Transmissible gastroenteritis virus nsp7 protein localized in the cytoplasm down-regulates interleukin 8 expression in porcine intestinal epithelial cell

Q. ZHANG[#], J. L. HUANG[#], Y. B. LIANG[#], Y. P. HE, D. W. TONG[*], X. G. XU[*]

College of Veterinary Medicine, Northwest A&F University, Yangling, Shaanxi 712100, P. R. China

Summary. – Transmissible gastroenteritis virus (TGEV) is an important pathogen in swine that is responsible for substantial economic losses. Previous studies suggest that the TGEV non-structural protein 7 (nsp7) plays an important role in the viral assembly process. However, the subcellular localization and other functions of the TGEV nsp7 protein are still unclear. In this study we have examined the subcellular localization and other functions of TGEV nsp7 protein through analysis of its effects on cell growth, cell cycle progression, interleukin 8 (IL-8) expression, and NF-κB activation. Our results showed that the nsp7 protein is localized in the cytoplasm and has no effect on intestinal epithelial cells (IECs) growth, cell cycle, and cyclin A expression. Further studies showed that TGEV nsp7 protein had no effect on GRP78 expression, could not induce endoplasmic reticulum (ER) stress and activate NF-κB activity. Interestingly, the IECs expressing nsp7 protein secreted lower levels of IL-8 than control cells. This is the first report to demonstrate the subcellular localization and novel functions of TGEV nsp7 protein. These findings provide novel information about the function of the poorly characterized TGEV non-structural protein 7.

Keywords: transmissible gastroenteritis virus; nsp7 protein; endoplasmatic reticulum stress; interleukin 8; NF-κB

Introduction

Transmissible gastroenteritis (TGE) is a fatal and highly contagious enteric infection of swine, which is characterized by vomiting, and severe watery diarrhea, associated with high morbidity in swine of all ages and high mortality in neonatal piglets (Wesley *et al.*, 1991). The causative agent of TGE is transmissible gastroenteritis virus (TGEV). TGEV belongs to the genus *Coronavirus*, the family *Coronaviridae*. TGEV like other coronaviruses is a pleomorphic enveloped virus that contains a large, single-stranded, positive-sense 28.5 kb RNA genome (Wesley *et al.*, 1990). Genome contains a 5' cap and a poly (A) tail at 3' end and has 7 open reading frames

(ORFs) (Hiscox *et al.*, 1995). The genes of TGEV are arranged in the order of 5'-Rep-S-3a-3b-E-M-N-7-3'. Four structural proteins include spike protein (S), membrane protein (M), envelope protein (E), and nucleoprotein (N); the other four proteins, Rep, 3a, 3b and 7 are non-structural proteins (Ribes *et al.*, 2011; Lan *et al.*, 2011). About two-thirds of the entire RNA comprises of ORFs 1a and 1b, encoding the replicase (Eleouet *et al.*, 1995).

The functions of four structural proteins have been intensively studied (Eleouet *et al.*, 2000; Godet *et al.*, 1992). As for TGEV non-structural proteins, it has been reported that TGEV non-structural protein 7 (nsp7) is encoded by 237 bp long open reading frame 7 (ORF7) at the 3' end of the viral genome. The nsp7 is 78 amino acids long, hydrophobic protein with molecular weight of 9.1 kDa (Tung *et al.*, 1992) expressed during virus replication, which is associated with the endoplasmic reticulum and cell surface membrane of infected cells. TGEV gene 7 is not an essential gene for virus viability but its deletion influences replication and attenuates virus virulence in piglets (Ortego *et al.*, 2003;

Park *et al.*, 2008). According to Snijders study, coronavirus nsp7 to nsp16, the cleavage products of two large replicase polyproteins translated from the virus genome, can direct coronavirus RNA synthesis and processing with nsp7 to nsp10 acting as crucial cofactors of these enzymes (Snijder *et al.*, 2016). Additionally, some reports show that the deletion of gene 7 in feline coronavirus (FCoVs), another member of the *Coronavidae* family, attenuated the viral virulence (Herrewegh *et al.*, 1995; Kennedy *et al.*, 2001). The nsp7 in porcine reproductive and respiratory syndrome virus (PRRSV), member of the *Nidovirales* order, can facilitate humoral immune system to produce anti-nsp7 antibodies, which are highly expressed in PRRSV-infected swine. Despite the studies mentioned above, the functions of TGEV non-structural proteins still requires further research.

Recently, no data are reported about the subcellular localization of TGEV nsp7 protein, its effects on cell growth and cell cycle progression. The intestine is the target organ of TGEV in piglets (Hooper *et al.*, 1966; Cornelius *et al.*, 1968). The porcine intestinal epithelial cells (IECs) are the target cells of TGEV. The epithelial cells in the gut serve as a physical barrier, restricting the movement of components and the passage of potentially harmful microorganisms between the lumen and the underlying mucosa (Schierack *et al.*, 2006).

In this study, we have analyzed the subcellular localization of TGEV nsp7 and elucidated the effects and mechanisms of this protein on cell cycle and cell growth. The results show that the expression of TGEV nsp7 has no effect on cell growth, cell cycle and cyclin A expression in IEC. Furthermore, we have shown that TGEV nsp7 is localized in the cytoplasm and does not induce ER stress. Our study also showed that the degradation of TGEV nsp7 is not via the proteasome pathway. In addition, the results also show that TGEV nsp7 protein can down-regulate IL-8 expression. These results have potentially important implications for understanding the molecular mechanisms of TGEV pathogenesis. To our knowledge, this is the first report about TGEV nsp7 protein function in porcine intestinal epithelial cells.

Materials and Methods

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 TGEV Shannxi strain was isolated from intestinal tract contents of TGEV infected piglets in Shannxi Province of China and nsp7 gene of TGEV was amplified as described previously (Ding L *et al.*, 2011). The IECs which were kindly provided by Prof. Yan-Ming Zhang, College of Veterinary Medicine, Northwest A&F University, were cultured as described previously (Wang *et al.*, 2010). IECs were grown in Dulbecco's modified eagle medium (DMEM) (Gibco BRL, USA) supplemented with 10%

heat-inactivated new born calf serum (FCS, Gibco BRL, USA), 100 IU of penicillin and 100 μg/ml of streptomycin at 37°C in a 5% CO_2 atmosphere incubator. The culture medium was replaced every 3 days.

Antibodies and reagents. Mouse monoclonal antibodies against GRP78, cyclin A, NF-κB p65, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, Inc., USA), Horseradish peroxidase (HRP)-conjugated secondary antibody was purchased from Pierce (Pierce, USA). The MG132 proteasome inhibitor was purchased from Calbiochem (USA) and the nuclear staining dye Hoechest 33342 and ER-Tracker™ Red probe were obtained from Invitrogen (USA).

Construction of recombinant plasmid. The primers used for TGEV ORF7amplification were as follows: forwards primer (TGEV-XhoI), 5'-CCG CTCGAGATGCTCGTCCTCCTCCATGC-3' (28032–28051 of TGEV TS strain) and reverse primer (TGEV-EcoRI), 5'-CGGGAATTCCGTGGTGGTGGTGGTGGTGCAT AATATGACTAACAATC-3' (28249–28268 of TGEV TS strain). The restriction sites are underlined. The reverse primer contained six his tags. The primers were designed according to the archived TGEV TS strain nucleotide sequence (GenBank: DQ201447.1) and synthesized by Shanghai Invitrogen (China). The PCR products were separated on 1.0% agarose gel electrophoresis, purified from the gel, digested with restriction enzymes and cloned into the pEGFP-N1 expression vector. The recombinant plasmid was designated as p7-EGFP and recovered from transformed *E. coli* using a Plasmid mini-kit (Axygen, China). Plasmids were verified by enzyme digestion and DNA sequencing.

Transfection. IECs were seed into 6-well plates 24 h before transfection (up to 70–80% confluence). Cells were transfected with p7-EGFP and pEGFP-N1 control vector using Lipofectamine 2000 (Invitrogen, USA) and maintained in selection media containing 1200 μg/ml G418 for two weeks.

Nsp7 protein degradation characteristics. The cell lines expressing p7-GFP protein and GFP were seeded into 6-well plates at a suitable concentration. After incubation at 37°C with 5% CO_2 for 24 h, the culture medium was replaced with fresh medium containing 20 μmol/l MG132, and then incubated in a CO_2 incubator at 37°C for 24 h. After 24 h incubation, the cells were washed twice with PBS and incubated with Hoechest 33342 at 37°C for 15 min. Images were analyzed by Fluorescence microscope (Model TE2000, Nikon, Japan).

Confocal microscopy analysis. To examine the expression and subcellular localization of TGEV nsp7 protein, the stable cell lines expressing p7-GFP protein or control cells (GFP and untransfected cells) were grown on glass cover slips. Cells washed with PBS were incubated with Hoechest 33342 at 37°C for 25 min, washed with PBS and incubated with ER Tracker Red probe (Invitrogen, USA) at 37°C for 25 min and washed with PBS again. Images were viewed by laser confocal scanning microscopy (Model LSM510 META, Zeiss, Germany).

Western blot analysis. Cells were harvested and washed with ice-cold PBS, then treated with ice-cold RIPA lysis buffer with 1mmol/l

Table 1. The primers for real-time PCR

Gene	Forward primer (5'–3')	Reverse primer (5'–3')	Product (bp)	Acc. 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

phenylmethyl sulfonfluoride (PMSF). Cell lysates were centrifuged at 12,000 × g at 4°C for 10 min. Protein concentrations were measured by BCA protein assay (Pierce, USA). Equivalent amounts of proteins were loaded and separated on 8–12% SDS-PAGE. Subsequently, proteins were transferred to PVDF membranes (Millipore Corp, USA). The membranes were blocked with 5% non-fat milk at room temperature for 1 h, and then incubated with specific primary antibodies over night at 4°C, followed by incubation with HRP-conjugated secondary antibody at room temperature for 1 h. The signal was detected by enhanced chemiluminescence (ECL) reagents (Pierce, USA).

Cell proliferation assay. The MTT cell proliferation assay was performed to determine the growth properties of TGEV nsp7 expressing and control cells according to the manufacturer's instructions. Briefly, cells were seeded in 96-well culture plates at a concentration of 4×10^3 cells per well in 200 μl of culture medium. After incubation at 37°C with 5% CO_2 for 24 h, 48 h, 72 h and 96 h, the culture medium was carefully replaced with 200 μl of a fresh medium without disturbing the cells. 20 μl of 3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenylte-trazolium bromide (MTT, 5mg/ml) (Sigma, USA) reagent was added to each well and incubated in a CO_2 incubator at 37°C for 4 h. After 4 h incubation, the reactions were stopped by addition of 100 μl of DMSO into each well. The absorbance at wavelength of 490 nm (A_{490}) was read by a microplate reader (Model 680, Bio-Rad, USA) at appropriate time intervals. The experiments were independently repeated three times.

Cell cycle analysis by flow cytometry. The cell cycle was analyzed by using propidium iodide (PI) staining. Briefly, approximately 2×10^6 cells of the p7-GFP expressing cell line and control cells were treated with trypsin, washed with PBS, resuspended in 75% ethanol and fixed at 4°C for 3 days. Cells were washed with PBS and resuspended in PBS containing 20 μg/ml of RNase A and 50 μg/ml of PI and incubated at 4°C for 30 min in the dark. Finally, the nuclear DNA content was determined by a Coulter Epics XL flow cytometer (Beckman Coulter, USA).

Real-time quantitative PCR analysis. Total RNA was extracted from cells using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions and reverse transcribed with M-MLV reverse transcriptase (Thermo Fisher Scientific, USA), oligo(dT)18 primers and 2 μg of total RNA. The expression of genes was quantified using Bio-Rad iQ5 Real Time PCR system (USA). The primers for qPCR used in this study are shown in the Table 1. Reactions were carried out in 25 μl volume containing SYBR Premix Ex Taq

TM II (Takara, China), sense and anti-sense primers (0.4 μmol/l) 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 specificity of amplification reactions was checked by melting curve (Tm value) analysis. The individual samples were normalized for genome equivalents using the respective CT value for the β-actin housekeeping gene. The relative quantification of gene expression was analyzed by the two-ddCt method as described previously (Livak *et al.*, 2001).

Detection of NF-κB activity. To determine the alteration of NF-κB activity by GFP and p7-GFP proteins in IECs, the level of NF-κB activity was measured using the NF-κB p65 TransAM kit (Active Motif) according to the manufacturer's instructions. Briefly, nuclear extracts were prepared by using the Nuclear extract kit (KeyGEN, China) and protein concentrations were measured by the BCA protein assay. Lysates (50 μg total proteins) were incubated in 96-well plates coated with the oligo-nucleotide probes recognized by active p65. Protein 65 was detected by a specific antibody against p65, followed by a horseradish peroxidase (HRP)-conjugated secondary antibody. The colorimetric reaction was measured at 450 nm (A_{450}). This experiment was repeated three times.

Enzyme-linked immunosorbent assay (ELISA). The stable TGEV ORF7 gene expressing cells and the control cells were seeded in 24-well plates at a density of 1×10^5 cells/ml in DMEM with 10% FCS and cultured for 48 h. 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, USA).

Statistical analysis. Data are shown as the means ± SD of three independent experiments done in triplicate. For each assay, student's *t*-test was used for statistical comparison. A value of $P < 0.05$ was considered significant.

Results

Confirmation of TGEV nsp7 protein expression in IEC

Western blot analyses showed that the cells transfected with p7-EGFP and pEGFP-N1 plasmid expressed protein of molecular mass of approximately 36 kDa and 27 kDa that were detected by anti-EGFP monoclonal antibodies (Fig. 1).
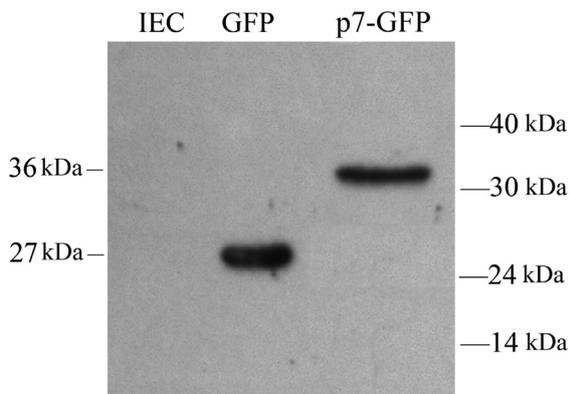
Fig. 1

Detection of p7-GFP fusion protein expression
Western blot analyses of whole extracts of cell lines expressing p7-GFP protein and control IECs. The proteins were detected by anti-GFP antibody.

Since the molecular mass of GFP is known to be approximately 27 kDa, this is in an agreement with the predicted size of the nsp7 protein. However, there was no signal in negative IEC control cells.

TGEV nsp7 protein degradation characteristics

The degradation characteristic of nsp7 protein was investigated using fluorescence microscope. The results showed that p7-GFP protein as well as GFP protein expression had no difference in the cell lines treated with MG132 and untreated cells (Fig. 2). The results showed that TGEV nsp7 protein degradation could not be inhibited by MG132 proteasome inhibitor and that the nsp7 protein degradation was not by the proteasome pathway.

TGEV nsp7 protein subcellular localization

The subcellular localization of nsp7 protein was investigated by confocal fluorescence microscopy. The results showed that p7-GFP protein was localized in the cytoplasm. By contrast, the GFP protein was distributed throughout the whole cell (Fig. 3).

TGEV nsp7 protein has no influence on cell proliferation

Compared with the GFP protein expressing and the control cells, the nsp7 protein expressing cells, showed a slight change in mitotic activity which caused only low change
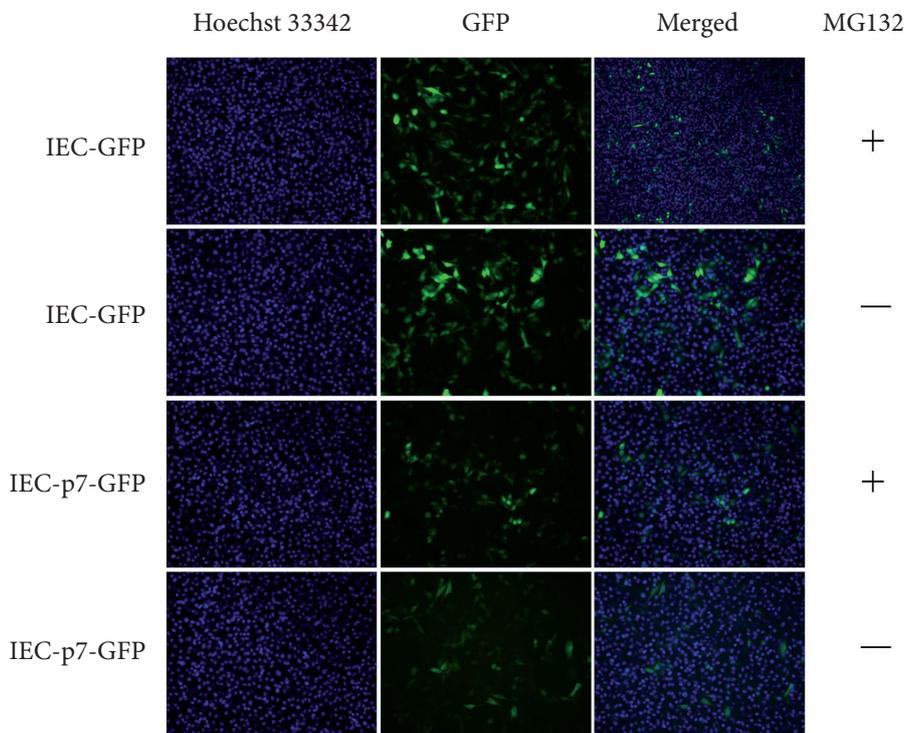

Fig. 2

Fluorescence detection of GFP and p7-GFP fusion protein expressed in IECs (100×)
All the cell lines were stained by Hoechst 33342. The images show that expression of p7-GFP in IECs treated with MG132 is the same as in untreated cells.
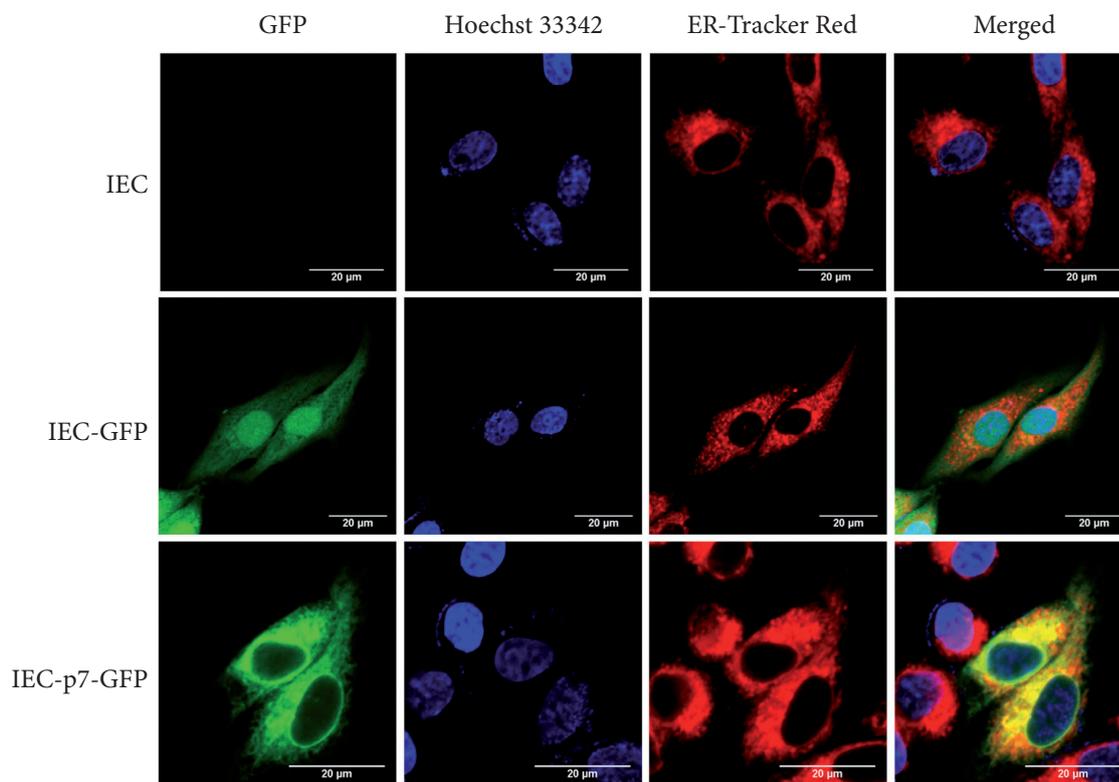
GFP	Hoechst 33342	ER-Tracker Red	Merged

Fig. 3

Subcellular localization of p7-GFP fusion protein in IECs by confocal microscopy
All the cell lines were stained by Hoechst 33342 and ER-Tracker ™ Red. Merged images show co-localization of p7-GFP protein in the ER. Bar = 20 μm.

in cell number. The results suggest that cell proliferation of nsp7 protein expressing cells did not change over a time course (Fig. 4).

TGEV nsp7 protein does not affect cell cycle

To investigate whether TGEV nsp7 expression has effect on cell cycle, flow cytometric analysis was performed based on DNA content in nuclei stained with PI. The proportions of G0/G1 phase, S-phase and G2/M phases for the control cells were 54%, 37.89% and 8.11%, respectively. For IECs expressing GFP, the proportions of the phases were G0/G1: 56.22%, S-phase: 38.01% and G2/M phase: 5.76%, whereas for p7-GFP expressing IECs, the proportions were G0/G1: 55.18%, S-phase: 35.49% and G2/M phase: 9.33%. The histograms were quantitatively analyzed according to the data above to determine the percentage of cells in G0/G1, S, and G2/M phases, where G0/G1 phase cells show 2 N DNA content and G2/M phase cells show 4 N DNA content (Fig. 5). The results showed that relatively to control cells, TGEV nsp7 protein expression caused a slight increase in the number of cells in the each phase. Taken together,
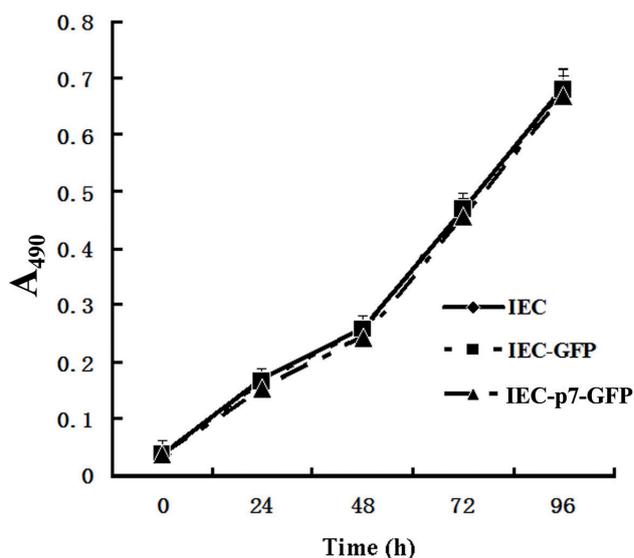

Fig. 4

Cell proliferation assay of the IECs expressing nsp7
The MTT assay was used to measure proliferation of IECs during 96 h. Each data set represents the mean ± SD of six replicates.
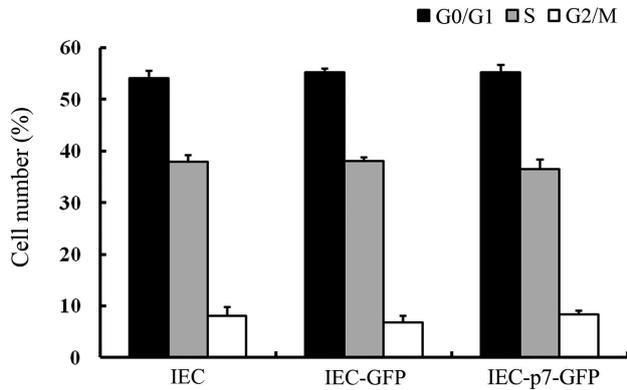
Fig. 5

The analysis of cell division stages of IECs expressing TGEV nsp7 protein by flow cytometry

The percentage analysis of the cells in each cell cycle phase. The results are mean ± SD from three independent experiments.

these results strongly suggested that TGEV nsp7 had no effect on cell cycle.

TGEV nsp7 protein has no effect on cyclin A expression

One of the key regulators of cell cycle progression from the S phase to the G2/M phase is cyclin A. To understand the effect of nsp7 protein expression on cell cycle, we examined cyclin A mRNA expression in transfected and control cells by qPCR. The results showed that there was no significant difference of cyclin A mRNA level between the p7-GFP
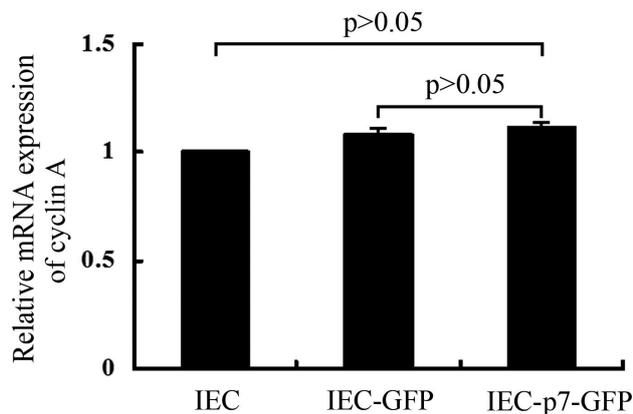
expressing cells and cells in both control groups (Fig. 6). The western blot assay showed that cyclin A expression level was only slightly increased in cells that expressed nsp7 protein compared with control cells (data not shown), and corresponded with qPCR results, indicating that the expression of nsp7 protein had no significant effect on cyclin A expression (P >0.05).

TGEV nsp7 has no effect on GRP78 expression and NF-κB activation

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; Li *et al.*, 2008; Quinones, de Ridder, and Pizzo, 2008). The GRP78 transcription level detected by qPCR assay showed a slight increase in nsp7 expressing cell line (Fig. 7). The western blot assay also did not show any GRP78 expression level change between the cells that expressed nsp7 protein and cells in both control groups (data not shown), corresponding with the result of qPCR assay. Analysis of the activity of NF-κB in p7-GFP expressing cell lines demonstrates that NF-κB was slightly activated compared with control cells (Fig. 8). Together, these analyses show that expression of TGEV nsp7 has no significant influence on GRP78 and NF-κB, suggesting that the TGEV nsp7 has no significant effect on ER stress activation, though the subcellular localization of nsp7 in the cytoplasm.

TGEV nsp7 down-regulates IL-8 expression

Secretion of IL-8 in the supernatant from untransfected and transfected cells was examined using ELISA assay. P7-
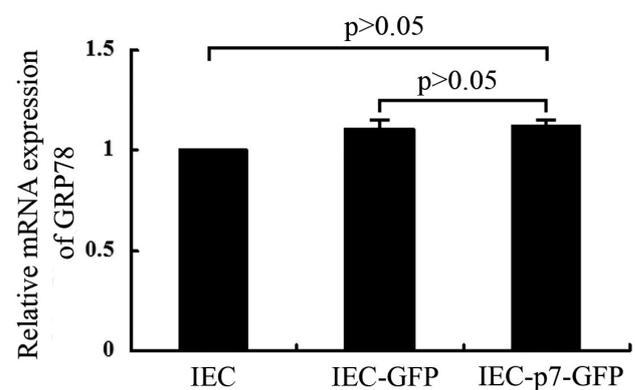

Fig. 6

The effect of TGEV nsp7 expression on the cyclin A protein expression

Total RNA was extracted from cells expressing GFP alone, p7-GFP or untransfected cells. Cyclin A mRNA levels were normalized to the corresponding CT value of β-actin mRNA. The results are mean ± SD from three independent experiments.

Fig. 7

The effect of TGEV nsp7 expression on the GRP78 expression

Total RNA was extracted from cells expressing GFP alone, p7-GFP or untransfected cells. GRP78 mRNA levels were normalized to the corresponding CT value of β-actin mRNA. The results are mean ± SD from three independent experiments.
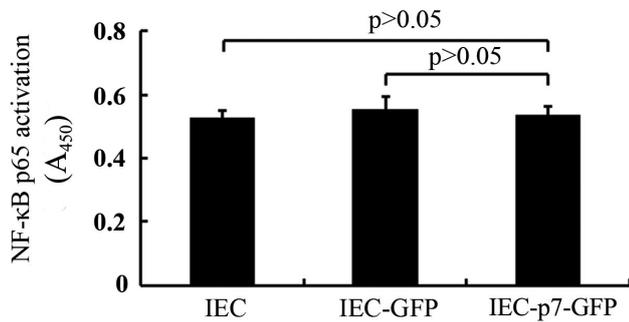
Fig. 8

The effect of TGEV nsp7 expression on the NF-κB activity in IECs
NF-κB p65 activation was determined using the TransAM assay. The data represent the mean and standard deviation from three different experiments.

GFP expressing cells were found to express lower levels of IL-8 compared to GFP expressing cells and the untreated cells (Fig. 9a). Moreover, the mRNA level of IL-8 was detected by qPCR assay and the results showed that TGEV nsp7 expression down-regulated the transcriptional level of IL-8 in comparison with the control cells (Fig. 9b). Thus, these results suggest that TGEV nsp7 expression is responsible for the down-regulation of IL-8 expression.

Discussion

In recent years, many studies were focused on the function of structural proteins, gene sequence analysis of TGEV, and genetic and phylogenetic analysis based on ORF7 genes. Some of them have reported that ORF7 influences the virulence of TGEV (Ortego *et al.*, 2003). Despite this vast amount of research, the subcellular localization and function of the TGEV nsp7 protein still remains unclear. Although some functions of this protein have been studied, the influence on cell cycle by nsp7 protein is still unknown. Moreover, the function of nsp7 protein is yet to be determined, particularly with regard to its effect on host cell physiological changes. In this study, we constructed a eukaryotic expression vector and generated TGEV nsp7-GFP fusion protein expressing cell line that allowed analysis of its properties. Co-localization studies clearly showed that TGEV 7 protein is localized in the cytoplasm. In this study, the effect of TGEV nsp7 protein on IECs proliferation was determined. MTT assay showed that TGEV nsp7 protein had no influence on cell proliferation and cell cycle. Cyclin A is very important for cells in transition from S phase to G2/M phase. QPCR results showed that there was no significant difference of cyclin A mRNA level between the p7-GFP expressing cells and cells in both control groups, which corresponded to the result of western blot. These results suggested that the TGEV nsp7 protein had no significant effect on host cell proliferation and cell cycle.
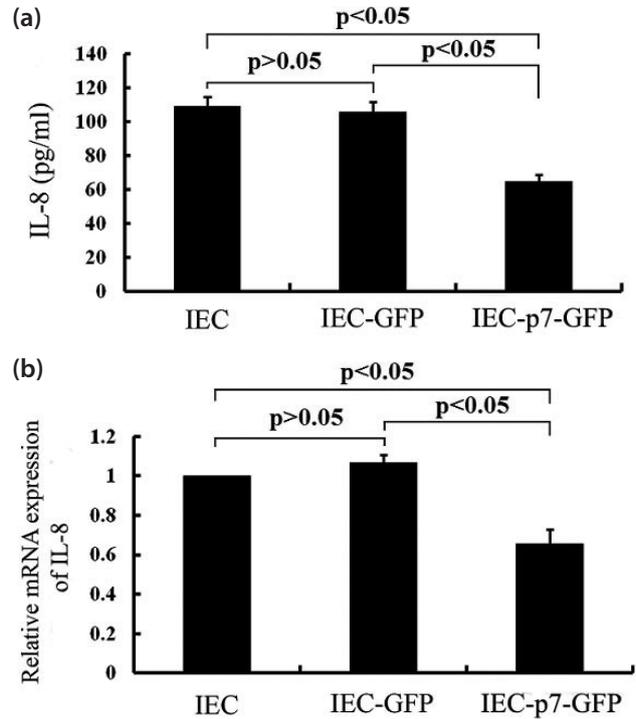

Fig. 9

The effect of TGEV nsp7 expression on IL-8 expression
(a) The concentrations of IL-8 in p7-GFP expressing cells or control cells supernatants were analyzed by ELISA. Data are mean ± SD. (b) Effect of TGEV nsp7 expression on porcine IL-8 transcription in IECs. Total RNA was extracted from cells expressing GFP alone, p7-GFP or untransfected cells. IL-8 mRNA levels were normalized to the corresponding CT value of β-actin mRNA. The results are mean ± SD.

In this study, it was shown that TGEV nsp7 protein was localized in the cytoplasm and was not able to induce ER stress, as indicated by the low up-regulation of the molecule chaperon GRP78, one of the typical markers of ER stress. The ER plays an essential role in multiple cellular processes that are important for cell survival and normal cellular functions (Anelli *et al.*, 2008). Viruses use the ER as an integral part of their replication strategy, they must contend with the ER stress response and the downstream consequence of ER stress signaling, including the initiation of an inflammatory response through the activation of NF-κB (Park *et al.*, 2008; Herrewegh *et al.*, 1995; Cruz *et al.*, 2011). As a pro-inflammatory neutrophil chemotactic factor, IL-8 plays an important role in the promotion of cell survival signaling and antagonizes the anti-viral activities of interferon. Our previous study suggested that TGEV N protein could induce ER stress and significantly activate NF-κB, which leads to the promotion of IL-8 transcription, while TGEV nsp7 protein as shown in this study could not. In fact, this study showed that TGEV nsp7 protein could even down-regulate the IL-8 expression in IECs.

There are a number of studies which have revealed that nidovirus nsp7 is involved in the immune interactions between nidoviruses and host cells. For instance, porcine epidemic diarrhea virus (PEDV) nsp7 can inhibit the IFN-β and IRF3 promoter activities, thus impairing host cells' innate immune response towards the virus (Zhang Q *et al.*, 2016). However, PRRSV nsp7 was reported to induce autophagy of the host cells, which enhanced cells' innate defense against infection (Cottam *et al.*, 2014). As for TGEV nsp7, it was reported that the absence of TGEV nsp7 led to increased pro-inflammatory responses and acute tissue damage after infection both *in vitro* and *in vivo*, with some pro-inflammatory up-regulated genes, such as IL-8 and NF-κB inhibitor alpha (NF-κBIA) (Cruz *et al.*, 2013). These results showed that the presence of nsp7 reduced inflammatory changes after TGEV infection in both *in vitro* and *in vivo*, indicating that TGEV nsp7 could counter-act the host antiviral response and modulate host innate immune response, which are similar to PEDV nsp7 (Cruz *et al.*, 2013). Corresponding to the results above, ours showed that the TGEV nsp7 expression in IECs could not activate NF-κB activity but could inhibit IL-8 expression on both mRNA and protein level. TGEV nsp7 may inhibit the expression of IL-8 by mechanisms that can be an important part of TGEV immune escape mechanism and requires further research.

One previous study reported that there was no difference of PKR-like endoplasmic reticulum kinase (PERK) activity between cells infected by the TGEV ORF7 deletion mutant virus and the parental virus, which suggests that the deletion of TGEV ORF7 could not induce the ER stress (Cruz *et al.*, 2011). Our observations also showed that nsp7 protein could not influence the GRP78 activity on neither transcription level nor protein level. In conclusion, all data show that TGEV nsp7 has no influence on ER stress response.

To conclude, localized in the cytoplasm but with no influence on GRP78 expression and ER stress, TGEV nsp7 has no effect on the IEC growth, cell cycle and cyclin A expression, either. In addition, though TGEV nsp7 does not influence the NF-κB activity, it can down-regulate the expression level of IL-8. This study provides some new features about the function of the TGEV nsp7 protein which are likely to be very useful in understanding the molecular mechanisms of TGEV pathogenesis.

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