



## CRISPR/Cas-9 vector system: targets UL-39 and inhibits *Simplexvirus humanalpha1* (HSV-1) replication *in vitro*

Jéssica Vasques Raposo<sup>1</sup>, Luciana Rodrigues Carvalho Barros<sup>2,4</sup>, Lauana Ribas Torres<sup>1</sup>,  
Rafaela Barbosa da Silva Pinto<sup>1</sup>, Amanda de Oliveira Lopes<sup>1</sup>, Elen Mello de Souza<sup>1</sup>, Martin Hernan Bonamino<sup>2,3</sup>,  
Vanessa Salete de Paula<sup>1\*</sup>

<sup>1</sup> Molecular Virology and Parasitology Laboratory, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil

<sup>2</sup> Program of Immunology and Tumor Biology. Division of Experimental and Translational Research. Brazilian National Cancer Institute – INCA

<sup>3</sup> Vice Presidency of Research and Biological Collections. Fundação Oswaldo Cruz - FIOCRUZ

<sup>4</sup> Faculdade de Medicina FMUSP, Universidade de Sao Paulo, Sao Paulo, SP, BR

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### ABSTRACT

*Simplexvirus humanalpha1* (HSV-1) affects approximately 67% of the world's population. Here, we sought to use the CRISPR / Cas9 system with the UL39 target, essential for virus replication. The sgRNA sequence was inserted into the plasmid (PX459-UL39). Vero cells were transfected with PX459-UL39, and inhibition of viral replication was assessed 24 and 48 hours later using plaque assays and fluorescence and qPCR. Fluorescence analyses revealed the presence of anti-HSV-1 CRISPR/Cas9 within Vero cells, and qPCR showed that the viral load decreased by > 95% of cells transfected with anti-HSV-1 CRISPR / Cas 9. Our data demonstrate the usefulness of the PX459-UL39 to inhibit HSV-1 infection.

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### Introduction

*Simplexvirus humanalpha1* (HSV-1) infection occurs via small lesions in the epithelium, especially around mucous membranes. Once inside the body, the virus establishes a productive infection that results in a localized lesion (1). These can occur around the lips and/or genitals, where they are referred to as cold sores. HSV-1 produces an acute primary infection (90% of the time asymptomatic), followed by the establishment of latent infection in the sensory ganglia, with periods of recurrence. Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated (CRISPR/Cas), an ancient antiviral system that was discovered in bacteria has shown tremendous potential as a precise, invariant genome-editing tool (2). Since it was discovered with a genetic editing technique, the CRISPR/Cas9 mechanism as an antiviral has been used frequently in studies *in vitro* and *in vivo*. Some studies have already used this technique to inhibit HBV, EBV, HPV and even HSV-1 (3-5) Recent data suggest that HSV-1, even during lytic infection, may be an excellent target for treatment using a CRISPR/Cas-based approach (2). One of these proteins is ICP6, transcribed from the UL39 gene encoding the larger subunit of the enzyme ribonucleotide reductase, which functions to convert ribonucleotide into deoxyribonucleotides (6). Here, we constructed a CRISPR/Cas9 anti-HSV-1 complex that targets UL39.

### Materials and Methods

Vectors based on PX459 (Addgene, #62988) containing the sgRNA were constructed. sgRNA, formed by annealing forward and reverse gRNA-17-UL39, was cloned into the *BbsI* (Bpil; ThermoFisher Scientific, MA, USA) restriction site in the PX459 plasmid according to the Ran protocol (7). Cloned plasmids were transformed into competent DH5a *Escherichia coli*, after which single clones were isolated and evaluated by restriction enzyme digestion and Sanger-based sequencing. The 41-bp target UL39 sequence (oligonucleotides pRGS-CR-GFP).

To determine the effect of CRISPR/Cas9 targeting of UL39 during HSV-1 replication, we infected VERO cells with HSV-1 and subsequently transfected them with pRGD-UL39 plasmid. Specifically, Vero cell cultures were infected with HSV-1 at MOIs of 0.1 and 0.001 for 24 h at 37 °C and then were transfected with a pRGD-UL39 plasmid containing an out-of-frame eGFP sequence. The ability of the CRISPR-carrying plasmid to edit the DNA sequence of the target UL39 was evaluated by confirming eGFP expression derived from indels, which promote in-frame GFP expression. Because eGFP was originally out of frame, one-third of genetic modifications in pRGD-UL39 would place the eGFP gene in-frame, leading to eGFP expression.

For each sample to be transfected, the DNA (0.5 - 5 µg / µL) was diluted in 50 µL of serum-free medium. Li-

\* Corresponding author. Email: [vdpaula.fiocruz@gmail.com](mailto:vdpaula.fiocruz@gmail.com)

pofectamine 2000 (Thermo Fischer) was also diluted in 50 µL of medium and incubated for five minutes at room temperature. Then, the diluted DNA was combined with Lipofectamine 2000, forming a total volume of 100 µL. The DNA + Lipofectamine 2000 complex was mixed gently and incubated for twenty minutes. The complex was added to each well that already contained cells and culture medium. To ensure the mixing and functioning of the complex, the plate was incubated at a temperature of 37 °C, with the presence of CO<sub>2</sub>, for 24 hours.

According to manufacture instructions for the lipofectamine 2000 transfection kit, the reaching of ~80% confluence and density of 1x10<sup>5</sup> cell/well were confirmed before the cells were infected with HSV-1 (10<sup>9</sup> copies/mL and 10<sup>7</sup> PFU/mL) at MOIs of 0.1 and 0.001 for 1 h, and 24 h later were transfected with pRGD-UL39 plasmid (0.5–5 µg/ µL) using Lipofectamine. Infected/ non-transfected cultures, uninfected/ transfected cultures and uninfected/ non-transfected cultures were used as controls. The culture supernatants were collected, and cells were fixed.

After transfection with the pRGD-UL39 plasmid, variously treated VERO cells plated on coverslips were fixed in 4% paraformaldehyde. The coverslips were washed three times with 1X phosphate-buffered saline (PBS) and incubated with 10 µg/mL of 4,6-diamidino-2-phenylindol (DAPI; Sigma-Aldrich, St. Louis, MO, USA) for 5 min to stain cell nuclei. Coverslips were subsequently slide-mounted with 2.5% 1,4-diazabicycles (2,2,2)-octane (DABCO; Sigma-Aldrich) and immediately analyzed using an AxioObserver photomicroscope (Zeiss Inc., New York, NY, USA) equipped with a 40x objective lens.

Cell viability was assessed using MTT (3-[4,5-dimethylthiazol-2-Yl]-2,5-diphenyltetrazolium bromide) assays. Uninfected Vero cells were seeded into 96-well plates at 30 x 10<sup>3</sup> cells/well and 24 h after plating were incubated with different amounts (0.1, 0.2, 0.4 and 0.6 µL/well) of Cas9 master mix for 24 and 48 h at 37°C. Thereafter, cells were incubated with the MTT reagent for 4 h at 37°C, and cell viability, expressed as a percentage, was assessed by measuring the color intensity of formazan crystals produced by metabolically viable cells at 570 nm using a spectrophotometer. Vero cells were plated onto 96-well plates at 10 x 10<sup>4</sup> cells/well in 500 µL of growth medium.

Inhibition of HSV-1 replication was assessed by directly measuring a 92-bp HSV-1 DNA fragment by quantitative polymerase chain reaction (qPCR), using previously described UL39 gene primers and probe-target; results were quantified by reference to a standard curve to gD HSV-1 region (8) qPCR was performed in a total volume of 20 µL using the AgPath-ID PCR kit (Life Technologies, Carlsbad, CA, USA). Each reaction consisted of 12.5 µL of 1x buffer (PCR Buffer), 2.5 µL of sense primers (1 µM),

2.5 µL of antisense primers (1 µM), 2 µL (0.4 µM) of the specific probe (Table 1) and 1 µL of 1x enzyme. In addition, to test samples, qPCR was performed on negative controls and known quantities of the 92-bp HSV-1 DNA fragment used to generate the standard curve.

Single guide RNAs (sgRNAs) targeting the sequence of UL39 were designed using the online tool, CRISPOR (<http://crispor.tefor.net>). The genomic region surrounding UL39 targeted by the CRISPR guide RNA, gRNA-17 (gRNA-17-UL39), was amplified using primers synthesized by Integrated DNA Technologies (IDT), as described in Table 1. Inhibition of viral replication was assessed using plaque assays and fluorescence and qPCR analyses.

## Results

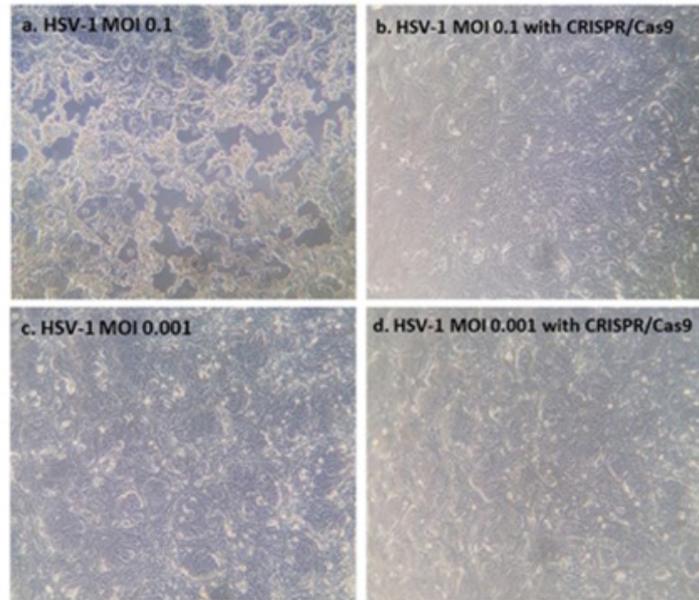
Cytotoxic effects of pRGD-UL39 plasmid on VERO cells were assessed 24 and 48 h post-transfection (hpt) by MTT assay. The results of MTT cell viability assays revealed negligible decreases in the percentage of viable cells compared with controls at 24 hpt (0.5%) and 48 hpt (2.6%), indicating that the pRGD-UL39 plasmid is relatively non-toxic. A light microscopy analysis at 48 hpt showed that most cells exhibited no changes in morphology and revealed very few floating dead cells. Viral growth kinetics were determined over a single infection cycle. Viral load at 48 hpi, measured by qPCR, was substantially reduced in cells in which UL39 was targeted by our CRISPR construct. In the absence of HSV-1, viral growth was higher in cells infected at an MOI of 0.1 than in those infected at an MOI of 0.001 (Figura 1a). At both MOIs, no replication of HSV-1 was detected by qPCR (two replicate experiments) in cells transfected with the pRGD-UL39 plasmid carrying the CRISPR/Cas9 anti-HSV complex (Figure 2).

We further showed that it is capable of inhibiting HSV-1 replication and thus is a potential candidate for herpes therapy. For infection, the medium was removed and Vero cells at ~80% confluence were infected with HSV-1 strain KOS for 1 h at a multiplicity of infection (MOI) of 0.1 and 0.001 in RPMI medium at 37°C in a humidified 5% CO<sub>2</sub> environment. Thereafter, cell cultures were maintained at 37°C for 24 and 48 h in 5% CO<sub>2</sub>, and the cytopathic effect was assessed by light microscopy. The culture supernatant was subsequently collected, centrifuged at 250 × g for 10 min to remove cellular debris, and stored at -70°C. The eGFP fluorescence monitored 24 h after transfection showed that cells were efficiently transfected and the UL39 target sequence was successfully edited (Figure 1b). From these data, we conclude that UL39 targeting by our CRISPR-carrying plasmid inhibits HSV-1 replication *in vitro*.

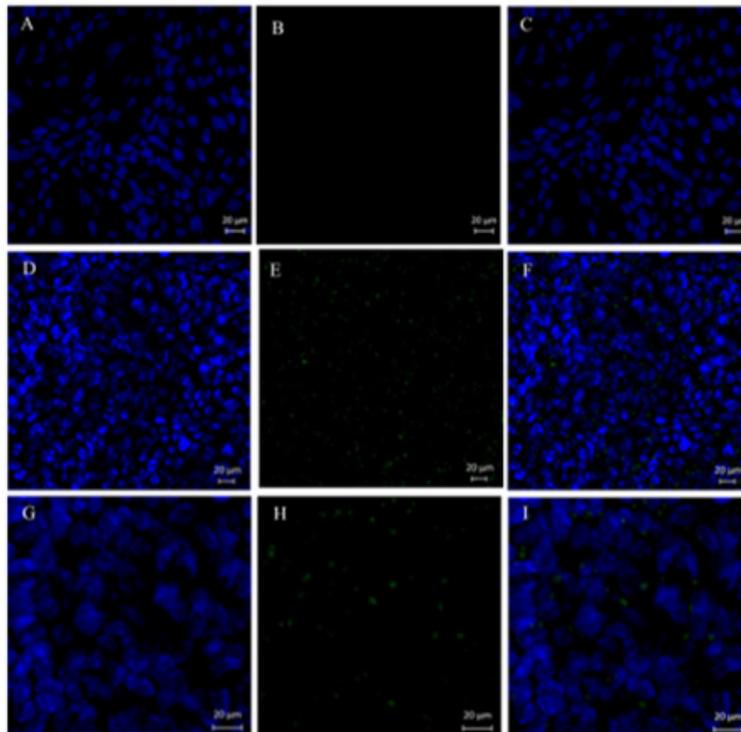
**Table 1.** Oligonucleotides were used in the study.

Name	Sequence
gRNA 17 UL-39 forward	5' CACCGCGCTTCGACGGGAGAGGATG 3'
gRNA 17 UL-39 reverse	5' AAACCATCCTCTCCCGTCGAAGCGC 3'
pRGS-CR-GFP forward	5' GGACATCACCTCCCACAAC 3'
pRGS-CR-GFP reverse	5' GTTTACGTGCGCCGTCCAG 3'
Primer HHV-1 UL-39 forward	5'-CCAAGTGCACCATGATCATCGA-3'
Primer HHV-1 UL-39 reverse	5'-GATGTTTGTACCCGCAACGAA-3'
HHV-1 UL-39 probe	FAM 5'-CCCGCGCACACGTC-3' MGB

**Figure 1a**



**Figure 1b**

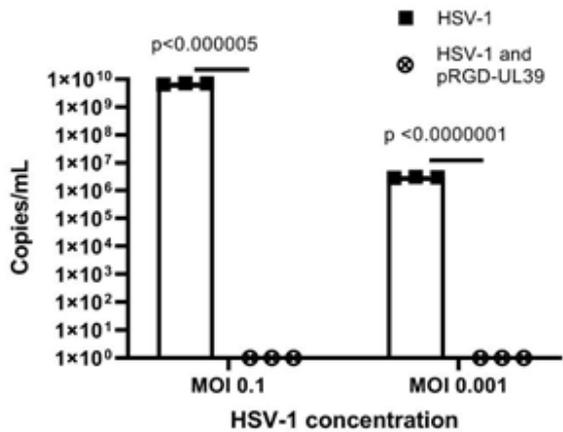


**Figure 1.** **Figure 1a.** Suppression of HSV-1 replication in Vero cells transfected with the pRGD-UL39 plasmid. (a and c) Untreated Vero cell infected with HSV-1. (b and d) Vero cells infected with HSV-1 and treated with pRGD-UL39. **Figure 1b.** GFP fluorescence in Vero cells. (a, b and c) Uninfected Vero cells transfected with pRGD-UL39 plasmid, showing DAPI-stained cell nuclei (blue) (a); absence of GFP (green), indicating no shift in the GFP reading frame (b); and merged image (c). (d, e and f) VERO cells transfected with pRGD-UL39 plasmid and infected with HSV-1, showing DAPI-stained cell nuclei blue (d); the presence of green fluorescence, indicative of effective shifting of the GFP reading frame(e); and merged image (f). (g, h and i) Higher-magnification images (magnification, 40×) of panels d (g), e (h) and f (i).

## Discussion

The standard treatment for herpes is antiviral drugs, but the continued use of these compounds can lead to the development of viral resistance, especially in immunocompromised patients. Previous studies (9-13) have shown

that the use of the CRISPR/Cas9 system is a promising approach for investigating the structure and functions of important HSV-1 genes, including in viral replication. Although some CRISPR/cas9 anti-HSV-1 studies have been previously reported, the current understanding of viral proteins and their functions is far from complete, accounting



**Figure 2.** Changes in the load viral of HSV1-infected cells in cultures transfected with pRGD-UL39 plasmid. Control, non-transfected Vero cells infected with HSV1 at MOI 0.1 and 0.001 PFU/cell.

for difficulties in treating diseases caused by a herpesvirus and developing effective vaccines against them; these deficiencies also highlight the need for more research in this area (14). The gRNA component of CRISPR/Cas9 directs Cas9 to the target DNA site containing a complementary sequence, where it promotes double-strand cleavage, thereby potentially preventing the production of viral particles containing the complete genome or cleaving genes that produce proteins essential for viral replication (11, 15, 16) as previously described, other genetic editing techniques used for HSV-1 have efficiencies of less than 1%, whereas the CRISPR/Cas9-mediated mutagenesis system is more than 50% efficient (17). In the current study, we tested the ability of the CRISPR/Cas9 system to suppress HSV-1 infection through inhibition of HSV-1 replication and the viral load decreased in > 95% of cells transfected with CRISPR/Cas9 anti-HSV-1. Inhibition of HSV-1 has previously been demonstrated by Roehm et al., who targeted ICP0, an immediate early protein that participates in the induction of HSV infection by activating the expression of viral and cellular genes (13) and Karpov et al. similarly demonstrated HSV-1 inhibition by targeting three genes—UL8, UL29, and UL52—involved in HSV-1 replication during lytic infection (14). In contrast to these other studies that used CRISPR/Cas9 as a genetic editing technique to block HSV-1 replication (13,14), we chose the ICP6 protein, encoded by the UL39 gene, as our target for editing. This gene was chosen because it encodes the larger subunit of the ribonucleotide reductase enzyme, which functions to convert ribonucleotides into deoxyribonucleotides and thus is directly related to viral replication (6). The limitation of our study was not using a scrambled nucleotide sequence of the UL39.

Accordingly, inhibition of ICP6 expression is expected to interfere with the replication of HSV-1. A previous study conducted by our research group specifically targeted the HSV-1 UL39 gene using the small interfering RNA (siRNA) technique in BALB/c mice infected with HSV-1 and herpetic encephalitis showed that the inhibition of viral replication in the brain of mice were from 43.6 to 99.9%; it also decreased symptoms of the disease and increased survival of the animals (8). These results confirm that UL39

is an essential region for HSV-1 replication, and thus is a viable target of CRISPR-based therapies. The choice of the gRNA sequence is a very important determinant of the effectiveness and specificity of CRISPR/Cas9 system activity. In the present study, gRNA sequences were designed using CRISPOR software, which calculates all available scores and allows the most appropriate selection for the specific test (18). The selected sequences yielded good scores, bound closer to the beginning of the target sequence, 1 off-target was found (0-0-0-1-0) in the position strand 938 FW and MIT specificity of 100. Overall, the cytotoxicity of CRISPR and reporter constructs following transfection was negligible (0.5% and 2.6% at 24 and 48 hpt, respectively), a finding in line with previous reports (14) that supports the use of Vero cells for functional assays of HSV-1 productive infections.

The efficiency of transfection was evaluated by detecting plasmid-derived GFP in Vero cells. Cells that were infected with HSV-1 and transfected with our CRISPR construct 24 h later displayed GFP signals perinuclear, indicating that the CRISPR system could act in these cells, as a repórtor (19). CRISPR/Cas9 anti-HSV-1 activity was investigated in cells infected with HSV-1, we infected Vero cells with HSV-1 (MOIs, 0.1 and 0.001) and transfected them with the CRISPR system (pRGD-UL39 plasmid) 24 h later. The qPCR analyzes were performed 24h and 48h after pRGD-UL39 plasmid transfection and indicated that HSV-1 viral activity at both an MOI of 0.1 and 0.001 viral load decreased by >95% of cells transfected with CRISPR anti-HSV-1 / Cas9. In our study, we also did the transfection before replication, however, the decrease in viral load was only 24% (data not shown). The best results were observed when the transfection was performed 24 h after the infection, simulating the action of using an antiviral. These findings corroborate previous reports that inhibition of UL39 results in impaired HSV-1 replication (8). Our results also compare favorably with previous reports on the use of the CRISPR/Cas9 system to inhibit HSV-1 replication. In the study that targeted the genes UL8, UL29 and UL52, HSV-1 reproduction was suppressed in 90% of infected Vero cells (14), whereas the study that targeted the ICP0 protein demonstrated viral suppression in 85–90% of cells (13). These findings reinforce the importance of studying different genes involved in HSV-1 replication and underscore the potential of the CRISPR/Cas9 anti-HSV-1 system to inhibit viral replication.

Our findings showed that a CRISPR/Cas9 system targeting the UL39 gene suppressed HSV-1 reproduction in infected Vero cells. The importance of evaluating the duration of antiviral action is well known. In our study, an evaluation of antiviral action at 2 d revealed potent viral inhibition. However, it has been previously reported that the anti-HSV-1 activity of CRISPR/Cas9 can be maintained for at least 6 d after HSV-1 infection. However, the effect disappeared completely after 2 days (14).

## Conclusion

In summary, our findings demonstrate that, in addition to exhibiting low cell cytotoxicity and localization to the cell perinuclear, our CRISPR Cas9/gRNA editing system targeting the UL39 gene successfully inhibited viral replication 48 h after infection at different MOIs. Thus, the genetic constructs used here could represent a promising

approach for future *in vivo* studies as well as new antiviral drug development efforts.

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### Conflicts of Interest

No reported conflicts. Conflicts that the editors consider relevant to the content of the manuscript has been disclosed.

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