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CRISPR/Cas9 produces anti-hepatitis B virus effect in hepatoma cells and transgenic mouse

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ABSTRACT

Chronic infection of hepatitis B virus (HBV) is at risk of liver cirrhosis and hepatocellular carcinoma and remains one of the major public health problems worldwide. It is a major barrier of persistence HBV cccDNA under current antiviral therapy as novel strategies of disrupting HBV cccDNA is pressing. The (CRISPR)/Cas9 system is presently emerging in gene editing and we also apply it for targeting and deleting the conserved regions of HBV genome. Two homologous sequences of HBV S and X genes were carried with CRISPR/Cas9 endonuclease to build pCas9 constructs, which may mediate anti-HBV effects of *in vitro* and *in vivo* systems in this study. The results showed the better anti-HBV productions by pCas9-2 and without significant differences in between Huh7 and HepG2 cells. CRISPR/Cas9 direct cleavage and mutagenesis were further analyzed of *in vitro* system. In the M-TgHBV mouse model of HBV, injection of pCas9 constructs by hydrodynamics decreased HBsAg of sera and liver HBcAg. In conclusion, this designed CRISPR/Cas9 system can induce anti-HBV effects and potentially consider as a novel therapeutic agent against chronic HBV infection.

1. Introduction

Hepatitis B virus (HBV) infection remains one of the major public health problems worldwide. Although prophylactic vaccines are available for many years, there are approximately 2 billion people to be infected, and more than 350 million being chronic carriers of HBV per year (Trepo et al., 2014). Current antiviral agents such as nucleos(t)ide analogues (NAs) or interferon (IFN) could not eliminate HBV, because covalently closed circular DNA (cccDNA) of HBV localizes persistently in the nucleus of hepatocytes (Dandri et al., 2000; Belloni et al., 2012). It is obvious that NAs show little or no effect on the removing of HBV cccDNA, and the staggering stability of HBV cccDNA provides a major barrier to eradicate virus (Sung et al., 2005). Hence, it needs so life-long treatment to cure chronic hepatitis B (CHB) (Werle-Lapostolle et al., 2004). In addition, High-dose INF-α can degrade HBV cccDNA with limited effects in clinic, but the side-effect of high doses INF-α are intolerance by most patients (Belloni et al., 2012; Lucifora et al., 2014). Therefore, it is important to find novel therapeutic options of eradicating HBV cccDNA to reach the cure of CHB clinically.

It is potential to eliminate HBV cccDNA by curative therapy strategies such as targeted endonucleases. These nucleases can introduce double strand breaks (DSBs) into viral DNA by nonhomologous end joining (NHEJ) pattern, enough to mutate HBV genes and inhibit its functions (Gaj et al., 2013). To this point, two targeted nucleases including zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), can damage the HBV genome in a site-specific fashion (Cradick et al., 2010; Bloom et al., 2013), which are composed of sequence-specific DNA-binding modules and nonspecific DNA cleavage domain (Gaj et al., 2013). Recently, Type II bacterial clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR – associated (Cas) system from *Streptococcus pyogenes* is a sequence-specific DNA nuclease by RNA-guided for multiplex genome editing (Cho et al., 2013; Cong et al., 2013). The delivery of single-guide RNAs (sgRNAs) can provide a powerful tool to edit DNA sequences, also in application for eliminating HBV cccDNA of our assumption.

HBV cccDNA is well considered as the template for HBV transcription of pregenomic RNA (pgRNA) and subgenomic RNAs (Quasdorff and Protzer, 2010). There are four open reading frames (ORFs) including precore/core, polymerase, preS1/S2/S and X genes in an overlapping manner of HBV genome, with four distinct

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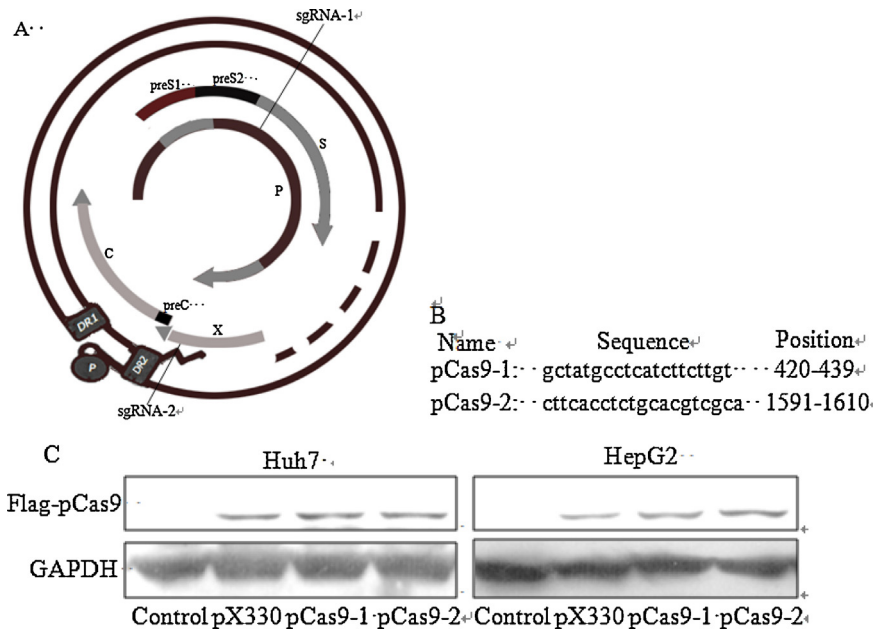

Fig. 1. CRISPR/Cas9 system was designed for targeting the HBV genomes. (A) Physical map of HBV genome and the targeting HBV sites of sgRNA. (B) The targeting HBV sequence of designed pCas9 constructs. (C) The expression of designed pCas9 constructs in transfected Huh7 and HepG2 cells.

promoters regulating independently (Yang and Kao, 2014). So far, we have selected S and X genes as well as their only homologous sequences from genotype A to H of HBV to be targeted. Based on these characterizations, we have designed two another specific and conserved sgRNAs which is distinct from the previous publication (Liu et al., 2015) correspondingly to carry endonuclease from CRISPR/Cas9 system, and investigated their anti-HBV effects of *in vitro* and *in vivo* systems in this study.

2. Materials and methods

2.1. Plasmids and reagent

HBV1.3 (GenBank: U95551.1, genotype D, serotype ayw) of AAV was purchased from Beijing Five Plus Molecular Medicine Institute (Beijing, China), and then was cloned into pcDNA3.1 (+) plasmid as previously described which is named for pcHBV1.3 (Christman et al., 1982). The human codon-optimized Cas9(hCas9) −pX330 expression vectors were purchased from Addgene (Cambridge, MA, USA) plasmid (Cong et al., 2013). We designed two another conserved sequences that is different from the similar publication (Liu et al., 2015) including 20 nucleotides of HBV S and X gene among different genotypes (Supplementary data1), which can be targeted for the potential sgRNA sites followed by a 5′-NGG or 5′-NAG PAM motifs using online software on http://crispr.mit.edu website. LPS (O55:B5) of *E. coli* was purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2. Cell culture and transfection experiments

The human hepatoma cell lines Huh7 and HepG2 were originated from the Type Culture Collection of the Chinese Academy of Sciences, and cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (Life Technologies, California, USA) and antibiotics at 37 °C in an incubator with 5% CO_2/air. Huh7 and HepG2 cells were co-transfected with pcHBV1.3 and pCas9 constructs at ratio of 1:1 for 48 h on each place using Lipofectamine 3000 (Life Technologies, California, USA) in the light of the manufacturer's instructions.

2.3. Deliver pCas9 by hydrodynamics into M-TgHBV mice

All experimental protocols were consented to this study by the Tongji University Ethics and Uses Committee of Laboratory Animals in research. Two pairs of M-TgHBV mice at about 40 days were generated by routine microinjection of linearized HBV genome from the cutting site at 1806 nt (GenBank AF461363.1, genotype C, serotype adr) DNA into fertilized eggs chromosome 9 in the second exon (gene AI604832) of C57BL/6J mice, which were purchased from Shanghai Research Center for Model Organism and reproduced under specific pathogen-free facilities at six- to seven-week old (Ren et al., 2006). Dilutions of 10 μg pX330 or pCas9 constructs were injected by hydrodynamics into the tail vein of mouse one by one, and mice were sacrificed post-injection 10 days. Sera were collected to detect HBsAg by ELISA, and liver samples were prepared to detect HBcAg.

2.4. Western blot

To examine pCas9 (only in hepatoma cells) and HBcAg (both in hepatoma cells and livers of M-TgHBV mice) expressions, total protein were extracted using RIPA kits (Biosynthesis Biotechnology Co., LTD, Beijing, China) from Huh7 and HepG2 cells after transfection with pCas9 constructs or pX330 vector for 48 h as well as liver samples of M-TgHBV mice post-injection 10 days by tail vein with pCas9 constructs or pX330 vector. Proteins were separated on 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and transferred onto the polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). After blocking, the membrane was incubated with a dilution (1:1000) of primary mouse antibody anti-Flag (Abcam, Cambridge, UK), anti-HBcAg (Santa Cruz, Dallas, USA) at 4 °C overnight. After washing, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary anti-mouse antibodies (1:5000; Bioworld Technology Co., Minion, USA) for 1 h at room temperature. The band of membrane was scanned and quantified by VersaDoc imaging system (Bio-Rad, California, USA) after ECL treatment, which was normalized to GAPDH (Santa Cruz, Dallas, USA).

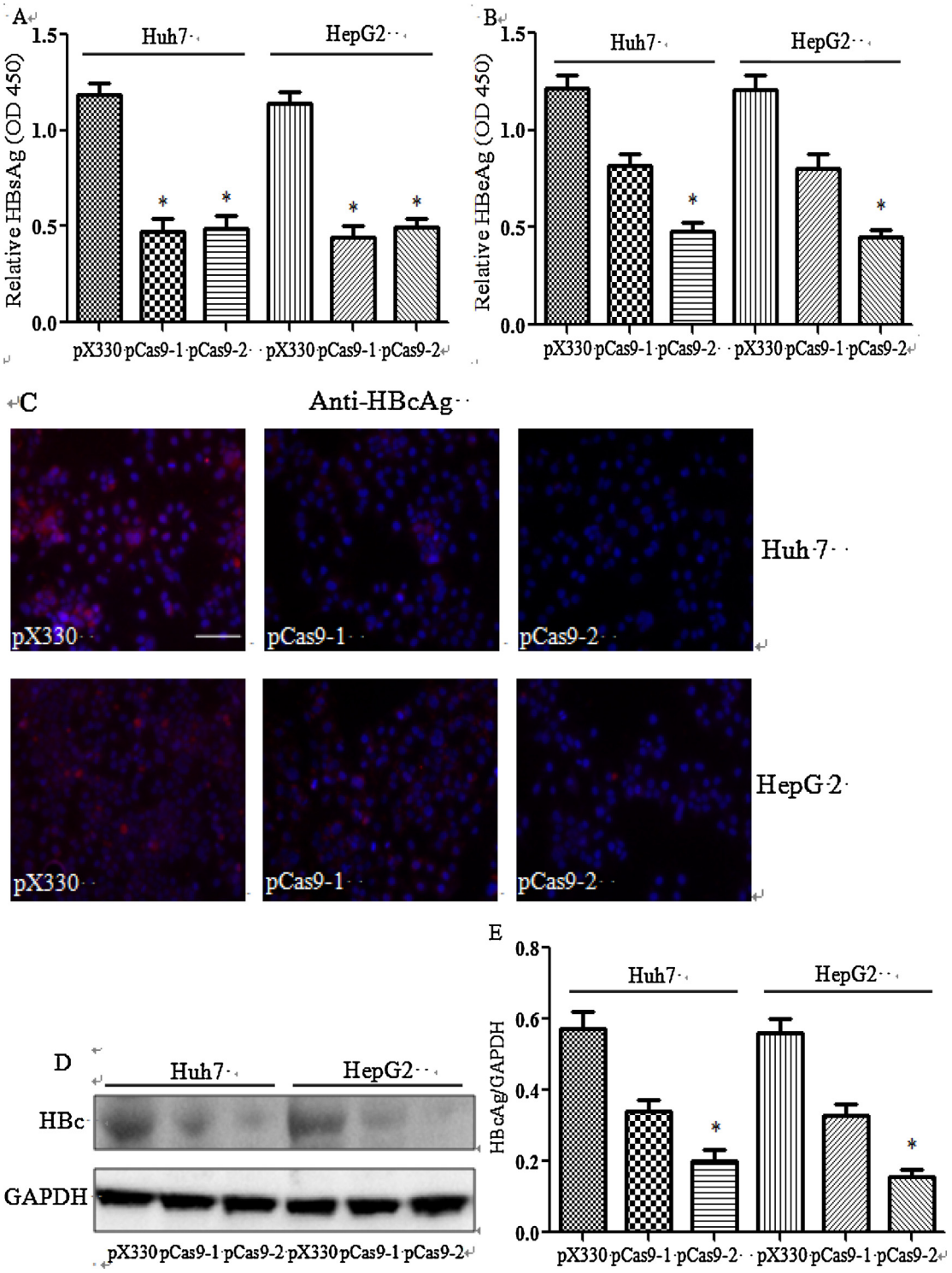

Fig. 2. Induction of pCas9 to reduce HBV productions. (A) and (B) The levels of HBsAg and HBeAg in supernatants of Huh7 and HepG2 cells at 48 h after transfection were estimated by ELISA. (C) IF analysis with HBcAg-specific antibody was determined after transfection in Huh7 and HepG2 cells for 48 h. DAPI was blue and HBcAg was red. Scale bar = 100 μm. (D) and (E) HBcAg expression of Huh7 and HepG2 cells at 48 h after transfection were analyzed by Western blot. The data were showed as means ± SDM. *$p < 0.05$ significant difference from the control of pX330. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.5. Enzyme-linked immunosorbent assay (ELISA)

The cell cytotoxicitys of Huh7 and HepG2 after transfection with pCas9 or pX330 for 48 h were analyzed by LDH ELISA kit (Jianglai, Shanghai, China) for measuring supernanant LDH level. Treatment of 200 ng/mL LPS on Huh7 and HepG2 for 48 h was considered as the positive control. All values (cytotoxicity%) were calculated by the positive control value as the protocol described.

The supernatant levels of HBsAg and HBeAg in Huh7 and HepG2 cells 48 h post-transfection with pcHBV1.3 and pCas9 (pX330 control) constructs as well as sera HBsAg of M-TgHBV mice by hydrodynamics for 10 days were determined using the

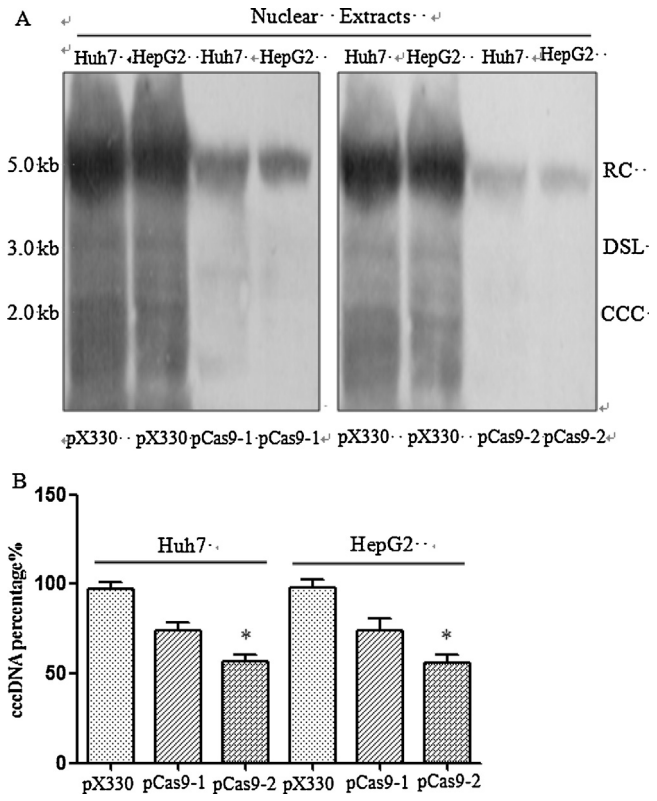

Fig. 3. Interruption of HBV cccDNA by pCas9. (A) The levels of HBV cccDNA and replicate intermediates (RC: relaxed circular, DSL: double-stranded linear) in nuclear extracts of Huh7 and HepG2 cells after transfection for 48 h were estimated by Southern blot. (B) The HBV cccDNA levels of nuclear extracts of Huh7 and HepG2 cells after transfection for 48 h were determined by real-time PCR. The data were showed as means ± SDM. $^{*}p < 0.05$ significant difference from the control of pX330.

commercial ELISA kit (Kehua Biotech, Shanghai, China) according to the instructions. In addition, sera IFN-γ, IL-1β and TNF-α of M-TgHBV mice post- hydrodynamics 12 h by pX330, pCas9 constructs and LPS (1 mg/kg body weight) were examined using ELISA kit (**Jianglai, Shanghai, China**) manually.

2.6. Hybridization of HBV DNA

The virion RC DNA is delivered into the nucleus after entry into the host cells, and then converted directly to cccDNA. In addition, all hepadnaviruses generate the double-stranded linear (DSL) DNA lightly, which can be converted to cccDNA in duck HBV and woodchuck hepatitis virus, but this conversion is currently imprecise (Gao and Hu, 2007; Yang and Summers, 1995; Yang and Summers, 1998). Hence, we used southern blot method to describe HBV replication intermediates including DSL DNA and cccDNA as well as RC DNA which can be interrupted by pCas9 constructs of *in intro* system (Dong et al., 2015). Each plate (5×10^5 cells) of Huh7 and HepG2 cells after 48 h co-transfection with pcHBV1.3 and pCas9 constructs were harvested and lysed in buffer including 50 mM Tris-HCl, 1 mM EDTA, 1% NP-40, and 1 × protease inhibitor cocktail (Sigma, MO, USA). Pelleted nuclei after brief centrifugation at 10,000*g* for 5 min were resuspended in SDS lysis buffer of 10 mM Tris-HCl, 10 mM EDTA, 150 mM NaCl, 0.5 mg/mL proteinase K (Life Technologies, California, USA) and 0.5% SDS, and then incubated at 37 °C overnight. The lysate was centrifuged at 12,000*g* for 15 min, and nucleic acids of supernatant was further extracted with phenol and chloroform (1:1), followed by ethanol precipitation. Total DNA were treated with DpnI (Beyotime, Haimen, China) at 37 °C for 1 h, and further digested with Plasmid-safe DNase I (Beyotime, Haimen, China). Viral DNA was separated by 1% agarose gel with blotting onto the nylon membrane, and then incubated with a DIG-labeled HBV probe of full-length genome according to DIG Probe Synthesis Kit (Roche, Basel, Switzerland). Results of Southern blot were analyzed by VersaDoc imaging system (Bio-Rad, California, USA) (Gao and Hu, 2007; Chen et al., 2014).

2.7. Quantification of HBV cccDNA

To qualify molecular basis of HBV persistence, cccDNA was examined by Real-time PCR also to support the southern blot results. Real-time PCR reaction was mixed in 20 μl system including 0.1 μg extracted DNA from the previous statement, 0.2 μM of forward and reverse primers, 12.5 μl Realtime PCR Master Mix (Toyobo, Tokyo, Japan), 0.2 μM of SYBR Green I probe (Life Technologies, California, USA) and added with sterile water. Primers for HBV cccDNA amplification are: forward (5′-CTCCCCGTCTGTGCCTTCT-3′), reverse (5′-GCCCCAAAGCCACCCAAG-3′). Amplifications were performed as follows: 95 °C for 10 min then 45 cycles of 95 °C for 10 s, 56 °C for 10 s, 10 s at 60 °C, 72 °C for 20 s. Serial dilutions of the pcHBV1.3 constructs were standardized as quantification (Chen et al., 2014; Pollicino et al., 2011).

2.8. T7endonuclease 1(T7E1) analysis

Huh7 and HepG2 cells were transfected with pcHBV1.3 and pCas9-2 constructs at 48 h. A pair of primers (forward: 5′- GTCTGCCGTTCCGACCGACC-3′; reverse: 5′-TGCATGGTGCTGGTGCGCAG-3′) was designed for pCas9-2 target site, and PCR productions (300 bp) were presented by the following: 95 °C for 3 min, 36 cycles of 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 30 s, and 72 °C for 5 min with the extracted HBV DNA templates, and melted to anneal and form heteroduplex DNA. The annealed DNA of T7E1 (New England BioLabs, New England, USA) treatment was separated by 1% agarose gel and visualized to sequence as previously described (Zhu et al., 2013).

2.9. Immunostaining

Huh7 and HepG2 cells were plated into coverslides of six-well plates (5×10^5 cells/well) and transfected with pcHBV1.3 and pCas9 constructs for 48 h. Cells on coverslides were fixed in 4% paraformaldehyde for 20 min, and permeabilized by the additional 0.1% Triton X-100, and then blocked in 5% skim milk of PBST. After incubation with anti-HBcAg mouse monoclonal antibody (Santa Cruz, Dallas, USA) of 1:100 dilution at 4 °C overnight, the cells were incubated with anti-mouse Alexa Fluor® 647 of secondary antibodies (Abcam, Cambridge, UK) at 1:500 dilution for 1 h, and restained with DAPI (Beyotime, Haimen, China) on nuclei for 5 min at room temperature. Fluorescence was analyzed with a metaconfocal microscope (Nikon, Tokyo, Japan).

Liver samples were gathered and frozen from mice sacrificed after hydrodynamic injection for 10 days. Frozen sections of liver were fixed and blocked similar to cell coverslides, and then incubated with anti-HBcAg primary antibody (1:100, Santa Cruz, Dallas, USA) and horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody (Beyotime, Haimen, China) and visualized.

2.10. Statistics

Data are presented as means ± the standard deviation (SD). One-way analysis of variance (ANOVA) in SPSS 17 (Microsoft, Redmond, WA) was used for statistical analysis as well as $P < 0.05$ was showed significant differences.

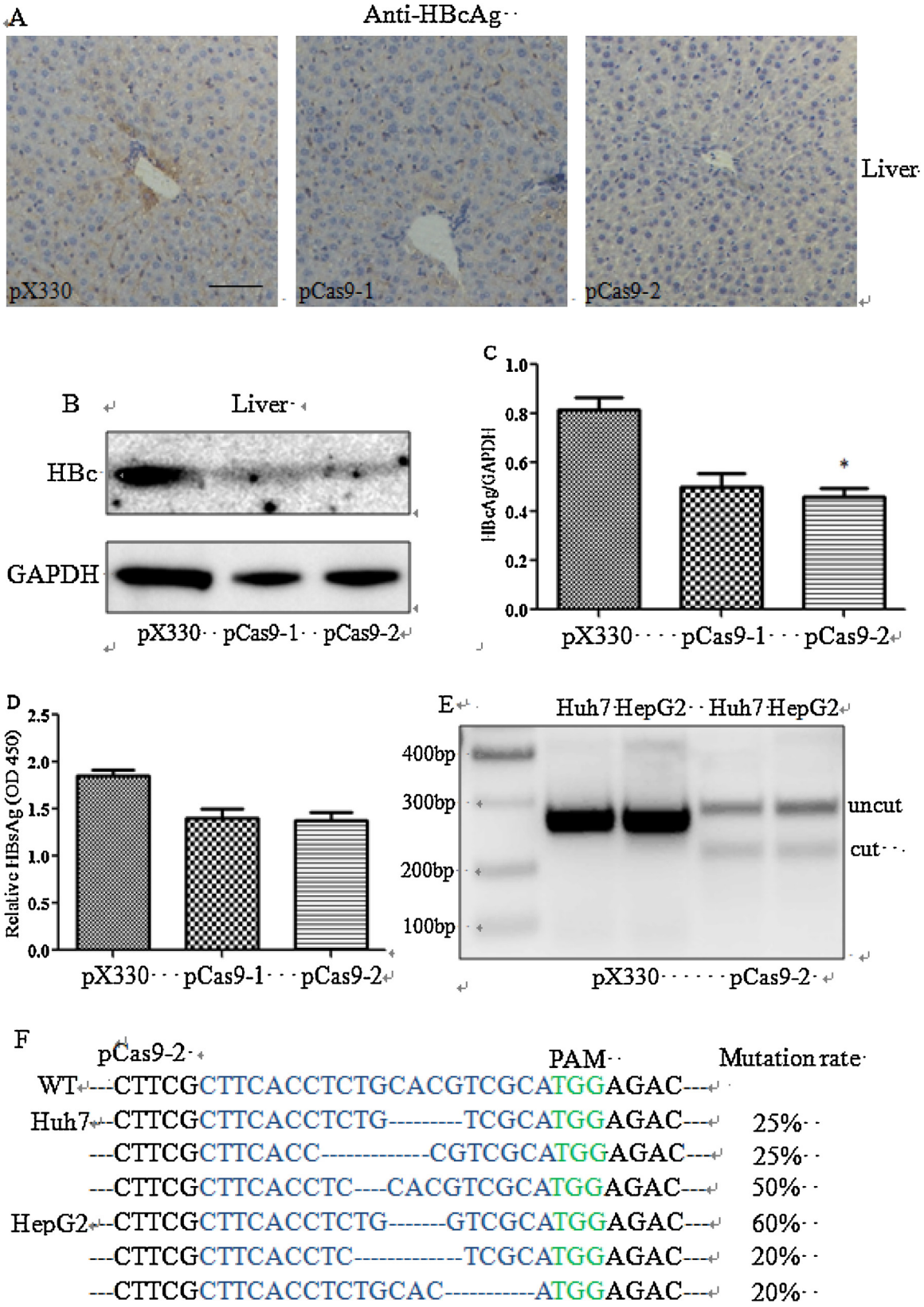

Fig. 4. Evaluation of the anti-HBV effect on M-TgHBV mice and mutagenesis by pCas9 constructs. (A) IHC analysis of HBcAg was detected after pCas9 delivery for 10 days in liver samples of M-TgHBV mice. Scale bar = 100 μm. (B)-(C) HBcAg expression of M-TgHBV mice after pCas9 delivery for 10 days were analyzed by Western blot. (D) Levels of serum HBsAg of M-TgHBV mice were determined post 10 days pCas9 delivery by ELISA. (E) Analysis of the pCas9-2-mediated cleavage by T7E1 assay in Huh7 and HepG2 cells. (F) Sequence analysis of pCas9-2 targeting HBV DNA from (E). Amplification of pCas9-2 target and deletions are showed in blue, as PAM sequence is colored in green. The data were showed as means ± SDM. $^{*}p < 0.05$ significant difference from the control of pX330. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Design for target sites of HBV genome

HBV is at least with eight genetically distinct genotypes from A to H, which contains >8% divergence among other genomes as well as exhibits diverse geographical and ethnic distributions (Schaefer, 2005). To potentially target multiple HBV isolates by sgRNA-Cas9, we identified another conserved sequences among usually different HBV genotypes from A to H (Supplementary data1) based on the available database of different HBV isolate sequences (Hayer et al., 2013). Two conserved sequences were identified according to computational ranking among HBV genotypes, which located in the open reading frame (ORF) S or X to overlap with ORF P as seen in Fig. 1A. The highly conserved sgRNA (20 bp) was designed on this basis and flanked by proto-spacer adjacent motifs (PAM) (3-bp NGG) on the 3′ end (Cho et al., 2013). The accurate targeting sites and sequences of HBV genome are described in Fig. 1A and B, which is also consistent to other genotype HBV genome as we selected in Supplementary data1. The FLAG-tagged Cas9 of the pX330 was expressed and determined by Western blot in Huh7 and HepG2 cells after indicated plasmids transfection for 48 h (Fig. 1C). The LDH assays results proofed that cell survival of cytotoxicity did not show significant differences between pX330 and pCas9 constructs groups, and both pX330 and pCas9 constructs groups decreased significantly compared to LPS treatment (positive control) of Huh7 and HepG2 cells (Supplementary data2).

3.2. Suppression of HBV production with Cas9/sgRNA combinations

The anti-HBV effect was estimated by the designed CRISPR/Cas system in hepatoma cells of Huh7 and HepG2. The pcHBV1.3 plasmid and sgRNA-Cas9 (pCas9) constructs were co-transfected into Huh7 and HepG2 cells, and cccDNA, replication intermediate, HBcAg, HBsAg, HBeAg secretions of HBV were measured. In addition, non-specific (N.S) sgRNA of px330 vector was also transduced into Huh7 and HepG2 cells with pcHBV1.3 plasmid as a negative control. The HBeAg concentration of culture supernatant decreased at 48 h post-transfection with sgRNA-Cas9 compared to those transfected with px330, and significantly reduced with pCas9-2 (Fig. 2B). In HBsAg assay, their supernatant concentration reduced significantly with sgRNA-Cas9 compared with positive control (Fig. 2A), indicating that the two pCas9 constructs displayed anti-HBV surface antigen effects. In the immunostaining detection, HBcAg was decreased by pCas9 of significant reduction by pCas9-2 compared to px330 treatment in Huh7 and HepG2 cells after transfection for 48 h (Fig. 2C).

HBV cccDNA, a template for viral and pregenomic messenger RNA, is hard to eliminate completely by Lamivudine or IFN-α (Belloni et al., 2012; Lai et al., 1997). The intracellular cccDNA levels from nuclear extracts of Huh7 and HepG2 cells were determined by real-time PCR with cccDNA quantitation primers. The pCas9 can decrease HBV cccDNA especially with significant reduction by pCas9-2 at 48 h after transfection compared to positive control (Fig. 3B). In the southern blot result (Fig. 3A), among cccDNA, relaxed circular (RC) and double-stranded linear (DSL) of HBV were damaged by pCas9 contrary to px330 treatment and with significance by pCas9-2 in nuclear extracts of Huh7 and HepG2 cells with pcHBV1.3 plasmid at 48 h post-transfection.

3.3. Cas9/sgRNA produce anti-HBV effect in transgenic mouse

In order to study anti-HBV effect of the designed pCas9 constructs, we have used M-TgHBV mouse as the *in vivo* system of expressing HBV production (Ren et al., 2006), and delivered pCas9 constructs by hydrodynamics. After 10 day of pCas9 injection, levels of serum HBsAg were decreased without significance by pCas9 constructs compared with px330 treatment in M-TgHBV mouse (Fig. 4B). In accordance with *in vitro* immunostaining, liver HBcAg of immunohistochemistry was decreased significantly by pCas9-2 compared with px330 treatment in M-TgHBV mouse at 10 days post-injection (Fig. 4A). To get rid of antiviral cytokines effects by expression of pCas-9, sera IFN-γ, IL-1β and TNF-α of M-TgHBV mice were determined, and sera cytokines of both pX330 and pCas9 constructs groups were less than LPS treatment (positive control) significantly as well as no significance exists in between pX330 and pCas9 constructs groups (Supplementary data3).

3.4. HBV mutational deactivation with Cas9/sgRNA constructs

As CRISPR/Cas-induced DSBs *via* non-homologous end-joining (NHEJ) (Cong et al., 2013), we have selected and assessed the factual mutations of HBV targeting sequences related to pCas9-2 according to its better anti-HBV effect. Amplified productions after T7E1 treatment was presented in Fig. 4C and various deletions were sequenced in Fig. 4D of Huh7 and HepG2 cells 48 h post-transfection with pcHBV1.3 and pCas9-2 constructs.

4. Discussion

The persistence of HBV cccDNA can reactivate after treatment withdrawal and drug resistance, although NAs and IFN play a key role in blocking the new HBV DNAs' formation (Dandri et al., 2000; Belloni et al., 2012). To eradicate HBV completely, genome editing technologies such as ZFNs, TALENs and CRISPR/Cas were used to target and edit persistent cccDNA. Meanwhile, The CRISPR/Cas system is better than ZFNs and TALENs because it is not necessary to produce customized proteins for binding specific sites (Sander and Joung, 2014). In this study, we have developed a CRISPR/Cas9 system to target with the HBV genomic DNA and decreased efficiently productions of viral genes, which can imply the potential antiviral therapy of CRISPR/Cas for cure of HBV infection.

It is important to select modest *in vitro* and *in vivo* system which can efficiently infect HBV, as well as the length of HBV plays a central role in this process. Yan et al. have reported that stable HepG2-hNTCP cells can infect 1.05 copies of HBV genome in 5–10% of the cells by HBsAg staining compared to parental HepG2 cells without NTCP transfection (Yan et al., 2012). However, there are some publications using HepG2 and Huh7 cells with 1.2 or 1.3 copies of HBV genome according to their practices and conveniences (Lin et al., 2014; Liu et al., 2015; Dong et al., 2015). In this study, we have delivered 1.3 copies of HBV genome into hepatoma cells and showed that no significant difference of anti-HBV effect by pCas9 constructs exists in between HepG2 and Huh7 hepatoma cells. In addition, primary human hepatocytes and stable HBV expression of hepatoma cells can provide several advantages contrary to HepG2 and Huh7 cells, but there are some general and common difficulties of our *in vitro* system of HBV.

There exist four ORFs (C, P, S, X shortly) by overlapping way of HBV genome for candidate (Yang and Kao, 2014), and we have selected two different target sites of HBV genome corresponding to ORF S, X overlapped with P, C respectively, which were located at 420–439, 591–1610 bp of HBV genotype D genome (Accession No. U95551.1) with high conservation on HBV genotype A-H genome. Many studies have previously demonstrated that HBx protein plays a central role in transcription of preC/C RNA according to episomal cccDNA templates (Belloni et al., 2009; Lucifora et al., 2011), and truncation of HBx may result in the reduction of Core protein (Seeger and Sohn, 2014). As well, the pre-S1 region of the LHBs protein is a critical determinant to bind cellular receptor and mediate

the entry of HBV on hepatocytes (Le et al., 2009; Sureau and Salisse, 2013). The CRISPR/Cas9 system is regarded as an originally bacterial adaptive immune system to defense against invading plasmids and phages, which can create DSBs at specific DNA locations to produce frameshifts and/or premature stop codons by NHEJ pathway, finally result in the formation of insertions and deletions of target gene templates (Gaj et al., 2013; Barrangou et al., 2007). Our results indicate that both of pCas9 can contribute to inhibit HBsAg secretion, and pCas9-2 of targeting X gene is better than pCas9-1 of targeting S gene to reduce HBV cccDNA, replication intermediate, HBcAg and HBeAg of both HepG2 and Huh7 cells. Dong et al. also suggest that pCas9 for X gene may provide better anti-HBV effect compared with pCas9 for C gene in Huh7 cells with HBV 1.3 (Dong et al., 2015). To determine the putative cleavage site, we have used T7E1 nuclease assay to study the pCas9-2 of targeting X gene in both HepG2 and Huh7 cells with HBV 1.3 transfection.

Furthermore, it is hopeful to confirm the anti-HBV effect of pCas9 on *in vivo* system. The mouse model is considered as the most suitable laboratory animal also in HBV system, and there seem to be the hydrodynamics and transgenic mice of HBV in well-established model (Ren et al., 2006; Huang et al., 2006). It is innovative to use M-TgHBV mouse as the *in vivo* system which can be affected by pCas9 constructs to produce anti-HBV effect, while normal mouse was delivered transiently by hydrodynamics with pHBV constructs in other related publication (Lin et al., 2014; Liu et al., 2015; Dong et al., 2015). In this study, we chose M-TgHBV mouse but not hydrodynamics mouse to avoid co-delivery both HBV and pCas9 constructs. Similar to results of hepatoma cells, pCas9 can decrease serum HBsAg and HBcAg of liver with particular significance of pCas9-2 for targeting X gene. There is a weakness that it is hard to detect HBV cccDNA because linearized HBV genome was transferred into this mouse model (Ren et al., 2006). Therefore, our designed pCas9 constructs can play an anti-HBV effect *in vivo* of targeting chromosomal DNA. Ramanan et al. indicate that Cas9/sgRNAs for targeting Core gene may induce the better effective reduction HBsAg and viral DNA level of HBV hydrodynamics-mouse serum (Ramanan et al., 2015).

Presently, our pCas9 constructs to target HBV have selected another conserved sequence by comparison from A to H genotype of HBV as well as M-TgHBV mouse was used to support the anti-HBV effect of our pCas9 constructs, which is different from other matched publications. There is also one limitation of the delivery system of pCas9 without stable effect similar to paralleled statements. In summary, we have developed a specific CRISPR/Cas9 to induce anti-HBV effect of *in vitro and in vivo system*, which can provide a potential strategy for application in curing chronic HBV infection in the future.

Conflict of interest

None

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.virusres.2016.04.003.

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