Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
Critical role of cytochrome c1 and its cleavage in porcine reproductive and respiratory syndrome virus nonstructural protein 4-induced cell apoptosis via interaction with nsp4

ZHANG Feng, GAO Peng, GE Xin-na, ZHOU Lei, GUO Xin, YANG Han-chun

Key Laboratory of Animal Epidemiology of the Ministry of Agriculture, College of Veterinary Medicine, State Key Laboratory of Agrobiotechnology, China Agricultural University, Beijing 100193, P.R.China

Abstract
Porcine reproductive and respiratory syndrome virus (PRRSV) actively induces cell apoptosis both in vitro and in vivo, which can contribute critically to viral pathogenesis. Previous studies have shown that the PRRSV nonstructural protein 4 (nsp4) is an important mediator of this process, but the underlying molecular details remain poorly understood. In this study, we found that the PRRSV nsp4 interacted with the mitochondrial inner membrane protein cytochrome c1 (cyto.c1) and induced its proteolytic cleavage. Interestingly, the cleaved N-terminal fragment of cyto.c1 was found to exert apoptotic activity, which could cause mitochondrial fragmentation, resulting in apoptotic cell death. And RNA interference (RNAi) silencing experiments further confirmed the crucial role which cyto.c1 played in nsp4- and PRRSV-induced cell apoptosis. Thus, our data provide an important piece of mechanistic clues for PRRSV-induced cell apoptosis and also elucidate a novel mechanism for the 3C-like proteases in this finding.

Keywords: PRRSV, nonstructural protein4 (nsp4), cytochrome c1 (cyto.c1), interaction, cleavage, apoptosis

1. Introduction
Apoptosis is an intrinsic cellular defense to restrict virus infection. Accordingly, many viruses have evolved to encode gene products that can efficiently limit this mechanism long enough for production of enough amounts of progeny virions (Teodoro and Branton 1997; O’Brien 1998; Roulston et al. 1999; Galluzzi et al. 2008; Neumann et al. 2015). On the other side, some viruses have developed specific mechanisms to actively induce apoptotic cell death (Teodoro and Branton 1997; O’Brien 1998). This process often takes place in the late stage of virus infection, which not only facilitates virus release and spread but also avoids triggering an inflammatory response (Teodoro and Branton 1997). A growing number of viruses, including arteriviruses (Miller and Fox 2004), coronavirus (Lin et al. 2006), and picornaviruses (Barco et al. 2000; Zaragoza et al. 2006), have been added to this list. The focus of this report is porcine reproductive and respiratory syndrome virus (PRRSV), an enveloped RNA virus of positive polarity from the family Arteriviridae in the order Nidovirales (Kuhn et al. 2016).
PRRSV is an economically critical pathogen of swine, which mainly causes reproductive failure in pregnant sows and respiratory distresses in young pigs, namely the porcine productive and respiratory syndrome (PRRS) (Albina 1997; Rossow 1998; Zhou and Yang 2010). Consequently, the disease costs pork producers 500–600 million USD each year in North America (Neumann et al. 2005). In Asia, the swine industry has been hit hard by the Chinese highly pathogenic PRRSV (HP-PRRSV) and the most recent NADC30-like variants (Tian et al. 2007; Zhou and Yang 2010; Zhao et al. 2005). The Chinese HP-PRRSV exhibits enhanced virulence and expanded tissue, and the infections of pigs often cause lesions of multiple organs (Tian et al. 2007; Wang et al. 2011, 2013; He et al. 2012).

In particular, the HP-PRRSV infections often cause severe thymus atrophy of piglets, leading up to 90% reduction in size characterized by cortical depletion of thymocytes and a significant drop of the CD4+CD8+ subpopulation (Wang et al. 2011; He et al. 2012; Guo et al. 2013; Li et al. 2014a). PRRSV infections also result in apoptosis of macrophages in lungs and lymph nodes as well as the germ cells in testes (Sur et al. 1997, 1998; Sirinarumitr et al. 1998; Feng et al. 2002; Wang et al. 2011, 2013; He et al. 2012). In particular, the HP-PRRSV infections often cause severe thymus atrophy of piglets, leading up to 90% reduction in size characterized by cortical depletion of thymocytes and a significant drop of the CD4+CD8+ subpopulation (Wang et al. 2011; He et al. 2012; Guo et al. 2013; Li et al. 2014a).

The molecular details of PRRSV-induced apoptosis remain poorly understood. An earlier study identified the viral major envelope glycoprotein GP5 as an apoptosis inducer (Suarez et al. 1996; Fernandez et al. 2002; Gagnon et al. 2002), but several later studies could not repeat this observation (Lee et al. 2004; Mu et al. 2015). While our work was underway on screening of viral components responsible for apoptotic cell death (Yuan et al. 2016), Ma et al. (2013) reported a link to apoptosis induction by PRRSV nsp4, a 3C-like protease that has homologues across diverse families of positive-stranded RNA viruses and is required for viral replicase polyprotein maturation.

In this report, we extended this observation and went on further to show that the PRRSV nsp4 could interact with the mitochondrial inner membrane protein cytochrome c1 (cyto.c1) and induced its cleavage. Unexpectedly, the cleaved N-terminal fragment of cyto.c1 was found to be a strong apoptosis inducer.

2. Materials and methods

2.1. Cells and viruses

MARC-145, HEK-293FT and Vero cells were all maintained at 37°C in Dulbecco’s Modified Eagle medium (DMEM) supplemented with 10% FBS and penicillin (50 U mL⁻¹)-streptomycin (50 mg mL⁻¹). Primary porcine pulmonary alveolar macrophages (PAMs) were prepared from the lung lavage fluids of 5 to 6 week-old specific-pathogen-free (SPF) pigs as previously described (Zhang et al. 2009). PAMs were maintained at 37°C in RPMI 1640 medium (Invitrogen, CA, USA) supplemented with 10% FBS and penicillin (50 U mL⁻¹)-streptomycin (50 mg mL⁻¹). The high pathogenic (HP) PRRSV JXwn06 and low virulent PRRSV strain HB-1/3.9 used in this study have been described previously (Zhou et al. 2009).

2.2. Antibodies and reagents

Mouse anti-HA monoclonal antibody (H3663), anti-Flag mAb (F9291), rabbit anti-Flag polyclonal antibody (SAB4301135), mouse anti-β-actin (A5441), and carbobenzoxy-valyl-alanaryl-aspartyl-[O-methyl]-fluoromethyl-yiketone (Z-VAD-FMK) (V116) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Rabbit anti-PARP mAb (9532) and anti-caspase-3 mAb (9665) were obtained from the Cell Signaling Technology (Danvers, MA, USA). Mouse anti-green fluorescent protein (GFP) mAb (66002-1-Ig) and rabbit anti-cytochrome c1 polyclonal antibodies (10242-1-AP) were purchased from Proteintech (Chicago, IL, USA). GFP bead was purchased from ChromoTek (Planegg-Martinsried, Germany). Staurosporine (S102392) was purchased from Aladdin (Shanghai, China). Mouse anti-PRRSV nsp4 mAb was prepared in our laboratory.

2.3. Plasmids construction

The plasmids pGBK77-nsp4 and pHA-nsp4 were created by cloning the nsp4 gene from HP-PRRSV JXwn06 strain into the vector pGBK77 and pCMV-HA, respectively (Clontech, CA, USA). In both cases, nsp4 was expressed as a fusion protein. In the plasmid pGBK77-nsp4, nsp4 was fused to the sequence coding for the DNA-binding domain of GAL4, whereas in the plasmid pHA-nsp4, it was in frame with the sequence for a HA epitope tag at its 5’ end. The recombinant plasmids pFlag-cyto.c1, pFlag-cyto.c1 (aa 1–230) and pFlag-cyto.c1 (aa 231–327) were generated by cloning the genes coding for pig cyto.c1 and the corresponding fragments into the vector pCMV-3×Flag. Cyto.c1 and its derivatives were expressed by fusion to an N-terminal Flag
epitope tag. The plasmid pGADT7-cyto.c1 was made by cloning the pig cyto.c1 gene into the plasmid pGADT7 AD (Clontech, CA, USA). Cyto.c1 was fused to the activation domain of GAL4. Site-directed mutagenesis was used to introduce point mutations in the nsp4-coding region of the plasmid pHAnsp4 to make three mutants: pHAnsp4 H39A, pHAnsp4 D64A and pHAnsp4 S118A. The cyto.c1 point mutation mutants (E230A, E252A, E254A, E280A and E282A) were generated by overlapping PCR based on the plasmid pFlag-Cyto.c1. In addition, the plasmid pHAnsp2 was constructed by cloning the nsp2 gene from PRRSV strain JXwn06 into the vector pCMV-HA.

2.4. Lentiviruses transduction

The lentiviral expression system containing pWPXL (foreign gene expressing plasmid), pMD2.G (VSV-G expressing plasmid), and psPAX2 (lentiviral packaging plasmid) was purchased from Addgene (Cambridge, MA, USA). The gene coding for nsp4 was cloned into pWPXL and expressed as a GFP fusion protein. The recombinant viruses were rescued by co-transfection of the three plasmids into HEK-293FT cells by using FuGENE HD Transfection Reagents (Promega, Madison, WI, USA) according to the methods described previously (Du et al. 2016). After a number of syncytia appeared, the supernatants were subsequently harvested, filtered, and concentrated by Amicon ultra-100 centrifuge tubes (Mick Millipore, Billerica, MA). The titers of lentiviruses were measured by a Quick Titer Kit (Cell Biolabs, San Diego, CA) according to the manufacturer’s instructions. To determine the role of cyto.c1 in nsp4-induced cell apoptosis, siRNA-treated MARC-145 cells were transduced with the lentiviruses expressing GFP or GFP-nsp4 in the presence of 8 µg mL⁻¹ polybrene (Sigma, MO, USA). At 48 h post-transduction, the cells were collected for Western blot analysis of caspase-3 and poly ADP-ribose polymerase (PARP) cleavage.

2.5. Yeast two-hybrid screening

Yeast two-hybrid screening for binding partners of HP-PRRSV strain JXwn06 nsp4 was performed according to the method as previously described (Wang et al. 2012). Briefly, the Matchmaker™ Gold Yeast Two-Hybrid System (Clontech, Shiga, Japan) was used according to the manufacturer’s instructions. The pGBKTT7-nsp4 plasmid expressing the fusion protein GAL4-BD-nsp4 was used as bait and the PAM cDNA expression library fusion to the GAL4-activation domain in the pGADT7 plasmid was used as prey. Positive yeast clones were selected on SD-/Adel/-His/-Leu/-Trp medium plates (QDO) containing 0.04 mg mL⁻¹ 5-bromo-4-chloro-3-indoyl-α-D-galactopyranoside (X-α-Gal, Clontech) and 0.07 µg mL⁻¹ aureobasinidin A (ABA, Clontech). Colonies that turned blue were cultured for plasmids extraction. Subsequently, the extracted plasmids were transformed into Escherichia coli DH5α cells for amplification prior to DNA sequencing and bioinformatics analysis with the NCBI BLAST Program. To further verify the results, the respective bait and prey plasmids were co-transformed into the yeast strain Y2HGold and selected on QDO/X/ABA plates.

2.6. Transfections

Vero, MARC-145 or HEK293T cells grown on coverslips in six-well plates were transfection when 60–70% confluent with 2–5 µg DNA per well in mixtures of 7–8 µL of lipofectamine in Opti-MEM. After incubation at room temperature (RT) for 20 min, the DNA-lipofectamine complexes were added directly to cells in antibiotics-free culture medium suplement with 10% FBS.

2.7. Co-immunoprecipitation

The protocol has been described elsewhere (Du et al. 2016). Briefly, for studying the interaction between nsp4 and cyto.c1, pHAnsp4 or the plasmids coding for its derivatives and pFlag-cyto.c1 were transfected into HEK293FT cells either singly or in combination. At 18–24 h post transfection, the cells were harvested and lysed in NP-40 lysis buffer containing 0.5% NP-40, 150 mmol L⁻¹ NaCl, 50 mmol L⁻¹ Tris-HCl (PH 8.0), and protease inhibitor cocktail (P8340, Sigma), and clarified by centrifugation at 12 000 r min⁻¹ for 20 min. The cell supernatants were then precleared with protein A/G sepharose beads (Santa Cruz, CA, USA) and immunoprecipitated using anti-HA beads (Thermo Scientific, Waltham, MA, USA) overnight at 4°C with gentle rotation. The beads were washed three times with the NP-40 lysis buffer and the proteins bound to the beads were separated by SDS-PAGE followed by Western blot analysis.

2.8. Western blot analysis

The amount of total proteins was quantified by BCA Protein Assay Kit (NCI3227CH, Pierce). The same amount of total proteins was subjected to Western blot analysis and β-actin served as a loading control. The protein samples were separated by SDS-PAGE, and then transferred onto PVDF membranes. The membranes were subsequently blocked with PBS-0.05% tween 20 (PBST) containing 5% skimmed milk for 1 h at RT before being probed with appropriate primary antibodies at RT for 2–3 h or at 4°C overnight. After being washed with PBST for 3 times with 10 min each, the membranes were incubated with the proper HRP-conjugated
secondary antibodies at RT for 1 h. Afterwards, the membranes were washed 3 times with 10 min each before being developed with the enhanced chemiluminescence (ECL) reagents (Pierce). HA antibodies were used at a dilution of 1:3000, Flag antibodies were used at a 1:2000 dilution, and cyto.c1 antibodies were used at a dilution of 1:500.

2.9. Confocal microscopy

The procedures have been described elsewhere (Du et al. 2016). Briefly, at 24 h post transfection, Vero or HeLa cells were fixed in 3.7% paraformaldehyde for 7 min, permeabilized for 10 min with PBS containing 0.1% Triton X-100 and 2% BSA at RT for 30 min. The samples were reacted with primary antibodies diluted in PBS containing 2% BSA in a humid chamber. The anti-HA epitope mouse monoclonal antibody was used in a 1:1000 dilution. The anti-Flag epitope antibodies were used in a 1:1000 dilution. After incubation for 1 h at RT, the cells were then rinsed three times with PBS for 5 min each, and reacted for another half hour with Alexa Fluor 488 F (ab’) 2 fragment of goat anti-mouse IgG (H+L) or Alexa Fluor 568 F (ab’) 2 fragment of goat anti-rabbit IgG (H+L) 1 h at RT, or in combination. The cells were washed once with PBS. Nuclear DNA was stained with DAPI (molecular probes) for 5 min. After three times rinses, the samples were mounted in aqua polypeanut media (Polysciences Inc., PA, USA) and examined under Nikon A1 confocal fluorescence microscope.

2.10. Flow cytometry analysis

To analyze the role of cyto.c1 in PRRSV-induced cell apoptosis, MARC-145 cells seeded on six-well plates were first transfected with small interfering RNA (siRNA) targeting cyto.c1 or scrambled control. At 24 h post transfection, the cells were infected with HP-PRRSV JXwn06 at an MOI of 0.1 or transduced with the lentivirus expressing GPF-nsp4. At 48 h post infection, the cell apoptosis was analyzed by Lipofectamine RNAiMax (Invitrogen) according to the manufacturer’s instructions. At 48 h post transfection, the cells were harvested and subject to Western blot analysis using an anti-cyto.c1 antibody. The β actin was used as an internal expression control. For transfection/infection assay, at 24 h post transfection of siRNA, MARC-145 cells are either infected with HP-PRRSV JXwn06 at an MOI of 0.1 or transduced with the lentivirus expressing GPF-nsp4. At 48 h post infection, the cell apoptosis was analyzed by either Western blot or FACS analysis.

3. Results

3.1. PRRSV nsp4 interacts with cyto.c1

PPRSV is capable of inducing apoptosis of in vivo and in vitro (Suarez et al. 1996; Lee and Kleiboeker 2007). Recently, the viral replicase protein nsp4 was reported to be a strong apoptosis inducer (Ma et al. 2013). Our studies on screening of viral components revealed the similar finding (Yuan et al. 2016). To seek the further clue for how PRRSV nsp4 might induce cell apoptosis, we turned to look for its cellular binding partners. We employed a Yeast Two-Hybrid System to screen a cDNA library generated from the porcine alveolar macrophages (PAMs) (Wang et al. 2012). There were 15 binding partners identified from the initial screening (Table 1), and one of them was cyto.c1, a key constituent in the mitochondrial electron transport chain (Hunte et al. 2003). There were three reasons why cyto.c1 was selected for further study. Firstly, it has been reported that PRRSV nsp4 might induce cell apoptosis, we turned to look for its cellular binding partners. We employed a Yeast Two-Hybrid System to screen a cDNA library generated from the porcine alveolar macrophages (PAMs) (Wang et al. 2012). There were 15 binding partners identified from the initial screening (Table 1), and one of them was cyto.c1, a key constituent in the mitochondrial electron transport chain (Hunte et al. 2003). There were three reasons why cyto.c1 was selected for further study. Firstly, it has been reported that PRRSV can induce cell apoptosis through the mitochondria-mediated pathway (Lee and Kleiboeker 2007). Secondly, cyto.c1 has been shown to be involved in cell apoptosis (Zhu et al. 2012). Thirdly, the sequence alignments show that cyto.c1 is highly conserved among human, pigs and monkeys (data not shown).

To further verify the interaction between nsp4 and cyto.c1, we carried out reverse yeast two-hybrid assay. When the plasmids pGBKTT7-nsp4 and pGADT7-cyto.c1 were co-transformed into the yeast Y2HGold cells, the colonies cultivated on the synthetic selection medium QDO/X/ABA plates turned blue (Fig. 1-A), indicating that there is a physical
Table 1  Identified novel binding partners for porcine reproductive and respiratory syndrome virus (PRRSV) nonstructural protein 4 (nsp4) by yeast two hybrid

| Protein name                              | Function                                      |
|-------------------------------------------|----------------------------------------------|
| Zinc finger protein 236                   | Zinc ion binding                             |
| Beta-2-microglobulin-like                 | Not yet                                      |
| RPS20                                     | Poly(A) RNA binding                          |
| LAMP1                                     | Autophagy                                     |
| CD40                                      | Apoptotic signaling pathway                   |
| TRAPPC9                                   | Cellular protein metabolic process            |
| Sodium/potassium-transporting ATPase subunit beta-3-like | ATPase activator activity                  |
| Cytochrome c1                             | Respiratory electron transport chain mitochondria damage |
| EIF4A                                     | Regulation of translational initiation        |
| KHDRBS1 (SAM68)                           | KH domain-containing, RNA-binding, signal transduction |
| Ran binding protein 9                     | GTP-binding nuclear protein                  |
| UBE2E1                                    | Ubiquitin-conjugating enzyme E2E1             |
| ATP1B1                                    | Sodium/Potassium-transporting ATPase         |
| Ferritin heavy chain                      | Cellular iron ion homeostasis                |
| LACTB                                     | Hydrolase activity                           |

interaction between nsp4 and cyto.c1. Next, Flag-cyto.c1 and HA-nsp4 in HEK 293 T cells were co-expressed and the interaction was examined by co-immunoprecipitation assay. As shown in Fig. 1-B, the antibodies to HA could pull down Flag-cyto.c1. Further truncation mutagenesis revealed two regions (aa 1–80 and 80–160) of nsp4 that could independently interact with cyto.c1 (Fig. 1-C).

To further substantiate the binding of nsp4 to cyto.c1, MARC-145 cells were transduced with lentiviruses that were expressing nsp4-GFP and GFP. The cell lysates were immunoprecipitated with an anti-GFP mAb, then detected by anti-cyto.c1 antibody. As shown in Fig. 1-D, the endogenous cyto.c1 could be probed in cells expressing the viral protein.

The interaction between nsp4 and cyto.c1 was also detected within mammalian cells by confocal microscopy (Fig. 1-E). When expressed alone, HA-nsp4 exhibited a diffusive distribution pattern with most in the nucleus where-as Flag-cyto.c1 was localized in the sites that resemble the structures of mitochondria. When co-expressed, nsp4 was re-localized to the puncta structures to become co-localized with Flag-cyto.c1, suggesting that they can find each other in mammalian cells. Together, we conclude that nsp4 and cyto.c1 can interact with each other both in mammalian cells and in vitro.

3.2. PRRSV nsp4 induces proteolytic cleavage of cyto.c1

Interestingly, while analyzing the interaction between nsp4 and cyto.c1, we noticed a fast-moving species of cyto.c1 in the cells co-expressed with nsp4 (Fig. 1-B, lane 1), but it was not present in the cells co-expressed with the vector (Fig. 1-B, lane 3). Since nsp4 possesses the protease activity, this result suggests that the fast migration band may be from the proteolytic cleavage by nsp4. This hypothesis was further confirmed by the following assays. Firstly, as shown in Fig. 2-A, the Flag-cyto.c1 in HEK293T cells were co-expressed with gradually increasing amount of HA-nsp4. And the cleavage of cyto.c1 was dependent on nsp4 in a dose-dependent manner, which suggested a critical role of nsp4 in the cleavage. Secondly, we introduced point mutations to the nsp4 catalytic triad His39-Asp64-Ser118 to construct three mutants: HA-nsp4 H39A, HA-nsp4 D64A, and HA-nsp4 S118A (Fig. 2-B). When tested in HEK293T cells, all three mutants failed to cleave cyto.c1 (Fig. 2-B), suggesting that the protease activity of nsp4 is required for the cleavage. In line with inability to cleave cyto.c1, the nsp4 catalytic mutants failed to induce cell apoptosis in MARC-145 cells (Fig. 2-C).

Thirdly, as a negative control, we investigated whether other PRRSV proteases are also able to cleave cyto.c1. PRRSV encodes a total of four proteases, including nsp1α, nsp1β, nsp2 and nsp4, but only nsp2 and nsp4 have trans-cleavage activity (Han et al. 2009; Tian et al. 2009). In this case, we found nsp2 failed to cleave cyto.c1 when co-expressed in HEK 293T cells (Fig. 2-D).

Fourthly, we mapped the nsp4 cleavage sites in cyto.c1 (Fig. 2-E). If nsp4 is directly involved in cleaving cyto.c1, mutating the nsp4 cleavage sites should be able to block the cleavage. Previous studies have shown that the PRRSV 3C-like serine protease has a strong preference for glutamic acid at P1 position of its substrate (Ziebuhr et al. 2000). As the cleaved product of cyto.c1 had a migration size of more than 20KD when probed with antibodies to the N-terminal tag, it suggests that the cleavage site is likely localized within the second half of cyto.c1. Bioinformatics analysis revealed 5 potential cleavage sites within this region (data not shown), including E230|G231, E252|V253, E254|F255, E280|P281,
and E282|H283. Subsequently, we introduced an E to A mutation to each of these sites in the coding sequence of Flag-cyto.c1. When tested in 293HEK T cells, only the E230A mutation conferred resistance to the nsp4-mediated cleavage (Fig. 2-E), suggesting that the cleavage takes places between E230 and G231. Consistent with this, the N-terminal fragment cyto.c1 aa 1–230 displayed the same migration rate as the fast-moving species on the SDS-PAGE (Fig. 2-F).

We also examined whether the cleavage of cyto.c1 is an event after the activation of caspase-3, the cleaved form of which is an indicator and executor of active cell apoptosis. To test this, we expressed HA-nsp4 and Flag-cyto.c1 either together or alone in HEK293 T cells (Fig. 2-G). The cells treated with staurosporine were used as a positive control for apoptosis-induction. At the same time, the cells were treated with pan-caspase inhibitor Z-VAD-FMK. At 30 h post transfection, the cells were examined for caspase-3 activation and cyto.c1 cleavage. As shown in Fig. 2-G, the cleavage of cyto.c1 by nsp4 was independent of Z-VAD-FMK treatment, suggesting that cyto.c1 cleavage is not a result of caspase-3 activation.

Last, we tested whether the cleavage is PRRSV strain-dependent (Fig. 2-H). In this case, Flag-cyto.c1 was co-expressed in HEK293T cells with nsp4 from a low pathogenic PRRSV strain HB-1/3.9. As shown in Fig. 2-H, the HB-1/3.9 strain nsp4 cleaved cyto.c1 as efficiently as that from JXwn06 strain.

### 3.3. The cyto.c1 cleavage occurs in the late stage of PRRSV infection

Having shown that the PRRSV nsp4 is responsible for cleaving cyto.c1 in co-transfected cells, we investigated

---

**Fig. 1** Porcine reproductive and respiratory syndrome virus (PRRSV) nonstructural protein 4 (nsp4) interacts with cytochrome c1 (cyto.c1). A, yeast two hybrid assay. The plasmids pGBKTT7-nsp4 and pGADT7-cyto.c1 were co-transformed into the yeast strain Y2HGold and selected on QDO/X/ABA plates. The top and second panels are the respective positive and negative controls. B, HEK 293T cells were transfected to express Flag-cyto.c1, or HA-nsp4 or together. At 24 h post-transfection, the cells were either subjected to direct Western blot analysis or lysed and immunoprecipitated with anti-HA antibodies. The proteins bound to sepharose beads were separated by SDS-PAGE, transferred to PVDF membranes, and probed with the antibodies to Flag and HA. C, the same as B except that nsp4 truncation mutants were used. D, MARC-145 cells were transduced with letiviruses that were expressing GFP or nsp4-GFP in the presence of 8 µg mL⁻¹ polybrene, respectively. The cells were harvested 48 h post-transduction and cell lysates were immunoprecipitated using anti-GFP beads. The proteins bound to sepharose beads were subjected to Western blot analysis using an anti-cytochrome c1 polyclonal antibody or anti-GFP mAb. E, PRRSV nsp4 interacts with cyto.c1 in mammalian cells. Vero cells grown on coverslips in six-well plates were transfected when 60–70% to express the indicated proteins, either alone or pairwise. At 18–24 h post-transfection, the cells were fixed, permeablized, and stained with antibodies to HA and Flag, and examined by confocal microscopy. For double transfections, the merged images are shown at the right.
Fig. 2 Porcine reproductive and respiratory syndrome virus (PRRSV) nonstructural protein 4 (nsp4) induces proteolytic cleavage of cytochrome c1 (cyto.c1).  A, dose-dependent response to cyto.c1 cleavage by PRRSV nsp4.  HEK 293T cells were transfected to express Flag-cyto.c1 together with increasing amount of HA-nsp4.  At 24 h post-transfection, the cleavage of cyto.c1 was analyzed by Western blot with antibodies to Flag, HA, and β-actin.  B, the nsp4 protease activity is required for cleaving cyto.c1.  The same as A, except that HEK 293T cells were transfected to express Flag-cyto.c1 with nsp4 mutants.  C, the nsp4 protease activity was required for activate caspase-3.  The same as A, except that MARC-145 cells were used.  D, nsp2 does not cleave cyto.c1.  HEK 293T cells were transfected to express Flag-cyto.c1 together with nsp2.  E, identification of nsp4 cleavage site in cyto.c1.  The same as A, except that HEK 293T cells were transfected to express HA-nsp4 together with cyto.c1 mutants.  F, size comparison of cyto.c1 (aa 1–230) with the cyto.c1 cleaved fragment.  HEK293 T cells were transfected to express Flag-cyto.c1 (aa 1–230) or co-express nsp4 and cyto.c1.  G, nsp4-induced cleavage does not depend on caspase-3 activation.  Transfected HEK293 T cells expressing nsp4 and cyto.c1 were either treated with Z-VAD-FMK or untreated (left).  Meanwhile, the untransfected cells were treated with STS at 6 h before cells were harvested.  At 30 h post transfection, the cells were analyzed by immunoblotting with indicated antibodies.  H, PRRSV HB-1/3.9 nsp4 induces cleavage of cyto.c1.  HEK293 T cells were transfected to co-express cyto.c1 and nsp4 from PRRSV strain HB-1/3.9, and the cleavage was analyzed by Western blot analysis of the whole cell lysate.
whether this event also occurs in PRRSV-infected cells. To this end, MARC-145 cells were infected with PRRSV strain JXwn06 at an MOI of either 0.1 or 1. At indicated times after infection, the cells were lysed and cyto.c1 was subject to Western blot analysis. In addition to the full-length cyto.c1, we observed the appearance of a fast-moving species of cyto.c1 at the late stage of PRRSV infection (Fig. 3), a time point that is coincident with the nsp4 expression and the caspase-3 activation. Moreover, this band was not due to the degradation by cellular proteases during cell disruption, as we did not see the same phenomenon in the mock-infected cells (Fig. 3, left panel). This result indicated that the fast-moving band should be from PRRSV infection, consistent with the above observation that nsp4 could mediate the cyto.c1 cleavage.

3.4. The cleaved form of cyto.c1 is capable of inducing cell apoptosis

Coincident with the cleavage of cyto.c1 by nsp4 (Fig. 2-C), Ma et al. (2013) reported that the 3C-like serine protease activity is required for nsp4-mediated cell apoptosis. Thus, it seems that this cleavage event is critical for nsp4-induced cell apoptosis. If this is the case, it suggests that the cleaved fragment(s) of cyto.c1 might have the apoptotic activity. To test this hypothesis, we made two additional constructs corresponding to the proteolytic products of cyto.c1, namely Flag-cyto.c1 (aa 1–230) and Flag-cyto.c1 (aa 231–327). The plasmids coding for Flag-cyto.c1 or its derivatives were used in equal amounts (µg) to transfact MARC-145 cells. In line with our hypothesis, Flag-cyto.c1 (aa 1–230) was able to induce significant cell apoptosis upon transfection of MARC-145 cells as measured by caspase-3 activation, whereas the level of apoptosis induction of the full-length cyto.c1 was much lower, although its expression level was much higher when compared to Flag-cyto.c1 (aa 1–230) (Fig. 4). Thus, we conclude that the proteolytic cleavage of cyto.c1 by nsp4 leads to shedding of an apoptosis inducer.

3.5. Cyto.c1 (aa 1–230) induces mitochondrial fragmentation

To understand how cyto.c1 (aa 1–230) might cause apoptosis, we tested its effect on mitochondrial integrity. To do that, we transfected Vero cells with the individual plasmids coding for Flag-cyto.c1 (aa 1–230), Flag-cyto.c1 (aa 231–327), or Flag-cyto.c1. At 24 h post transfection, the mitochondria were stained with mitotracker while the exogenous cyto.c1 was labeled with antibodies to the Flag tag. As expected, the normal mitochondria showed the filament- or tubule-like structure (Fig. 5-A, bottom). In contrast, in the cells expressing Flag-cyto.c1 (aa 1–230), the mitochondria structure became fragmented (Fig. 5-A, the second row). Moreover, the mitotracker staining was rather faint comparing to the surrounding cells not expressing foreign cyto.c1 (aa 1–230) (Fig. 5-A, the second row), suggesting that cyto.c1 (aa 1–230) is detrimental to mitochondria. On the other hand, Flag-cyto.c1 (Fig. 5-A, top) or Flag-cyto.c1 (aa 231–327) (Fig. 5-A, the third row) did not appear to have a very adverse effect on mitochondria morphology in Vero cells, and also there was no effect on the mitotracker staining. Representative examples with higher magnification were shown in Fig. 5-A (far right column). We also observed the similar detrimental effect of Flag-cyto.c1 (aa 1–230) on HeLa (Fig. 5-B) and MARC-145 (Fig. 5-C) cells. Thus, these data suggest that Flag-cyto.c1 (aa 1–230) has a dominant negative effect on mitochondrial function.

3.6. Cyto.c1 is critical for PRRSV- and nsp4-induced cell apoptosis

To support a critical role for cyto.c1 in PRRSV- or nsp4-induced apoptosis, we performed RNAi knockdown assay by designing three different siRNAs targeting the monkey cyto.c1 (siRNA-1, -2 and -3). The siRNAs were transfected into MARC-145 cells, and the knockdown efficiency was assessed at 48 h post transfection by Western blot analysis. As shown in Fig. 6-A, the siRNA-2 and -3 were most effective as compared to the scrambled control (siRNA-NC). When tested in the siRNA transfection/PRRSV infection assay, RNAi knockdown of cyto.c1 effectively decreased the percentage of apoptotic cells as measured by FACS analysis (Fig. 6-B), and significantly reduced the level (about 80%) of caspase-3 activation (Fig. 6-C). Similarly, depletion of cyto.c1 reduced PARP cleavage by about 80% when tested in the transfection/lentivirus transduction assay (Fig. 6-D). Together, these data support a critical role for cyto.c1 in nsp4- and PRRSV-induced apoptosis.

4. Discussion

Many positive-stranded RNA viruses can actively induce cell apoptosis. Previous studies have revealed that the 3C or 3C-like proteases play a critical role in the apoptosis induction (Barco et al. 2000; Lin et al. 2006; Zaragoza et al. 2006; Ma et al. 2013; Yuan et al. 2016). A salient example is coxsackievirus B3 3Cpro that targets IkB for cleavage (Zaragoza et al. 2006). The cleaved fragment then translocates into nucleus and forms a stable complex with NF-κB to inhibit its transactivation, leading to cell apoptosis (Zaragoza et al. 2006). In this study, our data show that the arterivirus 3Cpro uses a different mechanism by directly targeting a mitochondrial protein cyto.c1.

Cyto.c1 is one of the key constituents of multimeric
complex III (bc1 complex) in the mitochondrial electron transport chain. In the complex, the N-terminus of cyto.c1 faces the mitochondrial inter-membrane space and interacts with cytochrome c (Hunte et al. 2003; Crofts 2004). This interaction ensures the transfer of electrons from complex II to cytochrome c. Also, the bc1 complex is the major pathway for free radical generation within mitochondria (Cape et al. 2007). Previously, it was reported that PRRSV-induced cell apoptosis involves in the intrinsic (mitochondria) pathway (Lee and Kleiboeker 2007). Our studies here suggest that the mitochondria pathway plays an important role in PRRSV-induced apoptosis of MARC-145 cells, as knockdown of cyto.c1 greatly (about 80%) reduced PRRSV-induced caspase-3 activation (Fig. 6-C). Screening of viral components revealed that PRRSV nsp4 is a strong apoptosis inducer (Ma et al. 2013; Yuan et al. 2016). Mechanistically, our data support the notion that the proteolytic cleavage of cyto.c1 is a critical event for nsp4-induced cell apoptosis. There are several lines of evidence supporting this conclusion. First, the protease activity is absolutely required for nsp4-induced cell apoptosis (Fig. 2-C). Although it is possible that the mutations might affect an unknown function of nsp4 in addition to the protease activity, it is less likely that all three independent, relatively conserved mutations affect this single unknown function. Second, nsp4 induces specific cleavage of cyto.c1. This conclusion is derived by the key facts that the reaction was dose-dependent on nsp4 (Fig. 2-A) and that mutagenesis of either the nsp4 catalytic site (Fig. 2-B) or the nsp4 recognition site (Fig. 2-E) in cyto.c1 prevented the cleavage. Third, the cleaved N-terminal fragment cyto.c1 (aa 1–230) has a remarkable ability to induce apoptosis compared to the full-length cyto.c1 (Fig. 4). Moreover, in the singly transfected mammalian cells, expression of cyto.c1 (aa 1–230) causes mitochondria fragmentation with severely reduced mitochondria staining by mitotracker (Fig. 5), indicating that the cleaved fragment is detrimental to mitochondria.

Fig. 3 Cytochrome c1 (cyto.c1) is cleaved during porcine reproductive and respiratory syndrome virus (PRRSV) infection. MARC-145 cells were either mock-infected with Dulbecco’s Modified Eagle medium (DMEM) or infected with highly pathogenic (HP)-PRRSV at a multiplicity of infection (MOI) of 0.1 or 1. At different times after infection as indicated, the cells were harvested and subjected to Western blot analysis with antibodies to caspase-3, cyto.c1, nsp4 and β-actin.

Fig. 4 The cleaved N-terminal fragment of cytochrome c1 (cyto.c1) induces cell apoptosis. MARC-145 cells were transfected to express Flag-cyto.c1 or its derivatives as indicated. At 48 h post transfection, the cells were harvested and subjected to SDS-PAGE and Western blot analysis with antibodies to caspase-3, Flag and β-actin, respectively.
Interestingly, the property of cyto.c1 (aa 1–230) is well in line with a recent report regarding the feature of a cleaved form of cyto.c1 (Zhu et al. 2012). In that study, cyto.c1 was identified as a novel substrate for the activated caspase-3. The authors found that, during apoptosis, the activated caspase-3 can feed back to cleave cyto.c1 at the site of D106|H107 to release an N-terminal fragment cyto.c1 (aa 1–106). This fragment turned out to be detrimental and could cause further amplification of cyto.c release from mitochondrial to cytosol. When expressed alone in transfected cells, cyto.c1 (aa 1–106) results in profound mitochondria fragmentation and loss of membrane potential, leading to mitochondrial catastrophe (Zhu et al. 2012).

However, this specific cleavage by caspase-3 could be abrogated by caspase inhibitor Z-VAD-FMK. Interestingly, we did not detect the cleaved fragment by caspase-3 either in PRRSV-infected or in transfected cells. On the other hand, since cyto.c1 (aa 1–230) contains the sequence of cyto.c1 (aa 1–106), it is not surprising that the two share the similar property. However, cyto.c1 (aa 1–230) appears more detrimental than cyto.c1 (aa 1–106) since the cells expressing cyto.c1 (aa 1–230) lose most of the mitotracker staining (Fig. 5) while the latter does not (Zhu et al. 2012). Nevertheless, these indicate that PRRSV hijacks a cellular mechanism to induce cell apoptosis.

Bioinformatics analyses indicate that nsp4 is highly conserved among different PRRSV strains, and therefore it is no surprise that the nsp4 protein from low pathogenic PRRSV strain HB1/3.9 cleaved cyto.c1 as efficiently as that from HP-PRRSV JXwn06. This result is in stark contrast to the fact that HP-PRRSV strains induce much more pronounced cell apoptosis in multiple organs of pigs (He et al. 2012; Guo et al. 2013; Wang et al. 2014). However, this discrepancy can at least be partly explained by the much higher replication efficiency of HP-PRRSV strains in macrophages and pigs as well as the expanded tropism (Li et al. 2012; Li et al. 2014b). For the latter, HP-PRRSV has been reported to cause thymus atrophy but the low pathogenic PRRSV rarely does so (He et al. 2012; Guo et al. 2013). Unfortunately, since the protease activity is absolutely required for viral replicase maturation, it is difficult to directly test the role of nsp4 in virus-induced apoptosis by reverse genetics, unless specific residues important for the nsp4-cyto.c1 interaction can be identified. This task will be...
very challenging as there are two regions of nsp4 involved in interaction with cyto.c1 (Fig. 1). Nevertheless, our findings here reveal new information about the mechanistic clues for PRRSV-induced apoptosis and also indicate a novel mechanism for the 3C-like proteases of positive-stranded RNA viruses in induction of cell apoptosis.

5. Conclusion

PRRSV infection induces cell apoptosis in vitro and in vivo. And previous studies suggested that PRRSV nsp4 was responsible, but the exact molecular mechanisms of apoptosis induction are unclear. The major finding of this report is that the PRRSV nsp4 interacts with cyto.c1 and target the E230/G231 site of cyto.c1 to mediate its proteolytic cleavage. Furthermore, the cleaved N-terminal fragment of cyto.c1 was found to possess apoptotic activity that causes mitochondrial fragmentation, leading to apoptotic cell death. In addition, the RNAi silencing experiment suggests that cyto.c1 is critical for both PRRSV- and nsp4-induced apoptosis. Thus, our data provide a mechanistic clue for PRRSV-induced cell apoptosis and also indicate a novel mechanism for the 3C-like proteases in induction of apoptotic cell death.

Acknowledgements

This study was supported by the National 973 Program of China (2014CB542700), the National Natural Science Foundation of China (31330077, 31540004), and the earmarked fund for China Agriculture Research System (CARS-36).

References

Albina E. 1997. Epidemiology of porcine reproductive and respiratory syndrome (PRRS): An overview. Veterinary Microbiology, 55, 309–316.
Barco A, Feduchi E, Carrasco L. 2000. Poliovirus protease 3C(pro) kills cells by apoptosis. Virology, 266, 352–360.
Cape J L, Bowman M K, Kramer D M. 2007. A semiquinone intermediate generated at the Qo site of the cytochrome bc1 complex: importance for the Q-cycle and superoxide production. Proceedings of the National Academy of Sciences of the United States of America, 104, 7878–7892.
Choi C, Chae C. 2002. Expression of tumour necrosis factor-alpha is associated with apoptosis in lungs of pigs experimentally infected with porcine reproductive and respiratory syndrome virus. Research in Veterinary Science, 72, 45–49.
Crofts A R. 2004. The cytochrome bc1 complex: Function in the context of structure. Annual Review of Physiology, 66, 689–733.
Du J , Ge X, Liu Y , Wang Z, Zhang R, Zhou L, Guo X, Han J, Yang H. 2016. Targeting swine leukocyte antigen class I molecules for proteasomal degradation by the nsp1α replicase protein of the chinese highly pathogenic porcine reproductive and respiratory syndrome virus strain JXwn06. Journal of Virology, 90, 682–693.
Feng W H, Tompkins M B, Xu J S, Brown T T, Laster S M, Zhang H X, McCaw M B. 2002. Thymocyte and peripheral blood T lymphocyte subpopulation changes in piglets following in utero infection with porcine reproductive and respiratory syndrome virus. Virology, 302, 363–372.
Fernandez A, Suarez P, Castro J M, Tabares E, Diaz-Guerra...
Zhang Feng et al. / Journal of Integrative Agriculture 2017, 16(11): 2573–2585

M. 2002. Characterization of regions in the GP5 protein of porcine reproductive and respiratory syndrome virus required to induce apoptotic cell death. Virus Research, 83, 103–118.

Gagnon C A, Lachapelle G, Langelier Y, Massie B, Dea S. 2003. Adenoviral-expressed GP5 of porcine respiratory and reproductive syndrome virus differs in its cellular maturation from the authentic viral protein but maintains known biological functions. Archives of Virology, 148, 951–972.

Galluzzi L, Brenner C, Morselli E, Touat Z, Kroemer G. 2008. Viral control of mitochondrial apoptosis. PLoS Pathogens, 4, e1000018.

Guo B, Lager K M, Henningson J N, Miller L C, Schlink S N, Kappes M A, Kehrl M E, Brockmeier S L, Nicholson T L, Yang H C, Faaberg K S. 2013. Experimental infection of United States swine with a Chinese highly pathogenic strain of porcine reproductive and respiratory syndrome virus. Virology, 435, 372–384.

Han J, Rutherford M S, Faaberg K S. 2009. The porcine reproductive and respiratory syndrome virus nsp2 cysteine protease domain possesses both trans- and cis-cleavage activities. Journal of Virology, 83, 9449–9463.

He Y, Wang G, Liu Y, Shi W, Han Z, Wu J, Jiang C, Wang S, Hu S, Wen H, Dong J, Liu H, Cai X. 2012. Characterization of thymus atrophy in pigs infected with highly pathogenic porcine reproductive and respiratory syndrome virus. Veterinary Microbiology, 160, 455–462.

Hunte C, Palsdottir H, Trumpower B L. 2003. PROGRAMMATIC pathways and mechanisms in the cytochrome bc1 complex. FEBS Letters, 545, 39–46.

Kim T S, Benfield D A, Rowland R R. 2002. Porcine reproductive and respiratory syndrome virus-induced cell death exhibits features consistent with a nontypical form of apoptosis. Virus Research, 85, 133–140.

Kuhn J H, Lauck M, Bailey A L, Schectlin A M, Vishnevskaya T V, Bao Y, Ng T F, LeBreton M, Schneider B S, Gillis A, Tamoufe U, Diffo Jie D, Takuo J M, Kondov N O, Coffey L L, Wolfe N D, Delwart E, Clawson A N, Postnikova E, Bollinger L, et al. 2016. Reorganization and expansion of the nidoviral family Arteriviridae. Archives of Virology, 161, 755–768.

Labarque G, Van Gucht S, Nauwynck H, Van Reeth K, Persaet M. 2003. Apoptosis in the lungs of pigs infected with porcine reproductive and respiratory syndrome virus and associations with the production of apoptogenic cytokines. Veterinary Research, 34, 249–260.

Lee C, Rogan D, Ericksson L, Zhang J, Yoo D. 2004. Characterization of the porcine reproductive and respiratory syndrome virus glycoprotein 5 (GP5) in stably expressing cells. Virus Research, 104, 33–38.

Lee S M, Kleiboecker S B. 2007. Porcine reproductive and respiratory syndrome virus induces apoptosis through a mitochondria-mediated pathway. Virology, 365, 419–434.

Li L, Zhao Q, Ge X, Teng K, Kuang Y, Chen Y, Guo X, Yang H. 2012. Chinese highly pathogenic porcine reproductive and respiratory syndrome virus exhibits more extensive tissue tropism for pigs. Virology Journal, 9, doi: 10.1186/1743-422X-9-203.

Li Y, Wang G, Liu Y, Tu Y, He Y, Wang Z, Han Z, Li L, Li A, Tao Y, Cai X. 2014a. Identification of apoptotic cells in the thymus of piglets infected with highly pathogenic porcine reproductive and respiratory syndrome virus. Virus Research, 189, 29–33.

Li Y, Zhou L, Zhang J, Ge X, Zhou R, Zheng H, Geng G, Guo X, Yang H. 2014b. Nsp9 and Nsp10 contribute to the fatal virulence of highly pathogenic porcine reproductive and respiratory syndrome virus emerging in China. PLoS Pathogens, 10, e1004216.

Lin C W, Lin K H, Hsieh T H, Shiu S Y, Li J Y. 2006. Severe acute respiratory syndrome coronavirus 3C-like protease-induced apoptosis. FEMS Immunology and Medical Microbiology, 46, 375–380.

Ma Z, Wang Y, Zhao H, Xu A T, Wang Y, Tang J, Feng W H. 2013. Porcine reproductive and respiratory syndrome virus nonstructural protein 4 induces apoptosis dependent on its 3C-like serine protease activity. PLoS ONE, 8, e69387.

Miller L C, Fox J M. 2004. Apoptosis and porcine reproductive and respiratory syndrome virus. Veterinary Immunology and Immunopathology, 102, 131–142.

Mu Y, Li L, Zhang B, Huang B, Gao J, Wang X, Wang C, Xiao S, Zhao Q, Sun Y, Zhang G, Hiscox J A, Zhou E M. 2015. Glycoprotein 5 of porcine reproductive and respiratory syndrome virus strain SD16 inhibits viral replication and causes G2/M cell cycle arrest, but does not induce cellular apoptosis in Marc-145 cells. Virology, 484, 136–145.

Neumann E J, Kliebenstein J B, Johnson C D, Mabry J W, Bush E J, Seitzinger A H, Green A L, Zimmerman J J. 2005. Assessment of the economic impact of porcine reproductive and respiratory syndrome on swine production in the United States. Journal of the American Veterinary Medical Association, 227, 385–392.

O’Brien V. 1998. Viruses and apoptosis. The Journal of General Virology, 79, 1833–1845.

Rossow K D. 1998. Porcine reproductive and respiratory syndrome. Veterinary Pathology, 35, 1–20.

Roulaton A, Marcellus R C, Branton P E. 1999. Viruses and apoptosis. Annual Review of Microbiology, 53, 577–628.

Sirinarumit T, Zhang Y, Kluge J P, Halbur P G, Paul P S. 1998. A pneu-mo-virulent United States isolate of porcine reproductive and respiratory syndrome virus induces apoptosis in bystander cells both in vitro and in vivo. The Journal of General Virology, 79, 2989–2995.

Suarez P, Diaz-Guerra M, Prieto C, Esteban M, Castro J M, Nieto A, Ortin J. 1996. Open reading frame 5 of porcine reproductive and respiratory syndrome coronavirus 3C-like protease-induced apoptosis. FEBS Letters, 385–392.

Borner C. 2015. How do viruses control mitochondria-mediated apoptosis? Virus Research, 209, 45–55.

Sur J H, Doster A R, Christian J S, Galeota J A, Wills R W, Zimmerman J J, Osorio F A. 1997. Porcine reproductive
and respiratory syndrome virus replicates in testicular germ cells, alters spermatogenesis, and induces germ cell death by apoptosis. Journal of Virology, 71, 9170–9179.

Sur J H, Doster A R, Osorio F A. 1998. Apoptosis induced in vivo during acute infection by porcine reproductive and respiratory syndrome virus. Veterinary Pathology, 35, 506–514.

Teodoro J G, Branton P E. 1997. Regulation of apoptosis by viral gene products. Journal of Virology, 71, 1739–1746.

Tian K, Yu X, Zhao T, Feng Y, Cao Z, Wang C, Hu Y, Chen X, Hu D, Tian X, Liu D, Zhang S, Deng X, Ding Y, Yang L, Zhang Y, Xiao H, Qiao M, Wang B, Hou L, et al. 2007. Emergence of fatal PRRSV variants: Unparalleled outbreaks of atypical PRRS in China and molecular dissection of the unique hallmark. PLoS ONE, 2, e526.

Tian X, Lu G, Gao F, Peng H, Feng Y, Ma G, Bartlam M, Tian K, Yan J, Hilgenfeld R, Gao G F. 2009. Structure and cleavage specificity of the chymotrypsin-like serine protease (3CLSP/ nsp4) of porcine reproductive and respiratory syndrome virus (PRRSV). Journal of Molecular Biology, 392, 977–993.

Wang G, He Y, Tu Y, Liu Y, Zhou E M, Han Z, Jiang C, Wang S, Shi W, Cai X. 2014. Comparative analysis of apoptotic changes in peripheral immune organs and lungs following experimental infection of piglets with highly pathogenic and classical porcine reproductive and respiratory syndrome virus. Virology Journal, 11, doi: 10.1186/1743-422X-11-2

Wang G, Song T, Yu Y, Liu Y, Shi W, Wang S, Rong F, Dong J, Liu H, Cai X, Zhou E M. 2011. Immune responses in piglets infected with highly pathogenic porcine reproductive and respiratory syndrome virus. Veterinary Immunology and Immunopathology, 142, 170–178.

Wang L, He Q, Gao Y, Guo X, Ge X, Zhou L, Yang H. 2012. Interaction of cellular poly(C)-binding protein 2 with nonstructural protein 1beta is beneficial to Chinese highly pathogenic porcine reproductive and respiratory syndrome virus replication. Virus Research, 169, 222–230.

Yuan S, Zhang N, Xu L, Zhou L, Ge X, Guo X, Yang H. 2016. Induction of apoptosis by the nonstructural protein 4 and 10 of porcine reproductive and respiratory syndrome virus. PLOS ONE, 11, e0156518.

Zaragoza C, Saura M, Padalko E Y, Lopez-Rivera E, Lizarbe T R, Lamas S, Lowenstein C J. 2006. Viral protease cleavage of inhibitor of kappaBalpha triggers host cell apoptosis. Proceedings of the National Academy of Sciences of the United States of America, 103, 19051–19056.

Zhang H, Guo X, Ge X, Chen Y, Sun Q, Yang H. 2009. Changes in the cellular proteins of pulmonary alveolar macrophage infected with porcine reproductive and respiratory syndrome virus by proteomics analysis. Journal of Proteome Research, 8, 3091–3097.

Zhao K, Ye C, Chang X B, Jiang C G, Wang S J, Cai X H, Tong G Z, Tian Z J, Shi M, An T Q. 2015. Importation and recombination are responsible for the latest emergence of highly pathogenic porcine reproductive and respiratory syndrome virus in China. Journal of Virology, 89, 10712–10716.

Zhou L, Wang J, Ding Y, Ge X, Guo X, Yang H. 2015. NADC30-like strain of porcine reproductive and respiratory syndrome virus, China. Emerging Infectious Diseases, 21, 2256–2257.

Zhou L, Yang H. 2010. Porcine reproductive and respiratory syndrome in China. Virus Research, 154, 31–37.

Zhou L, Zhang J, Zeng J, Yin S, Li Y, Zheng L, Guo X, Ge X, Yang H. 2009. The 30-amino-acid deletion in the Nsp2 of highly pathogenic porcine reproductive and respiratory syndrome virus emerging in China is not related to its virulence. Journal of Virology, 83, 5156–5167.

Zhu Y, Li M, Wang X, Jin H, Liu S, Xu J, Chen Q. 2012. Caspase cleavage of cytochrome c1 disrupts mitochondrial function and enhances cytochrome c release. Cell Research, 22, 127–141.

Ziebuhr J, Snijder E J, Gorbalenya A E. 2000. Virus-encoded proteinases and proteolytic processing in the Nidovirales. The Journal of General Virology, 81, 853–879.

(Managing editor ZHANG Juan)