Porcine epidemic diarrhea virus ORF3 gene prolongs S-phase, facilitates formation of vesicles and promotes the proliferation of attenuated PEDV

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Received: 20 July 2015 / Accepted: 28 October 2015 / Published online: 4 November 2015
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Abstract Porcine epidemic diarrhea virus (PEDV) is a porcine enteropathogenic coronavirus that has received increasing attention since the emergence of a PEDV variant worldwide. Previous studies have shown that PEDV ORF3 encodes an ion channel protein. However, its influence on cell cycle and subcellular structure still require more research. In this study, we developed a Vero cell line that stably expresses PEDV ORF3 gene. Subcellular localization and influences of PEDV ORF3 on host cells were investigated. We further verified whether or not this gene enhances virus production. The results showed that PEDV ORF3 protein localizes in the cytoplasm and affects cell cycle progression by prolonging the S phase. In addition, the ORF3-expressing Vero cells had more vesicles than the host Vero cells. Furthermore, the attenuated PEDV rather than virulent PEDV could grow better in ORF3-expressing Vero cells. The expression level of the PEDV nucleocapsid protein also increased. These results provided information on the function of PEDV ORF3 and were helpful in understanding the mechanisms of PEDV replication.

Keywords PEDV · ORF3 · Virus replication · Cell cycle

Edited by William Dundon.

Electronic supplementary material The online version of this article (doi:10.1007/s11262-015-1257-y) contains supplementary material, which is available to authorized users.

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Introduction

Porcine epidemic diarrhea (PED) caused by porcine epidemic diarrhea virus (PEDV) is an acute intestinal infectious disease of pigs that is characterized by watery diarrhea, vomiting, and dehydration. It can affect pigs in all period, and be fatal to new-born piglets [1]. Since 2010, PED has been re-emerging in immunized swine herds in China, which were shown to be caused by variant PEDVs [2]. Recently, PED also reported in the United States, Mexico and Canada [3]. It has become an increasing problem in many swine-breeding countries worldwide, causing large economic losses.

PEDV is an enveloped positive-strand RNA virus that belongs to the family *Coronaviridae*. The genome structure of PEDV is similar to that of other coronaviruses, such as transmissible gastroenteritis coronavirus (TGEV) [4], severe acute respiratory syndrome-associated coronavirus (SARS-CoV) [5] and mouse hepatitis virus (MHV) [6]. The genome of PEDV contains seven open reading frames (ORFs), which encodes three non-structural proteins (ORF1a, ORF1b, and ORF3) and four structural proteins (spike glycoprotein, envelope protein, membrane glycoprotein, and nucleocapsid protein), arranged in the order 5'-ORF1a/1b-S-ORF3-E-M-N-3' [7, 8]. The ORF3 gene located between the S and E genes is conserved in three groups of coronaviruses [9]. This homology suggests that this gene may have similar functions.

Wild-type and attenuated PEDV have almost complete sequence identity, except for some variations and truncations in the ORF3 gene. A continuous 49 or 51 nucleotide region is deleted within the ORF3 gene when PEDV is continuously passaged in cell culture, indicating a possible involvement in viral pathogenicity [10, 11]. These features made ORF3 gene a potential target to conduct the

molecular epidemiology of PEDV [12, 13]. The ORF3a protein of SARS-CoV reportedly forms an ion channel and may modulate virus release [14]. Also, the SARS-CoV 3a protein is necessary for Golgi to plasma membrane transport [15]. Considering the similar genome structure between SARS-CoV and PEDV, a previous study adapted same methods used for the SARS-CoV 3a protein to demonstrate that the PEDV ORF3 protein also functioned as an ion channel and regulates virus production [16]. However, the mechanism by which the ORF3 gene affects PEDV replication remains unclear to date.

Presently, no data were reported about the influence of PEDV ORF3 on cell cycle and vesicles formation. In the present work, we developed a Vero cell line that could stably express the ORF3 protein of PEDV to explore its properties. Our results demonstrated that the ORF3 gene of PEDV could prolong S-phase, facilitate formation of vesicles and thus promote the proliferation of PEDV. These findings have potentially important implications for understanding the molecular mechanisms of PEDV pathogenesis.

Materials and methods

Cells and viruses

Vero cells were cultured in Minimum Essential Medium (MEM) (Gibco, USA) supplemented with 10 % fetal bovine serum (FBS, HyClone, USA) at 37 °C in a 5 % CO₂ incubator (Sanyo, Japan). In this study, PEDV field virus strain CH/YNKM-8/2013 (GenBank: KF761675.1) and attenuated virus strain AH-M (GenBank: KJ158152.1) were isolated from PED-affected piglets. The attenuated vaccine strain CV777 (GenBank: AF353511.1) was provided by Chengdu Tecbond Biological Product Co., Ltd (Chengdu, China).

Development of stable cell lines expressing the PEDV ORF3 protein

RT-PCR was performed to amplify the complete ORF3 gene (Genbank: KC342816.1) and then subcloned into Lenti-X vector. The vector was transfected into 293T cells using Xfect (Clontech) reagent in accordance with the manufacturer's instructions. The supernatant was collected and filtered through a 0.45 µm filter and then concentrated with ultracentrifugation. The concentration of puromycin was optimized in 24-well tissue culture plates with concentration gradient. Vero cells were infected by the recombinant lentivirus and screened with 2 µg/mL puromycin to obtain stable resistant Vero-ORF3 cells. Monoclonal Vero-ORF3 cells were selected by limiting-dilution

method. The insertion of PEDV ORF3 was verified by quantitative-PCR and sequencing. The expression level of ORF3 was detected by Western blot. β-actin was used as an internal control.

Cell cycle analysis

The cell cycle was measured by staining the DNA with propidium iodide (PI) as previously described [17]. In brief, the cells were harvested at 24 h post-seeding, washed with phosphate-buffered saline (PBS), and then fixed in 70 % cold ethanol at −20 °C. The fixed cells were washed with PBS, resuspended in PBS containing 20 µg/mL RNase A and 50 µg/mL PI, and then stained for 30 min at room temperature in the dark. The nuclear DNA content was examined by FACS caliber system (BD Biosciences, USA) with Cell Quest software.

Subcellular localization

Vero cells grow on cover slips for 60–70 % monolayer, and then transfected with the plasmid pDsRed-C1-ORF3, and pDsRed-C1 using lipofectamineTM 2000 reagent (Invitrogen) as the instruction. Twenty-four hours after transfection, cells were fixed in 4 % (w/v) paraformaldehyde for 15 min at room temperature, permeabilized with 0.2 % TritonX-100 for 10 min, and stained with DAPI for 15 min. Rinsed coverslips were used for confocal laser scanning microscopy.

Virus infection

The Vero cells and Vero-ORF3 cells were cultured in 24-well tissue culture plates at a density of 5×10^4 cells/well for approximately 24 h. When the cells reached monolayer, they were washed three times with serum-free medium, and then inoculated with 0.01 MOI PEDVs CH/YNKM-8/2013, AH-M, and CV777 strains at 37 °C for 1 h. Then the infected cells were maintained in MEM containing 6 µg/mL trypsin. The cells were scraped off at 24 h post infection and then stored at −80 °C.

RNA extraction and real-time PCR

Virus RNA was extracted using RNA extraction kit (Bioflux) in accordance with the manufacturer's instruction. The viral RNA was reverse transcribed into cDNA using PrimeScriptTM RT Master Mix (Takara). Reverse transcription conditions were 37 °C for 15 min, 85 °C for 5 s. Then the cDNA was used for quantitative real-time PCR on an ABI ViiA 7 real-time PCR system (Applied Biosystems, USA).

Quantitative real-time PCR was carried out using the FastStart Universal Probe Master (Rox) (Roche) in a total volume of 10 μ L in accordance with the manufacturer's instructions. The reaction conditions were at 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 56 °C for 30 s, and 72 °C for 31 s. The primers and probe are listed in Table 1. Serial dilutions of plasmid pMD18-T-M (constructed by inserting the PEDV M gene into pMD18-T) were used for standard curve preparation.

Relative real-time PCR was performed to detect ORF3 expression in Vero-ORF3 cells. SYBR Green (Takara, Japan)-based real-time PCR was carried out on an ABI 7300 instrument. The primers used are shown in Table 1. The reaction conditions were 30 s at 95 °C, followed by 40 cycles of 95 °C for 5 s and 60 °C for 31 s. Each sample was amplified thrice. β -actin was used as an internal control.

Western blot

Both parent Vero cells and Vero-ORF3 cells infected or mock infected with PEDV were treated with lysis buffer. Sodium dodecyl sulfate (SDS) loading buffer was added into the collected cell extracts and boiled for 10 min. Equivalent samples were loaded and electrophoresed on 12 % SDS-PAGE and then transferred to nitrocellulose membranes. After blocking with 5 % skim milk for 2 h at 37 °C, the NC membranes were incubated with primary antibodies over night at 4 °C, and then with HRP-conjugated secondary antibodies [18]. The signal was detected using a SuperSignal West Pico Luminol Kit (Pierce).

Electron microscopy

After 24 h, both host Vero and Vero-ORF3 cells were digested and then fixed in 2 % glutaraldehyde. The fixed cells were treated with 1 % osmium tetroxide, dehydrated in a graded ethanol series, and then embedded in epoxy resin. Ultrathin sections were prepared and stained with uranyl acetate and lead citrate. Samples were examined under a transmission electron microscope (Hitachi H-7650, Japan).

Statistical analysis

All the results were presented as the mean $\pm$ SD. SPSS statistics 17.0 was used for statistical analysis. Differences with a *P* value <0.05 and 0.01 were considered to indicate significant and highly significant, respectively.

Results

PEDV ORF3 expression in Vero cells

Four Vero cell clones that express whole ORF3 were obtained using the lentiviral vector system through the limiting-dilution method in the presence of 2 μ g/mL puromycin. Relative quantitative real-time PCR was performed to identify the expression level in the cell lines. ORF3 showed the highest expression level in Vero cell line clone3 (Fig. 1a). Thus, this cell line was used for the subsequent experiments. The selected cell clone (clone 3) was initially subjected to PCR and sequencing to identify the integration of the PEDV ORF3 gene. The Vero-ORF3 cell line showed a DNA band of approximately 675 bp (Fig. 1b). Western blot was performed to verify the expression level of ORF3 in Vero cells. As expected, Western blot results revealed a protein of approximately 26 kDa in the ORF3-expressing cells (Fig. 1c). However, no significant signal was detected in the Vero control cells.

Vero-ORF3 cells were serially passaged. Some of these cells were used for reverse transcription PCR to test whether or not the cell line is stable upon passaging. Results demonstrated that ORF3 expressions were stable in generations 5, 10, 15, 20, 25, 30, and 35 (Fig. S3a). Sequencing confirmed that this band was PEDV ORF3. These results indicated that the Vero-ORF3 cell line does not lose the expression of ORF3 upon passaging.

MTT assay was adapted to evaluate the cell proliferation and determine whether or not the stable cell line has similar replication kinetics to the parent cells. No obvious differences in cell proliferation were detected between the ORF3-expressing cells and the parent Vero cells (Fig. S3b). These results indicate that the constructed Vero-ORF3 cells stably expressed PEDV ORF3 and shared similar replication kinetics to the parent Vero cells.

Table 1 Primers and probe of relative real-time PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
ORF3	CGTTAGTAGCATTGACTTGTA	AGCATACTCGTCTAGTTGAA
β -actin	CCTGACTGACTACCTCATGAAGATC	ATCCACATCTGCTGGAAGGTGGA
PEDV	CGTACAGGTAAGTCAATTAC	GATGAAGCATTGACTGAA
Probe	FAM-TTCGTCACAGTCGCCAAGG-TAMRA

Fig. 1 Confirmation of PEDV ORF3 expression in Vero cells. **a** The relative mRNA amount of PEDV ORF3 in cell lines was detected by relative quantitative real-time PCR. β -actin was used as the reference gene. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with Vero cell). **b** ORF3 integration test by PCR. DL 2000 maker (Takara) was used. The size of DNA bands from top to bottom are 2000, 1000, 750, 500, 250, and 100 bp. The ORF3 gene is 675 bp in length. **c** ORF3 protein expression in the established cell line was identified by Western blot. The antibody against PEDV ORF3 was obtained from mouse serum immunized with prokaryotic expressed truncated ORF3 protein (1aa–70aa) (Supplementary Materials)

Influence of ORF3 on cell cycle

As published data showed that cell cycle deregulation was a common response to many infections [19, 20]. To determine whether the promotion of PEDV replication was caused by cell cycle arrest, Vero cells and Vero-ORF3 cells were analysed for cell cycle progression by flow cytometry (Fig. 2a). The histograms indicated the percentage of cells in each phase (Fig. 2b). In Vero cells, the distribution of cells at each phase was normal. Over 40 % of the Vero-ORF3 cells accumulated in the S-phase whereas only 28.03 % Vero cells accumulated in this phase. The percentage of cells in G2/M phase was decreased from 8.67 to 0.89 % when expressing ORF3. This result strongly indicated that ORF3 expression in Vero cells could prolong the S-phase, which was crucial for virus replication.

Influence of ORF3 on subcellular structure

Thin-section electron microscopy was carried out on Vero and Vero-ORF3 cells. The most obvious ultrastructural change between the cell lines was the proliferation of Golgi-related vesicles accompanied by swelling of some trans-Golgi sacs (Fig. 3). According to the comparison of Vero cells transfected with truncated ORF3 (Fig. 3c) and Vero-ORF3 (Fig. 3b), vesicles in truncated ORF3 transfected Vero cells were significantly less than Vero-ORF3 cells, suggesting that the increase of vesicle formation was due to the property of ORF3 expression instead of just protein aggregation effect. As shown in Fig. 4, ORF3 expression in Vero cells induced the formation of double membrane vesicles (DMVs) that were involved in coronavirus replication [21, 22]. Previous studies showed that the vesicles consist of a double membrane ranged from 200 to 350 nm [23].

Subcellular localization of PEDV ORF3 protein

In order to test the subcellular localization of ORF3 protein in Vero cells, the full length ORF3 was subcloned into pDsRed-C1 plasmid. The pDsRed-C1 vector and recombinant DsRed-ORF3 were transfected into Vero cells. The results showed that DsRed-ORF3 proteins localized in the cytoplasm, while the DsRed proteins distributed throughout the whole cell (Fig. 5).

Influence of ORF3 on PEDV proliferation in Vero cells

Previous study has shown that siRNA-induced ORF3 knockdown during PEDV infection reduces virus production [16]. In the present study, we developed a Vero cell line that stably expressed PEDV ORF3 to identify whether ORF3 enhances virus production and to elucidate how the ORF3 promotes PEDV replication. To investigate whether PEDV ORF3 promotes PEDV proliferation in Vero cells, Vero and Vero-ORF3 cells were infected with equal amounts (0.01 MOI) of CH/YNKM-8/2013, AH-M, and CV777 strains. Viral products were collected at 24 h post infection and quantified by real-time PCR (Fig. 6a), while TCID₅₀ was measured on Vero cells (Fig. 6b). Results of TCID₅₀ were in accordance with qPCR. Virus copies of the attenuated PEDV virus strain AH-M were significantly higher (tenfold) in Vero-ORF3 cells than in Vero cells. Similar results were observed in the attenuated strain CV777. The virulent PEDV strain CH/YNKM-8/2013 (YN for short in the figure) showed no significant difference between the two cell lines. These findings coincided with the immunofluorescence assay (Fig. S4). Basing on these results, we inferred that the ORF3 protein of PEDV

Fig. 2 Cell cycle distribution in Vero and Vero-ORF3 cells. **a** Flow cytometry analysis by PI staining. **b** The percentage of cells in each cell cycle phases. The results are presented as the mean $\pm$ SD of three independent experiments

Fig. 3 Ultrastructural morphology of parent Vero cell (**a**), Vero-ORF3 cell (**b**) and Vero cells transfected with truncated ORF3 (**c**). Bars 2 μ m

exerted a positive regulatory effect on attenuated PEDV proliferation.

Viral protein expression level on Vero and Vero-ORF3 cells

We further investigated the effect of ORF3 on viral protein expression level. Cell lysate was collected at 24 h post

infection. The level of N protein expression was detected by Western blot. GAPDH was used as an internal loading control. Western blot showed that the expression of the PEDV N protein was lower on Vero cells than on Vero-ORF3 cells (Fig. 7). Overall, these results indicated that PEDV ORF3 could promote the proliferation of attenuated PEDV.

Fig. 4 Formation of small double membrane vesicles (DMV). **a** Electron micrograph showing multiple DMVs in the cytoplasm of Vero-ORF3 cells. Bar, 2 µm. **b** Enlargement of the dotted line region in **a**. Bar 0.5 µm

Fig. 5 Detection of DsRed-ORF3 fusion protein subcellular localization in Vero cells. Merged images showed localization of DsRed-ORF3 out of nuclear. Scale bars 20 µm

Fig. 6 Influence of ORF3 on PEDV proliferation in Vero cells. The viral titer of AH-M, CV777, and CH/YNKM on Vero and Vero-ORF3 cells were quantified by real-time PCR (**a**) and TCID₅₀ (**b**). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with Vero)

Fig. 7 PEDV ORF3 promotes the expression level of nucleocapsid protein on PEDV-infected cells

Discussion

PED was generally considered to be under control or had only mild effects in swine herds in China before 2011 [24, 25]. The emergence of the PEDV variant in China, United State as well as other regions [26, 27] has drawn much attention on this disease. Many studies of PEDV ORF3 focused on gene sequence analysis. However, the function of this gene remains unclear compared to other coronaviruses.

In this study, we established cell lines that stably express PEDV ORF3, which allowed analysis of its properties. Lentiviral vector system was widely used as gene delivery vehicles because it can stably integrate foreign genes into the host cell genome [28, 29]. Our results showed that Vero cells expressing PEDV ORF3 were quite stable upon more than 30 passages. On the other hand, ORF3 expressing cells had similar replication kinetics as the parent cells. The established cells expressing PEDV ORF3 could be recognized by ORF3 antibodies in Western blot, suggesting that the expressed ORF3 of Vero cells was also identical to the native viral protein.

Cell cycle regulation and apoptosis are common responses of cells to many infections. Some viral proteins can affect the cell cycle such as the ORF7a of SARS-CoV [30] and the non-structural protein p28 of murine coronavirus [31]. The *S* phase is one of the most critical phases because it can provide a cellular environment that is beneficial for viral replication [32]. In the present study, ORF3 was found to prolong the *S* phase, which may associate with the virus replication.

Our observations indicated that the PEDV ORF3 was likely to be responsible for the formation of vesicles which were closely associated with the virus replication [33–35]. Virus assembly and RNA synthesis occur in DMVs for some RNA viruses [36]. This structure has been observed in cells infected with SARS-CoV [37] and Middle East respiratory syndrome (MERS)-CoV [38]. Similar DMV structure was found in our ORF3-expressing cells, indicating that ORF3 may have the similar function. Co-localization studies showed that DsRed-ORF3 (both full

length and truncated) localized in the cytoplasm, not observed in the cell nucleus.

Since ORF3 facilitates the formation of vesicles and prolongs the *S*-phase, which are both closely associated with virus replication, Vero-ORF3 and Vero cells were infected with different PEDV strains to test whether or not ORF3 can enhance virus production. Our results showed that attenuated CV777 and AH-M of PEDV strains generated more mRNA in Vero-ORF3 cells compared to the control parent cells. However, virulent PEDV showed no significant differences. These results demonstrated that ORF3 could promote the proliferation of attenuated PEDV but not essential. The complete ORF3 gene encodes a transmembrane protein that functions as an ion channel, while the truncated ORF3 protein of attenuated PEDV lacks the ion channel activity. A wide range of viruses encode proteins that function as ion channels. These proteins may influence viral replication and assembly, as well as virus entry and release from infected cells, and serve as promising anti-viral drug targets [39]. Ion channel proteins are important for virus replication, which may be associated with depolarization and ion balance.

In summary, the ORF3 gene of PEDV prolongs the *S*-phase cell cycle and facilitates the formation of vesicles. In addition, the attenuated PEDV rather than virulent PEDV could grow better in ORF3-expressing Vero cells. This work uncovered some novel features of PEDV ORF3, which would be helpful in understanding the molecular mechanisms of PEDV pathogenesis.

Acknowledgments This work was supported by the Grant of National Natural Sciences Foundation of China (31272572) and China Agricultural Research System (No. CARS-36).

Authors' Contributions Qigai He and Wentao Li conceived and designed the experiments. Shiyi Ye performed the majority of experiments and prepared the manuscript. Zhonghua Li, Fangzhou Chen, Han Hu, and Xiaozhen Guo participated part of the experiments. All the authors assisted in writing the manuscript and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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