

and clinical studies demonstrates that HCV infection causes excessive ROS production and decreased activity of antioxidant enzymes [Kato et al., 2001; Levent et al., 2006]. In addition, previous studies showed that aggravation of oxidative stress in hepatocytes infected with HCV is correlated with the iron overload, while phlebotomy improves oxidative stress markers and liver pathology [Serone et al., 2007]. Therefore, oxidative stress is a deleterious factor involved in the development of various hepatic diseases ranging from chronic hepatitis to hepatocellular carcinoma. In contrast, the influence of the intracellular redox state on HCV replication is controversial. Exogenous addition of either hydrogen peroxide or unsaturated fatty acid has been shown to induce oxidative stress and inhibit HCV replication in cell culture models [Choi et al., 2004; Huang et al., 2007]. Yano et al. [2007] reported previously that any of several nutrients (including vitamin E, a hydrophobic antioxidant) enhance HCV RNA replication. In contrast, overproduction of the antioxidant enzyme heme oxygenase-1 decreases HCV RNA replication in both full-length and subgenomic replicons [Zhu et al., 2008]. Despite these *in vitro* results, there have been no reports on the effect of antioxidant or pro-oxidant reagents on the life cycle of HCV in any animal models, such as chimeric mice harboring human hepatocytes infected with HCV.

BO-653 (2,3-dihydro-5-hydroxy-2,2-dipentyl-4,6-ditert-butylbenzofuran), a lipophilic (hydrophobic) antioxidant, was previously a clinical candidate for potential treatment of atherosclerosis and the prevention of post-angioplasty restenosis [Cynshi et al., 1998; Meng, 2003]. This compound is an effective inhibitor of lipid peroxidation and inhibits potently oxidation of lipids such as low-density lipoprotein [Noguchi et al., 1997; Tamura et al., 2003]. The present study examined the anti-HCV activity of BO-653 both *in vitro* and *in vivo*, and sought to clarify whether the antioxidant activity of the molecule was integral to the observed anti-HCV activity.

## MATERIALS AND METHODS

### Chemicals

BO-653 (molecular weight [MW], 388.6) was a gift of the Chugai Pharmaceutical company (Tokyo, Japan). Probucol [4,4'-(isopropylidenedithio)bis(2,6-di-*tert*-butylphenol)] was purchased from Wako Pure Chemical Industries (Osaka, Japan). *N*-acetyl cysteine and ascorbic acid (vitamin C) were obtained from Sigma-Aldrich (St. Louis, MO). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and *D*- $\alpha$ -tocopherol (vitamin E) were obtained from Calbiochem (San Diego, CA) and MP Biomedical LLC (Solon, OH), respectively.

### Viruses and Cells

Patients provided written informed consent prior to blood sample collection.

HuH-7 cells harboring a HCV subgenomic replicon (FLR3-1 cells; genotype 1b, Con-1 strain) were maintained at 37°C in 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium GlutaMAX-I (DMEM-GlutaMax I; Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS) and 0.5 mg/ml G418 [Inoue et al., 2007].

HuH-7 cells infected persistently with HCV (RMT-tri cells; genotype 1a) were generated in the laboratory as described below and were maintained in DMEM containing 10% FCS, nonessential amino acids, 10 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], and 0.4% glucose. Complementary DNA (cDNA) of the full-genome HCV (nucleotides 1–9,598; GenBank accession number AB520610) was prepared from the serum of a patient with acute hepatitis infected with HCV genotype 1a [Inoue et al., 2007]. The resulting serum harbored HCV at a titer of 10<sup>8.6</sup> copies/ml, as detected by a quantitative real-time polymerase chain reaction (qRT-PCR) as described previously [Takeuchi et al., 1999]. The sequence of the final cDNA construct was determined from a consensus of more than 10 clones, and was subcloned under the control of a T7 promoter (pHCV-RMT). The construct of HCV subgenomic replicon was generated from pHCV-RMT. HCV subgenomic RNA was transcribed using T7 RNA polymerase and the MEGAscript *in vitro* transcription kit (Ambion, Austin, TX) according to the manufacturer's instructions. The resulting synthetic RNA of the HCV subgenomic replicon was transfected into HuH-7 cells by electroporation. Following transfection, the HCV subgenomic replicon-bearing HuH-7 cells was established. Total RNA was extracted by the acid guanidinium-phenol-chloroform method from a sample of the HCV subgenomic replicon-bearing HuH-7 cells and reverse transcribed. Sequence of the resulting cDNA has three nonsynonymous substitutions compared to that of the original HCV subgenomic replicon. Next, three nonsynonymous substitutions were inserted into the original full-length HCV sequence to enhance the replication rate in HuH-7 cells. Full-length RNA was also transcribed as described above. The resulting synthetic RNA of full-length HCV was transfected into HuH-7 cells by electroporation. Following transfection, the HCV RNA level in the transfected cells was measured on a weekly basis, revealing persistent infection at a level of 10<sup>6.5</sup>–10<sup>6.8</sup> copies/ $\mu$ g total cellular RNA over the course of 50 days. Finally, the transfected cell line was designated as RMT-tri.

### Analysis of Anti-HCV Effect of BO-653 in FLR3-1 Cells

The anti-HCV activity of BO-653 in FLR3-1 cells was measured by inhibiting luciferase activity [Inoue et al., 2007]. In brief, FLR3-1 cells were seeded at 4  $\times$  10<sup>3</sup> cells/well in 96-well white plates. After 24 hr, the culture medium was replaced with fresh medium containing various concentrations of BO-653

(12–1,000  $\mu\text{M}$ ). The culture medium containing 1% MeOH was used as the negative control. After 72 hr incubation, the luciferase activity of the cells was measured using the Bright-Glo luciferase assay (Promega, Madison, WI) according to the manufacturer's instructions.

#### **Analysis of Anti-HCV Effect of BO-653 in RMT-Tri Cells**

RMT-tri cells were seeded at  $2.5 \times 10^4$  cells/well in 24-well plates. After 24 hr, the culture medium was replaced with fresh medium containing various concentrations of BO-653 (12–1,000  $\mu\text{M}$ ). The culture medium containing 1% MeOH was used as the negative control. After 72 hr incubation, the cell monolayer was harvested by adding 400  $\mu\text{l}$  of 5 M guanidine-isocyanate solution containing 5.6  $\mu\text{l}$  of 2-mercaptoethanol. The total RNA was extracted as above; HCV RNA was quantified by qRT-PCR.

#### **Comparison of Anti-HCV Activity of Lipophilic and Hydrophilic Antioxidants**

The anti-HCV activity of various antioxidants, including hydrophilic and lipophilic compounds, was compared in FLR3-1 cells. BO-653,  $\alpha$ -tocopherol, and probucol were used as lipophilic antioxidants; *N*-acetyl cysteine, ascorbic acid, and trolox were used as hydrophilic antioxidants. The anti-HCV activities of these compounds were determined by luciferase assays as described above.

#### **Cytotoxicity Testing**

Simultaneously with the luciferase assays, the cell viability was measured by using a WST-8 cell counting kit (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions.

#### **Western Blot Analysis**

FLR3-1 cells were treated with BO-653 as described above. After 96 hr, the cells were lysed with lysis buffer (protease inhibitor cocktail [Complete, Roche Diagnostics, IN] formulated according to the manufacturer's instructions in 10 mM Tris [pH 7.4], 150 mM NaCl, 1% sodium dodecyl sulfate (SDS), 0.5% Nonidet P-40). The cell lysates were resolved by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. After incubation with a blocking buffer consisting of 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 [TBS-T], the membranes were incubated with rabbit polyclonal anti-NS3 antibody (R212 clone) and goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugated IgG (GE Healthcare, Little Chalfont, Buckinghamshire, UK) as the primary and secondary antibodies, respectively. Labeling was visualized using the Immobilon Western system (Millipore, Billerica, MA). To provide a loading control,  $\beta$ -actin was detected using mouse anti- $\beta$ -actin monoclonal antibody (Sigma-Aldrich) and sheep anti-mouse IgG

HRP-conjugated IgG (GE Healthcare) as the primary and secondary antibodies, respectively.

#### **Immunofluorescent Staining**

FLR3-1 cells treated with 111  $\mu\text{M}$  BO-653 for 96 hr were probed with the primary antibody (anti-NS3) after blocking with TNB blocking buffer (PerkinElmer, Waltham, MA). An anti-rabbit IgG Alexa-Fluor 488 conjugate (Invitrogen, Grand Island, NY) was then applied as the secondary antibody.

#### **Measurement of Antioxidant Activity of BO-653, $\alpha$ -Tocopherol, and Probucol in Lipid Peroxidation**

Oxidation of methyl linoleate (10 mM) was carried out at 37°C under air in acetonitrile solution by adding 0.2 mM AMVN (2,2'-azobis-2,4-dimethylvaleronitrile) as a radical initiator in the presence of various concentrations of BO-653,  $\alpha$ -tocopherol, and probucol. These antioxidants were added at concentrations of 0.2–20  $\mu\text{M}$  to the reaction mixture; after 60 min incubation at 37°C, the reactions were stopped by chilling on ice.

The levels of lipid peroxidation were determined by the ferrous oxidation-xylenol orange (FOX) method [Nourooz-Zadeh et al., 1994]. In brief, completed methyl linoleate oxidation reactions were diluted 10-fold with MeOH containing 4.4 mM 2,6-di-*tert*-butyl-4-methylphenol (BHT). Each diluted solution (1.8 ml) was mixed with 0.1 ml of 2 mM xylenol orange solution (in 250 mM  $\text{H}_2\text{SO}_4$ ) and 0.1 ml of 5 mM ferrous chloride solution (in 250 mM  $\text{H}_2\text{SO}_4$ ). The mixture was incubated at room temperature for 60 min, at which point the absorbance at 570 nm was measured using a UV/visible light spectrophotometer. Cumene hydroperoxide was used to generate a standard curve for lipid hydroperoxidation.

#### **Pharmacokinetics of BO-653 in uPA/SCID Mice Harboring Human Hepatocytes**

Chimeric uPA/SCID mice harboring human hepatocytes were purchased from PhoenixBio (Hiroshima, Japan). All animal experiments were approved by the Ethics Committee of Tokyo Metropolitan Institute of Medical Science and were performed in accordance with the guidelines of the Animal Experimental Committee of Tokyo Metropolitan Institute of Medical Science. Two chimeric mice were administered BO-653 (at 800 or 2,000 mg/kg in 3% gum arabic solution) by single oral gavage. At 24 hr after administration, blood was collected and the plasma concentration of BO-653 was measured by high-performance liquid chromatography (HPLC). Aliquots of plasma (100  $\mu\text{l}$ ) were mixed with 50  $\mu\text{l}$  MeOH containing 10 mM ascorbic acid and 100  $\mu\text{l}$  acetonitrile containing 30  $\mu\text{g}/\text{ml}$  MeO-BO-653 as the internal control. The mixtures were centrifuged at 9,100g for 5 min, and the resulting supernatants of 30  $\mu\text{l}$  each were separated using

an octadecyl column (Capcell Pak C18 UG120, 3  $\mu$ m, 4.6 mm  $\times$  50 mm; Shiseido, Tokyo, Japan) at 30°C, a detection wavelength of 300 nm, and an eluent (acetonitrile) flow rate of 1.0 ml/min.

#### Treatment of HCV-Infected Chimeric Mice With BO-653 and/or PEG-IFN

Chimeric mice also were used as an *in vivo* model of persistent HCV infection, as described previously [Inoue et al., 2007]. uPA/SCID mice were engrafted with human hepatocytes; 6 weeks later, the chimeric mice were infected by intravenous (IV) injection with patient serum containing  $10^6$  copies of HCV genotype 1b (HCR6; GenBank accession no. AY045702). By 4 weeks after infection, the HCV RNA levels reached a plateau of  $10^6$ – $10^7$  copies/ml of mouse serum. To determine anti-HCV activity of BO-653 in the early phase of the treatment, the chimeric mice ( $n = 2$ – $5$  per group) infected with HCV were given once-daily oral gavage with 2,000 mg/kg BO-653 in 3% gum arabic, and/or twice weekly subcutaneous injection with 30  $\mu$ g/kg PEG-IFN $\alpha$ -2a (Chugai Pharmaceutical) as shown in Table I. Body weights were monitored daily, and blood for serum was collected prior to the start of treatment (Day-1) and once weekly thereafter (Days 8 and 14). Following the terminal bleed, animals were sacrificed and liver specimens were collected.

#### Quantitation of HCV RNA by qRT-PCR

After completion of the treatment, total RNA was purified from the serum and liver specimens by the acid guanidinium-phenol-chloroform method and qRT-PCR was used to quantify HCV RNA from the RNA samples corresponding to 1  $\mu$ l serum and about 5 mm<sup>3</sup> of liver.

#### Quantitation of Serum Human Albumin

The human albumin concentration in the blood of chimeric mice was measured in 2- $\mu$ l serum samples by using an Alb-II kit (Eiken Chemical, Tokyo, Japan) according to the manufacturer's instructions.

#### Statistical Analysis

Data are presented as mean  $\pm$  standard deviations (SDs). Statistical analysis was performed by using either Student's *t*-test or ANOVA, followed by Tukey's

test or Dunnett's test. A value of  $P < 0.05$  was considered statistically significant.

## RESULTS

### Inhibitory Effect of BO-653 on HCV Replication *In Vitro*

The anti-HCV activity of BO-653 (Fig. 1A) was investigated in cells harboring HCV subgenomic replicons (FLR3-1 cells). BO-653 suppressed the replication of HCV subgenomic replicons in a concentration-dependent manner (Fig. 1B). The half-maximal inhibitory concentration ( $IC_{50}$ ) of BO-653 in FLR3-1 cells was 36.0  $\mu$ M. In contrast, no cytotoxicity was observed with up to 1,000  $\mu$ M of BO-653 in FLR3-1 cells (Fig. 1B). Western blotting and immunofluorescent staining of FLR3-1 cells demonstrated that the level of HCV NS3 protein, but not that of  $\beta$ -actin, was reduced as the concentrations of BO-653 increased (Fig. 1C and D). A similar trend was seen in RMT-tri cells for the replication of full-genome HCV genotype 1a (Fig. 1E).

### Comparison of Anti-HCV Activity of Lipophilic Antioxidants

The antioxidant activity of BO-653 has been compared previously with that of probucol and  $\alpha$ -tocopherol [Cynshi et al., 1998]. Therefore, the anti-HCV activity of these three lipophilic antioxidants was compared in FLR3-1 cells. At a concentration  $>37$   $\mu$ M, BO-653 exhibited stronger inhibitory effects against HCV replication than did the two other compounds (Fig. 2A). In addition, the antioxidant activity of these compounds was determined by an *in vitro* lipid peroxidation system. BO-653 had the strongest antioxidant activity against lipid peroxidation in this *in vitro* assay (Fig. 2C).

### Comparison of Anti-HCV Activity of Hydrophilic and Lipophilic Antioxidants

The anti-HCV activities of some representative antioxidants were investigated further (Fig. 3A). As noted above, lipophilic antioxidants exhibited anti-HCV activity in cell culture; however, hydrophilic antioxidants (*N*-acetyl cysteine, ascorbic acid, and trolox) did not inhibit the replication of HCV subgenomic replicons at comparable concentrations. None of the

TABLE I. Schedule of Blood Collection and Drug Administration for Chimeric Mice Infected With HCV

	Day															
	-1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Collection of blood	B									B						B
BO-653		BO	BO	BO	BO	BO	BO	BO	BO	BO	BO	BO	BO	BO	BO	
PEG-IFN		I			I				I			I				
BO-653 + PEG-IFN		BO/I	BO	BO	BO/I	BO	BO	BO	BO/I	BO	BO	BO/I	BO	BO	BO	

B, sampling of blood; BO, orally administrated BO-653 (2,000 mg/kg); I, subcutaneous injection of PEG-IFN (30  $\mu$ g/kg).

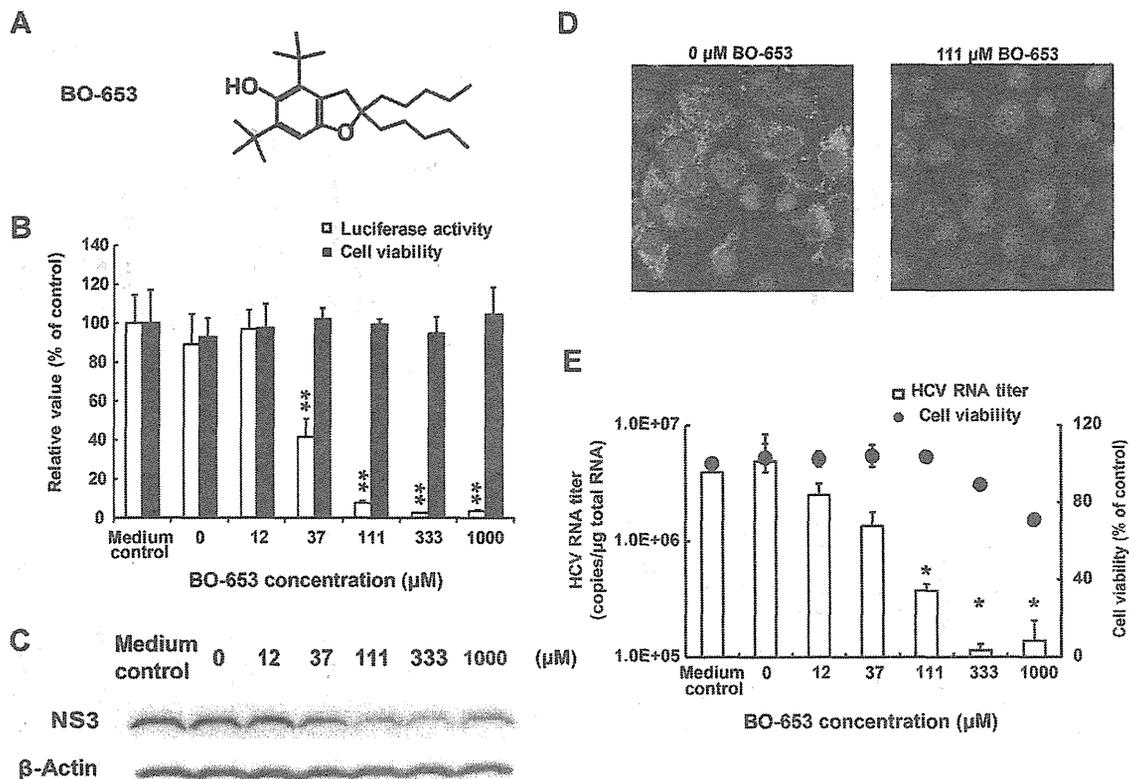


Fig. 1. In vitro anti-HCV activity of BO-653. A: The chemical structure of BO-653. B: The inhibitory effect of BO-653 on the replication of HCV subgenomic replicons in FLR3-1 cells. HCV replication (white bars) and cell viability (black bars) were determined after 72 hr of treatment; \*\* $P < 0.01$ , compared with the medium control. C: Effect of BO-653 on the levels of HCV NS3 protein and  $\beta$ -actin (Western blotting). D: Immunofluorescent staining of HCV

NS3 protein (green) in FLR3-1 cells in the absence or presence (111  $\mu\text{M}$ ) of BO-653; nuclei were stained with 4',6-diamidino-2-phenylindole (blue). E: Effect of BO-653 on the HCV RNA titer and cell viability of HuH-7 cells infected with HCV genotype 1a (RMT-tri) after 72 hr of treatment; \* $P < 0.05$ , compared with the medium control. Statistical analyses were performed by using ANOVA with post-hoc Dunnett's ( $n = 3$  replicates).

compounds had any cytotoxicity at the concentrations tested (Fig. 3B).

#### Anti-HCV Activity of BO-653 and PEG-IFN in Chimeric Mice Infected With HCV

As demonstrated above using an in vitro assay with FLR3-1 cells, lipophilic antioxidants, including BO-653, exhibited strong anti-HCV activity. The anti-HCV activity of BO-653 was assessed further in vivo by using the compound to treat humanized chimeric mice infected with HCV. First, to measure the pharmacokinetics of BO-653, two chimeric mice were administered orally BO-653 at 800 or 2,000 mg/kg. Twenty-four hours after administration, the mice had mean BO-653 plasma concentrations of 25.0 and 83.1  $\mu\text{M}$ , respectively (Table II). Thus, the BO-653 plasma concentration at the higher dose level exceeded the  $\text{IC}_{50}$  of BO-653 (36.0  $\mu\text{M}$ ) demonstrated previously by the in vitro assay (Fig. 1B), suggesting that oral administration of 2,000 mg/kg BO-653 might

be relevant therapeutically for chimeric mice infected with HCV. These mice were infected persistently with HCV genotype 1b by injection with the serum of a HCV-infected patient (see Materials and Methods Section). To determine the anti-HCV activity of BO-653 in early phase of treatment, BO-653 (oral) and/or PEG-IFN (subcutaneous) were then administered over a period of 14 days, according to the schedule shown in Table I, and serum and liver specimens were collected. No adverse effect of the treatment, such as loss of body weight or decreased human albumin secretion, was observed in any of the study groups (Fig. 4A and B). In the mice treated with PEG-IFN, which received a dose 20-fold higher dose than that used in the clinic, the serum HCV RNA titers fell approximately 30-fold and 50-fold in weeks 1 and 2, respectively. Treatment with BO-653 alone at 2,000 mg/kg orally once daily did not reduce the HCV RNA serum titers. However, the combination of BO-653 and PEG-IFN was effective, with the combination demonstrating 200-fold decrease in serum

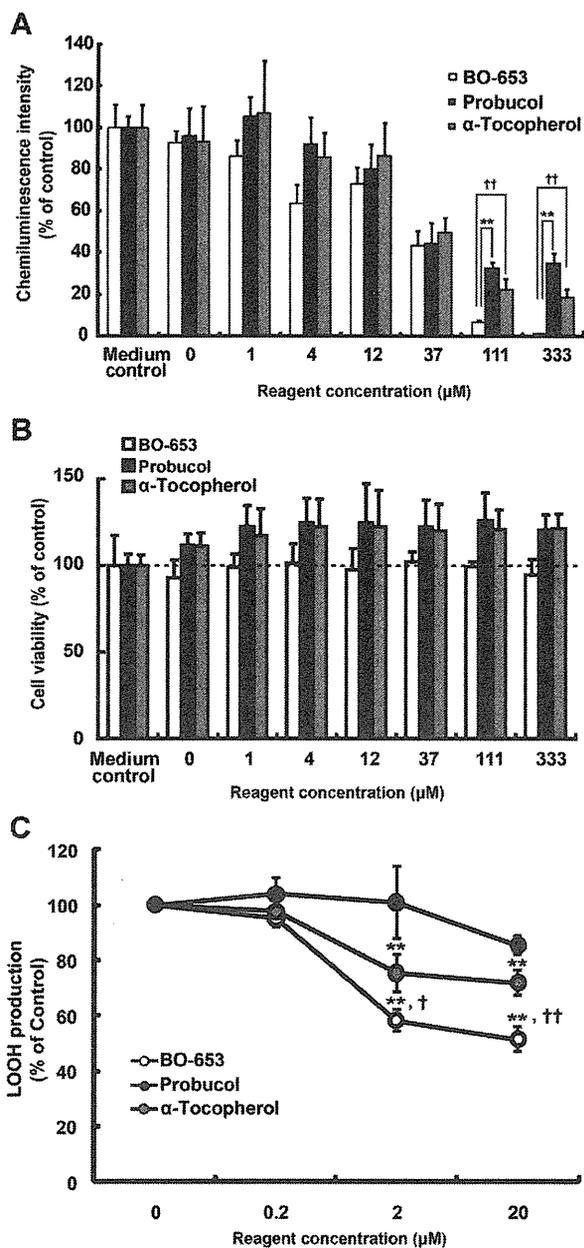


Fig. 2. Comparison of the in vitro anti-HCV activities of lipophilic antioxidants. A: The anti-HCV activity and (B) cytotoxicity of BO-653,  $\alpha$ -tocopherol, and probucol were measured in FLR3-1 cells after 72 hr exposure to the compounds;  $**P < 0.01$ , compared with probucol at the same concentration,  $^{\dagger\dagger}P < 0.01$ , compared with  $\alpha$ -tocopherol at the same concentration. C: Antioxidant activity of BO-653,  $\alpha$ -tocopherol, and probucol in the peroxidation of methyl linoleate with AMVN (FOX method);  $**P < 0.01$ , compared with probucol at the same concentration, and  $^{\dagger}P < 0.05$  and  $^{\dagger\dagger}P < 0.01$ , compared with  $\alpha$ -tocopherol at the same concentration. Statistical analyses were performed using ANOVA with post-hoc Tukey's.

HCV titer at 2 weeks; the effect was statistically significant compared to treatment with PEG-IFN alone (Fig. 4C). In the liver samples, a decrease in the titer of HCV RNA to 7–34% of the value in untreated mice

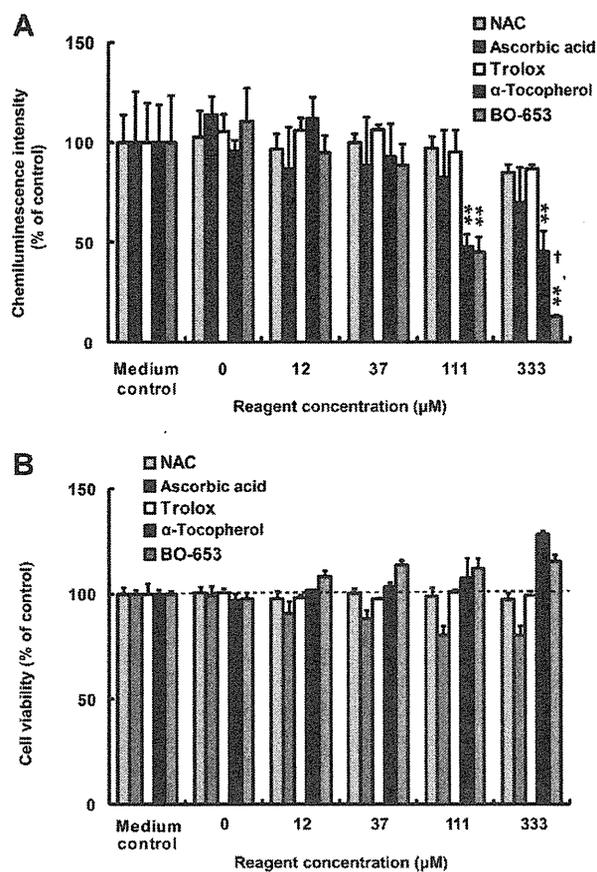


Fig. 3. Comparison of the anti-HCV activity of lipophilic and hydrophilic antioxidants. A: The anti-HCV activity and (B) cytotoxicity of BO-653 and  $\alpha$ -tocopherol (lipophilic antioxidants) and *N*-acetyl cysteine (NAC), ascorbic acid, and trolox (hydrophilic antioxidants) in FLR3-1 cells after 72 hr of exposure to the compounds.  $**P < 0.01$ , compared with the hydrophilic antioxidants at the same concentration, and  $^{\dagger}P < 0.05$ , compared with  $\alpha$ -tocopherol at the same concentration. Statistical analyses were performed using ANOVA with post-hoc Tukey's.

was also observed in the mice given the combination therapy (Fig. 4D).

DISCUSSION

Several lines of evidence indicate that chronic HCV infection is associated with persistently elevated levels of ROS, resulting in oxidative stress and thus contributing to the development of hepatic dam-

TABLE II. Concentration of BO-653 in Plasma

Dose	Mouse ID	Concentration of BO-653 in plasma ( $\mu$ M)	Mean concentration ( $\mu$ M)
800 mg/kg	1	30.2	25.0
	2	19.8	
2,000 mg/kg	1	100.9	83.1
	2	65.2	

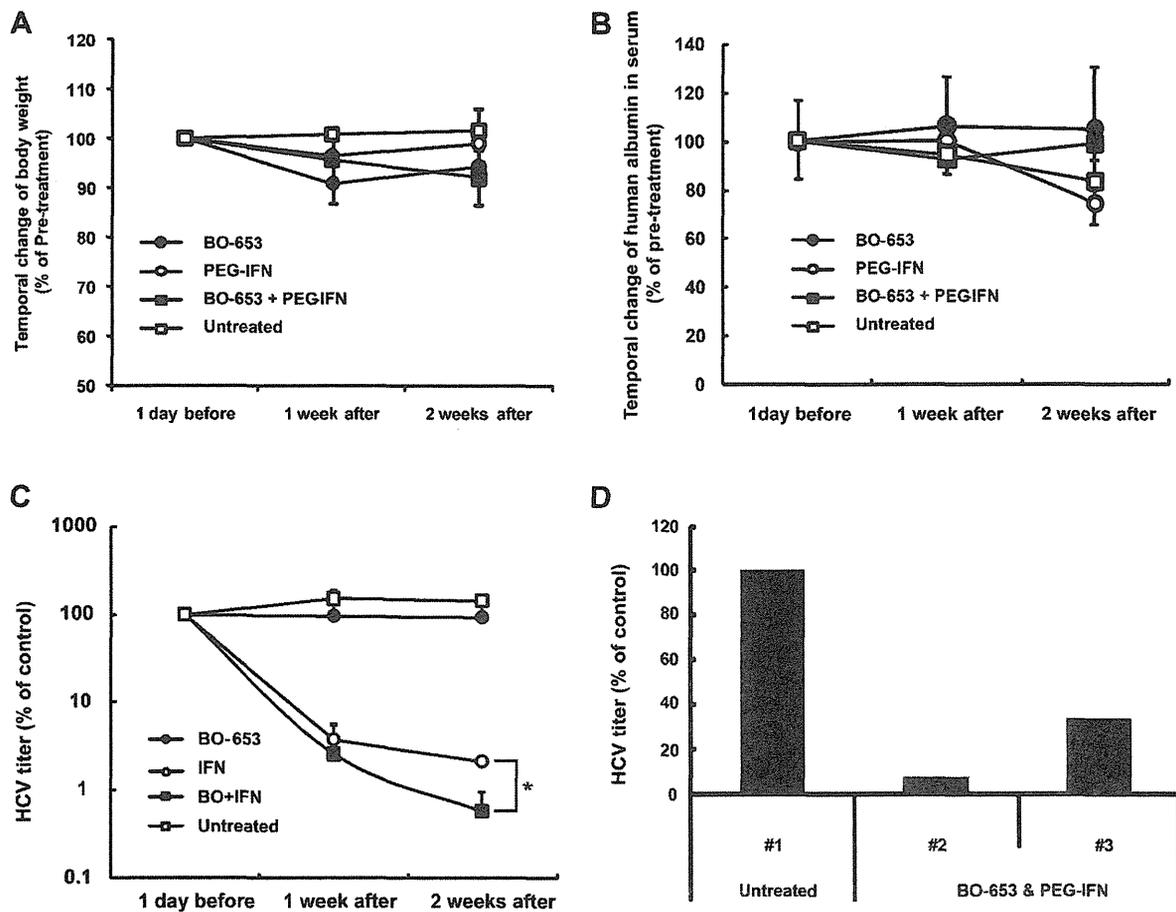


Fig. 4. Anti-HCV effect of BO-653 in chimeric mice infected with HCV. Temporal changes in the (A) body weight, (B) human albumin concentration in the serum, and (C) HCV RNA titer in the serum of chimeric mice infected with HCV after the indicated treatments. All groups included 3–5 mice, except for the untreated group (n = 2). The mice received once-daily oral gavage with 2,000 mg/kg BO-653 and/or twice-weekly subcutaneous injection with 30  $\mu$ g/kg PEG-IFN $\alpha$ -2a (see Table I). The HCV RNA titers were normalized to the

pre-treatment (Day-1) titer in the respective mouse. \* $P < 0.05$ , PEG-IFN and BO-653 combination treatment (n = 5) was compared with PEG-IFN monotherapy (n = 3) of the same duration. Statistical analyses were performed using Student's *t*-test. D: The HCV RNA titer in the liver of chimeric mice treated with or without PEG-IFN and BO-653 combination treatment (untreated group, n = 1; combination treatment group, n = 2).

age [Shimoda et al., 1994; Bureau et al., 2001; Gong et al., 2001; Waris et al., 2005; Levent et al., 2006]. On the other hand, the influence of the intracellular redox state on HCV replication is controversial. A previous study demonstrated inhibition of HCV subgenomic replicon replication by lipid peroxidation and restoration of the replication by treatment with vitamin E [Huang et al., 2007]. A similar result was reported by Choi et al. [2004], who showed inhibition of HCV replication by exogenous hydrogen peroxide treatment. These results indicate that elevated levels of ROS, higher than those induced by natural HCV infection, can lead to inhibition of HCV replication. Yano et al. [2007] reported that several antioxidants, including vitamin E and  $\beta$ -carotene, enhance the replication of HCV genome-length replicons at relatively

low concentrations (<10  $\mu$ M). In contrast, the antioxidant pyrrolidine dithiocarbamate can suppress HCV replication via the inhibition of STAT-3 activation [Waris et al., 2005]. Notably, these studies have been performed primarily in *in vitro* systems, using either subgenomic replicon-containing cells, full-genome replicon-containing cells, or cells persistently infected with HCV. Therefore, clarification of the effect of antioxidants on HCV replication will require further work, including the use of *in vivo* models.

BO-653 (2,3-dihydro-5-hydroxy-2,2-dipentyl-4,6-di-*tert*-butylbenzofuran), a lipophilic (hydrophobic) antioxidant, was investigated clinically for potential treatment of atherosclerosis and prevention of post-angioplasty restenosis [Cynshi et al., 1998; Meng, 2003]. Starting in November 2001, a phase II trial tested BO-653

for treatment of atherosclerosis and the prevention of post-angioplasty restenosis, but subsequent trials of the compound were suspended due to lack of efficacy. The present investigation of BO-653 as a potential anti-HCV drug reflects subsequent identification of the compound in random screening for antiviral activity.

In the present study, the anti-HCV activity of BO-653 was assessed in an *in vitro* system, and analyzed further in an *in vivo* system that used chimeric mice harboring human hepatocytes infected with HCV. In addition, the anti-HCV activity of BO-653 was compared with that of several other antioxidants, including hydrophilic and lipophilic compounds. Among these antioxidants, BO-653 had the strongest anti-HCV activity against HCV subgenomic replicons, followed by  $\alpha$ -tocopherol. A previous study showed that the replication of a HCV replicon was increased in the presence of lower concentrations of vitamin E [Yano et al., 2007]. However, the results of the present study suggest that vitamin E exhibits anti-HCV activity when present in cell culture at concentrations exceeding 37  $\mu$ M. Also, in chimeric mice infected persistently with HCV, the combination of PEG-IFN and BO-653 was more effective than PEG-IFN alone, although BO-653 alone did not have any anti-HCV activity in this mouse model. Monotherapy with DEBIO-025, a non-immunosuppressive cyclosporine A derivative and novel anti-HCV drug candidate, can decrease the viral load in patients with chronic HCV infection but not in chimeric mice infected with HCV [Inoue et al., 2007; Flisiak et al., 2009]; therefore, BO-653 monotherapy should be evaluated further in patients infected with HCV. Di Bona et al. [2006] reported impaired IFN- $\alpha$  signaling following oxidative stress, suggesting that oxidative stress causes resistance to the antiviral activity of IFN- $\alpha$  in patients infected with HCV. Taken together, these findings imply that lipophilic antioxidants such as BO-653 support the effects of PEG-IFN via antioxidant activity.

Several reports indicate that antioxidants, especially hydrophilic compounds, have antiviral activity [Docherty et al., 1999; Docherty et al., 2006; Ho et al., 2009; Geiler et al., 2010; Tian et al., 2010]. *N*-acetyl cysteine can inhibit the replication of H5N1 influenza A virus in a cell culture system [Geiler et al., 2010], and the antiviral effect of epigallocatechin gallate on enterovirus 71 may be associated with the modulation of the cellular redox state [Ho et al., 2009]. In contrast, the inhibitory effects of the lipophilic antioxidants on HCV RNA replication were stronger than those of the hydrophilic antioxidants. These lipophilic antioxidants inhibited the replication of HCV without cytotoxicity at concentrations higher than 37  $\mu$ M. The role of the redox state of the lipid membrane on HCV RNA replication is of great interest, given reports of the association of RNA replication with lipid rafts [Shi et al., 2003; Sakamoto et al., 2005]. Further studies will be needed to elucidate the precise

mechanism(s) underlying the suppression of HCV replication by BO-653.

In conclusion, the present study demonstrated that lipophilic antioxidants have stronger anti-HCV activity than hydrophilic antioxidants and that BO-653 has the strongest anti-HCV activity of the antioxidants tested. The combination of PEG-IFN and BO-653 was more effective than PEG-IFN alone in chimeric mice infected persistently with HCV. Further development of this compound would require additional considerations such as the route of administration and pharmacokinetics. Taken together, these findings provide insights into the influence of the intracellular redox state on the life-cycle of HCV.

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OPEN ACCESS

ORIGINAL ARTICLE

# Hepatitis C virus kinetics by administration of pegylated interferon- $\alpha$ in human and chimeric mice carrying human hepatocytes with variants of the *IL28B* gene

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## ABSTRACT

**Objective** Recent studies have demonstrated that genetic polymorphisms near the *IL28B* gene are associated with the clinical outcome of pegylated interferon  $\alpha$  (peg-IFN- $\alpha$ ) plus ribavirin therapy for patients with chronic hepatitis C virus (HCV). However, it is unclear whether genetic variations near the *IL28B* gene influence hepatic interferon (IFN)-stimulated gene (ISG) induction or cellular immune responses, lead to the viral reduction during IFN treatment.

**Design** Changes in HCV-RNA levels before therapy, at day 1 and weeks 1, 2, 4, 8 and 12 after administering peg-IFN- $\alpha$  plus ribavirin were measured in 54 patients infected with HCV genotype 1. Furthermore, we prepared four lines of chimeric mice having four different lots of human hepatocytes containing various single nucleotide polymorphisms (SNP) around the *IL28B* gene. HCV infecting chimeric mice were subcutaneously administered with peg-IFN- $\alpha$  for 2 weeks.

**Results** There were significant differences in the reduction of HCV-RNA levels after peg-IFN- $\alpha$  plus ribavirin therapy based on the *IL28B* SNP rs8099917 between TT (favourable) and TG/GG (unfavourable) genotypes in patients; the first-phase viral decline slope per day and second-phase slope per week in TT genotype were significantly higher than in TG/GG genotype. On peg-IFN- $\alpha$  administration to chimeric mice, however, no significant difference in the median reduction of HCV-RNA levels and the induction of antiviral ISG was observed between favourable and unfavourable human hepatocyte genotypes.

**Conclusions** As chimeric mice have the characteristic of immunodeficiency, the response to peg-IFN- $\alpha$  associated with the variation in *IL28B* alleles in chronic HCV patients would be composed of the intact immune system.

## INTRODUCTION

Hepatitis C is a global health problem that affects a significant portion of the world's population. The WHO estimated that, in 1999, 170 million hepatitis C virus (HCV)-infected patients were present worldwide, with 3–4 million new cases appearing per year.<sup>1</sup>

The standard therapy for hepatitis C still consists of pegylated interferon- $\alpha$  (peg-IFN- $\alpha$ ), administered once weekly, plus daily oral ribavirin for 24–48 weeks

## Significance of this study

### What is already known on this subject?

- Genetic polymorphisms near the *IL28B* gene are associated with a chronic HCV treatment response.
- HCV-infected patients with the *IL28B* homozygous favourable allele had a more rapid decline in HCV kinetics in the first and second phases by peg-IFN- $\alpha$ -based therapy.
- During the acute phase of HCV infection, a strong immune response among patients with the *IL28B* favourable genotype could induce more frequent spontaneous clearance of HCV.

### What are the new findings?

- In chronically HCV genotype 1b-infected chimeric mice that have the characteristic of immunodeficiency, no significant difference in the reduction in serum HCV-RNA levels and the induction of antiviral hepatic ISG by the administration of peg-IFN- $\alpha$  was observed between favourable and unfavourable human hepatocyte *IL28B* genotypes.
- By comparison of serum HCV kinetics between human and chimeric mice, the viral decline in both the first and second phases by peg-IFN- $\alpha$  treatment was affected by the variation in *IL28B* genotypes only in chronic hepatitis C patients.

### How might it impact on clinical practice in the foreseeable future?

- The immune response according to *IL28B* genetic variants could contribute to the first and second phases of HCV-RNA decline and might be critical for HCV clearance by peg-IFN- $\alpha$ -based therapy.

in countries where protease inhibitors are not available.<sup>2</sup> This combination therapy is quite successful in patients with HCV genotype 2 or 3 infection, leading to a sustained virological response (SVR) in approximately 80–90% of patients treated; however, in patients infected with HCV genotype 1 or 4, only approximately half of all treated individuals achieved a SVR.<sup>3,4</sup>

## Viral hepatitis

**Table 1** Characteristics of 54 patients infected HCV genotype 1

	<i>IL28B</i> SNP rs8099917			p Value
	TT (n=34)	TG (n=19) + GG (n=1)		
Age (years)	55.6±10.1	54.7±11.3		0.746
Gender (male %)	70	50		0.199
Body mass index (kg/m <sup>2</sup> )	24.6±3.1	24.7±3.3		0.870
Viral load at therapy (log IU/ml)	6.0±0.7	5.8±0.8		0.357
SVR rate (%)	50	11		0.012
Serum ALT level (IU/l)	100.3±80.8	79.3±45.0		0.226
Platelet count (×10 <sup>9</sup> /μl)	17.1±9.0	16.5±5.8		0.771
Fibrosis (F3+4 %)	42	40		0.877

HCV, hepatitis C virus; SNP, single nucleotide polymorphism; SVR, sustained virological response.

Host factors were shown to be associated with the outcome of the therapy, including age, sex, race, liver fibrosis and obesity.<sup>5</sup> Genome-wide association studies have demonstrated that genetic variations in the region near the interleukin-28B (*IL28B*) gene, which encodes interferon (IFN)-λ3, are associated with a chronic HCV treatment response.<sup>6–10</sup> Furthermore, it was demonstrated that genetic variations in the *IL28B* gene region are also associated with spontaneous HCV clearance.<sup>11–12</sup>

Interestingly, a recent report showed the effect of genetic polymorphisms near the *IL28B* gene on the dynamics of HCV during peg-IFN-α plus ribavirin therapy in Caucasian, African American and Hispanic individuals;<sup>13</sup> HCV-infected patients with the *IL28B* homozygous favourable allele had a more rapid decline of HCV in the first phase, which is associated with the inhibition of viral replication as well as the second phase associated with immuno-destruction of viral-infected hepatocytes.<sup>14</sup> However, it is unknown how a direct effect by the *IL28B* genetic variation, such as the induction of IFN-stimulated genes (ISG) or cellular immune responses, would influence the viral kinetics during IFN treatment. Over recent periods, engineered severe combined immunodeficient (SCID) mice transgenic for urokinase-type plasminogen activator (uPA) received human hepatocyte transplants (hereafter referred to as chimeric mice)<sup>15–17</sup> and are suitable for experiments with hepatitis viruses *in vivo*.<sup>18, 19</sup> We have also reported that these chimeric mice carrying human hepatocytes are a robust animal model to evaluate the efficacy of IFN and other anti-HCV agents.<sup>20, 21</sup>

The purpose of this study was to reveal the association between genetic variations in the *IL28B* gene region and viral decline during peg-IFN-α treatment in patients with HCV, and to clarify the association between different *IL28B* alleles of human hepatocytes in chimeric mice and the response to peg-IFN-α without immune response. These studies will elucidate whether the immune response by the *IL28B* genetic variation affects the viral kinetics during peg-IFN-α treatment.

## MATERIALS AND METHODS

### Patients

Fifty-four Japanese patients with chronic HCV genotype 1 infection at Nagasaki Medical Center and Nagoya City

University were enrolled in this study (table 1). Patients received peg-IFN-α2a (180 μg) or 2b (1.5 μg/kg) subcutaneously every week and were administered a weight-adjusted dose of ribavirin (600 mg for <60 kg, 800 mg for 60–80 kg, and 1000 mg for >80 kg daily), which is the recommended dosage in Japan. Patients with other hepatitis virus infection or HIV coinfection were not included in the study. The study protocol conformed to the ethics guidelines of the 1975 Declaration of Helsinki as reflected by earlier approval by the institutions' human research committees.

### Laboratory tests

Blood samples were obtained before therapy, as well as on day 1 and at weeks 1, 2, 4, 8 and 12 after the start of therapy and were analysed for the HCV-RNA level by the commercial Abbott Real-Time HCV test with a lower limit of detection of 12 IU/ml (Abbott Molecular Inc., Des Plaines, Illinois, USA). Genetic polymorphism in the *IL28B* gene (rs8099917), a single nucleotide polymorphism (SNP) recently identified to be associated with treatment response,<sup>6–8</sup> was tested by the TaqMan SNP genotyping assay (Applied Biosystems, Foster City, California, USA).

### HCV infection of chimeric mice with the liver repopulated for human hepatocytes

SCID mice carrying the uPA transgene controlled by an albumin promoter were injected with 5.0–7.5×10<sup>5</sup> viable hepatocytes through a small left-flank incision into the inferior splenic pole, thereafter chimeric mice were generated. The chimeric mice were purchased from PhoenixBio Co, Ltd (Hiroshima, Japan).<sup>17</sup> Human hepatocytes with the *IL28B* homozygous favourable allele, heterozygous allele or homozygous unfavourable allele were imported from BD Biosciences (San Jose, California, USA) (table 2). Murine serum levels of human albumin and the body weight were not significantly different among four chimeric mice groups, providing a reliable comparison for anti-HCV agents.<sup>22</sup> Three different serum samples were obtained from three chronic HCV patients (genotype 1b).<sup>21, 22</sup> Each mouse was intravenously infected with serum sample containing 10<sup>5</sup> copies of HCV genotype 1b. Administration of peg-IFN-α2a (Pegasys; Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) at the dose formulation (30 μg/kg) was consecutively applied to each mouse on days 0, 3, 7 and 10 (table 3).

### HCV-RNA quantification

HCV-RNA in mice sera (days 0, 1, 3, 7 and 14) was quantified by an in-house real-time detection PCR assay with a lower quantitative limit of detection of 10 copies/assay, as previously reported.<sup>21</sup>

### Quantification of IFN-stimulated gene-expression levels

For analysis of endogenous ISG levels, total RNA was isolated from the liver using the RNeasy RNA extraction kit (Qiagen, Valencia, California, USA) and complementary DNA synthesis

**Table 2** Four lines of uPA/SCID mice from four different lots of human hepatocytes (donor) containing various SNP around the *IL28B* gene

uPA/SCID mice	Donor	Race	Age	Gender	rs8103142	rs12979860	rs8099917
PXB mice	A	African American	5 Years	Male	CC	TT	TG
	B	Caucasian	10 Years	Female	CC	TT	TG
	C	Hispanic	2 Years	Female	TT	CC	TT
	D	Caucasian	2 Years	Male	TT	CC	TT

PXB mice; urokinase-type plasminogen activator/severe combined immunodeficiency (uPA/SCID) mice repopulated with approximately 80% human hepatocytes. SCID, severe combined immunodeficient; SNP, single nucleotide polymorphism.

**Table 3** Dosage and time schedule of pegIFN- $\alpha$ 2a\* treatment for HCV genotype 1b infected chimeric mice

Donor hepatocytes†	No of chimeric mice	Inoculum	Test compound	Dose			Frequency
				Level ( $\mu$ g/kg)	Concentration ( $\mu$ g/ml)	Volume (ml/kg)	
A	3	Serum A	Peg-IFN- $\alpha$ 2a	30	3	10	Day 0, 3, 7, 10
B	4	Serum A	Peg-IFN- $\alpha$ 2a	30	3	10	Day 0, 3, 7, 10
C	3	Serum A	Peg-IFN- $\alpha$ 2a	30	3	10	Day 0, 3, 7, 10
D	3	Serum A	Peg-IFN- $\alpha$ 2a	30	3	10	Day 0, 3, 7, 10
A	2	Serum B	Peg-IFN- $\alpha$ 2a	30	3	10	Day 0, 3, 7, 10
C	2	Serum B	Peg-IFN- $\alpha$ 2a	30	3	10	Day 0, 3, 7, 10
A	2	Serum C	Peg-IFN- $\alpha$ 2a	30	3	10	Day 0, 3, 7, 10
C	2	Serum C	Peg-IFN- $\alpha$ 2a	30	3	10	Day 0, 3, 7, 10

\*Pegasys; Chugai Pharmaceutical Co., Ltd., Tokyo, Japan.

†The *IL28B* genetic variation of the donor hepatocytes was indicated in table 2.

HCV, hepatitis C virus; peg-IFN- $\alpha$ , pegylated interferon  $\alpha$ .

was performed using 2.0  $\mu$ g of total RNA (High Capacity RNA-to-cDNA kit; Applied Biosystems). Fluorescence real-time PCR analysis was performed using an ABI 7500 instrument (Applied Biosystems) and TaqMan Fast Advanced gene expression assay (Applied Biosystems). TaqMan Gene Expression Assay primer and probe sets (Applied Biosystems) are shown in the supplementary information (available online only). Relative amounts of messenger RNA, determined using a FAM-Labeled TaqMan probe, were normalised to the endogenous RNA levels of the housekeeping reference gene, glyceraldehyde-3-phosphate dehydrogenase. The delta Ct method ( $2^{-(\text{delta } C_t)}$ ) was used for quantitation of relative mRNA levels and fold induction.<sup>23 24</sup>

### Statistical analyses

Statistical differences were evaluated by Fisher's exact test or the  $\chi^2$  test with the Yates correction. Mice serum HCV-RNA and intrahepatic ISG expression levels were compared using the Mann-Whitney U test. Differences were considered significant if p values were less than 0.05.

## RESULTS

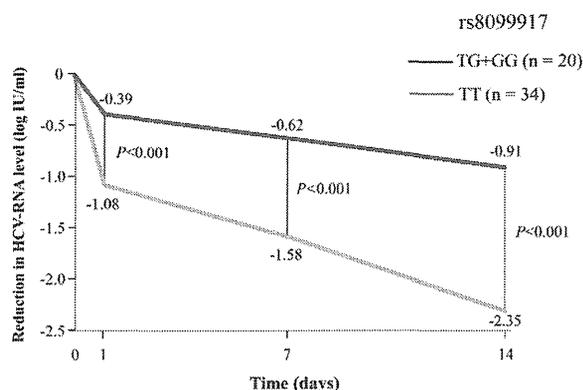
### Characteristics of the study patients

Genotypes (rs8099917) TT, TG and GG were detected in 34, 19 and one patient infected with HCV genotype 1, respectively. SVR rates were significantly higher in HCV patients with genotype TT than in those with genotype TG/GG (50% vs 11%,  $p=0.012$ ). The initial HCV serum load was comparable between

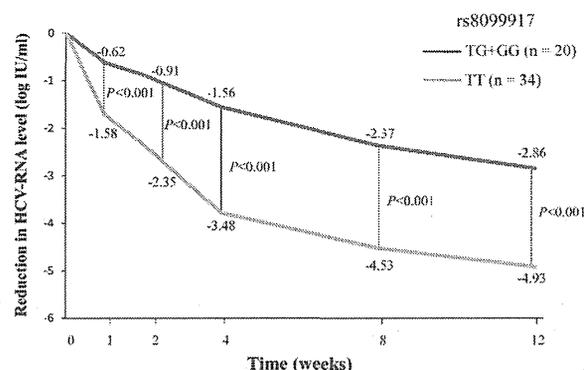
genotypes TT and TG/GG ( $6.0\pm 0.7$  vs  $5.8\pm 0.8$  log IU/ml). There were no significant differences in sex (male%, 70% vs 50%), age ( $55.6\pm 10.1$  vs  $54.7\pm 11.3$  years), serum alanine aminotransferase level ( $100.3\pm 80.8$  vs  $79.3\pm 45.0$  IU/L), platelet count ( $17.1\pm 9.0$  vs  $16.5\pm 5.8\times 10^4/\mu$ l) and fibrosis stages (F3/4%, 42% vs 40%) between HCV patients with the favourable (rs8099917 TT) and unfavourable (rs8099917 TG/GG) *IL28B* genotypes (table 1).

### Changes in serum HCV-RNA levels in patients treated by peg-IFN- $\alpha$ plus ribavirin

Figure 1 shows the initial change in the serum HCV-RNA level for 14 days after peg-IFN- $\alpha$  plus ribavirin therapy in patients infected with HCV genotype 1 based on the genetic polymorphism near the *IL28B* gene. The immediate antiviral response (viral drop 24 h after the first IFN injection) was significantly higher in HCV patients with genotype TT than genotype TG/GG ( $-1.08$  vs  $-0.39$  log IU/ml,  $p<0.001$ ). Figure 2 also shows the subsequent change in the serum HCV-RNA reduction after peg-IFN- $\alpha$  plus ribavirin therapy in patients infected with HCV genotype 1. Similarly, during peg-IFN- $\alpha$  plus ribavirin therapy, a statistically significant difference in the median reduction in serum HCV-RNA levels was noted according to the genotype (TT vs TG/GG). The median reduction in the serum HCV-RNA levels (log IU/ml) at 1, 2, 4, 8 and 12 weeks between genotypes TT and TG/GG was as follows:  $-1.58$  vs  $-0.62$ ,  $p<0.001$ ;  $-2.35$  vs  $-0.91$ ,  $p<0.001$ ;



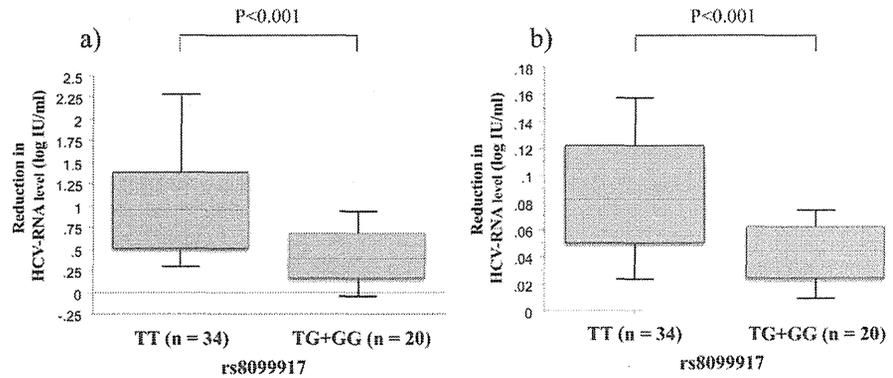
**Figure 1** Rapid reduction of median hepatitis C virus (HCV)-RNA levels (log IU/ml) at 1, 7 and 14 days between *IL28B* single nucleotide polymorphisms rs8099917 genotype TT (n=34) and TG/GG (n=20) in HCV genotype 1-infected patients treated with peg-IFN- $\alpha$  plus ribavirin.



**Figure 2** Weekly reduction of median hepatitis C virus (HCV)-RNA levels (log IU/ml) at 1, 2, 4, 8 and 12 weeks between *IL28B* single nucleotide polymorphisms rs8099917 genotype TT (n=34) and TG/GG (n=20) in HCV genotype 1-infected patients treated with pegylated interferon  $\alpha$  plus ribavirin.

## Viral hepatitis

**Figure 3** (A) The first-phase viral decline slope per day (Ph1/day) and (B) second-phase viral decline slope per week (Ph2/week) in hepatitis C virus (HCV) genotype 1-infected patients treated with pegylated interferon  $\alpha$  plus ribavirin. The lines across the boxes indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles for each group, respectively.

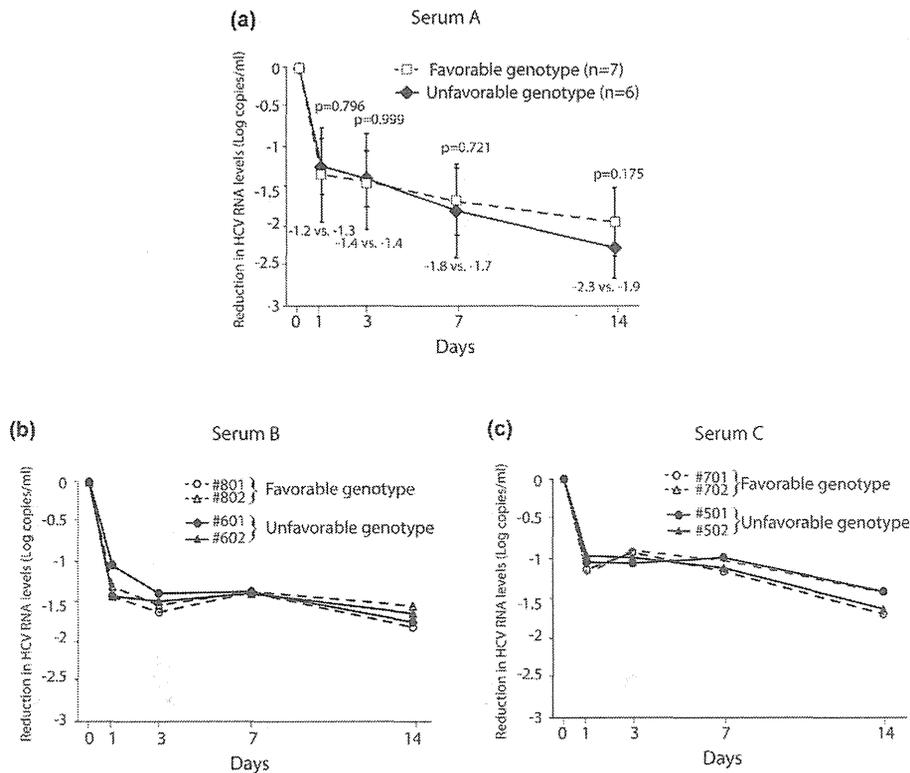


$-3.48$  vs  $-1.56$ ,  $p < 0.001$ ;  $-4.53$  vs  $-2.37$ ,  $p < 0.01$ ;  $-4.93$  vs  $-2.86$ ,  $p < 0.001$ . Furthermore, the initial first-phase viral decline slope per day (Ph1/day) and subsequent second-phase viral decline slope per week (Ph2/week) in TT genotype were significantly higher than in genotype TG/GG (Ph1/day  $0.94 \pm 0.83$  vs  $0.38 \pm 0.40$  log IU/ml,  $p < 0.001$ ; Ph2/week  $0.08 \pm 0.06$  vs  $0.04 \pm 0.03$  log IU/ml,  $p < 0.001$ ) (figure 3).

#### Changes in serum HCV-RNA levels in chimeric mice treated by peg-IFN- $\alpha$

In order to clarify the association between *IL28B* alleles of human hepatocytes and the response to peg-IFN- $\alpha$ , we prepared four lines of uPA/SCID mice and four different lots of human hepatocytes containing various rs8099917, rs8103142

and rs12979860 SNPs around the *IL28B* gene (table 2). The chimeric mice were inoculated with serum samples from each HCV-1b patient, and then HCV-RNA levels had increased and reached more than  $10^6$  copies/ml in all chimeric mice sera at 2 weeks after inoculation. After confirming the peak of HCV-RNA in all chimeric mice, they were subcutaneously administered with four times injections of the bolus dose of peg-IFN- $\alpha$ 2a for 2 weeks (table 3). Figure 4 shows the change in the serum HCV-RNA levels for 14 days during IFN injection into chimeric mice transplanted with *IL28B* favourable or unfavourable human hepatocyte genotypes. On peg-IFN- $\alpha$  administration, no significant difference in the median reduction in HCV-RNA levels in the serum A-infected<sup>22</sup> chimeric mice sera was observed between favourable ( $n=7$ ) and unfavourable



**Figure 4** Median reduction of hepatitis C virus (HCV)-RNA levels (log copies/ml) after administering pegylated interferon  $\alpha$  to chimeric mice having human hepatocytes containing various single nucleotide polymorphisms around the *IL28B* gene as favourable (rs8099917 TT) and unfavourable (rs8099917 TG) genotypes. Data are represented as mean+SD. Chimeric mice infected with a) serum A ( $n=7$ ; favourable genotype,  $n=6$ ; unfavourable genotype), (B) serum B ( $n=2$ , each genotype), and (C) serum C ( $n=2$ , each genotype). All serum samples were obtained from HCV-1b patients.

(n=6) *IL28B* genotypes on days 1, 3, 7 and 14 (-1.2 vs -1.3, -1.4 vs -1.4, -1.8 vs -1.7, and -2.3 vs -1.9 log copies/ml) (figure 4A). Moreover, we prepared two additional serum samples from the other HCV-1b patients (serum B and C)<sup>21</sup> to confirm the influence of *IL28B* genotype in early viral kinetics during IFN treatment. After establishing persistent infection with new HCV-1b strains in all chimeric mice, they were also administered four times injections of the bolus dose of peg-IFN- $\alpha$ 2a for 2 weeks (figure 4B,C). In a similar fashion, no significant difference in HCV-RNA reduction in chimeric mice sera was observed between favourable and unfavourable *IL28B* genotypes.

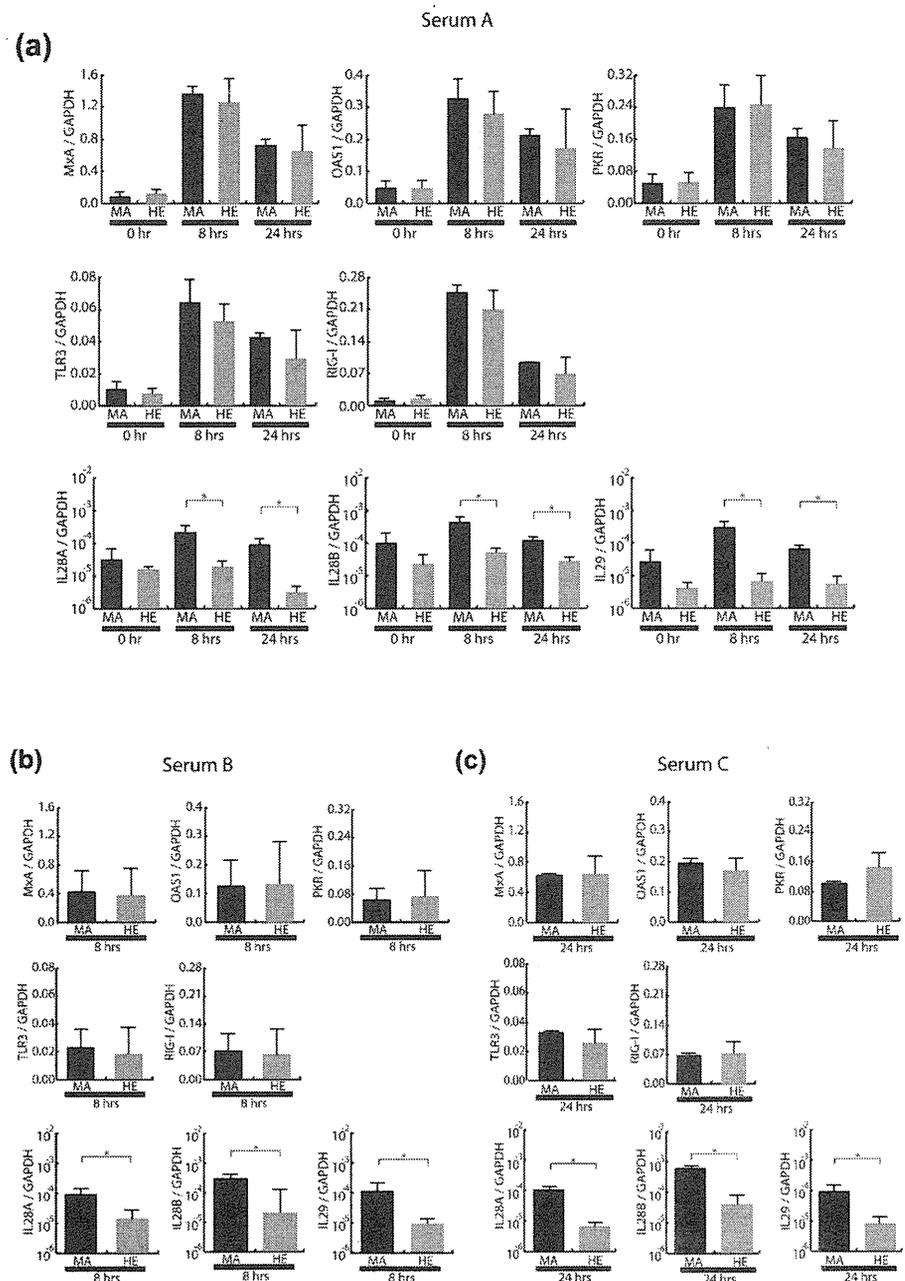
### Expression levels of ISG in chimeric mice livers

Because chimeric mice have the characteristic of severe combined immunodeficiency, the viral kinetics in chimeric mice

sera during IFN treatment could be contributed by the innate immune response of HCV-infected human hepatocytes. Therefore, ISG expression levels in mice livers transplanted with human hepatocytes were compared between favourable and unfavourable *IL28B* genotypes (figure 5).

As shown in figure 5A, ISG expression levels in mice livers were measured at 8 h and 24 h after IFN treatment. The levels of representative antiviral ISG (eg, myxovirus resistance protein A, oligoadenylate synthetase 1, RNA-dependent protein kinase) and other ISG for promoting antiviral signalling (eg, Toll-like receptor 3, retinoic acid-inducible gene 1) were significantly induced at least 8 h after treatment, and prolonged at 24 h. No significant difference in ISG expression levels in HCV-infected livers was observed between favourable and unfavourable *IL28B* genotypes. The other inoculum for persistent infection of HCV-1b also demonstrated no significant difference in ISG

**Figure 5** Intrahepatic interferon (IFN)-stimulated gene (ISG) expression levels in the pegylated interferon  $\alpha$  (peg-IFN- $\alpha$ )-treated chimeric mice having human hepatocytes containing homozygous favourable allele (rs8099917 TT; MA) and heterozygous unfavourable allele (rs8099917 TG; HE) were measured and expressed relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messenger RNA. Data are represented as mean+SD. (A) Time kinetics of ISG after administration of the peg-IFN- $\alpha$  in serum A-infected chimeric mice (n=3, each genotype). Comparison of ISG expression levels at (B) 8 h in serum B-infected mice and (C) 24 h in serum C-infected mice after administering peg-IFN- $\alpha$  (n=3, each genotype). Predesigned real-time PCR assay of *IL28B* transcript purchased from Applied Biosystems can be cross-reactive to *IL28A* transcript. \*p<0.05. MxA, myxovirus resistance protein A; OAS1, oligoadenylate synthetase 1; PKR, RNA-dependent protein kinase; RIG-I, retinoic acid-inducible gene 1; TLR3, Toll-like receptor 3.



## Viral hepatitis

expression levels between favourable and unfavourable *IL28B* genotypes (figure 5B,C). Interestingly, IFN- $\lambda$  expression levels by treatment of peg-IFN- $\alpha$  were significantly induced in HCV-infected human hepatocytes harbouring the favourable *IL28B* genotype (figure 5 A–C).

### DISCUSSION

Several recent studies have demonstrated a marked association between the chronic hepatitis C treatment response<sup>6–9</sup> and SNP (rs8099917, rs8103142 and rs12979860) near or within the region of the *IL28B* gene, which affected the viral dynamics during peg-IFN- $\alpha$  plus ribavirin therapy in Caucasian, African American and Hispanic individuals.<sup>13</sup>

It has been reported that when patients with chronic hepatitis C are treated by IFN- $\alpha$  or peg-IFN- $\alpha$  plus ribavirin, HCV-RNA generally declines after a 7–10 h delay.<sup>25</sup> The typical decline is biphasic and consists of a rapid first phase lasting for approximately 1–2 days during which HCV-RNA may fall 1–2 logs in patients infected with genotype 1, and subsequently a slower second phase of HCV-RNA decline.<sup>26</sup> The viral kinetics had a predictive value in evaluating antiviral efficacy.<sup>14</sup> In this study, biphasic decline of the HCV-RNA level during peg-IFN- $\alpha$  treatment was observed in both patients and chimeric mice infected with HCV genotype 1; however, in the first and second phases of viral kinetics, a difference between *IL28B* genotypes was observed only in HCV-infected patients; a more rapid decline in serum HCV-RNA levels after administering peg-IFN- $\alpha$  plus ribavirin was confirmed in patients with the TT genotype of rs8099917 compared to those with the TG/CG genotype.

On the other hand, in-vivo data using the chimeric mouse model showed no significant difference in the reduction of HCV-RNA titers in mouse serum among four different lots of human hepatocytes containing *IL28B* favourable (rs8099917 TT) or unfavourable (rs8099917 TG) genotypes, which was confirmed by the inoculation of two additional HCV strains. These results indicated that variants of the *IL28B* gene in donor hepatocytes had no influence on the response to peg-IFN- $\alpha$  under immunosuppressive conditions, suggesting that the immune response according to *IL28B* genetic variants could contribute to the first and second phases of HCV-RNA decline and might be critical for HCV clearance by peg-IFN- $\alpha$ -based therapy.

Two recent studies indeed revealed an association between the *IL28B* genotype and the expression level of hepatic ISG in human studies.<sup>27, 28</sup> Quiescent hepatic ISG before treatment among patients with the *IL28B* favourable genotype have been associated with sensitivity to exogenous IFN treatment and viral eradication; however, it is difficult to establish whether the hepatic ISG expression level contributes to viral clearance independently or appears as a direct consequence of the *IL28B* genotype. Another recent study addressed this question and the results suggested that there is no absolute correlation with the *IL28B* genotype and hepatic expression of ISG.<sup>29</sup> Our results on the hepatic ISG expression level in immunodeficient chimeric mice also suggested that no significant difference in ISG expression levels was observed between favourable and unfavourable *IL28B* genotypes. However, these results were not consistent with a previous report using chimeric mice that the favourable *IL28B* genotype was associated with an early reduction in HCV-RNA by ISG induction.<sup>30</sup> The reasons for the discrepancy might depend on the dose and type of IFN treatment, as well as the time point when ISG expression was examined in the liver. In addition, although IFN- $\lambda$  transcript levels measured in peripheral blood mononuclear cells or liver revealed inconsistent

results in the context of an association with the *IL28B* genotype,<sup>7, 8</sup> our preliminary assay on the *IL28A*, *IL28B* and *IL29* transcripts in the liver first indicated that the induction of IFN- $\lambda$  on peg-IFN- $\alpha$  administration could be associated with the *IL28B* genotype. Therefore, the induction of IFN- $\lambda$  followed by immune response might contribute to different viral kinetics and treatment outcomes in HCV-infected patients, because no difference was found in chimeric mice without immune response.

It has also been reported that the mechanism of the association of genetic variations in the *IL28B* gene and spontaneous clearance of HCV may be related to the host innate immune response.<sup>11</sup> Interestingly, participants with seroconversion illness with jaundice were more frequently rs8099917 homozygous favourable allele (TT) than other genotypes (32% vs 5%,  $p=0.047$ ). This suggests that a stronger immune response during the acute phase of HCV infection among patients with the *IL28B* favourable genotype would induce more frequent spontaneous clearance of HCV.

Taking into account both the above results in acute HCV infection and our results conducted on chimeric mice that have the characteristic of immunodeficiency, it is suggested that the response to peg-IFN- $\alpha$  associated with the variation in *IL28B* alleles in chronic hepatitis C patients would be composed of the intact immune system.

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**Contributors** YT and MM conceived the study. TW and FS and YT conducted the study equally. TW and FS coordinated the analysis and manuscript preparation. All the authors had input into the study design, patient recruitment and management or mouse management and critical revision of the manuscript for intellectual content. TW, FS and YT contributed equally.

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**Competing interests** None.

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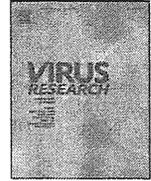
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## Short communication

## Translocase of outer mitochondrial membrane 70 induces interferon response and is impaired by hepatitis C virus NS3

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## ABSTRACT

Hepatitis C virus (HCV) elevated expression of the translocase of outer mitochondrial membrane 70 (Tom70). Interestingly, overexpression of Tom70 induces interferon (IFN) synthesis in hepatocytes, and it was impaired by HCV. Here, we addressed the mechanism of this impairment. The HCV NS3/4A protein induced Tom70 expression. The HCV NS3 protein interacted in cells, and cleaved the adapter protein mitochondrial anti-viral signaling (MAVS). Ectopic overexpression of Tom70 could not inhibit this cleavage. As a result, IRF-3 phosphorylation was impaired and IFN- $\beta$  induction was suppressed. These results indicate that MAVS works upstream of Tom70 and the cleavage of MAVS by HCV NS3 protease suppresses signaling of IFN induction.

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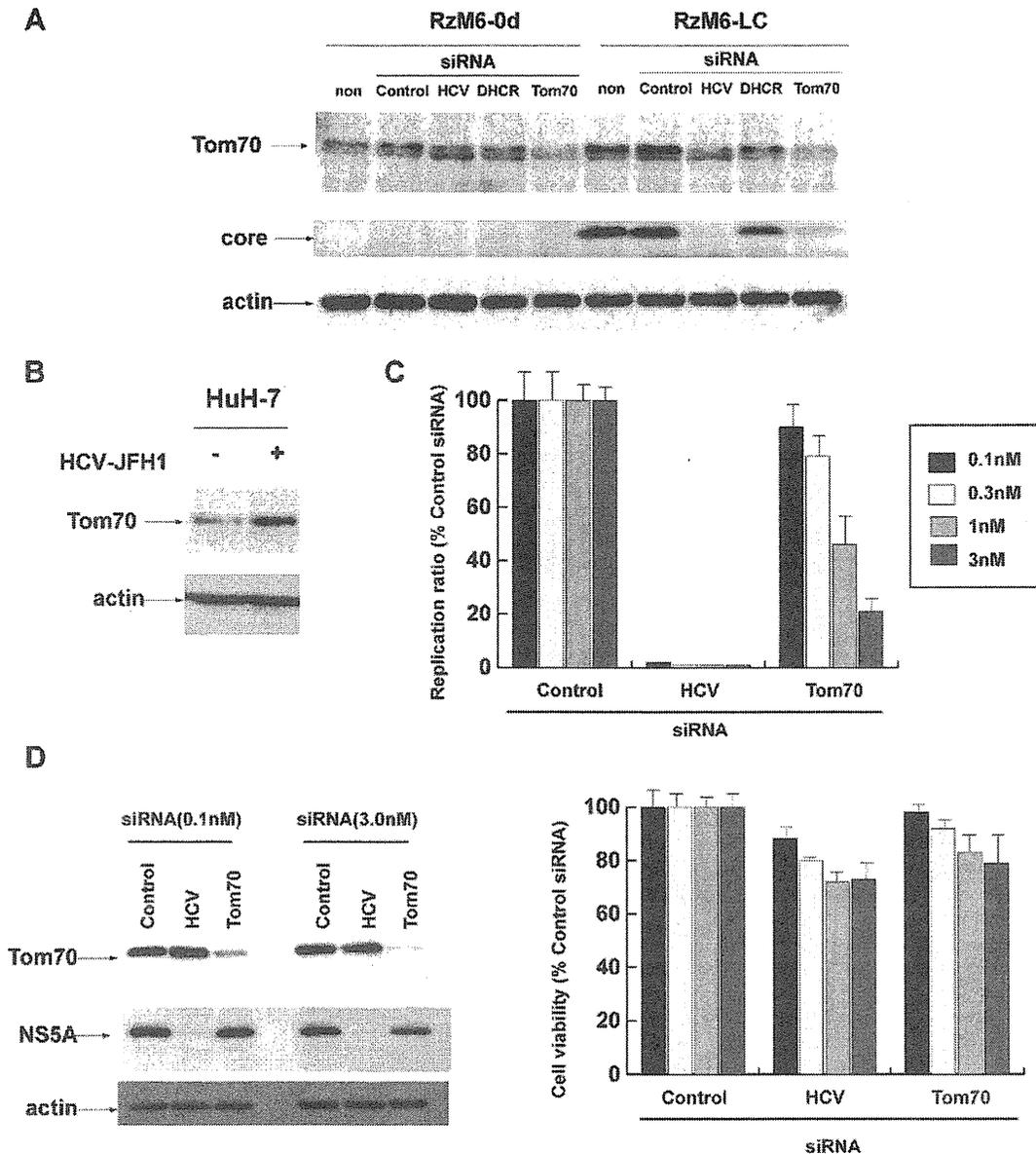
Type I interferon (IFN) induction is the front line of host defense against viral infection. Intracellular double-stranded RNA is a viral replication intermediate and contains pathogen-associated molecular patterns (PAMPS) (Saito et al., 2008) that are recognized by pathogen-recognition receptors (PRRs) to induce IFN. One PRR family includes the Toll-like receptors (TLRs), which are predominantly expressed in the endosome (Heil et al., 2004). Another route of IFN induction takes place in the cytosol through activation of specific RNA helicases, such as retinoic acid-inducible (RIG)-I and melanoma differentiation associated gene 5 (MDA5). The ligand for RIG-I is an uncapped 5' triphosphate RNA, which is found in viral RNAs of the *Flaviviridae* family, including hepatitis C virus (HCV), paramyxovirus, and rhabdoviruses (Kato et al., 2006). MDA5 recognizes viruses with protected 5' RNA ends, for example,

picornaviruses (Hornung et al., 2006). The adapter protein that links the RNA helicase to the downstream MAPK, NF- $\kappa$ B, and IRF-3 signaling pathways is referred to as the mitochondrial anti-viral signaling (MAVS) protein (Seth et al., 2005); alternative names include IPS-1, interferon-promoter stimulator 1; VISA, virus-induced signaling adaptor; and CARDIF, CARD adapter inducing IFN. HCV nonstructural protein 3 (NS3) possesses a serine protease domain at the N terminus (amino acids (aa) 1–180) and has been found to cleave adaptor proteins, MAVS at aa 508 (Meylan et al., 2005) and Toll/IL-1R domain-containing adapter inducing IFN- $\beta$ -deficient (TRIF at aa 372; Ferreón et al., 2005). These cleavages provoke abrogation of the induction of the IFN pathway.

The translocase of the outer membrane (TOM) is responsible for initial recognition of mitochondrial preproteins in the cytosol (Baker et al., 2007; Neupert and Herrmann, 2007). The TOM machinery consists of 2 import receptors, Tom20 and Tom70, and, along with several other subunits, comprises the general import pore (Abe et al., 2000). Recently, Tom70 was found to interact with MAVS (Liu et al., 2010). Ectopic expression or silencing of Tom70, respectively, enhanced or impaired IRF3-mediated gene expression and IFN- $\beta$  production. Sendai virus infection accelerated the

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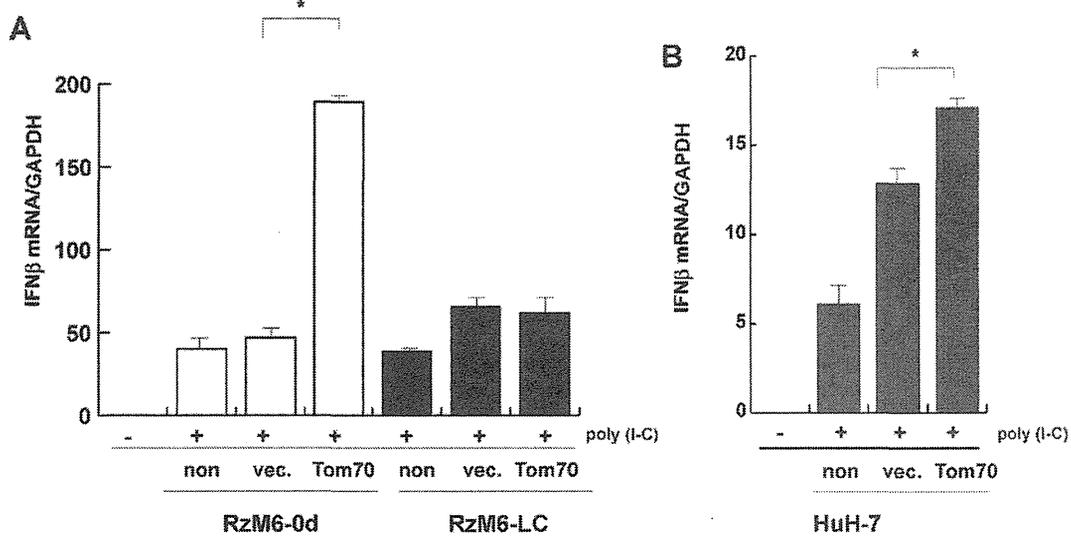
**Fig. 1.** HCV induces overexpression of Tom70 but impairs Tom70-induced IFN synthesis. (A) RzM6 cells (HCV<sup>-</sup>) and RzM6-LC cells (HCV<sup>+</sup>) were transfected with siRNAs of control (non-target siRNA#3: Thermo Fisher Scientific), HCV (R5: 5'-GUCUCGUAGACCGUGCAUCAuu-3'), DHCR24 (Nishimura et al., 2009), and Tom70 (Takano et al., 2011a). Control cells were mock-transfected. Tom70 protein was detected with Mab2-243a (Takano et al., 2011a) and actin protein was detected as an internal control (lower column). (B) HuH-7 cells were infected with HCV JFH-1 strain; Tom70 protein and actin protein were detected. (C) The HCV replicon cells (FLR3-1; Takano et al., 2011b) were transfected with siRNAs (control, HCV (R7: 5'-GUCUCGUAGACCGUGCACCAuu-3'), Tom70; 0.1, 0.3, 1, 3 nM) and HCV replication activity was measured with luciferase activity using the Bright-Glo Luciferase assay kit (Promega). Cell viability was measured using WST-8 (Dojindo) reagent. Ratio with those of control siRNA treatment was calculated. Vertical bars were S.D. (D) HCV replicon cells (FLR3-1) were transfected with control, HCV (R7) and Tom70 siRNAs (0.1, 0.3 nM) and Tom70, NS5A and actin proteins were detected.

Tom70-mediated IFN induction and the interaction of Tom70 with MAVS. These recent findings indicated that Tom70 might be a critical mediator during IFN induction (Liu et al., 2010).

We previously observed that HCV induces Tom70 and is related to the apoptotic response (Takano et al., 2011a). However, no synergistic effect was observed for IFN induction by Tom70 and HCV. Therefore, in the present study, we have investigated the mechanism of modification of the Tom70-induced IFN synthesis pathway by HCV and clarified a finely balanced system regulated by viral protein.

The expression of Tom70 protein was examined using western blotting and modification by HCV was characterized (Fig. 1A).

The level of Tom70 protein was increased in RzM6-LC cells compared with that in RzM6-Od cells (Tsukiyama-Kohara et al., 2004). The full-length HCV-RNA expression was induced by 4-hydroxy-tamoxifen (100 nM) and passed for more than 44 days in RzM6-LC cells, and HCV expression was not induced in RzM6-Od cells. Silencing of HCV expression by siRNA (R5; Thermo Scientific) abolished core protein expression, and decreased the level of Tom70 protein expression in RzM6-LC cells (Fig. 1A). Silencing of Tom70 by siRNA significantly decreased the level of HCV core protein expression in RzM6-LC cells (Fig. 1A). The siRNA against 3-beta-hydroxysterol-delta24 reductase (DHCR24) slightly decreased the level of Tom70 protein. In contrast, the



**Fig. 2.** Tom70-induced IFN synthesis was impaired by HCV. (A) RzM6-0d cells and LC cells were transfected with mock-vector, control pcDNA vector (vec.), or pcDNA-Tom70 expression vector, and the amount of IFN- $\beta$  mRNA was measured by RTD-PCR and normalized to the amount of GAPDH mRNA using Gene expression assay kit (GE-Healthcare). Poly(I-C) (GE Healthcare) (5  $\mu$ g) was transfected with RNAi Max reagent (Invitrogen) and IFN- $\beta$  mRNA was measured after 6 h of poly(I-C) treatment. Vertical bars indicate S.D. \* $p < 0.05$ . (B) HuH-7 cells were transfected with mock-vector, control vector, or Tom70 expression vector, and the amount of IFN- $\beta$  mRNA was measured by RTD-PCR and normalized to the amount of GAPDH mRNA. Vertical bars indicate S.D. \* $p < 0.05$ .

control siRNA did not have a significant effect on Tom70 protein expression.

We next examined the effects of HCV JFH-1 (Wakita et al., 2005) infection on Tom70 expression (Fig. 1B). Infection with HCV significantly increased the level of Tom70 protein expression. We also examine the role of Tom70 in HCV replication (Fig. 1C and D). Silencing of Tom70 by siRNA decreased the HCV replication in a dose dependent manner.

Thus, HCV induces Tom70 expression, and Tom70 is involved in viral replication.

It was recently shown that Tom70 recruits TBK1/IRF3 to mitochondria by binding to Hsp90 and inducing IFN- $\beta$  synthesis (Liu et al., 2010). Therefore, we examined the effects of Tom70 overexpression on IFN synthesis and modification by HCV (Fig. 2). Level of IFN- $\beta$  mRNA synthesis was quantitated by real-time detection (RTD) PCR. Overexpression of Tom70 by transfection of pcDNA6-Tom70 (Takano et al., 2011a) induced IFN- $\beta$  mRNA synthesis in the absence of HCV after poly(I-C) treatment (RzM6-0d cells). However, the Tom70-mediated induction of IFN- $\beta$  mRNA transcription was impaired in the presence of HCV (RzM6-LC cells) (Fig. 2A). Overexpression of Tom70 induced IFN- $\beta$  mRNA synthesis in HuH-7 cells (Fig. 2B). Induction of IFN- $\beta$  mRNA was lower in HuH-7 cells than HepG2 based RzM6 cells, which might be due to the defect in IFN induction system in HuH-7 cells (Preiss et al., 2008).

We have further addressed the mechanism of impairment of IFN- $\beta$  mRNA transcription by HCV.

To identify the viral protein that was responsible for the induction of Tom70, we examined the Tom70 protein expression levels in HCV core, E1, E2, NS2, NS3/4A, NS4B, NS5A, and NS5B protein-expressing cells (data not shown), and Tom70 protein expression level was highest in the NS3/4A-expressing cells than was observed in cells expressing other proteins (Fig. 3A, data not shown), indicating an effect of HCV NS3/4A protein on Tom70 expression.

The expression vector of Myc- and His-tagged Tom70 was transfected into the empty control or NS3/4A-expressing cells and immunoprecipitated with anti-Myc antibody (Suppl. Fig. 1A). Results showed that Myc-Tom70 was precipitated in both cells (right panel) and NS3 protein was specifically precipitated by

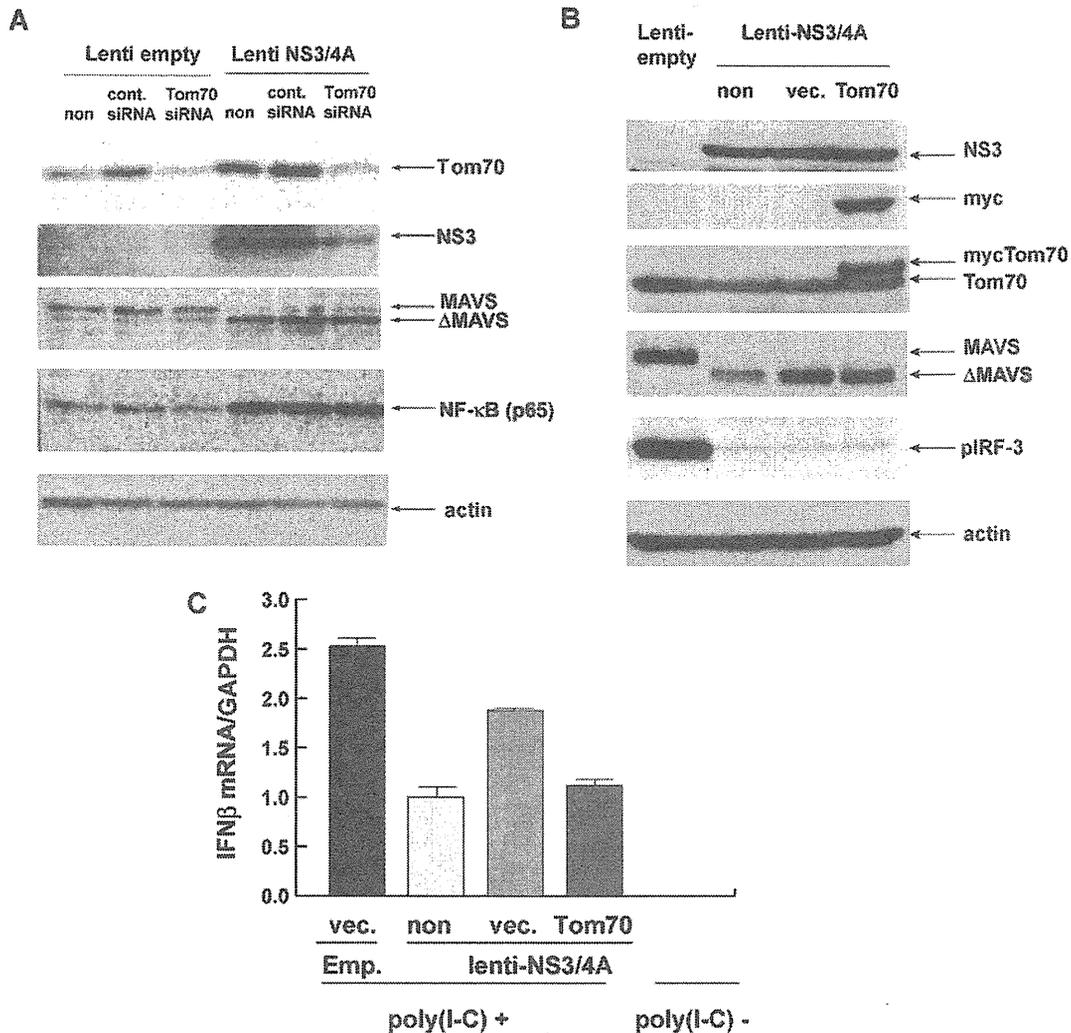
anti-Myc antibody in the NS3/4A-expressing cells (left panel). NS4A protein could not be detected (data not shown).

We next stained the NS3/4A-expressing cells with anti-NS3 and -Tom70 antibodies, and observed with confocal microscopy (Suppl. Fig. 1B). The signal of NS3 protein was clearly merged with that of Tom70, strongly supporting the possibility that the NS3 protein co-localizes with the Tom70 protein.

To clarify the effect of Tom70 on NS3, we transfected NS3/4A-expressing cells with the siRNA of Tom70 (Fig. 3A). Silencing of Tom70 decreased the level of NS3 protein in cells, but did not influence the levels of the MAVS and NF- $\kappa$ B proteins. These results suggest the possibility that Tom70 may increase the stability of NS3 protein in cells.

Tom70 reportedly interacts with MAVS during viral infection (Liu et al., 2010). Therefore, we examined the MAVS protein in cells expressing either the control empty or NS3/4A lenti-virus vector (Fig. 3B). Cleavage of MAVS (indicated as  $\Delta$ MAVS) was observed in NS3/4A protein-expressing cells, as was reported previously (Meylan et al., 2005). Overexpression of Tom70 did not have a significant effect on the MAVS expression level and did not prevent MAVS cleavage by NS3. IRF-3 phosphorylation was suppressed in NS3/4A-expressing cells and was not influenced by Tom70 overexpression. The induction of IFN- $\beta$  was impaired in NS3/4A-expressing cells, even in the presence of Tom70 overexpression (Fig. 3C). These data may indicate that MAVS exists upstream of Tom70 and that cleavage of MAVS by NS3/4A impaired the downstream signaling activation of IRF-3 phosphorylation (Suppl. Fig. 2).

Mitochondria provide a substantial platform for the regulation of IFN signaling. The MAVS adapter protein is a member of the family of RIG-I like receptors (RLRs), which links the mitochondria to the mammalian antiviral defense system (Seth et al., 2005). Proteomic studies have demonstrated that MAVS interacts with Tom70 (Liu et al., 2010). This interaction was accelerated by Sendai virus infection and synergized with ectopic expression of Tom70 to significantly increase the production of IFN- $\beta$  (Liu et al., 2010). The results of the present study revealed that infection with HCV induced Tom70 expression, but the presence of HCV impaired IFN



**Fig. 3.** Silencing of Tom70 decreased the level of NS3 and cleavage of MAVS by NS3/4A impaired IRF-3 phosphorylation even in the presence of Tom70. (A) Empty or NS3/4A-lenti virus vector expressing HepG2 cells were transfected with control siRNA and Tom70 siRNA or mock-transfected (non) as a control. MAVS, NS3, Tom70, and actin proteins were detected by western blot. (B) Empty or NS3/4A-expressing HepG2 cells were transfected with control pcDNA vector (vec.) and pcDNA6 (Invitrogen)-Tom70 or mock-transfected (non) as a control. NS3, Tom70, phosphorylated IRF-3, MAVS, and actin proteins were examined by western blot. (C) IFN-β mRNA was measured by RTD-PCR and normalized with GAPDH mRNA amount in empty or NS3/4A expressing cells with transfection of mock (non), pcDNA-vector (vec.) or pcDNA-Tom70 (Tom70). Poly(I-C) was treated, as described in the legend of Fig. 2.

induction. It has been reported that the C-terminal transmembrane domain (TM) of MAVS interacts with the N-terminal transmembrane domain of Tom70 (Liu et al., 2010). The HCV NS3 protein cleaves MAVS at residue 508 (Meylan et al., 2005), which should impair the interaction of MAVS and Tom70. This may attenuate the downstream signaling pathway (TBK-IRF3) and the induction of IFN synthesis (Suppl. Fig. 2). In our study, the level of NF-κB protein was not significantly influenced by Tom70 in the presence or absence of NS3. This may indicate that other pathways, such as TLR3 and downstream pathways, might compensate to maintain the NF-κB protein expression level in the absence of the MAVS-Tom70 signaling pathway.

Infection with HCV induced expression of Tom70, but the activation of the IFN signaling pathway was abrogated by the HCV NS3 protease. These findings indicate that recovery of the MAVS-Tom70 pathway may be a means to increase the efficacy of IFN therapy against HCV infection.

Recently, we observed that overexpression of Tom70 increased the resistance to the TNFα-induced apoptotic response (Takano

et al., 2011a), indicating that Tom70 overexpression might contribute to the apoptotic resistance of HCV-infected cells and the establishment of persistent HCV infection. Thus, Tom70 might be a novel target for the regulation of HCV infection.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2011.10.009.

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