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Porcine epidemic diarrhea virus N protein prolongs S-phase cell cycle, induces endoplasmic reticulum stress, and up-regulates interleukin-8 expression

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ABSTRACT

Porcine epidemic diarrhea (PED) is an acute and highly contagious enteric disease of swine caused by porcine epidemic diarrhea virus (PEDV). The porcine intestinal epithelial cell is the PEDV target cell. In this study, we established a porcine intestinal epithelial cell (IEC) line which can stably express PEDV N protein. We also investigate the subcellular localization and function of PEDV N protein by examining its effects on cell growth, cycle progression, interleukin-8 (IL-8) expression, and survival. The results show that the PEDV N protein localizes in the endoplasmic reticulum (ER), inhibits the IEC growth and prolongs S-phase cell cycle. The S-phase is prolonged which is associated with a decrease of cyclin A transcription level and an increase of cyclin A degradation. The IEC expressing PEDV N protein can express higher levels of IL-8 than control cells. Further studies show that PEDV N protein induces ER stress and activates NF-κB, which is responsible for the up-regulation of IL-8 and Bcl-2 expression. This is the first report to demonstrate that PEDV N protein can induce cell cycle prolongation at the S-phase, ER stress and up-regulation interleukin-8 expression. These findings provide novel information on the function of the PEDV N protein and are likely to be very useful in understanding the molecular mechanisms responsible for PEDV pathogenesis.

1. Introduction

Porcine epidemic diarrhea (PED) is an acute and highly contagious enteric disease in swine characterized by severe enteritis, vomiting, and watery diarrhea and has high mortality in piglets (Ducatelle et al., 1981). PED is one of the most important causes of economic loss in many swine-raising countries. This is mainly due to its high prevalence compared to the rare incidence of transmissible gastroenteritis (TGE) and the asymptomatic characteristics of the rotavirus (RV) infections (Carvajal et al., 1995). The causative agent of PED is the porcine epidemic diarrhea virus (PEDV), which belongs to the family *Coronaviridae* and was first reported in 1978 (Pensaert and de Bouck, 1978). PEDV is an enveloped virus possessing a single-stranded positive-sense RNA genome approximately 28 kb in size with a 5′ cap and a 3′ polyadenylated tail. The genome comprises a 5′ untranslated region (UTR), a 3′ UTR, and at least seven open reading frames (ORFs) that encode 4 structural proteins

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[spike (S, 150–220 kDa), envelope (E, 7 kDa), membrane (M, 20–30 kDa), and nucleocapsid (N, 58 kDa)] and three non-structural proteins (replicases 1a, 1b, and ORF3); these are arranged on the genome in the order 5′-replicase (1a/1b)-S-ORF3-E-M-N-3′ (Song and Park, 2012; Kocherhans et al., 2001; Yeo et al., 2003).

PEDV N protein binds to virion RNA and provides a structural basis for the helical nucleocapsid. Also, it can be used as the target for the accurate and early diagnosis of PEDV infection (Song and Park, 2012). It has been suggested that N protein epitopes may be important for induction of cell-mediated immunity (Curtis et al., 2002; Saif, 1993). To date, no data exists on the subcellular localization of PEDV N proteins and its effects on cell growth and cell cycle progression. Porcine intestinal epithelial cells (IECs) are the cells targeted by PEDV and the epithelial cells in the gut serve as a physical barrier which restricts the movement of components and passage of potentially harmful microorganisms between the lumen and underlying mucosa (Schierack et al., 2005).

In the present study, we demonstrate for the first time that PEDV N protein induces endoplasmic reticulum stress and up-regulates NF-κB, Bcl-2, and interleukin 8. In addition to above findings, we also uncovered that PEDV N protein prolongs the S phase of stage cell cycle. The results have potentially important implications for understanding the molecular mechanisms of pathogenesis for this economically important porcine disease.

2. Materials and methods

2.1. Vectors, plasmids and cells

The pEGFP-N1 eukaryotic expression vector was purchased from Clontech (USA) and *Escherichia coli* DH5α used for cloning were purchased from Tiangen Biotech (China). In this study, the PEDV Shaanxi strain was isolated from intestinal tract contents of PEDV infected piglets in Shaanxi Province of China and N gene of PEDV was amplified as described previously (Honglei et al., 2012). The established swine intestinal epithelial cell lines (IEC), which were kindly provided by Prof. Yan-Ming Zhang, College of Veterinary Medicine, Northwest A&F University, were cultured as described previously (Jing et al., 2010). Briefly, IEC cells were grown in Dulbecco's modified eagle medium (DMEM) (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated new born calf serum (Gibco BRL), 100 IU of penicillin and 100 μg of streptomycin per mL, at 37 °C in a 5% CO_2 atmosphere incubator.

2.2. Antibodies and reagents

Mouse monoclonal antibodies against cyclin A, GRP78, β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, Inc., CA, USA). Mouse anti-GFP monoclonal antibody was purchased from Millipore (Millipore, Temecula, CA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibody was purchased from Pierce (Pierce, Rockford, IL, USA). The MG132 proteasome inhibitor was purchased from Calbiochem (Calbiochem, San Diego, CA, USA) and the nuclear staining dye Hoechst33342 and ER-Tracker™ Red probe were obtained from Invitrogen (Invitrogen, Carlsbad, CA, USA).

2.3. Construction of recombinant plasmid and establishment of the stable cells expressing GFP-N and GFP protein

The primers used to amplify N gene of PEDV were as follows: forward primer (PEDV-XhoI), 5′-CCGCTCGAGATGGCTTCTGTCAGCTTTCA-3′ (26374–26393 of CV777 strain) and reverse primer (PEDV-Hind III), 5′-CCCAAGCTTATTTCCTGTATCGAAGAT-3′ (27679–27696 of CV777 strain). The restriction sites are underlined. The primers were designed according to the archived PEDV CV777 strain nucleotide sequence (GenBank: AF353511.1). The amplified product was cloned into the corresponding sites in the pEGFP-N1 expression vector. The recombinant plasmid was identified by enzyme digestion and DNA sequencing. The recombinant plasmid was named as pEGFP-N.

IEC cells were seeded into 6-well dishes 24 h before being transfected (up to 70–80% confluence). Cells were transfected with pEGFP-N and pEGFP-N1 control vector using Lipofectamine 2000 (Invitrogen) and maintained (up to 80–90% confluence) in selection media containing 1200 μg/mL G418 for two weeks. When all control cells had evidence of death in the presence of the selection agents, cultures transfected with pEGFP-N and pEGFP-N1 were propagated for two further weeks in medium containing 600 μg/mL G418. The resulting stably transfected cell lines expressing either GFP or GFP-N fusion proteins were used for subsequent analysis.

2.4. The observation of protein degradation characteristics

The stable cell lines expressing GFP-N protein and GFP were seeded respectively into 6-well dishes at a suitable concentration of cells each well. After incubation at 37 °C with 5% CO_2 for 24 h, the culture medium was replaced with fresh medium containing 20 μM MG132, and then incubated in a CO_2 incubator at 37 °C for 24 h. After 24 h incubation, the cells were washed with phosphate-buffered saline (PBS) for twice and incubated with Hoechst33342 at 37 °C for 15 min. Images were viewed after cells washed with PBS for twice by Fluorescence microscope (Model TE2000, Nikon, Japan).

2.5. Confocal microscopy

To examine the expression and subcellular localization of PEDV N protein, the cells expressing GFP-N protein or control cells (the cells expressing GFP and untransfected IEC cells) were grown on glass bottom dishes (35 mm) and washed with Hank's balanced salt solution (HBSS) and incubated with Hoechst33342 at 37 °C for 10 min, and then washed twice with HBSS. Cells were then incubated with ER-Tracker Red probe (Invitrogen) at 37 °C for 25 min and washed with HBSS for twice. Images were viewed by laser confocal scanning microscopy (Model LSM510 META, Zeiss, Germany).

2.6. Western blot analysis

Cells were harvested and treated with lysis buffer, equivalent amounts of proteins were loaded and electrophoresed on 12% SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% nonfat dry milk and then incubated with indicated primary antibodies over night at 4 °C, followed by HRP-conjugated secondary antibodies. The signal was detected using enhanced chemiluminescence (ECL) reagents (Pierce, Rockford, IL, USA).

2.7. Cell proliferation assay

The MTT cell proliferation assay was performed to determine the growth properties of cells expressing PEDV N protein and control cells as described previously (Li, 2012). Data are presented as a percentage of the control, and results are the mean ± SD of three independent experiments performed in duplicate.

2.8. Cell cycle analysis by flow cytometry

The cell cycle was measured by using propidium iodide staining as described previously (Xu et al., 2013; Tang et al., 2010). Briefly, approximately 2×10^6 cells of the stable cell lines and control cells were treated with trypsin, washed with phosphate-buffered saline (PBS) for twice, resuspended in 75% ethanol and fixed at 4 °C for 3 days. After washing with PBS, cell were resuspended in PBS containing 20 μg/mL of RNase A and 50 μg/mL of propidium iodide (PI) and incubated at 4 °C for 30 min in the dark. The nuclear DNA content was examined by a Coulter Epics XL flow cytometer (Beckman Coulter, USA).

2.9. Real-time quantitative PCR (qRT-PCR)

The procedures of Real-time quantitative PCR were described previously (Xu et al., 2013). Briefly, total RNA was extracted from cells using TRIzol agent (Invitrogen, California, USA), and 2 μg each RNA sample was reverse-transcribed using First-strand cDNA synthesis kit (Invitrogen, California, USA). The expression of genes was quantified using Bio-Rad iQ5 Real Time PCR System by means of a quantitative real-time PCR assay (qRT-PCR). The primers for qRT-PCR in this study were shown in Table 1. Reactions were carried out in 25 μL volume containing 1 × SYBR Premix Ex Taq™ II (Takara, Dalian, China), sense and anti-sense primers (0.4 μM) and target cDNA (4 ng). The cycling conditions were 95 °C for 5 min, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s. A negative control was included in each run and the specificity of amplification reaction was checked by melting curve (Tm value) analysis. The individual samples were normalized for genome equivalents using the respective CT value for the porcine β-actin housekeeping gene. The relative quantification of gene expression was analyzed by the two-ddCt method as described previously (Livak and Schmittgen, 2001).

2.10. Detection of NF-κB activity

To determine the alteration of NF-κB activity by GFP-N and GFP proteins in the established cell lines, the level of NF-κB activity was measured using the NF-κB p65 TransAM kit (Active Motif) according to the manufacturer's instructions. Briefly, cells nuclear extraction was prepared by using the Nuclear Extract Kit (KeyGEN, Nanjing, China) and protein concentrations were measured using the BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). Lysates (50 μg total proteins) were incubated in ELISA wells coated with the oligo-nucleotide motif recognized by active p65, then detected using a specific antibody against p65, followed by a horseradish peroxidase (HRP)-conjugated secondary antibody. The colorimetric reaction was measured at OD 450 nm.

2.11. Enzyme-linked immunosorbent assay

The stable PEDV N protein expressing cells and the control cells were seeded in 24-well plates at a density of 1×10^5 cells/mL in DMEM supplemented with 10% calf serum and cultured for 48 h. As suggested previously, MG132 that was discovered to block IL-8 expression was added after 24 h (Matsuo et al., 2009). The culture medium was then collected and centrifuged in a microcentrifuge at 1000 × *g* for 5 min to remove debris, the supernatants were then frozen at −80 °C until analyzed. The concentrations of IL-8 were measured using a swine IL-8 ELISA kit according to the manufacturer's instructions (Invitrogen).

2.12. Statistical analysis

All data were means ± SD from three independent experiments in triplicate. Results were analyzed by Student's *t*-test. *p* value less than 0.05 was considered to be statistically significant.

3. Results

3.1. Confirmation of PEDV N protein expression in IEC

Western blot analyses show that the cells transfected with pEGFP-N1-N and pEGFP-N1 plasmid expressed

Table 1
Sequences of primer pairs used for qRT-PCR.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product (bp)	Accession no.
Cyclin A	AAGTTTGATAGATGCTGACCCGTAC	GCTGTGGTGCTCTGAGGTAGGT	194	GQ265874
GRP78	AATGGCCGTGTGGAGATCA	GAGCTGGTTCTTGGCTGCAT	114	X92446
IL-8	CTGGCTGTTGCCTTCTTG	TCGTGGAATGCGTATTTATG	113	M86923
Bcl-2	TTGTGGCCTTCTTTGAGTTCG	CTACCCAGCCTCCGTTATCC	150	XM_003121700.1
β-Actin	GGACTTCGAGCAGGAGATGG	AGGAAGGAGGGCTGGAAGAG	138	XM_003124280.1

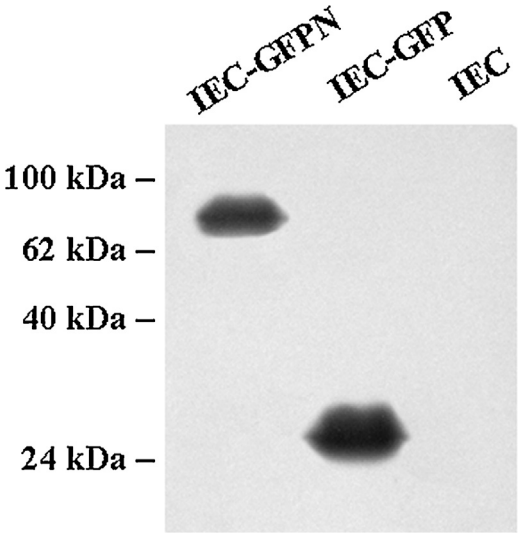

Fig. 1. Western blot analyses of expression products of GFP-N fusion protein in IEC cells. The cells were subjected to Western blot using anti-GFP antibody. Proteins were isolated from whole extracts of the stable cell lines expressing GFP-N protein and control cells.

molecular mass of approximately 82 kDa and 27 kDa protein respectively, that was detected by anti-EGFP monoclonal antibodies (Fig. 1). Since the molecular mass of GFP is known to be approximately 27 kDa, this is in good agreement with the predicted size of the N protein, viz. approximately 55–58 kDa. However, no signal was detected from the negative IEC control cells.

3.2. PEDV N protein degradation characteristics

The degradation characteristic of N protein was investigated using fluorescence microscope. The results show that GFP-N proteins were expressed in greater amounts in the cell lines treated with MG132 than untreated cells, while GFP protein expression has no change in the untreated cells and cells treated with MG132 (Fig. 2). The results show that PEDV N protein degradation was mainly via a proteasome pathway rather than a lysosomal one, and that the N protein degradation can be inhibited by MG132 proteasome inhibitor.

3.3. PEDV N protein subcellular localization

The subcellular localization of N protein was investigated by confocal fluorescence microscopy. The results show that GFP-N proteins are localized in the endoplasmic reticulum (ER), while the GFP protein was distributed throughout the whole cell (Fig. 3).

3.4. Inhibition of cell proliferation by PEDV N protein

Compared with pEGFP-N1 transfected and untransfected control cells, the N protein expressing cells divided much more slowly, leading to a significantly decreased cell number after a certain period of time. Cell proliferation of N expressing cells measured by the MTT assay was decreased by approximately 25% over a time course of 96 h (Fig. 4).

3.5. Expression of PEDV N protein prolongs the S-phase cell cycle

To investigate whether the growth inhibition of PEDV N expressing cells was due to arrest of the cell cycle at a particular phase or phases of the cell cycle progression, flow cytometric analysis was performed based on the DNA content in nuclei stained with PI. The proportions of G0/G1 phase, S-phase and G2/M phases for the control cells were 73%, 19.6%, and 7.3%, respectively. For IECs expressing GFP, the proportions of the phases were G0/G1: 75.8%, S-phase: 16.3%, and G2/M: 7.7%. On the other hand, for GFP-N-expressing IEC stable cells, the proportions were G0/G1: 69.3%, S-phase: 24.1%, and G2/M: 6.6% (Fig. 5A). Further quantitative analysis of the histograms was performed to determine the percentage of cells in each of the G0/G1, S, and G2/M phases (Fig. 5B).

The G0/G1 phase cells show a 2N DNA content and the G2/M phase cells show a 4N DNA content. These data suggest that the PEDV N protein prolongs the S-phase cell cycle and that prevents GFP-N-expressing cells from entering the G2/M phase. The results show that, relative to control cells, PEDV N protein expression causes a significant increase in the proportion of cells in the S-phase accompanied by a decrease in the proportion of cells in the G0/G1 phase. Taken together, these results strongly suggest that N protein causes the inhibition of cell growth by prolongation the S-phase cell cycle.

3.6. S-phase prolongation and cyclin A degradation

Cyclin A is a key regulator of the cell cycle progression from the S phase to the G2/M phase. To investigate the mechanism of N-induced S phase cell cycle prolongation, we first examined the cyclin A protein levels in transfected and control cells using western blot assay. As shown in Fig. 6A, the cyclin A expression level was significantly down-regulated in cell lines that expressed N protein compared with control cells. This indicates that it is the expression of N, rather than GFP, that prolongs the S phase cell cycle progression. To further support these findings, quantitative real-time RT-PCR was employed. The results show that cyclin A mRNA levels in the GFP-N-expressing cell lines were significantly lower than in control cells (Fig. 6B). This suggests that S-phase prolongation induced by PEDV N protein is associated with cyclin A protein degradation and a decrease in cyclin A transcription.

3.7. PEDV N protein induces ER stress via up-regulation of GRP78 and activation of NF-κB

To analyze the expression of glucose regulated protein 78 (GRP78), we chose a well characterized ER chaperone protein that is a marker of ER stress (Hong, 2005; Lee, 2005; Li et al., 2008). We examined the GRP78 protein levels in transfected and control cells by western blot assay. The GRP78 expression level was significantly up-regulated in cell lines that expressed N protein compared

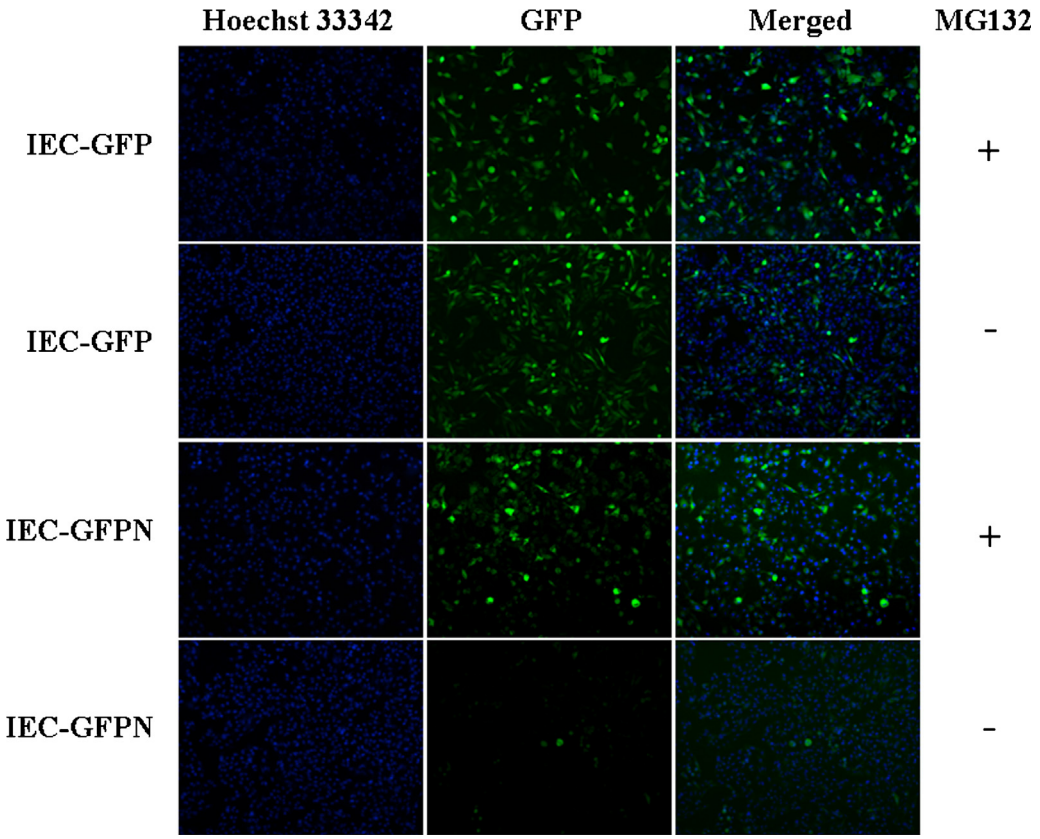

Fig. 2. Fluorescence detection of GFP and GFP-N fusion protein expressed in IEC cell lines (100×). All the cell lines were stained by Hoechst33342. Merged images show that expression of GFP-N is higher in IEC cells treated with MG132 than in untreated cells.

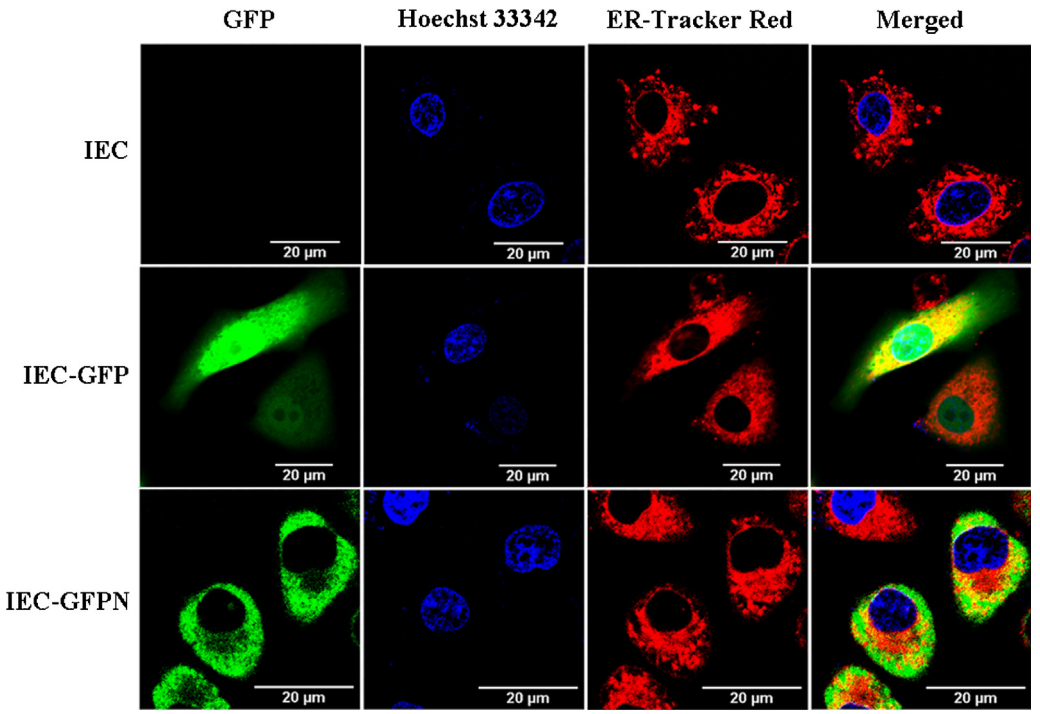

Fig. 3. Detection of GFP-N fusion protein subcellular localization in IEC cells by confocal microscopy. All the cell lines were stained by Hoechst33342 and ER-Tracker™ Red. Merged images showed co-localization of GFP-N protein in the ER. Bar = 20 μm for all the figures.

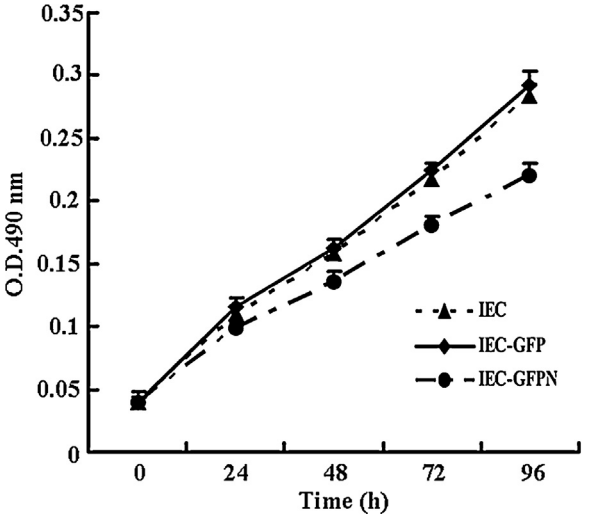

Fig. 4. Cell proliferation assays of the stable N protein expressing cells. The MTT assay was used to measure proliferation of 3×10^3 cells from IEC cell lines over time. Each data set represents the mean ± SD of six replicates.

with control cells (Fig. 7A). Moreover, the GRP78 transcription level detected using real-time PCR assay was also significantly increased in GFP-N-expressing cell line compared with controls (Fig. 7B).

Analysis of the activity of NF-κB in GFP-N-expressing cell lines demonstrates that NF-κB was significantly activated compared with control cells (Fig. 8). Together, these analyses show that expression of PEDV N protein results in the up-regulation of GRP78 and NF-κB, which suggests the N protein has a role in ER stress activation.

3.8. PEDV N protein up-regulates IL-8 expression

Secretion of IL-8 in the supernatant liquid from untransfected and transfected cells was examined using ELISA assay. As shown in Fig. 9A, GFP-N expressing cells were found to express higher levels of IL-8 compared to control GFP expressing cells and untransfected cells. After treatment with MG132, the level of IL-8 in the supernatants from control cells is significantly decreased. In contrast, there was no such change in supernatants from the GFP-N expressing cells. An investigation of the transcriptional levels of IL-8 using real-time quantitative PCR found that the mRNA levels of IL-8 in the GFP-N expressing cells is also higher than in the GFP transfected and untransfected cells (Fig. 9B). These results suggest that PEDV N up-regulates IL-8 expression in IECs. IL-8 expression is regulated by activation of NF-κB and, in turn, the activation of NF-κB is associated with ER stress during viral infection (Hoffmann et al., 2002; Waris et al., 2002). Thus, these results suggest that PEDV N expression results in ER stress and NF-κB activation which is responsible for the up-regulation of IL-8.

3.9. PEDV N protein up-regulates Bcl-2 expression

It is well-known that the anti-apoptotic molecule Bcl-2 is tightly regulated by the transcription factor NF-κb

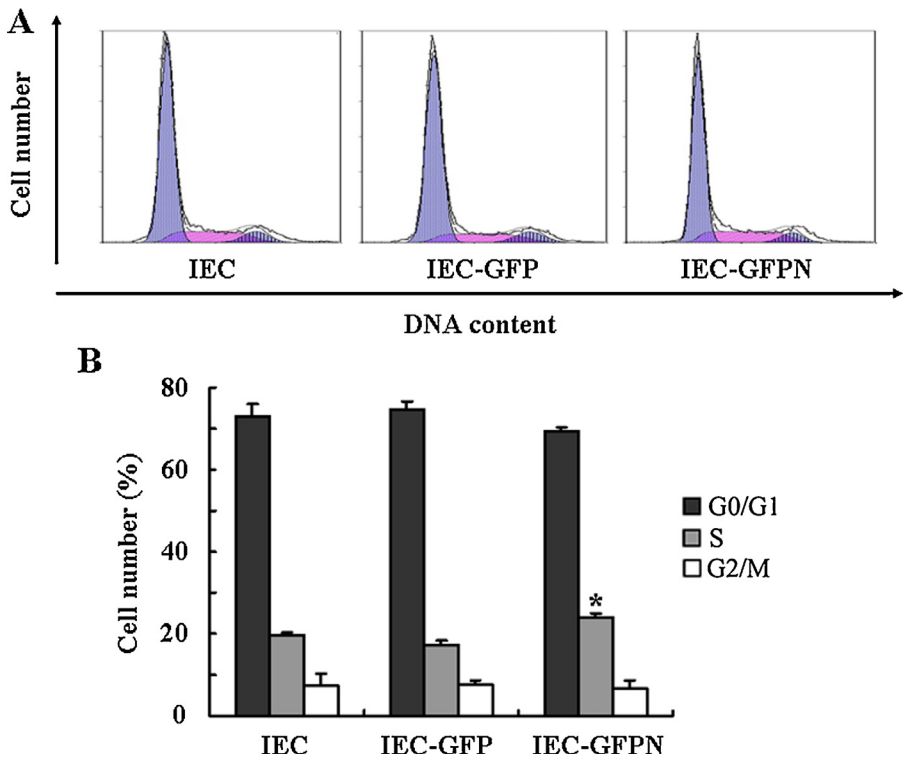

Fig. 5. The analysis of the stages of cell division of expressing PEDV N protein by flow cytometry. (A) Flow cytometry analysis of cells by propidium iodide staining. (B) The percentage of cells in each phase of the cell cycle from flow cytometry data. The results are mean ± SD from three independent experiments. $^*p < 0.05$ versus the control group (the cells expressing GFP and untransfected IEC cells).

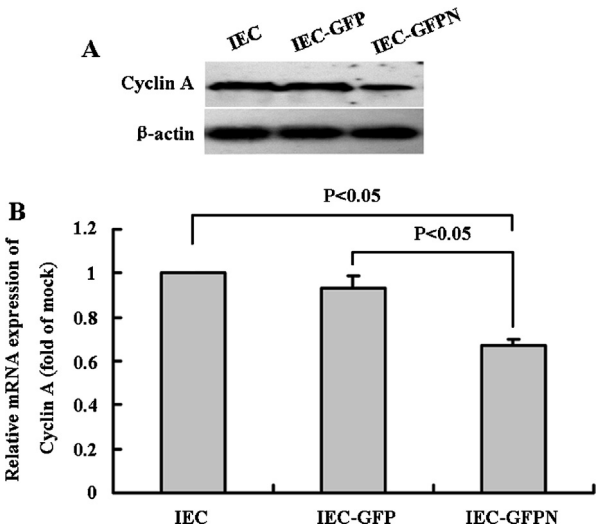

Fig. 6. The effect of PEDV N expression on the porcine cyclin A protein expression. (A) The level of cyclin A expression was determined by western blot with the mouse monoclonal antibodies against cyclin A in all cell lines. β-Actin was used as an internal loading control. (B) Total RNA was extracted from cells expressing either GFP alone, GFP-N or untransfected cells. Real-time PCR analysis of cyclin A mRNA levels were normalized to the corresponding CT value for porcine β-actin mRNA. The results are mean ± SD from three independent experiments.

(Fahy et al., 2005). Also, Bcl-2 as an anti-apoptotic molecule which is associated with cell survival (Batsi et al., 2009; Ricca et al., 2000; Seo et al., 2009). A quantitative real-time RT-PCR was employed and the

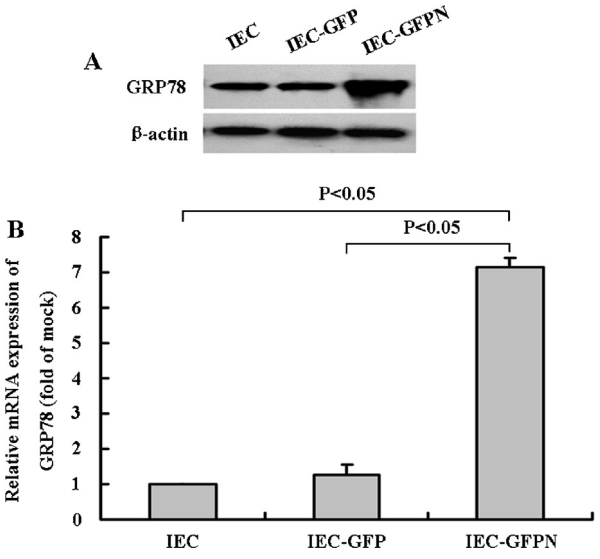

Fig. 7. Effect on porcine GRP78 expression by PEDV N expression in cultured IEC cells. (A) The level of GRP78 expression was determined by western blot with the mouse monoclonal antibodies against GRP78 in all cell lines. β-Actin was used as an internal loading control. (B) Total RNA was extracted from cells expressing either GFP alone, GFP-N or untransfected cells. Real-time PCR analysis of GRP78 mRNA levels was normalized to the corresponding CT value for porcine β-actin mRNA. The results are mean ± SD and representative of three independent experiments.

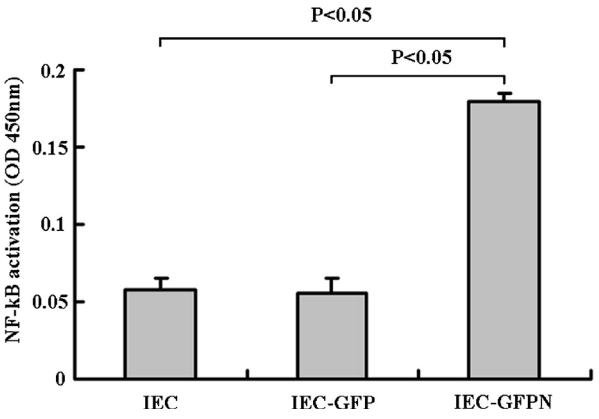

Fig. 8. PEDV N expression increases NF-κB activity in IEC cell lines. NF-κB p65 activation was determined using the TransAM assay. The data represent the mean and standard deviation from three different experiments.

results show that Bcl-2 expression in the GFP-N expressing cells is higher than in the control cells (Fig. 10). The results suggest that PEDV N protein may play a very important role in protecting the host cells from functional damage or apoptosis.

4. Discussion

Recent years, many studies were focus on the gene sequence analysis of PEDV. However, the subcellular localization and function of the PEDV N protein is still

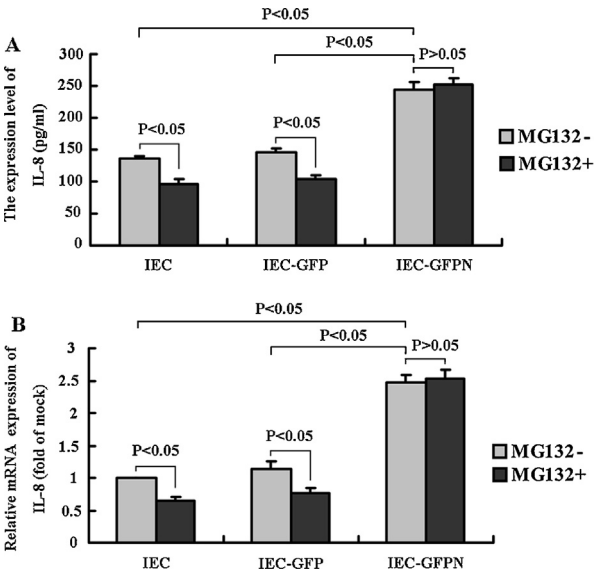

Fig. 9. PEDV N up-regulates IL-8 expression in IEC cells. (A) The concentrations of IL-8 in GFP-N expressing IEC or control cells (treated or untreated with MG132) culture supernatants were measured by ELISA. Data are mean ± SD and representative of three independent experiments. (B) The effect of PEDV N expression on porcine IL-8 transcription in cultured IEC cells. Total RNA was extracted from cells expressing either GFP alone, GFP-N fusion, or untransfected cells. Realtime RT-PCR analysis of IL-8 mRNA levels were normalized to the corresponding CT value for porcine β-actin mRNA. The results are mean ± SD and representative of three independent experiments.

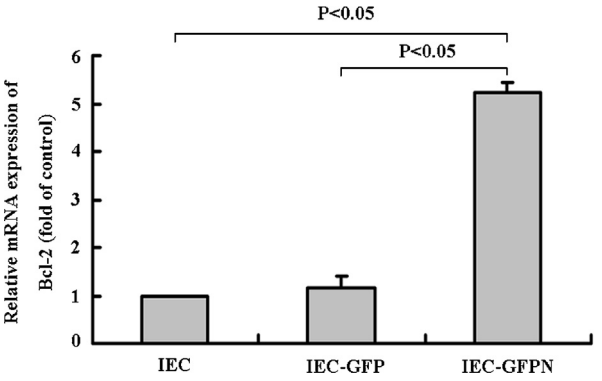

Fig. 10. Effect on porcine Bcl-2 expression by PEDV N protein in cultured IEC cells. Real-time PCR analysis of Bcl-2 mRNA levels were normalized to the corresponding CT value for porcine β-actin mRNA. Data are mean ± SD from three independent experiments.

unclear. Also, the function of this protein is yet to be determined, particularly with regard to its effect on host cell physiological changes. Recently, the N protein function of other coronaviruses also has been studied, especially in the N protein of transmissible gastroenteritis virus (TGEV) has extensively studied. The N protein of TGEV is an RNA chaperone and a naked plasmid DNA encoding the N protein of TGEV can elicit both humoral and cell-mediated immune (CMI) responses (Liu et al., 2001; Zúñiga et al., 2007). Previous study suggested a correlation among the replication cycle of SARS-CoV, subcellular localization of N, induction of apoptosis, and the subsequent activation of caspases leading to cleavage of the N protein of SARS-CoV (Diemer et al., 2008). In this study, we constructed a eukaryotic expression vector and generated stably expressing cell lines of PEDV N in fusion with the GFP protein that allowed the analysis of many of these properties. Co-localization studies clearly showed that GFP-N localized in the ER. It has been reported that the N proteins of MHV and IBV localized in the nucleolus of a small number of infected cells (Wurm et al., 2001). TGEV N protein was not observed in the nucleolus of infected ST cell lines (Calvo, 2005). In the case of our study, PEDV N protein was not observed in the nucleolus of the cells by confocal microscopy.

Viruses manipulate progression through the cell cycle and alter checkpoint signaling in order to provide a favorable environment for their own replication (Liu et al., 2005; Chulu et al., 2010). Recently, it was reported that the SARS-CoV N protein induced cell cycle arrest in the S phase through down-regulation of the S phase gene products expression (Surjit, 2006). One of the most critical phases during cell cycle progression is the S phase, since it can provide a cellular environment that is beneficial for viral replication. In this study, our findings show that the N protein of PEDV is able to inhibit the cell proliferation and prolongs the S-phase cell cycle. Cyclin A is very important in cells from the S phase to G2/M phase. In this study, the results of a western blot analysis show that cyclin A protein levels in PEDV N protein-expressing cell lines are significantly lower than in control cell lines. Furthermore, we also find that PEDV N protein significantly inhibits the transcription of cyclin A. These results show that the PEDV N protein plays an important role, not only in the cyclin A protein expression levels, but also in the transcription of cyclin A.

Our observations show that the PEDV N protein is likely to be responsible for inducing ER stress. We have shown that PEDV N protein localizes in the ER and is able to induce ER stress. This was indicated by the significant up-regulation of the molecule chaperon GRP78, a typical marker of ER stress. Under conditions of ER stress, mammalian cells accelerate the retrograde export of proteins from the ER to the cytosol for ubiquitylation and proteasome-mediated degradation (Friedlander et al., 2000; Rao and Bredesen, 2004; Schröder and Kaufman, 2005). Thus, we speculate that the PEDV N-expressing cells accelerate the retrograde export of cyclin A proteins from the ER to the cytosol for ubiquitylation and proteasome-mediated degradation.

The ER has essential roles in multiple cellular processes that are required for normal cellular functions and cell survival (Anelli and Sitia, 2008). Viruses use the ER as an integral part of their replication strategy and must therefore contend with the ER stress response and downstream consequences of ER stress signaling. This includes initiation of an inflammatory response through activation of NF-κB (Todd et al., 2008; Waris et al., 2002; Zhang and Kaufman, 2008). IL-8 as a pro-inflammatory neutrophil chemotactic factor plays an important role in the promotion of cell survival signaling and antagonizes the anti-viral activities of interferon. Our results show that PEDV N is able to up-regulate the expression of IL-8 in IEC cells. PEDV N protein induces ER stress and significantly activates NF-κB, which consequently leads to promotion of IL-8 transcription. Further research suggested that IL-8 production in control cells treated with MG132 was significantly lower than in untreated cells. However, IL-8 production from the GFP-N expressing cells treated with MG132 was not changed significantly compared with the untreated cells. Taken together, our findings suggest that MG132 is capable of inhibiting IL-8 production in control cells and PEDV N protein seems to antagonize the function of MG132.

In addition, NF-κB is a transcription factor that controls the expression of a variety of genes involved, not only in innate and adaptive immunity, but also in cell survival (Geng et al., 2009; Li and Verma, 2002; Wietek and O'Neill, 2007). As is known, Bcl-2 is an anti-apoptotic molecule which is associated with cell survival (Batsi et al., 2009; Ricca et al., 2000; Seo et al., 2009). Previous study suggests that the expression of Bcl-2 is regulated by the NF-κB (Fahy et al., 2005). In this study, the anti-apoptotic molecule Bcl-2 was significantly elevated in PEDV N protein expressing cells. Therefore, PEDV N protein may play an important role in protecting the host cells from morphological and functional damage or apoptosis.

In conclusion, PEDV N protein is localized in the ER. It inhibits IEC growth and prolongs the S-phase cell cycle. S-phase prolongation is associated with an increase in cyclin A degradation and a decrease in cyclin A transcription level. The PEDV N protein is able to up-regulate IL-8 expression in IECs. The up-regulation of IL-8 and Bcl-2 expression is ascribed to the ER stress response and

activation of NF-κB induced by PEDV N. Thus, the data suggest that PEDV N likely plays an important role in the inflammatory response and in persistent PEDV infection. This study has uncovered some novel features of the function of the PEDV N protein which are likely to be very useful in understanding the molecular mechanisms of PEDV pathogenesis.

Contributors

Xingang Xu and Honglei Zhang performed the majority of experiments and involved in manuscript preparation, Qi Zhang and Yong Huang participated in editing of the manuscript. Jie Dong and Yabing Liang participated part of the experiments. Dewen Tong and Hung-Jen Liu conceived of the study, participate in its design and coordination, and revised the manuscript. All authors read and approved the final manuscript.

Conflict of interest

There is no conflict of interest of any authors in relation to the submission.

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