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RESEARCH ARTICLE

HSV-2-encoded miRNA-H4 Regulates Cell Cycle Progression and Act-D-induced Apoptosis in HeLa Cells by Targeting CDKL2 and CDKN2A

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Abstract

MicroRNAs (miRNAs) encoded by latency-associated transcript are associated with both latent and acute stages of herpes simplex virus 2 (HSV-2) infection. In this study, miRNA-H4-5p and miRNA-H4-3p were ectopically expressed in HeLa cells to explore potential cellular targets of viral miRNAs and demonstrate their potential biological functions. The results showed that miRNA-H4-5p could reverse apoptosis induced by actinomycin D (Act-D) and promote cell cycle progression, but miRNA-H4-3p had no such obvious functions. Bioinformatics analysis, luciferase report assay, quantitative reverse transcription polymerase chain reaction (qRT-PCR), and Western blotting demonstrated that miRNA-H4-5p could bind to the 3′-untranslated region (UTR) of cyclin-dependent kinase inhibitor 2A (*CDKN2A*) and cyclin-dependent kinase-like 2 (*CDKL2*) to negatively regulate their expression. We verified that these two targeted genes were associated with cell apoptosis and cell cycle. Furthermore, in HeLa cells infected with HSV-2, we detected significantly reduced expression of CDKN2A and CDKL2 and demonstrated the negative regulation effect of miRNA-H4-5p on these two target genes. Our findings show that viral miRNAs play a vital role in regulating the expression of the host's cellular genes that participate in cell apoptosis and progression to reshape the cellular environment in response to HSV-2 infection, providing further information on the roles of encoded herpesvirus miRNAs in pathogen–host interaction.

Keywords Herpes simplex virus 2 (HSV-2) · MicroRNAs (miRNAs) · Anti-apoptosis · Cell cycle progression · Pathogen–host interaction

Introduction

The closely related herpes simplex viruses 1 and 2 (HSV-1 and HSV-2) are characterized by their ability to establish lifelong latent infection within sensory neurons. HSV-2 causes genital herpes as well as more serious illnesses such as encephalitis and neonatal herpes (Whitley 2015; Looker *et al.* 2017a, b). Additionally, HSV-2 is a cooperative factor for subsequent HIV acquisition (Looker *et al.* 2017a, b). The HSV-2 latency-associated transcript (LAT), which plays an important role in facilitating latent infection and reactivation, is the only readily detectable product during latency (Zheng *et al.* 2011; Liu *et al.* 2016). Emerging data have suggested that LAT is likely to restrict viral replication or inhibit cellular apoptosis via LAT-encoded miRNAs (Cullen 2009). Nonetheless, the detailed regulatory mechanisms of most LAT-encoded miRNAs remain enigmatic (Jurak *et al.* 2010; Tang *et al.* 2015).

miRNAs are small RNA molecules which can post-transcriptionally control gene expression by binding to target messenger RNAs (mRNAs) (Bartel 2009). Herpesviruses have been reported to be able to encode and express functional viral miRNAs that play critical roles in herpesvirus infection and pathogenesis (Cullen 2011; Grey 2015). Most identified targets of these miRNAs are virus-encoded, and it has been shown that HSV miRNAs down-regulate immediate early gene products such as ICP0, ICP4, and ICP34.5 (Tang *et al.* 2008; 2009; Umbach *et al.* 2008; Duan *et al.* 2012). For example, HSV-1 and HSV-2 miRNA-H3/4 inhibit the expression of ICP34.5 to shield

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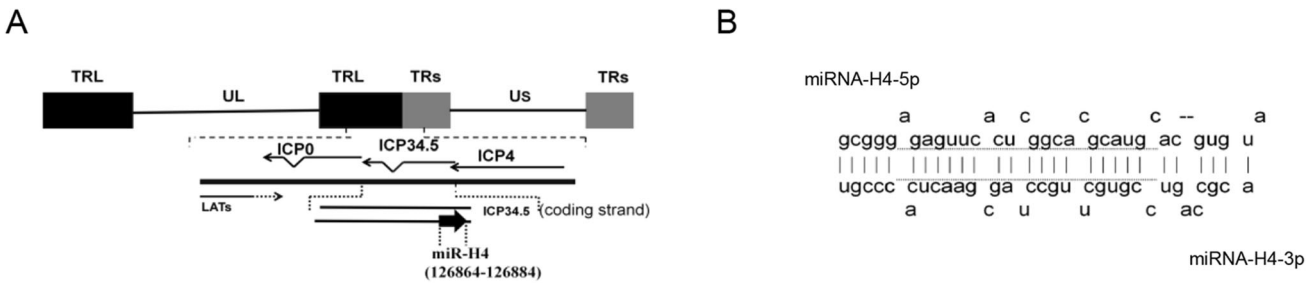

Fig. 1 Production of HSV-2 LAT-encoded miRNA-H4. **A** Schematic of the HSV-2 genome expanded with details of the LAT locus. miRNA-H4 maps with antisense to the noncoding region of ICP34.5. *L* long, *S* short, *IR* internal repeat, *TR* terminal repeat, *UL* unique long region, *US* unique short region. **B** Secondary structure adopted by pri-miRNA precursor of miRNA-H4 which includes miRNA-H4-5p and miRNA-H4-3p according to the miRbase.

latently infected neurons from cytopathic effects (Tang *et al.* 2008). The location of miRNA-H4 within the HSV-2 genome is illustrated in Fig. 1. Furthermore, viral miRNAs have recently been revealed to target several cellular genes involved in cell survival, proliferation, stress responses, and antiviral defense pathways (Cullen 2009). In particular, miRNA-LAT exerts an anti-apoptotic effect by down-regulating (TGF)-beta 1, SMAD3, and KLHL24 in response to HSV-1 infections (Gupta *et al.* 2006; Wu *et al.* 2013).

Based on this, we hypothesized that HSV-2 LAT-encoded miRNAs target host genes and lead to specific cellular effects associated with cell survival or cell progression. We used a stable HeLa cell line ectopically expressing miRNA-H4 as well as a HeLa cell line infected with HSV-2 to investigate the effects of miRNA-H4 in host cells. Bioinformatics analysis, dual luciferase assay, qRT-PCR, and Western blotting were performed to identify two cellular targets of miRNA-H4.

Materials and Methods

Cell Culture and Virus Infection

HeLa cells were obtained from the American Type Culture Collection (Manassas, USA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Takapuna, New Zealand) with 10% fetal bovine serum, 100 units/mL penicillin G, and 100 μg/mL streptomycin at 37 °C in 5% CO_2. HSV-2 strain 333 was obtained from American Type Culture Collection. Cells were infected with HSV-2 at a multiplicity of infection (MOI) of 2 for 16 h as described by Tang *et al.* (2009).

Construction of Plasmids

To construct a plasmid expressing miRNA-H4, a fragment containing miRNA-H4 precursors was amplified from HSV-2 333 genomic DNA. The amplified fragments were then cloned into a pmR-mCherry vector (Promega, Madison, USA), which was termed mCherry/miR-H4. The empty pmR-mCherry vector was used as the negative control. To construct 3′-UTR reporter plasmids, the 3′-UTR fragments of CDKN2A and CDKL2 mRNA, which contain the putative *miR-H4-5p* binding sites, were amplified from HeLa cells' cDNA, and cloned into the region downstream the synthetic *Renilla* luciferase reporter gene in the psiCHECK2 vector (Promega). To insert four point-mutations into the seed regions of the miRNA-H4-5p binding sites, primers of the seed sequences were mutated and mutated binding sites were cloned into the same site of the psiCHECK-2 vectors.

Cell Transfection

HeLa cells were transfected using mCherry/miR-H4 or pmR-mCherry using Lipofectamine LTX (Invitrogen, Carlsbad, USA). After 48 h, cells were continuously selected with 800 μg/mL G418 (Geneticin, Gibco-BRL, Gaithersburg, USA) for 2 weeks. At the end of the selection, qRT-PCR was used to confirm transfection efficiency. Stable HeLa cells expressing mCherry/miR-H4 were named HeLa-H4 cells, and HeLa cells transfected with empty pmR-mCherry were named HeLa-mCherry cells.

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted and reversed to cDNA using Super Script II (Invitrogen). qRT-PCR was performed using the ABI 7500 real-time PCR system (Applied Bio-Systems, Waltham, USA) and SYBR Green qPCR Super Mix (Invitrogen). Expression of miRNA-H4-5p, miRNA-H4-3p, CDKN2A, and CDKL2 was detected in transfected and infected cell lines, where U6 snRNA acted as the endogenous control to standardize the level of sample RNAs. Primers used for qRT-PCR are shown in Supplementary Table S1. qRT-PCR results were analyzed and

expressed as relative expression of CT (cycle threshold) value using the $2^{-\triangle\triangle CT}$ method (Livak and Schmittgen 2001).

miRNA and siRNA Transfection

miRNA-H4-5p mimics (MC-5p), miRNA-H4-5p inhibitor (MI-5p), miRNA-H4-3p inhibitor (MI-3p), miRNA inhibitor negative control (MI-NC), miRNA-H4 negative control (miRNA-NC), siRNAs against human CDKN2A (siRNA-p16), and CDKL2 (siRNA-p65) were all synthesized and purified by RiboBio (Guangzhou, China). siRNAs targeting nonspecific sequences were used as negative control (siRNA-NC). miRNA and siRNAs were all transfected using Lipofectamine 2000 (Invitrogen). The medium was replaced after 6 h. Final concentrations were, except where otherwise indicated, 100 mmol/L for MC-5p, 150 mmol/L for MI-5p and MI-3p, 100 mmol/L for siRNA-p16, and siRNA-p65. Efficiency of RNA transfection was approximately 80% and the overexpression of miRNAs or siRNAs persisted for at least 72 h.

Establishment of an Act-D-induced Apoptosis Model

A pilot study on HeLa cells treated with different concentrations of Act-D ranging from 0 to 2 μg/mL for periods ranging from 6 to 48 h, flow cytometry, and caspase-3 activity assay indicated that treatment with 1 μg/mL for 36 h was optimal for establishment of an Act-D-induced apoptosis model (data not shown). These specific conditions were therefore used in subsequent apoptosis induction experiments. Simultaneous gain-of-function and loss-of-function assays were performed by transfecting cells with or without related miRNA inhibitors. After 24 h, Act-D was added into the culture medium.

Caspase-3 Activity Detection, Hoechst Staining, Flow Cytometry Analysis, and Cell Proliferation Analysis

The activity of caspase-3 was detected using a caspase 3 activity apoptosis assay kit (Shanghai Bestbio Biological Technology, Shanghai, China). For Hoechst staining, 5 μg/mL Hoechst 33342 dye (Sigma, Bulington, USA) was added to the culture medium, and cells were further incubated for 20 min at 37 °C in the dark. Afterwards, digital images were obtained under an inverted fluorescent light microscope (Olympus, Tokyo, Japan). Apoptosis was evaluated using annexin V-PE/7-AAD staining followed by flow cytometry. Cells treated with Act-D were harvested and centrifuged at 200 *g* for 10 min, then were washed twice with cold phosphate buffered saline (PBS) to remove excessive culture medium. The cells were re-suspended at 1×10^6 cells/mL in binding buffer and incubated with annexin V-PE and 7-AAD (BD Biosciences Pharmingen, San Diego, USA) at 25 °C for 15 min in the dark. At least 5000 cells were collected and analyzed using flow cytometry (FACS Calibur, BD Biosciences Pharmingen). Cell proliferation was determined by MTT assay (Sigma) and EdU incorporation assay (RiboBio), according to manufacturers' instructions. EdU is a thymidine analog whose incorporation can be used to label cells undergoing DNA replication (Salic and Mitchison 2008).

Bioinformatics Analysis

To identify the cellular targets related to the anti-apoptotic function of viral miRNA-H4-5p, we used bioinformatics software RNAhybrid (http://bibisrv.techfak.uni-bielefeld.de/rnahybrid/submission.html) and TargetScan 11.0 (http://www.targetscan.org). Inputted the information of miRNA-H4-5p to the bioinformatics software to predict its possible target genes on the HeLa cell genome. Then selected the intersection of the predicted results from the two software.

Dual Luciferase Assay

Luciferase reporter assay was performed using the dual luciferase reporter assay system (Promega) according to the manufacturer's protocol. Cells were seeded in 24-well plates in 90% confluence. For the CDKN2A 3′-UTR and CDKL2 3′-UTR luciferase reporter assays, wild type (wt) and mutant (mut) reporter constructs were co-transfected into HeLa cells in the 24-well plates with 100 mmol/L miRNA-H4-5p and *Renilla* plasmids by using lipofectamine 2000 (Invitrogen). After 48 h, the reporter gene assay was analyzed using a GloMax bioluminescence detection instrument (Promega). Because of differences in transfection efficiency, firefly luciferase activity was normalized using the corresponding *Renilla* luciferase activity. All these experiments were performed at least three times.

Western Blot Analysis

Cells transfected for 48 h or infected with HSV-2 for 16 h were lysed using RIPA buffer (50 mmol/L Tris–HCl at pH 7.5, 150 mmol/L NaCl, 1% NP-40, 0.25% sodium deoxycholate). Lysates were then centrifuged to extract the proteins, which were then separated by gel electrophoresis and transferred to the PVDF membranes (Millipore, Billerica, USA). Following this, the membranes were washed by TBST and incubated in blocking buffer containing 5% defatted milk for 1 h at room temperature. This was followed by incubation with specific primary antibodies against CDKN2A (Cell Signaling Technology, Boston,

USA) or CDKL2 (Cell Signaling Technology). Subsequently, the membranes were incubated with peroxidase-conjugated polyclonal secondary antibody (Abcam, Cambridge, USA). All antibodies were diluted in PBST and incubated at room temperature for 2 h. In the end, membranes were washed in TBST and detected by enhanced chemiluminescence system Western Blotting Detection Reagents (Amersham Biosciences, Buckinghamshire, UK).

Statistical Analysis

All values are presented as mean ± standard deviation (SD). Each experiment was repeated at least three times. Student's *t* test was used to evaluate statistical significance, where $P < 0.05$ was considered significant. All statistical analyses were performed using SPSS v. 19.0 (International Business Machines, Armonk, USA).

Results

miRNA-H4-5p and miRNA-H4-3p are Ectopically Expressed in Stable Cell Line HeLa-H4

In the HeLa-H4 cell line, mature miRNA-H4-5p and miRNA-H4-3p were detected using qRT-PCR, and the expression of miRNA-H4-3p was lower than miRNA-H4-5p. In the subsequent experiment, we found that the MI-5p led to an 85% decrease in the level of miRNA-H4-5p ($P < 0.01$) (Fig. 2A), and MI-3p also significantly inhibited the expression of miRNA-H4-3p ($P < 0.01$) (Fig. 2B).

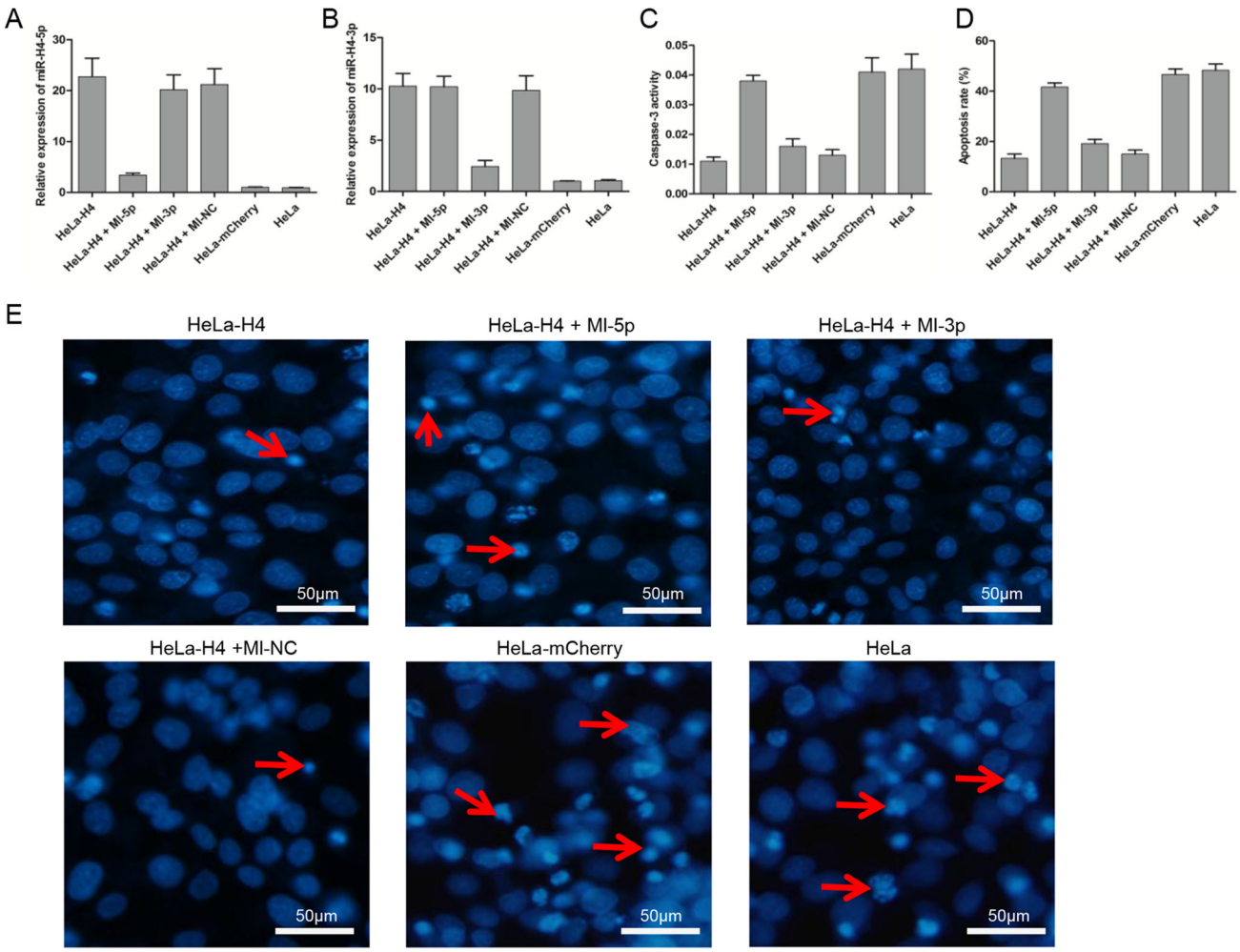

Fig. 2 miRNA expression and Act-D-induced apoptosis in HeLa-H4 cells. **A** Different cell lines were subjected to qRT-PCR to detect the relative expression of miRNA-H4-5p. **B** Different cell lines were subjected to qRT-PCR to detect the relative expression of miRNA-H4-3p. **C** The activity of caspase-3 in HeLa cells transfected with or without transfection, accompanied by Act-D treatment. **D** Apoptosis was assessed using FAM in HeLa cells with or without transfection, accompanied by Act-D treatment. **E** HeLa cells with or without transfection treated with Hoechst 33342 were imaged under a fluorescent microscope. Cells with bright blue nucleus were recognized as apoptotic.

Resistance of HeLa-H4 Cells to Act-D-induced Apoptosis is Correlated with Ectopic miRNA-H4-5p Expression

Act-D induces apoptosis in HeLa cells (Journey and Goldstein 1961; Goldberg and Rabinowitz 1962), and Yamasa *et al.* (2004) reported that Act-D causes caspase-3-dependent apoptosis in the induced cells. During earlier stages of programmed cell death, high levels of caspase-3 activity induce cells to undergo apoptosis (Kleeff *et al.* 2000; Lu *et al.* 2015). In Act-D-induced apotosis model, caspase-3 activity detection and flow cytometry assay (Fig. 2C, 2D) demonstrated that the level of apoptosis was lower in HeLa-H4 cells than that in HeLa cells and HeLa-mCherry cells ($P < 0.01$), and HeLa-H4 cells transfected with MI-5p led to a high level of apoptosis. This reversed the apoptosis resistance of HeLa-H4 cells ($P < 0.01$), while HeLa-H4 cells transfected with MI-3p or MI-NC showed a significantly lower rate of apoptosis ($P > 0.05$). In a concordant experiment treated with Hoechst 33342 (Fig. 2E), nucleic DNA of MI-5p-transfected HeLa-H4 cells was brighter and more intense than that in HeLa-H4 cells without MI-5p transfection. Although miRNA-H4-5p could not completely rescue the toxicity of Act-D treatment, these results demonstrated that miRNA-H4-5p plays a preventive role against Act-D-induced apoptosis.

miRNA-H4-5p Affects Cell Cycle Progression in HeLa Cells by Regulating G1-to-S Transition

To further study the effects of miRNA-H4 on cell cycle progression, EdU incorporation assay was performed. MI-5p and MI-3p were transfected into HeLa-H4 cells. As shown in Fig. 3, 41.8% of HeLa-H4 cells were in S phase, significantly higher than in HeLa cells (15.7%) and in HeLa-mCherry cells (16.1%) ($P < 0.01$), MI-5p-transfected HeLa-H4 cells in S phase were reduced to 17.2%, significantly lower than HeLa-H4 cells ($P < 0.01$), while MI-3p-transfected and MI-NC-transfected HeLa-H4 cells in S phase, representing 38.5% and 40.1%, were not statistically different from HeLa-H4 cells ($P > 0.05$). These results indicated that miRNA-H4-5p specifically affects the cell cycle by regulating the distribution of S phase, whereas miRNA-H4-3p has no obvious effects.

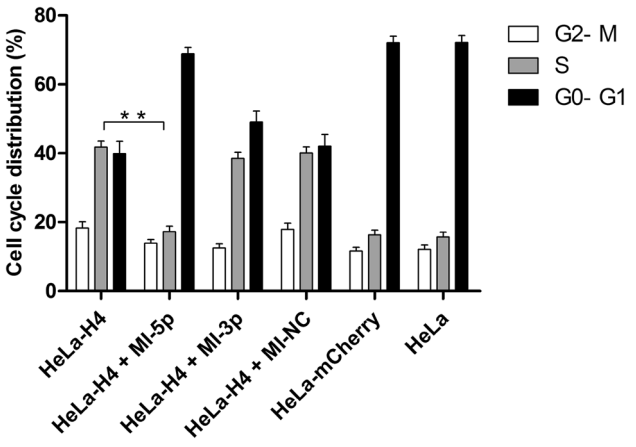

Fig. 3 Cells with or without transfection were subjected to cell cycle analysis using EdU incorporation. Cells with positive EdU were found to have accumulated in the S phase. Differences were judged to be statistically significant when $^{**}P < 0.01$.

Cellular CDKN2A and CDKL2 are the Targets of Viral miRNA-H4-5p in HeLa Cells

We analyzed and predicted the cellular target genes of miRNA-H4-5p by searching bioinformatics software RNAhybrid and TargetScan 11.0, the results showed that the sequence of miRNA-H4-5p paired well with the 3′-UTR of CDKN2A and CDKL2 (Fig. 4A). CDKN2A possesses conserved nucleotides (nts) known to be critical in regulating G_1-to-S cell cycle transition (Niederacher *et al.* 1999; Pal *et al.* 2016). Previous studies have shown that suppression of CDKL2 leads to prevention of cell apoptosis and transition of cell cycle (Li *et al.* 2014; Gomi *et al.* 2010). Given that miRNA-H4-5p protects against Act-D-induced cellular apoptosis and regulates cell cycle in HeLa cells, we hypothesized that CDKN2A and CDKL2 may be the targets of miRNA-H4-5p.

To test this hypothesis, the 3′-UTR of CDKN2A and CDKL2 or their mutant sequences were cloned into psi-CHECK-2 vector as described in the Materials and Methods section. The recombination vectors were then co-transfected with MC-5p or miRNA-NC, and the luciferase activity in the transfected HeLa cells was measured to determine whether the two candidates are the correct binding sites. Luciferase activity was dramatically suppressed in HeLa cells co-transfected with wild-type vector with MC-5p compared to those co-transfected with miRNA-NC (Fig. 4B, 4C). Compared with untransfected HeLa cells, there was no significant difference between HeLa cells stably co-transfected with mutant-type vectors with either MC-5p or miRNA-NC ($P > 0.05$). These data suggest that miRNA-H4-5p may interact with the 3′-UTR of CDKN2A and CDKL2 mRNA.

To directly assess the suppressive effect of miRNA-H4-5p on CDKN2A and CDKL2 expression, qRT-PCR assay and Western blotting were used in MC-5p-transfected HeLa cells (lane 4, Fig. 4D). The expressions of CDKN2A and CDKL2 proteins were significantly lower than in the control group ($P < 0.05$) (Fig. 4D, 4E, 4F). Rescue experiment was also performed by transfecting MC-5p-transfected HeLa cells with MI-5p (lane 3, Fig. 4D). As expected, blocking MC-5p resulted in a 50%–60% increase in the expression of CDKL2 and CDKN2A proteins ($P < 0.05$). These findings suggest that miRNA-H4-5p

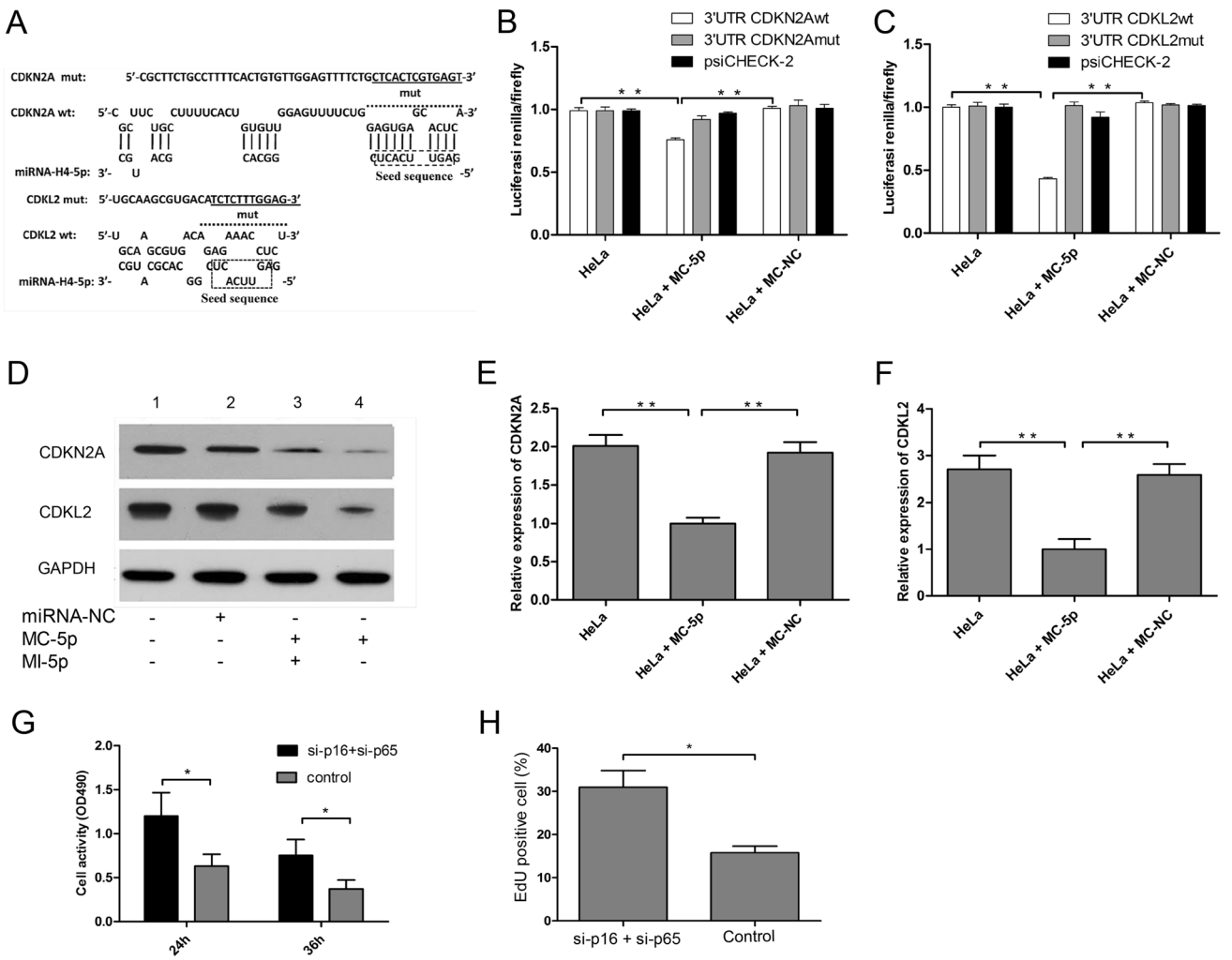

Fig. 4 Validation of miRNA-H4-5p targets. **A** Sequence alignment between miRNA-H4-5p and 3′-UTR of CDKN2A or CDKL2. Binding sites are indicated in dotted lines and the corresponding mutants are labeled in solid lines. All complementary pairing bases are indicated in the vertical curve. **B**, **C** Changes in luciferase enzyme viability. HeLa cells co-transfected with luciferase reporter containing either wild type (WT) or mutant type (mut) 3′-UTR of (B) CDKN2A or (C) CDKL2 along with MC-5p or miRNA-H4 negative control. Relative viability of luciferase enzyme = *Renilla reniformis*/firefly luciferase. **D** Effects of MC-5p or MI-5p on the expression of CDKN2A and CDKL2 in HeLa cells were examined using Western blot analysis with GAPDH as the internal control. Differences were judged to be statistically significant when $*P < 0.05$, $**P < 0.01$. **E**, **F** Expression of CDKN2A and CDKL2 mRNA was analyzed using qRT-PCR. **G** The effects of siRNAs on cell proliferation were determined by MTT assay 24 h and 36 h after transfection. **H** The relative number of EdU positive cells was estimated from siRNA-p16 and siRNA-p65 transfections, cells with positive EdU were found to have accumulated in the S phase.

negatively regulates CDKN2A and CDKL2 expression, consistent with the results of the luciferase reporter assay described above.

To address the biological functions of CDKL2 (p65) and CDKN2A (p16) in HeLa cells, siRNAs against p65 (siRNA-p65) and p16 (siRNA-p16) were transfected into HeLa cells. Data from the MTT assay showed that cell activity in HeLa cells transfected with siRNA-p65 and siRNA-p16 (1.204 at 24 h, 0.752 at 36 h) significantly higher than in HeLa cells (0.633 at 24 h, 0.373 at 36 h) ($P < 0.05$) (Fig. 4G). EdU incorporation assay showed a large number of HeLa cells transfected with siRNA-p65 and siRNA-p16 settled in the S phase (31.10%), significantly higher than untransfected HeLa cells (15.82%) ($P < 0.05$) (Fig. 4H). These findings strongly suggest that miRNA-H4-5p regulates cell cycle progression and inhibits cell apoptosis by targeting CDKL2 and CDKN2A genes.

The Regulatory Function of miRNA-H4-5p on Target Genes in HeLa Cells Infected with HSV-2

To test whether miRNA-H4-5p can regulate the expression of CDKN2A and CDKL2 in HSV-2-infected HeLa cells,

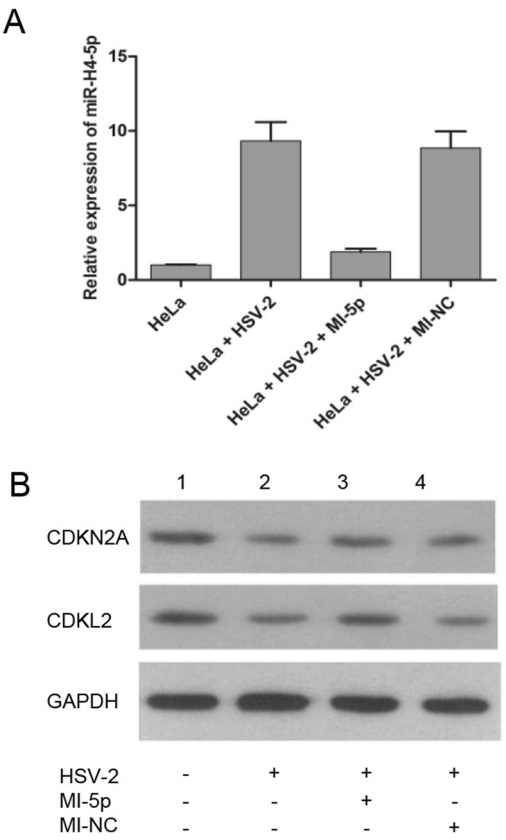

Fig. 5 miRNA expression and its regulatory function on target genes in HSV-2-infected HeLa cells. **A** Expression of miRNA-H4-5p in different cell lines after HSV-2 infection were detected using qRT-PCR. **B** Expression of CDKN2A and CDKL2 in different cell lines after HSV-2 infection were examined using Western blot analysis with GAPDH as the internal control.

MI-5p or MI-NC was transfected into HeLa cells 24 h before infection with HSV-2. Based on qRT-PCR data (Fig. 5A), the expression of miRNA-H4-5p was significantly lower after HSV-2 infection in MI-5p-transfected HeLa cells than in HeLa cells with or without MI-NC transfection ($P < 0.01$). At the same time, the expression of target genes CDKN2A and CDKL2 in different cell lines was detected by western blotting (Fig. 5B). Compared with uninfected HeLa cells, HSV-2 infection significantly reduced the expression of these target genes, while transfected with MI-5p increased the expression of these target genes in the infected cells. These results indicate that miRNA-H4-5p plays its regulatory role by downregulating the target genes CDKN2A and CDKL2 after infection of HSV-2.

Discussion

miRNAs are ~22 nt long noncoding RNAs that regulate gene expression post-transcriptionally, mainly by binding to the 3′-UTR of target mRNAs, resulting in translational inhibition or mRNA degradation (Bartel 2009; Afonso-Grunz and Müller 2015; Dalmay 2013). All metazoan organisms encode miRNAs, and miRNAs have recently been identified in several DNA viruses (Cullen 2009; Hooykaas *et al.* 2016; Pfeffer *et al.* 2004; Piedade and Azevedo-Pereira 2016; Du *et al.* 2015). Thus far, more than 200 viral miRNAs have been identified, predominantly in herpesviruses as well as in polyomaviruses, ascoviruses, and adenoviruses (Skalsky and Cullen 2010). miRNAs are non-immunogenic with less coding capacity and can evolve rapidly to target new transcripts. Therefore, in contrast to proteins, viral miRNAs are potentially ideal tools for viruses to modulate gene expression and may even directly regulate viral and/or host cell gene expression to benefit the virus (Gottwein and Cullen 2008).

HSV-1 and HSV-2 miRNA-H2 are both transcribed antisense to ICP0 and down-regulate ICP0 protein (Tang *et al.* 2009; Umbach *et al.* 2008, 2010), which is a viral immediate early transcriptional activator thought to play a key role in productive replication and reactivation from latency (Everett 2000). HSV-1 miRNA-H6 inhibits the expression of ICP4, another immediate early gene which down-regulates LAT and up-regulates genes of HSV-1, promoting the virus towards lytic infection (Duan *et al.* 2012; Tang *et al.* 2011). HSV-1 and HSV-2 LAT-encoded miRNA-H3/H4 inhibits the expression of ICP34.5, a key viral neurovirulence factor, exerting biological impact on facilitating latent infection and reactivation (Cullen 2009). Viral microRNAs can also target cellular transcripts, and regulate key regulators related to cellular processes including cell proliferation, metabolism, and apoptosis. However, the role of these viral miRNAs in host–pathogen interactions remains poorly studied, it is unclear whether HSV-2-encoded miRNAs can target host cells or regulate cellular gene expression.

In this study, we constructed mCherry/miR-H4 plasmids and used qRT-PCR and miRNA inhibitor transfections to examine the expression of two subtype miRNAs. Our results showed that miRNA-H4-5p and miRNA-H4-3p were ectopically expressed. To determine the functions of the miRNAs, we established an Act-D-induced apoptosis model in HeLa cells, then transfected them with mCherry/miR-H4 plasmids and relevant miRNA inhibitors. The results indicated that miRNA-H4-5p could dramatically reduce Act-D-induced apoptosis. In addition, cell proliferation analyses showed that miRNA-H4-5p could help HeLa cells enter S phase. In contrast, a different subtype,

miRNA-H4-3p, had no obvious function in apoptosis or cell cycle progression. To further demonstrate the underlying mechanism, we used bioinformatics prediction and pilot assay of several candidates closely related to cell cycle and apoptosis. Subsequently, using luciferase reporter assay, qRT-PCR, and Western blotting, we confirmed that CDKN2A and CDKL2 were cellular targets of viral miRNA-H4-5p. We then transfected siR-p16 and siR-p56 to HeLa cells to mimic the negative regulatory function of miRNA-H4-5p on these genes. Data from MTT and EdU incorporation assays showed that when these cellular targets were down-regulated, cell activity was increased and more HeLa cells were settled in the S phase. In the final stage of this study, we established a stable HeLa cell line transfected with or without MI-5p, and then infected the cells with HSV-2 and confirmed the inhibition effect of miRNA-H4-5p on target gene CDKN2A and CDKL2 after HSV-2 infection through qRT-PCR and Western-blot assays. This indicated that the expression of miRNA-H4-5p following HSV-2 infection could affect the cell cycle or apoptosis.

In summary, our results from this study demonstrate that HSV-2-encoded miRNA-H4-5p partly reverses Act-D-induced apoptosis and promotes the cell cycle into S phase via targeting *CDKN2A* and *CDKL2* genes, which are involved in cell activity and cycle distribution. However, the roles of miRNA-H4-3p and its potential cellular targets, and the effects of two targeted genes on viral life cycle in response to HSV-2 infection warrant further investigation. Our data further suggest that viral miRNAs play an important role in host cell regulation. Collectively, our studies provide insights into the understanding of the mechanisms related to HSV-2 LAT-mediated viral latency and recurrence.

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Author Contributions HY and JF designed the study; YZ, JY and YL performed the experiments; YZ and JY analyzed the data; YZ and YL wrote the manuscript; HY and JF finalized the manuscript. All the authors approved the final manuscript.

Compliance with Ethics standards

Conflict of interest The authors declare that they have no conflict of interest.

Animal and Human Rights Statement The authors declare that they have no conflict of interest. This article does not contain any studies with human or animal subjects performed by any of the authors.

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