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# Over-expression of human cytomegalovirus miR-US25-2-3p downregulates *eIF4A1* and inhibits HCMV replication

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ABSTRACT

**It has been reported that human cytomegalovirus (HCMV) miR-US25-2 reduces DNA viral replication including HCMV. However, the mechanism remains unknown. In our study, *eukaryotic translation initiation factor 4A1* (*eIF4A1*) was identified to be a direct target of miR-US25-2-3p. Small interfering RNA (siRNA) and miR-US25-2-3p mediated *eIF4A1* knockdown experiments revealed that high level of miR-US25-2-3p in MRC-5 cells decreased HCMV and host genomic DNA synthesis, and inhibited cap-dependent translation and host cell proliferation. However, *eIF4A1* up-regulation induced by miR-US25-2-3p inhibitor increased HCMV copy number. Therefore, the over-expression of miR-US25-2-3p and consequent lower expression of *eIF4A1* may contribute to the inhibition of HCMV replication.**



## 1. Introduction

Human cytomegalovirus (HCMV) is a β-herpesvirus that causes widespread, persistent human infection. MicroRNAs (miRNAs) are endogenous, non-coding RNA of about 22 nucleotides that regulates gene expression by controlling target mRNA translation or degradation [1]. So far, at least 19 miRNAs have been identified in HCMV [2]. Emerging evidences suggest that some HCMV miRNAs may regulate viral replication and play significant roles in determining the viral life cycle [3–5]. It has been reported that over-expression of miR-US25-2 can reduce the viral replication and DNA synthesis of DNA viruses including HCMV, and thus the authors hypothesized that miR-US25-2 could target host genes that are essential for virus replication [3]. However, the mechanism remains illusive.

In this study, we found that over-expression of miR-US25-2-3p, which is one miRNA derived from the precursor of miR-US25-2, obviously reduce HCMV replication. The targets of miR-US25-2-3p were screened using hybrid-PCR method [6], and *eukaryotic translation initiation factor 4A1* (*eIF4A1*), an ATP-dependent RNA helicase, which plays an important role in cap-dependent translation, cell proliferation and viral growth [7–14], was identified as a direct target of miR-US25-2-3p by luciferase reporter assay and functional analyses.

## 2. Materials and methods

### 2.1. Cell culture and virus

Human fetal diploid lung fibroblasts MRC-5 cells were maintained in modified Eagle's medium (MEM) with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 units/ml streptomycin. HCMV clinical strain Han was isolated from a urine sample of a 5-month-old infant hospitalized in Shengjing Hospital of China Medical University. The virus was inoculated in MRC-5 cells maintained in MEM supplemented with 2% FBS and penicillin–streptomycin, then the cell lysate containing virus was harvested and stored at −80 °C.

*Abbreviations:* HCMV, human cytomegalovirus; miRNA, microRNA; *eIF4A1*, *eukaryotic translation initiation factor 4A1*; MEM, modified Eagle's medium; FBS, fetal bovine serum; MOI, multiplicity of infection; UTR, untranslated region; FFL, firefly luciferase; siRNA, small interfering RNA; NC, negative control; qPCR, quantitative real-time polymerase chain reaction; RT, reverse transcription; snRNA, small nucleolar RNA; HRP, horseradish peroxidase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; IE, immediate-early; *VCAN V3*, *Versican, transcript variant 3*; *TGFBI*, *transforming growth factor, beta-induced, 68kDa*; *IER3*, *immediate early response 3*

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### 2.2. Hybrid-PCR

Stock virus was inoculated in MRC-5 at a multiplicity of infection (MOI) of 3–5 PFU per cell. To carry out early infection condition, 100 μg/ml phosphonoacetic acid (PAA) was added to the medium as the some time as virus inoculation. The MRC-5 cells were harvested at 48 h post infection (hpi) and total RNA was extracted with TRIzol (Invitrogen) and treated by TURBO DNA-free™ Kit (Ambion). Hybrid-PCR, an effective miRNA target screening method established in our laboratory [6], was carried out to screen putative targets of miR-US25-2-3p (Supplementary Fig. 1). A specific hybrid-primer of miR-US25-2-3p, 5′-GCGGGRGCTCTCCRR GTGGR-3′, was designed as follow: a reverse and complementary sequence of miR-US25-2-3p seed region (nucleotides 2–8) was located in the 3′-terminal of the hybrid-primer; R in the primer represents adenine (A) or guanine (G) due to G:U pair is allowed for the formation of miRNA:mRNA duplexes as described in the miRanda database (http://www.microrna.org/microrna/getMirnaForm.do). After reverse transcription using 3′-Full RACE Core Set kit (TaKaRa), sequences between miR-US25-2-3p binding sites and polyA signal were amplified with the hybrid-primer, the outer primer (5′-TACCGTCGTTCCACTAGTGATTT-3′) and the inner primer (5′-CGCGGATCCTCCACTAGTGATTTCACTATAGG-3′) provided in the kit. All PCR products were cloned into pMD 18-T vectors (TaKaRa), and were sequenced on an ABI 3730 automated sequencer. mRNA specific sequences located between the miR-US25-2-3p hybrid-primer, which is homologous to the miRNA binding site, and polyA tails were intercepted and used to identify the putative target genes (http://www.ncbi.nlm.nih.gov/blast).

### 2.3. Plasmid construction

3′-untranslated region (3′-UTR) of *eIF4A1* containing putative binding sites for miR-US25-2-3p was amplified from MRC-5 genome DNA (primers: sense, 5′-GGACTAGTGAACATTTTAGACAC CCTTTT-3′ and reverse, 5′-CCCAAGCTTGTTTCCAAGTCATTTTATTCA GA-3′), and cloned into the Spe I and Hind III sites of pMIR-REPORT firefly luciferase (FFL) vector (Promega), named as pMIR-*eIF4A1*-UTR. Mutant 3′-UTR of *eIF4A1* was constructed using two-step multi-site mutagenesis PCR (primers are listed in Supplementary Table), which generated a mutation of 7 bps from CCAGGUGG to *GUGCGGCA* in the predicted miR-US25-2-3p binding site. Recombinant plasmid containing the mutant 3′-UTR was named as pMIR-*eIF4A1*-UTR$^{M}$. According to the small interfering RNA (siRNA) sequence of *eIF4A1*, 5′-CUGGCCGUGUGUUUGAUAUGC-3′ [9], two oligonucleotides were designed and synthesized to form the hairpin siRNA template: 5′-GATCCGGCCGTGTGTTTGATATGCTTCAAGAGA GCATATCAAACACACGGCCAGA-3’ and 5′-AGCTTCTGGCCGTGTGTT TGATATGCTCTCTTGAAGCATATCAAACACACGGCCG-3′. The annealed double strand template with adherent ends was cloned into the *Bam*HI and *Hind*III sites of p*Silencer* 4.1-CMV vector (Ambion), and was named as sieIF4A1. All constructs were confirmed by DNA sequencing.

### 2.4. Virus DNA quantification

MRC-5 cells transfected with 60nM precursor molecules of miR-US25-2-3p (Ambion) or scrambled sequence pre-miR negative control (pre-miR-NC, Ambion) or specific microFF™ miR-US25-2-3p inhibitor (RiboBio), or 4 μg sieIF4A1 were infected with HCMV Han strain at MOI of 0.5 in 6-well plates. Lipofectamine 2000 (Invitrogen) was used for transfection as described by Matsuura et al. [15]. In the indicated time points, total cellular DNA was extracted for HCMV DNA quantification. The viral DNA copies were determined by fluorescence quantitative real-time polymerase chain reaction (qPCR) using a commercially available HCMV diagnostic kit (Da An Gene) [16].

### 2.5. RT-qPCR for miRNA quantification

Total RNA was isolated from virus infected cells with TRIzol (Invitrogen) and treated by TURBO DNA-free™ Kit (Ambion). Reverse transcription (RT) was performed using TaqMan miRNA reverse transcription kit (Applied Biosystems) and small RNA-specific primers of miR-US25-2-3p or small nucleolar RNA (snRNA) U6 (control) (Applied Biosystems). qPCR was carried out to measure the expression of mature miR-US25-2-3p and U6 using Universal PCR Master Mix Kit (Applied Biosystems) and specific TaqMan probe (Applied Biosystems) following the manufacture's instructions. The relative expression level of miR-US25-2-3p was normalized to U6 RNA by $2^{-\triangle\triangle CT}$ methods [17].

### 2.6. 3′-UTR luciferase reporter assay

MRC-5 cells were cultured in 24-well plates and transfected with 100 ng pMIR-*eIF4A1*-UTR or pMIR-*eIF4A1*-UTR$^{M}$ together with 100 ng pRL-TK-Renilla-luciferase plasmid (Promega) and 60 nM pre-miR-US25-2-3p or pre-miR-NC. 48 h later, luciferase activities were measured using Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. FFL activity was normalized to renilla luciferase activity.

### 2.7. Western blotting

MRC-5 cells in 60 mm plates were lysed and protein was harvested using PIPA buffer (Beyotime). Total protein levels were determined by bicinchoninic acid (BCA) analysis (Beyotime). Equal amount of the extracts were subjected to SDS–PAGE, transferred onto nitrocellulose membranes, and then protein was detected using primary antibodies specific to eIF4A1 (Abcam) or β-actin (Santa Cruz), and horseradish peroxidase (HRP)-conjugated secondary antibodies (Zhong Shan) with ECL regent (GE Bioscience).

### 2.8. Translation assay

For cap-dependent in vivo translation assay, relative FFL activity to its mRNA quantity was detected. 100 ng pMIR-REPORT luciferase vector and 60 nM pre-miR-US25-2-3p or pre-miR-NC or 0.8 μg sieIF4A1 were co-transfected into MRC-5 cells of 24-well plates. Luciferase activity was measured at 48 h and normalized to FFL mRNA levels. FFL cDNA copy number, as a measurement for its mRNA content, was determined by RT-qPCR using SYBR Premix Ex Taq kit (TaKaRa), and the pMIR-REPORT plasmid DNA was used as a standard. The primers for FFL cDNA detection were designed as reported before [18].

### 2.9. Cell proliferation assay

The in vitro cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. $1 \times 10^4$ cells/well of MRC-5 were seeded into 96-well plates, and were transfected with 60 nM pre-miR-US25-2-3p or pre-miR-NC or 0.2 μg sieIF4A1, respectively. At 0 h, 24 h, 48 h, and 72 h post transfection, 20 μl of 5 mg/ml MTT (Sigma) was added to the media and kept for 4 h incubation at 37 °C. Following removal of the culture medium, the remaining crystals were dissolved in 200 μl DMSO (Sigma) and the absorbance at 490 nm was measured.

### 2.10. Genome DNA quantification

To detect the genome DNA synthesis levels of host cells, MRC-5 were plated in 6-well plates, and transfected with 60 nM pre-miR-

US25-2-3p or pre-miR-NC or 4 μg sieIF4A1. Genomic DNA was extracted at 48 and 72 h post transfection using TIANamp Genomic DNA Kit (TIANGEN). The DNA quantities were estimated based on the volume and concentrations measured by the absorbance at 260 nm.

### 2.11. Statistics

Data are shown as mean ± SE. Statistical significance was determined by Student's *t*-test, with *P*-value of <0.05 considered to be statistically significant.

## 3. Results

### 3.1. Over-expression of miR-US25-2-3p reduces HCMV DNA replication

To validate the effect of miR-US25-2-3p on HCMV DNA replication, MRC-5 cells transfected with pre-miR-US25-2-3p or pre-miR-NC were infected by HCMV Han strain. As shown in Supplementary Fig. 2, over-expression of miR-US25-2-3p resulted in significant reductions of HCMV DNA compared with pre-miR-NC transfected cells at 96 hpi.

### 3.2. eIF4A1 is a direct target of miR-US25-2-3p

Using hybrid-PCR, 17 candidate target mRNAs were obtained from 68 sequenced clones (Table 1). One was from HCMV genome and the others were from human genome. Among the candidate targets, only *eIF4A1* has been reported to be positively associated with viral replication [11–14]. *eIF4A1* 3′-UTR has one putative target site of miR-US25-2-3p, which is also predicted by RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/submission.html) (Fig. 1A).

To validate whether or not eIF4A1 is a direct target of miR-US25-2-3p, luciferase reporter assay and western blot were performed. As shown in Fig. 1B, cotransfection of pMIR-*eIF4A1*-UTR with pre-miR-US25-2-3p resulted in a significant decrease (~60%) in luciferase activity; However, mutation of the miR-US25-2-3p binding site (Supplementary Fig. 3) in the *eIF4A1* 3′-UTR abolished this effect of miR-US25-2-3p. Moreover, ectopic

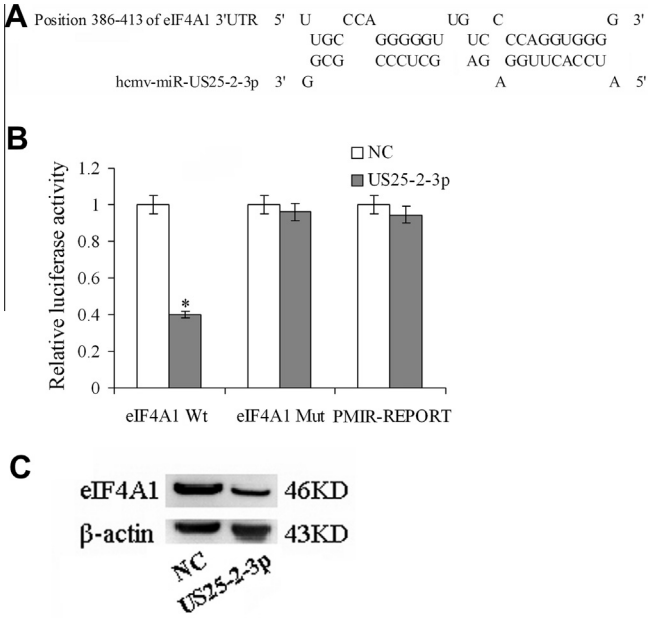


**Fig. 1.** miR-US25-2-3p inhibits *eIF4A1* expression by targeting *eIF4A1* 3′-UTR. (A) Schematic diagram of predicted target site of miR-US25-2-3p in the *eIF4A1* 3′-UTR. The predicted binding site of miR-US25-2-3p is indicated in the 386–413 of the *eIF4A1* 3′-UTR. The first nucleotide after the stop codon of *eIF4A1* is defined as "1". (B) Dual luciferase assay with cotransfection of the reporter vectors containing the wild type *eIF4A1* 3′-UTR (Wt) or mutant type *eIF4A1* 3′-UTR (Mut) or PMIR-REPORT vector, and pre-miR-NC or pre-miR-US25-2-3p in MRC-5 cells. The assays were performed in triplicate. $^{*}P < 0.01$. (C) Western blot analysis of eIF4A1 protein expression after transfection of pre-miR-NC or pre-miR-US25-2-3p in MRC-5 cells.

expression of miR-US25-2-3p markedly reduced the endogenous eIF4A1 protein expression in MRC-5 cells (Fig. 1C). These data demonstrate that miR-US25-2-3p inhibits *eIF4A1* expression directly by targeting *eIF4A1* 3′-UTR.

### 3.3. miR-US25-2-3p reduces HCMV replication by inhibiting eIF4A1 expression

To determine whether *eIF4A1* inhibition caused by miR-US25-2-3p could affect HCMV DNA synthesis, the expression of miR-US25-2-3p and *eIF4A1*, as well as HCMV copy numbers were detected in MRC-5 cells infected by HCMV Han strain during natural infection or after transfection of pre-miR-US25-2-3p, miR-US25-2-3p inhibitor, and sieIF4A1, respectively. As expected, transfection of pre-miR-US25-2-3p into MRC-5 cells resulted in substantially increase of miR-US25-2-3p expression compared to untreated cells (347-fold, 602-fold and 815-fold at 24, 48, and 72 hpi, respectively), while, miR-US25-2-3p expression was effectively blocked (0.02-fold, 0.03-fold and 0.17-fold at 24, 48, and 72 hpi, respectively) in miR-US25-2-3p inhibitor-transfected cells (Fig. 2A). The data showed that the natural expression of eIF4A1 in HCMV infected MRC-5 cells was upregulated compared with un-infected cells (Fig. 2B), and miR-US25-2-3p expression (Fig. 2A) and HCMV copy number were also exhibited time-dependent increase (Fig. 2C). However, miR-US25-2-3p inhibitor mediated miR-US25-2-3p silence in infected MRC-5 cells induced increased eIF4A1 expression at 48 and 72 hpi compared with the untreated cells (Fig. 2B), and the corresponding HCMV copy number was also increased (~1-fold) (Fig. 2C). Moreover, decrease of eIF4A1 expression caused by miR-US25-2-3p (Fig. 2B) and *eIF4A1* siRNA (Supplementary Fig. 4) markedly reduced HCMV DNA levels (Fig. 2C). These results indicated that miR-US25-2-3p may reduce HCMV replication by inhibiting *eIF4A1* expression.

**Table 1**
Putative miR-US25-2-3p targets identified by hybrid-PCR.

| Putative target mRNA | Accession Number |
|---|---|
| Echinoderm microtubule associated protein like 2 (*EML2*) | NM_001193268.1 |
| Chaperonin containing TCP1, subunit 8 (theta) (*CCT8*) | NM_006585.2 |
| Eukaryotic translation initiation factor 4A, isoform 1 (*eIF4A1*) | NM_001204510.1 |
| Ornithine decarboxylase antizyme 1 (*OAZ1*) | NM_004152.2 |
| Thioredoxin domain containing 12 (*TXNDC12*) | NM_015913.2 |
| Zinc finger protein 248 (*ZNF248*) | NM_021045.1 |
| Immediate early response 3 (*IER3*) | NM_003897.3 |
| Cytochrome c oxidase subunit IV isoform 1 (*COX4I1*) | NM_001861.3 |
| Oral-facial-digital syndrome 1(*OFD1*) | NM_003611.2 |
| Transforming growth factor, beta-induced, 68 kDa (*TGFBI*) | NM_000358.2 |
| Neighbor of BRCA1 gene 1 (*NBR1*), transcript variant 2 | NM_031858.2 |
| TSPY-like 2 (*TSPYL2*) | NM_022117.3 |
| Prefoldin subunit 1 (*PFDN1*) | NM_002622.4 |
| Ribosomal protein L4 (*RPL4*) | NM_000968.3 |
| versican (*VCAN*), transcript variant 3 | NM_001164097.1 |
| Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) | NM_002046.3 |
| HCMV *UL144* | FR677246.1 |

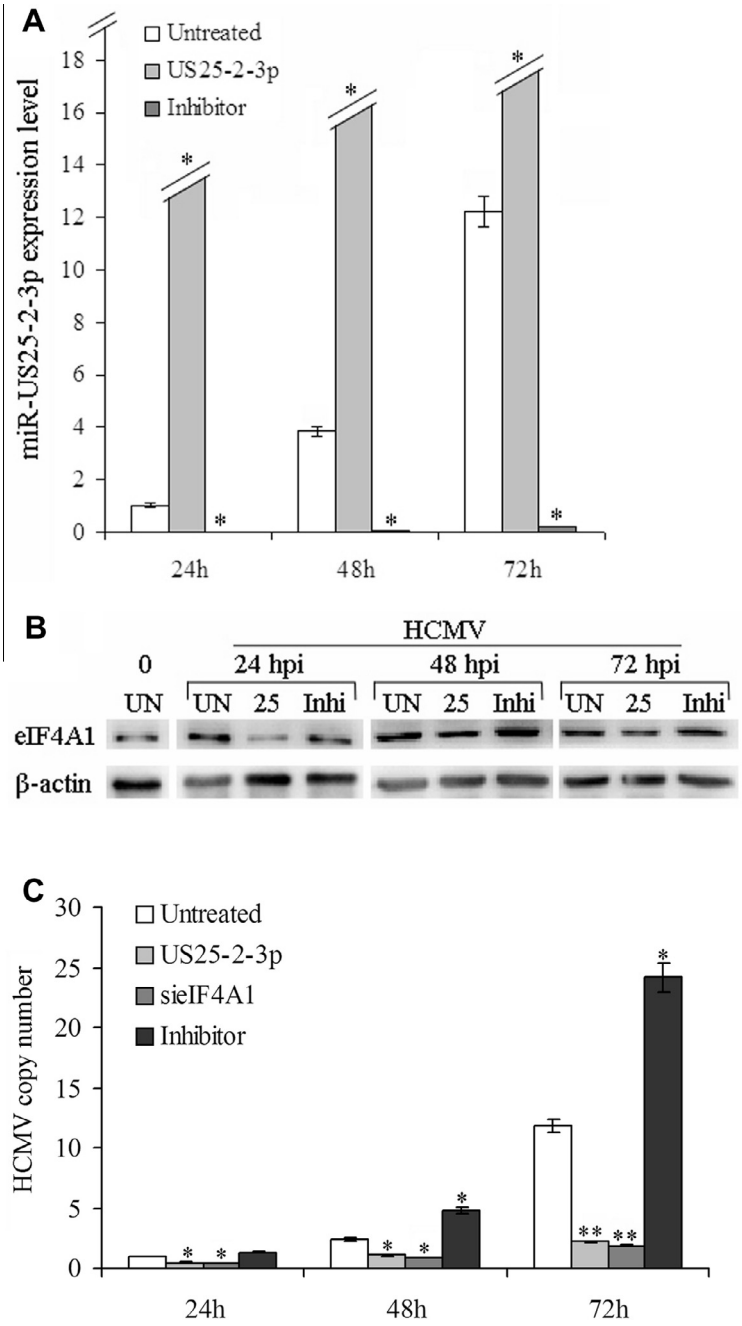


**Fig. 2.** miR-US25-2-3p reduces HCMV growth by suppressing *eIF4A1* expression (A) MRC-5 cells were transfected with pre-miR-US25-2-3p (25) or miR-US25-2-3p inhibitor (Inhi), and then were infected with HCMV Han at MOI, 0.5. At 24, 48, 72 hpi, mature miR-US25-2-3p was evaluated by TaqMan RT-qPCR, and normalized to that of untransfected cells (Untreated, UN) at 24hpi. The assays were performed in triplicate. $^{*}P < 0.01$. (B) MRC-5 cells were treated as (A). At 0, 24, 48, 72 hpi, Western blot analysis of eIF4A1 protein expression was performed. (C) MRC-5 cells were transfected with pre-miR-US25-2-3p or miR-US25-2-3p inhibitor or *eIF4A1* siRNA, and then were infected with HCMV Han at MOI, 0.5. At 24, 48, 72 hpi, viral DNA copy number was evaluated by RT-qPCR, and normalized to that of untransfected cells at 24 hpi. The assays were performed in triplicate. $^{*}P < 0.05$; $^{**}P < 0.01$.

### *3.4. miR-US25-2-3p inhibits eIF4A1-dependent translation*

Given the well-established role of *eIF4A1* in translation initiation, a putative effect of miR-US25-2-3p on translation was examined by FFL reporter assay. To directly measure the effect on protein synthesis and avoid bias by variations in transfection efficiencies or mRNA turnover, luciferase activity was normalized to luciferase mRNA levels. As expected, ectopic expression of miR-US25-2-3p, as well as knockdown of *eIF4A1* by *eIF4A1* siRNA in MRC-5 cells led to an approximate 68% and 70% decrease in the luciferase activity, respectively, compared to cells transfected with pre-miR-NC (Fig. 3). Therefore, miR-US25-2-3p may inhibit cellular translation by down-regulating *eIF4A1*.

### *3.5. miR-US25-2-3p suppresses host cell proliferation and genomic DNA synthesis*

The effect of miR-US25-2-3p on host cell proliferation and genomic DNA synthesis were evaluated by MTT and quantification of genomic DNA. As shown in Fig. 4A, the pre-miR-US25-2-3p- and *eIF4A1* siRNA-transfected MRC-5 cells exhibited significant growth suppression compared with the pre-miR-NC-transfected cells.

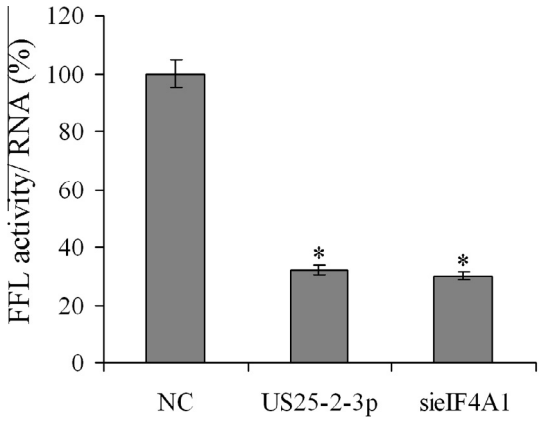


**Fig. 3.** miR-US25-2-3p inhibits *eIF4A1*-dependent translation. The FFL activity was normalized to the luciferase mRNA level. The data detected in MRC-5 cells expressing miR-US25-2-3p and *eIF4A1* siRNA was normalized to pre-miR-NC transfected MRC-5 cells. The assays were performed in triplicate. $^{*}P < 0.01$.

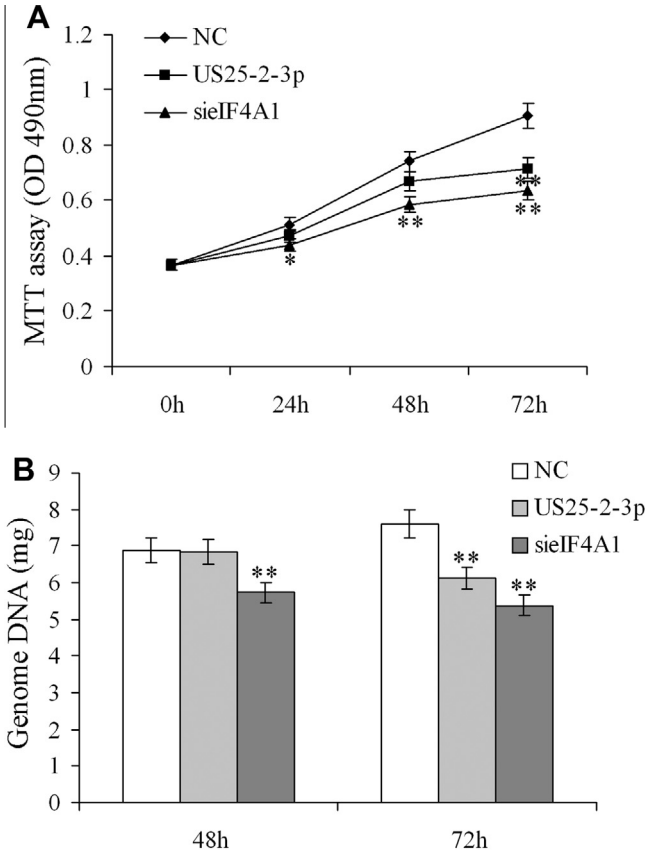


**Fig. 4.** miR-US25-2-3p inhibits cell proliferation and genomic DNA synthesis. *In vitro* cell proliferation assays after *eIF4A1* knockdown by pre-miR-US25-2-3p or siRNA in MRC-5 cells. The control cells were transfected with NC. The measurements were performed in triplicate. (B) The genomic DNA content in MRC-5 cells transfected as (A) was detected at 48 and 72 h post transfection, and normalized to that of pre-miR-NC transfected cells. The measurements were performed in triplicate. $^{*}P < 0.05$; $^{**}P < 0.01$.

Moreover, exogenous miR-US25-2-3p expression as well as *eIF4A1* siRNA transfection also significantly reduced genomic DNA synthesis of MRC-5 cells compared to the control cells (Fig. 4B). These results suggested that miR-US25-2-3p may mediate inhibition of cell growth and genomic DNA synthesis in an *eIF4A1*-dependent manner in MRC-5 cells.

## 4. Discussion

The discovery of small non-coding regulatory RNA molecules known as miRNAs is undoubtedly one of the most important findings in biological research in eukaryotic organisms and viruses, particularly in cytomegalovirus. miRNAs are non-immunogenic, so they may act as stealthy tools for viruses to regulate their as well as host gene expression.

HCMV miR-US25-2-3p is encoded by the intergenic region between US24 and US26, and forms the 3′ arm of the pre-miR-US25-2 stem-loop structure [19]. However, the functional knowledge about miR-US25-2-3p is very limited. Only one report from Stern-Ginossar et al. [3] indicated that miR-US25-2 reduced viral replication and DNA synthesis not only of HCMV but also of other DNA viruses (herpes simplex virus 1 and an adenovirus), but the mechanism is unknown. Our data further confirms that over-expression of miR-US25-2-3p, one miRNA derived from the precursor of miR-US25-2, can obviously inhibit HCMV DNA replication.

In our study, a host gene, *eIF4A1*, was screened out by hybrid-PCR and predicted by RNAhybrid to be a candidate target of miR-US25-2-3p. Literature review indicates that maintenance of cap-dependent translation is essential for DNA virus replication [11–14]. Due to the absence of their own translational machinery, HCMV must gain access to host cell ribosomes to synthesize their own proteins. eIF4A1, as an ATP-dependent RNA helicase, is crucial for the assembly of the translational active ribosome. eIF4A1 can unwind RNA secondary structures in the 5′-UTR of mRNAs which is necessary to allow efficient binding of the small ribosomal subunit, and subsequent scanning for the initiator codon [20]. eIF4A1, together with a cap-binding protein eIF4E [21] and a large scaffolding protein eIF4G [22], forms the translation initiation complex eIF4F [23,24], which recruits the 40S subunit to the 5′ cap of mRNA to facilitate cap-dependent translation. Besides cap-dependent translation, eIF4A1 has also been reported to play an important role in cell proliferation and DNA viral replication [9–14].

In our study, miR-US25-2-3p is the first identified miRNA to regulate *eIF4A1* expression. More importantly, we demonstrated for the first time that over-expression of miR-US25-2-3p has a negative effect on HCMV replication by inhibiting eIF4A1 protein expression. We found that the natural miR-US25-2-3p expression and HCMV copy number in infected MRC-5 cells showed time-dependent increase, and eIF4A1 protein expression also increased after HCMV infection compared to uninfected cells, which is consistent with previous reports [14,25,26]. No obvious correlation was observed between miR-US25-2-3p and *eIF4A1* expression during natural HCMV infection. However, silencing of miR-US25-2-3p by its inhibitor induced increased eIF4A1 protein expression and HCMV DNA synthesis in infected cells. Moreover, HCMV DNA copies were negatively associated with miR-US25-2-3p over-expression, and positively associated with eIF4A1 protein levels in the in vitro transfection study. Therefore, lower expression of *eIF4A1* regulated by miR-US25-2-3p may be a novel mechanism underlying HCMV replication decrease caused by miR-US25-2-3p over-expression. In addition, the fact that over-expression of miR-US25-2-3p and knockdown of *eIF4A1* by specific siRNA inhibited *eIF4A1*-dependent translation, cell growth and genomic DNA synthesis may contribute to the reduction of HCMV replication. Our findings suggest that inhibition of HCMV replication by high level of miR-US25-2-3p is probably mediated by suppression of *eIF4A1* in host cells.

Previous studies reveal that HCMV does not suppress the translation of host polypeptides. Many regulators, such as HCMV protein *immediate-early* (*IE*) *1*, *IE2* [14] and *UL69* [27], can increase translation initiation factor concentration to maintain effective viral replication, among which, the effect of miR-US25-2-3p could be relatively weak. As was seen in our study, the inhibitory effect of

*eIF4A1* expression and HCMV replication by relatively low level of natural expressed miR-US25-2-3p were not obvious, which was different from the observations in cells transfected with pre-miR-US25-2-3p. Moreover, considering other putative targets of miR-US25-2-3p, such as cell growth positive regulator-*versican, transcript variant 3* (*VCAN V3*) [28] and negative regulators-*transforming growth factor, beta-induced, 68kDa* (*TGFBI*) [29] and apoptosis induced factor- *immediate early response 3* (*IER3*) [30] might be involved in the regulation of viral DNA synthesis, the integrated effect of miR-US25-2-3p may be relatively mild. In our study, miR-25-2-3p over-expression only reduced HCMV copy number by 2 to 5-folds. So far, there has been no evidence that HCMV miRNAs are expressed during latency and would thus be present in a cell prior to the advent of lytic replication. Therefore, we suggests that miR-US-25-2-3p has only been shown to inhibit HCMV replication if expressed prior to infection, e.g., by transfection. Further study is needed to understanding the biological relevance of miR-US25-2-3p in the course of HCMV infection.

In conclusion, *eIF4A1* is the first identified target mRNA of miR-US25-2-3p, and the inhibition of HCMV replication by miR-US25-2-3p over-expression is mediated by suppression of *eIF4A1* in host cells. Although the biological significance of miR-US25-2-3p on inhibition of HCMV replication and underlying mechanisms regulating miR-US25-2-3p expression in infected host cells remains unknown, the discovery of negative effects of miR-US25-2-3p on viral replication may help to develop a miRNA-based therapeutic approach for reducing the severity of virus infection in immunocompromised individuals.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.05.057.

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