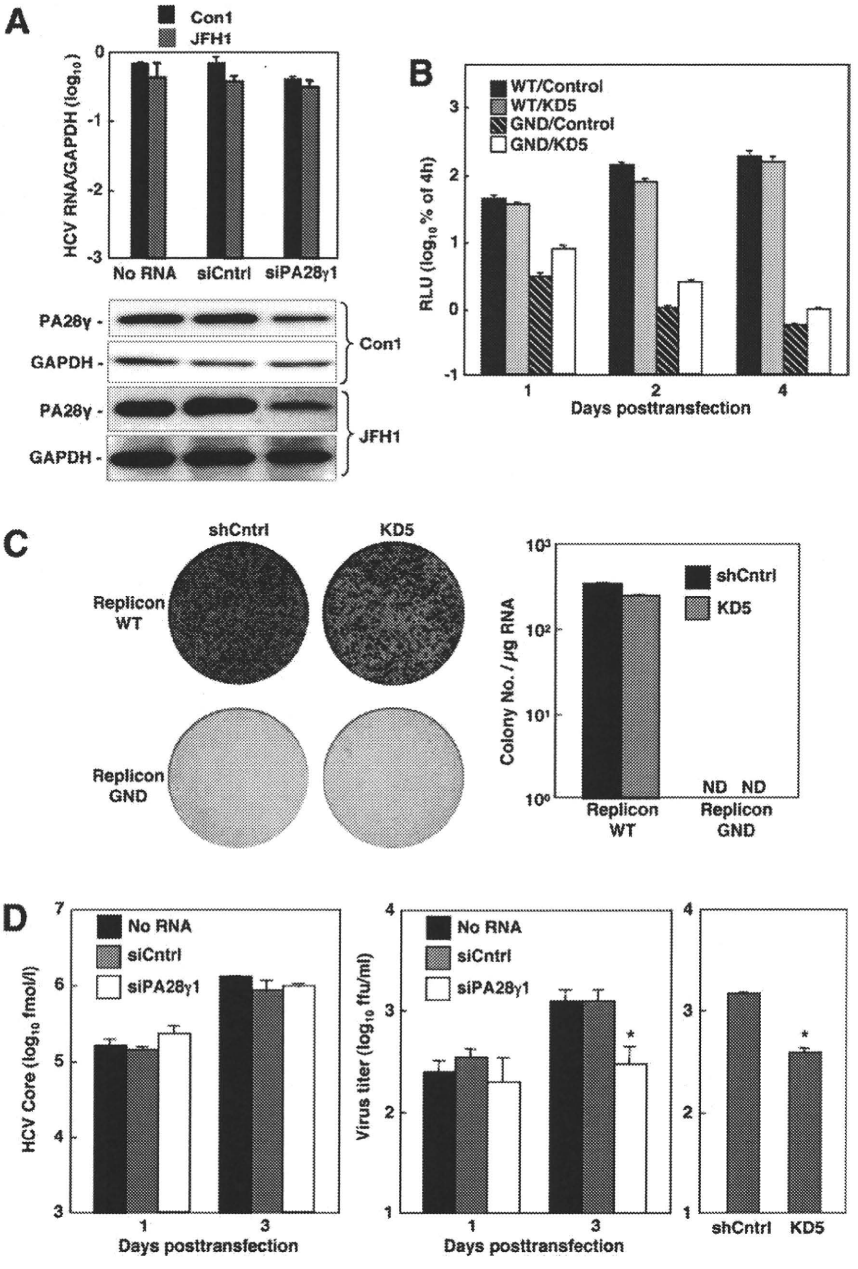

Fig. 3. Effect of PA28γ knockdown on HCV RNA replication. (A) The siCntrl or siPA28γ1 (10 nM) was transfected into the subgenomic HCV replicon cells derived from Con1 and JFH-1 strains. The transfected cells were harvested at 72 hours posttransfection. The replicon RNA was determined by quantitative reverse-transcription polymerase chain reaction at 72 hours posttransfection (upper). PA28γ or glyceraldehyde 3-phosphate dehydrogenase was detected by way of immunoblotting. Cell lysates were subjected to western blotting using antibodies to PA28γ and glyceraldehyde 3-phosphate dehydrogenase (lower). (B) The HCV replicon RNA encoding luciferase gene (WT) or the HCV replicon RNA that has a replication-deficient mutation (GND) was transfected into the shCntrl (Control) and KD5 cell lines. Relative luciferase activity was determined using the activity at 4 hours post-electroporation as a transfection efficiency. (C) Colony formation assay. Replicon RNA encoding the neomycin-resistance gene was transfected into the shCntrl and KD5 cell lines, and the remaining colonies were fixed with 4% paraformaldehyde at 4 weeks posttransfection and then stained with crystal violet. The number of colonies was counted (right). (D) Huh7OK1 cells transfected with 10 μg of *in vitro*-transcribed full-length JFH-1 viral RNA were further transfected with siCntrl or siPA28γ1 at 24 hours posttransfection of viral RNA. The level of HCV core protein in the cells was determined by way of ELISA at 1 and 3 days posttransfection (left). Infectious virus titers in the culture supernatants at 1 and 3 days posttransfection were determined by way of focus-forming assay (middle). Infectious viral titers in the shCntrl or KD5 cells transfected with 10 μg of the infectious viral RNA were determined at 5 days posttransfection (right). $^*P < 0.05$, $^{**}P < 0.01$ versus the control cells or cells transfected with siCntrl. Data are representative of three independent experiments.

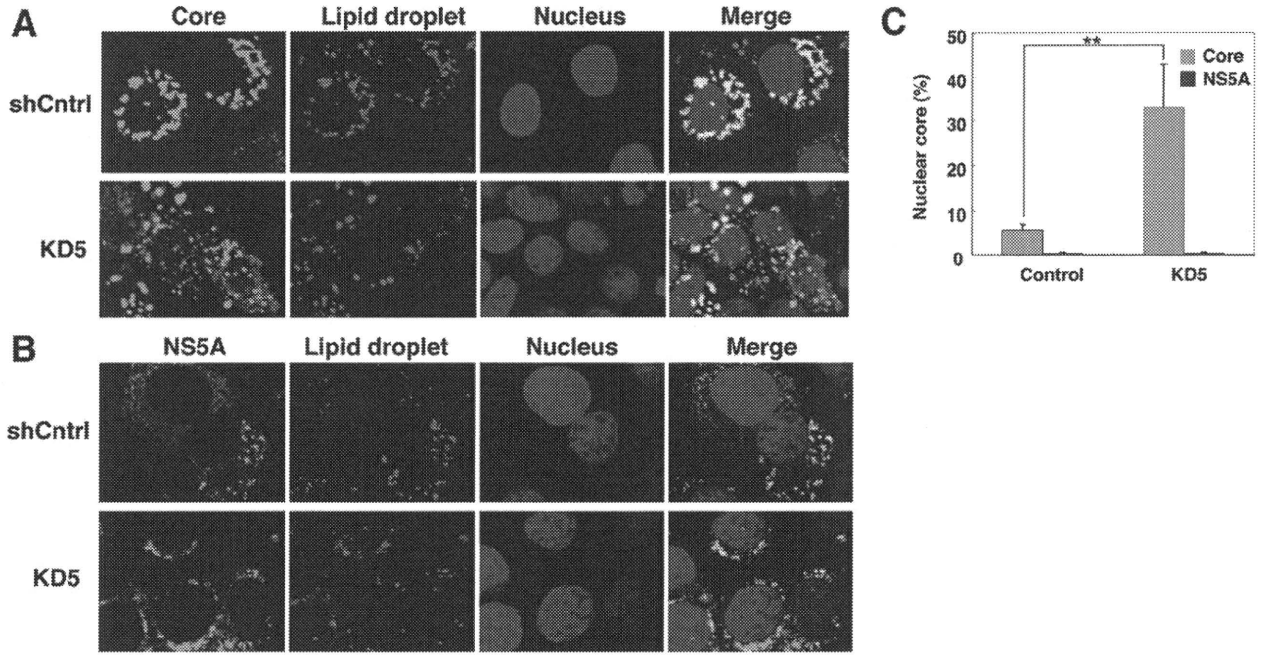

Fig. 4. Effect of PA28γ knockdown on the localization of HCV core protein and lipid droplets. The shCntrl and KD5 cell lines infected with JFH-1 virus were fixed with methanol or paraformaldehyde for 5 minutes at 4 days postinfection. HCV core (A) and NS5A (B) proteins were stained with rabbit antibodies raised against the proteins and Alexa Flour 488-conjugated goat anti-rabbit immunoglobulin G antibody. Lipid droplets were stained with Bodipy 558/568 C12. Nuclei were stained with 50 μM Heachest 33258 after treatment with 1 μg/mL of RNase A. Data are representative of three independent experiments. (C) The percentage of the area occupied by the core protein in nucleus and cytoplasm was calculated using the method described in Materials and Methods. The percentage of the nuclear NS5A to total NS5A was estimated by the same way as the ratio of the core protein. $^{**}P < 0.01$ versus control siRNA-transfected cells.

the PA28γ knockdown condition. These results suggest that PA28γ specifically regulates the postreplication steps in the life cycle of HCV.

Core Protein Is Partially Accumulated in the Nucleus of PA28γ Knockdown Cells. We reported previously that some fraction of HCV core protein migrates into the nucleus and is then degraded by a PA28γ-dependent proteasome pathway.[7] Furthermore, we have demonstrated that HCV core protein is clearly accumulated in the nucleus of the liver cells of PA28γ-knockout mice.[8] However, the role of PA28γ on the intracellular localization of HCV core protein in the infected HCV cells has not been characterized. HCV core protein was chiefly detected in cytoplasm of the control cell line infected with the JFH-1 virus, where it appeared around lipid droplets after staining with Bodipy 558/568 C12 (Fig. 4A, upper panels). In contrast, the core protein was detected not only in the cytoplasm around the surface of lipid droplets, but also in the nucleus in the KD5 cell line (Fig. 4A, lower panels). The NS5A protein was detected in the cytoplasm but not in the nucleus in both the shCntrl and KD5 cell lines (Fig. 4B). The percentage occupied by nuclear core protein to total core protein was increased by about six time levels in the KD5, while the ratio of nuclear NS5A to total NS5A exhibited no difference (Fig. 4C). These results suggest that PA28γ participates in the degradation of HCV core protein in the nucleus.

PA28γ Positively Regulates HCV Propagation by Inhibiting Ubiquitin-Dependent Degradation of Core Protein in Cytoplasm. We reported previously that HCV core protein is degraded by at least two distinct pathways: a ubiquitin-dependent proteasome pathway and a ubiquitin-independent proteasome pathway.[17] The ubiquitin E3 ligase, E6AP, can catalyze ubiquitin ligation of the core protein for ubiquitin-dependent degradation in the cytoplasm,[18] whereas PA28γ participates in the degradation of the core protein through a ubiquitin-independent pathway in the nucleus.[17] We have also demonstrated that PA28γ knockdown leads to enhanced ubiquitination of HCV core protein.[8] However, the interplay between these two pathways in cells infected with HCV has not been determined. To address this point, we examined the effects of knockdown of E6AP or PA28γ on the virus propagation and the ubiquitination of the core protein. JFH-1 virus was inoculated into E6AP- and/or PA28γ knockdown cell lines (Fig. 5A). Transfection of the plasmid encoding shRNA to E6AP into the control cells (shCntrl) increased virus production (Fig. 5A [C-E]) in comparison with that of the

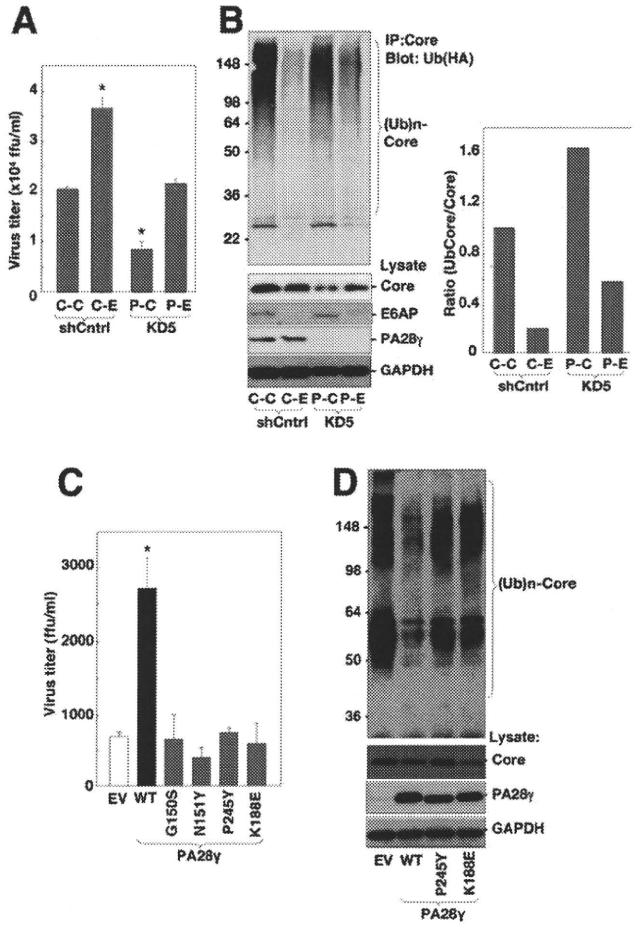

Fig. 5. PA28γ knockdown enhances E6AP-dependent ubiquitination of core protein and reduces virus titer. (A) shCntrl and KD5 cells transfected with plasmids encoding the negative control (C-C and P-C) or E6AP (C-E and P-E) shRNA were treated with puromycin for 2 days. The remaining cells seeded at 2.5×10^4 cells in a 24-well plate were infected with the JFH-1 virus at an moi of 0.05, and infectious virus titers in the supernatants were determined at 72 hours postinfection by way of focus-forming assay. (B) The cells transfected and infected as in (A) were further transfected with a plasmid encoding HA-tagged ubiquitin at 48 hours postinfection. The cells were treated with 10 μM MG132 for 5 hours at 72 hours postinfection and subjected to immunoprecipitation with anti-core monoclonal antibody and immunoblotting with anti-HA antibody. The ratio of ubiquitination of HCV core protein was assessed by the densitometries of the ubiquitinated and unubiquitinated core proteins. (C) KD5 cells transfected with plasmids encoding wild-type or mutant PA28γ were infected with the JFH-1 virus at an moi of 0.05 at 24 hours posttransfection, and the infectious titers in the supernatant were determined at 72 hours postinfection by way of focus-forming assay. (D) KD5 cells transfected with plasmids encoding HCV core protein and HA-tagged ubiquitin, together with wild-type or mutant PA28γ, were treated with 10 μM MG132 for 5 hours at 24 hours posttransfection and subjected to immunoprecipitation with anti-core monoclonal antibody and immunoblotting with anti-HA antibody. EV, empty vector; WT, plasmid encoding wild-type PA28γ. $^*P < 0.05$ versus shCntrl or KD5 cells transfected with the negative control or empty vector. Data are representative of three independent experiments.

control cells transfected with the plasmid encoding control shRNA (Fig. 5A [C-C]). Furthermore, the impaired virus production in the PA28γ knockdown cells (KD5) was restored by the transfection of the plasmid encoding shRNA to E6AP (Fig. 5A [P-E]). Cells expressing hemagglutinin (HA)-tagged ubiquitin infected with the JFH-1 virus were immunoprecipitated by the anti-core antibody, and the immunoprecipitates were analyzed by immunoblotting with anti-HA antibody (Fig. 5B). E6AP knockdown decreased the ratio of ubiquitination of HCV core protein, in contrast to the increase of that by PA28γ knockdown (Fig. 5B, lanes C-E and P-C). Furthermore, E6AP knockdown in the PA28γ knockdown cells restored the ubiquitination of the core protein to a certain extent (Fig. 5B, lane P-E). It was shown that Pro^{245} of PA28γ is critical for binding to the 20S proteasome, and that Gly^{150} and Asn^{151} of PA28γ are important for activation of the proteasome.[26] To further examine the functional significance of PA28γ on HCV propagation, expression plasmids encoding siRNA-resistant PA28γ mutants in which Gly^{150}, Asn^{151}, and Pro^{245} were replaced with Ser (G150S), Tyr (N151Y), and Tyr (P245Y), respectively, were transfected into KD5 cells and inoculated with JFH-1 virus at 24 hours posttransfection. The infectious virus titers in the culture supernatant were determined at 3 days postinfection (Fig. 5C). KD5 cells transfected with the plasmid encoding wild-type PA28γ exhibited a partial recovery of virus production, although those transfected with the plasmid encoding PA28γ G150S, N151Y, or P245Y or with an empty vector exhibited no effect on virus production. Replacing Lys^{188} with Glu in PA28γ (PA28γ K188E) confers the capability of proteasome-mediated cleavage after hydrophobic, acidic, and basic residues such as those exhibited by PA28α.[27] Expression of siRNA-resistant PA28γ K188E in KD5 cells could not restore virus production (Fig. 5D). The ubiquitination of HCV core protein was inhibited by expression of the wild-type PA28γ but not expression of the PA28γ mutants (P245Y or K188E) in KD5 cells (Fig. 5D). Collectively, these results suggest that PA28γ positively regulates HCV propagation by inhibiting degradation of HCV core protein by an E6AP/ubiquitin-dependent proteasome.

Discussion

To explore the role of PA28γ on the life cycle of HCV, we examined the effects of knockdown of PA28γ in Huh7OK1 cells infected with the JFH-1 virus. Knockdown of PA28γ in Huh7OK1 cells before or after infection with the JFH-1 virus impaired

production of infectious particles but did not impair viral RNA replication. However, PA28γ knockdown did not affect the production of JEV, of which the capsid protein does not interact with PA28γ, suggesting that PA28γ knockdown does not affect the general sorting pathway of flavivirus. These results suggest that PA28γ is specifically involved in the postreplication steps of HCV life cycle. Our previous report indicated that HCV core protein was accumulated in the nucleus of the hepatocytes of HCV core transgenic/PA28γ knockout mice.[8] PA28γ is located mainly in the nucleus, although a small portion is also located in the cytoplasm[7,28] and can up-regulate trypsin-like proteasome activity, which cleaves after basic amino acid residues.[27] Previous studies have shown that some fraction of HCV core protein is translocated into the nucleus and quickly degraded in the PA28γ-dependent proteasome pathway.[7,8,29] Miyanari et al.[30] demonstrated that the core protein is localized on the surface of lipid droplets and is surrounded by nonstructural proteins, suggesting that HCV particles are assembled near the surface of the lipid droplets. In the present experiments, although HCV core protein was detected on the surface of the lipid droplets in both control and PA28γ knockdown cell lines, it was partially localized in the nucleus in PA28γ knockdown cells but not control cells. Furthermore, localization of HCV core protein on the surface of lipid droplets was impaired in PA28γ knockdown cells (Fig. 4). These results suggest that HCV core protein is partially translocated into the nucleus and degraded in the PA28γ-dependent proteasome pathway in HCV-infected cells and that PA28γ does not directly participate in the particle formation of HCV.

HCV core protein is degraded by at least two proteasome pathways: a ubiquitin-dependent pathway and a ubiquitin-independent and PA28γ-dependent pathway.[17] The E3 ligase E6AP catalyzes ubiquitin ligation to HCV core protein, resulting in enhanced degradation of the core protein in the cytoplasm.[18] Knockdown of E6AP up-regulated virus production in cells infected with the JFH-1 virus,[18] suggesting that E6AP/ubiquitin-dependent degradation of the core protein contributes to an antiviral response. In contrast, knockdown of PA28γ induced up-regulation of the ubiquitination of HCV core protein and down-regulation of the viral production, suggesting that PA28γ-dependent proteasome activity contributes to the proviral response by suppressing E6AP-dependent degradation of the core protein, thereby enhancing viral particle formation. The wild-type PA28γ enhances the trypsin-like activity of proteasome that cleaves peptide bonds after basic residues of the substrates, whereas the PA28γ K188E mutant enhances the proteasome activity that cleaves peptide bonds after hydrophobic, acidic, and basic residues in the manner of PA28α.[27] Therefore, the sizes of fragments produced by the PA28γ-dependent proteasome should be different from those produced by the PA28α/β- or ubiquitination-mediated proteasome. It might be feasible to speculate that the peptide fragments of HCV core protein generated by the PA28γ-dependent proteasome or PA28γ *per se* may be directly or indirectly involved in the suppression of the E6AP-dependent ubiquitination of the core protein. Further studies will be needed to clarify the relationship between E6AP and PA28γ in the degradation and ubiquitination of HCV core protein. Figure 6 shows a schematic diagram of our hypothesis of the regulation of HCV propagation by PA28γ.

HCV core protein was found in not only nuclei but also cytoplasm of the infected KD5 cells (Fig. 4). The down-regulation of virus production should potently reduce a total amount of the core protein in KD5 cells before a clear accumulation of the core protein in nuclei. Furthermore, a small amount of PA28γ was found in the PA28γ knockdown cells, suggesting that E6AP-dependent degradation of HCV core protein is not potently suppressed in the PA28γ knockdown cells. If HCV core protein is constitutively expressed under the PA28γ knockout cells regardless of an amount of infected virus, a clear accumulation of the core protein in nuclei should be found without cytoplasmic expression of the core protein under the PA28γ knockout condition. We reported previously that HCC and liver steatosis in mouse are induced by the HCV core protein in the presence, but not the absence, of PA28γ.[8] Although HCV core protein is predominantly detected in the cytoplasm of the liver cells of $PA28\gamma^{+/+}$ mice,[8,31] HCV core protein was clearly accumulated in the nuclei, but clearly reduced in cytoplasm, of liver cells of $PA28\gamma^{-/-}$ mouse.[8] In addition, ubiquitination of HCV core protein was increased by PA28γ knockdown in the 293T cell line.[8] These results and the data in Fig. 5 suggest that the suppression of PA28γ function enhances the E6AP-dependent degradation of HCV core protein. Hence, the reason there is no difference between $PA28\gamma^{+/+}$ and $PA28\gamma^{-/-}$ mice with respect to the amount of core protein may be due to the competitive regulation of the core protein by E6AP- and PA28γ-dependent degradation mechanisms. E6AP-dependent degradation of HCV core protein in cytoplasm may be enhanced *in vivo* under the PA28γ knockout condition.

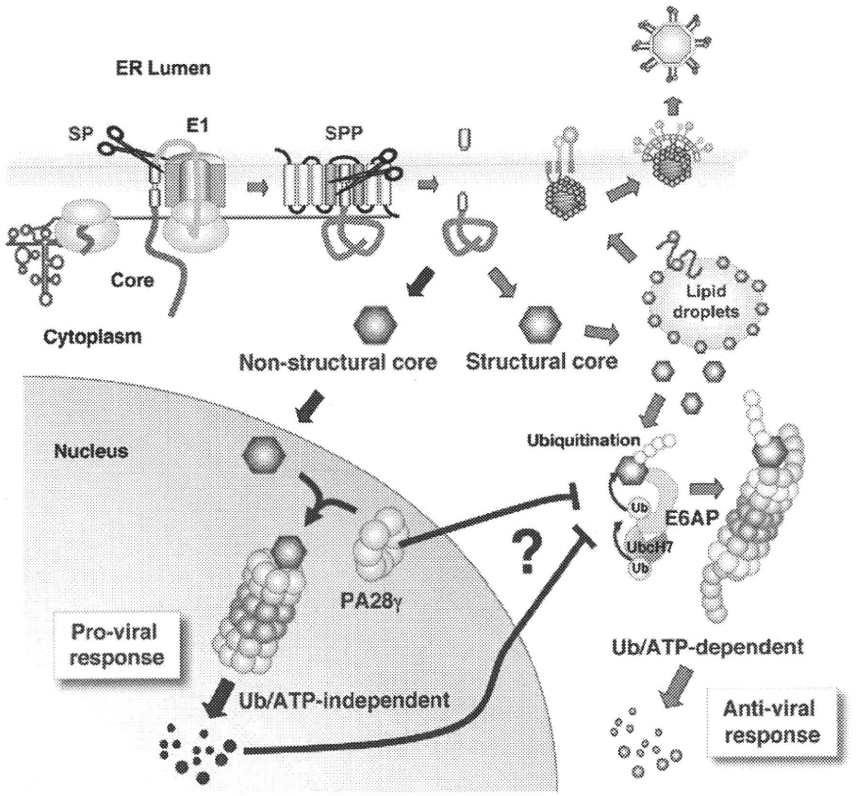

Fig. 6. Schematic diagram of the potential roles of PA28γ in HCV propagation. HCV core protein is cleaved off from the precursor polyprotein by signal peptidase (SP) and the signal sequence is further processed by signal peptide peptidase (SPP). The mature core protein mainly localizes on the lipid droplets close to the endoplasmic reticulum to form a nucleocapsid with the viral RNA genome and is incorporated into virus particles as a structural protein. In addition to the structural protein of HCV, the core protein has characteristics of a nonstructural protein. HCV core protein is degraded through ubiquitin-dependent and ubiquitin-independent proteasome pathways. E6AP catalyzes ubiquitin ligation to HCV core protein and promotes degradation in the cytoplasm, which contributes to the antiviral response. In contrast, the core protein partially migrates into the nucleus and is degraded through a ubiquitin-independent and PA28γ-dependent proteasome pathway, and the core protein fragments generated by the PA28γ pathway or PA28γ *per se* were suggested to participate in the suppression of E6AP-dependent ubiquitination of HCV core protein, which contributes to the proviral response.

In conclusion, in this study we demonstrated that the proteasome activator PA28γ positively regulates particle production of HCV by inhibiting E6AP-dependent ubiquitination of the core protein, in addition to our previous observation that PA28γ plays a crucial role in the development of liver pathology induced by HCV core protein.[8] PA28γ knockout mice exhibit only mild growth retardation.[15,16] Therefore, PA28γ may be a novel and promising antiviral target not only for elimination of HCV from hepatitis C patients but also for intervention in the progression of liver diseases induced by chronic HCV infection.

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