Effective inhibition of human cytomegalovirus gene expression by DNA-based external guide sequences

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To investigate whether a 12 nucleotide DNA-based miniEGSs can silence the expression of human cytomegalovirus (HCMV) UL49 gene efficiently, a HeLa cell line stably expressing UL49 gene was constructed and the putative miniEGSs (UL49-miniEGSs) were assayed in the stable cell line. Quantitative RT–PCR and western blot results showed a reduction of 67% in UL49 expression level in HeLa cells that were transfected with UL49-miniEGSs. It was significantly different from that of mock and control miniEGSs (TK-miniEGSs) which were 1 and 7%, respectively. To further confirm the gene silence directed by UL49-miniEGSs with human RNase P, a mutant of UL49-miniEGSs was constructed and a modified 5’ RACE was carried out. Data showed that the inhibition of UL49 gene expression directed by UL49-miniEGSs was RNase P-dependent and the cleavage of UL49 mRNA by RNase P was site specific. As a result, the length of DNA-based miniEGSs that could silence gene expression efficiently was only 12 nt. That is significantly less than any other oligonucleotide-based method of gene inactivation known so far. MiniEGSs may represent novel gene-targeting agents for the inhibition of viral genes and other human disease related gene expression.

Keywords DNA-based miniEGSs; RNase P; gene silence; human cytomegalovirus; antivirus

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Introduction

Nucleic acid-based gene interference strategies such as antisense oligonucleotides, ribozymes or DNAzymes, and RNA interference (RNAi) represent powerful research tools and promising therapeutic agents for human diseases [1–6]. The gene-targeting agents used can be a conventional antisense oligonucleotide, an antisense catalytic molecule (ribozyme or DNA enzyme) or an antisense molecule with an additional (guide) sequence that targets the mRNA for degradation by endogenous RNases such as RNase L and the RNA-induced silencing complexes [3–8]. Each of these approaches has its own advantages and limitations in terms of targeting efficacy, sequence specificity, toxicity, and delivery efficiency in vivo. The improvement in these current technologies and the development of new nucleic acid-based strategies will provide exciting tools and reagents for basic research and clinical applications including therapeutic interventions.

The endonuclease ribonuclease P (RNase P) processes tRNA by cleaving the 5’ leader sequence of the precursor, generating mature tRNA. Studies with chemically modified substrates have shown that the contact sites between pre-tRNA and some bacterial and Schizosaccharomyces pombe RNase P enzymes are located primarily in the aminoacyl acceptor stem, the T stem, and the T loop [9–13]. Hardt et al. [14] found that D stem deletions reduced cleavage by human and Thermus thermophilus RNase P, whereas these deletions affected the Escherichia coli enzyme to a lesser extent. Other studies demonstrated that RNase P cleavage of a small model substrate consisting only of the acceptor stem with a 5’ leader and the T stem and loop; all other domains of tRNA were replaced by a bulge. The most efficient cleavage was achieved with a bulge size of nine nucleotides. In tRNA, the acceptor stem and T stem form a consecutive helix that is the binding site for RNase P [15,16]. It was also demonstrated that RNase P recognizes bimolecular RNA structures that resemble tRNA precursors and subsequently cleave one
of the strands at a specific position [17,18]. The RNA strand cleaved was defined as the target strand, whereas the other strand was termed the external guide sequence (EGS). This discovery led to the idea of utilizing EGSs as oligonucleotide-based therapeutics [17–25]. Werner et al. [26] showed that the sequence UUCR adjacent to a 5- or 6-bp helix, which in turn is connected through a bulge with a second short helix (seven to eight nucleotides), is sufficient for the recognition and cleavage with human RNase P. This finding has a very important implication that we were able to design EGSs of a length of only 12 or 13 nt, which was named miniEGSs.

RNA-based EGSs have been expressed endogenously as transgene in both bacteria and mammalian cells [18,27], and were effective in inhibiting gene expressions of herpes simplex and influenza virus and in abolishing replication of influenza virus in human cells [28,29]. In vitro studies in human cells have shown that DNA-based EGSs, as well as EGS molecules with modified nucleotides, can direct M1 RNA and human RNase P to cleave an mRNA sequence, although their targeting efficiencies are lower than those of unmodified RNA-based EGSs. Previous studies showed that exogenous administration of chemically synthesized DNA-based EGSs is highly effective in treating human cytomegalovirus (HCMV)-infected cells and abolishing HCMV replication [17,30–32]. It is also unclear whether DNA-based miniEGSs can be exogenously administered into human cells for inhibiting gene expression.

HCMV, a human herpes virus, causes serious clinical manifestations in newborns and immunocompromised populations such as AIDS patients [33]. For example, this virus is the leading cause of congenital infections associated with mental retardation in newborns [34–38]. The development of effective antiviral compounds and approaches is central for the treatment and prevention of infections by HCMV as well as other herpes viruses. The UL49-deletion TowneBAC was significantly defective in growth in HFF. Thus, UL49 may serve as a target for novel drug development to combat HCMV infection [39].

In this study, we showed that a DNA-based miniEGS-induced RNase P to specifically cleave the UL49 mRNA in cell culture. Our study provided evidences that the RNase P-based approach is effective in inhibiting HCMV gene expression by targeting the UL49 mRNA. These results also demonstrated the feasibility of the active miniEGSs as a novel class of antiviral agents for treatment of human viral diseases.

Materials and Methods

Construction of plasmids
The DNA sequence that encodes HCMV UL49 was constructed by PCR using AD169 genomic DNA as a template and oligonucleotides UL49F: 5’-AGGCGAATTCGCCCAC CATGGCCAGTGTCTCCGA-3’ and UL49R: 5’-C GGCTCGAGGACATGGGCGACGGCCGTG-3’ as 5’ and 3’ primers, respectively. The reactions were performed for 30 s at 95°C, 1 min at 55°C, and 45 s at 72°C for 35 cycles. The PCR fragments were digested with BamHI and XhoI, and then cloned into pcDNA3.1/myc plasmid vector (Invitrogen, Karlsruhe, Germany). The restriction endonucleases BamHI and XhoI, TA Q polymerase, and T4 DNA ligase were purchased from Takara (Dalian, China). All oligonucleotides used as PCR primers were purchased from Genewindows (Guangzhou, China).

Construction of UL49-expressing cells
The protocol for the construction of HeLa cells expressing HCMV UL49 was modified based on the manual of pcDNA3.1/myc plasmid. In brief, HeLa cells (ATCC) were transfected with pcDNA3.1-UL49/myc vector DNA with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocols. The ratio of Lipofectamine 2000 to plasmid was 1 μl/μg. At 48–72 h after infection, cells were incubated in DMEM (Sigma-Aldrich, St. Louis, USA) containing 800 μg/ml neomycin. Cells were subsequently selected in the presence of neomycin for 3 week, and neomycin-resistant cells were cloned. Reverse-transcription PCR and western blotting were performed for UL49 expression analysis.

Design of DNA-based miniEGSs
The anti-UL49 DNA-based miniEGSs (UL49-miniEGSs) was designed as described by Werner et al. [26]. A search was conducted to find the pattern TTCR in the sequence of the HCMV cDNA (NC_001347). For the design of UL49-miniEGSs, the region between 71331 and 73043 bp was chosen. The putative EGSs were designed to hybridize as EGSs to different regions of the UL49 mRNA with an expected cleavage site 5’ to the double-stranded region. Each was designed for stem 1 to hybridize immediately adjacent to a TTCR sequence; the length of stem 1 was five or six nucleotides. Stem 2 for each EGS was designed so that a G residue was 3’ to the putative cleavage site; the helix length was seven or eight nucleotides. Consequently, the sizes of the bulges varied between 5 and 14 nucleotides, depending on the
positions of the G residues at the 3' end and the TTCR residues at the 5' end of the targeted region.

Two additional DNA-based miniEGSSs were designed. One was anti-TK DNA-based miniEGSSs (TK-miniEGSSs) that targeted the mRNA for thymidine kinase (TK) of herpes simplex virus (HSV-1) (NC_001806) and the other was 3' end mismatch mutant of anti-UL49 DNA-based miniEGSSs (mut-miniEGSSs). The two EGSs were used as controls to determine whether EGSs with an incorrect guide sequence could target UL49 mRNA in cell culture.

DNA-based miniEGSSs treatments of cells
Stable UL49-expressing HeLa cells were maintained at 37°C in a humidified atmosphere with 5% CO2 in DMEM (Invitrogen), supplemented with 10% fetal calf serum (FCS; Sijiqin, Hangzhou, China), penicillin (100 µg/ml), streptomycin (100 µg/ml), and neomycin (200 µg/ml) (Invitrogen). Cells were grown on collagen-treated plates and transfected with DNA-based miniEGSSs using the Lipofectamine 2000 (Invitrogen). For the knockdown of HCMV UL49 expression, UL49-miniEGSSs, mut-miniEGSSs, and TK-miniEGSSs were used. The corresponding amounts of EGS molecules were mixed with 25 µl of MEM (Invitrogen). In a separate tube, 2.5 µl of Lipofectamine 2000 per reaction were added to 50 µl of MEM and incubated for 5 min at room temperature. Both solutions were mixed and incubated for an additional 20 min at room temperature to allow complex formation. The solutions were then added to the cells in the 6-well plates to a final volume of 600 µl. Cells were incubated at 37°C in the presence of the mixed solution for 6 h.

Flow cytometry
To assay the transfection efficiency of these DNA-based miniEGSSs, some of miniEGSSs were 5' end fluorescein isothiocyanate (FITC) labeled. FITC-conjugated miniEGSSs were transfected into cells at 40 nM. Twenty-four hours post-transfection, cells were washed twice in PBS and finally resuspended in PBS containing 1% (v/v) of FCS. Samples were analyzed using an XL flow cytometer (Coultronics, Margency, France) equipped with an argon laser set at 488 nm. Untransfected cells were used as negative control. Green fluorescence was processed with a 520–530 nm band pass filter. Fluorescence was displayed on a monoparametric histogram (256 channels logarithmic scale) and was expressed as the mean intensity of fluorescence (MIF): MIF = e^[ln 10000/256][x], where 'x' is the mean peak channel on the logarithmic scale. A total of 10 000 cells were analyzed for each assay. Viable cells were selected using the biperametric histogram FLS × 90LS (size × granulometry).

5' RACE
The cleavage of HCMV UL49 mRNA was measured by 5' RACE. Stable UL49-expressing HeLa cells were transfected with 40 nM of UL49-miniEGSSs, 40 nM of mut-miniEGSSs, 40 nM of TK-miniEGSSs, and mock transfected. Total RNA was harvested 24 h after transfection using the TRIzol (Invitrogen) and DNase treated with RNA Free DNase I (Takara, Tokyo, Japan). Four micrograms of RNA were ligated to a synthetic DNA oligonucleotide adaptor (GCTGATTGTGATGAA CACTGCGTTTGCTGGTTGATGAAA) by treatment with T4 RNA ligase (2 units; New England Biolabs (Beijing) Ltd, Beijing, China) for 1 h at 37°C. After the ligation reaction, RNA was phenol/chloroform extracted and precipitated with ethanol. Ligation products were reverse-transcribed using oligo(dT) primer and MMLV RT (Toyobo, Tokyo, Japan) according to the manufacturer's guidelines. The resulting cDNAs were amplified by PCR using primer1 (5'CACGTTGTTTCTGG CTTTGTATG-3') and primer3 (5'CCTTAACCCCAGGG TTGGAGCCAGTG-3'), and primer2 (5'GACCTGCGCT GCCCCGTCTCTCA-3'), and primer3. As internal standard, a fragment of human endogenous β-actin was amplified simultaneously in each PCR. β-Actin sense primer: 5'-CACGCTTACACACACGATGTA CACACTGCGTTCGTTCTTGCGT-3', antisense primer: 5'-CAGGTAAAGCTCTGCTGGCTCAT-3'. RT-PCR products were examined by 1.5% agarose gel electrophoresis. The identity of specific cleavage products was confirmed by cloning of the PCR product and sequencing of individual clones.

Quantitative RT-PCR
Quantitative RT-PCR was performed using standard protocols on an Applied Biosystem's 7300 Sequence Detection System. Briefly, total RNA was extracted using TRIzol, then 5 µl of a 1/100 dilution of cDNA in water was added into 12.5 µl of the 2 × SYBR green PCR master mix (Takara), with 800 nM of each primer in a total volume of 25 µl. All reactions were run in triplicate using Applied Biosystem's 7300 Sequence Detection System. UL49 gene sense primer: 5'-GTTCTTGGCT CCTTACATCT-3'; anti-sense primer: 5'-CACAAAGTAG GGCTTGGTCTC-3'. As internal standard, a fragment of human endogenous β-actin was amplified simultaneously in each PCR. β-Actin sense primer: 5'-TCGTTCCACCGC 3'-ACTGTTGTTTCTGG CTTTGTATG-3'; anti-sense primer: 5'-CCTTAACCCCAGGG TTGGAGCCAGTG-3'.
AAATGCTTCTAG-3', anti-sense primer: 5'-ACTGCTGTCACCTTCACCGTCC-3'. Quantitative RT–PCR conditions: 95°C for 10 s 1 cycle, followed by 95°C for 5 s, 60°C for 20 s, 40 cycles for amplifying UL49 and β-actin.

Western blot analysis
For western blot analysis, cells were treated DNA-based miniEGSs with the same protocol in 6-well plates. After washing and lyses steps, proteins were separated using a 15% polyacrylamide gel (Bio-Rad, Munich, Germany). Electrophoresis was performed at 80 V for 30 min and 150 V for 1 h. Then, gels were transferred to a PVDF membrane (Amersham, Freiburg, Germany); Semi-electroblotting was carried out for 30 min at 27 V. Ponceau S solution was used for protein labeling. Blocking was performed for 8 h at 4°C with PBST/de-fatted milk 0.5%, and then primary antibody (1:1000 anti-myc antibody; Invitrogen) was added. After incubation for 2 h at 37°C and washing with PBST, incubation with the secondary antibody (1:5000 anti-rabbit IgG-HRP, Amersham Pharmacia) was performed for 45 min. After wash and removal of EDTA with PBS, the blots were incubated for 2 min with a luminescence solution ECL (Pierce, Rockford, USA) and exposed on a film in a darkroom. β-Actin (1:200) was used as positive control using 1:5000 anti-rabbit IgG-HRP (Amersham) as secondary antibody.

Results

Efficient expression of the UL49 in HeLa cells
The pcDNA3.1-UL49-myc recombinant plasmids were assayed by PCR, digestion of BamHI and XhoI, and sequencing (data not shown). Results showed that DNA fragment coding HCMV UL49 was cloned into pcDNA3.1/myc vector [Fig. 1(A)] and placed under the control of HCMV immediate-early (CMV) promoter for high-level expression in a wide range of mammalian cells. Only the plasmid that was successfully constructed could be used to establish UL49 stable expression HeLa cell lines.

The pcDNA3.1-UL49-myc plasmids were transfected into HeLa cells. Complete stable cell selection took up to 3 weeks of growth in neomycin-selective media. Neomycin-resistant cells were cloned. Reverse-transcription PCR and western blot were carried out to analyze selected cells. The data showed that UL49 stable expression cell lines was constructed successfully [Fig. 1(B,C)]. The cell lines expressing HCMV UL49 can be used for further studies in EGS assay experiment.

Design of DNA-based miniEGSs
On the basis of the principles of designing miniEGSs described above, one of the putative cleavage sites with the different EGSs was at position 603 of the 1713-bp UL49 mRNA [Fig. 2(A)]. The sequence UUCA adjacent to a 5 bp helix of UL49-miniEGSs GCACGGGTCCAC [Fig. 2(B)], which in turn is connected through a bulge with a second short helix (nine nucleotides) that is sufficient for recognition by human RNase P and induced targeted mRNA cleavage with human RNase P [Fig. 2(C)].
The mut-miniEGSs were the sequence of 3' end mutation of UL49-miniEGSs CAC to GTG [Fig. 2(D)] that is insufficient for recognition and cleavage with human RNase P. The TK-miniEGSs 5'-CCTGCGGAGAGC-3' [Fig. 2(F)] was designed to determine whether EGSs with an incorrect guide sequence could target UL49 mRNA in cultured cell.

**UL49-miniEGSs directed inhibition of UL49 expression**

To determine whether the UL49-miniEGSs directed human RNase P to cleave UL49 mRNA sequence efficiently, UL49-miniEGSs and TK-miniEGSs were transfected into stable UL49-expressing HeLa cells with mock transfection performed. The transfection efficiency of these DNA-based miniEGSs was assayed by flow cytometry. The transfection efficiency of UL49-miniEGSs, TK-miniEGSs, and mut-miniEGSs was 97.9 and 97.7, and 97.7%, respectively (Fig. 3). The results showed that these miniEGSs were transfected into cells successfully. That would make the data analyses in further study performed in the same background.

To assay the silencing efficiency of UL49 expression by UL49-miniEGSs, quantitative RT–PCR and western blot analyses were performed. A reduction of 67% in the levels of UL49 expression was observed in cells that transfected with 40 nM UL49-miniEGSs. It was significantly higher than those of mock and 40 nM TK-miniEGSs, which was 1 and 4%, respectively [Fig. 4(A,B)]. As a result, an efficient inhibition of HCMV UL49 gene expression was induced by UL49-miniEGSs.

**Inhibition directed by DNA-based miniEGSs is dose-dependent**

To determine whether the inhibition of UL49 expression directed by miniEGSs is depended on the dose of UL49-miniEGSs or not, the UL49-miniEGSs were transfected into stable cell lines at different concentrations (2.5, 5, 10, 20, 40, 60, and 80 nM). We consistently achieve an optimal transfection efficiency of about 97% (data not shown). To assay the silencing efficiency in different concentration, quantitative RT–PCR and western blot analyses were performed. The quantitative RT–PCR and western blot analysis [Fig. 5(A,B)] showed that when the concentration of UL49-miniEGSs was 2.5 nM, the inhibition efficiency was 17%; and then 5 nM was 20%, 10 nM was 33%, 20 nM was 56%, 40 nM was 70%, 60 nM was 87%, and 80 nM was 88%. As a result, the inhibition efficiency of UL49-miniEGSs was depended on concentration of UL49-miniEGSs. In another word, inhibition of HCMV UL49 expression directed by UL49-miniEGSs is dose dependent.

**Inhibition directed by DNA-based miniEGS is RNase P-dependent**

To determine whether the inhibition of UL49 expression directed by miniEGSs is depended on the sequence specificity of UL49-miniEGSs and RNase P-specific cleavage, the 40 nM mut-miniEGSs, which was mutant in RNase P cleavage site of the 3' end of miniEGSs, were transfected into the stable cell line. The transfection efficiency of mut-miniEGSs was 97.7% assayed by flow cytometry [Fig. 3(C)]. To assay the silence efficiency of mut-miniEGS, quantitative RT–PCR, and western blot analysis...
analyses were performed. The data showed that the inhibition efficiency of UL49 expression were 7% [Fig. 6(A,B)]. To further determine whether the inhibition of mut-miniEGS is dose dependent, the mut-miniEGSs were transfected into stable cell lines at different concentrations (20, 40, 60, and 80 nM). The quantitative RT–PCR and western blot analysis [Fig. 7(A,B)] showed that when the concentration of mut-miniEGSs was 20, 40, 60, and 80 nM, the inhibition efficiency of UL49 expression was 1, 2, 8, and 0%, respectively. There were significant differences compared with those of UL49-miniEGS. It showed that when the cleavage site of RNase P was mutational, the mutant of UL49-miniEGSs could not induce RNase P to cleave the targeted mRNA. The 3′ end sequence of EGSs is important for the cleavage of RNase P, and it means that the inhibition directed by miniEGSs is depended on mRNA cleavage induced by RNase P. In another word, inhibition of HCMV UL49 expression directed by UL49-miniEGSs is RNase P dependent.

Cleavage directed by DNA-based miniEGS is site specific
To make sure that the human RNase P-mediated cleavage of UL49 mRNA directed by UL49-miniEGSs is highly site specific, the modified 5′ RACE was carried out. A DNA adaptor was ligated to the 5′ end of cleavage site. When the UL49 transcripts were cleaved by RNase P in putative cleavage site, there are fragments by reverse-transcribed PCR using whatever paired primer1 and primer3 or paired primer2 and primer3. If the PCR fragments can be obtained only using primer2 and primer3, it means that transcripts were not cleaved [Fig. 8(A)]. The 5′ RACE results showed that using cDNA from the cells of mock, mut-miniEGSs and TK-miniEGSs transfected as templates, only primer2 and primer3 could get
the PCR fragments [lanes 1–3 and 7–9 in Fig. 8(B) and lanes 1–9 in Fig. 8(C)]. On the other hand, using cDNA from UL49-miniEGSs transfected cells as a template could get PCR fragments using both primer1 and primer3, and primer2 and primer3 [lanes 4–6 in Fig. 8(B)]. Identity of specific cleavage products was confirmed by cloning of the PCR product and sequencing of individual clones (data not shown). It means that cleavage of UL49 mRNA was highly specific directed by UL49-miniEGSs not by other irrelevant miniEGSs.

Discussion

The EGS-based technology represents an attractive approach for gene inactivation since it utilizes endogenous RNase P to generate highly efficient and specific cleavage of the target RNA [40,41]. In particular, RNase P is among the most ubiquitous and essential enzymes found in nature, as it is responsible for processing all tRNA molecules. Moreover, RNase P-mediated cleavage directed by EGSs is highly specific and does not generate ‘irrelevant cleavage’ that is usually associated with RNase H-mediated cleavage induced by conventional DNA-based oligonucleotides [40,42]. Thus, EGS molecules represent promising general gene-targeting agents that can be used in both basic research and clinical applications. Several criteria must be satisfied if successful targeting with the EGS technology is to be achieved. Among these are efficient delivery of the reagents, high efficiency of cleavage, and sequence specificity of the EGS.

Delivery of the EGS into the nuclear compartment is essential to the success of the EGS technology because RNase P is exclusively localized in nuclei [40]. To determine whether the EGSs were efficiently internalized into the cells, EGS molecules conjugated with a Cy3 label were included in the transfection mixture and were visualized by confocal microscopy. The majority of the EGS colocalized with those stained with DAPI (primarily staining genomic DNA in the nuclei; data not shown).
shown). It means that exogenous DNA-based EGSs can be delivered predominantly into the nuclei. The efficient delivery and proper localization of the EGS may be mediated by cellular tRNA-binding proteins, which may interact with the tRNA-like domains of the EGS and target the EGS to the nuclear compartment. Further exploitation of these interactions will facilitate the development of EGSs as novel gene-targeting agents for both basic research and clinical therapeutic applications.

The EGSs did not exhibit significant cytotoxicity, as cells treated with EGSs are indistinguishable from the cells treated with lipid complexes alone in the absence of the EGSs, in terms of cell growth and viability for 10 days (data not shown). The UL49-miniEGSs directed human RNase P to cleave UL49 mRNA sequence efficiently. In contrast, a reduction of $<8\%$ in the levels of UL49 expression was observed in cells that transfected control mut-miniEGSs. Mut-miniEGSs could bind efficiently to the UL49 mRNA sequence but contained point mutations that disrupted RNase P cleavage. A reduction of $<5\%$ in the levels of UL49 expression was observed in cells transfected with TK-miniEGSs that targeted TK1 mRNA. These results suggest that the overall observed inhibition with UL49 was primarily due to targeted cleavage by RNase P induced by UL49-miniEGSs, which is in contrast to the antisense effect or other non-specific effects of other EGSs.

The length of DNA-based miniEGSs was only 12 nucleotides. This is significantly shorter than others used in oligonucleotide-based method of gene inactivation known so far [43]. This new design of EGSs opens promising new perspectives for developing oligonucleotide-based antiviral or anti-human disease therapeutics, because shorter oligonucleotides are easier to synthesize.

HCMV is a member of the human herpes virus family, which includes seven other different viruses such as human syncytial virus (HSV) and Epstein–Barr virus [33]. HCMV infects 70–100% of adults in populations worldwide. Although the virus establishes life-long latency and persists after a primary infection, 60–90% of transplant recipients and AIDS patients may develop CMV infections due either to reactivation of latent virus
or to a new infection [44]. In such patients, CMV infection not only causes problems during the acute phase but also appears to increase the risk of long-term complications. These include transplant vascular sclerosis and other vascular complications in organ transplant recipients and autoimmune phenomena, such as chronic graft versus host disease in bone marrow transplant recipients and skin-related symptoms in AIDS patients. Thus, to find effective anti-HCMV drugs is important for these patients. The drug-resistant HCMV was found and choosing a new target for anti-virus therapy is essential. The UL49 is highly conserved among all herpes viruses and is considered as an ideal target for anti-HCMV therapy because the mutant HCMV with UL49 knockout is lethal in cultured cell [39] and our group found that silenced UL49 gene expression will inhibit virus growth (data not showed). Thus, UL49 may serve as a target for novel drug development to combat HCMV infection.

Further research of testing the efficiency of EGS-based gene inactivation in live pathogens should be the ultimate test for feasibility of using EGS in biomedical applications. Such further research to improve the nuclease resistance and binding affinity of the short EGSs by introducing further chemical modifications is currently ongoing. Further understanding of how the functional groups in the nucleotides of an EGS interact with human RNase P and the mRNA substrate will lead to the construction of highly active and stable EGSs with either different bases or modifications at these nucleotide positions. Moreover, engineering different designs of EGSs for increasing their targeting activity, as well as developing new means for improving their delivery, are needed to increase the efficacy of the EGSs in vivo. These studies will further facilitate the development of the EGS-based technology for gene-targeting applications in both basic research and clinical therapy, including the studies and treatment of HCMV infections.

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