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Aminopeptidase-N-independent entry of porcine epidemic diarrhea virus into Vero or porcine small intestine epithelial cells

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ABSTRACT

A monkey cell line Vero (ATCC CCL-81) is commonly used for porcine epidemic diarrhea virus (PEDV) propagation *in vitro*. However, it is still controversial whether the porcine aminopeptidase N (pAPN) counterpart on Vero cells (Vero-APN) confers PEDV entry. We found that endogenous expression of Vero-APN was undetectable in the mRNA and the protein levels in Vero cells. We cloned the partial Vero-APN gene (3340-bp) containing exons 1 to 9 from cellular DNA and subsequently generated two APN-knockout Vero cell lines by CRISPR/Cas9 approach. PEDV infection of two APN-knockout Vero cells had the same efficiency as the Vero cells with or without neuraminidase treatment. A Vero cells stably expressing pAPN did not increase PEDV production. SiRNA-knockdown of pAPN in porcine jejunum epithelial cells had no effects on PEDV infection. The results suggest that there exists an additional cellular receptor on Vero or porcine jejunal cells independent of APN for PEDV entry.

1. Introduction

Porcine epidemic diarrhea virus (PEDV) is a positive-sense single stranded RNA virus belonging to the alphacoronavirus genus in the subfamily *Coronavirinae* of the family *Coronaviridae* (Pensaert and de Bouck, 1978; Song and Park, 2012). PEDV causes acute enteric disease in swine, characterized by acute vomiting and watery diarrhea, which has high mortality rates in newborn piglets (Huang et al., 2013; Pan et al., 2012). PEDV was discovered in the United Kingdom in early 1970s and was subsequently identified in many European and Asian countries (Lee et al., 2010; Li et al., 2012; Pensaert and Callebaut, 1974). PEDV field strains isolated before 2010 and the derived vaccine strains belong to genogroup 1 (G1) (Kocherhans et al., 2001). Since late 2010, variant PEDV strains (genogroup 2 [G2]) have emerged in China and Southeast Asia that were fatal to young pigs (Huang et al., 2013; Li et al., 2012). In May 2013, PEDV G2 strains suddenly emerged in the United States, wiping out more than 10% of America's pig population in one year (Huang et al., 2013; Tian et al., 2014). PEDV, together with the other newly emerged swine enteric coronaviruses such as porcine deltacoronavirus (PDCoV) and swine enteric alphacoronavirus (SeACoV), are considered serious threats to the pork industry in Asia currently (Jung et al., 2016; Pan et al., 2017).

Aminopeptidase N (APN or CD13) is a type II zinc metalloprotease that mediates various cellular processes, including antigen presentation, cell differentiation, cell motility and coronavirus entry (Luan and Xu, 2007). Porcine APN (pAPN) was first identified as a major receptor for a porcine enteropathogenic alphacoronavirus, transmissible gastroenteritis virus (TGEV) (Delmas et al., 1992). A related human alphacoronavirus, HCoV-229E, utilizes the human APN (hAPN) to enter host cells (Yeager et al., 1992). The interactions between TGEV/HCoV-229E and pAPN/hAPN are highly natural host specific, in which TGEV can use pAPN but not hAPN as its cellular receptor, and HCoV-229E can use hAPN but not pAPN (Kolb et al., 1996). It was reported that transfection and expression of pAPN was sufficient to confer PEDV infection to a non-permissive canine kidney MDCK cell line, indicating pAPN is likely a functional receptor for PEDV as well (Li et al., 2007). Moreover, transgenic mice expressing pAPN confer susceptibility to PEDV (Park et al., 2015). It was also reported that, unlike TGEV or HCoV-229E, PEDV spike protein-mediated pseudovirus was able to enter non-permissive canine kidney MDCK cells exogenously expressing either hAPN or pAPN, suggesting that the PEDV-APN interaction is not species specific (Liu et al., 2015). Correspondingly, PEDV can infect various cell lines from human, monkey, pig and bat species (Liu et al., 2015), but whether species-specific APN functions as the PEDV entry receptor on the respective cell line has not been investigated. However, most recently, two groups independently demonstrated that neither hAPN nor pAPN is the functional receptor for PEDV by using more comprehensive analyses, including knockout of endogenous expression of hAPN or pAPN in human or porcine cell lines by the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-

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associated protein-9 nuclease (Cas9) system (Li et al., 2017; Shirato et al., 2016).

An African green monkey (*Chlorocebus sabaeus*) kidney cell line, Vero (ATCC CCL-81), is commonly used for PEDV adaption and culture in the presence of trypsin *in vitro* for research or vaccine production (Hofmann and Wyler, 1988; Pan et al., 2012). Green monkey specific APN expressed on Vero cells (Vero-APN/vAPN) may serve as the entry receptor for PEDV (Li et al., 2007). However, a few studies remarked Vero cells without APN expression, but no experimental evidences were provided in these publications (Li et al., 2016; Nam and Lee, 2010). Therefore, the aim of this study was to clarify the argument, investigating whether Vero-APN is indeed responsible for PEDV entry into Vero cells. Furthermore, we sought to test whether knockdown of pAPN expression on porcine small intestinal epithelial cell line, IPEC-J2, is correlated with PEDV infection in comparison with TGEV.

2. Results and discussion

We first aimed to confirm if the putative green monkey APN (vAPN) gene exists in Vero cellular genome DNA. The 5'-part of the vAPN gene (3,340 bp), which was not available on the public genome database, was cloned by genomic PCR from extracted Vero genomic DNA with a pair of PCR primers shared significant sequence homology with human, chimpanzee, rhesus monkey and pig APNs. As illustrated in Fig. 1, the partial cloned vAPN gene contains nine exons or coding DNA sequence (CDS). The start codon ATG is located at CDS1 having 617 base pair (bp), whereas the sizes of the other eight CDS (CDS2–9) vary from 66 to 155 bp (Fig. 1). The partial cloned vAPN gene and its encoding cDNA (1,572 bp in size) share 97.9% and 98.9% sequence homology with the available rhesus monkey APN gene and cDNA, respectively. Attempts to amplification of the entire or the 5'-partial vAPN mRNA by RT-PCR were failed. Therefore, we performed a quantitative real-time PCR (qRT-PCR) analysis to detect if vAPN mRNA is expressed endogenously in Vero cells. As a control we examined hAPN mRNA expression in Huh-7 cells. When we confirmed endogenous hAPN mRNA expression, which was in line with the previously publications (Li et al., 2017; Liu et al., 2015), relative quantification of vAPN mRNA level to the green monkey β-actin control in Vero cells did not give a convincingly positive value in a cell-culture period of 3 days (data not shown). The result indicated that vAPN mRNA was expressed under the detection limit or was deficient in expression.

We further examined endogenous and exogenous expression of APN in the protein level using a broadly reactive anti-APN antibody by immunofluorescence assay (IFA) and western blot (WB) analysis. For comparison, two recombinant APN expression plasmids were constructed. Plasmid pCI-pAPN harbored the full-length porcine APN cDNA (2,892 bp) whereas plasmid pRK-vAPN contained the nine CDS of vAPN without introns followed by a stop codon (1,575 bp) that were fused sequentially by overlapping PCR. Vero or Huh-7 cells were transfected with either of the two plasmids. At 48 hour post-transfection, transfected or untransfected cells were subjected to IFA or WB. Specific anti-APN fluorescence signal was detected in transfected Vero or Huh-7 cells as well as untransfected Huh-7 cells (though the intensity was weak), but not observed in untransfected Vero cells (Fig. 2A). The WB analysis was consistent with the IFA result, showing individual bands of distinct sizes representing the full-length APN (APN-FL) or the partial APN (vAPN-CDS1–9) in pCI-pAPN- or pRK-vAPN-transfected Vero cells, whereas no bands were detected in untransfected Vero cells (Fig. 2B). In contrast, Huh-7 cells displayed a band corresponding to APN-FL regardless whether transfection with pCI-pAPN- or pRK-vAPN or not (Fig. 2B). Theses results indicated that Huh-7 cells express hAPN endogenously while Vero cells probably do not express vAPN inherently.

To further examine the effects of anti-APN antibody on PEDV entry into these two cell lines, we carried out quantitative PEDV infection analysis with a recombinant PEDV-GFP (see "Materials and methods"). Either Vero or Huh-7 cells were infected efficiently by PEDV-GFP, showing clear GFP fluorescence co-localized with cytopathic effects (CPE), which could be assessed by counting the numbers of GFP-positive cell clusters (Fig. 3A). Moreover, PEDV-GFP infection of two cell lines could be neutralized by anti-PEDV-S IgM or IgG in a dose-dependent manner (Fig. 3B), indicating that it is an appropriate *in vitro* model for quantification of PEDV infection. When anti-APN antibody at 2 μg/ml or 20 mg/ml was incubated with the cells prior to PEDV-GFP infection, it did not block PEDV-GFP entry or infection in Vero cells at 12 or 24 hour post-infection (hpi; Fig. 3C). For Huh-7 cells, it was observed that pretreatment with the antibody significantly decreased GFP-positive cell cluster numbers at 12 hpi; however, there was no differences in comparison with untreated cells at 24 hpi (Fig. 3C). Shirato and his colleagues reported that pAPN promotes PEDV infection through its protease activity rather than its proposed receptor function (Shirato et al., 2016). Since the protease active sites are highly conserved between hAPN and pAPN (Delmas et al., 1994), it is hypothesized that the anti-APN antibody blocked PEDV infection by targeting the enzymatically catalytic sites of hAPN on Huh-7 cells in the early stage (0–12 hpi). The inhibited effect was likely diminished when PEDV continued to propagate and spread thereafter. These results demonstrated that anti-APN antibody had no effects on PEDV entry into Vero cells, probably due to the absence of vAPN expression.

In order to completely rule out the potential vAPN production in Vero cells that may be under the detection limit, we set out to generate vAPN-knockout cells using CRISPR/Cas9 system. A single-plasmid CRISPR/Cas9 approach was developed, in which two guide RNA (gRNA) molecules and the Cas9 enzyme followed by a puromycin resistance gene are simultaneously expressed (Fig. 4A). We designed a knockout strategy using an engineered pX480-vAPNKO plasmid by targeting the vAPN CDS1 with two gRNA/Cas9 complexes to delete a 160-bp fragment between nucleotides 290–449 downstream of the translation initiation site (Fig. 1). At 3 days post-transfection with the pX480-vAPNKO plasmid plus puromycin selection, the genomic DNA was isolated and tested for vAPN gene integrity by PCR. It was shown that two different cell pools (using the same approach in two batches) possessed the intended vAPN deletion fragment, displaying weak bands smaller than the intact CDS1 (Fig. 4B). From these cell pools, we generated single cell clones, designated as vAPN^{KO1} and vAPN^{KO2}, by limiting dilution with puromycin selection again. However, the intended vAPN deletion fragment was not visible in the clone vAPN^{KO1} (Fig. 4C). Sequence analysis of the PCR products of the clone vAPN^{KO1} revealed that there was only a 5-bp deletion (nt 290–294) downstream of the fist Cas9 cleavage site (Fig. 4D). The single cell clone vAPN^{KO2} displayed the intended vAPN deletion fragment that was subsequently confirmed to have the expected 160-bp deletion by sequencing (Fig. 4C and D). Nevertheless, either of the deletions in vAPN^{KO1} or vAPN^{KO2} would result in a frameshift of vAPN allele.

We next confirmed that these knockout cells, Vero-vAPN^{KO1} and Vero-vAPN^{KO2}, were deficient in vAPN expression by IFA (Fig. 5A). Upon PEDV-GFP infection, both Vero-vAPN^{KO1} and Vero-vAPN^{KO2} cells

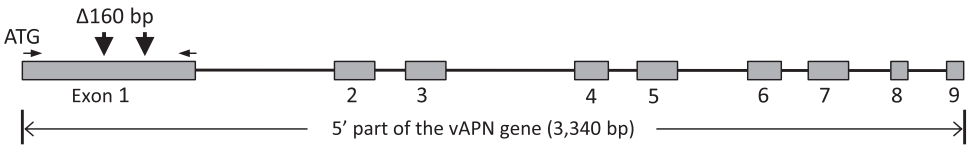

Fig. 1. Gene structure of the 5'-part of the vAPN gene containing the first nine exons (3,340 bp) determined in this study (GenBank accession number KX342855). Arrowheads indicate two Cas9 cleavage sites (nucleotides 290–449) downstream of the translation initiation site (ATG) within the exon 1 (CDS1). Deletion of a 160-bp fragment between these two sites will result in a frameshift. Arrows indicate PCR primers to assess gene integrity.