Hsa-let-7c-5p augments enterovirus 71 replication through viral subversion of cell signaling in rhabdomyosarcoma cells

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Abstract

Background: Human enterovirus 71 (EV71) causes severe hand, foot and mouth disease, accompanied by neurological complications. During the interaction between EV71 and the host, the virus subverts host cell machinery for its own replication. However, the roles of microRNAs (miRNAs) in this process remain obscure.

Results: In this study, we found that the miRNA hsa-let-7c-5p was significantly upregulated in EV71-infected rhabdomyosarcoma cells. The overexpression of hsa-let-7c-5p promoted replication of the virus, and the hsa-let-7c-5p inhibitor suppressed viral replication. Furthermore, hsa-let-7c-5p targeted mitogen-activated protein kinase kinase kinase 4 (MAP4K4) and inhibited its expression. Interestingly, downregulation of MAP4K4 expression led to an increase in EV71 replication. In addition, MAP4K4 knockdown or transfection with the hsa-let-7c-5p mimic led to activation of the c-Jun NH2-terminal kinase (JNK) signaling pathway, whereas the hsa-let-7c-5p inhibitor inhibited activation of this pathway. Moreover, EV71 infection promoted JNK pathway activation to facilitate viral replication.

Conclusions: Our data suggested that hsa-let-7c-5p facilitated EV71 replication by inhibiting MAP4K4 expression, which might be related to subversion of the JNK pathway by the virus. These results may shed light on a novel mechanism underlying the defense of EV71 against cellular responses. In addition, these findings may facilitate the development of new antiviral strategies for use in future therapies.

Keywords: Hsa-let-7c-5p, EV71, MAP4K4, JNK

Background

Human enterovirus 71 (EV71), which belongs to the genus Enterovirus in the family Picornaviridae, is a type of single-stranded positive-sense RNA virus that is known to be a major cause of hand, foot and mouth disease (HFMD) in young children. This virus may cause serious neurological complications compared with other pathogens associated with HFMD [1]. Since EV71 was first isolated in 1969 [2], several serious infectious outbreaks have occurred, placing heavy burdens on society [3–7]. However, no effective drug has been identified for clinical application. Therefore, EV71 infection remains a public health concern.

MicroRNAs (miRNAs), a class of highly conserved small non-coding-RNAs, are 18–25 nucleotides (nts) in length. They post-transcriptionally modulate gene expression via mRNA degradation, mRNA cleavage, and translational repression [8–10]. MiRNAs are involved in a number of biological processes, such as cell cycle progression, cell survival and apoptosis [11–14]. Increasing evidence indicates that miRNAs also play a role in the pathogenesis of viral infectious diseases. Both DNA and RNA viruses can act as boosters, destroyers and hijackers in the regulation of cellular miRNAs to facilitate progression of their life cycles [15, 16].

The lethal-7 (let-7) miRNA was first discovered in Caenorhabditis elegans (C. elegans), in which it acts as...
a key developmental regulator [17]. The Homo sapiens (H. sapiens) let-7 (hsa-let-7) family contains 13 individual members encoded on different chromosomes. The expression of these miRNAs, except for that of hsa-let-7c, is meticulously regulated by lin28 [18–20]. Hsa-let-7c is downregulated in most cancers and plays crucial roles in carcinogenesis and cancer metastasis [21–23]. In addition, it has been reported to be upregulated in response to RNA virus infection and to affect viral replication [24–26]. The miRNA let-7c has been shown to inhibit influenza virus replication by degrading viral gene M1 (+) cRNA in human lung epithelial cells [24]. Further, let-7c has been reported to suppress viral replication through targeting of the transcription factor BACH1 in dengue virus-infected human hepatoma Huh-7 cells [25]. Moreover, this miRNA may enhance HIV-1 infection by downregulating the cellular restriction factor p21 [26]. However, its roles in mediating virus-host interactions through the regulation of host pathways have not been previously investigated. In addition, the regulation of hsa-let-7c-5p in EV71 replication has not yet been reported.

In this study, we showed that the hsa-let-7c-5p-mediated regulation of mitogen-activated protein kinase kinase kinase kinase 4 (MAP4K4) might play an important role in EV71 replication. Hsa-let-7c-5p may modulate viral subversion of the c-Jun NH2-terminal kinase (JNK) signaling pathway and promote EV71 replication.

Results
Mature miRNA hsa-let-7c-5p expression is upregulated during EV71 infection
According to current and our previous studies, rhabdomyosarcoma (RD) cells and the Henrietta Lacks strain of cancer (HeLa) cells can be used as in vitro models to investigate the interplay between EV71 and its host [16, 27–29]. To determine whether hsa-let-7c-5p is involved in EV71 infection, we first examined the expression of mature hsa-let-7c-5p in uninfected and EV71-infected RD cells by stem-loop quantitative reverse transcription polymerase chain reaction (qRT-PCR). The effect of EV71 infection on hsa-let-7c-5p expression was detected at 12, 24 and 36 h post-infection (p.i.), and the results showed that hsa-let-7c-5p expression was detected at 12, 24 and 36 h post-infection (p.i.), and the results showed that hsa-let-7c-5p expression was significantly increased in EV71-infected RD cells compared with mock-infected cells at 24 h p.i. (1.9-fold change) and 36 h p.i. (2.7-fold change) (Fig. 1, p < 0.001). Similar hsa-let-7c-5p expression was detected in EV71-infected HeLa cells (Additional file 1: Figure S1). These data demonstrated that EV71 infection upregulated the expression of hsa-let-7c-5p.

The overexpression of hsa-let-7c-5p promotes EV71 replication
To investigate the role of hsa-let-7c-5p during EV71 infection, we first assessed the transfection efficiency of an hsa-let-7c-5p miRNA mimic in RD cells. A negative control (NC) miRNA mimic was also used in these experiments. Markedly increased hsa-let-7c-5p expression (by nearly 1000-fold) was observed in the hsa-let-7c-5p-overexpressing group compared with the NC group (Fig. 2a, p < 0.01). As shown in Additional file 1: Figure S2 and Fig. 2b, the hsa-let-7c-5p mimic at concentrations of 50 nM (p < 0.01), 70 nM (p < 0.001) and 100 nM (p < 0.001) increased the virus titer compared with the NC mimic, and the highest virus titer was detected following treatment with 100 nM of the hsa-let-7c-5p mimic. Thus, 100 nM of the hsa-let-7c-5p mimic was used in the following experiments. A substantially increased virus titer was observed in the hsa-let-7c-5p mimic group compared with the NC mimic and Mock-T groups (Fig. 2b, p < 0.001). Further, the levels of viral RNA and the structural protein VP1 were significantly increased in the hsa-let-7c-5p mimic group compared with the NC mimic and Mock-T groups (Fig. 2c, p < 0.001; Fig. 2d, p < 0.01 and p < 0.05, respectively). No difference in the virus titer, viral RNA level or VP1 protein level was observed between the NC mimic and Mock-T groups (Fig. 2b–d). Next, to exclude the possibility that hsa-let-7c-5p affects host cells, cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. No significant
A difference in cell viability was observed between hsa-let-7c-5p mimic- and NC mimic-transfected mock-infected RD cells (Fig. 2e). However, the hsa-let-7c-5p mimic reduced the survival of EV71-infected RD cells in contrast with the NC mimic (Fig. 2e, \( p < 0.05 \)). Taken together, these data demonstrated that an increased hsa-let-7c-5p level might promote EV71 replication and facilitate the viral impairment of host cells.
The inhibition of hsa-let-7c-5p expression represses EV71 replication

To further confirm the effect of hsa-let-7c-5p on EV71 replication, cells were treated with a miRNA inhibitor. This inhibitor, referred to as hsa-let-7c-5pi in the paper, is a synthetic oligonucleotide with a sequence that is exactly complementary to hsa-let-7c-5p. A negative control miRNA inhibitor (NCi) was also used, which is a miRNA oligonucleotide from C. elegans whose sequence shows almost no homology with the genomes of H. sapiens, Rattus norvegicus and Mus musculus.

To evaluate the effect of hsa-let-7c-5pi on hsa-let-7c-5p expression, 150 nM inhibitor oligonucleotide was transfected into RD cells. Stem-loop qRT-PCR revealed that hsa-let-7c-5p expression was significantly decreased in the hsa-let-7c-5pi group compared with the NCi group (Fig. 3a, \( p < 0.001 \)). In addition, the plaque assay results showed that hsa-let-7c-5pi significantly reduced the EV71 titer of the hsa-let-7c-5pi group compared with the NCi and Mock-T groups (Fig. 3b, \( p < 0.001 \)). Further, both the viral RNA and VP1 protein levels were significantly suppressed in the hsa-let-7c-5pi group compared with the NCi and Mock-T groups, as shown in Fig. 3c (\( p < 0.001 \)) and Fig. 3d (\( p < 0.01 \) and \( p < 0.05 \), respectively). Moreover, no significant differences in the virus titer, viral RNA level or VP1 protein level were detected between the NCi and Mock-T groups (Fig. 3b–d). MTT assays revealed no significant difference in cell viability between the mock-infected RD cells transfected with hsa-let-7c-5pi and those transfected with NCi (Fig. 3e). However, viability of the EV71-infected RD cells was enhanced in the hsa-let-7c-5pi group compared with the NCi group (Fig. 3e, \( p < 0.01 \)). These results showed that the hsa-let-7c-5pi inhibitor suppressed EV71 replication and protected host cells from EV71-induced cell death.

Hsa-let-7c-5p directly inhibits the expression of MAP4K4 by binding to its 3’UTR

To characterize the molecular mechanism associated with hsa-let-7c-5p function, we used an online miRNA prediction program, TargetScan, and the Starbase database to comprehensively analyze potential targets [30–32]. The results showed that MAP4K4 was a potential candidate target of hsa-let-7c-5p.

To date, the standard strategy used to validate miRNA targets involves artificial sensor assay, in which the 3′UTR of a gene of interest is coupled to a reporter plasmid. A schematic diagram of the binding site of hsa-let-7c-5p to the 3′UTR of the MAP4K4 mRNA is presented in Fig. 4a, and mutations in the sequence are highlighted in red and italic font. The luciferase assay results showed that the reporter activity of psi-MAP4K4-3′UTR was significantly decreased in the hsa-let-7c-5p mimic group compared with the NC group (Fig. 4b, \( p < 0.001 \)), whereas that of psi-MAP4K4-3′UTRmut did not significantly differ between the two groups (Fig. 4b).

To investigate the post-transcriptional regulation of hsa-let-7c-5p, we detected the MAP4K4 mRNA and protein levels by qRT-PCR and Western blotting, respectively. The hsa-let-7c-5p mimic at a concentration of 100 nM significantly reduced MAP4K4 mRNA expression compared with the NC mimic (Fig. 4c, \( p < 0.001 \)). In addition, the MAP4K4 protein level was obviously decreased in the hsa-let-7c-5p mimic group compared with the NC group (Fig. 4d, \( p < 0.001 \)). To further validate the miRNA-target interaction, cells were treated with an hsa-let-7c-5pi inhibitor to assess the regulatory effects of this miRNA at both the mRNA and protein levels. The hsa-let-7c-5p inhibitor oligonucleotide at a concentration of 150 nM increased the MAP4K4 mRNA and protein levels compared with the NC inhibitor (Fig. 4e, \( p < 0.05 \); Fig. 4f, \( p < 0.01 \)). These data demonstrated that MAP4K4 was a target of hsa-let-7c-5p and that hsa-let-7c-5p post-transcriptionally regulated MAP4K4 expression by directly binding to its 3′UTR.

MAP4K4 knockdown facilitates EV71 replication and activates the JNK signaling pathway

To detect whether MAP4K4 is involved in EV71 infection, we performed qRT-PCR and Western blotting. The results showed that MAP4K4 expression was significantly decreased at both the mRNA and protein levels in EV71-infected RD cells compared with mock-infected cells at 24 and 36 h p.i. (Fig. 5a, b; \( p < 0.001 \)). To further determine whether MAP4K4 affects EV71 replication, an shRNA pool was used to silence MAP4K4 expression. MAP4K4 expression was effectively silenced at both the mRNA and protein levels by the LV-shMAP4K4 vector in RD cells (Additional file 1: Figure S3). In addition, MAP4K4 expression was more strongly repressed by the LV-shMAP4K4-3 vector than by the other two vectors (Additional file 1: Figure S3). Thus, the LV-shMAP4K4-3 vector was used in the subsequent experiments. A significantly increased EV71 titer was detected in the LV-shMAP4K4-3-transfected group compared with the LV-shcontrol-transfected group (Fig. 5c, \( p < 0.001 \)). Moreover, EV71-infected RD cells transfected with the LV-shMAP4K4-3 vector exhibited significantly reduced MAP4K4 expression at both the mRNA and protein levels compared with those transfected with the LV-shcontrol vector (Fig. 5d, \( p < 0.001 \); Fig. 5e, \( p < 0.05 \)). Further, the EV71 RNA and VP1 protein levels were significantly enhanced in the LV-shMAP4K4-3-transfected RD cells compared with the LV-shcontrol-transfected cells (Fig. 5d, \( p < 0.001 \); Fig. 5e, \( p < 0.01 \)). The MTT assay results showed no significant difference in cell
viability between normal RD cells transfected with the LV-shMAP4K4-3 vector and those transfected with the LV-shcontrol vector (Fig. 5f). However, the viability of EV71-infected RD cells was significantly decreased in the LV-shMAP4K4-3-transfected group compared with the LV-shcontrol-transfected group (Fig. 5f, p < 0.05). Thus, we concluded that EV71 infection resulted in the downregulation of MAP4K4 expression and that knockdown of MAP4K4 promoted EV71 replication.

To further investigate the regulatory mechanism of EV71 replication by MAP4K4, the expression of proteins involved in the two major signaling pathways downstream of MAP4K4 (the JNK and NF-κB pathways) was assessed by Western blotting. The results showed that
the ratio of the phosphorylated JNK1/2 (p-JNK1/2) protein level to the total JNK1/2 protein level was higher in the LV-shMAP4K4-3-transfected group than in the LV-shcontrol-transfected group (Fig. 6a, \( p < 0.05 \)). The hsa-let-7c-5p mimic similarly increased the p-JNK1/2 protein level (Fig. 6b, \( p < 0.01 \)). In contrast, the p-JNK1/2 protein level was significantly reduced in the hsa-let-7c-5pi group compared with the NCI group (Fig. 6c, \( p < 0.01 \)). However, the phosphorylated NF-κB p65 (p-NF-κB p65) protein level was not significantly differ between RD cells transfected with the LV-shMAP4K4-3 vector and those with the LV-shcontrol vector (Additional file 1: Figure S3). Similar results were observed in the hsa-let-7c-5p mimic and hsa-let-7c-5pi groups compared with their
corresponding control groups (Additional file 1: Figure S3). These data showed that MAP4K4 knockdown and the hsa-let-7c-5p mimic activated the JNK signaling pathway but not the NF-kB pathway, whereas JNK pathway activation was suppressed by the hsa-let-7c-5p inhibitor.

Subversion of the JNK signaling pathway by EV71 promotes viral replication
As demonstrated above, the JNK signaling pathway is modulated by MAP4K4 and hsa-let-7c-5p. To investigate whether this pathway is involved in EV71 replication in
RD cells, we first detected JNK signaling pathway activity during EV71 infection by Western blotting. Total JNK1/2 expression remained unchanged in EV71-infected RD cells at 3, 6, 12, 24 and 36 h compared with that in mock-infected cells (Fig. 7a). However, the phosphorylated JNK1/2 level was increased in the EV71-infected cells at 3, 6, 12, 24 and 36 h compared with the mock-infected cells, with peak expression observed at 12 h (Fig. 7a, b). To determine whether JNK pathway activity is affected during the early stage of infection, cells were infected with UV-inactivated EV71. The results showed that UV-inactivated EV71 infection had no effect on the p-JNK1/2 or total JNK1/2 protein level at 1, 3, 6, 12 or 24 h.p.i. compared with mock infection (Fig. 7c, d). These data suggested that EV71 replication, but not EV71 infection, activated the JNK pathway.

To further confirm the link between EV71 replication and the JNK pathway in RD cells, we utilized a specific inhibitor of JNK, SP600125. No obvious cytopathic effect (CPE) on RD cells was observed at 24 or 36 h.p.i. in the SP600125-treated group compared with the dimethyl sulfoxide (DMSO)-treated group (Fig. 8a). Furthermore, the virus titer was significantly reduced in the SP600125-treated group compared with the DMSO-treated group (Fig. 8b, p < 0.001). Moreover, the EV71 RNA and VP1 protein levels were decreased by the SP600125 treatment compared with the DMSO treatment (Fig. 8c, p < 0.01; Fig. 8d). These data demonstrated that EV71-induced JNK pathway activation facilitated EV71 replication in RD cells.

**Discussion**

Here, we reported that EV71 infection resulted in upregulation of expression of the cellular miRNA hsa-let-7c-5p and that hsa-let-7c-5p overexpression promoted viral replication by targeting MAP4K4 to activate the JNK signaling pathway in RD cells. These results suggested that EV71 might utilize hsa-let-7c-5p to subvert signaling pathways for its own benefit.

It is important to elucidate the interactions between EV71 and host cellular miRNAs. Comprehensive miRNA profiling has been performed using a deep sequencing approach, and certain miRNAs have been proposed to participate in crucial interactions with EV71 [33, 34]. On the one hand, host miRNAs may target the viral genome directly to suppress viral replication. Wen et al. [35] have reported that miRNA-23b expression is downregulated by EV71 infection and that this miRNA inhibits viral replication via suppression of the structural protein VP1. In addition, Zheng et al. [36] have reported that hsa-miR-295-5p expression is upregulated by EV71 infection and that this miRNA suppresses viral replication by targeting the VP1 and VP3 coding regions. On the other hand, host miRNAs may regulate EV71 replication by targeting host factors and immune signaling pathways [37, 38]. Tang et al. [37] have reported that host miR-197 expression is downregulated by EV71 and that this miRNA inhibits viral replication by targeting the host RAN, member RAS oncogene family protein. In addition, Xu et al. [38] have reported that host miR-526a expression is upregulated by EV71 and that this miRNA attenuates viral replication by...
suppressing CYLD expression. Previously, we have found that miR-27a expression is downregulated and that hsa-let-7c-5p expression is upregulated in RD cells during EV71 infection. Further, we have reported that miR-27a overexpression suppresses viral replication through the regulation of EGFR-mediated pathways [16]. It is of interest to investigate the role of hsa-let-7c-5p miRNA upregulation in EV71 infection. In this study, we showed that the upregulation of hsa-let-7c-5p expression by EV71 promoted viral replication by activating the JNK signaling pathway downstream of MAP4K4.

MAP4K4, a member of the human STE20/mitogen-activated protein kinase kinase family of serine/threonine kinases, is involved in multiple cellular processes, such as cell motility, cytoskeletal rearrangement, and cell proliferation, as well as a complex network of signaling pathways. MAP4K4 is mainly associated with different types of cancer and diabetes [39, 40]. Notably, the roles of MAP4K4 in these biological processes and diseases involve the JNK and NF-κB signaling pathways [41–43]. MAP4K4 deficiency has been reported to stabilize TRAF2 and induce TRAF2/IL-6 upregulation, leading to insulin resistance [44]. In addition, MAP4K4 depletion has been shown to prevent primary CD4+ T cell proliferation and activation in vitro and to reduce the expression of IL-2 and IFN-γ [45]. Further, JNK phosphorylation has been demonstrated to be enhanced by MAP4K4 depletion in CD4+ T cells exposed to ozone-oxidized black carbon [46]. However, little is known regarding the function of MAP4K4 in EV71-infected RD cells. In our study, MAP4K4 knockdown also enhanced JNK1/2 phosphorylation. Moreover, EV71 infection resulted in the downregulation of MAP4K4 expression, and the silencing of MAP4K4 led to increased viral replication. These results implied that MAP4K4 might function as a novel antiviral regulator of EV71 replication.

Mammalian JNKs are encoded by three distinct genes, namely jnk1, jnk2 and jnk3. JNK1 and JNK2 are widely expressed in most cell types, and JNK3 expression is localized to the brain and testes [47]. JNK activation is

![Fig. 7 Modulation of JNK pathway activity by EV71 infection. a] The effect of EV71 infection on JNK activity was assessed by Western blotting. RD cells were infected with EV71 (MOI = 5) at the indicated time points, and the data were analyzed by comparison with the mock-infected group. The ratios of the p-JNK1/2 to total JNK1/2 signals are expressed as fold changes compared with the mock-infected group, as quantified using Image J software, as shown in b. c The effect of UV inactivation of EV71 on JNK activity was assessed by Western blotting. RD cells were infected with UV-inactivated EV71 at the indicated time points, and the data were analyzed by comparison with the mock-infected group. The ratios of the p-JNK1/2 to total JNK1/2 signals are expressed as fold changes compared with the mock-infected group, as quantified using Image J software, as shown in d. *p < 0.05; **p < 0.01.
mediated by inflammatory cytokines (such as TNF, IL-1 and TGF-β), Toll-like receptors and ligation of antigen receptors. The JNK pathway has been implicated in multiple diseases, including infectious diseases [48]. The JNK pathway has been shown to be required for the viral replication of herpes simplex virus, hepatitis B virus, hepatitis C virus, rotavirus, HIV-1 and human cytomegalovirus [49–54]. In addition, EV71 infection has been reported to activate the JNK pathway in immature dendritic cells and to promote the production of inflammatory cytokines, such as IL-6, IL-10 and TNF-α, whereas inhibition of this pathway results in the suppression of viral replication and the reduced secretion of cytokines [55]. In our study, EV71 replication activated the JNK pathway in RD cells, and blocking of the JNK pathway led to the inhibition of viral replication.

**Conclusions**

Collectively, the results of the present study have demonstrated that the hsa-let-7c-5p inhibitor and MAP4K4 might act as anti-EV71 modulators. Hsa-let-7c-5p might promote EV71 replication by targeting MAP4K4
expression. Moreover, hsa-let-7c-5p facilitates EV71 replication partly through the MAP4K4/JNK axis. EV71 infection may induce the upregulation of hsa-let-7c-5p, along with the downregulation of MAP4K4 and activation of the JNK pathway. These findings may indicate that EV71 uses an escape mechanism involving hsa-let-7c-5p-mediated regulation of the MAP4K4/JNK pathway to promote its own replication.

Methods

Cell culture, transfection and reagents
RD cells and African green monkey kidney (Vero) cells were both cultured in minimum essential medium (MEM; HyClone, South Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; PAN, Adenbach, Freistaat Bayern, Germany) and 100,000 U/L penicillin plus 100,000 g/L streptomycin at 37 °C and 5% CO₂. HeLa cells were grown in Dulbecco’s minimum essential medium (DMEM; HyClone, South Logan, UT, USA) supplemented with 10% heat-inactivated FBS and antibiotics (100,000 U/L penicillin and 100,000 g/L streptomycin) at 37 °C and 5% CO₂. All cell lines were purchased from the China Center for Type Culture Collection (CCTCC, Wuhan, China).

RD cells were transfected with synthetic oligonucleotides or DNA plasmids using TurboFect transfection reagent (Thermo Scientific, Waltham, MA, USA) according to the manufacturer’s protocol. Synthetic oligonucleotides representing the hsa-let-7c-5p mimic, negative control miRNA mimic, hsa-let-7c-5p inhibitor and negative control miRNA inhibitor were purchased from Ribo Biotech (Guangzhou, China), and they were dissolved in RNase-free H₂O at 20 μM for storage (at −80 °C).

A JNK inhibitor, SP600125, was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in DMSO (Sigma, St. Louis, MO, USA) at 10 mM, and this mixture was added to fresh culture medium (supplemented with 1% heat-inactivated FBS and antibiotics) at a final concentration of 20 μM for 1 h prior to viral infection and kept in the medium throughout the experiment.

EV71 infection

Cells were starved, infected with EV71 (BrCr strain, GenBank accession No. U22521) at a multiplicity of infection (MOI) of 5 and incubated for 1 h at 37 °C to allow for absorption of the virus. Then, the inoculum was removed, and incubation was continued in fresh culture medium supplemented with 1% heat-inactivated FBS and antibiotics at 37 °C and 5% CO₂. Infected cells and culture supernatants were collected together at the time points post-infection indicated in the text and figure legends and clarified by centrifugation (2000g). Finally, virus titers were determined by plaque assays.

To generate UV-inactivated virus, 2-mL aliquots of the virus were dispersed in a 6-cm tissue culture dish and then exposed to UV under a compact UV lamp (UVP, Upland, CA) for 1 h on ice. The remaining titer of the inactivated virus was determined by plaque assay [56].

Plasmid construction

To generate a psi-MAP4K4-3’UTR recombinant plasmid, a 927-nt partial fragment of the MAP4K4 3’UTR (GenBank accession No. NM_145686) was subcloned into a psiCHECK™-2 vector (Promega, Madison, WI, USA) using XhoI and NotI restriction sites. psi-MAP4K4-3’UTRmut was generated by overlapping PCR; this plasmid contained a mutated seed sequence, which was changed from CUACCUC to CAUCGAC. The restriction enzymes used in this experiment were purchased from Takara Bio (Dalian, China), and the LV-shMAP4K4 vector was obtained from Longqian Biotech (Shanghai, China). The primers used for plasmid construction are listed in Additional file 1: Table S1.

RNA extraction and qRT-PCR

Total RNA was extracted from cells with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), and 2 μg isolated RNA was then reverse transcribed using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA). qRT-PCR was performed with SYBR Green Real-Time PCR Master Mix (Toyobo, Osaka, Japan) according to the manufacturer’s instructions. The PCR conditions for measuring mRNA and EV71 RNA levels were as follows: 95 °C for 3 min, followed by 40 cycles at 95 °C for 10 s, 60 °C for 15 s and 72 °C for 20 s. GAPDH was used as an internal control, and the PCR primers used have been previously described [57]. In addition, the PCR conditions for measuring hsa-let-7c-5p expression were as follows, with U6 snRNA used as an internal control: 95 °C for 20 s, followed by 40 cycles at 95 °C for 10 s, 60 °C for 20 s and 70 °C for 10 s. The primers used for reverse transcription and qRT-PCR of U6 snRNA have been previously described [58]. The sequence of the primer used for the reverse transcription of hsa-let-7c-5p was 5’-GTCGTATCCAGTCGAGGTCTCGAGGTTATGCCACTGGATACGACAACCAT-3’. The qRT-PCR primers used are listed in Additional file 1: Table S2. The data were analyzed using the 2−ΔΔCt method.

Plaque assay

Vero cell monolayers were prepared by the addition of 0.5 mL cells to each well of 48-well plates at a concentration of 1 × 10⁵ cells per mL in MEM supplemented with 10% heat-inactivated FBS and antibiotics (Corning, NY, USA). The cells were incubated for 1 d before EV71 infection. Then, the medium was removed, and the cells...
were infected with 200 μL of a serial 10-fold-diluted viral suspension (with three replicates for each dilution). Next, the cells were incubated at 5% CO₂ and 37 °C for 1 h, and the viral solution was aspirated. Subsequently, 500 μL of MEM supplemented with 1% heat-inactivated FBS and antibiotics plus 1% carboxymethyl cellulose was added to the wells. Then, the plates were incubated at 37 °C and 5% CO₂ for 3 d, and the cells were fixed with methanol for 1 h at 4 °C and subsequently stained with crystal violet for 10 min. Plaques were counted manually.

**MTT assay**

RD cells were seeded in 96-well plates (Corning, NY, USA) at 1 × 10⁴ cells per well. Then, the cells were transfected with the hsa-let-7c-5p mimic, miRNA inhibitor, LV-shMAP4K4 plasmid or corresponding negative control according to the manufacturer’s instructions. After 4 h of incubation, the medium was aspirated, and then fresh MEM supplemented with 1% heat-inactivated FBS and antibiotics was added. After 48 h of transfection, the cells were further infected with or without EV71 for 24 h, followed by incubation with 20 μL of 5 mg/mL MTT for 4 h. A volume of 150 μL DMSO was used to solubilize the formazan converted by MTT, and the plates were shaken for 10 min. Absorbance was detected using a microplate reader (BioTek, VT, USA) at an OD of 490 nm.

**Luciferase assay**

RD cells were grown in 24-well plates (Corning, NY, USA) to 70% confluency and then transfected with a synthetic oligonucleotide or DNA plasmid, as indicated in the text and figure legends. At specific time points post-transfection, the cells were harvested and assayed using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer’s instructions.

**Western blotting analysis**

RD cells were lysed on ice with RIPA buffer (Biosharp, China) containing phenylmethanesulfonyl fluoride (PMSF; Biosharp, China), and then the cell lysates were centrifuged at 10,000g for 5 min at 4 °C, and the pellets were discarded. The protein samples were separated in 8–12% polyacrylamide gels and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Hertfordshire, UK). The membranes were blocked with 5% non-fat milk powder or bovine serum albumin (BSA) in Tris-buffered saline containing 0.1% Tween 20 (TBST) at room temperature for 2 h and then incubated overnight at 4 °C with the following primary antibodies (except for a membrane incubated with a mouse polyclonal antibody to enterovirus 71 for 48 h): a mouse polyclonal antibody to enterovirus 71 (1 μg/mL; ab 169442, Abcam, Cambridge, MA, USA), goat polyclonal antibody to human HGK (1 μg/mL; AF6027, R&D, Minneapolis, MN, USA), rabbit monoclonal antibody to phospho-SAPK/JNK (diluted 1:1000; Thr183/Tyr185, #4688, CST, MA, USA), rabbit anti-SAPK/JNK polyclonal antibody (diluted 1:1000; #9252, CST, MA, USA), rabbit polyclonal antibodies to NF-κB and phospho-NF-κB (diluted 1:1000; CST, MA, USA), and GAPDH mouse monoclonal antibody (diluted 1:5000; PMK043C, PMK, Wuhan, China). Blots were washed and incubated for 1–2 h at room temperature with goat anti-rabbit, rabbit anti-mouse and anti-goat horseradish peroxidase-labeled antibodies (diluted 1:25,000; PMK). Protein band signals were detected by enhanced chemiluminescence (ECL; Thermo scientific, Waltham, MA, USA). Band densities were measured by densitometry and quantified using Image J software (version 1.46r, Bethesda, MD, USA).

**Statistical analysis**

All experiments were performed in triplicate. The data are expressed as the mean ± standard derivation (SD) and were analyzed by analysis of variance (ANOVA) or Student’s t test. Significant differences were indicated by a p < 0.05.

**Additional file**

Additional file 1. Additional tables and figures.

**Abbreviations**

- EV71: enterovirus 71
- HFMD: hand, foot and mouse disease
- miRNAs: micro-RNAs
- RD: rhabdomyosarcoma
- MAP4K4: mitogen-activated protein kinase kinase kinase 4
- JNK: c-Jun NH₂-terminal kinase
- CPE: cytopathic effect
- MoI: multiplicity of infection
- MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

**Authors’ contributions**

Conceived and designed the experiments: LWH, HXH and ZBH. Performed the experiments: ZBH and CM. Analyzed the data: ZBH and LWH. Contributed reagents/materials/analysis tools: XSS, CX, LYJ, WZH and ZFF. Wrote the paper: LWH, ZBH, HXH, PBW, HS and YJ. All authors read and approved the final manuscript.

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**Competing interests**

The authors declare that they have no competing interests.

**Availability of data and materials**

Please see the Additional file 1. Additional data.
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References

1. Teoh HL, Mohammad SS, Britton PN, Kandula T, Lorentzos MS, Booy R, Jones CA, Rawlinson W, Ramachandran V, Rodriguez ML, et al. MicroRNA biogenesis, function and decay. Nat Rev Genet. 2010;11:597–610.

2. Escalera-Cueto M, Medina-Martinez I, del Angel RM, Sermeus A, Leonard E, Delaive E, Dieu M, Fransolet M, Arnould O, et al. MiR-129 triggers autophagic flux by regulating a novel Notch-1/2/3/4 autophagy regulatory network. Autophagy. 2014;10:1098–108.

3. Wu J, Feng H, Tao Y, et al. Tissue-specific expression of miRNAs in human liver. J Biomed Biotechnol. 2016;2016:7:10976–89.

4. Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR. Ruvkun G. The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature. 2000;403:978–83.

5. Viswanathan SR, Daley GQ, Gregory RI. Selective blockade of microRNA processing by Lin28. Science. 2008;320:970–1007.

19. Heo J, Joo C, Cho J, Ha M, Han J, Kim VN. Lin28 mediates the terminal uridylation of let-7 precursor MicroRNA. Mol Cell. 2008;32:276–84.

20. Rybak A, Fuchs H, Srinivasa L, Brandt C, Pohl EE, Nitsch R, Wulczyn FG. A feedback loop comprising lin-28 and let-7 controls pre-let-7 maturation during neural stem-cell commitment. Nat Cell Biol. 2008;10:987–93.

21. Namdini N, Tummalra L, Hou Z, Wu Y, Lui KB, Zhou X, Chen J, Zhang J, et al. MicroRNA let-7c is downregulated in prostate cancer and suppresses prostate cancer growth. PLoS ONE. 2012;7:e23823.

22. Zhao B, Han J, Chen J, Zhang Z, Li S, Fang F, Zheng Q, Ma Y, Zhang J, Wu N, et al. MicroRNA let-7c inhibits migration and invasion of human non-small cell lung cancer by targeting ITGB3 and MAP43K. Cancer Lett. 2014;342:43–51.

23. Lin KY, Yu H, Han BW, Wang WT, Pei PP, He B, Li XJ, Chen YQ. Genome-wide screen identified let-7c/miR-99a/miR-125b regulating tumor progression and stem-like properties in cholangiocarcinoma. Oncogene. 2016;35:3756–86.

24. Wright JH, Wang X, Manning G, LaMere BJ, Lei P, Zhu S, Khatry D, Flanagan PM, Bryant BJ, et al. Identification of microRNAs that modulate expression of the human genome by a deep sequencing approach. PLoS Pathog. 2013;9:e1003231.

25. Zheng Z, Ke X, Wang M, He S, Li Q, Zheng C, Zhang Z, Liu Y, Wang H, et al. MicroRNA-23b inhibits human microRNA hsa-miR-23b-5p suppresses enterovirus 71 infection by a deep sequencing approach. J Biomed. 2010;2010:425993.

26. Shao P, Zhou H, Ou LH, Yang JH, et al. miRNA-23a is required for the infection of enterovirus 71 to human cells. J Virol. 2015;89:10031–43.

27. Meier F, Li X, Wang J, Jin Q, Zhao Z. Enterovirus 71 infection by a deep sequencing approach. J Virol. 2015;89:10031–43.

28. Liu Y, Joo C, Cho J, Ha M, Han J, Kim VN. Lin28 mediates the terminal uridylation of let-7 precursor MicroRNA. Mol Cell. 2008;32:276–84.

29. Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR. Ruvkun G. The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature. 2000;403:978–83.

30. Viswanathan SR, Daley GQ, Gregory RI. Selective blockade of microRNA processing by Lin28. Science. 2008;320:970–1007.

31. Yang J, Joo C, Cho J, Han J, Kim VN. Lin28 mediates the terminal uridylation of let-7 precursor MicroRNA. Mol Cell. 2008;32:276–84.

32. Wu J, Feng H, Tao Y, et al. Tissue-specific expression of miRNAs in human liver. J Biomed Biotechnol. 2016;2016:7:10976–89.
41. Haas DA, Bala K, Busche G, Weidner-Glunde M, Santag S, Kati S, Gramolelli S, Damas M, Dittrich-Breiholz O, Kracht M, et al. The inflammatory kinase MAP4K4 promotes reactivation of Kaposi’s sarcoma herpesvirus and enhances the invasiveness of infected endothelial cells. PLoS Pathog. 2013;9:e1003737.

42. Liu AW, Cai J, Zhao XL, Jiang TH, He TF, Fu HQ, Zhu MH, Zhang SH. ShRNA-targeted MAP4K4 inhibits hepatocellular carcinoma growth. Clin Cancer Res. 2011;17:710–20.

43. Wang B, Shen ZL, Gao ZD, Zhao G, Wang CY, Yang Y, Zhang JZ, Yan YC, Shen C, Jiang KW, et al. MiR-194, commonly repressed in colorectal cancer, suppresses tumor growth by regulating the MAP4K4/c-Jun/MDM2 signaling pathway. Cell Cycle. 2015;14:1046–58.

44. Chuang HC, Sheu WH, Lin YT, Tsai CY, Yang CY, Cheng YJ, Huang PY, Li JP, Chiu LL, Wang X, et al. HGK/MAP4K4 deficiency induces TRAF2 stabilization and Th17 differentiation leading to insulin resistance. Nat Commun. 2014;5:4602.

45. Huang H, Tang Q, Chu H, Jiang J, Zhang H, Hao W, Wei X. MAP4K4 deletion inhibits proliferation and activation of CD4(+) T cell and promotes T regulatory cell generation in vitro. Cell Immunol. 2014;289:15–20.

46. Jin M, Chu H, Li Y, Tao X, Cheng Z, Pan Y, Meng Q, Li L, Hou X, Chen Y, et al. MAP4K4 deficiency in CD4(+) T cells aggravates lung damage induced by ozone-oxidized black carbon particles. Environ Toxicol Pharmacol. 2016;46:246–54.

47. Sukhumavasi W, Egan CE, Denkers EY. Mouse neutrophils require JNK2 MAPK for Toxoplasma gondii-induced IL-12p40 and CCL2/MCP-1 release. J Immunol. 2007;179:3570–7.

48. Weston CR, Davis RJ. The JNK signal transduction pathway. Curr Opin Cell Biol. 2007;19:142–9.

49. Song S, Qiu M, Chu Y, Chen D, Wang X, Su A, Wu Z. Downregulation of cellular c-Jun N-terminal protein kinase and NF-kappaB activation by berberine may result in inhibition of herpes simplex virus replication. Antimicrob Agents Chemother. 2014;58:5068–78.

50. Kang LJ, Choi YJ, Lee SG. Stimulation of TRAF6/TAK1 degradation and inhibition of JNK/AP-1 signalling by ginsenoside Rg3 attenuates hepatitis B virus replication. Int J Biochem Cell Biol. 2013;45:2612–21.

51. Rau SJ, Hildt E, Himmelsbach K, Thimme R, Wakita T, Blum HE, Fischer R. CD40 inhibits replication of hepatitis C virus in primary human hepatocytes by c-Jun N terminal kinase activation independent from the interferon pathway. Hepatology. 2013;57:23–36.

52. Holloway GC, Coulson BS. Rotavirus activates JNK and p38 signaling pathways in intestinal cells, leading to AP-1-driven transcriptional responses and enhanced virus replication. J Virol. 2006;80:10624–33.

53. Muthumani K, Wadsworth SA, Dayes NS, Hwang DS, Choo AY, Abeyesinghe HR, Siekerka JJ, Weiner DB. Suppression of HIV-1 viral replication and cellular pathogenesis by a novel p38/JNK kinase inhibitor. AIDS. 2004;18:739–48.

54. Zhang H, Niu X, Qian Z, Qian J, Xuan B. The c-Jun N-terminal kinase inhibitor SP600125 inhibits human cytomegalovirus replication. J Med Virol. 2015;87:2135–44.

55. Peng H, Shi M, Zhang L, Li Y, Sun J, Wang X, Xu X, Zhang X, Mao Y, Ji Y, et al. Activation of JNK1/2 and p38 MAPK signaling pathways promotes enterovirus 71 infection in immature dendritic cells. BMC Microbiol. 2014;14:147.

56. Wong WR, Chen YY, Yang SM, Chen YL, Horng JT. Phosphorylation of PI3K/Akt and MAPK/ERK in an early entry step of enterovirus 71. Life Sci. 2005;78:82–90.

57. Abe M, Ohira M, Kaneda A, Yagi Y, Yamamoto S, Kitano Y, Takato T, Nakagawa A, Ushijima T. CpG island methylator phenotype is a strong determinant of poor prognosis in neuroblastomas. Cancer Res. 2005;65:828–34.

58. Dai X, Zhang W, Zhang H, Sun S, Yu H, Guo Y, Kou Z, Zhao G, Du L, Jiang S, et al. Modulation of HBV replication by microRNA-15b through targeting hepatocyte nuclear factor 1alpha. Nucleic Acids Res. 2014;42:6578–90.