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

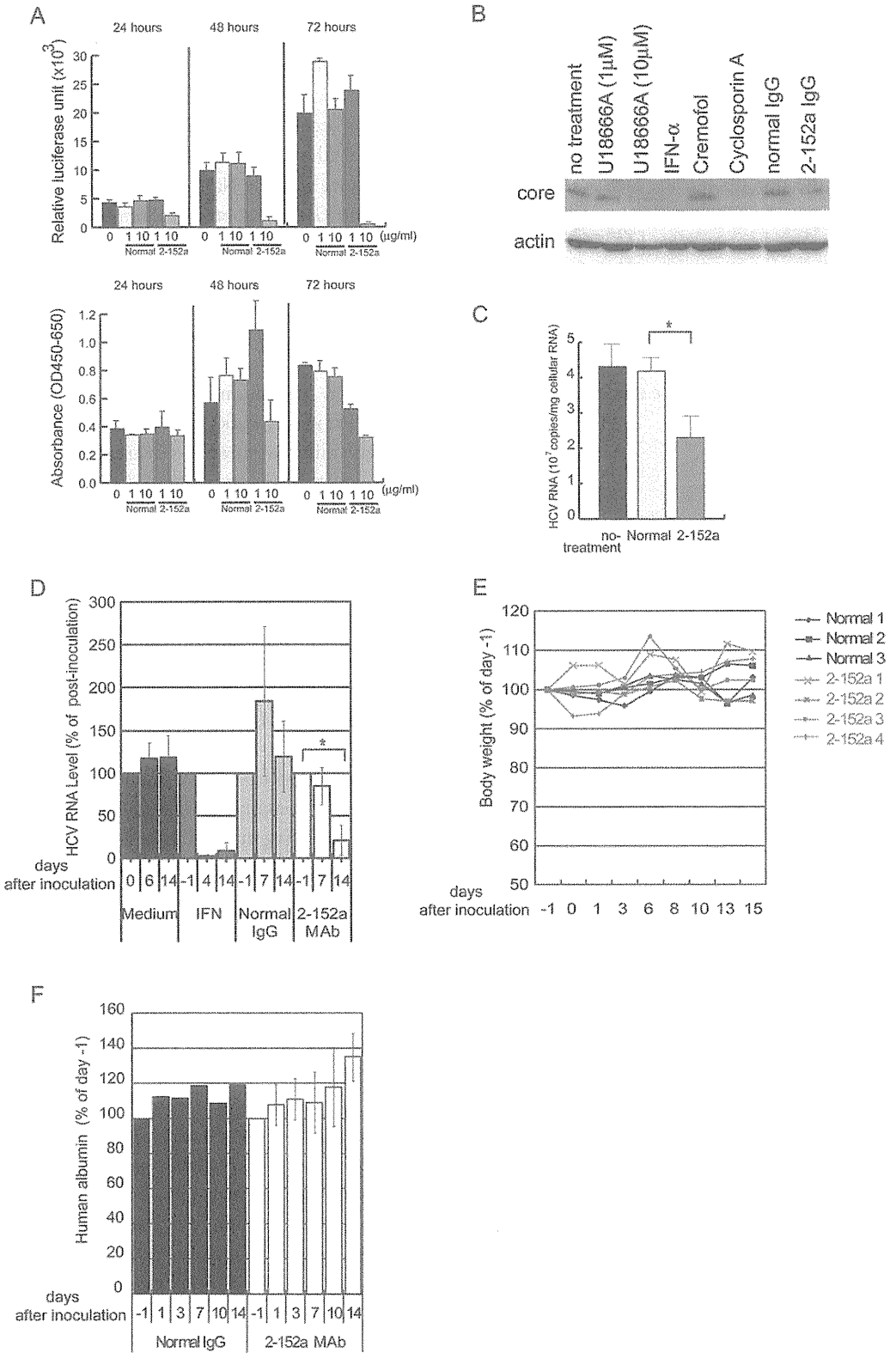

Figure 1. Anti-DHCR24 monoclonal antibody (2-152a MAb) suppresses HCV replication in vitro and in vivo. *A*, The effects of 2-152a MAb on HCV replication were measured by the luminescence activity and cell viability in FLR3-1 cells. The replicon cell line was incubated with IgG from normal mice or 2-152a MAb at 1 or 10 μg/mL for 24, 48, and 72 hours. The mean values from triplicate wells are indicated, and the vertical bars represent the standard deviation. The medium control (2% FCS-DMEM) without IgG is indicated as 0. *B*, The JFH/K4 cells were treated with cholesterol synthesis inhibitor U18666A (1 mM, 10 mM), IFN-α (250 IU/mL), Cyclosporin A (25 μM) and its solvent Cremophor, normal mouse IgG (10 μg/mL), and 2-152a IgG (10 μg/mL). HCV core and actin proteins were detected. *C*, HCV RNA copies were measured in JFH/K4 cells after treatment with normal or 2-152a IgG

(9E10; Cell Signaling Technology) and antiactin mouse monoclonal antibodies (Sigma-Aldrich) were utilized for detecting myc-fusion protein and normalization of the results, respectively.

cDNA Synthesis and Quantitative Reverse Transcriptase PCR

cDNA was synthesized from 0.5 or 1 mg of total RNA with a Superscript II kit (Invitrogen). TaqMan gene expression assays were custom designed and manufactured by Applied Biosystems. The expression was quantified with the ABI 7500 real-time PCR system (Applied Biosystems).

Microarray Analysis

For microarray analysis, total RNAs were extracted using RNAeasy kit (Qiagen), and RNA integrity was assessed using a Bioanalyzer (Agilent Technologies). cRNA targets were synthesized and hybridized with Whole Human Genome Oligo Microarray (G4112F; Agilent) according to the manufacturer's instructions.

RNA Interference, Expression Vector Construction, Transfection, and Rescue Experiments

Small interference RNA (siRNA) targeting betaine/GABA transporter-1 (BGT-1; nucleotides 120–144) was designed by using a program (https://rnaidesigner.invitrogen.com/) based on registered sequences in GenBank (5′-CAACAAGATGGAGT TTGTGCTGTCA-3′). Alternative siRNA (BGT-1-siRNA-362; nucleotides 362–386) was similarly designed. The HCV-siRNA (R7) sequence was 5′-GUCUCGUAGACCGUGCACCA dTdT-3′.

The coding region of the BGT-1 gene was obtained from RNA of R6FLR-N cells by reverse transcription–polymerase chain reaction (RT-PCR). The PCR products were inserted in *Eco*RV–*Xho*I sites of pcDNA6-myc His, version A (Invitrogen) after digestion of *Eco*RV–*Xho*I. To generate mutant plasmids that contained nucleotide substitutions in the siRNA-targeted site, we introduced point mutations into pcDNA-BGT-1 by using site-directed mutagenesis with a QuickChange multisite-directed mutagenesis kit (Stratagene), according to the manufacturer's instructions, and the following oligonucleotide primer: BGT-1-mut, 5′-CCAATGGACCAA-CAAGATGGAATTCGTTCTATCGGTGGCCGGGGAGCTC ATTGGG-3′ (the mutations introduced by mutagenesis are underlined).

Transfection of siRNAs was carried out by reverse transfection using Lipofectamine RNAiMAX according to the manufacturer's protocol (Invitrogen). Transfection of the expression vector was undertaken by using Lipofectamine LTX with Plus reagent (Invitrogen).

The rescue experiment was performed after reverse transfection of BGT-1 siRNA (1.5 nM) into R6FLR-N cells by using RNAiMAX reagent. After 48 hours, wild-type (wt) and mutant (mut) BGT-1 expression vectors (10 ng) were transfected by using Lipofectamine LTX, and the luciferase activity and cell viability were assessed by WST-8 assay (Dojindo) after 24 hours.

Analysis of HCV Infection and BGT-1 Expression

For infection assays, Cured/HuH-7 K4 cells were incubated with JFH/K4 cell-derived HCV (2.0×10^6 copies/mL). At 72 hours after incubation, HCV infection and BGT-1 expression were analyzed by real-time detection (RTD)-PCR and TaqMan expression assay, respectively, as described earlier.

Statistical Analysis

The Student t test was used to test the statistical significance of the results. P values $< .05$ were considered statistically significant.

Results

Inhibitory Effect of 2-152a MAb on HCV Replication In Vitro

We examined the effects of 2-152a MAb on HCV replication and the viability in HCV replicon cell lines. The treatment with 2-152a MAb significantly decreased HCV replication after 48 hours and cell viability after 72 hours (Figure 1*A*). To determine the recognition site of 2-152a MAb, we performed epitope mapping by using serial overlapping deletion mutants of the DHCR24 fusion protein (Supplementary Figure 1*A*). The recognition site was identified within amino acid residues 259–314 (Supplementary Figure 1*B*) and the predicted "Diminuto-like protein" homologous region [23] indicated in Supplementary Figure 1A.

Suppression of HCV Infection by 2-152a MAb

To determine the effects of 2-152a MAb on HCV infection, we inoculated the antibody into a persistently HCV-infected cell line (JFH/K4; Figure 1*B* and *C*) or uPA-SCID chimeric mice previously transplanted with human hepatocytes [20] and

Figure 1 continued. (10 μg/mL). The error bars indicate the standard deviation, and the asterisk indicates $P < .005$. *D*, Relative amounts of HCV RNA (% copies/mg total RNA on days −1 or 0) in the livers of chimeric mice inoculated with the control medium, PEGylated IFN-α, normal IgG, or 2-152a IgG were estimated by RTD-PCR. For normalization, the HCV RNA level 1 day before the inoculation (day −1) or on the day of inoculation (day 0) was defined as 100%. The graph shows the relative amounts of HCV RNA at −1 day (or day 0), 7 days (or 4 days), and 14 days. The error bars indicate the standard deviation, and the asterisk indicates $P < .005$. *E*, Ratio of body weight of mice inoculated with either normal IgG or 2-152a MAb IgG to that on day −1. *F*, Ratio of albumin concentration in serum samples of mice inoculated with 2-152a MAb IgG or normal IgG to that on day −1. The vertical bars indicate the standard deviation.

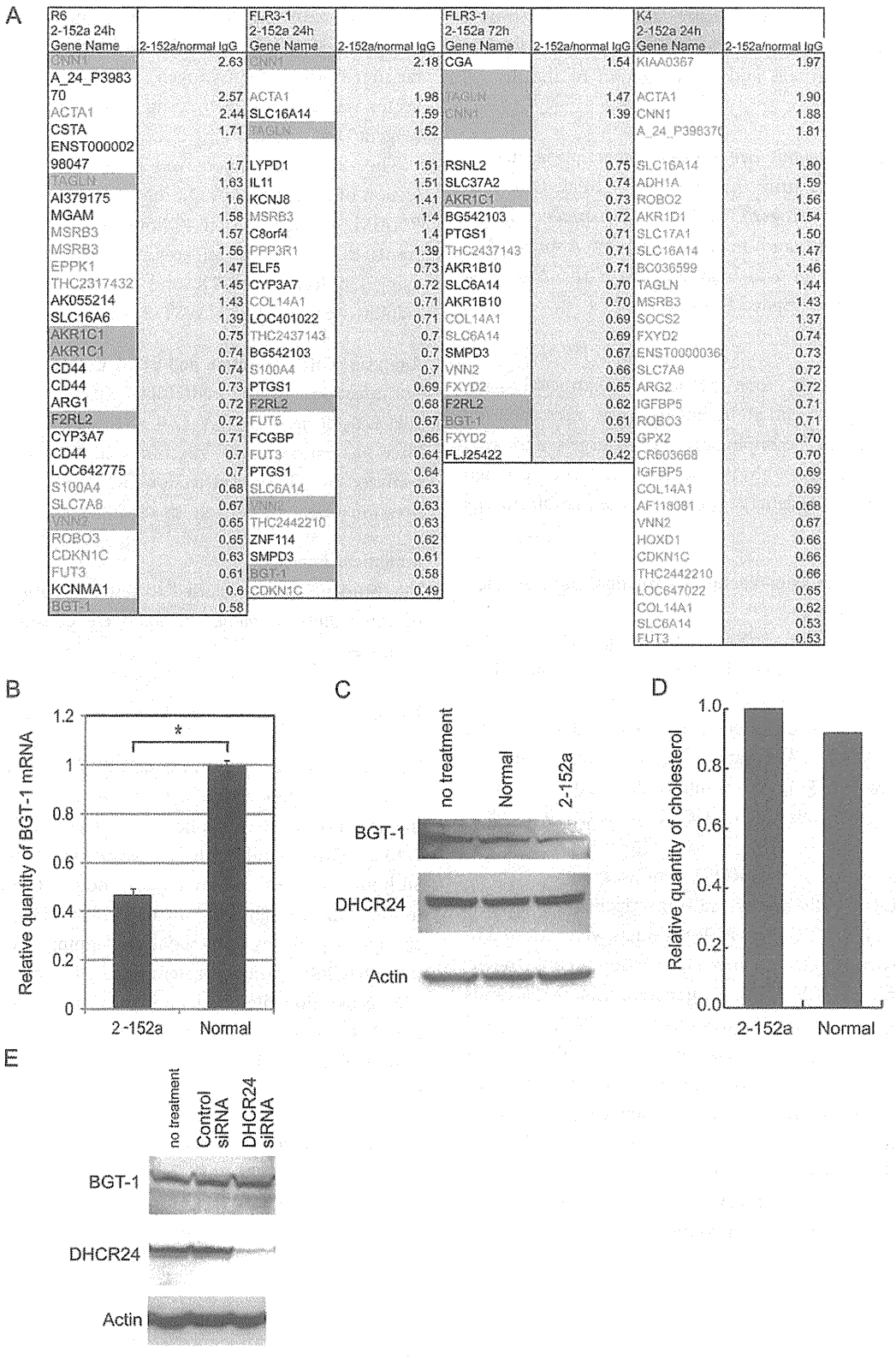

Figure 2. *A*, Genes that showed significant changes in expression after the 2-152a MAb treatment. HCV replicon cells (FLR3-1 and R6FLR-N) and K4 cells were treated with 2-152a MAb. The symbols shaded in gray indicate the genes that showed significantly changed expression commonly in R6FLR-N and FLR3-1 cells, and those shaded in orange indicate the genes that showed significantly changed expression in K4 cells. The amount of labeled probe for microarray analysis was 7-fold higher than that in the first experiment (Supplementary Table 1). Each value indicates the number of ratios of signal 2-152a MAb/normal IgG treatment. *B*, TaqMan expression assay of BGT-1 in samples of R6FLR-N cells treated with 2-152a MAb or normal IgG. BGT-1 mRNA (0.5 μg) samples treated with 2-152a MAb or normal IgG were transcribed by reverse transcriptase, and synthesized cDNAs were used for TaqMan

infected with HCV (Figure 1*D* and *F*). We detected viral protein (core) (Figure 1*B*) or viral RNA in cells (Figure 1*C*) and mouse blood by using RTD-PCR (Figure 1*D*). There was a significant reduction in the viral titers with 2-152a MAb treatment compared with that in normal IgG treatment (control) ($P < .005$, Figure 1*C* and *D*). No significant effects on body weight were observed by the inoculation of 2-152a MAb (Figure 1*E*). Further, no significant differences were found among the levels of human albumin in the sera of the normal IgG- and 2-152a MAb-inoculated mice (Figure 1*F*).

Expression of DHCR24 in Carcinoma Cells and on the Surface of HuH-7-Derived Cells

We observed abundant intracellular expression of DHCR24 in hepatoma cell lines in the previous study [12]; therefore, we characterized its expression on the surface of various carcinoma cell lines by flow cytometric analysis to clarify the mechanism of 2-152a MAb antiviral effects. In this analysis, DHCR24 expression was localized to the surface of the HuH-7 and HuH-7-based cell lines, HCV replicon cell lines (R6FLR-N, FLR3-1, and JFH-1), HCV persistently infected cell line (JFH/K4), and K4 cells; on the other hand, DHCR24 was not significantly expressed on the surface of the HepG2, Hep3B, RzM6-0d, RzM6-LC, WRL68, and PLC/PRF/5 cell lines (Supplementary Figure 1*C*). To confirm the expression of DHCR24 on the cell surface, we performed immunofluorescence staining (Supplementary Figure 1*D*). DHCR24 expression was detected in the HuH-7 cells without permeabilization.

Suppression of BGT-1 mRNA Expression in HCV Replicon Cell Lines After Treatment With 2-152a MAb

To determine the molecular mechanism underlying the effects of 2-152a MAb, we performed microarray analysis twice with different amounts of probes and evaluated the changes in gene expression associated with the 2-152a MAb treatment, which were specific to the HCV replicon cells rather than to the HCV-cured K4 cells. Using this methodology, we identified approximately 3–14 genes as upregulated and about 17–20 genes as downregulated following the treatment with 2-152a MAb, compared with the expressions in normal IgG-treated R6FLR-N, FLR3-1, and K4 cells (Figure 2*A*). Among these genes, the expression level of SLC6A12 (BGT-1; GenBank accession number NM_003044) showed significant downregulation in both the R6FLR-N and the FLR3-1 cell lines but not in the K4 cells (Figure 2*A*; Table 1). To validate this result, we tested BGT-1 mRNA expression in R6FLR-N cells treated with 2-152a MAb and normal IgG by using TaqMan expression assay. This assay demonstrated that the relative expression of BGT-1 was significantly suppressed by the treatment with 2-152a MAb ($P < .001$, Figure 2*B*). Significant downregulation of BGT-1 was also observed by treatment with 2-152a MAb in HCV-JFH-1-infected cells (Figure 2*C*).

Table 1. Screened Genes in HCV Replicon Cell Lines After Treatment of IgG

	Gene name	R6FLR-N 24 hours	FLR3-1 24 hours	FLR3-1 72 hours	HuH-7/K4 24 hours
Screened specifically in replicon cells[a]					
1st screening	AKR1C1	0.67	0.62	0.65	NS
	BGT-1	0.53	0.63	0.53	NS
2nd screening (7-fold)[a]	AKR1C1	0.74 or 0.75	NS	0.73	NS
	F2RL2	0.72	0.68	0.62	NS
	BGT-1	0.58	0.58	0.61	NS
Screened in replicon and cured K4 cells[b]					
1st screening	CNN1	2.75	0.6	1.62	1.9
2nd screening (7-fold)[c]	CNN1	2.63	2.18	1.39	1.88
	TAGLN	1.63	1.52	1.47	1.44
	VNN2	0.65	0.63	0.66	0.67

Abbreviations: HCV, hepatitis C virus; IgG, immunoglobin G; NS, not screened.

[a] Screened genes were significantly changed in HCV replicon cells but not in HuH-7/K4 cells; each value indicates ratio of signal 2-152a MAb IgG/normal IgG treatment.

[b] Screened genes were significantly changed in all cell lines, including replicon cells and HuH-7/K4 cells.

[c] Comparing to 1st screening, 7-fold amount of labeled probe was used for microarray.

We further addressed the mechanism of action of 2-152a MAb. Treatment with 2-152a MAb did not decrease the level of cholesterol (Figure 2*D*), and silencing of DHCR24 did not influence BGT-1 significantly (Figure 2*E*).

Inhibition of HCV Replication and Infection by siRNA Directed Against BGT-1

Because BGT-1 expression was suppressed by the treatment with 2-152a MAb, which had antiviral activity, we attempted BGT-1 silencing in HCV replicon cell lines by using designed siRNAs to examine the potential role of BGT-1 in HCV replication. BGT-1 silencing was confirmed by RT-PCR (Figure 3*A*). The effect of the siRNAs on HCV replication was examined by Western blotting with anti-NS5A antibody (Figure 3*B*) and measured by the luminescence level (Figure 3*C*, left panel) and cell viability (Figure 3*C*, right panel) in FLR3-1 cells. We also examined the effect of these siRNAs in R6FLR-N and JFH-1 cells (Supplementary Figure 2*A*) and observed similar inhibitory effects as

Figure 2 continued. gene expression assay. Each value was compensated with values of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA as the internal control and normal IgG. The asterisk indicates $P < .001$, and the vertical bars indicate the standard deviation. *C*, Level of BGT-1 and DHCR24 proteins detected in JFH/K4 cells after treatment with 2-152a or normal IgG (10 μg/mL). *D*, The relative cholesterol amount was measured in R6FLR-N cells treated with 2-152a or normal IgG (10 μg/mL). *E*, BGT-1 and DHCR24 proteins were detected in normal IgG- or 2-152a IgG-treated R6FLR-N cells.

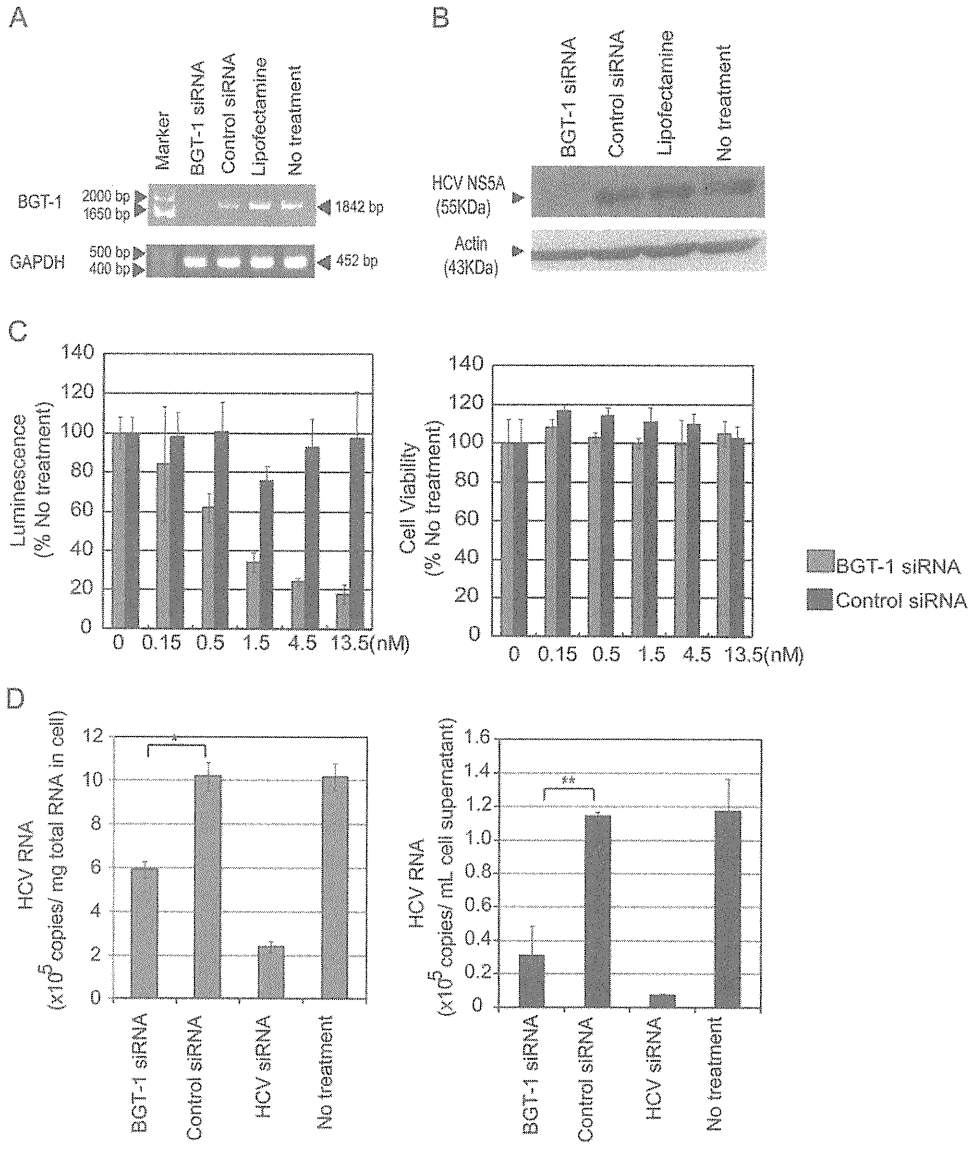

Figure 3. BGT-1 silencing by siRNA inhibits HCV replication in subgenomic HCV replicon cell lines and the persistently infected cell line. *A*, The siRNA targeting BGT-1 suppressed the expression of the corresponding mRNA. The mRNA of each sample was extracted 72 hours after siRNA (10 nM) transfection. Total RNA was transcribed and amplified by RT-PCR using primers specific to the open reading frame (ORF) of the BGT-1 (1842 bp) gene. The experiments were performed in triplicate, and the representative data are presented. *B*, The effects of BGT-1 siRNA (10 nM) on HCV were confirmed by Western blot analysis using an antibody against the HCV NS5A protein (55 kDa). The blots were striped and reprobed with an antibody directed against actin to examine protein loading in each lane. *C*, Levels of HCV replication (left panel) and cell viability (right panel) are presented according to serial concentrations of siRNA targeting BGT-1 and control siRNA in FLR3-1 cells (72 hours after transfection). The inhibition of replication or cell viability following siRNA targeting BGT-1 is defined relative to those of the cells that received no treatment (100%). The error bars represent the standard error of triplicate experiments. *D*, Quantification of HCV RNA by RTD-PCR in HCV persistently infected cells (JFH/K4) after treatment with BGT-1 siRNA. The cells were treated with siRNAs (10 nM) against BGT-1, control, and HCV (HCV R7) and harvested at 72 hours after transfection. TaqMan quantitative RT-PCR was performed for quantitation of HCV RNA in extracted RNA from cells (left panel) and their supernatants (right panel). The single asterisk (*) and double asterisk (**) indicate $P < .005$ and $P < .05$ against the control, respectively. The mean values from triplicate wells are indicated, and the vertical bars indicate the standard deviation.

those in FLR3-1 cells. The median inhibitory concentration (IC_{50}) values of BGT-1 siRNAs in various HCV replicon cell lines were as follows: FLR3-1 cells, 0.93 nM; R6FLR-N cells, 1.37 nM; JFH-1 cells, 5.95 nM. The cell viability was not significantly influenced by the siRNA treatment (Figure 3*C*, right panel; Supplementary Figure 2*A*, right panel).

Further, we monitored the levels of HCV RNA in JFH/K4 cells and their supernatants after BGT-1 silencing. Using RTD-PCR, we detected significant suppression in the HCV RNA levels by BGT-1 silencing in these cells ($P < .005$; Figure 3*D*, left panel) and their supernatants ($P < .05$; Figure 3*D*, right panel). These results were consistent with the strong inhibitory effects of

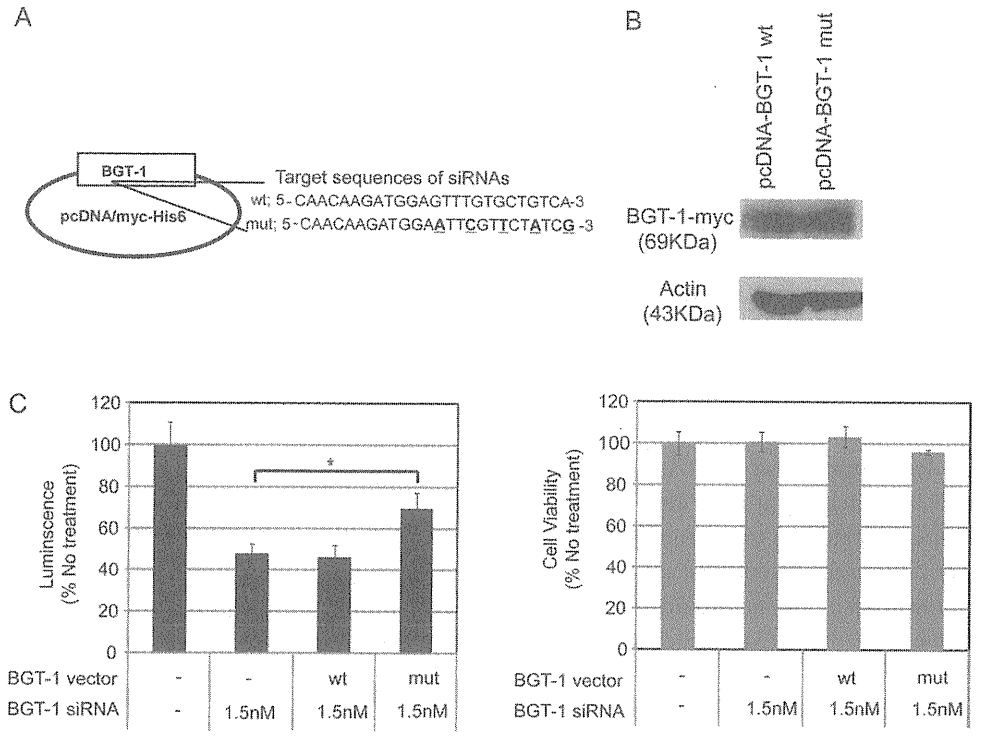

Figure 4. Validation of the inhibitory effects of BGT-1 siRNA on HCV replication in subgenomic HCV replicon cell lines. *A*, Schematic representation of the pcDNA-BGT-1 wild-type (wt) and mutant (mut) plasmids. The siRNA-targeted sites are indicated, and the underlined bold letters indicate the sequences induced by mutagenesis PCR. *B*, The wild type and mutant of the BGT-1-myc fusion protein were detected by using an anti-myc monoclonal antibody (9E10) in transfected R6FLR-N cells (upper panel). The blots were striped and reprobed with an antibody against actin to determine protein loading for each lane (lower panel). *C*, R6FLR-N cells were transfected with BGT-1 siRNA, and wild-type or mutant expression vectors were transfected after siRNA transfection. After 24 hours of vector transfection, the level of HCV replication (left panel) was measured by luminescence, and cell viability (right panel) was measured by WST-8 assay. The asterisk indicates $P < .05$ compared with transfection of siRNA alone. The mean values from triplicate wells are indicated, and the vertical bars represent the standard deviation.

BGT-1 siRNA on HCV replication, as shown in Figure 3*C*. We designed alternative siRNA targeting BGT-1 (BGT-1-siRNA-362) and observed its significant inhibitory effect on HCV replication without significant cytotoxicity (Supplementary Figure 2*B*).

Validation of the Anti-HCV Effects of siRNA Against BGT-1 by Rescue With Expression Vectors

To assess the specificity of BGT-1 silencing, we attempted to rescue HCV replication against the ectopic effects by this silencing. To examine the effect of the rescue, we constructed expression vectors of wild-type and mutant BGT-1 (Figure 4*A*) and confirmed the expression of each BGT-1-myc-fused protein (Figure 4*B*). The mutant BGT-1 vector contained 5 base mismatches within the site targeted by the BGT-1 siRNA without a change in the amino acid sequence of the protein (underlined in Figure 4*A*). We also transfected the pcDNA-BGT-1 plasmid after the siRNA treatment and observed significant recovery of HCV replication with mutant pcDNA-BGT-1 ($P < .05$; Figure 4*C*, left panel) without significant cytotoxicity (Figure 4*C*, right panel). BGT-1 expression was increased significantly in K4 cells in the presence of HCV ($P < .05$, Supplementary Figure 2*C*) at 72 hours after infection compared with the absence of HCV, and in RzM6-LC cells, which persistently express HCV [8], compared with RzM6-0d cells, which lack HCV expression ($P < .05$, Supplementary Figure 2*D*).

DISCUSSION

In this study, we determined that 2-152a MAb, which binds to but does not affect the activity of DHCR24, suppresses HCV replication and that BGT-1 is highly downregulated in HCV replicon cell lines treated with this antibody. Further, the efficient rescue of viral replication with a mutant expression vector indicates the specific inhibitory effect of BGT-1 silencing on HCV replication. Therefore, we hypothesize that BGT-1 plays an important role in HCV replication through a pathway that is likely independent of DHCR24, which in its own right can regulate the HCV life cycle [13].

BGT-1 is involved in sodium- and chloride-coupled betaine uptake, which helps in maintaining normal cellular conditions. Previous reports have described that the transcription of BGT-1 mRNA is regulated by a tonicity sensitive element (TonE) in response to hypertonic stress, a result that was first identified in the Madin-Darby canine kidney (MDCK) cell line [24]. BGT-1

is also thought to be responsible for the hyperosmotic stress response and in maintaining cell hydration. Denkert et al [25] reported that BGT-1 gene expression is induced by hyperosmolarity and inhibited by p38 mitogen-activated protein kinase ($p38^{MAPK}$) inhibitor SB20358. Further, several reports have evidenced that cell hydration affects viral replication and that viral replication increases during cell shrinkage due to hyperosmolarity, a result that was accompanied by increased BGT-1 mRNA expression [26]. Considering the reduction in HCV replication by the BGT-1 siRNA treatment, this treatment may prevent HCV replication by affecting hypoosmotic conditions in HCV-infected cells. Further studies are required to examine in detail the function of BGT-1 in HCV replication.

In summary, we demonstrated that the 2-152a monoclonal antibody inhibits HCV replication in HCV replicon cells and HCV infection in human hepatocytes transplanted into chimeric mice. The inhibitory effect of the monoclonal antibody on viral replication may be mediated by the suppression of BGT-1 expression. We propose BGT-1 as a key target for anti-HCV therapies.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (Supplementary Data).

Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Di Bisceglie AM, Carithers RL Jr, Gores GJ. Hepatocellular carcinoma. Hepatology **1998**; 28:1161–5.
2. Hayashi J, Aoki H, Arakawa Y, Hino O. Hepatitis C virus and hepatocarcinogenesis. Intervirology **1999**; 42:205–10.
3. Michielsen PP, Francque SM, van Dongen JL. Viral hepatitis and hepatocellular carcinoma. World J Surg Oncol **2005**; 3:27.
4. Mazzella G, Accogli E, Sottili S, et al. Alpha interferon treatment may prevent hepatocellular carcinoma in HCV-related liver cirrhosis. J Hepatol **1996**; 24:141–7.
5. Bruchfeld A, Stahle L, Andersson J, Schvarcz R. Ribavirin treatment in dialysis patients with chronic hepatitis C virus infection—a pilot study. J Viral Hepat **2001**; 8:287–92.
6. Kohara M, Tanaka T, Tsukiyama-Kohara K, et al. Hepatitis C virus genotypes 1 and 2 respond to interferon-alpha with different virologic kinetics. J Infect Dis **1995**; 172:934–8.
7. Nakamura H, Ogawa H, Kuroda T, et al. Interferon treatment for patients with chronic hepatitis C infected with high viral load of genotype 2 virus. Hepatogastroenterology **2002**; 49:1373–6.
8. Tsukiyama-Kohara K, Toné S, Maruyama I, et al. Activation of the CKI-CDK-Rb-E2F pathway in full genome hepatitis C virus-expressing cells. J Biol Chem **2004**; 279:14531–41.
9. Crameri A, Biondi E, Kuehnle K, et al. The role of seladin-1/DHCR24 in cholesterol biosynthesis, APP processing and Aβ generation in vivo. EMBO J **2006**; 25:432–43.
10. Waterham HR, Koster J, Romeijn GJ, et al. Mutations in the 3β-hydroxysterol Δ^{24}-reductase gene cause desmosterolosis, an autosomal recessive disorder of cholesterol biosynthesis. Am J Hum Genet **2001**; 69:685–94.
11. Lu X, Kambe F, Cao X, et al. 3β-Hydroxysteroid-Δ24 reductase is a hydrogen peroxide scavenger, protecting cells from oxidative stress-induced apoptosis. Endocrinology **2008**; 149:3267–73.
12. Nishimura T, Kohara M, Izumi K, et al. Hepatitis C virus impairs p53 via persistent overexpression of 3β-hydroxysterol Δ24-reductase. J Biol Chem **2009**; 284:36442–52.
13. Takano T, Tsukiyama-Kohara K, Hayashi M, et al. Augmentation of DHCR24 expression by hepatitis C virus infection facilitates viral replication in hepatocytes. J Hepatol **2011**; 55:512–21.
14. Lohmann V, Körner F, Koch J, Herian U, Theilmann L, Bartenschlager R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science **1999**; 285:110–3.
15. Watanabe T, Sudoh M, Miyagishi M, et al. Intracellular-diced dsRNA has enhanced efficacy for silencing HCV RNA and overcomes variation in the viral genotype. Gene Ther **2006**; 13:883–92.
16. Sakamoto H, Okamoto K, Aoki M, et al. Host sphingolipid biosynthesis as a target for hepatitis C virus therapy. Nat Chem Biol **2005**; 1:333–7.
17. Kato T, Date T, Miyamoto M, et al. Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. Gastroenterology **2003**; 125: 1808–17.
18. Blight KJ, McKeating JA, Rice CM. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. J Virol **2002**; 76:13001–14.
19. Wakita T, Pietschmann T, Kato T, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med **2005**; 11:791–6.
20. Mercer DF, Schiller DE, Elliott JF, et al. Hepatitis C virus replication in mice with chimeric human livers. Nat Med **2001**; 7:927–33.
21. Inoue K, Umehara T, Ruegg UT, et al. Evaluation of a cyclophilin inhibitor in hepatitis C virus-infected chimeric mice in vivo. Hepatology **2007**; 45:921–8.
22. Takeuchi T, Katsume A, Tanaka T, et al. Real-time detection system for quantification of hepatitis C virus genome. Gastroenterology **1999**; 116:636–42.
23. Sarkar D, Imai T, Kambe F, et al. The human homolog of Diminuto/Dwarf1 gene (hDiminuto): a novel ACTH-responsive gene overexpressed in benign cortisol-producing adrenocortical adenomas. J Clin Endocrinol Metab **2001**; 86:5130–7.
24. Takenaka M, Preston AS, Kwon HM, Handler JS. The tonicity-sensitive element that mediates increased transcription of the betaine transporter gene in response to hypertonic stress. J Biol Chem **1994**; 269: 29379–81.
25. Denkert C, Warskulat U, Hensel F, Häussinger D. Osmolyte strategy in human monocytes and macrophages: involvement of p38MAPK in hyperosmotic induction of betaine and myoinositol transporters. Arch Biochem Biophys **1998**; 354:172–80.
26. Häussinger D. The role of cellular hydration in the regulation of cell function. Biochem J **1996**; 313:697–710.

Research Article

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Augmentation of DHCR24 expression by hepatitis C virus infection facilitates viral replication in hepatocytes

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Background & Aims: We characterized the role of 24-dehydrocholesterol reductase (DHCR24) in hepatitis C virus infection (HCV). DHCR24 is a cholesterol biosynthetic enzyme and cholesterol is a major component of lipid rafts, which is reported to play an important role in HCV replication. Therefore, we examined the potential of DHCR24 as a target for novel HCV therapeutic agents.

Methods: We examined DHCR24 expression in human hepatocytes in both the livers of HCV-infected patients and those of chimeric mice with human hepatocytes. We targeted *DHCR24* with siRNA and U18666A which is an inhibitor of both DHCR24 and cholesterol synthesis. We measured the level of HCV replication in these HCV replicon cell lines and HCV infected cells. U18666A was administrated into chimeric mice with humanized liver, and anti-viral effects were assessed.

Results: Expression of DHCR24 was induced by HCV infection in human hepatocytes *in vitro*, and in human hepatocytes of chimeric mouse liver. Silencing of *DHCR24* by siRNA decreased HCV replication in replicon cell lines and HCV JFH-1 strain-infected cells. Treatment with U18666A suppressed HCV replication in the replicon cell lines. Moreover, to evaluate the anti-viral effect of U18666A *in vivo*, we administrated U18666A with or without pegylated interferon to chimeric mice and observed an inhibitory effect of U18666A on HCV infection and a synergistic effect with interferon.

Conclusions: DHCR24 is an essential host factor which augmented its expression by HCV infection, and plays a significant role in HCV replication. DHCR24 may serve as a novel anti-HCV drug target.

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Introduction

Extensive epidemiological studies have identified multiple risk factors for hepatocellular carcinoma (HCC), including chronic infection with hepatitis C virus (HCV), and hepatitis B virus (HBV), and cirrhosis due to non-viral etiologies, such as alcohol abuse and aflatoxin B1 exposure [1,2]. Of these factors, HCV appears to be the dominant causative factor for HCC in many developed countries. The World Health Organization estimates that 170 million people worldwide are infected with HCV and are, therefore, at risk of developing liver cirrhosis and HCC [3]. The combination of pegylated interferon-α (PEG-IFN-α) and ribavirin is currently the standard treatment regimen for patients with chronic HCV infection. However, viral clearance is achieved in only 40% to 60% of patients and depends on the HCV genotype with which the patient is infected [4].

We previously established the RzM6 cell line, a HepG2 cell line in which the full-length HCV genome (HCR6-Rz) can be conditionally expressed under control of the Cre/*lox*P system and is precisely self-trimmed at the 5′ and 3′-termini by ribozyme sequences [5]. Anchorage-independent growth of these cells accelerates after 44 days of continuous passaging, during which the Cdk-Rb-E2F pathway is activated [5]. In a previous study, we developed monoclonal antibodies (MoAbs) against cell surface antigens on HCV-expressing cells that had been passaged for over 44 days [6]. One of the targets of these MoAbs was 24-dehydrocholesterol reductase (DHCR24 is also called 3-β-hydroxysterol-Δ-24-reductase, seladin-1, desmosterol delta-24-reductase), a molecule that is frequently overexpressed in the hepatocytes of HCV-infected patients.

Keywords: Hepatitis C virus; Replication; DHCR24; U18666A.
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Abbreviations: DHCR24, 24-dehydrocholesterol reductase; HCV, hepatitis C virus; MoAb, monoclonal antibody; HCC, hepatocellular carcinoma; HBV, hepatitis B virus.

DHCR24 confers resistance to apoptosis in neuronal cells [7]. It also regulates the cellular response to oxidative stress by binding to the amino terminus of p53, thereby displacing mouse double minute 2 homolog isoform MDM2 (*Homo sapiens*) (MDM2) from p53 and inducing the accumulation of p53 in human embryonic fibroblasts [8].

DHCR24 is a cholesterol biosynthetic enzyme that is also called desmosterol reductase [9,10]. Cholesterol is a major component of lipid rafts, which are reported to play an important role in HCV replication [11]. Therefore, we characterized the role of DHCR24 in HCV replication and evaluated its potential as a target for novel HCV therapeutic agents. We also examined the synergistic antiviral effect of U18666A which is an inhibitor of both DHCR24 [12] and cholesterol synthesis [13] with IFN-α in the treatment of HCV.

Materials and methods

Cells and plasmids

Cell culture methods of the HuH-7 [14], HepG2 [15], hybridoma and myeloma PAI cells, RzM6 cells [5], and the HCV subgenomic replicon cells lines FLR3-1 (genotype 1b, strain Con-1; [16]), R6FLR-N (genotype 1b, strain N; [17]), and Rep JFH Luc3-13) genotype 2a, strain JFH-1 [18]) were utilized to evaluate HCV replication [19] are described in Supplementary data.

The *DHCR24* cDNA was synthesized and amplified by PCR using Phusion™ DNA polymerase (Finnzymes) and cloned into the pcDNA3.1 vector (Invitrogen) or lentivirus vector, as described previously [6].

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry analysis

The detailed procedures are described in Supplementary data [20].

Immunohistochemistry and Western blot analysis

The detailed procedures are described in Supplementary data.

The antibodies used in this experiment were: anti-Core, anti-NS3, anti-NS4B, anti-NS5B [5], and anti-NS5A (kindly provided by Dr. Matsuura, Osaka University), and anti-actin (Sigma).

Inhibition of DHCR24 by siRNA

We synthesized two siRNAs that were directed against human *DHCR24* mRNA: siDHCR24-417 and siDHCR24-1024. The target sequence of siDHCR24-417 was 5′-GUACAAGAAGACACACAAATT-3′, while that of siDHCR24-1024 was 5′-GAGAACUAUCUGAAGACAATT-3′. Additionally, we used siRNAs targeted against the HCV genome (siE-R7 and siE-R5) [17,21]. The siCONTROL Non-Targeting siRNA #3 (Dharmacon RNA Technologies) was used as the negative control siRNA. The chemically synthesized siRNAs were transfected into cells using Lipofectamine RNAiMAX (Invitrogen) and Opti-MEM (Invitrogen) by reverse-transfection. Cells were characterized 72 h after transfection.

Inhibition of viral replication by U18666A

U18666A (Calbiochem) was utilized to treat HCV replicon cells at a concentration of 62.5–1000 nM and chimeric mice at a concentration of 10 mg/kg (i.p.).

To determine whether cholesterol can reverse the U18666A treatment by the addition of cholesterol, we performed the experiments using HCV replicon cells (4×10^3 cells/well in a 96-well white plate, SUMILON). Culture medium was replaced after the cells had spread (at 24 h), and LDL (Calbiochem) was added to reach a final cholesterol concentration of 50 μg/ml. After a 24 h-incubation, U18666A (62.5, 125, 250, 500, and 1000 nM) was added to each well, and the cells were incubated for an additional 48 h. HCV replication activity was measured by luciferase assay, and cell viability was measured with the WST-8 cell counting kit according to the manufacturer's instructions (Dojindo Laboratories). Cholesterol measurements are described in Supplementary data.

Inhibition assay of HCV replication in replicon cells and persistent infected cells

For evaluation of the anti-HCV replication effect of the inhibitor U18666A in replicon cells and HCV persistently infected cells are described in Supplementary data.

Real-time detection (RTD)-PCR

Total RNA was purified from JFH-K4 cells that had been treated with siRNA or U18666A by the acid guanidium–phenol–chloroform method. HCV RNA was quantified by RTD-PCR as previously described [22].

HCV infection of chimeric mice with humanized liver and mRNA quantification by RTD-PCR

We used chimeric mice that were created by transplanting human primary hepatocytes into severe combined immunodeficient mice carrying a urokinase plasminogen activator transgene [23,24] that was controlled by the albumin promoter. These hepatocytes had been infected with plasma from a HCV-positive patient HCR6 (genotype 1b) [19]. The HCV 1b RNA level reached 2.9–18.0×10^6 copies/ml in mouse sera after 1–2 months of infection. HCV RNA in the mouse serum or total RNA from liver tissue from humanized chimeric mice with/without HCV infection was extracted using the acid guanidium–phenol–chloroform method. HCV RNA and *DHCR24* mRNA levels were quantified by RTD-PCR [22]. The primers and probes for HCV were prepared as previously described [22], and the primers and probes for *DHCR24* were prepared using TaqMan® Gene Expression assays (Applied Biosystems) according to the manufacturer's instructions. PEG-IFNα-2a (Chugai) was administered subcutaneously at a concentration of 30 μg/kg, at day 1, 4, 8, and 11 (the amount of PEG-IFN-α administered to the chimeric mice was 20-fold relative to that used in humans), and U18666A was administered intraperitoneally at a concentration of 10 mg/kg, every day for 2 weeks (Fig. 6A). The protocols for the animal experiments were approved by the local ethics committee.

Human serum albumin in the blood of humanized chimeric mice was measured using a commercially available kit, according to the manufacturer's instructions (Alb-II kit; Eiken Chemical).

Results

Identification of DHCR24

We inoculated mice (BALB/c) with RzM6 cells that expressed HCV protein and had been cultured for over 44 days (denoted as RzM6-LC cells); mice were inoculated at least seven times over a 2-week period. We then fused the splenocytes from mice that had been immunized with RzM6-LC cells to myeloma cells to establish hybridomas. Characterization of the culture supernatant from more than 1000 hybridoma cells by ELISA (data not shown) revealed that one MoAb clone (2-152a) recognized a molecule of approximately 60 kDa in various cells (Supplementary Fig. 1A and B. This molecule was more highly expressed in RzM6-LC cells (Supplementary Fig. 1A), HeLa cells, and HCC cell lines (HepG2, HuH-7, Hep3B, and PLC/PRF/5) than in HEK293 cells and several normal liver cell lines (NKNT, TTNT, and WRL68) (Supplementary Fig. 1B). To further characterize this molecule, we performed matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and obtained seven peptide sequences (Supplementary Fig. 1C, underlined). These peptide sequences suggested that the molecule that was recognized by the 2-152a antibody was DHCR24. We constructed a lentivirus expression vector containing myc-tagged DHCR24 (DHCR24-myc) and transduced it into HepG2 cells. By western blot analysis with 2-152a and anti-Myc antibody, we then confirmed that DHCR24 was expressed in the transduced cells (Supplementary Fig. 1D). We found that the 2-152a antibody specifically recognized DHCR24.

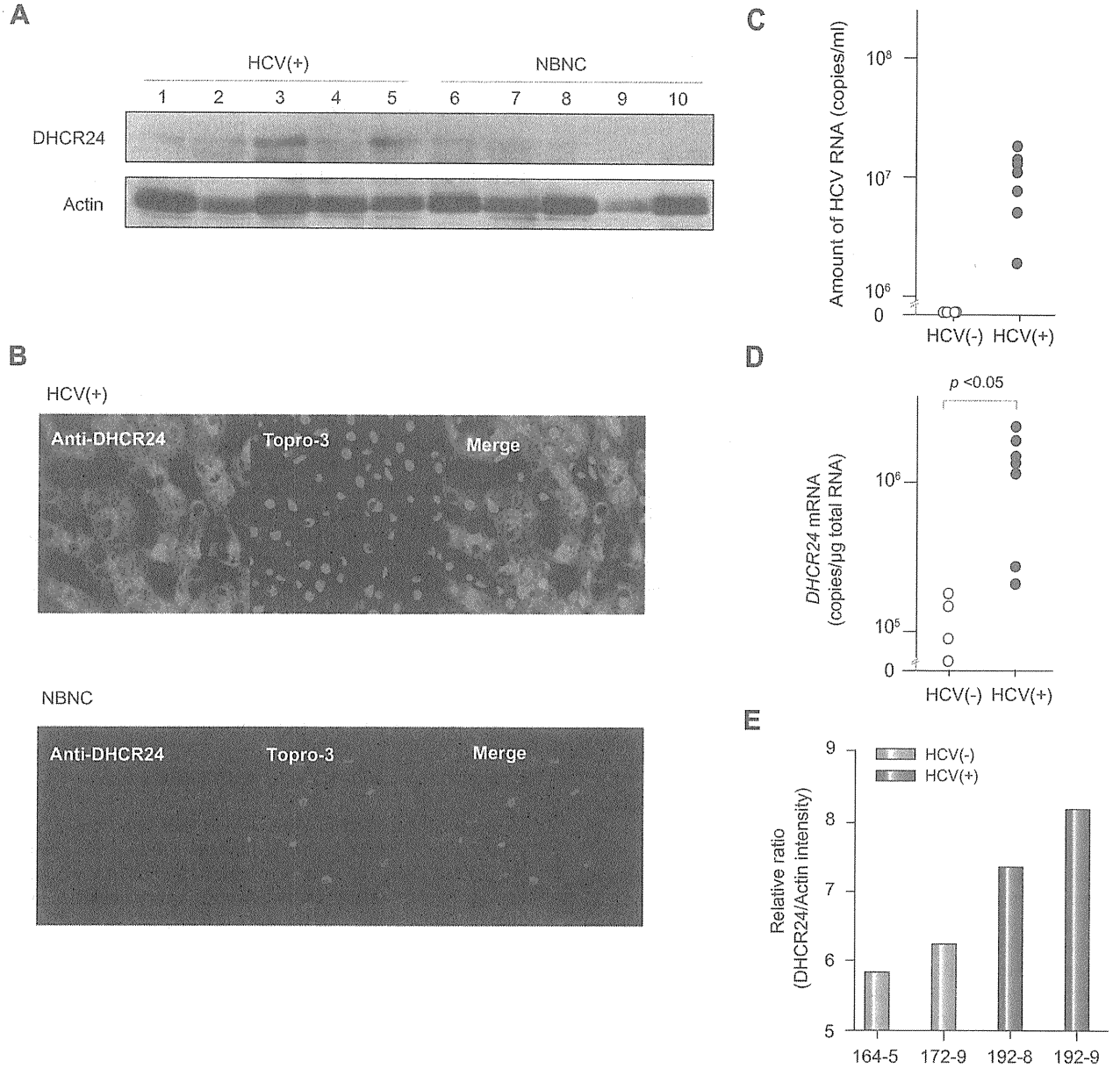

Fig. 1. HCV induces DHCR24 overexpression *in vitro* and *in vivo*. (A) Expression of DHCR24 in non-cancerous regions of livers of HCV-infected (+) and NBNC-HCC patients. Lysates (25 μg/lane) of non-cancerous liver tissues from HCC patients were analyzed by Western blot analysis using MoAb 2-152a. The patient numbers (Supplementary Table 1) are indicated at the top of the blot. (B) Immunohistochemical staining of HCV-infected non-cancerous tissues derived from an HCC patient using the monoclonal antibody 2-152a (Alexa488), anti-TO-PRO-3, or a merge (600× magnification) (upper panel). Tissues from an NBNC patient stained with the monoclonal antibody 2-152a (Alexa488) as well as TO-PRO-3 (640× magnification) (lower panel). (C) The amount of HCV RNA that was present in the HCV-R6 (genotype 1b)-infected chimeric mice with the humanized liver was quantified using RTD-PCR. The results of HCV uninfected ($n = 4$) and infected ($n = 7$) is indicated. (D) The amount of *DHCR24* mRNA present in total RNA isolates of HCV-R6 (genotype 1b)-infected chimeric mice with the humanized liver was quantified using RTD-PCR. *$p < 0.05$ (Mann–Whitney test). The results of HCV uninfected ($n = 4$) and infected ($n = 7$) are indicated. (E) DHCR24 protein was detected by Western blot analysis using MoAb 2-152a as a probe, and quantitated by LAS3000. Protein levels are normalized to actin and ratio is indicated.

HCV infection in vivo induces persistent overexpression of DHCR24

We next examined whether HCV infection could induce DHCR24 expression in human hepatocytes. DHCR24 was overexpressed more frequently in liver tissues from HCV-positive patients than in tissues from HBV- and HCV-negative (NBNC) patients (Fig. 1A and Supplementary Table 1). The liver tissue from HCV-positive patients stained more strongly for DHCR24 expression than the liver tissue from NBNC patients (Fig. 1B). We inoculated chimeric mice [19,23,25] with HCV ($10^{6.2}$ copies/ml) that had been isolated from the plasma of HCV-infected patients (patient R6, HCV genotype 1b). The serum concentration of human albumin (Supplementary Fig. 2A) in the chimeric mice after transplantation of hepatocytes indicated that human hepatocytes had engrafted in the mouse livers. Thirty days after transplantation, mice were infected with HCV, and HCV and RNA titers were analyzed both

before and after inoculation (Supplementary Fig. 2B). The average amount of HCV RNA that was present in the serum of the infected chimeric mice at 28 days post-infection was 1.1×10^7 copies/ml (Fig. 1C and Supplementary Fig. 2B). The *DHCR24* mRNA levels in the livers of the chimeric mice were also quantified at 28 days post-infection by real-time detection (RTD)-PCR [22]. The results revealed that there was a significant increase in *DHCR24* expression as measured by mRNA levels in HCV infected chimeric mice (Fig. 1D). Next, we examined the extent to which translation of DHCR24 occurred in the chimeric mice (Fig. 1E), higher DHCR24 protein levels were present in hepatocytes from HCV-infected mice (Nos. 192-8 and 192-9) than in those of uninfected mice (Nos. 164-5 and 172-9). These findings indicate that expression of DHCR24 is significantly up-regulated by HCV infection in human hepatocytes.

Role of DHCR24 in HCV replication

Since augmentation of DHCR24 expression was observed by HCV infection in humanized chimeric mice, we next examined whether DHCR24 was involved in HCV replication or not. We transfected siRNA into HCV replicon cell lines FLR3-1 (Fig. 2A and B) and R6FLR-N (Fig. 2C and D). Treatment with either two different *DHCR24* siRNA molecules (siDHCR24-417 or -1024) decreased HCV replication in a dose-dependent manner (Fig. 2A and C) but did not appear to have a significant effect on cell viability (Fig. 2B and D). Western blot analysis using HCV subgenomic replicon cell lines confirmed these findings (Fig. 2E and F). We also transfected the *DHCR24* siRNAs into HCV JFH-1 strain [18]-infected HuH7/K4 cell lines and found, by Western blot analysis, that the siRNAs inhibited HCV protein expression (Fig. 2G and H). These results indicate that DHCR24 may play a role in HCV replication.

The expression level of DHCR24 is linked to intracellular cholesterol levels

Human DHCR24 is involved in cholesterol biosynthesis [10]. It participates in multiple steps of cholesterol synthesis from lanosterol [26] (Fig. 3A). To examine the effect of cholesterol on the DHCR24 expression level in HuH-7 cells, we added cholesterol to cultured cells and determined the DHCR24 expression level (Fig. 3B). Expression levels of DHCR24 in HuH-7 cells were decreased approximately 50% by addition of cholesterol compared to that of the untreated control (Fig. 3B). On the other hand, that of DHCR24 in HepG2 cells was increased 2.5-fold by depletion of cholesterol using methyl-β-cyclodextrin (M-β-CD) (Fig. 3C).

These results indicate that the expression of DHCR24 in a cell correlates with the cholesterol level in that cell. Furthermore, silencing *DHCR24* reduced the cholesterol level in cells compared to control cells (Fig. 3D), suggesting that DHCR24 is essential for cholesterol synthesis.

Effect of U18666A on HCV replication in vitro

We further examined the role that DHCR24 plays in HCV replication by treating cells with U18666A. Treatment with U18666A (62.5, 125, 250, 500, and 1000 nM) of HCV replicon cells (FLR3-1) decreased HCV replication in a dose-dependent manner as shown by luciferase assay (Fig. 4A) and Western blot analysis (Fig. 4B). Notably, DHCR24 protein appeared as doublet bands in the absence of U18666A, but the lower band shifted to the upper band after treatment with U18666A (Fig. 4B). U18666A also suppressed HCV replication in other replicon cell lines (R6FLR-N and Rep JFH Luc 3-13; Fig. 4C and D). Treatment with U18666A (<250 nM) suppressed viral replication without producing significant cytotoxicity. We also examined the effect of 7-dehydrocholesterol reductase (DHCR7) (Fig. 3A) on HCV replication using the specific inhibitor BD1008 [26]. Treatment with BD1008 also suppressed HCV replication, but the concentration required was much higher than that needed in the U18666A assays (Fig. 4E); the concentration also greatly exceeded the intrinsic IC_{50} value for inhibition of σ-receptor binding (47 ± 2 nM) [27]. Therefore, DHCR24 may play a more significant role than DHCR7 in HCV replication. We next evaluated the compensatory effect that the addition of cholesterol had on cells treated with U18666A (Fig. 4F and G) by examining low density lipoprotein (LDL)-replaceable dissolved cholesterol levels as described in Supplementary data. Treatment with cholesterol led to partial restoration of HCV replication (Fig. 4F). These results suggest that U18666A suppresses HCV replication by depleting cellular cholesterol stores.

Next, we characterized the effect that U18666A had on HCV JFH-1 infection. Adding U18666A (62.5, 125, 250, and 500 nM) to HCV JFH-1-infected cell lines for 72 h, reductions of NS5B protein level were observed in cells treated more than 500 nM of U18666A (Fig. 5A and B). Additionally, the HCV RNA copy number in infected cells was suppressed by addition of 250 or 500 nM of U18666A (Fig. 5C). Examination of the cytotoxicity that U18666A (62.5–500 nM) had on infected cells revealed that it had little effect on cell viability (Fig. 5D). These results demonstrate that inhibition of DHCR24 by U18666A suppresses viral replication in HCV replicon cells and HCV-infected cells.

Evaluation of the anti-HCV effect of U18666A in vivo

To examine the effect of U18666A on HCV infection *in vivo*, we administered U18666A to HCV-infected chimeric mice with the humanized liver. The mice were infected with HCV via inoculation of patient serum HCR6 5 weeks after transplantation of human hepatocytes. U18666A (10 mg/kg) and PEG-IFN-α (30 μg/kg) were then administrated to these mice for 2 weeks (Fig. 6A). HCV RNA quantity (Fig. 6B) and serum human albumin levels (Fig. 6C) were measured in the mice after 1, 4, and 14 days of HCV infection. Treatment with U18666A alone significantly decreased HCV RNA levels in the serum (from 1×10^8 to 3×10^5 copies/ml) after 2 weeks, and its suppressive effect was more pronounced than that of PEG-IFN-α alone (8×10^5 copies/ml; Fig. 6B). Moreover, co-administration of U18666A and PEG-IFN-α synergistically (combination index <1) enhanced the antiviral effect of PEG-IFN-α (5×10^4 copies/ml). Treatment with these drugs did not significantly affect the serum human albumin concentrations in treated mice (Fig. 6C).

Discussion

The results of this study revealed that DHCR24, an enzyme that participates in cholesterol synthesis (last step; Fig. 3A), also plays a significant role in HCV replication. To our knowledge, this is the first report that this molecule is involved in HCV infection. The mevalonate route of the cholesterol synthesis pathway (starting from acetyl Co-A) has previously been reported to be involved in

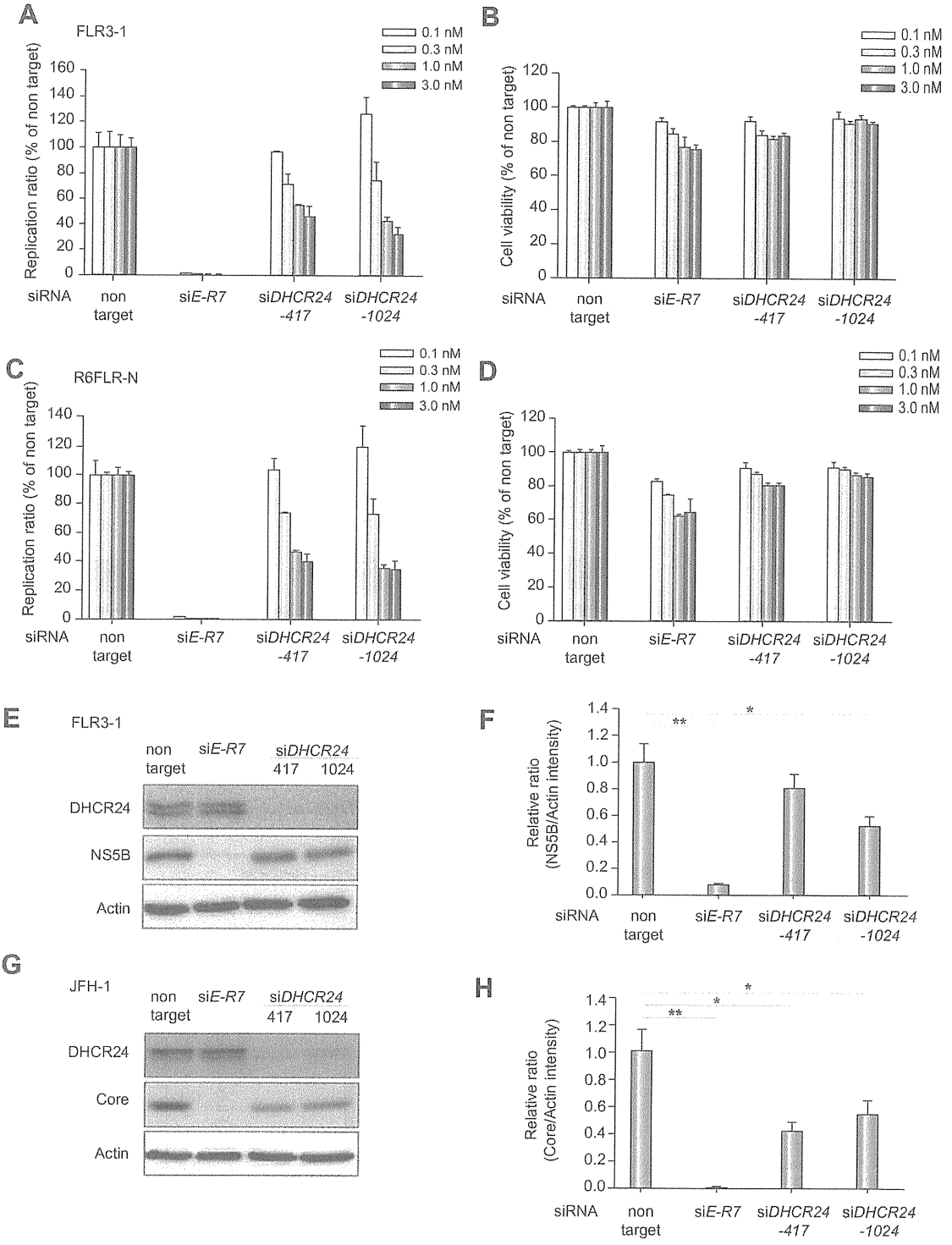

Fig. 2. Effect of DHCR24 knockdown on HCV replication. (A–D) Effect of DHCR24 knockdown on HCV replication in HCV replicon cells (FLR3-1 and R6FLR-N) at 72 h after the anti-*DHCR24* siRNAs (417 and 1024), siRNAs against HCV (siE-R7 for FLR3-1 and JFH-1; siE-R5 for R6-FLR-N), or non-target control siRNAs were transfected into HCV replicon cells. Replication activity was examined by luciferase assay (A and C), and cell viability was measured by the WST-8 assay (B and D). The data represent the mean of three experiments, and the bars indicate SD values. The Western blot analysis (E) and relative intensity of HCV-NS5B protein band was measured by LAS3000 and normalized with that of actin (F) after the treatment with siRNAs targeted against *DHCR24* (siDHCR24-417 and 1024) or HCV (si*E-R7*) in FLR3-1 replicon cells. (G and H) In HCV JFH-1-infected cells, DHCR24 knockdown by si*DHCR*24-417 and 1024 and HCV knockdown by siE-R7 were performed, and DHCR24 and HCV core protein expressions were confirmed by Western blot analysis. The relative intensity ratio of core protein to actin is indicated (H). The data represent the mean of three experiments, and the bars indicate SD values. $^{*}p$ <0.05, $^{**}p$ <0.01 (two-tailed Student's t test).

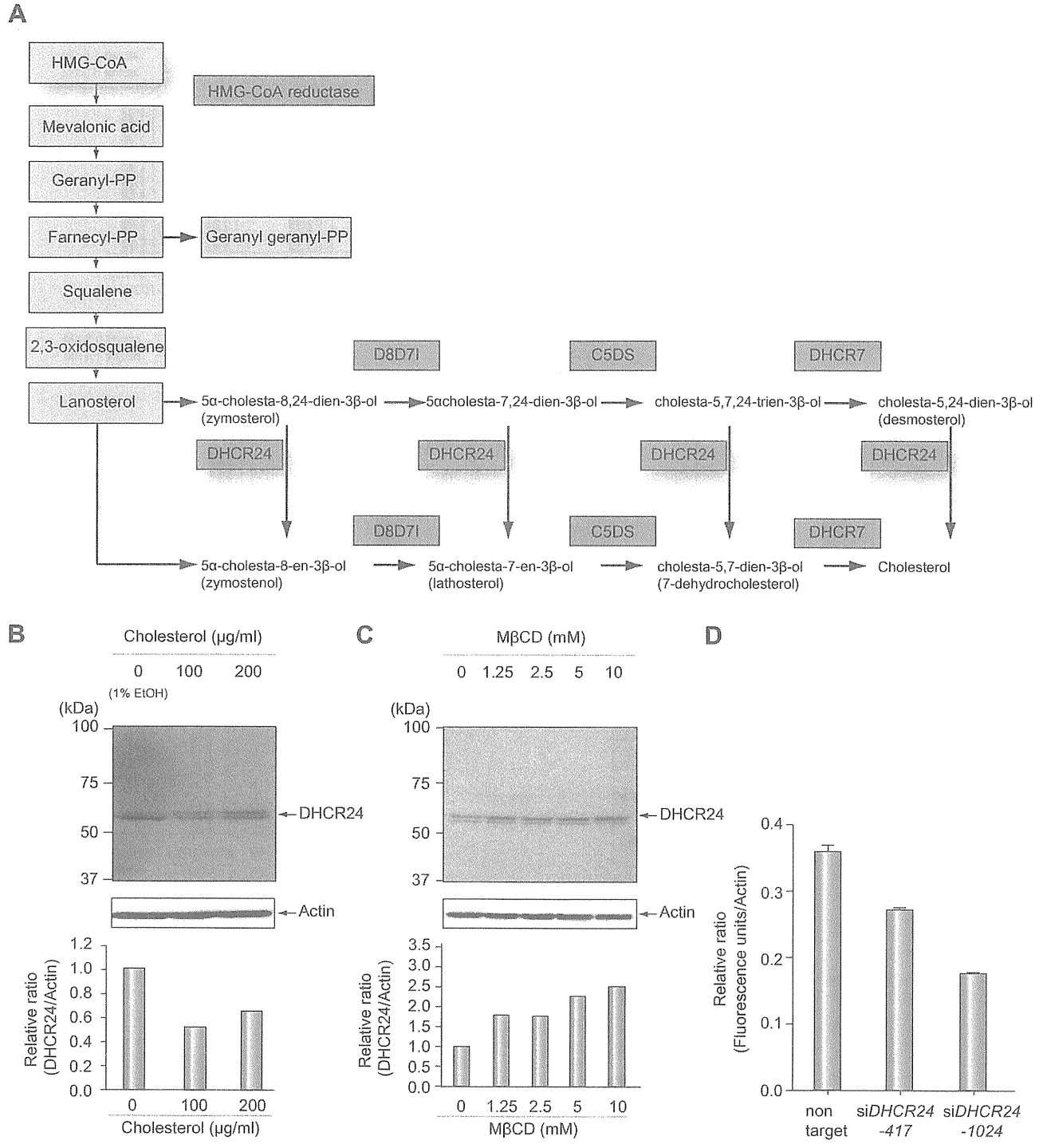

Fig. 3. The level of cholesterol and DHCR24 expression. (A) Cholesterol synthesis pathway, starting from HMG-CoA [26]. The abbreviations used are: D8D7I, 3β-hydroxysterol-delta(8)-delta(7)-isomerase; and C5DS, 3β-hydroxysterol-C^5-desaturase. (B) Cholesterol (0, 100, and 200 μg/ml) was added to HuH-7 cells, and, after 24 h, DHCR24 protein was detected by Western blot analysis using anti-DHCR24 MoAb and protein band intensity was measured and normalized to actin (lower panel). (C) HepG2 cells were treated with MβCD (0, 1.25, 2.5, 5, and 10 mM) for 30 min. After 72 h, these cells were harvested and examined by Western blot analysis with the anti-DHCR24 MoAb and relative intensity was measured as described in (B) (lower panel). (D) Cholesterol concentration in R6FLR-N cells was measured after treatment with non-targeting siRNA and *DHCR24* siRNA (417 and 1024). The cholesterol contents were measured by Amplex Red cholesterol assay, plotted based on fluorescence units and normalized to actin which was measured by Western blot analysis, and the relative ratio was then calculated. The data represent the mean of three experiments, and the bars indicate the SD values.

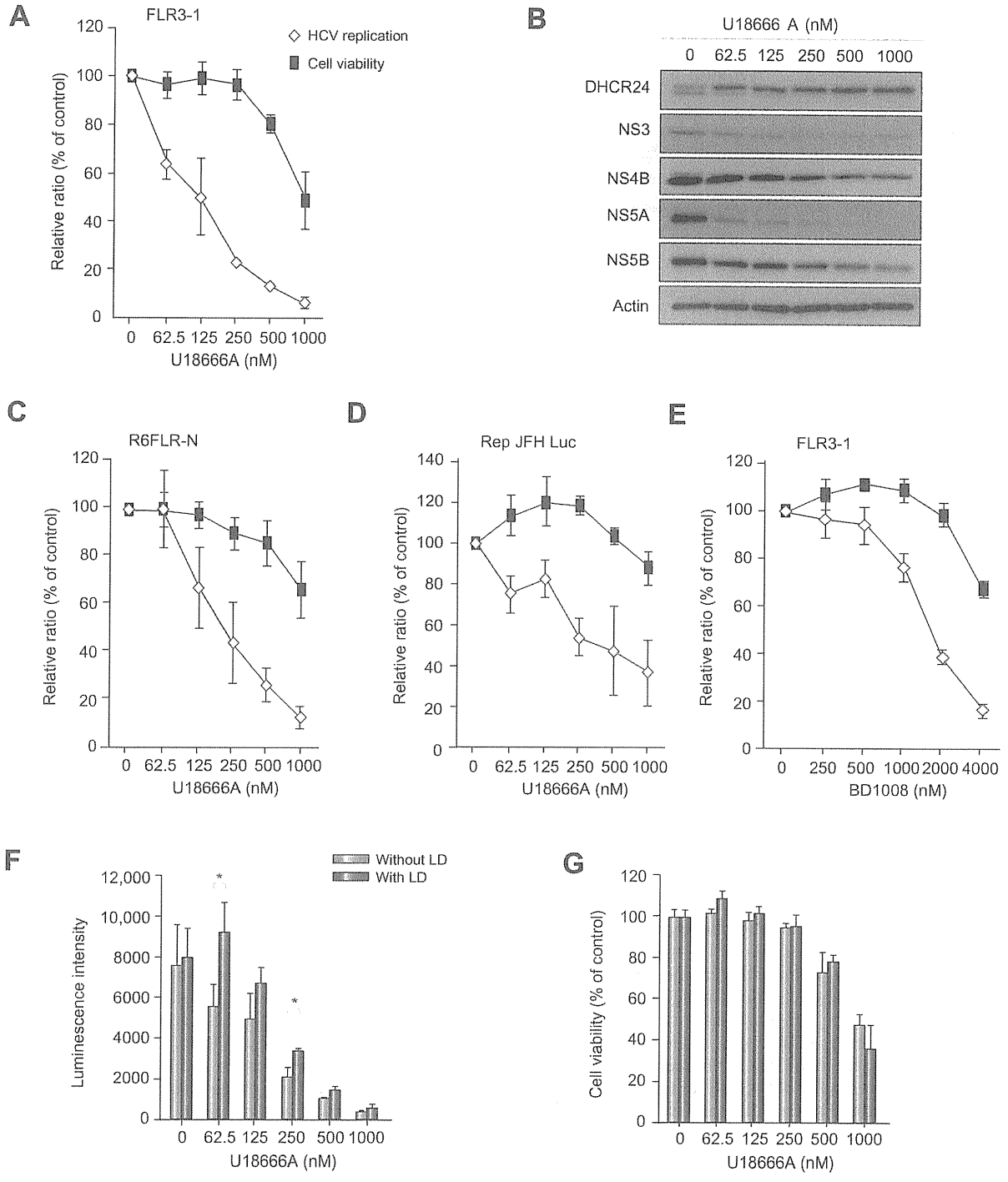

Fig. 4. Effect of U18666A on HCV replication. (A) Addition of U18666A to FLR3-1 cells and subsequent examination of HCV replication by the luciferase assay. Cell viability was measured by WST-8 assay. HCV replication and cell viability were measured 48 h after addition of U18666A. The bars indicate SD values. Open diamonds indicate the relative ratio of viral replication, and black squares indicate the cell viability in relation to untreated controls (A and C–E). (B) Treatment of FLR3-1 cells with U18666A decreased the expression of HCV proteins in a dose-dependent manner, as determined by Western blot analysis. (C and D) Effect of U18666A on HCV replication in other HCV replicon cells (C, R6FLR-N cells; D, Rep JFH Luc 3-13 cells). HCV replication and cell viability analyses were performed as described above. (E) The effect of the DHCR7 inhibitor BD1008 on HCV replicon cells (FLR3-1). Replication activity was examined by the luciferase assay, and cell viability was measured by the WST-8 assay. HCV replication and cell viability analyses were performed 48 h after the addition of U18666A. (F and G) FLR3-1 cells (5×10^3 cells/well) were treated with U18666A alone (light blue, or), low density lipoprotein (LDL) (final cholesterol concentration, 50 μg/ml), and U18666A (dark blue). HCV replication was determined by the luciferase assay 48 h later (F), and cell viability was measured by the WST-8 assay (G). *p <0.05 (two-tailed Student's t-test). The data represent the mean of three experiments, and the bars indicate SD values.

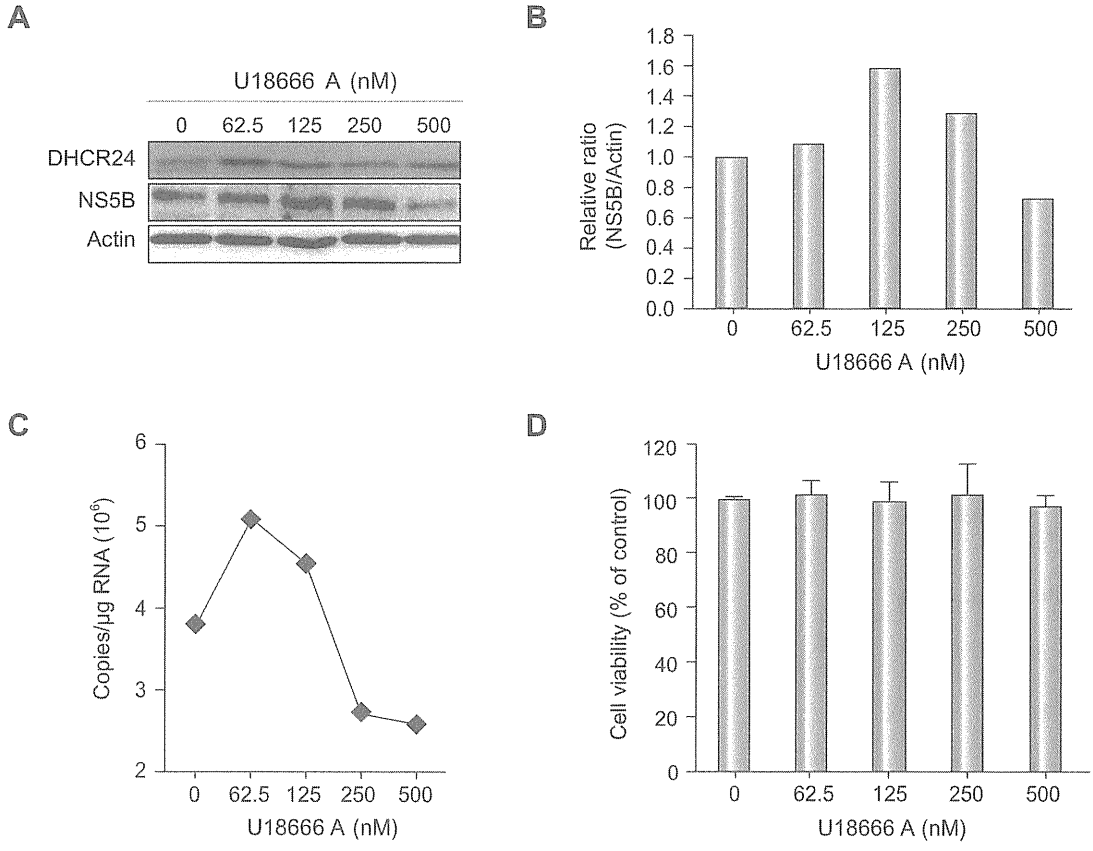

Fig. 5. Effect of U18666A on cells infected with HCV JFH-1. HCV JFH-1-infected cells treated with U18666A were examined 72 h after treatment. (A) Expression of HCV-NS5B protein with or without U18666A treatment, analyzed by Western blot analysis. (B) The intensity of HCV-NS5B protein expression is represented graphically. (C) HCV RNA in HCV JFH-1-infected cells with or without U18666A treatment was measured by RTD-PCR as described in Materials and methods. (D) Cell viability was measured by the WST-8 assay.

HCV replication [28]. The present findings are the first evidence that overexpression of one of the enzymes downstream of the mevalonate pathway, i.e., DHCR24, can be induced by HCV infection. In a previous study, 3-hydroxy 3-methyl-glutaryl Co-A (HMG-CoA) reductase was found to be inhibited by lovastatin, subsequently resulting in suppression of HCV replication [28]. The product of the mevalonate pathway that is required for HCV replication is reported to be a geranyl geranyl lipid [29]. Many lipids are crucial to the viral life cycle, and inhibitors of the cholesterol/fatty acid biosynthetic pathway inhibit viral replication, maturation, and secretion [30,31]. We found that inhibition of DHCR24 down-regulated HCV replication. DHCR24 catalyzes the reduction of the delta-24 bond of the sterol intermediate and works further downstream of farnesyl pyrophosphate (Fig. 3A) and, therefore, does not influence geranyl–geranylation. Thus, our findings indicate the existence of regulatory pathway of HCV replication by cholesterol synthesis and trafficking through DHCR24 rather than by protein geranyl–geranylation. DHCR24 deficiency reduces the cholesterol level and disorganizes cholesterol-rich detergent-resistant membrane domains (DRMs) in mouse brains [32]. Additionally, the HCV replication complex has been detected in the DRM fraction [11]. Therefore, a deficiency in DRM, induced by silencing *DHCR24*, may suppress HCV replication.

We demonstrated that the addition of cholesterol to HCV-infected hepatocytes treated with U18666A led to partial recovery of HCV replication, which suggests that cholesterol may be an important factor in HCV replication. U18666A impairs the intracellular biosynthesis and transport of cholesterol and inhibits the action of membrane-bound enzymes, including DHCR24, during sterol synthesis [33]. Moreover, the DHCR7 inhibitor BD1008 also suppresses HCV replication. Thus, the findings in this study further substantiate the fact that cholesterol plays an important role in HCV replication and infection.

Although monotherapy with statins is reportedly insufficient to induce anti-viral activity in HCV-infected patients [34], a synergistic action between statins and IFN has been observed [35]. The effect of the statin is thought to be mainly mediated by the depletion of geranyl geranyl lipids. It is important to note that higher doses of statins may increase the risk of myopathy, liver dysfunction, and cardiovascular events [36]. Moreover, the EC_{50} values of the statins that are associated with a reduction in HCV replication are reported to be 0.45–2.16 μM, while the IC_{50} of U18666A was estimated to be 125 nM in the present study. Therefore, U18666A may serve as a novel anti-HCV drug that could be utilized with IFN as a combined therapeutic regimen.

In summary, we demonstrated that the expression of DHCR24 is induced by infection with HCV and that DHCR24 is an essential host factor that is required for HCV replication. HCV may increase cholesterol synthesis in cells via the action of a host regulatory factor, such as DHCR24, that is correlated with cholesterol

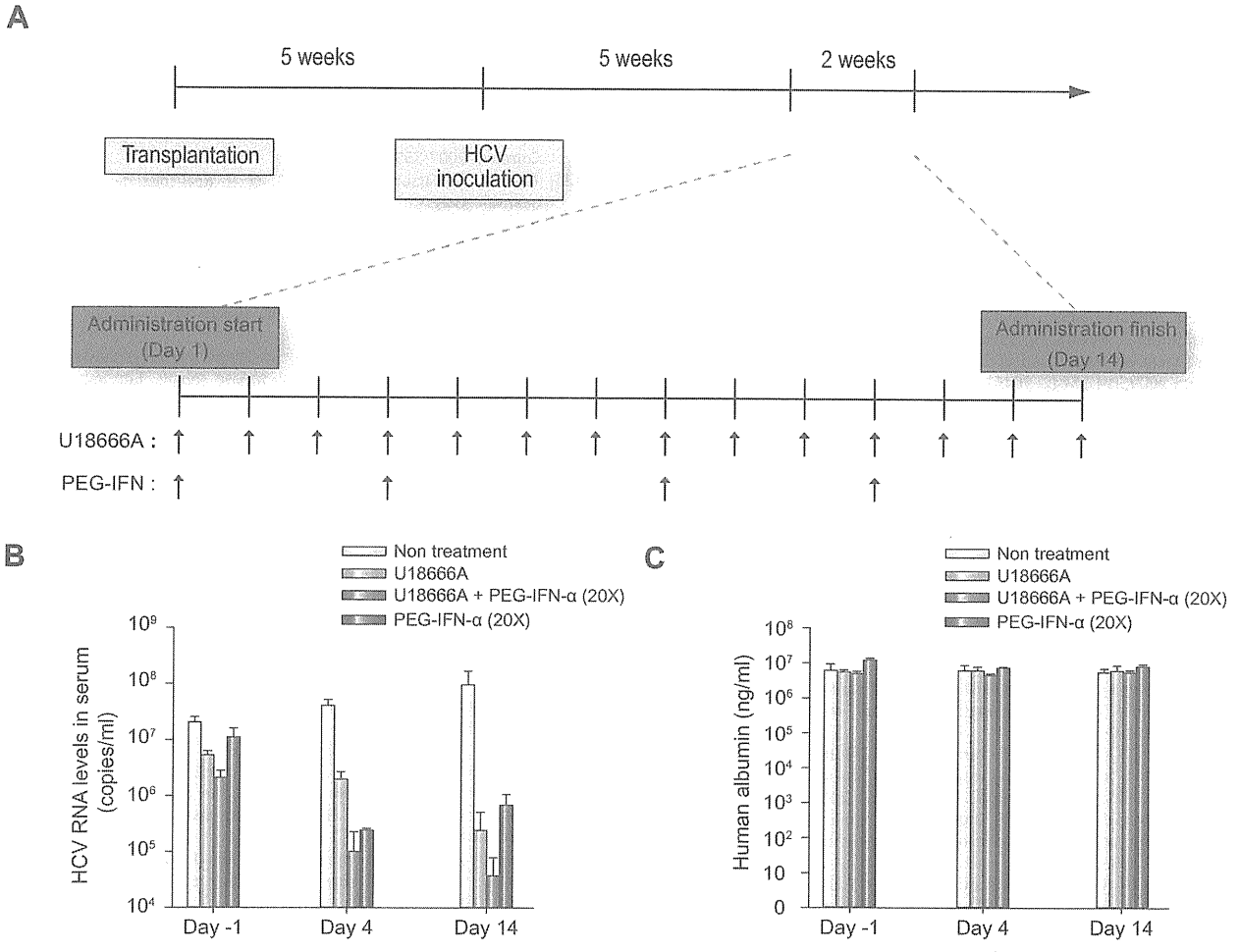

Fig. 6. Evaluation of the anti-HCV effect of U18666A in chimeric mice. (A) Diagram of the schedule that was followed to produce chimeric mice with the humanized liver, perform blood sampling, and administer drugs to chimeric mice infected with HCV. Four groups of three chimeric mice with the humanized liver were treated intraperitoneally with U18666A (10 mg/kg) and/or subcutaneously with PEG-IFN-α (30 μg/kg) at 2-day intervals for 2 weeks. (B) The effect of U18666A and/or PEG-IFN-α on HCV replication in chimeric mice with the humanized liver was determined by quantification of HCV-RNA using RTD-PCR. The bars indicate SD values ($n = 12$). (C) Human albumin concentrations in the sera of chimeric mice with the humanized liver. The bars indicate SD values ($n = 12$).

synthesis and is also directly involved in replication. Genome-wide analysis of the host response to HCV infection revealed the upregulation of genes related to lipid metabolism [37]. DHCR24 expression was found to be upregulated in the cDNA microarray analysis of chronic hepatitis C cases [38]. Future studies are needed to examine the detailed mechanism by which HCV infection augments DHCR24 expression in hepatocytes.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2010.12.011.

References

[1] Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. Lancet 2003;362:1907–1917.
[2] Tsukuma H, Hiyama T, Tanaka S, et al. Risk factors for hepatocellular carcinoma among patients with chronic liver disease. N Engl J Med 1993;328:1797–1801.
[3] Wasley A, Alter MJ. Epidemiology of hepatitis C: geographic differences and temporal trends. Semin Liver Dis 2000;20:1–16.
[4] Kohara M, Tanaka T, Tsukiyama-Kohara K, et al. Hepatitis C virus genotypes 1 and 2 respond to interferon-alpha with different virologic kinetics. J Infect Dis 1995;172:934–938.
[5] Tsukiyama-Kohara K, Tone S, Maruyama I, et al. Activation of the CKI-CDK-Rb-E2F pathway in full genome hepatitis C virus-expressing cells. J Biol Chem 2004;279:14531–14541.
[6] Nishimura T, Kohara M, Izumi K, et al. Hepatitis C virus impairs p53 via persistent overexpression of 3beta-hydroxysterol Delta24-reductase. J Biol Chem 2009;284:36442–36452.
[7] Greeve I, Hermans-Borgmeyer I, Brellinger C, et al. The human DIMINUTO/DWARF1 homolog seladin-1 confers resistance to Alzheimer's disease-associated neurodegeneration and oxidative stress. J Neurosci 2000;20:7345–7352.
[8] Wu C, Miloslavskaya I, Demontis S, Maestro R, Galaktionov K. Regulation of cellular response to oncogenic and oxidative stress by Seladin-1. Nature 2004;432:640–645.
[9] Waterham HR, Koster J, Romeijn GJ, et al. Mutations in the 3beta-hydroxysterol Delta24-reductase gene cause desmosterolosis, an autosomal recessive disorder of cholesterol biosynthesis. Am J Hum Genet 2001;69:685–694.
[10] Wechsler A, Brafman A, Shafir M, et al. Generation of viable cholesterol-free mice. Science 2003;302:2087.
[11] Aizaki H, Lee KJ, Sung VM, Ishiko H, Lai MM. Characterization of the hepatitis C virus RNA replication complex associated with lipid rafts. Virology 2004;324:450–461.
[12] Di Stasi D, Vallacchi V, Campi V, et al. DHCR24 gene expression is upregulated in melanoma metastases and associated to resistance to oxidative stress-induced apoptosis. Int J Cancer 2005;115:224–230.
[13] Bierkamper GG, Cenedella RJ. Induction of chronic epileptiform activity in the rat by an inhibitor of cholesterol synthesis, U18666A. Brain Res 1978;150:343–351.
[14] Nakabayashi H, Taketa K, Miyano K, Yamane T, Sato J. Growth of human hepatoma cells lines with differentiated functions in chemically defined medium. Cancer Res 1982;42:3858–3863.
[15] Knowles BB, Howe CC, Aden DP. Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. Science (New York, NY) 1980;209:497–499.
[16] Sakamoto H, Okamoto K, Aoki M, et al. Host sphingolipid biosynthesis as a target for hepatitis C virus therapy. Nat Chem Biol 2005;1:333–337.
[17] Watanabe T, Sudoh M, Miyagishi M, et al. Intracellular-diced dsRNA has enhanced efficacy for silencing HCV RNA and overcomes variation in the viral genotype. Gene Ther 2006;13:883–892.
[18] Wakita T, Pietschmann T, Kato T, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 2005;11:791–796.
[19] Inoue K, Umehara T, Ruegg UT, et al. Evaluation of a cyclophilin inhibitor in hepatitis C virus-infected chimeric mice in vivo. Hepatology 2007;45:921–928.
[20] Jensen ON, Wilm M, Shevchenko A, Mann M. Sample preparation methods for mass spectrometric peptide mapping directly from 2-DE gels. Methods Mol Biol (Clifton, NJ) 1999;112:513–530.
[21] Nakagawa S, Umehara T, Matsuda C, et al. Hsp90 inhibitors suppress HCV replication in replicon cells and humanized liver mice. Biochem Biophys Res Commun 2007;353:882–888.
[22] Takeuchi T, Katsume A, Tanaka T, et al. Real-time detection system for quantification of hepatitis C virus genome. Gastroenterology 1999;116:636–642.
[23] Mercer DF, Schiller DE, Elliott JF, et al. Hepatitis C virus replication in mice with chimeric human livers. Nat Med 2001;7:927–933.
[24] Tateno C, Yoshizane Y, Saito N, et al. Near completely humanized liver in mice shows human-type metabolic responses to drugs. Am J Pathol 2004;165:901–912.
[25] Umehara T, Sudoh M, Yasui F, et al. Serine palmitoyltransferase inhibitor suppresses HCV replication in a mouse model. Biochem Biophys Res Commun 2006;346:67–73.
[26] Kedjouar B, de Medina P, Oulad-Abdelghani M, et al. Molecular characterization of the microsomal tamoxifen binding site. J Biol Chem 2004;279:34048–34061.
[27] John CS, Lim BB, Geyer BC, Vilner BJ, Bowen WD. 99mTc-labeled sigma-receptor-binding complex: synthesis, characterization, and specific binding to human ductal breast carcinoma (T47D) cells. Bioconjug Chem 1997;8:304–309.
[28] Ye J, Wang C, Sumpter Jr R, et al. Disruption of hepatitis C virus RNA replication through inhibition of host protein geranyl-geranylation. Proc Natl Acad Sci USA 2003;100:15865–15870.
[29] Kapadia SB, Chisari FV. Hepatitis C virus RNA replication is regulated by host geranyl-geranylation and fatty acids. Proc Natl Acad Sci USA 2005;102:2561–2566.
[30] Aizaki H, Morikawa K, Fukasawa M, et al. Critical role of virion-associated cholesterol and sphingolipid in hepatitis C virus infection. J Virol 2008;82:5715–5724.
[31] Syed GH, Amako Y, Siddiqui A. Hepatitis C virus hijacks host lipid metabolism. Trends Endocrinol Metab 2010;21:33–40.
[32] Crameri A, Biondi E, Kuehnle K, et al. The role of seladin-1/DHCR24 in cholesterol biosynthesis, APP processing and Abeta generation in vivo. EMBO J 2006;25:432–443.
[33] Cenedella RJ. Cholesterol synthesis inhibitor U18666A and the role of sterol metabolism and trafficking in numerous pathophysiological processes. Lipids 2009;44:477–487.
[34] Bader T, Fazili J, Madhoun M, et al. Fluvastatin inhibits hepatitis C replication in humans. Am J Gastroenterol 2008;103:1383–1389.
[35] Ikeda M, Abe K, Yamada M, et al. Different anti-HCV profiles of statins and their potential for combination therapy with interferon. Hepatology (Baltimore, MD) 2006;44:117–125.
[36] Argo CK, Loria P, Caldwell SH, Lonardo A. Statins in liver disease: a molehill, an iceberg, or neither? Hepatology (Baltimore, MD) 2008;48:662–669.
[37] Su AI, Pezacki JP, Wodicka L, et al. Genomic analysis of the host response to hepatitis C virus infection. Proc Natl Acad Sci USA 2002;99:15669–15674.
[38] Honda M, Yamashita T, Ueda T, et al. Different signaling pathways in the livers of patients with chronic hepatitis B or chronic hepatitis C. Hepatology 2006;44:1122–1138.

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Translocase of Outer Mitochondrial Membrane 70 Expression Is Induced by Hepatitis C Virus and Is Related to the Apoptotic Response

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The localization of hepatitis C virus (HCV) proteins in cells leads to several problems. The translocase of outer mitochondrial membrane 70 (TOM70) is a mitochondrial import receptor. In this study, TOM70 expression was induced by HCV infection. TOM70 overexpression induced resistance to tumor necrosis factor-alpha (TNF-α)-mediated apoptosis but not to Fas-induced apoptosis in HepG2 cells. TOM70 was found to be induced by the HCV non-structural protein (NS)3/4A protein, and silencing of TOM70 decreased the levels of the NS3 and Mcl-1 proteins. These results indicate that TOM70 can directly interact with the NS3 protein. In hepatoma cells, silencing of TOM70 induced apoptosis and increased caspase-3/7 activity but did not modify caspase-8 and caspase-9 activity. TOM70 silencing-induced apoptosis was impaired in HCV NS3/4A protein-expressing cells. Thus, this study revealed a novel finding, that is, TOM70 is linked with the NS3 protein and the apoptotic response. ***J. Med. Virol. 83:801–809, 2011.***
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KEY WORDS: hepatitis C virus; translocase of outer mitochondrial membrane 70; apoptosis; non-structural protein 3; tumor necrosis factor-alpha

INTRODUCTION

Hepatitis C virus (HCV) infection causes acute and chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) [Seeff, 2002]. HCV easily establishes chronic infection, and localization of HCV proteins is reported to induce several disturbances in cells. One of the major target organelles of HCV is the mitochondrion, and HCV non-structural protein (NS)3/4A protease cleaves the mitochondrial antiviral signaling (MAVS)/IPS-1/VISA/Cardif protein, thereby impairing interferon signaling [Li et al., 2005] and influencing apoptotic responses [Nomura-Takigawa et al., 2006; Deng et al., 2008; Lei et al., 2009].

Most mitochondrial proteins are synthesized in the cytosol as preproteins, targeted to the mitochondria by cytosolic factors such as HSP70 and mitochondrial import stimulation factor (MSF), and transported to the intramitochondrial compartments by the preprotein import machineries of the outer and inner membranes (TOM and TIM complexes, respectively) [Mihara and Omura, 1996; Schatz, 1996; Neupert, 1997; Pfanner and Meijer, 1997]. The TOM machinery consists of two import receptors, namely, TOM20 and TOM70, and several other subunits that are arranged in a tightly bound complex termed the general import pore [Pfanner and Geissler, 2001; Hoogenraad et al., 2002; Stojanovski et al., 2003]. TOM70 was identified in *Saccharomyces cerevisiae* as a 70-kDa protein with no known function [Truscott et al., 2001]. TOM70 is recognized as the primary receptor for proteins with internal targeting signals, such as the F_1-ATPase β-subunit

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and cytochrome c_1 [Truscott et al., 2001]. TOM70 interacts with human myeloid cell leukemia-1 (Mcl-1), a Bcl-2 family member, and this interaction facilitates the mitochondrial targeting of Mcl-1 [Chou et al., 2006]. Mcl-1 can interact with the HCV core protein and suppresses core-induced apoptosis [Mohd-Ismail et al., 2009].

In the present study, it was found that TOM70 activity was enhanced by HCV. This study addresses TOM70 modification by HCV and its role in the apoptotic response.

MATERIALS AND METHODS

Cells

WRL68, HepG2, HuH-7, and HepG2 cells expressing non-structural proteins (Lenti-NS3/4A-HepG2, Lenti-NS4B-HepG2, Lenti-NS5A-HepG2, Lenti-NS5B-HepG2, and Lenti-empty-HepG2) were maintained and established as described previously [Tsukiyama-Kohara et al., 2004; Nishimura et al., 2009; Saitou et al., 2009]. The Cre/loxP conditional expression system for full-length HCV cDNA (*HCR6-Rz*) in RzM6 cells [Tsukiyama-Kohara et al., 2004] was induced using 100 nM of 4-hydroxytamoxifen (Sigma–Aldrich, St. Louis, MO) and passaged for 8 days (RzM6-8d) or for more than 44 days (RzM6-LC) [Nishimura et al., 2009] (Supplementary Fig. 1). Cell viability was measured using WST-8 (Dojindo, Kumamoto, Japan).

Purification and Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) Analysis of p70 and TOM70 Expression Vector

p70 was identified using MALDI-TOF-MS. The p70 band was excised, alkylated using 40 mM iodoacetamide/0.1 M NH_4HCO_3, and digested using trypsin. The p70 peptides were purified using an UltiMate capillary high-performance liquid chromatography system (Dionex) and analyzed using a 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA), as described previously [Jensen et al., 1999]. An expression vector with myc and His tags was constructed for TOM70 as follows: Total RNA was isolated from HuH-7 cells (10^6) by using the ISOGEN reagent (Nippon Gene, Tokyo, Japan). Purified RNA (2 μg) was reverse transcribed using SuperScriptIII (Invitrogen, Carlsbad, CA) and oligo$(dT)_{12-18}$ primer (Invitrogen), according to the manufacturer's protocol. The coding region of TOM70 cDNA was amplified by polymerase chain reaction (PCR) with LA *Taq* polymerase (Takara Bio, Shiga, Japan) and TOM70-F2 (5′-GGATCCGCAGAGGACACTTGTCATGGC-3′), which contained a *Bam*HI restriction site (underlined), as the forward primer and TOM70-R2 (5′-GCTGGAGTGCAGTGGCTATTC-3′) as the reverse primer. The amplified TOM70 cDNA was subcloned into the pCR2.1-TOPO vector. *Bam*HI–*Eco*RI-digested TOM70 cDNA was subcloned into pcDNA6/Myc-His(+) (Invitrogen) (TOM70-pcDNA6).

Immunoprecipitation (IP) and Western Blotting (WB)

The cells were solubilized in lysis buffer (20 mM HEPES-NaOH [pH 7.5], 1 mM EDTA [pH 7.5], 1 mM dithiothreitol, 1 μM diisopropylfluorophosphate, 150 mM NaCl, and 1% TritonX-100). Samples were centrifuged at 20,400*g* for 10 min at 4°C, and the supernatants were used for IP. Protein-G sepharose 4B beads (GE Healthcare, Piscataway, NJ; 20 μl) were washed, mixed with 2-243a antibody (2 μg) in 1% BSA-phosphate-buffered saline (PBS), and placed on a rotary shaker at 4°C for 1 hr. Next, the beads were washed three times with lysis buffer and treated with the cell lysate (4°C, overnight). The IP mix was washed four times with lysis buffer and solubilized with 2× SDS sample buffer (150 mM Tris [pH 6.8], 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.2% bromophenol blue). WB was performed as described previously [Nishimura et al., 2009]. Anti-myc monoclonal antibody (mAb) (9E10; Santa Cruz Biotechnology, Santa Cruz, CA), anti-HCV core mouse mAb (31-2), and anti-NS3 rabbit polyclonal antibody (R212) were used to examine the interaction between NS3 and myc-TOM70. Anti-Mcl-1 antibody (S-19; Santa Cruz Biotechnology) and anti-MAVS antibody (ab25084; ChIP grade; Abcam, Cambridge, MA) were also used. Professor Mihara (Kyusyu University) kindly provided anti-rat TOM70 polyclonal antibody (rTOM70).

Immunofluorescence Assay (IFA)

For mitochondrial staining, MitoRed (Dojindo) was added to the cell culture medium and incubated for 1 hr. The cells were fixed in 4% paraformaldehyde. The slides were then washed with PBS, permeabilized with 1% Triton X-100; and reacted with 2-243a mAb (1 μg/ml) and a polyclonal antibody against the endoplasmic reticulum (ER) (anti-PDI; 1:1,000; Stressgen Bioreagent, Kampenhout, Belgium) in 0.025% Tween-20 PBS, followed by reaction with FITC-conjugated goat anti-mouse IgG mAb (1:1,000; Cappel Products, Portland, ME) and Alexa 568-conjugated goat anti-rabbit IgG $(Fab')_2$ fragment (Invitrogen) in 0.025% Tween-20 PBS. The slides were covered with Vector Shield (Vector Laboratories, Burlingame, CA) and observed under an Olympus Fluoview laser-scanning microscope (Olympus, Tokyo, Japan).

Evaluation of Cell Death by Assessing Fas or Tumor Necrosis Factor (TNF)-α

The cells were plated in a 96-well plate (10^4 cells/well; Becton Dickinson, Franklin Lake, NJ) and transfected with empty pcDNA6 or TOM70-pcDNA6 (40 ng/well) by using the Lipofectamine 2000 reagent (Invitrogen). After 48 hr, the cells were treated with anti-Fas