



Novel pH-sensitive multifunctional envelope-type nanodevice for siRNA-based treatments for chronic HBV infection

Naoki Yamamoto^{1,†}, Yusuke Sato^{2,†}, Tsubasa Munakata¹, Masakazu Kakuni³, Chise Tateno³, Takahiro Sanada¹, Yuichi Hirata¹, Shuko Murakami⁴, Yasuhito Tanaka⁴, Kazuaki Chayama⁵, Hiroto Hatakeyama², Mamoru Hyodo², Hideyoshi Harashima², Michinori Kohara^{1,*}

¹Department of Microbiology and Cell Biology, Tokyo Metropolitan Institute of Medical Science, Tokyo 156-8506, Japan; ²Laboratory of Innovative Nanomedicine, Faculty of Pharmaceutical Sciences, Hokkaido University, Hokkaido 060-0812, Japan; ³PhoenixBio Co., Ltd., 3-4-1, Kagamiyama, Hiroshima 739-0046, Japan; ⁴Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya 467-8601, Japan; ⁵Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Minami-ku, Hiroshima 734-8551, Japan

Background & Aims: Antiviral agents including entecavir (ETV) suppress the replication of the hepatitis B virus (HBV) genome in human hepatocytes, but they do not reduce the abundance of viral proteins. The present study focused on effectively reducing viral protein levels.

Methods: We designed siRNAs (HBV-siRNA) that target consensus sequences in HBV genomes. To prevent the emergence of escaped mutant virus, we mixed three HBV-siRNAs (HBV-siRNAmix); the mixture was encapsulated in a novel pH-sensitive multifunctional envelope-type nanodevice (MEND), a hepatocyte-specific drug delivery system. Coagulation factor 7 siRNA was used to assess delivery and knockdown efficiencies of MEND/siRNA treatments in mice. The potency of MEND/HBV-siRNAmix was evaluated in primary human hepatocytes and in chimeric mice with humanized liver persistently infected with HBV.

Results: Effective knockdown of targets, efficient delivery of siRNA, and liver-specific delivery were each observed with MEND. MEND/HBV-siRNA caused efficient reduction of HBsAg and HBeAg *in vitro* and *in vivo*. However, ETV treatment did not efficiently reduce HBsAg or HBeAg when compared with a single

MEND/HBV-siRNAmix treatment. Furthermore, the suppressive effects of a single dose of MEND/HBV-siRNAmix persisted for 14 days *in vitro* and *in vivo*.

Conclusion: We demonstrated that MEND/HBV-siRNA controlled HBV more efficiently than did ETV. Furthermore, the effect of a single dose of MEND/HBV-siRNA persisted for a long time. These results indicated that MEND/HBV-siRNA may be a promising novel HBV treatment that is more effective than reverse transcriptase inhibitors.

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Introduction

Hepatitis B virus (HBV) is a noncytopathic, hepatotropic virus of the *Hepadnaviridae* family that causes either acute or chronic hepatitis in humans [1]. Chronic infection is mostly asymptomatic, but HBV carriers are at risk of developing cirrhosis and hepatic carcinoma [2]. Despite the availability of prophylactic vaccines, HBV is estimated to infect approximately 350 million people and cause 600,000 hepatitis B-related deaths annually worldwide. The HBV genome is a relaxed circular, partially double-stranded DNA molecule, surrounded by the HBV core antigen (HBcAg) and an envelope containing the HBV surface antigen (HBsAg) [3]. The negative strand has an invariable length of about 3.5 kb, and the positive strand is shorter than the negative strand. Following infection, covalently closed circular DNA (cccDNA) is synthesized and maintained at low copy numbers in the nuclei of infected hepatocytes; cccDNA serves as the transcription template for all viral RNAs [4]. Thus, cccDNA is responsible for the establishment of viral infection and persistence.

Currently, interferon (IFN)- α is used for hepatitis B therapy, and IFN- α treatment can result in virus clearance in some cases, but its efficacy is limited, and most patients cannot tolerate high doses [5,6]. Other clinically available HBV drugs are nucleoside or nucleotide analogues that inhibit the viral reverse transcriptase

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* Corresponding author. Address: Department of Microbiology and Cell Biology, Tokyo Metropolitan Institute of Medical Science, 2-1-6, Kamikitazawa, Setagaya-ku, Tokyo 156-8506, Japan. Tel.: +81 3 5316 3232; fax: +81 3 5316 3137.

E-mail address: kohara-mc@igakuken.or.jp (M. Kohara).

[†] These authors contributed equally to this work.

Abbreviations: HBV, hepatitis B virus; ETV, entecavir; MEND, pH-sensitive multifunctional envelope-type nanodevice; cccDNA, covalently closed circular DNA; HBsAg, hepatitis B virus surface antigen; HBeAg, hepatitis B virus e antigen; HBcAg, hepatitis B virus core antigen; IFN, interferon; RT, reverse transcriptase; PHH, primary human hepatocytes; PTH, primary tupaia hepatocytes; NTCP, sodium taurocholate cotransporting polypeptide; siRNA, short-interfering RNA; Geq, genome equivalents; pDNA, plasmid DNA; CTLs, cytotoxic T lymphocytes.



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(RT), but they reportedly result in viral resistance [7]. Moreover, RT inhibitors only control HBV infection, but never lead to elimination of HBV because HBV mRNAs derived from cccDNA are continuously produced even under treatment. Consequently, novel approaches for the treatment of HBV-infected individuals are needed. However, the absence of a robust cell culture system for HBV infection has stymied both virus lifecycle analyses and drug discovery efforts.

Primary human hepatocytes (PHH) and primary Tupaia hepatocytes are susceptible to HBV infection, but use of either cell type is hampered by limited availability of and unpredictable variability among liver specimens, human or Tupaia [8]. HepaRG cells are also susceptible to HBV infection, but this susceptibility is strictly dependent on HepaRG differentiation state [9]. Recently, sodium taurocholate cotransporting polypeptide (NTCP) has been identified as a HBV receptor [10]. Although overexpression of NTCP in HepG2 and HuH7 cells can render these cells able to support HBV infection and production, NTCP-transfected HepG2 or HuH7 cells are much less efficient model cells than PHH or primary Tupaia hepatocytes cells.

Here we have used PXB cells, which are primary hepatocytes derived from chimeric mice with humanized liver tissues [11], to develop a novel therapy for HBV treatment. PXB cells are susceptible to HBV infection, and more available and consistent than PHH cells. To find a cure for HBV infection, we have focused on short-interfering RNAs (siRNAs) that target HBV-specific RNAs to suppress production of HBV proteins. Several HBV proteins have been shown to downregulate the adaptive immune response to HBV [12,13]. Elimination of HBV proteins may be overcome by suppression of CD8⁺ T cell responses in chronic HBV-infected patients. One of the most challenging issues with siRNA-based therapies for HBV is targeting the delivery of the siRNAs to hepatocytes. The siRNA carrier we used is a novel pH-sensitive multifunctional envelope-type nanodevice (MEND). Previously, a MEND was able to deliver siRNA specifically to the liver in an animal model [14]. We have found that selected siRNAs that target consensus sequences of HBV RNAs, downregulated levels of HBV DNA, HBsAg and HBeAg *in vitro* and *in vivo*. These findings indicated that our approach could be the basis for a novel class of anti-HBV drug that is superior to RT inhibitors.

Materials and methods

Synthesis of compound 2

DLin-ketone was synthesized from linoleic acid as described previously [15]. *tert*-Butyl diethylphosphonoacetate (2.14 ml, 9.11 mmol) and *t*BuOK (937 mg, 8.35 mmol) were successively added to dry *t*BuOH (40 ml); the mixture was stirred at room temperature (r.t.) for 5 min; DLin-ketone (4.00 g, 7.59 mmol) dissolved in dry *t*BuOH (10 ml) was added. The mixture was refluxed for 3 h and then concentrated. The residue was dissolved in EtOAc (50 ml), and the organic layer was washed with saturated NH₄Cl (2 × 50 ml). The organic phase was dried over anhydrous Na₂SO₄. Evaporation of the solvent resulted in a yellowish oily residue. The residue was purified using an automated Teledyne ISCO combiflush Rf chromatography system (hexane/EtOAc). This process yielded 3.54 g (75%) of compound 2 as a colorless oil with the following properties: ¹H NMR (500 MHz, CDCl₃) δ: 5.52 (1H, s), 5.32–5.37 (8H, m), 2.76 (4H, t), 2.53 (2H, t), 1.97–2.09 (10H, m), 1.43 (9H, s), 1.20–1.40 (36H, m), 0.87 (6H, t) ppm.

Synthesis of compound 3

Compound 2 (453 mg, 0.725 mmol) was dissolved in 10% (v/v) of trifluoroacetic acid in dichloromethane (DCM). The mixture was stirred at r.t. for 4 h. EtOAc

(50 ml) was added to the mixture that was then washed with HCl solution (1 M, 50 ml × 2) and brine (50 ml). The organic phase was dried with Na₂SO₄. Evaporation of the solvent gave a pale yellow oily residual crude, which was used in subsequent experimental steps without further purification.

Synthesis of compound 4 (YSK13-C3)

Compound 3 (3.00 g, 5.28 mmol) was dissolved in DCM (26 ml); DMAP (323 mg, 2.64 mmol) and EDCI (2.53 g, 13.2 mmol) were then added to this solution. The mixture was stirred for 1 h at r.t., and then 3-(dimethylamino)-1-propanol (1.54 ml, 13.2 mmol) was added. This reaction mixture was stirred at r.t. overnight. The reaction mixture was diluted with DCM (100 ml) and washed with NaOH solution (1 M, 2 × 100 ml) and brine (100 ml). The organic phase was dried with Na₂SO₄, and solvents were removed *in vacuo*. The crude product was purified using an automated Teledyne ISCO combiflush Rf chromatography system (DCM/MeOH). This process yielded 1.90 g of YSK13-C3 as a pale yellow oil with the following properties: ¹H NMR (500 MHz, CDCl₃) δ: 5.60 (1H, s), 5.29–5.40 (8H, m), 4.10 (2H, t), 2.76 (4H, t), 2.57 (2H, t), 2.33 (2H, t), 2.21 (6H, s), 2.12 (2H, t), 1.97–2.09 (8H, m), 1.81 (2H, m), 1.20–1.45 (36H, m), 0.88 (6H, t) ppm.

Preparation and characterization of MEND/siRNA

The *t*BuOH dilution method was used to prepare MEND/siRNA. Briefly, a 90% aqueous *t*BuOH solution containing YSK13-C3, cholesterol (SIGMA Aldrich, St. Louis, MO), and mPEG_{2k}-DMG (NOF Corporation, Tokyo, Japan) (molar ratio: 70/30/3) was mixed with siRNA solution in a ratio of 2:1 (v/v). The lipid/siRNA solution was diluted in 20 mM citrate buffer (pH 4.0) with vigorous mixing. Ultrafiltration was used to remove the *t*BuOH, adjust the pH to 7.4, and concentration the products. Dil- or DiD-labeled MEND was prepared by adding Dil or DiD (0.5 mol% of total lipid, Invitrogen, Carlsbad, CA, USA) to the lipid mixture. Size and polydispersity of the MEND/siRNA were measured using a Zetasizer Nano ZS ZEN3600 instrument (Malvern Instruments, Worcestershire, UK). Ribogreen (Molecular Probes, Eugene, OR, USA) was used to determine the total siRNA concentration and the ratio of encapsulated siRNA to total siRNA. A negatively charged fluorescent dye 6-(*p*-toluidino)-2-naphthalenesulfonic acid (TNS) (Wako Chemicals, Osaka, Japan) was used to measure the apparent pKa of the MEND.

Evaluation of F7 gene silencing activity

MEND/siF7 at the indicated doses was intravenously administered to ICR mice (Japan SLC, Shizuoka, Japan). Mice were anesthetized 48 h after siRNA treatment, and blood samples were collected via cardiac puncture and processed to plasma with heparin. Biophen F7 chromogenic assay kits (Aniara Corporation) were used according to the manufacturer's protocol to measure plasma F7 activity.

Evaluation of biodistribution

DiD-labeled MEND/siRNA was administered to ICR mice at a dose of 0.5 mg/kg ICR. Liver, lung, spleens, and kidneys were collected 30 min after treatment; a Precellys 24 was used to homogenize each tissue sample in 1% sodium dodecylsulfate solution. VarioskanFlash (Thermo Scientific) was used to measure the fluorescence intensity at λ_{ex} = 648 nm and λ_{em} = 669 nm of each homogenate.

Observation of intrahepatic distribution

Dil-labeled MEND/Cy5-labeled siRNA was administered to ICR mice at a dose of 1 mg/kg. Each treated mouse was then injected with 40 μg FITC-conjugated Isolectin B4 (Vector Laboratories, Burlingame, CA, USA) 25 min after MEND treatment, and liver tissues were collected 30 min after MEND treatment. Hoechst33342 (Dojindo Laboratories, Kumamoto, Japan) was used to stain cell nuclei in liver specimens; a Nikon A1 (Nikon Co. Ltd., Tokyo, Japan) was then used to visualize the intrahepatic distribution of the MEND/siRNA. Images were captured with a 20× objective.

Preparation of siRNA for HBV knockdown

Each siRNA was designed to target sequences that are highly conserved among HBV 17 strains (Genotype A: AB246338, AY161149, X51970, AM282986, AF297621, Genotype B: AB246341, AB073858, AB033554, D00329, D00330, AF100309, Genotype C: AB246345, AB048704, AB014381, AY123041,

AB033556, AB231908). Chemically synthesized HBV-siRNAs and a control siRNA were purchased from Invitrogen (Carlsbad, CA, USA) and Thermo Scientific (Walsham, MA, USA), respectively. The sequences of these siRNAs were:

5'-UUGAGAGAAGUCCACCACGAGUCUA-3' (si249),
 5'-AAUUGAGAGAAGUCCACCACGAGUC-3' (si251),
 5'-UCAAGAUGUUGUACAGACUUGGCC-3' (si758),
 5'-CAGAGGUGAAGCGAAGUGCACACGG-3' (si1575),
 5'-GUGAAAAAGUUGCAUGGUGCUGUG-3' (si1804),
 5'-UUUCCGAAGUGUAGAUAGAG-3' (si2312),
 5'-UAAAGUUUCCACCUUAGAGUCCA-3' (si2460)
 5'-CUAAUACAGCCAAUACAUUU-3' (Negative control siRNA) (only the antisense strands are shown).

The HBV-siRNAmix was a mixture comprising equal moles of individual siRNAs; HBV-siRNAmix was prepared from si251, si1804, and si2312.

Primary human hepatocytes (PXB cells)

A monolayer of primary human hepatocytes derived from chimeric mice with human liver were obtained from PhoenixBio Co., Ltd (Hiroshima, Japan) and grown in collagen-coated plates (Corning Life Science, Tewksbury, MA, USA) [11,16,17]. Hepatocytes were cultured in dHCGM medium consisting of Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY, USA) with 10% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, USA), 20 mM HEPES (Life Technology), 44 mM NaHCO₃, antibiotics (100 U/ml penicillin G and 100 µg/ml streptomycin, Grand Island, NY, USA), 15 µg/ml L-proline (Wako, Osaka, Japan), 0.25 µg/ml Insulin (SIGMA, St Louis, MO, USA), 50 nM dexamethazone (SIGMA), 5 ng/ml epidermal growth factor (Millipore, Bedford, MA, USA), 0.1 mM L-ascorbic acid (Wako), and 2% dimethyl sulfoxide (SIGMA).

Preparation of HBV inoculum and infection *in vitro*

Sera for HBV inocula were obtained from chimeric mice infected with HBV-containing culture medium (genotypes: Ae_JPN, Bj_JPN35, and C_JPNAT; accession numbers: AB246338, AB246341, and AB246345, respectively). SMITEST EX-R&D (MBL; Medical & Biological laboratories Co., LTD, Nagano, Japan) was used to isolate HBV DNA from sera, and HBV DNA copy number/sample was determined by real-time PCR. Primary hepatocytes were infected with HBV at 5-genome equivalents (Geq)/cell in the presence of 4% polyethylene glycol 8000 (PEG8000, Promega, Madison, WI, USA). Inoculated cells were washed 1 and 2 days after infection; medium was harvested every 5 days after that.

In vitro HBV suppression assay

MEND/siRNAs were diluted in FBS and then mixed with dHCGM medium that contained 20% FBS. After 24 h treatment, the medium was replaced with dHCGM. Baraclude (Entecavir; ETV, Bristol-Myers Squibb, NY, USA) was dissolved in DMSO; this solution was added to the dHCGM during the examination.

DNA extraction and quantification of HBV DNA

SMITEST EX-R&D was used to extract HBV DNA from either 30 µl of culture medium or 1 µl of humanized-mouse serum. SMITEST EX-R&D was also used according to the manufacturer's instructions to extract intracellular HBV DNA from cell lysates. Quantification of HBV DNA was performed as described previously [18]. The mean value and standard deviation of three independent experiments are shown. The mean value of each control siRNA-treated or untreated sample was defined as the baseline.

Detection of HBsAg and HBeAg

Supernatants of cultures were harvested 7 and 14 days after treatment with MEND/HBV-siRNAs, MEND/HBV-siRNAmix, or entecavir. In treatment of MEND/HBV-siRNAs, supernatants of three independent experiments were pooled. Sera of chimeric mice were collected one day before and 1, 3, 5, 7, 10 and 14 days after MEND/HBV-siRNAmix treatment. Each supernatant sample was diluted 15-fold with saline. Each sera sample was diluted 300 or 600-fold with saline. Architect HBsAg QT and Architect HBeAg QT (Abbott Japan Co., Ltd., Tokyo) in SRL, Inc.

(Tokyo, Japan) were used to measure HBsAg and HBeAg, respectively, in each supernatant or sera sample (Tokyo, Japan). The mean values and standard deviations of three independent experiments are shown. The mean value of each control siRNA or untreated sample was defined as the baseline.

Generation of chimeric mice infected with HBV

Chimeric mice infected with HBV were prepared as previously described [19]. In brief, human hepatocytes were transplanted into uPA^{+/+}/SCID mice in PhoenixBio (Hiroshima, Japan) [11]. Then mice aged more than 12 weeks were injected with sera from chimeric mice infected with HBV (Genotype C_JPNAT, 1x10⁶ copies per dose). Kinetics of virus titers in sera were observed for 42 days. At the time of ETV or MEND/HBV-siRNAmix administration, serum levels of HBV genomic DNA were 1.5 × 10⁹ to 5.6 × 10⁹ or 3.3 × 10⁷ to 9.1 × 10⁸ copies/ml, respectively.

Treatment of chimeric mice

Chimeric mice infected with HBV each were randomly allocated into four groups (Genotype A MEND/HBV-siRNAmix; n = 2, MEND/control siRNA; n = 3, Genotype C MEND/HBV-siRNAmix; n = 3, MEND/control siRNA; n = 2 ETV; n = 5, Untreated; n = 5). Starting on day 0, mice were given a single intravenous dose of MEND/HBV-siRNAmix or MEND/control siRNA (5 mg/kg). ETV was dissolved in DMSO and then diluted with saline to 3 µg/ml. The dose formulation of ETV was administered to five mice via oral gavage once a day (30 µg/kg). Human serum albumin in the blood was measured using Alb-II kit (Eiken Chemical, Tokyo, Japan) according to the manufacturer's instructions.

Results

Development of optimal MEND/siRNA for hepatocytes

The capacity of an siRNA carrier to escape from the endosomal compartment is important for efficient siRNA delivery to hepatic tissue [20]. In a previous study, we developed a pH-sensitive cationic lipid, designated YSK05, which exhibited higher fusogenic activity with biomembranes than conventional lipids, and it mediated higher gene silencing activity [21]. Here, we designed a new pH-sensitive cationic lipid, designated YSK13-C3, to further improve gene silencing activity in hepatocytes. YSK13-C3 was synthesized from DLin-ketone in 3 steps (Fig. 1A). The MEND used in this study was formulated using YSK13-C3, cholesterol, mPEG_{2k}-DMG, and siRNA in a tBuOH dilution procedure. The mean diameter of the MENDs was ~75 nm, and the MEND size distribution was narrow (polydispersity: ~0.08). The *in situ* pKa analysis using a TNS indicated that the apparent pKa of the MENDs was 6.45, which is within the optimal range for hepatic gene silencing *in vivo* [22]. To evaluate the gene silencing activity with this MEND, siRNA targeting coagulation factor 7 (siF7) was formulated with the MEND and intravenously injected to mice. Dose-dependent F7 silencing was observed (Fig. 1B), and the ED₅₀ (the dose that causes 50% gene silencing) was 0.015 mg/kg, which is approximately 4-fold less than for YSK05-MEND. A biodistribution study revealed that more than 80% of the MEND accumulated in the liver, and only around 1% of the MEND was detected in any other single tissue including lung, spleen, or kidneys (Fig. 1C). We also examined the intrahepatic distribution of MEND/siRNA. In this experiment, the lipid membrane and siRNA were labeled with DiI and Cy5, respectively. Both MEND lipid and the siRNA had accumulated throughout liver tissue (Fig. 1D), and for the most part, MEND lipid and siRNA colocalized. These results indicated that the MEND that contained YSK13-C3 specifically accumulated in liver and exhibited efficient hepatic gene silencing with a single dose.

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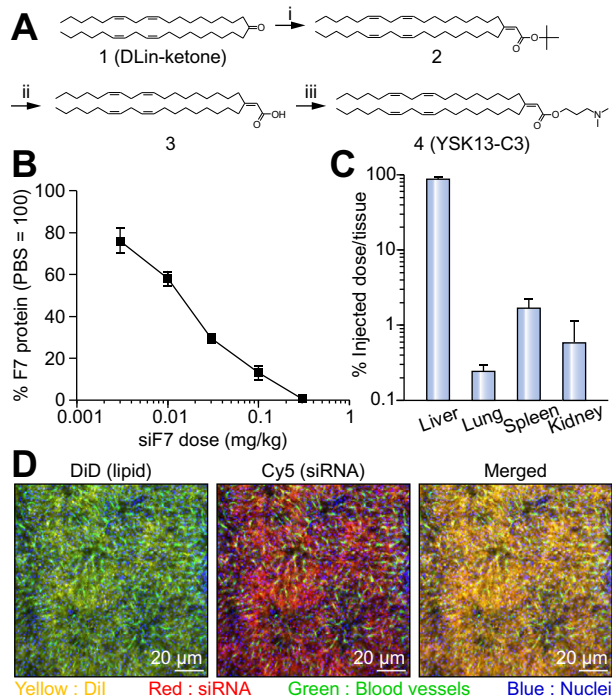


Fig. 1. Development of a new pH-sensitive cationic lipid YSK13-C3 and characterization of MEND/siRNA. (A) Synthesis of YSK13-C3. Reagents: (i) 1, *tert*-butyl diethylphosphonoacetate, *t*BuOH, *t*BuOK; (ii) TFA/DCM 1/9; (iii) 3-(dimethylamino)-1-propanol, EDC, DMAP, DCM. (B) Dose-dependent F7 gene silencing activity of MEND/siF7 in normal mice. (C) Quantification of biodistribution of MEND/siRNA. Lipid membrane of MEND was labeled with DiD. (D) Observation of intrahepatic distribution of MEND/siRNA. Lipid membrane and siRNA were labeled with DiI and Cy5, respectively.

Preparation of siRNAs that target HBV mRNA

HBsAg was translated from 2.1 and 2.4 kb transcripts, and HBeAg was derived from 3.5 kb transcripts (Fig. 2A). In order to reduce the amounts of HBsAg and HBeAg, we prepared seven siRNAs that targeted highly conserved regions of HBV mRNAs. The si249, si251, and si758 were designed to target 2.1, 2.4, and 3.5 kb transcripts. Target sequences of si1575 and si1804 were located in all transcripts, whereas those of si2312 and si2460 were located in 3.5 kb transcript. Indeed, the six siRNAs completely matched the viral sequence used in this experiment (Fig. 2B).

MEND/HBV-siRNA reduces the HBV antigens and HBV DNA *in vitro*

We developed an *in vitro* assay system to evaluate the suppressive effect of MEND/HBV-siRNA on HBV antigens. PHH designated PXB cells were derived from chimeric mice with humanized liver; PXB cells were seeded into culture plates and infected with HBV at 5 Geq/cell (Fig. 3A). HBV genotypes A, B, and C were propagated for more than 40 days in these cell cultures (Fig. 3B). Quantification of HBV DNA demonstrated that this culture system was suitable for the maintenance of long-term HBV infection, which would mimic chronic infection in patients.

The persistently infected cells were treated with the indicated MEND/HBV-siRNAs or a MEND/control siRNA for 24 h, and the amounts of HBsAg and HBeAg in medium were measured on days 7 and 14 (Fig. 3C, D). HBsAg levels in culture supernatant were

reduced by each of three siRNAs (si251, si1575, or si1804) for cells infected with genotype A, B, or C; moreover, these suppressive effects of the MEND/HBV-siRNAs continued for 14 days. In contrast, the two siRNAs (si2312 and si2460) that targeted sites located outside of the transcribed region of HBsAg had no suppressive effects on HBsAg levels for cells infected with A, B, or C. HBeAg levels in culture supernatant were reduced by each of five siRNAs (si251, si1575, si1804, si2312, or si2460) for cells infected with genotype A or C (Fig. 3D). HBeAg of genotype B could not be detected in this study, which depended on the Architect HBeAg QT (Abbott Japan Co., Ltd., Tokyo) assay. These results indicated that individual siRNAs that targeted different HBV mRNA led to a reduction of the respective antigen, HBsAg or HBeAg, and that these suppressive effects persisted for 14 days.

A HBV transcript is used as a template for synthesis of genomic HBV DNA. In addition, RT itself is translated from the full-length HBV transcript. Thus, we next investigated whether MEND/HBV-siRNAs affected HBV DNA amount. HBV-DNAs in the supernatant were reduced by si251, si1575, si1804, si2312, or si2460 siRNAs in the supernatant of the cells infected with genotype A, B or C (Fig. 3E). These results indicated that a single dose of the MEND/HBV-siRNAs could reduce the amount of HBV DNA and the amount of HBV antigens for 14 days.

Mixture of siRNAs leads to efficient and simultaneous reduction of both HBV antigens and of HBV DNA *in vitro*

Viruses replicated via RT are subject to high mutation rates [23–25]. In the present study, in order to prevent emergence of escaped mutant viruses, we prepared a MEND/HBV-siRNAmix that contained three siRNAs (si251, si1804, and si2312), each targeted to a different transcribed region. The suppressive effect of the MEND/HBV-siRNAmix on both HBV antigens and on HBV DNA levels was evaluated *in vitro* (Fig. 4A). Primary hepatocytes were treated with MEND/HBV-siRNAmix for 24 h. Additionally, to compare the effects of MEND/HBV-siRNAmix on HBV, parallel cell cultures were treated with ETV for 14 days. Rapid and efficiently reductions of HBsAg and HBeAg in the supernatant were observed with a single treatment of MEND/HBV-siRNAmix (Fig. 4B). HBsAg levels were suppressed to less than 9% of non-treated levels by administration of 22.5 nM MEND/HBV-siRNAmix. MEND/HBV-siRNAmix dramatically reduced HBeAg by day 7. In contrast, ETV did not efficiently reduce the amounts of HBsAg or HBeAg *in vitro* (Fig. 4B), but ETV did significantly reduce HBV DNA amounts (Fig. 4C, D). Suppressing effects of a single dose of MEND/HBV-siRNAmix on extracellular and intracellular levels of HBV DNA were comparable to those of ETV for 7 days. These results indicated that MEND/HBV-siRNAmix could reduce the amount of HBV antigens as well as that of viral DNA. In contrast, ETV had little ability to reduce HBV antigens *in vitro*.

MEND/HBV-siRNAmix leads to decreased levels of both HBV DNA and HBV antigens *in vivo*

Because the MEND/HBV-siRNAmix suppressed the production of viral antigens in primary hepatocytes, we evaluated the effects of this treatment on HBV antigens *in vivo*. Before conducting the experiment, we analyzed viral proliferation in chimeric mice. On 4 weeks post-infection, viral proliferation approached a plateau (Fig. 5B). Chimeric mice persistently infected with HBV were given a single intravenous dose of MEND/HBV-siRNAmix

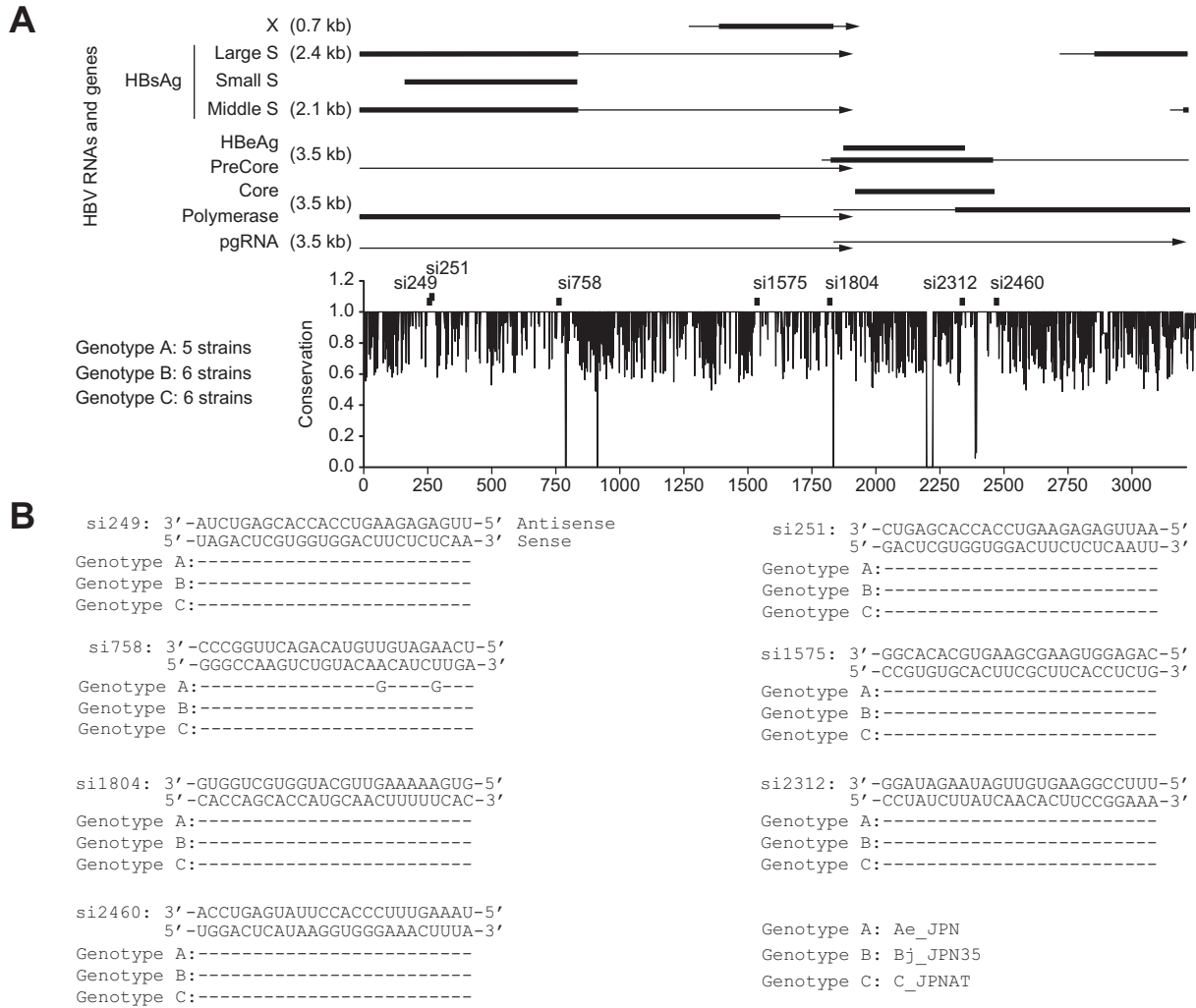


Fig. 2. Selection of siRNA targeting HBV mRNAs. (A) Conservation of HBV sequences and target regions for siRNAs. The conservation rate of nucleotide sequences was calculated based on sequences from 16 strains (each genotype A, B, or C). Seven siRNA target sequences were selected from highly conserved regions. Numbering of HBV bases begins with position 1 at the EcoRI site. (B) Alignment of HBV sequences in siRNAs target regions. Dashed lines indicate identical nucleotides among siRNA target sequences.

(Fig. 5A). The levels of HBsAg and HBeAg were rapidly reduced by a single dose of MEND/HBV-siRNAmix, and these suppressive effects continued for 14 days (Fig. 5C). Furthermore, HBV DNA levels in sera were reduced (Fig. 5D). MEND/HBV-siRNAmix-treated mice did not exhibit decrease human albumin levels (Fig. 5E). These results indicated that a single administration of MEND/HBV-siRNAmix could efficiently suppress the levels of HBV antigens as well as those of HBV DNA *in vivo*.

A reverse transcriptase inhibitor does not effectively suppress HBV antigens

The RT inhibitor ETV can reduce the amount of HBV DNA in sera of HBV-infected patients, but ETV has only a small effect on HBV antigen levels [26]. Here, we evaluated the anti-HBV effects of ETV in chimeric mice with humanized liver tissue that was chronically infected with HBV genotype C (Fig. 5A). Although daily administration of ETV reduced the amount of HBV DNA in the sera of these mice, no efficient reduction of HBsAg or HBeAg

was observed even when ETV was administered for 35 days (Fig. 5F). These results are consistent with the previous findings that ETV does not efficiently decrease HBsAg levels in HBV-infected patients [26].

Discussion

Pegylated-IFN (Peg-IFN) and RT inhibitors such as ETV have been used for the treatment of HBV. Peg-IFN can reduce the amount of viral antigens and DNA [27]. However, the antiviral effect of Peg-IFN is limited, and side effects are observed in patients treated with Peg-IFN. The point of action of ETV is the RT process; consequently, ETV is not expected to reduce the amounts of viral proteins. Indeed, little reduction of viral antigens was observed in chimeric mice with humanized liver tissue during 35 days of daily administration of ETV (Fig. 5F). Here, we aimed to use a liver-specific drug delivery system, designated MEND, together with an effective siRNAmix to suppress production of viral

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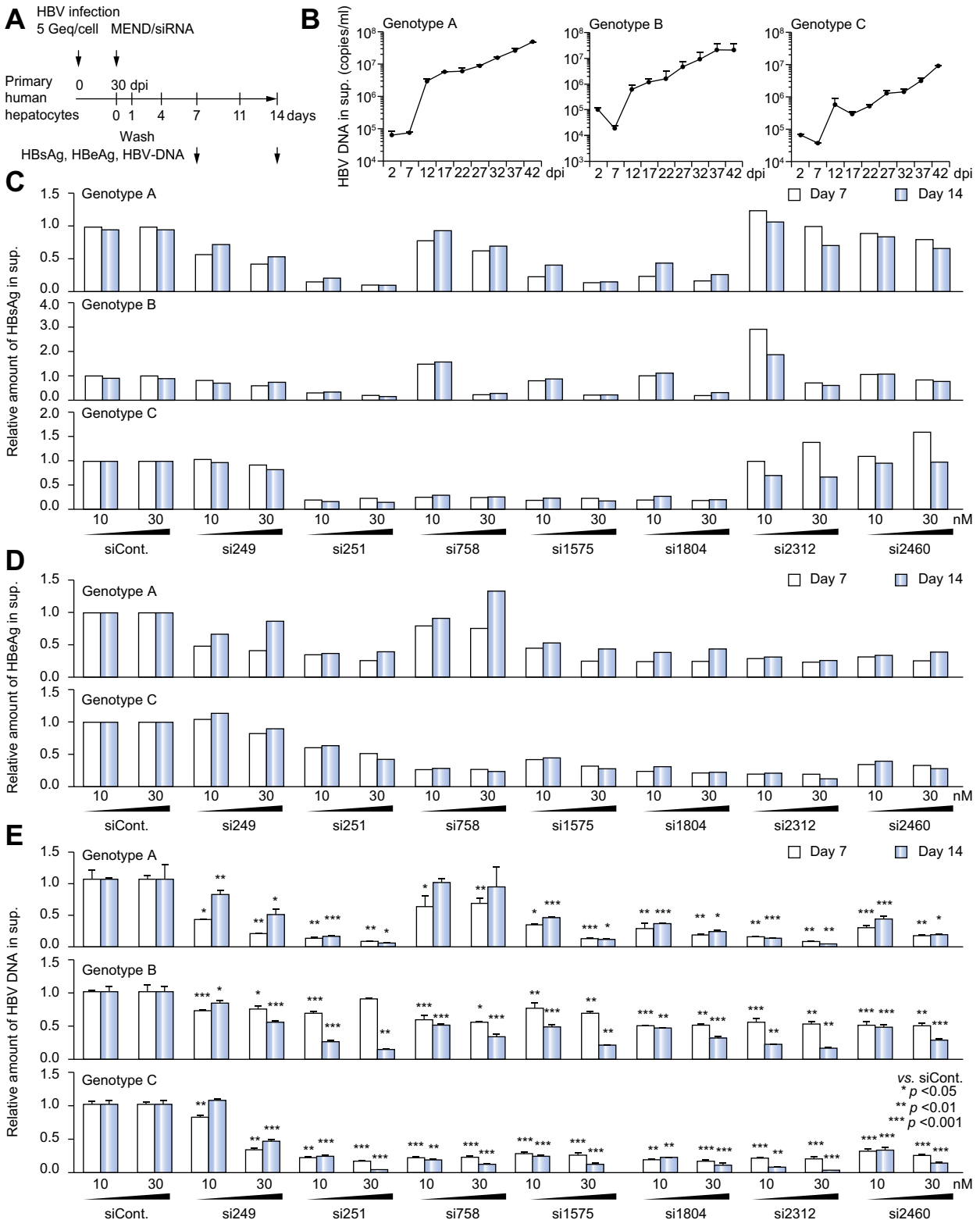


Fig. 3. Efficacy of MEND/HBV-siRNA containing a single siRNA in primary hepatocytes infected with HBV. (A) Experimental schedule for primary hepatocytes infected with HBV. (B) HBV DNA kinetics in the supernatant of primary hepatocytes inoculated with serum derived from HBV-infected mice. (C and D) Relative amounts of HBsAg and HBeAg. The supernatants (sup) from three independent experiments were pooled and analyzed. The mean value of each control siRNA was defined as the baseline. (E) Relative amount of HBV DNA. The mean values and standard deviations are shown based on the results of three independent experiments. The mean values for each control siRNA were defined as the baselines. Statistical comparison was performed using the *t* test. dpi, days post-infection.

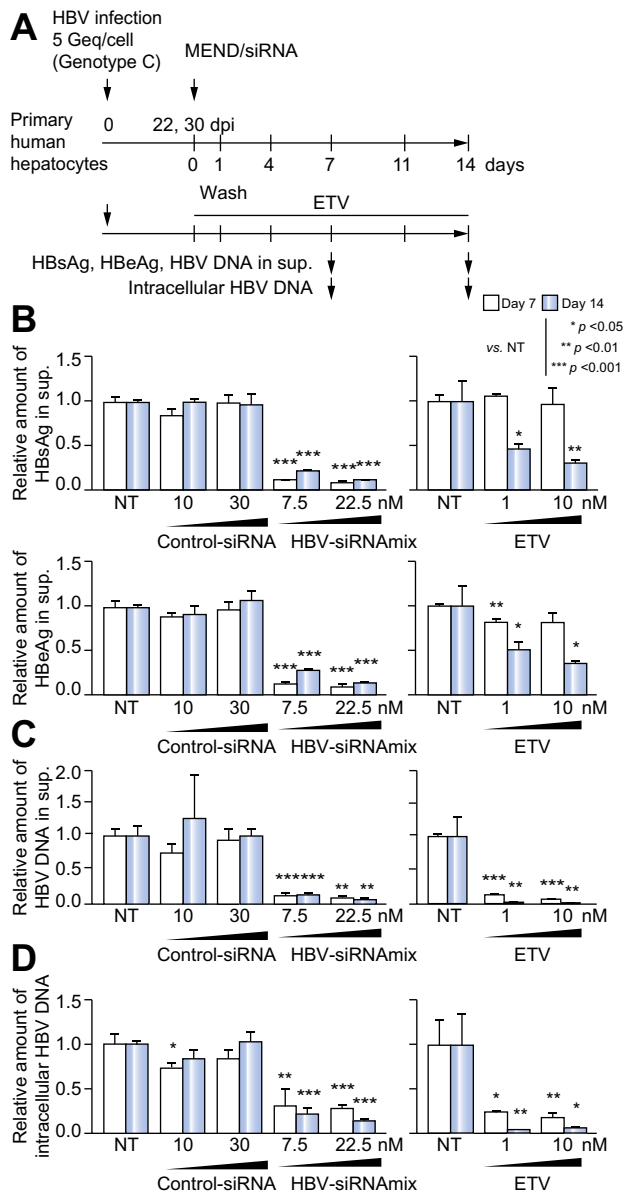


Fig. 4. Efficacy of MEND/HBV-siRNAmix containing the siRNAmix *in vitro*. (A) Experimental schedule for primary hepatocytes infected with HBV genotype C. (B) Relative amounts of HBsAg and HBeAg in the medium. (C and D) Relative amounts of extracellular and intracellular levels of HBV genomic DNA. The mean values and standard deviations are shown based on the results of three independent experiments. The mean values for non-treated (NT) were defined as the baselines. Statistical comparison was performed using the *t* test. dpi, days post-infection.

antigens. The MEND/HBV-siRNAmix suppressed HBsAg and HBeAg levels as well as HBV DNA levels *in vitro* and *in vivo* (Fig. 4B–D, and Fig. 5C, D). Therefore, the MEND/HBV-siRNAmix was superior to ETV for controlling HBV.

In a previous study, we developed a pH-sensitive cationic lipid, designated YSK05, and succeeded in efficient F7 gene silencing in mice (ED_{50} : 0.06 mg/kg) [14,21]. However, improvement of efficiency of endosomal escape remains challenging. Therefore, we designed a new pH-sensitive cationic lipid, designated YSK13-C3, based on the following strategies to improve

the efficiency of endosomal escape. First, YSK13-C3, like YSK05, has two long and unsaturated acyl chains to enhance the bulkiness of the hydrophobic region. Second, a double bond between acyl chains and an ester linker were also included to enhance the bulkiness of the hydrophobic region; notably, the bond angle of sp^2 carbon was fixed at 120° , which is larger than that of an sp^3 carbon ($\sim 109.5^\circ$). Also, an ester bond was chosen as the linker between hydrophobic region and the amine moiety to ensure that the MEND would be biodegradable. As expected, the MEND containing YSK13-C3 achieved strong F7 gene silencing (ED_{50} : 0.015 mg/kg), which is approximately 4-fold more efficient compared to that previously reported for YSK05-MEND [14]. The MEND containing YSK13-C3 specifically accumulated in liver, and its distribution throughout the liver was uniform. Also, observation of intrahepatic distribution of dual-labeled MEND demonstrated that siRNA and MEND lipid were localized in a uniform way and were almost always colocalized, indicating that the MEND/HBV-siRNAmix was taken up by most hepatocytes as an entire particle. These results indicated that the MEND containing YSK13-C3 was a suitable siRNA carrier for treatment of HBV infection.

RNA interference (RNAi) is based on the nucleotide sequence similarity between the siRNA and the target mRNA, and RNAi can be used to inhibit HBV replication *in vitro* and *in vivo* [28]. However, viruses that have a RT reaction in the life cycle are capable of producing escape mutations [23–25]. Additionally, although RNAi therapy with an effective drug delivery system for liver tissue has been reported, it was necessary to chemically modify the siRNA with cholesterol or saccharide [28]. To prevent the emergence of escape mutations, we prepared a mixture of three siRNAs that each targeted a different region of HBV genome. In addition, since MEND-siRNA particles were produced by simply encapsulating the siRNA within the MEND, it should be possible to easily obtain custom MEND-siRNA units that are optimized for individual patients. Furthermore, since the MEND encapsulates nucleic acids within the lipid membranes, the physical properties of the MEND are not affected by the type of the nucleic acids. The nucleic acids which can be delivered by the MEND are not restricted only to siRNA, and the MEND can efficiently deliver various kinds of nucleic acids including antisense oligonucleotides and plasmid DNAs (pDNAs) as well as siRNAs to hepatocytes. Indeed, we previously succeeded in efficient delivery of the anti-microRNA oligonucleotides against microRNA-122 to hepatocytes *in vivo*, and recently reported that intravenous administrations of the MEND encapsulating pDNA coding HBV 5'- ϵ region-derived RNA successfully abolished the HBV replication through induction of type III interferons in chimeric mice with humanized liver tissue [29]. Therefore, the MEND system would be able to deliver these various nucleic acids simultaneously to hepatocytes and offer the multi-target therapy against HBV infection.

HBsAg is translated from 2.1 and 2.4 kb mRNAs, and HBeAg is translated from a 3.5 kb full-length mRNA [30]. No reduction of HBsAg was observed following treatment with si2312 or si2460 (Fig. 3C), indicating that it is necessary to prepare siRNAs, which target mRNAs that encode each virus antigen. In contrast, HBeAg is translated from a 3.5 kb mRNA; consequently, each perfect-matching siRNAs reduced it (Fig. 3D). In addition, HBV DNA is produced from the full-length viral RNA by the viral RT. Therefore, HBV DNA reductions correlated with HBeAg reductions in our culture system (Fig. 3D, E).

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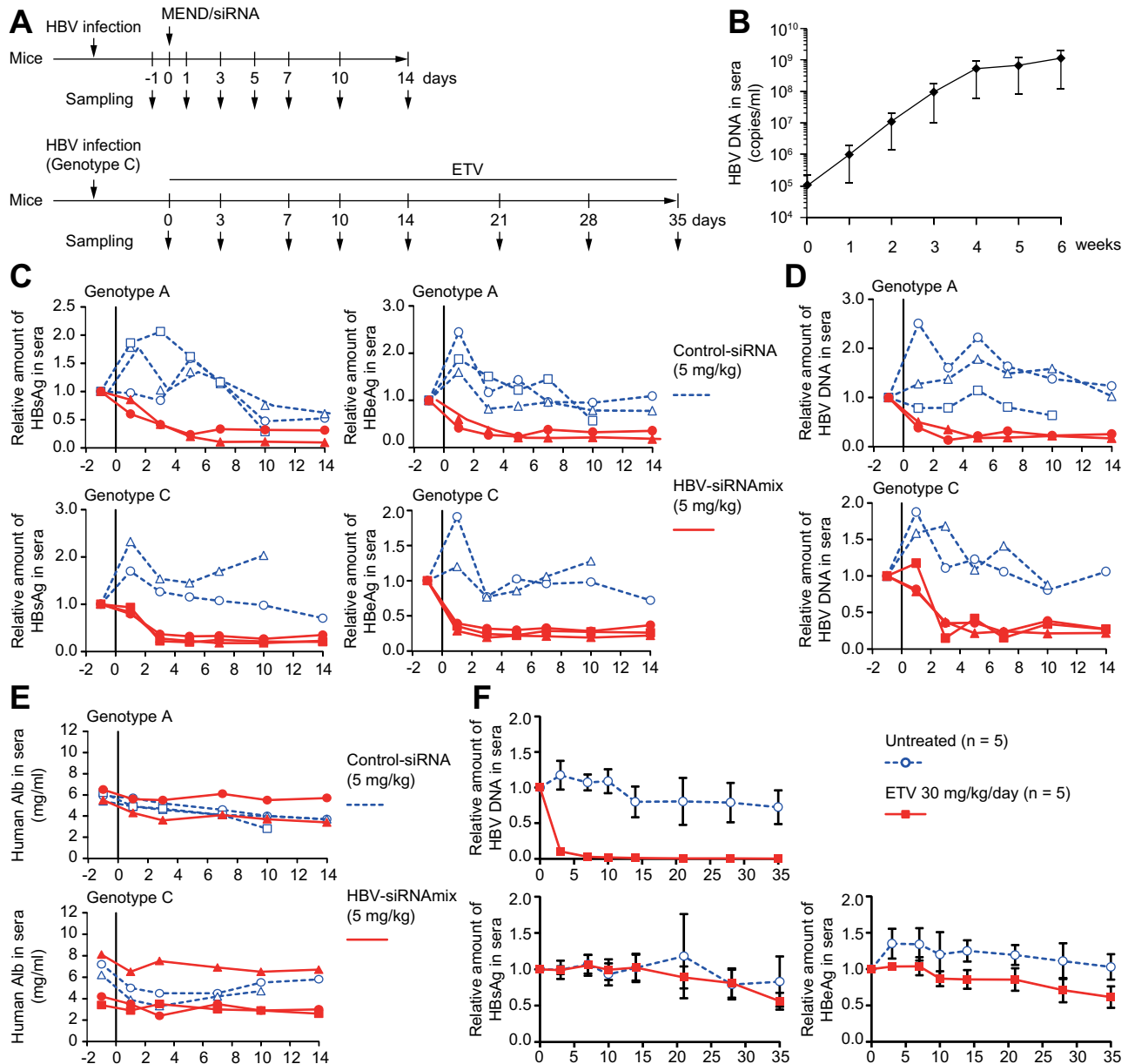


Fig. 5. Efficacy of MEND/HSV-siRNAmix in vivo. (A) Experimental schedule for chimeric mice with humanized liver. (B) Viral proliferation of a genotype C virus in chimeric mice. (C) Relative amounts of HBsAg and HBeAg in sera. (D) Relative amount of HBV DNA. (E) The amounts of human albumin (Alb) in sera. (F) Relative amounts of HBV DNA, HBsAg, and HBeAg in sera.

Cytotoxic T lymphocytes (CTLs) that specifically target HBV antigen are responsible for clearing HBV from patients [31]. However, CTLs do not respond properly in chronically infected patients [32]. A single dose of MEND/HSV-siRNAmix efficiently suppressed HBsAg and HBeAg as well as HBV DNA *in vivo* (Fig. 5C, D). Because HBV antigens are involved in the suppression of the immune response [12,13,33–35], effective suppression of viral proteins could activate HBV-specific acquired immunity and release from HBV persistence.

Here, we demonstrated that a MEND/HSV-siRNAmix could control HBV more efficiently than did ETV. The effect of a single dose of MEND/HSV-siRNAmix continued for a long time, indicating the burden on the patient would be reduced with

this treatment. Based on these findings, further investigations involving an animal model of acquired immunity are warranted.

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Conflict of interest

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

Authors' contributions

Conceived the study: M. Kohara.

Performed the experiments: N. Yamamoto, Y. Sato, M. Kakuni, C. Tateno, and T. Sanada.

Wrote the paper: N. Yamamoto, T. Munakata, Y. Sato, and M. Kohara.

Analyzed data: N. Yamamoto, T. Munakata, Y. Sato, Y. Hirata, M. Kakuni, C. Tateno, S. Murakami, Y. Tanaka, K. Chayama, H. Hatakeyama, Mamoru Hyodo and M. Kohara.

Coordinated the manuscript preparation: M. Kohara and H. Harashima.

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Author names in bold designate shared co-first authorship.

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