Ectromelia virus upregulates the expression of heat shock protein 70 to promote viral replication

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Abstract. The ectromelia virus (ECTV) is a mouse specific *Orthopoxvirus* that causes lethal infection in some mouse strains. ECTV infection of these mouse strains has been used as a valuable model for understanding the interplay between *Orthopoxvirus* species and their hosts, including variola virus in humans. Although poxviruses encode numerous proteins required for DNA and RNA synthesis, and are less dependent on host functions than other DNA viruses, a detailed understanding of the host factors required for the replication of poxviruses is lacking. Heat shock protein 70 (Hsp70) isoforms have been reported to serve various roles in the replication cycle of numerous viruses. In the present study, microarray and reverse transcription-quantitative polymerase chain reaction analysis were conducted to investigate the host gene expression profiles following ECTV infection in mice and cell cultures. The results indicated that one Hsp70 isoform, Hsp70 member 1B (*Hspa1b*), was highly upregulated during ECTV infection *in vitro* and *in vivo*. Subsequently, overexpression of Hspa1b protein and small interfering RNA-mediated gene silencing of *Hspa1b* revealed that Hspa1b is required for efficient replication of ECTV. Furthermore, the results demonstrated that ECTV replication may be significantly suppressed by two chemical Hspa1b inhibitors: Quercetin and VER155008. In conclusion, the present study clearly demonstrated that ECTV infection upregulates the expression of *Hspa1b* in order to promote its replication. The dependence on Hsp70 may be used as a novel therapeutic target for the treatment of *Orthopoxvirus* infection.

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ECTV, ectromelia virus; FBS, fetal bovine serum; hpi, hours post-infection; Hsc70, heat shock cognate 70; Hsp, heat shock protein; MOI, multiplicity of infection; MPXV, monkeypox virus; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; RNAi, RNA interference; VARV, variola virus; VACV, vaccinia virus; $TCID_{50}$, 50% tissue culture infective dose

Key words: ectromelia virus, Hspa1b, small-molecule inhibitor, viral replication

Introduction

Smallpox is a devastating disease in humans caused by variola virus (VARV), which was eradicated worldwide in the late 1970s (1,2). However, poxvirus infection, particularly *Orthopoxvirus* infection, remains a significant public health concern due to the bioterror threat concerning the release of VARV and the emergence of zoonotic poxvirus infections caused by monkeypox virus (MPXV) (3-6). Since VARV is a strict human pathogen, much of our current understanding of the pathogenesis of smallpox and the host response to infection is the result of studies using vaccinia virus (VACV) infection and other types of *Orthopoxvirus*, such as ectromelia virus (ECTV), MPXV and cowpox virus (6-8). Due to genetic similarity and the common features of the resulting disease with VARV, ECTV has been used as a model for the study of *Orthopoxvirus* infection, including pathogenesis of viral infection, viral immunology, genetic resistance to disease, and antiviral vaccine discovery (9-11). ECTV has a restricted host range, in that it only infects rodents and causes mousepox (10). Viral infection begins through abrasions in the skin, and then disseminates via the afferent lymphatic system. Viraemia occurs when the virus is released into the bloodstream, which permits infection of the spleen, liver and other organs (7).

The complex cell-virus interactions involved in poxvirus morphogenesis remain incompletely understood. Although poxvirus has a greater reliance on viral proteins compared with other double-stranded DNA viruses, host factors still serve a major role during replication. Heat shock protein 70 (Hsp70) family proteins are molecular chaperones that function to

fold nascent proteins, refold misfolded proteins and transport proteins between cellular compartments (12-14). Under stress conditions, including heat, oxidative stress and infection, Hsp70 proteins are activated and expressed at high levels (14-16). During viral infection, Hsp70 proteins are frequently recruited to the viral replication sites and are involved in various steps of the life cycle of numerous DNA and RNA viruses (14-19). For example, viruses can recruit Hsp70 proteins to promote folding of viral proteins involved in the cell-to-cell movement of the virus, and interaction with viral proteins or virions (20-23). In addition, Hsp70 proteins have been reported to be involved in assembly of the replicase complex to enhance viral genome replication (15,24). Conversely, Hsp70 proteins interfere with the polymerase activity of influenza virus and negatively regulate viral RNA replication, thus highlighting the complexity of the virus-chaperone interaction (25,26). Furthermore, Hsp70 proteins have the ability to enhance protective antiviral immunity by inducing the production of interferon-β (27,28).

The present study demonstrated that the expression levels of several isoforms of *Hsp70* were highly elevated during ECTV infection *in vivo* and *in vitro*, as determined by microarray and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Subsequently, the present study aimed to investigate the effects of Hspa1b on ECTV replication, via overexpression and RNA interference (RNAi) of Hsp70 member 1B (*Hspa1b*), and treatment with inhibitors of cytosolic Hspa1b. The results demonstrated that Hspa1b may have an important role in promoting the replication of ECTV.

Materials and methods

Animals, cell culture and viral infection. Specific-pathogen-free male BALB/c mice (age, 9 weeks; weight, 25.74±0.25 g) were purchased from the Experimental Animal Center of Lanzhou University (Lanzhou, China). Upon arrival, mice were housed in a biosafety level 3 room under the following conditions: Controlled temperature, 23±5˚C; humidity 45-55%; 12-h light/dark cycle. The mice were given free access to commercial mouse chow and water. After a 1-week acclimation period, mice were randomly assigned into three groups (n=5 mice/group). All mice were handled in accordance with the Good Animal Practice Requirements of the Animal Ethics Procedures and Guidelines of the People's Republic of China (29). The present study was reviewed and approved by the Animal Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Science (Lanzhou, China; permit no. LVRIAEC2016-005).

The African green monkey kidney cell line Vero, the murine fibroblast cell line NIH3T3, and the BALB/c mouse-derived embryonic fibroblast cell line BALB/3T3 clone A31 were obtained from China Center for Type Culture Collection (Wuhan, China). All cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 IU/ml penicillin and 100 μg/ml streptomycin at 37˚C in the presence of 5% CO_2. The wild-type strain of ECTV was originally isolated from a naturally infected laboratory mouse and propagated in Vero cells (30). The virus was confirmed by PCR using specific primers (forward, 5'-ATGGACGGA ACTCTTTTC-3'; reverse, 5'-AACTTCATCGTTGCGTTT AC-3'). The PCR was performed in a total volume of 25 μl containing 2 μl viral genomic DNA, 2 μl of each forward and reverse primers (10 μM), 4 μl PrimeSTAR Buffer (5X, including Mg^{2+}; Takara Biotechnology Co., Ltd., Dalian, China), 1.5 μl dNTP mixture, 0.2 μl PrimeSTAR HS DNA Polymerase (Takara Biotechnology Co., Ltd.) and 8.3 μl diethylpyrocarbonate water. PCR conditions were as follows: Denaturation at 95˚C for 4 min, 35 cycles at 98˚C for 10 sec, 60˚C for 15 sec and 72˚C for 2 min, with final extension at 72˚C for 10 min. The PCR products were analyzed by 1.0% agarose gel electrophoresis with 10 μg/ml ethidium bromide (Sangon Biotech Co., Ltd., Shanghai, China), and the positive products underwent Sanger sequencing by Genescript Co., Ltd. (Nanjing, China). Experimental infection of susceptible BALB/c mice exhibited less virulence than the ECTV-Moscow strain (30). Plaque-purified ECTV was serially passaged and viral titer was measured using a 50% tissue culture infective dose ($TCID_{50}$) assay.

Viral infection and microarray analysis. To examine the host transcriptome profiles of cells following ECTV infection, $5x10^5$ BALB/3T3 cells were seeded in each well of a 6-well plate and were infected in triplicate with ECTV at a multiplicity of infection (MOI) of 1. Control cells were mock infected with virus-free DMEM. The infected and control cells were incubated in complete DMEM at 37˚C in an atmosphere containing 5% CO_2 and were harvested for total RNA extraction at 6, 12, 24, 48 and 96 h post-infection (hpi). For cell harvesting, cells were washed three times with cold PBS and lysed with 1 ml TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.).

In view of the high transfection efficiency in NIH3T3 cells, NIH3T3 cells were chosen to carry out *in vitro* tests. Briefly, $7x10^5$ NIH3T3 cells were cultured in a 6-well plate and were infected in triplicate with ECTV (MOI, 1) for 2 h. Subsequently, the media were replaced with fresh media containing 2% FBS and cells were harvested for total RNA extraction at 2, 4, 8, 12, 24 and 48 hpi to detect the transcription levels of *Hspa1b* by qPCR.

With regards to the infection of BALB/c mice, 10 mice were individually anesthetized and infected subcutaneously in the abdomen with a single dose of 10^4 $TCID_{50}$/ml ECTV (in 100 μl PBS). Furthermore, 5 uninfected mice served as a control group, which were euthanized by cervical dislocation, after which spleen tissues were isolated. On days 3 and 10 post-infection, 5 mice in 3 and 10 dpi group were euthanized by cervical dislocation and whole spleen tissues were harvested, respectively. All of the spleen tissues from each group were pooled in a cell culture plate and cut into sections (0.2 cm) using surgical scissors. A total of 2.0 g pooled spleen tissues used for RNA extraction with TRIzol were separately stored in four tubes and maintained at -70˚C following freezing in liquid nitrogen.

Spleen tissue samples and BALB/3T3 cells were sent to Beijing CapitalBio Technology Co., Ltd. (Beijing, China) for microarray analysis. Briefly, total RNA was extracted from the samples, and the quality and quantity of RNA were assessed by formaldehyde agarose gel electrophoresis and spectrophotometry. Gene expression analysis was

performed using the Affymetrix GeneChip Mouse Genome Arrays (GeneChip® Mouse Genome 430 2.0; Affymetrix; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Microarray data were analyzed using Bio Molecule Annotation System 3.0 software (Beijing CapitalBio Technology Co., Ltd.). Using the cutoff criterion of a fold-change ≥2 or ≤0.5, differentially expressed genes were screened and clustered. All data were deposited into the Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/gds/) under the accession nos. GSE100644 (30) and GSE102850. The differential expression of 10 isoforms of the HSP70 family contained in the genechip were filtered out for hierarchical clustering using Cluster 3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm), and displayed by using Java TreeView (http://jtreeview.sourceforge.net/).

Plasmids and transfection. The murine *Hspa1b* gene (National Center for Biotechnology Information accession no. NM_010478) was synthesized from C57BL/6 mouse cDNA stored in our laboratory at -80˚C (29), and was cloned into the pCMV-Tag2b vector (Stratagene; Agilent Technologies, Inc., Santa Clara, CA, USA) with a FLAG tag at the N-terminus. Subsequently, the plasmids were transfected into *Escherichia coli* (Takara Biotechnology Co., Ltd.). The nucleotide sequences of the plasmids were confirmed using DNA sequencing, and the plasmid was extracted from the confirmed clone of *E. coli* using Endo-Free Plasmid Maxi kit (Omega Bio-Tek, Inc., Norcross, GA, USA). For transfection of cells with the plasmid, FuGENE HD transfection reagent (Promega Corporation, Madison, WI, USA) was used according to the manufacturer's protocol. A total of 3.0 μg pCMV-HSPa1b or pCMV-Tag2b plasmid mixed with 8.5 μl FuGENE HD reagent was transfected into $7x10^5$ NIH3T3 cells cultured in a 6-well plate; the control cells were incubated with 8.5 μl FuGENE HD reagent only. After 24 h transfection at 37˚C, the cells were collected for western blot analysis or were infected with ECTV (MOI, 1 or 5) for 24 h. Furthermore, NIH3T3 cells were transfected with various amounts (1.0, 2.0 and 3.0 μg) of pCMV-Hspa1b plasmid or empty vector followed by ECTV infection (MOI, 1) for 24 h to explore the effects of Hspa1b on ECTV replication.

RNAi assay. Gene silencing of Hspa1b by small interfering (si)RNA was conducted in NIH3T3 cells. siRNA sequences (Shanghai GenePharma Co., Ltd., Shanghai, China) targeting Hspa1b were as follows: S1, 5'-GAUUACUGUCAAGGUUAUUTT-3'; S2, 5'-AUCUGCUUGUCCAUGUUAATT-3'; and negative control (NC) siRNA, 5'-UUCUUCGAACGUGUCACGUTT-3'. A total of $7x10^4$ cells were seeded into 12-well plates and were transfected with a final concentration of 100 nM Hspa1b siRNA or NC siRNA using the FuGENE HD transfection reagent. Subsequently, the cells were incubated at 37˚C for 36 h in an atmosphere containing 5% CO_2 prior to determination of knockdown efficiency by qPCR or infection with ECTV (MOI, 1 or 5) for 24 h.

RT-qPCR assays. Total RNA was extracted from cellular or tissue samples using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and first-strand cDNA was synthesized using PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd.) according to the manufacturer's protocol. The mRNA expression levels of *Hspa1b* were detected using specific primers (sense, 5'-TGGTGCAGTCCGACATGAAG-3' and antisense, 5'-AGGTCGGTGTGAACGGATTTG-3'). *GAPDH* (sense, 5'-TCTTCCAGCCCTCCTTCCT-3' and antisense, 5'-TGTAGACCATGTAGTTGAGGTCA-3') was used as the internal amplification control. qPCR was performed in a final volume of 20 μl containing 10 μl 2X SYBR-Green qPCR Master Mix (Takara Biotechnology Co., Ltd.), 0.8 μl each primer (10 μM), 7.2 μl nuclease-free water and 2 μl cDNA. PCR amplification was performed as described previously (30). Relative gene expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (31).

Virus titration. To determine viral titers at various time-points post-infection, cells were lysed by three cycles of freeze-thawing and the supernatant was clarified by centrifugation at 1,000 x g for 10 min. Viral titers were determined in Vero cells according to the $TCID_{50}$ assay (32). Briefly, $2x10^4$ Vero cells in 100 μl DMEM supplemented with 10% FBS were seeded in each well of a 96-well plate and incubated at 37˚C in an atmosphere containing 5% CO_2. After 24 h of incubation, 25 μl serial 10-fold dilutions of viral samples were added to each well, with eight replicates per dilution. The plates were incubated for 7 days at 37˚C and checked daily for characteristic cytopathic effects. $TCID_{50}$ end point titers were calculated according to the Reed and Muench method (32).

Viral genome DNA copy number measurements. Genomic DNA was extracted from the NIH3T3 cell culture supernatants and whole-cell lysates using a viral RNA/DNA extraction kit (Takara Biotechnology Co., Ltd.) according to the manufacturer's protocol. ECTV genomic DNA copy numbers were determined by qPCR using ECTV P4b gene specific primers: Forward, 5'-GTAGAACGACGCCAGAATAAGATA-3' and reverse, 5'-AGAAGATATCAGACGATCCACAATC-3'. PCR amplification was performed as described previously (33). A standard curve was established from a cloned DNA fragment of the ECTV P4b gene (33). Quantification cycle values obtained by qPCR were plotted on the standard curve to calculate the viral DNA copy number.

Western blot analysis. Protein expression levels of Hspa1b were determined through the anti-FLAG tag by western blot analysis of total cell proteins. Cells were lysed using radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Beijing, China) and the protein concentration of cell lysates was determined using the bicinchoninic acid (BCA) assay (QuantiPRO BCA Assay kit; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) according to the manufacturer's protocol. Equal amounts of total protein (20 μg) were resolved by electrophoresis on 10% bis-Tris polyacrylamide gels and transferred to polyvinylidene fluoride membranes. Membranes were blocked for 1 h in 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween-20 (TBST) and were then probed with the following primary antibodies: Anti-FLAG (F3165; 1:10,000; Sigma-Aldrich; Merck KGaA) and anti-β-actin (60008-1; 1:2,000; ProteinTech Group, Inc., Chicago, IL, USA). Membranes were incubated with the primary antibodies diluted in 5% (wt/vol) bovine serum albumin (Sangon Biotech Co., Ltd.) and 1X TSBT at 4˚C overnight. Antibody

signals were detected by enhanced chemiluminescence detection kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) following incubation with secondary antibodies conjugated to horseradish peroxidase (A9044; 1:8,000; Sigma-Aldrich; Merck KGaA) at room temperature for 30 min.

Quercetin and VER155008 treatment. Quercetin (Sigma-Aldrich; Merck KGaA) and VER155008 (VER; Sigma-Aldrich; Merck KGaA) were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA) to produce a 10 mM stock solution, which was further diluted in DMEM supplemented with 2% FBS to the appropriate working concentrations. NIH3T3 or Vero cells were seeded in 12-well plates and incubated at 37˚C until the cells reached 60-80% confluence. Subsequently, cells were infected with ECTV at an MOI of 1 for 2 h, after which the media were replaced with fresh media containing the appropriate concentrations (10, 50 and 100 μM in NIH3T3 cells; 10, 20 and 50 μM in Vero cells) of quercetin and VER. After 24 h incubation, cell culture supernatants and whole-cell lysates were harvested for virus titration. To investigate the effects of the two Hspa1b inhibitors on viral growth kinetics, NIH3T3 cells were infected with ECTV at an MOI of 1 for 2 h, after which the media were replaced with fresh media containing 50 μM quercetin, VER, DMSO or DMEM media (Mock). Subsequently, cell culture supernatants and whole-cell lysates were harvested at the indicated time points.

Cell viability assay. Cell viability was determined using the MTT Cell Proliferation and Cytotoxicity Assay kit (Beyotime Institute of Biotechnology). Briefly, NIH3T3 cells were incubated in a 96-well plate and the medium was replaced with fresh medium containing various concentrations of quercetin, VER or DMSO. After 24 h, cell culture supernatants were replaced with 50 μl MTT (2 μg/ml) and incubated for 4 h at 37˚C. Subsequently, 100 μl formazan-dissolving solution (Beyotime Institute of Biotechnology) was added to each well and incubated at 37˚C until the crystals dissolved. Finally, the absorbance of the cell solution was measured using a universal microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 570 nm wavelength.

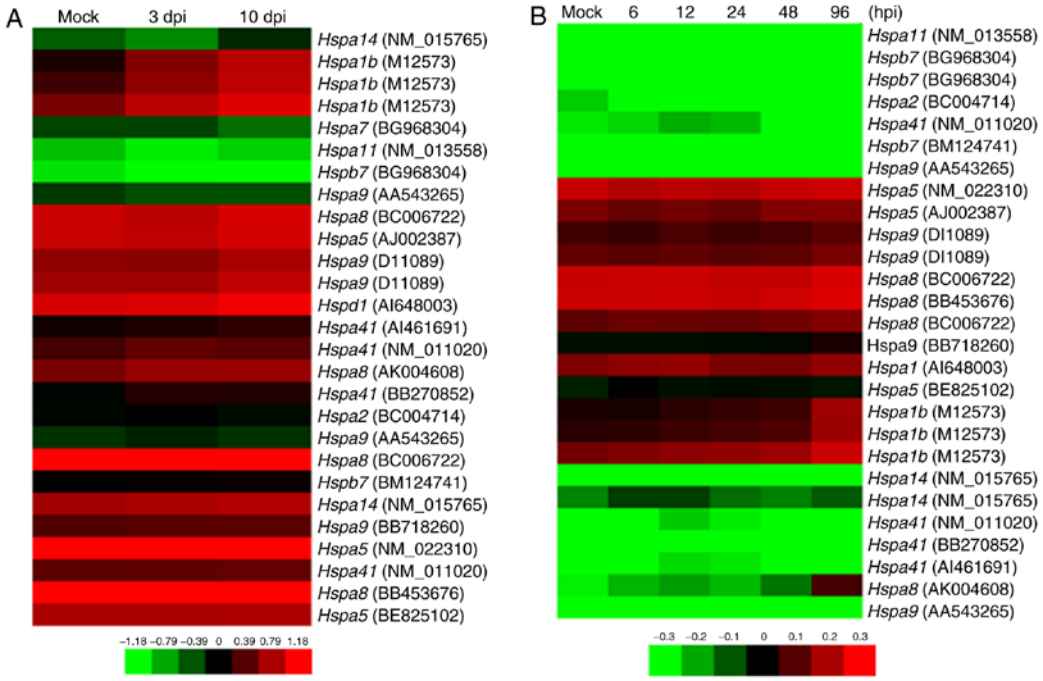
Statistical analysis. Data are presented as the means ± standard deviation from at least three independent experiments and were analyzed using Student's t-test, or one-way analysis of variance followed by Duncan's multiple range test. Statistical analyses were conducted using SPSS software (SPSS 18.0 for Windows; SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Elevated Hsp70 expression during ECTV infection in vitro and in vivo. ECTV has a restricted host range and infection of BALB/c mice with ECTV is associated with a high mortality rate. The natural route of infection is believed to be through abrasions in the skin, after which the virus uncontrollably replicates in the spleen and the liver (10). In the present study, infected mice began to exhibit disease symptoms starting at 7 days post-infection (dpi) and one mouse succumbed to the disease at 10 dpi (data not shown). To elucidate alterations in the host transcriptome profile caused by ECTV infection, microarray analysis was used to examine the spleen cells from ECTV-infected BALB/c mice and BALB/3T3 cells. Following normalization, the results demonstrated that *Hspa1b* was one of the most upregulated genes in spleen tissues of infected mice and BALB/3T3 cells (30). To examine whether other isoforms of the Hsp70 family were upregulated during ECTV infection, the differential expression of 10 isoforms of the HSP70 family contained in the genechip were analyzed. Compared with in the mock control groups, the heat maps demonstrated that the expression levels of numerous Hsp70 isoforms were induced *in vivo* (Fig. 1A) and *in vitro* (Fig. 1B), including *Hspa1b*, *Hspa5*, *Hspa8* and *Hspa9*. To examine the level of induction, the ratio of Hsp70 isoform mRNA expression levels in the infected samples compared with the mock control samples was determined; the results indicated that *Hspa1b* was the most highly induced isoform *in vivo* and *in vitro* (Fig. 1C). To validate the microarray data, the transcription levels of *Hspa1b* were determined in the spleen tissues by RT-qPCR analysis. As shown in Fig. 1D, the results of RT-qPCR were consistent with the microarray data. Furthermore, the transcription levels of *Hspa1b* were detected in NIH3T3 cells at various time-points. As demonstrated in Fig. 1E, the expression levels of *Hspa1b* were upregulated post-infection; the highest expression was detected at 48 hpi.

Overexpression of Hspa1b enhances ECTV replication. To investigate the effects of Hspa1b on ECTV replication, Hspa1b was overexpressed in NIH3T3 cells using the pCMV-Hspa1b plasmid; the empty pCMV-Tag2b vector was used as a negative control. A total of 24 h post-transfection, cells were infected with ECTV (MOI, 1 or 5), and the protein expression levels of FLAG (Fig. 2A) and viral titers were determined. As shown in Fig. 2B, the viral yields from Hspa1b-transfected cells were significantly higher compared with the control cells. Cells overexpressing Hspa1b that were infected with ECTV at an MOI of 5 also exhibited a significantly higher viral yield (Fig. 2C). To further confirm the positive effects of Hspa1b on ECTV replication, NIH3T3 cells were transfected with various amounts of pCMV-Hspa1b plasmid, followed by ECTV infection (MOI, 1). As shown in Fig. 2D (lower panel), the protein expression levels of FLAG were increased with the amount of transfected pCMV-Hspa1b plasmid. The results demonstrated that Hspa1b enhanced ECTV replication in a dose-dependent manner (Fig. 2D, upper panel).

RNAi-mediated depletion of Hspa1b reduces ECTV viral yields. To further investigate the effects of Hspa1b on ECTV replication, *Hspa1b* expression in NIH3T3 cells was suppressed following transfection with siRNAs for 36 h; the effects of Hspa1b knockdown on viral yield were subsequently determined. NIH3T3 cells were transfected with siRNAs, which either targeted the Hspa1b sequence (S1 and S2) or a nonspecific sequence (NC), and knockdown efficiency was confirmed by RT-qPCR analysis. siRNAs that targeted Hspa1b significantly reduced Hspa1b mRNA expression compared with in cells transfected with NC siRNA; knockdown with S1 was more pronounced compared with in cells transfected with S2 (57% for S1 and 36% for S2; Fig. 3A). Silencing Hspa1b



C

	Infection to mock ratio (spleen)		Infection to mock ratio (BALB/3T3)	
Probe set ID	3 d	10 d	48 h	96 h
1427126_at	4.39	12.66	1.46	4.93
1427127_x_at	3.47	8.83	1.51	4.04
1452318_a_at	3.41	7.79	1.75	3.58

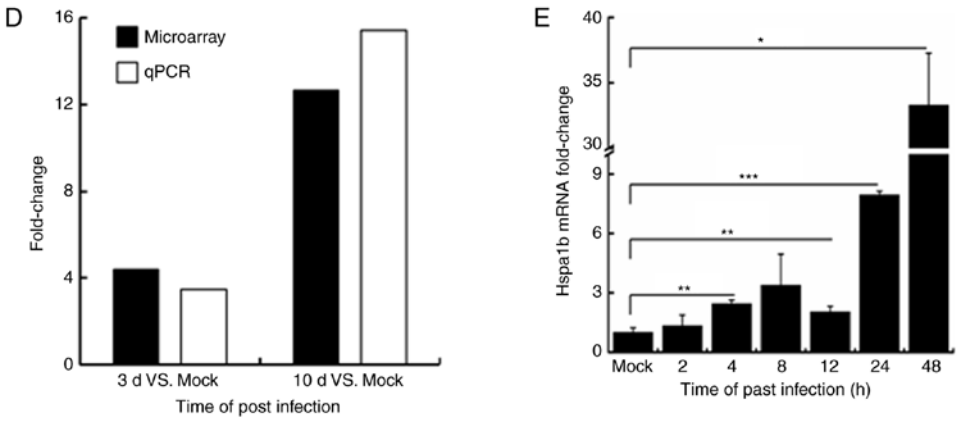


Figure 1. ECTV infection induces upregulation of *Hsp70* expression *in vitro* and *in vivo*. (A) Heat map corresponding to Hsp70 mRNA homologs differentially expressed in mock-infected spleen tissues compared with in ECTV-infected tissues at 3 and 10 dpi. The read values for individual transcripts at a given probe were transformed by $\log_2$ and constructed into a heat map using Cluster 3.0 software. Red represents upregulated genes, green represents downregulated genes. (B) Heat map corresponding to *Hsp70* mRNA homologs differentially expressed in mock-infected BALB/3T3 cells compared with in ECTV-infected cells at 6, 12, 24, 48 and 96 hpi. (C) Fold-changes in three *Hspa1b* probes in spleen tissues and BALB/3T3 cells are displayed in the tabulation. (D) Relative fold-change of *Hspa1b* gene expression as determined by RT-qPCR and microarray. The RT-qPCR values were normalized to GAPDH mRNA. (E) Relative fold-change of *Hspa1b* mRNA expression in ECTV-infected NIH3T3 cells. Cells were infected with ECTV at a multiplicity of infection of 1 and harvested at the indicated time-points. Samples were then subjected to RT-qPCR in triplicate. Expression levels were normalized to those of GAPDH. *P<0.05; **P<0.01; ***P<0.01. dpi, days post-infection; ECTV, ectromelia virus; hpi, hours post-infection; Hsp, heat shock protein; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

with two siRNAs significantly impaired ECTV replication at 24 hpi. A marked decrease in viral yield was detected in Hspa1b siRNA-transfected cells following infection with two doses of ECTV compared with in the control group (Fig. 3B and C).

Inhibitors of Hspa1b suppress ECTV replication. To further examine the importance of Hspa1b in ECTV replication, the effects of two Hspa1b inhibitors, quercetin and VER, were determined on ECTV replication. Quercetin is one of the most

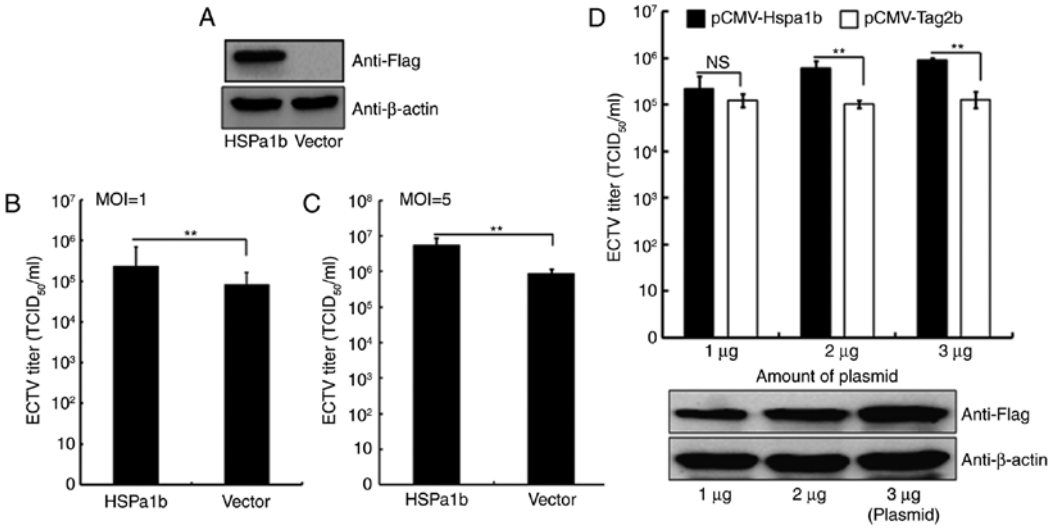


Figure 2. Overexpression of Hspa1b enhances ECTV replication. (A) Western blot analysis of FLAG-labeled Hspa1b protein and β-actin in NIH3T3 cells. Cells were transfected with 3.0 μg pCMV-Hspa1b or empty vector plasmid for 24 h and were then harvested for western blot analysis using FLAG or β-actin antibodies. (B and C) Viral titers in the supernatants and cells were detected and presented as $TCID_{50}$/ml. Cells were transfected with pCMV-Hspa1b or empty vector for 24 h following ECTV infection at an MOI of (B) 1 and (C) 5. After 2 h incubation, the supernatants were replaced with fresh media supplemented with 2% fetal bovine serum and collected at 24 h post-infection. (D) NIH3T3 cells transfected with various amounts of pCMV-Hspa1b plasmid were infected with ECTV (MOI, 1); after 48 h, viral titer was determined. The blots exhibit the protein expression levels of FLAG-labeled Hspa1b. **P<0.01. ECTV, ectromelia virus; Hspa1b, heat shock protein member 1B; MOI, multiplicity of infection; n.s., not significant; $TCID_{50}$, 50% tissue culture infective dose.

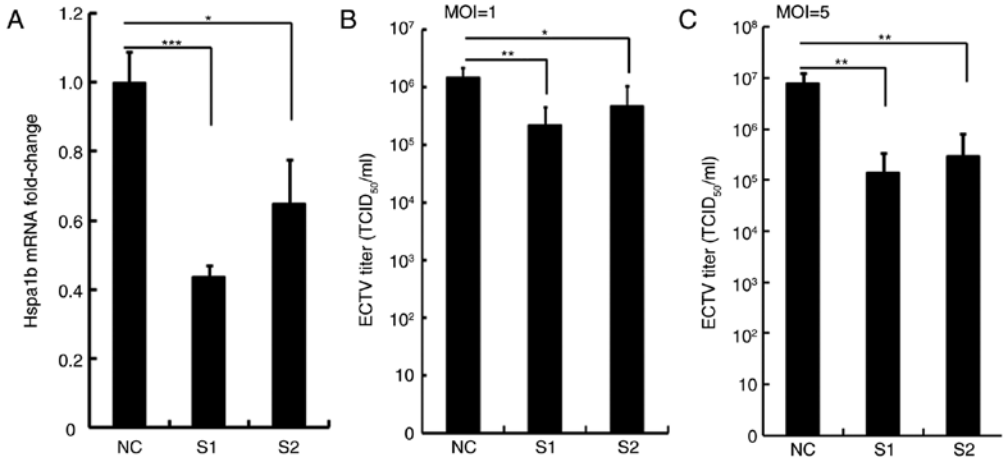


Figure 3. RNA interference-mediated depletion of Hspa1b reduces ECTV viral yield. (A) Cells were transfected with 100 nM S1, S2 or NC for 36 h and were collected for reverse transcription-quantitative polymerase chain reaction to detect the knockdown efficiency. (B and C) NIH3T3 cells pretreated with 100 nM S1, S2 or NC for 36 h were infected with ECTV at an MOI of (B) 1 and (C) 5 for 24 h. Viral titers in the supernatants and cells are presented as $TCID_{50}$/ml. *P<0.05; **P<0.01; ***P<0.01. ECTV, ectromelia virus; Hspa1b, heat shock protein member 1B; MOI, multiplicity of infection; NC, negative control; S1/S2, Hspa1b small interfering RNA 1/2; $TCID_{50}$, 50% tissue culture infective dose.

ubiquitous flavonoids, and as a Hsp inhibitor, its function is not limited to Hsp70 (34,35). VER is a small-molecule inhibitor of Hsp70, which can specifically bind to the ATPase domain of Hsp70 consequently inhibiting its function (36). In order to assess the effects of quercetin and VER on ECTV replication, the present study examined their cytotoxic effects on NIH3T3 and Vero cells by MTT assay. NIH3T3 or Vero cells were incubated with various concentrations of quercetin and VER. As shown in Fig. 4A, the viability of NIH3T3 cells was not significantly affected by treatment with ≤100 μM quercetin and VER. In addition, no cytotoxicity was detected following treatment of Vero cells with ≤50 μM quercetin and VER, whereas higher concentrations of quercetin and VER exhibited cytotoxic effects in Vero cells (Fig. 4B). Therefore, the present study determined the ability of VER and quercetin at 10, 20 and 50 μM to interfere with viral replication in Vero, and VER and quercetin at 10, 50 and 100 μM in NIH3T3 cells. Treatment of ECTV-infected NIH3T3 and Vero cells with various doses of quercetin or VER for 22 h resulted in a reduction of viral replication in a dose-dependent manner (Fig. 4C and D). In addition, treatment with 100 μM quercetin in NIH3T3 cells led to a significant reduction (~70-fold) in viral replication compared with in the DMSO-treated control group. Under the same conditions, VER (100 μM) only inhibited viral replication by ~15-fold. A similar inhibition was observed in Vero cells (Fig. 4D).

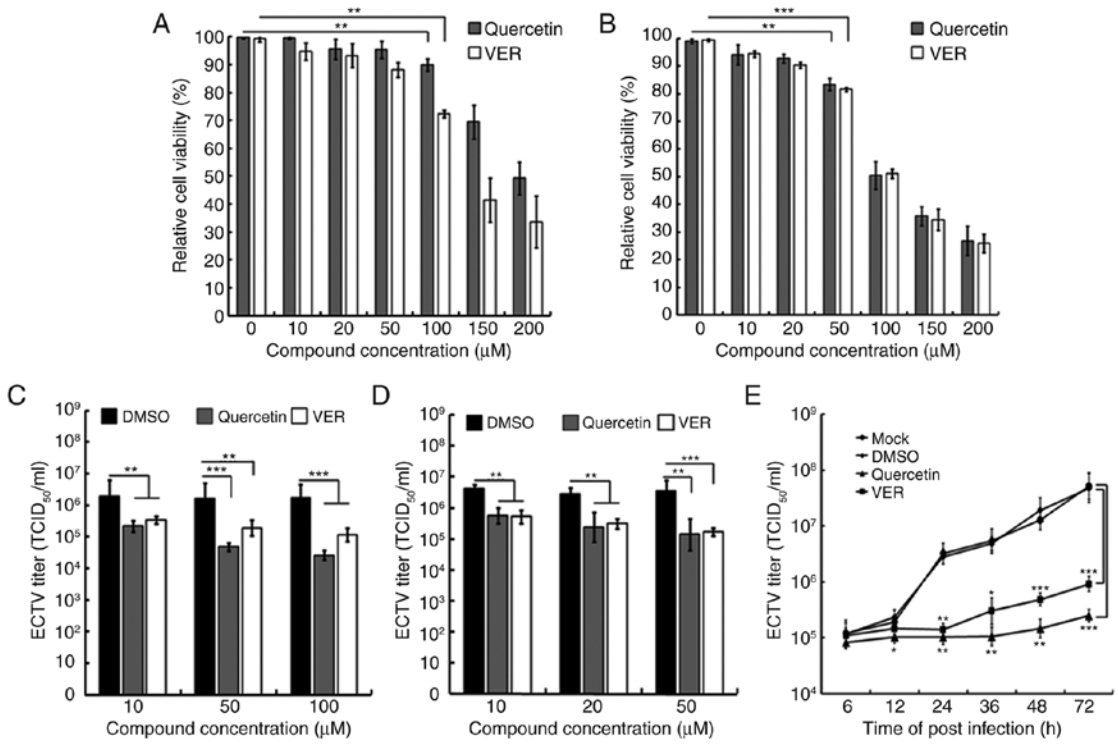


Figure 4. Effects of Hsp70 inhibitors VER and quercetin on ECTV replication. Cytotoxicity of quercetin and VER in (A) NIH3T3 or (B) Vero cells, as assessed by MTT assay following treatment with various concentrations of quercetin and VER for 24 h in the absence of ECTV. Effects of quercetin and VER treatment on ECTV replication in (C) NIH3T3 or (D) Vero cells were determined by $TCID_{50}$, which revealed a reduction in ECTV titers following treatment with subcytotoxic concentrations of quercetin and VER. (E) NIH3T3 cells incubated with ECTV (multiplicity of infection, 1) for 2 h were treated with 50 μM quercetin, 50 μM VER, DMSO control or mock treatment. Samples were collected at several time-points (6, 12, 24, 36, 48 and 72 hpi) for virus titration. *P<0.05; **P<0.01; ***P<0.01. DMSO, dimethyl sulfoxide; ECTV, ectromelia virus; $TCID_{50}$, 50% tissue culture infective dose; VER, VER155008.

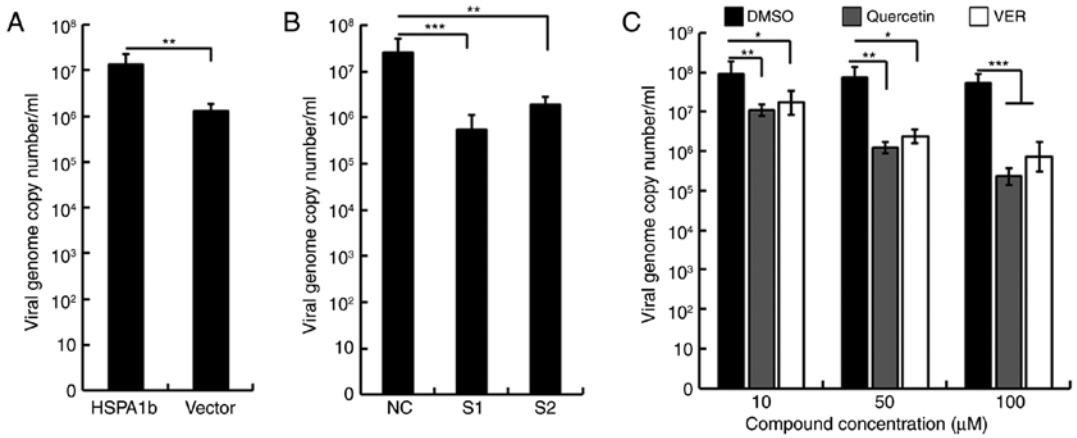


Figure 5. Cellular Hspa1b promotes viral DNA replication. (A) NIH3T3 cells were transfected with pCMV-Hspa1b or empty vector for 24 h followed by ECTV infection at an MOI of 1 for a further 24 h. (B) NIH3T3 cells were pretreated with 100 nM S1, S2 or NC for 36 h and were infected with ECTV for 24 h at an MOI of 1. (C) NIH3T3 cells incubated with ECTV (multiplicity of infection, 1) for 2 h were treated with 50 μM quercetin, 50 μM VER or DMSO (control). The supernatants and whole cells were harvested at 24 hpi for the determination of viral genome DNA copy numbers by qPCR. *P<0.05; **P<0.01; ***P<0.01. DMSO, dimethyl sulfoxide; ECTV, ectromelia virus; Hspa1b, heat shock protein member 1B; MOI, multiplicity of infection; NC, negative control; S1/S2, Hspa1b small interfering RNA 1/2.

To further assess the effects of the two Hspa1b inhibitors on viral growth kinetics, NIH3T3 cells were infected with ECTV at an MOI of 1 for 2 h, after which the media were replaced with fresh media containing 50 μM quercetin or VER. Infected cells and supernatants were harvested for virus titration at 6, 12, 24, 36, 48 and 72 hpi. Compared with in the DMSO-treated cells, the viral titer of quercetin- or VER-treated cells began to reduce at 12 hpi; quercetin and VER treatment reduced viral titers by ~2.3- and 1.7-fold, respectively. At 72 hpi, the effects were enlarged to ~200-fold in quercetin-treated cells and ~52-fold in VER-treated cells (Fig. 4E). These results further confirmed the importance of Hspa1b in ECTV replication and indicated that quercetin is a much more potent inhibitor than VER in NIH3T3 cells.

Cellular Hspa1b promotes viral DNA replication. It has previously been reported that Hsp70 is required in the genome replication of numerous viruses (18,37-39). To investigate whether Hspa1b affects the genome replication of ECTV, the present study examined the viral genome copy number of ECTV following overexpression or knockdown of Hspa1b. The viral genome copy number was significantly increased when Hspa1b was overexpressed in NIH3T3 cells (Fig. 5A). Cells transfected with two Hspa1b siRNAs exhibited a marked decrease in viral genome copy number compared with in the NC control group (Fig. 5B). Consistent with the viral yields presented in Fig. 3B, the viral genome was more impaired in S1-transfected cells compared with in cells transfected with S2. Furthermore, the present study determined the effects of quercetin and VER on viral genome replication. A significant decrease in viral genome copy number was observed in a dose-dependent manner, with ~30 and 25% reductions in viral genome following treatment with 100 μM quercetin and VER, respectively (Fig. 5C). Taken together, these results suggested that cellular Hspa1b may promote ECTV DNA replication. However, due to underdeveloped technological skills and unavailable ECTV antibodies, the exact mechanisms underlying how Hspa1b affects ECTV replication require further study.

Discussion

ECTV infection in mice is a valuable model to investigate the interplay between *Orthopoxvirus* species and their hosts, such as the host immune response to poxvirus infection, the host genetic control of antiviral resistance and the development of small-molecule antivirals (40,41). The present study used microarrays to examine the host gene expression profile in response to ECTV infection *in vivo* and *in vitro*. The results of the genetic analysis demonstrated that *Hspa1b* was one of the most significantly upregulated genes at various time-points. To validate the microarray data, RT-qPCR was performed to quantify the mRNA expression levels of *Hspa1b* using the same samples as those used for microarray analysis. In *in vivo* and *in vitro* samples, RT-qPCR analysis confirmed the results of the microarray analysis, in that Hspa1b was significantly upregulated during ECTV replication. Therefore, these observations suggested that Hspa1b may have an important role in ECTV replication. Previous studies regarding the transcriptome of host cells during VACV infection also indicated that *Hspa1b* was upregulated, and data from RNAi screening suggested that Hspa1b may have a necessary role in *Orthopoxvirus* infection, although the candidate genes were not further validated (42-44).

The Hsps form an evolutionarily well-conserved family of regulatory proteins, which are classified according to their molecular weight; e.g. Hsp40, Hsp60, Hsp70, Hsp90, Hsp100, Hsp110 and small Hsps (<34 kDa) (12). The Hsp70 family contains four major isoforms: The constitutively expressed heat shock cognate 70 (Hsc70), the stress-inducible Hsp70, the endoplasmic reticulum resident glucose-regulated protein (Grp)78 and the mitochondrial form Grp75 (45). These chaperone proteins are involved not only in cellular protein quality control, but also in response to stress conditions. Growing evidence has indicated that members of the Hsp70 family functionally interact with viral proteins to regulate viral replication (15,21-23,36). The majority of previous studies have focused on Hsp70/Hsc70, which positively regulates viral replication through the transcription and replication of viral RNA/DNA. Viruses use Hsp70 isoforms to aid in several stages of viral replication, including viral entry, transcription, envelope protein maturation, morphogenesis or DNA replication (46). Furthermore, the antiviral ability of Hsp70, via the innate immune system, has also been detected in a mouse model of measles virus brain infection and a model of vesicular stomatitis virus infection (28,47). To investigate the functional effects of Hspa1b on ECTV replication, the present study employed plasmid-mediated overexpression and siRNA-mediated gene silencing of Hspa1b in NIH3T3 cells. The results confirmed that Hspa1b expression may benefit ECTV replication in a dose-dependent manner. Viral genome copy number was altered by overexpression or suppression of Hspa1b in NIH3T3 cells, thus suggesting a role for Hspa1b in viral DNA replication. However, the role of this chaperone in other aspects of the viral life cycle and the exact mechanisms by which HSPa1b affects replication of ECTV require further exploration. Data from VACV infection models have provided evidence to suggest that Hspa1b is upregulated and heat shock factor 1, which is a regulator of Hsp70, is critical for *Orthopoxvirus* replication (44). Furthermore, a previous report established that Hsp90 was required for VACV to grow in cultured cells (48). Hsp90 interacts with VACV core protein 4a (A10L) and colocalizes with the viral factory during specific stages of the viral lifecycle (48). Hsp70 family proteins favor viral replication through interaction with viral proteins at several stages of viral replication. For example, Hsp72/Hsp70 interacts with NS5 proteins of members of the *Flaviviridae* family, such as classical swine fever virus, Japanese encephalitis virus and dengue virus (22-23,49). The N protein of Crimean-Congo hemorrhagic fever virus and PB2/PB1 protein of influenza A viruses can also interact with Hsp70 to enhance viral replication, respectively (15,26,36). It remains to be identified which ECTV proteins may interact with Hsp70 or other chaperones to facilitate viral replication.

To further confirm the effects of Hspa1b on ECTV replication, the present study analyzed the effects of two Hsps/Hsp70 inhibitors, quercetin and VER, on ECTV replication. Treatment with these inhibitors suppressed viral replication in a dose-dependent manner, and the effects of quercetin at the same concentration were more noticeable. One of the possible explanations is that besides Hsp70, quercetin may also inhibit the function of other Hsp members, which may be involved in ECTV infection, whereas VER only targets the Hsp70 family.

In conclusion, the present study identified a host cell protein, Hspa1b, which may promote ECTV replication. Notably, the Hsp chaperone involved in ECTV replication is a potential target for the development of antiviral therapies, since two Hsp70 inhibitors, quercetin and VER, were able to significantly suppress ECTV replication in cell culture.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

WC, ZJ, JC and XW designed the study and critically revised the manuscript. WC, HJ, XH and QJ performed the experiments, analyzed the data and drafted the manuscript.

Ethics approval and consent to participate

All mice were handled in accordance with the Good Animal Practice Requirements of the Animal Ethics Procedures and Guidelines of the People's Republic of China. The present study was reviewed and approved by the Animal Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Science (Lanzhou, China; permit no. LVRIAEC2016-005).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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