

were separated by polyacrylamide gel electrophoresis, and the proteins were visualized by autoradiography.

**Supplementary Fig. 6: Differential involvement of MDA5 and MyD88 in EMCV-mediated IFN production in cDCs and pDCs.**

DCs were induced from bone marrow cells obtained from MyD88<sup>+/-</sup>, MyD88<sup>-/-</sup>, MDA5<sup>+/-</sup> and MDA5<sup>-/-</sup> mice by cultivating in the presence of Flt3L. At day 7, B220<sup>+</sup>CD11c<sup>+</sup> pDCs and B220<sup>-</sup>CD11c<sup>+</sup> cDCs were purified by MACS, and infected with EMCV for 24h. IFN- $\alpha$  production in the culture supernatants was measured by ELISA. Error bars indicate  $\pm$  S.D. of triplicates.

**Supplementary Fig. 7: The survival of MDA5<sup>-/-</sup>, RIG-I<sup>-/-</sup> or IFN $\alpha$ / $\beta$ R<sup>-/-</sup> mice in response to VSV infection.**

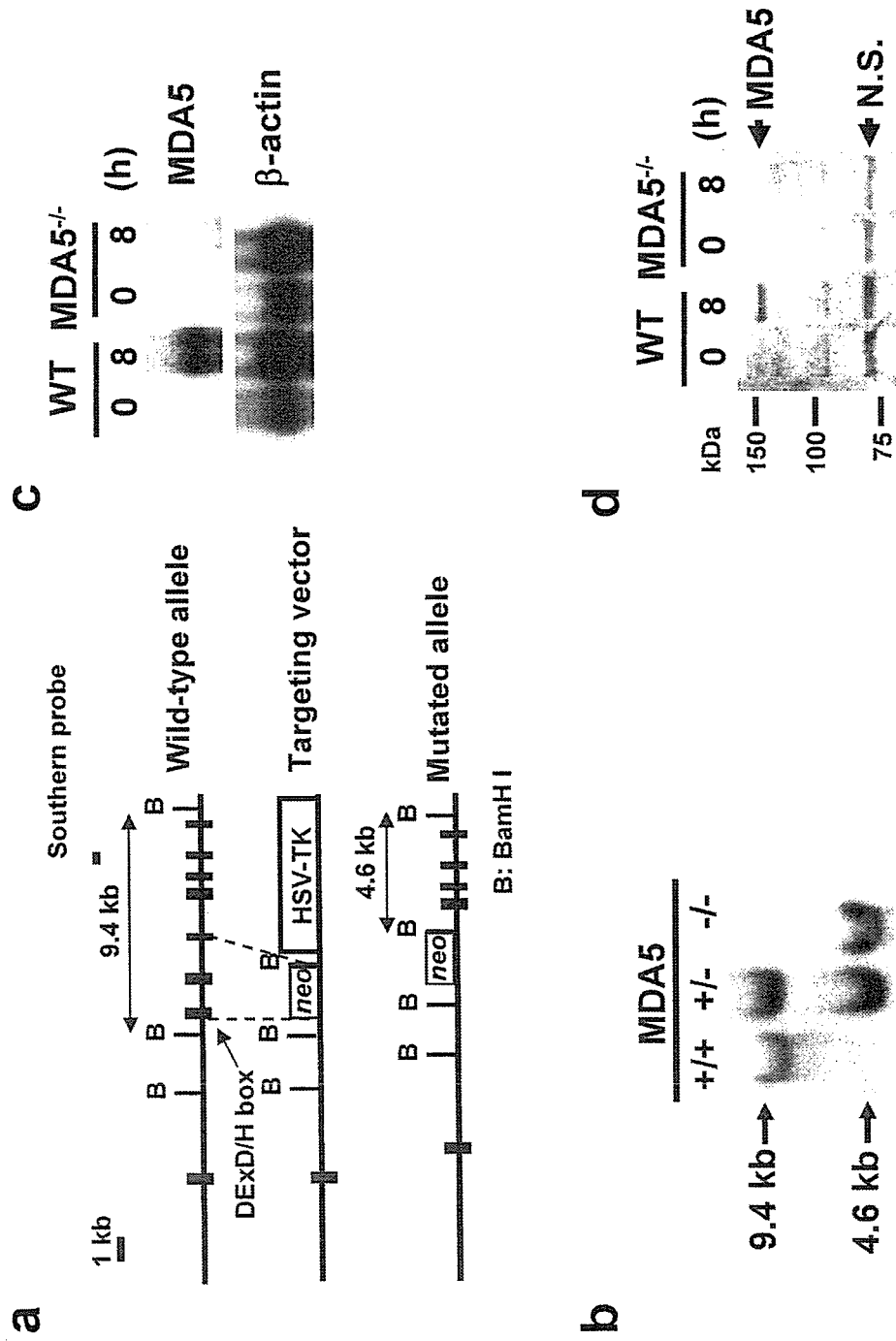
The survival of the mice (3-weeks old) infected with  $4 \times 10^6$  pfu VSV intranasally was monitored for 9 days ( $p < 0.01$  by the generalized wilcoxon test between RIG-I<sup>-/-</sup> mice and their littermate controls).

**Supplementary Fig. 8: Responses of MDA5<sup>-/-</sup> mice against EMCV infection**

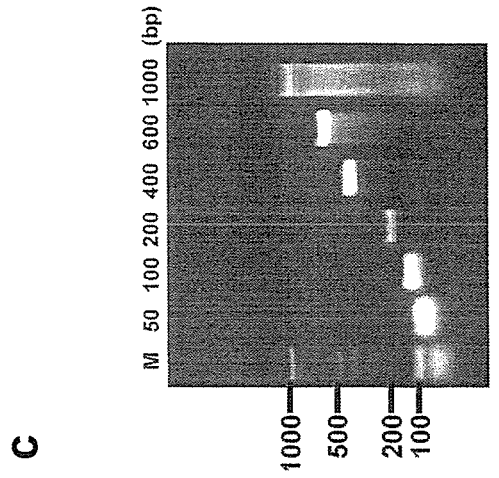
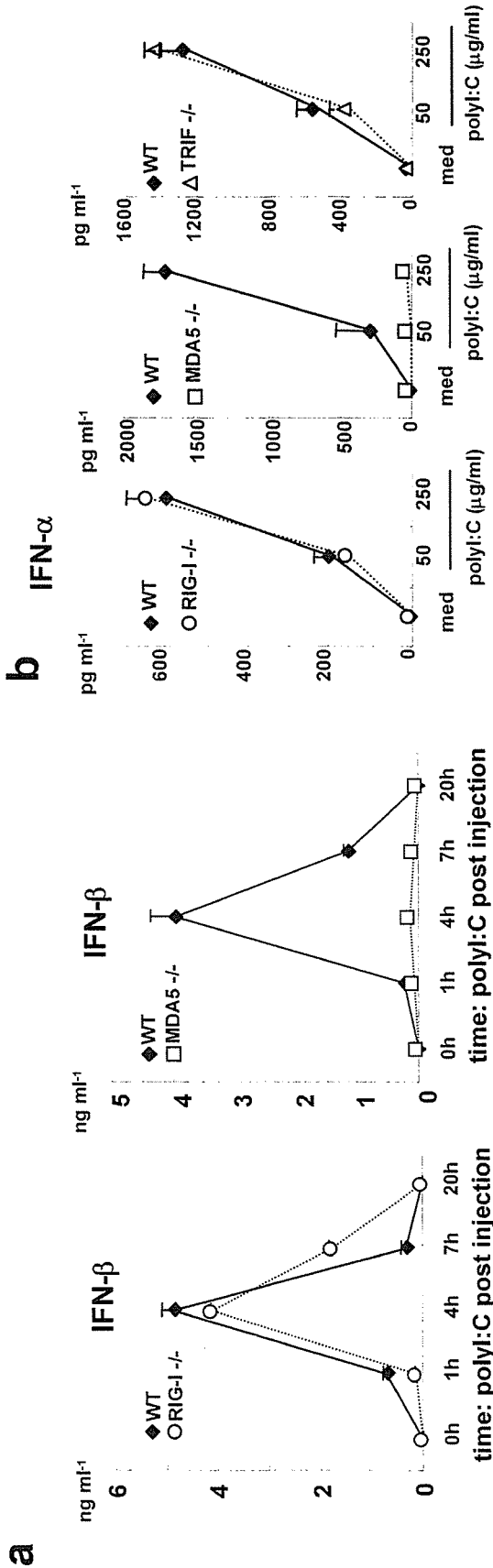
- (a) MDA5<sup>+/-</sup> and MDA5<sup>-/-</sup> mice (n=5) were intravenously inoculated with  $1 \times 10^7$  pfu EMCV. Sera were taken at 4 h after injection, and IFN- $\beta$ , Rantes and IL-6 production levels were determined by ELISA. \*,  $P < 0.05$  versus controls by the student's t-test.
- (b) Cardiac function of the mice 48 h after EMCV infection was assessed by echocardiography. Transthoracic M-mode echocardiographic tracings from MDA5<sup>+/-</sup> and MDA5<sup>-/-</sup> mice 48 h after EMCV infection.

**Supplementary Table I: Genotypes of mice derived from RIG-I<sup>+/-</sup> intercrosses or crosses between RIG-I<sup>+/-</sup> and RIG-I<sup>-/-</sup> mice.**

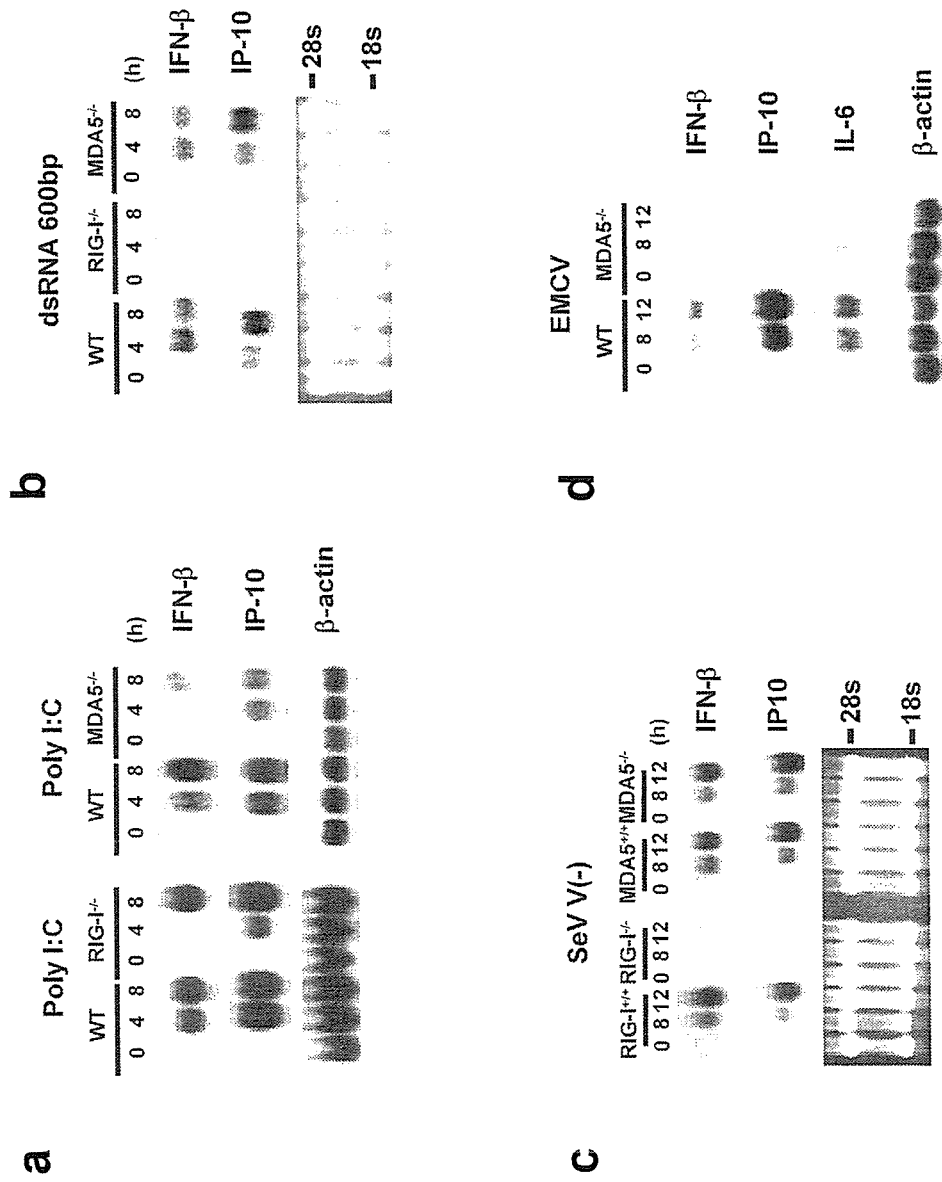
# Supplemental Figure 1.



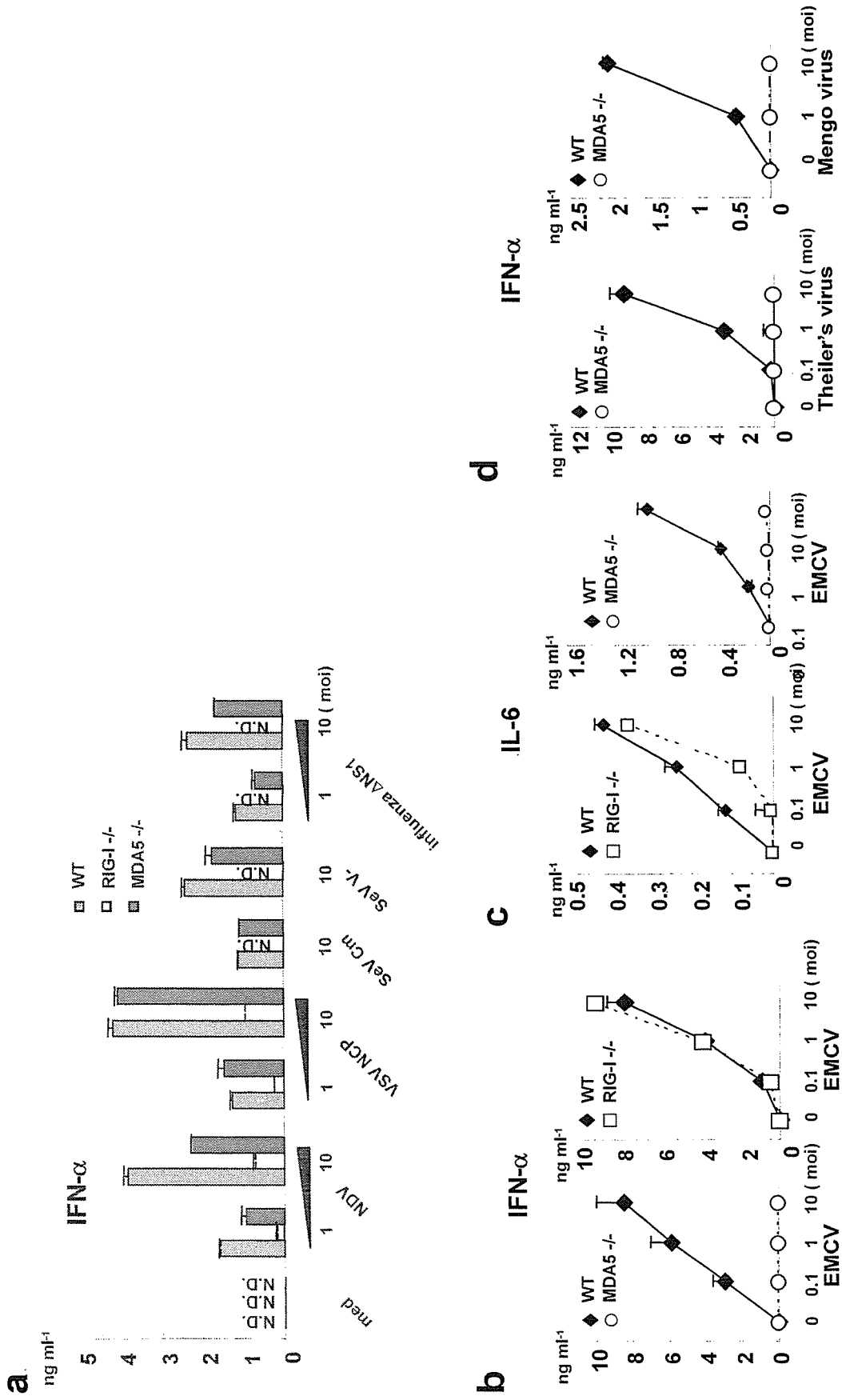
# Supplemental Figure 2.



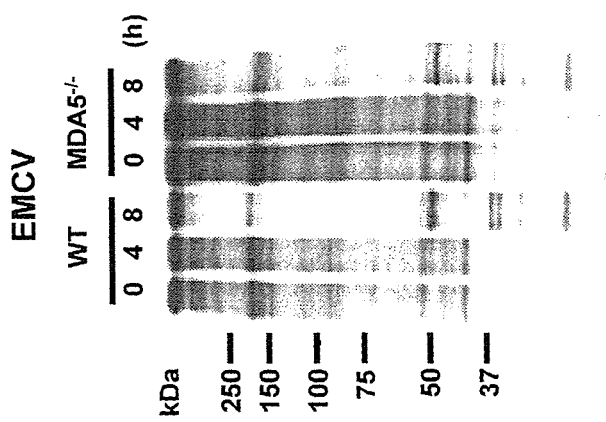
# Supplemental Figure 3.



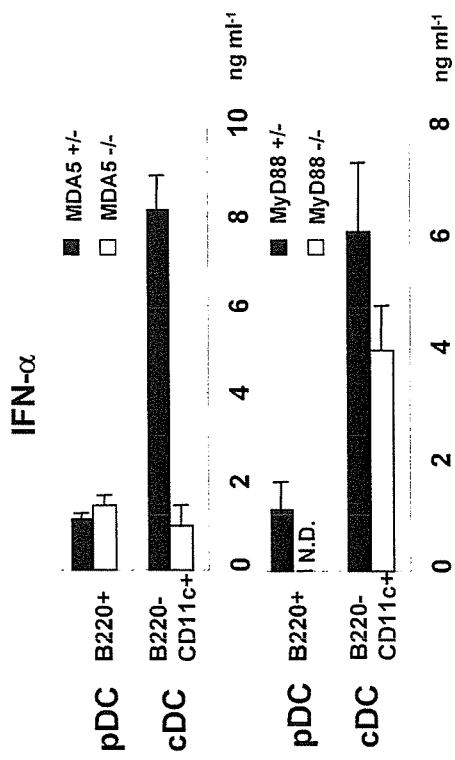
# Supplemental Figure 4.



# Supplemental Figure 5.

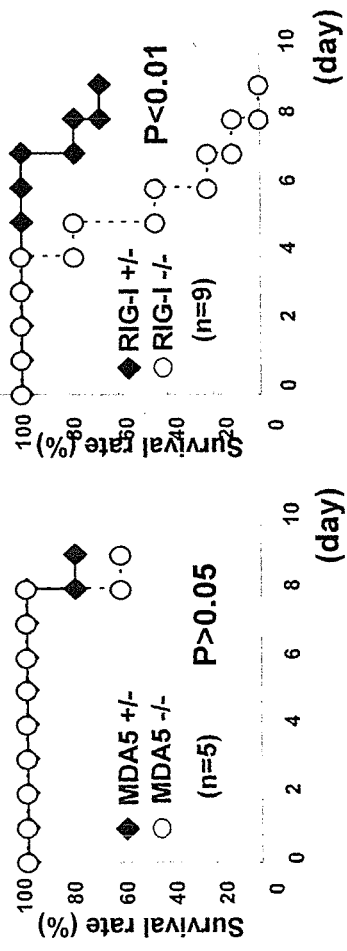


# Supplemental Figure 6.



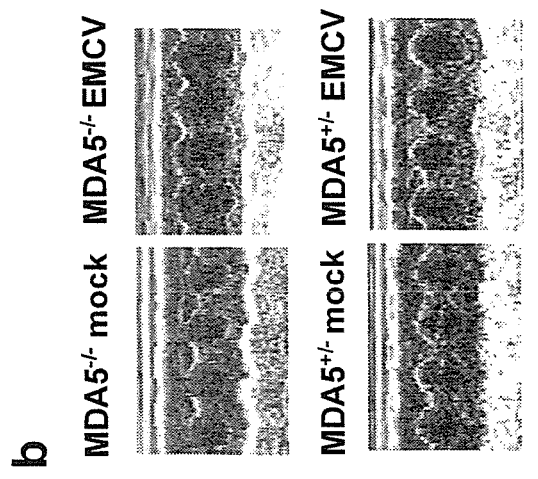
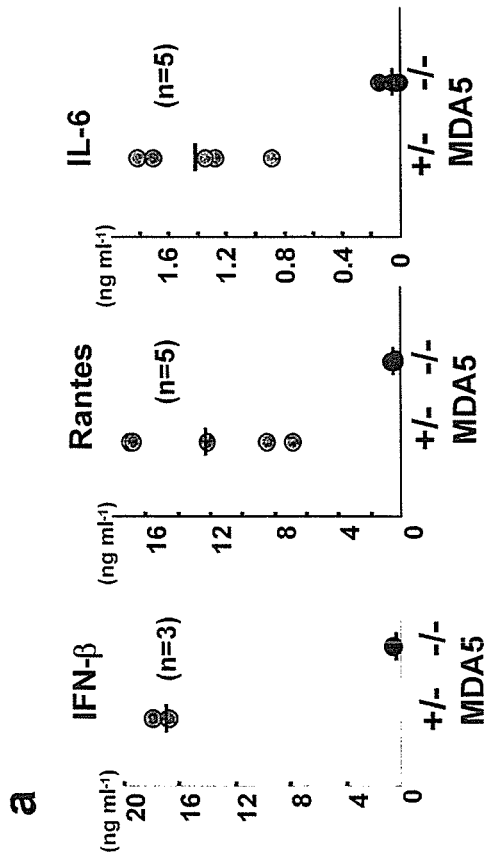
Supplemental Figure 7.

VSV infection





# Supplemental Figure 8.



**Supplemental Table 1.**

**a**

	RIG-I <sup>+/+</sup>	RIG-I <sup>+/-</sup>	RIG-I <sup>-/-</sup>	Total (%)
Postnatal (6w~)	16 (34)	28 (60)	3 (6)	47 (100)

**b**

	RIG-I <sup>+/-</sup>	RIG-I <sup>-/-</sup>	Total (%)
Postnatal (6w~)	108 (83)	22 (17)	130 (100)

## Involvement of the PA28 $\gamma$ -Dependent Pathway in Insulin Resistance Induced by Hepatitis C Virus Core Protein<sup>∇</sup>

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The hepatitis C virus (HCV) core protein is a component of nucleocapsids and a pathogenic factor for hepatitis C. Several epidemiological and experimental studies have suggested that HCV infection is associated with insulin resistance, leading to type 2 diabetes. We have previously reported that HCV core gene-transgenic (PA28 $\gamma^{+/+}$  CoreTg) mice develop marked insulin resistance and that the HCV core protein is degraded in the nucleus through a PA28 $\gamma$ -dependent pathway. In this study, we examined whether PA28 $\gamma$  is required for HCV core-induced insulin resistance in vivo. HCV core gene-transgenic mice lacking the PA28 $\gamma$  gene (PA28 $\gamma^{-/-}$  CoreTg) were prepared by mating of PA28 $\gamma^{+/+}$  CoreTg with PA28 $\gamma$ -knockout mice. Although there was no significant difference in the glucose tolerance test results among the mice, the insulin sensitivity in PA28 $\gamma^{-/-}$  CoreTg mice was recovered to a normal level in the insulin tolerance test. Tyrosine phosphorylation of insulin receptor substrate 1 (IRS1), production of IRS2, and phosphorylation of Akt were suppressed in the livers of PA28 $\gamma^{+/+}$  CoreTg mice in response to insulin stimulation, whereas they were restored in the livers of PA28 $\gamma^{-/-}$  CoreTg mice. Furthermore, activation of the tumor necrosis factor alpha promoter in human liver cell lines or mice by the HCV core protein was suppressed by the knockdown or knockout of the PA28 $\gamma$  gene. These results suggest that the HCV core protein suppresses insulin signaling through a PA28 $\gamma$ -dependent pathway.

Hepatitis C virus (HCV) is the causative agent in most cases of acute and chronic non-A, non-B hepatitis (15). Over one-half of patients with the acute infection evolve into a persistent carrier state (24). Chronic infection with HCV frequently induces hepatic steatosis, cirrhosis, and eventually hepatocellular carcinoma (22) and is known to be associated with diseases of extrahepatic organs, including an essential mixed cryoglobulinemia, porphyria cutanea tarda, membranoproliferative glomerulonephritis, and type 2 diabetes (13).

HCV is classified into the genus *Hepacivirus* of the family *Flaviviridae* and possesses a viral genome consisting of a single positive-strand RNA with a nucleotide length of about 9.5 kb. This viral genome encodes a single polyprotein composed of approximately 3,000 amino acids (9). The polyprotein is post-translationally cleaved by host cellular peptidases and viral proteases, resulting in 10 viral proteins (6, 10, 12). The HCV core protein is known to interact with viral-sense RNA of HCV to form the viral nucleocapsid (44). The HCV core protein is cleaved off at residue 191 by the host signal peptidase to release it from the E1 envelope protein and then by the host signal peptide peptidase at around amino acid residues 177 to 179 within the C-terminal transmembrane region (30, 39, 40). The mature core protein is retained mainly on the endoplasmic reticulum, although a portion moves to the nucleus and mitochondria (11, 51).

Recent epidemiological studies have indicated that type 2

diabetes is an HCV-associated disease (7, 29). However, it remains unclear how insulin resistance is induced in patients chronically infected with HCV, since there is no suitable model for investigating HCV pathogenesis. Type 2 diabetes is a complex, multisystemic disease with pathophysiology that includes a high level of hepatic glucose production and insulin resistance, which contribute to the development of hyperglycemia (8, 18). Although the precise mechanism by which these factors contribute to the induction of insulin resistance is difficult to understand, a high level of insulin production by pancreatic  $\beta$  cells under a state of insulin resistance is common in the development of type 2 diabetes. The hyperinsulinemia in the fasting state that is observed relatively early in type 2 diabetes is considered to be a secondary response that compensates for the insulin resistance (8, 18).

The HCV core protein is also known as a pathogenic factor that induces steatosis and hepatocellular carcinoma in mice (33, 35). Previously, we reported that insulin resistance occurs in HCV core gene-transgenic mice due at least partly to an increase in tumor necrosis factor alpha (TNF- $\alpha$ ) secretion (47) and that the HCV core protein is degraded through a PA28 $\gamma$ /REG $\gamma$  (11S regulator)-dependent pathway in the nucleus (32). It is well known that PA28 $\gamma$  enhances latent proteasome activity, although the biological significance of PA28 $\gamma$  is largely unknown, with the exception that PA28 $\gamma$  is known to regulate steroid receptor coactivator 3 (28). Although several reports suggested that the degradation of insulin receptor substrate (IRS) proteins by a ubiquitin-dependent proteasome activity contributes to insulin resistance (43, 50), the involvement of the HCV core protein in cooperation with PA28 $\gamma$  in the stability of IRS proteins and in the development of insulin resis-

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tance is not known. In this study, we examined the involvement of PA28 $\gamma$  in the induction of insulin resistance by the HCV core protein *in vivo*.

#### MATERIALS AND METHODS

**Preparation of PA28 $\gamma$ -knockout HCV core gene-transgenic mice.** C57BL/6 mice carrying the gene encoding IICV core protein genotype 1b (PA28 $\gamma^{+/+}$  CoreTg) line C49 and PA28 $\gamma^{-/-}$  mice have been described previously (35, 36). These two genotypes were crossbred to create PA28 $\gamma^{+/-}$ CoreTg mice. PA28 $\gamma^{+/-}$ CoreTg mice were bred to generate PA28 $\gamma^{-/-}$ CoreTg mice (35, 36). The HCV core gene and the target sequence to knock out the PA28 $\gamma$  gene were identified by PCR. The mice were given ordinary feed (CRF-1; Charles River Laboratories, Yokohama, Japan) and were maintained under specific-pathogen-free conditions.

**Glucose tolerance test.** The mice were fasted for more than 16 h before glucose administration. D-Glucose (1 g/kg body weight) was intraperitoneally administered to the mice. Blood samples were taken from the orbital sinus at the indicated time points. The plasma glucose concentration was measured by means of a MEDI-SAFE Mini blood glucose monitor (TERUMO, Tokyo, Japan). The serum insulin level was determined by a Mercodia (Uppsala, Sweden) ultrasensitive mouse insulin enzyme-linked immunosorbent assay (ELISA).

**Insulin tolerance test.** The mice were fed freely and then fasted during the study period. Human insulin (2 U/kg body weight) (Humulin; Eli Lilly, Indianapolis, IN) was intraperitoneally administered to the mice. The plasma glucose concentration was measured at the indicated time and was normalized based on the glucose concentration at the time just before insulin administration.

**Histological analysis of pancreatic islets.** Pancreas tissues were fixed with paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The relative islet area and islet number were determined with Image-Pro PLUS image analyzing software (NIPPON ROPER, Tokyo, Japan).

**Estimation of tumor necrosis factor alpha and HCV core protein.** Mouse TNF- $\alpha$  was measured by using a mouse TNF- $\alpha$  ELISA kit (Pierce, Rockford, IL) and normalized based on the amount of total protein in each sample. The protein concentration was estimated by using a BCA protein assay kit (Pierce). The amount of HCV core protein in the liver tissues was determined by using an ELISA system as described previously (4).

**In vivo insulin stimulation and immunoblot analysis.** Mice were fasted for more than 16 h before insulin stimulation and then anesthetized with ketamine and xylazine. Five units of insulin were injected into the mice via the interior vena cava. Livers of the mice were collected 5 min after the insulin injection and frozen in liquid nitrogen. Immunoblot analyses of the HCV core protein, PA28 $\gamma$ , and each of the insulin-signaling molecules were carried out with the liver tissue homogenates prepared in the homogenizing buffer containing 25 mM Tris-HCl (pH 7.4), 10 mM Na<sub>2</sub>VO<sub>4</sub>, 100 mM NaF, 50 mM Na<sub>2</sub>P<sub>2</sub>O<sub>7</sub>, 10 mM EGTA, 10 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P40 supplemented with Complete Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany) (53). Tissue lysates were subjected to sodium dodecyl sulfate-2% to 15% gradient polyacrylamide gel electrophoresis (PAG Mini DAIICHI 2/15 13W; Daiichi Diagnostics, Tokyo, Japan) and electrotransferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA). The protein transferred onto the membrane was reacted with rabbit anti-HCV core (32), rabbit anti-Akt (Cell Signaling, Danvers, MA), rabbit anti-phospho-Ser473-Akt (Cell Signaling), rabbit anti-IRS1 (Upstate, Lake Placid, NY), rabbit anti-phospho-Tyr608 mouse insulin receptor substrate 1 (Sigma, St. Louis, MO), or rabbit anti-IRS2 (Upstate) polyclonal antibody and then incubated with horseradish peroxidase-conjugated anti-rabbit antibody. Blotted protein was visualized using Super Signal Femto (Pierce) and an LAS3000 imaging system (Fuji Photo Film, Tokyo, Japan).

**Quantitative reverse transcription-PCR (RT-PCR).** Total RNA was isolated from mouse liver using an RNeasy kit (QIAGEN, Valencia, CA). The RNA preparation was treated with a TURBO DNA-free kit (Ambion, Austin, TX) to remove DNA contamination in the samples. The first-strand cDNAs were synthesized by a first-strand cDNA synthesis kit (Amersham Biosciences, Franklin Lakes, NJ). The targeted cDNA was estimated by using Platinum SYBR Green qPCR Super Mix UDC (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The fluorescent signal was measured by using an ABI Prism 7000 (Applied Biosystems, Foster City, CA). The genes encoding mouse TNF- $\alpha$ , IRS1, IRS2, and hypoxanthine phosphoribosyl transferase were amplified with the following primer pairs: 5'-GGTACAACCCATCGGCTGGCA-3' (forward) and 5'-GCGACGTGGAAGTGGCAGAAG-3' (reverse) for TNF- $\alpha$ , 5'-ATAG

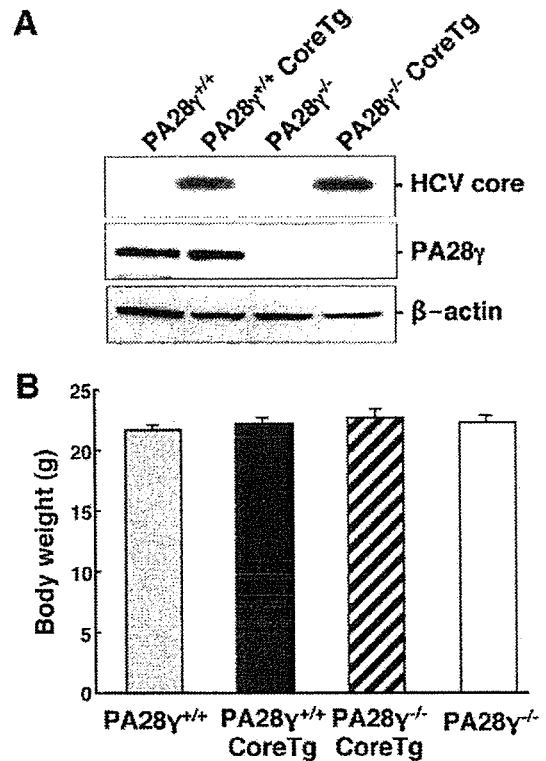


FIG. 1. Characterization of HCV core gene-transgenic mice deficient in the PA28 $\gamma$  gene. (A) Expression of the HCV core protein and PA28 $\gamma$  in the livers of PA28 $\gamma^{+/+}$ , PA28 $\gamma^{+/+}$ CoreTg, PA28 $\gamma^{-/-}$ , and PA28 $\gamma^{-/-}$ CoreTg mice. Lysates obtained from liver tissues of the mice (100  $\mu$ g protein/lane) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting using antibodies to the HCV core protein, PA28 $\gamma$ , and  $\beta$ -actin. (B) Body weights of the mice. Body weights of 2-month-old mice were measured ( $n = 7$  in each group). There were no statistically significant differences in body weights among the mice ( $P > 0.05$ ).

CTCTGAGACCTTCTCAGCACCTAC-3' (forward) and 5'-GGAGTTGCCCT CATTGCTGCCTAA-3' (reverse) for IRS1, 5'-AGCCTGGGGATAATGGTG ACTATACCGA-3' (forward) and 5'-TTGTGGGCAAAGGATGGGGACAC T-3' (reverse) for IRS2, and 5'-CCAAGCAAGCTTGCAACCTTAACCA-3' (forward) and 5'-GTAATGATCAGTCAACGGGGGAC-3' (reverse) for hypoxanthine phosphoribosyl transferase. Each PCR product was found as a single band with the correct size by agarose gel electrophoresis (data not shown).

**Reporter assay for TNF- $\alpha$  promoter activity.** The promoter region of the TNF- $\alpha$  gene (located from residues -1260 to +140) was amplified from mouse genomic DNA and was then introduced into the KpnI and BglII sites of pGL3-Basic (Promega, Madison, WI) (25). The resulting plasmid was designated as pGL3-tnf- $\alpha$ Pro. The gene encoding the HCV core protein was amplified from HCV strain J1 (genotype 1b) and cloned into pCAG-GS (1, 38). To avoid contamination with endotoxin from *Escherichia coli*, the plasmid DNA was purified by using an EndoFree Plasmid Maxi kit (QIAGEN). The total amount of transfected DNA was normalized by the addition of empty plasmids. Plasmid vector was transfected into hepatoma cell lines by lipofection using Lipofectamine 2000 (Invitrogen). Cells were harvested at 24 h posttransfection. Luciferase activity was determined by using the Dual-Luciferase Reporter Assay system (Promega). Firefly luciferase activity was normalized to coexpressed *Renilla* luciferase activity. The amount of firefly luciferase activity was presented as the increase ( $n$ -fold) relative to the value for the sample lacking the HCV core protein, which was taken to be 1.0. PA28 $\gamma$ -knockdown cell lines were established by using pSilencer 2.1 U6 Hygro (Ambion) according to the manufacturer's protocol.

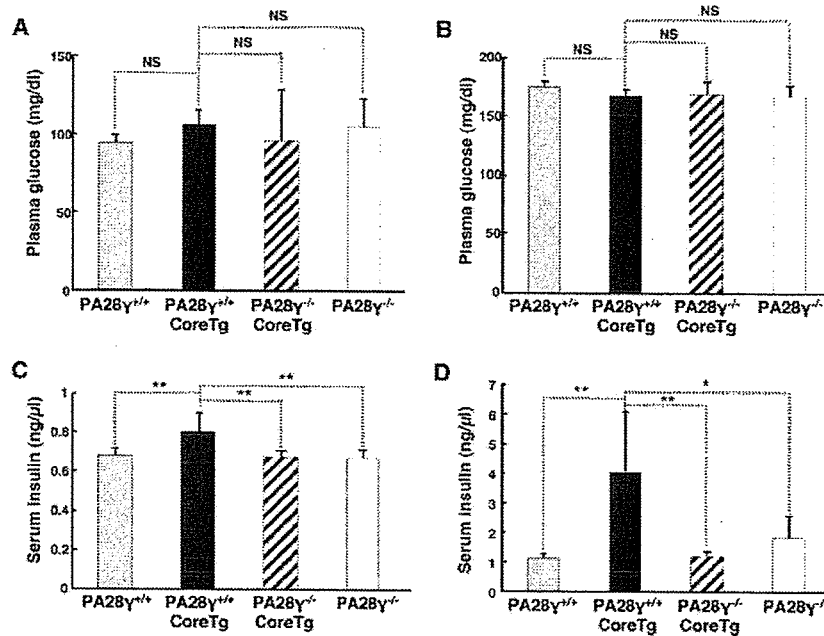


FIG. 2. Knockout of the PA28 $\gamma$  gene inhibited the hyperinsulinemia induced by HCV core protein. Plasma glucose levels of PA28 $\gamma^{+/+}$ , PA28 $\gamma^{+/+}$ CoreTg, PA28 $\gamma^{-/-}$ CoreTg, and PA28 $\gamma^{-/-}$  mice under fasting (A) or fed (B) conditions ( $n = 7$  in each group) are shown. Serum insulin levels in fasting (C) or fed (D) mice ( $n = 7$  in each group) are also shown. Values are represented as means  $\pm$  standard deviations. \* $P < 0.05$ ; \*\* $P < 0.01$ . NS, not statistically significant.

**Statistical analysis.** The results are presented as means  $\pm$  standard deviations. The significance of the differences was determined by Student's  $t$  test.  $P$  values of  $<0.05$  were considered statistically significant.

**RESULTS**

**HCV core gene-transgenic mice deficient in the PA28 $\gamma$  gene.**

To investigate the role of PA28 $\gamma$  in the development of insulin resistance in HCV core gene-transgenic (PA28 $\gamma^{+/+}$ CoreTg)

mice, we generated HCV core gene-transgenic mice deficient in the PA28 $\gamma$  gene (PA28 $\gamma^{-/-}$ CoreTg). A PA28 $\gamma^{+/+}$ CoreTg mouse expressing an amount of PA28 $\gamma$  equal to that of its normal littermates (Fig. 1A) was crossbred with a PA28 $\gamma^{-/-}$  mouse to generate a PA28 $\gamma^{+/-}$ CoreTg mouse. PA28 $\gamma^{+/-}$ CoreTg mice were bred with each other, and a PA28 $\gamma^{-/-}$ CoreTg mouse was selected by PCR. The HCV core protein was expressed in PA28 $\gamma^{+/+}$ CoreTg and PA28 $\gamma^{-/-}$ CoreTg

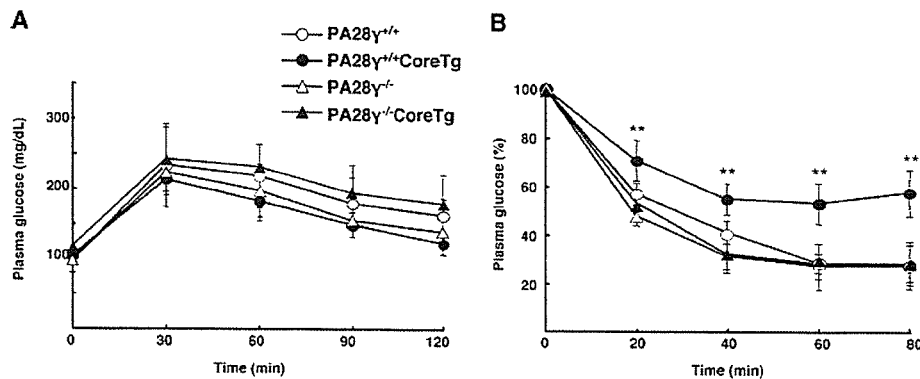


FIG. 3. Knockout of the PA28 $\gamma$  gene inhibits the insulin resistance induced by the HCV core protein. (A) Glucose tolerance test. D-Glucose was intraperitoneally administered to mice fasted for more than 16 h at 1 g/kg of body weight. Plasma glucose levels were estimated at the indicated times ( $n = 5$  in each group). There were no significant differences in glucose levels among the mice ( $P > 0.05$ ). (B) Insulin tolerance test. Human insulin (2 units/kg body weight) was intraperitoneally administered to the mice, and the plasma glucose levels were estimated at the indicated times. Values were normalized to the baseline glucose concentration at the time of insulin administration ( $n = 5$  in each group). The values for the PA28 $\gamma^{+/+}$  (open circles), PA28 $\gamma^{+/+}$ CoreTg (closed circles), PA28 $\gamma^{-/-}$  (open triangles), and PA28 $\gamma^{-/-}$ CoreTg (closed triangles) mice are represented as means and  $\pm$  standard deviations. Significant differences in insulin sensitivity ( $P < 0.01$ ) in PA28 $\gamma^{+/+}$ CoreTg mice compared to that in PA28 $\gamma^{+/+}$ , PA28 $\gamma^{-/-}$ , or PA28 $\gamma^{-/-}$ CoreTg mice are indicated by double asterisks (\*\*). There were no significant differences among PA28 $\gamma^{+/+}$ , PA28 $\gamma^{-/-}$ , and PA28 $\gamma^{-/-}$ CoreTg mice ( $P > 0.05$ ).

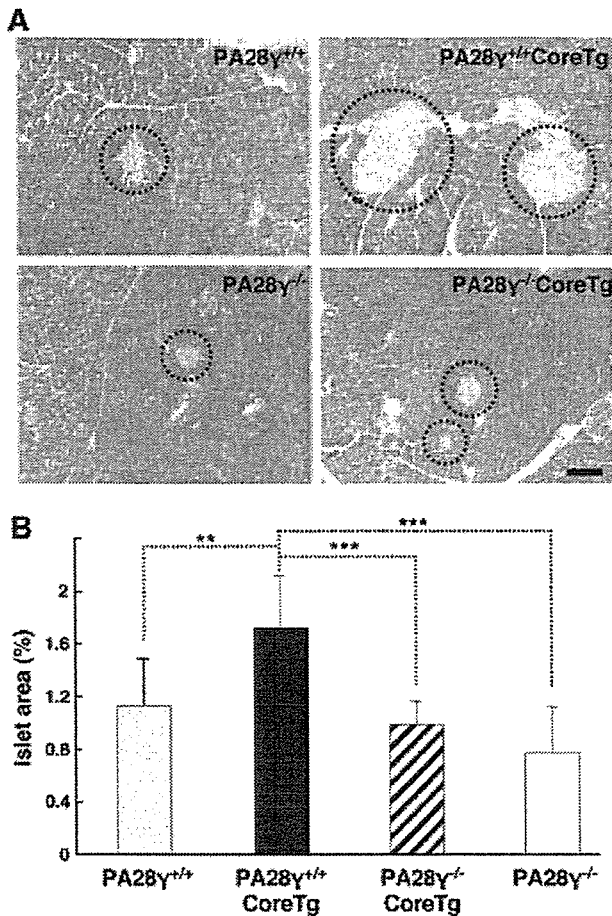


FIG. 4. PA28 $\gamma$  participated in the enlargement of pancreatic islets induced by the HCV core protein. (A) Histological sections prepared from pancreas tissues of PA28 $\gamma^{+/+}$ , PA28 $\gamma^{+/+}$ CoreTg, PA28 $\gamma^{-/-}$ , and PA28 $\gamma^{-/-}$ CoreTg mice were stained with hematoxylin and eosin. Dotted circles indicate pancreatic islets. (B) The area occupied by pancreatic islets was measured by computer software in three different fields of every six randomly selected sections of 10 mice per genotype and is represented as a percentage of the total pancreatic area. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . The scale bar indicates 100  $\mu$ m.

mice but not in PA28 $\gamma^{+/+}$  (normal littermates) or PA28 $\gamma^{-/-}$  mice. PA28 $\gamma$  was found at a similar level in PA28 $\gamma^{+/+}$ CoreTg and PA28 $\gamma^{+/+}$  mice but was not present in either PA28 $\gamma^{-/-}$  or PA28 $\gamma^{-/-}$ CoreTg mice (Fig. 1A). The expression of the HCV core protein in the livers of 2-month-old male mice was slightly higher in PA28 $\gamma^{-/-}$ CoreTg ( $1.36 \pm 0.44$  ng/mg of total protein;  $n = 7$ ) than in PA28 $\gamma^{+/+}$ CoreTg ( $1.23 \pm 0.22$  ng/mg of total protein;  $n = 7$ ) mice, but these values were not significantly different ( $P > 0.05$ ). Insulin sensitivity is dependent on several conditions such as body weight, obesity, and liver steatosis (26). PA28 $\gamma^{-/-}$  mice were slightly smaller than their normal littermates (PA28 $\gamma^{+/+}$ ) at more than 3 months old, as described previously (36), but this was not significantly different in 2-month-old mice (Fig. 1B). PA28 $\gamma^{+/+}$ CoreTg mice exhibited severe hepatic steatosis from 4 months of age (35). To avoid the influence of hepatic steatosis and body weight on the examination of insulin resistance, 2-month-old mice were

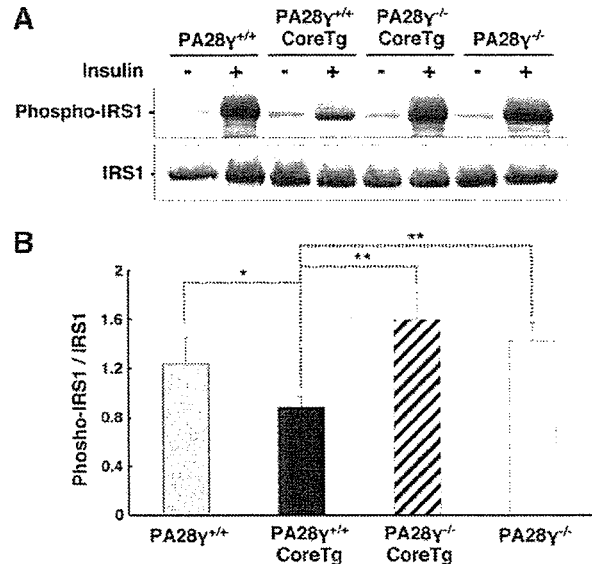


FIG. 5. PA28 $\gamma$  participated in the inhibition of the tyrosine phosphorylation of IRS1 induced by the HCV core protein. Liver tissues from PA28 $\gamma^{+/+}$ , PA28 $\gamma^{+/+}$ CoreTg, PA28 $\gamma^{-/-}$ , and PA28 $\gamma^{-/-}$ CoreTg mice were prepared after administration of insulin (+) or phosphate-buffered saline (-). The samples (100  $\mu$ g of total protein) were examined by immunoblotting with antibodies against IRS1 and phospho-Tyr608 of mouse IRS1 (A). Phosphorylated IRS1 was estimated from the density on the immunoblotted membrane by using computer software (B) ( $n = 5$  in each group). The data presented are representative of three independent experiments. \* $P < 0.05$ ; \*\* $P < 0.01$ .

used in this study. Figure 1B shows the body weights of 2-month-old mice. There were no significant differences in body weight among PA28 $\gamma^{+/+}$ CoreTg, PA28 $\gamma^{-/-}$ CoreTg, PA28 $\gamma^{-/-}$ , and PA28 $\gamma^{+/+}$  mice. Steatosis was not detected in the livers of the 2-month-old mice (data not shown).

PA28 $\gamma$  is involved in the development of hyperinsulinemia and insulin resistance in PA28 $\gamma^{+/+}$ CoreTg mice. In our previous study, we found a significant difference in serum insulin levels, but not in plasma glucose levels, between PA28 $\gamma^{+/+}$ CoreTg mice and normal littermates (47). To determine the involvement of PA28 $\gamma$  in the development of insulin resistance in PA28 $\gamma^{+/+}$ CoreTg mice, we examined here the plasma glucose and insulin levels in the mice under fasting and fed conditions. Although no significant difference in plasma glucose levels was observed in the mice under either fasting (Fig. 2A) or fed (Fig. 2B) conditions, serum insulin levels were significantly higher in PA28 $\gamma^{+/+}$ CoreTg mice than in PA28 $\gamma^{+/+}$  mice under both conditions (Fig. 2C and D), as described previously (47). In contrast, the serum insulin concentration in PA28 $\gamma^{-/-}$ CoreTg mice was recovered to a normal level similar to that of PA28 $\gamma^{+/+}$  and PA28 $\gamma^{-/-}$  mice under either fasting (Fig. 2C) or fed (Fig. 2D) conditions.

To determine the glucose intolerance among the mice, glucose was administered to the mice after fasting, and the plasma glucose level was then determined. There was no significant difference among the genotypes at any time point in the glucose tolerance test (Fig. 3A), suggesting that the volume of glucose was maintained at a normal level by the higher concentration of insulin in PA28 $\gamma^{+/+}$ CoreTg mice. In our previ-

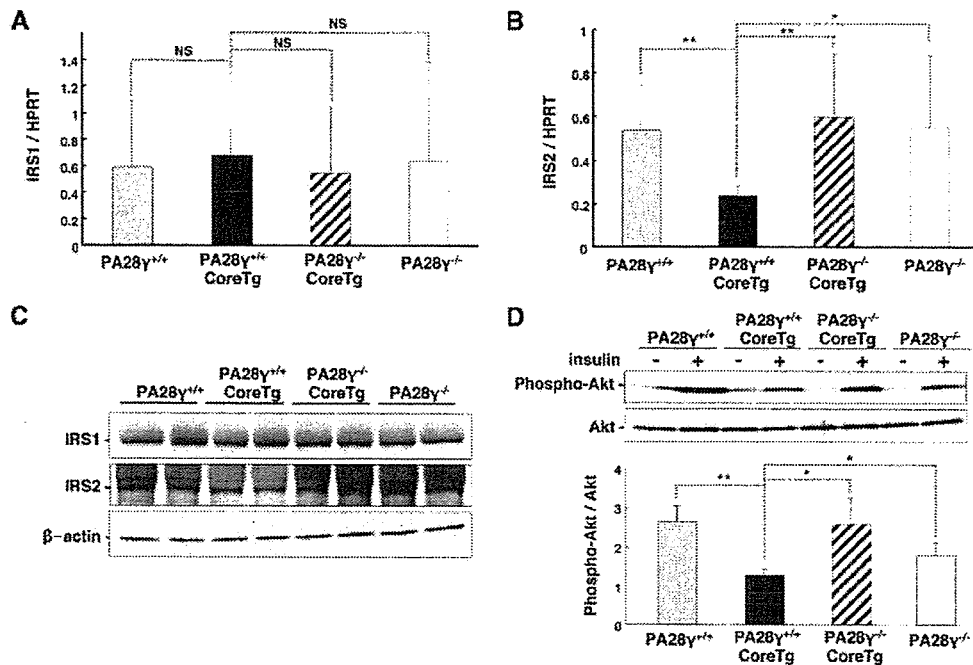


FIG. 6. PA28 $\gamma$  participated in the inhibition of the IRS2 expression and Akt phosphorylation induced by HCV core protein. The transcription of IRS1 (A) and IRS2 (B) was estimated by quantitative RT-PCR ( $n = 5$  in each group). (C) The expression levels of IRS1 and IRS2 in the livers of the mice were determined by immunoblotting with specific antibodies. (D) Phosphorylation of Akt in the livers of the mice was examined by immunoblotting with antibodies against Akt and phosphorylated Akt. The ratio of Akt phosphorylation was determined by computer software based on the densities of phosphorylated Akt and a total amount of Akt ( $n = 3$  in each group). The data presented are representative of three independent experiments. \* $P < 0.05$ ; \*\* $P < 0.01$ . NS, not statistically significant; HPRT, hypoxanthine phosphoribosyl transferase.

ous study, the reduction in the plasma glucose concentration after insulin administration was impaired in PA28 $\gamma^{+/+}$  CoreTg mice (47). In this study, PA28 $\gamma^{-/-}$  CoreTg mice exhibited a normal insulin level comparable to those of PA28 $\gamma^{+/+}$  and PA28 $\gamma^{-/-}$  mice by an insulin tolerance test, in contrast to PA28 $\gamma^{+/+}$  CoreTg mice, in which a high concentration of plasma glucose was detected at all time points, as previously reported (Fig. 3B). These data suggest that hyperinsulinemia was induced in PA28 $\gamma^{+/+}$  CoreTg mice to compensate for insulin resistance and retain a physiological level of plasma glucose and that PA28 $\gamma$  participates in the development of hyperinsulinemia and insulin resistance in PA28 $\gamma^{+/+}$  CoreTg mice.

**Morphology of pancreatic islets.** Hyperinsulinemia and insulin resistance are expected to enlarge the pancreatic islet mass due to the overexpression of insulin. Our previous report showed the enlargement of the pancreatic islets in PA28 $\gamma^{+/+}$  CoreTg mice. To clarify whether a knockout of the PA28 $\gamma$  gene restores the enlarged pancreatic islets to their normal size, the morphology of the pancreatic islets of the mice was evaluated by histologic examination (Fig. 4A). The relative islet area in the pancreatic cells of the PA28 $\gamma^{-/-}$  CoreTg mice was smaller than that of PA28 $\gamma^{+/+}$  CoreTg mice and comparable to that of PA28 $\gamma^{+/+}$  and PA28 $\gamma^{-/-}$  mice (Fig. 4B). Infiltration of inflammatory cells within or surrounding the islets was not found in all genotypes of mice. These results suggest that PA28 $\gamma$  also participates in the enlargement of pancreatic islets induced in PA28 $\gamma^{+/+}$  CoreTg mice.

**PA28 $\gamma$  impairs the insulin-signaling pathway through the suppression of both tyrosine phosphorylation of IRS1 and expression of IRS2.** Insulin binds to insulin receptors, resulting in the activation of downstream signaling (26). The activated insulin receptors phosphorylate themselves, IRS1, and IRS2. Phosphorylated IRS1 and IRS2 can activate phosphatidylinositol 3 (PI3)-kinase signaling, leading to the activation of glucose metabolism and cell growth. Our previous report showed that tyrosine phosphorylation of IRS1 is suppressed in the livers of PA28 $\gamma^{+/+}$  CoreTg mice and that the administration of anti-TNF- $\alpha$  antibody restores insulin sensitivity (47). We examined whether a knockout of the PA28 $\gamma$  gene could restore the tyrosine phosphorylation of IRS1. Tyrosine phosphorylation of IRS1 was suppressed in the livers of PA28 $\gamma^{+/+}$  CoreTg mice in response to insulin stimulation, whereas it was recovered in PA28 $\gamma^{-/-}$  CoreTg mice to levels comparable to those in PA28 $\gamma^{+/+}$  and PA28 $\gamma^{-/-}$  mice (Fig. 5).

Chronic hyperinsulinemia downregulates the expression of IRS2, which is one of the essential components of the insulin-signaling pathway in the liver (46). However, in our previous study, we showed that there was no significant difference in the phosphorylation of IRS2 between PA28 $\gamma^{+/+}$  CoreTg mice and their normal littermates (47). To gain more insight into the mechanisms of regulation of IRS expression, we determined the transcription and translation of IRS1 and IRS2 in the livers of the mice by real-time PCR and Western blotting, respectively. Although there was no significant difference in IRS1 expression at either the transcriptional or translational level among the mice

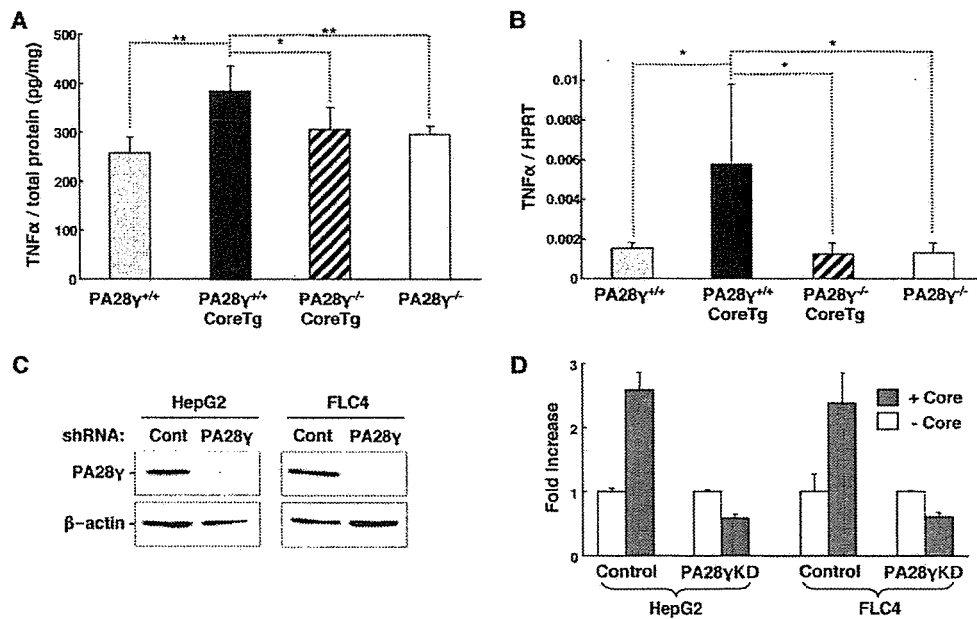


FIG. 7. PA28 $\gamma$  was required for activation of the TNF- $\alpha$  promoter by the HCV core protein. (A) Expression of TNF- $\alpha$  in the livers of mice was determined by ELISA ( $n = 5$  in each group). (B) TNF- $\alpha$  mRNA in the livers of mice was examined by quantitative RT-PCR ( $n = 5$  in each group). (C) Knockdown of the expression of PA28 $\gamma$  in the HepG2 and FLC-4 cell lines by the introduction of a plasmid encoding a short hairpin RNA (shRNA) targeted to the PA28 $\gamma$  gene. The expression levels of PA28 $\gamma$  and  $\beta$ -actin were determined by immunoblotting with specific antibodies. (D) Promoter activity of TNF- $\alpha$  in the presence or absence of the HCV core protein was determined by luciferase assay in the PA28 $\gamma$ -knockdown and control cell lines. The data presented are representative of three independent experiments. HPRT, hypoxanthine phosphoribosyl transferase.

(Fig. 6A and C), the expression of IRS2 was clearly impaired in PA28 $\gamma^{+/+}$  CoreTg mice at both the transcriptional and translational levels compared with that in other mice (Fig. 6B and C). The serine/threonine protein kinase Akt is phosphorylated by phosphoinositide-dependent kinase 1 (PDK1) under the activated condition of IRS family proteins (26). The insulin-induced phosphorylation of Akt was suppressed in the livers of PA28 $\gamma^{+/+}$  CoreTg mice but not in those of PA28 $\gamma^{+/+}$ , PA28 $\gamma^{-/-}$ , or PA28 $\gamma^{-/-}$  CoreTg mice (Fig. 6D). These results suggest that the expression of the HCV core protein in the livers of mice in the presence of PA28 $\gamma$  impairs the insulin-signaling pathway through the suppression of both the tyrosine phosphorylation of IRS1 and the expression of IRS2.

**PA28 $\gamma$  is required for activation of the TNF- $\alpha$  promoter by HCV core protein.** TNF- $\alpha$  is an adipokine (54) and suppresses the signaling pathway of IRS1 and IRS2 (14, 42). Several reports suggested that the serum TNF- $\alpha$  level is higher in HCV patients than in healthy individuals (19, 37). Elevations of TNF- $\alpha$  levels have also been demonstrated in the livers of PA28 $\gamma^{+/+}$  CoreTg mice (47). To determine the involvement of PA28 $\gamma$  in the enhancement of TNF- $\alpha$  expression, the expression of TNF- $\alpha$  in the livers of each genotype was determined by ELISA and real-time PCR (Fig. 7A and B). Transcription and translation of TNF- $\alpha$  were increased in the livers of PA28 $\gamma^{+/+}$  CoreTg mice but were restored in the livers of PA28 $\gamma^{-/-}$  CoreTg mice to levels comparable to those of PA28 $\gamma^{+/+}$  and PA28 $\gamma^{-/-}$  mice. To determine the effect of PA28 $\gamma$  expression on the promoter activity of TNF- $\alpha$  in human liver cells, PA28 $\gamma$ -knockdown human hepatoma cell lines HepG2 and FLC4 were

established by the introduction of a plasmid encoding a short hairpin RNA targeting the PA28 $\gamma$  gene in the cell lines. The expression of PA28 $\gamma$  was clearly suppressed in the cell lines (Fig. 7C). The expression of HCV core protein in the hepatoma cell lines potentiated TNF- $\alpha$  promoter activity, whereas the promoter activation by the HCV core protein was suppressed in the PA28 $\gamma$ -knockdown cell lines (Fig. 7D). These results suggest that PA28 $\gamma$  is required for the activation of the TNF- $\alpha$  promoter induced by the expression of the HCV core protein in human hepatoma cell lines.

## DISCUSSION

HCV infection has a close association with type 2 diabetes, which is a polygenic disease with a pathophysiology that includes a defect in insulin secretion, increased hepatic glucose production, and resistance to the action of insulin (2, 8, 18). Insulin binds to insulin receptors, which exhibit tyrosine kinase activity, leading to the autophosphorylation and phosphorylation of IRS (56). Tyrosine phosphorylation in IRS proteins leads to the interaction between IRS proteins and the regulatory subunit p85 of PI3-kinase, which enhances glucose uptake and inhibits lipolysis (21). Activated PI3-kinase phosphorylates phosphatidylinositol 4,5-bisphosphate to produce phosphatidylinositol 3,4,5-triphosphate, which contributes to the activation of PDK1 (55). Activated PDK1 phosphorylates downstream substrates including Akt and other kinases (55). A diabetic phenotype that included insulin resistance was found in IRS2-knockout mice with normal growth (57), although a



knockout of the IRS1 gene has been shown to lead to growth retardation and insulin resistance but not overt diabetes (5, 52). The double knockdown of IRS1 and IRS2 genes in the liver induces hyperinsulinemia and insulin resistance in mice (53). The reduction of both IRS1 and IRS2 under conditions of insulin resistance and hyperinsulinemia (3) and in the livers of *ob/ob* mice, an obese diabetic mouse model (20), has been reported previously. In the present study, the expression of the HCV core protein reduced the phosphorylation of tyrosine on IRS1 and the production of IRS2 in the livers of mice but did not completely abolish the activities of these genes, suggesting that residual activities of IRS transfer a faint signal to the downstream region of IRS. Therefore, PA28 $\gamma^{+/+}$  CoreTg mice may exhibit a milder phenotype than IRS1- and/or IRS2-knockout mice. In this study, knockout of the PA28 $\gamma$  gene restored the insulin sensitivity and signaling of IRS1 and IRS2 in PA28 $\gamma^{+/+}$  CoreTg mice, suggesting that the expression of the HCV core protein leads to the dysfunction of both IRS1 and IRS2 through a PA28 $\gamma$ -dependent pathway.

Our previous study suggested that the induction of TNF- $\alpha$  by the HCV core protein plays a role in insulin resistance (47). An increase in TNF- $\alpha$  levels has been correlated with obesity and insulin resistance in animal models and humans (14, 42). However, the mechanism by which TNF- $\alpha$  induces insulin resistance is not completely known. The expression of TNF- $\alpha$  has been shown to be increased in PA28 $\gamma^{+/+}$  CoreTg mice, resulting in the suppression of phosphorylation of IRS1, and insulin sensitivity in PA28 $\gamma^{+/+}$  CoreTg was improved by the administration of an anti-TNF- $\alpha$  antibody (47). In the present study, the expression level of TNF- $\alpha$  in PA28 $\gamma^{-/-}$  CoreTg mice was similar to that in PA28 $\gamma^{-/-}$  mice or their normal littermates. The expression of the HCV core protein enhanced the promoter activity of the TNF- $\alpha$  gene in human liver cell lines but not in those with a knockdown of the PA28 $\gamma$  gene by RNA interference (Fig. 7D). These data suggest that PA28 $\gamma$  plays a crucial role in HCV core-induced expression of TNF- $\alpha$ . Sterol regulatory element-binding proteins (SREBPs) were shown to be increased at the stage of viremia in HCV-infected chimpanzees (49). SREBPs are known to regulate not only the biosynthesis of lipid but also the transcription of IRS2 and TNF- $\alpha$  (17, 45). Therefore, it might be feasible to speculate that the HCV core protein may cooperate with PA28 $\gamma$  to regulate the expression of SREBPs.

Houstis et al. previously reported that reactive oxygen species (ROS) are increased in both cellular and mouse models of insulin resistance induced by treatment with TNF- $\alpha$  or dexamethasone and that insulin sensitivity was restored by treatment with small antioxidant molecules (16). The HCV core protein potentiates ROS production in hepatoma cells and HCV core gene-transgenic mice (23, 34, 41). Accelerated production of ROS results in mitochondrion dysfunction, which contributes to a decrease in fatty acid oxidation. Defects in mitochondrial fatty acid oxidation enhance the production of intracellular fatty acyl coenzyme A (CoA) and diacylglycerol (48, 58). Mitochondrion dysfunction and accumulation of lipid droplets in mice expressing the HCV core or the full-length HCV polypeptide have been reported (27, 34). An increase in lipid droplets also leads to the accumulation of fatty acid CoA and diacylglycerol (48, 58). Fatty acyl CoA and diacylglycerol nonspecifically activate the Ser/Thr kinase cascade, leading to the enhancement of the serine phosphorylation of IRS1 (26). Serine phosphorylation on IRS1 blocks the tyrosine

phosphorylation of IRS1 by insulin receptors (26). In the present study, however, serine phosphorylation of IRS1 in PA28 $\gamma^{+/+}$  CoreTg mice was similar to that in PA28 $\gamma^{-/-}$  CoreTg mice (data not shown). TNF- $\alpha$  signaling pathways other than the accumulation of ROS and fatty acid intermediates may also participate in the inhibition of tyrosine phosphorylation on IRS1 in PA28 $\gamma^{+/+}$  CoreTg mice.

How does the HCV core protein induce TNF- $\alpha$  production? Our previous report suggests that the HCV core protein is degraded through a PA28 $\gamma$ -dependent pathway (32). Recently, PA28 $\gamma$  has been shown to participate in the proteasome-dependent degradation of steroid receptor coactivator 3 (28). Degradation products of the HCV core protein via the PA28 $\gamma$ -dependent pathway may regulate the promoter activity of the TNF- $\alpha$  gene. PA28 proteins are necessary and sufficient to fully reconstitute Hsp90-initiated refolding together with Hsc70 and Hsp40 (31). Therefore, it might also be feasible to speculate that the HCV core protein refolded by an Hsp90/PA28 $\gamma$ -dependent pathway activates the promoter of the TNF- $\alpha$  gene together with an unknown transcription factor(s) or regulator(s).

In conclusion, the data obtained in this study suggest that the expression of the HCV core protein enhances the production of TNF- $\alpha$  and suppresses the phosphorylation of tyrosine on IRS1 and the production of IRS2 through a PA28 $\gamma$ -dependent pathway, thereby leading to insulin resistance. PA28 $\gamma$  may be a novel target for the treatment of HCV-induced diabetes.

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## E6AP Ubiquitin Ligase Mediates Ubiquitylation and Degradation of Hepatitis C Virus Core Protein<sup>∇</sup>

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Hepatitis C virus (HCV) core protein is a major component of viral nucleocapsid and a multifunctional protein involved in viral pathogenesis and hepatocarcinogenesis. We previously showed that the HCV core protein is degraded through the ubiquitin-proteasome pathway. However, the molecular machinery for core ubiquitylation is unknown. Using tandem affinity purification, we identified the ubiquitin ligase E6AP as an HCV core-binding protein. E6AP was found to bind to the core protein *in vitro* and *in vivo* and promote its degradation in hepatic and nonhepatic cells. Knockdown of endogenous E6AP by RNA interference increased the HCV core protein level. *In vitro* and *in vivo* ubiquitylation assays showed that E6AP promotes ubiquitylation of the core protein. Exogenous expression of E6AP decreased intracellular core protein levels and supernatant HCV infectivity titers in the HCV JFH1-infected Huh-7 cells. Furthermore, knockdown of endogenous E6AP by RNA interference increased intracellular core protein levels and supernatant HCV infectivity titers in the HCV JFH1-infected cells. Taken together, our results provide evidence that E6AP mediates ubiquitylation and degradation of HCV core protein. We propose that the E6AP-mediated ubiquitin-proteasome pathway may affect the production of HCV particles through controlling the amounts of viral nucleocapsid protein.

Hepatitis C virus (HCV; a single-stranded, positive-sense RNA virus that is classified in the family *Flaviviridae*) is the main cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (5, 26, 45). More than 170 million people worldwide are chronically infected with HCV (41). The approximately 9.6-kb HCV genome encodes a unique open reading frame that is translated into a polyprotein (5, 54). The polyprotein is cleaved cotranslationally into at least 10 proteins by viral proteases and cellular signalases (6, 10).

The HCV core protein represents the first 1 to 191 amino acids (aa) of the polyprotein and is followed by two glycoproteins, E1 and E2 (6). The core protein plays a central role in the packaging of viral RNA (25, 40); modulates various cellular processes, including signal transduction pathways, transcriptional control, cell cycle progression, apoptosis, lipid metabolism, and the immune response (9, 40); and has transforming potential in certain cells (43). Mice transgenic for the HCV core gene develop steatosis (32) and later hepatocellular carcinoma (31). These findings suggest that HCV core protein plays a crucial role in hepatocarcinogenesis.

Two major forms of the HCV core protein, p21 (mature form) and p23 (immature form), can be generated in cultured cells (60). Cellular signal peptidase cleaves at the junction of the core/E1, releasing the immature form of the core protein from the polypeptide (12, 46). Signal peptide peptidase cleaves just before the signal sequence, liberating the mature form of the HCV core protein at the cytoplasmic face of the endoplasmic reticulum (29). Several different sites have been proposed as potential cleavage sites of signal peptide peptidase, such as Leu-179 (15, 29), Phe-177 (36, 37), Leu-182 (15), and Ser-173 (46). Further processing of the HCV core protein yields a 17-kDa product with a C terminus at around amino acid 152. A truncated form of the core protein, p17, was found in transfected cells (42, 52) and liver tissues from humans with hepatocellular carcinoma (59). The majority of this protein translocates to the nucleus. The C terminus of the core protein is important for regulating the stability of the protein (20, 52).

We previously showed that the C-terminally truncated forms of the core protein are degraded through the ubiquitin-proteasome pathway (52). We found that the mature form of the core protein, p21, also links to a few ubiquitin moieties, suggesting that the ubiquitin-proteasome pathway involves proteolysis of heterologous species of the core protein (52). Overexpression of PA28 $\gamma$  (a REG family proteasome activator also known as REG $\gamma$  or Ki antigen) enhances the proteasomal degradation of the HCV core protein (30). A recent study has shown that

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