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Inhibition of hepatitis B virus (HBV) gene expression and replication by *HBx* gene silencing in a hydrodynamic injection mouse model with a new clone of HBV genotype B

Lei Li^{1,2}, Hong Shen¹, Anyi Li⁴, Zhenhua Zhang¹, Baoju Wang¹, Junzhong Wang¹, Xin Zheng¹, Jun Wu¹, Dongliang Yang¹, Mengji Lu⁵ and Jingjiao Song^{3*}

Abstract

Background: It has been suggested that different hepatitis B virus (HBV) genotypes may have distinct virological characteristics that correlate with clinical outcomes during antiviral therapy and the natural course of infection. Hydrodynamic injection (HI) of HBV in the mouse model is a useful tool for study of HBV replication *in vivo*. However, only HBV genotype A has been used for studies with HI.

Methods: We constructed 3 replication-competent clones containing 1.1, 1.2 and 1.3 fold overlength of a HBV genotype B genome and tested them both *in vitro* and *in vivo*. Moreover, A HBV genotype B clone based on the pAAV-MCS vector was constructed with the 1.3 fold HBV genome, resulting in the plasmid pAAV-HBV1.3_B and tested by HI in C57BL/6 mice. Application of siRNA against *HBx* gene was tested in HBV genotype B HI mouse model.

Results: The 1.3 fold HBV clone showed higher replication and gene expression than the 1.1 and 1.2 fold HBV clones. Compared with pAAV-HBV1.2 (genotype A), the mice HI with pAAV-HBV1.3_B showed higher HBsAg and HBeAg expression as well as HBV DNA replication level but a higher clearance rate. Application of two plasmids pSB-HBxi285 and pSR-HBxi285 expressing a small/short interfering RNA (siRNA) to the *HBx* gene in HBV genotype B HI mouse model, leading to an inhibition of HBV gene expression and replication. However, HBV gene expression may resume in some mice despite an initial delay, suggesting that transient suppression of HBV replication by siRNA may be insufficient to prevent viral spread, particularly if the gene silencing is not highly effective.

Conclusions: Taken together, the HI mouse model with a HBV genotype B genome was successfully established and showed different characteristics *in vivo* compared with the genotype A genome. The effectiveness of gene silencing against *HBx* gene determines whether HBV replication may be sustainably inhibited by siRNA *in vivo*.

Keywords: Hydrodynamic injection, HBV mouse model, HBV genotype B, *HBx* gene silencing, Antiviral research

* Correspondence: jjsong@tjh.tjmu.edu.cn

³Division of Clinical Immunology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, P.R. China
Full list of author information is available at the end of the article

Introduction

Hepatitis B virus (HBV) causes acute and chronic infection in the human liver and subsequently hepatic cirrhosis and hepatocellular carcinoma (HCC) that severely affects human health [1-3]. Although a highly effective vaccine is now available for the prevention of new HBV infections, about 400 million people worldwide have already been chronically infected and suffer from chronic liver injury [4].

An immunologically well-characterized small animal model for HBV infection remains unavailable due to the strict host specificity of HBV infection, which greatly hampers HBV-related research. The laboratory mouse is genetically and immunologically defined, and a large collection of genetically modified animals exists. However, mice can not be infected with HBV. Several lines of transgenic mice with replication competent HBV genomes have been established and represented to be powerful tools for HBV research [5]. However, HBV replication in the transgenic mice is generated from the integrated HBV sequence harbored in all hepatocytes, which is different from what occurs during a natural infection [5-8]. The presence of HBV genomes in these mouse lines inevitably induces immune tolerance to the HBV antigens. In addition, the capability of production of transgenic mouse model is not readily available in ordinary laboratory conditions. Transplant mouse models were established and used for different studies [9-11]. However, the models are based on immunodeficient mouse strains and difficult to handle in the laboratory.

Hydrodynamic injection (HI) of replication-competent HBV DNA into the tail veins of mice can establish HBV replication in the mouse liver [12,13]. In 40% of injected C56BL/6 mice, the persistence of HBV surface antigenemia (HBsAg) was greater than 6 months. HBV persistence is determined by the mouse genetic background and plasmid backbone [12]. The HBV HI mouse model is a highly interesting model for testing vaccination strategies and mechanisms of viral persistence [14-18]. This model may be used to the study replication competence of HBV constructs [15]. Previously, the established HBV HI mouse models were based on HBV genotype A replication-competent clones [12,13]. As HBV genotypes B and C are highly prevalent in Asia and may have distinct virological characteristics that correlate with clinical outcomes in the natural course of infection and antiviral therapy, it is warranted to establish HBV HI mouse models with characterized HBV strains.

Patients with chronic HBV infection are currently treated with interferon alpha (IFN- α) or nucleotide analogs such as entecavir and tenofovir. However, the current therapies have only a limited success rate and frequent viral recurrence after cessation of therapy, therefore, new antiviral strategies are required [19,20].

RNA interference has been developed as potential therapeutic approaches [21-24]. In previous studies, siRNAs targeting different regions of the HBV genome were used. The *HBx* gene encodes a small non-structural protein that has diverse functions and is required for HBV efficient replication [25,26]. The *HBx* mRNA contains the common sequence of the four transcripts of HBV [25,27,28], which makes it a potentially useful target for antiviral therapy.

In this study, we addressed the question whether a replication competent HBV genotype B clone could replicate transiently and persistently in HI mouse model. Plasmids pSB-HBxi285 (non-viral vector) and pSR-HBxi285 (retroviral vector) that express siRNA targeting the *HBx* gene were constructed. Using these constructs, we asked how the gene silencing by *HBx* siRNA of genotype B influences the course of HBV replication and persistence in the HI mouse model.

Results

Identification of different folds of HBV DNA expression plasmids

The 1.1, 1.2 and 1.3 fold over-length HBV genome DNA including nt 1658-3215-1986, nt 1360-3215-1986 and nt 1040-3215-1986 were cloned into the pBluescript II KS (+) vector separately. The construction procedure is shown in Additional file 1: Figure S1A and schematic representation of the 1.1, 1.2 and 1.3 fold HBV genome are shown in Additional file 1: Figure S1B. The plasmids were analyzed by restriction enzyme digestion with *Pst*I and *Sac*I. *Pst*I and *Sac*I restriction digestion of pBS-HBV1.1_B, pBS-HBV1.2_B and pBS-HBV1.3_B resulted in the appearance of additional bands of 3.5, 3.8 and 4.2 kb in addition to the pBluescript II KS (+) vector, respectively (Additional file 2: Figure S2). Consistently, *Pst*I restriction digestion of pBS-HBV1.1_B, pBS-HBV1.2_B and pBS-HBV1.3_B confirmed that these vectors have the sizes of 6.5, 6.8 and 7.2 kb, respectively (Additional file 2: Figure S2). The HBV 1.3 fold over length genome DNA was amplified and sub-cloned into the *Not*I site of the pAAV-MCS vector (Agilent technologies, La Jolla, USA). The construction procedure of pAAV-HBV1.3_B is shown in Additional file 1: Figure S1C. The plasmid pAAV-HBV1.3_B was identified by *Not*I restriction digestion and an additional band of 4.16 kb (data not shown).

pBS-HBV1.3_B shows high replication competence both *in vitro* and *in vivo*

To investigate the functionality of pBS-HBV1.1_B, pBS-HBV1.2_B and pBS-HBV1.3_B in hepatoma cells, the plasmids were transfected into Huh-7 cells. At 48 h post-transfection, the culture supernatants of the transfected cells were harvested and subjected to HBsAg and HBeAg detection. As shown in Figure 1A, the HBsAg and HBeAg

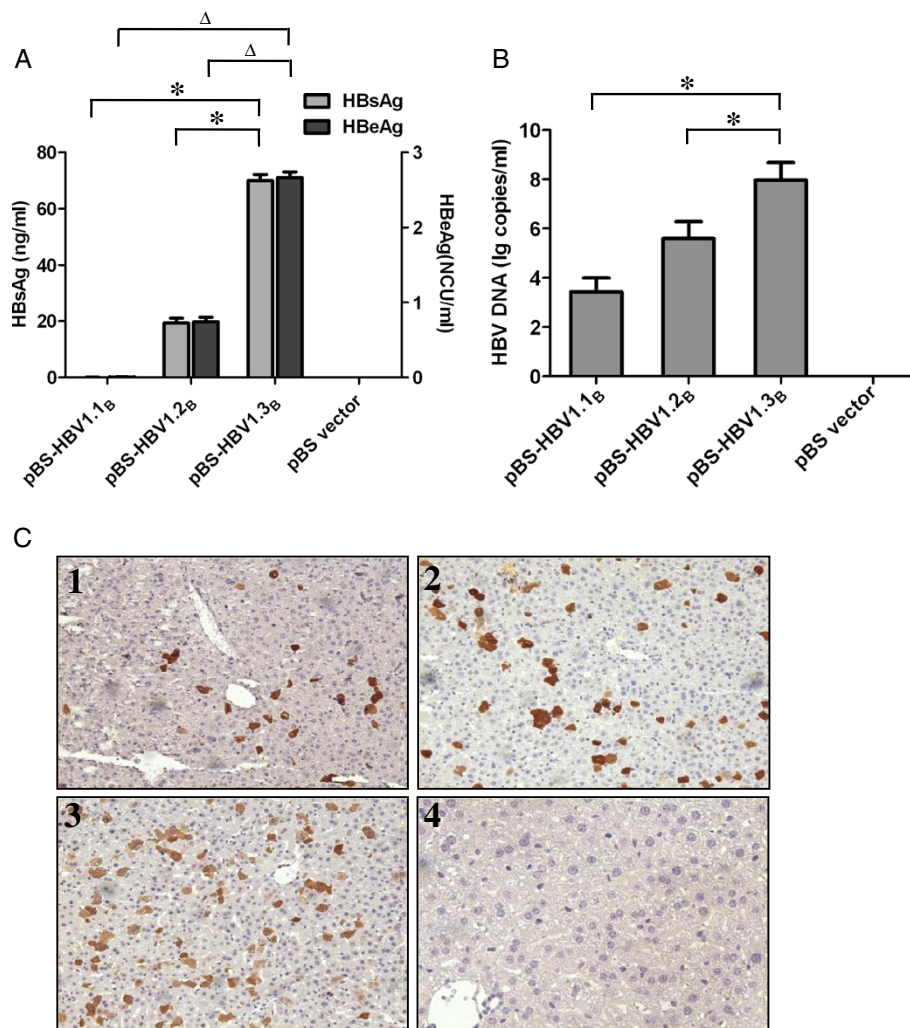


Figure 1 Comparison of the *in vitro* and *in vivo* replication competence of pBS-HBV1.1_B, pBS-HBV1.2_B and pBS-HBV1.3_B. 0.8 μg of plasmid DNA were transfected into Huh-7 cells which were seeded in 24-well plates at approximately 60% confluence and 10 μg of plasmid DNA were injected into the tail veins of BALB/c mice. Each group included 10 mice. **(A)** Titers of HBsAg (ng/ml) and HBeAg (NCU/ml, National Clinical Unit/ml) in the supernatants of pBS-HBV1.1_B, pBS-HBV1.2_B and pBS-HBV1.3_B transfected Huh-7 cells at 48 h post-transfection. The data were analyzed by one-way ANOVA, and the differences were statistically significant (* and ^Δ mean $p < 0.01$). **(B)** Real-time PCR detection of HBV DNA in mouse sera at day 7 after HI of pBS-HBV1.1_B, pBS-HBV1.2_B and pBS-HBV1.3_B (the number of the mice ≥ 3). The data were analyzed by one-way ANOVA, and the differences were statistically significant (* means $p < 0.01$). **(C)** Immunohistochemical staining of the liver sections for HBeAg in hepatocytes of pBS-HBV1.1_B- (1), pBS-HBV1.2_B- (2), pBS-HBV1.3_B- (3) and pBS (4)-injected mice at 7 day post injection (Original magnification: 200X).

levels in the culture supernatant of pBS-HBV1.3_B transfected Huh-7 cells were significantly higher than those with the of pBS-HBV1.1_B and pBS-HBV1.2_B plasmids ($p < 0.01$).

To further compare the replication competence of pBS-HBV1.1_B, pBS-HBV1.2_B and pBS-HBV1.3_B *in vivo*, BALB/c mice were subjected to HI with these plasmids. Each group contained 10 mice. At 7 dpi, mouse serum samples were collected for HBV DNA quantification. The liver tissue was collected for immunohistochemical staining of HBeAg. Figure 1B showed that HI with pBS-HBV1.3_B resulted in a significantly higher HBV DNA level in mouse serum samples compared with HI with

pBS-HBV1.1_B and pBS-HBV1.2_B ($p < 0.01$). Immunostaining of HBeAg demonstrated that HBeAg positive hepatocytes could be detected in the mouse liver after HI with each of the three plasmids but absent in the control mice. Moreover, the HBeAg positive hepatocytes were most abundant in the mouse liver of pBS-HBV1.3_B transfected mice (Figure 1C).

The influence of the host genetic background and plasmid backbone on HBV persistence

It is reported that HBV persistence after HI in mice depends on the host genetic background and plasmid backbone [12].

Firstly, the 1.3 fold over-length HBV genome was cloned into pAAV vector to generate pAAV-HBV1.3_B. HI with pAAV-HBV1.3_B was performed in C57BL/6 mice to examine whether this HBV isolate of genotype B is able to establish persistent replication *in vivo*. In parallel, pAAV-HBV1.2 described by Huang et al. (2006) was used as a reference. pAAV-HBV1.3_B group included 14 mice and pAAV-HBV1.2 group included 9 mice. The serum HBV DNA levels in C57BL/6 mice with pAAV-HBV1.3_B were determined at the indicated time points up to week 48 and were higher than those in mice with pAAV-HBV1.2 (Figure 2A).

The liver tissue was collected from C57BL/6 mice injected with pAAV-HBV1.3_B at 70, 168 and 252 dpi or pAAV-HBV1.2 at 70 dpi, 168 dpi and 340 dpi. HBV DNA was extracted from the liver tissue samples and assayed for HBV DNA by Southern blot analysis. Bands corresponding to the expected size of the input HBV plasmids and HBV replication intermediates including relaxed circular (RC) and single-stranded (SS) HBV DNAs could be detected in the liver of HBsAg-positive C57BL/6 mice hydrodynamically injected with pAAV-HBV1.3_B or pAAV-HBV1.2 (Figure 2B). HBV replication intermediates were at a higher level in mice injected with pAAV-HBV1.2 than in those with pAAV-HBV1.3_B. Intrahepatic HBV DNA levels also detected by real-time PCR and the results were consistent with the Southern-blot results (Figure 2C). Immunohistochemical staining of the liver sections for HBcAg revealed that both cytoplasmic and nucleic HBcAg were detected in the liver of HBsAg-positive mice hydrodynamically injected with pAAV-HBV1.3_B or pAAV-HBV1.2 (Figure 2D). In contrast, no HBcAg positive cell was present in the liver of the control mice and HBsAg-negative mice. All results obtained in this experiment indicate that HBV genotype B construct was replication competent in the mouse liver.

To investigate whether the vector backbone and the host genetic background also influence persistence of the HBV isolate of genotype B, 10 µg of the pBS-HBV1.3_B or pAAV-HBV1.3_B plasmids were injected hydrodynamically into the tail veins of male C57BL/6 or BALB/c mice. Each group included 5 mice. After HI, the mice were regularly bled and the temporal changes in HBsAg, HBeAg, HBcAb, HBsAb and HBV DNA levels were monitored. In BALB/c mice injected with pBS-HBV1.3_B, the HBsAg level increased in the first three days but dropped quickly afterward and all mice became negative at 1 wpi. However, the HBsAg level in BALB/c mice injected with pAAV-HBV1.3_B declined slowly with time and 75% of the mice were HBsAg positive at day 10 dpi. HBsAg became undetectable in all mice at 4 wpi (Figure 3A). In contrast to BALB/c mice, the HBsAg level decreased much more slowly after injection of the same plasmids in C57BL/6

mice. 50% of C57BL/6 mice injected with pBS-HBV1.3_B were HBsAg-positive at 1 wpi and all mice became negative at 2 wpi. Moreover, 75% of C57BL/6 mice injected with pAAV-HBV1.3_B were still HBsAg-positive at 2 wpi. HBsAg remained detectable in 50% of the mice after 10 weeks (Figure 3A). These results confirmed that the host genetic background as well as the vector backbone influenced HBV persistence.

In addition, we also compared the kinetics of viremia between pAAV-HBV1.3_B and pAAV-HBV1.2 in the HI C56BL/6 mouse model. The serum HBV DNA level in pAAV-HBV1.3_B injected mice was higher than that in pAAV-HBV1.2 injected mice (Figure 2A). However, the C57BL/6 mice received pAAV-HBV1.3_B had a lower positive rate of HBsAg compared with that received pAAV-HBV1.2. 20% of the mice are HBsAg positive at week 24 after HI with pAAV-HBV1.3_B, which was only half of that in mice injected with pAAV-HBV1.2 (Figure 3B).

None of the C57BL/6 mice injected with pAAV-HBV1.2 produced HBsAb after 28 dpi, although all of them produced HBcAb after the day 7 (Table 1). However, 3 of 14 C57BL/6 mice received pAAV-HBV1.3_B produced HBsAb after the 28 dpi. Moreover, the raise of HBsAb in pAAV-HBV1.3_B injected mice was faster than that in pAAV-HBV1.2. As shown in Table 1, for those mice producing HBsAb, they were HBsAg negative. All mice developed HBcAb after 7 dpi. Thus, different replication competent HBV genomes in the AAV vector may differ in their ability to establish the persistent replication in mice.

Impaired HBcAg specific cellular immunity in pAAV-HBV1.2 and pAAV-HBV1.3_B injected C57BL/6 mice during initial activation

Huang et al. (2006) confirmed that the tolerance toward HBV surface antigen in this model was due to an insufficient cellular immunity against hepatitis B core antigen. To confirm this result, HBV-specific CTL responses against full length HBcAg peptide were detected at 3dpi and 10 dpi after HI by ELISPOT assay of IFN-γ producing cells. At 3 dpi we almost could not detect any significant levels of HBcAg specific IFNγ-producing cells in the splenocytes (Figure 4A). However, the average number of IFNγ secreting cells was 463 and 653 in 1×10⁶ splenocytes in pAAV-HBV1.2 and pAAV-HBV1.3_B injected mice at 10dpi, respectively (Figure 4B). There is no significant difference between those two groups ($p > 0.05$). The result indicates that HBcAg-specific immune response may play a key role in clearing HBV infection at early stage in the HI mouse model.

HBxi285 inhibited HBV replication *in vitro*

To apply RNAi *in vivo*, the vector based expression of siRNAs was explored in the HI mouse model. For these

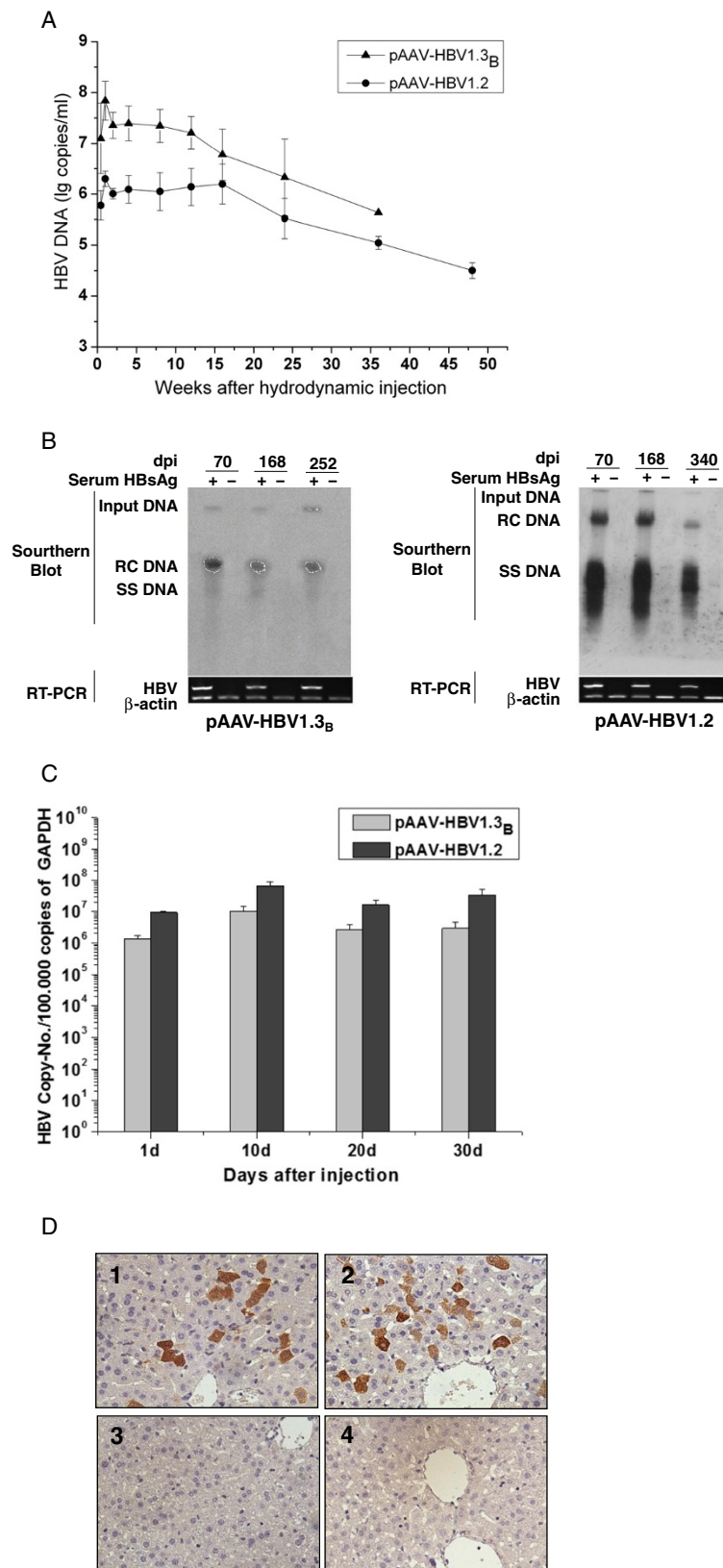


Figure 2 (See legend on next page.)

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Figure 2 HBV replication and gene expression *in vivo* after HI with pAAV-HBV1.3_B or pAAV-HBV1.2. 10 µg of pAAV-HBV1.3_B or pAAV-HBV1.2 were injected into the tail veins of C57BL/6 mice. pAAV-HBV1.3_B group included 14 mice and pAAV-HBV1.2 group included 9 mice. **(A)** Real-time detection of HBV DNA in mouse sera at the indicated time points (the number of the mice ≥ 3). **(B)** Southern blot analysis with 20 mg of total DNA extracted from liver tissues of the mice HI with pAAV-HBV1.3_B or pAAV-HBV1.2 at different time points after HI. All DNA samples were treated with RNase before subjected to agarose gel electrophoresis. Bands corresponding to the expected size of the input HBV plasmids, relaxed circular (RC) and single-stranded (SS) HBV DNAs are indicated. **(C)** Real-time PCR detection of HBV DNA levels in the liver of the mice after HI. **(D)** Immunohistochemical staining of the liver sections for HBcAg in hepatocytes of HBsAg-positive or HBsAg-negative mice at day 252 after HI with pAAV-HBV1.3_B (1, HBsAg positive mice and 3, HBsAg negative mice) and at day 340 after HI with pAAV-HBV1.2 (2, HBsAg positive mice and 4, HBsAg negative mice) (Original magnification: 400X).

purpose, two different vectors pSuper.retro (pSR) and pSuper.basic (pSB) were tested. Two different target sequences in the HBV *x* gene were selected and cloned into the vectors (see Material and Methods).

The antiviral effect of pSR-HBxi314 and pSR-HBxi285 was investigated *in vitro*. As shown in Figure 5A, the HBsAg and HBeAg levels decreased by 80.2% and 78.7% when pAAV-HBV1.3_B and pSR-HBxi285 were co-transfected into Huh-7 cells, respectively. However, when pAAV-HBV1.3_B and pSR-HBxi314 were co-transfected into Huh-7 cells, the HBsAg and HBeAg levels were only inhibited by 10.5% and 30.3%, respectively. Different concentrations of pSR-HBxi285 were used to test whether the antiviral effect was dose-dependent. Figure 5B and C showed that pSR-HBxi285 inhibited HBsAg and HBeAg expression as well as HBV DNA in a concentration-dependent manner in the range of 0.1–0.5 µg. The 0.5 µg dose of pSR-HBxi285 was most effective for the reduction of HBsAg, HBeAg and HBV DNA in transient transfection.

To compare the antiviral effects of pSB-HBxi285 and pSR-HBxi285 *in vitro*, pAAV-HBV1.3_B and different concentrations of pSB-HBxi285 or pSR-HBxi285 were co-transfected into Huh-7 cells. Figure 5D shows that pSB-HBxi285 and pSR-HBxi285 inhibited HBsAg and HBeAg in a dose-dependent manner. However, there was no significant difference in their antiviral effect ($P > 0.05$).

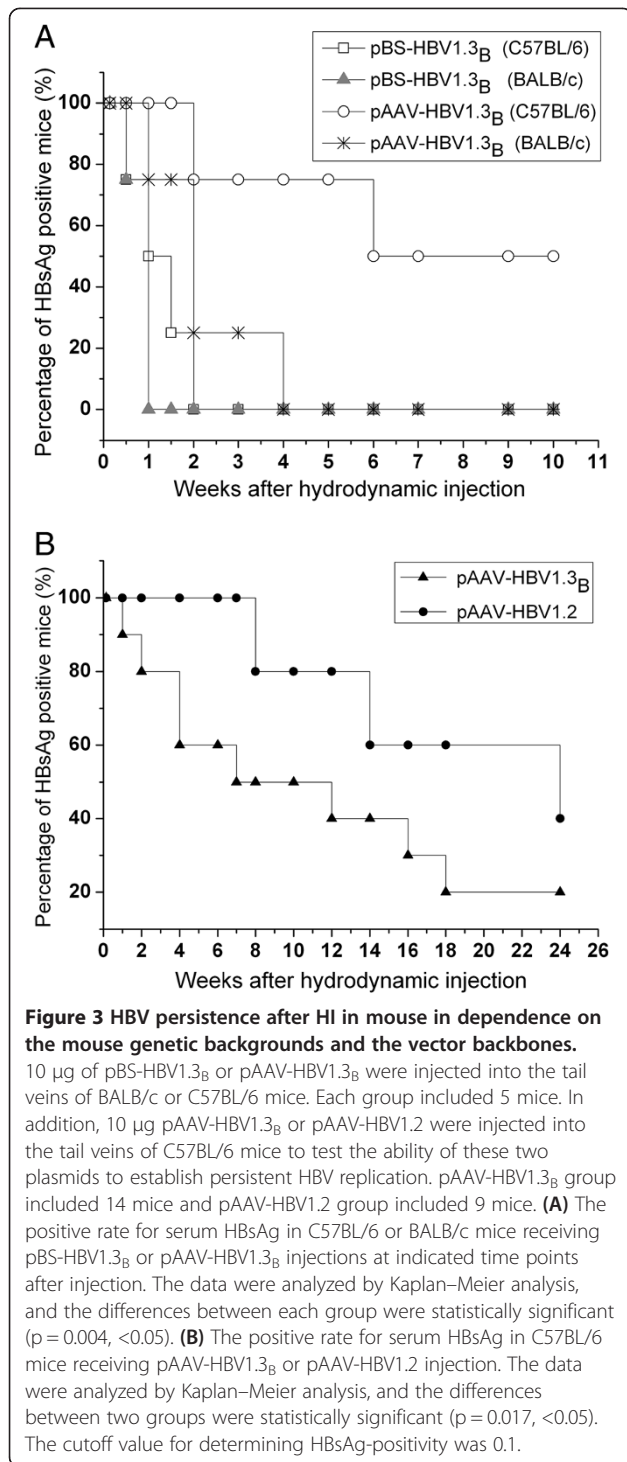
HBxi285 reduced serum HBsAg and HBV DNA levels in C57BL/6 mice after HI

Based on the previous experiments, 10 µg of pAAV-HBV1.3_B and 10 µg pSB-HBxi285 or pSR-HBxi285 were co-injected into C57BL/6 mice to determine the antiviral effect of HBxi285 *in vivo*. Each group contained 10 mice. Initially, HBsAg levels in mice were significantly reduced by HI in the presence of pSB-HBxi285 or pSR-HBxi285 plasmids at 1 dpi ($p < 0.05$), compared with the control groups receiving empty vectors (Figure 6A). However, the HBsAg levels in pSB-HBxi285 treated mice quickly increased during the follow up and reached the levels of the control at 14 dpi. In contrast, the HBsAg levels in pSR-HBxi285 treated mice were inhibited to a significantly lower degree before the 28 dpi compared with the

control groups ($p < 0.05$). HBsAg became detectable in some mice at 42 dpi. It is worthy to note that the majority (7 of 10) of the mice received pSR-HBxi285 were HBsAg negative during the whole experimental period (Table 2). HBV DNA concentrations in mouse sera were determined and were well correlated with HBsAg levels (Figure 6B). Figure 6C shows that the HBsAg positive rate in pSB-HBxi285 or pSR-HBxi285 treated mice was significantly decreased in comparison to the controls at 1 dpi. Thereafter, the positive rate of HBsAg in pSB-HBxi285 treated mice increased gradually and showed no significant difference in comparison to the control at 7 dpi. The positive rate of HBsAg in pSR-HBxi285 treated mice remained at a lower level (18.2–27.3%) than other groups until the 4th week post injection (wpi) but showed similar levels to the other groups in the 5th wpi. Consistently, immunostaining of HBcAg in the liver section of mice after HI demonstrated that the co-application of pSR-HBxi285 or pSB-HBxi285 abolished HBcAg expression in the mouse liver at 3 dpi (Figure 6D). However, HBcAg may become detectable in the mouse liver which received pSR-HBxi285 similar to those of control mice 4 weeks after HI. The results indicated that HBxi285 could significantly inhibit the HBsAg production and HBV replication. However the vector pSR-HBxi285 was more efficient in reducing HBsAg and HBV DNA levels than that of pSB-HBxi285.

Discussion

In the present study, an HBV genotype B HI mouse model was established. After HI with pAAV-HBV1.3_B, HBsAg and HBV DNA were detected in peripheral blood. Southern blot and immunohistochemical staining analysis revealed that HBV DNA and HBcAg were detected in the liver tissue of HBsAg-positive mice. Different hepatitis B virus genotypes may have distinct virologic characteristics, which may correlate with antiviral curative effects and clinical outcome [29,30]. The majority of HBV infections are distributed in the Asia-Pacific region. In these regions, patients are mainly infected with genotypes B and C. Previous studies showed that in China and Taiwan, approximately 90% of patients under thirty-five years old who are infected with



HBV would develop HCC and most of them carried an HBV genotype B infection [30,31]. Now, we have a system at hand to examine the replication of HBV genotype B clone in the mouse models.

In present study, we investigated the effect of the HBV genome length on HBV replication competence to find the optimal structure of replication competent HBV

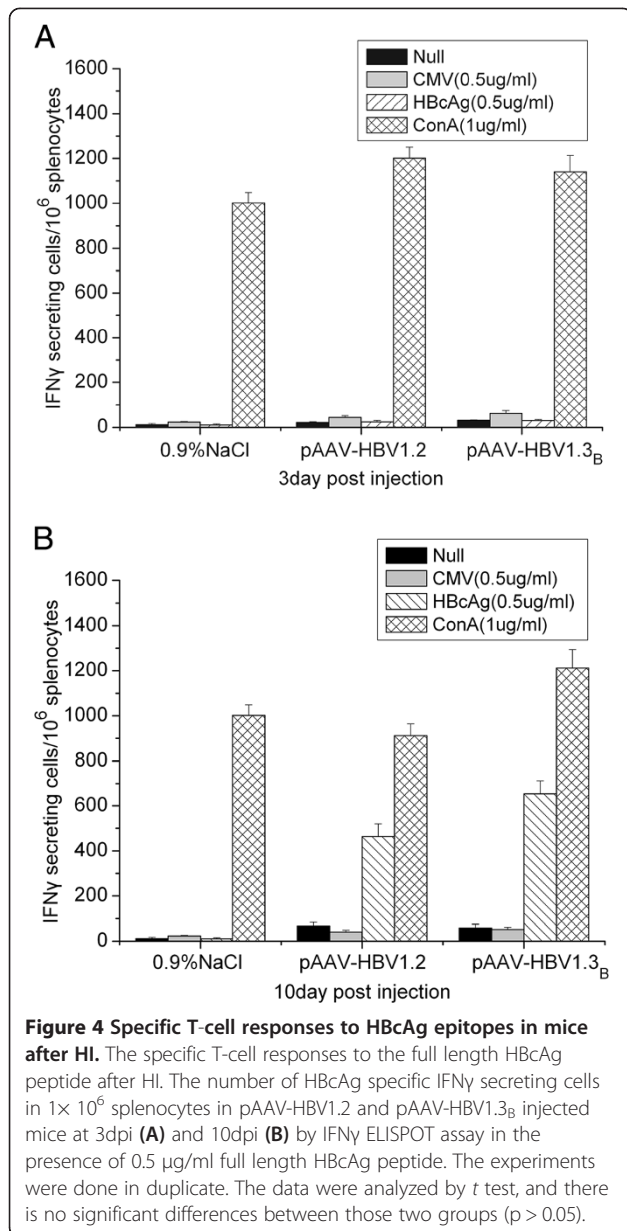
Table 1 Appearance of anti-HBc and anti-HBs antibodies in C57BL/6 mice after HI of HBV plasmids

HBV clone	Anti-HBc antibodies		Anti-HBs antibodies		
	Day 1	day 7	Day 7	Day 28	Day 168
pAAV-HBV1.3 _B	0/14	14/14	0/14	3/14	6/8
pAAV-HBV1.2	0/9	9/9	0/9	0/9	4/8

The number of C57BL/6 mice in each group positive for anti-HBc or anti-HBs antibodies after injected hydrodynamically with 10 µg of pAAV-HBV1.3_B or pAAV-HBV1.2 plasmids. All the mice with HBsAb are HBsAg negative.

genotype B clone. Three clones with 1.1, 1.2 and 1.3 fold over length HBV genotype B genomic DNA were constructed based on the pBluescript II KS (+) vector. Schematic representation of the 1.1, 1.2 and 1.3 fold HBV genome used in this study are shown in Additional file 1: Figure S1B. The plasmid with 1.3 fold genomes containing a complete extra copy of the entire EnhI, Enh II and HBx gene as shown in Additional file 1: Figure S1B which is a suitable HBV construct for transfection and HI experiments. When the cloned plasmids were transfected into Huh-7 cells or hydrodynamically injected into BALB/c mice, pBS-HBV1.3_B demonstrated significantly high-level replication and gene expression in comparison to pBS-HBV1.1_B and pBS-HBV1.2_B (Figure 1). Increasing the length of the 1.3 fold over length HBV genome did not further improve the replication competence. Nevertheless, the replication competence declined and could not reach the original level of HBV in nature infection state when the length is less than 1.2 fold of the HBV genome. Durantel et al. and other groups used 1.1 fold over length HBV genome for studies on drug resistant HBV isolates [32,33]. However, they added strong foreign promoters to enhance the HBV transcription and replication. In conclusion, the 1.3 fold HBV genome length containing a complete extra copy of the entire EnhI, EnhII and HBx gene as described in the present study could be regarded as an optimal length for HBV replication constructs with the authentic HBV sequence, independent on a foreign promoter.

To establish a persistent replication HBV mouse model with the HBV genotype B clone, 1.3 fold over length HBV genotype B genome was sub-cloned into the pAAV-MCS vector and introduced into C57BL/6 mice by HI. Huang et al. (2006) found that the AAV vector contributed to the persistence of HBV in mouse liver. Therefore, we used a similar vector pAAV-MCS, to construct HBV genotype B replicational clone in this study. This vector also has the ITR (inverted terminal repeats) structure, which was considered as a key factor for its stable existence *in vivo* by integration or non-integration way. Because the vector does not express other proteins except that encoded by the target genes, the vector backbone has no direct effect on the immune system.

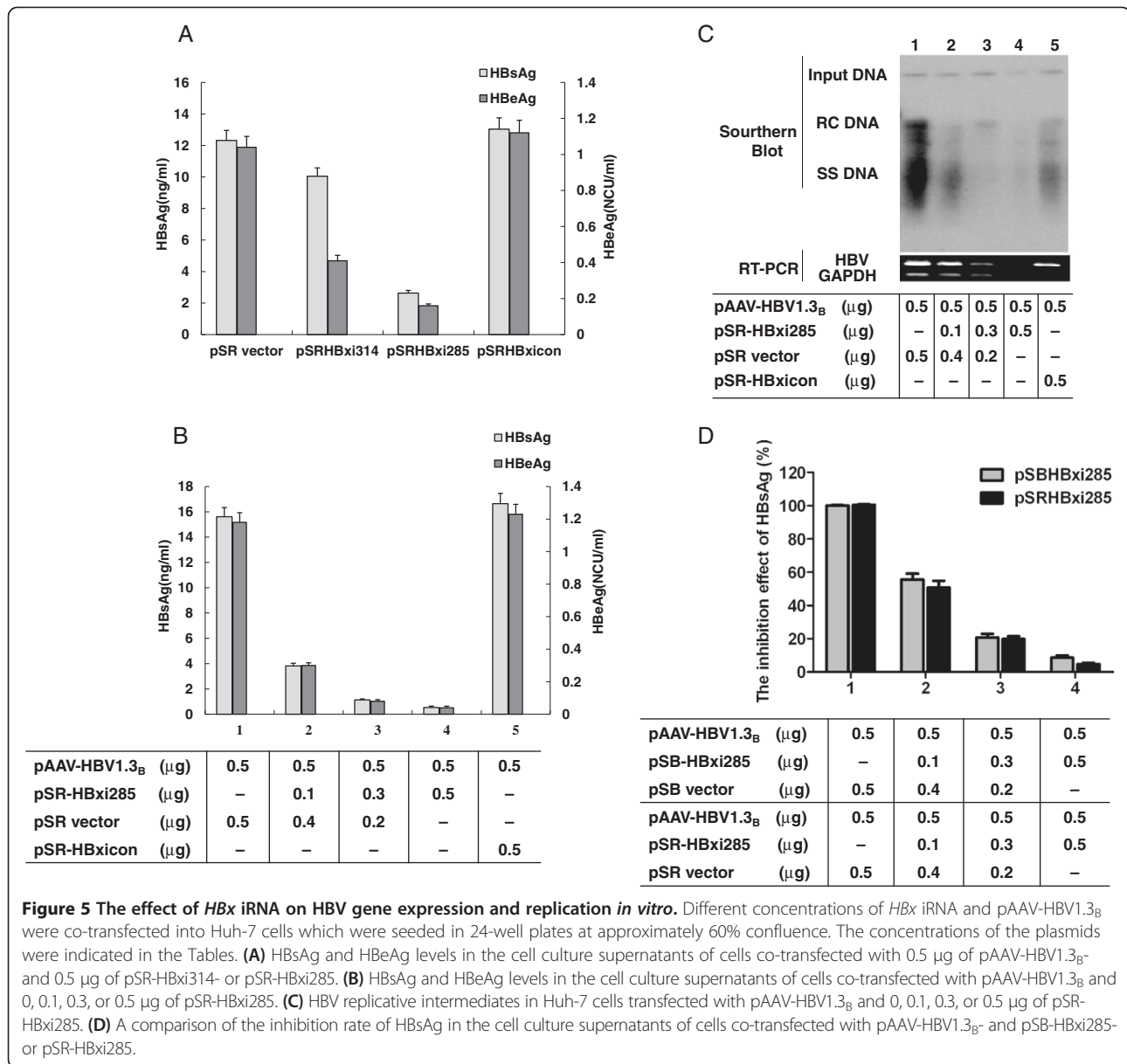


After HI, the mice presented higher HBV DNA level, but a lower positive rate for HBsAg in serum compared with pAAV-HBV1.2 injected mice [12]. Apparently, HBV replication is not strictly correlated with HBsAg production, as known for patients [34-38]. The result indicated that the model is more robust when pAAV-HBV1.2 genotype A is used as compared with pAAV-HBV1.3_B. Huang et al. (2006) confirmed that the tolerance toward HBV surface antigen in this model was due to an insufficient cellular immunity against hepatitis B core antigen, as documented in humans. We repeated some experiments and obtained the same results (Figure 4A and B). The difference in plasmid backbones between both clones needs to be investigated further.

The antigenemia and production of HBV particles were highly reproducible in the persistent HBV replication mouse model based on HI making this model appropriate for the evaluation of the efficacy of anti-viral drugs. Therefore, we further investigated the anti-viral effect of vector-expressed *HBx* siRNA in this model. Co-application of the *HBx* siRNA expression plasmids treatment of these mice induced a significant inhibition of HBV replication and expression. When pAAV-HBV1.3_B was co-injected into the tail veins of C57BL/6 mice with either pSB-HBxi285 or pSR-HBxi285, HBV expression and replication were efficiently inhibited. There are two methods commonly used to silence target genes. Chemical synthesized siRNAs have a good specificity and are commonly used on cells by transfection. However, such siRNAs, if not chemically modified, are unstable and eliminated rapidly *in vitro* and *in vivo*. The production of siRNAs, particularly with specific chemical modifications, is very expensive. Another approach is to use siRNA transcription vectors. The disadvantages to use such as retroviral vectors are, for example, the lower transfection efficiency and slow action compared with chemical synthesized siRNA. However, the vector-based approach has some advantages regarding the stability and costs. It is important to prolong the expression of siRNAs to maintain the inhibition of HBV. Moreover, when the pSuper.Retro vector was transfected into a packaging cell line such as 293 T cell line, it could produce retroviral supernatants and obtain a higher rate of stable cell integration (The Manual of pSuper RNAi system, OligoEngine, Washington, USA) [39,40].

HI of naked plasmid DNA is a simple yet effective *in vivo* gene delivery method into hepatocytes. The early reports suggested that a large portion of hepatocytes could be reached with this technique [41-44]. Therefore, it could be a useful method to test the efficacy of RNAi based antiviral therapies in the mouse model. Certainly, there is still a challenge how to transfer the knowledge gained in this system into human situation.

In this study, non-viral (pSuper.Basic) and retroviral vectors (pSuper.Retro) were used to construct shRNA (small hairpin RNA) expression plasmids [45] targeting the *HBx* gene of HBV. The pSuper.Retro vector contains the H1 promoter which is a RNA polymerase III like promoter and can transcript shRNA steadily, and showed persistent inhibitory effect *in vitro* [46,47]. The pSuper.Retro vector includes 3' and 5' terminal LTR structure of murine stem cell virus. The pSuper.Retro vectors can be transfected into a packaging cell line to produce retroviral supernatants and obtain a higher rate of stable cell integration. In addition, this vector contains an incomplete packaging signal and could not produce recombinant retroviruses (The Manual of pSuper RNAi system, OligoEngine, Washington, USA). pSuper.Basic is



a pUC origin vector (The Manual of pSuper RNAi system, OligoEngine, Washington, USA) and used to compare the stability with pSuper.Retro *in vivo*. The difference of both vectors in the gene silencing effects is likely due to their stability *in vivo*. HBx protein plays an important role in HBV replication and HBV induced HCC [26,48]. Therefore, *HBx* became a good target gene for gene silencing in recent years. Importantly, as all HBV RNA transcripts share the same sequence in the *HBx* due to its unique transcription mechanism, siRNAs targeting the *HBx* region actually knock down all HBV RNAs, as shown in the previous studies [28,49,50].

The result showed that the siRNA targeting the region nt 285–303 of the *HBx* coding region has a stronger antiviral effect than that targeting the region nt 314–

332. It is known that siRNA-specific features such as low G/C content, a bias towards low internal stability at the sense strand 3'-terminus, lack of inverted repeats, and sense strand base preference, interaction with the cellular machinery called RISC and so on are likely to contribute to efficient RNAi process. The relative influence of these parameters is not fully understood and now under investigation by many groups [51-56]. When pAAV-HBV1.3_B and pSB-HBxi285 or pSR-HBxi285 was co-injected into the tail veins of C57BL/6 mice, HBV expression and replication could be significantly inhibited by the first week. Thereafter, the antiviral effect declined in the pSB-HBxi285 treated mice, whereas the antiviral effect of pSR-HBxi285 was maintained for at least 4 weeks. These results indicated that the retroviral

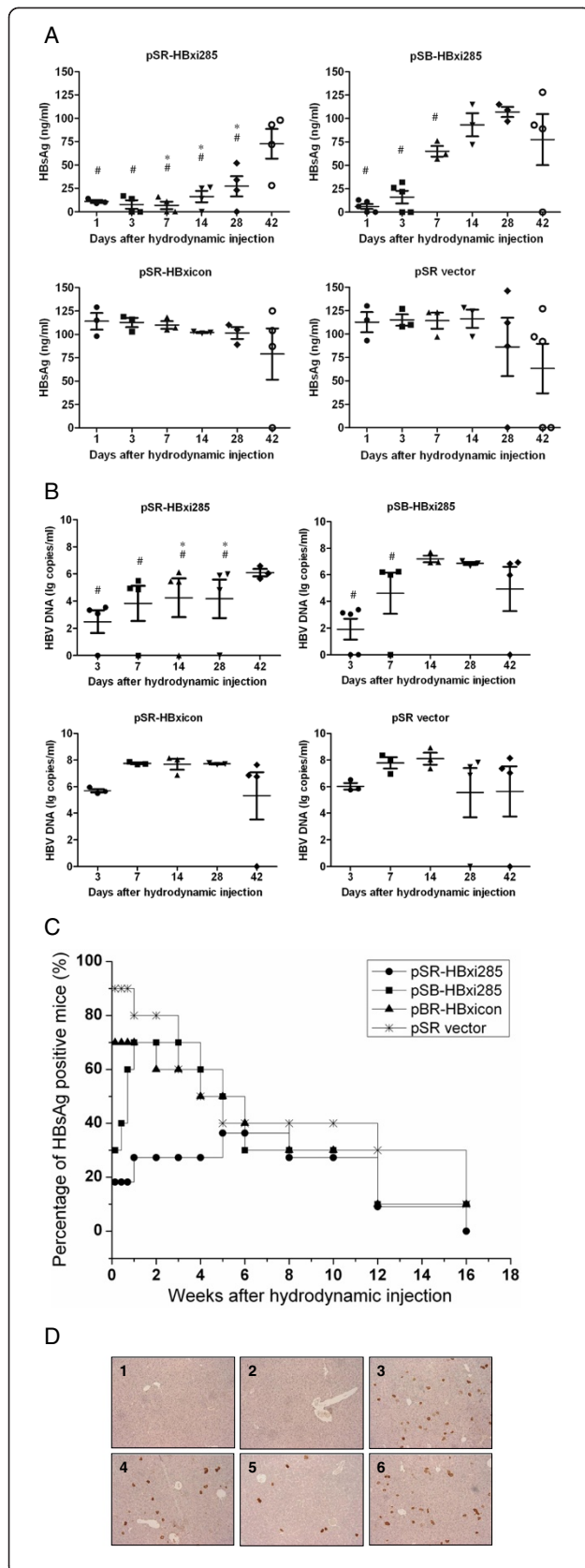


Figure 6 The effect of HBxi285 on HBV gene expression and replication *in vivo*. 10 μ g pAAV-HBV1.3_B and 10 μ g pSB-HBxi285, pSR-HBxi285 or control plasmids were co-injected into the tail veins of C57BL/6 mice. Each group included 10 mice. **(A)** HBsAg levels, **(B)** HBV DNA and **(C)** the positive rate of HBsAg in the serum of C57BL/6 mice treated with pAAV-HBV1.3_B and pSR-HBxi285 or pSB-HBxi285. Value 0 of HBV DNA means the copy number of HBV DNA is lower than detection limit. The data were analyzed by one-way ANOVA, and the differences were statistically significant (# : $p < 0.05$ pSR-HBxi285 group vs. pSR-HBxicon group; * : $p < 0.05$ pSR-HBxi285 group vs. pSB-HBxi285 group by one-way ANOVA) **(D)** Immunohistochemical staining for HBCAg in hepatocytes of C57BL/6 mice treated with pSR-HBxi285, pSB-HBxi285, pSR-HBxicon or pSR vector separately at 3 day post injection (1–4) and C57BL/6 mice treated with pSR-HBxi285 or pSR-HBxicon in the 4th wpi (5–6) (Original magnification: 200X).

vector based *HBx* siRNA expression plasmid could much more effectively inhibit HBV expression and replication than the non-viral vector based *HBx* siRNA expression plasmids. Among the mice which received pSR-HBxi285, HBV replication was completely suppressed in the majority of mice, as no HBsAg was detectable for the whole experimental period of 16 weeks. However, HBV gene expression and replication may resume in some mice despite an initial delay, indicating that transient suppression of HBV replication by *HBx* siRNA may also fail to clear HBV completely in some individuals. It is worth to mention that the change of serum HBsAg and HBV DNA in the mice after the application with *HBx* siRNAs occurred with different kinetics. The reason may be the different requirement of HBsAg translation and HBV DNA replication. It is well established that only a small fraction of HBsAg is required for virion production [57]. In the patients with chronic HBV infection, HBV DNA loads do not correlated with HBsAg [58]. Our results suggest that measures to prolong the antiviral effect of the siRNA should be investigated in future studies.

Conclusions

In summary, our established HBV genotype B HI mouse model is reliable and could be applied for studies on genetic and antiviral research of chronic HBV infection. The retroviral vector based *HBx* siRNA expression plasmids could efficiently inhibit HBV expression and replication, suggesting that an siRNA-based treatment strategy may be an additional therapeutic option for future clinical use in patients with chronic HBV infection.

Materials and methods

Construction of replication competent HBV genotype B clones

The plasmid pAAV-HBV1.2 which contains an 1.2 fold over-length HBV genotype A genome (nt 1400-3182-1987)

Table 2 HBsAg detection in HBxi285 treated and control mice

Mouse group	Persistent negative	Negative → positive ^a	Persistent positive or positive → negative ^b
pSR-HBxi285	7/10	2/10	1/10
pSB-HBxi285	3/10	4/10	3/10
pSR-HBxicon	3/10	0/10	7/10
pSR vector	1/10	0/10	9/10

^a Those mice with initial negative HBsAg became HBsAg positive. ^b Those mice with initial positive HBsAg became HBsAg negative during the whole experimental period of 16 weeks after HI. The cutoff value for determining HBsAg-positivity was 0.1.

[12], was kindly provided by Prof. Pei-Jer Chen. The plasmid pUC-HBV containing a full length genotype B HBV genome (1820-3215-1824 nt, GenBank accession number: AY220698.1) was kindly provided by Prof. Jiming Zhang (Fudan University, China). The construction procedure of pBS-HBV1.1_B, pBS-HBV1.2_B and pBS-HBV1.3_B is shown in Additional file 1: Figure S1A. First, the large fragments of the genotype B HBV DNA nt 1658-3215-247, nt 1360-3215-247 and nt 1040-3215-247 were amplified by PCR with primers *hbv1.1LF* (tagged with *PstI* site) and *hbvLR* (tagged with *XbaI* site), *hbv1.2LF* (tagged with *PstI* site) and *hbvLR*, *hbv1.3LF* (tagged with *PstI* site) and *hbvLR*, respectively. Those three fragments were inserted into the pBluescript II KS (+) (Invitrogen, Camarillo, USA) vector digested with *PstI* and *XbaI*. The small fragment of HBV DNA nt 247–1986 was amplified using primers *hbvSF* (tagged with *XbaI* site) and *hbvSR* (tagged with *SacI* site), and was inserted into the the pBluescript II KS (+) vector digested with *XbaI* and *SacI*. The resulting plasmids including nt 1658-3215-1986, nt 1360-3215-1986 and nt 1040-3215-1986 of HBV genome were named as pBS-HBV1.1_B, pBS-HBV1.2_B and pBS-HBV1.3_B, respectively. Schematic representation of the 1.1, 1.2 and 1.3 fold HBV genome are shown in Additional file 1: Figure S1B. All primers used to amplify HBV fragments are listed in Table 3. All DNA fragments in the final transfer vectors were amplified with Pyrobest DNA polymerase (TAKARA, Dalian, China) and cloned into the commonly used, commercially available pGEM-T vector (Promega, Madison, USA) for PCR cloning and sequencing. The correctness of the sequences of cloned DNA fragments were confirmed by sequencing. To establish HI mouse model with a high positive rate of HBsAg, we cloned the 1.3 fold HBV genome into the vector including the backbone of adeno-associated virus, similar to the described procedure [12]. The HBV 1.3 fold over length genome DNA was amplified from pGEM-HBV1.3_B with primers *hbv1.3 F* (tagged with *NotI*) and *hbv1.3R* (tagged with *NotI*), and sub-cloned into the *NotI* site of the pAAV-MCS vector (Agilent technologies, La Jolla, USA). The resulting plasmid was designated as pAAV-HBV1.3_B. The construction procedure of pAAV-HBV1.3_B is shown in Additional file 1: Figure S1C.

Cell culture and transfection

Huh-7 cells (Human hepatocarcinoma cell line) were maintained in 1640 medium supplemented with 10% of fetal bovine serum, 100 µg/ml penicillin and 100 µg/ml streptomycin. Cells were seeded in 24-well plates at approximately 60% confluence. After 24 h, cells were transfected with Lipofectamine 2000 (Invitrogen, Camarillo, USA) according to the manufacturer's instructions. For pBS-HBV1.1_B, pBS-HBV1.2_B and pBS-HBV1.3_B, 0.8 µg of plasmid DNA and 2 µl of lipofectamine 2000 were placed in each well of a 24-well plate. The efficiency of our transfection procedure was verified and controlled by transfection of a GFP expressing vector, as described previously [59].

To prove the antiviral effect of *HBx* siRNA, 0.5 µg of pAAV-HBV1.3_B and 0.5 µg of pSR-HBxi314 or pSR-HBxi285 were co-transfected into Huh-7 cells. The dose dependent antiviral effect of pSR-HBxi285 was tested by co-transfection of 0.5 µg pAAV-HBV1.3_B with 0, 0.1, 0.3 and 0.5 µg of pSR-HBxi285 into Huh-7 cells. To compare the antiviral effects of pSB-HBxi285 and pSR-HBxi285, 0.5 µg pAAV-HBV1.3_B combined with 0, 0.1, 0.3 and 0.5 µg of pSB-HBxi285 or pSR-HBxi285 were co-transfected into Huh-7 cells in parallel. All cell transfection experiments were performed three times.

HI experiments

The replication competence of the overlength HBV genomes *in vivo* was tested by HI with pBS-HBV1.1_B, pBS-HBV1.2_B or pBS-HBV1.3_B plasmids in BALB/c mice (female, 6–8 weeks old, from the breeding colonies of the experimental animal center in Hubei Province, China), with 10 mice per group. C57BL/6 mice (male, 6–8 weeks old, from the breeding colonies of the experimental animal center in Shanghai, China), were subjected to HI with pAAV-HBV1.3_B or pAAV-HBV1.2 to test the ability of these plasmids to establish persistent HBV replication. pAAV-HBV1.3_B group included 14 mice and pAAV-HBV1.2 group included 9 mice. To investigate the factors affecting HBV persistent in the mouse liver, we injected pBS-HBV1.3_B or pAAV-HBV1.3_B into BALB/c or C57BL/6 mice separately, with 5 mice per group. HI experiments were carried out as described previously [17],

Table 3 Primers for amplification of 1.3, 1.2, and 1.1 fold over length HBV replication-competent clones

Primer	Position	Sequence	Reference ^a
hbv1.1LF	HBV 1658-1677	5'-aac <i>tgc aga aga gga ctc ttg gac ttt c</i> -3'	AY220698.1
hbv1.2LF	HBV 1360-1379	5'-aac <i>tgc aga gta tac atc att tcc atg g</i> -3'	AY220698.1
hbv1.3LF	HBV 1040-1058	5'-tgc aac <i>tgc agt gga tat cct gct tta atg</i> -3'	AY220698.1
hbvLR	HBV 253-233	5'-gct <i>cta gac tct gtg gta ttg tga</i> -3'	AY220698.1
hbvSF	HBV 247-264	5'-ggc tag <i>tct aga ctc gtg gtg g</i> -3'	AY220698.1
hbvSR	HBV 1986-1969	5'-acc <i>gag ctc tcg aat aga agg aaa gaa</i> -3'	AY220698.1
hbv1.3 F	pGEM-T 4-39	5'-ata <i>aga atg cgg ccg ccg aat tgg gcc cga cgt cg</i> -3'	X65308.2
hbv1.3R	pGEM-T 117-81	5'-ata <i>gtt tag cgg ccg cgt tgg gag ctc tcc cat atg</i> -3'	X65308.2

Restriction sites are shown in italics. The gene sequences included in the primers are indicated by underling. ^a The Genbank accession numbers of the used reference sequences are given.

10 µg of HBV plasmid DNA was injected into the tail veins of mice in a volume of 0.9% NaCl, equivalent to 8% of the mouse body weight. The total volume was delivered within 5–8 s. For *HBx* siRNA experiments, 10 µg pAAV-HBV1.3_B and 10 µg pBS-HBxi285, pSR-HBxi285 or control plasmids were co-injected into the C57BL/6 mice. Each group included 10 mice. The control mice were injected only with the volume of 0.9% NaCl, equivalent to 8% of the mouse body weight.

Detection of HBsAg, HBeAg, HBsAb and HBcAb

Culture supernatants of pBS-HBV1.1_B, pBS-HBV1.2_B or pBS-HBV1.3_B transfected Huh-7 cells were harvested at 48 h post-transfection. Mouse sera were collected at the 1d (day), 3d, 7d, 10d, 2w (week), 3w, 4w, 5w, 6w, 7w, 8w, 9w, 10w... after HI. The analysis of HBsAg (ng/ml), HBeAg (NCU/ml, National Clinical Unit/ml), HBsAb (mIU/ml, Milli-International Units) and HBcAb (S/Co, Sample/Cutoff value) levels were determined by using the Architect system and CMIA kits (Abbott Laboratories, Wiesbaden-Delkenheim, DE) according to the manufacturer's instructions.

Isolation and analysis of HBV DNA in mouse sera

HBV DNA was extracted from 40 µl of mouse serum samples. The protocol was described previously [17]: 40 µl of samples were treated with 10 µg DNase I for 16 h at 37°C. 100 µl of lysis buffer (20 mM Tris-HCl, 20 mM EDTA, 50 mM NaCl and 0.5%SDS) containing 50 µg proteinase K were added. After incubation at 65°C for 3 h, viral DNA was isolated by phenol/chloroform extraction and ethanol precipitation. The DNA pellet was rinsed with 70% ethanol and resuspended in 10 µl of ddH₂O.

The quantification of HBV DNA was performed by using a routine real time PCR procedure, described previously [34,60-64]. The HBV copy numbers were determined by SYBR Green Real time PCR Master Mix (commercially available assay kit, TOYOBO, Osaka, Japan). Melt curve analysis and agarose gel electrophoresis were used to verify the specificity of the real-time PCR. The

following primers were used: forward primer: 5'-CTG CAT CCT GCT GCT ATG-3' (nt 408–425), reverse primer: 5'-CAC TGA ACA AAT GGC AC-3' (nt 685–701) according to the reference sequence with Genbank accession number (AY220698.1). Serum containing a known concentration of HBV DNA was used as a positive control.

Immunohistochemistry

The liver tissue was taken from the mice receiving HI with pBS-HBV1.1_B, pBS-HBV1.2_B or pBS-HBV1.3_B at 7 day post injection (dpi) and the mice receiving HI with pAAV-HBV1.3_B at 70 dpi, 168 dpi and 252 dpi or pAAV-HBV1.2 at 70 dpi, 168 dpi and 340 dpi, which was then used for immunohistochemical staining of the HBcAg in hepatocytes. The liver tissue of the mice received 0.9% NaCl was used as negative control. Liver tissue was collected from the mice and embedded in paraffin. Intrahepatic HBcAg was visualized by immunohistochemical staining with rabbit anti-HBc (Dako) of the liver tissue sections. The liver tissue sections were also stained with hematoxylin.

Purification and Southern blot analysis of HBV DNA from the liver tissue of mice

Total DNA from the pAAV-HBV1.3_B and *HBx* siRNA expression plasmids co-transfected Huh-7 cells and the liver tissue of mice was isolated using commercial kits (OMEGA, Norcross, USA). The isolated HBV DNA was subjected to agarose gel electrophoresis followed by denaturation and Southern blot analysis. All DNA samples were treated with RNase before gel electrophoresis. HBV DNA was detected by hybridization with a digoxigenin (DIG) (Roche, Lewes, UK)-labeled full length HBV probe. The hybridization signals were visualized on x-ray film using an alkaline phosphatase-anti-DIG-Fab conjugate in the presence of the chemiluminescence substrate CDP-Star (Roche).

Enzyme-Linked Immunospot (ELISPOT) assay

ELISPOT assay was carried out using the mouse IFN-γ precoated ELISPOT Kit (Dakewe, Shenzhen, China)

according to the manufacturer's instructions. Briefly, 96-well flat-bottomed microtiter plates were preincubated with the coating antibody (anti-IFN- γ monoclonal antibody) at 4°C overnight and blocked for 2 hr at 37°C. Mouse splenocytes at the density of 1×10^6 cells per well were added to wells in triplicate with final concentration of 0.5 $\mu\text{g}/\text{ml}$ full length HBcAg peptide (ProSpec, Ness-Ziona, Israel) separately and incubated at 37°C, 5% CO₂ for 24 hr. 1 $\mu\text{g}/\text{ml}$ of ConA (Sigma, St. Louis, USA) was used as positive control. Thereafter, cells were removed. Wells were washed ten times with PBS containing 0.05% Tween-20 (PBS-T) and incubated with 100 μl of biotinylated anti-IFN γ antibody for 1 hr. The plates were washed again with PBS-T and incubated with 50 μl HRP-streptavidin solution at 37°C for 1 hr. Spot-forming cells were counted and analyzed with an ELISPOT plate reader (BioReader 4000, Biosys, Germany). Results were presented as spot-forming cells per 1×10^6 cells.

Construction of HBx siRNA expression plasmids

Two pairs of DNA sequences targeting the nt 285–303 and nt 314–332 of the HBx coding region of HBV (genotype B, adw) were designed in accordance with the protocols for the siRNA expression vectors pSuper.Basic and pSuper.Retro (OligoEngine, Washington, USA), and the two RNAi design server (http://www.ambion.com/techlib/misc/siRNA_tools.html and <http://www.dharmacon.com/DesignCenter>). The DNA sequences were as follows: HBxi314F, 5'-GAT CCC CCG ACC GAC CTT GAG GCA TAT TCA AGA GAT ATG CCT CAA GGT CGG TCG TTT TTA-3', HBxi314R, 5'-AGC TTA AAA ACG ACC GAC CTT GAG GCA TAT CTC TTG AAT ATG CCT CAA GGT CGG TCG GGG-3'; HBxi285F, 5'-GAT CCC C GA GGA CTC TTG GAC TTT CAT TCA AGA GAT GAA AGT CCA AGA GTC CTC TTT T TA-3', HBxi285R, 5'-AGC TTA AAA AGA GGA CTC TTG GAC TTT CAT CTC TTG AAT GAA AGT CCA AGA GTC CTC GGG-3', the HBx iRNA control sequences were: HBxiconF, 5'-GAT CCC CGC ACC TAT AAC AAC GGT AGT TCA AGA GAC TAC CGT TGT ATA GGT GCT TTT TA-3', HBxiconR, 5'-AGC TTA AAA AGC ACC TAT AAC AAC GGT AGT CTC TTG AAC TAC CGT TGT TAT AGG TGC GGG-3' (the sequences targeting HBx gene are shown in italics). The single strand DNA products were purchased from Invitrogen (Shanghai, China).

The above complementary sequences were phosphorylated and ligated into *Bgl*II and *Hind*III digested pSuper.Basic or pSuper.Retro vector. The resulting plasmids were named as pSB-HBxi314, pSB-HBxi285, pSB-HBxicon, pSR-HBxi314, pSR-HBxi285 and pSR-HBxicon. The clones were sequenced to confirm the correctness of the sequences.

Statistical analysis

The data were evaluated by Student's t-test or one-way ANOVA followed by Tukey's post hoc test. The statistical significance of the data was considered at $p < 0.05$.

Approval of the ethics committee

The animal experiments were carried out in concordance with the guidelines established by the Institutional Animal Care and Use Committee at the Huazhong University of Science and Technique and the Tongji Hospital of Tongji Medical College. The mice used in this study were anesthetized with ketamine and xylazine.

Additional files

Additional file 1: Figure S1. Construction procedure for the pBS-HBV1.1_B, pBS-HBV1.2_B, pBS-HBV1.3_B and pAAV-HBV1.3_B vectors. **(A)** The fragments containing 1.1, 1.2 and 1.3 fold over length HBV genomes were amplified from the plasmid pUC-HBV containing a full length genotype B HBV genome (1820-3215-1824 nt, GenBank accession number AY220698.1) and inserted into the *Pst*I and *Sac*I sites of the plasmid pBluescript II KS (+). **(B)** Schematic representation of the 1.1, 1.2 and 1.3 fold HBV genome used in this study are shown. HBV ORFs as well as enhancers are indicated in different patters. **(C)** The HBV 1.3 fold over length genome DNA was amplified from pGEM-HBV1.3_B and sub-cloned into the *Not*I site of the pAAV-MCS vector.

Additional file 2: Figure S2. Identification of pBS-HBV1.1_B, pBS-HBV1.2_B and pBS-HBV1.3_B. pBS-HBV1.1_B, pBS-HBV1.2_B and pBS-HBV1.3_B were identified by restriction enzyme analysis. Lines 1/9: 1 kb DNA ladder; Lines 2–4: pBS-HBV1.1_B, pBS-HBV1.2_B and pBS-HBV1.3_B were digested with *Pst*I and *Sac*I respectively; Lines 5: pBluescriptII KS (+) was digested with *Pst*I and *Sac*I; Lines 6–8: pBS-HBV1.1_B, pBS-HBV1.2_B and pBS-HBV1.3_B were digested with *Pst*I.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LL, JS, DY, and ML conceived and designed the experiments. LL, JS, HS, and ZZ performed the experiments. LL, JS, DY, and ML analyzed the data. AL, BW, XZ, JW, JJW, DY, and ML contributed reagents/materials/analysis tools. JS, DY, and ML wrote the paper. All authors read and approved the final manuscript.

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Author details

¹Department of Infectious Diseases, Union Hospital of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, P.R. China. ²Department of Infectious Disease, Anhui Provincial Hospital, No.9 Lujiang Road, Hefei, P.R. China. ³Division of Clinical Immunology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, P.R. China. ⁴Animal Center, Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, P.R. China. ⁵Institute of Virology, University Hospital of Essen, University Duisburg-Essen, Essen, Germany.

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