

Original Research

LncRNA-SUSAJ1 Activates the ER Stress Pathway Inhibiting JEV Proliferation by Promoting PK15 Cells Apoptosis

Qiongyu Yuan^{1,†}, Jinyun Fan^{1,†}, Han Wang¹, Xiangchen Li¹, Songbai Yang¹, Ayong Zhao^{1,*}, Xiaolong Zhou^{1,*}

¹Key Laboratory of Applied Technology on Green Eco-Healthy Animal Husbandry of Zhejiang Province, Zhejiang Provincial Engineering Laboratory for Animal Health Inspection & Internet Technology, College of Animal Science and Technology & College of Veterinary Medicine of Zhejiang A&F University, 311300 Hangzhou, Zhejiang, China

*Correspondence: zay503@zafu.edu.cn (Ayong Zhao); zhouxiaolong@zafu.edu.cn (Xiaolong Zhou)

[†]These authors contributed equally.

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Abstract

Background: Epidemic encephalitis B is a common zoonosis that threatens both pigs and humans. Effective prevention and control of epidemic encephalitis B is difficult. The cellular defence mechanism is closely related to the body's resistance to viral invasion. Long non-coding RNAs (lncRNAs) are involved in regulating various cellular activities. We previously found that lncRNA-SUSAJ1 could inhibit the proliferation of Japanese encephalitis virus (JEV). However, the mechanism underlying this suppression remains unclear. **Methods:** We performed Western blotting and quantitative reverse-transcription polymerase chain reaction (RT-qPCR) analyses, as well as mitochondrial membrane potential, flow cytometry, terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL), RNA pull-down, and RNA immunoprecipitation assays. **Results:** JC-1 cationic dye staining showed that lncRNA-SUSAJ1 promoted the depolarisation of mitochondrial membrane potential; H2DCFDA probe staining showed that lncRNA-SUSAJ1 enhanced the level of reactive oxygen species in PK15 porcine kidney cells. qRT-PCR and Western blotting revealed the expression levels of associated mRNAs and proteins, and the TUNEL and flow cytometry assays detected cell apoptosis. Their results showed that lncRNA-SUSAJ1 promoted the expression of pro-apoptotic genes and inhibited the expression of anti-apoptotic genes. RNA pull-down experiments using biotin-labelled lncRNA-SUSAJ1 showed colocalisation between lncRNA-SUSAJ1 and the 70 kDa heat shock protein (Hsp70). lncRNA-SUSAJ1 also activated unfolded protein response-related pathways, regulated protein degradation, and promoted apoptosis via the endoplasmic reticulum stress response, thereby inhibiting viral replication. **Conclusions:** The findings of this study provide insight into the specific molecular mechanism of lncRNA-SUSAJ1 resistance to viral proliferation by promoting cell apoptosis, clarify the antiviral effect of lncRNA-SUSAJ1 on JEV.

Keywords: lncRNA-SUSAJ1; JEV; endoplasmic reticulum stress response; apoptosis

1. Introduction

Japanese encephalitis is usually caused by Japanese encephalitis virus (JEV), a common zoonosis. It poses a major threat to pigs and humans [1]. JEV belongs to the Flaviviridae family. It is a single-stranded RNA virus with neurotoxic and neuroinvasive characteristics. Functional proteins encoded during JEV replication [2,3] include the NS3 protein, which has three distinct enzyme activities that contribute to viral replication and assembly [4]. Infection with JEV results in acute central nervous system diseases in humans. JEV infection outcomes in pigs do not vary by breed or sex [5]. JEV infection occurs frequently in pigs in summer, and leads to nerve injury and reproductive disorders that cause substantial economic losses for the pig industry [6].

In the human genome, approximately 93% of DNA can be transcribed into RNA; 98% of RNAs are long non-coding RNAs (lncRNAs). lncRNAs are RNA molecules with lengths of 200–100,000 nt and no ability to translate

protein [7]. Additionally, lncRNAs regulate various cellular activities [8,9]. Recently, lncRNAs have been shown to play important roles in the host response and natural immunity caused by viral infection [10,11]; these findings have attracted considerable attention in the context of disease resistance breeding [12,13]. However, the relationship between lncRNAs and JEV infection has been unclear.

lncRNAs play important roles in many processes regulating gene expression. For example, they may interfere with the nuclear factor-kappa binding (NF- κ B) pathway, affecting the expression of genes involved in inflammation and carcinogenesis [14–16]. The NF- κ B pathway is essential for the transcriptional induction of several mRNAs encoding proteins involved in inflammation and cell survival, and NF- κ B signal transduction can regulate the incubation period of virus particles. lncRNAs in inflammatory cytokines and tumor cells are upregulated by NF- κ B and bind to the NF- κ B/I κ B complex to mask the phosphorylation site of I κ B, preventing the transmission of NF- κ B signals [17].



An association between the lncRNA Lethe and RELA (NF- κ B/Rel family) was found to inhibit the binding of RELA to DNA, reducing the transcriptional activity of RELA.

Apoptosis, a type of programmed cell death, is a mechanism that allows cells to self-destruct in the presence of appropriate stimuli. Distinct morphological changes during apoptosis include cell contraction, nuclear condensation, and nuclear pyknosis, along with biochemical phenomena (e.g., DNA cleavage between nucleosomes). Some studies [18–21] have shown that lncRNAs can effectively regulate the energy required for viral proliferation from host cells, by regulating the apoptosis of virus-infected cells; this promotes resistance to viral infection.

There is a close relationship between mitochondrial health and apoptosis. Apoptosis can be caused by activation of the mitochondrial pathway (endogenous pathway), which involves caspase-9. A change in mitochondrial membrane potential displaces the pro-apoptotic protein Bax, releasing cytochrome C into the cytoplasm, activating caspases, and leading to programmed cell death. Apoptosis is also closely related to the levels of reactive oxygen species (ROS) [20,22,23]; mitochondria are sensitive to changes in ROS within cells. Importantly, mitochondrial status directly determines cellular fate [24].

The endoplasmic reticulum (ER) is the main site of eukaryotic protein synthesis and processing. During viral replication, many viral proteins must be synthesized. When ER proteins cannot be correctly folded and modified, the ER stress response and unfolded protein response (UPR) are triggered to clear unfolded proteins and restore ER homeostasis [25]. The ER stress response contributes to viral infection; it can inhibit viral replication by regulating autophagy, thereby degrading proteins [26,27]. However, the molecular mechanism underlying the regulation of viral replication has not yet been elucidated.

In this study, we investigated the antiviral effect of lncRNA-SUSAJ1 on JEV infection, with the aim of better understanding the role of lncRNA in JEV infection and providing new insights into the clinical prevention and treatment of JEV infection.

2. Materials and Methods

2.1 Cells, Virus, and Antibodies

PK15 porcine kidney cells and BHK-21 hamster kidney cells were purchased from the American Type Culture Collection (ATCC, USA). PK15 and BHK-21 cells were both grown in high-glucose Dulbecco's modified Eagle medium (hgDMEM; Gibco, USA) containing 10% foetal bovine serum; they were incubated at 37 °C (5% CO₂ and 95% humidity).

JEV strain SA14-14-2 was amplified in BHK-21 cells. All infections were carried out by incubating the cells with virus at the MOI = 1, then the inoculum was removed, the cells were washed three times with PBS and fresh medium was added. The infection was performed and the infected

PK15 cells were maintained in DMEM supplemented with 2% FBS without penicillin/streptomycin mixtures.

For Western blotting and/or immunofluorescence, primary antibodies were purchased with specificity for the following proteins: eIF2 α (Bioworld, Cat:BS3651, Nanjing, Jiangsu, China); p-eIF2 α (Abcam, Cat:ab32157, Cambridgeshire, UK); GRP78 (Proteintech, Cat:11587-1-AP, Chicago, IL, USA); NS3 (GeneTex, Cat:GTX125868, SAN Antonio, TX, USA); Hsp70 (Abcam, Cat:ab47454, Cambridgeshire, UK); IRE1 (Bioss, Cat:bs-8680R, Beijing, China); p-IRE1 (Abcam, Cat:ab48187, Cambridgeshire, UK); cleaved Caspase-3 (ABclonal, Cat:A11021, Wuhan, Hubei, China); NF- κ B (Proteintech, Cat:10745-1-AP, Chicago, IL, USA); p-NF- κ B (Cell Signaling Technology, Cat:#3033S, Boston, MA, USA); TBB5 (Abgent, AM1031A, Shanghai, China). The following secondary antibodies were purchased: IgG-horseradish peroxidase mouse anti-rabbit (Cat:sc-2357) and mouse IgG kappa binding protein (Cat:sc-516102) coupled with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA); QuickBlock™ Primary Antibody Dilution Buffer for Western Blot (Beyotime, Cat:P0256, Shanghai, China); Bovine serum albumin (Sangon Biotech, Cat:A500023, Shanghai, China).

2.2 Transfection

PK15 cells were seeded on plates for 24 h. When the cell growth density reached 80–90%, using Lipofectamine 3000 (Invitrogen, USA), 2 μ g pcDNA 3.1 lncRNA-SUSAJ1 (the sequence of lncRNA-SUSAJ1 can be found in **Supplementary file**) or empty plasmid (negative control; NC) was transfected into each well in accordance with the manufacturer's instructions. Short interfering RNAs (siRNA) targeting lncRNA-SUSAJ1 was transfected along with si-NC and antisense oligonucleotides (ASO) [28] at a final concentration of 100 nM per well. JEV was used to infect the cells at 6 h after transfection.

2.3 Western Blotting

PK15 cells were seeded on 12-well plates for 24 h, then transfected using Lipofectamine. After 6 h of transfection, JEV infection was conducted. At 24 and 48 h after infection, 195 μ L radioimmunoprecipitation assay lysis buffer, 1 μ L protease inhibitor cocktail, 2 μ L phenylmethylsulphonyl fluoride, and 2 μ L phosphatase inhibitor were added to each well for 30 min; the contents of each well were then collected. Subsequently, protein lysates were mixed with sodium dodecyl sulphate–polyacrylamide gel electrophoresis loading buffer and incubated at 100 °C for 10 min. Protein samples (10 μ L each) were separated by electrophoresis on 12% polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 1% bovine serum albumin in Tris-buffered saline-Tween-20 (TBST) at room temperature for 2 h. Membranes were then incubated overnight at 4 °C

with primary antibodies. Three 10-min washes in TBST were performed; membranes were then incubated with secondary antibodies at room temperature for 45 min. Finally, three 10-min washes in TBST were performed; membranes were incubated with chromogenic solution for chemiluminescence analysis. Images were obtained using a chemiluminescence imaging system. The chemiluminescence intensity of each protein band was quantified using the Image J (1.4.3.67, Broken Symmetry Software, USA), and then protein levels were normalized by the amount of TBB5 protein.

2.4 Flow Cytometry

Apoptosis of JEV-infected cells was detected by flow cytometry. Cells were stained using a fluorescein isothiocyanate annexin V apoptosis detection kit (CA1020; Solarbio, China) in accordance with the manufacturer's instructions. The cells were analysed using a BD-FACS Canto-II flow cytometer (BD Biosciences, USA) and the percentage of apoptotic cells was quantified. Annexin V-fluorescein isothiocyanate-positive cells were classified as early apoptotic cells; PI-positive cells were classified as necrotic cells. Cells with both markers were classified as late apoptotic cells. All data were analysed by FlowJo software (TreeStar FlowJo 10.4.0, USA).

2.5 Real-Time Quantitative PCR Analysis

RNA was extracted from PK15 cells using TRIzol (Invitrogen); RNA quality was evaluated by spectrophotometry (Nano-300; Hangzhou Allsheng, China). RNA was reverse-transcribed to cDNA using PrimeScript RT Master Mix (Perfect Real Time; TaKaRa, Japan), in accordance with the manufacturer's instructions. Glyceraldehyde 3-phosphate dehydrogenase was used as the internal control for both lncRNAs and mRNA. SYBR Green was used for RT-qPCR on a real-time fluorescent thermocycler (CFX96 Touch; Bio-Rad, USA). The primer sequences used in this study are listed in **Supplementary Table 1**.

2.6 TUNEL Staining

A commercial one-step terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) apoptosis detection kit (C1088; Beyotime, China) was used for TUNEL staining in accordance with the manufacturer's instructions. Cells grown in 12-well plates were incubated with 50 μ L TUNEL solution per well. After incubation at 37 °C for 60 min, three 5-min washes with phosphate-buffered saline were conducted. For nuclear staining, cells were incubated with 4',6-diamidino-2-phenylindole (DAPI) at 37 °C for 10 min; anti-fluorescence quenching agent was added after three 5-min washes with phosphate-buffered saline. Apoptosis was observed using an inverted fluorescence microscope.

2.7 Mitochondrial Membrane Potential

A mitochondrial membrane potential detection kit (JC-1 cationic dye; M8650; Solarbio) was used to measure the mitochondrial membrane potential of PK15 cells, in accordance with the manufacturer's instructions. The following fluorescence values were detected using the Synergy-4 multi-functional enzyme reader (Synergy H1; BioTek, USA): 490 nm excitation wavelength/530 nm emission wavelength, and 525 nm excitation wavelength/590 nm emission wavelength. The mitochondrial membrane potential was determined on the basis of the red/green fluorescence ratio. Red and green fluorescence intensities were analysed to determine the mitochondrial membrane potential.

2.8 ROS Assay

The ROS-sensitive probe 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) was used for the detection of intracellular ROS levels. Cells in 12-well plates were washed twice with phosphate-buffered saline, and then incubated for 30 min at 37 °C with 0.5 mL of test solution per well (1:1000 H₂DCFDA; HY-D0940; MCE, USA) in DMEM). Cells were then washed three times with phosphate-buffered saline to remove excess H₂DCFDA. Furthermore, Hoechst 33342 trihydrochloride (HY-15559A; MCE) was used to stain the nuclei of PK15 cells; fluorescence intensities were observed directly under a fluorescence microscope to determine the levels of ROS.

2.9 RNA Pull-Down Assay

Synthesis of lncRNA-SUSAJ1 was conducted using a MAXIscript Kit (K0441; Thermo Fisher Scientific, USA) in accordance with the manufacturer's instructions. The interaction between lncRNA-SUSAJ1 and unknown proteins was examined using the Pierce Magnetic RNA-Protein Pull-Down Kit (20164; Thermo Fisher Scientific, USA), in accordance with the manufacturer's instructions.

2.10 RNA Immunoprecipitation Assay

The SimpleCh (R) Kit (Cell Signaling Technology) was used for RNA immunoprecipitation (RIP) assay, to assess the binding between the Hsp70 protein and lncRNA-SUSAJ1. Dithiothreitol (Beyotime) was added to the lysate to prevent RNA degradation; TRIzol was used to extract total RNA. Finally, RT-qPCR was performed on the Hsp70 protein-containing RNA mixture after adsorption with BeyoMag™ Protein A+G Magnetic Beads (P2108; Beyotime); this confirmed that the target lncRNA-SUSAJ1 was present in the pull-down mixture.

2.11 Statistical Analysis

SPSS (mac 26.0.0.2; SPSS Inc., USA) was used for data analysis. All data are presented as means \pm standard errors of the mean. The *t*-test was used to compare two groups; *P* < 0.05 was considered to indicate statistical sig-

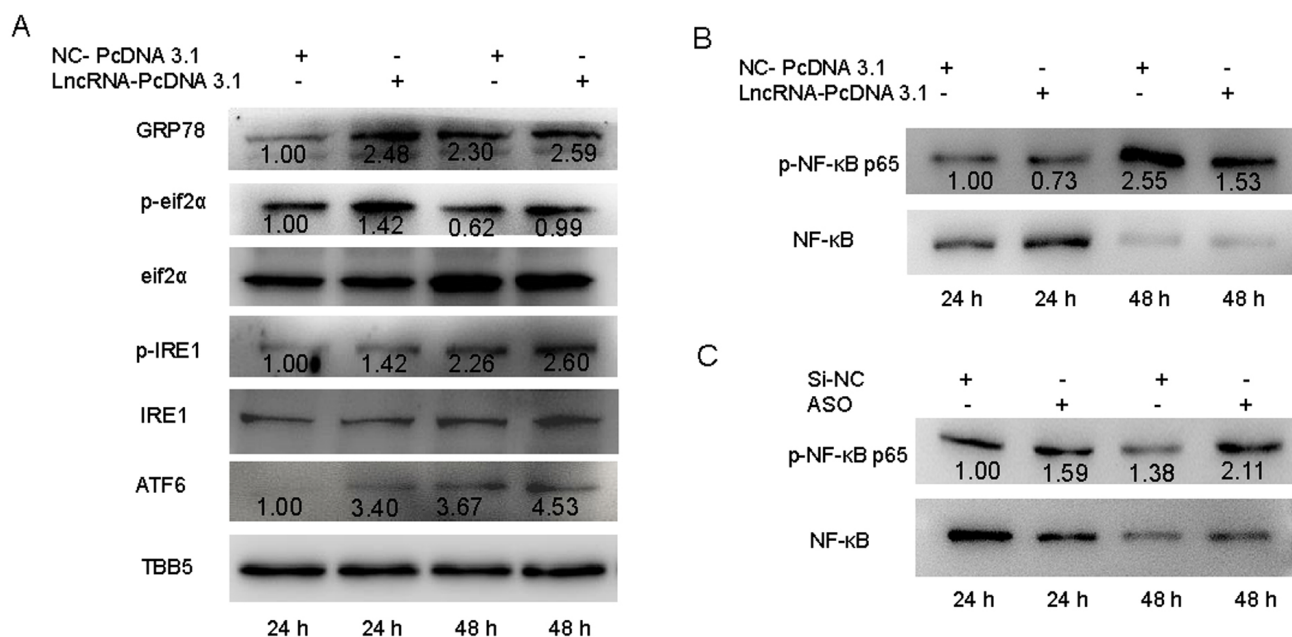


Fig. 1. The effect of lncRNA-SUSAJ1 on ER stress. (A) Overexpression of lncRNA-SUSAJ1 affects the expression level of ER stress-related proteins. Overexpression of lncRNA-SUSAJ1 in PK15 cells by pcDNA3.1. Whole-cell lysates were harvested from the treatment groups as indicated. Immunoblotting was performed to detect ER stress related signal protein levels using TBB5, eIF2 α and IRE1 as an internal control. (B) Overexpression of lncRNA-SUSAJ1 in PK15 cells by pcDNA3.1. Whole-cell lysates were harvested from the treatment groups as indicated. Immunoblotting was performed to detect NF- κ B p65 phosphorylation and NF- κ B protein levels. (C) Knockdown of lncRNA-SUSAJ1 in PK15 cells by ASO. Whole-cell lysates were harvested from the treatment groups as indicated. Immunoblotting was performed to detect NF- κ B p65 phosphorylation and NF- κ B protein levels.

nificance. GraphPad Prism 8.0 (GraphPad Software Inc., USA) was used to generate visual summaries of the data. Unless otherwise specified, the analytical data in all experiments were obtained from more than three independent experiments.

3. Result

3.1 lncRNA-SUSAJ1 Activates the ER Stress Pathway

In a previous study, we found that lncRNA-SUSAJ1 resisted the proliferation of JEV [28], although the molecular mechanism remained unclear. ER stress is an important pathway for regulating virus assembly and proliferation [29–32], and leads to the activation of the UPR pathway, which includes three main signalling pathways: protein kinase RNA-like ER kinase (PERK), IRE1, and activating transcription factor 6 (ATF6).

To investigate the effect of lncRNA-SUSAJ1 on ER stress, we performed Western blotting analysis. PK15 cells were transfected with plasmid for 6 h, and then infected with JEV for 24 and 48 h; total protein samples were extracted for Western blotting. lncRNA-SUSAJ1 overexpression promoted the expression of the ER stress marker protein GRP78 and induced phosphorylation of the eIF2 α and ATF6 pathways but did not activate the phosphorylation of IRE1 (Fig. 1A). Phosphorylated eIF2 α inhibits protein synthesis and promotes apoptosis, and NF- κ B is a crucial

protein involved in ER stress-induced apoptosis [33–35]. In this study, we examined the expression of the anti-apoptotic protein NF- κ B, and found that lncRNA-SUSAJ1 overexpression inhibited the phosphorylation of NF- κ B subunit p65, whereas lncRNA knockdown promoted its phosphorylation (Fig. 1B).

3.2 Interaction between Hsp70 Protein and lncRNA-SUSAJ1

HSP70 inhibits cell apoptosis by inhibiting pro-apoptosis factors [36–38]. In this study, we investigated the relationship between lncRNA-SUSAJ1 and HSP70. An RNA pull-down assay was performed to explore interactions in PK15 cells infected with JEV for 36 h. In JEV-infected PK15 cells, the amount of Hsp70 protein pulled down by lncRNA-SUSAJ1 was higher than that in non-infected JEV cells (Fig. 2A). RNA samples from the experimental Hsp70-RNA complex group and control IgG-RNA complex group were isolated and purified using the RIP assay, and then verified by qRT-PCR. The level of lncRNA-SUSAJ1 expression was significantly higher in the Hsp70 group than in the IgG group (Fig. 2B). Then the qRT-PCR product was diluted 100-fold and used for nucleic acid electrophoresis to confirm the presence of lncRNA-SUSAJ1 (Fig. 2C).

PK15 cells were transfected with plasmid for 6 h, and then infected with JEV for 24 and 48 h; total pro-

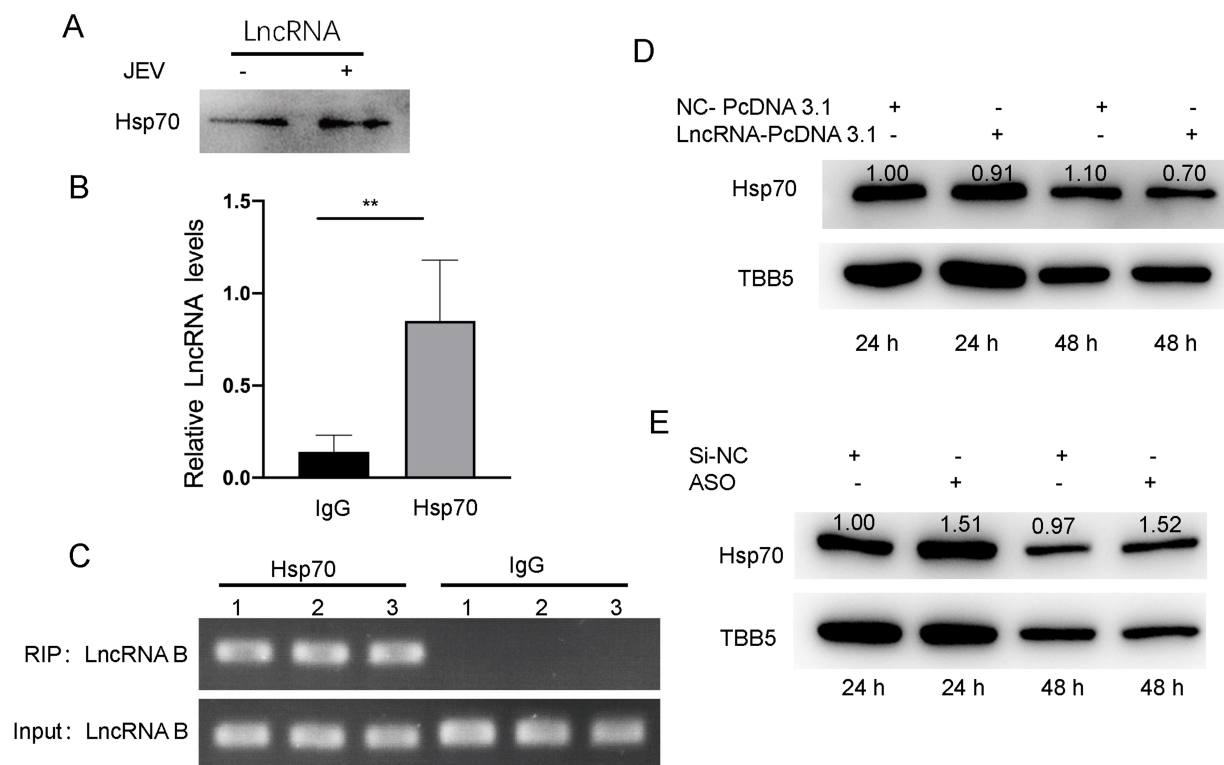


Fig. 2. The interaction between lncRNA-SUSAJ1 and HSP70. (A) The interaction of sense lncRNA-SUSAJ1 with HSP70 was determined by RNA-pull down and western blot. (B) The interaction between HSP70 and lncRNA-SUSAJ1 was determined by RNA-RIP. IgG was used as a negative control. (C) The agarose gel of lncRNA-SUSAJ1 after RNA-rip by HSP70. (D) Overexpression of lncRNA-SUSAJ1 in PK15 cells by pcDNA3.1. Whole-cell lysates were harvested from the treatment groups as indicated. Immunoblotting was performed to detect HSP70 protein levels using TBB5 as an internal control. $*p < 0.05$ and $**p < 0.01$ VS pcDNA3.1-NC. (E) Knockdown of lncRNA-SUSAJ1 in PK15 cells by ASO. Whole-cell lysates were harvested from the treatment groups as indicated. Immunoblotting was performed to detect HSP70 protein levels using TBB5 as an internal control. $*p < 0.05$ and $**p < 0.01$ VS si-NC.

tein samples were extracted for Western blotting. At 24 and 48 h after infection, pcDNA 3.1 lncRNA-SUSAJ1 inhibited Hsp70 protein expression compared to the control group NC, whereas lncRNA-SUSAJ1 knockdown promoted its expression (Fig. 2D,E). These results show that Hsp70 protein interacted with lncRNA-SUSAJ1 and that lncRNA-SUSAJ1 inhibited the expression of Hsp70 protein. Therefore, we conclude that lncRNA-SUSAJ1 may facilitate apoptosis in PK15 cells.

3.3 lncRNA-SUSAJ1 Regulates the Expression of Pro-Apoptotic and Anti-Apoptotic Genes in PK15 Cells

To study the effects of lncRNA-SUSAJ1 on apoptosis in JEV-infected PK15 cells, we transfected the pcDNA 3.1 lncRNA-SUSAJ1 and NC into the cells according to the manufacturer's instructions. After 6 h of transfection, JEV infection was conducted for 24 and 48 h. qRT-PCR was performed to detect the mRNA expression levels of apoptosis-related genes. After the transfection of pcDNA 3.1 lncRNA-SUSAJ1, the expression level of lncRNA-SUSAJ1 was significantly higher than that of NC (Fig. 3A). At 48 h after infection, the expression levels

of the pro-apoptotic genes caspase-3, caspase-8, caspase-9, BCL2L11, Bax, and TP53 were significantly increased (Fig. 3B–F,H); at 24 h after infection, the expression level of the anti-apoptotic gene BCL2 was significantly decreased (Fig. 3G).

3.4 lncRNA-SUSAJ1 Overexpression Promotes the Depolarisation of Mitochondrial Membrane Potential

Mitochondrial membrane potential was measured using the JC-1 cationic dye staining method. After transfection with lncRNA-SUSAJ1 overexpression and NC plasmids for 6 h, cells were infected with JEV for 24 and 48 h; they were then subjected to JC-1 cationic dye staining analysis.

When the mitochondrial membrane potential is normal, direct observation using a fluorescence-inverted microscope shows accumulation of JC-1 cationic dye in the mitochondrial matrix, forming a polymer that emits strong red fluorescence; when depolarised, the dye exists in the cytoplasm as a monomer that produces green fluorescence. At 24 and 48 h after infection, red and green fluorescence intensities indicated promotion of mitochondrial membrane

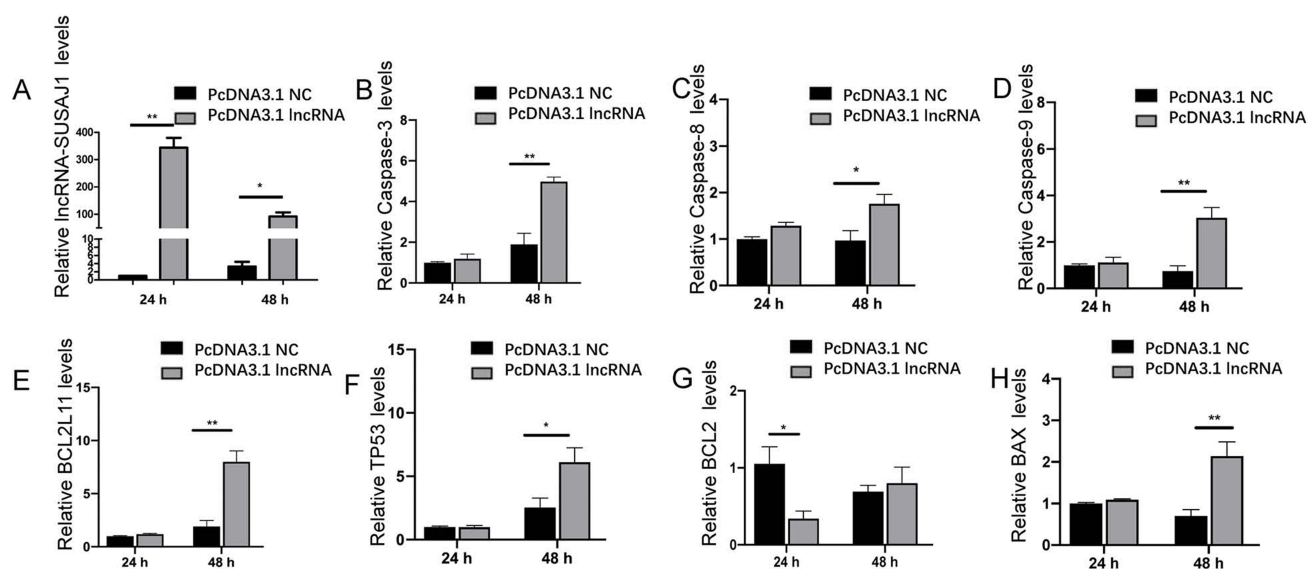


Fig. 3. IncRNA-SUSAJ1 overexpression elevates the mRNA levels of apoptosis-related genes. (A–H) PK15 cells were transfected with pcDNA3.1 IncRNA-SUSAJ1. Time course of IncRNA-SUSAJ1, Caspase-8, Caspase-9 *et al.* mRNA levels were determined by RT-qPCR after JEV infection (n = 3 per group). * $p < 0.05$ and ** $p < 0.01$ VS negative control (NC).

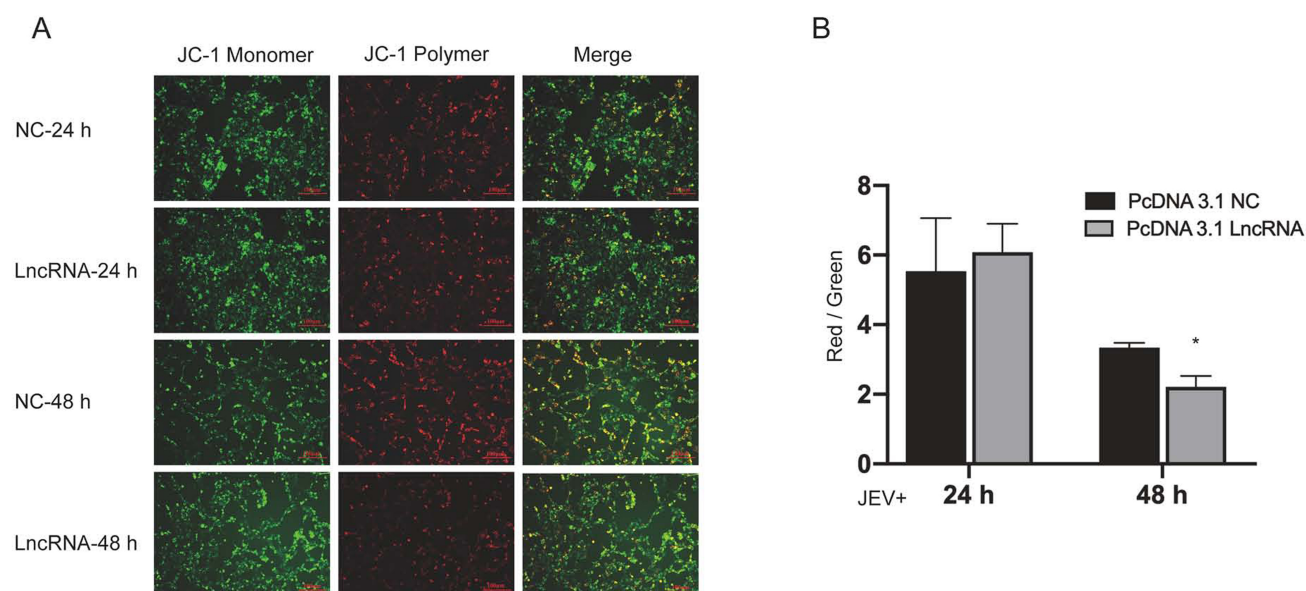


Fig. 4. Effects of IncRNA-SUSAJ1 overexpression on mitochondrial membrane potential (MMP). (A) PK15 cells were transfected with pcDNA3.1 IncRNA-SUSAJ1 and stained by JC-1 after JEV infected. Time course of MMP were stained by JC-1 dye, red bar = 100 μm . (B) Time course of MMP levels were determined by Automatic microplate reader (n = 3 per group), * $p < 0.05$ VS NC.

potential depolarisation (Fig. 4A).

Enzyme labelling analysis showed that the degree of mitochondrial membrane potential depolarisation at 48 h after infection was higher in the IncRNA-SUSAJ1 overexpression group than NC control group (Fig. 4B).

3.5 IncRNA-SUSAJ1 Overexpression Affects ROS Levels and Promotes Apoptosis in JEV-Infected PK15 Cells

After IncRNA-SUSAJ1 overexpression, ROS levels in JEV-infected PK15 cells were examined by H2DCFDA

and Hoechst 33342 staining. Blue-green fluorescence intensity was analysed in merged images. Compared with the uninfected group, the green fluorescence signal at 48 h was significantly increased in the infected group (Fig. 5A), indicating that IncRNA-SUSAJ1 overexpression significantly increased ROS levels in PK15 cells after JEV infection.

TUNEL analysis was used to identify late apoptotic cells. After IncRNA-SUSAJ1 overexpression and 48 h of JEV infection, the proportion of late apoptotic PK15 cells significantly increased (Fig. 5B). The results of TUNEL

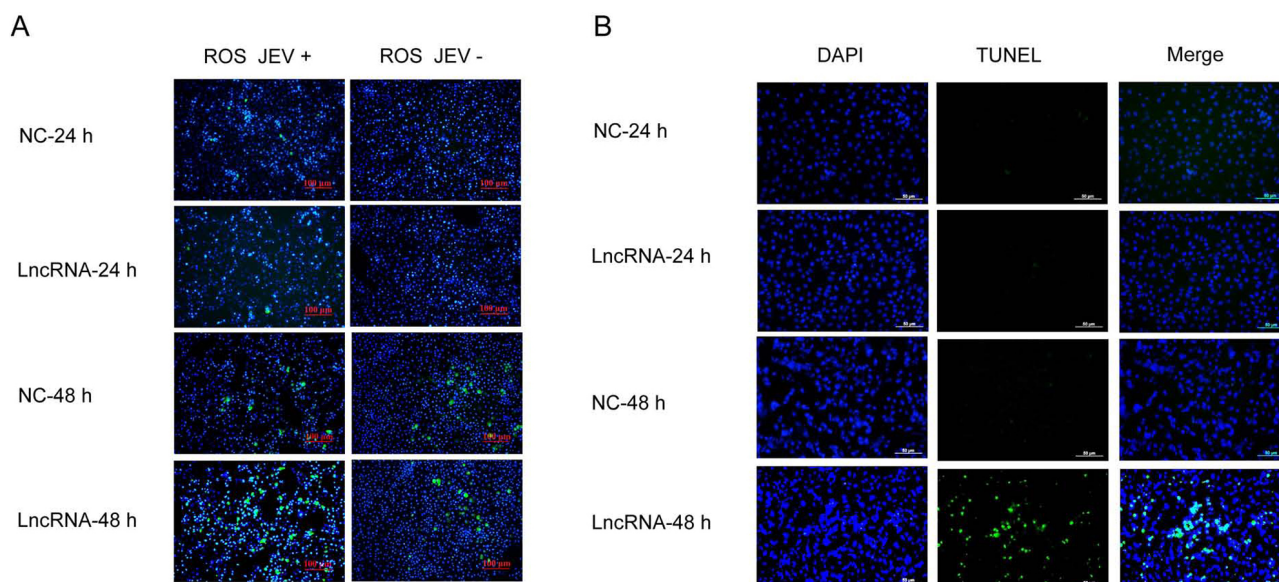


Fig. 5. Effects of lncRNA-SUSAJ1 overexpression on ROS level and cell apoptosis. (A) PK15 cells were transfected with pcDNA3.1 lncRNA-SUSAJ1 and stained by the ROS reagent after JEV infected. Time course of ROS levels were determined by immunofluorescence, red bar = 100 μm . (B) The apoptosis of PK15 cells were determined by TUNEL assay. Time course of cell apoptosis levels were stained by TUNEL reagent, white bar = 50 μm .

analysis were consistent with the those of flow cytometry analysis. With increasing JEV infection time, the proportion of late apoptotic cells increased among PK15 cells with lncRNA-SUSAJ1 overexpression.

Apoptosis was investigated by flow cytometry analysis. Among cells overexpressing lncRNA-SUSAJ1, after 24 h of JEV infection, the proportion of viable cells decreased by 11.5% ($p < 0.01$), the proportion of early apoptotic cells increased by 17.7%, and the proportion of late apoptotic cells decreased by 5.79% (Fig. 6A–C). After 48 h of JEV infection, the proportion of viable cells decreased by 15.6% ($p < 0.01$), while the proportion of early apoptotic cells increased by 4% ($p < 0.01$) (Fig. 6D,F) and the proportion of late apoptotic cells increased by 12.7% ($p < 0.01$).

Caspase-3 is closely associated with apoptosis [39]; levels of cleaved caspase-3 protein were detected by Western blotting in this study. lncRNA-SUSAJ1 strongly affected cleaved caspase-3 protein expression. At 24 and 48 h after infection, lncRNA-SUSAJ1 overexpression promoted the expression of cleaved caspase-3 protein (Fig. 6G), whereas lncRNA-SUSAJ1 knockdown inhibited the expression of cleaved caspase-3 protein (Fig. 6H).

4. Discussion

Although there is increasing evidence that lncRNAs are involved in regulating the host innate immune response and expression of virus-related genes and proteins, the specific mechanism underlying the effects of lncRNA-SUSAJ1 on JEV replication has not been elucidated.

You *et al.* [14] found that lncRNAs can serve as tar-

gets for new antiviral drugs. Zhang *et al.* [21] used a comprehensive and in-depth sequencing approach to confirm that lncRNAs participate in host antiviral responses during porcine reproductive and respiratory syndrome virus infection; in particular, lncRNAs regulate the apoptosis signalling pathway. Meng *et al.* [15] investigated the effects of lncRNAs on the immune response and viral replication; they identified key roles for lncRNAs in host–virus interactions. Torkzaban *et al.* [18] demonstrated that lncRNA-U1 directly upregulated the expression of NPBWR1 and induced apoptosis during infection with human immunodeficiency virus. Zou *et al.* [20] examined the role of lncRNA AX800134 in hepatitis B virus-related hepatocellular carcinoma. Viral proliferation and replication often require host cell components and energy sources. During apoptosis, infected cells die and the virus cannot use nutrients from these cells. This inhibits viral replication, transmission, and persistence. Here, we hypothesised that lncRNA-SUSAJ1 promotes apoptosis in JEV-infected PK15 cells, thereby inhibiting viral proliferation and replication.

To explore the effects of lncRNA-SUSAJ1 on JEV proliferation and replication, we examined apoptosis-related genes via RT-qPCR. We found that lncRNA-SUSAJ1 overexpression promoted the expression of proapoptotic genes and inhibited that of anti-apoptotic genes. We analysed apoptosis in PK15 cells that had been transfected with pcDNA3.1 lncRNA-SUSAJ1 and NC plasmid using flow cytometry and TUNEL staining. Flow cytometry showed that lncRNA-SUSAJ1 promoted apoptosis in PK15 cells. TUNEL analysis showed that lncRNA-SUSAJ1 promoted late apoptosis [40], and the proportion

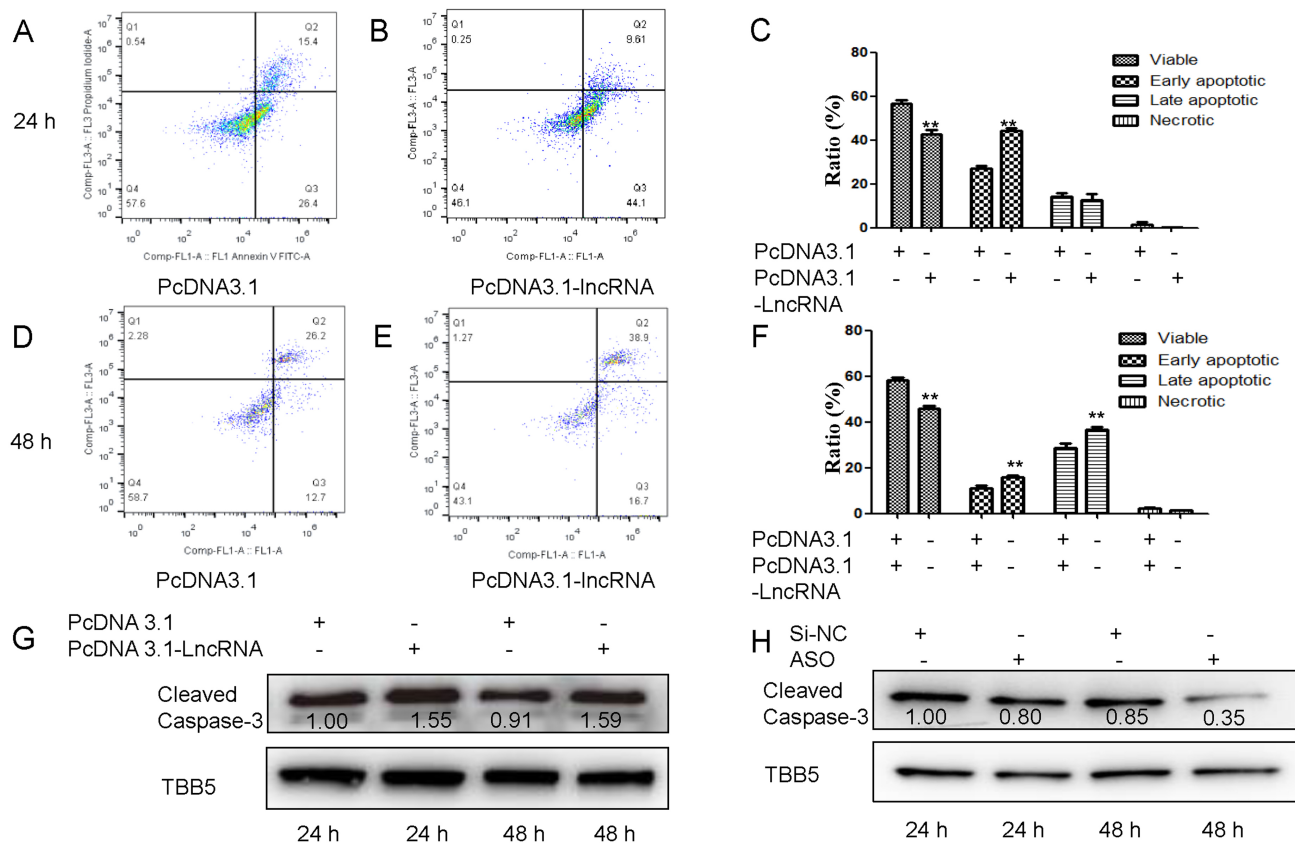


Fig. 6. Effects of lncRNA-SUSAJ1 overexpression on cell Annexin V and Caspase-3 levels. (A–B,D–E) PK15 cells were transfected with pcDNA3.1 lncRNA-SUSAJ1 and stained by the annexin V and PI reagent after JEV infected. The annexin V and PI levels were determined by Flow cytometry. (C and F) The ratios of viable, early apoptosis, late apoptosis, and necrotic cells detected by Flow cytometry, C (24 h), D (48 h), $**p < 0.01$ VS pcDNA3.1-NC. (G) PK15 cells were transfected with pcDNA3.1 lncRNA-SUSAJ1. Whole-cell lysates were harvested from the treatment groups as indicated. Immunoblotting was performed to detect Caspase-3 protein levels using TBB5 as an internal control. (H) Knockdown of lncRNA-SUSAJ1 in PK15 cells by ASO. Whole-cell lysates were harvested from the treatment groups as indicated. Immunoblotting was performed to detect Caspase-3 protein levels using TBB5 as an internal control.

of late apoptotic PK15 cells increased with increasing JEV infection duration ($p < 0.01$).

lncRNA-SUSAJ1 did not cause abnormal ROS accumulation in normal PK15 cells; however, in JEV-infected PK15 cells, lncRNA-SUSAJ1 promoted apoptosis, resulting in a significant increase in the proportion of cells experiencing late apoptosis, causing abnormal mitochondrial membrane potential and promoting the level of ROS produced by cell metabolism, perhaps due to lncRNA-SUSAJ1 interaction with novel proteins that may be present at very low levels in normal PK15 cells but are upregulated by JEV infection. This hypothesis requires further study.

Apoptosis is a type of programmed cell death; mitochondria-dependent apoptosis is the most common subtype. Mitochondria are sensitive to changes in ROS in cells. Some studies have shown that lncRNAs are involved in regulating cellular stress responses [41], such as oxidative stress and ER stress, which induce an antiviral response in the host. During oxidative stress, metabolism of ROS leads to mitochondrial damage [42].

The depolarisation of mitochondrial membrane potential displaces the pro-apoptotic protein Bax, releases cytochrome C from the inner membrane of mitochondria into the cytoplasm, activates the caspase-3 signalling pathway, and triggers a mitochondria-dependent apoptosis response. Therefore, caspase-3 is the most important enzyme in apoptosis. In this study, Western blotting showed that lncRNA-SUSAJ1 overexpression promoted the expression of cleaved caspase-3 protein in JEV-infected PK15 cells, while lncRNA-SUSAJ1 knockdown inhibited the expression of cleaved caspase-3 protein in JEV-infected PK15 cells. Furthermore, we demonstrated that lncRNA-SUSAJ1 overexpression promoted the release of ROS produced by PK15 cellular metabolism and reduced the mitochondrial membrane potential in JEV-infected PK15 cells.

HSP70 is a vital molecular chaperone that reduces UPR and helps proteins to fold correctly [36], resulting in the suppression of ER stress and promoting JEV proliferation. It remains unclear whether lncRNA-SUSAJ1 can inhibit JEV proliferation and replication by trigger-

ing a cellular stress response. To explore this, we performed RNA pull-down experiments, using biotin-labelled lncRNA-SUSAJ1 to analyse JEV-infected and uninfected PK15 cells after 36 h of incubation. Mass spectrometry analysis of the pull-down protein mixture indicated that lncRNA-SUSAJ1 has a regulatory role, in combination with the antioxidant protein Hsp70, which plays an important role in oxidative stress [43], when JEV infects PK15 cells. To verify the interaction between lncRNA-SUSAJ1 and Hsp70, we performed RIP and Western blotting experiments. The results revealed an interaction between lncRNA-SUSAJ1 and Hsp70, such that lncRNA-SUSAJ1 negatively regulated the Hsp70 expression.

ER stress and the UPR are involved in the regulation of viral replication [44,45]. The ER is an important organelle for viral replication and maturation. After viral infection of host cells, numerous viral proteins are synthesised in the ER cavity; this increases the protein load in the ER, leads to the accumulation of unfolded and misfolded proteins, induces the ER stress response, and activates the UPR [46]. Three UPR signalling pathways have been identified: ATF6, PERK, and IRE1 [47]. In this study, Western blotting was used to detect the marker protein GRP78, which is activated by the ER stress response. The results showed that lncRNA-SUSAJ1 antiviral activity was closely associated with the ER stress response. lncRNA-SUSAJ1 overexpression significantly increased the expression of GRP78 protein; it also inhibited JEV proliferation and replication by promoting activation of the ER stress response. Subsequently, the specific pathway underlying the promotion of UPR activation was examined. The expression levels of PERK, IRE1 and ATF6 pathway-related proteins were detected by Western blotting. Notably, lncRNA-SUSAJ1 positively regulated the ER stress response, promoted the expression of IRE1, and promoted the phosphorylation of eIF2 α , an important marker of PERK pathway activation [48]; however, it did not affect the ATF6 pathway.

NF- κ B is a protein complex that controls the transcription of various genes and participates in cellular responses to stress, free radicals, ROS, and cytokines; it also inhibits apoptosis [43]. ER stress regulates the NF- κ B pathway; the activation of PERK-eIF2 α inhibits NF- κ B phosphorylation, thereby promoting apoptosis [49]. Hsp70 also plays a key regulatory role in the NF- κ B signalling pathway, and thus in the tolerance of oxidative stress [50]; in particular, it regulates activation of the NF- κ B signalling pathway [51–53]. Furthermore, mir-192-3p can activate the NF- κ B signalling pathway, thereby promoting hepatitis B viral replication [54]. Because NF- κ B is the crucial pathway for cell apoptosis regulation, we speculated that lncRNA-SUSAJ1 may inhibit JEV proliferation via regulating cell apoptosis. Therefore, we investigated whether the mechanism of lncRNA-SUSAJ1 virus inhibition was related to regulation of the NF- κ B signalling pathway. We performed pcDNA3.1 lncRNA-SUSAJ1 ASO and NC plas-

mid transfection, followed by JEV infection for 24 and 48 h; we found that lncRNA-SUSAJ1 overexpression inhibited phosphorylation of the NF- κ B subunit p65, while lncRNA-SUSAJ1 knockdown promoted phosphorylation of the NF- κ B subunit p65. These findings indicate that lncRNA-SUSAJ1 can inhibit JEV proliferation and replication by regulating activation of the NF- κ B signalling pathway.

5. Conclusions

We comprehensively explored the mechanism by which lncRNA-SUSAJ1 influences the proliferation and replication of JEV in PK15 cells. When PK15 cells were infected with JEV, lncRNA-SUSAJ1 promoted the ROS response and increased the production of oxygen free radicals via cellular metabolism; lncRNA-SUSAJ1 also inhibited the expression of Hsp70 and promoted the phosphorylation of eIF2 α . These effects combined to inhibit activation of the NF- κ B pathway and accelerate apoptosis in PK15 cells, thereby inhibiting JEV proliferation and replication (Fig. 7).

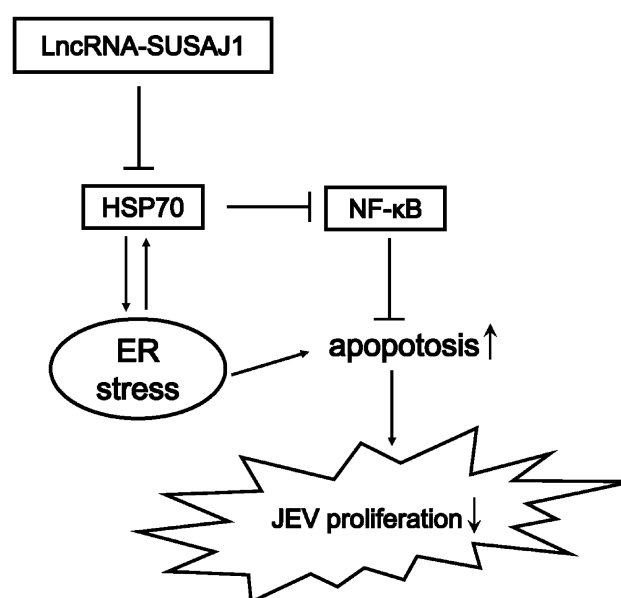


Fig. 7. Model of lncRNA-SUSAJ1 activated the ER stress promoting apoptosis in PK15 cells.

Abbreviations

JEV, Japanese encephalitis virus; lncRNAs, long non-coding RNAs; siRNAs, short interfering RNAs; ASO, antisense oligonucleotides; TBST, Tris-buffered saline-Tween-20; MMP, mitochondrial membrane potential; ROS, reactive oxygen species; ER, The endoplasmic reticulum; UPR, unfolded protein response; RIP, RNA immunoprecipitation.

Author Contributions

XZ and AZ conceived and designed the study project. QY and JF performed the experiments and edited most of the manuscript. QY analyzed the data with support from HW, XL and SY revised the manuscript content. XZ, SY, and AZ contributed to result discussion and data interpretation. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/j.fbl2709260>.

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