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Porcine parvovirus replication is suppressed by activation of the PERK signaling pathway and endoplasmic reticulum stress-mediated apoptosis

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ABSTRACT

Endoplasmic reticulum (ER) stress is associated with numerous mammalian diseases, especially viral diseases. Porcine parvovirus (PPV) is the causative agent of reproductive failure in swine. Here, we observed that the PPV infection of porcine kidney 15 and porcine testis cells resulted in the activation of ER stress sensors mediated by protein kinase R-like ER kinase (PERK), but not inositol-requiring enzyme 1 and activating transcription factor 6 (ATF6). ER stress activation obviously blocked PPV replication. Depletion of proteins, such as PERK, eukaryotic initiation factor 2, and ATF4, by small interfering RNA significantly enhanced PPV replication. Moreover, the pro-apoptotic factor C/EBP homologous protein was identified a key factor in the inhibition of PPV replication. These data demonstrate that PPV infection activates ER stress through the PERK signaling pathway and that ER stress inhibits further PPV replication by promoting apoptosis.

1. Introduction

Porcine parvovirus (PPV) is a small, non-enveloped, negative and single-stranded DNA virus that is a member of the *Parvovirus* genus in the *Parvovirinae* subfamily of the *Parvoviridae* family. Infection with PPV represents a major cause of reproductive failure in swine (Mayr and Mahnel, 1964) that is characterized by stillbirth, fetal mummification, embryonic death, and infertility. PPV was first isolated from sows in Germany in 1965 (Mengeling et al., 1991) and has subsequently been reported in many other countries. Outbreaks of PPV have severely hindered the development of the swine industry worldwide, resulting in huge economic losses.

The endoplasmic reticulum (ER) is a membrane-bound compartment mainly responsible for protein processing and transport, lipid metabolism, and calcium homeostasis (Hetz, 2012; Kim et al., 2008; Ron and Walter, 2007). Although the ER has a very strong homeostasis system, it can be destroyed by external factors, such as pathogen invasion, chemical insult, energy or nutrient deprivation, destruction of calcium homeostasis, oxidative stress, and accumulation of unfolded, misfolded, or excessive proteins (Dimcheff et al., 2004; Iwata and Koizumi, 2012; Kim et al., 2008; Ye et al., 2011; Zhang and Wang, 2012). The disturbance of ER homeostasis induces a stress response, known as ER stress. In response to ER stress, cells trigger a unique intracellular signaling pathway, termed the unfolded protein response (UPR), which reestablishes homeostasis in the ER or triggers apoptosis if ER stress is severe or prolonged. The UPR is mediated by three distinct signaling pathways: protein kinase R-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring protein-1 (IRE1) (Pahl, 1999; Ron and Walter, 2007; Shen et al., 2002). Under normal physiological conditions, these three proximal UPR sensors associate with glucose-regulated protein 78 KD (GRP78), also known as immunoglobulin binding protein (Ni et al., 2011; Quinones et al., 2008). GRP78 locates in the ER as a chaperone and belongs to the heat shock protein 70 family. During ER stress, GRP78 binds to misfolded proteins and releases PERK, ATF6, and IRE1. Activated PERK phosphorylates the alpha subunit of eukaryotic translation initiation factor 2 (eIf2α), which, in turn, activates the downstream signaling of activating transcription factor 4 (ATF4), leading to attenuated global translation initiation and protein synthesis (Shi et al., 1998). ATF4 stimulates the expression of the pro-apoptotic factor C/EBP homologous protein (CHOP), growth arrest, and the activation of DNA damage-inducible protein 34 and other genes that trigger oxidative stress and programmed cell death (Novoa et al., 2003; Zinszner et al., 1998). CHOP is a nuclear transcription factor downstream of ATF4 that can induce apoptosis in response to ER stress; however, overexpression will lead to down-regulated expression of the anti-apoptotic protein Bcl2. When

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released from GRP78, ATF6 translocates from the ER to the Golgi apparatus. Activated ATF6 is cleaved by proteases, generating the transcriptionally active form of ATF6 (ATF6p50) (Li et al., 2000). Activated IRE1 splices the full-length X-box binding protein 1 (XBP1) mRNA and removes 26 base pairs, resulting in the frameshift conversion of the premature unspliced XBP1 protein (XBP1U) to the spliced XBP1 protein (XBP1S) (Lee et al., 2003). ATF4, ATF6p50, and XBP1S migrate to the nucleus, where they promote the expression of a distinct set of UPR target genes, including those encoding molecular chaperones and proteins contributing to ER-associated degradation.

During viral infection, a large number of viral proteins are synthesized in the ER, which destroys the cellular translation machinery, resulting in the induction of ER stress and subsequent facilitation of viral infection (Zhang and Wang, 2012). It was reported that the SAT protein (SATp) of the attenuated PPV strains NADL-2 and Kresse accumulates in the ER and that the SAT deletion mutant of both of these strains induces a slow-spreading phenotype (Meszaros et al., 2017; Zadori et al., 2005). ER stress activation promoted viral egress of porcine testis (PT) fibroblasts infected with the Kresse strain of PPV at a high multiplicity of infection (MOI) (Meszaros et al., 2017). The early stage of infection with the attenuated PPV NADL-2 strain was almost completely abolished by the ER inducer MG132 (Boisvert et al., 2010). A similar inhibitory effect of MG132 treatment of infection with PPV strain Kresse was detected at a low MOI (Meszaros et al., 2017). These data suggested that ER stress has both negative and positive effects on PPV replication related to the strain and dose of the virus. However, the potential mechanism of how ER stress modulates PPV replication remains largely unclear. Several reports have suggested an interaction between viral replication and the UPR pathway (Zhang and Wang, 2012). In 2015, we isolated a PPV strain from the liver of an aborted fetus in Tianjin, China, named strain TJ (Cao et al., 2017). In the present study, we characterized the ER stress response in porcine kidney 15 (PK-15) and PT cells infected with PPV strain TJ. We observed that PPV strain TJ infection leads to an active ER stress pathway mediated by PERK/eIF2α, but not XBP1 and ATF6. ER stress-inducing drugs can inhibit PPV strain TJ infection. Depletion of the proteins PERK, eIF2α, and ATF4 enhanced viral titers, demonstrating that ER stress and the UPR play essential roles against PPV strain TJ infection.

If ER homeostasis is not restored, the UPR can trigger apoptosis to reduce or eliminate excessively stressed cells. Apoptosis, a programmed cell death, is an important channel for virus-induced pathophysiological cellular response and cytopathic effects (Kennedy, 2015). Previous evidence has shown that PPV infection induced apoptosis in PK-15 and swine testicular (ST) cells, and identified that p53, the mitochondria pathway, and ROS accumulation modulated PPV-induced apoptosis (Barber et al., 2017; Huang et al., 2014). However, the involvement of ER stress-induced apoptosis in PPV replication is still poorly understood. The results of the present study showed that ER stress-induced apoptosis primarily functions to antagonize PPV replication.

2. Materials and methods

2.1. Cells and viruses

PK-15 and PT cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's minimum essential medium supplemented with 8% fetal bovine serum under an atmosphere of 5% CO_2 at 37 °C. PPV strain TJ (Genebank accession no. KX233726) was isolated in our laboratory (Cao et al., 2017). Viral titers were determined by the Reed–Muench method.

2.2. Reagents and antibodies (Abs)

The ER stress-specific inducers thapsigargin (Tg) and tunicamycin (Tu), and rabbit polyclonal Ab against ATF4, mouse polyclonal Ab against ATF6, and mouse monoclonal Ab against β-actin were purchased from Sigma–Aldrich Corporation (St. Louis, MO, USA). Tg and Tu were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 10 mM, and 2 mg/mL, respectively. The apoptosis-specific inducer nutlin-3 was purchased from Selleck Chemicals LLC (Houston, TX, USA) and dissolved in DMSO to a concentration of 10 mM. Mouse monoclonal Abs against GRP78, PERK, and Bcl2 were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA, USA). Rabbit polyclonal Ab against PERK (phospho T982), mouse monoclonal [eIF2α] Ab against eIF2S1, and rabbit monoclonal [E90] Ab against EIF2S1 (phospho S51) were purchased from Abcam (Cambridge, MA, USA). The FITC Annexin V Apoptosis Detection Kit I was purchased from BD Biosciences (San Jose, CA, USA). IRDye 800CW goat anti-rabbit Ab against immunoglobulin (Ig) G and IRDye 680RD goat anti-mouse Ab against IgG were purchased from LI-COR Biosciences (Lincoln, NE, USA). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ab against IgG and tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse Ab against IgG were purchased from Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd. (Beijing, China). Anti-PPV VP2 monoclonal Ab (McAb) 8E4, which recognizes the PPV VP2 and VP1 proteins, was prepared in our laboratory (Sun et al., 2015).

2.3. Reverse transcription-PCR (RT-PCR) and real-time PCR

Cells were seeded into the wells of 24-well plates at a concentration of 1×10^5 cells/well. After grown in monolayers, the cells were mock-infected or infected with PPV. Cells were harvested at 12, 24, 36, and 48 h post-infection (hpi) and total RNA was extracted using an RNeasy mini kit (Qiagen, Hilden, Germany) and reverse-transcribed into complementary DNA (cDNA) using the PrimeScript™ II 1st strand cDNA synthesis kit (Takara, Dalian, China). To detect whether XBP1 splicing occurred in PPV-infected cells, RT-PCR was performed using specific primers for XBP1 as previously described (Xue et al., 2018). The PCR products were digested with *PstI* and separated by electrophoresis in 2% agarose gels. To detect the relative expression levels of GRP78, ATF4, and CHOP, the cDNA was subjected to real-time PCR with primer pairs specific for the indicated genes. The GRP78-specific primers were 5′ TCTACTCGCATCCCAAAG 3′ and 5′ CTCCCACGGTTTCAATAC 3′, the ATF4-specific primers were 5′ GAGATAGGAAGCCAGAC 3′ and 5′ AGATGCCACTGTCATTAT 3', the CHOP-specific primers were 5′ CTCA GGAGGAAGAGGAGG AAG 3′ and 5′ GCTAGCTGTGCCACTTTCCTT 3′, and the β-actin primers were 5′ GGCTCAGAGCAAGAGAGGTATCC 3′ and 5′ GGTCTCAAACATGATCTGAGTCATCT 3'. Amplification was performed using the Roche LightCycler 480 Real-Time PCR System (F. Hoffmann-La Roche AG, Basel, Switzerland) and the mRNA levels of GRP78, ATF4, and CHOP were calculated using β-actin as an internal reference according to the $2^{-\Delta\Delta CT}$ method.

2.4. Plasmids and RNA interference

The porcine CHOP (Genebank accession No: XM_005674378.2) gene was cloned from PK-15 cells by RT-PCR using the specific primer pair 5′- CCGGAATTCATGGCAGCTGAGTCATTGCC-3′ and 5′- CGGGGT ACCTCATGCTTGGTGCAGATTAA-3′. Porcine CHOP was inserted into the plasmid pCAGGS-HA using the restriction enzymes *EcoRI* and *KpnI*. The vector pCAGGS-HA was kept in our laboratory. Specific short hairpin RNA (shRNA) targeting PERK and CHOP, small interfering RNA (siRNA) molecules targeting ATF4 and eIF2α, and a scrambled control (NC) were obtained from GenePharma Co., Ltd. (Shanghai, China) (Table 1). In 6-well plates, PK-15 cells were transfected with 100 pmol siRNA molecules, or 4 μg of the empty vector pCAGGS-HA, or 4 μg of pCAGGS-HA-CHOP (HA-CHOP) with the use of Lipofectamine 2000 transfection reagent (Invitrogen Corporation, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. At 24 h after transfection, cells were infected with PPV at an MOI of 0.1 for an addition 24 h. Afterward, the cell lysates and cell-free culture supernatants were collected for protein and viral titration analyses.

Table 1
Sense strand sequences of siRNA targeting ATF4 and eIF2α, and shRNA targeting PERK and CHOP used in this study.

Gene name	code	Sense strand sequence (5′-3′)
PERK	1#	UGAUUUUUGCUCUACUUAAGG
	2#	CUGCAGAUUGUGGAGGCGGUA
	3#	AGGUCUAGGGAGCGAACCUCC
	4#	CGACAACCCGAAUUACAACAA
ATF4	1#	GAGAAGGUGGUAGCAGCAA
	2#	CAGAUAAUGACAGUGGCAU
	3#	GCCAUCUCCCAGAAAGUUU
eIF2α	1#	GUUGUCAUUAGAGUGGACA
	2#	GCAUUGAUGCCGUGAAAGA
	3#	GAAACCAUGCCCAUCAAGA
CHOP	1#	CACUGUCCAGCUGGGAGCUGG
	2#	GAAAGUGGCACAGCUAGCUGA
	3#	CCAGGGAAGUGGAGGCGACUC
NC		UUCUCCGAACGUGUCACGUTT

2.5. Cytotoxicity assay

The cytotoxicity of chemical drugs, such as Tg, Tu, and nutlin-3, were determined using the CCK-8 assay (Beyotime Institute of Biotechnology, Haimen, China) in accordance with the manufacturer's instructions. Briefly, cells were seeded into the wells of 96-well plates at 2×10^4 cells/well for 24 h and then treated with serially-diluted chemical drugs for 24 h. Afterward, 10 μl of enhanced CCK8 solution was added to each well and the plate was incubated for an additional 2 h. The optical density at 450 nm (OD450) was determined using a microplate reader and cell viability was calculated as follows: (OD450 treatment)/ (OD450 control) × 100.

2.6. Immunofluorescence assay (IFA)

PPV was mixed with DMSO, Tg (1μM), or Tu (2 μg/mL), and added to the PK-15 or PT cells for 1 h. Then, the cells were washed with phosphate-buffered saline (PBS) three times and maintained of the infection cells with that concentration. At 24 hpi, the cells were fixed with 4% formaldehyde for 30 min and permeabilized with 0.1% Triton X-100 for 10 min at room temperature. Fixed cells were incubated with PPV VP2 McAb (dilution, 1:500) and subsequently incubated with FITC-conjugated anti-mouse Ab against IgG (1:200) at 37 °C for 1 h. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) at room temperature for 10 min. Afterward, the cells were washed three times with PBS and examined under a fluorescence microscope (AMG EVOS F1; Advanced Microscopy Group, Bothell, WA, USA).

2.7. Apoptosis assessment by flow cytometric analyses

PK-15 cells were treated with Tg or nutlin-3 during PPV infection. At 24 hpi, the cells were harvested with trypsin-EDTA, centrifuged for 5 min at 1000 × *g*, washed twice with ice-cold PBS, and then re-suspended in 1 × annexin V binding buffer at a concentration of 1×10^6 cells/mL. The solution (100 μL, 1×10^5 cells) was then transferred to a 1.5-mL culture tube and stained with 5 μL of Annexin V-FITC and 5 μL of propidium iodide (PI), vortexed gently, and incubated for 15 min at room temperature in the dark. After staining, 400 μL of 1 × annexin V binding buffer was added to each tube, and then analyzed by flow cytometry for 1 h. The data were analyzed using FlowJo_v10 software.

2.8. Western blot analysis

Total cellular proteins were extracted using radioimmunoprecipitation assay buffer lysis buffer supplemented with 1 mM phenylmethylsulfonyl fluoride (Beyotime Institute of Biotechnology). After centrifugation at 12,000 × *g* for 15 min, lysate supernatants were boiled for 10 min and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Separated protein bands were then transferred onto a nitrocellulose membrane, which was blocked with 5% (w/v) bovine serum albumin in PBS containing 0.05% Tween 20 (PBST) at room temperature for 1 h. Next, the membranes were incubated with the indicated Abs at 4 °C overnight. After washing three times with PBST, the membranes were incubated with IRDye-conjugated secondary Abs in the dark at room temperature for 1 h. The membranes were washed as described above and then scanned using an Odyssey infrared imaging system (LI-COR Biosciences).

2.9. Statistical analysis

The Student's *t*-test was used to analyze statistical significance. A probability (*p*) value of less than 0.05 was considered statistically significant.

3. Results

3.1. PPV infection activates ER stress

Previous studies have shown that PPV infection can induce ER stress (Meszaros et al., 2017; Zadori et al., 2005). However, little is known about the potential mechanisms involved. *In vitro*, PK-15 and PT cells could infect PPV virus. A one-step growth curve of PPV in PK-15 and PT cells showed that the viral titer reached a maximal constant value at 60 hpi, and then gradually decreased (Fig. 1A). GRP78 is a sensitive marker of ER stress. We first assessed the mRNA and protein expression levels of GRP78 following PPV infection in PK-15 and PT cells. As shown in Fig. 1B and C, when compared with the mock-infected control group, PPV strain TJ induced GRP78 gene transcription at 12, 24, and 36 hpi in PK-15 cells, and at 24, 36, and 48 hpi in PT cells. The mRNA levels of GRP78 peaked in PPV-infected PK-15 and PT cells at 24 and 48 hpi. Similarly, the protein levels of GRP78 were increased in virus-infected PK-15 cells at 12, 24, and 36 hpi, and at 36 and 48 hpi in PT cells (Fig. 1D and E). These results clearly suggest that PPV infection induces ER stress in PK-15 and PT cells.

3.2. PPV infection activates ER stress via the PERK pathway

Under ER stress, the three UPR signaling pathways (PERK, ATF6, and IRE1) become activated. To identify whether the PERK and ATF6 signaling pathways are involved in the activation of ER stress during PPV infection, western blot analysis was used to investigate the protein changes of PERK, phosphorylated PERK (p-PERK), eIF2α, phosphorylated eIF2α (p-eIF2α), and cleaved ATF6 (50-kDa protein, p50ATF6) during the course of PPV infection. As shown in Fig. 2A and B, as compared with uninfected control cells, the amount of p-PERK and p-eIF2α increased as PPV infection progressed in PK-15 and PT cells, whereas the amount of total PERK and eIF2α was unaltered. Increased p-PERK and p-eIF2α concentrations were apparent at 12, 24, and 36 hpi in PK-15 cells and at 24, 36, and 48 hpi in PT cells. However, the changes in p50ATF6 protein expression could not be detected in PK-15 and PT cells (Fig. 2A and B). As expected, the mRNA levels of ATF4, and CHOP were significantly elevated during PERK-eIF2α activation by PPV (Fig. 2C and D). Elevated ATF4 and CHOP mRNA levels peaked at 24 hpi in PK-15 cells and at 48 hpi in PT cells. Taken together, these results indicate that PPV infection activates the PERK-eIF2α-ATF4-CHOP pathway, but not the ATF6 pathway.

When the IRE1 signaling pathway is activated by ER stress, XBP1 mRNA is spliced to a 26-nucleotide (nt) intron by the endonuclease domain of IRE1. XBP1 has a *PstI* restriction site, which located within the 26-nt region of XBP1 cDNA. RT-PCR was performed to determine whether PPV infection can induce splicing of XBP1 mRNA. The ER stress inducer Tg was used as a positive control. Total XBP1 cDNA was

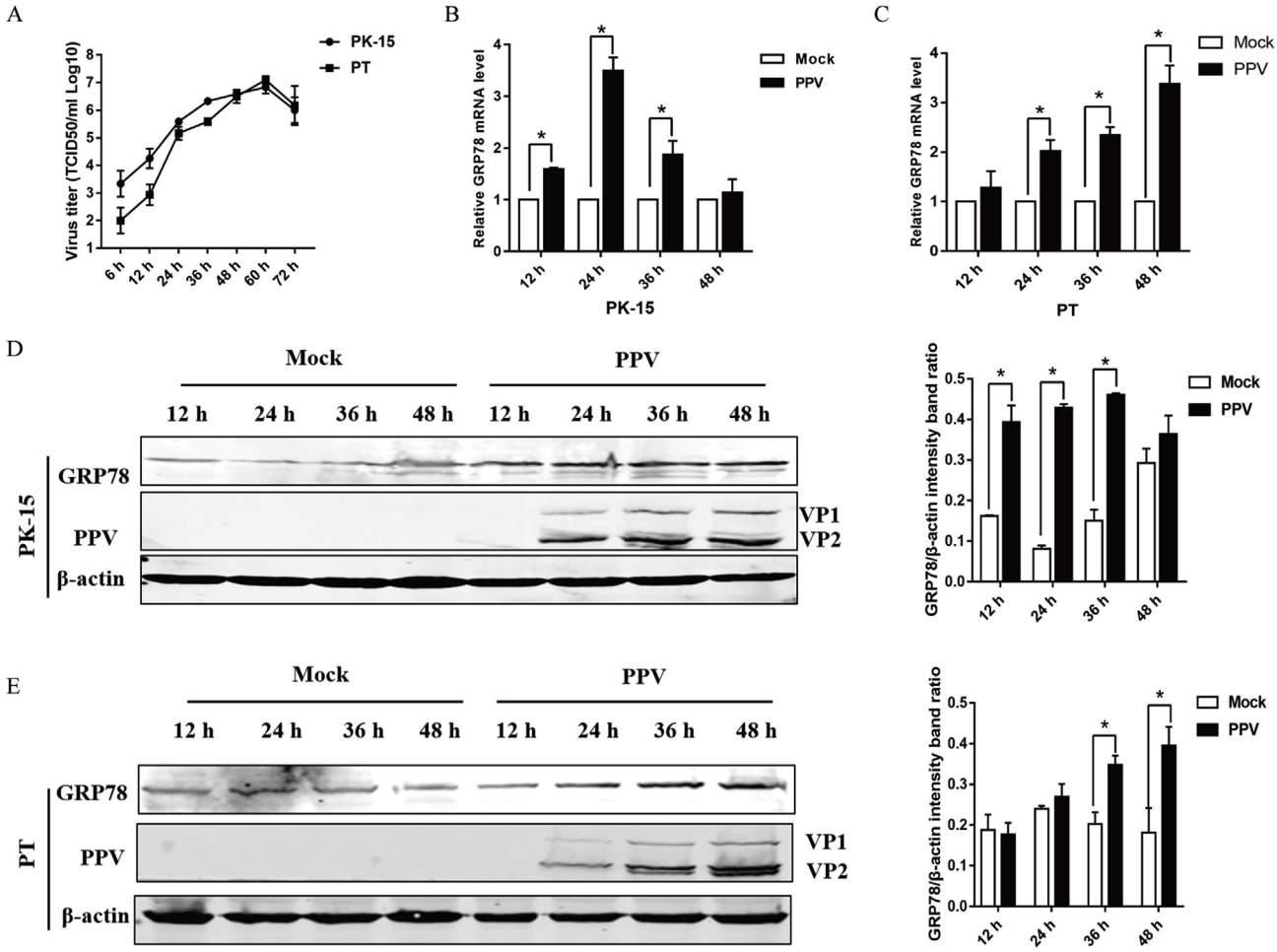

Fig. 1. PPV infection induces ER stress in PK-15 and PT cells. (A) Growth curves of PPV strain TJ in PK-15 and PT cells. PK-15 and PT cells were inoculated with PPV at an MOI of 0.1 and 0.01, respectively. At 6, 12, 24, 36, 48, 60, and 72 hpi, the viruses were harvested for virus titration by TCID50. (B and C) Real-time PCR analysis of GRP78 mRNA from mock- or PPV-infected PK-15 and PT cells at an MOI of 0.1 or 0.01, respectively, and harvested at 12, 24, 36, or 48 hpi. The fold induction of GRP78 was calculated using β-actin as an internal reference and normalized to the mock-infected samples. *$p < 0.05$ vs. the mock-infected control. (D and E) Western blotting was performed with GRP78 and PPV VP2 or VP1 proteins using lysates from mock- or PPV-infected PK-15 or PT cells. The band intensities of GRP78 were determined by densitometry and normalized to the intensities of the corresponding β-actin bands. *$p < 0.05$ vs. the mock-infected control. The experiment was repeated three times.

amplified by RT-PCR and digested with *PstI*. As shown in Fig. 2E and F, the spliced form of the XBP1 product (XBP1S) was increased, while the expression of unspliced XBP1 (XBP1U) was decreased in the control cells treated with Tg, whereas no increase of XBP1S was observed in cells infected with PPV or the mock infection groups. Taken together, the combined real-time PCR, RT-PCR, and western blot results suggest that ER stress induction was correlated with PPV infection, which was mainly mediated through the PERK pathway, but not the IRE1 and ATF6 pathways.

3.3. Activation of ER stress inhibits PPV replication

To determine the impact of ER stress activation on infection with PPV strain TJ, the infected cells were treated with the ER stress-specific inducers Tg and Tu. Tg inhibits sarcoendoplasmic reticulum Ca^{2+} ATPase, which induces ER stress by increasing intracellular free Ca^{2+} levels (Ali et al., 1985). Tu is an antibiotic that induces ER stress by blocking the formation of N-glycosidic linkages in newly synthesized polypeptides (Oyadomari and Mori, 2004). The cytotoxic effects of Tg and Tu were examined using the CCK-8 assay. Incubation of PK-15 and PT cells with 1 μM Tg or 2 μg/mL Tu for 24 h resulted in no significant toxicity (Fig. 3A). Treatment of PK-15 and PT cells with Tg and Tu significantly enhanced the expression of GRP78, as compared with DMSO, suggesting that Tg and Tu could activate ER stress in PK-15 and PT cells (Fig. 3B and C). To evaluate the effects of Tg and Tu on viral infection, IFA was used. As shown in Fig. 3D, treatments with Tg and Tu obviously decreased the sizes of the fluorescent foci both in PPV-infected PK-15 and PT cells, and the inhibitory effect of Tg was more obvious than that of Tu. This result clearly indicates that activation of ER stress with ER stress-specific inducers inhibited the production of PPV strain TJ progeny.

3.4. Inhibition of PERK, eIF2α, and ATF4 expression promotes PPV infection

Our studies revealed that PPV infection induces ER stress through the PERK signaling pathway. To investigate the effect of the PERK signaling pathway on PPV replication, specific shRNA targeting PERK, and specific siRNA targeting eIF2α and ATF4 were designed and synthesized. The knockdown efficiency of shRNA against PERK and siRNA against eIF2α and ATF4 was confirmed by western blot analysis (Fig. 4A, D, and G). Because the data indicated that knockdown efficiency was greatest with PERK-specific shRNA #4 (shPERK-#4), eIF2α-specific siRNA #2 (sieIF2α-#2), and ATF4-specific siRNA #2 (siATF4-#2), these specific siRNA were used in the following experiments. Next, PK-15 cells were transfected with specific siRNAs or NC, followed by

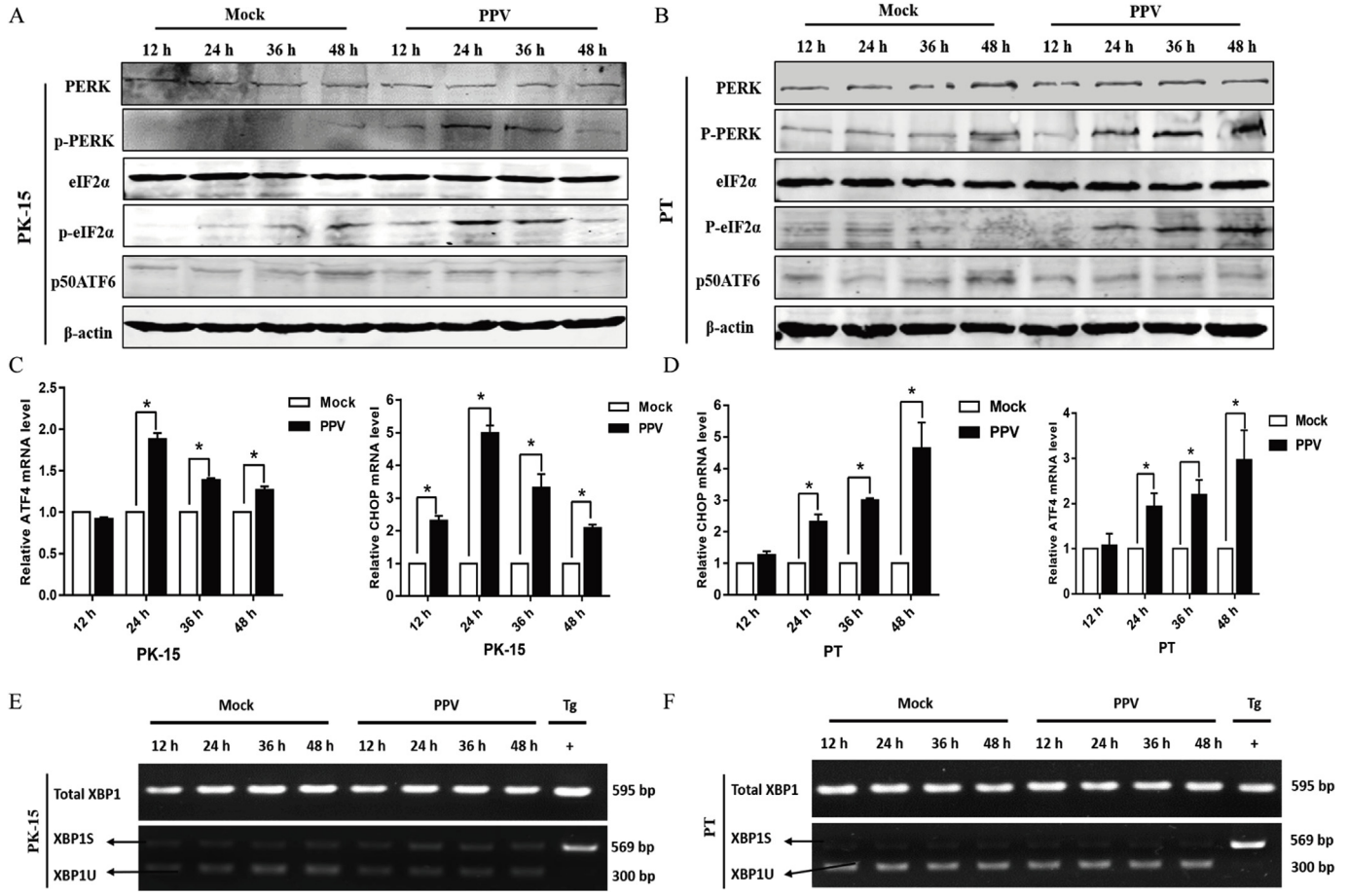

Fig. 2. PPV infection induces ER stress through the PERK signaling pathway. PK-15 and PT cells were mock infected or infected with PPV at an MOI of 0.1 or 0.01, respectively, and harvested at 12, 24, 36, or 48 hpi. (A and B) Western blotting was performed for PERK, p-PERK, eIF2α, p-eIF2α, and p50ATF6 proteins using lysates from PPV mock-infected or infected cells. β-actin was used as a loading control. (C and D) Real-time PCR analysis of ATF4 and CHOP mRNA from PK-15 and PT cells mock infected or infected with PPV. The fold inductions of ATF4 and CHOP were calculated using β-actin as an internal reference and normalized to the mock-infected samples. $^{*}p < 0.05$ vs. the mock-infected control. (E and F) RT-PCR analysis of XBP1 splicing in PK-15 and PT cells infected with PPV. As a positive control, PK-15 and PT cells were treated with 1 μM Tg for 6 h. The PCR products were digested with *PstI* and the digested PCR products resolved by electrophoresis with 2% agarose gels. XBP1S (569 bp) is spliced and XBP1U (300 bp) is unspliced. The experiment was repeated three times.

PPV infection. As shown in Fig. 4B, E, and H, knockdown of PERK, eIF2α, and ATF4 significantly increased PPV VP2 protein expression. $TCID_{50}$ assays also showed that the viral titers increased during depletion of endogenous PERK, eIF2α, and ATF4 (Fig. 4C, F, and I).

3.5. Effect of the pro-apoptotic gene CHOP on viral replication

CHOP is a nuclear transcription factor downstream of ATF4 that can induce apoptosis under prolonged or severe ER stress (Marciniak et al., 2004; Zinszner et al., 1998). To investigate the role of CHOP in the replication of PPV, we analyzed the relationship between its expression and viral infection in PK-15 cells. PK-15 cells were transfected transiently with the empty vector pCAGGS-HA or HA-CHOP and subsequently infected with PPV. Western blot results showed that PPV VP2 protein expression was significantly decreased in HA-CHOP-transfected cells (Fig. 5A). As expected, PPV infection and overexpression of CHOP led to a decrease in Bcl2 protein expression, suggesting that PPV and CHOP can induce apoptosis (Fig. 5A). We also examined the effect of HA-CHOP overexpression on PPV titers with the use of the $TCID_{50}$ assay. As shown in Fig. 5B, PPV titers were $10^{5.8}$ $TCID_{50}$/mL in the vector-transfected cells, but were obviously reduced to $10^{4.3}$ $TCID_{50}$/mL in PPV-infected cells transfected with HA-CHOP. These observations suggest that overexpression of the CHOP protein is capable of blocking PPV replication in PK-15 cells.

To further elucidate the importance of CHOP in the inhibition of PPV replication, we disrupted CHOP expression with the use of shRNA. PK-15 cells were transfected with shRNA targeting the CHOP gene. The expression of CHOP mRNA was determined by real-time PCR. As shown in Fig. 5C, the mRNA expression of CHOP was significantly suppressed in cells transfected with shCHOP, as compared with NC. The knockdown efficiency of shCHOP-#3 was greatest and used in the following experiments. The western blot data showed that the PPV VP2 and Bcl2 proteins were dramatically increased in CHOP shRNA-transfected cells (Fig. 5D). Additionally, the PPV titer in CHOP knockdown cells was confirmed by measuring the $TCID_{50}$. Silencing the endogenous expression of CHOP in PK-15 cells increased the efficiency of PPV infection (Fig. 5E). Taken together, these data demonstrate that CHOP is a key factor and can inhibit PPV replication.

3.6. PPV-induced ER stress accelerates apoptotic cell death and suppresses viral replication

Previous studies have reported that PPV infection can induce apoptosis through the activation of mitogen protein kinases, p53, and mitochondria-mediated pathway (Zhao et al., 2016; Zheng et al., 2018). Nutlin-3 is an agonist that can induce p53- and caspase-dependent cell apoptosis (Thompson et al., 2004; Vassilev et al., 2004). The cytotoxic effects of nutlin-3 were examined using the CCK-8 assay. Incubation of

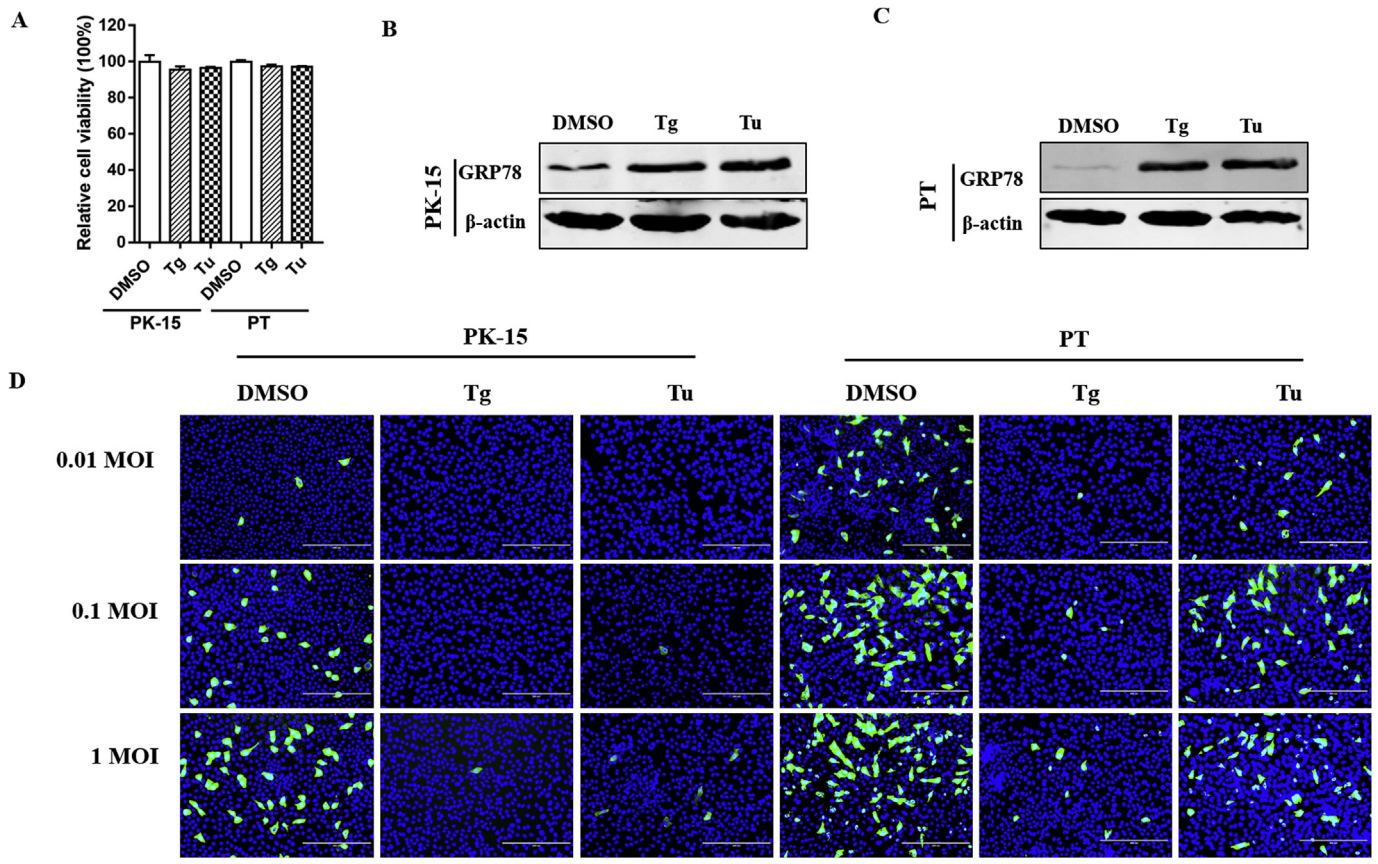

Fig. 3. ER stress activation inhibited PPV infection of both PK-15 and PT cells. (A) Cell viability was detected using the CCK-8 assay after treatment with 1 μM Tg or 2 μg/mL Tu for 24 h. (B and C) PK-15 and PT cells treated with 1 μM Tg or 2 μg/mL Tu for 6 h were analyzed by western blotting for GRP78 and β-actin. (D) IFA to detect PPV infection. DMSO, 1 μM Tg, or 2 μg/mL Tu was added during viral adsorption and maintained at that concentration after infection. PK-15 and PT cells were infected with PPV at an MOI of 0.01, 0.1, or 1. At 24 hpi, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and then stained with PPV VP2 McAb (green). Nuclei were stained with DAPI (blue).

PK-15 cells with 10 or 20 μM nutlin-3 for 24 h resulted in no significant toxicity (Fig. 6A). Next, we examined the effect of nutlin-3 on its ability to induce apoptosis in mock- and PPV-infected PK-15 cells. As shown in Fig. 6B, the apoptosis rate of mock- and PPV-infected cells increased with increasing nutlin-3 concentrations (10 and 20 μM). The apoptotic rates were 5.58% and 14.15% in mock- and PPV-infected cells treated with DMSO, respectively. These data suggest that the PPV strain TJ can induce apoptosis of PK-15 cells. When the PK-15 cells were treated with 10 and 20 μM nutlin-3, the apoptosis rates were 8.05%, and 9.3% in mock-infected cells, and 16.67%, and 24.12% in PPV-infected cells, respectively. In addition, nutlin-3 treatment decreased PPV replication and VP2 protein production (Fig. 6C and D). PPV titers were $10^{5.8}$ $TCID_{50}$/mL in DMSO-treated control cells, while the PPV titers of infected cells treated with nutlin-3 (10 and 20 μM) were significantly reduced to 10^{5} and $10^{3.8}$ $TCID_{50}$/mL, respectively. These results demonstrated that PPV strain TJ could induce apoptosis, which negatively regulates PPV replication.

Having found that PPV replication is blocked by the death-related transcription factor CHOP, we suspected that apoptosis has a defensive role against PPV infection under ER stress. Here, we confirmed the relationship between PPV-induced ER stress, apoptosis, and viral replication. As shown in Fig. 6E, Tg resulted in increased GRP78 and decreased Bcl2 protein levels in mock and PPV-infected cells, which caused Tg reduction of PPV VP2. As expected, the enhancement of ER stress significantly promoted apoptosis and inhibited PPV infection. Flow cytometric analysis was performed to clarify whether PPV-induced ER stress accelerates apoptotic cell death. As shown in Fig. 6F, Tg treatment significantly increased the apoptosis rate of both PPV- and mock-infected cells when compared with DMSO-treated control cells. Annexin V and PI staining showed that the apoptosis rates were 4.77% and 13.53% in DMSO and Tg-treated mock-treated cells, and 14.26% and 18.29% in DMSO- and Tg-treated PPV-infected cells, respectively. These data indicate that ER stress promotes apoptotic cell death, which was significantly enhanced during PPV infection. Collectively, the data demonstrate that PPV-induced ER stress accelerates apoptotic cell death and blocks viral replication.

4. Discussion

During viral infection, the virus/host cell interaction can trigger cellular stress responses, including the innate immune response, as well as oxidative- and ER stress-related responses, which result in antiviral responses as well as promote viral replication and maximize viral progeny production. However, despite years of study, little is known about the cellular stress responses to PPV infection in PPV replication and pathogenesis. The results of the present study demonstrated that PPV activates ER stress in PK-15 and PT cells, and that the potential mechanisms of PPV-mediated ER stress activation are regulated by the PERK signaling pathway. Furthermore, PPV-induced ER stress accelerates apoptotic cell death and suppresses PPV replication (Fig. 7).

ER stress and the UPR facilitate the replication of many viruses. For instance, bovine viral diarrhea virus (Jordan et al., 2002), Japanese encephalitis virus (Wu et al., 2011), dengue virus (Wati et al., 2009), hepatitis E virus (Nair et al., 2016), porcine circovirus 2 (Zhou et al., 2016), African swine fever virus (Netherton et al., 2004), and human cytomegalovirus (Isler et al., 2005) have evolved strategies to exploit

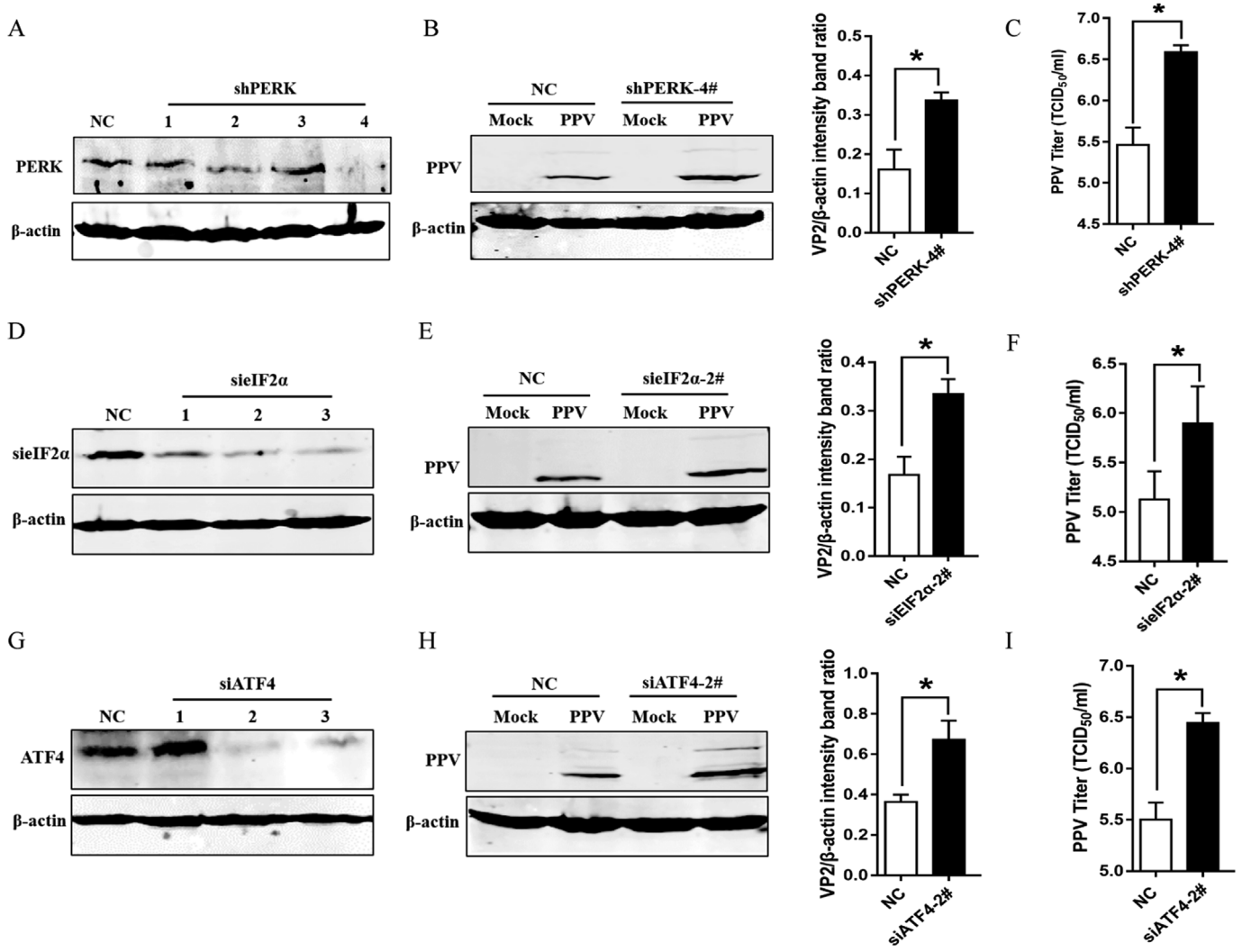

Fig. 4. Knockdown of endogenous PERK, eIF2α, and ATF4 expression by shRNA or siRNA promoted PPV replication. (A, D, and G) The silencing efficiency of PERK, eIF2α, and ATF4 by shRNA or siRNA were analyzed by western blot analysis. Detergent lysate from PK-15 cells transfected with PERK-specific shRNA, eIF2α, ATF4-specific siRNA or NC for 24 h were subjected to immunoblotting with Abs against PERK, eIF2α, ATF4, or β-actin (loading control). (B, E, and H) PPV VP2 protein was increased in PERK, eIF2α, and ATF4-knockdown cells. PK-15 cells were transfected with shPERk-#4, sieIF2α-#2, siATF4-#2, or NC for 24 h and then infected with PPV at an MOI of 0.1. At 24 hpi, the expression of PPV VP2 was detected by western blot analysis. Relative levels of VP2 were estimated by densitometric scanning after normalization against β-actin. $^*p < 0.05$ vs. the NC group. (C, F, and I) PPV titers on PK-15 cells transfected with shPERK-#4, sieIF2α-#2, siATF4-#2, or NC were calculated and expressed as $TCID_{50}$/mL. The results are presented as the mean ± SD of three independent experiments. $^*p < 0.05$ vs. the NC groups.

ER stress and the UPR to optimize replication and maximize viral progeny production. However, a study of vesicular stomatitis virus demonstrated that ER stress activation inhibited viral infection (Baltzis et al., 2004). Previous studies have shown that treatment with the ER stress inducer MG132 inhibited infection by PPV strain NADL-2, similar to the results observed with PPV strain Kresse infection at a low MOI (Boisvert et al., 2010; Meszaros et al., 2017). Meszaros et al. also showed that the specific ER stress inducer Tg inhibited infection by PPV strain Kresse. However, ER stress-inducing chemicals (MG132 and dithiothreitol) accelerated the release and spread of PPV strain Kresse at a high MOI (Meszaros et al., 2017). In our study, inducing the ER stress pathway by ER stress-inducing chemicals (Tg and Tu), interfered with PPV strain TJ infection of PK-15 and PT cells at MOIs of 0.01, 0.1, and 1, suggesting that activation of the ER stress pathway is essential to prevent viral infection. During PPV infection of cells at a high MOI (e.g., MOI of 3), Tg had an inhibitory effect on viral infection in both PK-15 and PT cells. However, the inhibitory effect of Tu on viral infection occurred in PK-15 cells, but not PT cells. Tu treatment of PPV strain TJ infection at a high MOI in PT cells had similar effect or even enhanced viral infection, as compared with DMSO-treated control cells (data not shown). This observation suggests that the mechanisms by which ER stress regulates PPV replication are related to the virus strain, the dose of the virus, and the infected cells.

Downstream signaling of the UPR involves three distinct pathways (PERK, IRE1, and ATF6), which are activated in response to ER stress (Ron and Walter, 2007). Viruses regulate ER stress activation via different mechanisms. Activation of ER stress is usually mediated by all three signaling pathways or one or two pathways during virus infection. Transmissible gastroenteritis virus, Borna disease virus, and dengue virus activate ER stress by targeting three branches of the UPR (Lee et al., 2018; Williams and Lipkin, 2006; Xue et al., 2018). Newcastle disease virus induces activation of ER stress via the PERK and ATF6 pathways, but not XBP1 (Cheng et al., 2016). ER stress activation by Herpes simplex virus 1, bovine viral diarrhea virus, and porcine circovirus 2 are mediated mainly by the PERK signaling pathway (Cheng et al., 2005; Jordan et al., 2002; Zhou et al., 2016). IRE1-alpha (IRE1α) and XBP1 play important roles in the activation of the hepatitis B virus S promoter by ER stress (Huang et al., 2005). Moreover, IRE1α is

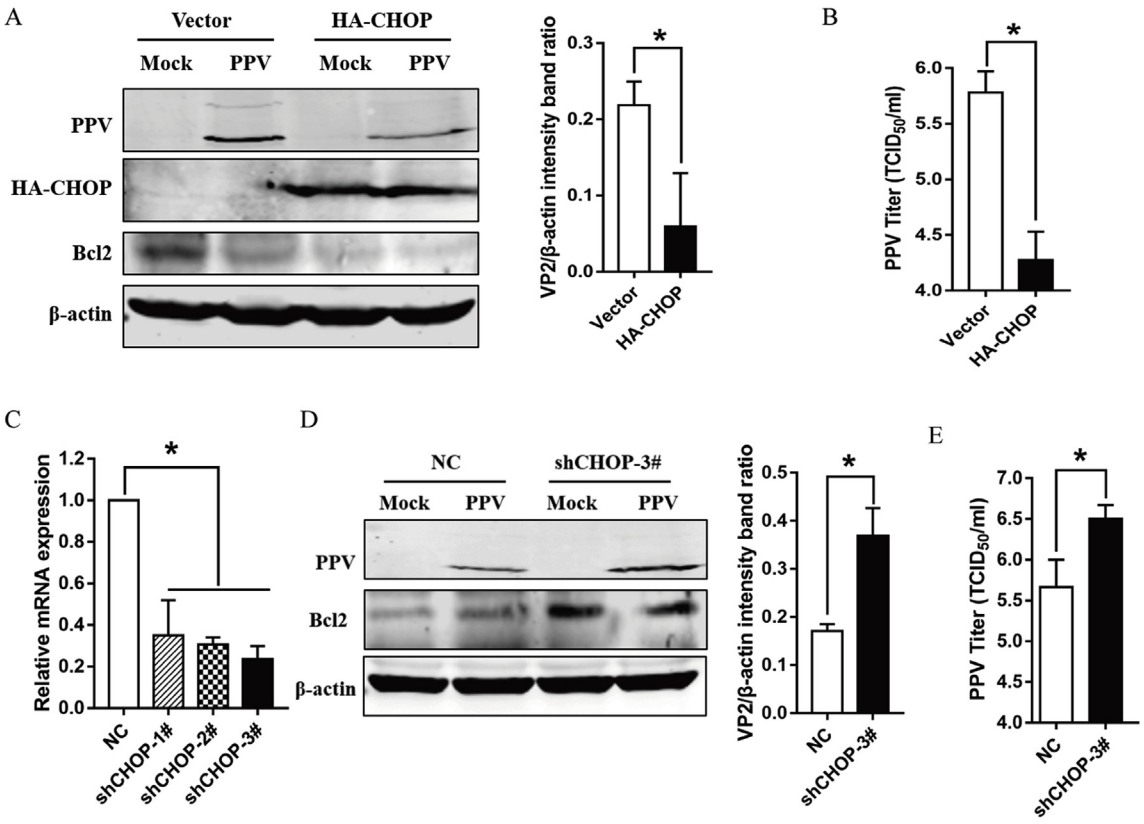

Fig. 5. Effects of CHOP overexpression or knockdown on PPV replication. PK-15 cells were transfected with a vector expressing CHOP (HA-CHOP) or CHOP shRNA. After 24 h of transfection, the cells were infected or mock-infected with PPV at an MOI of 0.1 for an additional 24 h. (A) Western blot analysis indicated that CHOP overexpression decreases the level of PPV VP2 and Bcl2 expression. Whole cell lysates were subjected to western blot analysis of PPV VP2, Bcl2, HA tag, and β-actin. Relative levels of VP2 were estimated by densitometric scanning after normalization against β-actin. $*p < 0.05$ vs. the NC group. (B) CHOP overexpression inhibited PPV replication as determined by the $TCID_{50}$ assay. (C) CHOP knockdown efficiency was determined by real-time PCR. (D) Knockdown of CHOP increased the expression of PPV VP2 and Bcl2. Relative levels of VP2 were estimated by densitometric scanning after normalization against β-actin. $*p < 0.05$ vs. the NC group. (E) Knockdown of CHOP increased PPV replication as determined with the $TCID_{50}$ assay. The results are presented as the mean ± SD of three independent experiments. $*p < 0.05$ vs. the NC group.

activated in cells infected with the avian coronavirus infectious bronchitis virus and protects the infected cells from apoptosis by modulating the UPR (Fung et al., 2014). Recent evidence indicates that PPV strain Kresse infection could activate XBP1 or CHOP expression (Meszaros et al., 2017). Our study demonstrated that PPV infection enhanced the mRNA levels of the UPR marker molecules ATF4 and CHOP, but not that of spliced XBP1 (Fig. 2). Therefore, it is possible that the activation of XBP1 has a different mechanism for the regulation of different PPV strains. Western blot analysis showed that PPV infection increased the phosphorylation levels of PERK and its downstream molecule eIF2α (Fig. 2). However, the ATF6 protein was not cleaved during PPV infection. This result indicates that the PERK signaling pathway is involved in ER stress activation in PK-15 and PT cells infected with PPV. By identifying the signaling events of the UPR pathway in the regulation of PPV replication, we found that PERK, eIF2α, and ATF4 promoted PPV replication using siRNAs. It has been shown that PPV primarily interacts with the PERK signaling molecules eIF2α and ATF4 to suppress viral replication.

In response to prolonged and severe ER stress, the UPR can trigger apoptosis. Previous studies have shown that PPV infection induces both ER stress and apoptosis (Barber et al., 2017; Boisvert et al., 2010; Huang et al., 2014; Meszaros et al., 2017). However, there is no evidence of the relationship among PPV-induced ER stress, apoptosis, and viral replication. In our case, PPV strain TJ induced apoptosis of PK-15 cells at a rate of 14.26% at 24 hpi (Fig. 6B). Also, apoptotic cell death was promoted by the apoptosis inducer (nutlin-3), which inhibited PPV strain TJ infection of PK-15 cells, suggesting that apoptosis negatively regulates PPV infection (Fig. 6C and D). CHOP is a death-related transcription factor that can promote cell apoptosis upon ER stress activation (Marciniak et al., 2004; Zinszner et al., 1998). It was clear that PPV strain TJ infection could increase the expression of CHOP (Fig. 2C and D). Overexpression of porcine CHOP clearly inhibited PPV replication and the titer was decreased by more than 32-fold (Fig. 5B). Moreover, transfection of PK-15 cells with shRNA targeting the CHOP gene promoted viral replication and increased the titer by 6.3-fold (Fig. 5E). Taken together, these data suggest that PPV infection can lead to the induction of CHOP and that CHOP has an essential role in the cellular antiviral response. It is likely that the expression of CHOP is increased in infected cells, which leads to accelerated apoptosis of infected cells and inhibits viral replication and viral progeny production. In addition, PPV strain TJ infection induced ER stress-accelerated apoptotic cell death, which contributes to the inhibition of PPV replication (Fig. 6E and F). Therefore, we identified a mechanism whereby PPV strain TJ-induced ER stress blocked viral replication by accelerating apoptotic cell death.

In summary, the aim of the present study was to expound the potential mechanism by which PPV strain TJ infection induces ER stress activation *in vitro* and observe the effect of ER stress on PPV replication. Our studies provide the first evidence that the PPV strain TJ activates ER stress via the PERK/eIF2α/ATF4/CHOP signaling pathway in PK-15 and PT cells, and that ER stress activation inhibits PPV replication *in vitro* by promoting apoptotic cell death. Depletion of the endogenous signaling events of the PERK signaling pathway, including PERK, eIF2α, ATF4, and CHOP significantly increase viral replication. These data lay

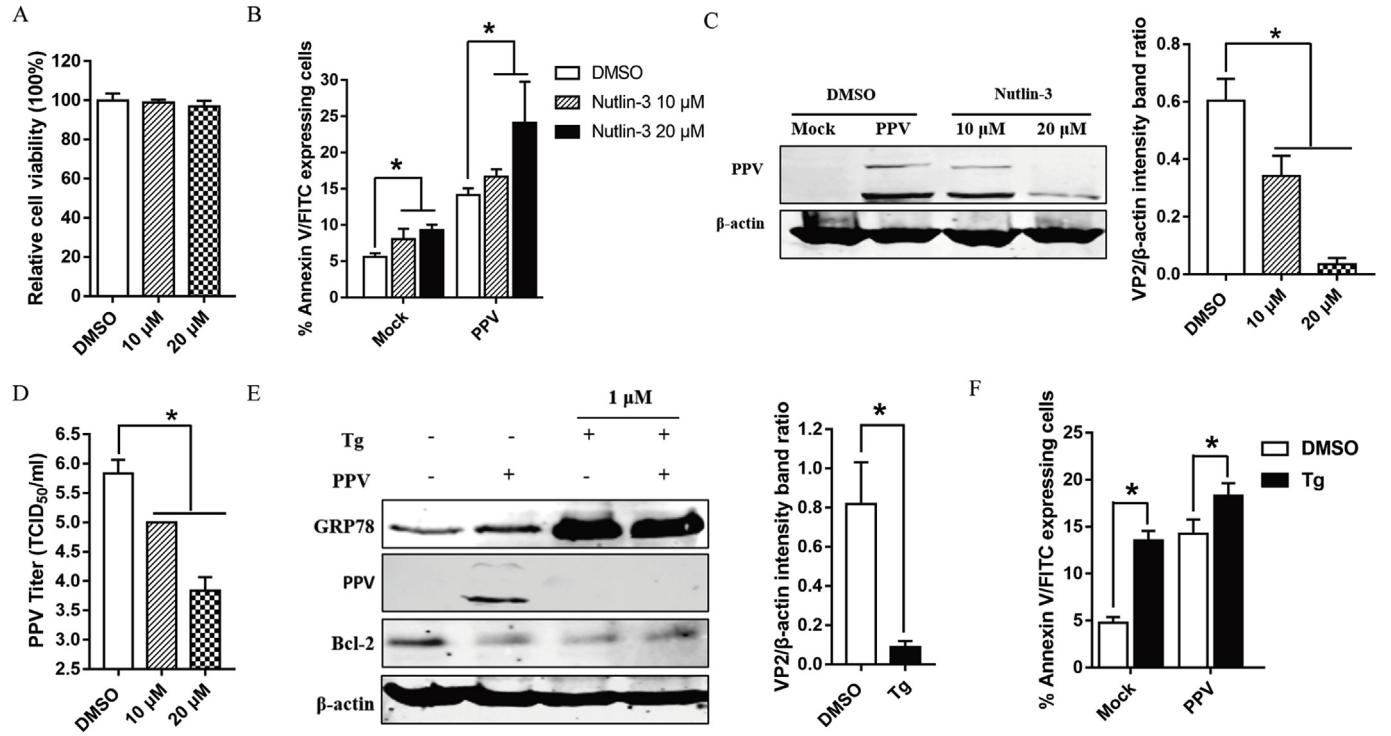

Fig. 6. PPV induced ER stress promoted apoptosis and affected the viral titer. (A) Cell viability was detected using the CCK-8 assay after treatment with 10 or 20 μM nutlin-3 for 24 h. (B) The apoptotic rate of mock-infected and PPV-infected cells induced by nutlin-3. Cells infected with 0.1 MOI of PPV and treated with 10 and 20 μM nutlin-3 were harvested, stained with Annexin V-FITC and PI at 24 hpi, and analyzed by flow cytometry. Annexin V- and PI-positive cells were regarded as apoptotic. (C) Levels of PPV VP1 or VP2 and β-actin proteins as determined by western blot analysis. Cells infected with 0.1 MOI of PPV and treated with 10 and 20 μM nutlin-3. At 24 hpi, cells lysates were subjected to western blot analysis. Mock-infected and PPV-infected DMSO-treated cells served as the negative and positive controls, respectively. The band intensities of PPV VP2 determined by densitometry and normalized to the intensities of the corresponding β-actin bands. (D) Viral titers were determined with the $TCID_{50}$ assay. Representative data from three independent experiments are presented as the mean ± SD. $*p < 0.05$ vs. the DMSO control. (E) PK-15 cells were treated with Tg and infected with PPV at an MOI of 0.1. The lysates of PK-15 cells were subjected to western blot analysis using specific Abs against GRP78, Bcl2, PPV VP2, and β-actin. Relative levels of VP2 were estimated by densitometric scanning after normalization against β-actin. $*P < 0.05$ vs. the NC group. (F) Apoptosis rate of PPV-infected PK-15 cells treated with Tg. Cells infected with 0.1 MOI of PPV and treated with DMSO or 1 μM Tg. Cells were harvested and stained with Annexin V-FITC and PI at 24 hpi and analyzed by flow cytometry. Cells positively stained with annexin V and PI were regarded as apoptotic.

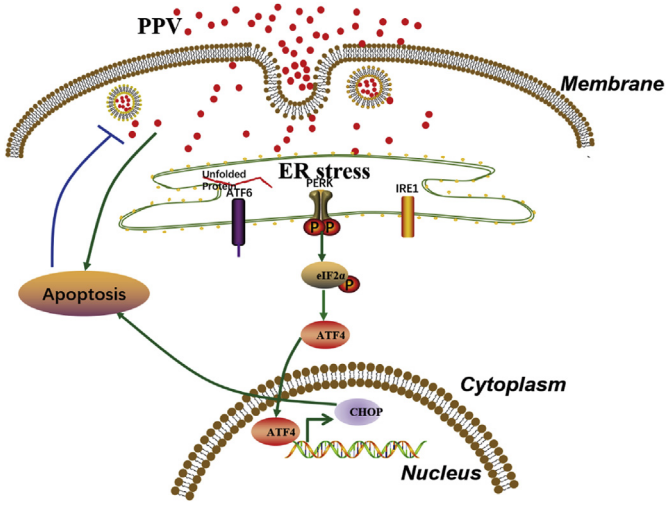

Fig. 7. A schematic model of the viral inhibition by the PERK-eIF2α-ATF4-CHOP pathway during PPV infection. PPV strain TJ infection induces ER stress and apoptosis. ER stress induction was associated with PPV infection and mainly mediated by the PERK-eIF2α-ATF4-CHOP pathway, but not the IRE1 and ATF6 pathways. PPV-induced ER stress accelerates apoptotic cell death and suppresses PPV replication.

the foundation for future studies to better understand the biological characteristics in relation to PPV infection and develop new antiviral drugs against PPV.

Declaration of competing interest

The authors declare that they have no conflicts of interest about this article.

Acknowledgments

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