Inhibition of hepatitis B virus by the CRISPR/Cas9 system via targeting the conserved regions of the viral genome

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Hepatitis B virus (HBV) remains a global health threat as chronic HBV infection may lead to liver cirrhosis or cancer. Current antiviral therapies with nucleoside analogues can inhibit the replication of HBV, but do not disrupt the already existing HBV covalently closed circular DNA. The newly developed CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR-associated 9) system is a powerful tool to target cellular genome DNA for gene editing. In order to investigate the possibility of using the CRISPR/Cas9 system to disrupt the HBV DNA templates, we designed eight guide RNAs (gRNAs) that targeted the conserved regions of different HBV genotypes, which could significantly inhibit HBV replication both in vitro and in vivo. Moreover, the HBV-specific gRNA/Cas9 system could inhibit the replication of HBV of different genotypes in cells, and the viral DNA was significantly reduced by a single gRNA/Cas9 system and cleared by a combination of different gRNA/Cas9 systems.

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INTRODUCTION

Hepatitis B virus (HBV) belongs to the family of Hepadnaviridae and replicates in human hepatocytes. There are ~2 billion people who have been infected by HBV worldwide, among which 350 million people are chronic carriers. HBV chronic carriers suffer from a higher risk of liver diseases, including liver cirrhosis and cancer (Perz et al., 2006). The HBV virion contains a partially double-stranded genomic DNA comprising a complete coding strand (negative strand) and an incomplete non-coding strand (positive strand), i.e. relaxed circular DNA (rcDNA). Upon infection of hepatocytes, the viral rcDNA is released into the nucleus and converted into covalently closed circular DNA (cccDNA), which serves as the template for transcription of viral pregenomic RNA (pgRNA) and protein-coding mRNAs, including precore RNA, S RNAs and X RNA. The viral transcripts are transported into the cytoplasm and translated into viral proteins. Subsequently, the pgRNA is encapsidated by viral core and polymerase protein, and reverse transcribed into new viral rcDNA. The DNA-containing nucleocapsids are either enveloped and secreted as progeny virus or recycled back to the nucleus to amplify the cccDNA pool (Schädler & Hildt, 2009).

Current antiviral drugs against HBV include IFN-α to adjust the host antiviral immune response and nucleoside/nucleotide analogues as reverse transcriptase inhibitors (Ayoub & Keeffe, 2011). However, the efficiency of IFN-α is limited and the high dosage of IFN-α is not tolerated by patients (Jablonskiowski, 2003). Nucleoside analogues can only control but not functionally cure HBV infection due to the persistence of HBV cccDNA, which serves as the template for transcription of viral pgRNA and mRNAs (Liu et al., 2014b). Therefore, patients need long-term treatment, which is expensive and may lead to concomitant drug resistance (Januszkiewicz-Lewandowska et al., 2014; Xie et al., 2014). Moreover, as a type of retrovirus, HBV is likely to generate mutations during the reverse transcription and this subsequently increases the chance of escaping traditional antiviral drug treatment (Chan, 2011). As the cccDNA of HBV exhibits staggering stability and declines slowly under antiviral therapy, the efficient and non-toxic clearance of the cccDNA is a major goal for HBV therapy (Werle-Lapostolle et al., 2004).

In recent years, several genome-editing methods have been established based on sequence-specific endonucleases, including zinc-finger nucleases (ZFNs) (Mani et al., 2005), transcription activator-like effector nucleases (TALENs) (Joung & Sander, 2013) and the newly developed CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR-associated 9) system (Gaj et al., 2013). The CRISPR/Cas9

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Two supplementary tables are available with the online Supplementary Material.
system is derived from the adaptive immune system of bacteria and archaea. The Cas9 protein belongs to the type II CRISPR/Cas system, which cleaves DNA in a sequence-specific manner with the guidance of CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA). The cleavage of Cas9 requires the PAM sequence (NGG) downstream of the target DNA and creates DNA double-strand breaks (DSBs). Then the DSBs are typically repaired by non-homologous end-joining (NHEJ) or homology-directed repair. The error-prone NHEJ pathway may introduce insertions/deletions (indels) or mutations, eventually leading to frameshift mutations and gene knockout (Hsu et al., 2014). Moreover, the crRNA–tracrRNA complex can be fused into a single guide RNA (gRNA) (Hsu et al., 2014). After simplification, the CRISPR/Cas9 system requires the design of only a single sequence that matches the DNA targeted for cleavage. Compared with ZFN- and TALEN-based genome editing, the CRISPR/Cas9 system is easier to use. Therefore, great advances in genome engineering have been made based on the CRISPR/Cas9 system in recent years.

Recently, the gRNA-guided CRISPR/Cas9 (gRNA/Cas9) system has been successfully applied to many fields of biological and medical sciences. The application of this technology to virus research has also been reported, such as the genome editing of Epstein–Barr virus and herpes simplex virus (Yuen et al., 2015), and the eradication of human immunodeficiency virus infection (Craddock et al., 2013; Ebina et al., 2013). In the present study, we provide evidence that the gRNA/Cas9 system could profoundly inhibit HBV replication both in vitro and in vivo by directly disrupting the viral genome with HBV-specific gRNAs or their mixtures which target the conserved regions of the HBV genome. Moreover, HBV-specific gRNA/Cas9 systems could inhibit the replications of HBV of different genotypes in cells. The viral genome is significantly reduced by a single gRNA/Cas9 system and eliminated by a combination of different gRNA/Cas9 systems. This study shows the possibility to inhibit viral replication and clear the cccDNA of HBV of different genotypes that might be used for functional anti-HBV therapy, avoiding the concomitant drug resistance caused by mutations during viral replication.

RESULTS

Design and construction of HBV-specific gRNA/Cas9 systems

In order to design HBV-specific gRNAs, we searched potential 20-base sequences on the HBV genome (genotype D) via the online gRNA design tool designed by the Feng Zhang Laboratory (crispr.mit.edu). This tool can scan sequence for possible CRISPR guides (20 nt followed by a PAM sequence) in the input DNA sequence and ensure selected sequences with the fewest potential off-target matches throughout the selected host genome (Hsu et al., 2013). To ensure that the gRNAs could target different HBV genotypes and reduce missing targets caused by viral genome mutations, we aligned the sequences of 26 representative HBV genotypes of the World Health Organization reference panel (Chudy et al., 2012) to identify the relatively conserved genomic regions among different HBV genotypes, which also implied the low variable regions of the viral template (Fig. 1a). The sequences of gRNAs were chosen and modified to match those conserved regions with as little variation as possible (Fig. 1a). As shown in Fig. 1, eight gRNAs that targeted different regions of HBV genome sequence were selected (Fig. 1b, Table 1) and constructed into the chimeric gRNA/Cas9-expressing vector pX330 (Hsu et al., 2014) under the control of the U6 promoter of RNA polymerase III (Fig. 1c). The constructed HBV-specific gRNA/Cas9 systems targeting different regions of HBV genome were sequentially named T1–T8 (Table 1).

HBV-specific gRNA/Cas9 systems inhibit HBV replication

To explore the inhibitory effects of the constructed HBV-specific gRNA/Cas9 systems, the vectors carrying T1–T8 or their mixture (Tmix) were co-transfected into liver-derived HepG2 cells with the HBV genotype D replication-competent plasmid (pHBV1.3) and pSV-β-gal (as internal control). As shown in Fig. 2, the levels of HBV RNA transcripts in the transfected HepG2 cells were detected by Northern blotting and significantly suppressed by T1–T8 or Tmix gRNA/Cas9 systems compared with the negative control GFP-specific gRNA (T GFP) (Fig. 2a). Furthermore, the HBV replication intermediate DNA in the transfected cells (Fig. 2b) and the offspring virion DNA in the culture supernatant (Fig. 2c) were extracted and determined using real-time quantitative PCR (qPCR) 4 days after co-transfection. All gRNA/Cas9 T1–T8 systems could dramatically suppress HBV replication (Fig. 2b, c). Impressively, the cells transfected with the gRNA/Cas9 Tmix system showed up to 100-fold inhibition compared with T GFP control on both extracellular and intracellular HBV DNA (Fig. 2a–c). To evaluate the side effects of the gRNA/Cas9 systems due to the potential off-target gRNAs, cell viability was tested by CCK8 assays and showed that they, including gRNA/Cas9 Tmix, had no cytotoxicity (Fig. 2d). Moreover, two representative genomic homologous sequences of gRNA/Cas9 T1 and T7, which had the highest scores when predicted by the online software (crispr.mit.edu), were amplified and sequenced. As shown in Table S1 (available in the online Supplementary Material), there was no detectable mutation caused by the gRNA/Cas9 T1 or T7 systems, indicating that the off-target effect was indistinct.

HBV templates are cleaved and cleared by HBV-specific gRNA/Cas9 systems

To investigate whether the HBV-specific gRNA/Cas9 systems could directly target the HBV-expressing plasmid pHBV1.3, which represents the viral template, we re-extracted pHBV1.3 from the HepG2 cells at different time

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points from 6 to 24 h post co-transfection with gRNA/Cas9 T1. Cellular pHBV1.3 was linearized at the unique site of endonuclease HindIII (Fig. 3a) and subjected to Southern blotting (Fig. 3b). Interestingly, an increasing amount of 5 and 1.8 kb DNA bands, which were produced by the cleavage of gRNA/Cas9 T1 and endonuclease HindIII, were observed as time passed (Fig. 3b). This might be explained by the fact that insufficient or delayed NHEJ would not be able to rejoin the abundant DSBs cleaved by the gRNA/Cas9 system in time and therefore part of the viral templates were cleaved but not rejoined by NHEJ. Moreover, cellular pHBV1.3 plasmids from HepG2 cells co-transfected with gRNA/Cas9 GFP, T1,T7 (as representatives) or Tmix were prepared and detected as described above at 48 h post-transfection. As shown in Fig. 3(c), the amounts of cellular pHBV1.3 cleaved by gRNA/Cas9 T1,T7 or Tmix were significantly reduced compared with the T GFP control. In particular, it was almost undetectable for the plasmid cleaved by gRNA/Cas9 Tmix at multiple sites (Fig. 3c), which might lead to the degradation of linearized DNA in vivo. To evaluate the mutagenesis rate of cellular pHBV1.3 treated by the HBV-specific gRNA/Cas9 systems in HepG2 cells, we re-extracted the remaining plasmids at 48 or 96 h post co-transfection with gRNA/Cas9 T1, T7 and T GFP. The plasmids were amplified and subjected to Sanger sequencing. As shown in Table 2, no mutations were detected. These results indicated that most of the cellular pHBV1.3 plasmids cleaved by the HBV-specific gRNA/Cas9 systems might not be rejoined by the NHEJ in time and subsequently cleared in HepG2 cells.

To further demonstrate the effect of HBV-specific gRNA/Cas9 systems on HBV DNA, core-associated HBV DNA was extracted from HepG2 cells at different time points, digested at the unique EcoRI endonuclease site (Fig. 3a) and subjected to Southern blotting analysis. As shown in Fig. 3(d), treatment with gRNA/Cas9 T1 significantly repressed the accumulation of cellular HBV DNA, including
Table 1. Sequences of HBV-specific gRNAs

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1 (gRNA1)</td>
<td>Forward caccTACCGCAGCTAGACTCG</td>
</tr>
<tr>
<td>T1 (gRNA1)</td>
<td>Reverse aaccCGAGTCAGACTCGTGAGTT</td>
</tr>
<tr>
<td>T2 (gRNA2)</td>
<td>Forward caccGTAGCTCCAGTTGCAGGGTAA</td>
</tr>
<tr>
<td>T2 (gRNA2)</td>
<td>Reverse aaccACGTCAGACTCGTGAGTT</td>
</tr>
<tr>
<td>T3 (gRNA3)</td>
<td>Forward caccGTAGCTCCAGTTGCAGGGTAA</td>
</tr>
<tr>
<td>T3 (gRNA3)</td>
<td>Reverse aaccACGTCAGACTCGTGAGTT</td>
</tr>
<tr>
<td>T4 (gRNA4)</td>
<td>Forward caccGTAGCTCCAGTTGCAGGGTAA</td>
</tr>
<tr>
<td>T4 (gRNA4)</td>
<td>Reverse aaccACGTCAGACTCGTGAGTT</td>
</tr>
<tr>
<td>T5 (gRNA5)</td>
<td>Forward caccGTAGCTCCAGTTGCAGGGTAA</td>
</tr>
<tr>
<td>T5 (gRNA5)</td>
<td>Reverse aaccACGTCAGACTCGTGAGTT</td>
</tr>
<tr>
<td>T6 (gRNA6)</td>
<td>Forward caccGTAGCTCCAGTTGCAGGGTAA</td>
</tr>
<tr>
<td>T6 (gRNA6)</td>
<td>Reverse aaccACGTCAGACTCGTGAGTT</td>
</tr>
<tr>
<td>T7 (gRNA7)</td>
<td>Forward caccGTAGCTCCAGTTGCAGGGTAA</td>
</tr>
<tr>
<td>T7 (gRNA7)</td>
<td>Reverse aaccACGTCAGACTCGTGAGTT</td>
</tr>
<tr>
<td>T8 (gRNA8)</td>
<td>Forward caccGTAGCTCCAGTTGCAGGGTAA</td>
</tr>
<tr>
<td>T8 (gRNA8)</td>
<td>Reverse aaccACGTCAGACTCGTGAGTT</td>
</tr>
</tbody>
</table>

The capital letters are guide RNA sequences, and the lower case letters are sticky ends for cloning.

Both rcDNA and replication intermediates, as compared with that of the gRNA/Cas9 TGF control. However, the gRNA/Cas9 T1 and EcoRI cleavage products of HBV DNA were not detected due to the low amount of cellular HBV DNA accumulated. Taken together, these results are consistent with those of Fig. 2, which indicated that the gRNA/Cas9 systems were able to cleave the HBV templates and led to their clearance.

HBV-specific gRNA/Cas9 systems inhibit the replication of HBV of different genotypes

The gRNAs were designed according to the conserved regions of different HBV genotypes. It is reasonable to speculate that the CRISPR/Cas9 system inhibits not only the replication of HBV genotype D that we investigated above, but also other genotypes. In order to confirm this hypothesis, we examined the antiviral effects of gRNA/Cas9 T1, T7 or Tmix on HBV genotypes A, B and C. At 2 days after co-transfection of HepG2 cells with the corresponding plasmids, the relative levels of HBV E antigen (HBeAg) in culture supernatants were measured by ELISA (Fig. 4a) and the relative levels of viral RNA transcripts were determined by Northern blotting (Fig. 4b). As shown in Fig. 4, gRNA/Cas9 T1, T7 and...

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Fig. 2. Anti-HBV effects of the gRNA/Cas9 system in HepG2 cells. HepG2 cells were co-transfected with HBV-specific gRNA/Cas9 expression plasmids and pHBV1.3. (a) At 48 h post-transfection, the expression levels of HBV transcripts were measured by Northern blotting. (b, c) At 96 h post-transfection, intracellular HBV replication intermediates (b) and extracellular virion DNA (c) were extracted and expression levels measured by qPCR. (d) Potential cytotoxicity of the CRISPR/Cas9 systems was determined by CCK8 at 48 h after transfection. The gRNA targeting GFP (TGF) served as irrelevant control. Tmix represents the mixture of eight different HBV gRNAs. In each of the transfections, β-galactosidase-expressing plasmids were included to normalize the transfection efficiencies. The results of the CCK8 assay and qPCR were calculated from three independent experiments and data are presented as mean ± SEM.

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Tmix could inhibit HBeAg expression and viral RNA transcript levels of HBV with genotypes A, B and C to varying degrees. Moreover, gRNA/Cas9 Tmix showed the most stable suppression efficiency on all of the three HBV genotypes as well as HBV genotype D.

### Inhibition of HBV in vivo by the gRNA/Cas9 system induces clearance and error-prone NHEJ of viral DNA

To demonstrate the anti-HBV efficiency of HBV-specific gRNA/Cas9 systems in vivo, pHBV1.3 (genotype D) together with gRNA/Cas9 T1, T7 or Tmix were injected into BALB/c mice through hydrodynamic injection (HDI), which was established as a method of inducing the mouse infection model. At 4 days post-HDI, mice sera were harvested to quantify the serum HBV DNA equivalents through qPCR (Fig. 5a) and the serum HBV S protein antigen (HBsAg) (Fig. 5b) and HBeAg (Fig. 5c) by ELISA. Compared with the gRNA/Cas9 T GFP control, serum HBV DNA was decreased strikingly after administration of T1, T7 or Tmix (Fig. 5a). The S ORF-targeted gRNA/Cas9 T1 (Fig. 1b) could effectively suppress the expression of HBsAg, but not HBeAg (Fig. 5b, c). In contrast, gRNA/Cas9 T7 was designed to target the 3’ end of precore ORF (Fig. 1b). Although the precore ORF encoded both the HBV core protein and E protein, the C terminus of the E protein was 34 aa shorter than the core protein. After cleavage by gRNA/Cas9 T7, part of the coding region of E protein was preserved. Therefore, gRNA/Cas9 T7 could inhibit the expression of HBeAg modestly (Fig. 5c) and effectively suppress the expression of core protein in mice liver (Fig. 5d), but not that of HBsAg (Fig. 5b). Moreover, gRNA/Cas9 Tmix could significantly inhibit the secretion of both HBsAg and HBeAg, and the expression of HBV core protein antigen (HBcAg) (Fig. 5b–d). The differences amongst the repression effects of gRNA/Cas9 T1 and T7 on the expression of HBsAg, HBeAg and HBcAg might be explained by the specific site-targeting effect of the

### Table 2. On-target mutations in the re-extracted pHBV1.3 plasmids

<table>
<thead>
<tr>
<th>CRISPR/Cas9 system</th>
<th>Amplification primer</th>
<th>Transfection time (h)</th>
<th>Mutation frequency (mutated/WT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T GFP</td>
<td>HBV1.3-T1</td>
<td>48</td>
<td>0/46</td>
</tr>
<tr>
<td>T1</td>
<td>HBV1.3-T1</td>
<td>48</td>
<td>0/46</td>
</tr>
<tr>
<td>T7</td>
<td>HBV1.3-T7</td>
<td>48</td>
<td>0/46</td>
</tr>
<tr>
<td>T mix</td>
<td>HBV1.3-Tmix</td>
<td>48</td>
<td>0/46</td>
</tr>
</tbody>
</table>

Fig. 3. Clearance and cleavage of the HBV templates by HBV-specific gRNA/Cas9 systems. (a) Schematic diagram of the cleavage sites of endonuclease HindIII and T1 on the pHBV1.3 sequence (upper panel) and EcoRI and T1 on the HBV DNA (lower panel). (b) Cleavage of pHBV1.3 by the indicated gRNA/Cas9 systems. The transfected plasmids were re-extracted at 6, 9, 12 and 24 h post co-transfection, and then detected by Southern blotting. (c) Clearance of pHBV1.3 by gRNA/Cas9 systems. HepG2 cells were co-transfected with pHBV1.3 plasmids and the indicated gRNA/Cas9 systems. At 48 h post-transfection, the plasmids were re-extracted from transfected cells by Hirt extraction and the remaining pHBV1.3 plasmids were determined by Southern blotting with a HBV-specific probe. (d) Repression of HBV DNA accumulation by gRNA/Cas9 T1. HBV DNA was extracted at 48, 72 and 96 h post-transfection, and then subjected to Southern blotting. RC, rcDNA; RI, replication intermediates.
gRNA/Cas9 system. Interestingly, the intrahepatic HBV RNA transcripts were obviously decreased by $T_1$ or $T_7$ and almost eliminated by $T_{mix}$ as compared with control $T_{GFP}$ (Fig. 5e). This result was consistent with phenomena observed in vitro (Fig. 2), which might be explained by the difference between partial NHEJ after single cleavage and clearance post multiple cleavages of viral templates (Fig. 3).

To further illustrate the hypothesis above, the mutagenic efficiencies of $T_1$, $T_7$ or $T_{mix}$ were determined in the liver of the HDI mouse model. As shown in Fig. 6(a), the T7 endonuclease 1 (T7E1) assay was used to characterize the efficiencies of target-specific mutagenesis of DNA extracted from mice livers which had been treated by $T_1$, $T_7$ or $T_{mix}$. In the $T_1$- and $T_7$-treated mice, $\sim 11.0 \%$ of amplified HBV sequences contained mutations, whilst mutations in $T_{mix}$-treated mice were undetectable (Fig. 6a). Sequencing of HBV DNA amplified from hepatic extracts confirmed that the deletions within the target sites were the predominant mutations, and the mutation rate in $T_1$- and $T_7$-treated mice was 9.7 and 11.3 $\%$, respectively, which is in accordance with data from analysis of the T7E1 assay. Although the mutation rate of the HBV genome is modest, the decrease of HBV DNA is quite remarkable in vivo (Fig. 5c). This inconsistency might be attributed to the fact that the fragments of HBV template cleaved by the gRNA/Cas9 system were not all repaired by NHEJ and most of them were cleared by undefined mechanisms.

In conclusion, these results demonstrated that HBV-specific gRNA/Cas9 systems could effectively suppress HBV replication in mouse liver by inducing clearance and error-prone NHEJ of viral DNA templates.

**DISCUSSION**

HBV is likely to mutate at an estimated rate of $1 \times 10^{-5}$ to $3 \times 10^{-5}$ nucleotide substitutions per site per year during reverse transcription, which increases the chance of escaping traditional antiviral drug treatment (Chan, 2011). Due to the high heterogeneity of the HBV genome, HBV was defined as different genotypes or subgenotypes with a sequence divergence $> 8 \%$ or $>4 \%$, respectively. During the preparation of our manuscript, several papers related to harnessing HBV with CRISPR/Cas9 systems were published (Kennedy et al., 2014; Lin et al., 2014; Seeger & Sohn, 2014; Zhen et al., 2015). However, the high heterogeneity of the HBV genome was not considered during the selection of their gRNA targets. This might be a major obstacle to applying their gRNAs to individuals in clinical research, because HBV exists in a quasispecies status for different patients, which shows the high complexity of the viral genomes (Li et al., 2015). Therefore, the HBV-specific gRNA/Cas9 systems were designed to target the relatively conserved regions of 26 different HBV genotypes in our study (Fig. 1). This might be a benefit because: (1) one gRNA/Cas9 could target the viral template of different HBV genotypes, e.g. the gRNA/Cas9 systems could inhibit the replication of HBV genotypes A, B, C and D (Figs 2 and 4); (2) we could avoid the missing targets of the gRNA/Cas9 systems caused by mutations during the replication of HBVs because the relatively conserved regions also imply low variable regions of the viral template; and (3) we could increase the inhibitory efficiency of the gRNA/Cas9 systems due to the importance of the conserved regions after natural selection during viral evolution. In addition, the application of multiple gRNA/Cas9 systems, such as $T_{mix}$ could further avoid HBV escaping from the inhibition of a single gRNA/Cas9 system and enhance the inhibition efficiency. In this study, $T_{mix}$ was indeed able to significantly inhibit the replication of HBV of all the genotypes that we used (Figs 2 and 4). Thus, the strategy of simultaneously targeting the conserved regions of HBV using multiple gRNAs is more effective in inactivating HBV replication and can prevent the escape from treatment brought about by viral mutation, which is common for nucleoside analogue therapies.

Although the mutation rate of HBV-specific gRNA/Cas9 systems in HepG2 cells was too low to be detected (Table 2), the T7E1 assay and sequencing showed that the gRNA/Cas9 $T_1$
or T7 systems could introduce ~11% mutagenesis in vivo (Fig. 6). By re-extracting the transfected plasmids from the transfected cells at different time points, we demonstrated that the HBV templates were cleaved and partially cleared by the treatment with T1 or T7 and almost eliminated by the treatment with Tmix (Fig. 3). However, the mutation and clearing rates of viral templates do not correspond with the suppression effect on HBV replication (Figs 2 and 5). In fact, this discrepancy has also been observed in several other papers regarding targeting HBV with ZFNs, TALENs and CRISPR (Bloom et al., 2013; Lin et al., 2014; Weber et al., 2014). However, this interesting phenomenon was not discussed in previous studies. Usually, the DSBs caused by cleavage of gRNA/Cas9 should be repaired through the NHEJ pathway. However, due to the abundant HBV templates in a single cell, the cellular NHEJ machinery might not be able to repair most of them in time. Thus, the fragments of HBV templates generated by multiple gRNA/Cas9 Tmix system cleavage might be degraded by cells, which will eliminate the viral templates and subsequently inactivate HBV RNA transcription (Figs 2a, 3c, 4b and 5e). In contrast, the partial clearing and mutagenesis of viral templates caused by a single gRNA/Cas9 system (such as T1 or T7) (Fig. 6) will influence both HBV RNA transcription and the function of transcripts (Figs 2a, 4b and 5e), which finally inhibit HBV replication (Figs 2b, c and 5a).

As the current antiviral therapies for HBV infection cannot cure the virus due to the stable viral cccDNA, researchers are looking for new strategies to resolve this problem. Directly targeting the HBV genome DNA via endonucleases is a potential strategy. At present, there are three well-known genome-editing technologies, i.e. ZFNs, TALENs and CRISPR/Cas9...

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**Fig. 5.** Anti-HBV effects of the gRNA/Cas9 systems in vivo. Four groups of mice, each comprising seven animals, were subjected to HDI with solutions containing pHBV1.3, pSV-β-gal and HBV-specific CRISPR/Cas9 system T1, T7 or Tmix. Mice sera and livers were harvested on day 4 following HDI. (a–c) Levels of HBV DNA (a), and HBsAg (b) and HBeAg (c) in the sera were measured by ELISA or qPCR. (d) Immunohistochemical detection of HBcAg in the livers of mice subjected to HDI; ×100 and ×400 fields are shown. (e) Analysis of HBV transcripts in mice livers by Northern blotting. Data were calculated from seven independent mice per item and presented as mean ± SEM.
systems. Although targeting the HBV genome by these genome-editing technologies has been reported previously (Bloom et al., 2013; Kennedy et al., 2014; Lin et al., 2014; Seeger & Sohn, 2014; Weber et al., 2014; Zhen et al., 2015), here we would like to propose a way to inhibit HBV with different genotypes by using the same gRNA/Cas9 system, and explore the possibility and efficiency of multiple gRNAs/Cas9 systems. With this study, we provide the possibility to inhibit viral replication and clear the cccDNA of HBV of different genotypes, which might be a functional anti-HBV therapy to avoid the mutations during viral replication.

**METHODS**

**Plasmids.** The human codon-optimized SpCas9 and chimeric gRNA expression plasmid pX330 were obtained from Addgene (plasmid 42230). The HBV (genotype D: GenBank accession number V01460.1) replication-competent plasmid pHBV1.3 was a generous gift of Dr Guangxia Gao (Institution of Biophysics, Chinese Academy of Sciences) (Liu et al., 2014a). pAAV/HBV1.2 (genotype A: GenBank accession number AF305422.1) was a generous gift of Dr Pei-jer Chen (National Taiwan University) (Huang et al., 2006). The HBV replicons (genotype B: GenBank accession number AY305422.1; genotype C: GenBank accession number FJ895793.1) were generous gifts of Dr Ying Zhu (Wuhan University). pSV-β-gal plasmid was obtained from Promega.

Fig. 6. Mutagenic effects of CRISPR/Cas9 systems in the mouse liver. (a) Total DNA was extracted from the livers of mice subjected to HDI. Mutagenic efficiencies of T₁ (left panel) or T₇ (right panel) were analysed by T7E1 assay. Intensities of bands were determined by Quantity One and mutagenesis ratios are shown at the bottom of each lane. Each lane represents data obtained from an individual animal. ND, Not detected. (b, c) Sequencing of T₁ (b) and T₇ (c) target regions following HDI. Amplified DNA from each of the groups of seven mice was pooled and subjected to deep sequencing. Sequences targeted by CRISPR/Cas9 systems are shown in blue and the PAMs are shown in red. Red arrowheads denote predicted Cas9 cutting sites. The dashed lines denote deletions; the bases highlighted in yellow and green denote an insertion and a mutation, respectively. Commonly detected sequences are shown together with the number of sequence reads indicated on the right.
Design and cloning of HBV-specific gRNA/Cas9 plasmids. The candidate 20-base gRNA sequences were derived from the HBV genome sequence of pHBV1.3 (genotype D). We used the CRISPR/Cas9 design tool of the Feng Zhang Laboratory (crispr.mit.edu) to search the functional gRNA sequences, which contained the downstream 3' PAM with GG dinucleotides (N20-NGG). The 5' 20-base nucleotides were inserted to pX330 and named pX330-U6-HBVgRNA (Tn for short). The Tn mix was mixture of eight pX330-U6-HBVgRNA plasmids in equal amount. The 20-base gRNA of T GFP was derived from the EGFP gene sequence.

Cell culture and transfection. Human hepatoma cell-derived HepG2 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 U penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹ at 37 °C and 5% CO₂. HepG2 cells were seeded in 24-well plates 24 h prior to transfection and transfected using Neonect (Neofect Biotech) according to the manufacturer’s instructions. The pX330-U6-HBVgRNA/HBV-expressing plasmid/pSV-β-gal plasmid ratio was 8 : 1 : 1.

Cell viability assay. HepG2 cells were seeded into 96-well plates on the day prior to transfection. Groups of replicates included cells that were mock transfected (10 ng pHBV1.3, 10 ng pSV-β-gal, 80 ng pX330-U6-GFPgRNA) or transfected with the CRISPR/Cas9 systems (10 ng pHBV1.3, 10 ng pSV-β-gal, 80 ng pX330-U6-HBVgRNA). Cell viabilities were determined using a CCK8 kit according to the manufacturer's instructions (Dojindo).

Analysis of secreted HBV antigens. At the indicated time points, cell culture supernatants or mice sera were collected to detect the levels of HBsAg and HBeAg by a commercial ELISA kit (Kehua Bioengineering). All values were normalized against β-galactosidase activities in the cell lysates measured by a Beta-Glo kit (Promega).

HBV nucleic acids analysis. HepG2 cells were co-transfected with pX330-U6-HBVgRNAs (400 ng each for pHBV1.3 (50 ng) and pSV-β-gal plasmids (50 ng). At 48 h post-transfection, the cellular RNA was extracted and analysed by Northern blotting as described previously (Hao et al., 2015). At 96 h post-transfection, the viral replication intermediate DNA (Cao et al., 2014) and extracellular virion RNA (Tian et al., 2011) were extracted as described previously. The intracellular replication intermediates or extracellular virion DNA were then analysed by qPCR; the primers (2RC/CCS and 2RC/CCAS) were adopted from a previously published paper (Werle-Lapostolle et al., 2004).

Recovery of transfected DNA and Southern blotting. This assay was conducted according to a protocol described previously (Wilson & Patient, 1991). At the indicated time points, the transfected cells were lysed by adding 250 μl Hirt lysis buffer (0.6% SDS/10 mM EDTA) per well and incubated at room temperature (15–25 °C) for 15 min. Then, the genomic DNA and cellular debris were precipitated by adding 0.1 ml 5 M NaCl (incubated overnight) and centrifugation at 4 °C, and the supernatants were transferred to fresh tubes. After phenol/chloroform extraction, ethanol precipitation and washing, the DNA pellets containing the recovered plasmid DNA were resuspended in 20 μl Tris/EDTA buffer. The extracted DNA was then digested with HindIII (Thermo Scientific) and analysed by Southern blotting as described previously (Hao et al., 2015).

Hydrodynamics-based transfection in mice. For the in vivo experiments, 5-week-old male BALB/c mice were used and separated in to four groups (seven mice each). pHBV1.3 (5 μg) and pSV-β-gal (5 μg) were injected into the tail vein of mice together with pX330-U6-HBVgRNA plasmids (Tn, T7 and Tn mix) or pX330-U6-GFPgRNA plasmid (T GFP) (25 μg) within 8 s in a volume of saline equivalent to 10% of the mouse body weight. Animals were sacrificed 4 days after HDL. Sera were taken for analysis of HBsAg, HBeAg and HBV DNA. For Northern blotting analysis, a piece of liver tissue was homogenized in 1 ml TRIZol reagent (Life Technologies) and total RNA was isolated following the manufacturer’s protocol. Total RNA (5 μg) of each mouse was subjected to Northern blotting analysis as described above. All mice were housed in a pathogen-free mouse colony and the animal experiments were performed according to the 1998 Guide for the Care and Use of Medical Laboratory Animals (Ministry of Health, China). Core antigen expression in mice livers was analysed with immunohistochemical staining as described previously (Hao et al., 2015).

T7E1 assay. To confirm that cleavage and targeted sequence disruption occurred at the intended target site for the constructed CRISPR/Cas9 systems, we used a mismatch-sensitive T7E1 (New England Biolabs). Genomic DNA was prepared from mouse liver tissues with a TIANcomb DNA Lys&Det PCR kit (Tiangen) following the manufacturer’s instructions. Regions spanning the T1 and T7 target sequences were amplified using KOD-Plus-Neo DNA polymerase (Toyobo) with the primer sets HBV (1–24) F/HBV (794–818) R and HBV (2027–2051) F/HBV (346–370) R. The PCR products (200 ng for each sample) were subjected to heteroduplex formation after annealing and treated with 10 U T7E1 in 1 × NEB Buffer 2 (New England Biolabs). After incubation at 37 °C for 30 min, the products were resolved electrophoretically. Image software (Quantity One; Bio-Rad) was used to measure band intensities and cleavage efficiencies (% indels) were calculated as previously described (Hsu et al., 2013).

Sequencing

Deep sequencing. Target loci were amplified by the specific primer sets T1 F/T1 R and T7 F/T7 R as shown in Table S2. Before being sequenced on an illumina HiSeq 2500 platform, the PCR products were purified, end-repaired and connected with sequencing primer. For the sequences gained by sequencing, low-quality and joint pollution data were removed to obtain reliable target sequences (clean reads) for subsequent analysis. The corresponding Read1 and Read2 (sequences gained from the 5' and 3' ends) were respectively spliced. ClustalX2 software was then used for sequence alignment.

Sanger sequencing. Target and off-target loci were amplified by the specific primer sets HBV1.3-T1 F/HBV1.3-T1 R, HBV1.3-T7 F/HBV1.3-T7 R, T1-off-target 1 F/T1-off-target 1 R, T1-off-target 2 F/T1-off-target 2 R, T7-off-target 1 F/T7-off-target 1 R and T7-off-target 2 F/T7-off-target 2 R as shown in Table S2. The PCR products were purified and cloned into pLB vector (Tiangen) following the manufacturer’s instructions. For each sample, 50 clonal amplicons were sequenced and analysed.

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