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Involvement of PA28 γ in the Propagation of Hepatitis C Virus

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We have reported previously that the proteasome activator PA28 γ 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 PA28 γ in the propagation of HCV has not been clarified. PA28 γ is an activator of proteasome responsible for ubiquitin-independent degradation of substrates in the nucleus. In the present study, knockdown of PA28 γ 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 PA28 γ knockdown cells was restored by the expression of an small interfering RNA (siRNA)-resistant PA28 γ . Although viral proteins were detected in the cytoplasm of cells infected with HCV, suppression of PA28 γ 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 PA28 γ 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 PA28 γ knockdown cells was restored through knockdown of E6AP or expression of the siRNA-resistant wild-type but not mutant PA28 γ incapable of activating proteasome activity. **Conclusion:** Our results suggest that PA28 γ 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 Phe¹⁷⁷ and Leu¹⁷⁸ 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 PA28 γ /REG γ in the nucleus and is digested by a PA28 γ -dependent proteasome activity.⁷ *In vivo* experiments in a mouse model suggest that PA28 γ plays a critical role in the pathogenesis induced by HCV core protein.^{8,9} PA28 γ forms a homoheptamer in the nucleus and enhances the proteasome-mediated cleavage after basic amino acid residues, whereas PA28 α and PA28 β exhibit 41% and 34% homology to PA28 γ , 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 PA28 γ 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 PA28 γ are largely unknown *in vivo*, because PA28 γ -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 PA28 γ -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 PA28 γ in the propagation of HCV has not yet been characterized. In this study, we examined the biological significance of PA28 γ 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 PA28 γ 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 siPA28 γ 1 and siPA28 γ 2, 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% CO₂. 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 RNA²⁰ 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-shPA28 γ 4 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 μ g/mL. Huh7OK1 cells transfected with the control plasmid were selected with puromycin and designated as shCtrl, whereas those transfected with pSilencer-shPA28 γ 4 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 PA28 γ complementary DNAs resistant to siRNA against PA28 γ were prepared by using the silent mutations as reported.⁸ These plasmids were transfected into Huh7OK1 cells and cultivated in medium containing 0.1 μ g/mL of puromycin for 2 days. The surviving cells were used for virus infection. The shCtrl and KD5 cells were transfected with pSilencer shE6AP or pSilencer 3.1 H1 puro negative control (Ambion) and treated with 0.1 μ g/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 assay²¹ 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 described²³ 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 C₁₂ according to the method of Targett-Adams et al.²⁴ at 4 days postinfection, and then fixed at 4°C for 30 minutes with 4% paraformaldehyde in phosphate-buffered saline. After treatment of cells with 1 μ g/mL of RNase A, nuclei were stained with 50 μ M Hechst 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 PA28 γ 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 Huh7OK1 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 Huh7OK1 cell line exhibits highly permissive to JFH-1 virus infection has not been clarified yet. Two siRNAs were used to knock down PA28 γ , but only one, siPA28 γ 1, was used because the other had off-target effects (Supporting Fig. 1). To examine the effect of PA28 γ on the propagation of HCV, siPA28 γ 1 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

PA28 γ 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

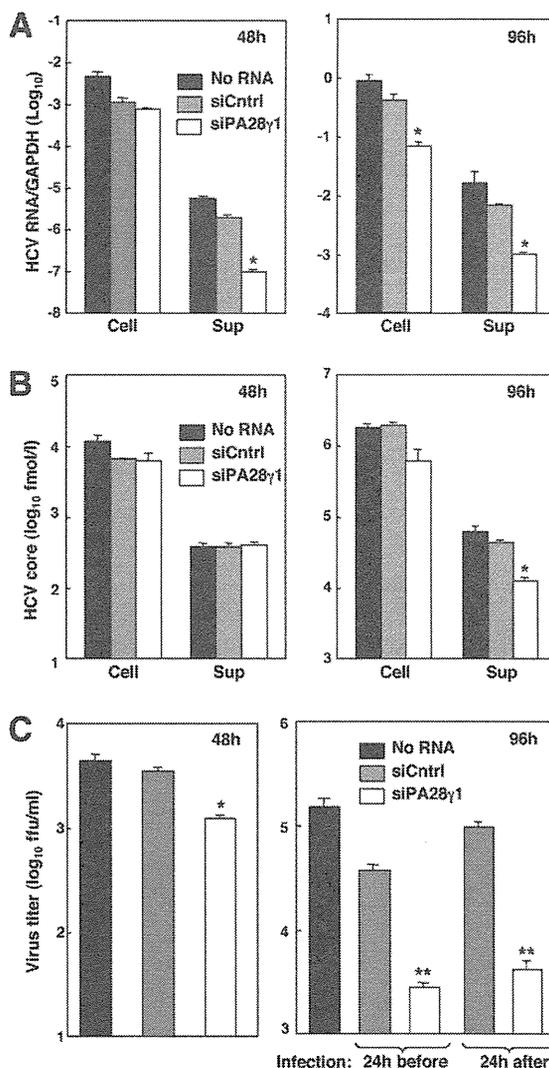


Fig. 1. Transient knockdown of PA28 γ before or after infection with HCV reduces particle production. (A) Huh7OK1 cells transfected with a control siRNA (siCntrl) or PA28 γ siRNA1 were infected with JFH-1 virus at 24 hours posttransfection and then harvested at 48 hours (left panel) and 96 hours postinfection (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 PA28 γ 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 PA28 γ 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 siPA28 γ 1 into cells even at 24 hours postinfection (Fig. 1C, right panel). These results suggest that PA28 γ participates in the regulation of HCV propagation in postentry steps.

Stable Knockdown of PA28 γ Impairs Viral Propagation. To establish the PA28 γ knockdown cell lines, Huh7OK1 cells were transfected with a plasmid encoding a short hairpin RNA (shRNA) targeted to PA28 γ and selected with hygromycin, resulting in two clones—KD5 and KD7—that exhibited a clear reduction of PA28 γ expression (Fig. 2A). Although the suppression of PA28 γ 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 PA28 γ 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 PA28 γ in PA28 γ 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 PA28 γ in control cells slightly enhanced virus production (Fig. 2E). Our previous data suggest that capsid protein of JEV does not bind to PA28 γ .⁷ To examine whether PA28 γ 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 PA28 γ does not participate in the virus production pathway of JEV. These results further support the notion that PA28 γ participates in HCV propagation.

Knockdown of PA28 γ Exhibits No Effect on Viral RNA Replication. Although knockdown of PA28 γ resulted in the suppression of viral particle and RNA production in the culture supernatant at 48 hours postinfection with JFH-1 virus, viral RNA in the cells was not reduced (Fig. 1), suggesting that PA28 γ does not participate in viral replication. To gain more insight on this point, we examined the effect of PA28 γ knockdown on RNA replication in replicon cells. Transient knockdown of PA28 γ through introduction of siPA28 γ into the subgenomic HCV replicon cells

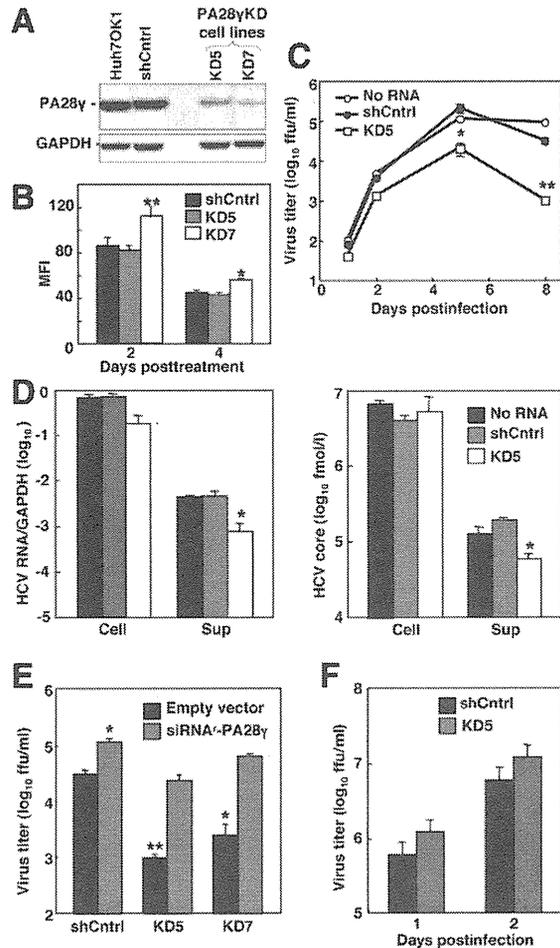
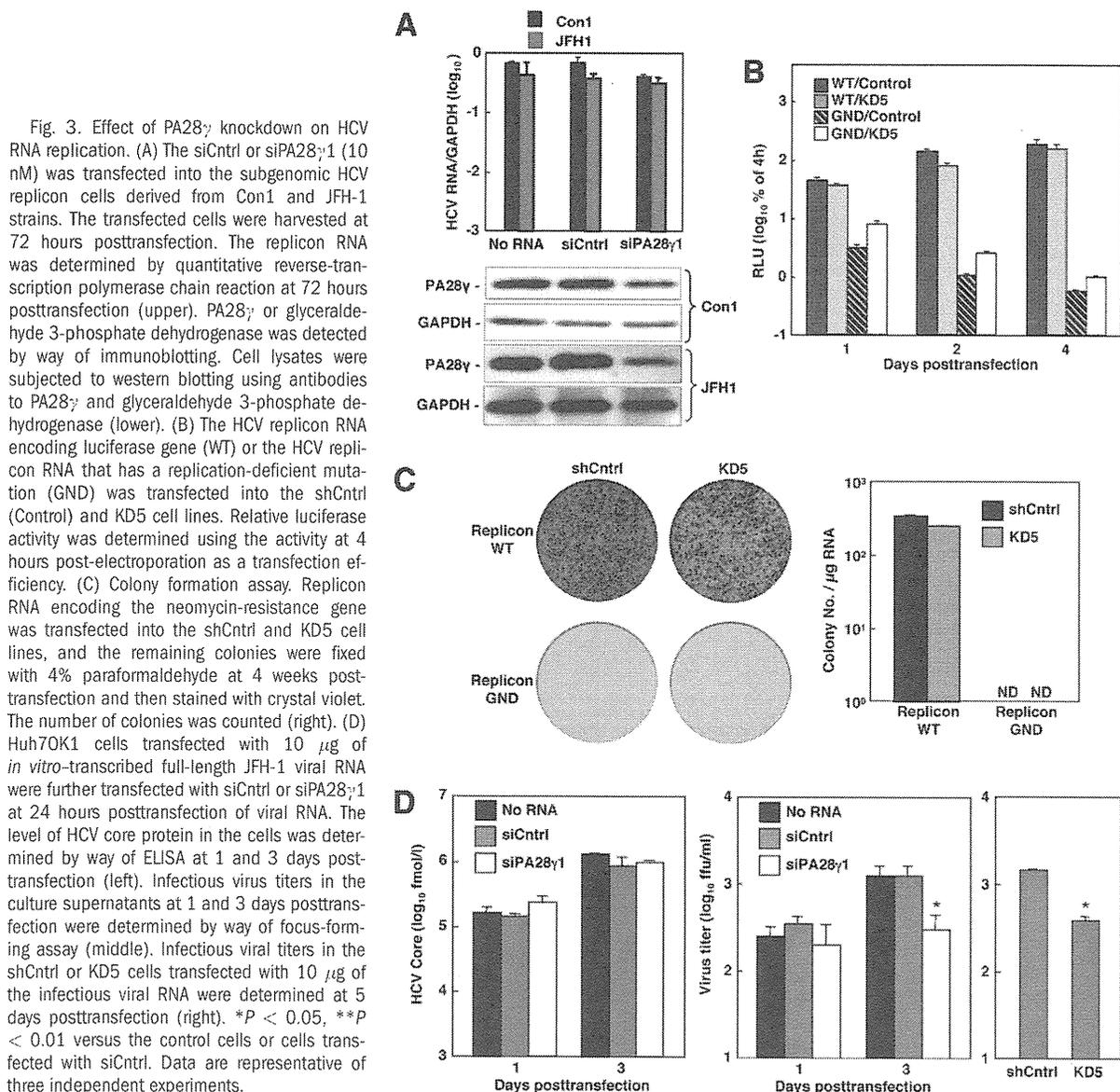


Fig. 2. Establishment of PA28 γ knockdown cell lines and propagation of HCV. (A) Huh7OK1 cells were transfected with pSilencer shPA28 γ or control plasmid and selected by hygromycin at 48 hours posttransfection. Two PA28 γ knockdown cell lines (KD5 and KD7) and one control cell line (shCntrl) were established, and PA28 γ 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-1 virus 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 PA28 γ 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.

derived from the Con1 or JFH-1 strain induced no significant reduction of HCV RNA (Fig. 3A). Furthermore, luciferase activities in the stable PA28 γ

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 PA28 γ 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 PA28 γ on the postreplication steps,

in vitro transcribed full-length viral RNA was transfected into Huh7OK1 cells, and siPA28 γ 1 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 siPA28 γ 1 (Fig. 3D, left panel). However, infectious virus titers in the supernatant were significantly decreased by the transient and stable knockdown of PA28 γ compared with control cells (Fig. 3D, middle and right panels). Furthermore, PA28 γ did not contribute to the virus production of JEV (Fig. 2F), suggesting that the general sorting pathway of the flavivirus is functional under



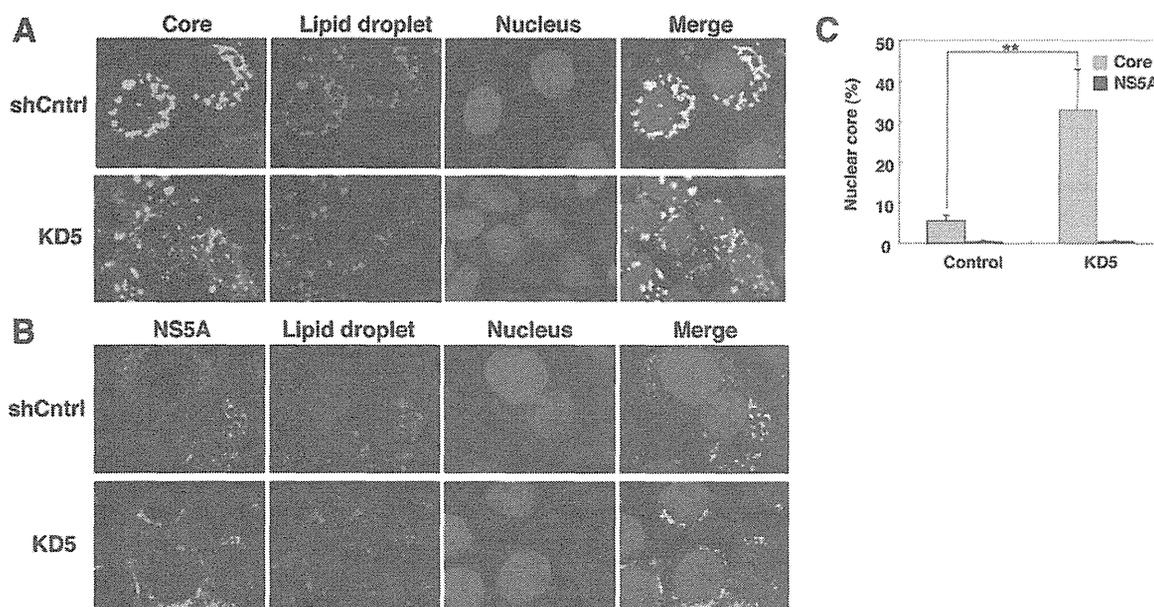


Fig. 4. Effect of PA28 γ knockdown on the localization of HCV core protein and lipid droplets. The shCntrl and KD5 cell lines infected with JFH-1 virus were fixed with methanol or paraformaldehyde for 5 minutes at 4 days postinfection. HCV core (A) and NS5A (B) proteins were stained with rabbit antibodies raised against the proteins and Alexa Fluor 488-conjugated goat anti-rabbit immunoglobulin G antibody. Lipid droplets were stained with Bodipy 558/568 C12. Nuclei were stained with 50 μ M Hechst 33258 after treatment with 1 μ g/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 PA28 γ knockdown condition. These results suggest that PA28 γ specifically regulates the postreplication steps in the life cycle of HCV.

Core Protein Is Partially Accumulated in the Nucleus of PA28 γ Knockdown Cells. We reported previously that some fraction of HCV core protein migrates into the nucleus and is then degraded by a PA28 γ -dependent proteasome pathway.⁷ Furthermore, we have demonstrated that HCV core protein is clearly accumulated in the nucleus of the liver cells of PA28 γ -knockout mice.⁸ However, the role of PA28 γ 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 PA28 γ participates in the degradation of HCV core protein in the nucleus.

PA28 γ 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 PA28 γ participates in the degradation of the core protein through a ubiquitin-independent pathway in the nucleus.¹⁷ We have also demonstrated that PA28 γ 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 PA28 γ on the virus propagation and the ubiquitination of the core protein. JFH-1 virus was inoculated into E6AP- and/or PA28 γ 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

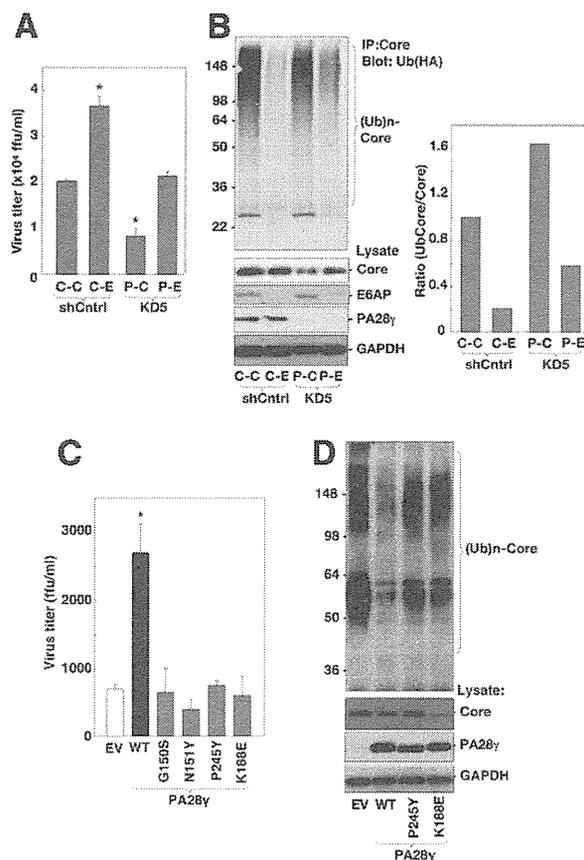


Fig. 5. PA28 γ knockdown enhances E6AP-dependent ubiquitination of core protein and reduces virus titer. (A) shCntrl and KD5 cells transfected with plasmids encoding the negative control (C-C and P-C) or E6AP (C-E and P-E) shRNA were treated with puromycin for 2 days. The remaining cells seeded at 2.5×10^4 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 $10 \mu\text{M}$ 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 PA28 γ 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 PA28 γ , were treated with $10 \mu\text{M}$ 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 PA28 γ . * $P < 0.05$ 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 PA28 γ 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 PA28 γ knockdown (Fig. 5B, lanes C-E and P-C). Furthermore, E6AP knockdown in the PA28 γ knockdown cells restored the ubiquitination of the core protein to a certain extent (Fig. 5B, lane P-E). It was shown that Pro²⁴⁵ of PA28 γ is critical for binding to the 20S proteasome, and that Gly¹⁵⁰ and Asn¹⁵¹ of PA28 γ are important for activation of the proteasome.²⁶ To further examine the functional significance of PA28 γ on HCV propagation, expression plasmids encoding siRNA-resistant PA28 γ mutants in which Gly¹⁵⁰, Asn¹⁵¹, and Pro²⁴⁵ 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 PA28 γ exhibited a partial recovery of virus production, although those transfected with the plasmid encoding PA28 γ G150S, N151Y, or P245Y or with an empty vector exhibited no effect on virus production. Replacing Lys¹⁸⁸ with Glu in PA28 γ (PA28 γ K188E) confers the capability of proteasome-mediated cleavage after hydrophobic, acidic, and basic residues such as those exhibited by PA28 α .²⁷ Expression of siRNA-resistant PA28 γ 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 PA28 γ but not expression of the PA28 γ mutants (P245Y or K188E) in KD5 cells (Fig. 5D). Collectively, these results suggest that PA28 γ positively regulates HCV propagation by inhibiting degradation of HCV core protein by an E6AP/ubiquitin-dependent proteasome.

Discussion

To explore the role of PA28 γ on the life cycle of HCV, we examined the effects of knockdown of PA28 γ in Huh7OK1 cells infected with the JFH-1 virus. Knockdown of PA28 γ 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, PA28 γ knockdown did not affect the production of JEV, of which the capsid protein does not interact with PA28 γ , suggesting that PA28 γ knockdown does not affect the general sorting pathway of flavivirus. These results suggest that PA28 γ 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/PA28 γ knockout mice.⁸ PA28 γ 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 PA28 γ -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 PA28 γ knockdown cell lines, it was partially localized in the nucleus in PA28 γ knockdown cells but not control cells. Furthermore, localization of HCV core protein on the surface of lipid droplets was impaired in PA28 γ knockdown cells (Fig. 4). These results suggest that HCV core protein is partially translocated into the nucleus and degraded in the PA28 γ -dependent proteasome pathway in HCV-infected cells and that PA28 γ 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 PA28 γ -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 PA28 γ induced up-regulation of the ubiquitination of HCV core protein and down-regulation of the viral production, suggesting that PA28 γ -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 PA28 γ enhances the trypsin-like activity of proteasome that cleaves peptide bonds

after basic residues of the substrates, whereas the PA28 γ K188E mutant enhances the proteasome activity that cleaves peptide bonds after hydrophobic, acidic, and basic residues in the manner of PA28 α .²⁷ Therefore, the sizes of fragments produced by the PA28 γ -dependent proteasome should be different from those produced by the PA28 α/β - or ubiquitination-mediated proteasome. It might be feasible to speculate that the peptide fragments of HCV core protein generated by the PA28 γ -dependent proteasome or PA28 γ *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 PA28 γ 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 PA28 γ .

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 potentially 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 PA28 γ was found in the PA28 γ knockdown cells, suggesting that E6AP-dependent degradation of HCV core protein is not potentially suppressed in the PA28 γ knockdown cells. If HCV core protein is constitutively expressed under the PA28 γ 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 PA28 γ 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 PA28 γ .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 PA28 γ knockdown in the 293T cell line.⁸ These results and the data in Fig. 5 suggest that the suppression of PA28 γ 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 PA28 γ -dependent degradation mechanisms. E6AP-dependent degradation of HCV core protein in cytoplasm may be enhanced *in vivo* under the PA28 γ knockout condition.

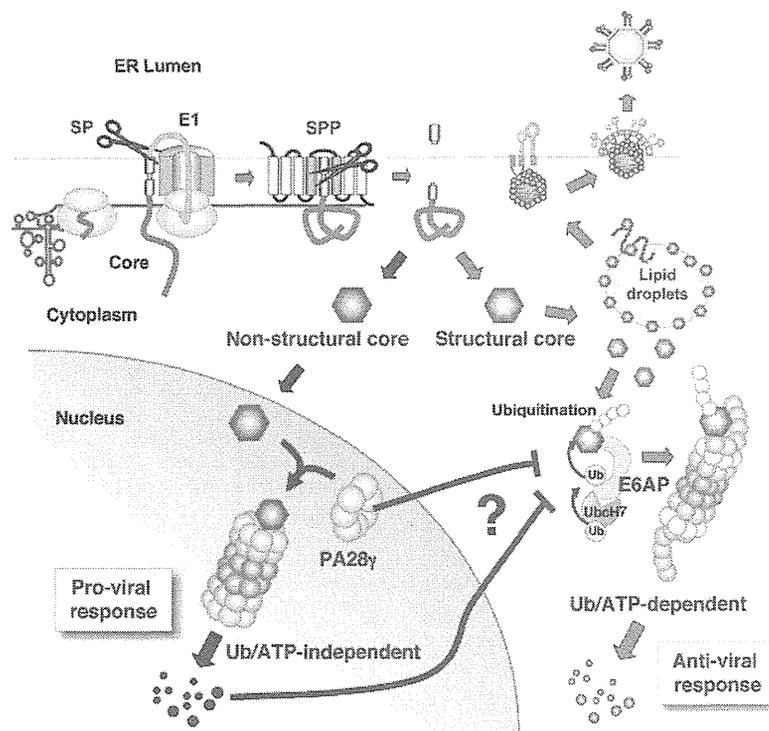


Fig. 6. Schematic diagram of the potential roles of PA28 γ in HCV propagation. HCV core protein is cleaved off from the precursor polyprotein by signal peptidase (SP) and the signal sequence is further processed by signal peptide peptidase (SPP). The mature core protein mainly localizes on the lipid 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 PA28 γ -dependent proteasome pathway, and the core protein fragments generated by the PA28 γ pathway or PA28 γ *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 PA28 γ positively regulates particle production of HCV by inhibiting E6AP-dependent ubiquitination of the core protein, in addition to our previous observation that PA28 γ plays a crucial role in the development of liver pathology induced by HCV core protein.⁸ PA28 γ knockout mice exhibit only mild growth retardation.^{15,16} Therefore, PA28 γ 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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Involvement of PA28 γ in the Propagation of Hepatitis C Virus

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We have reported previously that the proteasome activator PA28 γ 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 PA28 γ in the propagation of HCV has not been clarified. PA28 γ is an activator of proteasome responsible for ubiquitin-independent degradation of substrates in the nucleus. In the present study, knockdown of PA28 γ 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 PA28 γ knockdown cells was restored by the expression of an small interfering RNA (siRNA)-resistant PA28 γ . Although viral proteins were detected in the cytoplasm of cells infected with HCV, suppression of PA28 γ 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 PA28 γ 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 PA28 γ knockdown cells was restored through knockdown of E6AP or expression of the siRNA-resistant wild-type but not mutant PA28 γ incapable of activating proteasome activity. **Conclusion:** Our results suggest that PA28 γ 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 Phe¹⁷⁷ and Leu¹⁷⁸ 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 PA28 γ /REG γ in the nucleus and is digested by a PA28 γ -dependent proteasome activity.⁷ *In vivo* experiments in a mouse model suggest that PA28 γ plays a critical role in the pathogenesis induced by HCV core protein.^{8,9} PA28 γ forms a homoheptamer in the nucleus and enhances the proteasome-mediated cleavage after basic amino acid residues, whereas PA28 α and PA28 β exhibit 41% and 34% homology to PA28 γ , 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 PA28 γ 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 PA28 γ are largely unknown *in vivo*, because PA28 γ -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 PA28 γ -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 PA28 γ in the propagation of HCV has not yet been characterized. In this study, we examined the biological significance of PA28 γ 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 PA28 γ 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 siPA28 γ 1 and siPA28 γ 2, 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% CO₂. 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 RNA²⁰ 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-shPA28 γ 4 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 μ g/mL. Huh7OK1 cells transfected with the control plasmid were selected with puromycin and designated as shCtrl, whereas those transfected with pSilencer-shPA28 γ 4 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 PA28 γ complementary DNAs resistant to siRNA against PA28 γ were prepared by using the silent mutations as reported.⁸ These plasmids were transfected into Huh7OK1 cells and cultivated in medium containing 0.1 μ g/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 μ g/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 assay²¹ 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 described²³ 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 C₁₂ according to the method of Targett-Adams et al.²⁴ at 4 days postinfection, and then fixed at 4°C for 30 minutes with 4% paraformaldehyde in phosphate-buffered saline. After treatment of cells with 1 μ g/mL of RNase A, nuclei were stained with 50 μ M Hechst 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 PA28 γ 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 Huh7OK1 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 Huh7OK1 cell line exhibits highly permissive to JFH-1 virus infection has not been clarified yet. Two siRNAs were used to knock down PA28 γ , but only one, siPA28 γ 1, was used because the other had off-target effects (Supporting Fig. 1). To examine the effect of PA28 γ on the propagation of HCV, siPA28 γ 1 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

PA28 γ 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

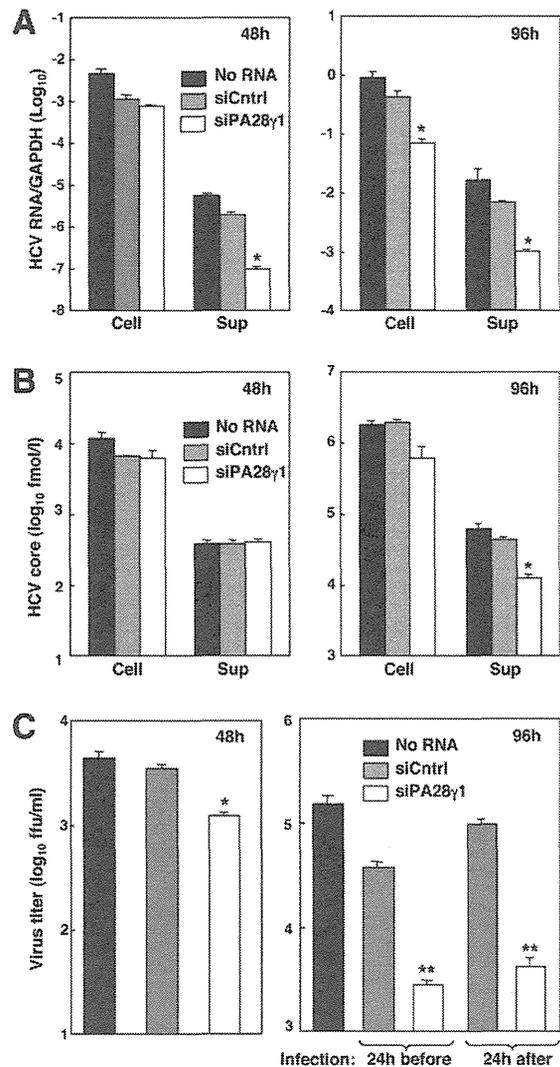


Fig. 1. Transient knockdown of PA28 γ before or after infection with HCV reduces particle production. (A) Huh7OK1 cells transfected with a control siRNA (siCntrl) or PA28 γ siRNA1 were infected with JFH-1 virus at 24 hours posttransfection and then harvested at 48 hours (left panel) and 96 hours postinfection (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 PA28 γ 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 PA28 γ 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 siPA28 γ 1 into cells even at 24 hours postinfection (Fig. 1C, right panel). These results suggest that PA28 γ participates in the regulation of HCV propagation in postentry steps.

Stable Knockdown of PA28 γ Impairs Viral Propagation. To establish the PA28 γ knockdown cell lines, Huh7OK1 cells were transfected with a plasmid encoding a short hairpin RNA (shRNA) targeted to PA28 γ and selected with hygromycin, resulting in two clones—KD5 and KD7—that exhibited a clear reduction of PA28 γ expression (Fig. 2A). Although the suppression of PA28 γ 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 PA28 γ 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 PA28 γ in PA28 γ 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 PA28 γ in control cells slightly enhanced virus production (Fig. 2E). Our previous data suggest that capsid protein of JEV does not bind to PA28 γ .⁷ To examine whether PA28 γ 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 PA28 γ does not participate in the virus production pathway of JEV. These results further support the notion that PA28 γ participates in HCV propagation.

Knockdown of PA28 γ Exhibits No Effect on Viral RNA Replication. Although knockdown of PA28 γ resulted in the suppression of viral particle and RNA production in the culture supernatant at 48 hours postinfection with JFH-1 virus, viral RNA in the cells was not reduced (Fig. 1), suggesting that PA28 γ does not participate in viral replication. To gain more insight on this point, we examined the effect of PA28 γ knockdown on RNA replication in replicon cells. Transient knockdown of PA28 γ through introduction of siPA28 γ into the subgenomic HCV replicon cells

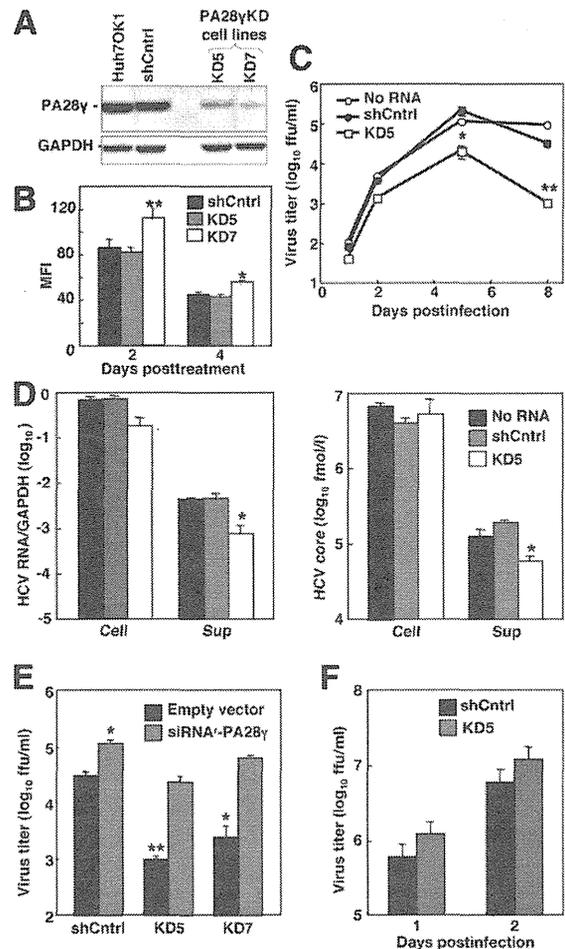


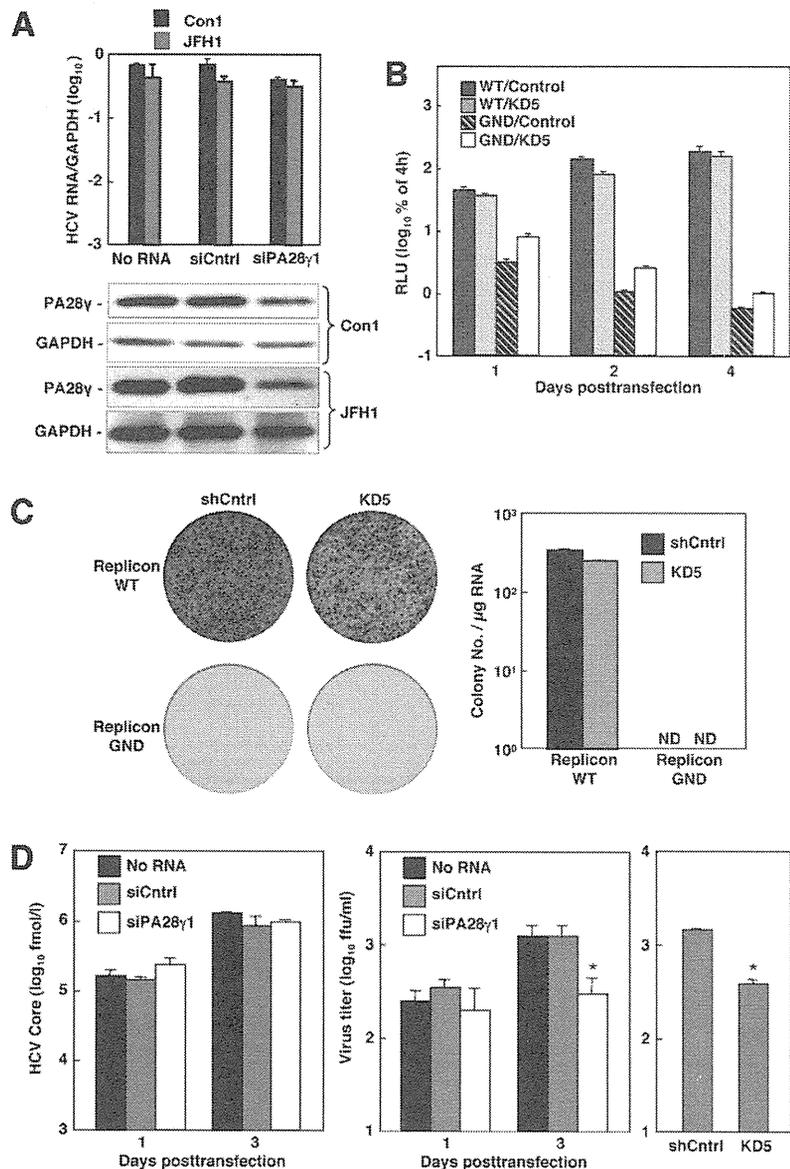
Fig. 2. Establishment of PA28 γ knockdown cell lines and propagation of HCV. (A) Huh7OK1 cells were transfected with pSilencer shPA28 γ or control plasmid and selected by hygromycin at 48 hours posttransfection. Two PA28 γ knockdown cell lines (KD5 and KD7) and one control cell line (shCntrl) were established, and PA28 γ 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-1 virus 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 PA28 γ 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.

derived from the Con1 or JFH-1 strain induced no significant reduction of HCV RNA (Fig. 3A). Furthermore, luciferase activities in the stable PA28 γ

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 PA28 γ 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 PA28 γ on the postreplication steps,

in vitro transcribed full-length viral RNA was transfected into Huh7OK1 cells, and siPA28 γ 1 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 siPA28 γ 1 (Fig. 3D, left panel). However, infectious virus titers in the supernatant were significantly decreased by the transient and stable knockdown of PA28 γ compared with control cells (Fig. 3D, middle and right panels). Furthermore, PA28 γ 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 PA28 γ knockdown on HCV RNA replication. (A) The siCntrl or siPA28 γ 1 (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). PA28 γ or glyceraldehyde 3-phosphate dehydrogenase was detected by way of immunoblotting. Cell lysates were subjected to western blotting using antibodies to PA28 γ 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 μ g of *in vitro*-transcribed full-length JFH-1 viral RNA were further transfected with siCntrl or siPA28 γ 1 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 μ g 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.



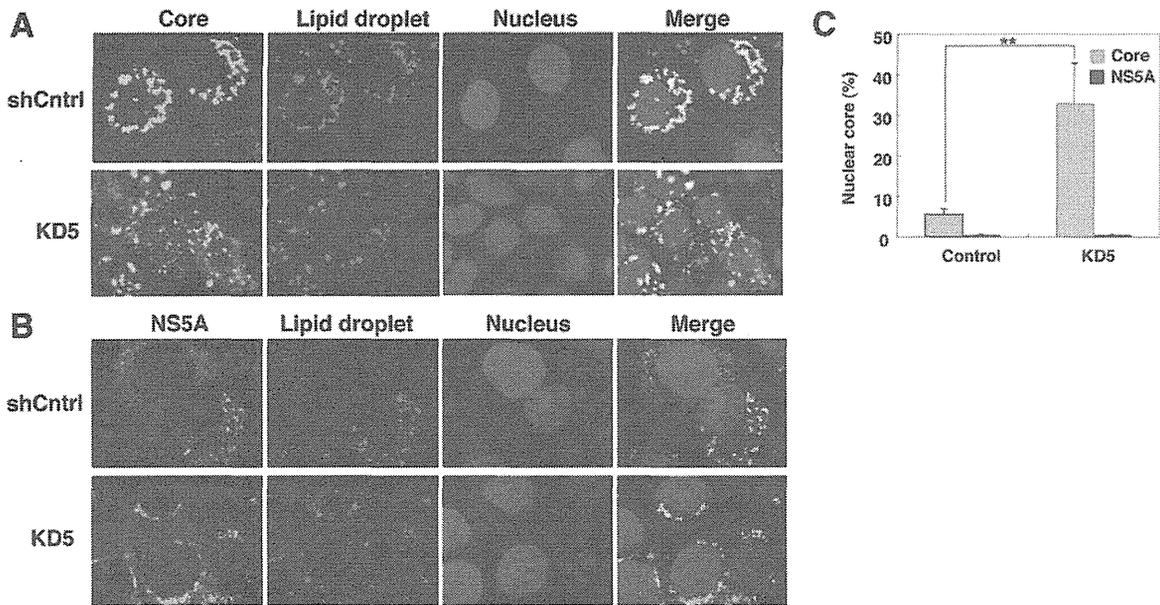


Fig. 4. Effect of PA28 γ knockdown on the localization of HCV core protein and lipid droplets. The shCntrl and KD5 cell lines infected with JFH-1 virus were fixed with methanol or paraformaldehyde for 5 minutes at 4 days postinfection. HCV core (A) and NS5A (B) proteins were stained with rabbit antibodies raised against the proteins and Alexa Fluor 488-conjugated goat anti-rabbit immunoglobulin G antibody. Lipid droplets were stained with Bodipy 558/568 C12. Nuclei were stained with 50 μ M Hoechst 33258 after treatment with 1 μ g/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 PA28 γ knockdown condition. These results suggest that PA28 γ specifically regulates the postreplication steps in the life cycle of HCV.

Core Protein Is Partially Accumulated in the Nucleus of PA28 γ Knockdown Cells. We reported previously that some fraction of HCV core protein migrates into the nucleus and is then degraded by a PA28 γ -dependent proteasome pathway.⁷ Furthermore, we have demonstrated that HCV core protein is clearly accumulated in the nucleus of the liver cells of PA28 γ -knockout mice.⁸ However, the role of PA28 γ 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 PA28 γ participates in the degradation of HCV core protein in the nucleus.

PA28 γ 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 PA28 γ participates in the degradation of the core protein through a ubiquitin-independent pathway in the nucleus.¹⁷ We have also demonstrated that PA28 γ 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 PA28 γ on the virus propagation and the ubiquitination of the core protein. JFH-1 virus was inoculated into E6AP- and/or PA28 γ 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

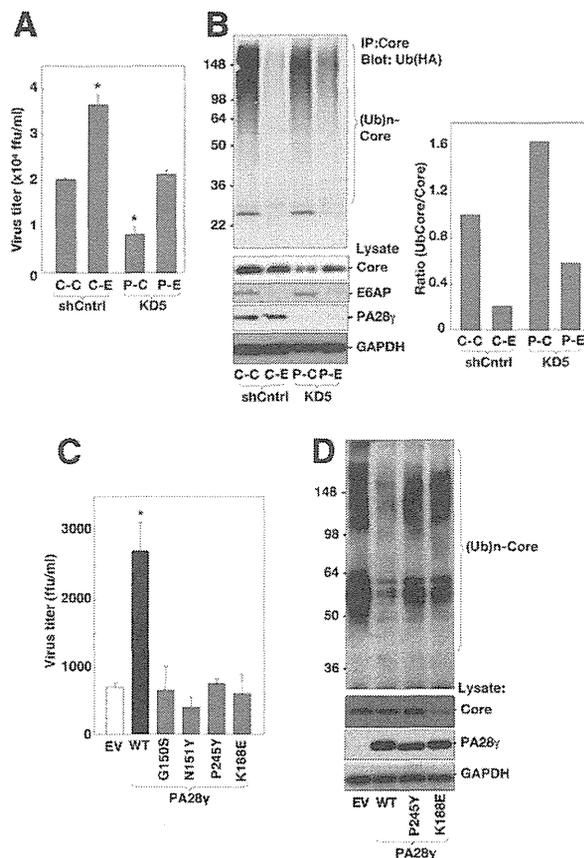


Fig. 5. PA28 γ knockdown enhances E6AP-dependent ubiquitination of core protein and reduces virus titer. (A) shCntrl and KD5 cells transfected with plasmids encoding the negative control (C-C and P-C) or E6AP (C-E and P-E) shRNA were treated with puromycin for 2 days. The remaining cells seeded at 2.5×10^4 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 $10 \mu\text{M}$ 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 PA28 γ 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 PA28 γ , were treated with $10 \mu\text{M}$ 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 PA28 γ . * $P < 0.05$ 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 PA28 γ 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 PA28 γ knockdown (Fig. 5B, lanes C-E and P-C). Furthermore, E6AP knockdown in the PA28 γ knockdown cells restored the ubiquitination of the core protein to a certain extent (Fig. 5B, lane P-E). It was shown that Pro²⁴⁵ of PA28 γ is critical for binding to the 20S proteasome, and that Gly¹⁵⁰ and Asn¹⁵¹ of PA28 γ are important for activation of the proteasome.²⁶ To further examine the functional significance of PA28 γ on HCV propagation, expression plasmids encoding siRNA-resistant PA28 γ mutants in which Gly¹⁵⁰, Asn¹⁵¹, and Pro²⁴⁵ 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 PA28 γ exhibited a partial recovery of virus production, although those transfected with the plasmid encoding PA28 γ G150S, N151Y, or P245Y or with an empty vector exhibited no effect on virus production. Replacing Lys¹⁸⁸ with Glu in PA28 γ (PA28 γ K188E) confers the capability of proteasome-mediated cleavage after hydrophobic, acidic, and basic residues such as those exhibited by PA28 α .²⁷ Expression of siRNA-resistant PA28 γ 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 PA28 γ but not expression of the PA28 γ mutants (P245Y or K188E) in KD5 cells (Fig. 5D). Collectively, these results suggest that PA28 γ positively regulates HCV propagation by inhibiting degradation of HCV core protein by an E6AP/ubiquitin-dependent proteasome.

Discussion

To explore the role of PA28 γ on the life cycle of HCV, we examined the effects of knockdown of PA28 γ in Huh7OK1 cells infected with the JFH-1 virus. Knockdown of PA28 γ 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, PA28 γ knockdown did not affect the production of JEV, of which the capsid protein does not interact with PA28 γ , suggesting that PA28 γ knockdown does not affect the general sorting pathway of flavivirus. These results suggest that PA28 γ 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/PA28 γ knockout mice.⁸ PA28 γ 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 PA28 γ -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 PA28 γ knockdown cell lines, it was partially localized in the nucleus in PA28 γ knockdown cells but not control cells. Furthermore, localization of HCV core protein on the surface of lipid droplets was impaired in PA28 γ knockdown cells (Fig. 4). These results suggest that HCV core protein is partially translocated into the nucleus and degraded in the PA28 γ -dependent proteasome pathway in HCV-infected cells and that PA28 γ 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 PA28 γ -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 PA28 γ induced up-regulation of the ubiquitination of HCV core protein and down-regulation of the viral production, suggesting that PA28 γ -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 PA28 γ enhances the trypsin-like activity of proteasome that cleaves peptide bonds

after basic residues of the substrates, whereas the PA28 γ K188E mutant enhances the proteasome activity that cleaves peptide bonds after hydrophobic, acidic, and basic residues in the manner of PA28 α .²⁷ Therefore, the sizes of fragments produced by the PA28 γ -dependent proteasome should be different from those produced by the PA28 α/β - or ubiquitination-mediated proteasome. It might be feasible to speculate that the peptide fragments of HCV core protein generated by the PA28 γ -dependent proteasome or PA28 γ *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 PA28 γ 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 PA28 γ .

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 potentially 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 PA28 γ was found in the PA28 γ knockdown cells, suggesting that E6AP-dependent degradation of HCV core protein is not potentially suppressed in the PA28 γ knockdown cells. If HCV core protein is constitutively expressed under the PA28 γ 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 PA28 γ 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 PA28 γ .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 PA28 γ knockdown in the 293T cell line.⁸ These results and the data in Fig. 5 suggest that the suppression of PA28 γ 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 PA28 γ -dependent degradation mechanisms. E6AP-dependent degradation of HCV core protein in cytoplasm may be enhanced *in vivo* under the PA28 γ knockout condition.

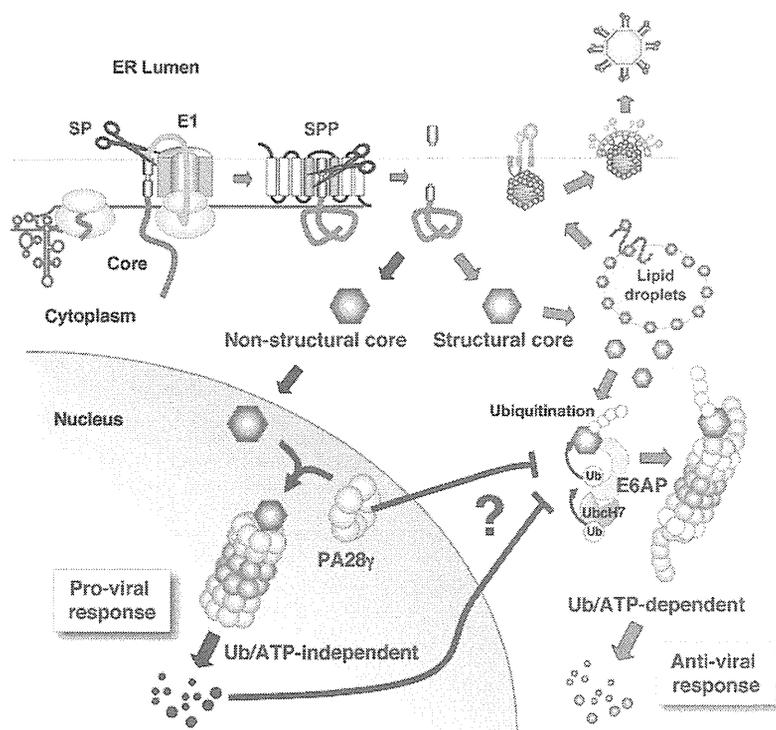


Fig. 6. Schematic diagram of the potential roles of PA28 γ in HCV propagation. HCV core protein is cleaved off from the precursor polyprotein by signal peptidase (SP) and the signal sequence is further processed by signal peptide peptidase (SPP). The mature core protein mainly localizes on the lipid 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 PA28 γ -dependent proteasome pathway, and the core protein fragments generated by the PA28 γ pathway or PA28 γ *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 PA28 γ positively regulates particle production of HCV by inhibiting E6AP-dependent ubiquitination of the core protein, in addition to our previous observation that PA28 γ plays a crucial role in the development of liver pathology induced by HCV core protein.⁸ PA28 γ knockout mice exhibit only mild growth retardation.^{15,16} Therefore, PA28 γ 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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