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Cellular microRNA bta-miR-2361 inhibits bovine herpesvirus 1 replication by directly targeting EGR1 gene

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ABSTRACT

Bovine herpesvirus 1 (BHV-1) is an economically important pathogen of cattle and has led to significant consequences on the cattle industry worldwide. MicroRNAs (miRNAs) are a class of regulators that play critical roles in virus and host interaction. However, the roles of host miRNAs in BHV-1 infection remain largely unclear. In this study, a set of differentially expressed miRNAs by small RNA deep sequencing were analyzed in the Madin-Darby Bovine Kidney Cells (MDBK) infected with BHV-1 after 12 h, 24 h and 48 h post-infection compared to mock infection, and it was confirmed that bta-miR-2361 was significantly down-regulated. Moreover, bta-miR-2361 mimics transfection could inhibit BHV-1 replication. Combined with up-regulated genes from BHV-1-infected MDBK cells by deep RNA-sequencing and predicted by bioinformatics tools, early growth response 1 (EGR1) was putative target of bta-miR-2361. Furthermore, EGR1 was up-regulated during BHV-1 infection, and overexpression of EGR1 promoted BHV-1 replication whereas knockdown of EGR1 had the opposite effects. Subsequently, the target association between bta-miR-2361 and 3′UTR of EGR1 was further validated using a dual-luciferase reporter assay. In addition, overexpression of bta-miR-2361 resulted in decreased EGR1 mRNA and protein levels. Further mechanistic study showed that EGR1 stimulated BHV-1 UL46 promoter activity, but overexpression of bta-miR-2361 suppressed the production of UL46 gene. Collectively, this is the first study to reveal that bta-miR-2361 as a novel host factor regulates BHV-1 replication via directly targeting the EGR1 gene, which is a transcription factor that regulates viral UL46 gene of BHV-1. These results provide further insight into the study of BHV-1 pathogenesis.

1. Introduction

Bovine herpesvirus 1 (BHV-1), a member of the *alphaherpesvirus* family, is identified as the causative agent of one of the most economically significant viral pathogens in cattle (Muylkens et al., 2007; Hou et al., 2017). BHV-1 infection can cause a diverse range of clinical syndromes including respiratory symptoms, gastrointestinal symptoms, genital disorders, and abortions. In addition, cattle become highly susceptible to secondary infection by diverse pathogens due to the immunosuppressive properties of the virus, which consequently lead to significant and economic losses to the cattle industry worldwide (Jones and Chowdhury, 2007; Salimena et al., 2016; Hou et al., 2018a; Zhao et al., 2018a, b).

MicroRNAs (miRNAs) are short noncoding single-stranded RNAs that consists of 18–25 nucleotides. Typically, miRNAs can be produced to suppress gene expression by host or virus via the translational inhibition or degradation of the 3′ untranslated region (3′ UTR) of their target messenger RNAs (mRNAs) in a sequence-specific manner (Huang et al., 2013). The genome of BHV-1 has been reported to encode at least 10 miRNAs. Studies have shown that viral miR-B8-3p and miR-B9 of BHV-1 can suppress BHV-1 replication (Kanokudom et al., 2018). There are growing evidences that not only miRNAs encoded by DNA viruses but also cellular miRNAs play central roles in viral infection by regulating the expression of key genes (Hou et al., 2018b; Trobaugh and Klimstra, 2017). In the *alphaherpesvirus*, miRNA-649 can promote HSV-1 replication by targeting MALT1 (Zhang et al., 2017), and miR-23a was found to facilitate HSV-1 replication by targeting interferon regulatory factor 1 (IRF1) and inhibiting the antiviral innate immune pathway (Ru et al., 2014). Thus, cellular miRNAs represent such host dependencies and play important roles in regulating viral infection. In

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addition, recent reports have demonstrated that numerous miRNAs exhibit altered expression profiles in MDBK cells during viral infection. For instance, the up-regulated bta-miR-29b and bta-miR-2411 upon bovine viral diarrhea virus (BVDV) infection attenuate BVDV replication by reducing viral infection-related autophagy and reducing Pelota gene expression, respectively (Fu et al., 2015; Shi et al., 2018). Moreover, cellular microRNA bta-miR-222 suppresses caprine parainfluenza virus type 3 replication via down-regulation of interferon regulatory factor 2 (Li et al., 2018). However, the interaction between BHV-1 and host miRNAs in MDBK cells has not been reported.

Early growth response 1 (EGR1), also named Krox24, zif268 and NGFI-A, is a multifunctional transcription factor that expresses rapidly after a broad range of extracellular stimuli (Pagel and Deindl, 2012). EGR1 belongs to a family of zinc finger DNA-binding proteins that is involved in diverse biologic functions and signal transduction cascades according to its various target genes (Liang et al., 2010; Pagel and Deindl, 2012). Mounting evidences have shown that EGR1 can participate in a variety of viral infection diseases. For instance, EGR1 can promote the replication of herpes simplex virus 1 (HSV-1) (Yao et al., 2012), Epstein-Barr virus (EBV) (Vockerodt et al., 2013), Kaposi's sarcoma-associated herpes virus (KSHV) (Sarkar R1 and SC, 2017) and enterovirus 71 (EV71) (Song et al., 2015) by regulating their viral genes or genome. Nevertheless, it is not clear whether EGR1 could regulate BHV-1 replication. In addition, it has been reported that EGR1 can mediate the effect of miRNA on virus replication. For example, the induction of miR-141 by EGR1 has a certain effect on enterovirus infection (Ho et al., 2011). However, there is no report that miRNAs affect viral replication by directly regulating the expression of EGR1.

In this study, we firstly investigated bta-miR-2361 screened by high-throughput miRNA deep sequencing was significantly down-regulated in MDBK cells infected with BHV-1. Bta-miR-2361 mimics were transfected into MDBK cells to evaluate whether bta-miR-2361 expression had potential effects on BHV-1 replication. Then, expression patterns of EGR1 gene, one of predicted target genes of bta-miR-2361, was detected in MDBK cells after BHV-1 infection, and effect of EGR1 gene expression on BHV-1 replication was determined in MDBK cell lines with stable expression or knockdown of EGR1 gene. In addition, bta-miR-2361 targeting the 3′UTR of EGR1 was validated using a dual-luciferase reporter assay. And the negative expression of EGR1 mRNA and protein levels regulated by bta-miR-2361 were verified. What's more, we also introduced EGR1 as an important mediator of this process by stimulating BHV-1 UL46 promoter activity. Our findings probably provide new insights into virus and host interactions during BHV-1 infection.

2. Materials and methods

2.1. Virus and cell lines

BHV-1/BarthaNu/67 strain (HVRIIBRV0004) obtained from China Veterinary Culture Collection Center (CVCC), was stored by the Ruminant Disease Research Center, Shandong Normal University, Jinan, Shandong Province, China, and the 50% tissue culture infectious dose ($TCID_{50}$) of BHV-1 determined by the Reed Muench method was $5.0 \times 10^{7.5} TCID_{50}$/0.1 mL. The 293 T (GDC0067) originating from China Center for Type Culture Collection (CCTCC), and the Madin-Darby Bovine Kidney Cells (MDBK) lines provided by American Type Culture Collection (ATCC CCL-22) were preserved in our laboratory. MDBK cells and 293 T cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS, Gibco, USA) at 37 °C, 5% CO_2.

2.2. Analysis of miRNA and mRNA expression profling

For miRNA expression profling analysis, the preparation of sample was performed based on an established method with modifications (Hou et al., 2018b). Briefly, MDBK cells were infected with BHV-1 at a multiplicity of infection (MOI) of 0.1 or mock infected for 12 h, 24 h or 48 h, and then the cells were harvested. Total cellular RNA was isolated using Trizol Reagent (Invitrogen, NY), and the quality and quantity of total RNA were evaluated by using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA) and Agilent RNA 6000 Nano kit as previously described by Piechotta and Su (Piechotta et al., 2014; Su et al., 2011), and then cDNA libraries were prepared and sequenced by BGI Co., Ltd (BGISEQ, BGI, Shenzhen).

For mRNA expression profling analysis, total RNA from control MDBK or 0.1MOI BHV-1 infected cells for 24 h was extracted using Trizol (Invitrogen, NY). The purity, concentration and integrity of the extracted total RNA were measured as described above. The preparation of cDNA libraries were performed according to previously described (Jin et al., 2018) and sequenced by BGI Co., Ltd (BGISEQ, BGI, Shenzhen). Three biological replicates were used for every experiment. Visualization of differentially expressed miRNAs and mRNA with the cut-off condition for absolute fold change ≥ 2 and $P < 0.05$ was done by using Hemi (Cuckoo group China).

2.3. Quantification of differentially expressed miRNAs by RT-qPCR

MDBK was infected with the BHV-1 at a MOI of 0.1, and total cells collected at 12 h, 24 h and 48 h post infection were subjected to miRNAs isolation by using a miRcute miRNA isolation kit (Tiangen Biotech Co., Ltd., Beijing, China). Reverse-transcribed to cDNA from 2 μg of total RNA was conducted according to the manufacturer's instructions of the miRcute miRNA first-strand cDNA synthesis kit (Tiangen, Beijing, China). Quantification of mature miRNA was performed using the miRcute miRNA real-time quantitative PCR (RT-qPCR) detection kit (SYBR Green) (Tiangen Biotech Co., Ltd., Beijing, China) on a Light Cycler 480 real time quantitative PCR system (Roche Applied Science) with a universal reverse primer and a specific forward primer as shown in Table 1. Amplification was performed for 15 min at 95 °C, followed by 40 cycles of 94 °C for 20 s, 65 °C for 34 s, and dissociation at 95 °C for 15 s, 65 °C for 60 s, and 95 °C for 30 s. The relative expression of mature miRNA was quantified within each sample using the $2^{-\triangle\triangle Ct}$ method, and 5S rRNA served as an internal control (Li et al., 2018).

2.4. RT-qPCR for monitoring EGR1 mRNA levels

The level of EGR1 mRNA was determined by RT-qPCR as described previously (Hou et al., 2018c). Briefly, MDBK cells were infected with BHV-1 at a MOI of 0.1, and total cells were collected at different time points post-infection. Total RNA was extracted from treated cell samples using RNAprep Pure Cell Kit (QIAGEN, Valencia, CA, USA). To exclude genomic DNA (gDNA) contamination in RNA samples, the RNA concentration was measured with a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientifc, Waltham, MA), and the A260/280 and A260/230 ratios were both approximately 2 (Zhu et al., 2017). Reverse transcription of RNA was performed using PrimeScript RT reagent Kit (TaKaRa, Japan). The SYBR Green-based RT-qPCR was carried out using a Premix Ex Taq kit according to the manufacturer's protocols (TaKaRa, Japan). Roche LightCycler 480 Real Time PCR System (Roche Applied Science, Germany) was used to detect mRNA expression with the primers of EGR1-F and EGR1-R (Table 1). Meanwhile, a PCR control without reverse transcription was performed for confirming DNA contamination in my RNA prepare by analysis of dissolution curve. The threshold cycle (Ct) value related to RNA levels of each target gene was normalized to GAPDH expression and the relative expression of each sample was analyzed by the $2^{-\triangle\triangle Ct}$ method.

2.5. Generation of stable expression of EGR1 gene cell lines

EGR1 gene was amplified from the cDNA of MDBK cells by PCR with

Table 1
List of primers used in this study.

Primer name	Sequence (5'-3')	Application
bta-miR-2361	GGGGGTTGTGTTGTTTTTTTTTGT	RT-qPCR detection of miRNAs
bta-miR-339b	CGGGTCCCTGTCCTCCAGGA	
bta-miR-92b	CGCATTGCACTCGTCCCG	
bta-miR-1468	GGGGCTCCGTTTGCCTGTTT	
5S RNA-F	ATACCACCCTGAACGCGCCC	
5S RNA-R	TATCCCAGGCGGTCTCCCAT	
EGR1-F	AGGAGCGATGAACGCAAGA	RT-qPCR detection of EGR1
EGR1-R	ACGGGCGATGGGTATGAG	
S-EGR1 (*EcoR* I)	CCGGAATTCGCCACCATGGCCGCG GCCAAGGCCGAAA	PLVX-IRES-puro-EGR1-HA
AS-EGR1(*Xba* I)	TGCTCTAGA*Ttaagcgtagtctggggacgtcgtatgg*GTAGCAAATTCAATTGTCCTGG	
S-UL46 (*Kpn* I)	CGGGGTACCTGCTTGGGCCGCTCGTGGACAT	pGL3-UL46
AS-UL46 (*Xho* I)	CCGCTCGAGGGTATCGTCGTCGGCGTCTTTCG	
S-ICP22 (*Kpn* I)	CGGGGTACCCACTCGCTTGCCACGCGACC	pGL3-ICP22
AS ICP22 (*Xho* I)	CCGCTCGAGGCAGCGGAAGAGGGTGTTGACG	
$S^{-3'}$UTR	AGGGAA AGGAAAAGGGAGAA	Cloning EGR1 3'UTR WT
$AS^{-3'}$UTR	TTTTCCCAAAAGTGGATTA	
$S^{-3'}$UTR-M	AGGGAAAGGAAAAGGGAGAAAATGA**TTGTGTT**	Cloning EGR1 3'UTR mutation sequences
$AS^{-3'}$UTR-M	TTTTCCCAAAGTGAGATTCTA	

Note: Underline, underline stands for the restriction endonuclease enzyme cutting sites; Small letter, HA-Tag sequence; Bold, mutation sites.

the *EcoR* I and *Xba* I restriction enzyme sites introduced into the upstream and downstream primers, respectively. Then EGR1 fragment was cloned into eukaryotic expression vector PLVX-IRES-puro (Clontech Laboratories, Mountain View, CA) with HA Tag at the C-terminus as previously described (He et al., 2016). Subsequently, cell lines stably expressing EGR1 gene were established using a lentivirus-mediated transduction strategy according to the manufacturer's protocol. Briefly, lentiviruses expressing EGR1-HA were generated by transfecting pLVX-EGR1-HA-IRES-Puro into 293 T cells along with the packaging vector pLP1, pLP2 and pLP/VSVG (Invitrogen). The produced lentiviruses were harvested at 72 h post-transfection, filtered with a 45-μm filter, and transferred to infect MDBK cells for 48 h and then the culture medium was removed from the wells and replaced with fresh medium containing puromycin (2 to 5 μg/mL) every 3 to 4 days until resistant colonies were obtained. Subsequently, puromycin-resistant colonies were picked, and the expression of EGR1 gene was determined with anti-EGR1 rabbit antibody (Abcam, Cambridge, MA) by western blot.

2.6. Short hairpin RNA for stable knockdown of EGR1

To make a stable knockdown of the endogenous EGR1 expression in MDBK cells using a lentiviral system, the lentiviral vector for delivering short hairpin RNA (shRNA) targeting EGR1 gene was constructed based on an established method with modifications (Patel et al., 2015). Briefly, two small interfering RNA sequences (1: 5′- GCA TCA GCA TGC GCA ACT TCA-3′, 2: 5′-GGC ATA CCA AGA TCC ACT TGC-3′) targeting EGR1 gene and the negative control sequences (NC: 5′-ACGUGACACG UUCGGAGAA-3′) were designed, synthesized and cloned into the pYr-Lvsh lentiviral vector. Then four plasmid-based lentiviral packaging system (pLP1, pLP2, pLP/VSVG) was used to transfect 293 T cells to package lentiviruses according to the manufacturer's recommendations (Clontech, Palo Alto, CA). The supernatants containing viruses were harvested to detect the viral titer. MDBK cells were infected with shRNA-lentiviruses at MOI of 10 in the presence of polybrene (10 mg/L), and puromycin-resistant colonies were screened as described above. The knockdown efficiency of shRNA-mediated EGR1 gene was detected with rabbit anti-EGR1 (Abcam, Cambridge, MA) and β-actin (Abways, China) as internal control.

2.7. Target gene prediction of miRNA

The potential targets of bta-miR-2361 (miRBase accession number MIMAT0011899 in the microRNA databases (http://www.mirbase.org/)) were predicted using miRNA target gene prediction tools with TargetScan (http://www.targetscan.org/) and RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/).

2.8. Measurement of BHV-1 replication in MDBK cells

MDBK cells with stable overexpression or knockdown of the endogenous EGR1 gene, and MDBK cells transfected with 50 nM of bta-miR-2361 mimics or corresponding non-targeting negative control (bta-miR-NC) oligonucleotides purchased from RiboBio (Guangzhou, China) for 24 h were infected with BHV-1 at a MOI of 0.01 for another 24 h. Cells and culture medium were harvested for virus titration to determine BHV-1 replication levels that expressed as the $\log_{10}TCID_{50}$/mL by the Reed-Muench endpoint method (Yu et al., 2012)

2.9. Western blot analysis of EGR1 expression

MDBK cells were infected with BHV-1 at a MOI of 0.1, and total cells collected at 12 h, 24 h and 48 h post infection were subjected to 10% SDS gel electrophoresis and transferred onto PVDF membranes. The blots were blocked using TBST with 5% non-fat dry milk. Anti-EGR1 antibody (Abcam, Cambridge, MA) was used at a dilution of 1:1 000. Anti-β-actin rabbit antibody (Abways, China) was added at a dilution of 1:20 000. The membranes were washed as before and visualized using enhanced-chemiluminescence reagents according to the manufacturer's protocol.

2.10. Luciferase reporter assay

To examine the transcriptional activity of the promoter sequences recognized by EGR1, the promoter plasmid of UL46 or ICP22 containing EGR1 binding site (EBS) and without EBS were cloned into pGL3 basic vector with the primers listed in Table 1. All constructs were verified by DNA sequencing analysis. Subsequently, 293 T cells were plated into 96-well plates and then pLVX-IRES-puro-EGR1-HA, PGL3-UL46 or pGL3-ICP22 together with the pRL-TK Renilla-reporter vector as the internal reporter control were transfected into 293 T cells to detect the luciferase activity driven by the promoter. In addition, the plasmid of pGL3-ICP27 group from HSV-1 was used for the positive control (Yao et al., 2012), the empty plasmid of pGL3 group was used for the negative control, and non-transfected 293 T cells were used as the blank control.

The EGR1 3′UTR sequence (EGR1 3′UTR WT) containing putative bta-miR-2361 targeting seed sequence 5′-AACACAA-3′ was amplified

from genomic cDNA of MDBK cells by PCR with the primer S^{-3}'UTR and AS^{-3}'UTR, and the corresponding mutation sequence 5′-TTGTGTT-3′ (EGR1 3′UTR MUT) was created by overlap extension of PCR with the primer S^{-3}'UTR-M and AS^{-3}'UTR-M. A fragment consisting of wild-type or site-directed mutagenesis 3′UTR of EGR1 was inserted into the dual-luciferase reporter vector pmirGLO (Promega Corporation, Madison, WI, USA). The primers for amplification of the EGR1 3′UTR WT and EGR1 3′UTR MUT gene were shown in Table 1. The protocols for luciferase assays were performed using the dual luciferase reporter assay kit (Promega) using a Spectra Max M5 microplate reader (Molecular Devices Instruments Inc, USA) as previously described (Hou et al., 2018b). Briefly, 293 T cells were plated into 96-well plates and then bta-miR-2361mimic or its negative control miRNA (bta-miR-NC) was cotransfected into 293 T cells with EGR1 3′UTR WT or EGR1 3′UTR MUT recombinant plasmids using the Attractene Transfection Reagent (Qiagen, Germany) for 36 h. The empty vector pmirGLO group was used for the negative control, and non-transfected 293 T cells were used as the blank control. Then Firefly and Renilla luciferase activities were measured respectively and Firefly luciferase activities were normalized against Renilla luciferase activities as the relative fluorescence intensity.

2.11. Statistical analyses

GraphPad Prism software (version 5.0; San Diego, CA, USA) was used to perform statistical analysis. Two-way ANOVA multiple comparisons test was used to calculate statistical significance and multiple samples was calculated by one-way ANOVA multiple comparisons test. The results were presented as the mean values ± standard deviation (SD) from at least three independent experiments, and P value below 0.05 was considered statistically significant ($^{*}P < 0.05$; $^{**}P < 0.01$).

3. Results

3.1. BHV-1 infection down-regulates bta-miR-2361 expression

In order to explore whether BHV-1 infection altered the expression level of cellular miRNAs, we analyzed the miRNA expression profile in MDBK cells during BHV-1 infection at 12 h, 24 h and 48 h post-infection (hpi) by small RNA deep sequencing. We obtained the average of 24,117,983, 24,120,488 and 23,673,903 high quality reads from the Conrtol_12 h, Conrtol_24 h, Conrtol_48 h, and 24,136,662, 24,137,272 and 24,112,667 from BEFV_12 h, BEFV_24 h, BEFV_48 h, respectively (Table S1). After filtering low quality reads, the adaptor sequences, contaminants, polyNs and reads of < 18 nt, a total of 23,762,022, 24,010,902, and 23,540,301 clean reads remained from the Conrtol_12 h, Conrtol_24 h, Conrtol_48 h, and 24,015,223, 24,022,934 and 23,995,409 from BEFV_12 h, BEFV_24 h and BEFV_48 h were mapped to the bovine genome, respectively (Table S1). The differentially expressed miRNAs were selected with the cut-off condition for absolute $\log_2$ Fold Change (FC) ≥2 and $P < 0.05$ following BHV-1 infection compared to the mock-treated cells as shown by heat map (Fig.1A). Among those differentially expressed miRNAs, the majority of miRNAs such as bta-miR-2361, bta-miR-339b, bta-miR-92b and bta-miR-1468 showed similar expression patterns at the three time points. Subsequently, these candidate miRNAs were further confirmed using RT-qPCR, and the results showed that bta-miR-2361 was significantly decreased at three time points after BHV-1 infection compared to the control group, while other miRNAs were found somewhat inconsistently expressed at three time points (Fig. 1B). Therefore, our results suggested that bta-miR-2361 might have potential function in BHV-1 infection.

3.2. Bta-miR-2361 inhibits BHV-1 replication in MDBK cells

To investigate the effect of bta-miR-2361 on BHV-1 infection, MDBK cells were transfected with bta-miR-2361 mimics or a mimic negative control (bta-miR-NC). The transfection efficiency of bta-miR-2361 mimics was confirmed through RT-qPCR assay as previously reported (Gong et al., 2018; Hou et al., 2018b). Therein, bta-miR-2361 was successfully overexpressed by mimics as compared to cells transfected with bta-miR-NC group (Fig. 2A). Furthermore, the viral titer (shown as the $\log_{10}TCID_{50}$/mL) was determined in bta-miR-2361 mimic transfected MDBK cells as compared to NC groups upon BHV-1 infection. The results showed that the viral titer of BHV-1 had a decrease in addition of bta-miR-2361 mimic by > 10-fold relative to that in NC mimic transfected MDBK cells (Fig. 2B). These data demonstrated that bta-miR-2361 mimics can negatively regulate viral replication.

3.3. BHV-1 infection up-regulates EGR1 expression

It is common knowledge that miRNA plays its role by inhibiting its target genes. Thus, we hypothesized that reduction of bta-miR-2361 expression induced by BHV-1 infection may conversely promote the expression of host gene required for viral replication. To search the target genes modulated by bta-miR-2361, first of all, we performed RNA sequencing using next-generation sequencing (NGS) technology to test the alteration of mRNA expression profile in BHV-1 infected MDBK cells at 24 hpi based on viral replication dynamics analysis. Among these, we focus on analysis of 176 genes that were up-regulated expression during BHV-1 infection (Fig. 3A). Simultaneously, we predicted bta-miR-2361 targets with bioinformatics tools such as Targetscan (http://www.targetscan.org) and RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/) with minimum cut-off free energy (MFE) gained from miRNA-target formation kept as -20 kcal/mol. This led to the identification of 106 potential genes which have at least one consensus seed sequence at 3′UTR. In combination with the binding prediction for bta-miR-2361 using bioinformatics software and the differential expression of the prospective target gene, EGR1 gene, one of predicted targeting gene, contained the target site of bta-miR-2361 at the 3′UTR of EGR1 (Fig. 3B). Therefore, we focused on EGR1 for further study.

Accordingly, the expression levels of the EGR1 gene in BHV-1 infected MDBK cells at different time points were determined by RT-qPCR and western blot. As shown in Fig. 3C, the mRNA level of EGR1 was dramatically up-regulated compared with mock infection. Moreover, an increased expression of EGR1 protein level was also observed in BHV-1 infected MDBK cells at 12 h, 24 h and 48 h (Fig. 3D). Taken together, these data demonstrated that BHV-1 infection up-regulated EGR1 gene expression.

3.4. EGR1 promotes BHV-1 replication in MDBK cells

In order to investigate whether viral induction of EGR1 gene affected BHV-1 replication, we generated MDBK cell lines stably expressing the HA epitope-tagged EGR1 (EGR1-HA), and empty plasmid vector (HA-EV). Western blot analysis was performed to identify the EGR1 gene expression probed with anti-EGR1 antibody. A significantly up-regulated expression of EGR1 gene was observed in stably expressing EGR1-HA cell lines compared to HA-EV-expressed cells (Fig. 4A), suggesting that the stable ectopic expression of EGR1 gene cell lines were successfully established. Meanwhile, stable knockdown of EGR1 gene by shRNA recombinant lentivirus mediated MDBK cell lines, along with their corresponding negative control cell lines were constructed. In virtue of low expression of endogenous EGR1, shRNA-mediated EGR1 knockdown cell lines were infected with BHV-1 at a MOI of 0.1 for 24 h, and then the inhibiting efficiency was confirmed by western blot. This results showed that the expression of EGR1 gene was effectively inhibited in shRNA-mediated knockdown cell lines (Fig. 4B), and the more efficient EGR1-shRNA-2 was used in the following experiments.

Subsequently, the stable overexpression or knockdown of EGR1 gene cells lines were infected with an equal amount of BHV-1

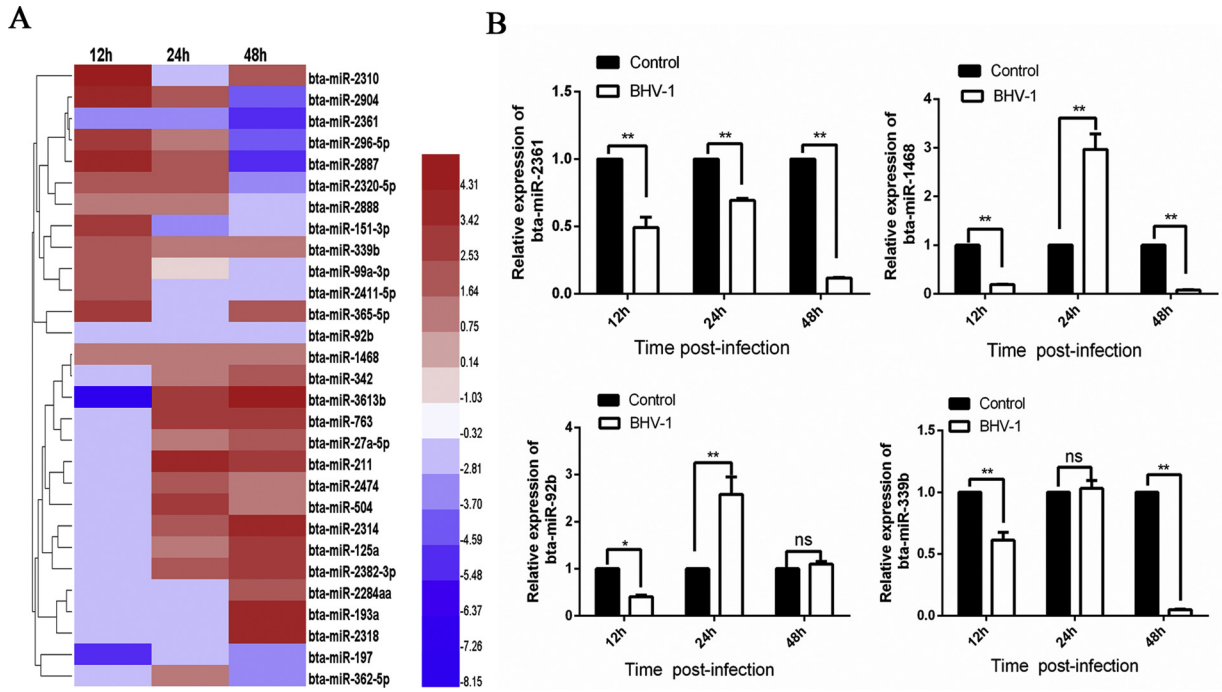

Fig. 1. BHV-1 infection down-regulates bta-miR-2361 expression. (A) Heatmap of miRNA expression in MDBK cells infected with BHV-1 (0.1MOI) at 12, 24, 48 hpi, color indicates the differentially expressed miRNAs with the cut-off condition for absolute fold change ≥2 and $P < 0.05$ following BHV-1 infection compared to the mock-treated cells. (B) Relative quantitation analysis of endogenous bta-miR-2361, bta-miR-339b, bta-miR-92b and bta-miR-1468 in MDBK cells infected with BHV-1 (0.1MOI) for 12 h, 24 h or 48 h by RT-qPCR. The relative expression of miRNA was quantified using the $2^{-\triangle\triangle Ct}$ method, and 5S rRNA served as an internal control. Data were shown as the mean ± SD from three independent experiments, $*P < 0.05$, $**P < 0.01$.

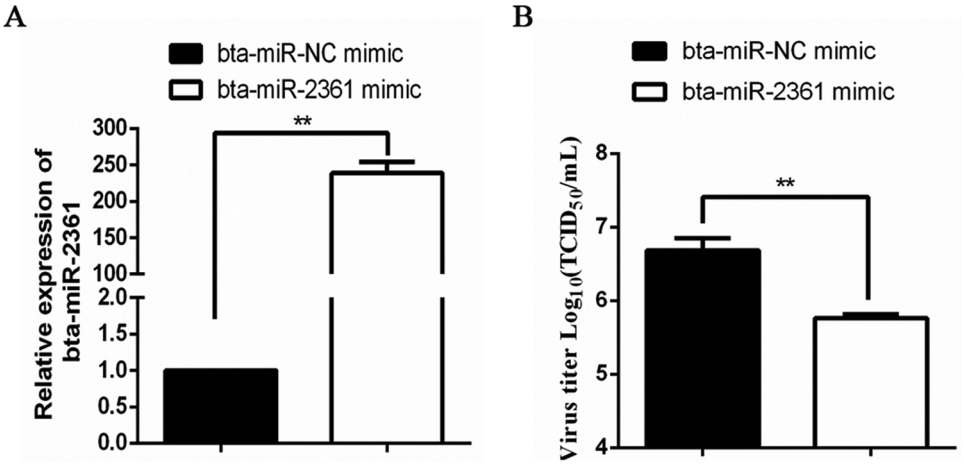

Fig. 2. Bta-miR-2361 inhibits BHV-1 replication in MDBK cells. (A) MDBK cells were transfected with bta-miR-2361 mimics or bta-miR-NC for 24 h, and the levels of bta-miR-2361 expression in MDBK cells was monitored by RT-qPCR. (B) MDBK cells were transfected with bta-miR-2361 mimics or bta-miR-NC respectively and then infected with 0.01MOI BHV-1 at 24 h post transfection. Cells were collected at 24 hpi and repeated freezing and thawing 3 times. The titer of BHV-1 was determined by $TCID_{50}$ assay. The mean ± SD of the data were reported as three independent experiments, $*P < 0.05$, $**P < 0.01$.

(0.01MOI) for 24 h, and viral titers were determined to analyze whether EGR1 affected the replication of BHV-1. As shown in Fig. 4C, overexpression of EGR1 significantly enhanced replication of BHV-1, whereas the knockdown of EGR1 gene had the opposite effects (Fig. 4D). Collectively, the results indicated that EGR1 induction contributed to BHV-1 replication.

3.5. EGR1 promotes the expression of viral UL46 gene

Previous studies have indicated that EGR1 as a zinc-finger DNA-binding protein functions in the regulation of HSV-1 or EBV replication by directly binding to promoter sequences of target viral genes (Bedadala et al., 2007; Chang et al., 2006). Next, we investigated whether EGR1 increased BHV-1 replication by activating the expression of viral genes. We conducted a search of EGR1-binding sequences in the genome of BHV-1 (https://www-bimas.cit.nih.gov/molbio/proscan/) and found promoters of UL46 and ICP22 containing EGR1-binding sequences (5′-CGCCCGCGC-3′). Then we determined the promoter activity of viral UL46 and ICP22 by the luciferase reporter assay (Alcaraz-Pérez et al., 2008). As illustrated in Fig. 5A, viral UL46 and ICP22 promoter regions were cloned into the pGL3-basic vector (Promega, Madison, WI) to drive the firefly luciferase gene. And pGL3-UL46, pGL3-ICP22 or pGL3-ICP27 of HSV-1 being used as a positive control recombinant plasmid together with EGR1 pLVX-IRES-puro-EGR1-HA and the internal control plasmid PRL-TK were cotransfected into 293 T cells, respectively. Luciferase activity was measured at 24 h after transfection. The results showed that overexpression of EGR1 significantly increased the luciferase activities controlled by UL46 promoter, which contain EGR1 binding sequences ($P < 0.05$), but failed to alter luciferase activities driven by ICP22 promoter, as compared to the control (Fig. 5B). Furthermore, the activation of the EGR1 on pGL3-UL46 reporter appeared to be specific because cotransfection with the

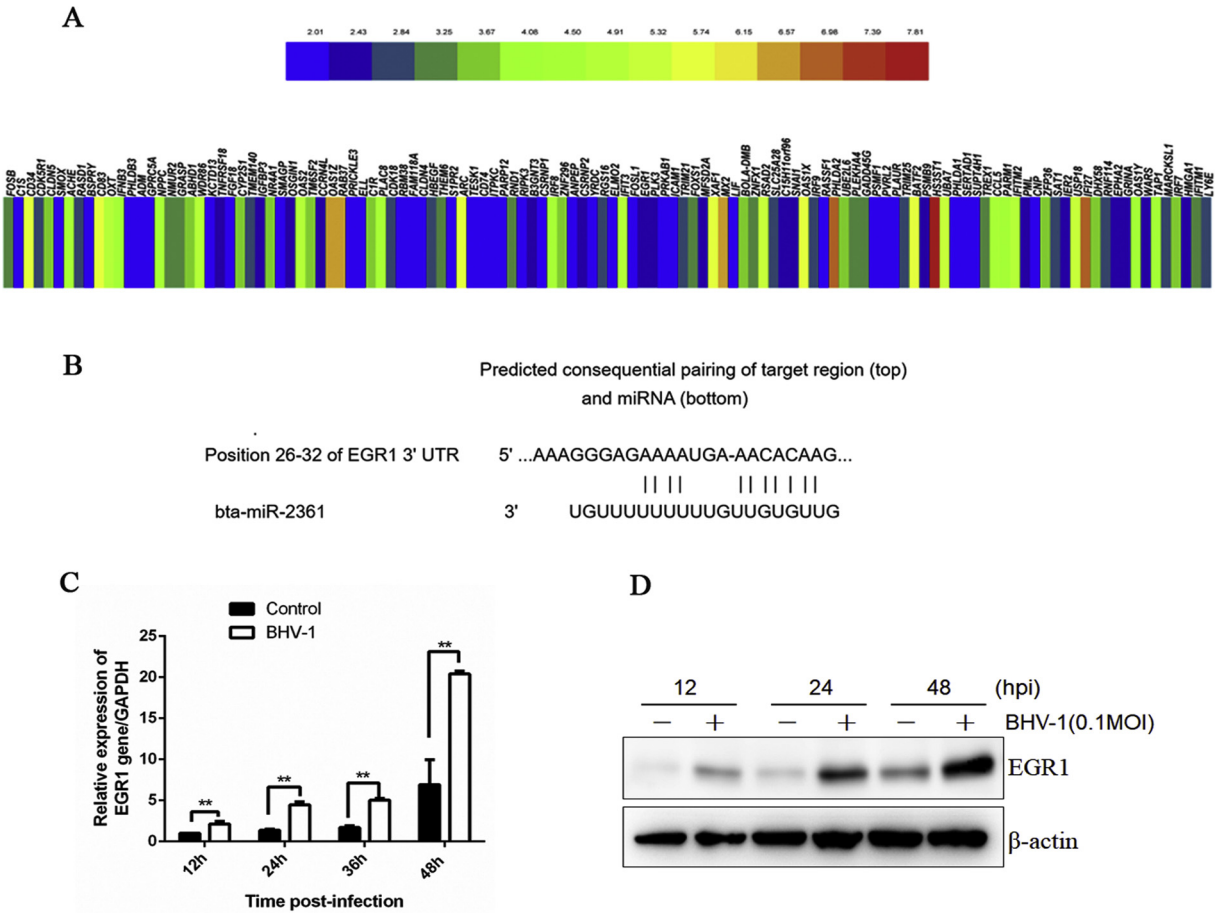

Fig. 3. BHV-1 infection up-regulates the expression of EGR1 gene. (A) Hierarchical clustering analysis revealed up-regulated genes in MDBK cells infected with BHV-1 (0.1MOI) for 24 h, color indicates the $\log_2$ ratios of BHV-1 infection vs mock infection with the cut-off condition for fold change ≥2 and $P < 0.05$. (B) Schemata showed the binding sites of bta-miR-2361 in EGR1 gene. (C) The expression of EGR1 gene in MDBK cells infected with BHV-1 (0.1MOI) at 12, 24, 36 and 48 hpi was monitored by RT-qPCR, and GAPDH was used as internal control. Data are expressed as relative fold change compared with mock infection. The mean ± SD of the data were expressed as three independent experiments, $^*P < 0.05$, $^{**}P < 0.01$. (D) The expression of EGR1 protein in MDBK cells infected with BHV-1 at a MOI of 0.1 or mock infection at 12, 24 and 48 hpi was determined by western blot with anti-EGR1 antibody, and β actin was used as an internal standard.

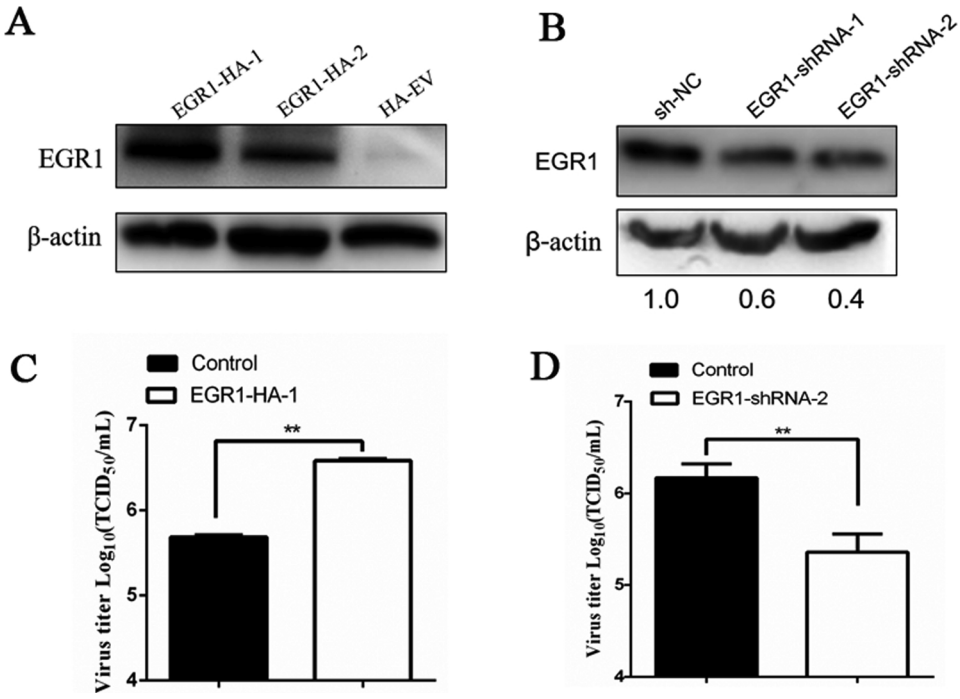

Fig. 4. EGR1 promotes BHV-1 replication in MDBK cells. (A) The expression level of EGR1 protein in stable expressing cell lines was assayed by western blot with anti-EGR1 antibody, and β actin was used as an internal standard. (B) The knockdown efficiency of EGR1-shRNA in MDBK cell lines upon BHV-1 infection at a MOI of 0.1 for 24 h was determined by western blot with anti-EGR1 antibody compared with control shRNA cell lines, and β actin as an internal standard. Numbers below the image was EGR1/β-actin ratios of band optical density values from Image *J* software. The stably expressing EGR1 gene cell lines EGR1-HA1 (C), shRNA2-mediated knockdown cell lines EGR1-shRNA-2 (D) and their corresponding control groups were inoculated with BHV-1 (0.1MOI) for 24 h, then the titers of BHV-1 infected cells were determined by $TCID_{50}$ assay. The data were means with SD from three independent experiments, $^*P < 0.05$, $^{**}P < 0.01$.

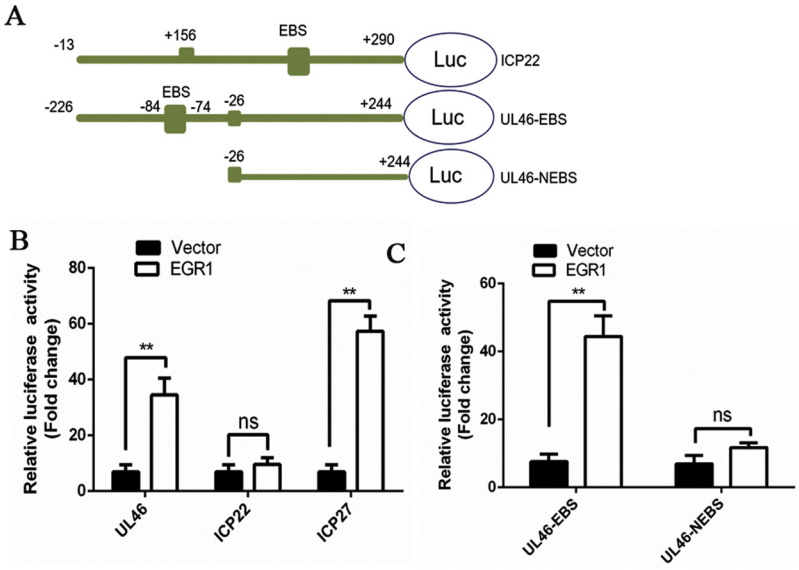

Fig. 5. EGR1 regulates expression of viral gene UL46. (A) Schematic of putative EGR1-binding sites within UL46 and ICP22 promoter were shown and UL46 and ICP22 promoter vectors containing EGR1 binding sequences (EBS) and UL46 promoter vectors without EGR1 binding sequences (NEBS) were constructed. (B) pGL3-UL46 or pGL3-ICP22 plasmid encoding firefly luciferase together with a plasmid encoding EGR1 or empty control vector that absence of EGR1 plasmids, and the internal control plasmid PRL-TK encoding Renilla luciferase were cotransfected into 293 T cells, respectively. The relative luciferase activities were measured in 293 T cells at 24 h. The ICP27 group from HSV-1 was used for the positive control. Data was presented as means ± SD of three independent experiments. $*P < 0.05$, $**$ $P < 0.01$, ns, not significant. (C) 293 T cells co-transfected with the plasmid of UL46 promoters with the EGR1 binding site (UL46-EBS) or without EGR1 binding sequence (UL46-NEBS) and recombinant plasmid of pLVX-IRES-puro-EGR1-HA were subjected to luciferase reporter assay. Relative firefly luciferase unit normalization with Renilla activity was analyzed. The mean ± SD of the data were expressed. The experiments were repeated three times, $*P < 0.05$, $**P < 0.01$; ns, not significant.

empty vector pLVX-IRES-puro-HA (without expressing the EGR1) did not have any effect on luciferase activity (Fig. 5B). To further identify the functional importance of the EGR1 binding site for activating the viral promoter, UL46 luciferase reporter plasmids deleting EGR1 binding site (UL46-NEBS) was constructed (Fig. 5A) and UL46 luciferase reporter plasmid containing promoter region with or without EGR1 binding site respectively was cotransfected with plasmid pLVX-IRES-puro-EGR1-HA into 293 T cells, and luciferase activity was determined. As shown in Fig. 5C, UL46 promoter activity of promoter region deleting EGR1 binding site (UL46-NEBS) was relatively reduced as compared to the promoter region with EGR1 binding site (UL46-EBS). In sum, these findings suggested that EGR1 up-regulated expression of UL46 via specifically binding to its corresponding sequences in the viral gene promoters.

3.6. Bta-miR-2361 directly targets the EGR1 gene

Since bta-miR-2361 was down-regulated and EGR1 was up-regulated in MDBK cells infected with BHV-1, and EGR1 gene was one of predicted target candidate genes of bta-miR-2361 (Fig. 3B), it is of interest to investigate whether bta-miR-2361 inhibited BHV-1 replication via directly target EGR1 gene. The wild-type or mutant with base pair mutations in the 3′UTR seed region of EGR1 recombinant pmirGLO plasmids were constructed (Firefly-EGR1 3′ UTR (WT or MUT)-Renilla) (Fig. 6A) and cotransfected into 293 T cells with bta-miR-2361 mimics or bta-miR-NC mimics for measuring of luciferase activity respectively. And decreased luciferase activity signal was observed following wild-type EGR1 reporter plasmid transfected with the bta-miR-2361 mimics in comparison to cells transfected with bta-miR-NC mimics (Fig. 6B), whereas luciferase activity of 3′UTR mutant plasmid yielded a mild change after cotransfection with bta-miR-2361 mimics or bta-miR-NC mimics (Fig. 6B), indicating that bta-miR-2361 was likely to directly interact and inhibit the EGR1 expression after binding to the seed sequence. As expected, over-expression of bta-miR-2361 inhibited EGR1 expression in MDBK cells at the levels of mRNA and protein (Fig. 6C, E). What's more, bta-miR-2361 mimic transfection during BHV-1 infection significantly reduced the expression of UL46 gene (Fig. 6D) and inhibited BHV-1 replication (Fig. 2B). Together, the data generated a schematic map that illustrates bta-miR-2361 function and mechanism of action. We demonstrated that bta-miR-2361 acting as a negative regulator of BHV-1 replication could regulate EGR1 gene via targeting 3′UTR of EGR1. In addition, BHV-1 infection up-regulated EGR1 expression that promoted viral replication by regulating the expression of viral gene UL46 (Fig. 6E).

4. Discussion

Cellular miRNAs are a class of small RNAs that have been recognized as key regulators of gene expression, and there is increasing evidence that miRNA machinery play a critical role in host and pathogen interaction by modulating both host biological pathways and regulating viral life cycle (Chiang et al., 2013; Jin et al., 2013; Shirasaki et al., 2013). However, the role of cellular miRNAs in the process of BHV-1 infection has not been reported. In order to identify cellular miRNAs that may be involved in BHV-1 replication, the NGS technology was used to analyze an integrated miRNA expression profile in BHV-1 infected MDBK cells. And altered miRNA expression may have broader implications for the miRNA-dependent gene silencing and virus replication. Bta-miR-2361, one of the differentially expressed genes, is a novel miRNA molecule, and its role and mechanism in viral infection and replication has not been evaluated so far. Here, bta-miR-2361 was confirmed to be down-regulated after BHV-1 infection by RT-qPCR, and miRNA functional analysis indicated that bta-miR-2361 was antagonistic to viral replication when it is present at greatly elevated levels. Nevertheless, the increase in titer of BHV-1 appeared modest in cells transfected with the miR-2361 inhibitor (data not shown), this suggested that the virus-induced reduction in bta-miR-2361 levels observed during BHV-1 infection probably had some biological impact of the down regulation of miR-2361 achieved by the inhibitor.

Mounting evidence suggests that cellular miRNAs exert their biological functions through regulating the expression of multiple genes by binding to 3′UTR of target genes (Guo et al., 2010; Li et al., 2010; Ma et al., 2012; Xie and Zhou, 2018). We predicted the potential target genes of bta-miR-2361 using various miRNA prediction tools, but there were too many candidate genes to be confirmed. It has been reported that the high-throughput sequencing technique is a highly efficient tool in screening of miRNA targets, for example, a large-scale proteomic study has been reported as a useful approach for the identification of targets of cellular miR-197 downregulated by Enterovirus A71(Tang et al., 2018). In our study, high-throughput transcriptome sequencing was performed on MDBK cells infected with BHV-1 and uninfected MDBK cells. Up-regulated mRNAs were analyzed to narrow candidate miRNA targets as the expression of bta-miR-2361 was decreased in MDBK cells infected with BHV-1. We subsequently predicted binding sites in target gene, and further identify the EGR1 gene as one of bta-miR2361 targets by 3′ UTR dual luciferase reporter system. Moreover,

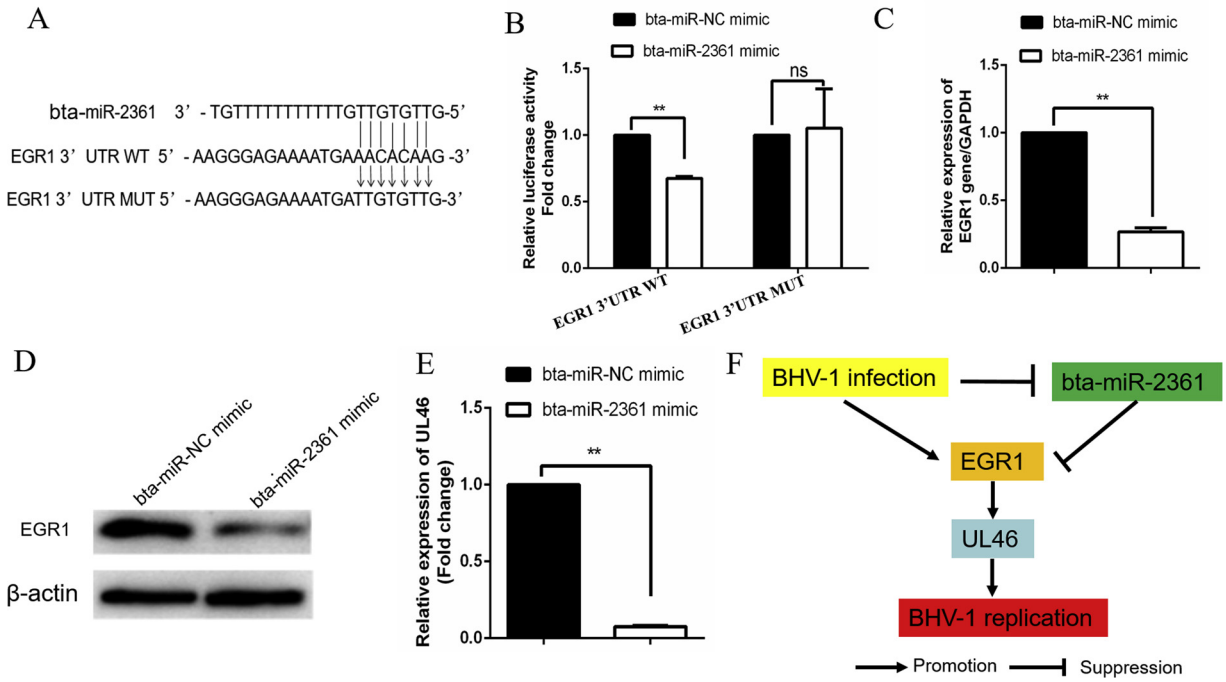

Fig. 6. Bta-miR-2361 directly targets the EGR1. (A) Schemata showed the wild-type 3′UTR of EGR1 gene complemented with bta-miR-2361 seed sequence, and its mutant of putative bta-miR-2361 targeting sequences. The luciferase reporter plasmid containing bta-miR-2361 binding site (Firefly-EGR1-3′UTR-WT-Renilla) or mutant binding site at 3′UTR of EGR1 (Firefly-EGR1-3′UTR-MUT-Renilla) was constructed. (B) 293 T cells were cotransfected with bta-miR-2361 and EGR1 3′UTR WT or EGR1 3′UTR MUT, and the relative luciferase activities were measured with dual-luciferase assay. *$P < 0.05$, **$P < 0.01$, ns, not significant. (C) MDBK cells transfected with bta-miR-2361 mimics or bta-miR-NC mimics for 24h. Then, the cells were collected and mRNA levels of EGR1 were evaluated by RT-qPCR. (D) MDBK cells transfected with bta-miR-2361 mimics or bta-miR-NC mimics for 24h and then infected with BHV-1 for 24h. The expression of EGR1 protein was evaluated by western blot. (E) MDBK cells were transfected with bta-miR-2361 mimics or bta-miR-NC mimics, and then infected with 0.1MOI BHV-1 at 24 h post transfection. The expression of UL46 gene was assessed by RT-qPCR. The mean ± SD of the data were expressed as three independent experiments,*$P < 0.05$, **$P < 0.01$. (E) A schematic representation of the role of bta-miR-2361 in modulating BHV-1 replication.

the proposed model of the relationship between bta-miR-2361 and EGR1 gene during BHV-1 infection was supported by the following observations. First, EGR1 expression was up-regulated in MDBK cells infected with BHV-1 at the level of mRNA and protein. In addition, overexpression of EGR1 gene promoted viral replication, whereas inhibiting expression of EGR1 gene could suppress the BHV-1 replication. Most importantly, bta-miR-2361 can decrease the expression of EGR1 gene. Therefore, bta-miR-2361 may inhibit viral replication by regulating the expression of EGR1 gene. However, there is no information on how the reduced bta-miR-2361 levels is brought about during BHV-1 infection, and future experimentation is needed to confirm it.

Several studies have demonstrated that EGR1 plays an important role in various virus replication. For example, EGR1 suppresses foot-and-mouth disease virus replication by enhancing type I interferon pathway signal transduction (Zhu et al., 2018). EGR1 modulates pro-apoptotic pathway and promotes Venezuelan equine encephalitis virus (VEEV) replication (Baer et al., 2016). Yet there is growing evidence that EGR1 acting as a transcription factor, contains a DNA binding domain that specifically binds to the viral genome and regulates the viral gene expression. Previous reports showed that EGR1 plays a significant role in the life cycle of herpesvirus replication including HSV-1, EBV and KSHV (Bedadala et al., 2007; Chang et al., 2006; Dyson et al., 2012; Hsia et al., 2013). Here, we found that EGR1 can promote the replication of BHV-1 and further demonstrated that EGR1 could directly bind to the viral gene UL46 promoter, but not bind to the viral gene ICP22. This result is inconsistent with previous reports that EGR1 could regulate expression of ICP22 gene of HSV-1 although BHV-1 and HSV-1 belong to herpes virus family. Furthermore, UL46 gene, which is present only in alphaherpesviruses, is one of the most abundant tegument proteins of BHV-1, but the physical function of UL46 gene during BHV-1 infection remains unclear. One reported role for UL46 protein of several alphaherpesviruses in viral replication is to enhance the transactivation of viral immediate early genes (Murphy et al., 2008). So some inferences drawn from the data indicate that EGR1 enhances the functional role of viral reactivation by facilitating the transcription of UL46. Moreover, HSV-1 UL46 has been reported to function in the inactivation of the STING DNA-sensing pathway that plays a major role in antiviral responses (Deschamps and Kalamvoki, 2017), which provides another possible mechanistic explanation for the expression of UL46 producing a more favorable environment for viral replication. However, whether the tegument protein UL46 shares similar features with other well-characterized UL46 of HSV-1 is a subject for further investigation.

These findings provided one of the potential molecular mechanisms of bta-miR-2361 in regulating BHV-1 replication and identified EGR1 as a key mediator of this process. Nevertheless, there is still much study needed to explore the in-depth mechanisms about how bta-miR-2361 functions. For instance, judging from the result of the reduction in titer of BHV-1 in MDBK cells transfected with the bta-miR-2361, despite > 200-fold higher levels of the miRNA expression compared to control groups, the inhibition efficiency appears modest. Is there any other targets of bta-miR-2361 antagonizing it? What's more, whether other factors have the similar effects with bta-miR-2361 on EGR1 or whether EGR1 participates in BHV-1 replication by regulating host genes is an interesting and important question deserving further research.

In summary, based on the data from high-throughput sequencing, a novel bta-miR-2361 and its candidate target EGR1 gene was further studied in MDBK cells during BHV-1 infection, the EGR1 gene promoted BHV-1 replication via regulating of viral UL46 gene, whereas bta-miR-2361 inhibited viral replication through down-regulated expression of EGR1 gene. To our knowledge, this study is the first to reveal that bta-miR-2361 could inhibit BHV-1 replication through directly targeting

EGR1, which potentially provides a new perspective on the therapeutic strategies of BHV-1.

Competing interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Authors' contributions

H.PL and Z.M performed the experiments and drafted manuscript, H. WQ analyzed the data, H.HB and W.HM designed and instructed the experiments. All authors have read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.05.004.

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