Title: Carcinoembryonic Antigen-related Cell Adhesion Molecule 5 (CEACAM5) Is an Important Surface Attachment Factor Facilitating the Entry of the Middle East Respiratory Syndrome Coronavirus (MERS-CoV)

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Running title: CEACAM5 Facilitates MERS-CoV Entry


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Abstract

The spike proteins of coronaviruses are capable of binding to a wide range of cellular targets, which contribute to the broad species tropism of coronaviruses. Previous reports have demonstrated that Middle East respiratory syndrome coronavirus (MERS-CoV) predominantly utilizes dipeptidyl peptidase-4 (DPP4) for cell entry. However, additional cellular binding targets of the MERS-CoV spike protein that may augment MERS-CoV infection have not been further explored. In the current study, using the virus overlay protein binding assay (VOPBA), we identified carcinoembryonic antigen-related cell adhesion molecule 5 (CEACAM5) as a novel cell surface binding target of MERS-CoV. CEACAM5 co-immunoprecipitated with the spike protein of MERS-CoV in both overexpressed and endogenous settings. Disrupting the interaction between CEACAM5 and MERS-CoV spike with anti-CEACAM5 antibody, recombinant CEACAM5 protein, or siRNA knockdown of CEACAM5 significantly inhibited the entry of MERS-CoV. Recombinant expression of CEACAM5 did not render the non-permissive baby hamster kidney (BHK21) cells susceptible to MERS-CoV infection. Instead, CEACAM5 overexpression significantly enhanced the attachment of MERS-CoV to the BHK21 cells. More importantly, the entry of MERS-CoV was increased when CEACAM5 was overexpressed in permissive cells, which suggested that CEACAM5 could facilitate MERS-CoV entry in conjunction with DPP4 despite not being able to support MERS-CoV entry independently. Taken together, our study identified CEACAM5 as a novel cell surface binding target of MERS-CoV that facilitates MERS-CoV infection through augmenting the attachment of the virus to the host cell surface.
Importance

Infection with the Middle East respiratory syndrome coronavirus (MERS-CoV) is associated with the highest mortality rate among all known human-pathogenic coronaviruses. Currently, there are no approved vaccines or therapeutics against MERS-CoV infection. The identification of carcinoembryonic antigen-related cell adhesion molecule 5 (CEACAM5) as a novel cell surface binding target of MERS-CoV advanced our knowledge on the cell binding biology of MERS-CoV. Importantly, CEACAM5 could potentiate the entry of MERS-CoV through functioning as an attachment factor. In this regard, CEACAM5 could serve as a novel target in addition to dipeptidyl peptidase-4 (DPP4) in the development of antiviral strategies for MERS-CoV.
Introduction

Coronaviruses are enveloped, positive-sense, and single stranded RNA viruses with genome size of approximately 30kb. They belong to the Coronaviridae family under the order of Nidovirales and are currently classified into four major genera, including the α, β, γ, and δ genus (1). Coronaviruses can infect a wide range of mammals as well as birds (2). The broad species tropism is predominantly attributed to the high diversity in receptor usage across different coronaviruses. To date, six coronaviruses are known to infect humans and they utilize different surface molecules for cell entry. In particular, human coronavirus 229E (HCoV-229E) binds aminopeptidase N (APN) (3) and human coronavirus OC43 (HCoV-OC43) binds O-acetylated sialic acid (4). Severe acute respiratory syndrome coronavirus (SARS-CoV) (5) and human coronavirus NL63 (HCoV-NL63) (6) both binds angiotensin I converting enzyme 2 (ACE2). The receptor for human coronavirus HKU1 (HCoV-HKU1) has not been defined. However, O-acetylated sialic acid has been suggested as an attachment factor that contributes to the binding of HCoV-HKU1 to the cell surface (7). The Middle East respiratory syndrome coronavirus (MERS-CoV) is the sixth coronavirus known to cause infection in humans (8). Intriguingly, MERS-CoV utilizes a unique cellular receptor, dipeptidyl peptidase-4 (DPP4), for virus entry (9). The host cell receptors for a number of animal coronaviruses have also been identified. For instance, porcine transmissible gastroenteritis coronavirus (TGEV) binds APN (10) and the prototype β-coronavirus mouse hepatitis virus (MHV) uses carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) for entry (11).
Coronaviruses have evolved complex receptor recognition patterns. In addition to the defined receptors essential for virus entry into host cells, multiple co-receptors or attachments factors have been reported to play critical roles in the propagation of coronaviruses. In this regard, sialic acids (12) and glycoproteins (13) facilitate the binding of TGEV to target cells in addition to APN. Similarly, HCoV-NL63 utilizes heparan sulfate proteoglycans for attachment to target cells (14). Apart from ACE2, SARS-CoV can also enter cells through liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin (L-SIGN) (15) and dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) (16). Additionally, we have previously identified major histocompatibility complex class I C (HLA-C) as an attachment factor for HCoV-HKU1 that facilitates the entry of the virus (17).

The emerging MERS-CoV is associated with the highest mortality rate of more than 30% among all known human-pathogenic coronaviruses in inflicted patients and there is as yet no approved treatment regimens or vaccine for MERS (18, 19). As of May 16th 2016, MERS-CoV has caused 1733 laboratory-confirmed cases of human infection, including at least 628 deaths (20). Clinical features of severe MERS include high fever, pneumonia, acute respiratory distress syndrome (ARDS), as well as extrapulmonary manifestations including gastrointestinal symptoms, lymphopenia, acute kidney injury, hepatic inflammation, and pericarditis (21). In agreement with these clinical observations, recent in vitro and in vivo studies have highlighted the extraordinarily wide range of tissue and cell tropism of MERS-CoV, which is unparalleled by other coronaviruses (22-24). Following the identification of DPP4 as the receptor of MERS-CoV, the broad tissue
tropism of MERS-CoV infection is in part explained by the ubiquitous cellular expression of DPP4. However, alternative factors may exist and potentiate the infection of MERS-CoV either in conjunction or independently of DPP4. In this study, we employed the virus overlay protein binding assay (VOPBA) followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) approach to identify novel cell surface binding targets of MERS-CoV. Our data demonstrated carcinoembryonic antigen-related cell adhesion molecule 5 (CEACAM5) as a novel attachment factor that facilitated MERS-CoV entry. Importantly, interrupting the interaction between CEACAM5 and MERS-CoV-spike inhibited virus entry. Overexpression of CEACAM5 did not grant entry but significantly increased the attachment of MERS-CoV on non-permissive cells. Collectively, our study identified CEACAM5 as an important cell surface binding target for MERS-CoV-spike that facilitates host cell entry for MERS-CoV.

**Materials and Methods**

**Cells.** A549, AD293, Huh7, Caco2, and VeroE6 cells were maintained in Dulbecco’s Modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 unit/ml penicillin and 100 µg/ml streptomycin. BEAS2B and Calu3 cells were maintained in DMEM/F12 supplemented with 10% heat-inactivated FBS, 100 unit/ml penicillin and 100 µg/ml streptomycin. BHK21 cells were maintained in Minimum Essential medium (MEM) supplemented with 10% heat-inactivated FBS, 100 unit/ml penicillin and 100 µg/ml streptomycin. Human primary T cells were isolated from PBMCs with negative selection.
using the Dynabeads Untouched Human T cells Kit (Invitrogen, USA) as we previously described (25). Isolated T cells were maintained in Roswell Park Memorial Institute medium (RPMI-1640) supplemented with 10% FBS, 100 µg/ml streptomycin, 100 U/ml penicillin, 1% sodium pyruvate, 1% non-essential amino acids, and were used immediately for infection.

Virus. The EMC/2012 strain of MERS-CoV (passage 8, designated MERS-CoV) was provided by Dr. Ron Fouchier (Erasmus Medical Center) and cultured with VeroE6 cells with serum free DMEM supplemented with 100 unit/ml penicillin and 100 µg/ml streptomycin. Three days post virus inoculation, culture supernatants were collected, aliquoted, and stored at -80°C. To determine virus titer, aliquots of MERS-CoV were used for plaque assays on confluent VeroE6 cells in 24-well plates. In brief, MERS-CoV stocks were 10-fold serially diluted in DMEM. Diluted MERS-CoV was then added to duplicate wells of 24-well plates at a volume of 200µl. After inoculating for 1 hour at 37°C, the inoculum was removed from the wells and an agarose overlay was added to the cells. The cells would then be incubated for approximately 72 hours. To count plaques, the cells were fixed with 4% paraformaldehyde for 5 hours and stained with crystal violet.

Antibodies. Rabbit anti-human DPP4, rabbit anti-human CEACAM5, rabbit control IgG (Abcam, USA), and mouse anti-human β-actin (Immunoway, USA) were used in the relevant experiments. MERS-CoV NP was detected with the guinea pig anti-MERS-CoV NP serum as we previously described (25). MERS-CoV spike was detected either with
the in-house mouse anti-MERS-CoV spike immune serum or with a rabbit anti-MERS-CoV spike antibody (Sino Biological, China). Secondary antibodies including Alexa Fluor 488/647 goat anti-guinea pig and Alexa Fluor 488/647 goat anti-rabbit (Life Technologies, USA) were used for flow cytometry. Goat anti-mouse HRP (Abcam, USA) was used for Western blot.

**Plasmid construction.** The synthetic human codon-optimized S gene was used as a template for the construction of all MERS-CoV-S plasmids. For pcDNA-MERS-CoV-S used in producing MERS-S-pseudotyped virus, the full-length S from the N-terminal KpnI site to the C-terminal XhoI site was subcloned to generate full-length S in pcDNA 3.1(+) For the construction of the S1 plasmid (amino acids 1-925), the 5’ forward primer sequence containing a BSSHII site (5’GCGCGCCACCATGATACACTCAGTGTTTCTACTGATGTTC) was used together with the 3’ reverse primer (5’-GGGCCCATCACCGTCTTCCCACACAGTGGATG) with a Apal site. The fragment was PCR amplified and cloned into the pSFV1 vector (kindly provided by Dr. P. Liljestrom) with the C terminus fused in-frame with the FLAG sequence (DYKDDDDK) and resulting in the plasmid pSFV-MERS-CoV-S1-FLAG. For the construction of the pSFV-DPP4-6xHis plasmid, forward primer containing a BSSHII site (5’-GCGCGCCACCATGAAGACACCGTGGAAGGTTCT) and reverse primer with a Apal site (5’-GGGCCAGGTAAAGAAACATTGTTTTATG) were used. The fragment was cloned into the pSFV1 vector with the 6x His sequence (HHHHHH) fused in-frame at the C-terminus. For the construction of the pcDNA-DPP4, the forward primer containing a KpnI site (5’-TAAGCAGGTACCAGCCACCATGAAGACACCGTGGAAGGTCT) and
the reverse primer containing a EcoRI site (5’-TGCTTAGAATTCTTAAGGTAAAGAAACATTGTTTTATGAAGTG) were used to insert the full-length human DPP4 into the pcDNA3.1(+) vector between the KpnI and the EcoRI sites. For the construction of pcDNA-CEACAM5-V5, the forward primer (5’GATATCCACCATGGAGTCTCCCTCGGCCCCTCCCCAC’) contains the N-terminal signal sequence, the EcoRV site, and the Kozak sequence. The reverse-primer (5’-CTCGAGTATCAGAGCAACCCCAACCAGCCTCC) contains the XhoI site at the C-terminal cytoplasmic domain. The amplified fragment was used to generate full length CEACAM5 in pcDNA 3.1(+) with a V5 tag (GKPIPPLLGLDST) in-frame at the C-terminus, resulting in pcDNA-CEACAM5-V5.

Production of MERS-S-pseudotyped virus. Lentivirus-based MERS-S-pseudotyped virus was generated by co-transfecting 293FT cells with pcDNA-MERS-full length spike in combination with pNL4-3-R’.E’, which is a HIV backbone plasmid bearing the luciferase reporter gene. pNL4-3-R’.E’ was obtained through the NIH AIDS research and reference reagents program. The viral particles in supernatant were harvested at 48 hours post transfection by ultra-centrifugation in 30% sucrose solution in Beckman rotor SW32Ti at the speed of 32,000 rpm for 1 hour at 4°C (17). The p24 concentrations were quantified with a p24 antigen enzyme-linked immunosassay kit (Cell Biolabs, USA) and stored in aliquots at -80°C. Pseudovirus titer was quantified in unit of lentiviral particle (LP) per ml according to the manufacturer’s instruction.
Virus Overlay Protein Binding Assay (VOPBA) and Western Blot. Confluent A549 cells in 75 cm² tissue flask were washed three times in chilled PBS, surface-biotinylated, and extracted using Pierce Cell Surface Protein Isolation Kit (Thermo Scientific, USA) according to the manufacturer’s protocol. Biotinylated membrane extracts were bound onto neutrAvidin agarose resin (Thermo Scientific, USA), washed, and eluted in LDS sample buffer (Invitrogen, USA). After electrophoresis into 4-12% gradient NuPAGE SDS-PAGE Gel (Invitrogen, USA) under reducing condition, biotinylated surface extracts were electro-transferred onto Hybond-PVDF membranes (GE Healthcare, UK) for 2 hours at constant voltage of 30 V at 4°C. Membrane was blocked in 10% skim milk (Oxoid, UK) in PBS at 4°C overnight on a rotator. The membrane was then incubated with 1% skim milk solution containing MERS-S-pseudotyped virus at 10⁹ lentiviral particles/ml for 2 hours. Membrane was washed twice in 10% skim milk, once in PBS and incubated with rabbit antiserum raised against MERS-CoV spike protein at 1 μg/ml for 2 hours at room temperature. After incubating with the spike-specific antibody, we washed the membrane twice in 10% skim milk, once in PBS, and incubated with PBS containing 1:5000 goat anti-rabbit-800 infra-red dye (LI-COR, USA). Membranes were scanned with an Odyssey Imaging System (LI-COR, USA). The visualized reactive protein detected by VOPBA on Western blot was excised from the gel. The gel piece was inserted into dialysis tubing and filled with 1X MOPS SDS running buffer. Protein contents were electro-eluted at a constant voltage of 50V for 6 hours, collected, dialyzed overnight at 4°C against PBS in Slide-A-Lyzer Cassette (Thermo Scientific, USA) and concentrated using Microcon columns (Millipore, USA). Eluted contents were electrophoresed and confirmed by VOPBA. The gel fragment was excised for LC-
MS/MS analysis carried out in the Center for Genomic Sciences at the University of Hong Kong as we previously described (26).

**Co-immunoprecipitation (co-IP).** Co-IP was performed using anti-FLAG M2 affinity gel beads (Sigma, USA) or V5 antibody pre-adsorbed to Protein A+G sepharose (Thermo Scientific, USA). Cell lysates were harvested in lysis buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 0.1% NP-40, protease and phosphatase inhibitors cocktails, Roche). Lysed cells were incubated on ice for 15-30 min and then centrifuged. Supernatant was added to antibody-coupled beads or anti-FLAG affinity gel beads and incubated overnight at 4ºC. Beads were washed three times with lysis buffer and Western blot was performed. Immunoprecipitated products or cell lysates were mixed with SDS loading buffer and heated at 95ºC for 10 min. Samples were fractionated in 10% SDS-PAGE and then transferred onto PVDF membranes (GE Healthcare, UK). After blocking overnight with 10% skim milk, membranes were incubated with corresponding primary antibodies and then with infra-red dye conjugated secondary antibodies (LI-COR, USA). Membranes were scanned with an Odyssey Imaging System (LI-COR, USA).

**Quantitative RT-PCR.** Cells were lysed in RLT buffer with 40 mM DTT and extracted with the RNeasy mini kit (Qiagen, USA). Viral RNA in the supernatant was extracted with the PureLink Viral RNA/DNA mini kit (Life Technologies, USA). Reverse transcription (RT) and quantitative polymerase chain reaction (qPCR) were performed with the Transcriptor First Strand cDNA Synthesis kit and LightCycler 480 master mix from Roche as we previously described (27, 28). In the RT reactions, reverse primers
against the NP gene of MERS-CoV were used to detect cDNA complementary to the positive strand of viral genomes. The following sets of primers were used to detect NP in qPCR.

(F) 5'- CAAAACCTTCCCTAAGAAGGAAAAG-3'
(R) 5'- GCTCCTTTGGAGGTTCAGACAT-3'
(Probe) FAM 5'-ACAAAAGGCACCAAAAGAAGAATCAACAGACC-3' BHQ1

Antibody blocking assays. The antibody blocking assay based on the MERS-S-pseudotyped virus was performed by measuring the infection of target cells with the use of luciferase as a reporter gene (17). In brief, antibody was diluted to 2µg/ml in serum free culture medium and incubated with Calu3 cells for 1 hour at 37°C. Pseudotyped virus was added to target cells at a ratio of 100 lentiviral particle (LP) per cell and the inoculum was replaced with fresh medium with 10% FBS after 1 hour. Luciferase activity was determined at 48 hours post infection. For the antibody blocking assay based on the infectious MERS-CoV, Calu3 cells were pre-incubated with rabbit polyclonal anti-CEACAM5 at different concentrations ranging from 0.5-4 µg/ml. Rabbit polyclonal anti-DPP4 at 4 µg/ml and rabbit IgG at 4µg/ml were included as controls. After pre-incubating with the antibodies for 1 hour at 37°C, the cells were challenged with MERS-CoV at 1 MOI for 1 hour at 37°C in the presence of antibodies. The cells were subsequently washed with PBS and lysed with RLT with 40mM dithiothreitol (DTT). To determine the impact of antibody blocking on MERS-CoV multi-cycle replication, Huh7 cells were pre-incubated with 2 µg/ml anti-CEACAM5 antibody, anti-DPP4 antibody, or rabbit IgG. After the pre-incubation, the cells were infected with MERS-CoV at 0.0005 MOI for 1
hour at 37°C in the presence of antibodies. The cells were then washed in PBS and replenished with DMEM with blocking antibodies. Infected cells were incubated at 37°C and harvested at 1, 24, and 48 hours post infection. The virus copy number was determined with qPCR.

**Human recombinant protein blocking assays.** Human recombinant proteins (DPP4, CEACAM5, and control IgG) were obtained from Sino Biological. MERS-CoV was pre-incubated with human recombinant proteins (5 µg/ml to 40 µg/ml for CEACAM5, 40 µg/ml for DPP4, and 40 µg/ml for control IgG) for 1 hour before inoculating onto Calu3 and Huh7 cells. The protein-virus complexes were then incubated with Calu3 and Huh7 cells for 2 hours at 37°C. After the incubation period, the inoculum was discarded and the cells were washed with PBS followed by lysing in RLT with 40mM DTT. The virus copy number was determined with qPCR.

**siRNA knockdown.** Silencer Select siRNA human CEACAM5 siRNA, Silencer Select siRNA human DPP4 siRNA, and Silencer Select siRNA negative control were obtained from Life Technologies. Transfection of siRNA on Huh7 cells was performed using Lipofectamine 3000 (Thermo Fisher, USA) following manufacturer’s manual. In brief, Huh7 cells were transfected with 100µM siRNA for two consecutive days. At 24 hours post the second siRNA transfection, the cells were challenged with MERS-CoV at 1 MOI for 2 hours at 37°C. Following the incubation, the cells were washed with PBS and lysed in RLT with 40mM DTT. The virus copy number was determined with qPCR.
Flow cytometry. All samples were detached with 10mM EDTA and fixed in 4% paraformaldehyde. Cell permeabilization for intracellular staining was performed with 0.1% Triton X-100 in PBS. Immunostaining for flow cytometry was performed following standard procedures as we previously described (29). The flow cytometry was performed using a BD FACSCanto II flow cytometer (BD Biosciences, USA) and data was analyzed using FlowJo vX (Tree Star, USA).

Flow cytometry of BHK21 cells with overexpression of DPP4 and CEACAM5.

BHK21 cells were transfected with pSFV-DPP4 or pcDNA-CEACAM5. The transfected cells were inoculated with MERS-CoV at 48 hours post transfection. To determine virus entry, the cells were inoculated with MERS-CoV at 5 MOI at 37°C for 2 hours. After 2 hours, the cells were washed with PBS and incubated for another 4 hours. At 6 hours post-infection, the cells were washed extensively with PBS, fixed in 4% paraformaldehyde, and immunolabeled for flow cytometry. To determine virus attachment, the cells were inoculated with MERS-CoV at 30 MOI at 4°C for 2 hours. After 2 hours, the cells were washed with PBS, fixed in 4% paraformaldehyde, and immunolabeled for flow cytometry.

Confocal microscopy. This study was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster. Normal human lung sections were deparaffinized and rehydrated following standard procedures. Antigen unmasking was performed by boiling tissue sections with the antigen unmasking solution from Vector Laboratories. Goat anti-DPP4 was obtained from R&D and rabbit anti-
CEACAM5 was obtained from Abcam. Secondary antibodies were obtained from Life Technologies. Mounting and DAPI staining were performed with the Vectashield mounting medium (Vector Laboratories). Images were acquired with a Carl Zeiss LSM 780 system.

**Statistical analysis.** Data on figures represented means and standard deviations. Statistical comparison between different groups was performed by Student’s *t*-test using GraphPad Prism 6. Differences were considered statistically significant when *p* < 0.05.

**Results**

**Identification of CEACAM5 as a cell surface binding protein of MERS-CoV**

To probe for potential cell surface factors that can interact with MERS-CoV, we employed the virus overlay protein binding assay (VOPBA), which we have previously utilized to identify host cell surface binding proteins of the influenza A virus (26). In brief, membrane proteins on A549 cells were selectively biotinylated and separated from non-biotinylated intracellular proteins by binding to avidin agarose (23). The biotinylated A549 surface extracts separated in 4-12% gradient SDS NuPAGE gel were transferred to PVDF membrane before incubating with MERS-S-pseudotyped viruses for 2 hours at room temperature (Figure 1A). Subsequently, reactive signals were revealed with a rabbit polyclonal antibody against MERS-CoV spike. As shown in figure 1B, VOPBA revealed several reactive bands from A549 surface extracts including two prominent bands at molecular sizes of approximately 120 and 90 kDa (lane 1) but not from the control NIH3T3 cell (lane 2), which is not permissive for MERS-CoV infection (30). In order to
confirm the immunoreactivity of these two proteins to MERS-S-pseudotyped virus, the relevant fractions from the SDS-PAGE gel was excised, electro-eluted, and hybridized with MERS-S-pseudotyped virus. Although both proteins reacted with the virus, the protein at around 90 kDa returned a much stronger signal and was selected for subsequent analyses (Figure 1C). Tryptic peptides mass fingerprints derived from the excised band revealed the protein to be human carcinoembryonic antigen-related cell adhesion molecule 5 (CEACAM5) using the MASCOT search engine against NCBI and Swiss-Prot database (Figure 1D).

**CEACAM5 is expressed on cell types that are highly susceptible to MERS-CoV**

Most CEACAMs are considered as modulators of general cellular processes including proliferation, motility, apoptosis, attachment, as well as cell-cell interaction. In addition, CEACAM5 may also play roles in innate immune defense by binding and trapping microorganisms (31). To verify the relevance of CEACAM5 in MERS-CoV entry, we investigated the expression of CEACAM5 on a number of cell types. To this end, different cell types in 6-well plates were detached with EDTA, washed, and fixed in 4% paraformaldehyde. Immunostaining for surface CEACAM5 and DPP4 expression were performed without cell permeabilization. The percentage of positive cells and the surface mean fluorescent intensities (MFIs) were determined with flow cytometry. In agreement with previously published reports, DPP4 was detected ubiquitously on different human cell lines (Figure 2A) as well as human primary T cells (Figure 2B). In addition, the polyclonal antibody against human DPP4 also cross-reacted with the DPP4 on a number of non-human mammalian cells, which included VeroE6 (monkey), BHK21 (hamster),
and L929 (mouse) cells (Figure 2C). Notably, despite being recognized by the human
DPP4 antibody, the DPP4 on hamster and mouse cells do not support MERS-CoV entry
(30). In contrast to DPP4, our data demonstrated that the expression of CEACAM5 was
more restricted. Among the six human cell lines we analyzed, CEACAM5 expression
was detected on A549, Calu3, and Huh7 cells (Figure 2A). At the same time, little to no
CEACAM5 expression was detected on AD293, BEAS2B, and Caco2 cells (Figure 2A).
Importantly, CEACAM5 expression was also detected on human primary T cells (Figure
2B). However, no human CEACAM5 ortholog was expressed on VeroE6, BHK21, and
L929 cells (Figure 2C). Quantitatively, CEACAM5 expression was detected
predominantly on cells that are highly susceptible to MERS-CoV infection, which
include Calu3, Huh7, A549, and T cells (Figure 2D and Figure 2E). Thus, our data
supported the notion that CEACAM5 might be an important surface binding protein,
which could facilitate MERS-CoV entry.

CEACAM5 is co-expressed with DPP4 in human lung tissues
To further verify the physiological relevance of CEACAM5 in MERS-CoV infection, we
evaluated the expression of CEACAM5 in human lung tissues and compared the
expression with that of DPP4 by immunofluorescent microscopy. Our result revealed that
CEACAM5 could be readily detected in the epithelial cells in various regions of the
human lung tissues (Figure 3). In particular, colocalization between CEACAM5 and
DPP4 was observed in the bronchial epithelium (Figure 3A) but appeared to be more
extensive in the epithelium of small airways (Figure 3B). The colocalization between
CEACAM5 and DPP4 was also detected in alveoli (Figure 3C) including the alveolar
macrophages (Figure 3C, arrowheads). Collectively, the detection of CEACAM5 in the epithelial cells of the human lung tissues and its co-expression with DPP4 in these samples demonstrated the potential capacity of CEACAM5 in facilitating MERS-CoV infection in the lower respiratory tract.

**CEACAM5 interacts with MERS-CoV spike**

To validate our VOPBA-LC-MS/MS findings, the direct interaction between MERS-CoV S1 and CEACAM5 was characterized by co-immunoprecipitation (co-IP). BHK21 cells were either transfected with a CEACAM5-V5 expression vector or a control vector tagged with V5. The surface and intracellular expression of transfected CEACAM-V5 was verified with flow cytometry (Figure 4A). Transfected BHK21 cells were then immunoprecipitated with either MERS-CoV S1-FLAG or E. coli bacterial alkaline phosphatase-FLAG (BAP-FLAG) pre-adsorbed onto anti-FLAG M2 agarose beads. The bound protein complexes were spun down, washed, and subjected to Western blot detection with the anti-FLAG (Figure 4B, upper panel) or the anti-V5 antibody (Figure 4B, lower panel). As demonstrated in figure 4B, CEACAM5 bound specifically to MERS-CoV S1 (lower panel, lane 1). In contrast, CEACAM5 did not bind to the control bait protein (lower panel, lane 2). At the same time, CEACAM5 was not detected in samples transfected with the empty vector (lower panel, lane 3). In parallel, we performed reciprocal co-IP experiments, in which CEACAM5-V5 was used as the bait instead of MERS-CoV S1-FLAG (Figure 4C). To this end, CEACAM5-V5 or empty vector transfected BHK21 cell lysates were immunoprecipitated with anti-V5 pre-adsorbed protein A/G sepharose, spun down, washed, and incubated with purified
MERS-CoV S1-FLAG or BAP-FLAG. Western blot detection with anti-FLAG showed that CEACAM5-V5 interacted with MERS-CoV S1-FLAG (Figure 4C, upper panel, lane 1) but not BAP-FLAG (Figure 4C, upper panel, lane 2). As a negative control, the V5 empty vector did not pull down MERS-CoV S1 (Figure 4C, upper panel, lane 3). In line with the flow cytometry data from Figure 2C, the antibody against human CEACAM5 did not pick up any signal from the BHK21 cell lysates (Figure 4C, lower panel, lane 3).

To further validate our findings and avoid the use of overexpressed or tagged fusion proteins, we performed endogenous co-IP on non-transfected Huh7 cells infected with MERS-CoV using antibody against CEACAM5 or MERS-CoV spike. As demonstrated in figure 4D, MERS-CoV spike was detected in infected samples immunoprecipitated with the anti-CEACAM5 antibody. In contrast, MERS-CoV spike was not detected from uninfected samples or from infected samples immunoprecipitated with non-specific rabbit IgG. The interaction between MERS-CoV spike and CEACAM5 was confirmed with reciprocal co-IP using antibody against MERS-CoV spike. In this regard, endogenous CEACAM5 was detected from infected samples immunoprecipitated with antibody against MERS-CoV spike but not from uninfected samples or from infected samples immunoprecipitated with non-specific rabbit IgG (Figure 4D). Take together, our co-IP results demonstrated that MERS-CoV S1 could bind specifically to CEACAM5.

CEACAM5-specific antibody blocks MERS-CoV entry and spread

After confirming the direct interaction between CEACAM5 and MERS-CoV S1, we next investigated the potential functional role of CEACAM5 in MERS-CoV entry. We first assessed the capacity of CEACAM5 antibody in blocking the entry of MERS-S-
pseudovirus. In this setting, Calu3 cells were pre-incubated with polyclonal antibody against human CEACAM5, or with rabbit IgG and polyclonal antibody against DPP4 as negative and positive controls, respectively. After the pre-incubation, MERS-S-pseudoviruses were inoculated on to the cells for another hour in the presence of the CEACAM5 antibody. Inoculum was replaced with fresh medium with 10% FBS after 1 hour and the luciferase activity was determined at 48 hours post infection. As demonstrated in figure 5A, polyclonal antibody against human CEACAM5 at 2 μg/ml significantly reduced the entry of MERS-S-pseudovirus. Next, the antibody blocking experiment was further validated using infectious MERS-CoV under a titration of different CEACAM5 antibody concentrations ranging from 0.5 μg/ml to 4 μg/ml. Our result demonstrated that CEACAM5 antibody blocked the infection of MERS-CoV in a dose-dependent manner (Figure 5B). As a control, antibody added after MERS-CoV inoculation did not affect virus entry (Figure 5C). At the same time, addition of the CEACAM5 antibody did not prevent the entry of MERS-CoV in Caco2 cells, which expressed a low level of CEACAM5 (Figure 5D). To determine the impact of CEACAM5 inhibition on multi-cycle spread of MERS-CoV, we extended the antibody blocking experiment to Huh7 cells. In this scenario, Huh7 cells were pre-incubated with the CEACAM5 antibody for 1 hour and inoculated with MERS-CoV at low MOI in the presence of the CEACAM5 antibody. Infected cells were then washed and incubated in DMEM culture media in the presence of the CEACAM5 antibody. Remarkably, our data suggested that the CEACAM5 antibody significantly reduced MERS-CoV propagation in both cell lysates (Figure 5E) and supernatants (Figure 5F) of infected Huh7 cells. Overall,
we demonstrated that CEACAM5 was important for MERS-CoV entry as well as in multi-cycle viral spread.

**CEACAM5 recombinant protein and siRNA knockdown of CEACAM5 inhibits MERS-CoV entry**

In addition to antibody blocking, we sought to further verify the impact of CEACAM5 inhibition on MERS-CoV infection with alternative approaches including protein blocking and siRNA knockdown. In protein blocking assays, MERS-CoV was pre-incubated with human recombinant CEACAM5 protein ranging from 5 μg/ml to 40 μg/ml. Human recombinant DPP4 protein and human recombinant IgG were included as controls. Calu3 or Huh7 cells were subsequently inoculated for 2 hours with the virus-protein mixes. After inoculation, the cells were washed and harvested for viral load quantification with qPCR analysis. Remarkably, our data demonstrated that the human CEACAM5 recombinant protein blocked the entry of MERS-CoV in a dose-dependent manner in both Calu3 (Figure 6A) and Huh7 cells (Figure 6B). As a control, CEACAM5 protein added after MERS-CoV inoculation had no effect on virus entry (Figure 6C).

Next, we depleted the endogenous expression of CEACAM5 in Huh7 cells with siRNA. The reduction of CEACAM5 surface expression was examined with flow cytometry with no cell permeabilization (Figure 6D). As illustrated in figure 6E and figure 6F, siRNA treatment significantly diminished the surface expression of CEACAM5 both in terms of the percentage of CEACAM5 positive cell (Figure 6E) as well as the mean fluorescent intensity (MFI) (Figure 6F). Notably, when the CEACAM5 siRNA-treated Huh7 cells were challenged with MERS-CoV, we detected a significant decrease in MERS-CoV...
entry (Figure 6G). Overall, our data from protein blocking and siRNA knockdown experiments corroborated with the findings of the antibody blocking assays, which supported CEACAM5 to be an important cell surface protein in MERS-CoV infection.

CEACAM5 is an attachment factor for MERS-CoV

To further define the role of CEACAM5 in MERS-CoV infection, we examined the capacity of CEACAM5 in supporting MERS-CoV entry and attachment. To assess the role of CEACAM5 in MERS-CoV entry, CEACAM5-overexpressing BHK21 cells were inoculated with MERS-CoV at 37°C for 2 hours. The cells were then washed and incubated at 37°C for 4 additional hours before harvesting for flow cytometry. Next, to assess the role of CEACAM5 in MERS-CoV attachment, CEACAM5-overexpressing BHK21 cells were inoculated with high MOI MERS-CoV at 4°C for 2 hours. The infected cells were subsequently washed, harvested, and immunolabeled for flow cytometry. In both cases, DPP4-overexpressing BHK21 cells were included as controls.

The result of the entry assessment recapitulated the finding that human DPP4 overexpression could support MERS-CoV entry in the otherwise non-permissive BHK21 cells. In stark contrast, human CEACAM5-expressing BHK21 cells failed to sustain the entry of MERS-CoV (Figure 7A and Figure 7B). In the attachment assessment, our data suggested that while MERS-CoV could attach to the cell surface of BHK21 cells, human DPP4 overexpression increased the amount of attached virus particles by around 3-folds. Intriguingly, in human CEACAM5-expressing BHK21 cells, the amount of attached virus particles was also significantly increased by more than 2-folds (Figure 7C and Figure 7D). To further verify the potential contribution of CEACAM5 in MERS-CoV entry, we
overexpressed CEACAM5 in BHK21 and AD293 cells followed by MERS-CoV infection. In corroboration of the result from figure 7A, our data showed that while

CEACAM5 enhanced virus attachment to BHK21 cells (Figure 8B), it could not function as an independent receptor for MERS-CoV entry in the non-permissive BHK21 cells (Figure 8A). Perhaps most importantly, overexpression of CEACAM5 increased the entry of MERS-CoV in permissive cells (Figure 8C) in addition to enhancing the attachment of the virus (Figure 8D), which suggested that CEACAM5 could contribute to MERS-CoV entry in conjunction with DPP4. To further investigate if CEACAM5 could facilitate the entry of other human coronaviruses, we challenged CEACAM5-transfected VeroE6 cells (Figure 9A) with SARS-S-pseudovirus and compared pseudovirus entry with that from the empty vector-transfected VeroE6 cells. Our result demonstrated that CEACAM5 overexpression did not significantly enhance the entry of SARS-S-pseudovirus (Figure 9B). Taken together, our data identified CEACAM5 as an important cell surface binding protein for MERS-CoV that could facilitate the entry of MERS-CoV by acting as an

attachment factor.

Discussion

The spike proteins of coronaviruses are known to bind a broad range of cellular targets including sialic acids, sugars, and proteins (32). It is now clear that MERS-CoV predominantly utilizes DPP4 for cell entry (9). However, additional cellular binding targets of the MERS-CoV spike protein that may augment MERS-CoV entry have not been further explored. In the current study, using the VOPBA-LC-MS/MS approach, we unveiled CEACAM5 as a novel cell surface binding protein of MERS-CoV (Figure 1).
Intriguingly, expression analysis revealed that CEACAM5 were expressed on cell types that are highly permissive to MERS-CoV infection, including Calu3, Huh7, A549, and primary T cells (Figure 2). CEACAM5 and DPP4 co-expressed in the human lung, which highlighted the physiologically relevance of CEACAM5 in MERS-CoV entry (Figure 3). Subsequently, a series of co-IP studies demonstrated the capacity of CEACAM5 in binding to the spike protein of MERS-CoV either in overexpressed or endogenous settings (Figure 4). Remarkably, when the interaction between CEACAM5 and MERS-CoV spike was perturbed with anti-CEACAM5 antibody (Figure 5), recombinant CEACAM5 protein (Figure 6), or CEACAM5 siRNA knockdown (Figure 6), the infection of MERS-CoV was significantly inhibited. Notably, recombinant expression of CEACAM5 did not confer susceptibility of non-permissive BHK21 cells to infection by MERS-CoV (Figure 7A). Instead, CEACAM5 overexpression significantly enhanced the attachment of MERS-CoV to the BHK21 cells (Figure 7B). Perhaps most importantly, the entry of MERS-CoV was increased when CEACAM5 was overexpressed in AD293, which suggested that CEACAM5 could facilitate MERS-CoV entry in conjunction with DPP4 despite being unable to support MERS-CoV entry independently (Figure 8). The interaction between CEACAM5 and MERS-CoV appeared to be specific since CEACAM5 did not facilitate the entry of SARS-CoV (Figure 9). Taken together, our study identified CEACAM5 as a novel cell surface binding protein of MERS-CoV that facilitates MERS-CoV infection through augmenting the attachment of the virus to the cell surface.
CEACAM family members including CEACAM5 are heavily glycosylated cell surface proteins (33, 34). Currently, 12 members of the CEACAM family have been identified in human. Most CEACAMs are considered as modulators of general cellular processes including proliferation, motility, apoptosis, attachment, as well as cell-cell interaction (35). Notably, aberrant CEACAM function is associated with tumor progression and a number of CEACAMs are utilized as clinical biomarkers. In addition, members of the CEACAM family have been implicated in signal transduction and the binding of bacteria to host target cells. In particular, certain CEACAMs including CEACAM5 are expressed on the apical membrane of epithelial cells and are exploited as receptors or binding factors for a number of pathogenic bacteria (35). Engaging surface CEACAMs by these bacteria not only provides a portal of attachment but also triggers internalization and entry of the bacteria into epithelial cells.

In the context of coronaviruses, previous reports have identified murine CEACAM1 as the principal cellular receptor of the mouse hepatitis virus (MHV) (11). The discovery of CEACAM5 as a cellular binding target of MERS-CoV spike suggested that perhaps cell surface CEACAMs are common binding targets of coronavirus spikes that can be exploited for attachment or entry. It will be tempting to speculate that other members of the coronavirus family can also make use of certain CEACAMs to facilitate infection. Notably, the spike proteins of coronaviruses are capable of binding to multiple cellular targets. As an example, SARS-CoV binds to ACE2 with the spike S1 C-terminal domain (CTD) (36). At the same time, the SARS-CoV spike can also bind DC-SIGN (16), DC-SIGNR (16), and L-SIGN (15). Similarly, TGEV binds to APN with the spike S1 CTD...
but the virus is also capable of binding to cell surface sugars and sialic acids using the N-terminal domain (NTD) of S1 (37). Since MHV binds CEACAM1 at the first 330 amino acids of the S1 NTD (38), one may postulate that MERS-CoV spike may similarly bind CEACAM5 with the NTD of S1. In this regard, the CEACAM5-S1 NTD interaction may orchestrate with the DPP4-S1 CTD interaction (36, 39) and facilitates the infection of MERS-CoV. Importantly, the epitopes from the receptor-binding domain of MERS-CoV S1 are known to trigger the production of potent neutralization antibodies. It is potentially feasible to include additional epitopes derived from the CEACAM5-binding domain in future vaccine designs, which may further enhance the efficiency of the produced neutralizing antibodies at the same time avoiding potential immunopathological effects as observed from using the full length spike protein (40).

By definition, a receptor is a cellular factor essential for viral entry and bound by the viral glycoproteins whereas an attachment factor is a cellular factor that can increase the efficiency of virus entry but is not sufficient to render otherwise refractory cells susceptible to infection. In line with the definitions, CEACAM5 expression was predominantly detected in cell types highly susceptible to MERS-CoV infection, including Huh7, Calu3, and T cells. Therefore, the expression of CEACAM5 may in part contribute to the permissiveness of a certain cell type in addition to DPP4. Our data showed that the expression of CEACAM5 did not confer infectivity by MERS-CoV to the non-permissive BHK21 cells. However, the expression of CEACAM5 markedly increased the amount of virus particles that were bound to the cell surface. Subsequent analyses confirmed that CEACAM5 expression could enhance MERS-CoV entry in
AD293 cells, which expressed endogenous DPP4. Therefore, mechanistically CEACAM5 may help to concentrate the virus particles at the cell surface and render the virus particles a higher accessibility to DPP4, which can then be used to initiate the cell entry process.

In conclusion, in this study we identified CEACAM5 as a novel surface binding protein for MERS-CoV that could facilitate MERS-CoV entry through serving as an important attachment factor. This novel cell surface factor could be a potential antiviral target in the combat against MERS-CoV infection.

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Potential conflicts of interest. All authors: No reported conflicts.

Reference


Drosten C. 2012. Human coronavirus EMC does not require the SARS-coronavirus receptor and maintains broad replicative capability in mammalian cell lines. MBio 3.


Figure Legends

Figure 1. CEACAM5 is a cell surface binding protein of MERS-CoV. (A) A549 and NIH3T3 biotinylated cell membrane proteins were extracted, separated by 4-12% gradient gel, and transferred to PVDF membrane. (B) Membrane proteins were probed with MERS-S-pseudotyped virus followed by detection of virus binding by incubating with immune serum directed against the MERS-CoV spike protein (lane 1). Non-susceptible cell membrane extracts from NIH3T3 was included as a negative control (lane 2). (C) The gel fractions corresponding to the positive signal at approximately 90kDa was cut, electro-eluted, and confirmed by VOPBA prior to submission for MS protein identification. (D) The identified amino acid sequences and their corresponding positions were illustrated. The position of CEACAM5 in (B) and (C) was indicated with *.

Figure 2. Surface expression of CEACAM5 on mammalian cells. (A) The indicated human cell lines were fixed in 4% paraformaldehyde and immunolabeled for surface DPP4 and CEACAM5 expression. The shaded curve and the solid line represented isotype and antigen-specific staining, respectively. The same fixation and immunostaining procedures were performed for human primary T cells (B) as well as a number of non-human mammalian cells (C). (D) The average percentage of
DPP4/CEACAM5 positive cells was illustrated. (E) The average DPP4/CEACAM5 mean fluorescent intensity (MFI) was quantified. Rabbit isotype IgG was used in place of antigen-specific antibodies for the controls. Data in (D) and (E) represented mean and standard deviation from three independent experiments.

**Figure 3. The expression of CEACAM5 in human lung tissues.** Immunostaining of CEACAM5 and DPP4 were performed on paraffin slides of normal human lung tissues. Representative images of CEACAM5 and DPP4 expression in various regions of the human lung were illustrated, which included the bronchi (A), small airways (B), and alveoli (C). Arrowheads indicated alveolar macrophages with CEACAM5 and DPP4 co-expression. Bars represented 50μm.

**Figure 4. CEACAM 5 interacts with MERS-CoV spike.** (A) Surface and intracellular expression of CEACAM5-V5 were verified with flow cytometry. (B) For co-IP, BHK21 cells were transfected with CEACAM5-V5 (lane 1 and lane 2) or empty vector (lane 3). The cell lysate was immunoprecipitated with MERS-CoV-S1-FLAG (lane 1 and 3) or E. coli bacterial alkaline phosphatase (BAP)-FLAG protein (lane 2) pre-adsorbed onto anti-FLAG M2 agarose beads prior to SDS-PAGE. The protein complex was detected by using the anti-FLAG antibody or the anti-V5 antibody. (C) Reciprocal co-IP was performed using CEACAM5-V5 as the bait. Purified MERS-CoV-S1-FLAG (lane 1 and 3) or BAP-FLAG proteins (lane 2) were immunoprecipitated with overexpressed CEACAM5-V5 or pcDNA-V5 protein pre-adsorbed onto anti-V5 sepharose beads. Western blots were detected with the anti-FLAG or the anti-CEACAM5 antibody. (D)
Endogenous co-IP was performed from MERS-CoV-infected or mock-infected Huh7 cell lysates. Immunoprecipitation was performed using the rabbit anti-CEACAM5 antibody, the rabbit anti-MERS-CoV spike antibody, or the rabbit isotype IgG. Western blots were detected with the rabbit anti-MERS-CoV spike antibody or the rabbit anti-CEACAM5 antibody.

**Figure 5. CEACAM5-specific antibody blocks MERS-CoV entry and replication.**

(A) The antibody blocking assay was performed in Calu3 cells using MERS-S-pseudotyped virus. Antibodies were diluted to 2 µg/ml and incubated with Calu3 cells for 1 hour at 37°C. Pseudotyped viruses were then added at a ratio of 100 lentiviral particle (LP) per cell for 1 hour. Luciferase activity was determined at 48 hours post infection and was normalized to that of the mock-treated cells. (B) The antibody blocking assay was performed in Calu3 cells using MERS-CoV. Calu3 cells were pre-incubated with antibodies at the indicated concentrations for 1 hour at 37°C. The cells were then inoculated with MERS-CoV at 1 MOI for 1 hour at 37°C in the presence of the antibodies. After 1 hour, the cells were washed and harvested. MERS-CoV entry was assessed with qPCR and the result was normalized to that of the mock-treated cells. (C) Calu3 cells were treated with CEACAM5 antibody for a total of 2 hours during pre-incubation and virus inoculation (-1 - 1) or after virus inoculation (1 - 3). MERS-CoV entry was assessed with qPCR. (D) The antibody blocking assay was performed in Caco2 cells. (E and F) The impact of CEACAM5 inhibition on MERS-CoV replication was investigated in Huh7 cells. Huh7 cells were pre-incubated with antibodies at 2 µg/ml for 1 hour at 37°C. The cells were then inoculated with MERS-CoV at 0.0005 MOI for 1
hour at 37°C in the presence of the antibodies. At the end of the inoculation period, the inoculum was replaced with culture media containing the indicated antibodies. Cell lysates (E) and supernatants (F) were harvested at 1, 24, and 48 hours post infection. The virus genome copy number was determined with qPCR. ND indicated that virus was not detected in the corresponding samples. In all panels, data represented mean and standard deviation from three independent experiments. Results from the anti-DPP4-, anti-CEACAM5-, and control IgG-treated samples were compared with that of the mock-treated samples. Statistical analyses were carried out using Student’s t-test. Statistical significance was indicated by asterisk marks when p < 0.05.

Figure 6. CEACAM5 recombinant protein and siRNA knockdown of CEACAM5 inhibits MERS-CoV entry. (A and B) MERS-CoV at 0.1 MOI was pre-incubated with human recombinant proteins at the indicated concentrations for 1 hour at 37°C. After the pre-incubation, the protein-virus mix was added to Calu3 cells (A) or Huh7 cells (B) for 2 hours at 37°C. The cell lysates were subsequently harvested for qPCR analysis. Human recombinant DPP4 and human recombinant IgG were included as positive and negative controls, respectively. Results from the human recombinant DPP4-, human recombinant CEACAM5-, and control human recombinant IgG-treated samples were compared with that of the mock-treated samples. (C) Huh7 cells were treated with CEACAM5 protein for a total of 3 hours during pre-incubation and virus inoculation (-1 - 2) or after virus inoculation (2 - 5). MERS-CoV entry was assessed with qPCR. (D) Huh7 cells were treated with 100uM gene-specific or scrambled siRNA for two consecutive days. (E and F) The reduction of surface CEACAM5 and DPP4 expression was summarized. (G)
siRNA-treated Huh7 cells were then subjected to MERS-CoV infection at 1 MOI for 2 hours at 37°C. Cell lysates were subsequently harvested for qPCR analysis and the result was normalized to that of the mock-treated cells. Results from the DPP4 siRNA-, CEACAM5 siRNA-, and scrambled siRNA-treated samples were compared with that of the mock-treated samples. In all panels, data represented mean and standard deviation from three independent experiments. Statistical analyses were carried out using Student’s t-test. Statistical significance was indicated by asterisk marks when \( p < 0.05 \).

**Figure 7. CEACAM5 is an attachment factor for MERS-CoV.** (A and B) To assess whether CEACAM5 is important for MERS-CoV entry, human CEACAM5 was overexpressed in BHK21 cells. Human CEACAM5-expressing BHK21 cells were then challenged with MERS-CoV at 5 MOI for 2 hours at 37°C. The inoculum was then replaced with culture media and the cells were incubated for another 4 hours before harvesting for flow cytometry. (C and D) To assess whether CEACAM5 is important for MERS-CoV attachment, human CEACAM5-expressing BHK21 cells were challenged with MERS-CoV at 30 MOI for 2 hours at 4°C before harvesting for flow cytometry. Human DPP4-expressing BHK21 cells were included as controls for both entry and attachment assays. Data in (B) and (D) represented the percentage of MERS-CoV NP positive BHK21 cells after infection with or without DPP4/CEACAM5-overrepression. In both panels, mean and standard deviation were derived from three independent experiments. Statistical analyses were carried out using Student’s t-test. Statistical significance was indicated by asterisk marks when \( p < 0.05 \).
Figure 8. CEACAM5 overexpression does not confer infectivity by MERS-CoV to non-permissive cells but enhances MERS-CoV entry in permissive cells. (A and C) To verify the role of CEACAM5 in MERS-CoV entry, human CEACAM5 was overexpressed in BHK21 cells or AD293 cells. The cells were then challenged with MERS-CoV at 1 MOI for 2 hours at 37°C. After 2 hours, the cells were washed extensively and harvested for qPCR analysis. (B and D) To verify the role of CEACAM5 in MERS-CoV attachment, human CEACAM5-expressing BHK21 cells or human CEACAM5-expressing AD293 cells were challenged with MERS-CoV at 1 MOI for 2 hours at 4°C. The cell lysates were then harvested for qPCR analysis. DPP4- and empty vector (pcDNA3.1)-expressing BHK21 cells were included as controls. In all panels, data represented mean and standard deviation from three independent experiments. Results from the human DPP4-, human CEACAM5-, and empty vector-overexpressing samples were compared with that of the mock-treated samples. Statistical analyses were carried out using Student’s t-test. Statistical significance was indicated by asterisk marks when \( p < 0.05 \).

Figure 9. CEACAM5 overexpression does not facilitate SARS-CoV entry in VeroE6 cells. (A) VeroE6 cells were transfected with a CEACAM5-expressing plasmid or an empty vector. (B) The cells were challenged with SARS-S-pseudovirus or VSV-G-pseudovirus at a ratio of 100 lentiviral particle (LP) per cell for 1 hour. Luciferase activity from CEACAM5-transfected VeroE6 cells was determined at 48 hours post pseudovirus challenge and was normalized to that of the empty vector-transfected VeroE6 cells. Mean and standard deviation in panel B were derived from three
independent experiments. Statistical analyses were carried out using Student’s $t$-test.

Statistical significance was indicated by asterisk marks when $p < 0.05$. 
### A

<table>
<thead>
<tr>
<th>Condition</th>
<th>Mock Transfection</th>
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<th>CEACAM5 Transfection</th>
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<tr>
<td><strong>MERS-CoV NP</strong></td>
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### B

**Entry**

- DPP4: - + - -
- CEACAM5: - + - +
- MERS-CoV: + + + +

**% NPS Positive**

- **DPP4**: 31.8
- **CEACAM5**: 56.1
- **MERS-CoV**: 78.9

**Entry**

- **DPP4**: - + - -
- **CEACAM5**: - + - +
- **MERS-CoV**: + + + +

**Percentage**

- **DPP4**: 99.4
- **CEACAM5**: 99.7
- **MERS-CoV NP**: 99.4

**Attachment**

- **DPP4**: 12.0, 12.0
- **CEACAM5**: 20.0, 20.0
- **MERS-CoV**: 50.5, 50.5