Cell Research (2017) 27:440-443.

www.nature.com/cr

LETTER TO THE EDITOR

A non-viral CRISPR/Cas9 delivery system for therapeutically targeting HBV DNA and *pcsk9 in vivo*

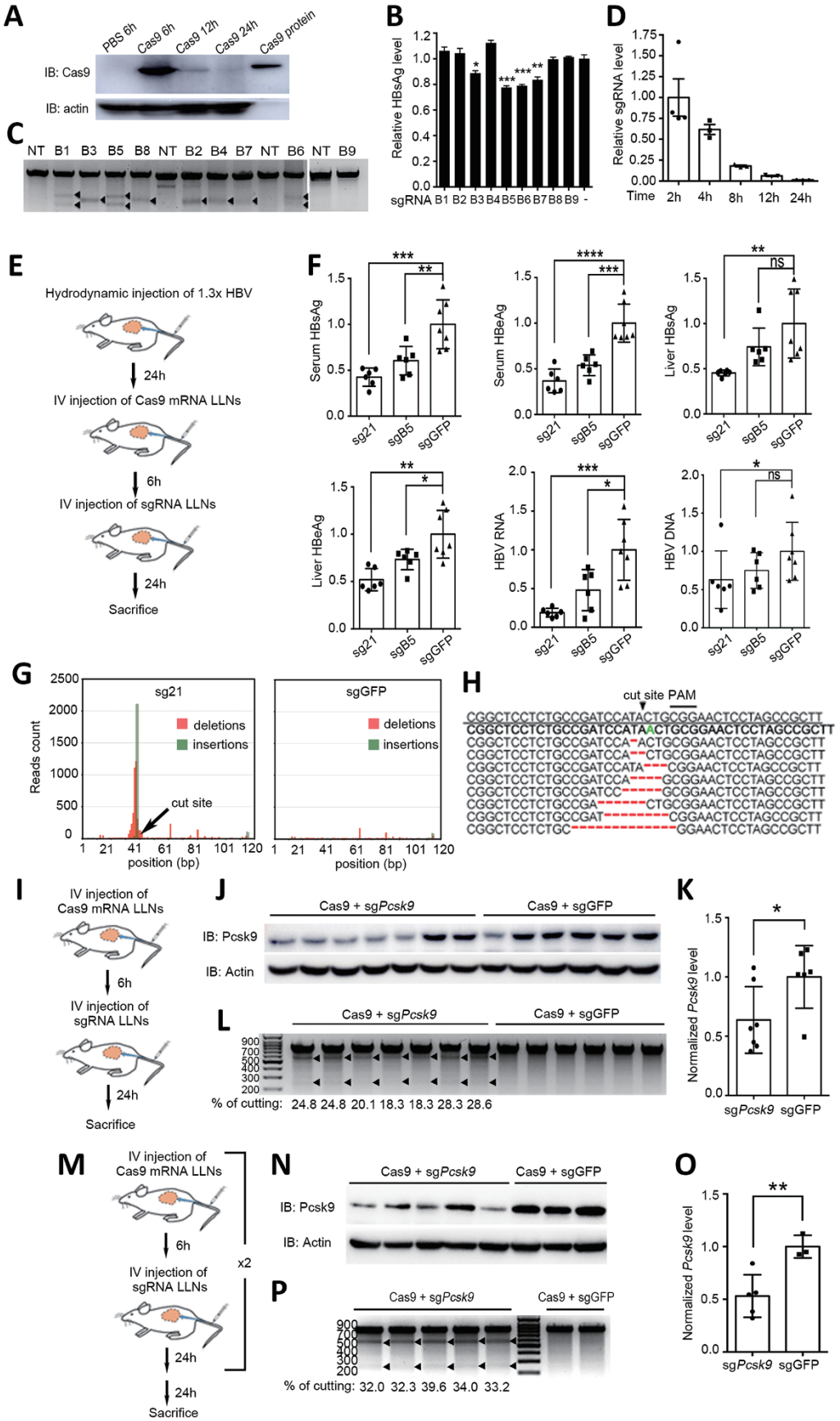
Cell Research (2017) **27**:440-443. doi:10.1038/cr.2017.16; published online 24 January 2017

Dear Editor,

The clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein (Cas) system has revolutionized biomedical research and facilitated the development of new therapies based on genome editing [1]. A major roadblock to achieve the therapeutic potential of the CRISPR/Cas system is the lack of a safe and effecting *in vivo* delivery method. Adeno-associated virus (AAV)-assisted delivery of the CRISPR/Cas9 system has shown gene targeting efficacy *in vivo*, however, the long persistence and immunogenicity of AAV in the host prevent the wide therapeutic application of AAV-based CRISPR/Cas9 delivery [2]. A previous study has combined lipid nanoparticle and AAV vector to deliver CRISPR/Cas9 system components [3]. Here we show that by using an optimized formula of our newly developed lipid-like nanoparticles (LLNs) [4], we are able to effectively deliver Cas9 mRNA and single-guide RNA (sgRNA) to the liver and achieve *in vivo* targeting of HBV DNA and the *proprotein convertase subtilisin/kexin type 9* (*pcsk9*) gene, a therapeutic target for treating hypercholesterolemia [5]. To the best of our knowledge, this is the first report on non-viral delivery of CRISPR/Cas9 system components (mRNA + sgRNA) in adult animals.

Previously, we reported the development and optimization of TT3 LLNs (Supplementary information, Figure S1A), which are capable of efficiently delivering mRNA both *in vitro* and *in vivo* [4]. To optimize the LLN formulation for Cas9 mRNA delivery, we designed 27 formulas of TT3 LLNs (Supplementary information, Table S1) and examined their ability to delivery Cas9 mRNA into HEK293T cells stably expressing EGFP and an sgRNA targeting EGFP [3]. Forty-eight hours after the delivery, we quantified the percentage of EGFP-positive cells by fluorescence-activated cell sorting (FACS) analysis. Formula 3 showed the highest delivery efficiency, reducing the percentage of EGFP-positive cells by over 50% (Supplementary information, Figure S1B). Next we performed *in vivo* delivery of TT3 LLNs of formula 3 into C57BL/6 mice via tail vein injection. Western blot analysis of liver lysates revealed that Cas9 protein was robustly expressed 6 h post injection, and the protein level rapidly decreased at 12 h and became undetectable at 24 h (Figure 1A). Consistently, the mRNA level of Cas9 was high at 6 h and gradually decreased afterwards (Supplementary information, Figure S1C). This time course suggests that the expression of Cas9 mRNA delivered by TT3 LLNs can last no more than 24 h.

HBV covalently closed circular DNA (cccDNA), the major form of the viral genome in infected hepatocytes, represents an intractable barrier to eradication of the virus and is resistant to the currently available therapies [6]. Several previous studies have explored the potential of the CRISPR/Cas9 system to target HBV cccDNA in mice by hydrodynamic injection of plasmid DNA [7-11]; however, therapeutically relevant *in vivo* delivery methods are still lacking [12]. To systematically identify potential sgRNAs targeting HBV DNA, we used the CRISPR DESIGN tool (http://crispr.mit.edu) to design potential sgRNAs. To achieve a broad-spectrum antiviral activity, we selected 9 sgRNAs that target regions with the highest levels of conservation among HBV strains (Supplementary information, Figure S1E) [13]. We tested the sgRNAs using an *in vitro* transfection assay in HepAD38 cells, a hepatocyte line with an integrated HBV genome. We found that when each combined with Cas9, several sgRNAs efficiently diminished HBV protein production as measured by ELISA of HBV surface antigen (HBsAg) (Figure 1B). We also performed T7E1 assays in a cell model that produces episomal HBV cccDNA [14], and found that together with Cas9, all sgRNAs except for B9 induced indels in the HBV DNA (Figure 1C). We selected the most potent sgRNA B5 (sgB5) based on its performance in ELISA for further analysis. We found that sgB5 delivered by TT3 LLNs via tail vein injection can be detected in the mouse liver


A
PBS 6h
Cas9 6h
Cas9 12h
Cas9 24h
Cas9 protein
IB: Cas9
IB: actin
B
Relative HBsAg level
sgRNA B1 B2 B3 B4 B5 B6 B7 B8 B9 -
D
Relative sgRNA level
Time 2h 4h 8h 12h 24h
C
NT B1 B3 B5 B8 NT B2 B4 B7 NT B6 NT B9
E
Hydrodynamic injection of 1.3x HBV
24h
IV injection of Cas9 mRNA LLNs
6h
IV injection of sgRNA LLNs
24h
Sacrifice
F
Serum HBsAg
Serum HBeAg
Liver HBsAg
Liver HBeAg
HBV RNA
HBV DNA
sg21 sgB5 sgGFP
ns
G
sg21
sgGFP
deletions
insertions
cut site
Reads count
position (bp)
H
cut site PAM
I
IV injection of Cas9 mRNA LLNs
6h
IV injection of sgRNA LLNs
24h
Sacrifice
J
Cas9 + sgPcsk9
Cas9 + sgGFP
IB: Pcsk9
IB: Actin
K
Normalized Pcsk9 level
sgPcsk9 sgGFP
L
% of cutting: 24.8 24.8 20.1 18.3 18.3 28.3 28.6
M
IV injection of Cas9 mRNA LLNs
6h
IV injection of sgRNA LLNs
24h
x2
24h
Sacrifice
N
IB: Pcsk9
IB: Actin
O
Normalized Pcsk9 level
sgPcsk9 sgGFP
P
% of cutting: 32.0 32.3 39.6 34.0 33.2

by qRT-PCR and its level decreased significantly 4-8 h post injection (Figure 1D).

To evaluate the anti-HBV effect of the CRISPR/Cas9 system delivered by TT3 LLNs, we used a well-established mouse model of HBV replication [15]. The plasmid encoding 1.3 times of the HBV genome (1.3x HBV; mimicking the plasmid-like HBV cccDNA) was delivered into mice using hydrodynamic tail vein injection (Figure 1E). Twenty-four hours later, TT3 LLNs with Cas9 mRNA were administered via regular tail vein injection. To maximize the formation of Cas9/sgRNA complex, we injected sgRNA 6 h post the injection of Cas9 mRNA, which resulted in the strongest reduction of liver HBsAg levels in a preliminary test (Supplementary information, Figure S2A). We sacrificed the mice 24 h post the sgRNA injection and measured the HBV burden. We used an sgRNA targeting GFP (sgGFP) and a previously reported sgRNA targeting HBV DNA (sg21), both validated in our *in vitro* test, as negative and positive controls, respectively (Supplementary information, Figure S2B). We found that Cas9 (0.56 mg/kg) and sg21(0.25 mg/kg) significantly decreased all measurements of HBV viral loads, including liver and serum HBsAg levels, liver and serum HBV e antigen (HBeAg) levels, and liver HBV RNA and DNA levels, when compared with sgGFP (Figure 1F). The efficacy of Cas9+sg21 LLNs in reducing HBV viral loads is comparable to a previous study using hydrodynamic injection of a plasmid encoding Cas9 and sg21 [11]. sgB5 identified by our screening had a significant effect in 4 out of the 6 measurements (Figure 1F). To verify that the reduction is due to CRISPR/Cas9-mediated HBV DNA cleavage, we sequenced the HBV DNA extracted from the liver of Cas9+sg21 LLNs-treated mice and detected multiple indels near the sg21 cutting site, while no obvious enrichment of indels near the cutting site was observed in the Cas9+sgGFP group, supporting a specific cleavage of HBV DNA by Cas9+sg21 (Figure 1G and 1H). We also assessed the potential off-target effects of Cas9+sg21 TT3 LLN treatment by sequencing the top three predicted off-target sites and found no obvious indels, suggesting a low off-target rate (Supplementary information, Figure S2C).

To test whether CRISPR/Cas9 components delivered by our method can efficiently edit endogenous genes, we targeted mouse *pcsk9* gene, which is a drug target for treating hypercholesterolemia [5]. We selected an sgRNA targeting *pcsk9* (sgPcsk9) based on an *in vitro* transfection assay in NIH3T3 cells (Supplementary information, Figure S2D). For *in vivo* test, one injection of Cas9 mRNA LLNs followed by an injection of sgRNA LLNs 6 h later resulted in a significant reduction of Pcsk9 protein level in C57BL/6 mice compared to the control group injected with Cas9 mRNA and sgGFP LLNs (Figure 1I-1K). T7E1 assays using genomic DNA from the mouse liver revealed a cleavage in sgPcsk9-targeting region in all samples examined (Figure 1L). It is worth noting that Pcsk9 protein level can vary in normal mice, which might explain the occasional discordance between Pcsk9 protein level and the cutting efficiency (e.g., lanes 7 and 8 in Figure 1J and 1L). Hematoxylin and eosin staining shows no significant liver damage in Cas9/sgPcsk9/LLN-treated mice (Supplementary information, Figure S2E). To increase the targeting efficiency, we doubled

Figure 1 Delivery of the CRISPR/Cas9 system by optimized TT3 LLNs can suppress HBV and *Pcsk9* gene expression in mice. **(A)** Time course of Cas9 expression in mouse liver after tail-vein injection of Cas9 LLNs. Western blotting of liver lysates was performed using anti-Cas9 antibody. Lysates of cells with stable Cas9 expression served as a positive control. Actin: loading control. **(B)** Transfection of HepAD38 cells with Cas9 mRNA and sgRNAs to screen for effective sgRNAs. **P* < 0.05, ***P* < 0.01,****P* < 0.001 (two-tailed *t*-test; *n* = 3). **(C)** Cutting efficiency of Cas9 and sgRNAs in an episomal HBV DNA model. Huh7 cells stably expressing Cas9 and different sgRNAs were transfected with prcccDNA and pCMV-Cre. Seventy two hours later, liver cccDNA was extracted and T7E1 assay was then performed. NT: non-targeting control sgRNA. **(D)** sgRNA B5 levels in mouse liver at the indicated time points after tail-vein injection determined by qRT-PCR. sgRNA levels were normalized to the mRNA levels of *Gapdh*. **(E)** Scheme of the experiment to test the effect of TT3 LLN-mediated CRISPR/Cas9 delivery on HBV replication. **(F)** Levels of serum and liver HBsAg and HBeAg determined by ELISA and levels of liver HBV RNA and DNA determined by qRT-PCR. All values shown were normalized to those of the sgGFP group. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001 (two-tailed *t*-test). ns: *P* > 0.05. **(G)** Plot of read counts from the deep sequencing results of the PCR products of the sg21 target region from a Cas9+sg21 LLNs-treated mouse (left) and a Cas9+sgGFP LLNs-treated mouse (right). **(H)** Sequence alignment showing indels near the cutting site detected in a Cas9+sg21 LLNs-treated mouse. **(I)** Scheme of the mouse experiment performed to knock down *Pcsk9* gene expression in mice. **(J)** Western blotting of mouse liver lysates (one sample per mouse). **(K)** Densitometry of western blot bands in **J**. The protein level of Pcsk9 was normalized to that of actin in each sample and the average was then calculated. Data shown represent the average level of Pcsk9 in the sgPcsk9 group normalized to that of the sgGFP group. **P* < 0.05 (two-tailed *t*-test). **(L)** T7E1 assay of the sgPcsk9 target region. Triangles indicate the specific bands resulting from T7E1 cutting, which are of the expected sizes (555 bp + 247 bp). Cutting efficiency was determined by band densitometry using ImageJ. **(M**-**P)** A similar procedure was performed as in **I**, except for that the injections of LLNs were repeated once 24 h after the injection of sgRNA LLNs. Pcsk9 protein expression **(N**, **O)** and cutting efficiency **(P)** were then determined. ***P* < 0.01 (two-tailed *t*-test).

the injections (Figure 1M) and found that the Pcsk9 protein level was further reduced (Figure 1N and 1O). The T7E1 assay also demonstrated a higher cutting ratio compared with that of the single treatment (Figure 1P). These results demonstrate that our TT3 LLN system can effectively deliver CRISPR/Cas9 components to target endogenous genes.

In summary, we have demonstrated that optimized TT3 LLNs are capable of effectively delivering Cas9 mRNA and sgRNA to the mouse liver for gene targeting. We show that the targeting is effective for both episomal and chromosomal DNA. Since Cas9 mRNA/protein and sgRNA are degraded within one day in mice, our method provides a temporarily controllable way for *in vivo* genome editing. Based on our previous study, LLN-based delivery is highly specific to the liver [4]. Thus, LLN-mediated CRISPR/Cas9 delivery possesses the potential to treat a wide range of liver-related diseases.

Acknowledgments

We thank Drs X Wen, D Anderson, G Ou, J Hu, and Q Deng for providing reagents and advice. This work was supported by grants from the State Key Research Development Program of China (2016YFC1200300), the Young Thousand Talent Program and the Bill & Mellinda Gates Foundation to XT. YD acknowledges the following supports: the Early Career Investigator Award from the Bayer Hemophilia Awards Program, Research Awards from the National PKU Alliance, New Investigator Grant from the AAPS Foundation, Maximizing Investigators' Research Award (1R35GM119679) from the National Institute of General Medical Sciences and the start-up fund from the College of Pharmacy at The Ohio State University.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)