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Efficient inhibition of duck hepatitis B virus DNA

by the CRISPR/Cas9 system

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Abstract . Cu r rent therapeut ic st rateg ies cannot erad icate

polyme ra se cha i n reac t ion. T he comb i ned i n h ib it ion of

hepa t it is B v i r us cova lent ly closed c i rcu la r DNA (HBV

CRISPR /Cas9 system and entecavir (ETV) was also assessed.

cccDNA), which accounts for the persistence of HBV infec-

Two sgRNAs, sgRNA4 and sgRNA6, exhibited efficient inhi-

tion. Very recently, the clustered regula rly interspaced shor t

bition on DHBV total DNA (77.23 and 86.51%, respectively),

pa l indrom ic repeat (CR ISPR)/CR ISPR-associated protein 9

cccDNA (75.67 and 85.34%, respectively) in PDHs, as well as

(Cas9) system has been used as an efficient and powerful tool

DHBV total DNA in the culture medium (62.17 and 59.52%,

for viral genome editing. Given that the pr imary duck hepa-

respectively). The inhibition remained or enhanced from day

tocy te (PDH) infected with duck hepatitis B vi rus (DHBV)

5 to day 9 fol lowing t ransfect ion. The combinat ion of the

has been widely used to study human HBV infection in vitro,

CRISPR /Cas9 system and ETV fur ther increased the inhibi-

the present study aimed to demonstrate the targeted inhibition

tory effect on DHBV total DNA in PDHs and culture medium,

of DHBV DNA, especia l ly cccDNA, by the CR ISPR /Cas9

but not cccDNA. The CRISPR /Cas9 system has the potential

system using this model. We designed six single-guide RNAs

to be a useful tool for the suppression of DHBV DNA.

(sgRNA1-6) targeting the DHBV genome. The sgRNA /Cas9

plasm id was t ransfec ted into DHBV-in fec ted PDHs, and

Introduction

then DHBV tota l DNA (in culture medium and PDHs) and

cccDNA were quantified by reverse transcription‑quantitative

Hepat it is B vi r us (HBV ) in fect ion rema ins a major publ ic

hea lth problem worldwide at present. Patients with ch ron ic

HBV in fect ion a re at h igh r isk of prog ressing to ci r rhosis,

hepatocellular carcinoma and liver failure. It is estimated that

~800,000 people die from HBV-related diseases per year (1).

A lthough the int roduct ion of the hepat it is B vaccine into

national immunization programs has dramatically reduced the

incidence of HBV infection, the rate of ver tical transmission,

especially for hepatitis B virus e antigen-positive mothers, is

up to 9.8% in China in 2002 (2). Nucleoside analogues (NAs),

such as entecavir (ETV), lam ivudine and adefovir dipivoxil

can inhibit the reverse transcription of HBV mRNA and have

been used as pr ima ry ant ivi ra l agents for the t reatment of

HBV infection in clinical practice. Never theless, they cannot

radically eliminate HBV covalently closed circular (cccDNA)

in the nucleus of hepatocytes, which is the template for HBV

replication (3). Moreover, drug resistance may occur following

long‑term use of NAs. Thus, it is urgent to find out new efficient

methods to eliminate HBV cccDNA.

The clustered regu la rly interspaced shor t pa l ind rom ic

repeat (CRISPR)/CRISPR-associated (Cas) system, originally

identified in bacter ia and a rchaea, is the th i rd generation of

genome - ed it ing technology (4). The type I I CR ISPR /Cas

system from Streptococcus pyogenes and its simplified deriva-

tive, the Cas9/single guide RNA (sgRNA) system (5,6), has

emerged as a potent new tool for targeted gene modification

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Abbreviations: HBV, hepatitis B virus; cccDNA, covalently closed

circular DNA; CRISPR, clustered regularly interspaced shor t

palindromic repeat; Cas9, CRISPR‑associated protein 9; PDH,

pr imary duck hepatocyte; DHBV, duck hepatitis B virus; sgRNAs,

single‑guide RNAs; ETV, entecavir; NAs, nucleoside analogues;

sgNS, one nonsense sgRNA; ZFN, zinc finger nucleases; TALEN,

transcr iption activator-like effector nucleases

Key words: CRISPR /cas9, duck hepatitis B virus, hepatitis B virus,

covalently closed circular DNA, antivirus

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in huma ns a nd severa l o ther sp ec ies (7-10). Since 2013,

CRISPR /cas9-mediated genome editing has been successfully

fin ished on severa l human viruses, such as papilloma virus,

human immunodeficiency virus and Epstein‑Barr virus (11‑13).

So far, several repor ts have indicated that HBV total DNA

and HBV cccDNA in in fected hepatocy tes can be reduced

by the CRISPR /cas9 system (14 -16). However, most of these

studies have used HBV-infected human hepatoma cell lines,

wh ich a re not considered as optima l cell models for human

HBV infection, to evaluate drug efficacy. Therefore, it is essen-

tia l to eva luate the inh ibition efficacy of the CR ISPR /Cas9

system in the setting of primary hepatocytes with natural HBV

infection. As a model vi rus of HBV, duck hepatitis B vi rus

(DHBV ) sha res the sim i la r vi r us st r uctu re and repl icat ion

features with HBV (17). In this respect, primary duck hepato-

cytes (PDHs) naturally infected with DHBV provide valuable

model systems for studying HBV infection (18). In l ight of

this, the authors hypothesized that the CRISPR /Cas9 system

may suppress DHBV DNA. In order to validate the hypothesis,

firstly, DHBV‑specific sgRNA /Cas9 dua l expression vector

was constructed and transfected into DHBV-infected PDHs.

Second ly, the inh ibit ion efficacy on DHBV tota l DNA and

cccDNA by the CRISPR /Cas9 system was evaluated. Finally,

the combined inhibition of CRISPR /cas9 system and ETV was

assessed.

Materials and methods

Des ign and con s t ruc t ion of DHBV‑ spec if ic sgRNA /Ca s9

pla sm ids. sgRNAs were designed using the CR ISPR /Cas

system (Cas9/gRNA) Off-Targeter (CasOT) tool (http://casot.

cbi.pku.edu.cn /; Pek ing Un iversity, Beijing, Ch ina) to m in i-

mize potential off-target effects. The sequences of six sgRNAs

t a rge t ing DHBV genome (GenBa n k : K01834.1) a nd one

nonsense sgRNA (sgNS) are presented in Table I.

The PSpCas9(BB)-2A-GFP (PX458) plasmid was obtained

from Addgene, Inc. (Cambridge, MA, USA). The plasmid was

extracted using the EndoFree Min i Plasm id K it II (Tiangen

Biotech Co., Ltd., Beijing, China). PX458 was digested with

BbsI (New England Biolabs, Inc., Ipswich, MA, USA), and

then the linearized vector was purified using the Gel Extraction

k it (Omega Bio-tek, Inc., Norcross, GA, USA) according to

the manufactu rer 's inst r uct ions. Each pa i r of sgRNAs was

annealed to double strands, which was ligated to the linearized

vector with T4 DNA ligase (New England Biolabs, Inc.). The

co ‑expression plasm id sgRNA /Cas9 was identified th rough

sequencing (Fig. 1).

Isola t ion , in fect ion and t ransfect ion of PDHs. Duck l ings

(1-day-old) were purchased from Qianjin Duck Farm (Beijing,

Ch ina). A l l an ima l ca re and exper imenta l procedures were

performed with the approval of the Institutional Animal Care

and Use Comm it tee at Beijing Youan Hospita l affil iated to

Capital Medical University (Beijing, China) according to the

Guide for the Care and Use of Laboratory Animals (National

Inst itutes of Hea lth, Bethesda, MD, USA). DHBV-posit ive

and ‑negative duck lings were identified by polymerase chain

reac t ion (PCR) using Ex Taq DNA polyme ra se (Ta ka ra

Biotechnology Co., Ltd., Dalian, China) using serum samples.

Sample processing, the cor responding pr imers (DHBV2548

and DHBV2840R), PCR reaction m ixture and amplification

cycle was descr ibed previously (18). The PCR product was

verified by agarose gel electrophoresis (Fig. 2).

PDHs were isolated f rom 7-day-old DHBV-f ree Pek in

ducklings (19). Isolated PDHs were seeded in a 24-well plate at

1.0x105/well, and were cultured in L‑15 medium (Invitrogen;

T h e r mo F i sh e r S c i en t i f ic, I n c. , Wa l t h am , M A , USA)

supplemented with 10% feta l bovine ser um (Hyclone; GE

Healthcare Life Sciences, Logan, UT, USA), 10 uM hydrocor-

tisone (Sigma‑Aldr ich; Merck KGaA, Darmstadt, Germany),

10 µg /m l insu l in (Cel l Appl icat ions, Inc., San D iego, CA,

USA), 20 mM HEPES, and 1% penicillin /streptomycin (both

from Sigma‑Aldrich; Merck KGaA) in a humidified chamber

at 39˚C without CO2. The next day, PDHs were infected with

DHBV-positive serum (~4x106 copies/well). On the third day

fol lowing seed ing, DHBV-in fected PDHs were t ransfected

with DHBV‑specific sgRNA /Cas9 dual expression vector using

Lipofectam ine 2000 ( Invit rogen; Thermo Fisher Scient ific,

Inc.). The ratio between Lipofectamine 2000 (µl) and plasmid

(µg) was 3:1. Some wells were treated with ETV (China Food

and Drug Administration, Beijing, China) at 0.13 nM (20) on

days 3, 5, and 7 following transfection. The culture medium

and cells were harvested on day 5 or day 9 following transfec-

tion for analyses.

Ext ract ion of DHBV tota l DNA and cccDNA. DHBV tota l

DNA in the culture medium was extracted using TIANamp

Virus DNA /RNA k it (Tiangen Biotech Co., Ltd.). Following

the remova l of the cu ltu re med ium, the cel ls were lysed

and to t a l DNA wa s ex t rac ted using T IANamp Genom ic

DNA k it (Tiangen Biotech Co., Ltd.). For the pur ification of

DHBV cccDNA, DHBV total DNA was fur ther treated with

Plasmid‑Safe™ ATP‑Dependent DNase (Epicenter; Illumina,

Inc., San Diego, CA, USA) at 37˚C for 30 m in fol lowed by

70˚C for 30 min to digest linear double‑stranded DNA, and the

resulting product was recycled using Cycle Pure k it (Omega

Bio-tek, Inc.) according to the instructions of manufacturer.

Quan t if ica t ion of DHBV DNA in the culture medium and

PDHs. DHBV total DNA and cccDNA were then quantified

by reverse transcription‑quantitative PCR with specific primers

and TaqMan MGB probes (Sangon Biotech Co., Ltd, Shanghai,

China). The sequences of the pr imers and the cor responding

TaqMan probes are displayed in Table II. PCR was performed

using the StepOnePlus Rea l- t ime PCR system (Appl ied

Biosystems; Thermo Fisher Scientific, Inc.). In a final volume of

20 µl, the following was added: 10 µl SsoAdvanced™ Universal

Probes SuperMix (Bio-Rad Laboratories, Inc., Hercules, CA,

USA), 2 µl DNA template, 2 µl forwa rd and reverse m ixed

primers (5 mM), 1 µl TaqMan probe (2.5 mM) and 5 µl double

distilled water. Amplification was performed under the following

conditions: 95˚C for 30 sec, 40 cycles of 95˚C for 5 sec, 60˚C for

30 sec, and 72˚C for 30 sec. Duck β-globin gene was used as an

internal reference. The relative quantification of total DNA and

cccDNA was standardized to that of the sgNS group.

Statistical analysis. Normally distributed data were presented

as mean ± standard error of the mean and analyzed by Student's

t-test. Non-norma lly distr ibuted data were expressed as the

median (range) and was analyzed by the Mann-Whitney U test.

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Table I. Sequence of the protospacer and protospacer adjacent motif targeted by DHBV‑specific sgRNAs in the DHBV genome.

Name

Nucleotide position

Gene region

Sequence (GN18-20NGG)

sgRNA1

1,310-1,332

S

F: 5'-GAGCTGGCCTAATCGGATTACTGG-3'

R: 5'-CCAGTAATCCGATTAGGCCAGCTC-3'

sgRNA2

1,293-1,315

S

F: 5'-GACCTTCGGGGGAATACTAGCTGG-3'

R: 5'-CCAGCTAGTATTCCCCCGAAGGTC-3'

sgRNA3

1,363-1,385

S

F: 5'-GAAATACTGAGGAGGCTAGATTGG-3'

R: 5'-CCAATCTAGCCTCCTCAGTATTTC-3'

sgRNA4

1,449-1,472

S

F: 5'-GCAAATCTCTCCACATTACGTAGG-3'

R: 5'-CCTACGTAATGTGGAGAGATTTGC-3'

sgRNA5

2,738-2,760

C

F: 5'-GAAGACGCTTTAGAGCCTTATTGG-3'

R: 5'-CCAATAAGGCTCTAAAGCGTCTTC-3'

sgRNA6

29-51

P

F: 5'-GTTAACGAGGAATCACTGGATAGG-3'

R: 5'-CCTATCCAGTGATTCCTCGTTAAC-3'

sgNS

F: 5'-GAAATCCTGCAGAAAGACCTGG-3'

R: 5'-CCAGGTCTTTCTGCAGGATTTC-3'

sgRNA, single‑guide RNA; DHBV, duck hepatitis B virus.

Figu re 2. The ident ificat ion of DHBV‑posit ive serum by PCR. The size of

the amplified PCR product was 292 bp. Lane 1, DHBV positive serum; and

lane 2, DNA marker. DHBV, duck hepatitis B virus; PCR, polymerase chain

reaction.

detected at var ious time points following transfection. Fig. 3

indicated that there was no significant difference among the

three groups: No treatment group, Lipofectamine 2000 group

and sgNS group. Compa red with the sgNS group, sgRNA4

and sgRNA6 exhibited higher efficacy in suppressing DHBV

DNA on day 5 following transfection. For sgRNA4, DHBV

tota l DNA in PDHs was reduced by 77.64%, (for example,

from 1.30x103 copies/cell to 2.96x102 copies/cell). A sim ila r

reduct ion (60.19%) was observed for sgRNA6. In add it ion,

DHBV cccDNA was also suppressed significantly by sgRNA4

and sgRNA6 (60.19 and 68.82%, respectively). The inhibition

efficacy of sgRNA4 and sgRNA6 on DHBV tota l DNA and

cccDNA presented a sign if icant d ifference compa red with

sgNS (P= 0.002 and 0.015 respectively for sgRNA4, P= 0.003

and 0.004 respectively for sgRNA6).

The inhibition efficacy of sgRNA4 and sgRNA6 remained or

improved on day 9 following transfection. From day 5 to day 9

fol lowing t ransfect ion, the inh ibit ion on DHBV tota l DNA

remained (from 77.64 to 77.23%) by sgRNA4, but increased

(from 73.51 to 86.51%) by sgRNA6. DHBV cccDNA in PDHs

was fur ther inhibited by sgRNA4 (from 60.19 to 75.67%) and

sgRNA6 (from 68.82 to 85.34%). Moreover, DHBV total DNA

F ig u r e 1. T h e c o n s t r u c t e d c o - ex p r e s s io n p l a sm id s DH BV- s p e c i f i c

sgRNA /Cas9 and sgNS/Cas9 were ident ified th rough sequencing. DHBV,

duck hepatitis B virus; sgRNA, single‑guide RNAs.

Statistical analysis was conducted using SPSS software (version,

19.0; IBM SPSS, Chicago, IL, USA). A two‑sided P<0.05 was

considered to indicate a statistically significant difference.

Results

sgRNA4 and sgRNA6 significantly suppressed DHBV DNA on

day 5 following transfection. To analyze the inhibition efficacy

of six sgRNAs, the levels of DHBV DNA in PDHs were

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Table II. DHBV primers for polymerase chain reaction.

Name

Primer

cccDNA

Forward

Reverse

TaqMan probe

Forward

Reverse

TaqMan probe

Forward

Reverse

TaqMan probe

Total DNA

β-globin

Sequence (5'-3')

TGCCATAAGCGTTATCAGACGTT

GGCTAAGGCTCTAGAAGCATTGA

ATATAATCCTGCTGACGGCC

TTCGGAGCTGCTTGCCAA

TCATACACATTGGCTAAGGCTCT

CGTCTACATTGCTGTTGTCGTGTGTGAC

AGCAGTTGTTGGAGCAGGAA

TCTTTGGCTGTTGGCATCTA

AGAGGAGTGATGAGCAAGAGACAGTGGC

DHBV, duck hepatitis B virus; cccDNA, covalently closed circular DNA.

Figu re 3. sgRNA4/Cas9 and sgRNA6/Cas9 efficient ly inh ibit the repl icat ion of DHBV on day 5 fol lowing t ransfect ion. The relat ive quant ificat ion of

(A) total DNA and (B) cccDNA was standardized to that of the sgNS group. The percentages of DHBV total DNA and cccDNA expression were presented

as mean ± standard er ror of the mean. Compar isons were performed between the sgRNAs groups and the sgNS group. \*P<0.05. ns, no significant difference

among the three groups: No treatment group, the Lipofectam ine 2000 group and sgNS group. sgRNA, single‑guide RNAs; Cas9, CRISPR‑associated protein 9;

DHBV, duck hepatitis B virus; sgNS, one nonsense sgRNA; cccDNA, covalently closed circular DNA; PDHs, pr imary duck hepatocytes.

in the cu ltu re med ium was reduced by 62.17 and 59.52%,

respectively for sgRNA4 and sgRNA6 (Fig. 4).

ETV enhanced the suppression on DHBV DNA accumulation

by CR ISPR /Cas9 system. Consider ing that the suppression

on DHBV DNA by CRISPR /Cas9 system was incomplete, it

would be interesting to fur ther assess the combined inhibition

of CR ISPR /Cas9 system and ETV, the f i rst-l ine t reatment

option for patients with HBV infection. As presented in Fig. 5,

on day 9 fol low ing t ransfec t ion (the sixth day fol low ing

ETV t rea tment), t he i n h ib it ion ef f icacy on DHBV to t a l

DNA in PDHs was h igher in sgRNA4+ETV (97.52%) and

sgRNA6+ETV (96. 57%) g roups compa red w ith sgRNA4

(77.23%) and sgRNA6 (86.51%) groups (P= 0.006 and 0.005

respectively). Similarly, the inhibition efficacy on DHBV total

DNA in the cu ltu re med ium was h igher in sgRNA4+ETV

(85.45%) and sgRNA6+ETV (78.29%) groups compared with

sgRNA4 (62.17%) and sgRNA6 (59.52%) groups (P=0.006 and

0.011, respectively). However, ETV treatment did not enhance

t he i n h ib it ion on DHBV cccDNA i n PDHs by sgRNA4

(sgRNA4 vs. sgRNA4+ETV: 75.67% vs. 73.90%; P= 0.144)

and sgRNA6 (sgRNA6 vs. sgRNA6+ETV: 85.34% vs. 82.60%;

P= 0.144). Thus, the combination of ETV and CR ISPR /Cas9

system led to a fur ther reduction of DHBV total DNA in PDHs

and culture medium, but not DHBV cccDNA.

Discussion

In the present study, two sgRNAs (sgRNA4 and sgRNA6)

targeting the DHBV genome were demonstrated to suppress

DHBV total DNA and cccDNA successfully. ETV enhanced

the inh ibit ion of the CR ISPR /Cas9 system on DHBV tota l

DNA. The cur rent findings suggested that the CRISPR /Cas9

system targeting specific sites of the DHBV genome may be

an effective technology to inhibit DHBV infection in PDHs.

To the best of the authors' knowledge, the present study is the

first repor ting the ta rgeted inhibition of DHBV DNA by the

CRISPR /Cas9 system.

Genet ic mod if icat ions have been ach ieved successfu l ly

using genome - ed it ing technolog ies, includ ing z inc f inger

nucleases (ZFN ) (21), t ransc r ipt ion act ivator -l ike ef fector

nucleases (TALEN) (22) and the CRISPR /Cas9 system (23).

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Figure 4. The inhibition on DHBV DNA, especially cccDNA, increases from day 5 to day 9 following transfection. The levels of DHBV (A) total DNA and

(B) cccDNA on day 5 and day 9 following transfection were compared. \*P<0.05. ns, no significant difference between the two groups. DHBV, duck hepatitis B

virus; cccDNA, covalently closed circular DNA; PDHs, pr imary duck hepatocytes.

Figure 5. ETV enhances the inhibition of sgRNA4 and sgRNA6 on DHBV total DNA, but not cccDNA. On the day 9 following transfection, DHBV (A) total

DNA in PDHs and (B) culture medium as well as (C) cccDNA in PDHs were measured by reverse transcr iption-quantitative polymerase chain reaction. The

relative quantification of total DNA and cccDNA was standardized to that of the sgNS group. The percentages of total DNA and cccDNA expression were

presented as mean ± standa rd er ror of the mean. The statistica l ana lysis was per formed between the sgRNA groups and the sgNS group. \*P<0.05. ns, no

significant difference between the two groups. ETV, entecavir; sgRNA, single‑guide RNAs; DHBV, duck hepatitis B virus; PDHs, pr imary duck hepatocytes;

cccDNA, covalently closed circular DNA; sgNS, one nonsense sgRNA.

As the classical method for genome engineering, ZFN is used

to inva l idate the H IV co -receptor C-C chemok ine receptor

type 5, wh ich is cur rently in cl in ica l t r ia ls (NCT01252641,

NCT00842634 and NCT01044654). It is repor ted that TALEN

plasm ids for 18,740 human protein-coding genes have been

assembled (24). However, both ZFNs and TALENs ut i l ize

protein‑based programmable, sequence‑specific DNA‑binding

modules, whose construction is usually complex. The emer-

gence of the CRISPR /Cas9 system in 2013 (25) makes it a facile

and efficient alternative to ZFNs and TALENs. Only sgRNAs

targeting specific genes are required for highly efficient gene

modification using the CRISPR /Cas9 system (26,27).

In the present study, the effective sgRNAs ta rgeting the

DHBV genome were located at the S and P regions. Previous

studies also screened out effective sgRNAs located at different

reg ions of HBV genome, such as X, core, polymerase and

su r face ORFs (14,15,28). sgRNAs ta rget ing the conserved

HBV sequence were effective for HBV genomes of different

genotypes (29). Thus, it may be necessa ry to const ruct the

sgRNAs library targeting different regions of HBV genome in

order to screen out the most efficient sgRNAs.

Severa l p rev ious repor t s used either Huh7 cel ls (29)

transfected with the HBV-expression vector pAAV/HBV1.2

(genotype A) or HepAD38 cells (30) stably expressing HBV

DNA to evaluate the targeted inhibition on HBV genome by

the CRISPR/Cas9 system. In terms of biological characteristics

and HBV infection mode, PDHs infected with DHBV-positive

serum have advantages over immor talized cell lines, because

the former may mimic the natural process of HBV infection. In

the present study, we chose DHBV-infected PDHs as an in vitro

model to assess the inhibition efficiency of the CRISPR/Cas9

system. In this regard, the result that the CRISPR/Cas9 system

can efficiently inh ibit DHBV cccDNA is more close to the

rea l-world HBV in fec t ion, thus ex h ibit ing g rea t cl in ica l

impor tance. Moreover, the study investigated whether ETV

can improve the anti-viral effects of CRISPR/Cas9 system. The

results indicated that the combination of the CR ISPR /Cas9

system and ETV enhanced the suppression of DHBV tota l

DNA, but not cccDNA. One possible explanation is that ETV,

as a nucleoside reverse t ranscr iptase inh ibitor, cou ld on ly

reduce DHBV total DNA, but has little or no effect on DHBV

cccDNA. Therefore, it was specu lated that the combined

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application of the CRISPR/Cas9 system and ETV may have the

potential to control DHBV infection more effectively.

There a re two l im itat ions in the present study. Fi rst ly,

sgRNAs ta rgeting different regions of DHBV genome need

to be designed in order to screen out the most effective ones.

Second ly, in vivo stud ies need to be per formed to va l idate

the inh ibitory effect of the CR ISPR /Cas9 system on DHBV

cccDNA.

A lthough g rea t advancement s have been made in the

prevention and treatment of HBV infection, the high morbidity

of HBV-associated compl icat ions is st i l l a huge th reat for

human hea lth. The present study is thought to be the f i rst

to demonst rate the efficient inh ibition of the CR ISPR /Cas9

system on DHBV cccDNA. Even though fu r ther study is

required to improve the inhibitory efficiency, the current find-

ings pave the way for el im inat ing HBV cccDNA using the

CRISPR /Cas9 system in clinical practice in the future.

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