

A modified murine model based on hydrodynamic injection for the analysis of chronic human hepatitis B virus infection

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Abstract. Hepatitis B virus (HBV) is a persistent pathogen that causes acute and chronic necroinflammatory liver disease and is attributable to ~1 million deaths per year. In the present study, a conventional murine model was introduced based on the hydrodynamic injection of engineered replication-competent HBV DNA into the tail veins of C57BL/6 mice. In a previous study, nine in-frame ATG (start) codons in the S open reading frame (S1-S9) were analyzed. The highly conserved ATG S5 was mutated to ACG by T378C, which led to the substitution sM75T and inhibition of the production and secretion of the hepatitis B surface antigen (HBsAg), and subsequent inhibition of HBV replication. In the present study, T378C was introduced into the pAAV-HBV1.3 plasmid and was confirmed to affect HBsAg production and secretion, and HBV replication *in vivo*, which was in agreement with the previous *in vitro* results. Furthermore, the murine model was improved by co-injection of the replication-competent HBV plasmid DNA with Lipofectamine 2000 (LP). In this model, LP not only significantly enhanced HBV replication in mice, but also upregulated the expression of HBsAg and the hepatitis B core antigen. The current modified murine model was superior to the conventional murine HBV model based on HBV challenge by hydrodynamic injection.

Introduction

Approximately 2 billion people worldwide are infected with human hepatitis B virus (HBV) (1) and >350 million are chronically infected and at high risk of progression to cirrhosis, liver failure or hepatocellular carcinoma (2,3). More than half of all liver cancers are attributable to HBV infection;

and HBV-related liver diseases remain a major public health concern, leading to ~1 million deaths per year (3).

Advances in established cost-effective and reliable experimental models have accelerated the development of therapeutic modalities for these life-threatening viral infections (3). Although progress has been made, *in vitro* and *in vivo* HBV assays are being developed, but are impeded by a lack of understanding of HBV specificity for and entry into human liver cells, as well as reliable cell and animal infection models (4). In addition to humans, only chimpanzees and a small mammal, the treeshrew (families Tupaiidae and Ptilocercidae), are known to be susceptible to human HBV infection (5-7). Previously, basic studies of HBV and the development of antiviral therapeutics were hindered by the lack of suitable *in vitro* infection systems and animal models (8). Currently, only suboptimal cell transfection- and murine hydrodynamic injection (HI)-based assays are available for HBV research (9).

To analyze clinical HBV replication, isolates are obtained by the transfection of replicative recombinant HBV DNA into hepatoma cell lines or HI of replication-competent replicons into the tail veins of C57BL/6 mice which are able to replicate and secrete HBV virions (10,11). Previously, in order to obtain a HBV murine model, 10 mg HBV plasmid DNA was injected into the tail veins of mice in a volume of phosphate-buffered saline equivalent to 8% of the mouse body weight, with the total volume being delivered within 5-8 sec (12-14). In a previous study, the plasmid, pAAV-HBV1.3, did not replicate properly in mice, thus it was difficult to detect HBV DNA in the liver and serum (14). The HBV core antigen (HBcAg) was challenging to detect, particularly in HBV mutants with weaker replication capacity compared with wild-type (WT) HBV (14). In order to enhance HBV replication in mice, 10 mg plasmid pAAV-HBV1.3 and 20 μ L Lipofectamine 2000 (LP) transfection reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) were mixed and administered via HI into the tail veins of C57BL/6 mice. Plasmid DNA is well distributed and packaged with LP in liposomes, which facilitates the transfer of DNA to the murine liver cells. The optimized murine HBV-LP model was superior to the murine HBV model without LP based on HBV challenge via HI in mice, at the DNA and protein levels.

HBV has a 3.2-kb, relaxed circular (RC), partially double-stranded overlapped DNA genome containing four overlapping open reading frames (ORFs): P, S, C and X (15,16).

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HBV possesses the smallest known DNA viral genome, the latter of which optimized genomic organization through an overlapping gene strategy (17). The S ORF encodes the surface proteins, L, M and S (the HBsAg), through alternative translation initiation from three in-frame start codons (18). In our previous study, a methionine (M) to threonine (T) substitution at residue 75 (sM75T) was found to be associated with reduced HBV replication and HBsAg expression *in vitro*. In the present study, the previously modified HBV murine model was used to determine whether sM75T influenced HBV replication and HBsAg expression *in vitro* similar to that *in vivo*. The results obtained were as expected.

Materials and methods

Cell culture and treatment. Huh7 human hepatocarcinoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) at 37°C in a 5% CO₂ atmosphere supplemented with 10% fetal bovine serum (Gibco-BRL, Carlsbad, CA, USA), 2 mM/l glutamine, 100 IU/ml penicillin, and 100 IU/ml streptomycin. Huh7 cells were seeded in 6-well plates and transfected with 2 µg plasmids per well using LP (19). Female C57BL/6 (H-2b) mice (age, 6–8 weeks) were raised under specific pathogen-free conditions in the Central Animal Laboratory of Shaoxing Centre for Disease Control and Prevention (Shaoxing, China) and were handled following animal ethics guideline standards, according to the principles of animal protection, animal welfare and ethical inspection. The study was approved by the Medical Laboratory Animal Management Committee of Zhejiang Province (Department of Health of Zhejiang Province, Zhejiang, China). C57BL/6 mice were challenged with HBV replication-competent plasmids with or without LP administered by HI. The mouse assay was repeated three times, six mice were used in each group and there were seven groups in total: negative control, pBSK-HBV1.3, pAAV-HBV1.3, pAAV-HBV1.3-LP, pBSK-HBV1.3-T378C, pAAV-HBV1.3-T378C and pAAV-HBV1.3-T378C-LP.

Plasmid constructs. HBV mutants were constructed using fusion polymerase chain reaction (PCR), with primers carrying aimed mutations and WT pBSK-HBV1.3 as a template, which is a replication-competent plasmid 1.3-fold greater in length than the HBV genome. The sM75T was included in the pBSK-HBV1.3 plasmid to construct pBSK-HBV1.3-sM75T in our previous study (19). For the *in vivo* assay, pAAV-HBV-1.3 and pAAV-HBV1.3-rtXs were constructed based on pHBV1.3 and pHBV1.3-rtXs, respectively. pHBV1.3-WT/MT and pAAV were digested with the *Sac*I and *Sac*II restriction endonuclease (Takara Bio, Dalian, China), then end-filled with T4 and Klenow DNA polymerase (Takara Bio), respectively (14). The recovered products were digested by *Hind*III (Takara Bio) and ligated by T4 ligase (New England BioLabs, Beijing, China) to generate pAAV-HBV1.3 and pAAV-sM75T. All primers used in our study were manufactured by Sangon Biotech (Shanghai, China).

Enzyme-linked immunosorbent assay (ELISA). Huh7 cells were transfected with the indicated HBV-bearing plasmids and the hepatitis B surface antigen (HBsAg) and the hepatitis B

e antigen (HBeAg) in the supernatant at 96 h post-transfection (hpt) and were detected using an ELISA kit for the detection of HBsAg/HBeAg (Shanghai Kehua Diagnostic Medical Products Co., Ltd., Shanghai, China), according to the manufacturer's instructions (20).

qPCR. When the cell lysis solution had been treated with DNase I (Roche Applied Science, Penzberg, Germany) at 37°C for 30 min to digest the plasmid DNA, total HBV DNA was purified from the lysates of Huh7 cells 96 hpt and used as the template for qPCR which was conducted using SYBR-Green I (Roche, Mannheim, Germany) on a Light Cycler real-time PCR unit (Applied Biosystems, Inc., Foster City, CA, USA) according to the manufacturer's instructions. Primers (sense: 5'-GTTGCCCGTTGTCCTCTAATTC-3' and antisense: 5'-GGAGGGATACATAGAGGTTCCCTT-3' for RC) hybridized to the HBV surface gene were designed to quantify the HBV DNA RC genomes (100-bp fragments) by qPCR relative to an external plasmid DNA standard.

Analysis of encapsidated HBV DNA. Replication-competent HBV WT and mutant-type (MT) plasmids were transfected into Huh7 cells or injected into C57BL/6 mice, which were previously challenged via HI of replication-competent pAAV-HBV1.3 or -T378C, respectively, with or without LP, as described previously (12). HBV DNA replicative intermediates from intracellular core particles were extracted and subjected to agarose gel electrophoresis, followed by denaturation and southern blotting with a ³²P-labeled full-length HBV probe, as described previously (14). Hybridization signals were visualized and analyzed using a phosphoimager (Cyclone® Plus Storage Phosphor System; Parkard Instrument Company, Inc., Meriden, CT, USA). Data from the densitometric analyses were quantified using OptiQuant software (PerkinElmer, Inc., Waltham, MA, USA).

Immunohistochemical staining. Liver tissues were collected from mice sacrificed at the indicated time points. Intrahepatic HBcAg of mice was visualized by immunohistochemical staining of formalin-fixed paraffin-embedded tissues by rabbit anti-HBc antibodies (DAKO) and secondary goat antirabbit IgG HRP antibody (DAKO), using an Envision System (DAKO). The Envision system is also known as the enhanced labeled polymer system. The liver sections were also stained with hematoxylin and eosin (12,14).

Statistical analysis. The statistical analysis was conducted using GraphPad (GraphPad software, San Diego, CA, USA). Differences in multiple comparisons were determined for statistical significance using the Student's t-test. P<0.05 was considered to indicate a statistically significant difference. Results are presented as the mean ± SD.

Results

Replication and protein expression of WT and MT plasmids. The plasmid pBSK-HBV1.3 bearing a replication-competent WT HBV, which was 1.3-fold greater in length than the genotype A genome (GenBank accession no. U95551, ayw), was used as a backbone to construct mutant pBSK-HBV1.3.

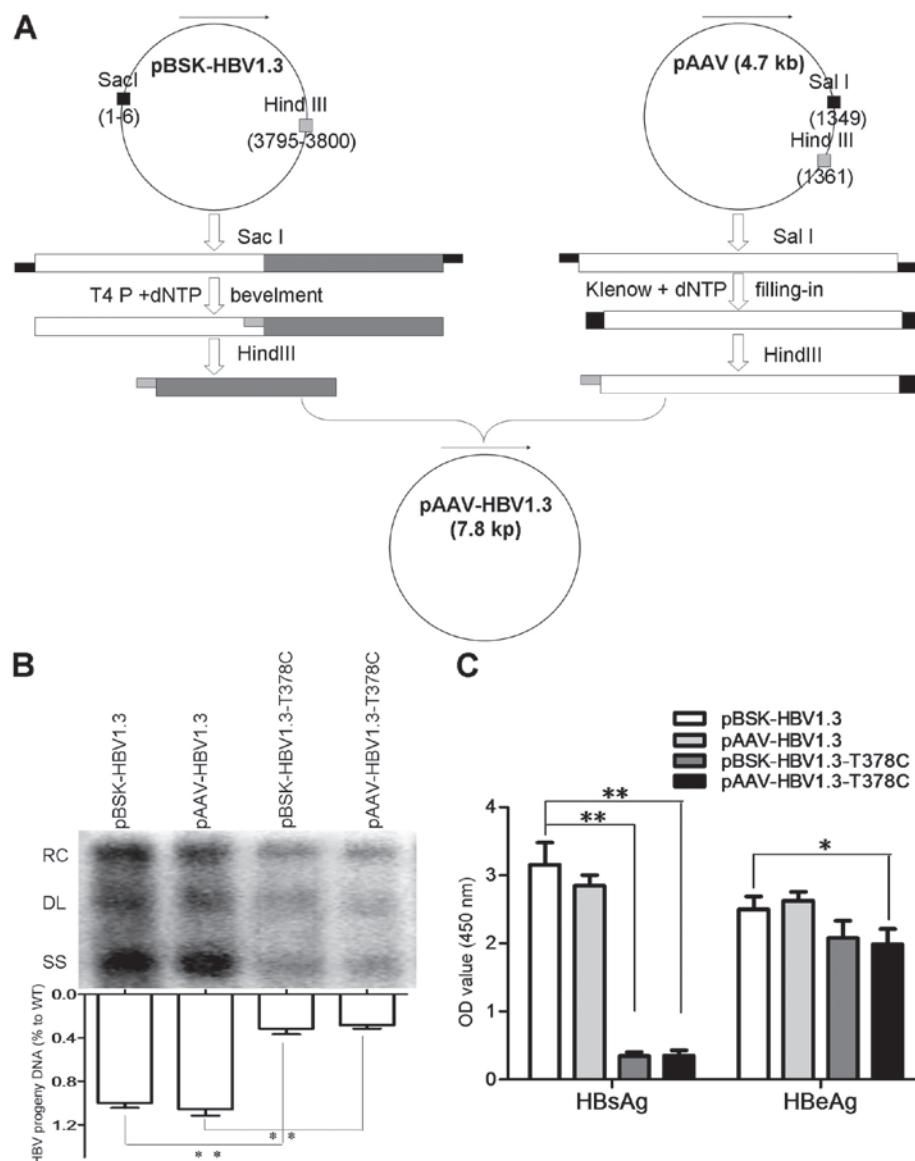


Figure 1. Construction strategy of pAAV-HBV1.3, replication and protein expression of WT and MT plasmids. (A) For the *in vivo* assay, pAAV-HBV-1.3 and pAAV-HBV1.3-T378C were constructed based on pHBV1.3 and pHBV1.3-T378C plasmids, respectively. The pHBV1.3/pHBV1.3-T378C and pAAV plasmids were digested with *Sac*I, then end-filled with T4 and Klenow DNA polymerase, respectively. The recovered products were digested with *Hind*III and ligated by T4 ligase to generate pAAV-HBV-1.3 or pAAV-HBV1.3-T378C. (B) Detection of HBV replication intermediates by southern blot analysis. HBV replication RC, DL, and SS HBV DNAs are indicated (upper panel). Intracellular encapsidated HBV DNA levels of each construct were compared with that of the WT genome (set as 100%, lower panel). (C) The expression of HBsAg and HBeAg were measured using commercial enzyme-linked immunosorbent assay kits. Each value is presented as the mean of three independent experiments. The error bars represent the standard deviation. *P<0.05 and **P<0.01. HBV, hepatitis B virus; WT, wild-type; MT, mutant-type; RC, relaxed circular; DL, double-stranded linear; SS, single-stranded; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen.

The sM75T in the *s* gene was introduced into pBSK-HBV1.3 to obtain pBSK-HBV1.3-sM75T. For the *in vivo* assay, pAAV-HBV-1.3 and pAAV-HBV1.3-sM75T were constructed based on plasmids pBSK-HBV1.3 and pBSK-HBV1.3-sM75T, respectively, as described above (Fig. 1A). Plasmids pBSK-HBV1.3, pBSK-HBV1.3-sM75T, pAAV-HBV1.3 and pAAV-HBV1.3-sM75T were transfected into Huh7 cells and the HBsAg and HBeAg in the culture medium were detected by ELISA. Replicative HBV intermediates from intracellular core particles were extracted from Huh7 cells and detected by southern blot analysis, as previously described (14). The data indicated that pBSK-HBV1.3 and pAAV-HBV1.3 had similar replication levels of HBs/eAg, while pBSK-HBV1.3-sM75T

and pAAV-HBV1.3-sM75T had similar replication levels to HBs/eAg. In addition, the pBSK-HBV1.3-sM75T and pAAV-HBV1.3-sM75T exhibited moderately decreased levels of HBV DNA and HBeAg compared with the WT and a markedly decreased expression level of HBsAg (Fig. 1B and C).

LP enhances HBV replication in mice. The LP transfection reagent is a proprietary formulation for transfecting nucleic acids (DNA, RNA and mRNA) into a wide range of eukaryotic cells and was used in the present study to package HBV plasmid DNA allowing entry into the Huh7 cells. However, it is not clear whether LP promotes HBV replication when mixed and co-injected with pAAV-HBV1.3 into

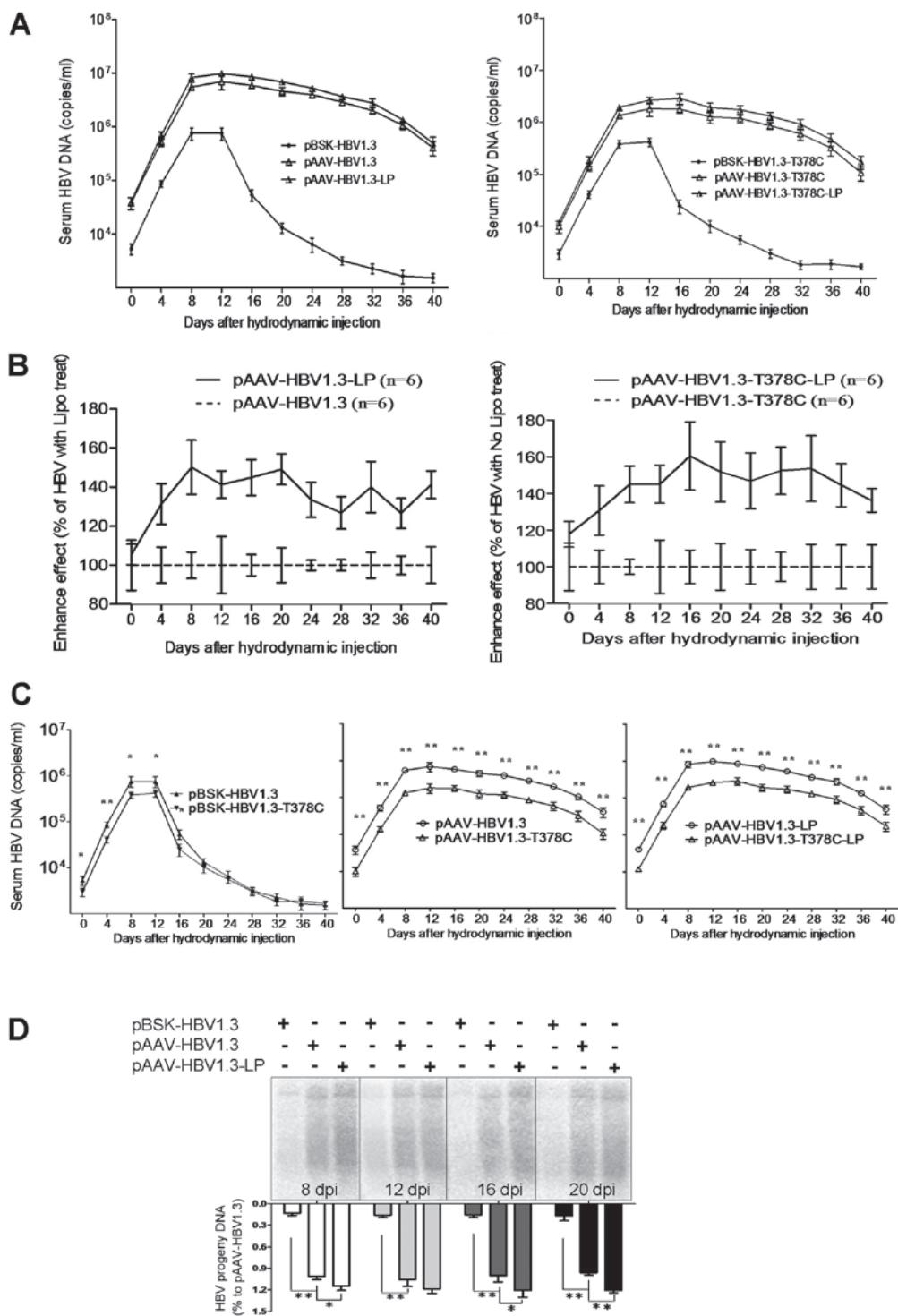


Figure 2. Replication of pBSK/pAAV-HBV1.3 and -T378C in the murine model. C57BL/6 (H-2b) mice were challenged with pBSK/pAAV-HBV1.3 and -T378C by tail vein administration of hydrodynamic injection. At the indicated time points, HBV DNA and proteins in the sera and liver samples were measured by qPCR targeted to HBV RC DNA, southern blot analysis, enzyme-linked immunosorbent assay and immunohistochemical analysis, respectively. (A-C) HBV DNA serum analysis of mice from the pBSK/pAAV-HBV1.3 and -T378C groups. The kinetics of HBV replication expression of each group, which contained pBSK, pAAV and pAAV-Lipo subgroups. (B) The enhanced HBV replication effect of LP *in vivo* is shown as the upregulation of serum HBV DNA in LP-mixed mice compared with that of mice without LP. The average HBV DNA copy number of the unmixed control group at each of the indicated time points was set as 100%. (C) The effect of T378C *in vivo* on the pBSK, pAAV and pAAV-Lipo subgroups is shown. (D) To detect HBV DNA in the liver, mice from each group were sacrificed at the indicated time points. Total DNA was isolated from the liver tissue and subjected to southern blot analysis. HBV, hepatitis B virus; LP, Lipofectamine 2000.

mice tail veins. C57BL/6 mice were respectively challenged with pBSK-HBV1.3, -HBV1.3-sM75T, pAAV-HBV1.3 and -HBV1.3-sM75T with or without LP by HI. HBV DNA in the mouse sera and liver samples were measured by qPCR

targeted to RC and southern blot analysis at the indicated time points, respectively. Based on the *in vitro* results, LP significantly increased HBV replication in the sera (Fig. 2A and B) and liver (Fig. 2D) samples. The mouse model was analyzed

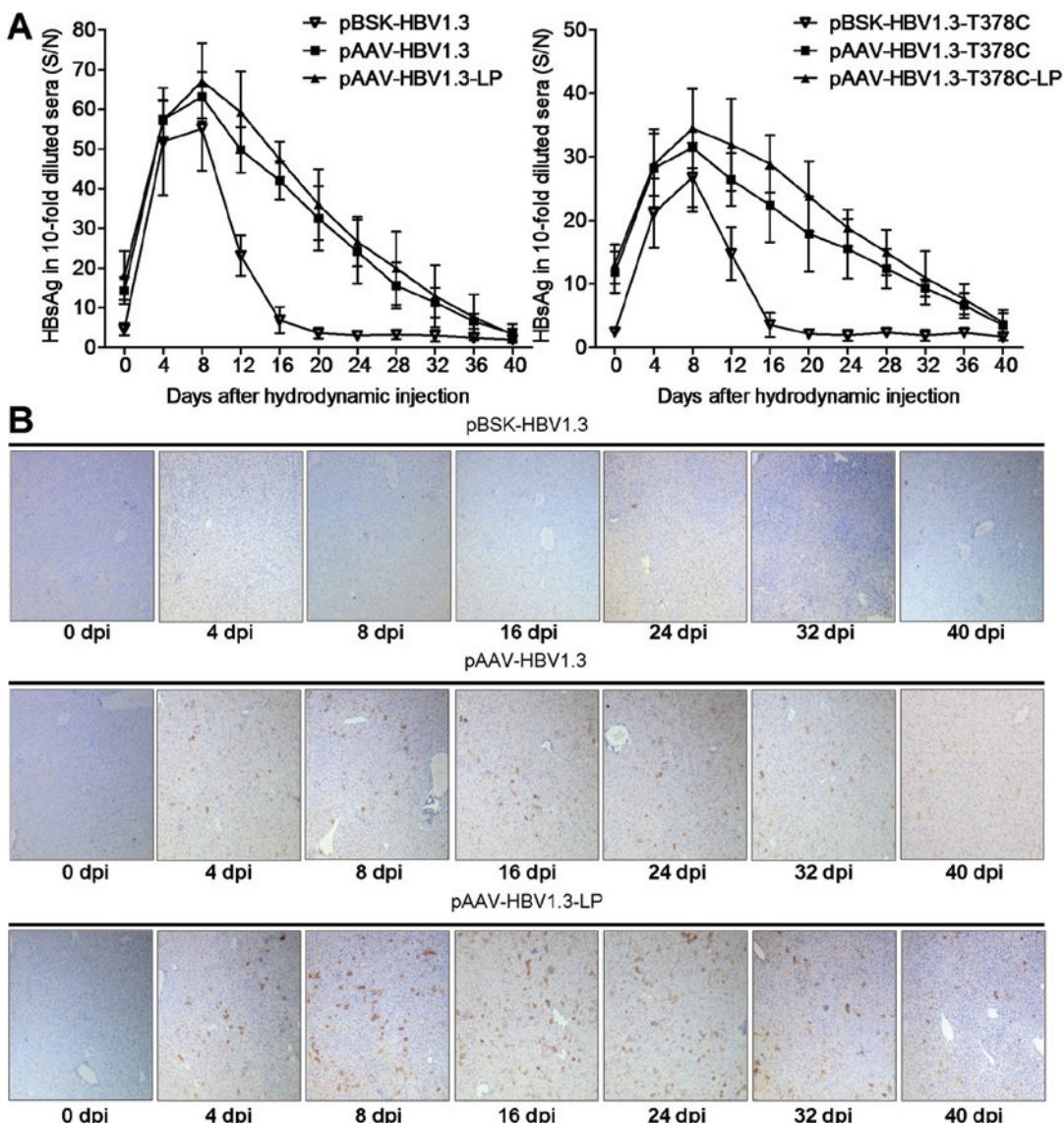


Figure 3. Kinetics of HBsAg and HBcAg expression. (A) The HBsAg level was determined using a commercial enzyme-linked immunosorbent assay kit and presented as the optical density value at 450 nm. (B) Immunohistochemical staining of HBcAg in the mouse liver following administration of pBSK-HBV1.3, pAAV-HBV1.3 or pAAV-HBV1.3-LP. Magnification, $\times 100$. LP, Lipofectamine 2000; S/N, samples/negative.

following HI to confirm the results of our previous study. In line with our previous observations at the cellular level, the sM75T substitution also significantly depressed HBV replication *in vivo* at all time points (Fig. 2C), particularly in the pAAV and LP pAAV groups. Serum HBV DNA reached the highest level at 12 days postinfection (dpi) in all groups. By contrast, the pBSK group demonstrated a maximum viral DNA level at 12 dpi, which rapidly decreased thereafter. Moreover, on the background of the sM75T substitution, LP enhanced HBV replication.

LP enhances HBsAg and HBcAg expression in mice. The HBV HBsAg level peaked at 8 dpi and then decreased gradually in mouse sera following HI with pBSK-HBV1.3-sM75T or pAAV-HBV1.3-sM75T (Fig. 3A), which was comparable with previously published data (10,17). Serum HBsAg of the pAAV group remained positive for the complete observation period of 5 weeks, but only for ~2 weeks in the pBSK group.

Similar results were observed in the -sM75T group (Fig. 3A). The intrahepatic HBcAg was further analyzed by immunohistochemical staining with specific antibodies (Fig. 3C). HBcAg was expressed in hepatic cells in the pAAV-HBV1.3 group at 4 dpi and persisted for at least 5 weeks, particularly in the pAAV-HBV1.3-LP group, while no obvious HBcAg expression was identified in the pBSK-HBV1.3 group at any of the time points. HBcAg expression was not detected in livers of the mice that were administered with pAAV-HBV1.3-sM75T and pBSK-HBV1.3-sM75T via HI (data not shown).

Discussion

Approximately 2 billion people worldwide are infected with human HBV, of which >350 million are chronically infected and at high risk of progression to cirrhosis, liver failure or cancer. Over 50% of liver cancers worldwide are attributable to HBV infection. Thus, HBV-related liver diseases remain a

major public health concern, with ~1 million mortalities annually. Individuals co-infected with HBV and HDV are at greater risk for rapid progression and severe disease.

LP is a proprietary formulation for transfecting nucleic acids (DNA, RNA and mRNA) into a wide range of eukaryotic cells and was used in our previous study to package HBV plasmid DNA and allowing entry into Huh7 cells (11). However, it is not clear whether LP promoted HBV replication when mixed and co-injected with pAAV-HBV1.3 into mice via the tail vein. According to the results of the present study, the pAAV vector was confirmed to be more suitable than pBSK for *in vivo* studies of HBV replication.

The HBV life cycle is a complicated process that is regulated by various host and viral factors (21,22). Although the surface proteins predominantly assist viral envelopment and secretion, there are also studies that have indicated that the surface proteins may be involved in replication regulation (23). In our previous study, the T378C substitution led to sM75T within the HBsAg encoding gene and was identified to reduce cellular HBsAg expression and HBV replication (19), which was also demonstrated in our *in vitro* experiments. Thus, our previous and present results confirmed that surface proteins are not essential for HBV DNA replication *in vivo* and *in vitro*.

The *in vivo* HBV model based on the hydrodynamic injection of an engineered, replication-competent HBV DNA into the tail veins of C57BL/6 mice was used to evaluate the anti-HBV effect of nucleos(t)ide analog and study resistance mutation of HBV *in vivo* in our previous study (14). The greatest problem is that the HBV replication capacity in this mice model was too low for southern blot analysis. In the present study, we modified the mice model by injecting the mixture of LP and replication-competent HBV plasmid DNA. Based on our data, LP significantly enhanced the HBV replication capacity, the level of HBsAg and HBcAg in mouse liver. This improved model is more conducive to HBV study *in vivo*. In the future, we aim to achieve an improved standardization *in vivo* model and use it to perform HBV resistant phenotype analysis.

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