CRISPR-Cas13a Cleavage of Dengue Virus NS3 Gene Efficiently Inhibits Viral Replication

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The CRISPR-Cas9 system has been applied to DNA editing with precision in eukaryotic and prokaryotic systems, but it is unable to edit RNA directly. A recently developed CRISPR-Cas13a system has been shown to be capable of effectively knocking down RNA expression in mammalian and plant cells. In this study, we employ the CRISPR-Cas13a system to achieve reprogrammable inactivation of dengue virus in mammalian cells. Quantitative reverse transcription PCR (qRT-PCR), fluorescence-activated cell sorting (FACS), and plaque assays showed that CRISPR RNA (crRNA) targeting the NS3 region led to the greatest viral inhibition among 10 crRNAs targeting different regions along the dengue viral genomic RNA. Deletions and insertions had also been found adjacent to the NS3 region after NS3-crRNA/Cas13a complex transfection. Our results demonstrate that the CRISPR-Cas13a system is a novel and effective technology to inhibit dengue viral replication, suggesting that such a programmable method may be further developed into a novel therapeutic strategy for dengue and other RNA viruses.

INTRODUCTION

Dengue virus (DENV) is a single-stranded positive sense RNA virus and a member of the genus *Flavivirus* within the family Flaviviridae. The DENV genome is approximately 11,000 nt, consisting of a 5′ untranslated region (UTR), an open reading frame (ORF) encoding a polyprotein, C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5, and a 3′ UTR. There are four DENV serotypes, DENV-1, DENV-2, DENV-3, and DENV-4. Infection with DENV in humans mostly results in a mild and self-limiting febrile disease, dengue fever (DF), but sometimes more severe disease forms such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS).[1] With increased global travel, dengue is becoming a more notable global health problem, especially in tropical and subtropical regions.[2] The development of a preventive vaccine against DENV has been hindered by the phenomenon of antibody-dependent enhancement (ADE),[3] and thus it is more urgent to develop novel therapeutics for dengue treatment.

Designing and screening novel drugs by targeting key steps of virus replication based on structural and mechanistic insights has been a major direction for anti-viral drug development.[4,5]

Several compounds, including nucleoside/nucleoside analogs, non-nucleoside inhibitors, and small interfering RNA (siRNA) targeting important genes or proteins, have been tested experimentally, but there is unpredictable toxicity *in vivo* for nucleoside/nucleoside analogs, due possibly to mitochondrial dysfunction and renal toxicity.[6–8] The other approaches have also not yielded satisfactory antiviral drugs.

The clustered regularly interspaced short palindromic repeats (CRISPR)-associated system (Cas) was originally identified as a part of an adaptive immune system against bacteriophage infections in prokaryotes such as bacteria and archaea.[9] Based on this system, the CRISPR-Cas9 genome-editing technique has emerged and been used for the development of therapeutics for many viral diseases caused by pathogenic viruses that make a double-stranded RNA (dsDNA) intermediate in their replication cycles,[10] such as DNA hepatitis B virus (HBV),[11–15] human papillomavirus (HPV),[16,17] and Epstein-Barr virus (EBV),[18,19] and the proviral DNA genome of RNA virus, such as HIV.[20,21] However, CRISPR-Cas9 cannot edit the RNA virus genome directly, limiting the scope of its use. The discovery of CRISPR-Cas13 (known previously as C2c2), a class 2 type VI-A ribonuclease that contains two higher eukaryote and prokaryote nucleotide-binding (HEPN) RNase domains that are capable of targeting and cleaving single-stranded RNA (ssRNA) molecules of the phage genome,[22] unwrapped new promise for its application in editing cellular RNA and RNA viruses. Indeed, a single-component programmable RNA-guided RNA targeting CRISPR
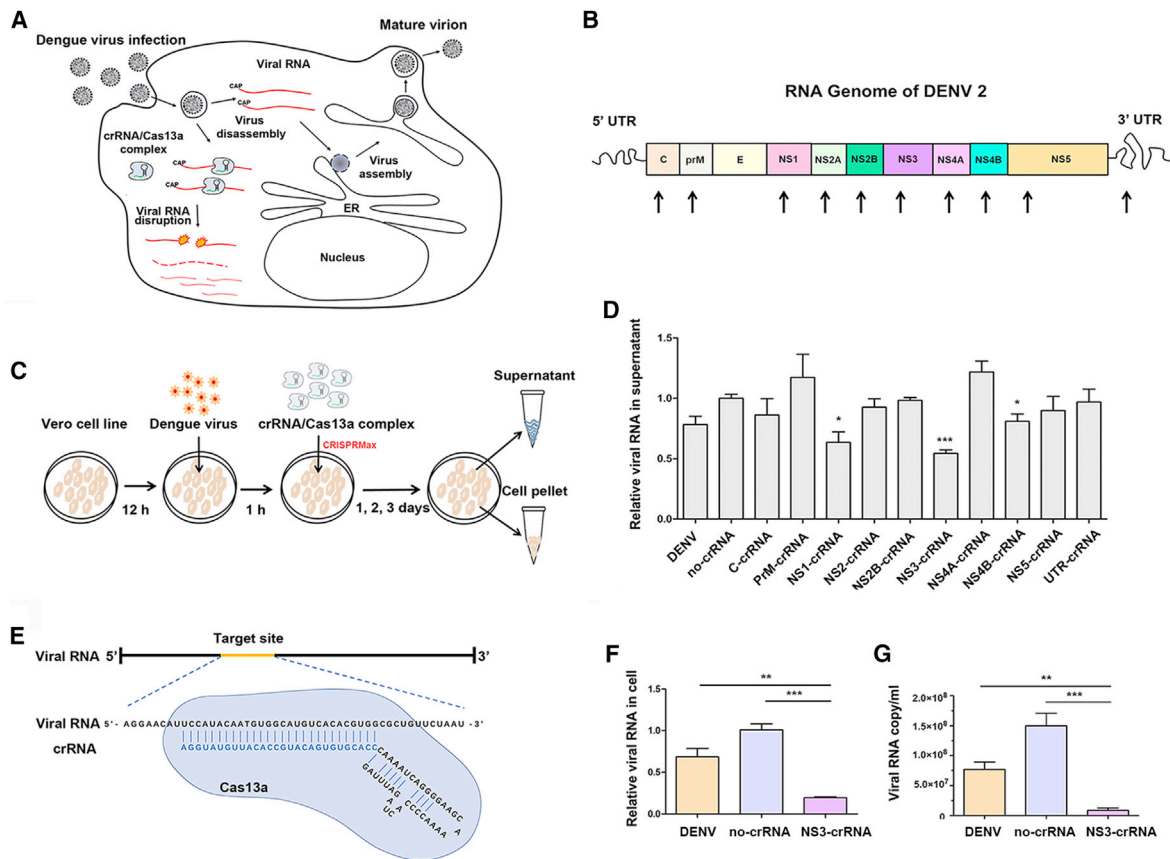
Figure 1. Schematic and Screening of crRNAs for Inhibiting DENV2 Infection Using the CRISPR-Cas13a System

(A) Schematic of the DENV life cycle and putative anti-DENV mechanism of the CRISPR-Cas13a system. Cas13a-mediated cleavage of the target site might disrupt viral RNA, potentially leading to indel formation or degradation. (B) Schematic of 10 target sites in conserved regions of the DENV-2 gene. Ten targets conserved among the four serotypes of DENV were chosen for CRISPR-Cas13a-targeted inhibition; the specific sites are indicated with arrows. (C) Procedures of the anti-DENV experiments showing transfection of the crRNA/Cas13a complex and collection of cells and supernatants for assessment. (D) Screening the effectiveness of different crRNAs. The inhibitory effect of crRNA on DENV infection was screened by detecting DENV RNA copies in the supernatant by qRT-PCR with the 3′ UTR primers. Results are shown as the ratio of viral RNA copies for each treatment, compared with the no-crRNA group, which was set as 1. Any value <1 suggests an inhibition. (E) Schematic of viral RNA targeted via the specific crRNA/Cas13a complex. The target site is highlighted in yellow. (F and G) Evaluation of the inhibitory effect of NS3-crRNA on viral RNA in both cell pellets (F) and supernatant (G) by qRT-PCR. Bars represent mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

effectors, CRISPR-Cas13a, has been shown to be capable of inducing ssRNA cleavage *in vitro* in prokaryotes.[23,24] Furthermore, engineered Cas13a from *Leptotrichia wadei* (LwaCas13a) has been demonstrated to be capable of knocking down mammalian RNA as efficiently as does the RNA interference method, with even better specificity and fewer off-target effects.[25] These new findings led to the application of CRISPR-Cas13a in a turnip mosaic virus (TuMV) interference experiment in plants.[26] To investigate whether it is possible to extend its use to RNA viruses that cause human diseases, we adapted the CRISPR-Cas13a system to DENV and discovered a crRNA that is capable of efficiently suppressing DENV replication in a cell culture system. We hypothesize that the CRISPR-Cas13a system could suppress DENV infection by mutagenizing critical genomic elements or degrading viral genome RNA through specifically targeting the DENV genome RNA (Figure 1A).

RESULTS

Screening for Vulnerable Sites in the Dengue Viral Genome for CRISPR/Cas13a Targeting

To determine sites that can be efficiently targeted by CRISPR/Cas13a and that are conserved among four different DENV serotypes, we first compared the genomic RNA sequences from four viral strains each representing one dengue serotype (GenBank: DENV-1/AF180817, DENV-2/U87411, DENV-3/KU725665, DENV-4/KP704160). The 10 most conserved regions located ahead of nine individual protein coding regions (i.e., Capsid, PrM, NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5), and one UTR at the 3′ end (3′ UTR), had been selected as targets for CRISPR-Cas13a editing. All of these 10 regions have an exact match on DENV-2/16681 (GenBank: U87411), which was used in this study because this strain has been studied extensively and for which historical data are abundant. The selected crRNA sequences

Table 1. RNA Sequences of DENV2 Targeted by CRISPR-Cas13a

Target	RNA Sequence (5′→3′)	Site[a]
crRNA-capsid	UCAAUAUGCUGAAACGCGAGAGAAACCG	133–161
crRNA-PrM	AACAUGGAUGUCAUCAGAAGGGGCCUGG	761–789
crRNA-NS1	CGUGCACACAUGGACAGAACAAUACAA	2491–2519
crRNA-NS2a	UUACAAAACGCAUGGAAAGUGAGUUGCA	3955–3985
crRNA-NS2b	AUGAUAUUCCCAUGACAGGACCAUUAGU	4202–4230
crRNA-NS3	UCCAUACAAUGUGGCAUGUCACACGUGG	4657–4685
crRNA-NS4a	AAUUUGCAGCCGGAAGAAAGUCUCGAC	6355–6383
crRNA-NS4b	CAAAAGCAACCAGAGAAGCUCAGAAAAG	7204–7233
crRNA-NS5	GUGUGUACAACAUGAUGGGAAAAAGAGA	8917–8945
crRNA-3′UTR	AACAAAAAACAGCAUAUUGACGCUGGGA	10647–10675

[a]Site of sequence was obtained from GenBank: U87411 (DENV-2).

and corresponding matches on the RNA genome of DENV-2/16681 are shown in Table 1 and Figure 1B.

These designed crRNAs were synthesized, formulated with purified LwaCas13a protein into the crRNA/Cas13a complex, and then used to transfect DENV-2-infected Vero cells using Lipofectamine CRISPRMAX. Supernatants from treated cells were collected on day 3 after viral infection for assessing antiviral activity by quantitative reverse transcription PCR (qRT-PCR) quantification of viral RNA copy numbers (Figure 1C). As shown in Figure 1D, compared to control cells treated with LwaCas13a alone (no-crRNA), in which the viral copy number was designated as 1, three treatments targeting NS1, NS3, and NS4B showed significantly lower levels of viral copy numbers ($p < 0.05$, $p < 0.01$, and $p < 0.05$, respectively). The other seven crRNA/Cas13a complexes showed no significant effect on virus replication. Because the NS3-crRNA (Figure 1E) showed the greatest viral inhibition (45.54% ± 5.82%) among 10 crRNAs, it was chosen for further investigation.

Specificity of Dengue Viral Inhibition Mediated by NS3-crRNA In Vitro

Since NS3-crRNA is the most effective one to inhibit dengue viral infection using the CRISPR-Cas13a system, further experiments using optimized conditions showed that viral RNA copies in both cells (Figure 1F) and supernatant (Figure 1G) decreased significantly ($p < 0.001$) in the NS3-crRNA-treated group compared with the no-crRNA-treated group (relative cellular RNA, 0.19 ± 0.02 versus 1.00 ± 0.14; supernatant RNA, 8.56 ± 4.23 × 10^6 versus 1.50 ± 0.21 × 10^8 copies/mL), demonstrating the high efficiency of NS3-crRNA to inhibit dengue viral replication. Furthermore, we examined cell viability using the Cell Counting Kit-8 (CCK-8) method to ascertain whether the inhibition of DENV was due to the suppression of cell viability caused by the collateral cleavage of non-specific Cas13a. The results showed that relative to the viability in the control group (noCRISPR-Cas13a system transfection), cell viability was not affected in a statistically significant manner by any of the activated or unactivated Cas13a proteins (Figure S2).

To confirm the above observed inhibition of DENV RNA replication by LwaCas13a/NS3-crRNA (herein called NS3-crRNA), we performed FACS and plaque assays to detect DENV infection on day 3 post-infection, using LwaCas13a without crRNA as a negative control (herein called no-crRNA). FACS analysis by intracellular staining using an anti-DENV monoclonal antibody (mAb) found that the percentage of DENV-positive cells was significantly lower in those treated with NS3-crRNA (17.48% ± 1.83%) than with no-crRNA control (48.80% ± 3.05%) ($p < 0.001$) (Figures 2A–2C). Moreover, a plaque assay (Figures 2D and 2E), which reflects the infectious DENV in supernatant, showed that viruses in the supernatant of the NS3-crRNA-treated group (averaging at 4,000 ± 1,474 focus-forming units (FFU)/mL) were lower than those treated with no-crRNA only (24,333 ± 1,528 FFU/mL) and those from DENV infection only (46,667 ± 8,083 FFU/mL) ($p < 0.001$, for both comparisons). These results indicated that NS3-crRNA not only inhibited dengue viral replication, but it also suppressed the formation of infectious viral particles, which will benefit the inhibition of viral infection in the next cycle of virus replication.

Viral RNA Copies and Plaque Numbers in the Supernatant of NS3-crRNA-Treated Cells Had Different Kinetics Compared with the Control Groups

To further explore the characteristics of the CRISPR-Cas13a system at inhibiting dengue viral replication, we then compared the kinetics of RNA copies and infectious viral particles on days 1, 2, and 3 post-infection (p.i.) after different treatments. Both RNA copy and plaque number in supernatant of the NS3-crRNA-treated group increased with much lower speed than did those of the two control groups. Compared to the no-crRNA treated group, viral RNA copy (Figure 3A) in the NS3-crRNA treated group on day 1 p.i. was 33.5% lower, while the plaque number (Figure 3B) in two groups was not significantly different. Since day 2 p.i., both RNA copy and plaque number in the NS3-crRNA-treated group were significantly lower than those of the no-crRNA-treated group and DENV infection group. Viral RNA copy decreased about 56% and plaque number decreased about 70% on day 2 p.i., further decreasing to 95% for RNA copy and 84% for plaque on day 3 p.i. These results indicated that NS3-crRNA was capable of inhibiting dengue viral infection from the early stage of viral replication, and this significant inhibitory effect remained at least 3 days p.i., suggesting that the efficacy of NS3-crRNA on DENV inhibition probably resulted from efficiently cleaving the targeted viral genome RNA.

NS3-crRNA Cleaved the NS3 Region of DENV with High Efficiency

To further evaluate the efficiency of target region disruption, a pair of target-specific primers, NS3F (forward) and NS3R (reverse), were designed, and the reverse primer was located exactly at the NS3-crRNA target region (Figure 3C). As a control, 3′ UTR-derived primers were used to measure the total viral RNA copies. As shown in Figure 3D, the transcript ratio of 3′ UTR to NS3 in the NS3-crRNA-treated group peaked on day 1 and decreased gradually with time, staring from 19.00 ± 3.46 on day 1 p.i. to 13.49 ± 2.60 on day 2, and to
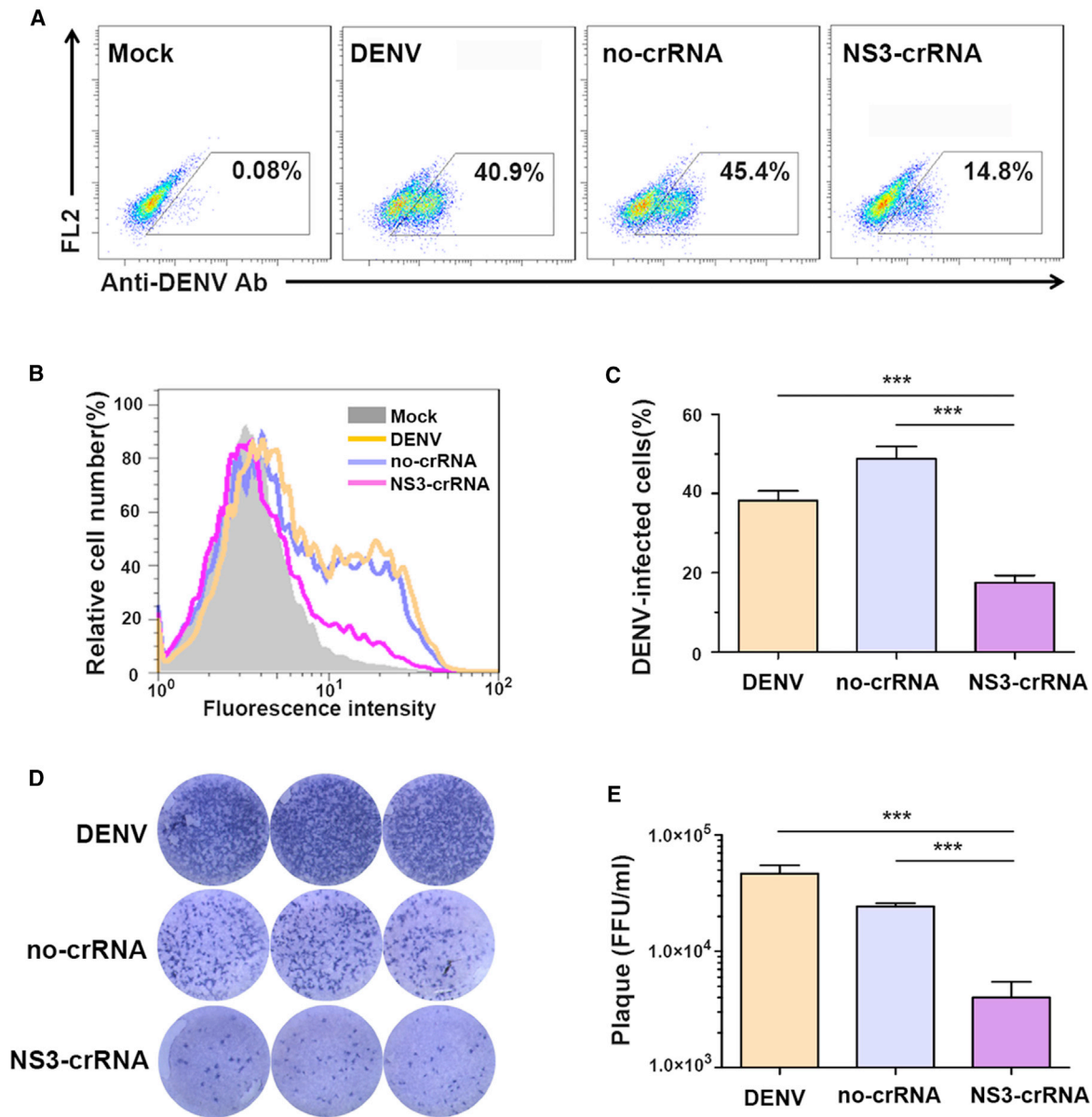
Figure 2. Evaluation of the Inhibitory Effect of NS3-crRNA on DENV Replication by FACS and Plaque Assay

Vero cells were treated according to the description in Materials and Methods. Cell pellets were harvested for infected Vero cell number by FACS. (A) Representative FACS pseudo-color plot of each treatment. (B) Representative FACS results shown as overlay histograms from different treatments. (C) Average percentage of infected cells of each group detected by FACS. (D) Representative data of plaque assay detecting infectious virions in supernatant. (E) Average infectious virions under each condition quantified by plaque assay. ***$p < 0.001$.

3.63 ± 0.44 on day 3. In the two control groups, treated with either no-crRNA or DENV, the ratio of 3′ UTR to NS3 remained at constant low levels over time. Figure 3E directly showed the difference of 3′ UTR and NS3 transcript levels in the NS3-crRNA-treated group compared to that in no-crRNA-treated control (in which viral copy was designated as 1): the relative NS3 transcript level was only 0.043 ± 0.017 on day 1 p.i., and remained at low levels for the next 2 days; in contrast, the relative 3′ UTR transcript level decreased grad-

ually over time, from 0.639 ± 0.065 on day 1 p.i. to 0.146 ± 0.015 on day 3 p.i.. These results indicate that NS3-crRNA significantly and specifically cleaved the viral RNA NS3 target at the first steps of the virus replication cycle, allowing transcripts of other regions to be made at the early steps.

To assess the disruption on the DENV RNA genome after NS3-crRNA/Cas13a transfection, total RNA in supernatants was extracted
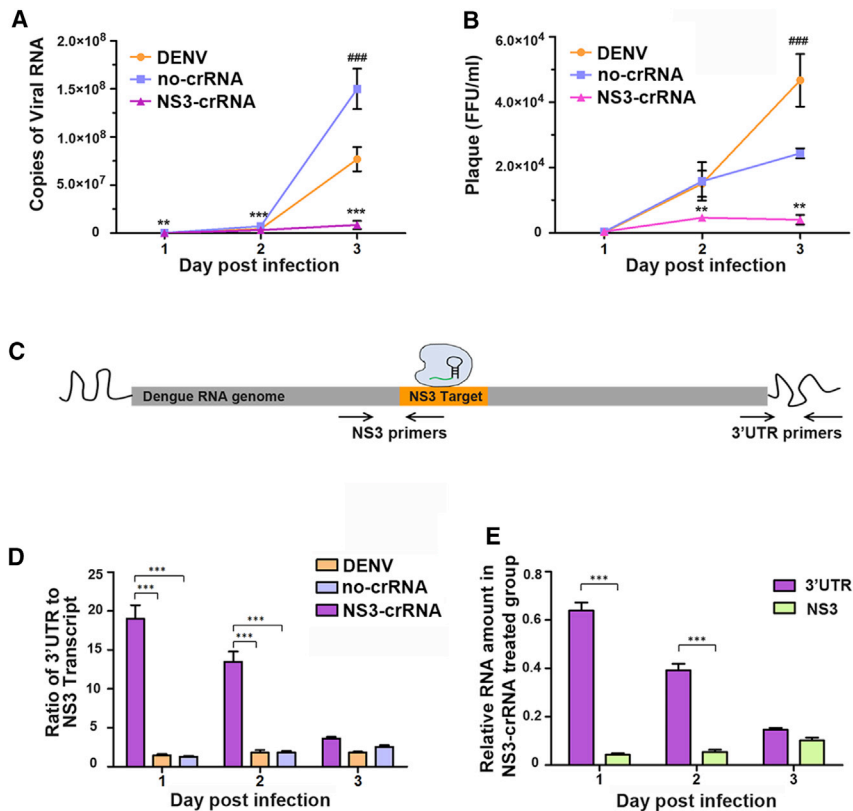
Figure 3. Kinetics of Dengue Viral Replication in Cells under Different Treatment Conditions as Detected by RT-PCR and Plaque Assays

Supernatant from each treatment was collected on days 1, 2 and 3, viral copies were detected by qRT-PCR using two pairs of primers, and infectious virions were measured by a plaque assay. (A) Quantitative RNA copies by RT-PCR using the 3′ UTR primers. RNA copies were expressed as copies/mL. (B) Infectious virions (FFU/mL) by plaque assay. In (A) and (B): **$p < 0.01$, ***$p < 0.001$ for NS3-crRNA versus no-crRNA; ###$p < 0.001$ for NS3-crRNA versus DENV. (C) Schematic of the two pairs of primers: one pair targets 3′ UTR as above, and another pair targets the NS3-crRNA-targeting region. (D) Ratio of RNA copies measured by the 3′ UTR primers to those by NS3 primers at each time point; the higher value indicates a higher number of mutations. (E) Relative RNA copy in NS3-crRNA-treated group at each time point compared with the no-crRNA-treated group; the value of the no-crRNA-treated group was set as 1 at each time point; the lower value stands for a higher level of DENV inhibition. ***$p < 0.001$ between two groups in (D) and (E).

DISCUSSION

CRISPR systems had been demonstrated to efficiently edit DNA sequences and thus they were explored for use in the treatment of genetic diseases and DNA viral replications in the past few years.[9,27–29] The recent establishment of the CRISPR-Cas13a system has expanded its application to RNA editing.[23–25] It is worth mentioning that the RNA-targeting CRISPR-Cas13a system may avoid permanent off-target genetic lesions in DNA-mediated CRISPR-based therapeutics. Here, to extend the utility of the LwaCas13a system for RNA targeting, we examined the antiviral activity of the CRISPR-Cas13a system against dengue viral replication for the first time. Our results showed that the CRISPR-Cas13a system could effectively cleavage viral genome at designed targeting sites, leading to inhibition of viral replication. As a proof-of-concept study, we demonstrated the potential use of the CRISPR-Cas13a system as a novel programmable antiviral strategy.

Because of the length limitation of crRNA, it is critical to determine its specific targeting site. In the absence of any published data regarding its application in DENV, we selected a systematic approach to go over the entire genome to screen for venerable sites through which crRNA targeting may work. We covered each DENV ORF, which together encode 10 mature proteins: three structural proteins, including capsid (C), premembrane (PrM), and envelope (E), and seven nonstructural proteins, including NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. The nonstructural proteins have been investigated as important targets for drug development.[30] Inhibition of viral enzymes has also been proven to be one of the most successful antiviral approaches, as demonstrated by the clinically approved antiviral drugs for HIV, hepatitis C virus (HCV), and other viruses.[6,31] In agreement with these other publications, we demonstrated that targeting the NS3

on days 1, 2, and 3 p.i., the NS3-crRNA target region was amplified using RT-PCR, and PCR products were cloned into the pEasy-T cloning vector. In 4 out of 10 TA clones (2, 3, 4, and 5) on day 1 p.i., the PCR products appeared to have insertion or deletion at the NS3 region (Figure 4A), and the proportion of TA clones containing insertion or deletion of NS3 decreased over time (2 out of 10 on day 2 p.i., and 1 out of 10 on day 3 p.i.). Such insertion or deletion was not observed in either the no-crRNA or DENV control groups (Figure 4A). These results suggest that those virions with specific mutation in the NS3 region at the early stage might lose their infectivity.

To examine the specific sequence changes induced by NS3-crRNA treatment, Sanger sequencing was used. Results showed that deletions ranged from 191 to 404 bp, and an insertion of 24 bp, in the NS3 target region (Figure 4B). Interestingly, the length of deletions at the upstream of the NS3-crRNA target site is much longer than that at the downstream, indicating that the upstream of the target site is more prone to NS3-crRNA cleavage. After analyzing the deletion sequences, we found that not only the NS3 coding region, but also a part of the NS2B coding region, had been deleted, leading to a compromise in their normal transcription and the formation of the NS2B-NS3Pro complex. Thus, NS3-crRNA may inhibit DENV replication by directly acting on the NS3 region and indirectly on other replication machineries.
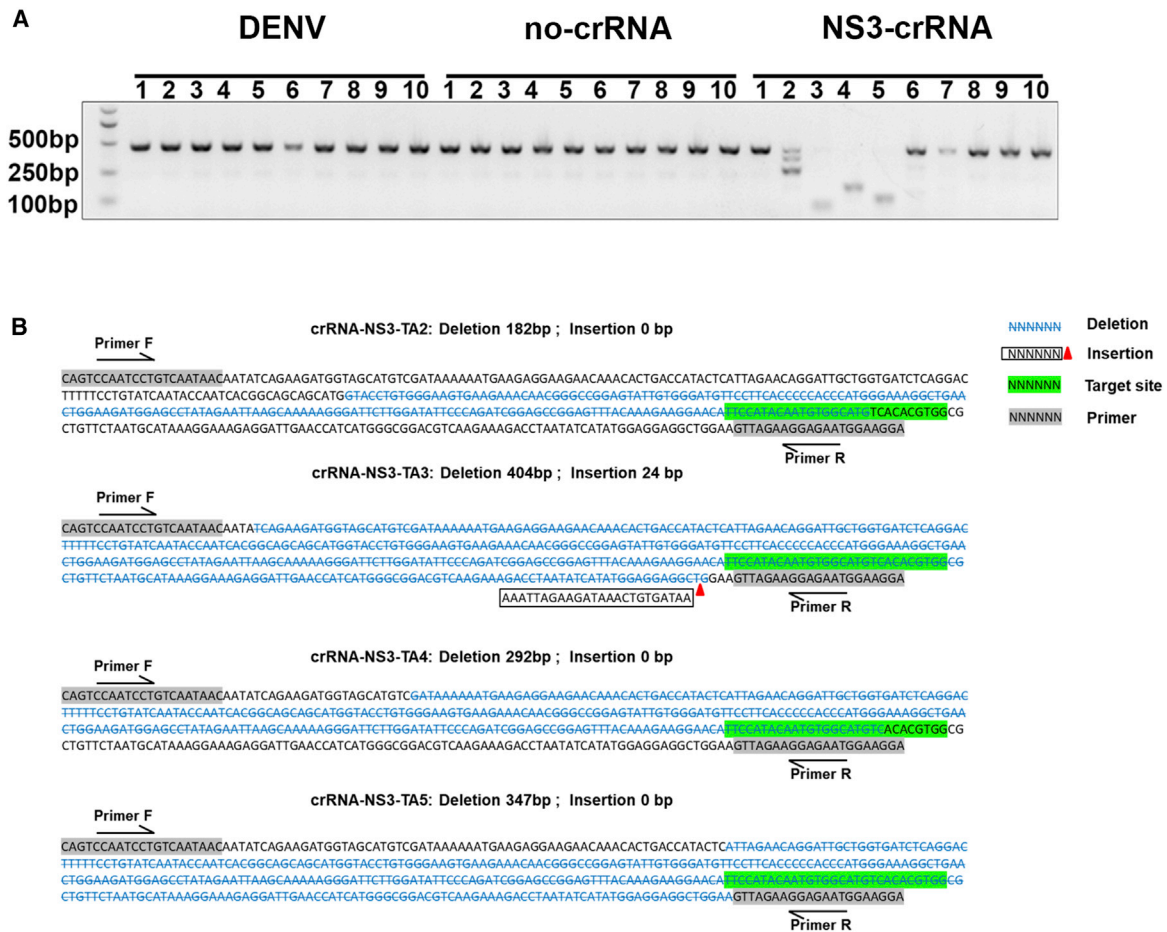
Figure 4. Analysis of Dengue Viral RNA Cleavage by NS3-crRNA

Vero cells infected with DENV-2/16681 at an MOI of 0.1 were transfected with either NS3-crRNA or no-crRNA in a 24-well tissue culture plate. RNA was extracted from supernatants of each group on days 1, 2, and 3 post-infection (p.i.). RT-PCR was performed using a pair of primers targeting NS3, and TA clones were constructed with the PCR products. (A) Agarose gel electrophoresis of PCR products of 10 clones of each treatment. Analyses showed that target genes treated with NS3-crRNA yielded various sizes of DNA products, whereas control samples without treatment with NS3-crRNA had an intact DNA fragment of 500 bp. (B) Four representative sequences derived from 10 clones treated with NS3-crRNA on day 1 p.i., using the Sanger sequencing method. Results show primer sites (gray), the NS3-crRNA targeting site (green), and various sizes of viral RNA genome elimination (range, 191–404 bp, as indicated by strikethrough). The red arrowhead indicates the insertion site.

gene utilizing the CRISPR-Cas13a system led to efficient inhibition of dengue viral replication.

The DENV NS3 gene is 1,854 bp in length, encoding a 618-aa residue protein that has multiple enzymatic activities, including serine protease, helicase, RNA 5′-triphosphatase (RTPase), and adenosine triphosphatase (ATPase) activities. Interactions between NS3 and NS5, NS2B, and NS4B are important for the entire viral life cycle,[30–32] especially viral replication and maturation steps, making it one of the most important targets for antiviral drugs. The N-terminal (1–180 aa) region of NS3 has been identified as the protease domain, together with a peptidic cofactor NS2B to form the NS2B-NS3Pro complex, which has been considered as a primary target for antiviral drugs.[33–35] In our current work, the NS3-crRNA targets nucleotides encoding a 10-aa residue (NS3$_{55-64aa}$, FHTMWHVTRG) of a serine

protease domain, which is highly conserved among the four DENV serotypes,[36] efficiently inhibiting the replication of DENV. As Cas13a protein undergoes further conformational changes since the target RNA is recognized and bound with the crRNA, the specific cleavage site of Cas13a on target RNA may be dependent on its configuration.[22] In this study, the sequencing results of the NS3-targeted region in DENV showed that NS3-crRNA/Cas13a specifically disrupted the crRNA target site ranging from 191 to 404 bp, and the length of deletions upstream of the target site is much longer than downstream of the target. These results suggest that the cleavage of target RNA by CRISPR-Cas13a is directional (Figure 1A). Incidentally, the NS3-crRNA not only deleted a part of the NS3 protease domain, but the deletion also encompasses the NS2B coding region. Taken together, both effects resulted in a greater inhibition of viral replication. Whether the same applies to other human flaviviruses

is yet to be determined, and the underlying mechanisms are unknown at present.

In this study, the plaque assay showed that the infectious DENV in supernatant of the Cas13a-treated group was lower than in the DENV infection group (Figure 2D), whereas the copies of DENV RNA inside cells of the Cas13a-treated group were higher than those from DENV infection (Figure 3A). This might be caused by the lipofection transfection that influenced the formation of infectious viral particles. Additionally, as siRNA derived from virus alone could disrupt the viral genome through Dicer or an antisense effect, to find out whether there is a multiple antiviral mechanism of the CRISPR-Cas13a system, we performed additional experiments to compare the inhibition efficiency of NS3-crRNA alone and that of the NS3-crRNA/Cas13a complex. Interestingly, NS3-crRNA alone also exhibited an antiviral effect as well, although the inhibition effect was slightly lower than that of the crRNA in company with Cas13a protein at the third day after transfection (about 2-fold difference, Figure S1). This indicates that the CRISPR-Cas13a system has multiple mechanisms such as virus-derived siRNA or the antisense effect of RNA interference,[37] except crRNA guided Cas13a disruption in antiviral progress, and further investigations are still needed.

In conclusion, as a proof-of-concept study, we provide results to demonstrate that the CRISPR-Cas13a system could be developed into a novel programmable antiviral strategy *in vitro*. However, an efficient and safe delivery system is a hurdle in developing gene therapy tools. Recently, Wang et al.[38] utilized the CRISPR-Cas13a system to inhibit the formation of glioma intracranial tumors in mice through the lentivirus vector, and Zhao et al.[39] knocked down mutant KRAS mRNA and elicited marked tumor shrinkage in mice by directly injecting the CRISPR-Cas13 RNPs and CRISPRMAX reagents into the tumor tissue. As safe and more practicable delivery systems or formulates with higher efficiency have been well developed, such as a recombinant adenovirus or nanoparticles,[40,41] this reprogrammable-specific antiviral strategy based on the CRISPR-Cas13a system is quite promising for the future.

MATERIALS AND METHODS
Cell Lines and DENV
Vero cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin and streptomycin (P/S), and 100 mg/mL L-glutamine (DMEM-10). DENV-2/16681 (kindly provided by Dr. Richard Kinney, CDC, Fort Collins, CO, USA) was propagated in C6/36 *Aedes albopictus* mosquito cells in minimum Eagle's medium (MEM) as previously reported.[1] The harvested viral culture supernatant was filtered through a 0.2-μm filter and titrated by a plaque assay on Vero cells, then aliquoted and stored at −80°C until further use.

crRNA Preparation and LwaCas13a Protein Purification
Ten 28-base protospacer sequences were derived from the DENV RNA genome (GenBank: U87411), and their reverse complementary DNA oligomers were synthesized as single-stranded DNA (ssDNA)

(Tianyi Huiyuan Biotechnology, Beijing, China) with an appended CRISPR repeat sequence. As templates, these oligomers were amplified with a forward primer (F-T7) and the corresponding reverse primers (Table S1) by PCR. The PCR products were transcribed into crRNAs by incubation with T7 polymerase overnight at 37°C using the HiScribeT7 quick high-yield RNA synthesis kit (New England Biolabs). Then, crRNAs were purified using RNAXP clean beads (Beckman Coulter) at a 2× ratio of beads to reaction volume, with an additional 1.8× supplementation of isopropanol (Sigma).

The LwCas13a expression vector Twinstrep-SUMO-huLwCas13 (pC013) (Addgene, plasmid #90097) was a gift from Dr. Feng Zhang (Harvard University, Boston, MA, USA). It was transformed into Rosetta 2(DE3)pLysS Singles Competent Cells (CoWin Biosciences). Protein expression was induced by supplementation with isopropyl β-D-thiogalactoside (IPTG) (Sigma) to a final concentration of 500 μM, and cells were cooled to 18°C for 16 h for protein expression. The LwCas13a protein was then purified through nickel-chelating affinity chromatography (GE Healthcare), SUMO protease digestion, and a HiTrap SP HP cation exchange column (GE Healthcare) via fast protein liquid chromatography (FPLC) (ÄKTA pure, GE Healthcare) according to the published protocol by Zhang and colleagues.[42] Aliquots of LwCas13a were kept at −80°C in storage buffer (600 mM NaCl, 50 mM Tris-HCl [pH 7.5], 5% glycerol, 2 mM DTT) until use.

DENV Infection
Vero cells were seeded onto a 24-well plate (2.5×10^5 cells per well in 1 mL of solution). On the second day, DENV-2/16681 at a multiplicity of infection (MOI) of 0.1 was added to 90% confluent cells and incubated for 60 min; after the media containing virus were removed, 500 μL of Opti-MEM was added to each well. During the virus adsorption period, a crRNA/Cas13a complex was prepared according to an optimized condition: 0.75 μL of Lipofectamine CRISPRMAX reagent (Life Technologies) and 25 μL of Opti-MEM in tube 1 were mixed well, and 500 ng of Cas13a protein, 125 ng of each DENV-specific crRNA, 0.5 μL of PLUS reagent, and 25 μL of Opti-MEM in tube 2 were mixed thoroughly and incubated at room temperature (RT) within 10 min, and then reagents in tubes 1 and 2 were put together and mixed completely and incubated for 25 min at RT. Then, complexes containing crRNA/Cas13a, Cas13a alone, or crRNA alone were added to each well containing DENV-infected cells and incubated for 4 h; finally, 1.5 mL of DMEM containing 2% FBS, 1% penicillin and streptomycin, and glutamine were added to each well, and cells were incubated at 37°C, 5% CO_2 for 24, 48, or 72 h. Cell pellets were harvested for detecting viral RNA copies by RT-PCR, enumeration of the number of DENV-infected Vero cells was accomplished by FACS, and culture supernatants were collected for detecting viral RNA copies by qRT-PCR and infectious virions by a plaque assay.

qRT-PCR
To quantify the DENV RNA, we first use a plasmid pEZ-3′ UTR that contained the 203 bp of the 3′ UTR gene as a standard to quantify

DENV RNA that was extracted from the supernatant of the DENV-infected Vero cells, thus obtaining the RNA copies in 1 μL; this quantified DENV RNA was used as the standard throughout our experiments. Viral RNA was extracted either from supernatant using the QIAamp viral RNA mini kit (QIAGEN), or from the cell pellet using the QIAamp RNA mini kit (QIAGEN), after which one-step real-time RT-PCR was performed using the Takara One-Step SYBR Prime-Script RT-PCR kit (Takara Biotech, Dalian, China) on an Eppendorf Realplex4 (Eppendorf, Hamburg, Germany). The reactions started at 37°C for 15 min, 85°C for 5 min for reverse transcription, and then 95°C for 30 s for denaturing, followed by 40 cycles of amplification at 95°C for 10 s and 60°C for 30 s. The primers were designed to target the highly conserved region of the 3′ UTR as follows: forward, 5′-AAGGACTAGAGGTTAGAGGAGACCC-3′ (nucleotides 10589–10613), reverse, 5′- CGTTCTGTGCCTGGAAT GATG-3′ (nucleotides 10697–10677),[43] β-Actin was used as an internal control for the amounts of cellular RNA.

FACS

The percentage of DENV-infected Vero cells was detected by intracellular staining of viral protein using FACS. Briefly, adherent Vero cells infected with DENV for 72 h were removed from each well with trypsin-EDTA, washed with PBS once, and subsequently treated with 1× BD lysing solution (BD Biosciences) and permeabilization buffer (eBioscience) at RT for 10 min; then, anti-DENV mAb D1-11 (Abcam) at 1:500 dilution was added to each tube for 40 min at RT. Following washing once with PBS, the cells were incubated with 1:200 diluted anti-mouse IgG-FITC (Abcam ab6785) for 30 min at RT, then washed once with PBS, fixed with 4% paraformaldehyde solution, and subjected to FACS analysis (Millipore, Guava easyCyte HT flow cytometer). Data were analyzed using FlowJo software.

Plaque Assay

Supernatants from different samples were 10-fold serially diluted (starting from 4-fold diluted supernatant) and then were added to Vero cells in a 96-well plate. After incubation at 37°C for 90 min to allow for virus attachment, 200 μL of 1.5% methylcellulose solution dissolved in DMEM supplemented with 2% fetal calf serum (FCS) was plated to each well. After incubation at 37°C for 4 days, the cell monolayer was treated with 4% paraformaldehyde and 0.1% Triton X-100 Tris-buffered saline (TBS), then blocked with 3% BSA-TBS for 20 min at RT, washed with PBS, and specific antibody D1-11 (Abcam ab9202, 1:1,000), m-IgGκ BP-B (Santa Cruz, 1:500), and alkaline phosphatase (AP)-streptavidin conjugate (Thermal Fisher Scientific, 1:1,000) were sequentially added to the plates with 2 h of incubation at RT for each reagent. Finally, BCIP (5-bromo-4-chloro-3-indolyl phosphate)/NBT (nitroblue tetrazolium)-plus (Mabtech) filtered with a 0.4-μm filter was incubated with the cell monolayer in the dark for 15–30 min to form virus foci. The titer of virus in the supernatants was expressed as FFU/mL.

Analysis of Genomic Region Modified by CRISPR-Cas13a

Two methods were utilized to analyze the efficiency of NS3-crRNA cleavage on target viral RNA. One method is qRT-PCR using a pair of specific primers targeting the NS3 segment (crRNA) (NS3 forward, 5′-ACTGGAAGATGGAGCCTATA-3′, NS3 reverse, 5′-AC ATGCCACATTGTATGGAA-3′). Because only the uncleaved RNA can be detected normally, the cleaved RNA will not be detected, thus indirectly reflecting the cleavage efficiency. As a control, 3′ UTR-derived primers were used to evaluate the replication of DENV as described above. The quantity of DENV RNA was used as a standard.

Another method is sequencing the RT-PCR products of dengue viral RNA extracted from supernatants of each treatment well. In brief, the dengue viral RNA genome in the supernatant of each group was extracted using the QIAamp viral RNA mini kit (QIAGEN) following the manufacturer's protocol. The RNA genomic region of NS3 in cell culture supernatant was reverse transcribed and amplified using a One-Step PrimeScript RT-PCR kit (Takara) with 5′-CAGTCCAATCCTGTCAATAAC-3′ (sense) and 5′-TC CTTCCATTCTCCTTCTAAC-3′ (antisense) as primers. The PCR products were ligated into T-cloning vector pEasy-T (TransGen Biotech), and 10 clones in each group were selected for Sanger sequencing.

Statistical Analysis

In each testing program, we used four biological replicates, and each experiment was repeated at least three times. Results are presented as either mean ± SD or geometric mean and error. To compare the statistical differences among experimental groups, a one-way or two-way ANOVA was used. A p value less than 0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION

AUTHOR CONTRIBUTIONS

Z.K., H.S., X.J., Y.Z., and H.L conceived this study. Z.K. and H.L. designed the experiments. H.L., Z.K., X.D., S.W., M.L., Q.L. Y.G., and J.L performed the experiments. H.L. and Z.K. conducted statistical analyses. H.L., Z.K., and X.J wrote the manuscript. H.S. and Y.Z. revised the manuscript.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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