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SHORT COMMUNICATION

# Subculturing cells have no effect on CRISPR/Cas9-mediated cleavage of UL30 gene in pseudorabies virus

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## Abstract

CRISPR/Cas9-mediated genome editing can inhibit virus infection by targeting the conserved regions of the viral genomic DNA. Unexpectedly, we found previously that pseudorabies virus (PRV) could escape from CRISPR/Cas9-mediated inhibition. In order to elucidate whether the escape of PRV from Cas9-mediated inhibition was due to cell deficiencies, such as genetic instability of sgRNA or Cas9 protein, the positive cells were passaged ten times, and PRV infection in the sgRNA-expressing cells was evaluated in the present study. The results showed that subculturing cells has no effect on Cas9-mediated cleavage of PRV. Different passages of PX459-PRV cells can stably express sgRNA to facilitate Cas9/sgRNA cleavage on the UL30 gene of PRV, resulting in a pronounced inhibition of PRV infection. Studies to elucidate the mechanism of PRV escape are currently in progress.

KEYWORDS
CRISPR/Cas9, pseudorabies virus (PRV), single-guide RNA (sgRNA), UL30 protein

## 1 | INTRODUCTION

CRISPR Cas9-mediated genome editing can inhibit virus infection by targeting the conserved regions of the viral genomic DNA.[1-6] Previously, we constructed a cell line stably expressing Cas9 endonuclease and sgRNA targeting the conserved UL30 gene of pseudorabies virus (PRV), and found that the sgRNA could specifically target and cleave the conserved UL30 gene, resulting in the robust suppression of PRV infection.[7] Unexpectedly, PRV could escape from CRISPRCas9-mediated inhibition.[7] There have been reports that Cas9-mediated cleavage can be disrupted by cell factors, such as interactions between genomic context and internal gRNA,[8] type I interferons,[9] and anti-CRISPR proteins.[10] In order to elucidate whether the escape of PRV from Cas9-mediated inhibition was due to cell deficiencies, such as genetic instability of sgRNA or Cas9 protein, the positive cells were passaged 10 times and PRV infection in the sgRNA-expressing cells was evaluated in the present study.

Lin-zhu Ren and Zhi-yuan Peng contributed equally to this work.

## 2 | METHODS

Previously, we found that there was no significant difference among PK-15, PX459, and PX459-PRV cells, and the expression of sgRNA had no notable effect or toxicity on cell viability.[7] In the present study, sgRNA-expressing cells (PX459-PRV) and control cells (PX459 and PK-15) were seeded onto 12-well plates. At 70%-80% confluency, the cells were infected with 500 μL of PRV (6 $TCID_{50}$). At 1 h post-inoculation (hpi), the cells were washed 3 times with 200 μL of PBS and maintained in fresh DMEM supplemented with 2% FBS at 37°C and 5% $CO_2$.The cytopathic effect (CPE) was observed at 0, 32, 48, and 72 hpi.

In order to evaluate whether the UL30 gene could be consistently edited by specific sgRNA, PX459-PRV, PX459 or PK-15 cells were serially passaged 10 times onto 60-mm dishes. Western blot was performed to examine the expression of Cas9 protein. Then, cells from passages 0, 2, 4, 6, 8, 10 were infected with PRV for 12 hours and the effect of the sgRNA on PRV infection was determined using real-time PCR and titration assays. To analyze the Cas9-mediated disruption of PRV DNA, viral DNA was extracted from passages 2 and 10 of the PRV-infected cells, and amplified. PCR products were purified and sequenced directly.

## 3 | RESULTS AND DISCUSSION

Comparing experiments on cells infected by PRV with control experiments, the results showed that CPEs were obvious in the control cells PX459 and PK-15 at 32, 48 and 72 hpi, while no CPE could be observed in PX459-PRV cells before 72 hpi (Figure 1), indicating that the PRV-induced cytopathic effect can be delayed in PX459-PRV cells. Furthermore, although the same cell densities were seeded on the plates, cell density increased in PX-459-PRV cells from 0 to 72 hpi, while cell densities decreased in PX-459 and PK-15 cells. Therefore, a slight CPE could be monitored in PX-459-PRV cells at 72 hpi because of the higher density. These results demonstrated that replication of PRV was markedly inhibited in PX459-PRV cells. At the same time, the results showed that Cas9 proteins were highly expressed in different passages of PX459-PRV and PX459 cells, while no obvious band could be detected in each passage of PK-15 cells (Figure 2A). The results also showed that PRV replication and yield were significantly decreased in each passage of PX459-PRV cells compared with that of PX459 and PK-15 cells (Figure 2B-C), indicating that the UL30-specific sgRNAs were stably expressed in each passage, and the UL30 gene of PRV was efficiently edited by the CRISPR Cas9 system, resulting in a pronounced decrease in viral replication.

As expected, the sequencing chromatogram of PCR products amplified from passages 2 and 10 of the PX459-PRV cells infected with PRV showed overlapping peaks, while those from PRV-infected PK-15 and PX459 cells showed well-formed and distinctive single colored peaks (Figure 2D). Moreover, the peaks overlapped from the target site to the end of the amplicon. These results demonstrated that multiple PCR products were amplified from passages 2 and 10 of the PRV-infected PX459-PRV cells, indicating that the viral DNA was successfully edited by the Cas9-mediated cleavage in different passages of PX459-PRV cells.

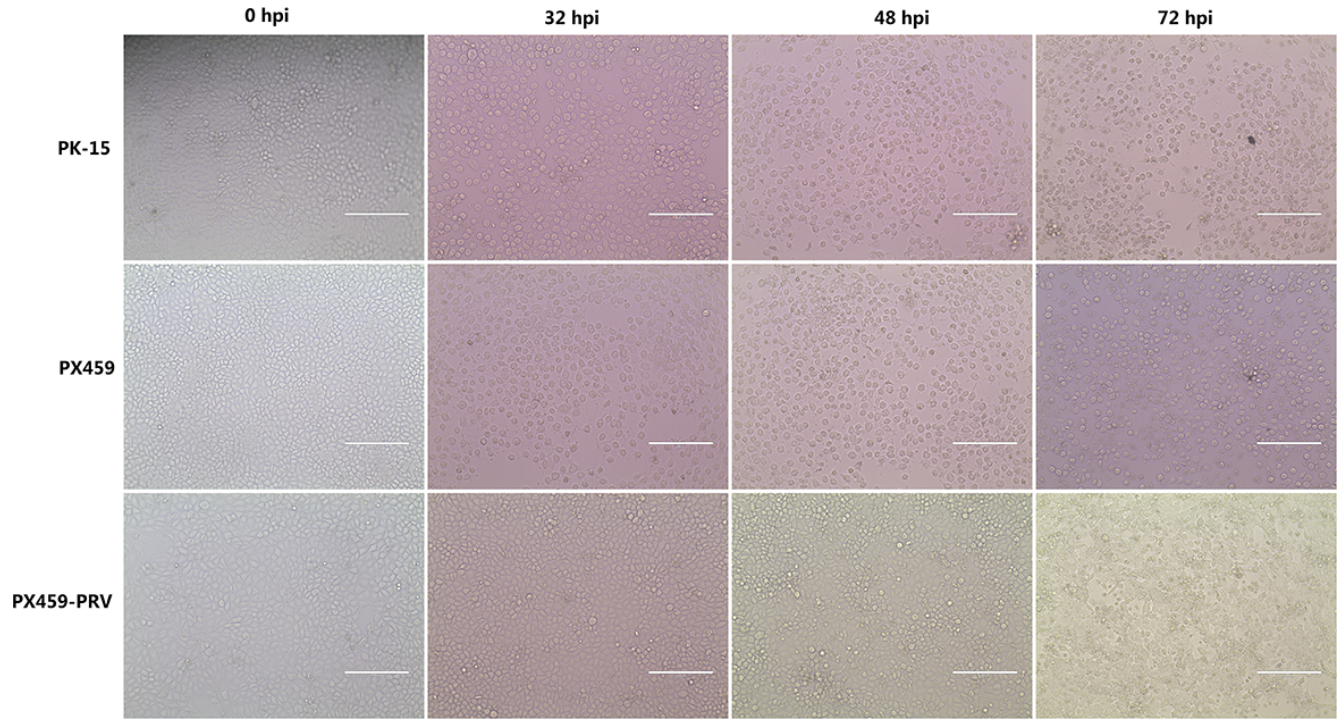


**FIGURE 1** Cytopathic effect. sgRNA-expressing cells (PX459-PRV) and control cells (PX459 and PK-15) were seeded onto 12-wells plate. At 70%-80% confluency, the cells were infected with 500 μL of PRV (6 $TCID_{50}$). At 1 h post-inoculation (hpi), the cells were washed three times with 200 μL of PBS and maintained in fresh DMEM supplemented with 2% FBS at 37°C and 5% $CO_2$. The CPE was observed at 0, 32, 48, and 72 hpi

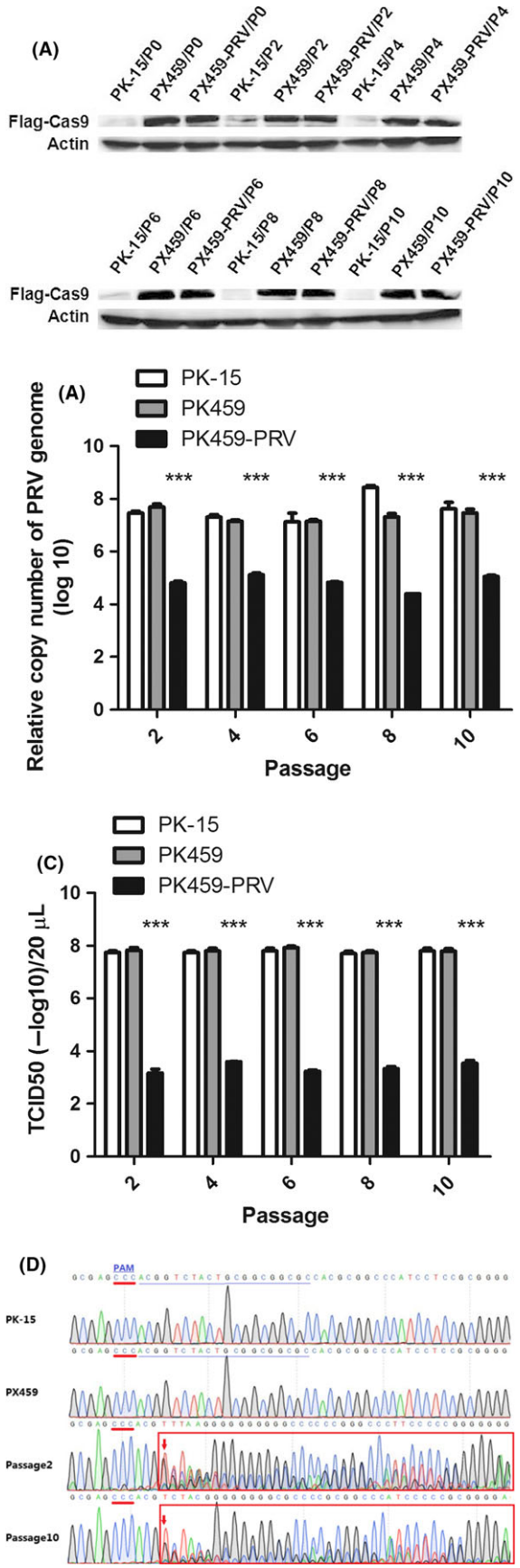


**FIGURE 2** Replication of PRV was inhibited in the cells stably expressing the specific sgRNA. PX459-PRV, PX459 or PK-15 cells were serially passaged 10 times onto 60-mm dish. Western blot was performed to examine the expression of Cas9 protein using Flag tag Antibody Rabbit polyclonal (Proteintech Group Inc., Wuhan, China) as primary antibody and HRP-conjugated goat anti-rabbit IgG (Beyotime, Jiangsu, China) as secondary antibody. Cells from passages 0, 2, 4, 6, 8, 10 were infected with PRV for 12 h and the effect of the sgRNA on PRV infection was determined using real-time PCR and titration assays according to the protocol described previously.[7] (A), Expression level of Cas9 Protein in each cell passage. (B), Copy number of PRV genome. (C), $TCID_{50}$. (D), Chromatogram characteristic of PCR products. Viral DNA was extracted from cells, and amplified using PRV-30-F3 and PRV-30-R, according to the protocol described previously.[7] PCR products were purified and sequenced directly

It has been reported that there are at least 2 opposite outcomes of Cas9/sgRNA cleavage, inactivation of the virus and acceleration of viral escape.[11] Nucleotide insertions, deletions, and substitutions around the Cas9/sgRNA cleavage site are typical for DNA repair by the nonhomologous end-joining pathway (NHEJ pathway).[12] Therefore, studies to elucidate the mechanism of PRV escape are urgently needed. We report here that subculturing cells has no effect on Cas9-mediated cleavage of PRV and we are currently investigating the mechanism of the NHEJ pathway involved in PRV escape. The occurrence of viral escape from CRISPR Cas9-mediated inhibition may limit the use of Cas9/sgRNA in virus control or therapy.[7] Combinations of two strong sgRNAs targeting different regions of the viral genome, or RNAi and CRISPR Cas9 can completely abrogate viral replication and prevent viral escape.[13,14] Therefore, research into the combination of 2 strong sgRNAs targeting different regions of the PRV genome is ongoing.

In conclusion, subculturing cells has no effect on Cas9-mediated cleavage of PRV. PX459-PRV cells from different passages can stably express sgRNA to facilitate Cas9/sgRNA cleavage on the UL30 gene of PRV.

## CONFLICT OF INTEREST

None.

## AUTHOR CONTRIBUTIONS

JYB, DXP, and HSO. conceived and designed the study; LZR, ZYP, XHL, XRC, LY, JWF and TO carried out experimental work and data analysis; LZR and JYB wrote the initial draft of the manuscript. All authors contributed to revising the manuscript. All authors gave final approval for publication.

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