

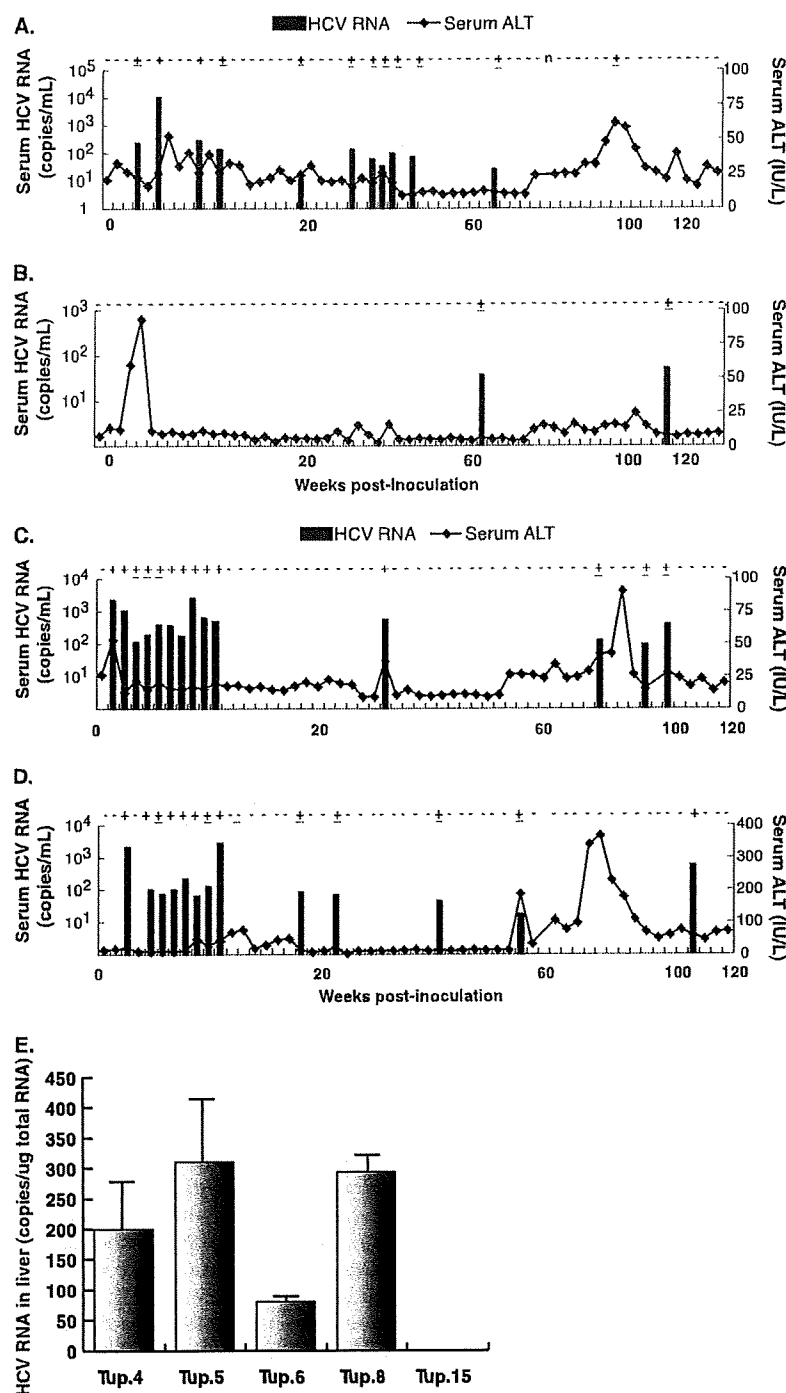


FIG. 1. Course of infection with patient serum HCR6 and RCV. (A) The results of quantitative RTD-PCR for HCV RNA and serum ALT concentrations were combined and plotted to show the course of infection in Tup.5. The bars and the ordinates on the left represent HCV RNA as genome equivalents/ml of serum. The curved line and the ordinates on the right represent serum ALT concentrations as IU/liter serum. (B) Serum HCV RNA and ALT concentrations for infection of Tup.6. (C) The graph for Tup.4. (D) The graph for Tup.8. The vertical axis for serum ALT in this graph is scaled differently from the others because of significant ALT elevation. (E) Quantification of HCV RNA in tupaia liver. HCV RNA in hepatocytes from tupaia (Tup.4, Tup.5, Tup.6, Tup.8, and Tup.15) livers was isolated 172 weeks after HCV infection and quantified by RTD-PCR. As few as 10 copies of the genome were detected, and the quantification range was between 10¹ and 10⁸ copies (26).

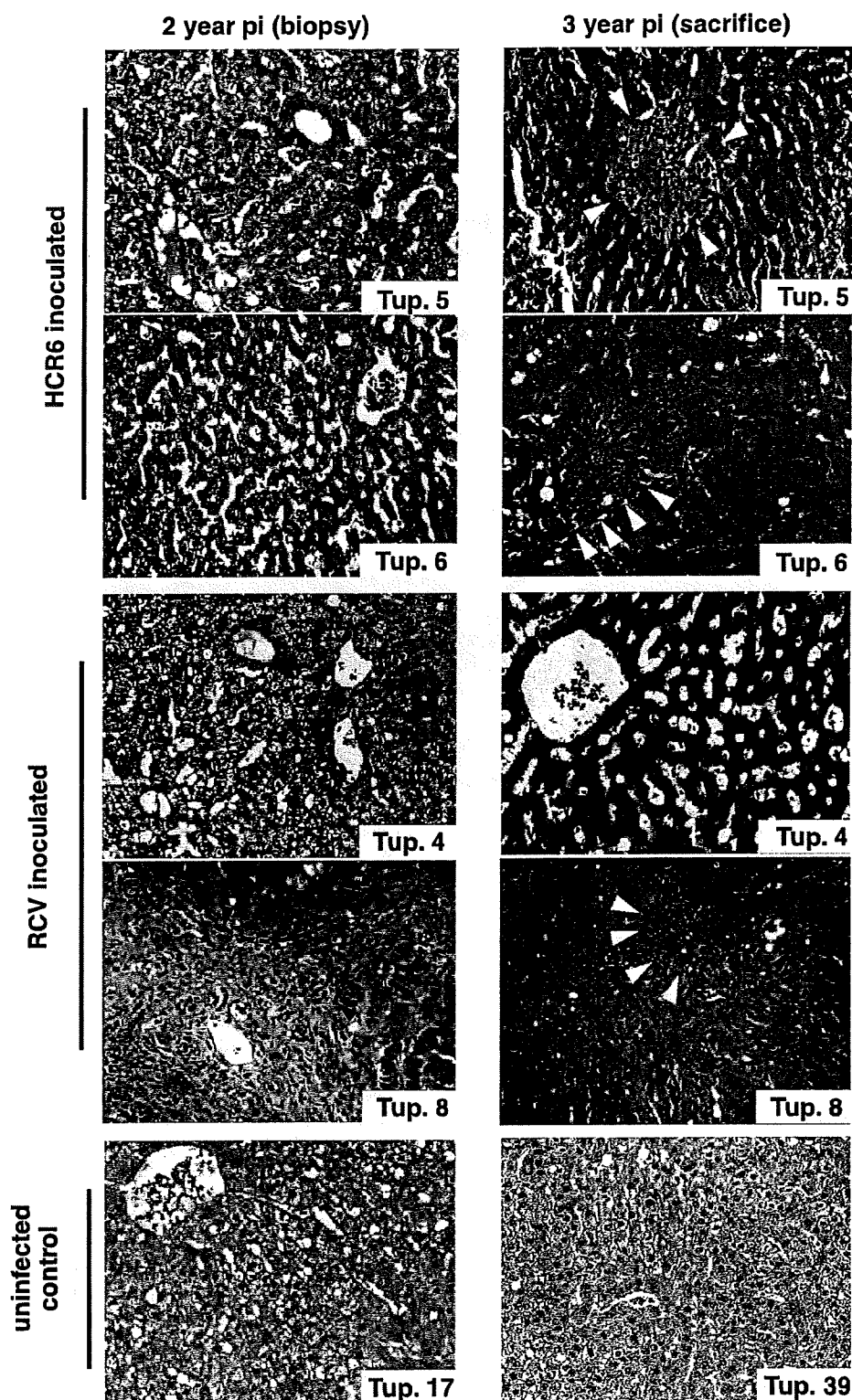


FIG. 2. Micrographs of liver specimens stained with H&E. Liver tissue from HCR6-inoculated tupaia (A to D) and RCV-inoculated tupaia (E to H) was obtained at 2 and 3 years postinoculation (pi). (I and J) Liver specimens from uninfected animals age matched to each inoculated animal were also obtained. The HCV-infected tupaia livers harbored infiltrating lymphocytes (white arrowheads) and fibrosis (broken lines and black arrowheads), which indicate chronic hepatitis.

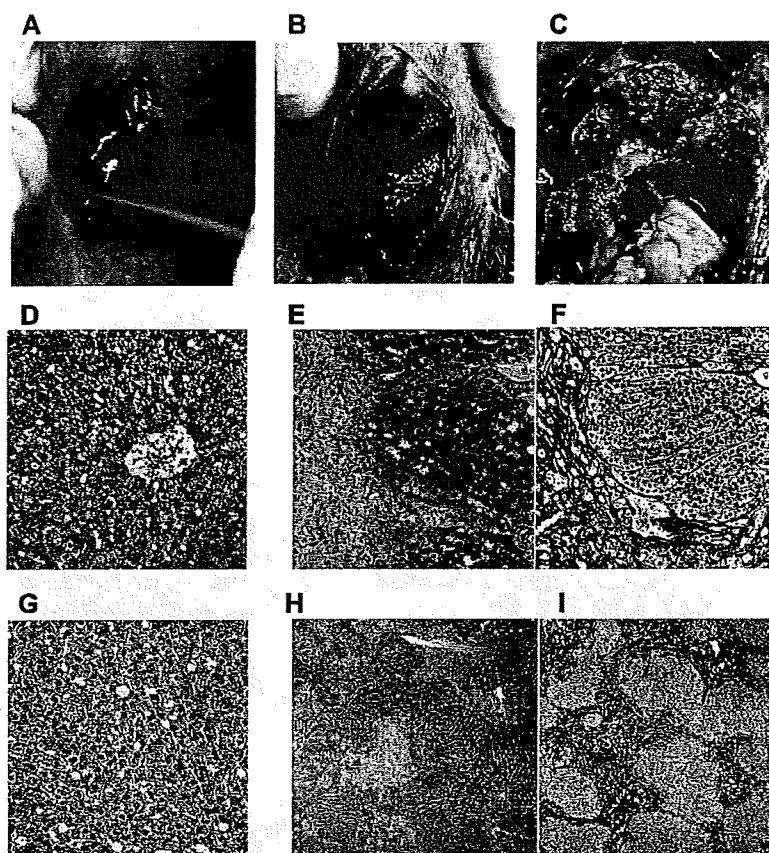


FIG. 3. Macro- and microscopic features of tupaia liver. (A) Infection-free control tupaia (Tup.15; 92 weeks). (B) RCV-infected animal displaying liver cirrhosis (Tup.8; 84 weeks postinoculation). (C) RCV-infected animal with massive surface nodules (Tup.8; 144 weeks postinoculation). (D and G) H&E staining of the uninfected Tup.15 at 92 weeks (D) and the uninfected Tup.39 at 242 weeks (G). (E, F, H, and I) H&E and silver staining of Tup.8 at 84 weeks postinoculation (E and F) or at 144 weeks postinoculation (H and I).

hepatitis had worsened with time in all HCV-infected tupaia (Fig. 2A to H and Table 2).

Fibrosis and cirrhosis were also examined. Mild fibrosis was seen in Tup.6, while severe fibrosis was seen in Tup.8. Cirrhosis was histologically investigated in all animals (Table 2). There was no significant difference between groups I and III at 94 weeks postinfection ($P = 0.194$), but at 144 weeks postinfection, a slight difference was observed ($P = 0.059$; SPSS 12.0). Macroscopic observation of the liver biopsy specimens (taken 2 years postinoculation) indicated liver cirrhosis in Tup.8 (Fig. 3B) compared with Tup.15 (uninfected control) (Fig. 3A), while silver staining of histology samples revealed fibrosis and cirrhotic nodules (Fig. 3E and F). Macroscopic observation upon sacrifice (3 years postinoculation) indicated that liver cirrhosis in Tup.8 had worsened (Fig. 3C). In contrast, age-matched infection-free negative control tupaia displayed none of these pathologies (Fig. 3A, D, and G).

Progressive lipid degeneration was noted in infected tupaia throughout the course of infection (Fig. 4). In particular, Tup.5 displayed microvesicular lipid droplets in the first biopsy specimens (at 2 years), which developed into macrovesicular droplets and foamy degeneration in biopsy specimens at 3 years (Fig. 4C and D). Liver specimens from other infected animals

displayed intracellular micro- and macrovesicular lipid droplets in hepatocytes at 3 years postinoculation (Fig. 4F, H, and J). These anomalies were not present in liver specimens from infection-free control animals (Fig. 4A and B).

Transmission of viral-RNA-positive serum to naive animals reproduces acute hepatitis and viremia. To confirm virion regeneration *in vivo*, and to exclude the possibility of false-positive serum HCV RNA results due to amplification of the original inocula, HCV RNA-positive sera from primary inoculated tupaia were used to inoculate naive tupaia. Three different sera were tested in this passage experiment, with two naive tupaia used as recipient animals for each trial (see Materials and Methods) (Table 1, group II).

In the first reinfection experiment, serum from Tup.5 (originally infected with patient serum HCR6) was collected at 5 weeks postinoculation and used to infect two naive animals. The recipient animals showed intermittent viremia over the subsequent 3 months (Fig. 5A). In the second and third cases of reinfection, sera from Tup.8 at 10 weeks postinoculation and from Tup.4 at 8 weeks postinoculation also induced viremia in the naive inoculated animals, similar to the first reinfection experiment (Fig. 5B and C). Furthermore, the PCR titers of the recipient tupaia were significantly greater than the inoc-

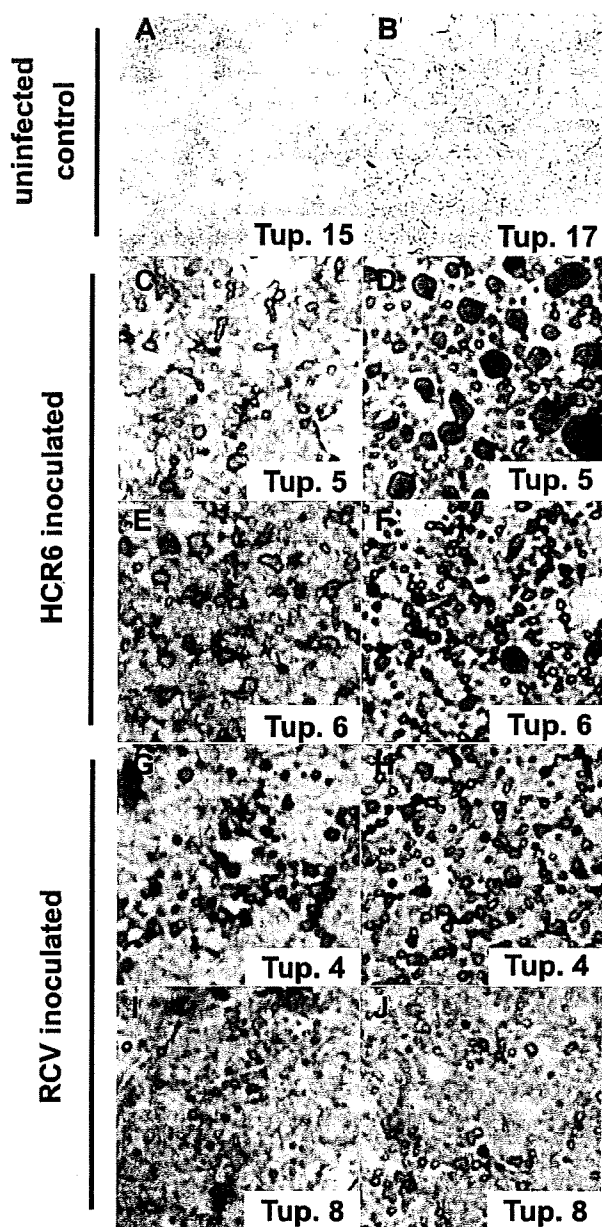


FIG. 4. Sudan IV-stained liver specimens exhibiting fatty liver degeneration. Cryosections of liver stained by Sudan IV as described in Materials and Methods show fatty liver degeneration. The left and right columns display biopsy specimens of infected animals (2 years postinoculation) and animals sacrificed at 3 years postinfection, respectively. (A and B) Uninfected controls at 2 years (Table 1 shows sample timing). (C to F) Patient serum HCR6-infected animals. (G to J) RCV-infected animals.

ulation titers (10^2 genome equivalents/animal) (Table 1). For Tup.11, serum from 4 weeks postinoculation contained almost 10^4 genome equivalents/ml of HCV RNA (Fig. 5B). In addition, significant increases in serum ALT accompanied detection of serum HCV RNA. These results indicate that HCV RNA-positive sera from group I actually contained infectious

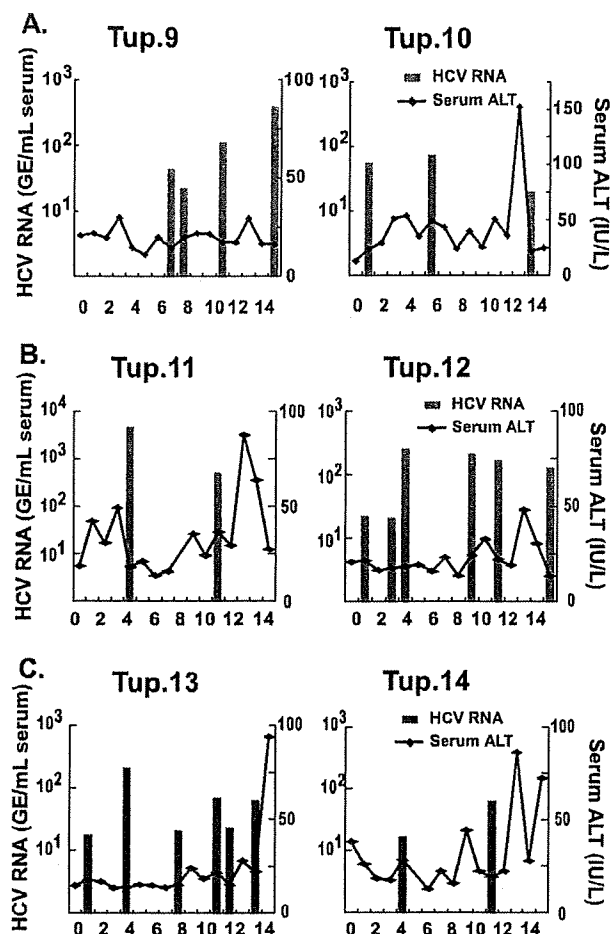


FIG. 5. Results of a reinfection experiment. (A) Quantitative RTD-PCR for HCV RNA and serum ALT levels are shown. Two naive animals were inoculated with tupaia serum (using serum taken at 5 weeks postinoculation from Tup.5, originally inoculated with patient serum HCR6) containing 100 genome equivalents (GE)/ml and were monitored for 15 weeks postinoculation (Table 1). (B) Tupaia serum (taken at 10 weeks postinoculation from Tup.8, originally inoculated with RCV) that was positive for HCV RNA was passed into two naive animals. The animals were inoculated with tupaia serum at 100 GE/animal and monitored for 15 weeks postinoculation. (C) Tupaia serum (taken at 8 weeks postinoculation from Tup.4, originally inoculated with RCV) that was positive for HCV RNA was passed into naive animals. The animals were inoculated with serum at 100 GE/animal and monitored for 20 weeks postinoculation.

virion particles. They also suggest that reconstituted HCV particles made from cDNA are infectious in tupaia.

We amplified a portion of the NS5A sequence, which is known as the interferon sensitivity determining region, by reverse transcription-PCR as described in the supplemental material. Each PCR product was subcloned and sequenced to compare the encoded amino acid sequences. For the purposes of this study, animals were inoculated with a molecular clonal virus consisting of a unique viral sequence of cDNA. The interferon sensitivity determining region sequences recovered from an animal infected with clonal inoculum (Tup.8 at 103 weeks postinoculation) were found to be heterogeneous, with

a few amino acid substitutions (K2212M for 2/10 cases, L2232P for 1/10 cases, and L2253S for 6/10 cases) (see Fig. S2E in the supplemental material). Interestingly, the codon for amino acid 2224 encodes valine, but it was found to be variant for alanine and valine in sequences from the original patient serum (HCR6). Tupaia infected with patient serum also exhibited variability at position 2224; valine occupancy was rare, as was seen in the original HCR6 population (see Fig. S2B and C in the supplemental material). On the other hand, this position was occupied solely by valine for sequences recovered from Tup.8 (see Fig. S2E in the supplemental material), indicating that genetic variations shown for Tup.8 originated from the pHCR6 cDNA sequence. Taken together, quasispecies detection of circulating virus represents further evidence demonstrating intrinsic replication of HCV in tupaia despite low levels and infrequent detection of viremia.

DISCUSSION

In the present study, we described persistent HCV infection in tupaia. Long-term follow-up was performed and revealed histological progression of HCV-related liver disorders in infected tupaia, including steatosis, fibrosis, and cirrhosis, in addition to acute and chronic hepatitis. HCV genomic RNA was detected in animal sera intermittently throughout the entire course of infection. However, HCV RNA was detected in the liver upon sacrifice (3 years postinoculation). Furthermore, HCV RNA in serum contained genomic variants that had diverged from the inoculated virus (see Fig. S1 and S2 in the supplemental material). These data strongly indicate an established persistent infection in the tupaia studied. All animals exhibited HCV viremia soon after inoculation, yet the viremia was intermittent and accompanied by relatively low RTD-PCR titers compared with equivalent human and chimpanzee infections. The discrepancy between humans and tupaia might be due to host-dependent differences in replication efficiency. Over the course of HCV infection in these tupaia, serum ALT profiles indicated repeated liver injury, probably due to host immune responses mediated by agents such as cytotoxic T lymphocytes rather than direct viral cytopathic effects.

In cases of tupaia infection, experimental inoculations rarely led to sustained viremia, which for most human cases lasts for the entire course of infection. Even the course of infection appeared transient and self-resolved. It seems likely that HCV replication is less compatible with the tupaia host environment. This possibility was substantiated by a previous report by Xu et al. (34), where tissue-cultured virions of cloned genotype 1b, referred to as HCVcc in the paper, could not cause chronic infection with sustained viremia in tupaia. Although HCVcc actually infected most of the inoculated tupaia (83%; 10/12), chronic infection was seen for only a fraction of them (20%; 2/10). In this study, we also tried to detect a humoral response to HCV core antigen. We found that tupaia sera were HCV positive for antibodies only at occasional time points, observable as intermittent steep responses (data not shown). Overall, sustained seroconversion was not seen in this study, probably because HCV propagation in vivo was so limited or well controlled by host immunity. Given that models of HCV propagation are severely limited, the most important and interesting finding of this study is the successful detection of HCV RNA in

livers of infected tupaia 3 years after inoculation, indicating that HCV persists in tupaia. Although the limited propagation of HCV in tupaia is a drawback of this model at the present time, the isolation of tupaia-adapted HCV may be feasible by performing multiple infection passages. This possibility is supported by both quasispecies development and successful reinfection.

The chimpanzee is the animal species most closely related to humans, and as a model, it has contributed significantly to our understanding of HCV infection and pathogenesis. However, reproducing HCV pathogenesis in humans or chimpanzees can take as long as 10 to 20 years. The chronically infected tupaia in the present study developed complicated liver disorders in a much shorter time. Using tupaia, with their relatively short life span (3 to 5 years in the laboratory), as a model of HCV infection, we can evaluate HCV pathogenesis and correlate senescence and duration of infection.

The recent development of a primary human hepatocyte xenograft-uPA/SCID mouse model opened up opportunities to test putative antivirals against HCV replication in vivo (10, 17). In this innovative model, human hepatocytes, which are transplanted into the lobe of a mouse liver, can support HCV replication effectively. As a result, the level of circulating HCV RNA is comparable to that of a human patient. However, this mouse model is immunodeficient, and thus, it lacks the interplay between host immunity and viral infection. Therefore, it does not provide a suitable platform for characterizing immune responses to HCV infection.

HCV infection in tupaia represents an important model of HCV infection, particularly for the study of key determinants controlling virus propagation in vivo. The pathogenesis of HCV infection can be substantially different among humans, chimpanzees, and tupaia, and the mechanisms governing these differences are of great interest. Comparative studies of HCV infection in these different species will help us to understand the basic mechanisms of persistent infection.

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Hepatitis C Virus Impairs p53 via Persistent Overexpression of 3 β -Hydroxysterol Δ 24-Reductase^{*[5]}

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Tomohiro Nishimura^{†§1}, Michinori Kohara^{¶1}, Kosuke Izumi^{||}, Yuri Kasama[‡], Yuichi Hirata[¶], Ying Huang^{||2}, Masahiro Shuda^{¶3}, Chise Mukaidani^{**}, Takashi Takano[¶], Yuko Tokunaga[‡], Hideko Nuriya[¶], Masaaki Satoh[‡], Makoto Saito[‡], Chieko Kai^{||}, and Kyoko Tsukiyama-Kohara^{†‡4}

From the [†]Department of Experimental Phylaxiology, Faculty of Medical and Pharmaceutical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto, Kumamoto 860-8556, the [‡]Chemo-Sero-Therapeutic Research Institute, Kikuchi Research Center, Kyokushi, Kikuchi, Kumamoto 869-1298, the [¶]Department of Microbiology and Cell Biology, Tokyo Metropolitan Institute of Medical Science, 1-6 Kamikitazawa 2-chome, Setagaya-ku, Tokyo 156-8506, the ^{||}Laboratory Animal Research Center, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokane-dai, Minato-ku, Tokyo 108-8639, and the ^{**}Study Service Department, PhoenixBio Company, Ltd., 3-4-1 Kagamiyama, Higashi-Hiroshima 739-0046, Japan

Persistent infection with hepatitis C virus (HCV) induces tumorigenicity in hepatocytes. To gain insight into the mechanisms underlying this process, we generated monoclonal antibodies on a genome-wide scale against an HCV-expressing human hepatoblastoma-derived cell line, RzM6-LC, showing augmented tumorigenicity. We identified 3 β -hydroxysterol Δ 24-reductase (DHCR24) from this screen and showed that its expression reflected tumorigenicity. HCV induced the DHCR24 overexpression in human hepatocytes. Ectopic or HCV-induced DHCR24 overexpression resulted in resistance to oxidative stress-induced apoptosis and suppressed p53 activity. DHCR24 overexpression in these cells paralleled the increased interaction between p53 and MDM2 (also known as HDM2), a p53-specific E3 ubiquitin ligase, in the cytoplasm. Persistent DHCR24 overexpression did not alter the phosphorylation status of p53 but resulted in decreased acetylation of p53 at lysine residues 373 and 382 in the nucleus after treatment with hydrogen peroxide. Taken together, these results suggest that DHCR24 is elevated in response to HCV infection and inhibits the p53 stress response by stimulating the accumulation of the MDM2-p53 complex in the cytoplasm and by inhibiting the acetylation of p53 in the nucleus.

Hepatitis C virus (HCV)⁵ is composed of a single-stranded RNA genome of positive polarity (1). Translation of viral pro-

teins is initiated from an internal ribosome entry site (2) and results in a single polypeptide that is subsequently cleaved by host and viral proteases to yield viable proteins (3). The HCV genome does not rely on canonical translation factors and can readily establish chronic infection without integrating into the host genome, resulting in hepatic steatosis and hepatocellular carcinoma (HCC) (4). More than 170 million people worldwide are infected with HCV (5); chronic HCV infection and aging are the major risk factors for HCC (6–8). Liver cancer is the fifth most common cause of cancer mortality worldwide (9). The frequent inactivation of p53 in human HCC suggests that the loss of p53-dependent apoptosis may promote hepatocarcinogenesis (10). Chronic HCV infection results in chronic liver inflammation and induces endoplasmic reticulum stress and oxidative stress, which are thought to induce hepatocarcinogenesis (11, 12). The mechanistic details underlying HCC development are not fully understood. To gain insight into the molecular mechanisms underlying HCV-induced pathogenesis, we previously established RzM6 cells (13), a human hepatoblastoma (HepG2)-derived cell line in which expression of the full-length HCV genome is controlled by a Cre/loxP system. Expression of the HCV genome promoted anchorage-independent growth of RzM6 cells after 44 days of culture from the onset of HCV expression (RzM6-44d cells) but not in RzM6 cells after 0 days (RzM6-0d cells) (13). In the present study, we generated monoclonal antibodies against RzM6 cells cultured for longer than 44 days (RzM6-LC cells) and then screened the antibodies for their ability to bind antigens overexpressed in these cells. We identified 3 β -hydroxysterol Δ 24-reductase (DHCR24) from this screen and characterized its role in the HCV-induced cell growth deregulation.

EXPERIMENTAL PROCEDURES

Cells, Growth Assay, and Plasmids—HepG2 human hepatoblastoma cells, HuH-7 human hepatoma cells, WRL68 human embryonic hepatic cells, HEK293 human embryonic kidney cells, and human WI38 fibroblast cells were purchased from the American Type Culture Collection. NIH3T3 mouse fibroblast cells were from Japanese Collection of Research Bioresource. Cells were cultured under the growth conditions described in the supplemental material. RzM6 cells were established by

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¹ Both authors contributed equally to this work.

² Present address: Liver Diseases Branch, NIDDK, National Institutes of Health, Bethesda, MD 20892.

³ Present address: Molecular Virology Program, University of Pittsburgh Cancer Institute, University of Pittsburgh, 5117 Centre Ave., Pittsburgh, PA 15213.

⁴ To whom correspondence should be addressed. Tel.: 81-96-373-5560; Fax: 81-96-373-5560; E-mail: kkohara@kumamoto-u.ac.jp.

⁵ The abbreviations used are: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; siRNA, small interfering RNA; HA, hemagglutinin; ELISA, enzyme-linked immunosorbent assay; HBV, hepatitis B virus.

Supplemental Material can be found at:
<http://www.jbc.org/content/suppl/2009/11/13/M109.043232.DC2.html>
<http://www.jbc.org/content/suppl/2009/10/27/M109.043232.DC1.html>

Impairment of p53 by HCV through DHCR24 Overexpression

transfection of HepG2 cells with the plasmid *HCR6-Rz*, which contains the full-length HCV cDNA (nucleotides 1–9611; GenBankTM accession number AY045702), and stably transformed cell lines were selected in media containing G418 (800 μ g/ml bioactive; Invitrogen). These cell lines, termed 2–18, were then transfected with pCAG-Mer-Cre-Mer (Cre/*loxP* system) and were selected in media containing puromycin (Sigma), as described previously (13), to generate the RzM6 cell line. HCV expression was induced by treatment with 4-hydroxy-tamoxifen (100 nM). Cells expressing HCV for 44 days (RzM6-44d cells) displayed augmented anchorage-independent cell growth. Cells expressing HCV for more than 44 days are referred to as RzM6-LC cells.

The tumor formation assay was performed by injecting RzM6-0d, RzM6-44d, or RzM6-LC cells in the exponential growth phase into nude mice. Cells in culture were harvested with trypsin, and 2×10^6 or 1×10^7 cells were subcutaneously injected into the backs of athymic nude mice (ICR strain, Charles River). HepG2 and WRL68 cells with plasmid DNA or small interfering RNA (siRNA) were transiently transfected using Lipofectamine 2000 or RNAi Max (Invitrogen). HepG2 cells transfected with the pcDNA3.1-based HA- and FLAG-tagged DHCR24 expression vector were selected in media containing 800 μ g/ml G418 (Invitrogen). The terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling assay was performed using the TMR red *in situ* cell death detection kit (Roche Applied Science).

Generation of Monoclonal Antibodies—BALB/c mice received seven or eight intraperitoneal injections of RzM6-44d cells (5×10^6 cells/injection) in RIBI adjuvant (trehalose dimycolate + monophosphoryl lipid A emulsion; RIBI ImmunoChemResearch) at 3–4-week intervals. At the end of this immunization regimen, the spleens were removed, and the splenocytes were fused with mouse myeloma PAI cells using polyethylene glycol 1500 (Roche Applied Science), as described previously (14). Hybridoma cells were selected in medium containing hypoxanthine, aminopterin, and thymidine (Invitrogen), and culture supernatants were collected for whole-cell enzyme-linked immunosorbent assay (ELISA) screening.

ELISA, Immunostaining, Northern Blotting, Western Blotting, and Immunoprecipitation—Immunofluorescence assays, whole-cell ELISA, standard ELISA, and immunostaining are described in the supplemental materials. Northern blotting was performed as described previously (13). For immunoprecipitation and Western blotting, frozen specimens were homogenized on ice using a Dounce homogenizer fitted with a type-A pestle (Wheaton Science Products) in radioimmune precipitation buffer (1% SDS, 0.5% (v/v) Nonidet P-40, 0.15 M NaCl, 10 mM Tris, pH 7.4, 5 mM EDTA, and 1 mM dithiothreitol). Western blotting was performed as previously described (13) with the following primary antibodies: anti-DHCR24 monoclonal antibody 2-152a, polyclonal anti-p53 (Cell Signaling Technologies), anti-MDM2 (murine double minute clone 2 oncoprotein) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-Myc (9E10) (Santa Cruz Biotechnology, Inc.), and anti-HCV core protein monoclonal antibody 515 (15) or 31-2. Anti-p53 monoclonal antibody (DO-1) and polyclonal antibody (FL-393) (Santa Cruz Biotechnology, Inc.) were used for immuno-

staining. Phosphorylation of p53 was characterized by Western blotting with antibodies against phosphorylated Ser⁶, Ser⁹, Ser¹⁵, Ser²⁰, Ser³⁷, Ser⁴⁶, and Ser³²⁹ (Cell Signaling Technologies). Acetylation of p53 was examined by immunoprecipitation with anti-p53 (DO-1) and the ExactaCruz immunoprecipitation reagent (Santa Cruz Biotechnology, Inc.), followed by Western blotting with antibodies against p53 (rabbit polyclonal; Cell Signaling) or acetylated p53 (Lys³⁷³/Lys³⁸²) (Upstate Biotechnology). The interaction between p53 and MDM2 was examined by immunoprecipitation with anti-p53 (FL-393) or anti-MDM2 (H221; Santa Cruz Biotechnology, Inc.) and protein A-Sepharose (GE Healthcare), followed by Western blotting with monoclonal antibodies against MDM2 (SMP14, Santa Cruz Biotechnology, Inc.) and p53 (DO-1), respectively. Polyclonal anti-actin (Santa Cruz Biotechnology, Inc.), anti-histone H1 (Santa Cruz Biotechnology, Inc.), and anti-heat shock protein 70 (HSP70) (Stressgen) primary antibodies were used for normalization of Western blots. Subcellular fractionation of RzM6-0d and LC cells was performed as previously described (15).

Cloning and Expression of DHCR24 and *In Vitro* Translation—Total RNA was isolated from 1×10^6 HuH-7 cells using ISOGEN Reagent (Nippon Gene). Purified RNA (2 μ g) was reverse-transcribed with Superscript II (Invitrogen) using random primers according to the manufacturer's protocol. The DHCR24 cDNA was then amplified by PCR with Phusion DNA polymerase (BioLabs). The following primers were used for the first round of amplification: D-5-1 (5'-CCCGGGCTGTGGGCTACAGG-3', forward) and D-3-1 (5'-CCAGGCCACTTTT-ATTAAAA-3', reverse). Primers for the second round of amplification were D-5-2 (5'-GTTCTCGAGCAGTGA-CAGGAGGCGCGAAC-3', forward) and D-3-2 (5'-GTTC-TCGAGTCCAGGCGGGCTCCAGCTCA-3', reverse). The amplified DHCR24 cDNA was subcloned into the pGEM-T easy vector (Promega). *In vitro* translation was performed using TNT(R) reticulocyte lysate (Promega) and the Express Protein Labeling Mix (New England Nuclear) in the presence of either [³⁵S]Met/Cys or non-radioactive methionine. Amplified DHCR24 cDNA was digested with XhoI and subcloned into the pCAG-PURO vector (16) for transfection into WRL68 cells using Lipofectamine 2000. Amplified DHCR24 cDNA was also subcloned into the pcDNA3.1 vector containing an HA or FLAG tag (kindly supplied by Dr. N. Takahashi, Tokyo University of Agriculture and Technology) for transfection into RzM6 or HepG2 cells using Lipofectamine LTX (Invitrogen). Transfected cells were selected in media containing G418.

The lentiviral vector, pCSII-EF-MCS-EMCV IRES-GFP (generous gift from Hiroyuki Miyoshi, RIKEN, Tsukuba, Japan), was modified by replacing the green fluorescent protein gene with the hygromycin phosphotransferase gene to construct pCSII-EF-MCS-EMCV IRES-Hygro. DHCR24 fused to the 5'-HA or 5'-FLAG tag-encoding sequence were cloned under the EF promoter. The resulting plasmid was cotransfected with packaging plasmid (pCAG-HIVgp and pCMV-VSVG-RSV-Rev) in 293FT cells (Invitrogen) to produce recombinant lentivirus. Following infection, cells were selected with hygromycin B (600 μ g/ml; Sigma).

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<http://www.jbc.org/content/suppl/2009/10/27/M109.043232.DC1.html>

Impairment of p53 by HCV through DHCR24 Overexpression

Silencing of DHCR24 and HCV by siRNA—The DHCR24 stealth siRNA was designed to target the human DHCR24 mRNA sequence 5'-GCAAGCUGAAUAGCAUUGGCAAUUA-3' (nucleotides 970–993) using the BLOCK-iT RNAi designer (Invitrogen). A mutated siRNA (5'-GCAGUCUAACGAUACGGAAGUUA-3') was synthesized as a control. An alternative siRNA, siDHCR24-1024, was designed as 5'-GAGAACUAUCUGAAGACAATT-3'. The HCV siRNA was synthesized as previously described (17). Cells were transfected with a 1 nM concentration of the chemically synthesized siRNAs using Lipofectamine 2000 or Lipofectamine RNAiMAX (Invitrogen) in Opti-MEM (Invitrogen) and then incubated for 4–6 h at 37 °C. Cells were characterized 48 h after transfection.

Caspase and Reporter Assays—Cells (1×10^4 cells/well) were seeded into white 96-well plates (Sumitomo Bakelite), treated with 1 mM H_2O_2 , and then lysed with caspase-Glo 3/7 buffer (Promega). Caspase 3/7 activity was determined by measuring the absorbance resulting from cleavage of a luminescent substrate containing the sequence DEVD (Promega) using a multilabel counter (PerkinElmer Life Sciences). The p21^{WAF1/CIP1} promoter activity was assayed in HepG2, RzM6-0d, or RzM6-LC cells transfected with pWWP-Luc (kindly supplied by Dr. Bert Vogelstein (The Johns Hopkins University)). Cells were cotransfected with phRL-TK(Int-) (Promega) for normalization of promoter activity. Cells were incubated for 2 days after transfection and were then treated with 1 mM H_2O_2 for 4 h. Promoter activity was measured using the Dual-Luciferase reporter assay system (Promega).

HCV Infection of Humanized Chimeric Mouse Liver and mRNA Quantification by Quantitative Reverse Transcription-PCR—Detailed procedures are described in the supplemental material.

Statistical Analysis—Student's *t* test was used to test the statistical significance of the results. *p* values less than 0.05 were considered statistically significant.

RESULTS

Expression of DHCR24 Parallels Hepatocarcinogenesis—RzM6 cells expressing full-length HCV were established using the Cre/loxP expression-switching system (13). RzM6 cells cultured for longer than 44 days (termed RzM6-LC) had a greater ability to form colonies (13) and to induce tumors in nude mice (Fig. 1A). We produced monoclonal antibodies against RzM6-LC cells (see supplemental materials) and screened them for their ability to bind RzM6 antigens overexpressed upon the onset of hepatocarcinogenesis. Antibody clone 2-152a bound to a ~60-kDa protein (p60) that was expressed at higher levels in RzM6-LC cells than in RzM6 cells before the onset of HCV expression (termed RzM6-0d) (13) (Fig. 1B). p60 was strongly expressed in hepatoma-derived HuH-7 cells but was less abundant in the less aggressive cancer cell line, HepG2, or in the normal embryonic cell lines WRL68, HEK293, or NIH3T3 (supplemental Fig. 1A). To identify p60, the protein was purified from RzM6-LC cells using immunoaffinity chromatography and subjected to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (supplemental Fig. 1B). Through this process, p60 was identified as DHCR24 (also

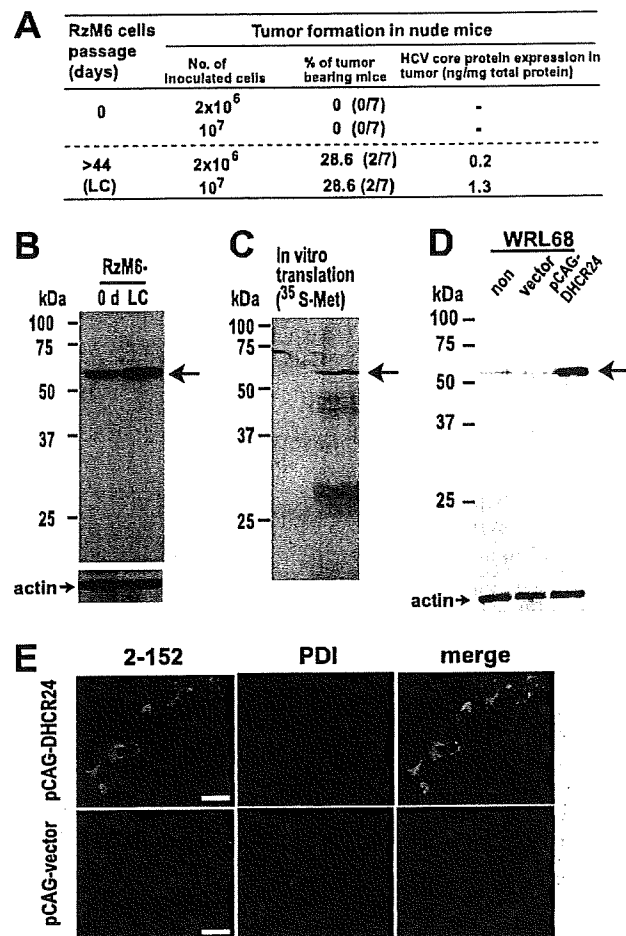


FIGURE 1. Analysis of RzM6 cell tumorigenicity and identification of DHCR24 overexpression in RzM6-LC cells. A, summary of tumor formation in nude mice injected with RzM6-0d or RzM6-LC cells. B, detection of a ~60-kDa protein (arrow, upper panel) in whole-cell lysates (30 μ g/lane) from RzM6-0d and RzM6-LC cells by Western blotting with monoclonal antibody 2-152a. Anti-actin antibody was used for normalization (arrow, lower panel). Data are representative of two independent experiments. C, empty vector (pGEM3, left lane) or pGEM-DHCR24 (right lane) was subjected to *in vitro* transcription/translation using rabbit reticulocyte lysates in the presence of [³⁵S]methionine. Samples were subjected to SDS-PAGE followed by autoradiography. D, cell lysates from untransfected WRL68 cells (non), WRL68 cells transfected with empty pCAG vector (vector), and WRL68 cells transfected with pCAG-DHCR24 vector were examined by Western blotting with monoclonal antibody 2-152a (upper panel) or monoclonal anti-actin antibody (lower panel). E, WRL68 cells were transfected with pCAG-DHCR24 or pCAG vector alone and subjected to immunocytochemistry with monoclonal antibody 2-152a (green) or anti-protein-disulfide isomerase antibody (PDI; red). Scale bars, 25 nm.

known as seladin-1) (18–20), an enzyme that catalyzes the reduction of the Δ -24 double bond of sterol intermediates during cholesterol biosynthesis and is up-regulated by oxidative stress (19, 21). DHCR24 cDNA was cloned and translated *in vitro* (Fig. 1C) and also expressed in human embryonic hepatic WRL68 cells (Fig. 1D), and the monoclonal antibody 2-152a reacted with the expressed protein (Fig. 1, D and E).

HCV Induces Persistent DHCR24 Overexpression—Since DHCR24 was up-regulated in RzM6-LC cells, we examined whether HCV can induce DHCR24 expression in human hepatocytes. We also compared the expression levels of DHCR24

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Impairment of p53 by HCV through DHCR24 Overexpression

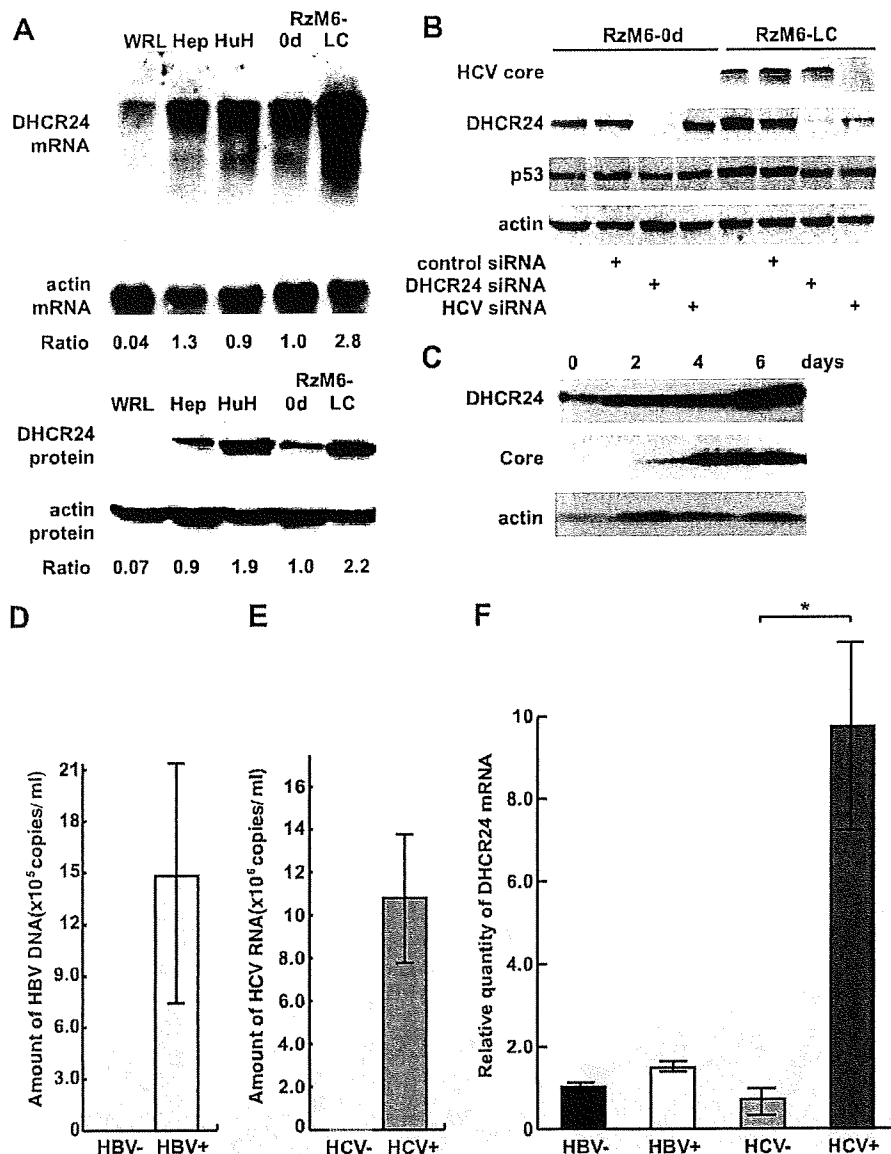


FIGURE 2. Induction of DHCR24 by HCV in human liver cell lines. *A*, expression of DHCR24 mRNA (Northern blot, upper panel) and protein (Western blot, lower panel) in WRL68 (WRL), HepG2 (Hep), HuH-7 (HuH), RzM6-0d, and RzM6-LC cells. Ratios indicate the amount of DHCR24 mRNA or protein in each cell type (quantified by densitometry) relative to that of RzM6-0d cells. *B*, Western blotting of HCV core protein, DHCR24, and p53 (DO-1 antibody) proteins in RzM6-0d and RzM6-LC cells following treatment with the indicated (+) siRNA. *C*, the induction of DHCR24 by HCV after 2, 4, and 6 days in RzM6 cells by treatment of tamoxifen (100 nM). Data are representative of two independent experiments, and anti-actin antibody was used as a loading control (*A–C*). *D*, amount of HBV DNA in HBV-infected human hepatocytes in chimeric mouse liver quantitated by RTD-PCR. *E*, amount of HCV RNA in HCV-infected human hepatocytes in chimeric mouse liver quantitated by RTD-PCR. *F*, the quantitation of DHCR24 mRNA in mock-infected, HBV-infected, and HCV-infected human hepatocytes in chimeric mouse liver. Vertical bars, S.D.; *, $p < 0.05$ (two-tailed Student's t test).

protein and mRNA in a panel of hepatic and embryonic cell lines (Fig. 2*A*). Northern blotting revealed that DHCR24 mRNA expression was notably higher in RzM6-LC cells than in RzM6-0d cells, indicating that induction of DHCR24 occurs at the transcriptional level. DHCR24 protein levels were also higher in HuH-7 and RzM6-LC cells relative to RzM6-0d cells (Fig. 2*A*). To examine whether persistent up-regulation of DHCR24 in RzM6-LC cells resulted from HCV expression, we utilized an siRNA to knockdown HCV expression (17) (Fig. 2*B*).

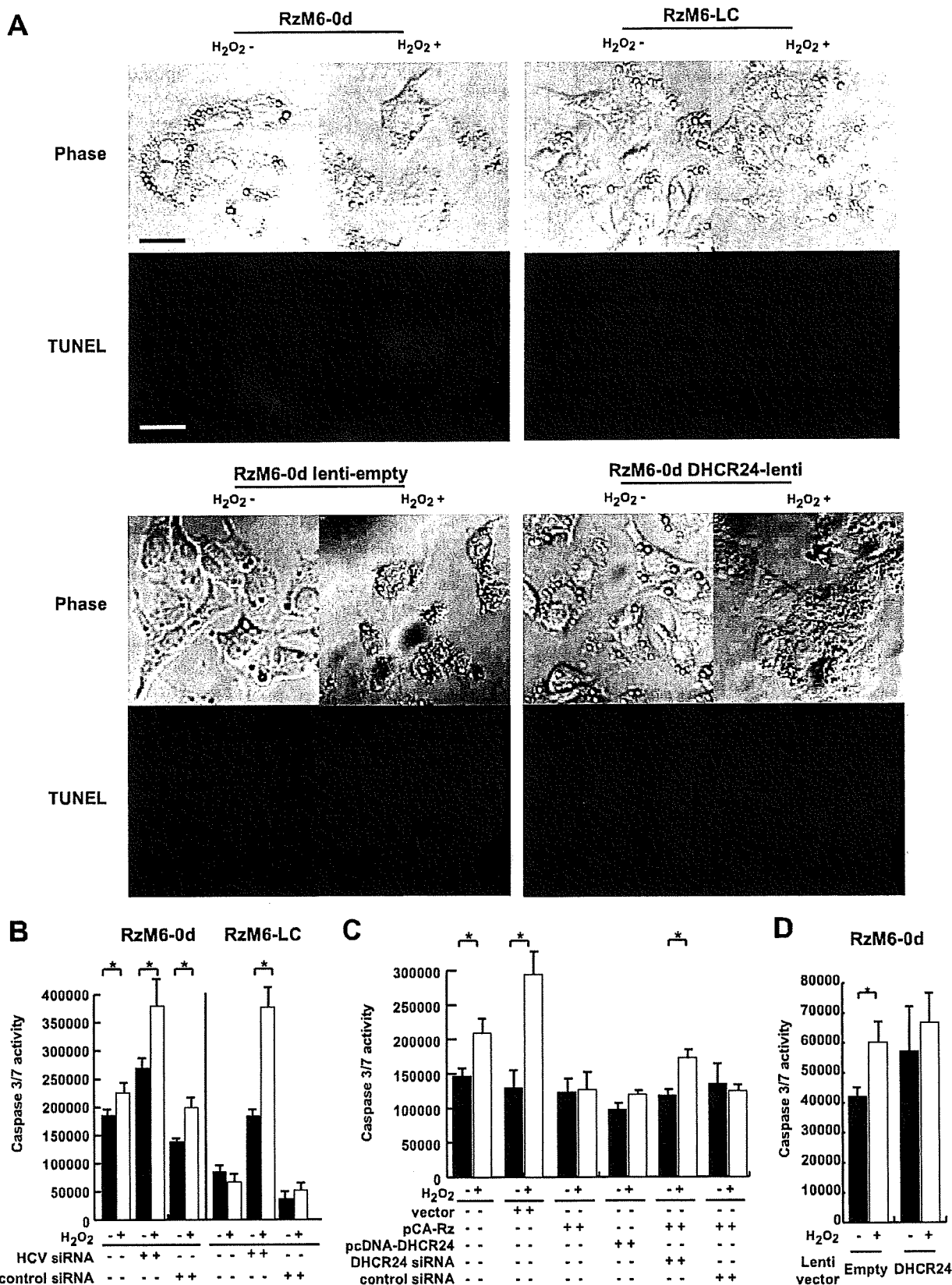
Silencing HCV by greater than 99% with siRNA reduced the expression of DHCR24 and p53 in RzM6-LC cells. When we induced HCV expression with tamoxifen, the induction of DHCR24 was observed after 2, 4, and 6 days (Fig. 2*C*). These results indicate that expression of the full-length HCV genome induced DHCR24 overexpression. DHCR24 was not induced in HuH-7 cells infected with the HCV strain JFH-1 (22) (data not shown). This result might be explained by the substantial endogenous expression of DHCR24 in HuH-7 cells (Fig. 2*A* and supplemental Fig. 1*A*). To examine whether HCV infection can induce DHCR24, human hepatocytes in chimeric mice were infected with hepatitis B virus (HBV) or HCV (Fig. 2, *D* and *E*). Notable up-regulation of DHCR24 mRNA was detected in HCV-infected human hepatocytes but not in HBV-infected human hepatocytes (Fig. 2*F*).

Persistent Overexpression of DHCR24 Induces Apoptotic Resistance to Oxidative Stress—As HCV infection increased the expression of DHCR24, we further examined the effect of DHCR24 on hepatocytes. Because DHCR24 regulates oxidative stress-induced apoptosis (19, 21, 23, 24), the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling assay was performed with RzM6 cells to examine the effect of DHCR24 overexpression on H_2O_2 -induced apoptosis (Fig. 3*A*). Fragmentation of genomic DNA was less pronounced in DHCR24-overexpressing cells (RzM6-LC cells and RzM6-0d cells transduced with DHCR24 lentivirus) than in RzM6-0d cells or RzM6-0d cells transduced with empty lentiviral vector. To quantify

the apoptotic response, we examined the effect of DHCR24 overexpression on caspase activity (Fig. 3, *B–D*). Caspase activation by H_2O_2 was suppressed in RzM6-LC cells compared with RzM6-0d cells (Fig. 3*B*). Transfection with HCV siRNA recovered the caspase response in RzM6-LC cells. Caspase 3/7 activity was also examined following the transfection of HepG2 cells with pCA-Rz (Fig. 3*C*). Induction of caspase activation by H_2O_2 was inhibited by expression of the HCV gene; the inhibition was partially recovered by transfection with DHCR24

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Impairment of p53 by HCV through DHCR24 Overexpression



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Impairment of p53 by HCV through DHCR24 Overexpression

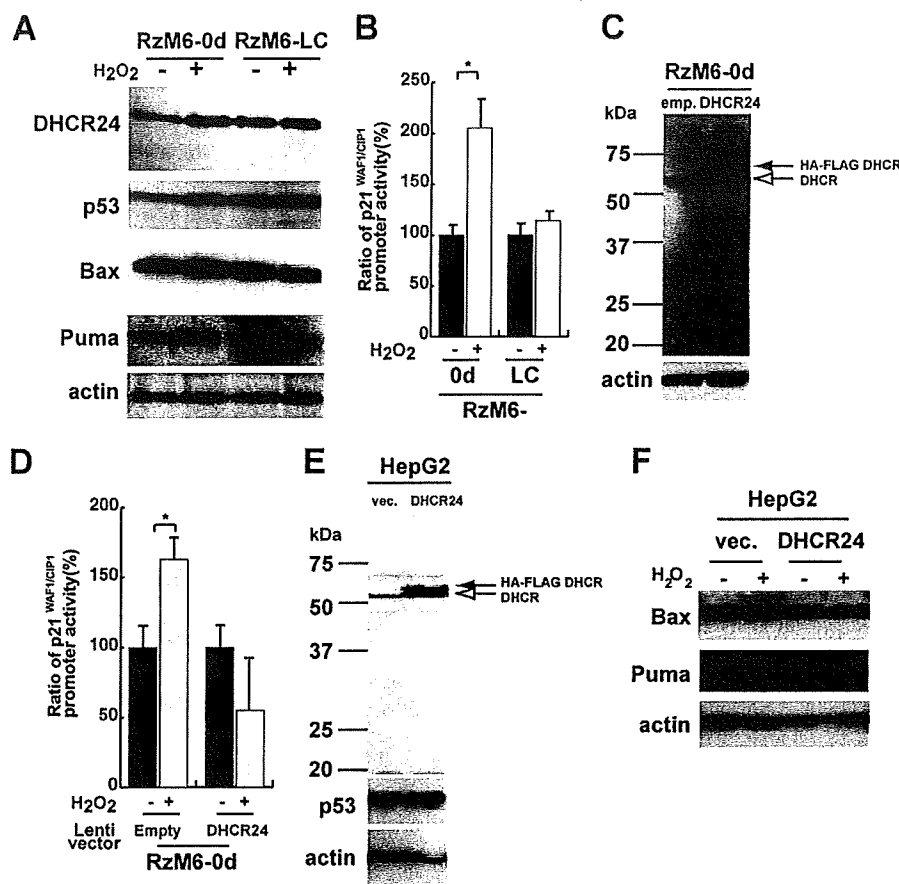


FIGURE 4. Persistent overexpression of DHCR24 impairs p53 activity. A, Western blotting of RzM6-0d and RzM6-LC cells treated with or without H₂O₂ was performed using antibodies against the proteins indicated on the left. B, p21^{WAF1/CIP1} promoter activity in RzM6-0d and RzM6-LC cells transfected with pWWP-Luc followed by treatment with or without H₂O₂. Promoter activity was calculated as the ratio of firefly luciferase activity to *Renilla* luciferase activity. The ratio of promoter activity with H₂O₂ treatment to without H₂O₂ treatment is indicated as a percentage. C, DHCR24 expression in RzM6-0d cells transfected with lenti-empty (*emp.*) or lenti-DHCR24 (with HA-FLAG tag) vector. D, p21^{WAF1/CIP1} promoter activity in RzM6-0d cells transfected with lenti-empty or lenti-DHCR24 vector followed by treatment with or without H₂O₂. Promoter activity was calculated as the ratio of firefly luciferase activity to *Renilla* luciferase activity. The ratio of promoter activity with H₂O₂ treatment to activity without H₂O₂ treatment is indicated as a percentage. E, HepG2 cells transfected with pcDNA vector (*vec.*) or DHCR24 expression vector (pcDNA-DHCR24 HA-FLAG tag; *DHCR24*) and selected by G418 were analyzed by Western blotting with DHCR24 monoclonal antibody 2-152a (*top*), p53 monoclonal antibody (DO-1) (*middle*), and actin (*bottom*). F, expression of Bax and Puma in HepG2 cells transfected with pcDNA vector (*vec.*) or pcDNA-DHCR24 vector followed by treatment with or without H₂O₂ was analyzed by Western blotting with antibodies to the proteins indicated at the left. Actin was analyzed as a control. In A–F, representative data from three independent experiments are shown; *, *p* < 0.05 (two-tailed Student's *t* test).

siRNA. Moreover, overexpression of DHCR24 from a lentivirus vector (Fig. 3D) (25) or from transfection with a DHCR24 expression vector (data not shown) impaired H₂O₂-induced caspase activation in RzM6-0d cells. Thus, enhanced expression of DHCR24 promotes resistance to H₂O₂-induced apoptotic responses, and HCV-induced apoptotic resistance is partially mediated by DHCR24.

and MDM2 was stronger in RzM6-LC cells than in RzM6-0d cells (Fig. 5, A and B). Lentiviral vector overexpression of DHCR24 in RzM6-0d cells increased the binding of p53 to MDM2 (data not shown). Furthermore, cell fractionation analysis revealed that the interaction between MDM2 and p53 mostly occurred in the cytoplasmic fraction, even after H₂O₂

Persistent Overexpression of DHCR24 Inhibits p53 Activity—H₂O₂ induces p53-dependent apoptosis (26). Expression of the p53-induced apoptotic response mediators, Bax (27) and Puma (28), did not increase after H₂O₂ treatment of RzM6-LC cells (Fig. 4A). Therefore, we examined p53 expression and function in DHCR24-overexpressing cells. H₂O₂ increased the expression of DHCR24 and p53 in RzM6-0d cells. By comparison, the expression of these proteins was already elevated prior to H₂O₂ treatment in RzM6-LC cells, and we found no further H₂O₂-induced increase in expression (Fig. 4A). Consistent with these findings, H₂O₂ activated transcription from the p21^{WAF1/CIP1} promoter in RzM6-0d cells but not in RzM6-LC cells (Fig. 4B). This response of the p21^{WAF1/CIP1} promoter is impaired in cells overexpressing DHCR24 via the lentivirus vector (Fig. 4, C and D) or via the expression vector (Fig. 4E). Induction of Bax and Puma expression after H₂O₂ treatment was decreased in DHCR24-overexpressing cells (Fig. 4F). These results indicate that although p53 expression is elevated in DHCR24-overexpressing cells, the function of p53 in the oxidative stress pathway is impaired.

Overexpression of DHCR24 Enhances the Interaction between p53 and MDM2—Since DHCR24 is a regulator of the p53-MDM2 interaction (21), we examined the interaction between p53 and its specific ubiquitin ligase, MDM2. Unexpectedly, the interaction between p53

FIGURE 3. Prior overexpression of DHCR24 inhibits H₂O₂-induced apoptosis. A, representative phase-contrast images of RzM6-0d and RzM6-LC cells (*upper panels*) or lenti-empty and lenti-DHCR24 vector-transduced RzM6-0d cells (*lower panels*) treated with or without H₂O₂ (1 mM, 4 h). *In situ* cell death was detected by the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay with tetramethylrhodamine. Scale bars, 25 nm. Representative data from three experiments are shown. B, caspase 3/7 activity (relative light units) in RzM6-0d and RzM6-LC cells treated with or without H₂O₂. Cells were transfected with or without HCV siRNA or with control siRNA as indicated. C, caspase 3/7 activity in HepG2 cells treated with or without H₂O₂. Cells were transfected with control pcDNA vector (*vector*), pCA-Rz, pcDNA-DHCR24, DHCR24 siRNA, or control siRNA. D, caspase 3/7 activity in RzM6-0d cells treated with or without H₂O₂. Cells were transfected with lenti-empty or lenti-DHCR24 vector. In B–D, data reflect the means ± S.D. of three independent triplicate experiments; *, *p* < 0.05 (two-tailed Student's *t* test).

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Impairment of p53 by HCV through DHCR24 Overexpression

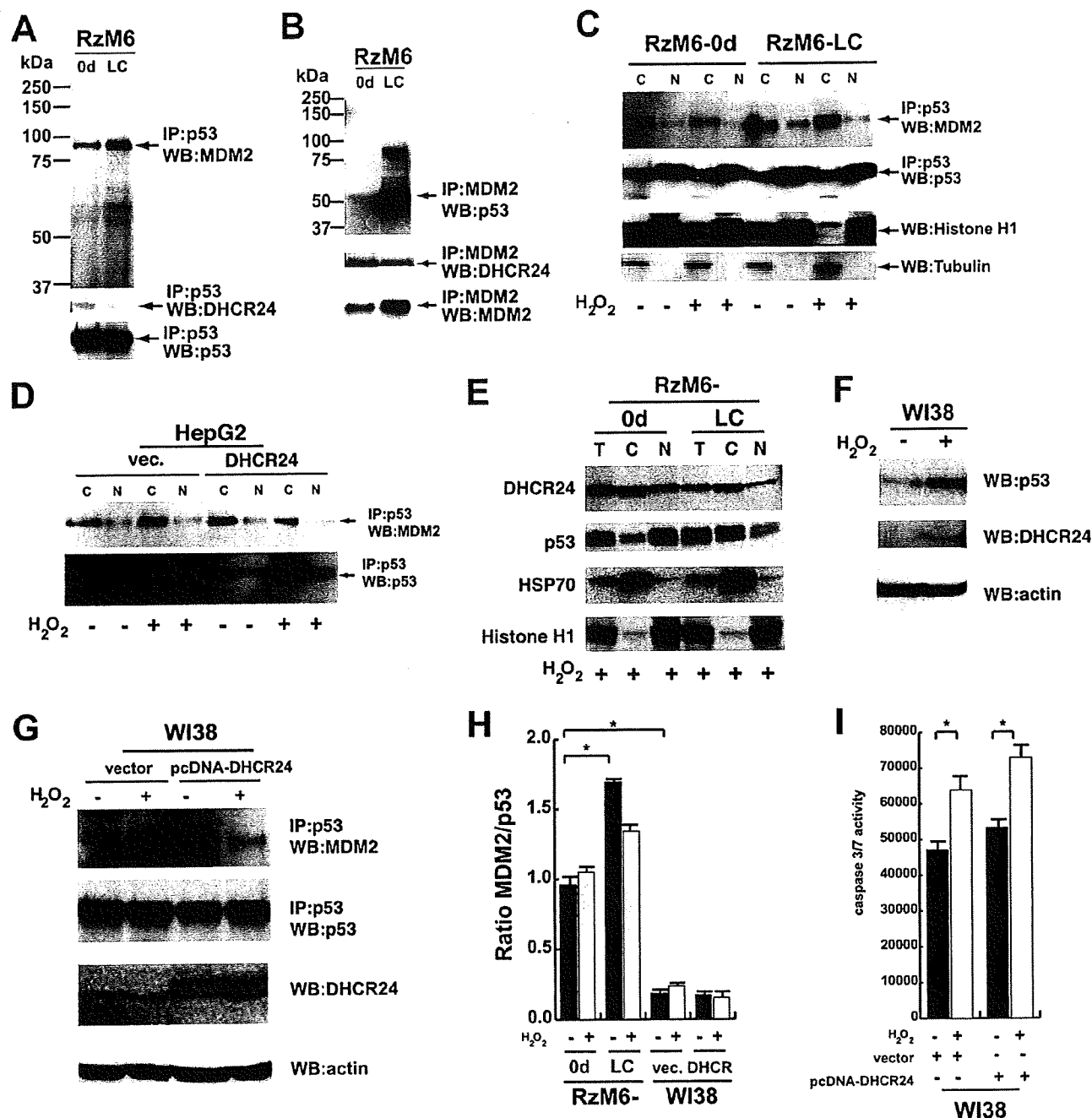
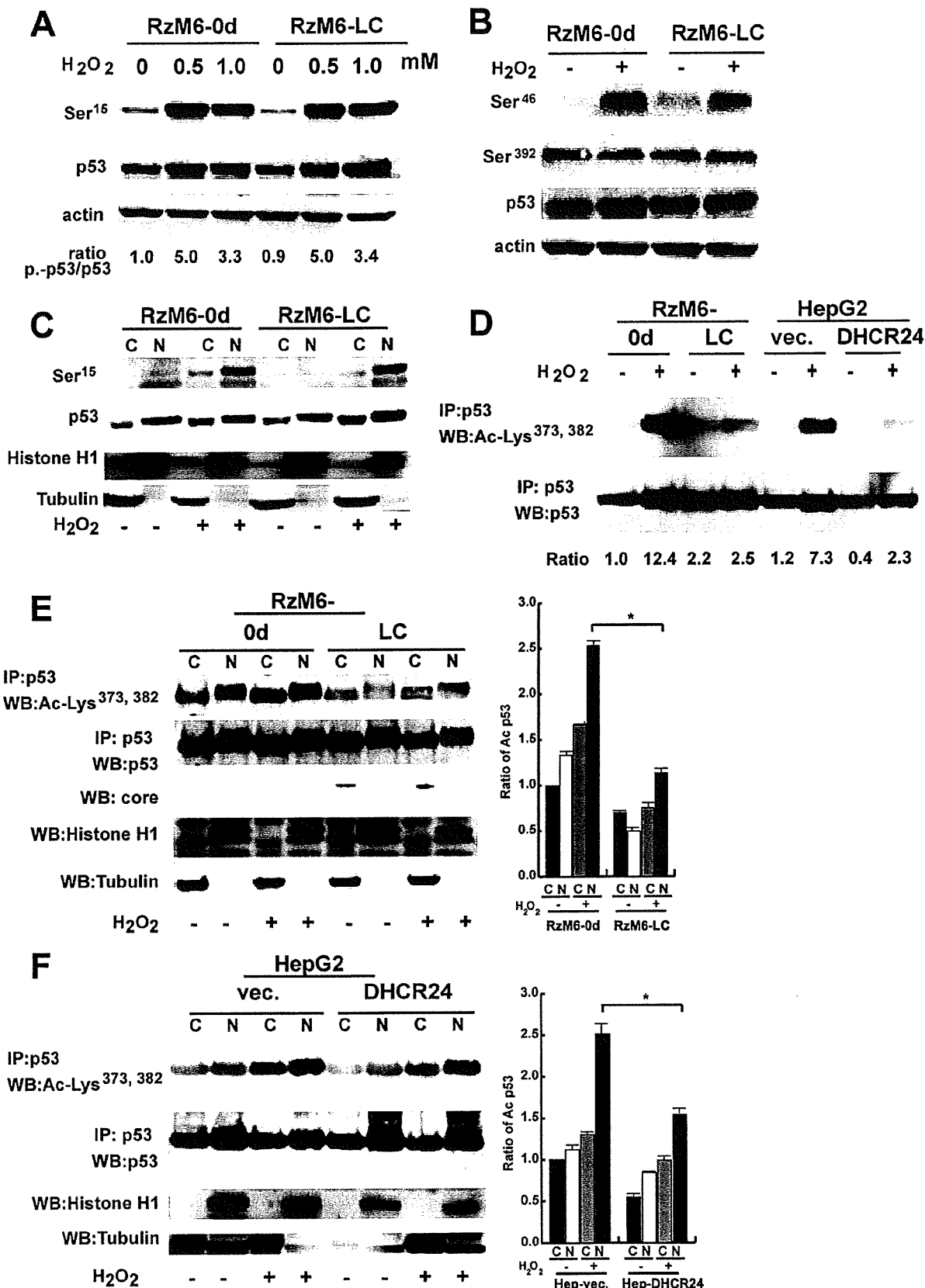


FIGURE 5. DHCR24 overexpression enhances the interaction between p53 and MDM2 in the cytoplasm. A, p53 was immunoprecipitated (IP) from RzM6-0d and RzM6-LC cells with polyclonal anti-p53 antibody (FL393) followed by Western blotting with monoclonal antibodies against MDM2 (top) and p53 or DHCR24 (bottom). B, MDM2 was immunoprecipitated from RzM6-0d and RzM6-LC cells using polyclonal anti-MDM2 antibody (H221) followed by Western blotting (WB) with monoclonal antibodies against p53 (top) and MDM2 or DHCR24 (bottom). C, p53 was immunoprecipitated from cytoplasmic (C) or nuclear (N) fractions of RzM6-0d and LC cells using anti-p53 antibody (FL393) followed by Western blotting with anti-MDM2 antibody (top) and anti-p53 antibody (bottom). Cell fractionation was confirmed by Western blotting with anti-histone H1 and tubulin (WB). D, p53 was immunoprecipitated from cytoplasmic or nuclear fractions of HepG2 cells transfected with control pcDNA (vec.) or pcDNA-DHCR24 expression vector using polyclonal anti-p53 (FL393) followed by Western blotting with antibodies against the proteins indicated at the right. Reaction with secondary antibodies (anti-rabbit or mouse IgG conjugated with horseradish peroxidase) alone did not show any signal (data not shown), and data representative of three independent experiments are shown (A–D and G). E, RzM6-0d or RzM6-LC cells with H_2O_2 treatment were fractionated into total (T), cytoplasmic, and nuclear fractions and subjected to Western blotting with the antibodies indicated on the left. F, Western blotting of WI38 cells with or without H_2O_2 treatment (0.5 mM, 4 h) with antibodies against p53, DHCR24, and actin. G, immunoprecipitation of p53 from cells transfected with pcDNA vector and pcDNA-DHCR24 (DHCR) with or without H_2O_2 treatment (0.5 mM, 4 h), followed by Western blotting with antibodies against MDM2 (first column) and p53 (second column). Cells were examined with Western blotting with anti-DHCR24 (third column) and anti-actin (fourth column). H, the average ratio of the quantified results of immunoprecipitation of p53 and Western blotting of MDM2 in RzM6-0d, RzM6-LC, and WI38 cells with transfection of pcDNA vector or pcDNA-DHCR24 with or without H_2O_2 treatment are indicated. Vertical bars, S.D. * $p < 0.05$ (two-tailed Student's *t* test). I, a caspase 3/7 assay was performed in WI38 cells with pcDNA vector (vector) and pcDNA-DHCR24 overexpression vector. Vertical bars, S.D. * $p < 0.05$ (two-tailed Student's *t* test).

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Impairment of p53 by HCV through DHCR24 Overexpression



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Impairment of p53 by HCV through DHCR24 Overexpression

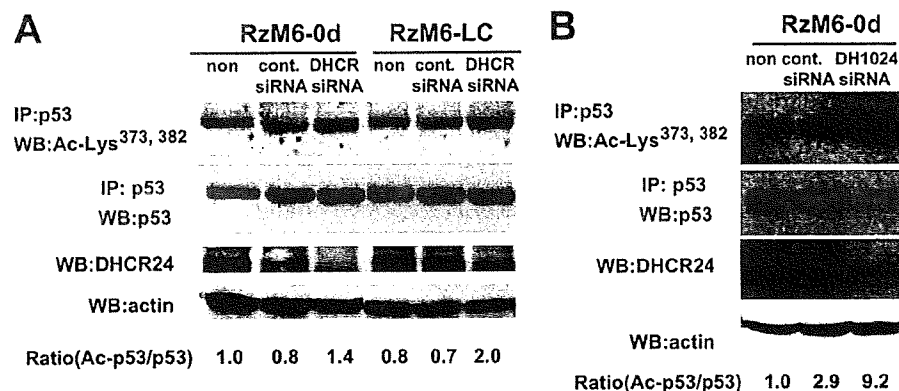


FIGURE 7. Silencing of DHCR24 increases p53 acetylation. A, RzM6-0d and RzM6-LC cells were untreated (non) or treated with control siRNA (cont. siRNA) or DHCR24 siRNA. Acetylated p53 Lys³⁷³ and Lys³⁸² (top) and total p53 (bottom) were immunoprecipitated (IP) with anti-p53 antibody (DO-1) followed by Western blotting (WB), and the expression of actin and DHCR24 by Western blotting (bottom) was examined. The average ratio of acetylated p53 to total p53 in RzM6-0d cells without treatment is indicated at the bottom. B, RzM6-0d cells were transfected with control siRNA (cont. siRNA), DHCR24 siRNA (DH1024 or DHCR), or untreated (non). Acetylated p53 Lys³⁷³ and Lys³⁸² (top) and total p53 (bottom) were immunoprecipitated with anti-p53 antibody followed by Western blotting, and the expression of actin and DHCR24 by Western blotting (bottom) was examined. Reaction with secondary antibodies (anti-rabbit or mouse IgG conjugated with horseradish peroxidase) alone did not yield any signals (data not shown), and data representative of three independent experiments are shown (A and B).

treatment (Fig. 5C). Silencing of DHCR24 with siRNA decreased the interaction between p53 and MDM2 (supplemental Fig. 2A). Overexpression of DHCR24 in HepG2 cells increased the interaction between p53 and MDM2 in the cytoplasm (HepG2-DHCR24; Fig. 5D and supplemental Fig. 2, B and C). Level of p53 in the nucleus after treatment with H₂O₂ was low in RzM6-LC cells (Fig. 5E).

In a previous *in vitro* study that used bacterially expressed and purified protein (21), the interaction between p53 and MDM2 was decreased when the amount of DHCR24 was increased. These discrepancies with our results may be due to the different experimental systems. p53 and DHCR24 were induced by H₂O₂ in WI38TERT cells (21), as observed in our system (Fig. 5F). However, the interaction between MDM2 and p53 was much lower in WI38 cells than in RzM6 cells (Fig. 5, G and H). Moreover, ectopic expression of DHCR24 did not inhibit apoptotic response to H₂O₂ in WI38 cells (Fig. 5I) but suppressed apoptosis in HepG2 cells (Fig. 3). This different response may be due to the different regulatory systems of p53 and MDM2 in the liver and lung; MDM2 phosphorylation at Ser¹⁶⁶ is regulated by the MEK-ERK (mitogen-activated protein

kinase/extracellular signal-regulated kinase kinase-extracellular signal-regulated kinase) pathway in hepatocytes and Akt in lung cells (29). The response of MDM2 at Ser¹⁶⁶ to H₂O₂ was significantly high in liver (HepG2) cells but was low in WI38 cells (supplemental Fig. 2, D and E), as previously observed in A549 cells (29). The up-regulation of MDM2 phosphorylation at Ser¹⁶⁶ accelerates its E3 ligase activity (30).

We also found that overexpression of DHCR24 did not up-regulate the transcription of p53 or MDM2 genes (supplemental Fig. 3, A and B). However, DHCR24 overexpression inhibited polyubiquitination in RzM6-LC cells and H358 (p53 null) cells (supplemental Fig. 3, C–E). This inhibition of polyubiquitination would inhibit p53 degradation,

resulting in an increased amount of p53 available to interact with MDM2.

Posttranslational Modification of p53 in DHCR24-overexpressing Cells—We did not detect any p53 nucleotide substitutions in RzM6-LC cells compared with the p53 in RzM6-0d cells (data not shown). Thus, we examined the posttranslational modification of p53, which is thought to be tightly connected to the regulation of its function and localization (31). We examined the phosphorylation of p53 at Ser¹⁵ (Fig. 6A); at Ser⁶, Ser⁹, Ser²⁰, and Ser³⁷ (supplemental Fig. 4A); and at Ser⁴⁶ and Ser³⁹² (Fig. 6B) by Western blotting and found no marked differences in phosphorylation after H₂O₂ treatment between RzM6-0d and RzM6-LC cells. Ser¹⁵-phosphorylated p53 was detected in the nuclear fraction of RzM6-0d and RzM6-LC cells after H₂O₂ treatment (Fig. 6C). We assessed the acetylation of p53 at Lys³⁷³ and Lys³⁸² by immunoprecipitation followed by Western blotting and found that acetylation of p53 was significantly low in RzM6-LC cells compared with RzM6-0d cells following H₂O₂ treatment (Fig. 6D). H₂O₂ induced the acetylation of p53 at Lys³⁷³ and Lys³⁸² in RzM6-0d cells (Fig. 6E). This acetylation

FIGURE 6. Posttranslational modification of p53 in cells overexpressing DHCR24. A, phosphorylation of p53 at Ser¹⁵ in RzM6-0d and RzM6-LC cells after treatment with 0, 0.5, or 1.0 mM H₂O₂ was examined by Western blotting with specific rabbit polyclonal antibodies. p53 was detected with anti-p53 monoclonal antibody (DO-1). Actin was analyzed as a control. B, phosphorylation of p53 at Ser⁴⁶ and Ser³⁹² in RzM6-0d and RzM6-LC cells after treatment with 0 or 1.0 mM H₂O₂ was analyzed by Western blotting with specific rabbit polyclonal antibodies. p53 was detected with anti-p53 monoclonal antibody. Actin was analyzed as a control. C, RzM6-0d or RzM6-LC cells with or without H₂O₂ treatment were fractionated into cytoplasmic (C) and nuclear (N) fractions that were subjected to Western blotting with the antibodies indicated on the left. D, acetylation of p53 Lys³⁷³ and Lys³⁸² (top) and total p53 (bottom) was characterized by immunoprecipitation (IP) with anti-p53 antibody followed by Western blotting (WB) with the rabbit polyclonal antibodies indicated on the left in RzM6-0d and RzM6-LC cells or HepG2 cells transfected with pcDNA vector (vec.) or pcDNA-DHCR24 expression vector (DHCR24). The average ratio of acetylated p53 to total p53 in RzM6-0d cells without H₂O₂ treatment is indicated at the bottom. E, acetylation of p53 Lys³⁷³ and Lys³⁸² (top) and total p53 (second panel) was characterized by immunoprecipitation with anti-p53 antibody followed by Western blotting with the rabbit polyclonal antibodies indicated on the left in RzM6-0d and RzM6-LC cells. HCV core protein was detected by Western blotting with monoclonal antibody (31, 32). Cell fractionation was confirmed with antibodies against histone H1 and tubulin. p53 and acetylated p53 were quantitated, and the average ratio of acetylated p53 to total p53 in the cytoplasmic (C) fraction of RzM6-0d cells is indicated. Vertical bars, S.D. *, *p* < 0.05 (two-tailed Student's *t* test). F, acetylation of p53 Lys³⁷³ and Lys³⁸² (top) and total p53 (second panel) was characterized by IP with anti-p53 antibody followed by Western blotting with the rabbit polyclonal antibodies indicated on the left in HepG2 cells transfected with pcDNA vector or pcDNA-DHCR24 vector with or without H₂O₂ treatment. Cell fractionation was confirmed with antibodies against histone H1 and tubulin. p53 and acetylated p53 were quantitated, and the average ratio of acetylated p53 to total p53 in the cytoplasmic fraction of Hep-vec cells is indicated. Vertical bars, S.D. *, *p* < 0.05 (two-tailed Student's *t* test). Reaction with secondary antibodies (anti-rabbit or mouse IgG conjugated with horseradish peroxidase) alone did not show any signals (data not shown), and data representative of three independent experiments are indicated (A–F).

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Impairment of p53 by HCV through DHCR24 Overexpression

was significantly inhibited in RzM6-LC cells (Fig. 6E). Persistent ectopic overexpression of DHCR24 also repressed H₂O₂-induced p53 acetylation in the nuclei of HepG2 cells (Fig. 6F). In addition, silencing of DHCR24 by siRNA caused up-regulation of p53 acetylation at Lys³⁷³ and Lys³⁸² in RzM6-0d and RzM6-LC cells (Fig. 7A). When the alternative siRNA for DHCR24 (DH1024) was transfected into RzM6-0d cells, the acetylation of p53 was accelerated (Fig. 7B). The mutant DHCR24 vector suppressed the effect of DHCR24 siRNA (supplemental Fig. 4B). These results indicate that overexpression of DHCR24 down-regulates p53 acetylation.

DISCUSSION

This study demonstrates that DHCR24 expression parallels hepatocarcinogenesis and that HCV induces overexpression of DHCR24 at both the mRNA and protein levels. Moreover, persistent DHCR24 overexpression suppresses the p53 response to H₂O₂. These findings are consistent with the previous report that inactivation and mutation of p53 plays a role in the development of HCC (32). Hepatocytes with p53 abnormalities are likely to escape from cell cycle check points and acquire resistance to apoptosis, thereby increasing their tumorigenic potential. Likewise, genetic inactivation of p53 is associated with late stage disease (32). HCV RNA levels are notably lower in cancerous tissues from HCV-positive HCC patients than in non-cancerous tissues (33). Thus, impairment of p53 function by HCV-induced DHCR24 overexpression might play a crucial role in early stage disease progression.

In DHCR24-overexpressing cells, p53 was mostly distributed in the cytoplasm (supplemental Fig. 2, A and B). This change in the distribution pattern of p53 might be caused by an increased interaction between p53 and MDM2, which might be induced by increased phosphorylation of MDM2 and suppression of polyubiquitination by overexpression of DHCR24. The up-regulation of the p53-MDM2 interaction negatively regulates p53 and shuttles p53 from the nucleus to the cytoplasm (34, 35). MDM2 inhibits both p53 transcriptional activation and p300-mediated p53 acetylation upon ternary complex formation with p300 and p53 (36–38). Acetylation of p53 by CBP/p300 mostly occurs in the nucleus (36). Therefore, the increase in cytoplasmic p53-MDM2 complexes in DHCR24-overexpressing cells may account for the observed suppression of p53 acetylation in the nucleus, even after treatment with H₂O₂ (supplemental Fig. 5). Impaired acetylation of C-terminal lysine residues decreases the sequence-specific DNA-binding activity (39) and the stability of p53 (37, 40). Taken together, our data suggest that DHCR24 overexpression may down-regulate p53 function by inhibiting degradation, increasing the formation of the p53-MDM2 complex in the cytoplasm, and suppressing acetylation of p53 in the nucleus.

In conclusion, we propose that HCV infection impairs the function of p53 through DHCR24 overexpression, which up-regulates the interaction between p53 and MDM2 in the cytoplasm and suppresses p53 acetylation in the nucleus. Because overexpression of DHCR24 is observed in other cancers, including melanoma (24) and prostate cancer (41), the findings from this study might provide a foundation for investigations into the mechanisms underlying the formation of these cancers.

We plan to examine the liver-specific regulatory network of the p53-MDM2 interaction by DHCR24 in a future study.

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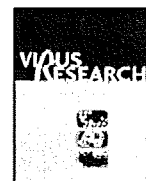
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Hepatitis C virus-core protein facilitates the degradation of Ku70 and reduces DNA-PK activity in hepatocytes

Kentarou Saitou^{a,b}, Kiyohisa Mizumoto^a, Tomohiro Nishimura^{c,d},
Chieko Kai^b, Kyoko Tsukiyama-Kohara^{c,*}

^a Department of Biochemistry, School of Pharmaceutical Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan

^b Laboratory Animal Research Center, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokane-dai, Minato-ku, Tokyo 108-8639, Japan

^c Department of Experimental Phylaxiology, Faculty of Medical and Pharmaceutical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto-City, Kumamoto 860-8556, Japan

^d The Chemo-Sero-Therapeutic Research Institute, Kikuchi Research Center, Japan

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ABSTRACT

We have established monoclonal antibodies (MoAbs) that are directed against hepatitis C virus (HCV)-expressing cells. They showed enhanced tumorigenicity after passage in culture for more than 44 days (RzM6-44d cells). To address the mechanism underlying this phenomenon, we characterized the MoAbs, and found that one of the clones recognized a molecule that was down-regulated in the RzM6-44d cells. This molecule was purified and identified as the 70-kDa thyroid autoantigen Ku70. Moreover, expression of the full-length HCV genome or HCV-core protein sequence in WRL68 human embryonic liver cells reduced the level of Ku70 protein, enhanced the ubiquitination of Ku70, and decreased the activity of DNA-activated protein kinase (DNA-PK). Therefore, it appears that the HCV-core protein facilitates the degradation of Ku70 and reduces DNA-PK activity in noncancerous liver cells.

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1. Introduction

Hepatitis C virus (HCV) causes chronic hepatitis, which may progress to cirrhosis and hepatocellular carcinoma (HCC) (Saito et al., 1990). HCV contains a single-stranded, 9600-nucleotide RNA genome with positive polarity, and the major proteins are the core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B. In the presence of *ras* expression, the HCV-core protein causes cellular transformation (Chang et al., 1998; Ray et al., 1996). Transgenic (Tg) mice that express the HCV-core protein show steatosis, and they develop HCC after a long period of latency (Moriya et al., 1998).

HCC may originate from dividing hepatocytes or liver stem cells that have accumulated genetic alterations (Lengauer et al., 1998). The expression of DNA repair enzymes is significantly down-regulated in HCV-associated HCC (Collier et al., 1996; Zekri et al., 2005). Moreover, the HCV-core protein impairs DNA repair (van Pelt et al., 2004) and transforms NIH3T3 cells by promoting genomic instability (Smirnova et al., 2006), E2-CD81 interaction to induce double-stranded DNA breaks (Machida et al., 2005), and overexpression of NS5A to induce chromosomal instability (Baek et al., 2006). To clarify the mechanism through which HCV infection mod-

ifies hepatocyte growth and causes HCC, we previously established liver cell lines (RzM6 cells) in which the HCV genome (*HCR6-Rz*) can be conditionally expressed and precisely self-trimmed at the 5'- and 3'-termini by ribozyme sequences (Tsukiyama-Kohara et al., 2004). When the RzM6 cells were passaged in culture for 44 days (termed RzM6-44d cells), tumorigenicity was enhanced due to the activation of the cyclin-dependent kinase-retinoblastoma protein-E2F pathway (Tsukiyama-Kohara et al., 2004). In the current study, we used monoclonal antibodies (MoAbs) raised against these passaged RzM6 cells to identify Ku70 as an antigen for which expression is repressed in the noncancerous liver tissues of HCV-infected patients. Since Ku70 is a subunit of DNA-activated protein kinase (DNA-PK), we examined HCV-mediated modification of DNA-PK activity in hepatocytes.

2. Materials and methods

2.1. Cells and plasmids

Previously, we established a Cre/loxP system to express conditionally the full-length HCV cDNA (*HCR6-Rz*) in HepG2 cells (Tsukiyama-Kohara et al., 2004). RzM6 cells were established by further transfection with the *Mer-Cre-Mer* unit (Zhang et al., 1996). Expression of the full-length HCV RNA by RzM6 cells is induced by treatment with 100 nM 4-hydroxytamoxifen (Sigma). Full-length

* Corresponding author. Tel.: +81 96 373 5560; fax: +81 96 373 5560.
E-mail address: kkohara@kumamoto-u.ac.jp (K. Tsukiyama-Kohara).

Table 1
Characteristics of the patients.

Patient	Gender	HBV ^a	HCV ^b	Type
1	M	–	+	HCC
2	F	–	+	HCC
3	M	–	+	HCC
4	M	–	–	HCC
5	M	–	–	CCC
6	M	–	–	HCC
7	F	–	–	HCC
8	F	–	–	HCC
9	M	–	+	HCC
10	M	–	+	HCC
11	M	+	–	HCC
12	M	+	–	HCC
13	M	+	–	HCC
14	F	+	–	HCC
15	M	+	–	HCC

M, male; F, female; CCC, cholangiocellular carcinoma.

^a HBV status: +, presence of HBs antigen, anti-HBs antibody or anti-HBc antibody.^b HCV (genotype 1b) antibody-positive.

HCV RNA was synthesized from a full-length HCV expression plasmid (pCA-Rz) that possesses a CAG promoter, and the plasmid DNA was digested at nucleotides 927, 1688, and 2594 with *FspI*, *MluI*, and *SexAI* to generate the HCV core, core-E1, and core-E2 protein regions, respectively. HepG2 cells and WRL68 cells were purchased from the American Type Culture Collection and were cultured in Dulbecco's modified Eagle's medium (DMEM; Nissui) that was supplemented with 10% fetal calf serum (FCS; Sigma), and further supplementation with 1 mM sodium pyruvate (GIBCO-BRL), and 0.1 mM nonessential amino acids (GIBCO-BRL) for the WRL68 cells.

2.2. Patients and specimens

Liver tissues were surgically obtained from 15 HCC patients (Table 1) who attended the Liver Unit of Tokyo Metropolitan Komagome Hospital. The subjects were randomly selected from chronic HCC patients of both sexes. Informed consent was obtained from all patients in accordance with the Helsinki Declaration prior to 2003.

2.3. Derivation of MoAbs

Hybridoma cells were established that produce MoAbs against RzM6 cells passaged for more than 44 days with HCV expression (RzM6-44d cells). BALB/c mice were immunized with seven or eight intraperitoneal injections of 5×10^6 RzM6-44d cells. The spleens were removed, and the splenocytes were fused with PAI cells using polyethylene glycol-1500 (Boehringer Mannheim) and selected in HAT medium (GIBCO-BRL), as described previously (Kohler and Milstein, 1975). Hybridoma cells were selected, and the reactivities of the culture supernatants with RzM6-44d cells were checked using a cell-based ELISA (Feit et al., 1983).

2.4. Electrophoresis and Western blotting

Cells were lysed in lysis buffer (10 mM Tris–HCl [pH 7.5], 1% sodium dodecyl sulfate [SDS], 0.5% NP-40, 0.15 M NaCl). Specimens from the patients were homogenized with a Dounce homogenizer (Wheaton Science Products) in RIPA buffer (1% SDS, 0.5% NP-40, 0.15 M NaCl, 10 mM Tris [pH 7.4]). Western blotting was performed, as described previously (Tsukiyama-Kohara et al., 2004), using anti-Ku70 antibodies (Santa Cruz Biotechnology) or an anti-Ku80 MoAb (Genetex). The HCV-core protein was detected using the anti-HCV core MoAb 31-2. Bound antibody was detected with a horseradish peroxidase-conjugated secondary antibody, followed

by visualization with the ECL reagents (Amersham Bioscience) and scanning with the LAS1000 scanner (Fujifilm). Densitometric analysis of protein bands was performed using the Image Quant software (Molecular Dynamics).

2.5. Affinity purification of P70

P70 was purified by affinity chromatography. Initially, a HiTrap NHS-activated HP column (Amersham Bioscience) was reacted with MoAb clone 2-344 (1.38 mg) and washed sequentially with coupling buffer (0.2 M NaHCO₃, 0.5 M NaCl [pH 8.3]), blocking buffer (0.5 M ethanolamine, 0.5 M NaCl [pH 8.3]), washing buffer (0.1 M acetate, 0.5 M NaCl [pH 4.0]), and again with blocking buffer. HuH-7 cells (10^9) were disrupted in lysis buffer (1% Triton X-100, 20 mM HEPES–NaOH [pH 7.5], 1 mM dithiothreitol [DTT], 1 mM diisopropyl fluorophosphate, 1 mM EDTA) and sonicated. The supernatant was passed through the affinity column, and bound protein was eluted with elution buffer (0.2 M glycine–HCl [pH 3.0]).

2.6. Matrix-assisted laser desorption/ionization-time of flight-mass spectroscopy (MALDI-TOF-MS)

The affinity column bound fraction was purified using the Ultimate capillary high-performance liquid chromatography system (Dionex) and analyzed with the 4700 Proteomics Analyzer (Applied Biosystems), as described previously (Jensen et al., 1999).

2.7. RNA isolation and Northern blot analysis

RNA was isolated using the TRIzol reagent (Nippon Gene) from WRL68 cells that were transfected with the HCV expression plasmid. Northern blot analysis was performed as described previously (Lehrach et al., 1977). The mRNA bands were analyzed by the Image Quant software (Molecular Dynamics), normalized by the 28S rRNA band, and the expression ratio was calculated by dividing the intensity of the each band by the intensity of the band for the mock-transfected WRL68 cells. The Ku70-specific probe was generated by PCR using the following primers: Ku70-for-895-914, 5'-TCTGGTCCAGAAGGCTCTCA-3'; and Ku70-rev-1580-1561, 5'-CTGCTGTTCGGCTCCATC-3'.

2.8. Ku70 stability assay

The half-life of Ku70 was determined in mock-transfected and HCV genome-expressing WRL68 cells that were treated with 10 µg/ml cycloheximide for the indicated times at 37 °C. The levels of Ku70 protein were determined by Western blotting, as described above.

2.9. Immunoprecipitation–Western blotting assay

Cells were lysed in 500 ml of NP-40 lysis buffer (1% NP-40, 20 mM Tris [pH 8.0], 150 mM NaCl, 10% glycerol, 20 mM NaF, 3 mM Na₃VO₄, 1× Complete Protease Inhibitor cocktail [Invitrogen]). Lysates were centrifuged at 15,000 × g for 15 min (4 °C), and the proteins were immunoprecipitated overnight at 4 °C using the anti-Ku70 antibody (C-19), which had been captured on 50% protein G-Sepharose. Immune complexes were washed with NP-40 lysis buffer, and Western blotting with the anti-ubiquitin MoAb P4D1 (Santa Cruz Biotechnology) was performed to detect protein ubiquitination.

2.10. Immunoprecipitation-kinase assay

Immunoprecipitation-kinase assays for the catalytic subunit of DNA-PK (DNA-PK_{cs}) were performed using the Signa TECT DNA-

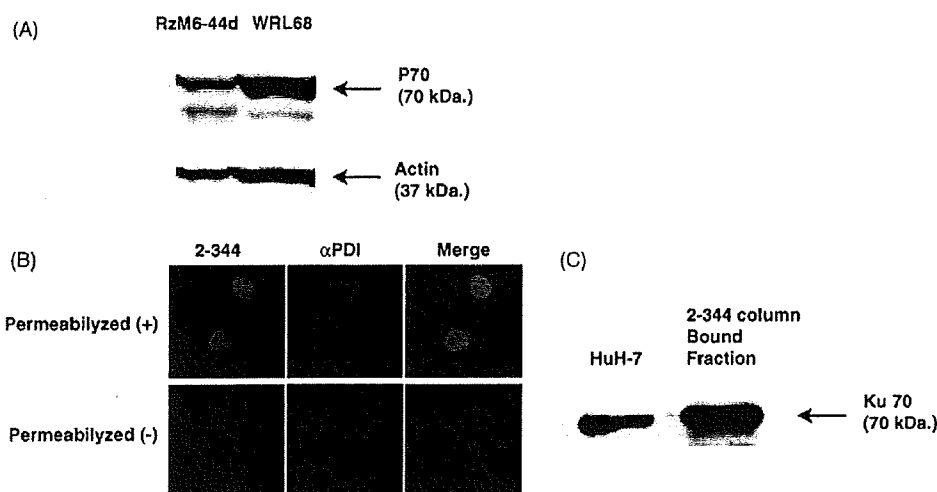


Fig. 1. Reactivity of MoAb clone 2-344 (A) Expression of P70 in RzM6-44d and WRL68 cells, (B) Localization of P70 in detergent-permeabilized (upper column) and unpermeabilized (lower column) WRL68 cells, as determined by immunofluorescence. Cells were co-stained with MoAb clone 2-344 (green) and anti-protein disulfate isomerase (PDI) antibody (red). (C) P70 was purified from HuH-7 cells by chromatography using an immobilized MoAb 2-344 column, and was reacted with a commercial anti-Ku70 antibody. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Dependent Protein Kinase Assay System (Promega). Briefly, WRL68 cells were transfected for 48 h with the HCV genome expression plasmids and lysed in NP-40 buffer (1% NP-40, 150 mM NaCl, 50 mM Tris [pH 7.8], 1 mM EDTA). The lysates were precleared, and DNA-PK α was immunoprecipitated from the cytosolic fraction using an anti-DNA-PK α antibody. The immunoprecipitates were washed three times with NP-40 lysis buffer and once with wash buffer (50 mM Tris [pH 7.4], 5 mM MnCl $_2$) and then resuspended in reaction buffer (50 mM Tris [pH 7.4], 5 mM MnCl $_2$, 5 mM DTT, 5 mM NaF, 40 mM MgCl $_2$, 1 mM Na $_3$ VO $_4$, 4 mM ATP, 50 mg/ml salmon sperm DNA) that contained 10 mCi of [γ - ^{32} P]ATP and 1 μ l of biotinylated p53-derived peptide substrate. After 5 min of incubation at 30°C, the reactions were terminated by boiling. Each supernatant (10- μ l aliquot) was spotted onto a square of SAM ^{2R} membrane. Incorporation of [γ - ^{32} P] into the peptide was measured using a phosphorimager (Fujifilm) or a scintillation counter.

3. Results

3.1. Establishment of MoAb that recognizes Ku70

We first developed MoAbs to the cellular antigens of RzM6-44d cells, which are HepG2 cells that express *HCR6-Rz* (Tsukiyama-Kohara et al., 2004). We found that MoAb clone 2-344 reacted with a 70-kDa protein (P70), and that P70 was down-regulated in RzM6-44d, as compared to the P70 level in human embryonic liver WRL68 cells (Fig. 1A). MoAb clone 2-344 strongly stained the nuclei of the WRL68 cells (Fig. 1B). Similar results were obtained for HuH-7 cells, non-passaged M6 cells (0 day), and 44-day-passaged M6 cells (data not shown). The molecules recognized by MoAb clone 2-344 were identified by antibody column purification and MALDI-TOF-MS analysis. The following peptides were identified: NIYVLQELD-NPGAK; ILELDQFK; ILELDQFKGQGGQK; IMLFTNEDNPHGNDSSAK; KPGGFDISLFYR; DIISIAEDEDLR; TFNTSTGGLLPSDTKR; NIPPYF-VALVPQEEELDDQK; IQVTPPGFQLVFLPFADDK; SDSFENPVLQGHFR; ELVYPPDYNPEGK; and VEYSEELKTHISK. Based on these results, we identified the 70-kDa thyroid autoantigen, also known as Ku70, as a candidate protein. Western blotting with a MoAb to Ku70 confirmed that the purified protein was Ku70 (Fig. 1C).

3.2. Levels of Ku70 in the tissues of patients with HCC

We examined the expression levels of Ku70 in the cancerous and noncancerous tissues of patients with HCC. Five of these patients were HCV-positive (nos. 1–3, 9, and 10), five were negative for HCV and HBV (nos. 4–8), and five were HBV-positive (nos. 11–15) (Table 1). Western blotting of tissue homogenates revealed that the levels of Ku70 were low in the noncancerous tissues of all the HCV-positive HCC patients. In contrast, two of the HBV-positive patients and one HBV-negative, HCV-negative HCC patient showed down-regulated expression of Ku70 (Fig. 2).

3.3. Effects of HCV on levels of Ku70 and Ku80 in WRL68 cells

We examined the effect of HCV on Ku70 expression in WRL68 cells (Fig. 3A and B), and found that the level of Ku70 was reduced 24–72 h after transfection with the full-length HCV genome. To

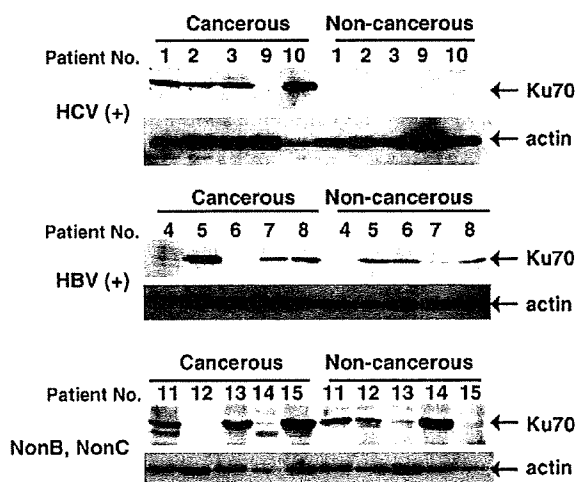


Fig. 2. Western blot analysis of the Ku70 expression levels in the cancerous and noncancerous tissues of HCC patients infected with HCV (HCV+) or HBV (HBV+), as well as in those of HCC patients who are not infected with either HCV or HBV (non-B/C).