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Aminopeptidase N is not required for porcine epidemic diarrhea virus cell entry

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ABSTRACT

Porcine epidemic diarrhea virus (PEDV) is an emerging pathogenic coronavirus that causes a significant economic burden to the swine industry. The virus infects the intestinal epithelium and causes villous atrophy, resulting in diarrhea and dehydration. Interaction of the viral spike (S) surface glycoprotein − through its S1 subunit − with the host cell receptor is the first step in infection and the main determinant for virus tropism. As for several other *alphacoronaviruses* including the porcine transmissible gastroenteritis virus (TGEV) and the human coronavirus 229E (HCoV-229E), the aminopeptidase N (APN) protein was reported to be a functional receptor for PEDV. In this study we examined the role of APN as a receptor. We show that overexpression of porcine APN renders MDCK cells susceptible to TGEV, but not to PEDV. Consistently, unlike TGEV-S1, PEDV-S1 exhibited no binding to cell-surface expressed APN or to a soluble version of APN. Moreover, preincubation of these viruses with soluble APN or pretreatment of APN expressing ST cells with soluble TGEV-S1 blocked TGEV infection, but had no effect on infection by PEDV. The combined observations indicated that APN is not required for PEDV infection. To definitively prove this conclusion, we applied CRISPR/Cas9 genome engineering to knock out APN expression in PEDV-susceptible porcine (ST) and human cell lines (Huh7 and HeLa). As a consequence these cells no longer bound TGEV-S1 and HCoV-229E-S1 at their surface and were resistant to infection by the corresponding viruses. However, genetic ablation of APN expression had no effect on their infectability by PEDV, demonstrating that APN is not essential for PEDV cell entry.

1. Introduction

Porcine epidemic diarrhea virus (PEDV) is a member of the *Coronaviridae* family (subfamily *Coronavirinae,* genus *Alphacoronavirus*) and causes mild to severe diarrheal disease in pigs. PEDV replicates efficiently in the small intestine and causes acute, severe atrophic enteritis inducing watery diarrhea, dehydration and vomiting, with particularly high mortality in sero-negative neonatal piglets (Debouck and Pensaert, 1980). PEDV was first reported in feeder and grower pigs in England in 1971 (Chasey and Cartwright, 1978). Subsequently, PEDV positive cases were reported in several other European countries. In Asia, PEDV was first identified in 1982, after which it remained endemic causing substantial economic losses to the pork industry (Song and Park, 2012). Since 2010, a large-scale outbreak of PEDV was reported in China. The severe outbreak was characterized by 80–100% morbidity and 50–90% mortality among infected suckling piglets (Li et al., 2012; Sun et al., 2012). Apart from some suspected cases in Canada in 1970s (Turgeon et al., 1980), North America was free of PEDV until April 2013, when a severe outbreak started which spread rapidly across the United States, causing high mortalities among infected piglets and substantial economic losses (Chen et al., 2014; Cima, 2013; Huang et al., 2013).

The interaction of viruses with their cellular receptor is the first and crucial step in the virus life cycle as it enables cell entry and productive infection. Receptor interaction of coronaviruses is mediated by the trimeric spike (S) glycoproteins that form the characteristic, large spikes on the coronaviral envelope (Gallagher and Buchmeier, 2001). Coronaviruses use a variety of cellular proteins as functional receptors for cell entry. The porcine *alphacoronavirus* transmissible gastroenteritis virus (TGEV), which is clinically indistinguishable from PEDV, utilizes aminopeptidase N (APN) as its receptor (Delmas et al., 1992), similar to other *alphacoronaviruses* including the human coronavirus 229E (HCoV-229E) (Yeager et al., 1992), the feline infectious peritonitis virus (FIPV) and the canine coronavirus (CCV) (Tresnan et al., 1996). An exception

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within the *alphacoronavirus* genus is the human coronavirus NL63 (HCoV-NL63) which employs angiotensin converting enzyme 2 (ACE2). The ACE2 receptor was earlier identified as a functional receptor for the *betacoronavirus* severe acute respiratory syndrome coronavirus (SARS-CoV) (Li et al., 2003). The *betacoronaviruses* mouse hepatitis virus (MHV) and Middle East respiratory syndrome coronavirus (MERS-CoV) mediate infection by binding to carcinoembryonic antigen-cell adhesion molecule (CEACAM1) and dipeptidyl peptidase 4 (DPP4) (Raj et al., 2013; Williams et al., 1991), respectively. Some coronaviruses, including human coronavirus OC43 (HCoV-OC43) and bovine coronavirus (BCoV) use acetylated sialic acids as functional receptors (Schultze et al., 1991; Vlasak et al., 1988).

PEDV has been reported to utilize APN, also known as CD13, as a functional cellular receptor (Li et al., 2007), underlining the more common use of this molecule as a receptor for *alphacoronaviruses*. APN is a 150 kDa glycosylated type II transmembrane protein that is highly abundant on the apical membrane of mature enterocytes. It was termed "moonlighting enzyme" referring to its multiple biological functions, including peptide metabolism, cell motility and adhesion (Mina-Osorio, 2008). In pigs, porcine APN (pAPN) is abundantly expressed on the brush border membrane of the intestine; additionally, it is also expressed on the surfaces of epithelial cells of kidneys and respiratory tract (Cong et al., 2015). Several lines of evidence support the view that APN is a functional receptor for PEDV. i) A ~150 kDa PEDV-binding protein was identified in swine testis (ST) cells by virus overlay protein binding assay (VOPBA), the interaction with which could be blocked by antibodies against pAPN (Oh et al., 2003). ii) pAPN overexpression in non- or poorly susceptible cell lines (i.c. Madin Darby canine kidney and ST cell) conferred susceptibility of these cells to PEDV infection (Nam and Lee, 2010). iii) Preincubation of cells with either pAPN antibodies or pAPN binding peptides prior to inoculation with PEDV interfered with infection (Li et al., 2007). iv) PEDV S1 was found to biochemically interact with soluble APN in a dot-blot assay and with cell surface expressed APN in a FACS assay (Deng et al., 2016; Liu et al., 2015). v) Transgenic mice expressing pAPN were rendered susceptible to PEDV infection (Park et al., 2015). Despite these findings, PEDV efficiently infects African green monkey kidney (Vero) cells (Hofmann and Wyler, 1988; Kusanagi et al., 1992), known not to express APN (Li et al., 2007; Shirato et al., 2011), questioning the role of pAPN as a functional receptor.

During our studies on the process of cell entry by PEDV, we made confusing observations that were at variance with the apparently accepted view on the essential role of APN in this process. These observations urged us to critically reassess APN's role in PEDV cell entry. The results of our studies, particularly the unimpaired infection of cells in which APN expression had been knocked out, lead us to conclude that APN is not the cell surface receptor for PEDV.

2. Materials and methods

2.1. Antibodies, cells and viruses

Polyclonal rabbit serum detecting HCoV-229E was kindly provided by Pierre J. Talbot (Breslin et al., 2003), mouse monoclonal antibody against TGEV (ab20301) and mouse anti-HA epitope tag antibody (ab130275) were purchased from Abcam. The 3F12 mouse monoclonal antibody recognizing the PEDV nucleocapsid protein was purchased from BioNote (Republic of Korea). Swine testis (ST), African green monkey kidney (Vero-CCL81) cells, Madin-Darby canine kidney (MDCK) cells, human hepatoma cells (Huh7), human embryonic kidney 293 cells stably expressing the SV40 large T antigen (HEK-293T), and derivatives of these cell lines were maintained in Dulbecco modified Eagle medium (DMEM, Lonza BE12-741F) supplemented with 10% fetal bovine serum (FBS). An MDCK derived cell line stably expressing the porcine aminopeptidase N (pAPN; GB: NP_999442.1) was made by the Moloney murine leukemia virus transduction system (Clontech) using the pQCXIN retroviral vector encoding the pAPN protein C-terminally extended with the HA-tag (YPYDVPDYA). Stably transfected MDCK-pAPN cells were selected and maintained with G418 (PAA). PEDV was grow in Vero cells in maintenance medium (αMEM-TPB), which was Eagle's minimum essential medium alpha (Life Technologies, 22571-020) supplemented with 0.3 % tryptose phosphate broth (TPB, Sigma T9157). Cell culture-adapted PEDV-DR13 (GB: JQ023162) and recombinant virus encoding the PEDV-DR13 S protein and the green fluorescent protein (GFP) (rPEDV-S^{DR13}-GFP) (Li et al., 2013) were propagated and titrated in aMEM-TPB supplemented with 20 mM HEPES in Vero cells. All other PEDV viruses were propagated and titrated in αMEM-TPB supplemented with 20 mM HEPES plus 15 μg/ml trypsin (Sigma T4799) in Vero cells. PEDV-CV777 (GB: AF353511; kindly provided by Dr. Kristin van Reeth, Ghent University), PEDV field isolates from China (strain GDU, GB: KU985230) and The Netherlands (strain UU, GB: KU985229). Recombinant rPEDV-S^{CV777}-GFP and rPEDV-S^{GDU}-GFP encoding spike proteins of different PEDV strains in the DR13 background were generated as described before (Li et al., 2016; Li et al., 2013; Wicht et al., 2014). The open reading frame 3 (ORF3) of these recombinant viruses was replaced with the GFP gene. TGEV strain Purdue (GB: ABG89335.1) was propagated and titrated on Porcine kidney (PD5) cells in DMEM with 1% of FBS.

2.2. Recombinant protein expression

pCAGGS expression vectors encoding PEDV-S1 (isolate GDU, residues 1–728; isolate DR13, residues 1–724; isolate CV777, residues 1–725) C-terminally tagged with the Fc domain of human or mouse IgG were generated as described before (Li et al., 2016). Similarly, expression plasmids were made encoding Fc-tagged S1 subunits of TGEV (isolate Purdue, GB: ABG89335.1, residues 1–785), HCoV-229E (GB: NP_073551.1, residues 1–537) and MERS-CoV (Raj et al., 2013), as well as constructs expressing Fc-tagged soluble (i.c. non-membrane anchored) form of porcine APN and human DPP4 (residues 39–766). These Fc-chimera's were expressed by transfection of the expression plasmids into HEK-293T cells and affinity purified from the culture supernatant using protein A sepharose beads (GE Healthcare). Purity and integrity of all purified recombinant proteins was checked by SDS-PAGE.

2.3. Immunofluorescence microscopy

For immunofluorescence staining, cells were washed twice with PBS and fixed with 3.7% formaldehyde (Merck, 1040031000) in PBS, followed by membrane permeabilization with 0.1% Triton-X-100 (Sigma, 93426) in PBS for 10 min at room temperature. Fixed cells were incubated with 3% Bovine serum albumin (BSA) (GE Healthcare Life Sciences) in PBS for 1 h followed by incubation with the primary antibody for 1 h in PBS with 1% BSA. After rinsing three times with PBS, staining was completed by Alexa Fluor®488-conjugated goat α rabbit antibody (Life Technologies, A11008) or Alexa Fluor®488-conjugated goat α mouse antibody (Life Technologies, A11001). Nuclei were visualized using DAPI nuclear counterstaining (Molecular Probes). Pictures of immunofluorescent cells were captured using an EVOS-fl fluorescence microscope (Advanced Microscopy Group) at 10 x magnification. Percentage of infected cells (relative to mock-treated) was calculated by counting infected cells in 10 x microscopic fields.

2.4. Flow cytometry

Adherent cells were rinsed with PBS and detached with cell culture dissociation solution (Sigma, C5914). Cells were resuspended in PBS supplemented with 2 % FBS and 0.02 % sodium azide, pelleted by centrifugation and fixed in 3.7 % formaldehyde in PBS. Subsequently, cells were incubated with 15 μg/ml of the indicated recombinant S1-Fc proteins, followed by incubation with Alexa Flour®488-labelled goat-

anti-human IgG antibody (Life Technologies, A11013) and analysed with the FACS-Calibur flow cytometer (BD Bioscience). FACS data analyses were performed using the FlowJo software (TreeStar Inc).

2.5. Western blot analysis

To detect APN expression in Vero-pAPN and MDCK-pAPN cells, cells were lysed in cell lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% deoxycholic acid) with protease inhibitors cocktail (Roche, USA) and subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (10% acrylamide separating gel). Next, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (BioRad, 162-0176) and membrane was blocked with 3 % BSA. Detection of the HA-tagged pAPN protein in cell lysates was done using the horseradish peroxidase (HRP) conjugated anti-HA monoclonal antibody (Abcam, ab1190; in PBS with 1% BSA and 0.1% Tween 20). Proteins were detected using the Amersham ECL Western Blotting Analysis System (GE healthcare, RPN2109) and the Odyssey® Fc Imaging System (LI-COR Ltd.).

2.6. CRISPR/Cas-mediated genome editing

Porcine and human APN knockout cells were generated by CRISPR/CAS9 genome editing as described earlier (Hsu et al., 2014; Langereis et al., 2015). To knockout the human APN gene in Huh7 and HeLa cells, two guide RNAs targeting exon 1 (nucleotides 190–209, 5′- CCTTGGACCAAAGTAAAGCG-3′) and intron 1 (nucleotides 152–171, 5′-AGCTGCAAATGCACCGAACT-3′) were used. Guide RNA targeting exon 1 (nucleotides 83–102, 5′-TCTGTCTGTGGTGTACGCCC-3′) of the porcine APN gene was used to construct $ST^{\Delta APN}$ cells. Single cell clones of APN knockout cells were obtained by limiting dilution and genotyped by PCR and DNA sequencing. Knockout of APN expression in the mutant cell lines was further confirmed by western blot, S1-Fc cell surface binding and virus infection assays for TGEV or HCoV-229E, that are known to use porcine and human APN as their receptor, respectively.

2.7. Virus entry in the presence of soluble porcine APN

PEDV (m.o.i. = 1) or TGEV (m.o.i. = 0.1) were preincubated with serially diluted soluble, porcine APN for 1.5 h at 37 °C after which virus-protein mixtures were supplemented with (inoculum with PEDV) or without (inoculum with TGEV) 8 μg/ml trypsin, and subjected to ST cells. Following a 2 h incubation, the inoculum was removed and cells were washed twice with PBS, and supplied with fresh DMEM medium containing 1% FCS (TGEV infected wells) or 2 μg/ml trypsin (PEDV infected wells). Cells were fixed at 16 h post inoculation and subjected to (immune) fluorescence staining.

2.8. Virus entry upon preincubation of cells with soluble TGEV-S1-mFc proteins

ST cells were preincubated with DMEM containing various amounts of soluble TGEV-S1-mFc proteins for 1.5 h at 37 °C after which an equal volume of PEDV (m.o.i. = 5) or TGEV (m.o.i. = 0.1) was added. PEDV infection was done in the presence of 8 μg/ml trypsin. After a 2 h incubation, the inoculum was removed and cells were washed twice with PBS, and supplied with fresh DMEM medium containing 1% FCS (in TGEV infected wells) or 2 μg/ml trypsin (PEDV infected wells). Cells were fixed at 16 h post inoculation and subjected to (immune) fluorescence staining.

2.9. Dot-blot hybridization assay

Coronavirus S1 binding to CoV receptors was assessed using s dot-blot hybridization assay as described by Peng et al. (Peng et al., 2011). Briefly, samples with 10 μg of hDPP4-Fc or pAPN-Fc proteins were spotted onto nitrocellulose membranes (Thermo Fisher Scientific Inc.). After air-drying, the membranes were blocked with 3% BSA at 4 °C overnight. The membranes were then incubated with 50 μg/ml coronavirus S1-mFc proteins at room temperature for 2 h, washed five times with PBS containing 0.1% Tween 20 and incubated with HRP-conjugated Goat Anti-Mouse IgG (H + L) (Thermo Fisher Scientific Inc., 31430) in PBS with 1% BSA and 0.1% Tween 20. Proteins were detected using the Amersham ECL Western Blotting Analysis System (GE healthcare, RPN2109) and the Odyssey® Fc Imaging System (LI-COR Ltd.).

2.10. Statistical analysis

Statistical analysis of the data was performed with Prism 6.05 software using an unpaired one tailed Student's *t*-test. Error bars indicate standard deviations. The level of significance was expressed as * $P < 0.05$, ** $P < 0.01$.

3. Results

3.1. Cellular overexpression of porcine APN does not confer susceptibility to PEDV infection

To verify the role of porcine APN (pAPN) as a functional receptor for PEDV, we attempted to rescue PEDV infection in non-susceptible MDCK cells by overexpression of pAPN on the cell surface. A stable cell line overexpressing porcine APN (MDCK-pAPN) was generated and pAPN expression was confirmed by RT-PCR (data not shown), western blotting and immunofluorescence staining. Western blot analysis detected a band of 150 kDa in MDCK-pAPN lysates corresponding to the molecular weight of porcine APN (Fig. 1a) and immunofluorescence staining detected pAPN expression in ± 90% of cells (Fig. 1b). The overexpression of pAPN rendered MDCK cells susceptible to TGEV infection, consistent with the role of pAPN as a cellular receptor during TGEV entry. To verify the functionality of pAPN as a receptor for PEDV, MDCK-pAPN cells, parental MDCK cells and Vero cells were inoculated with recombinant PEDV containing the spike genes of two classical strains (DR13 and CV777) and a contemporary strain (GDU). As expected, Vero-CCL81 cells could support infection by all the recombinant PEDV viruses. However, the expression of pAPN in MDCK cells failed to rescue infection of any of the recombinant PEDVs.

3.2. TGEV S1 − but not PEDV S1–can bind to porcine APN

To further check whether PEDV could biochemically interact with pAPN, we expressed and purified the S1 protein of PEDV and TGEV, both C-terminally tagged with the Fc-portion of human (Fc) or mouse (mFc) IgG for affinity purification and detection. S1-Fc binding to MDCK-pAPN and MDCK cells was analysed by flow cytometry. Binding of TGEV-S1-Fc was seen to the surface of MDCK-pAPN cells but not to that of parental MDCK cells. In contrast, overexpression of pAPN did not confer binding of PEDV-S1-Fc to MDCK cells (Fig. 2a). A similar binding pattern was seen upon pAPN overexpression in HEK293T cells (data no shown). Furthermore, using a dot blot hybridization assay as described by Peng et al. (Peng et al., 2011), we demonstrated binding of MERS-CoV-S1-mFc and TGEV-S1-mFc to their human Fc-tagged human DPP4 or porcine APN receptor, respectively (Delmas et al., 1992; Raj et al., 2013), whereas binding of the S1 of various PEDV strains to pAPN could not be detected (Fig. 2b).

3.3. TGEV infection − but not that of PEDV − can be inhibited by preincubation of virus with soluble pAPN, or of cells with TGEV S1

Swine testis (ST) cells endogenously express pAPN on their surface (Meng et al., 2014) and support PEDV and TGEV infection (Liu et al., 2015; Ma et al., 2014). We tested whether PEDV infection on these

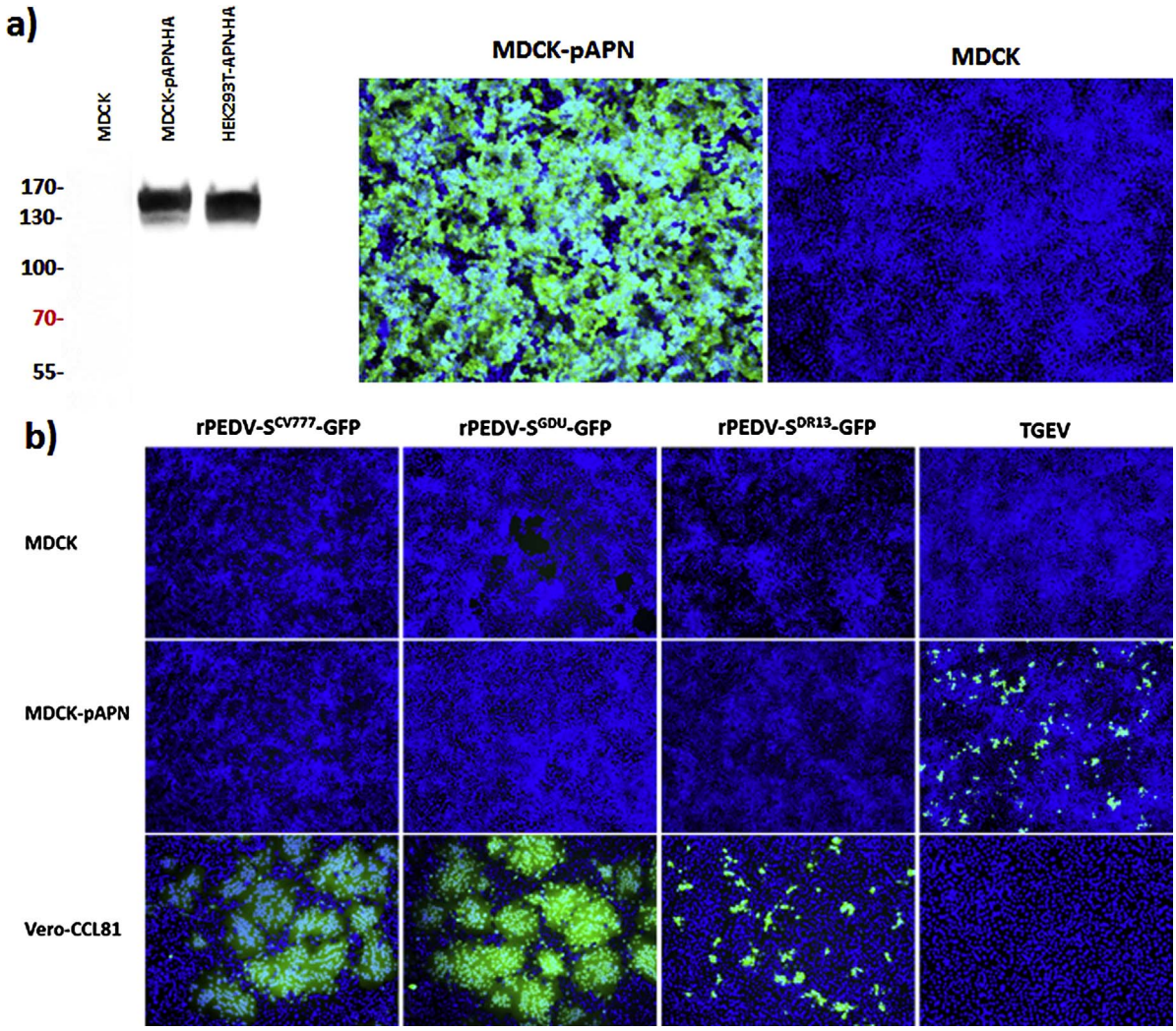

Fig. 1. Cellular overexpression of porcine APN renders MDCK cells susceptible for TGEV, but not for PEDV.(a) MDCK cells stably expressing HA-tagged porcine APN (MDCK-pAPN) were generated and pAPN expression was confirmed by Western blotting with HRP conjugated anti-HA tag antibody (left panel) and immunofluorescence assay (right panel) using mouse anti-HA antibody as primary antibody and Alexa Fluor 488-conjugated goat anti-mouse IgG (H + L) as secondary antibody. A lysate of HEK-293T cells transiently transfected with the HA-tagged pAPN encoding plasmid was taken along as a positive control. Sizes of marker proteins are indicated in kilodaltons. (b) Overexpression of pAPN makes MDCK cells susceptible to TGEV, but not to PEDV. MDCK, MDCK-pAPN and Vero-CCL81 cells were infected with GFP-encoding recombinant PEDV viruses carrying spike proteins of different strains (PEDV strains GDU, CV777 and DR13). The cells were stained with DAPI. The infection experiments were performed three times with similar results, representative images are shown.

porcine cells is compromised by preincubation with soluble pAPN (i.c. its non-membrane anchored form). PEDV was pre-incubated with Fc-tagged pAPN ectodomain (pAPN-Fc) at different concentrations, prior to inoculation. TGEV was taken along as a positive control. Soluble pAPN significantly inhibited infection of TGEV in a dose-dependent manner, whereas it failed to neutralize PEDV infection (Fig. 3**a and 3b**). It cannot be excluded that lack of inhibition by soluble APN is due to low affinity binding of PEDV S1 to pAPN. Hence we used TGEV S1 that displays high affinity pAPN binding to saturate TGEV binding sites on pAPN on the surface of ST cells, and assessed the effect on PEDV and TGEV infection. ST cells were preincubated with indicated concentration of TGEV-S1-mFc and subsequently infected with TGEV and PEDV (Fig. 3c and 3d). As expected, preincubation of cells with TGEV-S1-mFc could significantly inhibit TGEV infection in a dose dependent manner, blocking approximately 70% of TGEV infection at 200 μg/ml (Fig. 3d). However, PEDV infection was not affected by pretreatment of cells with TGEV-S1-mFc (Fig. 3c). The latter data suggest that PEDV and TGEV do not share the same receptor, or that they recognize different parts on the pAPN receptor.

3.4. Knockout of pAPN expression in ST cells inhibits TGEV, but not PEDV infection

To corroborate our observations, we constructed a mutant ST cell line lacking APN expression ($ST^{\Delta APN}$) using the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated 9) genome editing system (Hsu et al., 2014). The identity of the mutant cell line was confirmed by sequencing. The $ST^{\Delta APN}$ cell line harbors a 2 nucleotide out-of-frame deletion in exon 1 (Fig. 4a), resulting in the production of a 32 amino acid short truncated peptide that lacks "virus-binding motifs" of TGEV (Chen et al., 2012). Surface staining with the pAPN-dependent TGEV S1 was used to functionally assess APN knock-out in the $ST^{\Delta APN}$ cells. The HCoV-229E S1-Fc that can bind to human but not porcine APN was taken along as a negative control. Compared to parental ST cells, the $ST^{\Delta APN}$ cells lost their ability to bind TGEV-S1-Fc (Fig. 4b). Moreover, TGEV could only infect ST cells and not $ST^{\Delta APN}$ cells, confirming the knockout of pAPN expression in $ST^{\Delta APN}$ cells (Fig. 4c and d). We subsequently used different PEDV strains to infect ST and $ST^{\Delta APN}$ cells. No significant difference in PEDV infection was found (Fig. 4c and d), demonstrating that knockout of APN expression in ST cells does not block PEDV infection.

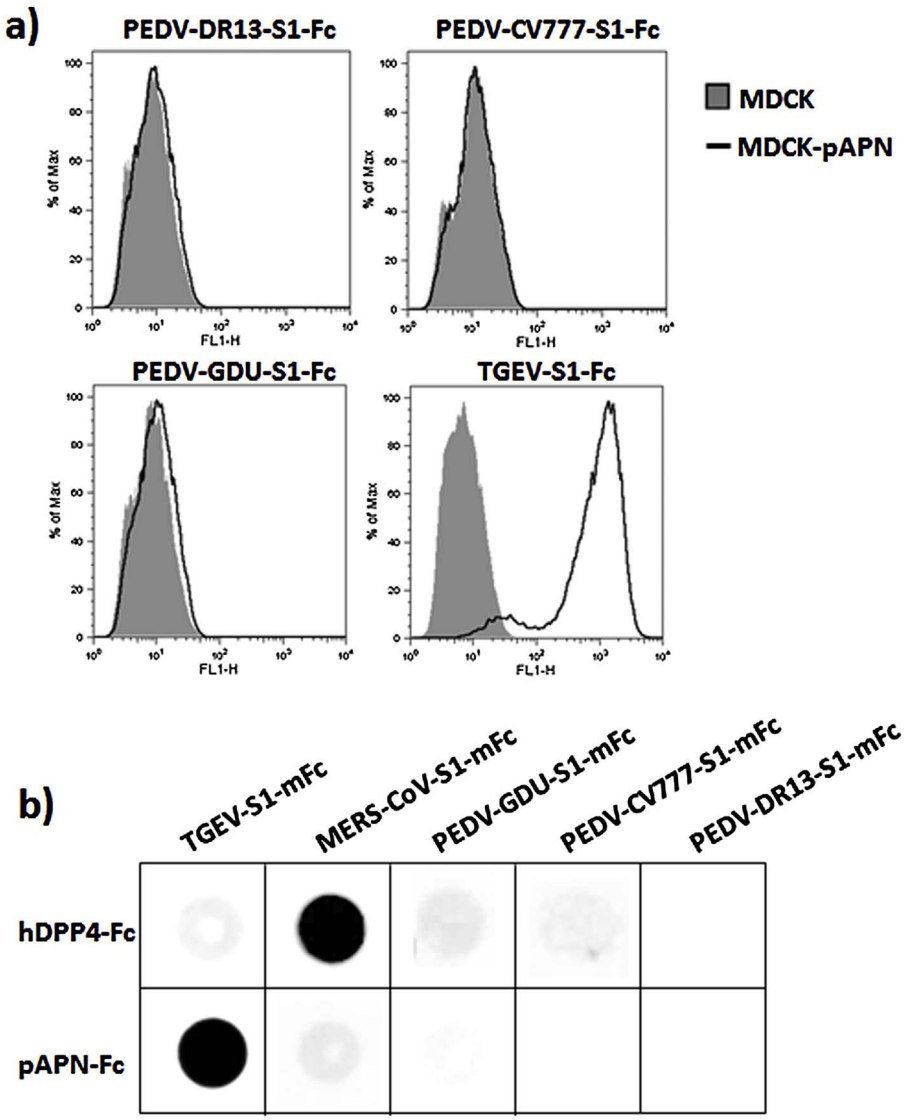

Fig. 2. PEDV S1 does not bind porcine APN.(a) Binding of S1-Fc protein of different PEDV strains to the cell surface of MDCK-pAPN (black line) and MDCK cells (grey shading) was measured by flow cytometry. The porcine APN-binding TGEV-S1-Fc was used as positive control. (b) Dot blot hybridization assay was used to detect binding of Fc-tagged S1 proteins of different PEDV strains to porcine APN (pAPN) spotted on nitrocellulose membrane. The human dipeptidyl peptidase 4 (hDPP4) was also spotted. The pAPN-binding TGEV-S1-Fc and hDPP4-binding MERS-CoV S1-Fc were taken along as positive controls. S1 proteins were C-terminally tagged with the Fc part of murine IgG1 and pAPN or hDPP4 were tagged with a C-terminal human IgG1 Fc. Binding of S1-Fc proteins to pAPN or hDPP4 was detected using antibodies against the murine Fc tag.

3.5. Knockout of APN gene in PEDV-susceptible human cell lines does inhibit HCoV-229E but not PEDV infection

Human cells have also been shown to be susceptible to PEDV (Liu et al., 2015). Human cervix cells HeLa cells can support PEDV infection, but seem to lack APN expression (Bausch-Fluck et al., 2015). HeLa cells do not support infection of the APN-dependent HCoV-229E, consistent with the lack of detectable APN expression in these cells. Moreover, genetic ablation of the APN gene in HeLa cells by CRISPR/Cas9 genome editing did not affect PEDV infection suggesting that human APN is not necessary in PEDV infection of HeLa cells (data not shown). Human hepatocellular carcinoma cell line Huh7 cells express human APN and are susceptible to PEDV and HCoV-229E (Tang et al., 2005). To check the APN dependency of PEDV infection in Huh7 cells, we created mutant Huh7 cells (Huh7$^{\Delta APN}$) in which the APN gene was disrupted by CRISPR/Cas9 genome engineering. Sequence analysis of the APN gene in the Huh7$^{\Delta APN}$ cell line indicated the occurrence of a 577 nucleotide out-of-frame deletion in exon 1 (Fig. 5a). The loss of APN expression was confirmed by western blotting (data not shown) and the lack of HCoV-229E-S1 staining (Fig. 5b), as well as by the lack of HCoV-229E infection (Fig. 5c). However, the APN gene knockout in Huh7 cells did not prevent infection of different strains of PEDV (Fig. 5c). Moreover, PEDV DR13 did display similar growth kinetics and reached similar titers on Huh7 and Huh7$^{\Delta APN}$ (Fig. 5d). Altogether, these results convincingly demonstrate that APN is not required for PEDV infection of porcine and human cells.

4. Discussion

Over the last 10 years a number of studies has suggested that PEDV – similar to porcine *alphacoronavirus* TGEV – uses porcine APN as a functional host receptor (Li et al., 2007; Li et al., 2009; Oh et al., 2003 Oh et al., 2003). However, pAPN overexpression in otherwise non-susceptible, receptor-negative cells was never found to robustly support virus infection (Li et al., 2007). In addition, African green monkey kidney (Vero) cells, which were historically used for PEDV isolation and propagation, do not express APN as inferred from mass spectrometry analyses of the Vero cell proteome, immunofluorescent staining (Guo et al., 2014; Li et al., 2007; Shirato et al., 2011; Zeng et al., 2015) and RT-PCR analysis (own observation). During our study to assess the role

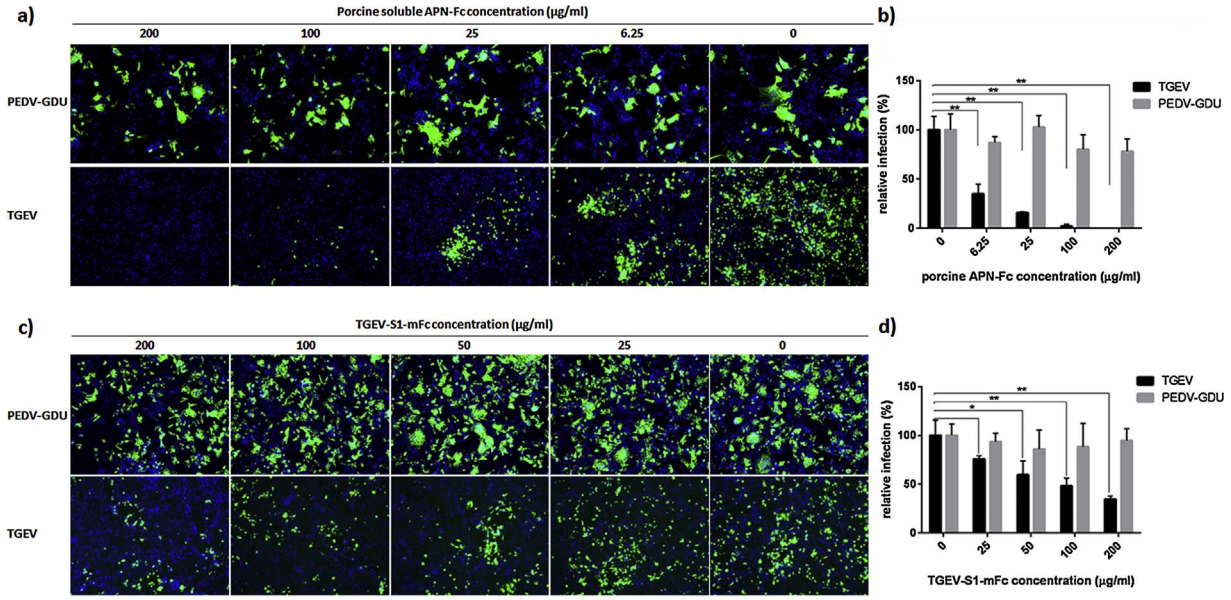

Fig. 3. Antagonizing APN binding does inhibit TGEV, but not PEDV.(a) PEDV (strain GDU) or TGEV virions were preincubated with various concentrations of soluble pAPN-Fc protein, prior to inoculation of ST cells. Cells were fixed at 16 h post infection and PEDV- or TGEV-infected cells were visualized by immunofluorescence staining using anti-TGEV or anti-PEDV antibody (green) and nuclei were stained with DAPI (blue). Experiment has been repeated twice, and representative pictures are shown. (b) Quantification of levels of infected cells (panel a) are shown relative to that for mock-treated viruses (0 μg/ml pAPN-Fc). (c) ST cells were preincubated with various concentrations of APN-binding TGEV-S1-mFc protein, followed by inoculation of ST cells with PEDV or TGEV. Cells were fixed at 16 h post infection and processed for immunofluorescence as described under (a). Experiments have been performed three times with similar results, and representative pictures are shown. (d) Quantification of levels of infected cells (panel c) relative to mock-treated cells (0 μg/ml TGEV-S1-mFc). The error bars represent standard deviations of the mean values. Statistical significance was assessed by unpaired one-tailed Student's test (* = $P < 0.05$, ** = $P < 0.01$).

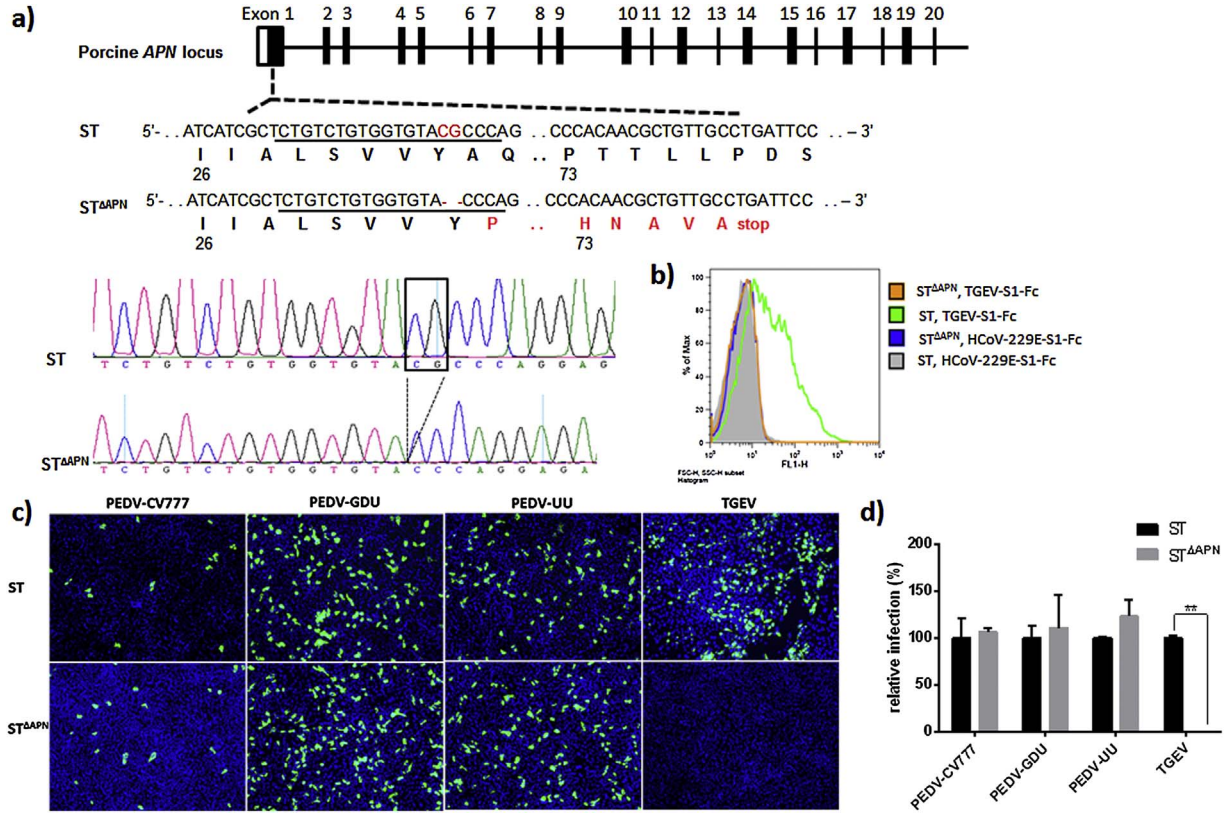

Fig. 4. Knockout of porcine APN expression in ST cells does inhibit TGEV, but not PEDV.(a) APN-expression in PEDV-susceptible porcine ST cells was knocked out by CRISPR/Cas9 genome engineering. Sequencing of the mutant $ST^{\Delta APN}$ cell line shows a 2-nucleotide out-of-frame deletion in exon 1 of the APN gene. The numbers below the protein sequence indicate the amino acid position. (b) Genetic ablation of APN in ST cells results in loss of binding by TGEV-S1-Fc, as shown by flow cytometry. HCoV-229E-S1-Fc that binds human but not porcine APN was taken along as a negative control. (c) Knockout of pAPN expression in ST cells does abrogate infection by TGEV, but not by (various strains of) PEDV. (d) Quantification of relative levels of infected cells on $ST^{\Delta APN}$ compared with the virus infection on ST cells. The error bars represent standard deviations of the mean values. Statistical significance was assessed by unpaired one-tailed Student's test (* = $P < 0.05$, ** = $P < 0.01$).

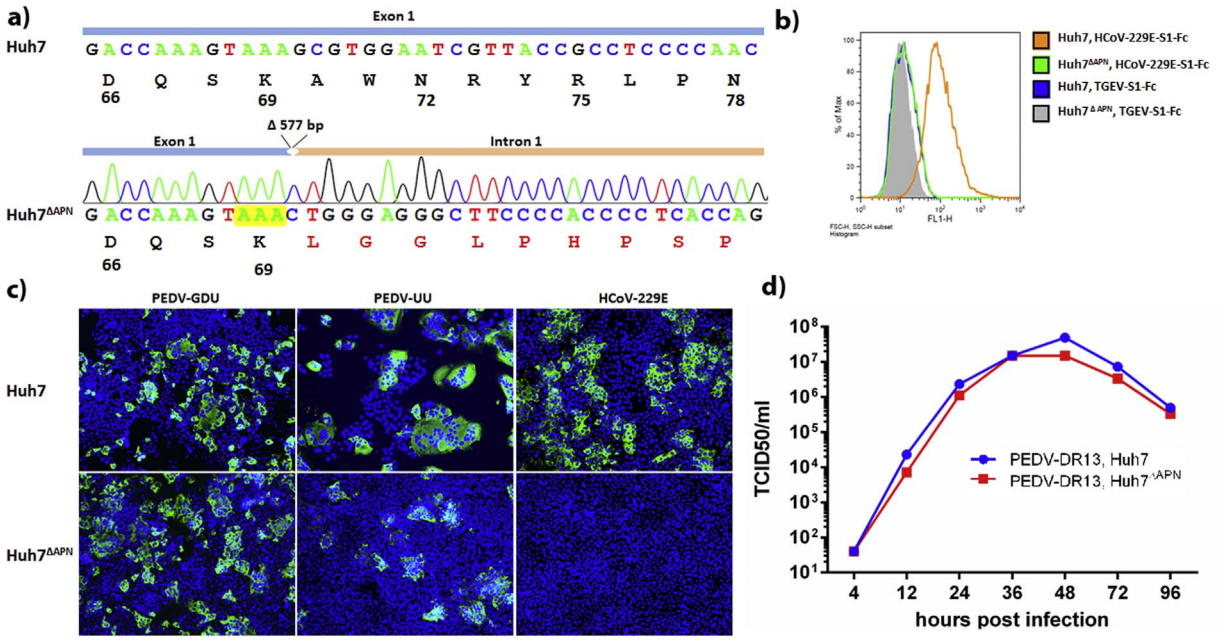

Fig. 5. Knockout of APN gene in PEDV-susceptible human cell line prevents infection by HCoV-229E, but not by PEDV.(a) Generation of APN knockout Huh7 cell line by the CRISPR/Cas9-mediated genome editing system. Sequencing of the APN allele of the Huh7$^{\Delta APN}$ cell line revealed a 577 nucleotide frameshift deletion covering the junction of exon 1 and intron 1. The numbers below the protein sequence indicate the amino acid position. (b) FACS analyses of HCoV-229E-S1-Fc binding to Huh7 and Huh7 $^{\Delta APN}$ cells. TGEV-S1-Fc − incapable of binding to human APN − was used as a negative control. (c) PEDV (strains GDU and UU) and HCoV-229E infection on Huh7 and Huh7$^{\Delta APN}$ cell line. Infected cells were visualized by immunofluorescence staining using anti-PEDV and anti-HCoV-229E antibodies and nuclei were counterstained with DAPI (blue). Infection experiments were repeated twice, and representative images are shown. (d) Growth kinetics of PEDV (strain DR13) on Huh7 and Huh7$^{\Delta APN}$ cells (MOI 0.01). Huh7 and Huh7$^{\Delta APN}$ cells were infected with PEDV DR13 (MOI = 0.01), washed after three hours and viral infectivity in the culture media was determined at different times p.i. by a quantal assay on Vero cells from which TCID50 (50% tissue culture infective dose) values were calculated.

of APN in PEDV entry, we established that overexpression of porcine APN in non-susceptible cells did not confer susceptibility to PEDV. No interaction of PEDV S1 to pAPN was found using biochemical and FACS-based assays. The recently established CRISPR/Cas9 genome editing system was used to study APN function during PEDV entry. It demonstrated that genetic ablation of APN in porcine or human cells susceptible to PEDV did not abrogate PEDV infection. In all these experiments we used multiple PEDV strains to exclude strain-specific artifacts in receptor usage and we exploited TGEV and HCoV-229E as a well-established control for APN receptor usage. From our combined results we therefore conclude that APN is not required as a functional receptor for PEDV entry.

During the completion of our studies a paper was published by Shirato et al. that lead to the same conclusion. It was largely based on similar approaches as ours except for the APN knock out experiments we performed to demonstrate that APN is not essential for PEDV entry. The authors demonstrated that overexpression of pAPN did not render cells susceptible to PEDV and showed that PEDV was unable to bind pAPN and could not be neutralized by treatment with soluble pAPN. These results are similar to our observations, but contrary to that of others (Cong et al., 2015; Deng et al., 2016; Liu et al., 2015; Nam and Lee, 2010). Interestingly, Shirato et al. also showed that overexpression of pAPN in porcine CPK cells facilitated PEDV entry and, moreover, that the enhancement was contributed by an enzymatic activity of APN (Shirato et al., 2016). This observation might explain the earlier reports that overexpression of pAPN in cells exerted positive effects on infection by PEDV (Cong et al., 2015; Liu et al., 2015; Nam and Lee, 2010).

With the exclusion of porcine APN as a functional receptor for PEDV, the actual main virus receptor still awaits its identification. Cell surface carbohydrates including heparan sulphate proteoglycans have been reported as an attachment factor for PEDV (Huan et al., 2015). In addition, sialoglycoconjugates exposed on cell surfaces can function as auxiliary receptors, though their usage varies among PEDV strains (Deng et al., 2016; Li et al., 2016; Liu et al., 2015). Intriguingly, PEDV can infect cells of many species, including those of pigs, monkeys, cats (Truong et al., 2013), ducks (Khatri, 2015), bats and humans (Xu et al., 2016). The broad cell species tropism of PEDV indicates that the virus binds a host cell surface molecule that is evolutionarily conserved among mammalian species.

Conflict of interest

The authors declare that there are no conflicts of interest.

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