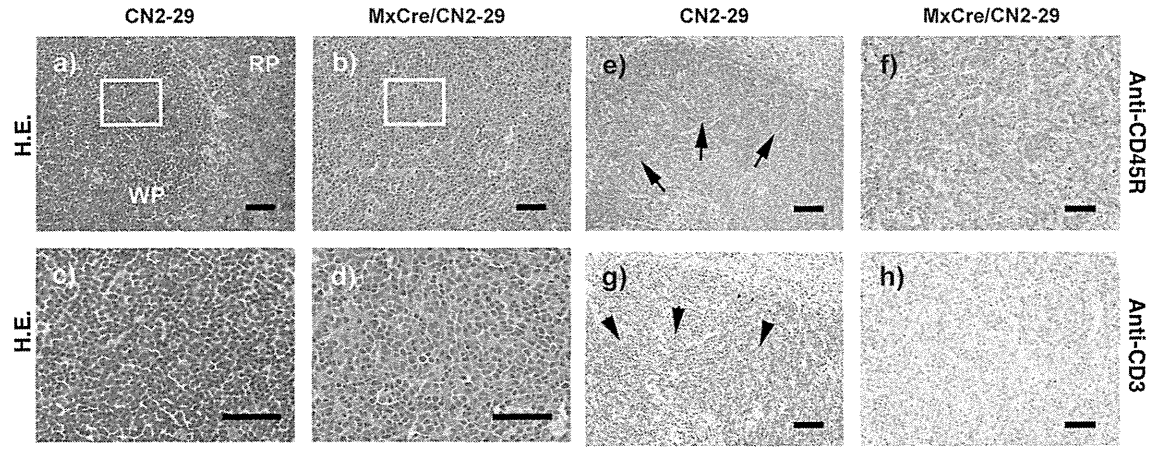

Fig. 4. Stable expression of HCV viral proteins induces lymphoproliferative diseases. The white pulp (WP) and red pulp (RP) comprise the components of the spleen in wild-type (WT) mice. The neoplastic cells replace the normal structures, such as the white pulp and red pulp. (c and d) The neoplastic cells are larger than lymphocytes (c), and the nuclei are irregular, round, oval, elongated, and polygonal (d). (e and g) The white pulp in WT mice consisted of both a B-cell-rich area (arrows, e) and a T-cell-rich area (arrowheads, g). (f and h) The neoplastic cells show staining for the B cell marker, CD45R, thereby supporting the diagnosis of B-cell lymphoma (f), while they do not show staining for the T-cell marker CD3 (h). Frames c and d are higher-magnification views of the boxed areas (white) in a and b, respectively.

The relationship between chronic HCV infection and lymphoma during interferon (IFN) disruption

It has been demonstrated that HCV infection causes lymphoproliferative diseases, such as B cell non-Hodgkin's lymphomas and mixed cryoglobulinemia [25, 39]. We established IFN regulatory factor-1-null (*irf-1*$^{-/-}$) mice with inducible and persistent expression of HCV structural proteins (*irf-1*/CN2 mice), in order to evaluate the molecular mechanisms of lymphoproliferation associated with the disruption of IFN signaling and chronic HCV infection [28]. *Irf-1*/CN2 mice had extremely high incidences of lymphomas and lymphoproliferative disorders and displayed increased mortality. Disruption of *irf-1* reduced their sensitivity to *Fas*-induced apoptosis and decreased the levels of caspase-3/7 and caspase-9 mRNA species and associated enzymatic activities. Furthermore, the *irf-1*/CN2 mice showed decreased activation of caspase-3/7 and caspase-9 and increased levels of interleukin (IL)-2, IL-10, and Bcl-2, which promote oncogenic transformation of lymphocytes. Disruption of IFN signaling resulted in the development of lymphomas, indicating that differential signaling occurs in lymphocytes rather than in the hepatocyte. *IRF-1*-inducible genes probably play essential roles in suppressing HCV-induced lymphomas and in eliminating HCV protein-expressing cells. Our transgenic mice provide evidence that the overexpression of apoptosis-related proteins, including Bcl-2, and/or aberrant cytokine production are primary events in HCV-induced lymphoproliferation.

HCV proteins expressed in B cells cause lymphoma

To extend the above-mentioned study with regard to the interaction of lymphoma and HCV infection, we established HCV transgenic mice that expressed the full HCV genome in B cells (RzCD19Cre mice) and observed a 25.0% incidence of diffuse large B cell non-Hodgkin's lymphomas within 600 days after birth [19]. The incidence of B cell lymphoma significantly correlated with the level of soluble IL-2 receptor alpha subunit (sIL-2Ralpha) in RzCD19Cre mouse serum. All RzCD19Cre mice with substantially elevated serum sIL-2Ralpha levels (>1,000 pg/ml) developed B cell lymphomas. Compared with tissues from control animals, the B cell lymphoma tissues of RzCD19Cre mice expressed significantly higher levels of sIL-2Ralpha. We showed that the expression of HCV in B cells promotes non-Hodgkin's-type diffuse B cell lymphoma, and therefore, the RzCD19Cre mouse is an appropriate model for studying

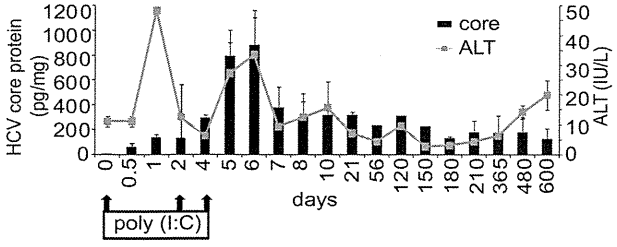

Fig. 5. Pathogenesis of the immunocompetent HCV persistent infection mouse model. HCV core protein expression was sustained for at least 600 days after poly(I:C) injection. Serum ALT levels also continued to elevate.

the mechanisms related to the development of HCV-associated B cell lymphoma [19].

Chronic persistent expression in HCV transgenic mice

We generated another switching system to study the expression of HCV proteins using Mx promoter-driven Cre recombinase with poly(I:C) induction. The *Mx* promoter is active in hepatocytes as well as in hematopoietic cells. We crossed CN2 mice with *Mx1-Cre* transgenic mice, in which Cre recombinase is expressed by the IFN-inducible *Mx1* promoter. Injection of *Mx1-Cre*/CN2-29 mice with poly(I:C) induces IFN production and the expression of CN2 gene products in hematopoietic cells (mainly in Kupffer cells and lymphocytes), livers, and spleens but not in most other tissues. As illustrated in Fig. 5, the serum alanine aminotransferase (ALT) levels increased, peaking at 24 h after the first poly(I:C) injection. These serum ALT levels then decreased until day 4, when they increased again until day 6, along with HCV core protein levels. Thereafter, HCV core protein was observed consistently for at least 600 days. We also showed that the serum ALT levels gradually increased after day 210 despite no change in the HCV core protein levels. Histological analysis showed that the HCV core protein was expressed in most hepatocytes of transgenic mice which also exhibited lymphocytic infiltration by the core protein (Fig. 5). These observations indicate that the expression of the HCV proteins caused chronic hepatitis in the CN2-29$^{(+/-)}$/*MxCre*$^{(+/-)}$ mice because of a weak and persistent immune response. We observed a number of other pathological changes in these mice, including swelling of hepatocytes, abnormal architecture of liver-cell cords, abnormal accumulation of glycogen, steatosis, fibrosis, and hepatocellular carcinoma. We are convinced that HCV transgenic mice are suitable for evaluating the mechanisms of persistent HCV infection and for assisting with the design of HCV vaccines.

Role of NK cells in the antiviral effect of HCV transgenic mice

The liver is enriched with NK cells and this intrahepatic population is embedded in the endothelial lining of the liver sinusoids. These NK cells were originally described as 'pit' cells [20]. Intrahepatic NK cells may behave differently to NK cells in other areas because of the 'tolerogenic' environment of the liver, with murine intrahepatic NK cells known to be hyporesponsive. They are less cytotoxic and have an altered cytokine profile producing lower levels of IFN-γ and greater levels of immunoregulatory cytokines, such as IL-10, than peripheral blood and splenic NK cells [24]. This hyporesponsive state has been described in the early stages of HBV infection and may contribute to the establishment of chronic viral infection [7]. Peripheral blood NK cell frequencies, both the absolute number and the percentage of the total lymphocyte population, are reduced in chronic HCV compared to healthy individuals [30]. In individuals with chronic HCV infection, NK cell frequency increases following successful antiviral therapy, while a reduction in peripheral blood NK cell frequency in individuals with chronic HCV as compared to spontaneous resolvers has also been noted [10]. Thus, NK cells may play key roles in suppressing HCV replication. We actually observed much higher levels of HCV core proteins in Tg mice with a depleted population of NK cells. Furthermore, Cre-mediated genomic DNA recombination efficiency in HCV-Tg mice was strong in NK cell-depleted mice between 0.5 and 1 day compared to untreated mice. These data indicate that NK cells participate in the elimination of core expressing hepatocytes during the innate immune response in the acute phase of HCV infection [41].

Chimeric Human Liver Mice Model

Mercer *et al.* generated mice with chimeric human livers by transplanting normal human hepatocytes into

SCID mice carrying a plasminogen activator transgene (*Alb-uPA*). Homozygosity of *Alb-uPA* was associated with significantly higher levels of human hepatocyte engraftment, and these mice developed prolonged HCV infections with high viral titers after inoculation with infected human serum [31].

We used the chimeric mice as they were a vast improvement over the originals, which had a high substitution rate of human hepatocytes [45], and examined the inhibitory effect of DEBIO-025, a novel non-immunosuppressive cyclophilin inhibitor derived from cyclosporin A, on naïve HCV genotypes 1a or 1b *in vivo* [14]. Collectively, this small animal model is useful for assessing the activity of antiviral compounds [33] and for evaluating protection and passive immunization studies of HCV [26, 32], but because they lack an immune system, this model is not suitable for studies of HCV pathogenesis.

A recent study showed that in $Fah^{-/-}Rag2^{-/-}Il2rg^{-/-}$ mice, the selection pressure for transplanted human hepatocytes can be regulated by the drug 2-(2-nitro-4-trifluoro-methylbenzoyl)-1,3-cyclohexanedione. In the absence of this drug, mouse hepatocytes die because of the accumulation of toxic tyrosine catabolites and a lack of fumarylacetoacetate hydrolase, while human hepatocytes remain healthy. These mice have a high level of human liver chimerism, they propagate both HBV and HCV, and the HCV-infected mice are responsive to antiviral treatment. It seems that this human liver chimeric mouse model will be useful for studying HBV and HCV infection, and it has already proven valuable in antiviral drug testing [2].

The development of molecular biological techniques has allowed us to generate transgenic mice. Using these techniques we are able to analyze the immune responses to various viral proteins in mice, even though the virus does not normally infect murine species. It is essential to generate an infectious HCV mouse model for a more precise analysis of the interaction between host and virus. The chimeric human liver mouse model would appear to be a powerful tool for evaluating the effects of antiviral drugs. It is hoped that an experimental mouse model for HCV will yield a number of useful insights into the immunopathogenesis of this viral infection, and assist in the development of antiviral drugs.

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Conditional gene expression in hepatitis C virus transgenic mice without induction of severe liver injury using a non-inflammatory Cre-expressing adenovirus

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ABSTRACT

We previously established inducible-hepatitis C virus (HCV) transgenic mice, which expressed the HCV gene (nucleotides 294–3435) encoding the core, E1, E2, and NS2 proteins. The expression of these proteins is regulated by the Cre/loxP system and an adenovirus vector (AdV) that expresses Cre DNA recombinase (Cre) controlled by the CAG promoter (AxCANCre). Recent studies have demonstrated that AxCANCre injection alone results in severe liver injury by induction of the adenovirus protein IX (Ad-pIX) gene. As a result, HCV protein expression in transgenic mice livers was only short-term. In contrast, the EF1α promoter-bearing AdV induces slight Ad-pIX gene expression without inducing severe liver injury. Therefore, in the present study, we developed a Cre-expressing AdV that bears the EF1α promoter (AxEFCre) to express HCV protein in the transgenic mouse livers. In the non-transgenic mice injected with AxCANCre, alanine aminotransferase (ALT) levels were elevated and severe liver inflammation occurred; this was not observed in AxEFCre-injected mice. In contrast, AxEFCre-injected HCV transgenic mice showed milder liver inflammatory responses that were clearly due to HCV protein expression. Moreover, the AxEFCre injection enabled the transgenic mice to persistently express HCV protein. These results indicate that use of AxEFCre efficiently promotes Cre-mediated DNA recombination *in vivo* without a severe hepatitis response to AdV. This inducible-HCV transgenic mouse model using AxEFCre should be useful for research on HCV pathogenesis.

1. Introduction

Infection with the hepatitis C virus (HCV) is a major global health problem, as persistent viral infection leads to liver cirrhosis and hepatocellular carcinoma (Goodman and Ishak, 1995; Shepard et al., 2005). The chimpanzee is the only validated animal model for *in vivo* studies of HCV infection, while *in vivo* studies on the pathogenesis of HCV have been conducted using new animal models (Kremsdorf and Brezillon, 2007). Several groups have established transgenic mice that constitutively express single or multiple HCV protein(s) in the liver (Lerat et al., 2002; Moriya et al., 1997). However, in these mice, HCV protein expression begins *in utero*; as a result, they develop immune tolerance to the HCV antigens, and HCV-specific cellular responses or liver inflammation cannot be induced. To overcome these obstacles, we previously developed immunocompetent HCV transgenic mice in which HCV protein expression was tightly regulated by the Cre/*lox*P system (Wakita et al., 1998).

The E1- and E3-deleted adenovirus vector (AdV) has been widely used for both basic studies of gene function and for gene therapy *in vivo*. To deliver the *Cre* gene into the livers of HCV transgenic mice, we used an AdV that carries the CAG promoter linked to a nuclear localization signal-tagged Cre (AxCANCre), which has been used for Cre-mediated DNA recombination (Baba et al., 2005; Kobayashi et al., 2000; Shintani et al., 1999; Wakita et al., 1998). While AdV is relatively efficient in inducing transgene expression, several studies have shown that the viral vector itself can induce strong inflammatory responses in murine livers (Kafri et al., 1998; Wakita et al., 2000). Moreover, expression of transgenes via AdVs persists for only 2–4 weeks due to elimination of infected cells through immune responses directed against the AdVs (Akagi et al., 1997; Bangari and Mittal, 2006; Kafri et al., 1998; Sun et al., 2005; Wakita et al., 2000). To address these problems, the viral

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genes that cause cellular immune responses have been investigated. Recently, Nakai et al. (2007) reported that co-expression of adenovirus protein IX (Ad-pIX) resulted in AdV-induced immune responses. However, AdVs that carried the EF1α promoter did not induce Ad-pIX or increase the alanine aminotransferase (ALT) level, facilitating long-term transgene expression in mice.

In the present study, we generated a Cre-expressing AdV bearing the EF1α promoter (AxEFCre) to enable the persistent expression of HCV protein in the livers of inducible-HCV transgenic mice regulated by the Cre/*lox*P recombination system. When this AdV was used to express Cre in the HCV transgenic mouse livers, it induced less severe inflammatory responses and improved the long-term expression of HCV proteins compared to CAG promoter-bearing AdVs. Thus, AxEFCre efficiently promotes Cre-mediated DNA recombination *in vivo* without a severe hepatitis response to AdV and should be useful for HCV gene expression in the HCV transgenic mice.

2. Materials and methods

2.1. Cells

The 293 cells [CRL-1573, a human embryonic kidney cell line that contains the Ad5 E1 region; American Type Culture Collection (ATCC)] and HepG2 cells (HB-8065, a human hepatocellular carcinoma cell line; ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical) supplemented with 10% fetal bovine serum (FBS; JRH Biosciences), 100 U/mL penicillin, and 100 μg/mL streptomycin (GIBCO) (10% FBS-DMEM). In addition, HepG2 cells containing the Cre reporter unit CALNLZ (Baba et al., 2005), termed Hep-CALNLZ cells, were selected for resistance to G418 (300 μg/mL; Sigma) and cultured in 10% FBS-DMEM.

2.2. Adenovirus vectors

E1- and E3-deleted AdVs derived from human adenovirus type 5 encoding expression units with a leftward orientation were used in this study. As expression units, untagged Cre or NLS-tagged Cre under the control of the CAG promoter (AxCANCre or AxCACre), untagged Cre or NLS-tagged Cre under the control of the EF1α promoter (AxEFNCre or AxEFCre), and untagged β-galactosidase (LacZ) under the control of the EF1α promoter (AxEFLacZ) were constructed (Fig. 1A). AxCANCre and AxCACre were generated as described previously (Kanegae et al., 1995). AxEFNCre, AxEFCre, and AxEFLacZ were constructed using pAxEFwtit2 DNA/RE Treatment (Nippon Gene). All of the AdVs were purified using two rounds of CsCl gradient centrifugation, and the titers of the concentrated and purified virus stocks were determined as described previously (Kanegae et al., 1994).

2.3. Animal procedures

HCV transgenic mice CN2-29 (C57BL/6 background) and normal C57BL/6 mice were used in the experiments. The CN2-29 transgenic mice express HCV genotype 1b proteins (core, E1, E2, and NS2 proteins) under the regulation of the Cre/*lox*P conditional switching system (Wakita et al., 1998). The transgenic mice were intravenously injected with each AdV at a dose of 1.0×10^9 plaque-forming units (PFU), and sacrificed 0.5, 7, or 21 days after injection for liver histology and biochemical analysis. All mice were bred in a pathogen-free facility and tested routinely for mouse hepatitis virus and other pathogens. All experiments using mice were approved by The Tokyo Metropolitan Institute of Medical Science Animal Experiment Committee and were performed in accordance with the animal experimentation guidelines of The Tokyo Metropolitan Institute of Medical Science.

2.4. Western blot detection of Cre and Ad-pIX

The HepG2 cells were placed in collagen-coated, 12-well plates and infected with the AdVs at a multiplicity of infection (MOI) of 20 or 100 for Western blot detection of Cre or Ad-pIX, respectively. After 24 h, the cells were washed with phosphate-buffered saline (PBS) and resolved in radioimmunoprecipitation assay (RIPA) buffer [10 mM Tris–HCl (pH 7.5), 0.15 M NaCl, 1% sodium dodecyl sulfate (SDS), 0.5% Nonidet P-40, protease inhibitor cocktail (Complete; Roche Molecular Biochemicals)]. The protein concentrations of the cell lysates were measured using the DC protein assay (Bio-Rad Laboratories). The cell lysates were electrophoresed on SDS–polyacrylamide gel, transferred to polyvinylidene difluoride membrane (GE Healthcare) activated with methanol, and blocked with 5% skim milk in PBS containing 0.1% Tween-20 (PBST). After washing with PBST, the membrane was incubated overnight at 4 °C in the presence of anti-Cre rabbit polyclonal antibody or anti-Ad-pIX rabbit polyclonal antibody (Nakai et al., 2007) prepared from hyper-immune rabbit sera, or anti-β-actin mouse monoclonal antibody (Sigma), followed by incubation with horseradish peroxidase (HRP)-conjugated $F(ab')_2$ of anti-rabbit or mouse IgG (GE Healthcare) for 1 h at room temperature. The expression levels of these proteins were visualized using the ECL system (GE Healthcare) and an LAS3000 imager (Fujifilm).

2.5. LacZ gene activation and cytotoxicity of Cre-expressing AdVs

The Hep-CALNLZ cells were cultured on collagen-coated, 96-well plates and infected with AdVs at various MOIs in four-fold serial dilutions. After 48 h, cytotoxicity assays were performed using the Cell Counting Kit-8 (Dojindo Molecular Technologies), according to the manufacturer's instructions. To detect *LacZ* expression, the cells were fixed with 4% paraformaldehyde in PBS for 10 min, washed with PBS, and incubated in X-Gal solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM $MgCl_2$ in PBS) containing 0.5 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; WAKO Pure Chemicals) at 37 °C overnight.

2.6. Extraction of total RNA and quantification of Ad-pIX mRNA levels

The HepG2 cells were infected with the AdVs at an MOI of 100 and were harvested after 24 h. The CN2-29 transgenic mice were injected with the AdVs at a dose of 1.0×10^9 PFU and were sacrificed to obtain their liver samples after 12 h. Total RNA was extracted from the cells or mouse livers using the RNeasy Mini Kit (Qiagen) and RNase-free DNase (Qiagen). Reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Copy numbers of the Ad-pIX cDNA were assessed by quantitative real-time detection polymerase chain reaction (RTD-PCR) with the specific probe AdIX-354-S25FT (5′-[FAM]-TCAGCAGCTGTTGGATCTGCGCCAC-[TAMRA]-3′); AdIX-327-S24 (5′-TTTGACCCGGGAACTTAATGTCGT-3′) and AdIX-387-R19 (5′-GGAGGAAGCCTTCAGGGCA-3′) were used as primers. The standard curve was generated using pAxEFLacZ. Analyses were conducted using an ABI PRISM 7700 Sequence Detection System with TaqMan Universal PCR Master Mix (Applied Biosystems).

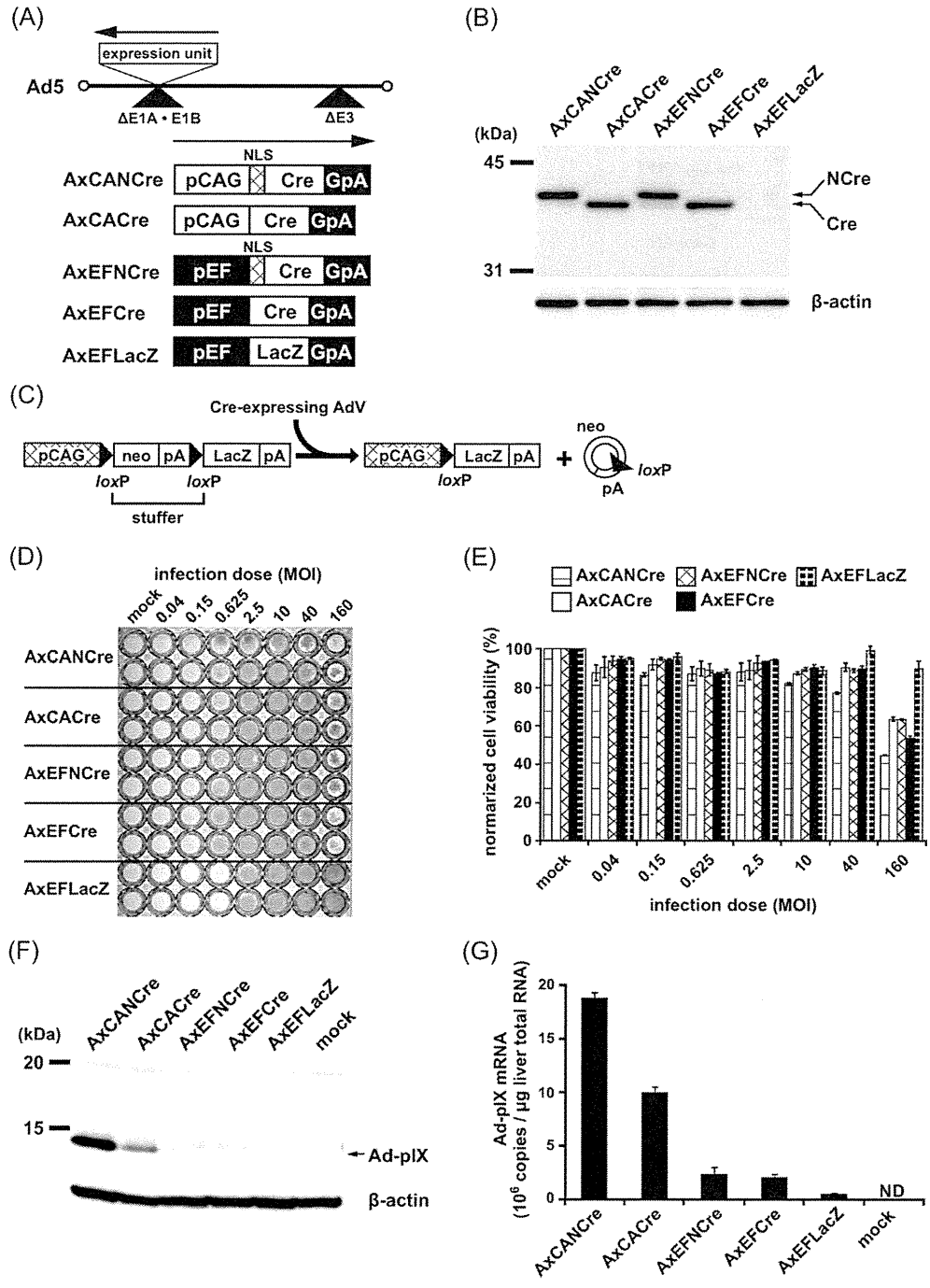

Fig. 1. Generation of AdVs expressing Cre under the control of different promoters. pCAG, CAG promoter; pEF, EF1α promoter; GpA, rabbit β-globin poly(A) signal; NLS, nuclear localization signal; neo, neomycin-resistance gene; pA, poly(A) signal; LacZ, β-galactosidase; Ad-pIX, adenovirus protein IX. (A) Structures of Cre-expressing AdVs. NLS-tagged Cre (NCre) or Cre were expressed under the control of the CAG or EF1α promoter. AxEFLacZ, which expresses LacZ under the control of the EF1α promoter, was used as a control. (B) Cre protein expression. HepG2 cells were infected with AdVs at an MOI of 20. After 24 h, total protein extracts from the cells were subjected to Western blotting. The detected β-actin protein is also shown. Note that mobility is slightly reduced when Cre is tagged with NLS (lanes, AxCANCre and AxEFNCre). (C) Schematic representation of *LacZ* transgene activation mediated by Cre-expressing AdV in Hep-CALNLZ cell chromosomes. Cre recognizes a pair of its target sequences *lox*P and removes the stuffer region as a circular DNA, resulting in expression of the transgene by the CAG promoter. (D) Cre recombination activity. Hep-CALNLZ cells were infected with Cre-expressing AdVs at the indicated dosages (four-fold serial dilutions; MOI range, 0.04–160). After 48 h, the cells were fixed and stained using X-gal staining. The first lane contains the mock-infected controls. The AxEFLacZ-infected lanes show the *LacZ*-gene-expressed control. (E) Cre cytotoxicities. Hep-CALNLZ cells were infected with AdVs at the indicated dosages (four-fold serial dilutions; MOI range, 0.04–160). After 48 h, cell viability was measured using a Cell Counting Kit-8. (F) Ad-pIX protein expression. HepG2 cells were infected with AdVs at an MOI of 100. After 24 h, total protein extracts from the cells were subjected to Western blotting. The detected β-actin protein is also shown. (G) mRNA expression of Ad-pIX. HepG2 cells were infected with AdVs at an MOI of 100. After 24 h, total RNA extracts from the cells were subjected to reverse transcription and quantitative RTD-PCR with an Ad-pIX-specific probe and a primer pair. ND, not detected.

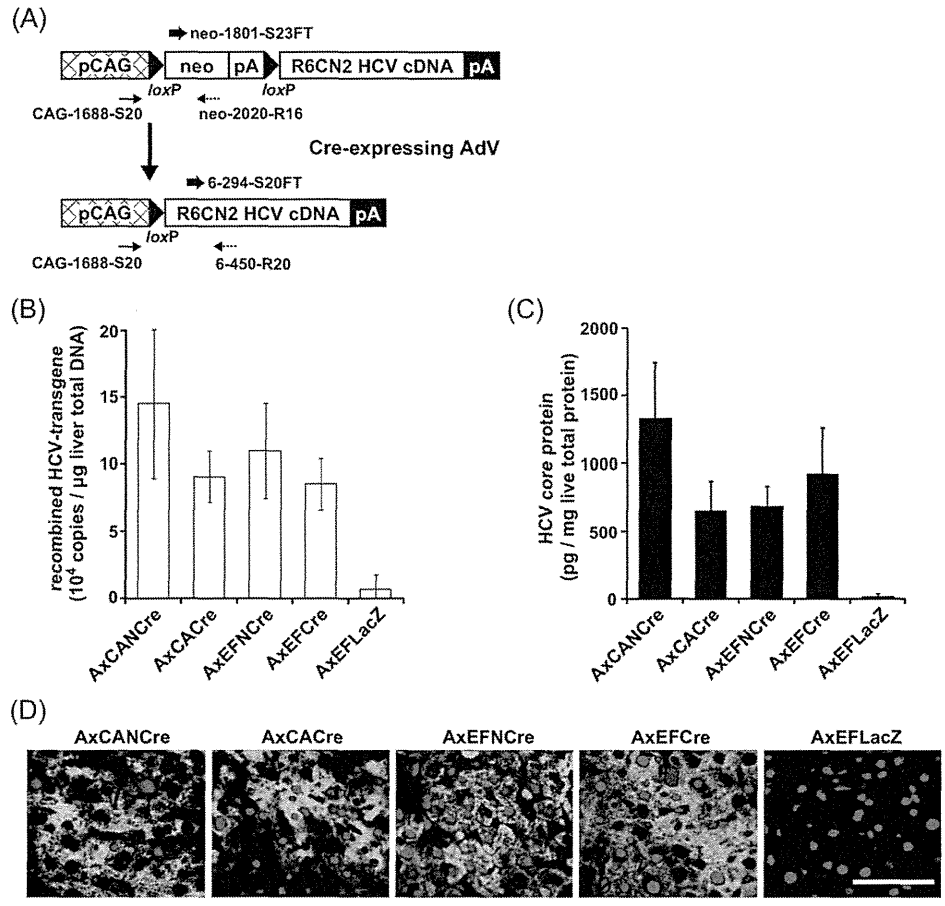

Fig. 2. Cre-mediated genomic DNA recombination and HCV core protein expression in transgenic mouse livers. (A) Structure of the Cre-mediated activation transgene unit CALNCN2 (Wakita et al., 1998). pCAG, CAG promoter; neo, neomycin-resistance gene; pA, poly(A) signal. The R6CN2 HCV cDNA (nucleotides 294–3435) contains the core, E1, E2, and NS2 regions. This construct does not allow HCV mRNA transcription prior to Cre-mediated DNA recombination, detected with the primer pair CAG-1688-S20 and neo-2020-R16. When Cre-expressing AdV is injected, the *neo* gene and poly(A) signal are removed by recombination between two *loxP* sequences. The recombined HCV transgene is detected with the primer pair CAG-1688-S20 and 6-450-R20. (B) Determination of Cre-mediated DNA recombination. CN2-29 transgenic mice were injected with 1.0×10^9 PFU for each AdV, and liver samples were harvested 7 days post-injection. Genomic DNA was extracted from the livers, and the numbers of copies of the recombined HCV-transgenes were determined using quantitative RTD-PCR with specific probes (6-294-S20FT) and a primer pair (CAG-1688-S20 and 6-450-R20). The values shown are means ± S.D. of more than three individual specimens. (C) Measurements of HCV core protein concentration in liver samples obtained from CN2-29 transgenic mice 7 days after injection of 1.0×10^9 PFU for each AdV. The samples were homogenized and the concentrations of HCV core protein were determined by EIA. The values shown are means ± S.D. of three individual specimens. (D) Immunofluorescence analysis of HCV core proteins. Liver sections of CN2-29 transgenic mice 7 days after injection of 1.0×10^9 PFU for each AdV were fixed and co-stained with rabbit anti-core polyclonal antibody (green) and DAPI (blue). Scale bar, 50 μm.

2.7. Determination of Cre-mediated HCV transgene recombination in mouse livers

The transgenic mouse livers were digested at 37 °C overnight in lysis buffer [50 mM Tris–HCl (pH 8.0), 0.1 M NaCl, 20 mM EDTA, 1% SDS] containing 1 mg/mL proteinase K. Total genomic DNA was then extracted using the phenol–chloroform extraction method. The copy numbers of the recombined HCV transgene in the livers were assessed via quantitative RTD-PCR (Takeuchi et al., 1999) with the specific probe 6-294-S20FT (5′-[FAM]-TGATAGGGTGCTTGCGAGTG-[TAMRA]-3′) and the primer pair CAG-1688-S20 (5′-GGTTGTTGTGCTGTCTCATC-3′) and 6-450-R20 (5′-ACAGGTAAACTCCACCAACG-3′) (Fig. 2A). The standard curve was generated using pCALNCN2/59-2 (Wakita et al., 1998) and quantitative RTD-PCR with the specific probe neo-1801-S23FT (5′-[FAM]-TCAAGAGACAGGATGAGGATCGT-[TAMRA]-3′) and the primer pair CAG-1688-S20 (5′-GGTTGTTGTGCTGTCTCATC-3′) and neo-2020-R16 (5′-TGCCTCGTCCTGCAGT-3′) (Fig. 2A). The *GAPDH* gene was used as an internal control for all samples. Analyses were carried out on an ABI PRISM 7700 Sequence Detection System with TaqMan Universal PCR Master Mix (Applied Biosystems).

2.8. Quantitation of HCV core proteins in mouse liver lysates

The transgenic mouse livers were homogenized in 0.5 mL RIPA buffer, and centrifuged at 15,000 rpm for 10 min at 4 °C. The protein concentrations of the supernatants were measured using the Bradford method (DC protein assay; Bio-Rad). The concentrations of HCV core proteins in the liver samples were determined using the Ortho HCV core protein ELISA kit (Eiken Chemical).

2.9. Biochemical analyses of mouse sera

Sequential blood samples were obtained by orbital bleeding after each AdV administration, and the sera were isolated by centrifugation at 10,000 rpm for 3 min at 4 °C. Serum ALT levels were determined using the Transaminase-CII Test A (Wako Pure Chemicals).

2.10. Histology and immunohistochemical staining

The liver samples were fixed with 4% paraformaldehyde in PBS, paraffin-embedded, sectioned at 4-μm thickness, and stained

with hematoxylin and eosin (H&E). Liver histology was evaluated according to modified Histology Activity Index (HAI) scores in three categories: piecemeal necrosis, spotty necrosis, and portal inflammation (Knodell et al., 1981; Yang et al., 1994).

The liver tissues were frozen in OCT compound (Tissue Tech) for immunohistochemical staining of HCV core proteins. The sections were fixed with a 1:1 solution of acetone:methanol at −20 °C for 10 min and then washed with PBS. Subsequently, the sections were incubated with the IgG fraction of an anti-HCV core rabbit polyclonal antibody (RR8) (Wakita et al., 1998) labeled with biotin in blocking buffer for 1 h at 4 °C. The sections were incubated with strept–avidin-conjugated horseradish peroxidase for 30 min at room temperature. Immunohistochemical staining was conducted using the Tyramide Signal Amplification Kit (Molecular Probes). Fluorescently labeled sections were stained with 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes) to stain the cell nuclei at room temperature before cover slipping. Fluorescence was observed under a fluorescence microscope (Carl Zeiss).

2.11. Statistical analysis

Data are shown as the mean ± S.D. Statistical analyses were performed using analysis of variance (ANOVA) followed by the Student–Newman–Keuls (SNK) test or analyzed using the unpaired Student's *t*-test. Statistical significance was established at $p < 0.05$.

3. Results

3.1. Generation of Cre-expressing AdVs

To enable HCV transgenic mice using the Cre/*lox*P system to express HCV protein persistently without severe inflammatory responses to AdV, we first constructed AdVs that expressed Cre with or without a nuclear localization signal (NLS) tag (AxEFNCre or AxEFCre, respectively) together with *LacZ* under the control of the EF1α promoter (AxEFLacZ) (Fig. 1A). AxCANCre and AxCACre were also generated to compare the impacts of using the CAG promoter and the EF1α promoter (Fig. 1A). Expression of Cre proteins from various AdVs was confirmed in human liver-derived HepG2 cells by Western blot analysis (Fig. 1B). Cre protein expression levels were not significantly different whether the gene was expressed under the control of the CAG or the EF1α promoter in the HepG2 cells (Fig. 1B). Next, we examined the recombination activities of Cre expressed via the AdVs using the Hep-CALNLZ cell line, HepG2 cells that express CALNLZ (Fig. 1C). When the Cre-expressing AdVs bearing the CAG or EF1α promoters infected these cells, the blue color produced by *LacZ* activation was observed for MOIs of 0.15–160. The cells infected with AxEFLacZ showed the blue staining in an MOI-dependent manner (Fig. 1D, lane AxEFLacZ). In contrast, the color faded for MOIs >40 when the Cre-expressing AdVs were used (Fig. 1D, lanes AxCANCre, AxCACre, AxEFNCre, and AxEFCre). At an MOI of 160, all of the Cre-expressing AdVs resulted in cytotoxicity, while the LacZ-expressing AdV did not affect cell viability (Fig. 1E).

AdV-induced immune responses are partly caused by co-expression of Ad-pIX (Nakai et al., 2007). To confirm the protein expression levels of Ad-pIX due to the AdVs, we performed Western blotting with anti-Ad-pIX sera (Fig. 1F). When HepG2 cells were infected with AdVs bearing the CAG promoter, significant amounts of Ad-pIX were detected as 14-kDa bands (Fig. 1F, lanes AxCANCre and AxCACre). In contrast, when using AdVs bearing the EF1α promoter, the 14-kDa band representing Ad-pIX was undetectable, as was the case for mock-infected HepG2 cells (Fig. 1F, lanes AxEFNCre, AxEFCre, AxEFLacZ, and mock). We also examined the mRNA expression levels of Ad-pIX and obtained similar results that correlated with the protein expression levels (Fig. 1G).

3.2. HCV gene expression and core protein production mediated by various Cre-expressing AdVs in transgenic mouse livers

The HCV transgenic mouse CN2-29 contains a reporter unit (CALNCN2) that is activated by Cre and conditionally expresses the HCV gene (Fig. 2A; Wakita et al., 1998). To assess the efficiency of Cre-expressing AdVs in promoting HCV gene expression, we intravenously injected the CN2-29 transgenic mice with various AdVs. At 7 days post-injection, Cre protein expression was confirmed by Western blot analysis of liver lysates (data not shown). The recombined HCV transgene levels in the livers were determined by quantitative RTD-PCR using specific probes and primer pairs, as described in Section 2 (Fig. 2A and B). When each Cre-expressing AdV was injected, the respective recombined HCV transgene was detectable; AxCANCre-injected CN2-29 transgenic mice expressed the highest levels of the recombined HCV transgene in their livers (Fig. 2B). CN2-29 transgenic mice injected with AdVs expressing NLS-tagged Cre had higher levels of the recombined HCV transgene in their livers (Fig. 2B, AxCANCre and AxEFNCre). This result suggests that NLS-tagged Cre efficiently translocated to the cell nucleus, which is consistent with our previous data (Baba et al., 2005). However, the levels of the recombined HCV transgene were not correlated with the expression level of HCV core protein (Fig. 2C).

The core protein levels in the livers were measured by enzyme immunoassay (EIA) as described in Section 2. The expression of the E1 and E2 proteins in the CN2-29 transgenic mouse livers has been shown previously (Wakita et al., 1998). The mean core protein level was 1.3 ng/mg total protein in the CN2-29 transgenic mouse livers 7 days after administration of AxCANCre (Fig. 2C). AxCACre- and AxEFNCre-injected mice expressed approximately one-half of the core protein levels resulting from AxCANCre injection (Fig. 2C).

Expression of core proteins in AdV-injected CN2-29 transgenic mouse livers was confirmed through immunofluorescence staining. Core proteins were expressed in the hepatocytes in the lobules of liver sections from Cre-expressing AdV-injected mice (Fig. 2D). In contrast, AxEFLacZ-injected transgenic mice did not express core proteins (Fig. 2C and D).

3.3. Liver injury and Ad-pIX expression in HCV transgenic mice injected with AdVs

To evaluate hepatocellular injury caused by expression of HCV proteins in CN2-29 transgenic mice injected with Cre-expressing AdVs, we serially estimated the serum ALT levels (Fig. 3A). For AxCANCre, the serum ALT level was elevated on day 5 and peaked 1–2 weeks post-injection (Fig. 3A, open triangle). ALT levels in AxCACre-injected transgenic mice were also elevated, although these levels declined over time (Fig. 3A, open circle). When AxEFNCre or AxEFCre was injected, ALT levels did not immediately increase, although they gradually increased after day 5 (Fig. 3A, closed triangle and closed circle, respectively). Injection of AxEFLacZ did not increase serum ALT levels in the CN2-29 transgenic mice (Fig. 3A, closed rectangle).

We also performed histological analyses of liver sections from CN2–29 transgenic mice 7 days after AdV injection (Fig. 3B). We found that severe inflammation with lymphocyte infiltration and spotty necrosis were diffusely observed in the livers of mice injected with the AdVs bearing the CAG promoter (AxCACre and AxCANCre) (Fig. 3B, a,b). In contrast, AxEFNCre-injected and AxEFCre-injected transgenic mouse livers exhibited mild inflammation without massive piecemeal necrosis on day 7 (Fig. 3B, c,d). No inflammation was observed in the AxEFLacZ-injected mice (Fig. 3B, e).

To confirm the expression levels of Ad-pIX in AdV-injected transgenic mice, we determined Ad-pIX mRNA in the liver using

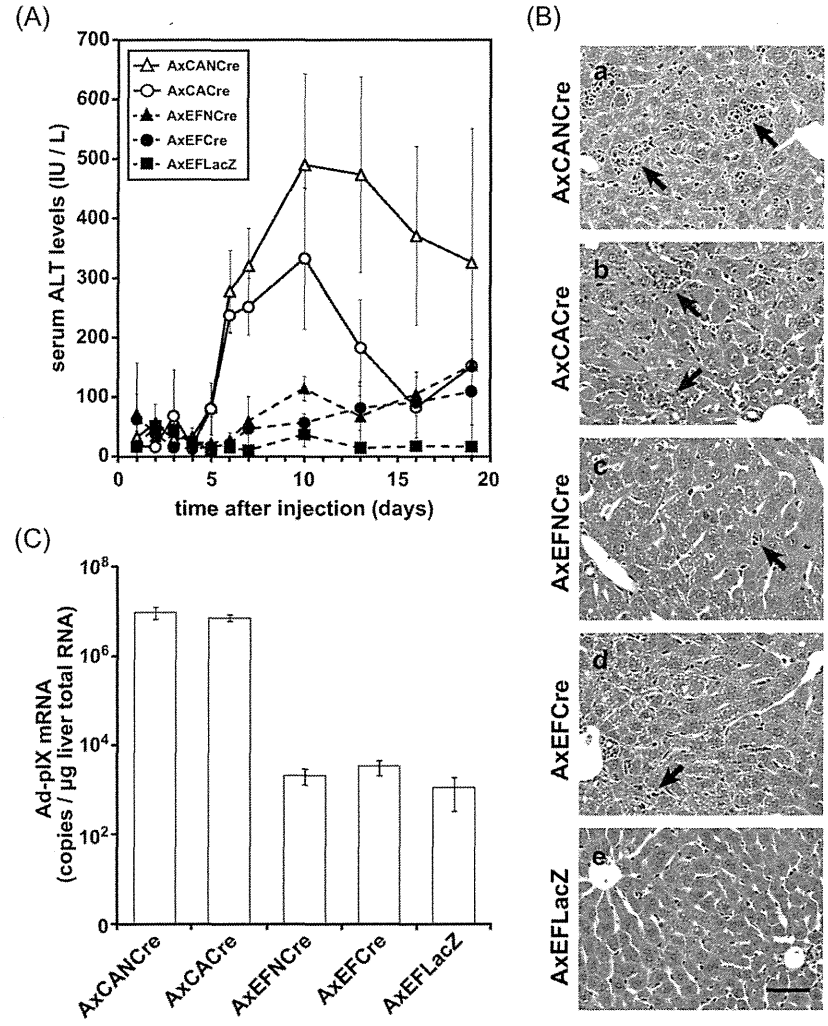

Fig. 3. Effects of AdVs on liver injuries in HCV transgenic mice. (A) Serum ALT levels were measured at the indicated time-points in CN2-29 transgenic mice injected with 1.0×10^9 PFU AxCANCre (open triangle), AxCACre (open circle), AxEFNCre (closed triangle), AxEFCre (closed circle), and AxEFLacZ (closed rectangle). The ALT levels are shown as means ± S.D. of three individual specimens. (B) Histopathologic changes in the livers of transgenic mice 7 days after injection of each AdV. The liver sections were stained with H&E. The arrows represent lymphocyte infiltrations. Scale bar, 50 μm. (C) mRNA expression of Ad-pIX in the livers. CN2-29 transgenic mice were injected with 1.0×10^9 PFU of the AdVs. After 12 h, the livers were harvested. The total RNA extracts from the livers were subjected to reverse transcription and RTD-PCR with an Ad-pIX-specific probe and a primer pair, as described in Section 2. The numbers of copies of Ad-pIX mRNA are shown as means ± S.D. of three individual specimens.

reverse transcription and quantitative RTD-PCR, as described under Section 2. The copy numbers of Ad-pIX mRNA were quite high in transgenic mice that were injected with AdV bearing the CAG promoter (Fig. 3C). The observed inflammation levels were consistent with the expression levels of Ad-pIX.

3.4. Liver inflammatory responses to the HCV protein inducibly expressed by AdVs in transgenic mice

Because our results indicated that severe liver injuries were caused by AdVs bearing the CAG promoter, we evaluated liver inflammatory responses to the HCV protein inducibly expressed by AdVs in transgenic mice 7 days post-injection according to the modified HAI scoring system (Fig. 4A) (Knodell et al., 1981; Yang et al., 1994). Among the transgenic mice, more severe liver damage was observed in those that were injected with Cre-expressing AdVs bearing the CAG promoter (Fig. 4A, AxCANCre and AxCACre) compared to those injected with Cre-expressing AdVs bearing the EF1α promoter (Fig. 4A, AxEFNCre and AxEFCre).

Because AxEFCre more efficiently expressed HCV proteins than AxEFNCre (Fig. 2C and D), we injected AxEFCre into transgenic mice and wild-type mice to examine the effects of HCV protein expression. The severity of liver inflammation in the AxEFCre-injected transgenic mice was significantly greater than in the AxEFCre-injected wild-type mice or the AxEFLacZ-injected transgenic mice (Fig. 4A and B).

Seven days after AdV administration, serum ALT levels of AxCANCre-injected wild-type mice were significantly higher than those of AxEFCre-injected wild-type mice (Fig. 4C). This ALT elevation was observed in both transgenic and wild-type mice injected with AxCANCre (Fig. 4C). In contrast, AxEFCre was injected into the two groups, transgenic mice expressing HCV proteins exhibited more severe liver injury than wild-type mice (Fig. 4C and D).

3.5. Effects of Cre-expressing AdV bearing the EF1α promoter on HCV protein expression in transgenic mouse livers

To investigate whether CN2-29 transgenic mice injected with AdVs bearing the EF1α promoter showed liver inflammation caused

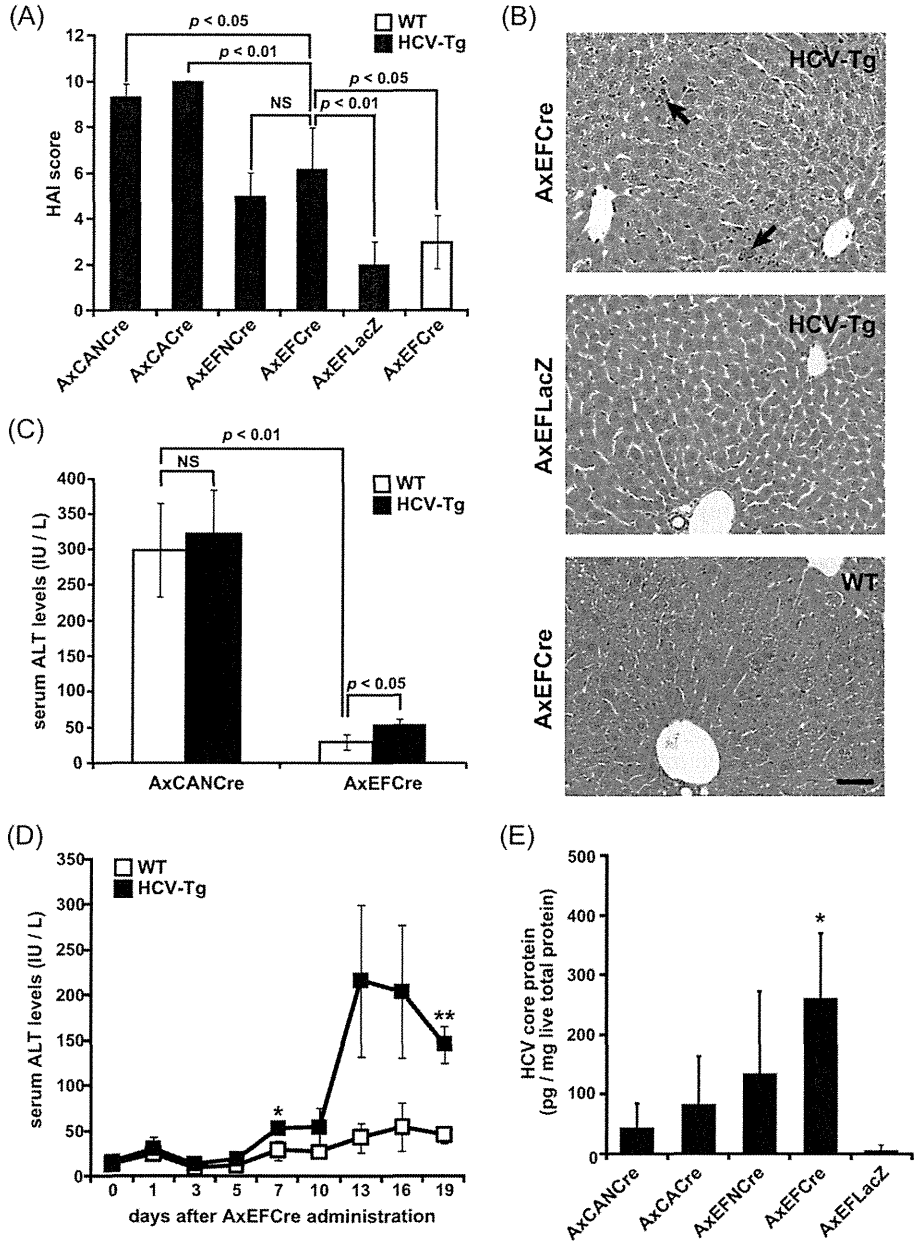

Fig. 4. Liver inflammatory responses due to HCV protein expression induced by AxEFCre. (A) Histopathology of mouse livers after injection of AdVs. Histopathologic features of the livers of CN2-29 transgenic mice (HCV-Tg, closed bars) injected with 1.0×10^9 PFU of AxCANCre, AxCACre, AxEFNCre, AxEFCre, or AxEFLacZ, and wild-type mice that were injected with 1.0×10^9 PFU of AxEFCre at day 7 post-injection (WT, opened bar). Pathologic changes were evaluated by light microscopy of H&E-stained sections of the mouse livers using the modified HAI scoring system. The extent of pathology was scored on a scale from 0 (none) to 12 (severe). All of the scores are means ± S.D. of more than three individual specimens. Statistical analysis was performed using an unpaired Student's *t*-test. NS, not significant. (B) Histopathologic changes resulting from HCV protein expression in mouse livers. CN2-29 transgenic mice (HCV-Tg) were injected with AxEFCre or AxEFLacZ and wild-type mice (WT) were injected with AxEFCre 7 days post-injection. The liver sections were stained with H&E. The arrows represent piecemeal necrosis. Scale bars, 50 μm. (C) Serum ALT levels with or without HCV protein expression 7 days after administration of the AdVs. Statistical analysis was performed using an unpaired Student's *t*-test between CN2-29 transgenic mice (HCV-Tg) and wild-type mice (WT). NS, not significant. (D) Sequential changes in serum ALT levels after AxEFCre administration. Serum ALT levels were measured at the indicated time-points in CN2-29 transgenic mice (HCV-Tg, closed square) or wild-type mice (WT, opened square) that were injected with 1.0×10^9 PFU AxEFCre. ALT levels are shown as means ± S.D. of more than three individual specimens. Statistical analysis was performed using an unpaired Student's *t*-test between CN2-29 transgenic mice (HCV-Tg) and wild-type mice (WT). $^*p < 0.05$; $^{**}p < 0.01$. (E) HCV core protein expression 21 days after AdV administration in transgenic mouse livers. CN2-29 transgenic mice were injected with 1.0×10^9 PFU for each AdV. After 21 days, the livers were harvested and homogenized. The concentrations of HCV core proteins in liver lysates were determined by EIA. The values shown are means ± S.D. of three individual experiments. Statistical analysis was performed using an ANOVA, followed by the SNK test. $^*p < 0.05$.

by persistently expressed HCV proteins, we evaluated core proteins by EIA in transgenic mouse livers 21 days post-injection of the AdVs (Fig. 4E). HCV core protein expression was scarcely detectable in the transgenic mice injected with AxCANCre, while the AxEFCre-injected transgenic mice showed significantly higher levels of core protein expression (Fig. 4E). Although, the AxCANCre injection was scarcely observed at day 21 in the transgenic mice (Fig. 4E), HCV core protein expression induced by AxEFCre injection was observed until at least day 56.

4. Discussion

In the present study, we demonstrated that Cre-expressing AdVs bearing the EF1α promoter induce HCV gene expression and

HCV protein production without induction of severe liver injury in inducible-HCV transgenic mice. We further observed that increases in serum ALT levels and liver inflammation were related to HCV protein expression mediated by AxEFCre injection. Moreover, AxEFCre injection enabled the transgenic mice to persistently express HCV proteins.

In previous studies, HCV transgenic mice constitutively expressing HCV proteins exhibited symptoms of steatosis and/or hepatocellular carcinoma, but did not show inflammatory or immunopathologic changes (Lerat et al., 2002; Moriya et al., 1997, 1998; Sun et al., 2001). Inducible-HCV transgenic mouse lineages, in which HCV protein expression is regulated, have enabled investigation of the immunopathogenesis of HCV protein expression. HCV transgenic mice regulated by the Cre/*lox*P system (Sun et al., 2005; Tumurbaatar et al., 2007; Wakita et al., 1998) or the tetracycline regulatory system (Ernst et al., 2007) exhibit inducible and liver-specific expression of HCV proteins. Inducible-HCV transgenic mice using the Cre/*lox*P system with an AdV that expresses Cre under the control of the CAG promoter (AxCANCre) exhibit HCV-specific immune responses (Wakita et al., 1998, 2000). The inducible-HCV CN2-29 transgenic mice, which express the core, E1, E2, and NS2 proteins, have HCV-specific cytotoxic T lymphocytes (Takaku et al., 2003; Wakita et al., 1998, 2000).

However, they show severe inflammatory responses to AxCANCre itself and thus, HCV protein expression is only transient (Wakita et al., 2000). These significant obstacles have limited the utility of inducible-HCV transgenic mice. Therefore, to deliver the *Cre* gene into the liver, non-adenoviral induction methods have been developed (Ho et al., 2008; Sun et al., 2005; Zhu et al., 2006). Meanwhile, adenoviral genes that cause cellular immune responses have been identified and modified AdVs that do not trigger host immune responses have been developed (Palmer and Ng, 2005). A recent study demonstrated that immune responses to AdVs bearing the CAG promoter were associated with co-expression of Ad-pIX, whereas immune responses were minimal when transgene expression was controlled by the EF1α promoter (Nakai et al., 2007). Therefore, we postulated that severe inflammation of mouse livers after administration of Cre-expressing AdVs bearing the CAG promoter (AxCANCre) might be caused by expression of Ad-pIX. In the present study, we generated Cre-expressing AdVs bearing the EF1α promoter (AxEFCre) and infected HCV transgenic mice. AxEFCre-injected mice expressed much less Ad-pIX mRNA and did not show the increased levels of ALT or severe liver inflammation as did Cre-expressing AdVs under the control of the CAG promoter (Fig. 3). In contrast, AxCANCre administration caused severe liver injury in both HCV transgenic mice and wild-type mice (Fig. 4D; Wakita et al., 2000). AxEFCre administration caused liver injury in the HCV transgenic mice, but not in the wild-type mice (Fig. 4A–D). These results suggest that AxEFCre alone induces only minimal host immune responses compared to AxCANCre; therefore, the liver inflammatory responses exhibited by AxEFCre-injected transgenic mice were clearly due to expression of HCV proteins. Because AxCANCre injection alone causes severe liver injuries, most of the hepatocytes infected with AxCANCre are eliminated and HCV protein expression in the livers of transgenic mice is only transient (Wakita et al., 2000). On the other hand, AxEFCre injection did not induce such severe liver injuries. The AxEFCre-injected HCV transgenic mice showed milder liver inflammation in response to expression of HCV proteins and persistently expressed HCV proteins without elimination of hepatocytes infected with AxEFCre.

In conclusion, HCV gene expression mediated by the Cre/*lox*P system and a Cre-expressing AdV that bears the EF1α promoter, AxEFCre, enables Cre-mediated recombination of transgenes in mice without inducing severe liver injury due to the AdV itself. Moreover, this inducible-HCV transgenic mouse model should be useful for investigation of liver injury due to HCV and the pathogenesis of HCV.

Acknowledgments

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MAJOR ARTICLE

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

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

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

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

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

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

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

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

Materials And Methods

Cell Lines and Reagents

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

Generation of 2-152a MAb

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

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

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

Replication Assay Using HCV Replicon Cells

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

Immunostaining and Antibodies

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

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

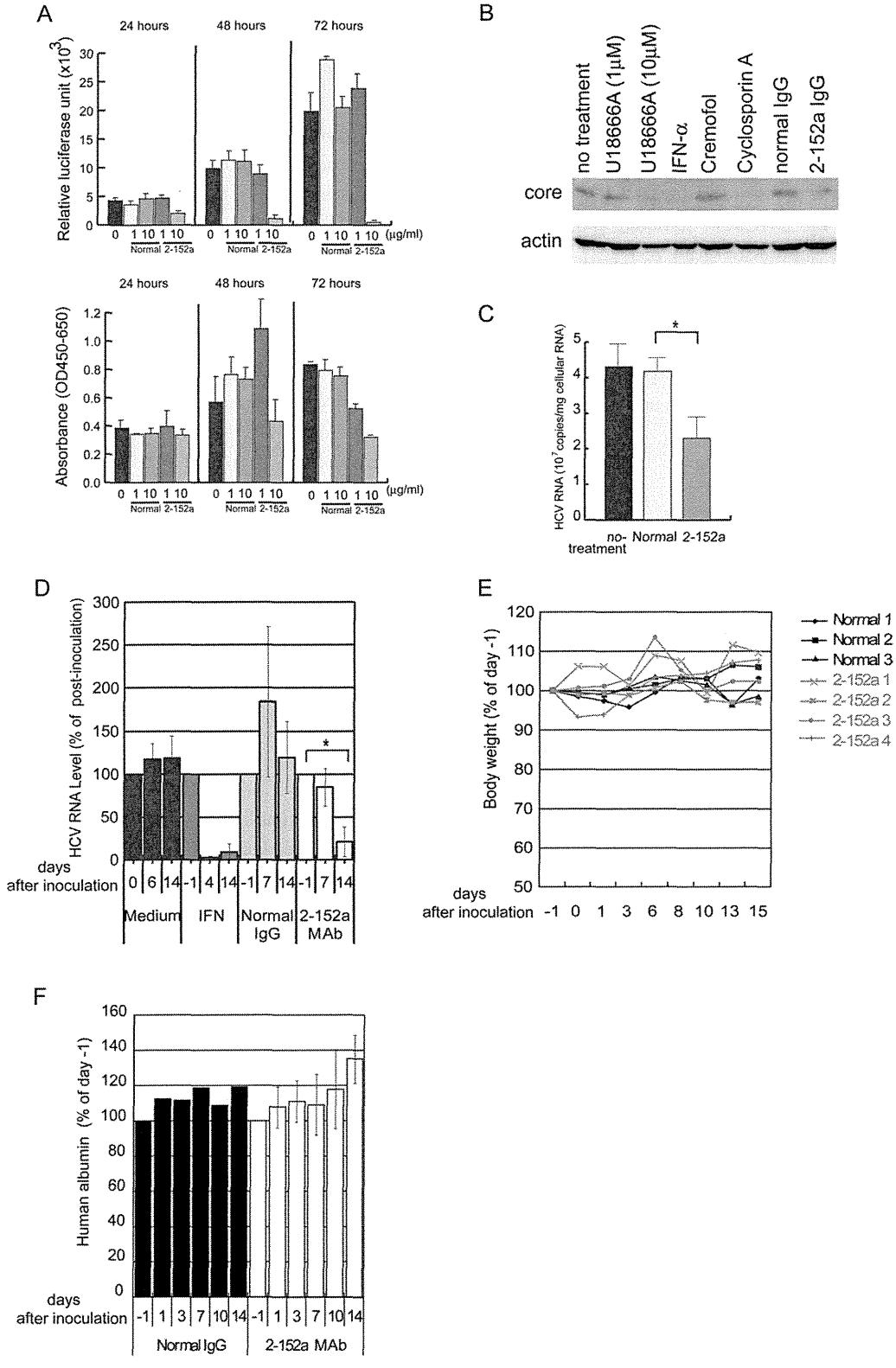


Figure 1. Anti-DHCR24 monoclonal antibody (2-152a MAb) suppresses HCV replication in vitro and in vivo. *A*, The effects of 2-152a MAb on HCV replication were measured by the luminescence activity and cell viability in FLR3-1 cells. The replicon cell line was incubated with IgG from normal mice or 2-152a 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.

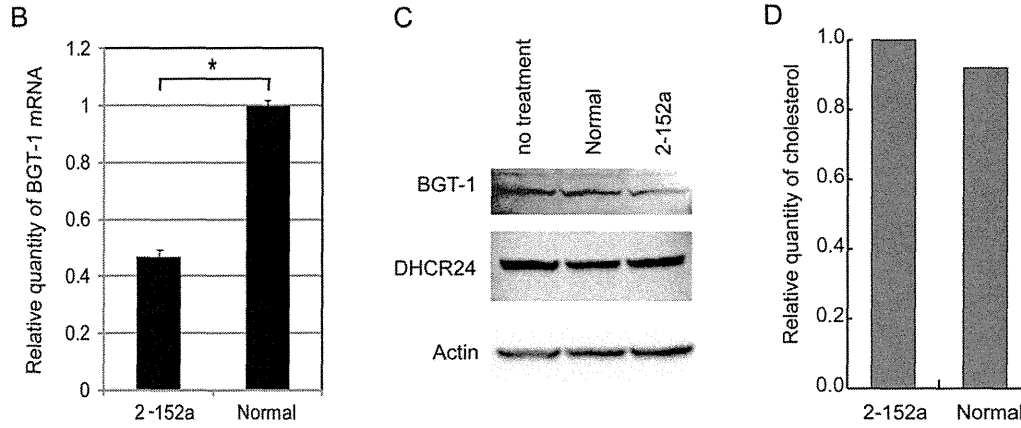
Suppression of HCV Infection by 2-152a MAb

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.

A

R6 2-152a 24h Gene Name	2-152a/normal IgG	FLR3-1 2-152a 24h Gene Name	2-152a/normal IgG	FLR3-1 2-152a 72h Gene Name	2-152a/normal IgG	K4 2-152a 24h Gene Name	2-152a/normal IgG
CNN1	2.63	CNN1	2.18	CGA	1.54	KIAA0367	1.97
A_24_P3983							
70	2.57	ACTA1	1.98	TAGLN	1.47	ACTA1	1.90
ACTA1	2.44	SLC16A14	1.59	CNN1	1.39	CNN1	1.88
CSTA	1.71	TAGLN	1.52			A_24_P39837	1.81
ENST000002							
98047	1.7	LYPD1	1.51	RSNL2	0.75	SLC16A14	1.80
TAGLN	1.63	IL11	1.51	SLC37A2	0.74	ADH1A	1.59
AI379175	1.6	KCNJ8	1.41	AKR1C1	0.73	ROBO2	1.56
MGAM	1.58	MSRB3	1.4	BG542103	0.72	AKR1D1	1.54
MSRB3	1.57	C8orf4	1.4	PTGS1	0.71	SLC17A1	1.50
MSRB3	1.56	PPP3R1	1.39	THC2437143	0.71	SLC16A14	1.47
EPPK1	1.47	ELF5	0.73	AKR1B10	0.71	BC036599	1.46
THC2317432	1.45	CYP3A7	0.72	SLC6A14	0.70	TAGLN	1.44
AK055214	1.43	COL14A1	0.71	AKR1B10	0.70	MSRB3	1.43
SLC16A6	1.39	LOC401022	0.71	COL14A1	0.69	SOCS2	1.37
AKR1C1	0.75	THC2437143	0.7	SLC6A14	0.69	FXYD2	0.74
AKR1C1	0.74	BG542103	0.7	SMPD3	0.67	ENST0000036	0.73
CD44	0.74	S100A4	0.7	VNN2	0.66	SLC7A8	0.72
CD44	0.73	PTGS1	0.69	FXYD2	0.65	ARG2	0.72
ARG1	0.72	F2RL2	0.68	F2RL2	0.62	IGFBP5	0.71
F2RL2	0.72	FUT5	0.67	BGT-1	0.61	ROBO3	0.71
CYP3A7	0.71	FCGBP	0.66	FXYD2	0.59	GPX2	0.70
CD44	0.7	FUT3	0.64	FLJ25422	0.42	CR603668	0.70
LOC642775	0.7	PTGS1	0.64			IGFBP5	0.69
S100A4	0.68	SLC6A14	0.63			COL14A1	0.69
SLC7A8	0.67	VNN2	0.63			AF118081	0.68
VNN2	0.65	THC2442210	0.63			VNN2	0.67
ROBO3	0.65	ZNF114	0.62			HOXD1	0.66
CDKN1C	0.63	SMPD3	0.61			CDKN1C	0.66
FUT3	0.61	BGT-1	0.58			THC2442210	0.66
KCNMA1	0.6	CDKN1C	0.49			LOC647022	0.65
BGT-1	0.58					COL14A1	0.62
						SLC6A14	0.53
						FUT3	0.53



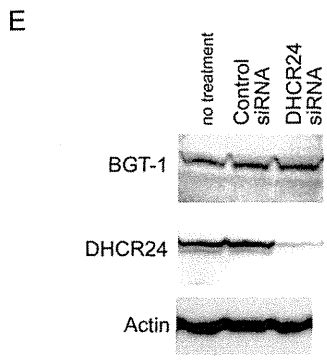


Figure 2. *A*, Genes that showed significant changes in expression after the 2-152a MAb treatment. HCV replicon cells (FLR3-1 and R6FLR-N) and K4 cells were treated with 2-152a MAb. The symbols shaded in gray indicate the genes that showed significantly changed expression commonly in R6FLR-N and FLR3-1 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.