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Integrin αvβ3 enhances replication of porcine epidemic diarrhea virus on Vero E6 and porcine intestinal epithelial cells

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ABSTRACT

The entry mechanism of porcine epidemic diarrhea virus (PEDV) remains unclear, especially the virus receptor. Our previous study revealed a potential correlation between integrin αvβ3 and PEDV infection. In the current study, the effect of overexpression, silencing, antibody inhibition, and co-expression with porcine aminopeptidase N (pAPN) of integrin αvβ3 on PEDV infection was investigated and analyzed in African green monkey Vero E6 cells and porcine intestinal epithelial cells (IECs) using the classical strain CV777 and variant strain HM2017 of PEDV. Integrin αvβ3 significantly enhanced the replication of the classical and variant strains of PEDV in Vero E6 cells and IECs. The integrin αv and β3 subunits were both involved in the enhancement of PEDV infection, the Arg–Gly–Asp peptides targeting integrin αvβ3 significantly inhibited replication of PEDV in Vero E6 cells, and co-expression of integrin αvβ3 with pAPN significantly enhanced replication of PEDV in Vero E6 and BHK-21 cells. These results demonstrate that integrin αvβ3 enhances PEDV replication in Vero E6 cells and IECs. These data provide novel insights into the entry mechanism of PEDV.

1. Introduction

Porcine epidemic diarrhea is a highly contagious, enteric disease caused by porcine epidemic diarrhea virus (PEDV) that is characterized by watery diarrhea, vomiting, dehydration, and a mortality rate of up to 90% in suckling piglets (Pensaert and de Bouck, 1978). PEDV is an enveloped, single-stranded, positive-sense RNA virus that belongs to the order *Nidovirales*, family *Coronaviridae*, and genus *Alphacoronavirus*. The major structural proteins of PEDV include the spike protein (S), membrane protein (M), envelope protein (E), and nucleocapsid protein (N). Since the end of 2010, several pig-producing provinces in Southern China have experienced PEDV outbreaks. Since then, the disease has spread throughout other provinces of China (Li et al., 2012). Thus far, although PEDV vaccines are widely used in domestic pig farming, PEDV infection still occurs frequently and has resulted in enormous economic losses to the pig industry (Sun et al., 2016; Wang et al., 2016).

Binding of viral attachment proteins to the host-cell receptor is the first step in viral infection. Li et al. (2007) reported that porcine aminopeptidase N (pAPN) serves as a functional receptor for PEDV entry into the host cell. Subsequent research has confirmed that pAPN is a receptor for PEDV (Nam and Lee, 2010; Kim and Lee, 2015; Li et al., 2016a, 2016b; Liu et al., 2016; Kamau et al., 2017; Zhang et al., 2018). However, other studies have countered that the pAPN is not a functional or required receptor for PEDV cell entry (Shirato et al., 2016; Li et al., 2017) and entry of PEDV into Vero cells or porcine small intestinal epithelial cells (IECs) is not dependent on pAPN (Ji et al., 2018). Moreover, Whitworth et al. (2018) revealed that knockout pigs possessing the null amino peptidase N phenotype retain susceptibility to infection with PEDV. Hence, the entry mechanism of PEDV remains unclear.

Integrins are cell surface αβ heterodimeric glycoproteins that contribute to a variety of cellular functions. Previous studies have shown that integrins act as receptors or co-receptors for viral infection (Stewart and Nemerow, 2007; Kotecha et al., 2017; Nestić et al., 2018). For example, integrin αvβ3 has been shown to serve as an entry receptor for various viruses. In our previous studies, integrins α3, αv, β1, β3, β4, and β6 were identified in Vero cells susceptible to PEDV infection (Guo et al., 2014). Further proteomics studies have shown that

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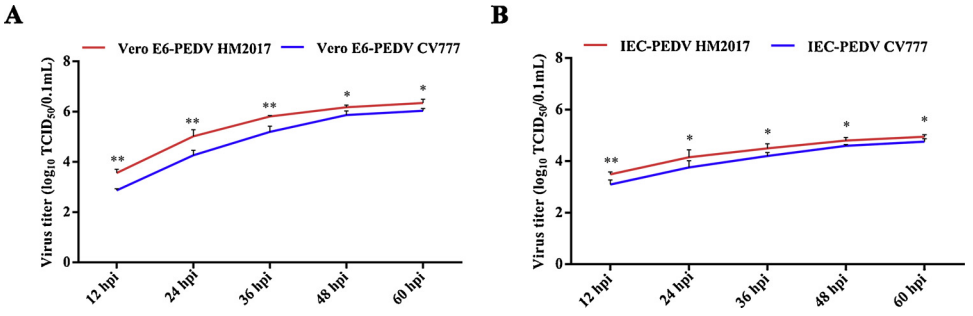

Fig. 1. Growth curves of PEDV strains HM2017 and CV777 in Vero E6 cells and IECs. (A) Growth curves of PEDV strains HM2017 and CV777 in Vero E6 cells. (B) Growth curves of PEDV strains HM2017 and CV777 in IECs. The cells were inoculated with PEDV strain HM2017 or CV777 at 0.1 MOI. The $TCID_{50}$ value was detected at different time points and the viral titers were calculated.

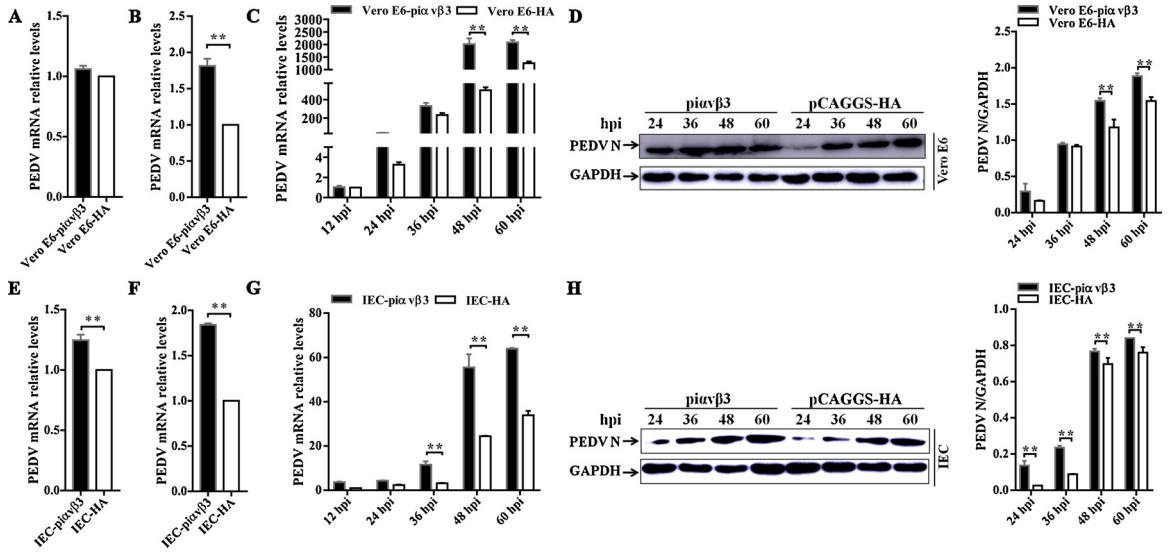

Fig. 2. Effect of integrin αvβ3 overexpression on PEDV infection of Vero E6 cells and IECs. (A) The mRNA levels of PEDV strain CV777 in piαvβ3-Vero E6 cells during viral binding. (B) The mRNA levels of PEDV strain CV777 in piαvβ3-Vero E6 cells during viral internalization. (C) The mRNA levels of PEDV strain CV777 in piαvβ3-Vero E6 cells during viral proliferation. (D) Viral protein expression levels of PEDV strain CV777 in piαvβ3-Vero E6 cells during viral proliferation. (E) Viral mRNA levels of PEDV strain CV777 in piαvβ3-IECs during viral binding. (F) Viral mRNA levels of PEDV strain CV777 in piαvβ3-IECs during viral internalization. (G) Viral mRNA levels of PEDV strain CV777 in piαvβ3-IECs during viral proliferation. (H) Viral protein expression levels of PEDV strain CV777 in piαvβ3-IECs during viral proliferation.

the integrin β3 subunit is potentially associated with PEDV infection (Sun et al., 2015). Therefore, the aim of the current study was to investigate the role of integrin αvβ3 in PEDV infection. The resulting data are expected to improve the current understanding of the entry mechanism of PEDV.

2. Materials and methods

2.1. Virus, cells, and sera

PEDV strain CV777 (GenBank accession no. KT323979; group I) was kindly provided by the Division of Swine Digestive System Infectious Diseases, State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences. PEDV variant strain HM2017 (GenBank accession no. MK690502; group II) was isolated from the intestinal tissue of a diarrheic piglet in China. African green monkey kidney epithelial (Vero E6) cells and porcine IECs were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). BHK-21-pAPN cells were provided by Prof. Luis Enjuanes (Canto Blanco University, Madrid, Spain; Santiago et al., 2017) and cultured in DMEM supplemented with 10% FBS, 2 μg/mL of G418, and 5 μg/mL of puromycin. All cells were cultured under a humidified atmosphere of 5% CO_2/95% air at 37 °C. Polyclonal antibodies (Abs) against the porcine integrin subunits αv (piαv) and β3 (piβ3) were prepared using recombinant proteins of the piαv and piβ3 subunits as immunogens, respectively (Gao et al., 2016; Wang et al., 2017).

2.2. Viral growth curve of PEDV strains CV777 and HM2017

Viral growth curves of PEDV strains CV777 and HM2017 in Vero E6 cells or IECs were constructed according to the median tissue culture infective dose ($TCID_{50}$), respectively. Briefly, Vero E6 cells or IECs were seeded into the wells of 96-well plates at a density of 10^5 cells per well in 100 μL of medium and incubated for 48 h at 37°C under an atmosphere of 5% CO_2/95% air. Afterward, the medium was removed and 100 μL of 10-fold serial dilutions of the virus (PEDV strain CV777 or HM2017) were added to each well. The cytopathic effect was examined every 12 h for 5 days post-inoculation. The viral titer was determined according to the Reed and Muench method.

2.3. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted from the cells using the RNAsimple Total RNA Kit (Tiangen Biotech (Beijing) Co., Ltd., Beijing, China). Complementary DNA synthesis was performed with random primers using the FastKing gDNA Dispelling RT SuperMix Kit (Tiangen Biotech (Beijing) Co., Ltd.). qRT-PCR analysis was performed with the SYBR Green I fluorescent dye assay and the Bio-Rad CFX Connect Real-Time System (Bio-Rad Laboratories, Hercules, CA, USA). The qRT-PCR reaction volume was 25 μL, which consisted of 12.5 μL of 2 × SYBR®

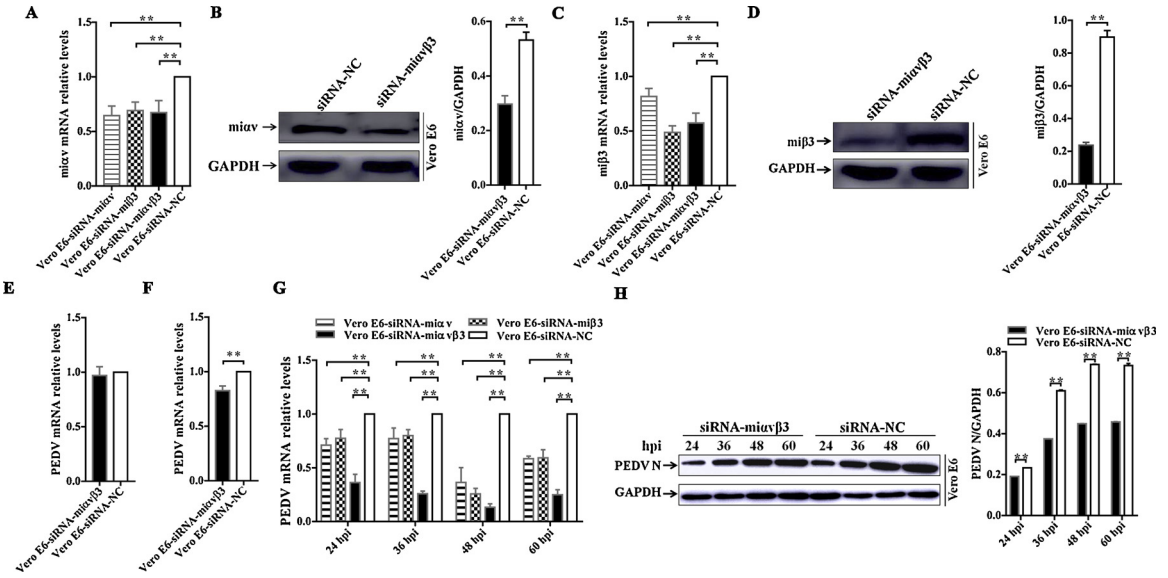

Fig. 3. Effects of monkey integrin αvβ3 siRNA on PEDV infection of Vero E6 cells. (A) qRT-PCR analysis of siRNA-miαv (5′-CCCUCUGACAUUGAUUGUUTT-3′). (B) Western blotting analysis of siRNA-miαv (5′-CCCUCUGACAUUGAUUGUUTT-3′). (C) qRT-PCR analysis of siRNA-miβ3 (5′-GCGAUCAGAUGGCUGCAUATT-3′). (D) Western blotting analysis of siRNA-miβ3 (5′-GCGAUCAGAUGGCUGCAUATT-3′). (E) Viral mRNA levels of PEDV strain CV777 in Vero E6-siRNA-miαvβ3 cells during viral binding. (F) Viral mRNA levels of PEDV strain CV777 in Vero E6-siRNA-miαvβ3 cells during viral internalization. (G) Viral mRNA levels of PEDV strain CV777 in Vero E6-siRNA-miαvβ3 cells during viral proliferation. (H) Viral protein expression levels of PEDV strain CV777 in Vero E6-siRNA-miαvβ3 cells during viral proliferation. *Note*. Vero E6-siRNA-miαvβ3 cells represent Vero E6 cells that were co-transfected with miαv and miβ3 siRNAs.

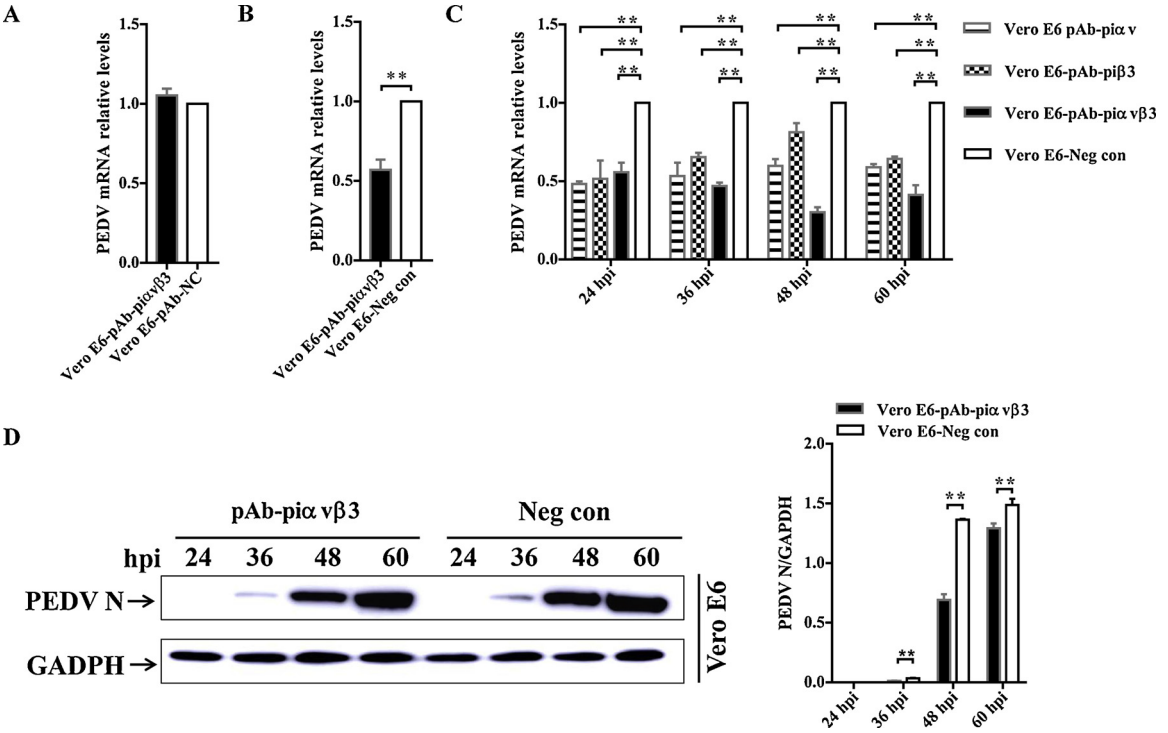

Fig. 4. Effects of inhibition of monkey integrin αvβ3 on PEDV infection of Vero E6 cells by pAbs against piαv and piβ3. (A) Viral mRNA levels of PEDV strain CV777 in Vero E6-pAb-piαvβ3 cells during viral binding. (B) Viral mRNA levels of PEDV strain CV777 in Vero E6-pAb-piαvβ3 cells during viral internalization. (C) Viral mRNA levels of PEDV strain CV777 in Vero E6-pAb-piαvβ3 cells during viral proliferation. (D) Viral protein expression levels of PEDV strain CV777 in Vero E6-pAb-piαvβ3 cells during viral proliferation. *Note*. Vero E6-pAb-piαvβ3 cells represent Vero E6 cells that were inhibited by pAbs against piαv and piβ3.

Premix Ex Taq (Takara Bio, Inc., Kusatsu, Japan), 0.5 μL (10 pmol/L) of the forward primer, 0.5 μL (10 pmol/L) of the reverse primer, 4 μL of template DNA, and 7.5 μL of sterile water. The reaction conditions were as follows: denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, and a final dissociation stage. Relative quantification analysis was performed using the cycle threshold ($2^{-\Delta\Delta Ct}$) method (Livak and Schmittgen, 2001; Luo et al., 2017).

2.4. Western blot analysis

Equivalent amounts of cellular lysates were separated by 12%

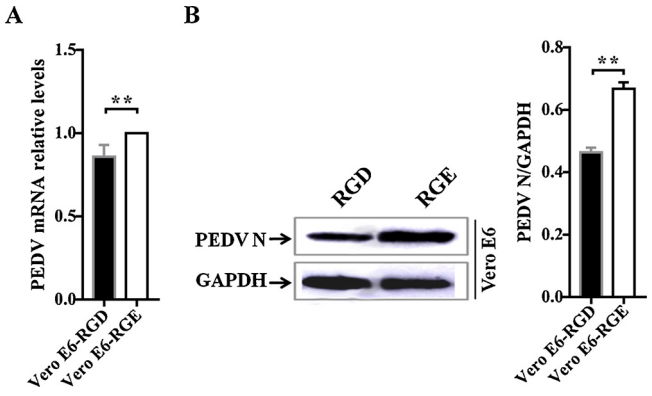

Fig. 5. Effects of the RGD peptides targeting integrin αvβ3 on PEDV infection of Vero E6 cells. (A) Viral mRNA levels of PEDV strain CV777 in Vero E6 cells. (B) Viral protein expression levels of PEDV strain CV777 in Vero E6 cells.

sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (0.45 μm), which were blocked with 5% (w/v) non-fat dried milk for 1 h and then incubated with primary monoclonal Abs (mAbs) against glyceraldehyde 3-phosphate dehydrogenase (ZSGB-BIO Technology Co., Ltd., Beijing, China) or PEDV N, or mouse polyclonal Abs (pAbs) against the piαv and piβ3 subunits at 37 °C for 1 h. After washing with 0.05% Tween-20 in phosphate-buffered saline (PBST), the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary Abs. After washing with 0.05% PBST, the Abs were detected with Luminata^{TM}Crescendo Western HRP substrate (Merck KGaA, Darmstadt, Germany) using the Amersham Imager 600 (GE Healthcare, Chicago, IL, USA). Protein levels were analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA).

2.5. Construction of eukaryotic expression plasmids of porcine integrin αvβ3

The nucleotide sequences of the piαv and piβ3 subunits containing the *Eco*RI and *Xho*I restriction sites at the 5′ and 3′ terminals were synthesized based on the codon usage bias of the eukaryotic genes according to nucleotide sequences of the piαv and piβ3 subunits (GenBank accession nos. EF474019 and NM_214002). The synthesized genes of the piαv and piβ3 subunits were cloned into the eukaryotic expression vector pCAGGS, which was tagged with human influenza hemagglutinin. The recombinant plasmids were extracted using the EndoFree Plasmid Maxi Kit (Qiagen, Hilden, Germany) and then were co-transfected into Vero E6 cells and IECs, respectively. Transient expression of porcine integrin αvβ3 (piαvβ3) in Vero E6 cells and IECs was identified by qRT-PCR, western blotting, and indirect immunofluorescence, respectively.

2.6. Effects of integrin αvβ3 overexpression on PEDV infection

To investigate the effects of integrin αvβ3 on the binding, internalization, and proliferation of PEDV, piαvβ3 was expressed on Vero E6 (piαvβ3-Vero E6) cells and IECs (piαvβ3-IECs) by co-transfection of recombinant plasmids of the piαv and piβ3 subunits, respectively. For the viral binding assay, piαvβ3-Vero E6 cells and piαvβ3-IECs were incubated with PEDV strain CV777 at a multiplicity of infection (MOI) of 1 at 4 °C for 2 h. After washing three times with ice-cold PBS, the cells were collected in PBS. For the viral internalization assay, piαvβ3-Vero E6 cells and piαvβ3-IECs were incubated with PEDV strain CV777 at 3 MOI at 37 °C for 2 h. After washing three times with PBS, the cells were digested with 0.25% trypsin and suspended in PBS. Following centrifugation, the cell pellets were washed three times and collected in PBS. For the viral proliferation assay, piαvβ3-Vero E6 cells and piαvβ3-IECs were incubated with PEDV strain CV777 at 1 MOI at 37 °C for 12, 24, 36, 48 and 60 h. Afterward, the samples were subjected to detection of PEDV RNA and protein by qRT-PCR and western blot analyses, respectively.

2.7. Effects of silenced monkey integrin αvβ3 on PEDV infection

To investigate the effect of the silenced monkey integrin αvβ3

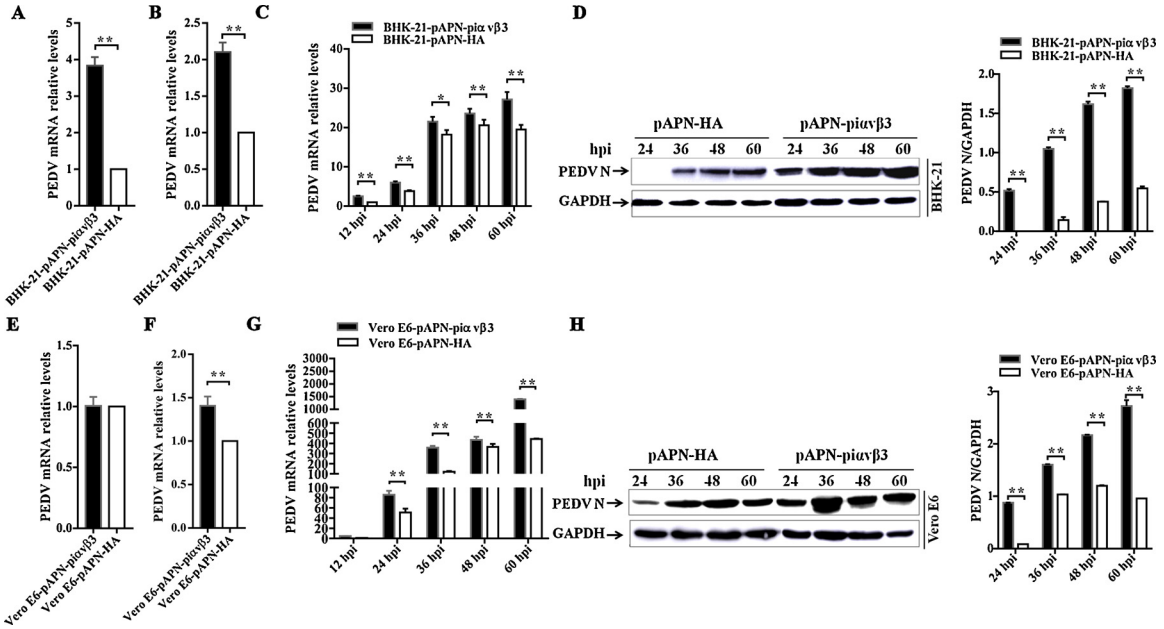

Fig. 6. Synergistic effect of pig integrin αvβ3 with pAPN for PEDV infection of BHK-21 and Vero E6 cells. (A) Viral mRNA levels of PEDV strain CV777 in BHK-21 cells during viral binding. (B) Viral mRNA levels of PEDV strain CV777 in BHK-21 cells during viral internalization. (C) Viral mRNA levels of PEDV strain CV777 in BHK-21 cells during viral proliferation. (D) Viral protein expression levels of PEDV strain CV777 in BHK-21 cells during viral proliferation. (E) Viral mRNA levels of PEDV strain CV777 in Vero E6 cells during viral binding. (F) Viral mRNA levels of PEDV strain CV777 in Vero E6 cells during viral internalization. (G) Viral mRNA levels of PEDV strain CV777 in Vero E6 cells during viral proliferation. (H) Viral protein expression levels of PEDV strain CV777 in Vero E6 cells during viral proliferation.

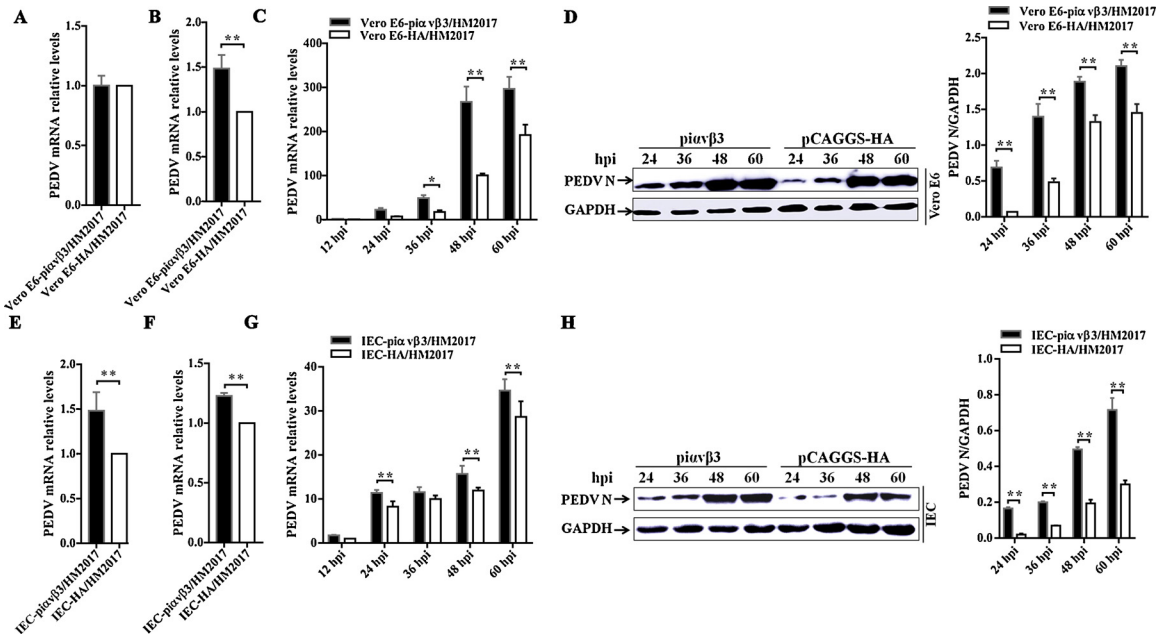

Fig. 7. Effect of pig integrin αvβ3 on PEDV variant strain HM2017 infection of Vero E6 cells and IECs. (A) Viral mRNA levels of PEDV strain HM2017 in Vero E6 cells during viral binding. (B) Viral mRNA levels of PEDV strain HM2017 in Vero E6 cells during viral internalization. (C) Viral mRNA levels of PEDV strain HM2017 in Vero E6 cells during viral proliferation. (D) Viral protein expression levels of PEDV strain HM2017 in Vero E6 cells during viral proliferation. (E) Viral mRNA levels of PEDV strain HM2017 in pig IECs during viral binding. (F) Viral mRNA levels of PEDV strain HM2017 in pig IECs during viral internalization. (G) Viral mRNA levels of PEDV strain HM2017 in pig IECs during viral proliferation. (H) Viral protein expression levels of PEDV strain HM2017 in pig IECs during viral proliferation.

(miαvβ3) on PEDV infection, small interfering RNA (siRNA) duplexes targeting the monkey integrin αv subunit (siRNA-miαv: 5′-CCCUCUG ACAUUGAUUGUUTT-3′) and β3 subunit (siRNA-miβ3: 5′-GCGAUCAG AUGGCUGCAUATT-3′) were synthesized, respectively. Vero E6 cells were transfected with siRNA-miav and siRNA-miβ3 using Attractene Transfection Reagent (Qiagen). The Vero E6 cells were incubated with PEDV strain CV777 at 1 MOI at 36 h after transfection with siRNA-miav and siRNA-miβ3. The effects of the silenced miαvβ3 on the binding, internalization, and proliferation of PEDV were analyzed according to the above methods (Section 2.6). Non-specific siRNAs (5′-UUCUCCGA ACGUGUCACGUTT-3′) were used as controls.

2.8. Effect of Abs and arginine–glycine–aspartate (RGD) peptides against integrin αvβ3 on PEDV infection

The pAbs against the piαv and piβ3 subunits and RGD peptides against miαvβ3 were chosen to further investigate the effects of integrin αvβ3 on PEDV infection. For Ab inhibition, Vero E6 cells were pretreated with Abs against the integrins subunits piαv and piβ3 (dilution, 1:20) at 37 °C for 1 h. For inhibition of RGD peptides, Vero E6 cells were pretreated with RGD peptides (40 μM) at 37 °C for 6 h. After treatment with the Abs or RGD peptides, Vero E6 cells were incubated with PEDV at 1 MOI. The binding, internalization, and proliferation of PEDV were analyzed according to the above methods (Section 2.6). Negative sera from healthy BALB/c mice and arginine–glycine–glutamate (RGE) peptides were used as controls.

2.9. Synergistic effects of pig integrin αvβ3 with pAPN on PEDV infection

BHK-21-pAPN cells expressing pAPN and Vero E6-pAPN cells expressing pAPN were used to investigate the synergistic effects of co-expression of piαvβ3 with pAPN on PEDV infection. Briefly, piαvβ3 was expressed by BHK-21-pAPN and Vero E6-pAPN cells by co-transfection of the recombinant plasmids of the piαv and piβ3 subunits according to the above methods (Section 2.5), respectively. BHK-21-pAPN cells expressing piαvβ3 (BHK-21-pAPN-piαvβ3) and Vero E6-pAPN cells expressing piαvβ3 (Vero E6-pAPN-piαvβ3) were incubated with PEDV strain CV777 at 1 MOI, respectively. The binding, internalization, and proliferation of PEDV were analyzed according to the above methods (Section 2.6). BHK-21-pAPN and Vero E6-pAPN cells were used as controls.

2.10. Effects of pig integrin αvβ3 on PEDV variant strain infection

To validate the effects of piαvβ3 on PEDV variant strain infection, piαvβ3-IECs and piαvβ3-Vero E6 cells were incubated with 1MOI of PEDV strain HM2017, which belongs to the variant group II strains of PEDV according to genotyping of the entire genome. The binding, internalization, and proliferation of PEDV strain HM2017 in piαvβ3-IECs and piαvβ3-Vero E6 cells were analyzed according to the above methods (Section 2.6).

2.11. Statistical analysis

All statistical analyses were performed using GraphPad Prism® 7.00 software (GraphPad Software, Inc., La Jolla, CA, USA). One-way analysis of variance and the *t*-test were used for data analysis and graph production. All data are presented as the mean ± standard deviation. A probability (p) value of < 0.05 was considered statistically significance and $p < 0.01$ as very significant.

3. Results

3.1. PEDV strains CV777 and HM2017 exhibit different in vitro growth kinetics

The growth curves of PEDV strains CV777 and HM2017 based on the $TCID_{50}$ at different hours post-infection (hpi) were visualized. Results indicated that the viral titers of PEDV strains CV777 and HM2017 rose rapidly from 12 hpi to 60 hpi in Vero E6 cells and IECs (Fig. A and B). In Vero E6 cells, the viral titers of HM2017 strain ranged from 3.83×10^4 to 1.33×10^7 $TCID_{50}$/mL at different hpi, while the viral titers of CV777 strain ranged from 6.81×10^2 to 3.16×10^6 $TCID_{50}$/mL at different hpi (Fig. 1A). In IECs, the viral titers of HM2017

strain ranged from 4.09×10^3 to 9.98×10^4 $TCID_{50}$/mL at different hpi, while the viral titers of CV777 strain ranged from 2.29×10^3 to 6.84×10^4 $TCID_{50}$/mL at different hpi (Fig. 1B). The viral titers of PEDV strains CV777 and HM2017 in Vero E6 cells were significantly higher than that in IECs (Fig. A and B). In Vero E6 cells and IECs, the viral titers of PEDV strain HM2017 were significantly higher than that of CV777 strain (Fig. A and B).

3.2. Overexpression of integrin αvβ3 enhances PEDV infection on Vero E6 cells and IECs

The piαv or piβ3 subunit was not expressed on Vero E6 cells and IECs when the recombinant plasmid was singly transfected into the target cells (data not shown). However, piαvβ3 was successfully expressed on Vero E6 cells and IECs when the recombinant plasmids of both subunits were simultaneously co-transfected into the target cells (data not shown). Next, the role of integrin αvβ3 on the binding, internalization, and proliferation of PEDV strain CV777 were analyzed in Vero E6 cells and IECs, respectively. The results indicated that in Vero E6 cells, piαvβ3 overexpression had significantly enhanced the internalization and proliferation of PEDV strain CV777 ($p < 0.05$) (Fig. 2B, C and D). In IECs, piαvβ3 overexpression significantly enhanced the binding, internalization, and proliferation of PEDV strain CV777 ($p < 0.05$) (Fig. 2E, F, G and H).

3.3. Silencing or inhibition of integrin αvβ3 inhibits PEDV infection

The siRNA-miαv targeting the miαv subunit significantly downregulated expression of the miαv and miβ3 subunits in Vero E6 cells ($p < 0.05$) (Fig. 3A and B). siRNA-miβ3 targeting the miβ3 subunit significantly decreased expression of the miαv and miβ3 subunits in Vero E6 cells ($p < 0.05$) (Fig. 3C and D). The silencing of miαvβ3 significantly decreased internalization of PEDV strain CV777 in Vero E6 cells, as compared to the control siRNAs ($p < 0.05$) (Fig. 3F). In Vero E6-siRNA-miαvβ3, Vero E6-siRNA-miαv, and Vero E6-siRNA-miβ3 cells, the viral RNA levels of PEDV strain CV777 at 24, 36, 48, and 60 hpi were significantly lower than that of Vero E6-siRNA-NC cells, respectively, in which the co-transfection of siRNA-miαv and siRNA-miβ3 (siRNA-miαvβ3) exhibited higher viral inhibition of PEDV strain CV777, as compared to single transfection of siRNA-miαv or siRNA-miβ3 (Fig. 3G and H). The Ab blocking results indicated that the pAbs against piαvβ3 significantly inhibited viral internalization of PEDV strain CV777 in Vero E6 cells, as compared to negative sera from healthy mice ($p < 0.05$) (Fig. 4B). During viral proliferation, pAbs against piαv, piβ3, and piαvβ3 exhibited significant viral inhibition by PEDV strain CV777 at 24, 36, 48, and 60 hpi, as compared to the control group, of which viral inhibition of the pAbs against piαvβ3 was higher at 36, 48, and 60 hpi than that of the pAbs against piαv or piβ3 (Fig. 4C and D). Moreover, the synthesized RGD peptides significantly inhibited replication of PEDV strain CV777 in Vero E6 cells, as compared to the RGE control peptides ($p < 0.05$) (Fig. 5).

3.4. Integrin αvβ3 and pAPN exhibit a synergistic effect on enhancement of PEDV replication

The synergistic effects of integrin αvβ3 and pAPN on PEDV infection were evaluated in BHK-21-pAPN-piαvβ3 cells and Vero E6-pAPN-piαvβ3 cells. The results indicate that the co-expression of pAPN and piαvβ3 on BHK-21 cells significantly increased the binding, internalization, and proliferation (12, 24, 36, 48 and 60 hpi) of PEDV strain CV777, as compared to the BHK-21-pAPN cells ($p < 0.05$) (Fig. 6A, B, C and D). Co-expression of pAPN and piαvβ3 on Vero E6 cells significantly enhanced the internalization and proliferation (24, 36, 48, and 60 hpi) of PEDV strain CV777, as compared to the Vero E6-pAPN cells ($p < 0.05$) (Fig. 6F, G and H).

3.5. Integrin αvβ3 facilitates replication of PEDV variant strain HM2017

The effects of piαvβ3 on PEDV variant strain infection were evaluated in piαvβ3-Vero E6 cells and piαvβ3-IECs. The results indicate that in piαvβ3-Vero E6 cells, the viral RNA levels of PEDV strain HM2017 exhibited a significant increase during viral internalization, as compared to piαvβ3-Vero E6 cells ($p < 0.05$) (Fig. 7B). Both viral RNA and protein levels of PEDV strain HM2017 also significantly increased during viral proliferation (24, 36, 48, and 60 hpi) ($p < 0.05$) (Fig. 7C and D). In the piαvβ3-IECs, the viral RNA levels of PEDV strain HM2017 were significantly higher than that of the control IECs during viral binding and internalization (Fig. 7E and F). Also, viral RNA and protein levels of PEDV strain HM2017 were significantly increased at 24, 48, and 60 hpi ($p < 0.05$) (Fig. 7G and H).

4. Discussion

The entry mechanism of PEDV has been the subject of extensive research in recent years. pAPN was initially identified as an entry receptor for PEDV, but its role has become controversial and even negated (Li et al., 2007; Nam and Lee, 2010; Shirato et al., 2016; Li et al., 2017; Ji et al., 2018; Whitworth et al., 2018). Other studies have reported that the tight junction protein occludin and type II transmembrane serine proteases TMPRSS2/MSPL enhance entry and replication of PEDV (Luo et al., 2017; Shi et al., 2017). These data suggest that additional cellular receptors should be explored to reveal the entry mechanism of PEDV. Integrins are common receptors for a variety of viruses and integrin αvβ3 is the cellular receptor for rotavirus, human cytomegalovirus, West Nile virus, and herpes simplex virus type 1 (Guerrero et al., 2000; Chu and Ng, 2004; Parry et al., 2005; Wang et al., 2005). The results of previous studies revealed that integrin αvβ3 was associated with PEDV infection (Guo et al., 2014; Sun et al., 2015). In the present study, the effects of integrin αvβ3 on PEDV infection were further investigated in PEDV-susceptible Vero E6 cells and IECs. Our results indicate that integrin αvβ3 enhanced the replication of classical and variant strains of PEDV in Vero E6 cells and IECs. These data provide novel insights into the entry mechanism of PEDV.

Vero cells from monkeys and IECs from pigs are commonly used for PEDV propagation. The results of the present showed that integrin αvβ3 could enhance replication of PEDV in Vero E6 cells and IECs, suggesting that integrin αvβ3 is a potential entry receptor for PEDV infection. However, integrin αvβ3 did not induce a cytopathic effect in non-susceptible HeLa and BHK-21 cells (data not shown), demonstrating that integrin αvβ3 is a co-receptor of PEDV, not a functional entry receptor. Moreover, siRNA targeting of the integrin αv subunit simultaneously downregulated expression of the integrin αv and β3 subunits in Vero E6 cells. The expression levels of the integrin αv and β3 subunits simultaneously decreased in Vero E6 cells following siRNA targeting of the integrin β3 subunit. This result demonstrates that expression of integrin αvβ3 is dependent on the co-expression of the two subunits. Furthermore, pAbs against the integrin αv or β3 subunit inhibited replication of PEDV. These data demonstrate that the integrin αv and β3 subunits simultaneously play roles in PEDV infection. The enhancement of PEDV infection mediated by integrin αvβ3 is dependent on the co-existence of the integrin αv and β3 subunits.

RGD is a peptide motif that can be specifically recognized by integrin αvβ3. Integrin αvβ3 along with the RGD motif plays a central role in cell adhesion as a prototype adhesion signal. Moreover, numerous pharmaceutical applications are also emerging for RGD (Dong et al., 2017). In the present study, RGD peptides significantly decreased replication of PEDV in Vero E6 cells. This result supports prior evidence that integrin αvβ3 enhances replication of PEDV in Vero E6 cells and IECs. Additionally, these data suggest that RGD peptides serve as potential therapeutic drugs for PEDV infection.

pAPN was initially reported as a cellular receptor of PEDV. However, subsequent studies have demonstrated that pAPN is not a

functional cell receptor for PEDV. Recent evidence suggests that PEDV infection of Vero cells, porcine jejunum epithelial cells, and pAPN-deficient pigs is not dependent on pAPN (Ji et al., 2018; Whitworth et al., 2018). These two studies almost negate the conclusion that pAPN is a functional cellular receptor of PEDV. In the present study, co-expression of piαvβ3 and pAPN on BHK-21 and Vero E6 cells significantly increased the internalization and proliferation of PEDV strain CV777, demonstrating that integrin αvβ3 and pAPN have a synergistic enhancing role in PEDV infection. As compared to pAPN, integrin αvβ3 plays a major role in enhancement of PEDV replication, while pAPN has only a weak role in PEDV infection, which is basically in agreement with the results of previous studies (Ji et al., 2018; Whitworth et al., 2018). These data suggest that entry of PEDV into host cells may possibly be dependent on multiple cellular receptors.

Since the PEDV outbreaks in China at the end of 2010, the virulence-determining S1 gene of PEDV epidemic strains has been undergoing variation in China and other countries (Wang et al., 2016; Su et al., 2018). The PEDV variant strains exhibit changes in their mechanisms of pathogenicity and infection (Li et al., 2016a, 2016b; Su et al., 2019). In the current study, PEDV strain HM2017, which a variant strain in China (phylogenetic group II), was used to determine whether integrin αvβ3 plays a role in infection of variant PEDV strains. The results indicate that integrin αvβ3 enhanced replication of PEDV strain HM2017 in Vero E6 cells and IECs. These data demonstrate that integrin αvβ3 is a common entry factor for classic and variant strains of PEDV into Vero E6 cells and IECs.

In conclusion, the results of the current study revealed that integrin αvβ3 enhances replication of the classical and variant strains of PEDV in Vero E6 cells and IECs, and integrin αvβ3 and pAPN have a synergistic effect on PEDV infection of Vero E6 cells and BHK-21 cells. These findings are expected to increase our understanding of the entry mechanism of PEDV and provide valuable information for designing antiviral drugs against PEDV infection.

Declaration of Competing Interest

The authors declare no conflict of interest.

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