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Original Research Article

siRNAs Targeting Viral Protein 5: The Major Capsid Protein of Herpes Simplex Virus-1 Affects its Propagation and Cytoskeleton

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Abstract

Purpose: *To investigate whether siRNA targeting viral protein 5 (VP5) can become a new treatment for herpes simplex virus type 1 (HSV-1).*
Methods: *Flow cytometry was performed to determine the ratio of siRNA and lipo2000 to reach the highest transfection efficiency. Western blot and q-PCR were performed to determine the knockdown efficiency of siRNA targeting UL19, as well as changes in cytoskeleton proteins. Plaque reduction assays and confocal microscopy were conducted to test the influence of VP5 knockdown on the HSV-1 life cycle and viral replication. F-actin organization was observed through confocal microscopy during HSV-1 infection when transfected with siRNA.*
Results: *Relative expression level of the UL19 gene dropped to 6 % while plaque formation inhibition rate rose to 85 % compared with virus control. Knocking down VP5 expression abrogated the changes to F-actin that were induced by HSV-1 infection.*
Conclusion: *Interfering with UL19 gene expression inhibits HSV-1 replication efficiently in vitro. The results indicate that the major capsid protein VP5 encoding gene UL19 may be a promising target for RNA interference-based therapeutic strategy against HSV-1.*

Keywords: *siRNA, Viral protein 5, Herpes simplex virus type 1, Gene expression, Propagation, Cytoskeleton rearrangement, F-actin, Transfection*

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INTRODUCTION

Herpes simplex virus type 1 (HSV-1), a neurotropic alpha – herpes virus, is a linear double-stranded DNA virus that occurs naturally in humans [1]. It can lead to a number of mild to severe diseases, and could even be life threatening when patients are immuno-compromised [2]. Aciclovir® (ACV) and other related nucleoside analogs have been approved worldwide for the treatment of the virus [3]. However, problems associated with toxicity and drug resistance have been reported [4]. Currently, there is no specific drug available for eliminating latent herpes simplex virus infections [5]. Therefore, the need for new, safe and effective antiviral drugs is imperative.

The viral caspid is an important structural component of HSV-1 virus particles. It plays a critical role in viral replication, assembly, maturation and infection. Assembly of herpesvirus capsids takes place in the nucleus. The procapsid is formed and the viral genome is

then packaged into it. The mature HSV-1 capsid comprises four proteins (VP5, VP23, VP19C and VP26) and is shaped like an icosahedral shell. It consists of 162 capsomeres, including 150 hexons, 11 pentons and one portal that consists of a dodecamer of UL16. VP5, encoded by the UL19 gene, is the major structural protein of hexons and pentons [6], and acts as a scaffold during the formation of the HSV-1 capsid. It also recruits other capsid proteins to the icosahedron and is a major antigenic component [7]. Interactions have been detected between VP5 and the scaffold protein, triplex protein and VP26. This interaction is important for closure of the capsid shell into an icosahedral structure [8]. These findings suggest that VP5 are necessary for HSV-1 to proliferate, and open up the possibility that VP5 could be a new target in anti-HSV therapies.

The use of RNA interference (RNAi) techniques could be a novel approach for anti-HSV therapies [9]. RNAi involves gene-silencing based on sequence-specific targeting and post-transcriptional mRNA degradation, induced by double-stranded RNAs [10]. Short interfering RNAs (siRNAs), generated by the ribonuclease III enzyme Dicer, that are 21-25 nucleotides act as functional intermediates in RNAi for inducing target mRNA cleavage by the RNA-induced silencing complex (RISC) [11]. This powerful technology has been widely used to manipulate gene expression, identify gene functions on a whole-genome scale, and develop antiviral strategies for the prevention and treatment of human viral diseases [12]. To date, RNAi has been employed against several human pathogens, including human immunodeficiency virus type 1, hepatitis C virus, hepatitis B virus, poliovirus, influenza virus A and Dengue virus [13]. In our current study, we applied siRNAs targeting UL19 of HSV-1 to investigate the antiviral activities of VP5.

EXPERIMENTAL

Materials

African green monkey kidney cells (Vero; ATCC CCL81), provided by the Wuhan Institute of Virology at the Chinese Academy of Sciences were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum (FCS) at 37 °C/5 % CO_2. HSV-1 strain F (ATCC VR-733) was obtained from Hong Kong University and propagated in Vero cells, then stored at -80 °C until required. Virus titers were determined using plaque assays. The primary antibodies used included a mouse monoclonal antibody (MAb) against the HSV-1 ICP5 major capsid protein (3B6; Abcam), an anti-cofilin rabbit antibody (3312; Cell Signaling Technology), an anti-p-cofilin rabbit antibody (3313: Cell Signaling Technology), an anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) rabbit MAb (14C10; Cell Signaling Technology). The secondary antibodies used included Alexa Fluor 488-conjugated goat anti-mouse IgG (H + L) (Invitrogen), Alexa Fluor 633-conjugated goat anti-mouse IgG (H + L) (Invitrogen).

RNAi

Lipofectanmine® RNAiMAX (Invitrogen, Carlsbad, California, America) was used to introduce siRNAs into cells. For transfection with the siRNA duplexes, vero cells were seeded into 24-well plates (8×10^4 cells/well) and incubated at 37 °C overnight. When the cultures reached 90 % confluence, the siRNA duplex was mixed with lipofectamine RNAiMAX and added to the culture medium. After 4 h, cells were washed with phosphate-buffered saline (PBS) and overlaid with 200 µl of virus diluent to achieve a multiplicity of infection (MOI) of 5 for 1.5 h. Cells were then washed and DMEM containing 2 % FCS. The UL19 siRNA duplexes were 5′-CUU CGC UGA UGA ACG UUG ATT-3′ (sense) and 5′-UCA ACG UUC AUC AGC GAA GGG-3′ (antisense). The scrambled siRNA duplexes were 5′-UUC UCC GAA CGU GUC ACG UTT-3′ (sense) and 5′-AGG UGA CAC GUU CGG AGA ATT-3′ (antisense); these did not target any known genes and were used as negative controls. All siRNAs were synthesized by Shanghai GenePharma Co Ltd.

RNA isolation, reverse transcription, and real-time quantitative PCR (qPCR)

Vero cells were grown in 6-well plates to 70-80 % confluence, then transfected with the various siRNAs (200 pmol) using lipofectamine RNAiMAX. After 4-6 h, cells were infected with HSV-1 at an MOI of 5. After 24 h, total RNA from infected cells was extracted using Trizol (Invitrogen). RNA concentrations were measured using a spectrophotometer (Beckman) at wavelengths of 260 and 280 nm. Extracted RNA (1 µg) was reverse-transcribed into cDNA using a PrimeScript RT reagent Kit (Takara). The qPCR assays were conducted using SsoFast™ EvaGreen® Supermix (Bio-Rad) according to the manufacturer's instructions. Primer pairs used were specific for cofilin1 (5′-GCC AGA CAA GGA CTG CCG CT-3′ and 5′-TCG GGG GCC CAG AAG ATG AAC A-3′), UL19 (5′-GAC CGA CGG GTG CGT TAT T-3′ and 5′-GAA GGA GTC

GCCATT TAG CC-3′), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 5′-CAC CAC CAA CTG CTT AGC C-3′ and 5′-CAG TGG ATG CAG GGA TGA TG-3′).

Immunoblotting analysis

Total proteins were extracted from cells using a RIPA total protein lysate kit, according to the manufacturer's instructions. Cell extracts were electrophoresed by 12 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to a polyvinylidene fluoride membrane (PVDF; Immobilon). After membranes were blocked with 5 % (w/v) non-fat dry milk (Difco) in TBS-T (137 mM NaCl, 20 mM Tris, and 0.1 % (v/v) Tween-20) at room temperature for 1 h, they were washed three times for 5 min with TBS-T. Membranes were incubated with appropriate primary antibodies diluted in TBS-T at 4 °C overnight and washed three times for 5 min with TBS-T. The membranes were then incubated with secondary antibodies at room temperature for 1 h and washed three times for 5 min with TBS-T. Positive signals were detected using enhanced chemiluminescence (ECL) reagents (Pierce, Rockford, IL). Differences in protein loading were normalized to GAPDH controls.

Plaque assays

Vero cells were grown in 24-well plates to 70 % confluence, and then transfected with the various siRNAs (40 pmol) using lipofectamine RNAiMAX. After 4 h, cells were infected with 30 plaque-forming units (PFUs) of HSV-1 strain F. The virus suspension was discarded 2 h later and cells washed with PBS, then overlaid with 1 ml of a 1:1 mixture of sodium carboxymethylcellulose (NaCMC) and DMEM lacking serum. At 72 h post-infection, plates were fixed with 10 % paraformaldehyde for 15 min and stained with 1 % crystal violet for 20 min. Plaques were enumerated in each well, overlapping plaques and plaques at the edges of a well were counted as a single plaque.

Flow cytometry

Flow cytometry (FCM) was used to test the transfection efficiency of siRNAs. After transfection with FAM labeled scrambled siRNA duplexes (F: 5′-UUC UCC GAA CGU GUC ACG UTT-3′ and R: 5′-AGG UGA CAC GUU CGG AGA ATT-3′) for 6 h, cells were washed with PBS and fixed for 5 min in 4 % formaldehyde - PBS. The fluorescence was then analyzed with a flow cytometer (Becton Dickinson, CA).

Laser-scanning confocal immunofluore-scence microscopy

Cells were seeded in 10-mm culture dishes and grown to 70 % confluence for siRNA transfection and HSV-1 infection at an MOI of 10. Cells were fixed with 4 % paraformaldehyde for 15 min, permeabilized with 0.1 % Triton X-100 for 5 min and blocked with 5 % bovine serum albumin (BSA) for 1 h. The Cells were then incubated with an appropriate primary antibody at room temperature, washed three times with PBS, and incubated with an Alexa Fluor 647-conjugated secondary antibody (1:1000 dilution) in 5 % BSA for 1 h at room temperature. Cells were washed three times with PBS, then TRITC-phalloidin and DAPI were used to label F-actin and cell nuclei, respectively. Fluorescence images were acquired using a confocal laser-scanning microscope (LSM 510; Zeiss).

Statistical analysis

The results are expressed as mean ± S.D. Statistical significance was determined using Student's t-test, with $p < 0.05$ considered statistically significant.

RESULTS

VP5 knockdown inhibits HSV-1 proliferation

The most efficient transfection rate was obtained using 10 pmol of siRNA mixed with 2 µl of lipofectamine RNAiMAX per square centimeter (Fig 1A). UL19 gene expression was inhibited by about 94 % (Fig 1B), and no influence was detected upon the other virus genes. Western blot analysis showed that the protein levels of VP5 were reduced to 18 % (Fig 1C). HSV-1 proliferation was significantly reduced (Fig 1D) and viral replication was apparently inhibited (Fig 2). At 16 h post-infection, VP5 was expressed at high levels and virus particle has begun to move from the nucleus to the cytoplasm. When the expression of VP5 was blocked by siRNAs, packaging of the virus in the nucleus was interrupted (Fig 2).

VP5 knockdown affects the depolymerization of F-actin

Distinct F-actin organization was observed during HSV-1 infection (Fig 3). The stress fiber was destroyed, appearing as sparse dots and with an irregular morphology. However, knockdown of VP5 expression abrogated the changes to F-actin that were induced by HSV-1 infection. HSV-1 infection adversely affected the filamentary

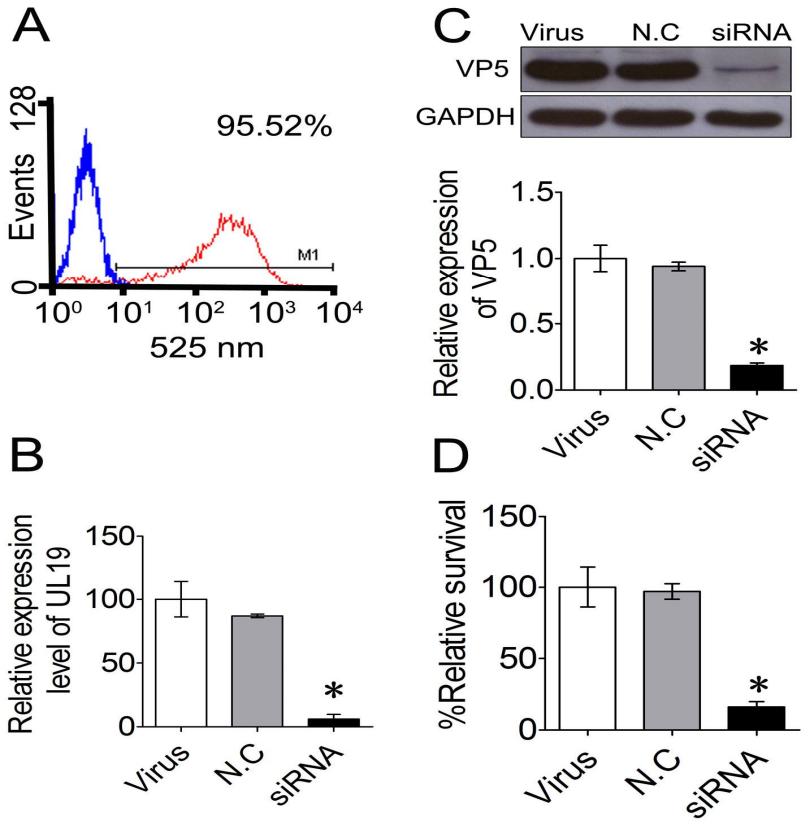

Figure 1: Knockdown of the VP5 major capsid protein using specific siRNAs. (A) Efficiency of transfection was assessed with flow cytometry. (B) Relative expression levels of UL19 was measured by qPCR. (C) Western blotting was conducted to determine silencing efficiency following transfection with siRNAs and infection with HSV-1 (MOI = 5). (D) Plaque formation assays were conducted to analyze the effects of siRNAs on HSV-1 proliferation

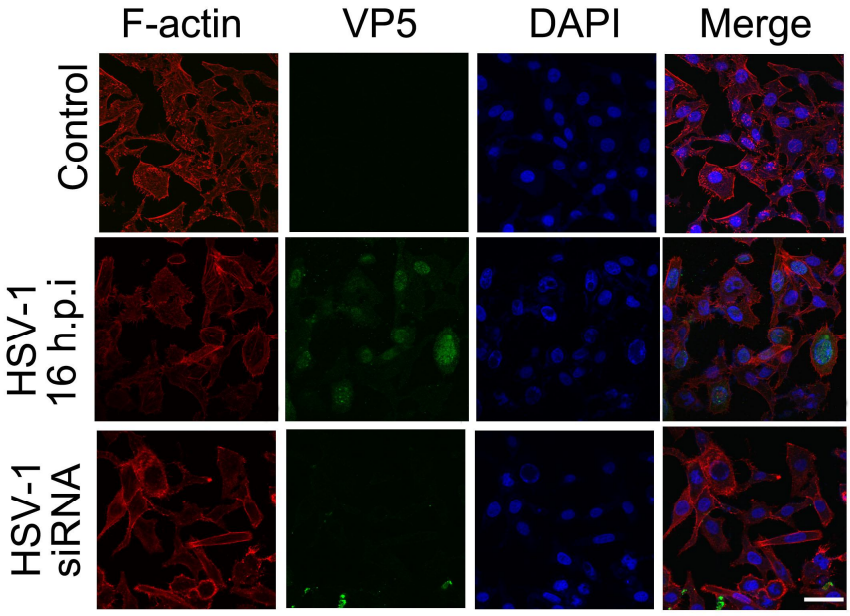

Figure 2: Knockdown of VP5 inhibits HSV-1 proliferation. Proliferation of HSV-1 (MOI = 10) in vero cells was observed using laser-scanning confocal microscopy. Vero (top), HSV-1 (middle), and siRNA (bottom). VP5 was detected using an anti-VP5 antibody (green), while F-actin was stained with TRITC-phalloidin (red), and nuclei stained with DAPI (blue). Bar, 20 μm

structures of F-actin, with the stress fibers in the nucleus becoming depolymerized. Following RNAi, the number of virus particles had decreased and the actin cytoskeleton appeared to be normal.

VP5 knockdown affects expression of cytoskeletal regulators

We used qPCR assays to determine the influence of VP5 down-regulation on the expression of genes related to cytoskeletal modulation. We assessed gene expression levels at 6 (early stage) and 12 h (late stage) post-infection, and found that UL19 expression was inhibited by 81 % and 85 %, respectively (Fig 4A). The expression of cofilin1 increased in conjunction with HSV-1 proliferation, but was decreased in the siRNA group (Fig 4B). The increase in cofilin1 expression promoted the depolymerization of F-actin, which was inhibited by siRNAs. In addition, western blotting was conducted to examine the expression levels of major cytoskeleton-related proteins (cofilin1, p-cofilin1, β-actin) during HSV-1 infection. At 6 h post-infection, the quantities of cofilin1 and p-cofilin1 had decreased. In the siRNA group, the decreases were to levels below those of the virus control. At 12 h post-infection, total expression levels for cofilin and p-cofilin1 were up-regulated compared with those at 6 h post-infection. However, this trend was not significant for the siRNA group (Figure 4C). These results show that the siRNAs targeting VP5 influenced changes in cytoskeleton proteins during retrograde transport and following HSV-1 infection.

DISCUSSION

In this study we evaluated the inhibitory effects of siRNA duplexes, specific for HSV-1 VP5, on HSV-1 replication and the cytoskeleton of cells. The siRNAs targeting UL19 were able to specifically and efficiently down-regulate VP5. Following transfection of the siRNAs, the proliferation and packaging of HSV-1 in the nucleus was significantly reduced. Knockdown of

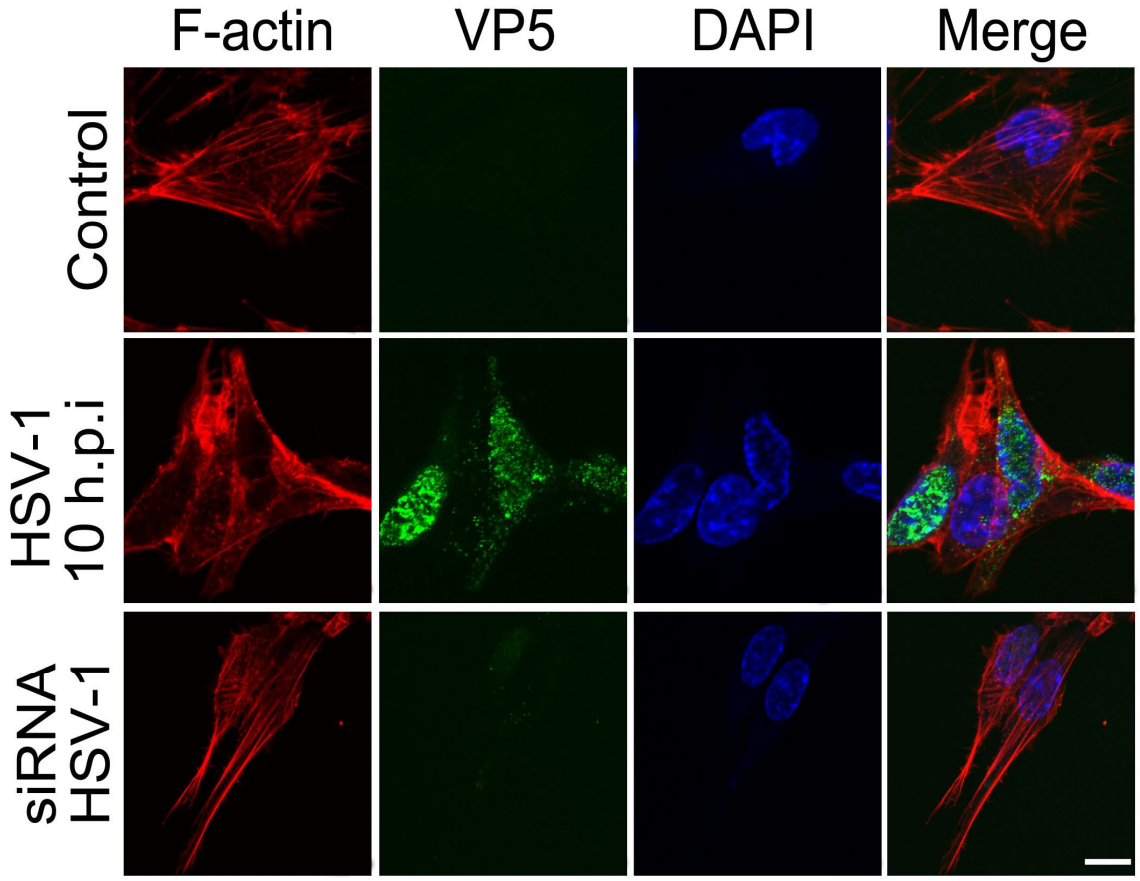

Figure 3: VP5 influences the depolymerization of F-actin following HSV-1 infection. F-actin (red), VP5 (green), and nuclei (blue). Bar, 10 μm

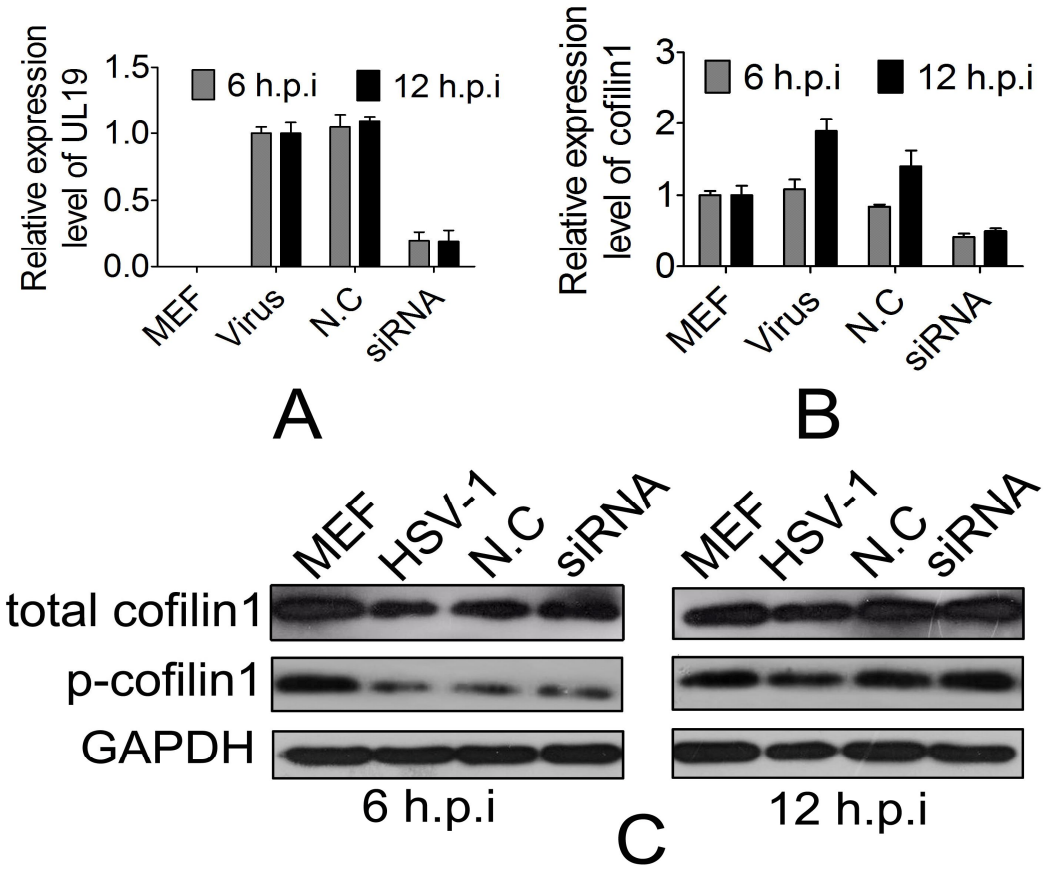

Figure 4: VP5 knockdown affects the expression of genes that regulate the cytoskeleton and affects rearrangement of the cytoskeleton. Cells transfected with siRNA were infected with HSV-1 (MOI = 5) over various durations (6 h and 12 h). Relative mRNA expression levels of *UL19* (A) and cofilin1 (B) were determined by qPCR. (C) Western blotting was used to determine the protein expression levels of cofilin1 and p-cofilin1

VP5 abrogated changes in F-actin that were induced by HSV-1 infection. This knockdown influenced changes in cytoskeleton proteins during retrograde transport [14], and could possibly lead to alterations in some cytoskeletal proteins following infection with HSV-1.

VP5 is a major capsid protein synthesized during the latter phases of HSV-1 gene expression. It is thought to be essential for the formation of the capsid [15]. This capsid protein plays a key role in HSV-1 packaging and maturity. Previous studies have shown that HSV-1 strains with mutated forms of VP5 fail to form plaques in cell lines [16]. These findings suggest that VP5 is necessary for HSV-1 to proliferate, and opens up the possibility that VP5 could be a new target in anti-HS-1 therapies [17].

RNAi has become an advanced tool for screening and identifying gene function. It may also play an important role in antiviral defense, with inhibition of viral replication demonstrated *in vitro* for a variety of RNA viruses, including rotaviruses, respiratory syncitial virus, and Dengue virus. The use of RNAi in the treatment of HSV infections is a definite possibility. Although siRNAs appear to have great promise in antiviral therapy, this technology has several limitations for clinical applications. Delivery of siRNAs is the biggest obstacle to the development of siRNA-based therapeutic agents [18]. Direct administration would require siRNAs that are modified to be resistant to nucleases, and perhaps conjugated with a ligand to target the siRNA to specific tissues. Additionally, there are problems related to the identification of effective target sites within the target gene, along with activation of interferons triggered by siRNAs, and the probability of escape mutants emerging [19].

CONCLUSION

The findings of this study show that siRNAs targeting VP5 confers excellent antiviral activity by inhibiting HSV-1 replication in cells. The findings also indicate that major capsid protein VP5 encoding gene UL19 may be a potential target for an RNA interference-based therapeutic strategy against HSV-1.

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