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Transmissible gastroenteritis virus N protein causes endoplasmic reticulum stress, up-regulates interleukin-8 expression and its subcellular localization in the porcine intestinal epithelial cell

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ABSTRACT

This essay focuses on transmissible gastroenteritis virus (TGEV), which is an enteropathogenic virus related to contagious and acute diseases in suckling piglets. Previous literature suggests that the TGEV nucleocapsid protein (N) plays a significant role in viral transcriptional process, however, there is a need to examine other functions of TGEV N protein in the porcine intestinal epithelial cell (IEC) which is the target cell of TGEV. In the present study, we investigated the degradation, subcellular localisation, and function of TGEV N protein by examining its effects on cycle progression, endoplasmic reticulum (ER) stress, interleukin-8 (IL-8) expression, and cell survival. The results showed that TGEV N protein localised in the cytoplasm, inhibited IEC growth, prolonged the S-phase cell cycle by down-regulating cell cycle protein cyclin A, and was mainly degraded through the proteasome pathway. Moreover, TGEV N protein induced ER stress and activated NF-κB, which was responsible for the up-regulation of IL-8 and Bcl-2 expression. This report mainly considers the functions of TGEV N protein in IEC. To be specific, in IEC, TGEV N protein induces cell cycle prolongation at the S-phase, ER stress and up-regulates IL-8 expression. These results provide a better understanding of the functions and structural mechanisms of TGEV N protein.

1. Introduction

TGEV belongs to the genus *Alphacoronavirus* of the family *Coronaviridae* (de Groot et al., 2011). The infection of transmissible gastroenteritis virus (TGEV) leads to severe diarrhoea especially in piglets at age of up to 2 weeks, resulting in significant economic loss in swine-producing areas around the world (Kim and Chae, 2001). TGEV contains a large, single-stranded, positive-sense RNA genome (Sandrine et al., 2012). About two-thirds of the TGEV genome (28.5 kb) encodes the replicase gene (rep) at the 5′ end, and other one-third of the viral genes encodes the structural, and non-structural, proteins at the 3′ end (Rota et al., 2003). TGEV has four main structural proteins: spike (S), integral membrane protein (M), nucleocapsid protein (N), and a small envelope protein (sM), following the order 5′-S-3a-3b-*E*-M-N-7-3′ (Penzes et al., 2001).

TGEV N protein accumulation level is an important factor in efficient transcription (Zúñiga et al., 2010). The N protein is located in the nucleolus of infected cells and might delay the cell cycle (Wurm et al., 2001). In addition, TGEV N protein causing cell cycle arrest in PK-15 cells has been reported (Ding et al., 2014). Infection by TGEV causes villous atrophy throughout jejunum and the ileum, in other words, infection by TGEV is observed mainly in the porcine intestinal epithelial cell (IEC) (Pospischil et al., 1981); however, other concerns about the TGEV N protein in IEC are not fully understood, particularly the N protein-effected physiological changes in the host cells.

In this research, we investigated that TGEV N protein locates in the cytoplasm, effects cell cycle progression by down-regulating cyclin A expression, causes endoplasmic reticulum stress, and up-regulates NF-κB, Bcl-2, and interleukin 8(IL-8) expression.

2. Materials and methods

2.1. Vectors, plasmids and cells

The TGEV Shaanxi strain was isolated from intestinal tract contents of TGEV infected piglets in Shaanxi Province of China (Ding et al.,

Abbreviations: ER, endoplasmic reticulum; IEC, intestinal epithelial cell line; IL-8, interleukin 8; N, nucleocapsid; ORFs, open reading frames; TGE, transmissible gastroenteritis virus; TGEV, TGE virus; UPR, unfolded-protein response

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

Gene	Forward primer(5′–3′)	Reverse primer (5′–3′)		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

2011). The established IEC line,which was a kind gift from Prof. Yan-Ming Zhang, was cultured as described normaly. (Wang et al., 2010).

2.2. Antibodies and reagents

Antibodies specific for cyclin A, GRP78, NF-κB, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, Inc., CA, USA). 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). Anti-TGEV N protein antibody was produced in our laboratory (Chang et al., 2015). The nuclear staining dye Hoechst33342 and ER-Tracker™ Red probe were obtained from Invitrogen (Invitrogen, Carlsbad, CA, USA). MG132 proteasome inhibitor was purchased from Calbiochem (Calbiochem, San Diego, CA, USA).

2.3. Recombinant plasmid's construction and transient transfection

The N gene of TGEV Shaanxi strain was PCR amplified, then cloned into the corresponding sites in the eukaryotic expression vector pEGFP-N1 (GFP). According to enzyme digestion and DNA sequencing, the recombinant plasmid (N-GFP) was confirmed. IEC was seeded into 6-well dishes, and transfected with N-GFP or GFP vector: after 48 h of transfection, N protein expression was directly observed by a fluorescence microscopy (Model TE2000, Nikon, Japan) and further confirmed by Western blot analysis.

2.4. The observation of the protein degradation characteristic

Transfected cells were cultured with fresh medium which contains 20 μM MG132 and incubated for 24 h, then the cells were rinsed with PBS and incubated with nuclear dye Hoechst33342 at 37 °C for 20 min. Images were collected, after the cells have been rinsed twice with PBS, using fluorescence microscopy.

2.5. Confocal microscopy

Transfected cells were fixed with 4% formaldehyde at room temperature for 30 min after 24 h incubation, and then rinsed with PBS. After steps mentioned above, Hoechst33342 were used to strain all cells at 37 °C for 20 min and rinsed with PBS. ER-Tracker Red probe were used to incubate IECs at 37 °C for 20 min followed by PBS rinsing. ER-Tracker Red is a red fluorescent probe of ER and it can be used for specific fluorescent staining of ER in live cells. To support the results further, transfected IECs were also incubated with anti-N antibody, followed by goat anti-rabbit secondary antibody. Using laser confocal scanning microscope(Model LSM510 META, Zeiss, Germany), images were viewed.

2.6. Western blot analysis

After rinsed with ice-cold PBS, transfected cells were harvested and then lysed with ice-cold RIPA buffer with 1 mM phenylmethyl sulphonylfluoride (PMSF). Protein concentrations were assayed using BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). Equivalent amounts of proteins were subjected to 8–12% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corp, Atlanta, GA., USA). The membranes were blocked in PBS buffer with 5% non-fat dry milk at room temperature for 1 h, and then incubated with indicated primary antibodies overnight at 4 °C, followed by HRP-conjugated secondary antibodies incubation at room temperature for 1 h. The signal was detected by increased chemiluminescence (ECL) reagents (Pierce, Rockford, IL., USA).

2.7. Cell cycle analysis

Based on the principle that DNA content in nuclei were stained with propidium iodide (PI), flow cytometric analysis was employed in order to determine cell cycle progression in IEC transfected with recombinant plasmid. Transfected cells were fixed in 75% ethanol for 5 days at 4 °C and stained with PI. Coulter Epics XL flow cytometer(Beckman Coulter, USA) were used to analyse DNA content.

2.8. Real-time quantitative PCR analysis

Total RNA was extracted from cells using Trizol reagent (Invitrogen, California, USA). Reverse transcription was performed with M-MLV reverse transcriptase, oligo (dT) 18 primers and 2 μg of total RNA, according to manufacturer's instructions. The primers for real-time quantitative PCR assay were shown in Table 1. Reactions were carried out in a 25 μl-mixture containing AccuPower 2× Greenstar qPCR Master Mix (Bioneer Corporation, South Korea), sense and anti-sense primers (0.4 μM) and target cDNA (4 ng). The reaction entails a 5 min initialisation at 95 °C and 40 cycles of 5 s-denaturation at 95 °C, and 30 s-annealing and elongation at 60 °C. A negative control was included in every test and melting curve (T_m value) were used to present each amplification reactions' specificity. Using the respective CT value for the porcine β-actin housekeeping gene to equalize genome, the individual samples were normalized. 2ˆ-ddCT method was used to determine target gene expression's relative quantification (Livak and Schmittgen, 2001).

2.9. NF-κB activity's detection

The GFP and N-GFP alteration proteins of NF-κB activity was measured using Western blot assay and the NF-κB p65 TransAM kit (Active Motif). Briefly, transfected cells' nuclear extractions protein were extracted using the Nuclear Extract Kit (KeyGEN, Nanjing, China) and BCA Protein Assay Reagent (Pierce, Rockford, IL, USA) were used to determine nuclear protein concentrations. Lysates (50 μg total proteins) were incubated in ELISA wells coated with the oligo-nucleotide motif recognised by active p65, followed by HRP-conjugated secondary antibody incubation. Finally, the sample was measured using colorimetric reaction at 450 nm.

2.10. Enzyme-linked immunosorbent assay

The culture medium of transfected IEC was harvested and analysed using swine IL-8 ELISA kit.

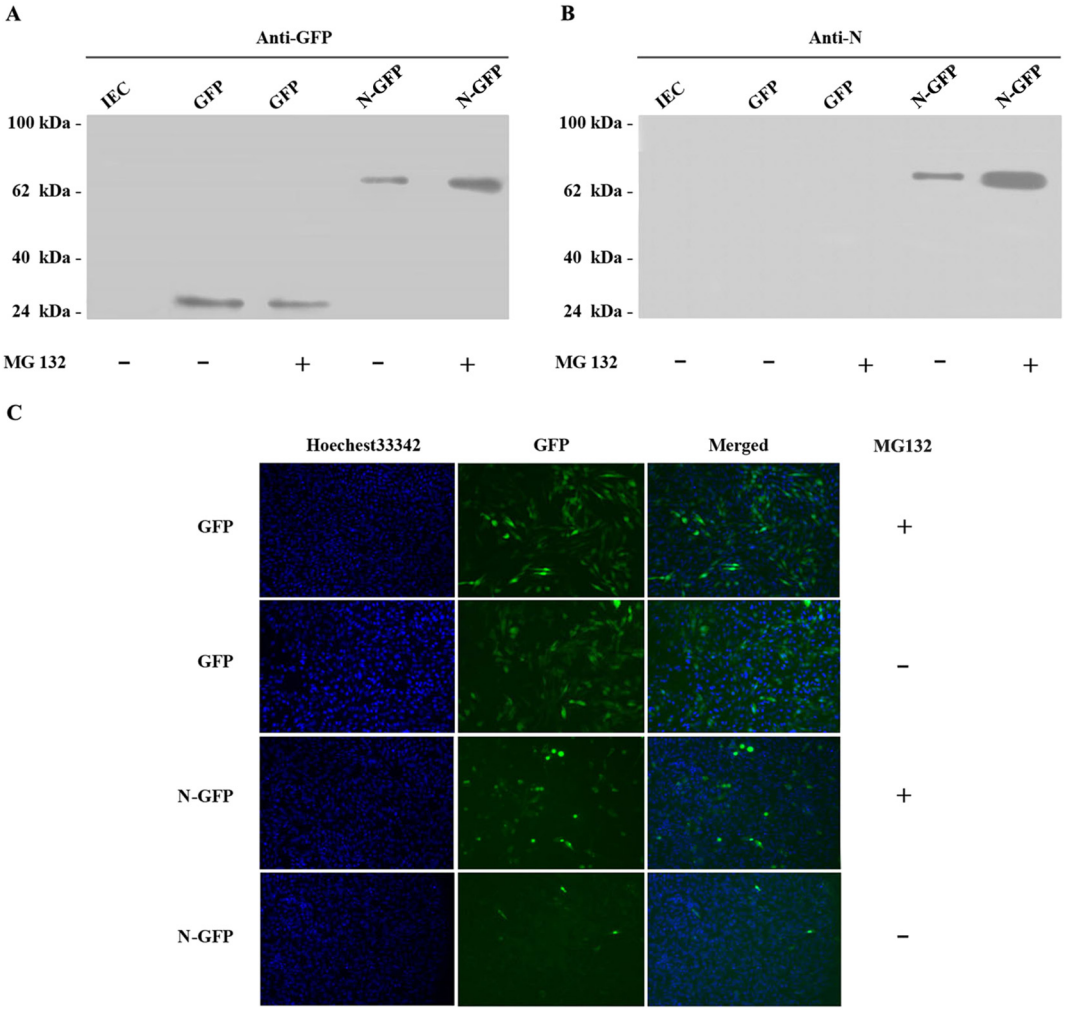

Fig. 1. The expression products and protein degradation characteristics of TGEV N protein in IEC.
Cells were transfected with N-GFP expression vector or GFP vector and treated with MG132 for 24 h. (A) The cells were subjected to Western blot analysis using anti-GFP antibodies. (B) The cells were subjected to Western blot analysis using TGEV N protein antibodies. (C) Protein degradation characteristics were observed by fluorescence microscopy. All the data shown are representative of three independent experiments.

2.11. Statistical analysis

All data are presented as the means ± SD of three independent experiments done in triplicate. For each assay, *t*-test was used for statistical comparison and a p value of < 0.05 is considered statistically significant.

3. Results

3.1. TGEV N protein expression and degradation characteristics

The Western blot analysis demonstrated that the protein molecular mass produced by the cells transfected with GFP-N plasmid is approximately 70 kDa, as was detected with anti-GFP monoclonal antibodies (Fig. 1A) and anti-TGEV N protein antibodies (Fig. 1B). The molecular mass of GFP is approximately 27 kDa, indicating that N protein, whose molecular mass is approximately 43 kDa, was successfully expressed. Meantime, no signal was detected from untransfected cells.

Fluorescence microscopyhe were used to observe N proteins' degradation characteristics (Fig. 1C) and its degradation level was detected by Western blot assays (Fig. 1A, B). The results show that GFP-N protein level in the cells treated with MG132 was higher than that in untreated cells, while there were no GFP protein expression differences between MG132 treated and untreated ones.

3.2. TGEV N protein subcellular localisation

Confocal fluorescence microscopy were used to investigate the subcellular localization of N protein. And results show that GFP-N proteins distribute predominantly in the cytoplasm, while the GFP protein was localised in the whole cell (Fig. 2A, B).

3.3. TGEV N protein prolongs the S-phase cell cycle by down-regulating cell cycle protein cyclin A

To investigate in which particular phase TGEV N-effected cell cycle arrest occurred, cell cycle profiles were analysed by flow cytometry (Fig. 3A) and cyclin protein were examined by RT-PCR and Western-blot assay (Fig. 3C, D). Besides, in order to confirm the percentage of cells in each phases of the G0/G1, S, and G2/M(Fig. 3B), histograms quantitative analysis was employed. These data suggest that the DNA content of S-phase increased, while the G0/G1 and G2/M phases showed slight changes and that TGEV N protein prolonged the S-phase cell cycle and prevented GFP-N expressing cells from entering the G2/M phase. The results show that TGEV N protein could cause S-phase prolongation. As shown in Fig. 3C, comparing with control cells, the Cyclin A protein level was significantly decreased in N protein

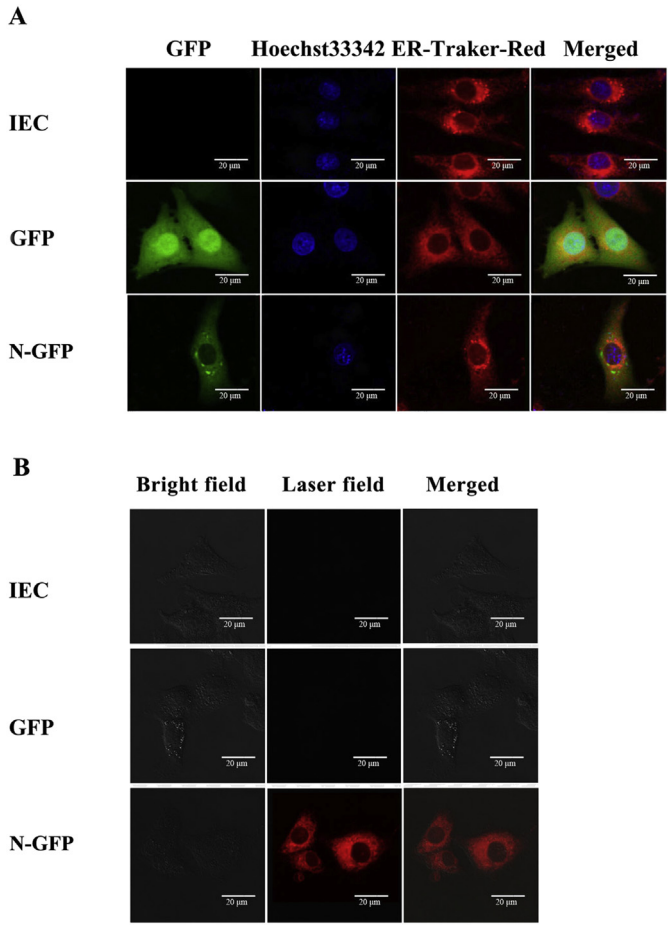

Fig. 2. Subcellular localization of TGEV N protein in IEC. Cells were transfected with N-GFP expression vector or GFP vector.
(A) Cells were stained by Hoechst33342 and ER-Tracker ™ Red. (B) Cells were stained with anti-N antibody, followed by goat anti-mouse antibody. Bar = 20 μm for all the figures. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

expressing cells. To further support these findings, real-time quantitative PCR assay were uesd to determine the cyclin A mRNA level. The results show that, in the GFP-N expressing cells, cyclin A mRNA levels are significantly lower than that in control cells (Fig. 3D), suggesting that S-phase prolongation induced by TGEV N protein is closely related to cyclin A protein degradation and can down-regulate cyclin A transcription.

3.4. TGEV N causes ER-stress and up-regulates expression of IL-8 and Bcl-2

GRP78 is widely used as a key regulator for ER stress. The levels of GRP78 expression in transfected and untransfected cells were detected by Western blot assays (Fig. 4A) and real-time PCR assay (Fig. 4B). The results show that GRP78 and expression level in transfected cells expressing N protein are both significantly higher than that in untransfected cells. Western Blot assay (Fig. 4C) and ELISA assay (Fig. 4D) results show that the NF-κB expression level presents the same trends as GRP78.

In this research, ELISA assay (Fig. 4E) and real-time PCR assay (Fig. 4F) results showed that TGEV N up-regulates IL-8 expression in IEC, suggesting that TGEV N expression result in ER stress, and NF-κB activation, are responsible for the up-regulation of IL-8.

In this research, Western blot(Fig. 4G) and quantitative real-time PCR (Fig. 4H) were used, showing that the expression of Bcl-2 in N-GFP protein expressing cells is higher than that in untransfected cells. The results suggest that TGEV N protein is able to up-regulate Bcl-2 expression both at the gene, and protein, levels which might be induced by ER stress response through the NF-κB signalling pathway.

4. Discussion

TGEV N protein has been reported to induce apoptosis, but its effects on host cell physiological changes are not clear. In this study, we investigated the TGEV N protein's degradation, subcellular localisation, and function by examining its effects on cycle progression, IL-8 expression, and ER stress in IEC.

Knowing the subcellular localisation of a protein enables us to have a better understanding of its function (Marcos et al., 2013). With the SARS-CoV N protein located in the cytoplasm of A549 and Vero E6 cells (Diemer et al., 2008), PEDV N protein in the ER (Xu et al., 2013), TGEV N protein in the nucleolus of a small number of infected cells (Wurm et al., 2001) and the Golgi–ERGIC of infected ST cell lines (Calvo, 2005), our study demonstrated that TGEV N protein was located in the cytoplasm of IEC. According to these results, we think that TGEV N protein may be located in different sub-cellular positions in a cell type-dependent way.

It has been shown that many coronaviruses' N protein could inhibit cell cycle progression although the mechanism governing this remained unclear. SARS-CoV N protein binds to, and subsequently inhibits activity of the cyclin-CDK complex, resulting in inhibition of S phase progression (Surjit et al., 2005). Chicken anaemic virus (CAV) apoptin protein and Herpes simplex type 1 (HSV-1) ICP0 protein block cell cycle progression at the G2/M phase (Teodoro et al., 2004; Lomonte et al., 2001). PEDV N protein prolongs the S phase of cell cycle (Xu et al., 2013). In this research, our results showed that TGEV N protein was able to prolong the S-phase cell cycle. The cyclin A levels in the transfected cells were lower than those in untransfected cells. This data suggested that TGEV N protein prolonged the S phase of cell cycle and played a crucial part in both cyclin A protein expression and cyclin A transcription.

GRP78, an ER resident protein belonging to Hsp70 family, is the major regulator of the cell's unfolded-protein response (UPR)of a cell, which is the cellular response to ER stress (Wu et al., 2011). Activated UPR can decrease protein translation and increase ER capacity, which leads to a GRP78 up-regulation, and thus relieves ER stress. It is important to notice that the UPR will induce apoptosis when ER homeostasis is not restored (Pfaffenbach and Lee, 2011). Our data showed that TGEV N protein up-regulate GRP78 expression, which suggested that TGEV N protein was responsible for ER stress, and might participate in cell apoptosis.

Over-expressed GRP-78 can cause an ER stress response (ER stress NF-κB3) through the NF- κB pathway, which leads to both an inflammatory response and cell survival signalling pathways (Baker et al., 2011). It also prevents apoptosis by increasing anti-apoptotic genes expression, such as that of Bcl-2 (Jang and Surh, 2004). Bcl-2 family members are important regulators for apoptosis, and Bcl-2 over-expression enhances NF-kB-dependent transcriptional activity (Ricca et al., 2000).Our data showed that TGEV N protein can up-regulate NF-κB and Bcl-2 expression. Therefore, TGEV N protein may play a crucial part in protecting the host cells from morphological and functional damage or apoptosis.

IL-8, as a prototypic human chemokine factor, plays an important role in the promotion of cell survival signalling and antagonises the anti-viral activities of interferon. Coronaviruses generally do not induce a high inflammatory response such as IL-6, IL-1, and IL-8 (Hoffmann et al., 2002). IL-8 production plays an important role in the over-expression and modulation of Bcl-2 (Escudero-Lourdes et al., 2012). In this study, we found that the TGEV N protein up-regulated IL-8 expression in host cells. The degradation of the TGEV N protein, when inhibited by MG132 proteasome inhibitor, was mainly through the proteasome pathway. More researches showed that, in untransfected

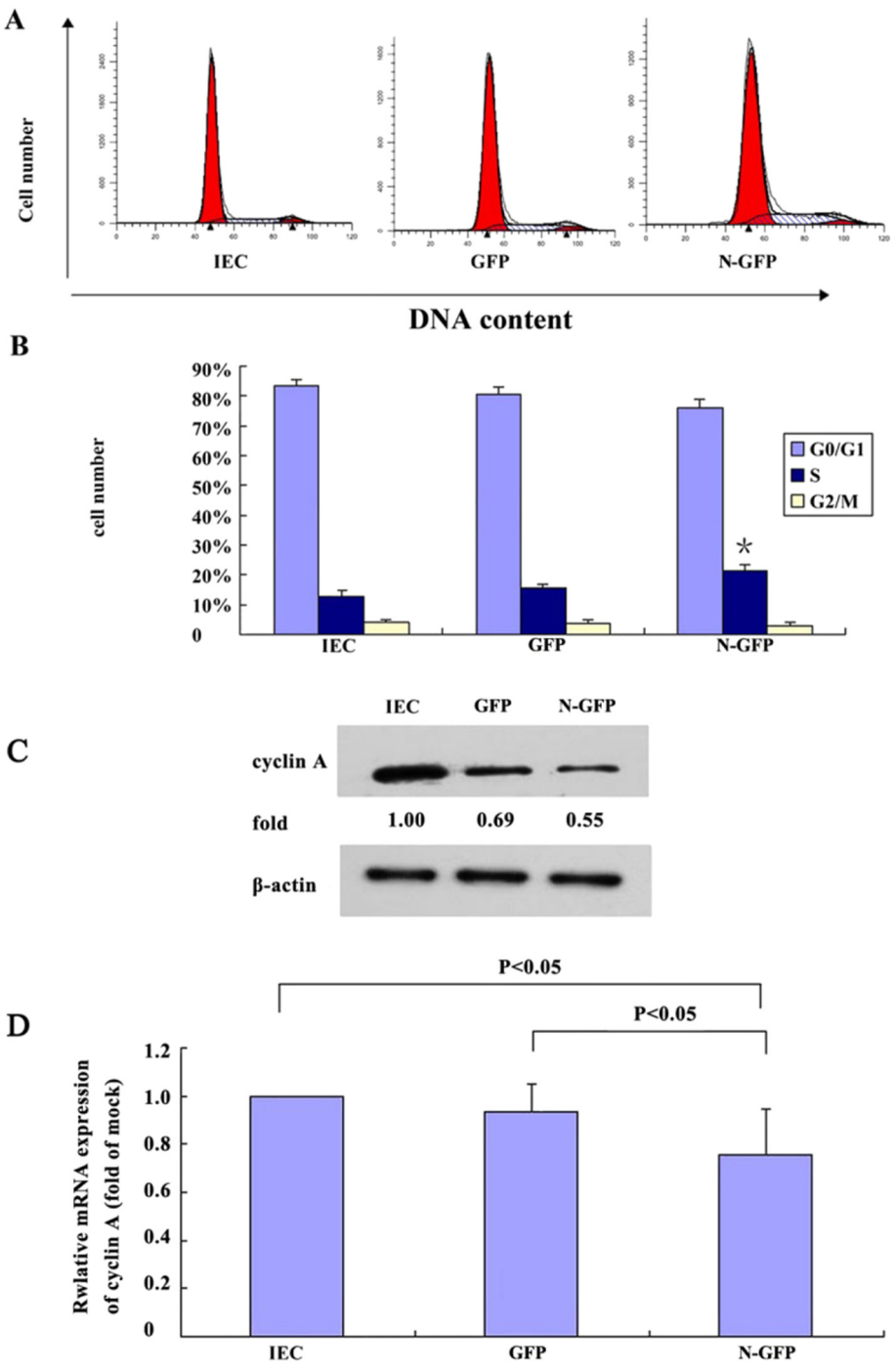

Fig. 3. Cell cycle arrest and the expression of cyclin A induced by TGEV N protein. Cells were transfected with N-GFP expression vector or GFP ector for 48 h. (A) Flow cytometry analysis of cells by propidium iodide staining. (B) The percentage of cells in each phase of the cell cycle from flow cytometry data. (C) The level of cyclin A expression was determined by western blot. β-actin was used as an internal loading control. (D) 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. * $p < 0.05$ versus the control groupversus the control group (the cells expressing GFP and untransfected IEC cells).

cells treated with MG132, IL-8 production was much lower than that in untreated cells; however, in the N-GFP expressing cells, IL-8 production showed a slight change than that in untreated cells, indicating that MG132 was able to inhibit IL-8 expression in untransfected cells and that TGEV N protein contributed to antagonism of the effects of MG132. Taken together, TGEV N protein can cause ER stress and activated NF-κB. As a result, the IL-8 expression was increased and thus enhanced the expression of Bcl-2.

In conclusion, the present study demonstrated that, following degraded through the proteasome pathway and localised in the cytoplasm of IEC, TGEV N protein could prolong the S-phase in the cell cycle, which was in the way of the degradation and decreased cyclin A transcription. NF-κB, IL-8 and Bcl-2 have closely relationship that regulate cells' survival, propagation and apoptosis. In our research, ER stress response and NF-κB activation up-regulated the expression of IL-8 and Bcl-2 in IEC, which induced by TGEV N protein. Results reveals a comprehensive understanding in cell cycle and ER stress induced by TGEV N protein, which may provide new insight into TGEV and IEC interaction.

Author's contribution

Qi Zhang, Ying Xu and Rong Chang performed the majority of experiments and involved in manuscript preparation. Xingang Xu and Dewen Tong conceived of the study, participate in its design and coordination, and revised the manuscript. All authors read and approved the final manuscript.

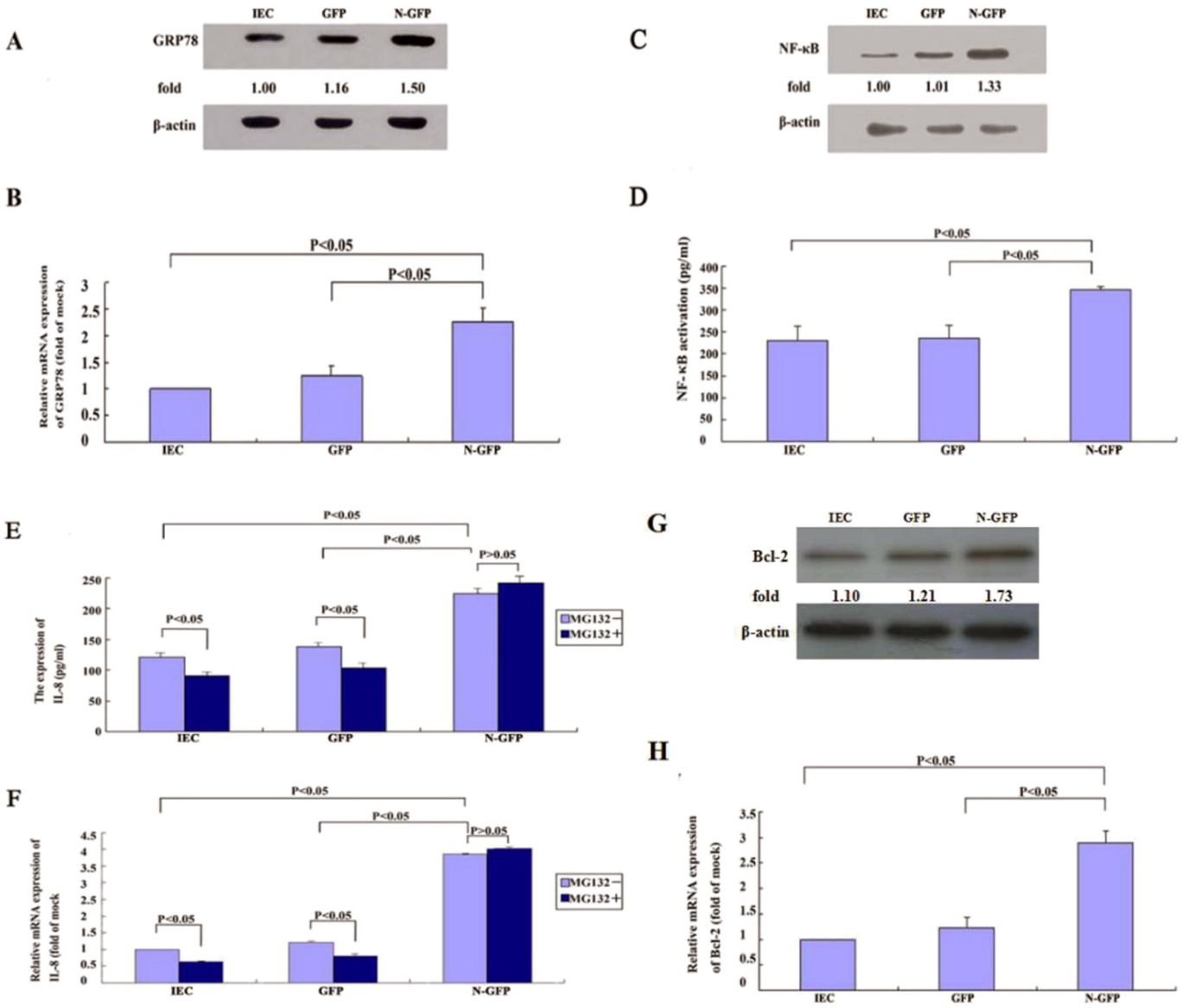

Fig. 4. The effect of TGEV N protein in IEC on NF-κB activity and the expression of GRP78, IL-8, Bcl-2, IL-8.
Cells expressing GFP or N-GFP for 48 h. (A) The level of GRP78 expression was determined by western blot. (B) Real-time PCR analysis of GRP78 mRNA levels. (C) The level of NF-κB expression was determined by western blot. (D) NF-κB p65 activation was determined using the ELISA assay. (E) The expression of IL-8 in N-GFP expressing IEC or untransfected cells (treated or untreated with MG132) culture supernatants were measured by ELISA. (F) IL-8 mRNA levels were analysed by Real-time PCR assay. (G) The level of Bcl-2 expression was determined by western blot. (H) Real-time PCR analysis of Bcl-2 mRNA levels. β-actin was used as an internal loading control. The results are mean ± SD and representative of three independent experiments.

Conflict of interest

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

Acknowledgements

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