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Research Paper

The CRISPR–Cas9 system for genome editing of the ASS1 gene in human cells to predict its effect on HSV-1 replication

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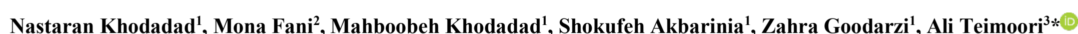

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ABSTRACT

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Herpes simplex virus type 1 (HSV-1) is a highly contagious pathogen that establishes lifelong latent infections. The replication of HSV-1 is potentially influenced by the arginine succinate synthase (ASS1) gene, a key regulator of cellular metabolism. This study utilized the CRISPR–Cas9 genome editing platform to specifically target and disrupt the ASS1 gene to examine its effect on viral propagation. A guide RNA (gRNA) was designed to complement a sequence within the ASS1 gene. A donor plasmid and the pCas-guide plasmid were cloned and cotransfected into the human embryonic kidney (HEK) cells with sheared adenovirus (Ad)5 DNA (HEK293-AD) cells. Potential ASS1-knockout clones were identified and validated via polymerase chain reaction (PCR) and DNA sequencing analysis. The impact on HSV-1 replication was quantified via a plaque assay to determine the viral titer. Sequencing data from ASS1-gRNA/Cas9-treated cells did not confirm successful gene knockout, as the intended ASS1 disruption was not achieved. The viral titer did not significantly differ between the HSV-1 infection group (MOI=0.01) and the control group. These findings indicate that the single gRNA designed for this study lacked sufficient specificity to elicit a CRISPR-Cas9-mediated gene knockout. Therefore, employing a set of more specific gRNAs is recommended to increase targeting efficiency. Further investigations are needed to elucidate the desired genetic modification and observe its subsequent effects on HSV-1.

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1. Introduction

HSV-1, a prevalent member of the Herpesviridae family, affects an estimated 64% of the global adult population [1,2]. Primary infection occurs at epithelial surfaces, followed by retrograde transport to sensory ganglia, where the virus establishes a permanent latent state within neuronal nuclei [3-5]. The metabolic landscape of the host cell is significantly altered by HSV-1, which increases glycolysis and modulates pyruvate carboxylase activity. These reprogramming channels carbon units into the tricarboxylic acid (TCA) cycle and bolsters de novo nucleotide synthesis, leading to an expanded pool of various nucleotides and an increased flux of carbon through aspartate [6,7]. Arginine, an indispensable amino acid in the human body, is involved in many biological functions, such as the activation of reproductive systems, hormone secretion, lipid metabolism, and wound healing. This amino acid is produced from citrulline via two steps that involve the enzymes arginine succinate synthase (ASS1) and arginine succinate lyase, with ASS1 acting as the limiting factor in this process [8,9]. The evidence suggests that small interfering RNA (siRNA)-mediated knockdown of ASS1 can increase HSV-1 production, whereas ASS1 overexpression reduces the viral yield in fibroblasts. Notably, ASS1-deficient cells remain permissive for viral replication and protein synthesis [6,10,11].

Clustered regularly interspaced short palindromic repeats (CRISPR) systems, which rely on CRISPR RNAs (crRNAs) along with CRISPR-associated (Cas9) proteins, represent defense mechanisms within bacterial and archaeal organisms that serve to direct the destruction of complementary sequences found within target DNA [12,13]. The CRISPR/Cas9 system is predicated upon the *Streptococcus pyogenes* type II CRISPR system, which comprises gRNA and the Cas9 nuclease. The gRNA and Cas9 nuclease collaborate to form an RNA–protein complex, which subsequently enables the digestion of exclusive target sequences [14-17]. The chimeric CRISPR/Cas9 system has been modified for use in the cells of eukaryotic organisms, leading to alterations in the genomes of various organisms, such as Drosophila, Zebrafish, *Caenorhabditis elegans*, yeast, plants, and even mammals [14,15,18].

In the present work, a monocistronic cassette harboring a cytomegalovirus (CMV) promoter, a red fluorescent protein (RFP) gene, and Simian vacuolating virus 40 (SV40) polyadenylation signals were constructed. This cassette was flanked by homology arms corresponding to the ASS1 locus to facilitate precise integration via CRISPR–Cas9-assisted homologous recombination. The objective of this research was to design a gRNA to direct Cas9 to the ASS1 gene, assess the inhibition of ASS1 expression, and monitor the consequent effects on the kinetics of HSV-1 replication.

2. Materials and Methods

2.1 Cell culture

The human embryonic kidney (HEK) cells with sheared adenovirus (Ad)5 DNA (HEK293-AD) were cultured in high-glucose Dulbecco's modified Eagle’s medium (DMEM) (Gibco, Gaithersburg, MD, USA) enriched with 10% fetal bovine serum (FBS) (Gibco, USA), streptomycin (100 µg/ml), and penicillin (100 IU/ml) (Sigma-Aldrich, St. Louis, MO, USA) and maintained at 37°C with 5% CO_2 [19].

2.2 Construction of plasmids

The plasmid GE100018 was utilized as a vector for gRNA (pCas-Guide-EF1a-GFP) (Rockville, MD, USA), and the plasmid pTZ57R/T (Thermo Fisher Scientific, USA) was employed as a donor vector for homologous recombination (HR). To establish the CRISPR–Cas9 system that targets exon 13 of the human ASS1 gene, we designed a gRNA sequence (5′-GATCGACACTCACGCGACTTCGTTCG-3′) [20] and inserted it into the gRNA expression cassettes of the pCas-Guide-EF1a-GFP (GE100018) vector, which includes the U6 promoter for gRNA transcription and the CMV promoter for Cas9 protein expression.

For the HR donor plasmid, the pTZ57/R vector was used as a backbone. The pCMV-DsRed-SV40polyA reporter cassette from the pDsRed-N1 vector (Takara Bio) was subcloned and inserted into this backbone. To generate compatible ends for TA cloning, the insert was amplified via Taq DNA polymerase. The multiple cloning site (MCS) region of the pDsRed-N1 fragment was excised via digestion with BamHI and BglII to avoid conflict with restriction sites in the HR vector MCS. Two homology arms designed to flank the Cas9 cleavage site were amplified via PCR. The pTZ57/R vector, with T-overhangs suitable for TA cloning, has an optimal packaging size for recombinant genomes between 4.1 and 4.9 kb; however, the targeting efficiency is highest, with homology arms exceeding 2 kb. This study utilized shorter arms of 0.6–0.9 kb [21, 22]. Specific primers with incorporated restriction sites (EcoRI/KpnI for the left arm; BamHI/HindIII for the right arm) were used to amplify the ASS1 homology arms (primer sequences in Table 1). The final ASS1 donor plasmid was assembled with a 5′ homology arm (791 bp), the pCMV-DsRed-SV40polyA cassette, and a 3′ homology arm (834 bp) [23-25]. A diagram of the plasmids is provided in Figure 1 and 2; both figures were generated using the SnapGene software 8.0 (GSL Biotech LLC, San Diego, CA, USA) [26].

2.3 Ligation, transformation, and colony PCR

The ligation step involved inserting the RFP gene into the pTZ57R/T vector via a TA cloning kit made by Thermo Fisher Scientific, USA.

Table 1. The primers used for amplification of homologous arms.

Primers	Sequences 5′-------> 3′	Enzyme sites
FleftASS1	*GAATTCCGG*AAGGACGTGCCCAAGGTCGCCCA	EcoRI
RleftASS1	*GGTACCCCG*GAAGTCGCGTGAGTGTCTGCAG	KpnI
FrightASS1	*GGATCCGCG*GTACATGAAGAGCTCCAAGGAGG	BamHI
RrightASS1	*AAGCTTGGG*GGATTAGCCGCCAACTTTGAACA	HindIII

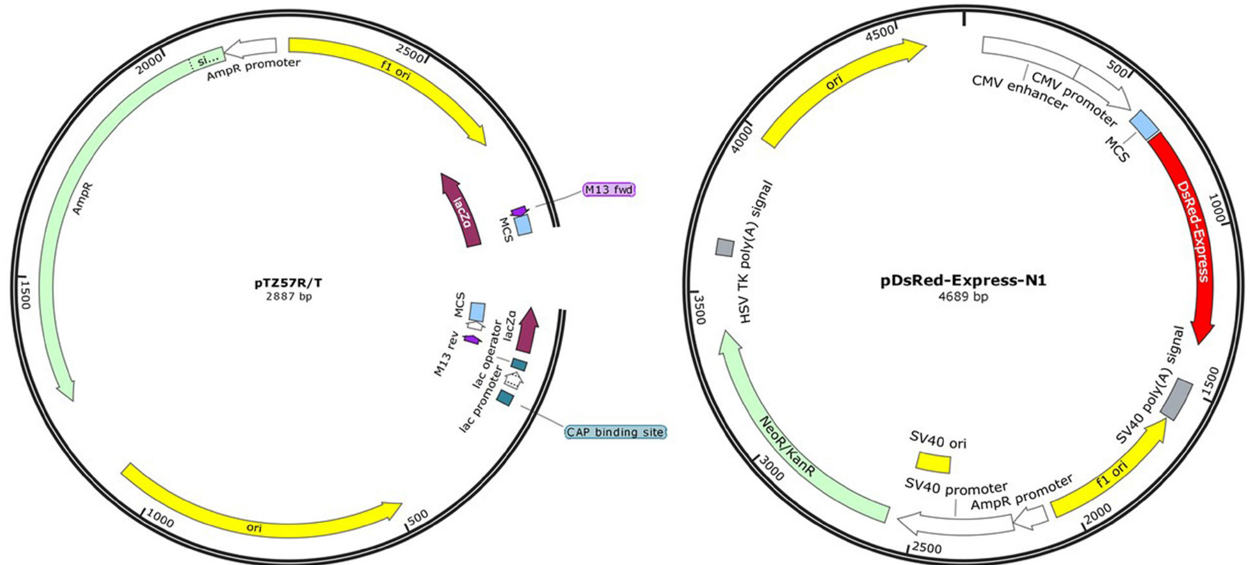

Figure 1. pTZ57R/T vector and pDsRed-N1 vector.

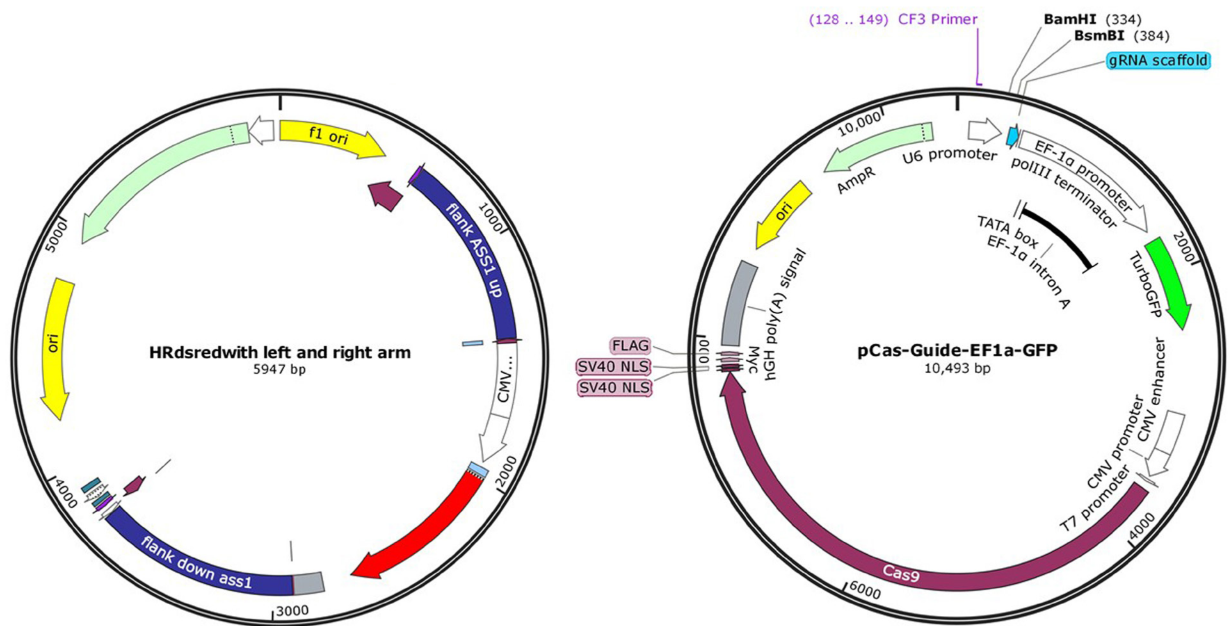

Figure 2. Homologous recombination (HR) plasmid and pCas-guide plasmid.

The reaction mixture was prepared in a 10 µl volume consisting of 3 µl of the insert gene (RFP), 3 µl of the pTZ57R/T vector, 1 µl of T4 ligase, 2 µl of 5x ligation buffer, and 1 µl of distilled water. The mixture was first kept at 22°C for one hour and then kept at 4°C overnight. The competent cells used in the transformation step were prepared via the calcium chloride method with the DH5α strain of *Escherichia coli* bacteria [27]. Specifically, *E. coli* cells were cultured in 5 ml of Luria–Bertani (LB) (Sigma-Aldrich, USA) broth and kept at 37°C overnight. The following day, 100 µl of these cells was transferred to 5 ml of LB broth and kept at 37°C until they grew to a certain density (OD 0.6). After incubation, the mixture was

centrifuged at 3000 rcf and 4°C for 5 minutes. The supernatant was subsequently removed, and the pellet was mixed with 1 ml of cold calcium chloride (0.1 M). The suspension was then kept at -20°C for 5 minutes, followed by centrifugation at 3000 rcf and 4°C for 5 minutes. This process was repeated twice with 750 µl and 250 µl of cold calcium chloride. Finally, 3 µl of the ligated DNA was mixed with 50 µl of the cell suspension and kept cold on ice for 30 minutes. After that, the suspension was quickly heated to 42°C for 90 s before being immediately returned to ice. Subsequently, 950 µl of LB broth without antibiotics was added, and the mixture was incubated at 37°C for 45 minutes. To assess successful transformation, 100 µl of these cells was plated on LB agar media supplemented with 100 mg/ml ampicillin, 20 mg/ml X-Gal, and 20 mg/ml IPTG (Thermo Fisher, US). The plates were maintained at 37°C for 18 hours and then transferred to a 4°C refrigerator for 2 hours. The recombinant plasmids (pTZ-RFP) were confirmed through colony PCR via M13 gene-specific primers [28].

A colony was removed via a loop and suspended in 50 µl of distilled water. Following centrifugation, 2 µl of the solution was used as a template for PCR amplification. The PCR protocol included initial denaturation (95°C for 5 minutes), followed by 35 cycles of amplification (94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute and 30 seconds), with a final extension step at 72°C for 10 minutes. The recombinant plasmids (pTZ-RFP) were removed from the white colonies with a plasmid extraction kit (Favorgen, Taiwan). Finally, to confirm the correct orientation of the RFP gene in the pTZ57R vector, the recombinant plasmid (pTZ-RFP) was digested with the xbaI enzyme (Thermo Fisher, USA) [29].

2.4 *Generation of ASS1-knockout cell lines*

To generate ASS1-knockout clones, HEK293-AD cells were cotransfected with the pCas-Guide-EF1a-GFP vector and the donor vector for ASS1. The transfection was carried out via calcium phosphate (Abcam, UK) according to the established protocols provided by the supplier. The cells were subsequently examined under a fluorescence microscope the following day. Once observed, the entire contents of each well were carefully removed from the 24-well plate, and the cells were then placed in a refrigerated centrifuge for 10 minutes at a rate of 10000. In the subsequent stage, DNA extraction was performed on the cells via deposition via a Yekta Tajhiz DNA Extraction Kit. However, the accuracy of the ASS1-knockout clone was verified by amplifying the DNA surrounding the targeted region via PCR, followed by sequencing to confirm the genetic modifications. The specific primers utilized for PCR and sequencing can be found in Table 2. Furthermore, HEK293-AD cells were used as a control in this study [25].

2.5 *Viral titer*

To conduct an experiment examining the efficiency of HSV-1 infection in ASS1 gRNA-cloned cells, we initiated the process by generating a series of dilutions using a multiplicity of infection (MOI) of 0.01 for the HSV-1 strain KOS. The HEK293 cells were subsequently subjected to infection with the aforementioned dilutions, specifically at a concentration of 2×10^5, for one hour at 37°C in DMEM. Upon completion of this step, the supernatant containing the viral particles was collected and subsequently supplemented with 5% DMEM in each well of the cells [30]. To determine the virus titer, we executed a plaque assay, which was based on our previously conducted research study [20,31,32]. By utilizing the dilution factor, the number of formed plaques, and the amount of virus, the viral titer was calculated in terms of plaque-forming units (PFUs) per milliliter.

3. Results

3.1 *Direct fluorescence assay*

After a period of 24 hours, the cells were transfected with Cas9 and gRNA plasmids. Subsequently, fluorescence screening was conducted via fluorescence microscopy to assess the transfection rate. A high percentage of cells treated with either the CRISPR-Cas9 constructs or the control plasmids exhibited both green and red fluorescence, confirming successful delivery and expression of the plasmids (Figure 3).

3.2 *Targeted disruption of the ASS1 gene via the CRISPR system*

The efficacy of ASS1 gene disruption was analyzed via PCR on genomic DNA from gRNA/Cas9-treated HEK-AD cells. The assay was designed to detect two distinct bands (1590 bp and 3111 bp) upon successful knockout. However, only the 1590 bp band, corresponding to the wild-type allele, was observed in all the samples. This result indicates that the CRISPR-Cas9 system did not disrupt the ASS1 gene. The control (untreated ASS1 gene) yielded a single PCR product of 412 bp.

3.3 *Sequencing of ASS1*

Plasmids containing the CRISPR–Cas9 system, as well as a negative control, were transfected into HEK293-AD cells. To further validate the impact of the CRISPR–Cas9 system, the ASS1 gene was sequenced in untreated cells and those treated with this system. The results indicated that the DNA segment remained unaltered by Cas9 (Figure 4). Hence, we confirmed that the designed CRISPR–Cas9 system did not effectively induce changes in the ASS1 gene.

Table 2. The primers used for PCR and sequencing of the ASS1-knockout clone.

Primers	Sequences 5′-------> 3′	Products length (bp)
FbeforeASS1	AGGTGCTGATACGGAGGGGAGGGC	1590 and 3111
RafterASS1	ATGATTTGGGGACTGTCTGGAAGA	

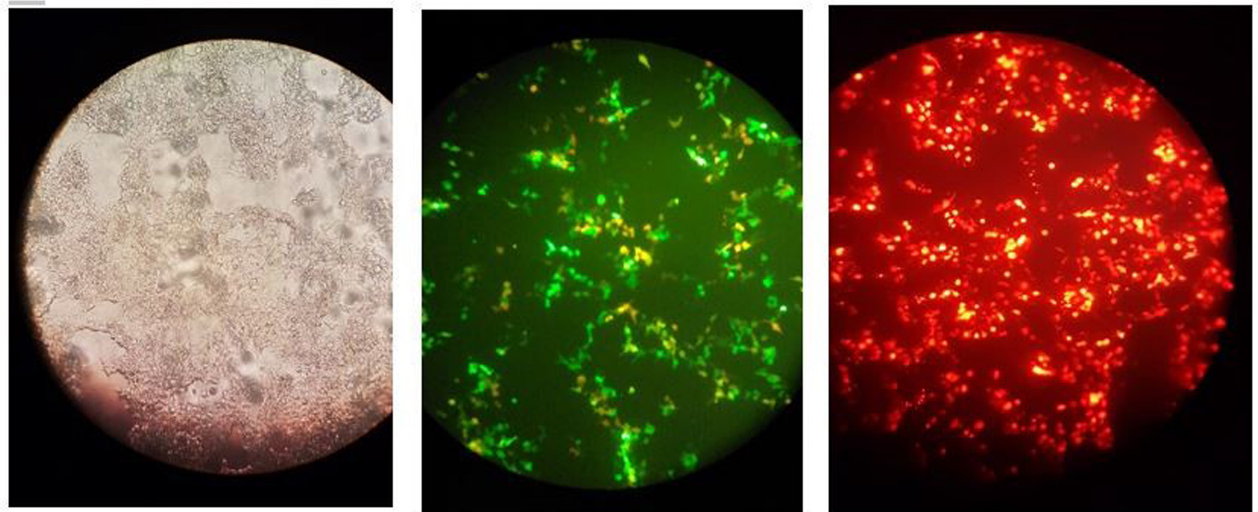

Figure 3. Results of the direct fluorescence assay of cells treated with the CRISPR–Cas9 system from left to right (Control plasmid, green fluorescent protein (GFP), and red fluorescent protein (RFP)).

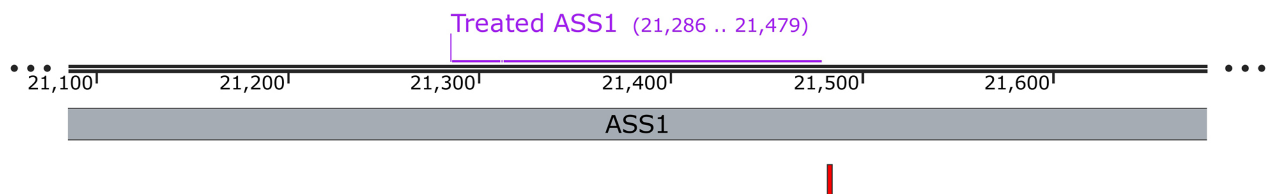

Figure 4. The sequencing results of the ASS1 gene treated with the CRISPR–Cas9 system generated with SnapGene software.

3.4 Viral titer

By observing the morphological changes in HSV-1-infected HEK293-AD cells, cytopathic effects were identified within a 24- to 48-hour period. According to the results of a plaque formation assay, no discernible alterations resulting from the editing system were observed in HSV-1 infection at an MOI of 0.01.

4. Discussion

The ASS1 gene, located on chromosome 9, encodes argininosuccinate synthetase. Its functional depletion typically hinders enzyme production, a condition previously associated with increased HSV-1 genome replication, infectivity, and viral titer [6,33,34]. To achieve ASS1 knockdown, we employed the CRISPR-Cas9 system and designed a single gRNA to target a specific sequence in exon 13. We then evaluated the system's capacity to inhibit ASS1 and modulate HSV-1 replication in HEK293-AD cells. Fluorescence-based assays confirmed successful transfection, as evidenced by green fluorescent protein (GFP), and red fluorescent protein (RFP) expression. However, PCR analysis of treated cells failed to detect any genetic disruption of the ASS1 locus.

This lack of editing was further corroborated by DNA sequencing, which revealed no mutations in the target sequence. Consequently, the viral titer at an MOI of 0.01 was unchanged following treatment with ASS1-gRNA-Cas9. Previous studies have revealed a correlation between intracellular arginine levels and HSV-1 replication efficiency, with arginine deprivation inhibiting viral propagation [35-37].

The restoration of normal virus production upon arginine supplementation underscores its critical role in the viral life cycle [36,38]. Early work by Wildy et al. (1982) suggested that arginase released from macrophages suppresses HSV-1 replication, suggesting that arginine modulation is an antiviral strategy [39]. In support of this, more recent research by Sanchez et al. (2016) demonstrated that treatment with pegylated arginase (peg-ARG1) potently inhibits HSV-1 replication, cell-to-cell spread, and cytopathic effects, resulting in superior anti-HSV-1 activity compared with that of acyclovir [40]. This research faced a main limitation due to the insufficient specificity and effectiveness of the single-gRNA designed to target the

ASS1 gene. The failure to produce a genetic knockout, as confirmed by PCR and sequencing analysis, prevented a clear assessment of the role of ASS1 in influencing HSV-1 replication in this cellular model. As a result, the viral titers could not be reliably compared between ASS1-knockout and wild-type cells. Additionally, using shorter homology arms (0.6–0.9 kb) in the donor plasmid, although faster, may have reduced the efficiency of homology-directed repair (HDR), which is known to work best with arms longer than 2 kb [41,42]. Relying on a single clonal cell line and using only one MOI for viral infection also restricts the broader applicability of the findings.

Future investigations should adopt a multifaceted gRNA strategy, using a combination of highly specific gRNAs targeting multiple exons of the ASS1 gene to increase the likelihood of successful and biallelic knockout. Employing computational tools for thorough off-target prediction and selecting gRNAs with the highest on-target scores is highly recommended. Additionally, constructing donor vectors with extended homology arms (>1.5 kb) can significantly increase HDR efficiency [43].

Subsequent studies should also incorporate robust functional validation methods, such as Western blotting, to verify the absence of the ASS1 protein alongside genetic confirmation. To thoroughly assess the impact on HSV-1, future work should evaluate viral replication kinetics across various MOIs and cell types, including primary cells, to confirm that the results are not cell line specific.

In conclusion, this study aimed to elucidate the functional relationship between the ASS1 gene and HSV-1 replication by employing CRISPR-Cas9-mediated gene editing. While the transfection protocol was successful, as evidenced by robust fluorescent reporter expression, the designed CRISPR system failed to introduce the intended genetic disruptions in the ASS1 locus. This lack of efficacy, attributed to the inadequate specificity of a single gRNA, resulted in no observable phenotypic change in the host cells and, consequently, no significant alteration in viral replication kinetics at the tested MOI. These findings underscore a critical technical challenge in CRISPR-based research: the absolute necessity of employing highly efficient and specific gRNAs to achieve meaningful genetic perturbations. Therefore, this work highlights that the potential role of ASS1 in HSV-1 replication remains an open question that requires further investigation with a more potent and validated knockout model to draw definitive conclusions.

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Authors' contributions

AT: contributed substantially to the conception and design of the study. NK, MF, MK, SA, ZG: contributed to data acquisition, analysis, and interpretation. NK, MF: drafted the manuscript, and AT, NK: critically revised it for important intellectual content. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work.

Conflict of interest

No potential conflict of interest was reported by the authors.

Ethical declarations

This study was conducted in accordance with the ethical principles of the Declaration of Helsinki and approved by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences (Approval No: IR.AJUMS.REC.1395.292). This article does not encompass any investigations involving individuals of the human participants executed by any of the authors.

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References

1. Dutton AJ, Hayes CK, Leib DA, Akhtar LN. Herpes Simplex Virus Neurovirulence Across the Human Lifespan. J Infect Dis. 2025;232(1):14-24. DOI: 10.1093/infdis/jiaf275 PMID: 40418737
2. Birkmann A, Saunders R. Overview on the management of herpes simplex virus infections: Current therapies and future directions. Antiviral Res. 2025;237:106152. DOI: 10.1016/j.antiviral.2025.106152 PMID: 40154924
3. Szpara ML, Parsons L, Enquist LW. Sequence variability in clinical and laboratory isolates of herpes simplex virus 1 reveals new mutations. J Virol. 2010;84(10):5303-13. DOI: 10.1128/JVI.00312-10 PMID: 20219902
4. Sawtell NM, Thompson RL. Alphaherpesvirus Latency and Reactivation with a Focus on Herpes Simplex Virus. Curr Issues Mol Biol. 2021;41:267-356. DOI: 10.21775/cimb.041.267 PMID: 32883886
5. Dochnal S, Merchant HY, Schinlever AR, Babnis A, Depledge DP, Wilson AC, et al. DLK-Dependent Biphasic Reactivation of Herpes Simplex Virus Latency Established in the Absence of Antivirals. J Virol. 2022;96(12):e0050822. DOI: 10.1128/jvi.00508-22 PMID: 35608347
6. Grady SL, Purdy JG, Rabinowitz JD, Shenk T. Argininosuccinate synthetase 1 depletion produces a metabolic state conducive to herpes simplex virus 1 infection. Proc Natl Acad Sci U S A. 2013;110(51):E5006-15. DOI: 10.1073/pnas.1321305110 PMID: 24297925
7. Guo YF, Liao MQ, Cai WL, Yu XX, Li SN, Ke XY, et al. Physical activity, screen exposure and sleep among students during the pandemic of COVID-19. Sci Rep. 2021;11(1):8529. DOI: 10.1038/s41598-021-88071-4 PMID: 33879822

8. Ohshima K, Nojima S, Tahara S, Kurashige M, Hori Y, Hagiwara K, et al. Argininosuccinate Synthase 1-Deficiency Enhances the Cell Sensitivity to Arginine through Decreased DEPTOR Expression in Endometrial Cancer. Sci Rep. 2017;7:45504. DOI: 10.1038/srep45504 PMID: 28358054
9. Jamehdor S, Sangtarash MH, Farivar S, Amini R, Teimoori A. Correlation of arginine metabolism with neurodegenerative diseases in infected cells with herpes simplex virus-1. Reviews and Research in Medical Microbiology. 2022;33(1):45-50. DOI: 10.1097/MRM.0000000000000264
10. Li T. An Essential Role of Argininosuccinate Synthase 1 in Kaposi's Sarcoma-Associated Herpesvirus-Induced Cellular Transformation: University of Southern California; 2016. URL: https://search.proquest.com/openview/761c21ac2e9910afc28e810f92f47a0c/1?pq-origsite=gscholar&cbl=18750
11. Dogrammatzis C, Waisner H, Kalamvoki M. "Non-Essential" Proteins of HSV-1 with Essential Roles In Vivo: A Comprehensive Review. Viruses. 2020;13(1):17. DOI: 10.3390/v13010017 PMID: 33374862
12. Khatun R, Sengupta S, Bhattacharya M. Bacterial Microbial Defense Systems: From CRISPR-Cas Mechanisms to Emerging Anti-Phage Strategies. IJoI. 2025;13(3):52-8. DOI: 10.11648/j.iji.20251303.12
13. Ganger S, Harale G, Majumdar P. Clustered regularly interspaced short palindromic repeats/CRISPR-associated (CRISPR/Cas) systems: Discovery, structure, classification, and general mechanism. CRISPR/Cas-Mediated Genome Editing in Plants: Apple Academic Press; 2023. p. 65-97. DOI: 10.1201/9781003331759
14. Ebina H, Misawa N, Kanemura Y, Koyanagi Y. Harnessing the CRISPR/Cas9 system to disrupt latent HIV-1 provirus. Sci Rep. 2013;3:2510. DOI: 10.1038/srep02510 PMID: 23974631
15. Song J, Kim S, Kim HY, Hur KH, Kim Y, Park HG. A novel method to detect mutation in DNA by utilizing exponential amplification reaction triggered by the CRISPR-Cas9 system. Nanoscale. 2021;13(15):7193-7201. DOI: 10.1039/d1nr00438g PMID: 33720266
16. Kozovska Z, Rajcaniova S, Munteanu P, Dzacovska S, Demkova L. CRISPR: History and perspectives to the future. Biomed Pharmacother. 2021;141:111917. DOI: 10.1016/j.biopha.2021.111917 PMID: 34328110
17. Vasques Raposo J, Rodrigues Carvalho Barros L, Ribas Torres L, Barbosa da Silva Pinto R, De Oliveira Lopes A, Mello de Souza E, et al. CRISPR/Cas-9 vector system: targets UL-39 and inhibits Simplexvirus humanalpha1 (HSV-1) replication in vitro. Cell Mol Biol (Noisy-le-grand). 2023;69(7):19-23. DOI: 10.14715/cmb/2023.69.7.3 PMID: 37715442
18. Sachkova M, Modepalli V, Kittelmann M. The Deep Evolutionary Roots of the Nervous System. Annu Rev Neurosci. 2025;48(1):311-329. DOI: 10.1146/annurev-neuro-112723-040945 PMID: 40198851
19. Graham FL, Smiley J, Russell WC, Nairn R. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J Gen Virol. 1977;36(1):59-74. DOI: 10.1099/0022-1317-36-1-59 PMID: 886304
20. Khodadad N, Fani M, Jamehdor S, Nahidsamiei R, Makvandi M, Kaboli S, et al. A knockdown of the herpes simplex virus type-1 gene in all-in-one CRISPR vectors. Folia Histochem Cytobiol. 2020;58(3):174-181. DOI: 10.5603/FHC.a2020.0020 PMID: 32937678
21. Kohli M, Rago C, Lengauer C, Kinzler KW, Vogelstein B. Facile methods for generating human somatic cell gene knockouts using recombinant adeno-associated viruses. Nucleic Acids Res. 2004;32(1):e3. DOI: 10.1093/nar/gnh009 PMID: 14704360
22. Bollen Y, Post J, Koo BK, Snippert HJG. How to create state-of-the-art genetic model systems: strategies for optimal CRISPR-mediated genome editing. Nucleic Acids Res. 2018;46(13):6435-6454. DOI: 10.1093/nar/gky571 PMID: 29955892
23. Liu YC, Cai ZM, Zhang XJ. Reprogrammed CRISPR-Cas9 targeting the conserved regions of HPV6/11 E7 genes inhibits proliferation and induces apoptosis in E7-transformed keratinocytes. Asian J Androl. 2016;18(3):475-9. DOI: 10.4103/1008-682X.157399 PMID: 26228041
24. Ge X, Xi H, Yang F, Zhi X, Fu Y, Chen D, et al. CRISPR/Cas9-AAV Mediated Knock-in at NRL Locus in Human Embryonic Stem Cells. Mol Ther Nucleic Acids. 2016;5(11):e393. DOI: 10.1038/mtna.2016.100 PMID: 27898094
25. Wang T, Wei JJ, Sabatini DM, Lander ES. Genetic screens in human cells using the CRISPR-Cas9 system. Science. 2014;343(6166):80-4. DOI: 10.1126/science.1246981 PMID: 24336569
26. GSL Biotech LLC. SnapGene Viewer (Version 8.0.4). 2023. URL: https://www.snapgene.com/snapgene-viewer
27. Sambrook J, Russell DW. Molecular Cloning: Ch. 8. In Vitro amplification of DNA by the polymerase chain reaction: Cold Spring Harbor Laboratory Press; 2001. URL: https://www.gob.mx/cms/uploads/attachment/file/685877/5098_-Sambrook_J.-_Molecular_Clonning_compressed.pdf
28. Okasha H, Samir S. Synthesis and molecular cloning of antimicrobial peptide chromogranin A N-46 gene using conventional PCR. Gene Reports. 2020;18:100571. DOI: 10.1016/j.genrep.2019.100571
29. Soltani M, Sadrebazzaz A, Nassiri M, Tahmoorespoor M. Cloning, Nucleotide Sequencing and Bioinformatics Study of NcSRS2 Gene, an Immunogen from Iranian Isolate of Neospora caninum. Iran J Parasitol. 2013;8(1):114-27. PMID: 23682269
30. Roehm PC, Shekarabi M, Wollebo HS, Bellizzi A, He L, Salkind J, et al. Inhibition of HSV-1 Replication by Gene Editing Strategy. Sci Rep. 2016;6:23146. DOI: 10.1038/srep23146 PMID: 27064617
31. Fani M, Khodadad N, Ebrahimi S, Ghafari S, Makvandi M, Teimoori A, et al. Zinc Sulfate in Narrow Range as an In Vitro Anti-HSV-1 Assay. Biol Trace Elem Res. 2020;193(2):410-413. DOI: 10.1007/s12011-019-01728-0 PMID: 31028520
32. Nahid Samiei R, Ebrahimi S, Fani M, Ghafari S, Makvandi M, Khodadad N, et al. In vitro effect of some nucleoside reverse transcriptase inhibitors against HSV-1 replication. Eur Rev Med Pharmacol Sci. 2020;24(3):1454-1459. DOI: 10.26355/eurrev_202002_20204 PMID: 32096195
33. Yuan F, Guo D, Liu Y, Xu Y, Gao G, Wu Y, et al. Generation of an ASS1 heterozygous knockout human embryonic stem cell line, WAe001-A-13, using CRISPR/Cas9. Stem Cell Res. 2018;26:67-71. DOI: 10.1016/j.scr.2017.11.007 PMID: 29247816
34. Ariav Y, Ch'ng JH, Christofk HR, Ron-Harel N, Erez A. Targeting nucleotide metabolism as the nexus of viral infections, cancer, and the immune response. Sci Adv. 2021;7(21):eabg6165. DOI: 10.1126/sciadv.abg6165 PMID: 34138729
35. Becker Y, Olshevsky U, Levitt J. The role of arginine in the replication of herpes simplex virus. J Gen Virol. 1967;1(4):471-8. DOI: 10.1099/0022-1317-1-4-471 PMID: 4295376
36. Inglis VB. Requirement of arginine for the replication of herpes virus. J Gen Virol. 1968;3(1):9-17. DOI: 10.1099/0022-1317-3-1-9 PMID: 4300568
37. Meingast C, Heldt CL. Arginine-enveloped virus inactivation and potential mechanisms. Biotechnol Prog. 2020;36(2):e2931. DOI: 10.1002/btpr.2931 PMID: 31622532
38. Grimes JM, Khan S, Badeaux M, Rao RM, Rowlinson SW, Carvajal RD. Arginine depletion as a therapeutic approach for patients with COVID-19. Int J Infect Dis. 2021;102:566-570. DOI: 10.1016/j.ijid.2020.10.100 PMID: 33160064
39. Wildy P, Gell PG, Rhodes J, Newton A. Inhibition of herpes simplex virus multiplication by activated macrophages: a role for arginase? Infect Immun. 1982;37(1):40-5. DOI: 10.1128/iai.37.1.40-45.1982 PMID: 6286497

40. Sanchez MD, Ochoa AC, Foster TP. Development and evaluation of a host-targeted antiviral that abrogates herpes simplex virus replication through modulation of arginine-associated metabolic pathways. Antiviral Res. 2016;132:13-25. DOI: 10.1016/j.antiviral.2016.05.009 PMID: 27192555
41. Jin YY, Zhang P, Liu DP. Optimizing homology-directed repair for gene editing: the potential of single-stranded DNA donors. Trends Genet. 2025;41(9):788-803. DOI: 10.1016/j.tig.2025.04.014 PMID: 40436684
42. Dickinson DJ, Ward JD, Reiner DJ, Goldstein B. Engineering the Caenorhabditis elegans genome using Cas9-triggered homologous recombination. Nat Methods. 2013;10(10):1028-34. DOI: 10.1038/nmeth.2641 PMID: 23995389
43. Doench JG, Fusi N, Sullender M, Hegde M, Vaimberg EW, Donovan KF, et al. Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. Nat Biotechnol. 2016;34(2):184-191. DOI: 10.1038/nbt.3437 PMID: 26780180