A cell line that expresses complete HCV genome (RzM6-LC) was established to investigate the effects of persistent expression of HCV on cell growth [Tsukiyama-Kohara et al., 2004]. A monoclonal antibody (2-152a mAb) against the RzM6-LC cell line was also developed to produce clones that recognize both cell surface and intracellular molecules. As a result, 3β-hydroxysterol Δ24-reductase (DHCR24) was identified as the target of 2-152a mAb [Nishimura et al., 2009].

DHCR24 is an oxidoreductase with a broad expression pattern and shares homology with a family of flavin-adenine dinucleotide-dependent reductases [Waterham et al., 2001]. In mammals, DHCR24 functions as an enzyme to catalyze the conversion of desmosterol to cholesterol in the post-squalene cholesterol biosynthetic pathway, and it is essential for normal tissue development and maintenance [Waterham et al., 2001; Crameri et al., 2006]. DHCR24 regulates cholesterol synthesis and promotes recruitment of domain components into detergent-resistant membrane fractions [Crameri et al., 2006]. An absence of DHCR24 leads to desmosterolosis—a rare disorder of cholesterol biosynthesis [Waterham et al., 2001]. Expression of DHCR24 is downregulated in areas of the brain affected by Alzheimer's disease [Greeve et al., 2000], suggesting that DHCR24 has alternative functions. Indeed, DHCR24 is also known as seladin-1 (the selective Alzheimer's disease indicator 1), reflecting the association between DHCR24/seladin-1 and the selective vulnerability of the neurons in the affected areas of the brain. High levels of DHCR24/seladin-1 exert protective effects, conferring resistance against oxidative stress and preventing apoptotic cell death [Greeve et al., 2000; Benvenuti et al., 2005; Di Stasi et al., 2005; Luciani et al., 2005; Lu et al., 2008]. Endogenous DHCR24/seladin-1 levels are upregulated in response to acute oxidative stress [Wu et al., 2004; Benvenuti et al., 2006; Kuehnle et al., 2008], whereas the expression declines to low levels upon chronic exposure [Benvenuti et al., 2006; Kuehnle et al., 2008]. Therefore, DHCR24/seladin-1 may be involved in integrating cellular responses to oxidative stress. DHCR24 also functions as a hydrogen peroxide scavenger [Lu et al., 2008]. Based on these findings, DHCR24 may play a crucial role in maintaining cellular physiology by regulating both cholesterol synthesis and cellular defense against oxidative stress.

HCV infection impairs apoptosis induced by oxidative stress and inhibits p53 function via overexpression of DHCR24 [Nishimura et al., 2009]. Augmented expression of DHCR24 also facilitates efficient replication of HCV [Takano et al., 2011b]. Since DHCR24 may play a significant role in viral replication and in the tumorigenicity of the hepatocellular carcinoma related to HCV, the molecular mechanism of overexpression of DHCR24 in response to HCV was examined in the present study.

MATERIALS AND METHODS

Cell Lines

The HepG2 hepatoblastoma cell line, the HepG2-derived RzM6 cell line, which is capable of conditional control of expression of HCV genome (genotype 1b) based on the Cre/*loxP* system (RzM6-0d, no switching; RzM6-LC, switching of full genome HCV induced by tamoxifen), and HepG2-derived CN5 cell line, in which all HCV proteins were expressed conditionally by cre adenovirus (CN5-Cre) [Tsukiyama-Kohara et al., 2004] were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Using a stable expression system based on lentiviral vectors, HepG2/Lenti cell lines (core, E1, E2, NS2, NS3/4A, NS4B, NS5A, and NS5B) were established [Takano et al., 2011a]. An additional cell line—HepG2-emp—was infected with an empty lentiviral vector. Cells from the human hepatoma HuH-7 cell line were maintained in DMEM supplemented with 10% FBS and 0.4% glucose. The cell lines harboring HCV replicon, namely, R6FLR-N (genotype 1b) and FLR3-1 (genotype 1b), which are derived from HuH-7 [Takano et al., 2011b], were maintained under selective pressure with G418 (500 μg/ml for R6FLR-N and FLR3-1) in DMEM GlutaMAX (Invitrogen, Carlsbad, CA) containing 10% FBS. Cured/HuH-7 K4 cells—cured of HCV by interferon-α treatment—were maintained in DMEM GlutaMAX containing 10% FBS without G418. The JFH/K4 cell line, which shows persistent infection with the HCV JFH-1 strain, was maintained in DMEM containing 10% FBS. The human fetal hepatic cell line WRL68 was obtained from the American Type Culture Collection and maintained in DMEM supplemented with 10% FBS, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids. The human hepatoma cell line PLC/PRF/5 was obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University, and cultured in Eagle's minimum essential medium supplemented with 10% FBS.

Construction of DHCR24 Promoter Reporter Plasmids

Genomic DNA was extracted from HepG2 cells, and the 5′-flanking sequence of the predicted transcription start site of *DHCR24* (~5 kb) was isolated. The genomic region was inserted upstream of the firefly luciferase gene in pGL3-Basic (Promega, Madison, WI). Deletion constructs of the *DHCR24* promoter region were constructed using restriction enzymes and PCR (sense primer for −4956, 5′-GATCCTCGAGCACTCCTGCTCACCACTGAT-3′; sense primer for −2982, 5′-GATCCTCGAGGAGGCTCACATTGTAGAAAG-3′; antisense primer, 5′-GTAGTAGATATCGAAGATAAGCGAGAGCGG-3′) and cloned into pGL3-Basic at the *Xho*I and *Nco*I sites.

Dual Luciferase Reporter Assay

HepG2 cells (1×10^4 cells/well in a 96-well plate) were transfected with each of the 3 *DHCR24* promoter reporter plasmids and their deletion constructs (0.25 μg/well) using cationic lipid (Lipofectamine LTX, Invitrogen). Samples were analyzed with the Dual-Glo Luciferase Assay System (Promega) at 48 h post-transfection, and luminescence was measured using a TriStar LB941 microplate reader (Berthold, Bad Wildbad, Germany). To account for differences in transfection efficiency, the luminescence produced by firefly luciferase (FL) was normalized to that produced by Renilla luciferase (RL), which was expressed by co-transfection with phRL-TK (Promega; 0.025 μg/well).

Electrophoresis Mobility Shift Assay

Nuclear extracts were prepared from 5×10^6 to 1×10^7 cells as described previously [Dignam et al., 1983]. Electrophoresis mobility shift assays (EMSAs) were performed by a nonradioactive method using the DIG Gel Shift Kit (Roche, Indianapolis, IN). Briefly, binding reactions were performed by mixing the following components: 1 μg of poly[d(I-C)], 0.1 μg of poly L-lysine, 40 fmol DIG-labeled double-stranded oligonucleotide probe (HCV response element −167/−140 [28-mer], 5′-CCCCCGCCTCGCGCGGCGGCGGGGAGAA-3′; Sp1 consensus sequence [22-mer], 5′-ATTCGATCGGGGCGGGGCGAGC-3′; MZF1.1-4 consensus sequence [21-mer], 5′-GATCTAAAAGTGGGGAGAAAA-3′; AP-2α consensus sequence [26-mer], 5′-GATCGAACTGACCGCCCGCGGCCCGT-3′), and 10 μg of the nuclear extract in binding buffer (10 mM Tris–HCl, pH 7.5; 50 mM NaCl; 5 mM $MgCl_2$; 0.5 mM EDTA). Where indicated, reactions were supplemented with unlabeled/competitive oligonucleotide at a 50-fold molar excess concentration before addition of the probe. Mithramycin A (MMA; Sigma, St. Louis, MO), which blocks the binding of Sp1 to target sequences, was added at different final concentrations (2.5, 5, and 10 μM) and incubated at 4°C for 1 h. For supershift assays, 1 μg of monoclonal anti-Myc Tag antibody (Upstate Biotechnology, Lake Placid, NY) was added 30 min prior to addition of the probe. Binding reactions were carried out at 25°C for 30 min and electrophoresed on 6% acrylamide–0.5× TBE gels, transferred to positively charged nylon membranes, and detected by a chemiluminescence method (Roche) and a LAS1000 scanner (Fujifilm Co., Tokyo, Japan).

Silencing of Sp1, HCV, and DHCR24 by siRNA

SP1 Validated Stealth RNAi™ siRNA (VHS40867, Invitrogen) was designed with the BLOCK-iT RNAi designer to target the human Sp1 mRNA sequence. RzM6-0d and RzM6-LC cells (1.5×10^6 cells in a 100-mm dish) were transfected with Sp1 siRNA (final concentration, 30 nM) using Lipofectamine RNAiMAX (Invitrogen) in Opti-MEM (Invitrogen) and incubated for 48 h at 37°C. The siRNAs specific for DHCR24 and HCV were designed and utilized as described previously [Nishimura et al., 2009].

Kinase Inhibitors

ATM kinase inhibitor KU55933 (Wako Pure Chemical Industry, Osaka, Japan; final concentration, 10 μM), PI3K inhibitor LY294002 (Cell Signaling Technology, Beverly, MA; 50 μM), and MEK1 inhibitor PD98059 (Cell Signaling Technology; 50 μM) were added to cell cultures, which were incubated for 8 h at 37°C.

Western Blotting

Western blotting was performed as described previously [Tsukiyama-Kohara et al., 2004] with the following primary antibodies: rabbit monoclonal anti-DHCR24/Seladin-1 (C59D8; Cell Signaling Technology); rabbit polyclonal anti-Sp1, anti-phospho-Akt (Ser473), and mouse monoclonal anti-phospho-ERK (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); mouse monoclonal anti-HCV core (clone 31-2), E1 (clone 384), E2 (clone 544), NS4A (c14II-2-1), NS5A (32-2), NS5B (14-5), rabbit polyclonal anti-NS2, NS3 (R212), and NS4B (RR10) [Tsukiyama-Kohara et al., 2004]. Phosphorylation of Sp1 was investigated by 5% SDS–PAGE and immunoblotting with a polyclonal antibody against Sp1 phosphorylated at Ser101 (Active Motif, Carlsbad, CA) or Thr453 (Abcam, Cambridge, MA). Detection of γH2AX was performed by 15% SDS–PAGE and immunoblotting with mouse monoclonal anti-phospho-histone H2AX (Ser139) (JBW301; Upstate Biotechnology). Phosphorylated ATM (Ser1981) and ATR (Ser428) were detected by specific antibodies (Cell Signaling Technology). Monoclonal anti-actin (Sigma), anti-histone H1 (Santa Cruz Biotechnology, Inc.), anti-HAUSP (Calbiochem, San Diego, CA), and anti-heat shock protein 90 (Stressgen, Victoria, BC, Canada) primary antibodies were used for normalization of Western blotting. Bound antibody was detected with a horseradish peroxidase-conjugated secondary antibody and visualization using ECL reagents (GE Healthcare, Piscataway, NJ) and an LAS1000 scanner (Fujifilm). Densitometric analysis of protein bands was performed with Image Quant software (Molecular Dynamics, Sunnyvale, CA).

Quantitative PCR and HCV Infection

Total RNA was extracted from cell lines using ISOGEN, and reverse transcription of total RNA (125 ng) was performed with SuperScript III Reverse Transcriptase and Random Primers (Invitrogen). Synthesized cDNA samples were subjected to a TaqMan gene expression assay (Applied Biosystems, Foster City, CA), and the level of expression of *DHCR24* mRNA in each sample was normalized to the level of expression of *GAPDH* mRNA and represented as a ratio of the control (Hep-emp, CN5-Hep, or RzM6-0d). Infection of the human hepatocytes from human liver-uPA/SCID chimeric mice with HCV was performed,

and HCV RNA, *DHCR24* mRNA, and 18S rRNA were measured by quantitative PCR (qPCR), as described previously [Takano et al., 2011a].

Statistical Analysis

The Student's *t*-test was used to analyze the statistical significance of the results; P values < 0.05 were considered statistically significant.

RESULTS

DHCR24 Expression Is Upregulated by the Complete HCV Genome But Not by Individual Viral Proteins

Overexpression of *DHCR24* in human hepatocytes from human liver-uPA/SCID chimeric mice has been observed after HCV infection (Fig. 1A). The overexpression of *DHCR24* in cells expressing HCV decreased to a similar extent as that observed in control cells following treatment with HCV siRNA (Fig. 1B). Since these findings suggest that overexpression of *DHCR24* is associated with the expression or infection by HCV, the identity of the viral factor involved in the augmentation of expression of *DHCR24* was examined. The level of expression of *DHCR24* mRNA was measured by quantitative RT-PCR (Fig. 1C) in HepG2-derived cell lines that stably express individual HCV proteins (core, E1, E2, NS2, NS3/4A, NS4B, NS5A, or NS5B; Supplementary Fig. 1). The level of expression of *DHCR24* mRNA was slightly higher in the cells expressing NS4B and NS5A than in control cells; however, there was no significant difference in the expression of *DHCR24* mRNA among these cell

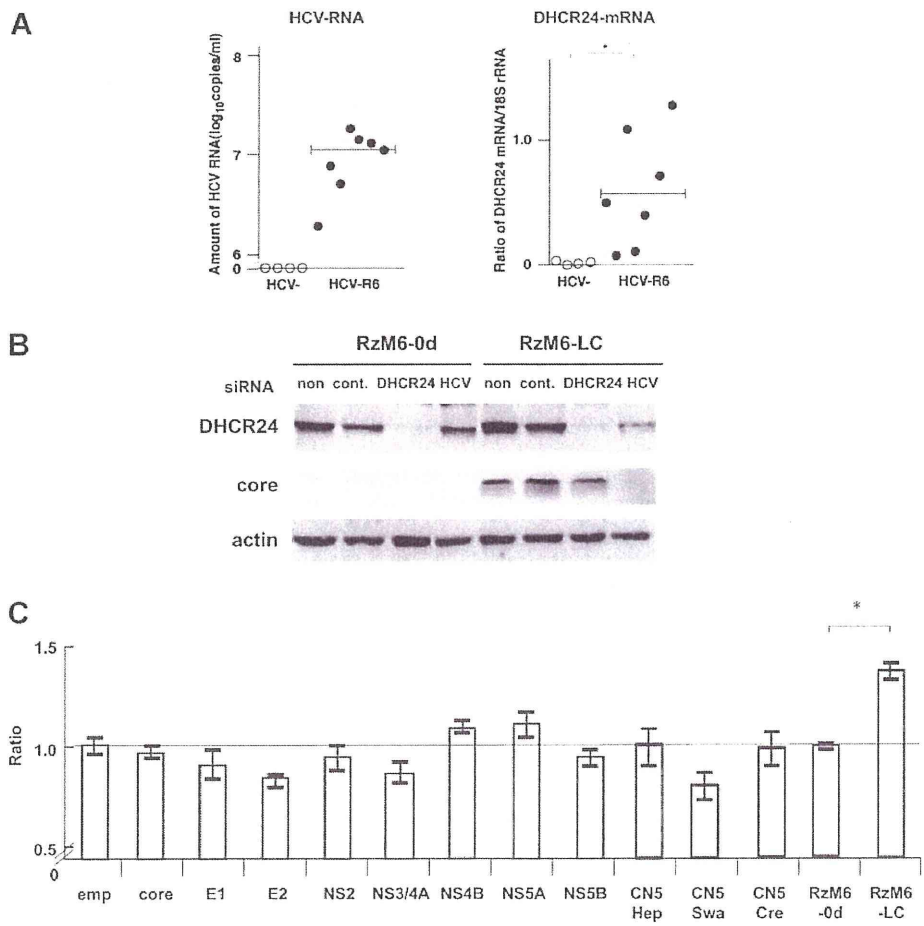

Fig. 1. *DHCR24* expression is induced in the presence of the complete HCV genome. **A**: The amount of HCV RNA in chimeric mice infected with HCV-R6 (genotype 1b) was quantified by qPCR (left panel). The amount of DHCR24 mRNA was measured, and the ratio to the amount of 18S rRNA was calculated in the tissues (right panel). **B**: Western blotting of DHCR24, HCV core, and actin protein in RzM6-0d and LC cells following treatment with the indicated siRNA. **C**: Level of *DHCR24* mRNA expression in cell lines with stable expression of individual HCV proteins, the HCV open reading frame, or the complete HCV genome. Total RNA from HepG2/Lenti cell lines (emp, core, E1, E2, NS2, NS3/4A, NS4B, NS5A, and NS5B), CN5 cell lines (CN5-Hep, CN5-Swa, and CN5-Cre), or RzM6 cell lines (RzM6-0d and RzM6-LC) were prepared, and reverse transcription was performed. Synthesized cDNA was subjected to quantitative PCR. The level of expression of *DHCR24* mRNA for each sample was normalized to that of *GAPDH* mRNA and represented as a ratio of HepG2-emp ($^*P < 0.05$).

lines. No significant upregulation of *DHCR24* mRNA was observed in the CN5-Cre cell line, which expresses all HCV proteins and is negative for viral replication [Tsukiyama-Kohara et al., 2004]. In contrast, significant upregulation of *DHCR24* was observed in a cell line that expresses the complete HCV genome (RzM6-LC) compared with the expression in HCV-negative control cells (RzM6-0d). Thus, expression of viral proteins alone is insufficient to reproduce the augmentation of expression of *DHCR24* induced by HCV.

DHCR24 Promoter Activity Is Potentiated by the Expression of HCV

The 5′-flanking region contains a number of possible transcriptional regulatory elements, including three candidate-binding motifs for the endoplasmic reticulum (ER) stress-responsive transcription factor, XBP1. Cellular ER stress is induced in response to the expression of the HCV gene and infection by HCV [Tardif et al., 2005]. Thus, to explore host factors involved in the transcriptional regulation of *DHCR24*, the 5′-flanking genomic region (~5 kb) of *DHCR24* was isolated. Subsequently, *DHCR24* promoter reporter plasmids that contain the 5′-flanking region of *DHCR24* and the firefly luciferase gene were constructed (Fig. 2A). Relevant regions of the promoter were defined by constructing deletion mutants of the 5′-flanking regions, which were analyzed by a dual luciferase reporter assay in the presence or absence of full-length HCV genome expression—resulting from transfection with pCA-Rz [Tsukiyama-Kohara et al., 2004] or the control pCAGGS vector, respectively (Fig. 2B). Progressive shortening of the 5′-flanking regions did not

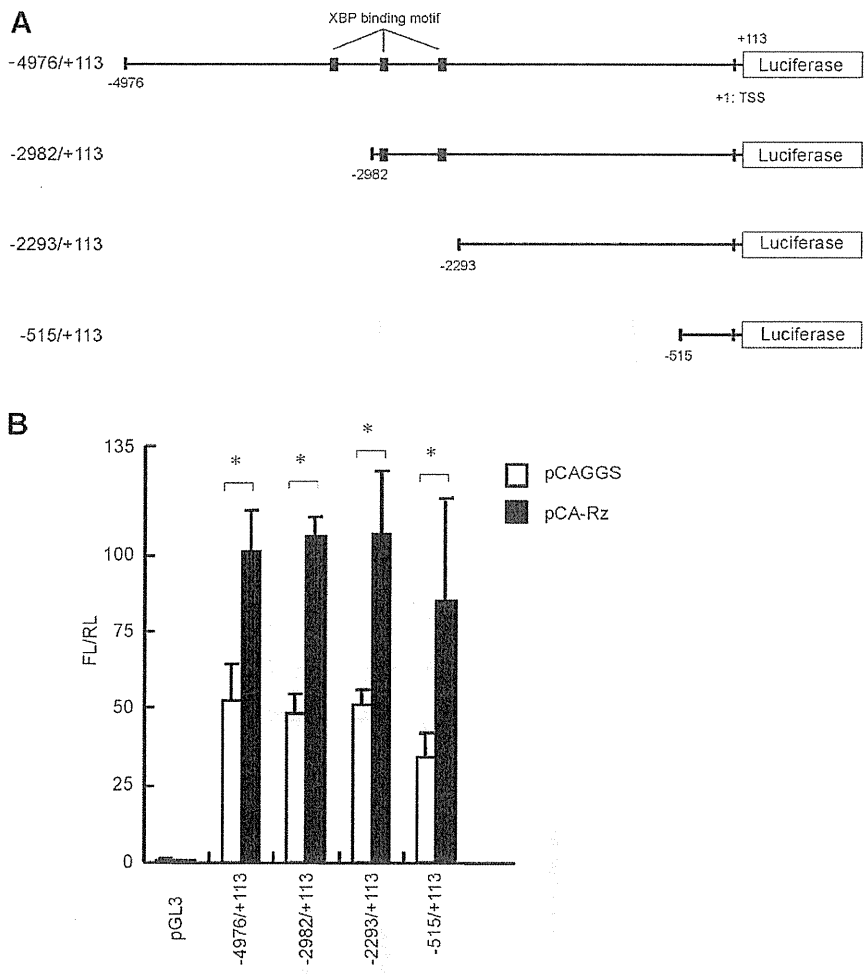

Fig. 2. *DHCR24* promoter activity is augmented by the expression of HCV. **A:** The 5′-flanking region of *DHCR24* was cloned from HepG2 and inserted upstream of the firefly luciferase gene in pGL3 (−4976/+113). A deletion series of the 5′-flanking regions was also constructed (−2982/+113, −2293/+113, and −515/+113). The black boxes indicate potential binding sites for the ER stress-responsive transcription factor, XBP-1. TSS, transcription start site (+1). **B:** HepG2 cells (1×10^4 cells/well in a 96-well plate) were co-transfected with each *DHCR24* promoter reporter plasmid (0.25 μg/well), a Renilla luciferase expression vector (phRL-TK; 0.025 μg/well), and either an expression vector containing the HCV full-length genome (pCA-Rz; 0.5 μg/well) or an empty expression vector (pCAGGS). Luciferase activity at 48 h post-transfection is shown as the ratio of firefly luciferase (FL) to Renilla luciferase (RL). Data are shown as the mean ± SD from 2 representative experiments performed in triplicate ($^*P < 0.05$).

result in significant differences in the basal promoter activity (Fig. 2B). The −515/+113 construct also produced a significant response in the presence of full-length HCV genome expression.

Additional reporter deletion mutants were constructed to define the region in the *DHCR24* promoter that is responsive to HCV expression. To this aim, potential binding motifs for transcription factors were predicted in the minimized *DHCR24* promoter sequence (nucleotides –515/+113; Fig. 3A), and a series of promoter mutants containing sequential 100-bp deletions was constructed. As shown in Fig. 3A, while the promoter activity of −515/+113, −400/+113, −300/+113, and −200/+113 constructs was increased significantly by expression of HCV ($^*P < 0.05$), the promoter activity of the −100/+113 construct was unchanged. Therefore, an HCV-responsive sequence appears to be located in the upstream region (−200 to −100 bp) from the transcriptional start site of *DHCR24*, which includes sequences with similarity to the consensus-binding motifs for AP-2α, Sp1, MZF-1, Pax-4, and NF-Y.

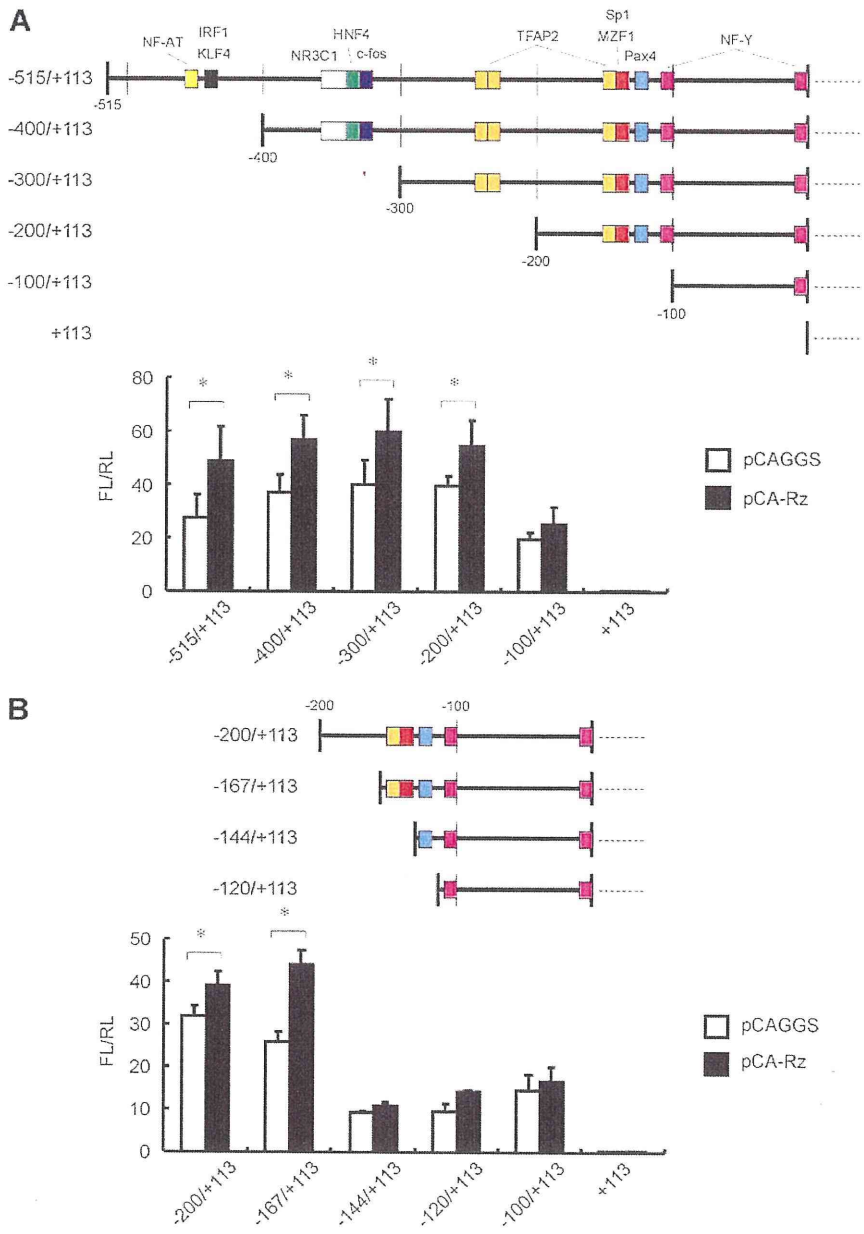

Fig. 3. Responsiveness of the *DHCR24* promoter to the expression of HCV. **A**: A *DHCR24* promoter series with sequential 100-bp deletions was constructed and analyzed as described in the legend to Fig. 2B ($^*p < 0.05$). **B**: An additional deletion series (−167/+113, −144/+113, and −120/+113) was constructed and analyzed as described in (A).

A more detailed deletion series (−167/+113, −144/+113, and −120/+113) was constructed (Fig. 3B) to determine the minimum-binding motif that responds to HCV expression. The responsiveness to the expression of HCV was lost with the removal of the proximal portion (−167 to −145), which includes candidate-binding motifs for AP-2α, Sp1, and MZF-1. Thus, the identified HCV response element in the *DHCR24* promoter represents the minimum element of DNA sequence required for the promotion of the expression of DHCR24 induced by HCV.

HCV Expression Augments the Interaction Between the HCV Response Element and the Binding Molecule(s)

Transcription of *DHCR24* is upregulated significantly in RzM6-LC cells that show persistent expression of HCV [Nishimura et al., 2009]. Therefore, the effect of expression of HCV on the interaction between the HCV response element and its related transcription factor(s) was examined. Nuclear extracts were prepared from RzM6-LC cells, and an electrophoretic mobility shift assay (EMSA) using a DIG-labeled double-stranded oligonucleotide corresponding to the response element (−167/−140, 28 bp; Fig. 4A) was performed. The interaction between the response element and the nuclear factor was increased significantly in nuclear extracts from RzM6-LC cells compared with that in RzM6-0d cells (Fig. 4B). Thus, the binding affinity or quantity of the nuclear factor may be increased by the expression of HCV. The shifted band corresponding to the Sp1 consensus sequence also increased in RzM6-LC cells compared with that in control RzM6-0d cells, whereas no difference was

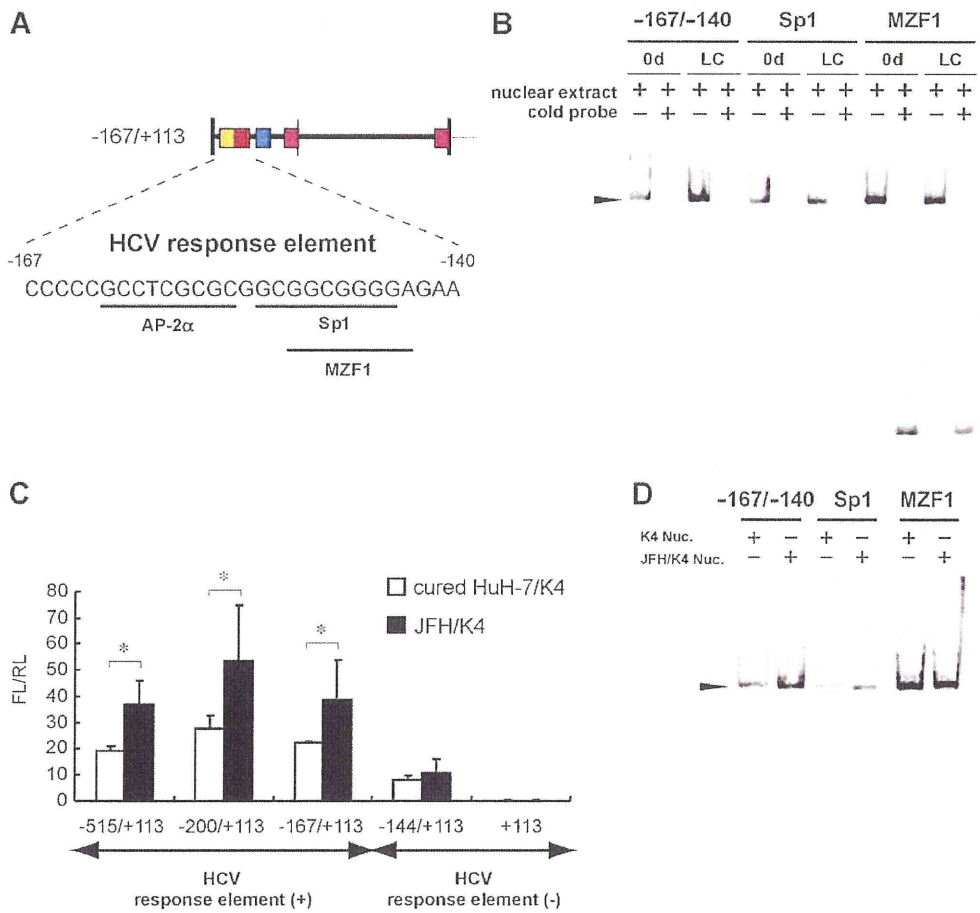

Fig. 4. The HCV response element mediates the overexpression of *DHCR24* induced by HCV. **A**: The HCV response element (−167/−140) in the 5′-flanking region of *DHCR24* includes sequences with similarity to the consensus-binding motifs for AP-2α, Sp1, and MZF-1. **B**: Nuclear extracts were prepared from RzM6-0d and RzM6-LC cells and subjected to an electrophoresis mobility shift assay (EMSA; 10 μg/sample) using the DIG-labeled HCV response element (28-bp), Sp1 (22-bp), or MZF-1 (21-bp) probes. Cold probe indicates unlabeled oligonucleotides. The arrowhead indicates the interaction between the binding factor(s) and each oligonucleotide. **C**: Cured HuH-7/K4 cells and JFH/K4 cells were co-transfected with each *DHCR24* promoter reporter plasmid (0.5 μg/well) and phRL-TK (0.05 μg/well) and analyzed as described in Fig. 2B ($^{*}P < 0.05$). (**D**) Nuclear extracts prepared from cured HuH-7/K4 cells or JFH/K4 cells were subjected to EMSA (25 μg/sample) using the DIG-labeled HCV response element, Sp1, or MZF-1 probes.

noted in the intensity of the shifted band for the MZF-1 sequence between the RzM6-LC and RzM6-0d cells (Fig. 4B). In contrast to Sp1, the ability of endogenous MZF-1 to bind to its target sequence (affinity and/or amount) in the RzM6-LC cells was approximately equivalent to that observed in the RzM6-0d cells. Thus, MZF-1 is not likely to be involved in the increase, mediated by HCV expression, in the shifted band corresponding to the response element.

HCV Infection Upregulates the Transcriptional Activity of the DHCR24 Promoter Through HCV Response Element

An in vitro model of HCV infection that replicates the entire HCV life cycle [Wakita et al., 2005] was used to confirm that transcription of *DHCR24* was mediated through the HCV response element. JFH/K4 cells, which show persistent infection with the HCV JFH-1 strain [Wakita et al., 2005], and control cells (cured HuH-7/K4) were transfected with the *DHCR24* promoter reporter plasmids, and promoter activity was measured. While *DHCR24* promoter reporters that included the HCV response element (−515/+113, −200/+113, and −167/+113) displayed significantly higher activity in JFH/K4 cells that in control cells, no difference was seen between the JFH/K4 cells and control cells transfected with the reporter lacking the HCV response element (−144/+113; Fig. 4C). These results suggest that the transcriptional activity of the *DHCR24* promoter was upregulated by HCV infection in a manner dependent on the response elements. Furthermore, augmentation of complex formation with the response element and the Sp1 probe was confirmed by EMSA using nuclear extracts from JFH/K4 and cured HuH-7/K4 cells (Fig. 4D).

Sp1 Binds to the HCV Response Element

The HCV response element (−167/−140) includes candidate-binding motifs for Sp1, MZF-1, and AP-2α (Fig. 4A). However, expression of AP-2 is repressed in the HepG2 cell line from which RzM6-LC cells are derived [Williams et al., 1988]. Thus, binding of AP-2α to the response element was investigated by a supershift assay using anti-Myc and nuclear extract from HepG2 cells transfected with a Myc-tagged AP-2α expression vector (Fig. 5A). The mobility of the DNA-AP-2α complex was supershifted by the addition of anti-Myc (lane 6) but not control IgG (lane 5), whereas an additional shifted band corresponding to the response element was not observed after addition of anti-Myc (lane 3). Therefore, although exogenous AP-2α protein expressed in HepG2 cells binds to the AP-2α consensus sequence, it does not bind to the HCV response element.

The ability of Sp1 to form a DNA–protein complex with the HCV response element was investigated by performing EMSAs in the presence of mithramycin A (MMA)—a GC-specific DNA-binding antibiotic that binds to the GC-box in the promoter to block binding of Sp1 or other Sp family proteins [Blume et al., 1991]. As shown in Fig. 5B, MMA (2.5, 5.0, and 10 μM) inhibited complex formation in a dose-dependent manner. In contrast, the formation of DNA–protein complexes with the MZF-1 probe was not affected by the addition of MMA, suggesting that the inhibition mediated by MMA was specific for the GC box-Sp1, and that complex formation with the response element requires the Sp1 binding site. A supershift assay using nuclear extract from HepG2 cells transfected with a Myc-tagged Sp1 expression vector and anti-Myc was also performed (Supplementary Fig. 2). The mobility of the HCV response element and the Sp1 consensus sequence was supershifted partially by addition of anti-Myc (lanes 3 and 6). The effect of silencing the expression of Sp1 with small interfering RNA (siRNA) was analyzed by EMSA using nuclear extracts from Sp1-knockdown RzM6-0d and RzM6-LC cells (Fig. 5C). DNA–protein complexes with the response element or the Sp1 probe were not observed (lanes 2, 4, 6, and 8); however, formation of DNA–MZF-1 complexes was not influenced by siRNA treatment (lanes 9–12). Immunoblotting was used to confirm efficient silencing of the Sp1 protein in cells used to generate the nuclear extracts (Fig. 5D). A significant decrease in the expression of DHCR24 was observed in the cytosolic fraction from RzM6 cells transfected with siRNA specific for Sp1 (Fig. 5D). Thus, these results indicate that Sp1, but neither AP-2α nor MZF-1, bound to the HCV response element, and that Sp1 may play an important role in the transcriptional regulation of *DHCR24*.

Transcriptional Regulation of DHCR24 Through the HCV Response Element Is Mediated by Oxidative Stress

DHCR24 functions as a mediator of the cellular response to oxidative stress [Greeve et al., 2000; Benvenuti et al., 2005; Di Stasi et al., 2005; Luciani et al., 2005; Lu et al., 2008] and is a hydrogen peroxide scavenger [Lu et al., 2008]. Expression of the *DHCR24* gene is also induced in response to oxidative stress [Wu et al., 2004; Benvenuti et al., 2006; Kuehnle et al., 2008]. Expression of the HCV gene elevates the level of reactive oxygen species (ROS) via dysregulation of ER-mediated calcium homeostasis, which results in oxidative stress [Tardif et al., 2005]. Therefore, the role of oxidative stress induced by HCV in the regulatory mechanism of the expression of *DHCR24* was examined. HepG2 cells were treated with hydrogen peroxide (H_2O_2) and transfected with reporter plasmids containing the *DHCR24* promoter deletion mutants. Measurement of promoter activity revealed a significant increase in transcription in response to oxidative stress (H_2O_2) for *DHCR24* promoters containing the HCV response element (−4976/+113, −2982/+113, −515/+113, and −167/+113) but not for the promoter lacking the response element (−144/+113; Fig. 6A). Therefore, enhanced transcription in response to oxidative stress by reporter constructs

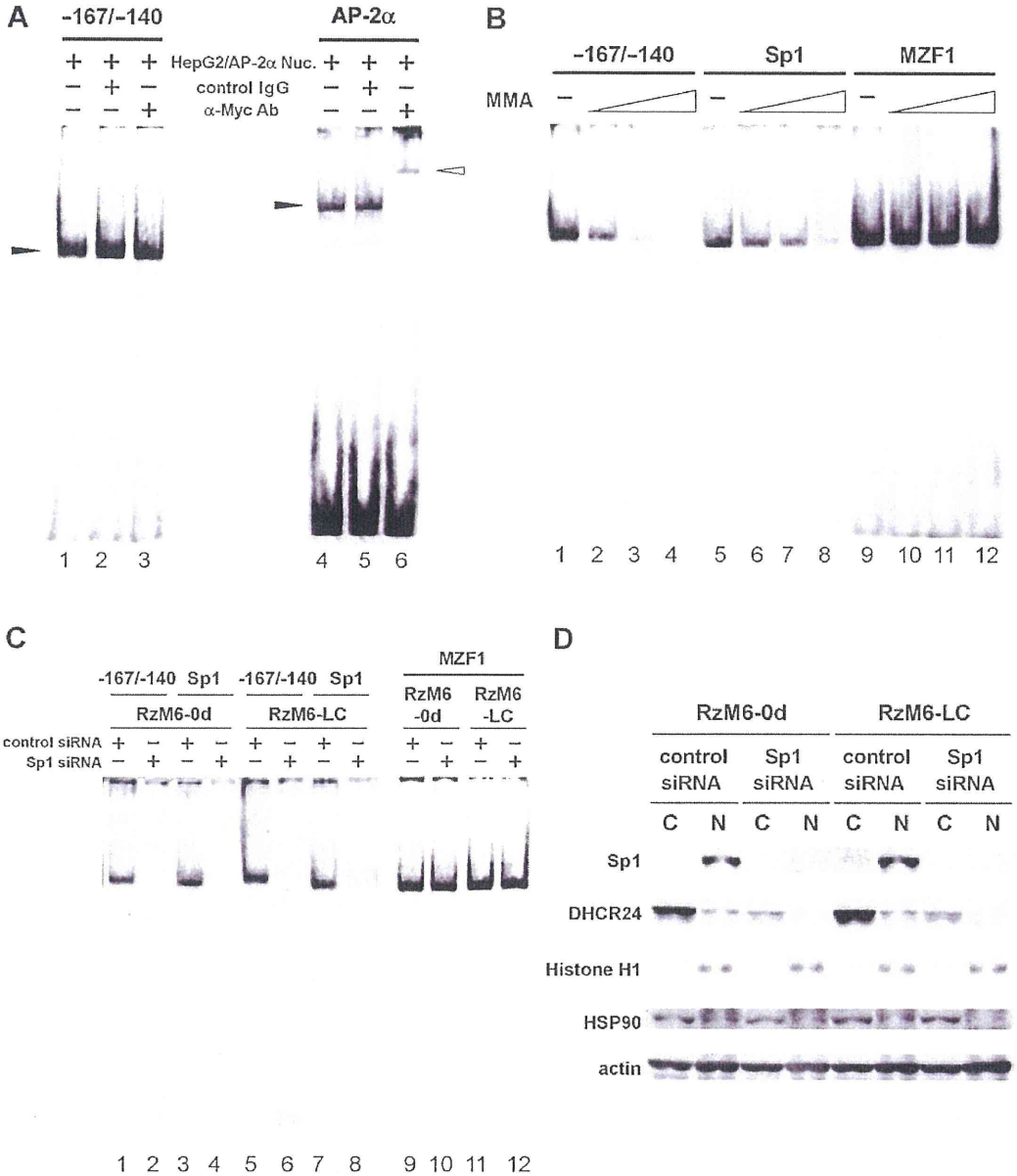

Fig. 5. Sp1 binds to the HCV response element. **A**: Nuclear extract was prepared from HepG2 cells transfected with pcDNA6-AP-2α-myc and subjected to EMSA (10 μg/sample) using DIG-labeled HCV response element or AP-2α probes (26-bp). For a supershift analysis of myc-tagged AP-2α, anti-Myc, or control IgG was added to the binding reaction. The closed arrowhead indicates the interaction between the binding factor(s) and each oligonucleotide, and an additional interaction with antibody is indicated by an open arrowhead. **B**: Nuclear extract from HepG2 cells was pre-incubated at 4 C for 1 h with different concentrations (2.5, 5, and 10 μM) of mithramycin A (MMA) and subjected to EMSA (10 μg/sample) using the DIG-labeled HCV response element, Sp1, or MZF-1 probes. **C**: Nuclear extracts were prepared from RzM6 cells transfected with Sp1 siRNA or control siRNA and subjected to EMSA (10 μg/sample) using the DIG-labeled HCV response element, Sp1, or MZF-1 probes. **D**: Expression of Sp1, DHCR24, and other proteins was detected in both the nuclear fraction (N), used for the EMSA shown in Fig. 4C, and in the cytosolic-membrane fraction (**C**).

containing the *DHCR24* promoter may be mediated through the HCV response element. The formation of complexes containing the response element or Sp1 probe was increased markedly in the nuclear extracts from the H_2O_2-treated HepG2 cells (Fig. 6B) or other hepatic cell lines (Supplementary Fig. 3), suggesting that oxidative stress enhances the binding affinity of Sp1 to the HCV response element.

Overexpression of DHCR24 in M6-LC Cells Is Blocked by an ROS Scavenger

The increase in the expression of DHCR24 induced by oxidative stress can be blocked by treatment with an ROS scavenger, *N*-acetylcysteine (NAC) [Wu et al., 2004], which is a precursor of the potent biological antioxidant glutathione. The H_2O_2-induced overexpression

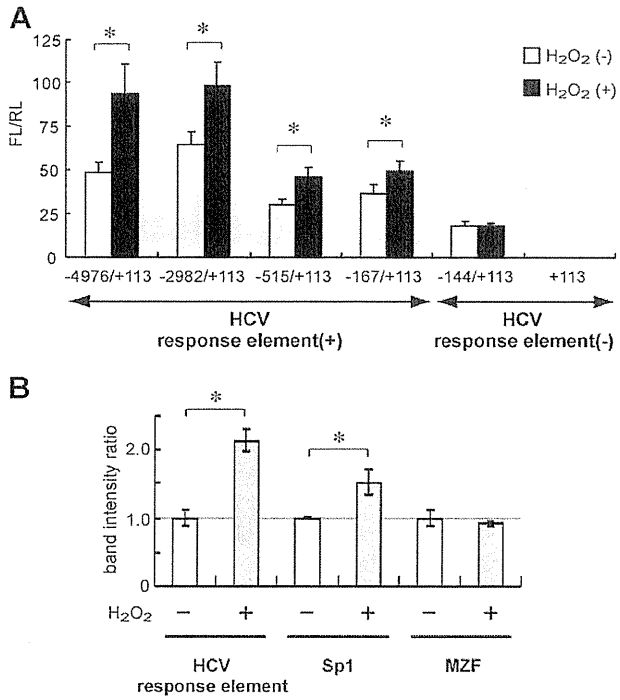

Fig. 6. Oxidative stress increases the transcription of *DHCR24* through the HCV response element and Sp1. **A**: HepG2 cells (1×10^4 cells/well in a 96-well plate) were co-transfected with individual *DHCR24* promoter reporter plasmids (0.5 μg/well) and phRL-TK (0.05 μg/well). Forty-four hours post-transfection, cells were treated with or without 1 mM H_2O_2 for 4 h and analyzed as described in Fig. 2B ($^*P < 0.05$). **B**: Nuclear extracts prepared from H_2O_2-treated (1 mM, 4 h) or untreated HepG2 cells were subjected to EMSA (10 μg/sample) using the DIG-labeled HCV response element, Sp1, or MZF-1 probes. Densitometric analysis of shifted bands was performed using the Image Quant software. Data are shown as the mean + SD from triplicate quantifications of two representative experiments ($^*P < 0.05$).

of DHCR24 was inhibited by pre-treatment with NAC and blocked partially by NAC treatment after the induction of oxidative stress (~50% suppression; Fig. 7A). The enhanced expression of DHCR24 in RzM6-LC cells decreased after 12 or 24 h of treatment with NAC without influencing the level of expression of HCV, suggesting that overexpression of DHCR24 in cells expressing HCV is mediated through oxidative stress.

Overexpression and Enhanced Phosphorylation of Sp1 in the Cells Expressing HCV

Sp1 is a transcription factor that is activated in response to a variety of cellular stressors, including oxidative stress [Schafer et al., 2003; Chu and Ferro, 2006; Dasari et al., 2006; Qin et al., 2009; Lin et al., 2011]. Thus, Sp1 may play an important role in linking oxidative stress and augmentation of *DHCR24* transcription in cells expressing HCV. Sp1 was overexpressed significantly in RzM6-LC cells treated with H_2O_2 compared with the control cells (Fig. 8A). Phosphorylation of Sp1 at Ser101 was also elevated under oxidative stress. Both the basal level and phosphorylation status of nuclear Sp1 were higher in the presence of HCV (RzM6-LC cells) than in the absence of HCV (RzM6-0d cells; Fig. 8B).

Phosphorylation of Sp1 at Ser101 is a target of the DNA damage signaling pathway mediated by ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3-related) kinases [Olofsson et al., 2007; Iwahori et al., 2008]. As shown in Fig. 8C, phosphorylation of Sp1 at Ser101 was no longer detectable following pre-treatment with an ATM kinase inhibitor (KU55933) before exposure to H_2O_2. In contrast, phosphorylation was not affected by other kinase inhibitors (phosphatidylinositol-3 kinase inhibitor, LY294002 or MEK1 inhibitor, PD98059). Similarly, phosphorylation of Sp1 at Thr453, which is important for transcriptional activation of Sp1 [Milanini-Mongiat et al., 2002; D'Addario et al., 2006; Hsu et al., 2006; Lin et al., 2011], was not seen in response to oxidative stress following treatment with KU55933 (Fig. 8C). The induction of expression of DHCR24 after H_2O_2 exposure was suppressed significantly by treatment with KU55933 or NAC, which corresponds with inhibition of Sp1 phosphorylation. In the presence of MMA, the phosphorylation of Sp1 was not inhibited. However, since MMA blocks the binding of Sp1 [Blume et al., 1991], the induction of expression of DHCR24 by H_2O_2 was inhibited. Impairment of DHCR24 induction by H_2O_2 was also observed after treatment with siRNAs targeting ATM (Supplementary Fig. 4).

Studies on the relationship between HCV and ATM have reported that the interaction of NS3/4A with ATM results in delayed de-phosphorylation of both phosphorylated ATM and phosphorylated histone H2AX at Ser139 (γH2AX), which acts as a substrate for ATM in response to DNA damage [Lai et al., 2008]. In the present study, delayed de-phosphorylation of γH2AX was also observed in HCV replicon cells (Supplementary Fig. 5), which corresponded with increased phosphorylation of the H2AX Ser139 residue in cells expressing HCV (Fig. 8). Similarly, phosphorylation of ATM was sustained in HCV replicon cells (Supplementary Fig. 6). Therefore, DNA repair may be impaired in cells expressing or replicating HCV, resulting in sustained DNA damage. As a result, downstream substrates such as Sp1 Ser101 and Thr453 residues or the H2AX Ser139 residue may be phosphorylated to a greater extent in cells expressing HCV compared with control cells in the basal state or cells under oxidative stress (Fig. 8A and B).

Taken together, these results indicate that the oxidative stress induced by HCV may produce quantitative as well as qualitative activation of Sp1, thereby resulting in augmentation of *DHCR24* transcription.

DISCUSSION

HCV establishes chronic infection and induces persistent overexpression of DHCR24 in human hepatocytes [Nishimura et al., 2009]. HCV also confers

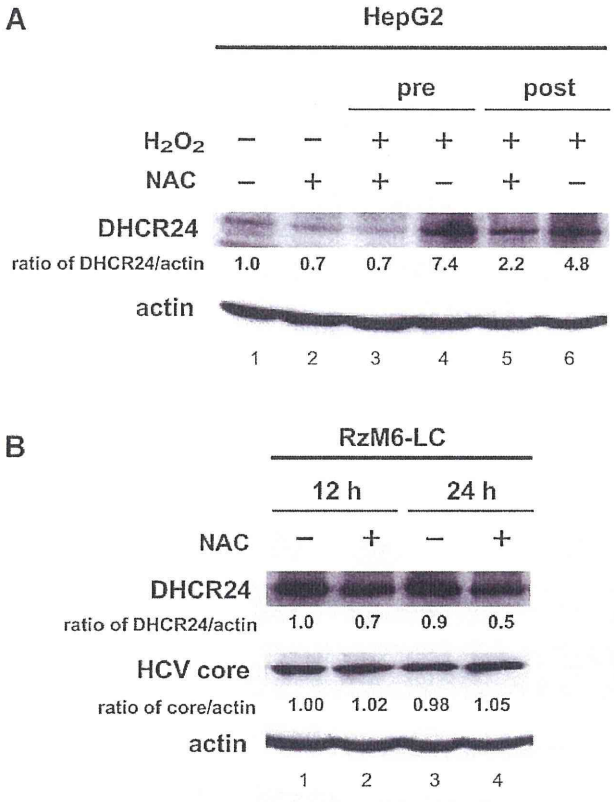

Fig. 7. Overexpression of DHCR24 in the cells expressing HCV is blocked by treatment with an oxidative stress scavenger. **A**: HepG2 cells were treated without (lanes 1, 4, and 6) or with (lanes 2 and 5) NAC (10 mM, 8 h). Cells treated with H_2O_2 (1 mM, 4 h) were also treated with 10 mM NAC for 8 h either before (pre; lanes 3 and 4) or after (post; lanes 5 and 6) H_2O_2 exposure. Whole-cell lysates (40 μg/lane) were analyzed by 10% SDS–PAGE and immunoblotting using a DHCR24/Seladin-1 mAb. Immunoblotting with an actin mAb served as the internal loading control. The ratio of DHCR24/actin was normalized to that of untreated cells (lane 1). **B**: RzM6-LC cells were treated with NAC (10 mM) for 12 h (lane 2) or 24 h (lane 4). Whole-cell lysates were analyzed as described in (A). The ratio of HCV core to actin protein was also calculated. Experiments were performed three times, and representative results are shown.

resistance to the apoptosis induced by oxidative stress and suppresses p53 activity by blocking nuclear p53 acetylation and increasing the interaction between p53 and HDM2 (p53-specific E3 ligase) in the cytoplasm, which may be mediated by inhibition of p53 degradation. Thus, the augmentation of DHCR24 by HCV reflects the tumorigenicity of hepatocytes. The present study identified the genomic region of *DHCR24* that is responsive to HCV, and showed that this response is mediated through the activation of Sp1 induced by oxidative stress. In general, expression of the HCV gene elevates the levels of ROS through dysregulation of ER-mediated calcium homeostasis [Tardif et al., 2005]. In healthy cells, ROS usually exist in equilibrium with antioxidants that scavenge ROS and prevent cellular injury. However, this critical balance may be disrupted in the cells infected with HCV, resulting in the accumulation of ROS and the development of constitutive oxidative stress.

Sp1 is a member of the Sp/KLF family of transcription factors that bind to GC elements of promoters [Black et al., 2001; Kaczynski et al., 2003; Chu and Ferro, 2005; Li and Davie, 2010]. Under a variety of endogenous and exogenous stimuli—including oxidative stress and DNA damage—activation of Sp1 may be mediated via induction of expression of Sp1 and post-translational modifications such as acetylation, sumoylation, O-linked glycosylation, and phosphorylation. Sp1 is phosphorylated by several kinases, including DNA-dependent protein kinase, casein kinase II, and cyclin A/cdk2, which exert both positive and negative effects on transcription [Jackson et al., 1990; Armstrong et al., 1997; Fojas de Borja et al., 2001; Ryu et al., 2003]. Sp1 is the only Sp/KLF family member to contain putative consensus SQ/TQ cluster domains within the transactivation domains, which suggests that Sp1 is a substrate of the PI3K-related kinases, for example, ATM, DNA-dependent protein kinase, and ATR. Indeed, Sp1 is a target of the ATM-dependent DNA damage response pathway [Iwahori et al., 2007, 2008; Olofsson et al., 2007]. ATM plays a central role in orchestrating molecular events involved in double-strand break signaling, which is mediated via the phosphorylation of a variety of substrate proteins—including p53 and BRCA1 transcription factors—involved in the DNA damage response. As a result, these phosphorylation events lead to cell cycle checkpoint activation, DNA repair, altered gene expression patterns, and/or apoptosis [Shiloh, 2006].

Given the role of Sp1 in oxidative stress [Schafer et al., 2003; Chu and Ferro, 2006; Dasari et al., 2006; Rojo et al., 2006; Qin et al., 2009; Lin et al., 2010], Sp1 may be regulated by the oxidative stress induced by HCV and the subsequent phosphorylation, which depends on ATM. However, little is known regarding the regulation of Sp1 in response to DNA damage. Although the precise role of phosphorylation of Sp1 at Ser101 in the DNA damage response is unclear, the similar kinetics of Sp1 and γH2AX phosphorylation [Olofsson et al., 2007] suggest that Sp1 is an early target of the DNA damage response pathway. Thus, Sp1 may be involved in modulating the cellular response to DNA damage to prevent cell death [Ryu et al., 2003]. Phosphorylation of Sp1 at Ser101 and histone H2AX, which occurs in parallel in response to oxidative stress, was enhanced in cells expressing HCV compared with that observed in control cells (Fig. 8A). Interestingly, augmentation of Sp1 phosphorylation in parallel with histone H2AX phosphorylation was also detected for cells expressing HCV in the basal state (Fig. 8A and B), which may be primarily due to the increase in endogenous Sp1 protein (Fig. 8A and B). In support of these results, enhanced phosphorylation of Ser101 on Sp1 occurs upon HSV-1 infection, and is mediated by ATM [Iwahori et al., 2007]. Thus, increased phosphorylation of Sp1 and γH2AX in cells expressing HCV is likely to reflect the higher activity

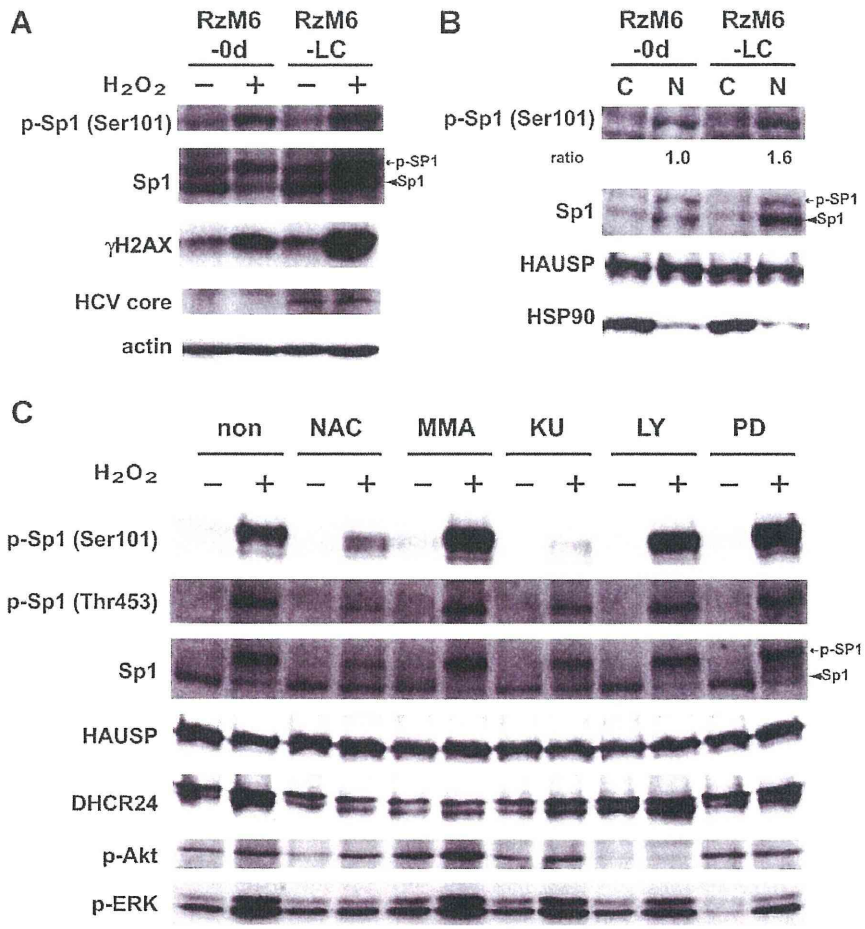

Fig. 8. Overexpression and elevated phosphorylation of Sp1 in the cells expressing HCV. **A**: RzM6-0d and RzM6-LC cells were treated with or without H_2O_2 (1 mM, 4 h). Whole-cell lysates (15 μg/lane) were analyzed by 15% SDS–PAGE and immunoblotting using phospho-H2AX (Ser139) (γH2AX) and HCV core mAbs. An actin mAb served as an internal loading control. Whole-cell lysates (25 μg/lane) were analyzed by 5% SDS–PAGE and immunoblotting using anti-Sp1 (phosphorylated Sp1 and native Sp1, as indicated) and anti-phospho-Sp1 (Ser101) was performed. **B**: RzM6-0d and RzM6-LC cells were fractionated to produce nuclear (N) and cytosolic-membrane fractions (**C**). Fractionated samples (15 μg/lane) were analyzed as described in (**A**). The ratio of phosphorylated Sp1 to Sp1 protein is indicated. Immunoblotting using anti-HAUSP served as a high-molecular-weight loading control. **C**: RzM6-0d cells were pre-treated for 8 h with NAC (10 mM), MMA (10 μM), KU55933 (KU; 10 μM), LY294002 (LY; 50 μM), or PD98059 (PD; 50 μM) and incubated for 4 h in the absence or presence of H_2O_2 (1 mM). Whole-cell lysates (40 μg/lane) were analyzed by 5% SDS–PAGE and immunoblotting using anti-phospho-Sp1 (Ser101), (Thr453), and polyclonal anti-Sp1 (white arrowhead, phosphorylated Sp1; black arrowhead, native Sp1). Detection of HAUSP was performed to confirm the quantity of loaded protein in each lane. Whole-cell lysates (25 μg/lane) were analyzed simultaneously by 10% SDS–PAGE and immunoblotting using anti-DHCR24/seladin-1 mAb, anti-phospho-Akt (Ser473), and anti-phospho-ERK antibodies.

of ATM, which may result from the accumulation and frequency of DNA damage caused by increased generation of endogenous ROS.

Oxidative stress is a common mechanism of liver injury [Loguercio and Federico, 2003] and is mediated by the direct effects of ROS on signal transduction pathways, including extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinases (MAPKs), which act as downstream kinases in the MAPK cascade to phosphorylate Sp1 Thr453/739 residues [Milanini-Mongiat et al., 2002; D'Addario et al., 2006; Hsu et al., 2006; Chuang et al., 2008; Lin et al., 2011]. These signal transduction pathways are also stimulated by oxidative stress in the hepatic cells expressing or replicating HCV, [Qadri et al., 2004; Burdette et al., 2010; Lin et al., 2010]. Therefore, oxidative stress in response to HCV may induce downstream signaling pathways, such as ERK1/2, JNK, and p38 MAPK as well as ATM/ATR, to activate Sp1 via post-translational modifications.

Sp1 is a host factor activated by several viral proteins, including HIV-1 Vpr, and HTLV-1 Tax [Peng et al., 2003; Amini et al., 2004; Chang et al., 2005; Zhang et al., 2009]. The HCV core and NS5A proteins also activate Sp1 [Lee et al., 2001; Xiang et al., 2010]. The HCV core upregulates the DNA-binding activity and phosphorylation of Sp1 [Lee et al., 2001], and NS5A may also exert a similar effect on Sp1 activity. However, a physical interaction between these

proteins and Sp1 has not yet been demonstrated. Both HCV core and NS5A proteins have a high potential for oxidative stress induction [García-Mediavilla et al., 2005; Dionisio et al., 2009], which may mediate activation of Sp1. On the other hand, individual viral proteins were insufficient to increase the expression of DHCR24 (Fig. 1A). Therefore, in addition to induction of oxidative stress by each viral protein, the persistence of the signaling pathways induced by oxidative stress, for example, ATM (Supplementary Fig. 6), may also be required for the Sp1-mediated increase in the expression of DHCR24.

The results of the present study revealed that knockdown of expression of Sp1 almost completely blocked the enhanced expression of DHCR24. Sp1 is expressed ubiquitously in various mammalian cells and is involved in regulating the transcriptional activity of genes implicated in many cellular processes [Black et al., 2001; Kaczynski et al., 2003; Chu and Ferro, 2005; Li and Davie, 2010]. Thus, Sp1 may represent an essential master regulator among the myriad of transcription factors involved in the direct regulation of *DHCR24* transcription.

In conclusion, HCV was shown to enhance the expression of DHCR24 via the activation of Sp1, which may shed light on the mechanism of tumorigenesis associated with HCV.

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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-β 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.

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-κB, 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-β-deficient (TRIF at aa 372; Ferreon 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-β production. Sendai virus infection accelerated the

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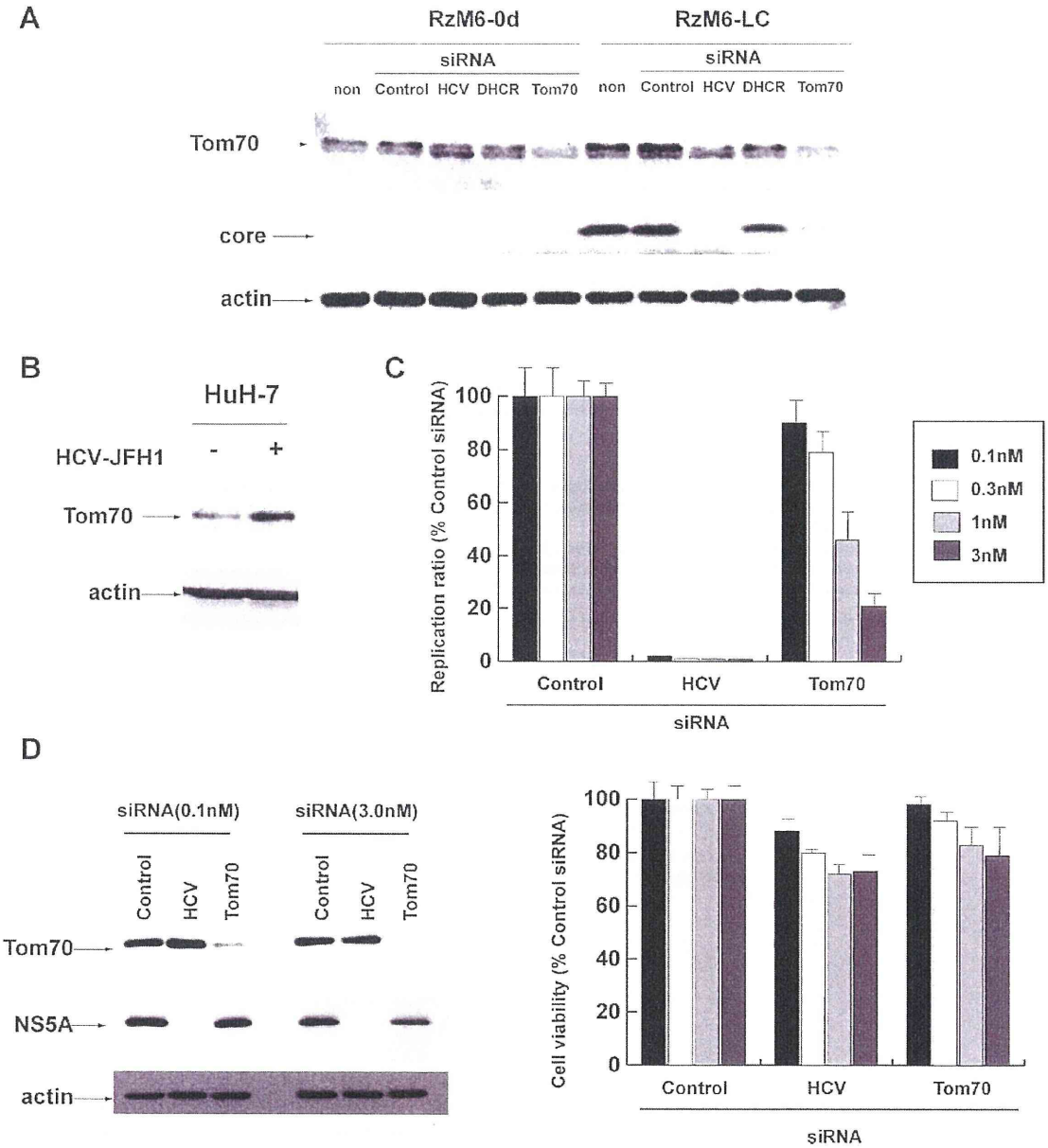

Fig. 1. HCV induces overexpression of Tom70 but impairs Tom70-induced IFN synthesis. (A) RzM6 cells (HCV-) and RzM6-LC cells (HCV+) were transfected with siRNAs of control (non-target siRNA#3: Thermo Fisher Scientific), HCV (R5: 5′-GUCUCGUAGACCGUGCAUCAuu-3′), DHCR24 (Nishimura et al., 2009), and Tom70 (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 RzM6-0d cells (Tsukiyama-Kohara et al., 2004). The full-length HCV-RNA expression was induced by 4-hydroxytamoxifen (100 nM) and passaged for more than 44 days in RzM6-LC cells, and HCV expression was not induced in RzM6-0d 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

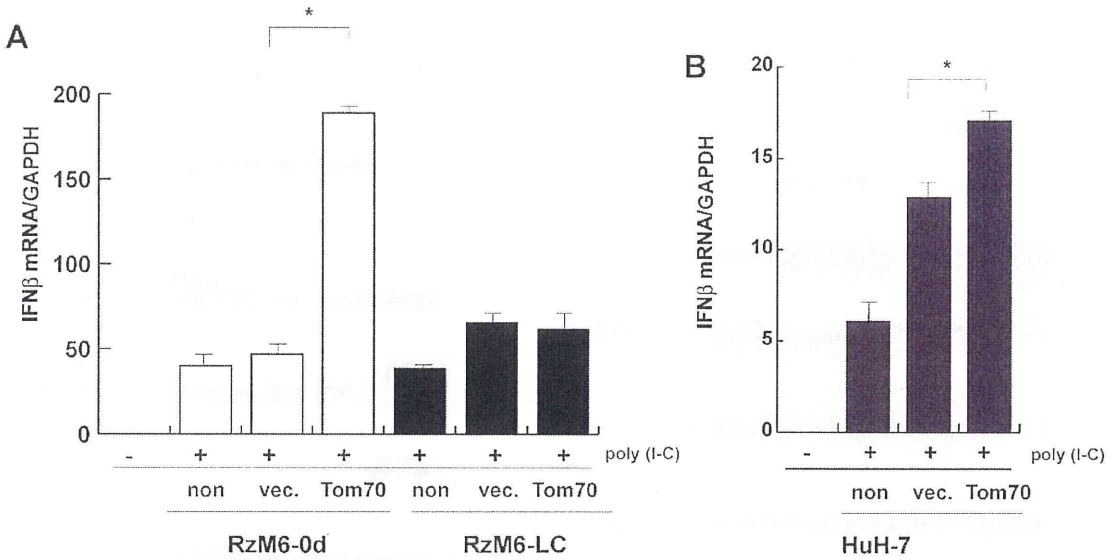

Fig. 2. Tom70-induced IFN synthesis was impaired by HCV. (A) RzM6-0d cells and LC cells were transfected with mock-vector, control pcDNA vector (vec.), or pcDNA-Tom70 expression vector, and the amount of IFN-β mRNA was measured by RTD-PCR and normalized to the amount of GAPDH mRNA using Gene expression assay kit (GE-Healthcare). Poly(I–C) (GE Healthcare) (5 μg) was transfected with RNAi Max reagent (Invitrogen) and IFN-βmRNA 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-β 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-β 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-β mRNA synthesis was quantitated by real-time detection (RTD) PCR. Overexpression of Tom70 by transfection of pcDNA6-Tom70 (Takano et al., 2011a) induced IFN-β mRNA synthesis in the absence of HCV after poly(I–C) treatment (RzM6-0d cells). However, the Tom70-mediated induction of IFN-β mRNA transcription was impaired in the presence of HCV (RzM6-LC cells) (Fig. 2A). Overexpression of Tom70 induced IFN-β mRNA synthesis in HuH-7 cells (Fig. 2B). Induction of IFN-β 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-β 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-κB 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 ΔMAVS) 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-β 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-β (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

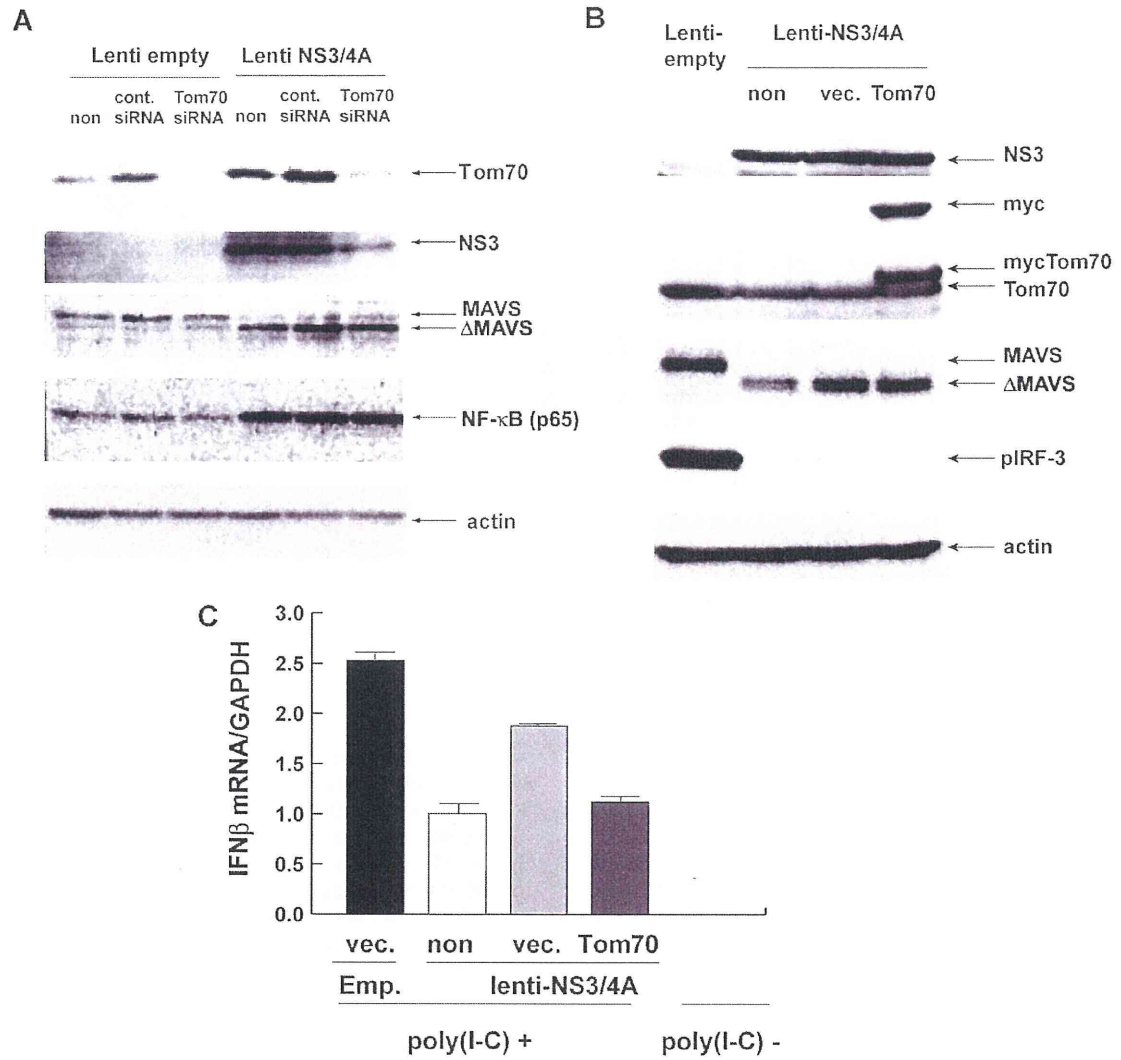

Fig. 3. Silencing of Tom70 decreased the level of NS3 and cleavage of MAVS by NS3/4A impaired IRF-3 phosphorylation even in the presence of Tom70. (A) Empty or NS3/4A-lenti virus vector expressing HepG2 cells were transfected with control siRNA and Tom70 siRNA or mock-transfected (non) as a control. 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.

Acknowledgements

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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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MAJOR ARTICLE

Monoclonal Antibody 2-152a Suppresses Hepatitis C Virus Infection Through Betaine/GABA Transporter-1

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Background. We recently established a monoclonal antibody (2-152a MAb) that binds to 3β-hydroxysterol-Δ24-reductase (DHCR24) by immunizing mice with cells (RzM6-LC) persistently expressing hepatitis C virus (HCV). Here, we aimed to analyze the activity of 2-152a MAb against HCV replication and explore the molecular mechanism underlying the antiviral activity.

Methods. We characterized the effects of 2-152a MAb on HCV replication and performed a microarray analysis of antibody-treated HCV replicon cells. The molecules showing a significant change after the antibody treatment were screened to examine their relationship with HCV replication.

Results. The antibody had antiviral activity both in vitro and in vivo (chimeric mice). In the microarray analysis, 2-152a MAb significantly suppressed the expression of betaine/GABA transporter-1 (BGT-1) in 2 HCV replicon cell lines but not in HCV-cured cells. Silencing of BGT-1 expression by small interfering RNA (siRNA) revealed significant suppression of HCV replication and infection without cytotoxicity. Further, BGT-1 expression was significantly increased in the presence of HCV ($P < .05$).

Conclusions. Our results suggest that 2-152a MAb suppresses HCV replication and infection through BGT-1. These findings highlight important roles of BGT-1 in HCV replication and reveal a possible target for anti-HCV therapy.

Hepatitis C virus (HCV) causes chronic hepatitis and hepatocellular carcinoma (HCC) [1–3]. Chronic HCV infection is a major global public health concern because it affects at least 170 million people worldwide [2]. The most effective treatment against HCV currently comprises a combination therapy of PEGylated α-interferon (IFN-α) and ribavirin [4, 5]. However, considering that sustained virological responses develop in only approximately half of the patients infected with HCV genotype 1, the clinical efficacy of this therapy is limited [6, 7]. Efforts to develop therapies against HCV are further hindered by the high level of viral variation and capacity of the virus to cause chronic infection. Therefore, there is an urgent need to develop effective treatments against chronic HCV infection.

In a previous study, we established a cell line expressing HCV (RzM6-LC) to investigate the effects of persistent HCV expression on cell growth [8]. We also established a monoclonal antibody (2-152a MAb) against the RzM6-LC cell line to produce clones that recognize both cell surface and intracellular molecules. Using this method, we identified 3b-hydroxysterol-D24-reductase (DHCR24) as the recognition molecule of this antibody.

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DHCR24 (also termed seladin-1) is an enzyme that catalyzes the conversion of desmosterol to cholesterol in the postsqualene cholesterol biosynthetic pathway [9, 10]. DHCR24 also acts as a hydrogen peroxide scavenger [11]. Therefore, DHCR24 may play a crucial role in maintaining cell physiology through cholesterol synthesis and oxidative stress. We previously demonstrated that HCV infection upregulates DHCR24 expression, and overexpression of DHCR24 inhibits apoptosis and inactivates the tumor suppressor gene p53 [12]. Moreover, silencing of DHCR24 suppressed HCV replication [13]. However, the precise mechanisms through which DHCR24 affects the HCV life cycle are unclear. In this study, we aimed to analyze the activity of 2-152a MAb against HCV replication and explore the molecular mechanism underlying the antiviral activity.

Materials And Methods

Cell Lines and Reagents

Human hepatoma cell line HuH-7 cell-based HCV replicon-harboring cell lines [14] R6FLR-N (genotype 1b) [15], FLR3-1 (genotype 1b) [16], and JFH-1 (genotype 2a) [17] were maintained in Dulbecco's modified Eagle's medium (DMEM) GlutaMAX (Invitrogen) containing 10% fetal calf serum (FCS; Sigma-Aldrich) in the presence of G418 (500 mg/mL for R6FLR-N and FLR3-1, 300 mg/mL for JFH-1; Invitrogen). Cured/HuH-7 histone H3 lysine 4 (K4) cells cured off HCV by interferon treatment [18] were maintained in DMEM GlutaMAX containing 10% FCS without G418. The JFH/K4 cell line persistently infected with the HCV JFH-1 strain and HuH-7 cell lines were maintained in DMEM containing 10% FCS [19]. The human hepatoblastoma HepG2 cell line was also maintained in DMEM containing 10% FCS.

Generation of 2-152a MAb

BALB/c strain of mice was immunized with 7–8 intraperitoneal injections of RzM6-LC cells (5×10^6) in RIBI adjuvant (trehalose dimycolate + monophosphoryl lipid A emulsion; RIBI ImmunoChem Research). After completion of the immunization regimen, their spleens were excised and splenocytes were fused with mouse myeloma plasminogen activator inhibitor (PAI) cells by using PEG1500 (Roche). Hybridoma cells were then selected with hypoxanthine, aminopterin, and thymidine (Invitrogen), and culture supernatants were collected for screening by whole-cell enzyme-linked immunosorbent assay (ELISA).

HCV Infection in Humanized Chimeric Mouse Liver and HCV mRNA Quantification by Real-time Detection Polymerase Chain Reaction

We purchased (from PhoenixBio Co.) chimeric mice that were established by transplanting human primary hepatocytes into severely combined immunodeficient (SCID) mice carrying a urokinase plasminogen activator (uPA) transgene controlled by an albumin promoter [20]. These mice were then infected with plasma isolated before 2003 from an HCV-positive patient (HCR6) [8, 21], in accordance with the Declaration of Helsinki. The protocols for the animal experiments were preapproved by the local ethics committee, and the animals were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. HCV genotype 1b RNA levels were established at $0.96–1.84 \times 10^7$ copies/mL in mouse serum samples before the antibody treatment. The antibody (2-152a MAb) and normal immunoglobin G (IgG, 400 mg/20 g body weight) were intraperitoneally injected into the mice ($n = 4$) at 2-day intervals over a period of 14 days. IFN-α (30 mg/kg) was administered subcutaneously at 2-day intervals over a period of 2 weeks. Human serum albumin in the blood of chimeric mice was measured by using an Alb-II kit according to the manufacturer's instructions (Eiken Chemical). HCV RNA levels in serum and JFH/K4 cells were measured by real-time detection polymerase chain reaction (real-time detection [RTD]-PCR) as described previously [22]. HCV RNA in the cell cultures and supernatants was extracted by using Isogene and Isogene LS (Nippon Gene), respectively.

Replication Assay Using HCV Replicon Cells

We used 3 HCV subgenomic replicon cell lines: R6FLR-N, FLR3-1, and JFH-1. They were seeded at a density of 5×10^3 cells/well in 96-well tissue culture plates in DMEM GlutaMAX (Invitrogen) containing 5% fetal bovine serum (Thermo Scientific). Following incubation for 24 hours at 37°C (in 5% CO_2), the medium was removed and serial dilutions of antibody were added. Luciferase activity was determined by using a Bright-Glo luciferase assay kit (Promega) after 72 hours according to the manufacturer's instructions. The results were calculated as the average percentage relative to the reactivity in untreated cells, which was set at 100%. The viability of the replicon cells was measured by using a WST-8 cell counting kit (Dojindo) according to the manufacturer's instructions.

Immunostaining and Antibodies

Cells were cultured on glass coverslips (1.0 cm diameter) and fixed with 1% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 10 minutes in 24-well plates. To permeabilize the cell membranes, the cells were treated with 1% Triton X-100 in PBS at room temperature for 10 minutes. After washing with 0.05% Tween-20 in PBS, the cells were incubated with 2-152a MAb, antiprotein disulfate isomerase (PDI) rabbit polyclonal antibody (Stressgen Bioreagents) or normal mouse IgG for 1 hour and washed with 0.05% Tween-20 in PBS. Alexa Fluor 488-labeled goat antimouse IgG was used as the secondary antibody.

Anti-NS5A antibody was provided by Dr Yoshiharu Matsuura (Osaka University). Anti-myc mouse monoclonal antibody