Gene Therapy (2015), 1–9

www.nature.com/gt

ORIGINAL ARTICLE

Harnessing the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated Cas9 system to disrupt the hepatitis B virus

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The current therapies to treat hepatitis B virus (HBV) infection are limited. Recently, clustered regularly interspaced short palindromic repeat (CRISPR) systems, originally identified in bacteria and archaea, have been found to consist of an RNA-based adaptive immune system that degrades complimentary sequences of invading plasmids and viruses. Here, we studied the effects of the CRISPR/CRISPR-associated Cas9 system that was targeted to the surface antigen (HBsAg)-encoding region of HBV, both in a cell culture system and *in vivo*. The HBsAg levels in the media of the cells and in the sera of mice were analyzed by a quantitative enzyme-linked immunosorbent assay. The HBV DNA levels were assessed by quantitative PCR and HBsAg expression in mouse livers was assessed by an immunohistochemical assay. The amount of HBsAg secreted in the cell culture and mouse serum was reduced by CRISPR/Cas9 treatment. Immunohistochemistry analyses showed almost no HBsAg-positive cells in the liver tissue of CRISPR/Cas9-S1+X3-treated mice. The CRISPR/Cas9 system efficiently produced mutations in HBV DNA. Thus, CRISPR/Cas9 inhibits HBV replication and expression *in vitro* and *in vivo* and may constitute a new therapeutic strategy for HBV infection.

Gene Therapy advance online publication, 5 February 2015; doi:10.1038/gt.2015.2

INTRODUCTION

Hepatitis B virus (HBV) is one of the major pathogens of human liver disease, and >360 million people worldwide are chronically infected.[1] Many of the individuals with HBV will eventually develop severe liver disease such as liver cirrhosis and hepatocellular carcinoma (HCC).[2–4] The similarity between the genographical distribution of chronic carriers of the viral surface antigen (HBsAg) and HCC sufferers supports this hypothesis. Furthermore, the observation that HBV infection precedes tumor development and the detection of HBV markers such as HBsAg or viral DNA sequences (a non-integrated form in the tumor tissue) also supports this idea.[5,6] Currently, chronic HBV infection is treated with interferon-α or nucleoside analogs such as lamivudine, entecavir and adefovir dipivoxil.[7–10] However, these treatments have several drawbacks, including interferon-related side effects and the development of escape mutants after a long period of lamivudine treatment. These side effects result in low efficacy of the current drugs and high rates of drug resistance.[11,12] Therefore, the development of novel therapeutic approaches that effectively inhibit HBV replication is urgently required.

HBV belongs to the hepadnavirus family, comprising hepatotropic DNA viruses that are able to infect mammalian and avian hosts. HBV exhibits most of the genetic structures and replicative characteristics observed in other viruses in this family[13] and is composed of an outer envelope, which is formed by HBsAg, and an inner nucleocapsid, which packages the viral genome and polymerase.[14] The HBV genome is thought to be a circular DNA molecule that has ~3200 nucleotides but is only partially double stranded. One strand (the long (L) strand) is complete but has a nick at a fixed position. The complementary strand (the short (S) strand) is incomplete, extending for a variable distance from a fixed 5′ terminus, which is ~ 250 nucleotides 5′ to the nick in the L strand.[15,16] The HBV genome is remarkably compact and is a partially double-stranded relaxed circular DNA molecule containing precore/core (C), polymerase (P), surface (S) and X open-reading frames (ORFs). The P-ORF is the largest ORF and encodes the enzyme responsible for the priming and reverse transcription of HBV pregenomic RNA. Pre S1, Pre S2 and S, which are in-phase start codons of the S-ORF, initiate the translation of the large, middle and major sAgs (HBsAgs), respectively.[17] The C-ORF contains core and precore initiation codons for the translation of the overlapping nucleocapsid protein and secrete HBV e antigen. X encodes a protein that regulates viral gene transcription,[18] is required for natural infection *in vivo*, and is implicated in hepatocarcinogenesis.[19,20] Each HBV ORF overlaps at least one other, and together, they comprise the entire genome. Regulatory cis-elements are included within the protein-coding regions in a highly compact arrangement, which restricts HBV sequence plasticity. Consequently, the emergence of escape mutants is limited, and HBV is particularly susceptible to the disabling effects of mutations.

The replication of HBV forms a crucial intermediate-covalently closed circular DNA (cccDNA). On infection, the relaxed circular DNA is transported into the nuclei and converted into cccDNA, which acts as the template for viral RNA transcription. Subsequently, a plus-strand DNA is synthesized using the minus-strand as a template by viral polymerase. Finally, the HBV genome

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Received 11 July 2014; revised 2 December 2014; accepted 8 December 2014

is either encapsulated to produce new virions and is secreted or re-enters the nuclei to maintain a steady pool of 5–50 cccDNA molecules per infected hepatocyte.[21,22] The elimination of chronic HBV infection requires the consequent cleavage of cccDNA from the infected hepatocytes.[23]

Over the last decade, novel genome-editing methods that utilize artificial nucleases such as zinc finger nucleases (ZFNs)[24] and transcription activator-like effector nucleases (TALENs)[25] have been developed. These molecularly engineered nucleases recognize and cleave specific nucleotide sequences in target genomes, resulting in various mutations such as substitutions, deletions and insertions, which are induced by the host DNA repair machinery. These technologies have enabled the production of genome-manipulated animals across a wide range of species such as *Drosophila*,[26] zebrafish[27] and rat.[28] Although Bloom *et al.*[17] previously demonstrated a targeted nuclease-mediated disruption of HBV cccDNA by TALENs, the difficulties related to the design, development and empirical testing in a cellular context has hampered the widespread use of this technique.[29]

Recently, a third genome-editing method was developed based on clustered regularly interspaced short palindromic repeat (CRISPR) systems. CRISPR systems, which are dependent on a complex consisting of CRISPR-coded RNAs (crRNAs) and CRISPR-associated (Cas) proteins, were originally identified in bacteria and archaea and have been found to act as an RNA-based adaptive immune system that degrades the complementary sequences of invading plasmids and viruses.[30–32] Three types of CRISPR–Cas systems (I, II and III) have been functionally identified across a wide range of microbial species, and each system contains a cluster of Cas genes and its corresponding CRISPR array. The type II CRISPR/Cas system consists of two short RNAs, the crRNAs, and the DNA endonuclease Cas9. The crRNA hybridizes with the tracrRNA to form a crRNA: tracrRNA duplex, which is loaded onto Cas9 to direct the cleavage of cognate DNA sequences bearing appropriate protospacer-adjacent motifs.[33] Notably, the combination of the humanized Cas9 protein with a synthetic single-guide RNA (single-gRNA), generated by fusing crRNA and trans-activating crRNAs, was able to reconstitute RNA-based nucleases and cause double-strand breaks in mammalian cells.[34] Ebina *et al.*[35] suggested that the CRISPR/Cas system, which is composed of a gRNA and a human codon-optimized Cas9 (hCas9) nuclease, may be a useful tool for curing HIV-1 infection. In addition, Wang *et al.*[36] reported that this approach is highly efficient for genome modification, and, furthermore, that double genomic mutations have been successfully introduced using two gRNAs targeting two different chromosomes simultaneously. These results suggest that the CRISPR/Cas system may be a useful tool for multiple genomic editing.

In this study, we have designed a CRISPR/Cas9 system that targets a few conserved and HBV-specific sites within the viral genome. We have shown that a CRISPR/Cas9 system that targets cognate sequences in the S- and X-ORFs can efficiently introduce HBV-disabling mutations at the intended target sites both in cell culture and in animal experiments.

RESULTS

Inhibition of HBV antigens in cell culture by CRISPR/Cas9

The supernatants of HepG2.2.15 cells that were co-transfected with a gRNA expression plasmid and an hCas9 expression plasmid were collected at 48 h after transfection, and the HBsAg levels were assessed by enzyme-linked immunosorbent assay. After treatment with CRISPR/Cas9, the HBsAg levels markedly decreased in the treated cells (by 61% for CRISPR+Cas9+S1-targeted gRNA and by 56% for CRISPR+Cas9+X3-targeted gRNA) compared with the control group (Blank; Figure 1). This finding indicates that the HBsAg concentrations in the culture supernatants significantly decreased in the cells that had been transfected with ORF S- and X-targeted gRNAs.

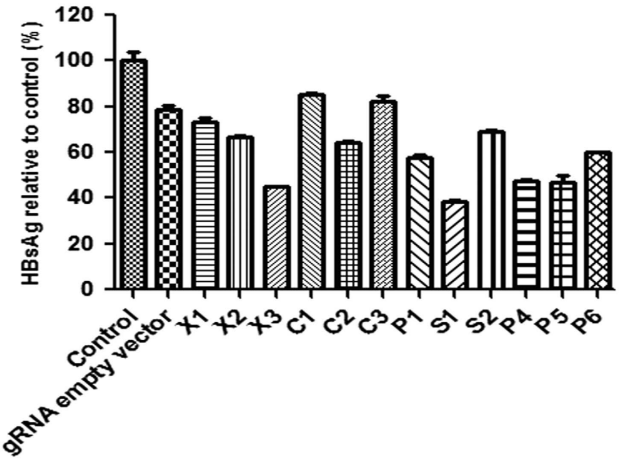

Figure 1. The reduction of HBsAg expression by CRISPR/Cas9. The CRISPR/Cas9 plasmids were transfected into HepG2.2.15 cells. At 48 h post transfection, the culture medium was collected and the HBsAg level was measured by ELISA.

CRISPR/Cas9-mediated targeted mutagenesis of cccDNA *in vitro*

We designed a gRNA expression vector to target the HBV genome under the control of the human U6 polymerase III promoter. U6 transcription of gRNA is initiated with guanine and requires the protospacer-adjacent motif-NGG followed by a 20-bp target sequence. Accordingly, the gRNA-expressing plasmids were generated for the target conserved domain of the P-, S-, X- and C-ORFs as described in the methods section (Figure 2a). The transfection efficiency of the hCas9 plasmid was examined by PCR using specific primers (Figure 3a) to ensure that the CRISPR/Cas9 system was transfected successfully.

To assess the targeted CRISR/Cas9-mediated mutagenesis of cccDNA, circul ar duplex DNA isolated from HepG2.2.15 cells and HepG2 cells transfected with pAAV-HBV1.3 were subjected to *Hin*dIII digestion, which is a restriction site that does not exist in the HBV genome. After digestion, the cccDNA was treated with ATP-dependent DNase (PSAD). PSAD has lower digestion activity towards macromolecules and linearized DNA, and the combination of these two enzymes improves the specificity of PSAD. The PCR-based analyses were initially conducted to assess the contamination of the cccDNA sample with cellular genomic DNA and HBV rcDNA (Figure 3b). To detect genomic DNA contamination, primer sets that amplify HBV DNA or control cellular genomic sequences located in the *A1AT* gene were used. With the *A1AT* gene primers, DNA was efficiently amplified from cellular genomic DNA but not when using the cccDNA preparation as a template (Figure 3b). The amplification of HBV S-sequences from the genomic template was expected and is likely to be derived from stable HBV integrants within the cells. These data indicate that the cccDNA preparations were not significantly contaminated with either genomic DNA or HBV rcDNA.

After quantitative analysis by quantitative PCR (qPCR), the relative expression of cccDNA was analyzed (Figure 4). These results showed 12 gRNAs that exhibited diverse efficacy in HepG2.2.15 cells, with 8 of the 12 showing significant knockdown efficacy compared with the control (blank) or gRNA empty and hCas9 plasmids (negative; $P < 0.05$). ORF S- and X-targeted gRNAs had a clear role in inhibiting the expression of cccDNA. We chose the gRNA2 S1 and X3 because of their higher suppressive ability.

Inhibition of the HBV antigen *in vivo* by CRISPR/Cas9

To determine the effect and the optimal concentration of CRISPR–Cas9 for HBV inhibition *in vivo*, the hydrodynamic injection (HDI) method was employed.[37] The HBsAg serum levels were reduced

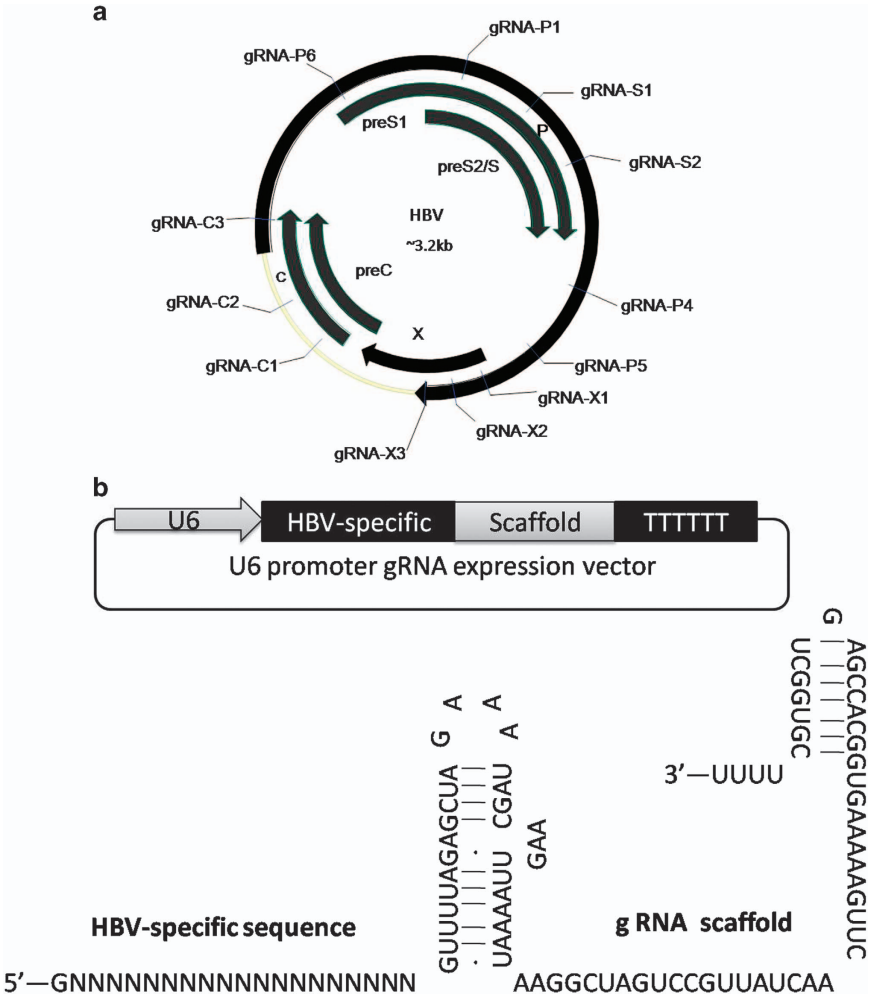

Figure 2. Designs of gRNAs targeting HBV-specific sequences. (**a**) An illustration of the gRNA-targeted sequences located in the HBV genome. (**b**) Chimeric HBV-specific gRNA designs.

by 85% for ORF S1/P2-targeted gRNA and 77% for ORF X3-targeted gRNA on day 1 compared with the levels induced by the gRNA-empty vector. A kinetic study revealed that the inhibitory effect of CRISPR/Cas9 on serum HBsAg levels was strongest for X3-gRNA+S1-gRNA (93%) on day 1 compared with the gRNA-empty vector, and diminished slightly thereafter, still attaining 89% inhibition on day 3. The inhibitory effect of CRISPR–Cas9–S1 and CRISPR–Cas9–X3, individually, on HBsAg levels was slightly weaker than the effect of the combined treatment on days 1 and 3 (Figure 5). The irrelevant control gRNA exhibited no effect on HBsAg levels, demonstrating the specificity of the gRNA effect.

To examine how long CRISPR/Cas9 inhibits HBV gene expression, the HBsAg concentrations in the sera of treated animals were measured on days 1, 2, 3, 4, 7, 8 and 10 after CRISPR–Cas9 (X3+S1) transfection. The treatment of HDI mice with CRISPR–Cas9 (X3+S1) resulted in the inhibition of the secreted HBsAg by 92%, 90% and 89% on days 1, 2 or 3, respectively, compared with the untreated control. No changes in HBsAg levels were found on days 4–10 (Figure 6).

Targeted disruption of HBV sequences *in vivo*

The cccDNA isolated from the mouse liver was analyzed using the procedures mentioned above. The T7 endonuclease 1 assay demonstrated efficient target-specific mutagenesis of DNA extracted from the livers of mice that had been treated with the S-/X-gRNA and hCas9 plasmids (Figures 7a and b). A detectable mutation in control mice was consistently $< 1\%$, whereas in the S- and X-gRNA/hCas9-treated mice 58–75% of the amplified HBV sequences contained mutations. These mutation patterns are observed as a result of DNA repair in the non-homologous end-joining (NHEJ) pathway and are typical after genome editing,[38] which strongly suggests that this CRISPR/Cas9 component generated double-stranded DNA breaks at the specific HBV target site that were repaired through the NHEJ pathway.

The sequencing of HBV DNA amplified from hepatic extracts confirmed that the intended target sites were mutated (Figure 8). These results showed that the cloned HBV DNA contained various mutations compared with the DNA of mice receiving the gRNA-empty plasmid (wild type). The clones harbored a combination of deletion and insertion mutations. These results clearly show that the CRISPR/Cas9 system efficiently produced mutations in the HBV DNA.

The effects of CRISPR/Cas9 on HBsAg expression in the liver of HBV-Tg mice

The effects of CRISPR/Cas9 on HBV antigens were determined by immunohistochemical staining of liver sections from treated and control mice in the HDI model and HBV transgenic (HBV-Tg) model. Compared with the findings for the control group, HBsAg-positive cells were absent in the livers of mice treated with CRISPR/Cas9-S1+X3 on day 1 (Figures 9 and 10).

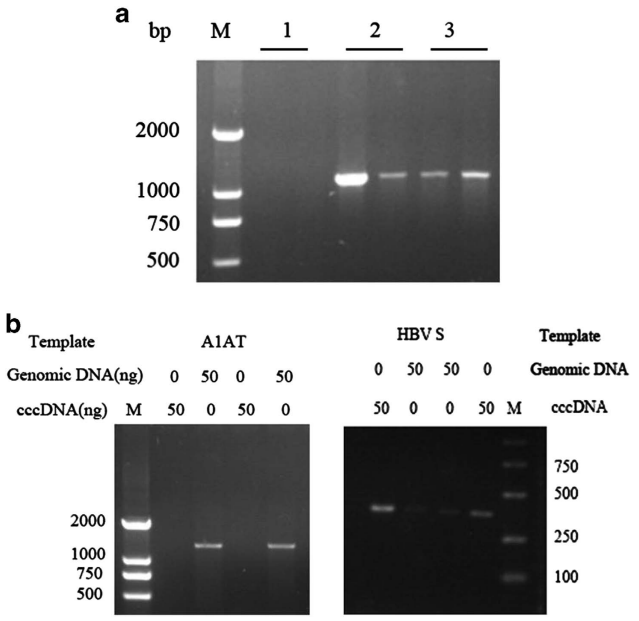

Figure 3. Targeted disruption of cccDNA extracted from HepG2.2.15 cells and HepG2 cells transfected with pAAV-HBV1.3. (**a**) The levels of intracellular Cas9 were analyzed by PCR 48 h post transfection of the following different cells: (1) cells without any transfection; (2) HepG2 cells transfected with pAAV-HBV1.3; (3) HepG2.2.15 cells (1.2 kb). (**b**) PCR analysis using A1AT and S-gene primer sets conducted on total genomic DNA or cccDNA isolated from cells. A1AT and S-gene amplicons of the expected sizes—1.2 kb and 300 bp, respectively. The DNA fragment sizes (bp) in the molecular weight marker lane (M) are shown.

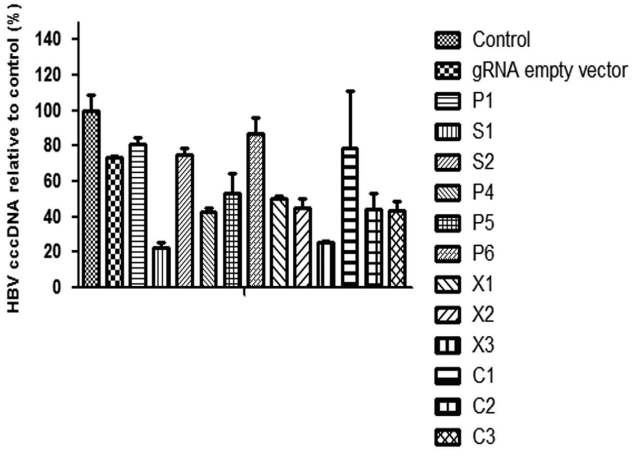

Figure 4. The inhibition of HBV cccDNA replication by the CRISPR/Cas9 system. The copies of HBV cccDNA indicates viral amplification activity. HBV cccDNA was isolated from the HepG2.2.15 cells and quantified by qPCR.

DISCUSSION

Genome modifications have laid the foundation for functional studies in modern biology and have led to significant discoveries.[39] Targeted editing of genomic sequences is now a rapidly expanding field. Recently reported successes of sequence-specific DNA modification have indicated that the technology holds considerable potential and may help treat a variety of diseases, including those caused by viral infections.[40] Although intended target sequence modification methods have been developed such as I-SceI,[41] chemical-based nucleases[42] and ZFNs,[43] considerable technical hurdles have impeded the widespread use of these methods. Further developments of this technology applying TALENs[44] and CRISPR-associated Cas9-based RNA-guided nucleases[45,46] have been particularly successful in the modification of genomic targets.[47] Moreover, recent results have suggested that CRISPR/Cas9-mediated disruption of viral sequences is more efficient and safer[48,49] than targeting viruses using ZFNs and TALENs as has been reported for HIV. Moreover, when using TALENs and ZFNs, two endonuclease-encoding constructs have to be designed per target against the 3′- and 5′-ends of a single target sequence. ZFNs and TALENs also only exert their genome modification function after dimerization on the target locus; thus, targeting multiple loci simultaneously by TALEN or ZFN results in an increase in nuclease concentration and may increase the risk of off-target effects. In addition, target modifications using these approaches have not always been satisfactory. Several reports have suggested that the mutation-creating rates of ZFN and TALEN are usually low and preparing two efficient pairs for two target loci simultaneously is difficult.[50,51] In contrast, Fujii *et al.*[52] indicated the availability of the Cas9 system for large-scale deletions and implied that the system can contribute largely to genomic studies. Furthermore, the crystal

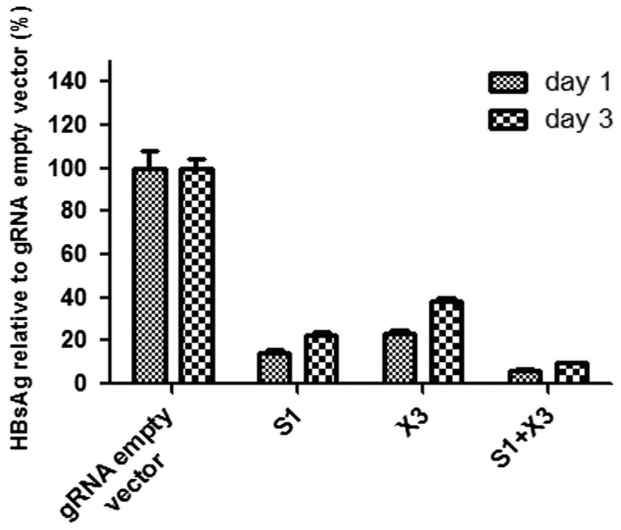

Figure 5. Serum HBsAg levels after CRISPR/Cas9 treatment in mice. The HBsAg levels were significantly reduced by CRISPR/Cas9 treatment in mice. HBsAg inhibition is specific and was detected for at least 3 days.

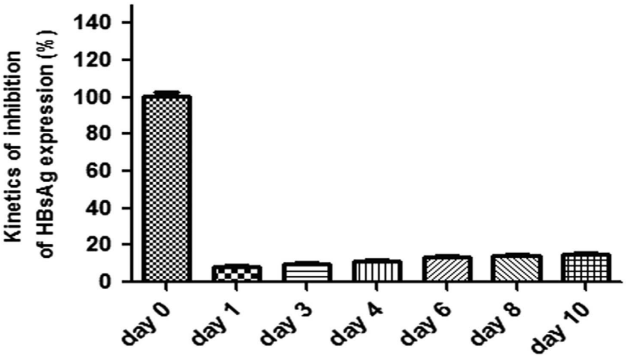

Figure 6. The HBsAg concentrations in the sera were measured on days 1, 2, 3, 4, 7, 8 and 10 after transfection with CRISPR–Cas9 (X3+S1). The data shown are mean ± s.d. based on three independent experiments.

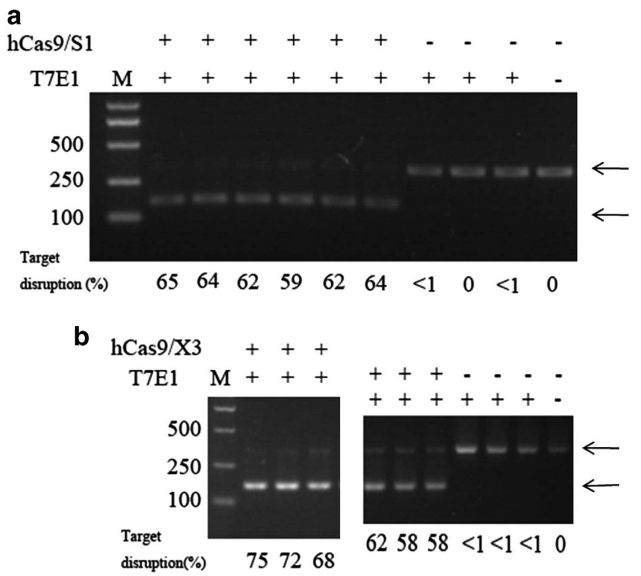

Figure 7. Targeted disruption of HBV DNA sequences *in vivo*. The T7 endonuclease 1 (T7E1) assay was conducted on hepatic DNA isolated from mice. Each lane represents data obtained from an individual animal. Heteroduplex DNA was treated in the presence (+) or absence (−) of T7E1. (**a**) The mice were treated with solutions containing negative control (−) or gRNA-empty/S1-gRNA–hCas9 plasmids (+). The arrows indicate the larger intact PCR product (373 bp) and a smaller digested fragment (122 bp). (**b**) The mice were subjected to solutions containing negative control (−) or gRNA-empty/X3-gRNA-hCas9 plasmids (+). The arrows indicate the larger intact PCR product (381 bp) and smaller digested fragment (112 bp). The measured target disruption is indicated below as a percentage.

S1	
CTCCTGTCCTC**CAACTTGTCCTGGTTATCGC**TGGATGT	Wild type
CTCCTGTCCTCCAACTgccacaacctttcaCGCTGGATGTG	S1(-12, +14)
X3	
GCTTCACCT**CTGCACGTCGCATGGAGCC**ACCGTGAAC	Wild type
GCTTCACCTCTGttaccaat TGGAGCCACCGTGAAC	X3(-9, +8)

Figure 8. Sequencing analysis of the CRISPR/Cas-target site. The HBV DNA target sequence is indicated in bold, and the protospacer-adjacent motif sequence is highlighted as underlined text. The deletion and insertions are highlighted as lower case letters. The net change in length caused by each indel mutation is to the right of each sequence (+, insertion; −, deletion). The WT sequence is shown on the top. Note that several alterations have both insertions and deletions of the sequence.

structure of Cas9 in complex with gRNA and target DNA has been reported,[33,53] which may facilitate improvement and further use of this tool.

In the present study, we have provided several lines of evidence that CRISPR/Cas9 directed against HBV sequences in the S1/X3 antigen/polymerase region is capable of inhibiting HBV gene expression and viral replication both in HepG2.2.15 cells, which support viral production and an *in vivo* HDI model. The observed inhibition was specific and was sustained for 3 days after CRISPR/Cas9 administration. Eleven gRNA candidates were evaluated. Overall, S1 targets the HBV S-region, is only less sensitive to mutation, and is also shared by the major viral transcripts.[54] In contrast, X encodes a protein that regulates viral gene transcription and exhibits the highest level of CRISPR/Cas9-mediated inhibition both *in vitro* and *in vivo*. Detailed analysis of the mouse or human genomes indicated that these organisms do not contain sites that would be predicted to be suitable X3 and S1 Cas9 targets, which suggests that unintended double stranded breaks are unlikely to occur in normal human and mouse genomes. The highly economical use of HBV genetic material limits the ability of the virus to escape the disabling effects of site-specific nucleases.[17] To date, most studies have been concerned with developing models for functional analysis, and little work has been conducted using CRISPR/Cas9 to disable pathology-causing genes such as those encoded by viruses. Our finding that CRISPR/Cas9 is capable of efficiently disabling HBV targets represents a substantial advance in the therapeutic application of designer nucleases.

Although cccDNA is not formed during HBV replication in the mice subjected to HDI, disruption of the intended HBV sequences without overt evidence of toxicity is a significant observation, and after quantitative analysis by qPCR, the relative expression of cccDNA was analyzed. The effect of CRISPR/Cas9 on viral DNA production is most likely direct. Although Southern blot analysis is well-suited for analyzing cccDNA, qPCR is now considered the gold standard for selective quantitation of cccDNA. Moreover, we demonstrated that targeting S1 and X3 by CRISPR/Cas9 induces a decrease in the expression of HBsAg *in vivo*. This interference with transgene and endogenous gene expression after CRISPR/Cas9 administration in mice has been reported by several groups. To the best of our knowledge, efficient functioning *in vivo*, coupled with cccDNA disruption in HepG2.2.15 cells, shows the potential antiviral therapeutic utility of engineered CRISPR/Cas9.

The present study showed that CRISPR/Cas9 can be used to inhibit the replication of a human pathogen in a mammalian model system and may be applicable as a therapeutic strategy for naturally occurring HBV infections. Several remaining issues still need to be addressed for further applications. For example, the efficiency of this system should be improved. In our hydrodynamics system, the serum HBsAg levels were reduced but still persisted following CRISPR/Cas9 treatment. To overcome this obstacle, an efficient delivery system with a high vector to target cell ratio, such as adenoviral or adeno-associated viral vectors, would be required to efficiently utilize the CRISPR/Cas9 system.

However, numerous hurdles must be cleared before utilizing genome editing for HBV eradication therapies. First, the efficiency of this system should be improved. In our hydrodynamics system, serum HBsAg levels were reduced, but the interference in HBV replication induced by CRISPR/Cas9 treatment was transient. To eradicate HBV infection, prolonging therapy and ensuring its delivery to every infected cell in the body, including extrahepatic viral reservoirs, are essential components to a successful system. An alternative approach would be to use an efficient delivery system with a high vector, such as adenoviral or adeno-associated viral vectors,[55] to achieve continuous and prolonged treatment. The use of immediate, prolonged treatments, and even the simultaneous use of several treatments, may be possible. The second hurdle concerns possible off-target effects, which are pertinent concerns for all genome-editing strategies that may lead to non-specific gene modification events. If Cas9 has off-target effects, eliminating this off-target activity prior to utilizing the CRISPR/Cas9 system for anti-HBV treatment may be best. However, a significant effort has been made to improve the specificity of CRIPSR/Cas9 with truncated gRNAs and paired nickase gRNAs.[53,56] The third hurdle to be overcome is to achieve safe and efficient delivery of the therapeutic transgenes, which still remains difficult. Engineering recombinant CRISPR/Cas9-expressing virus vectors and assessment in animal models where HBV cccDNA production occurs such as woodchucks infected with woodchuck hepatitis virus[57,58] will be important for preclinical evaluation. Despite these hurdles, the efficiency and specificity of HBV DNA-targeting CRISPR/Cas9 supports the use of these enzymes to inactivate the expression of pathology-causing genes.

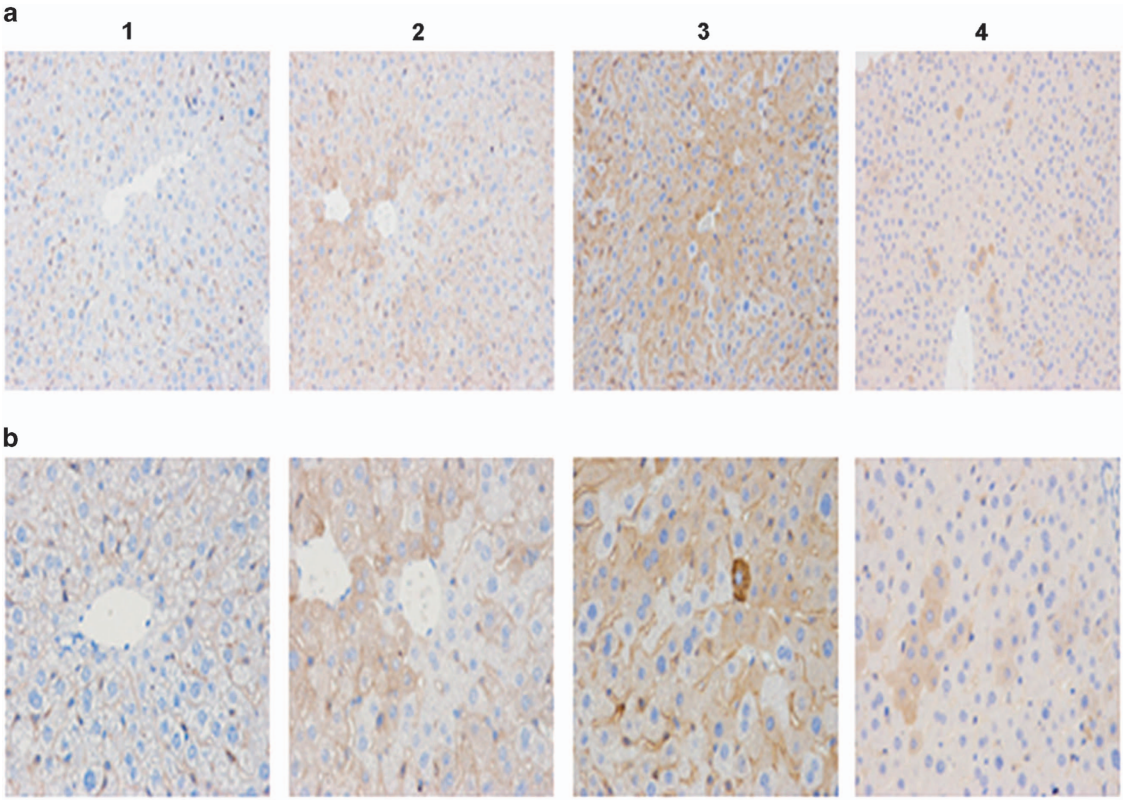

Figure 9. Immunohistochemical staining for HBsAg in the liver sections (HDI model). (**a**) ×100 and (**b**) ×400. CRISPR–Cas9Cas9 treatment reduced the number of HBsAg-positive cells on day 1 compared with the control. The staining was performed on tissue sections from the liver of animals per group. 1: without monoclonal antibody against HBsAg; 2: blank group; 3: gRNA-empty vector group; 4: CRISPR–Cas9 (X3+S1) group.

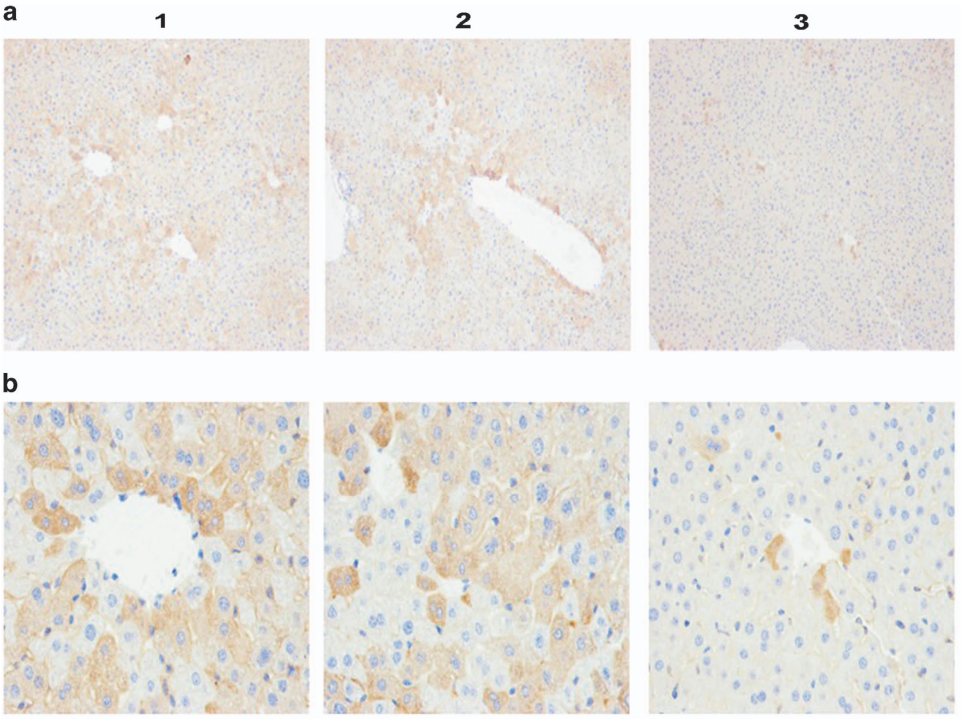

Figure 10. HBsAg immunohistochemical staining in the liver sections (HBV transgenic model). (**a**) ×100 and (**b**) ×400. CRISPR–Cas9Cas9 treatment reduces the number of HBsAg-positive cells on day 1 compared with the control. The staining was performed on tissue sections from the livers of animals per group. 1: blank group; 2: gRNA-empty vector group; 3: CRISPR–Cas9 (X3+S1) group.

In conclusion, we have shown that CRISPR/Cas9 can be designed to inhibit HBV replication and gene expression both *in vitro* and *in vivo*. These results indicate that the Cas9/gRNA system may provide an easy, inexpensive and short-term method for mammalian genome modification and that extensive genome modifications can be achieved by simultaneously using multiple gRNAs. This information should be useful in future studies for the assessment of the therapeutic potential of CRISPR/Cas9 in the treatment of acute and chronic infections by a pathogen such as HBV.

MATERIALS AND METHODS

Plasmids

The hCas9 expression vector (plasmid 41815) and gRNA cloning vectors (plasmid 41824) were obtained from Addgene (Cambridge, MA, USA). The plasmids were prepared by using the Qiagen Endofree Plasmid Kit (Qiagen, Hilden, Germany).

Design and cloning of HBV-specific gRNA

gRNA expression plasmids were constructed according to manufacturer's protocol,[59,60] and detailed BLAST searches of the human and murine genomes were conducted to identify potential off-target binding of HBV gRNAs. To assess the utility of HBV cccDNA-targeting gRNAs, 12 sets of oligonucleotides (Table 1) were designed to target the complete genomic HBV DNA (ORFs P, S, X and C). All oligonucleotides were synthesized and purified by Sangon Biotech Co (Shanghai, China). Briefly, to prepare a 100-bp double-stranded DNA insert fragment containing the target sequence (20 bp) and a protospacer-adjacent motif sequence, we used a set of oligonucleotides and generated the fragment using T4 PNK (NEB, Ipswich, MA, USA). The double-stranded DNA fragment was purified and inserted into the BbsI site of a gRNA cloning vector with T4 DNA ligase (NEB).

Cell culture and transfection

Two different liver-derived cell lines, HepG2 and HepG2.2.15, were used to determine CRISPR–Cas9 efficacy in culture. An HBV replication-competent plasmid, pAAV-HBV1.3, containing inverted terminal repeat elements of adeno-associated virus (AAV) and 1.3 copies of the HBV genome (ayw) was kindly provided by Professors Linsheng Zhang and Yusen Zhou and was used in transient cotransfections of HepG2 cells with plasmids expressing CRISPR/Cas9. The HepG2.2.15 human hepatoblastoma cell line was stably transfected with a head-to-tail dimer of the HBV DNA genome (strain ayw, U95551), and this line expressed all viral RNAs and proteins of the viral genome and secreted virus-like particles.[49] The cells were maintained in Dulbecco's modified Eagle's minimal essential medium, supplemented with 100 ml/l fetal calf serum (Hyclone, Logan, UT, USA), 400 μg ml^{-1} G418, 100 μg ml^{-1} streptomycin and 100 IU ml^{-1} penicillin at 37 °C in a humidified atmosphere containing 5% CO_2.

Table 1. The gRNA sequences and location

Name	*Genomic target*	*PAM*	*Target location*
P1	CCTCGAGAAGATTGACGATA	AGG	p114
S1	CAACTTGTCCTGGTTATCGC	TGG	s/p357
S2	CAGGTGCAATTTCCGTCCGA	AGG	s/p581
P4	GAAAGTATGTCAACGAATTG	TGG	p985
P4	AGGTTCCACGCACGCGCTGA	TGG	p1227
X1	AAACAAAGGACGTCCCGCGC	AGG	x/p1406
X2	CGCCCCGTGGTCGGTCGGAA	CGG	X1506
X3	GGTCTCCATGCGACGTGCAG	AGG	X1596
C1	TCTAGAAGATCTCGTACTGA	AGG	c1976
C2	ACTACTAGGTCTCTAGATGC	TGG	C2138
C3	GATTGAGATCTTCTGCGACG	CGG	c/P2414
P6	GGCTGGATCCAACTGGTGGT	CGG	p2894

Abbreviations: gDNA, guide RNA; PAM, protospacer-adjacent motif. The gRNA sequences and location.

The HepG2 cells were seeded in 12-well plates 24 h prior to transfection. Polyethylenimine was used to transfect cells with 300 ng pAAV-HBV1.3, 1 μg of the hCas9 expression vector (Plasmid 41815:hCas9 Addgene) and 1 μg of the gRNA expression vector.

The HepG2.2.15 cell line, a derivative of the human HepG2 hepatoma cell line that has been stably transformed with two copies of the HBV genome, constitutively produces HBV particles.[61] The cells were seeded into 12-well plates and grown until 60–80% confluency, after which the cells were transfected with 1 μg of hCas9 expression vector (Plasmid 41815:hCas9) and 1 μg of gRNA expression vector.

The control group was transfected with no vector, and the gRNA-empty vector group was transfected with 1 μg of hCas9 expression vector and 1 μg of gRNA-empty vector using Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Two days after transfection, the cells were harvested, and the HBsAg concentrations in cell culture medium were measured using the Monolisa HBsAg ULTRA kit (Bio-Rad, Hercules, CA, USA).

Extraction and quantitation analysis of HBV DNA

After CRISPR/Cas9 transfection, HBV cccDNA was extracted from HepG2.2.15 cells and mouse liver as previously described[62] with a minor modification. Specifically, the HBV genome was first digested with *HindIII* (NEB), which is a restriction site not present in HBV DNA or HBV cccDNA, at 37 °C for 1 h and 80 °C for 20 min to convert the cccDNA into a linear DNA fragment, followed by treatment with ATP-dependent DNase (Plasmid-safe, Epicenter Biotechnologies, Madison, WI, USA) at 37 °C for 1 h and 70 °C for 30 min. Genomic DNA was extracted from the HepG2.2.15 cells and mouse liver using the TIANGEN DNA Blood MINI Kit (TIANGEN, Beijing, China).

PCR analysis was used to confirm that the cccDNA was successfully isolated from HepG2.2.15 cells and mouse liver. The *alpha1 antitrypsin* (*A1AT*) genomic DNA sequence and HBV S-ORF were amplified using the methods previously detailed,[63] with the following primers: *A1AT*-forward (*A1AT*-F): 5′-TTCCCTGGTCTGAATGTGTG-3′ and *A1AT*-reverse *(A1AT*-R): 5′-ACTGTCCCAGGTCAGTGGTG-3′; S-F: 5′-ATCCTGCTATGCCTCATCTT-3′ and S-R: 5′-ACAGTGGGGGAAAGCCCTACGAA-3′. For amplification, 50 ng of either HepG2.2.15 genomic DNA or cccDNA preparation was used as a template.

QPCR was performed to quantify the amount of cccDNA extracted from HepG2.2.15 cells. The PCR reaction mixture (20 μl) contained 10 μl GoTaq qPCR Master Mix (Promega, Madison, WI, USA), 20 ng DNA, 0.5 μl (10 μM) forward and reverse primers and 0.5 μl FAM-5′-labeled probes (Sangon Biotech Co.). The forward primer was 5′-CTCCCCGTCTGTGCCTTCT (nt1548–1566); the reverse primer was 5′-GCCCCAAAGCCACCCAAG (nt1903–1886); and the TaqMan probe was FAM-caccgaatgttgcccaa ggtcttacata-BHQI.

In the HepG2 cell models, the total cellular DNA was extracted to confirm that the hCas9 expression vector was transfected into the cells, and the DNA samples were subjected to PCR using the following specific primers: the forward primer 5′-GCTTCCTAAACACTCTCTGCTGTA-3′ and the reverse primer 5′-TCTTGACAACTTCTTCTGAGGG -3′.

T7 endonuclease 1 assay

To confirm that CRISPR/Cas9 cleavage and targeted sequence disruption occurred at the intended target site, a mismatch-sensitive T7 endonuclease 1 was used (NEB). The DNA region encompassing the S- and X-target site was amplified under standard conditions using the following primers: S1 forward: 5′-GCCTCTCCCTTATCGTCAA-3′ (nt106–124) and reverse: 5′-GGAC AAACGGGCAACATA-3′ (nt461–479) and X3 forward: 5′-GCAAACATTATCGG GACTG-3′ (nt1335–1317) and reverse: 5′-AGGTCGGTCGTTGACATT-3′ (nt1698–1680). The PCR products were subjected to heteroduplex formation after denaturing 200 ng of amplified DNA at 95 °C for 5 min followed by slow cooling to 35 °C. The samples were treated with 5 U of T7 endonuclease 1 in 1 × NEB Buffer 2 (NEB), incubated at 37 °C for 30 min and then analyzed by agarose gel electrophoresis. Tanon electrophoretically software (Tanon Science & Technology Co., Ltd., Shanghai, China) was used to measure band intensities, and targeted disruption was previously described by Guschin *et al.*[64]

Sequencing

The PCR fragments containing each target locus were cloned into a plasmid using the pMD19-T for sequencing by Sangon Biotech Co.

The mutated sequences were identified by comparison with the wild-type unmodified sequence.

Animals and CRISPR/Cas9 treatment *in vivo*

All mouse experiments were conducted according to the National Institutes of Health Guidelines for Animal Care. All animal procedures were performed according to the guidelines developed by the China Council on Animal Care, and the protocol was approved by the Academy of Military Medical Science (Beijing, China).

For *in vivo* experiments, we used 6–7-week-old female BALB/c nude mice (18–22 g) from the Laboratorial Center of Experimental Animals, the Academy of Military Medical Science. HBV-Tg mice, which were purchased from the Infections Disease Center of No. 458 Hospital (Guangzhou, China), were also used. The HBV-Tg mouse lineage was initially produced in a BALB/c background. The transgene present in these mice consists of 1.3 copies of the HBV adr genome. The HBV-Tg mice express high levels of HBsAg in their serum and have detectable levels of HBV DNA in their serum.[65–67] All the mice used for experiments were 8-weeks-old.

HBV-Tg mice were randomly divided into four groups (eight per group): the empty vector group, CRISPR+Cas9+S1-treated group, CRISPR+Cas9 +X3-treated group and CRISPR+Cas9+S1+X3-treated group. CRISPR/Cas9 and gRNA plamids were delivered into mice using the hydrodynamic tail vein injection method.[68] Briefly, 40 μg Cas9 and 20 μg S1-gRNA and/or 20 μg X3-gRNA, dissolved in 1.5 ml Ringer's solution (147 mM NaCl, 4 mM KCl and 1.13 mM $CaCl_2$), were rapidly injected into the tail vein.

BALB/c nude mice were also randomly divided into four groups (eight per group) similar to the HBV-Tg mice. Plasmid HBV and CRISPR/Cas9 were delivered into mice using the hydrodynamic tail vein injection method.[68] Briefly, 60 μg pHBV, 40 μg Cas9 and 20 μg S-gRNA and/or 20 μg X-gRNA dissolved in 1.5 ml Ringer's solutions were rapidly injected into the tail vein.

On day 1 or 3 after the injection, the mice were bled and the sera were separated and examined for HBsAg or HBV DNA content. The livers of the mice were dissected into pieces, and a section was frozen immediately in liquid nitrogen, and another section was preserved in formalin for histological analysis.

HBsAg analysis

The HBsAg levels in the media of the transfected cells as well as in the sera of the treated mice were detected by a quantitative enzyme-linked immunosorbent assay, following the manufacturer's instructions. The suppression of HBsAg replication in the HBx-knockdown group was calculated using the following formula:

$$\text{Suppression rate} = [1 - \text{A value (HBsAg knockdown)}/\text{A value (negative control)}] \times 100\%$$

Immunohistochemistry

For histology and immunohistochemistry, formalin-fixed mice livers were embedded in paraffin using an automated device (Tissue-Tek VIP; Sakura, Torrance, CA, USA). The tissue sections were stained with hematoxylin and eosin. Immunostaining for HBsAg was performed using an automated staining device (IHC Staining System, Nexus, Ventana, Tucson, AZ, USA) with specific immunohistochemical stains for HBsAg (mouse, clone ZCH16, Code No. 1880023; Zymed, San Francisco, CA, USA).

Statistical methods

The results are presented as the mean ± s.d. The data were based on three independent experiments (*in vitro*) and on eight animals per group *(in vivo)*. The comparison between groups was performed by an independent samples *t*-test. The level of significance was set to $P < 0.05$.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Professor Linsheng Zhan and Professor Yusen Zhou for providing the plasmids; Professor Hui Zhong for help with the modification of the article; Ling-Zhen Li, Shao-Ming Guo and Cui-Li Zhu for their excellent technical assistance. This study was supported by grants from the National Natural Science Foundation of China (grant no. 81272701).

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