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# Host Species Restriction of Middle East Respiratory Syndrome Coronavirus through its Receptor Dipeptidyl Peptidase 4

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Abstract

Middle East Respiratory Syndrome coronavirus (MERS-CoV) emerged in 2012. Recently the MERS-CoV receptor dipeptidyl peptidase 4 (DPP4) was identified and the specific interaction of the receptor-binding domain (RBD) of MERS-CoV spike protein and DPP4 was determined by crystallography. Animal studies identified rhesus macaques but not hamsters, ferrets or mice to be susceptible for MERS-CoV. Here we investigated the role of DPP4 in this observed species tropism. Cell lines of human and non-human primate origin were permissive of MERS-CoV, whereas hamster, ferret or mouse cell lines were not, despite presence of DPP4. Expression of human DPP4 in non-susceptible BHK and ferret cells enabled MERS-CoV replication, whereas expression of hamster or ferret DPP4 did not. Modeling the binding energies of MERS-CoV spike protein RBD to DPP4 of human (susceptible) or hamster (non-susceptible) identified five amino acid residues involved in the DPP4-RBD interaction. Expression of hamster DPP4 containing the five human DPP4 amino acids rendered BHK cells susceptible to MERS-CoV, whereas expression of human DPP4 containing the five hamster DPP4 amino acids did not. Using the same approach, the potential of MERS-CoV to utilize the DPP4s of common Middle Eastern livestock was investigated. Modeling of the DPP4 MERS-CoV RBD interaction predicted the ability of MERS-CoV to bind the DPP4s of camel, goat, cow and sheep. Expression of the DPP4s of these species on BHK cells supported MERS-CoV replication. This suggests, together with the abundant DPP4 presence in the respiratory tract, that these species might be able to function as a MERS-CoV intermediate reservoir.

Importance

The ongoing outbreak of Middle East Respiratory Syndrome coronavirus (MERS-CoV) has caused 184 laboratory confirmed cases to date, with 80 fatalities. Although bats and dromedary camels have been identified as potential MERS-CoV hosts, the virus has so far not been isolated from any species other than humans. The inability of MERS-CoV to infect commonly used animal models such as hamster, mice and ferrets, indicates the presence of a species barrier. We show that the MERS-CoV receptor DPP4 plays a pivotal role in the observed species tropism of MERS-CoV and subsequently identified the amino acids in DPP4 responsible for this restriction. Using a combined modeling and experimental approach we predict that based on the ability of MERS-CoV to utilize the DPP4 of common Middle East livestock species, such as camels, goats, sheep and cows, these form a potential MERS-CoV intermediate host reservoir species.

54 Introduction

55

56 The Middle East respiratory syndrome coronavirus (MERS-CoV) was first identified in 2012 in a patient
57 from Saudi-Arabia (1). To date, 496 laboratory confirmed cases have been reported in eight different
58 countries, with an estimated 20-25% case fatality rate (2). MERS-CoV is a positive strand RNA virus
59 belonging to the C lineage within the *Betacoronavirus* genus and is genetically closely related to
60 coronavirus sequences obtained from insectivorous bats originating from Europe, Asia, Africa and the
61 Middle East (1, 3-5). The detection of MERS-CoV neutralizing antibodies as well as the recovery of viral
62 sequences and virus in dromedary camels across the countries of the Middle East suggests the potential
63 involvement of an intermediate reservoir in the emergence of MERS-CoV in humans (6-10).
64 Phylogenetic analysis of MERS-CoV genomes obtained from 43 human cases in Saudi Arabia suggests
65 the occurrence of multiple zoonotic spill-over events (11, 12).
66 Similarly to Severe Acute Respiratory Syndrome coronavirus (SARS-CoV), another *Betacoronavirus*
67 which caused the SARS pandemic, MERS-CoV appears to primarily target the lower respiratory tract
68 causing acute respiratory distress in severe human cases (2, 13, 14). However, in contrast to SARS-CoV,
69 which uses angiotensin converting enzyme 2 (ACE2) as its cellular host receptor (15), MERS-CoV
70 utilizes dipeptidyl peptidase 4 (DPP4, also known as CD26) (16). Interaction of the receptor binding
71 domain (RBD) of the MERS-CoV spike protein with DPP4 initiates attachment to the host cell and
72 subsequent virus internalization. The RBD was mapped to be a 231 amino acid region in the S1 subunit of
73 the spike protein (17). DPP4 is a type II transmembrane glycoprotein, involved in cleavage of dipeptides
74 and degradation of incretins (18). DPP4 is widely expressed in different tissues, such as lungs and kidney
75 and the cells of the immune system, although a detailed description of DPP4 expression in the human
76 respiratory tract and kidney is currently not available. DPP4 is relatively conserved between mammalian
77 species, allowing the MERS-CoV spike protein to bind to both bat and human DPP4 (16, 18).
78 *In vitro* studies using a variety of different primary and immortalized cell lines reported a broad tropism
79 of MERS-CoV (19-22). Most cell lines with a human-, bat-, non-human primate- or swine-origin were

found to be susceptible to infection with MERS-CoV. In contrast, cell lines originating from mice, hamsters, dogs and cats were not susceptible to MERS-CoV infection (16, 19). *In vitro* data on the species tropism of MERS-CoV appears to correlate with the *in vivo* host restriction of MERS-CoV; rhesus macaques can be experimentally infected with MERS-CoV, whereas inoculation of other commonly used animal models such as the Syrian hamster, mouse or ferret did not result in efficient viral replication (23-27). Recent studies suggest that DPP4 plays an important role in the non-susceptibility of the mouse and ferret to MERS-CoV (28-30).

Here, we investigated the host species restriction of MERS-CoV and the role of the DPP4 receptor in this observed species tropism. Differences in DPP4 between MERS-CoV permissive and non-permissive species were identified to be responsible for the ability of DPP4 to function as the MERS-CoV receptor.

## Materials and methods

### Biosafety statement

All infectious work with MERS-CoV was performed in a high containment facility at the Rocky Mountain Laboratories (RML), Division of Intramural Research (DIR), National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH). The work was approved by the RML Institutional Biosafety Committee (IBC) at biosafety level 3 (BSL3).

### Ethics statement

Fresh animal tissues were obtained from local slaughter facilities (cow, goat and sheep), from an in-house tissue repository (rhesus macaque and mouse) or collected under a tissue sampling protocol (hamster and ferret) approved by the Institutional Animal Care and Use Committee of the Rocky Mountain Laboratories, and the collection was performed following the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC) by certified staff in an AAALAC approved facility.

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107 Cells and virus

108 Huh-7 (human carcinoma), Vero (African green monkey kidney), BHK (baby hamster kidney), 3T3
109 (mouse embryonic fibroblast) and MEF C57Bl6 (mouse embryonic fibroblast) cells were maintained in
110 Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovin serum (FBS), 2 mM L-
111 Glutamine, 50 U/ml penicillin and 50 µg/ml of streptomycin (culture DMEM). Primary ferret kidney cells
112 were generated as follows: within 30 minutes of tissue collection, the fibrous capsule, adjacent medulla
113 and any fat, blood cloths and connective tissue were dissected from the ferret kidney which was
114 subsequently cut into small pieces. The tissue sample was washed with ice-cold Hank's balanced salt
115 solution (HBSS) containing 10 mM EGTA until the supernatant was clear and further cut into 1 $mm^3$
116 pieces. Hereafter, the tissue sample was incubated at 37°C for 20 minutes whilst rolling in 25 ml of warm
117 non-supplemented DMEM/F12 GlutaMAX media containing 1 mg/ml collagenase (Worthington) and
118 passed through a 100 µm sieve, a 70 µm sieve and a 40 µm sieve. Supernatant was centrifuged at 400 g
119 for 5 min at 4 °C and washed 3 times with HBSS. The pellet was then resuspended in DMEM/F12
120 GlutaMAX media supplemented with 10% FBS, 50 U/ml penicillin, 50 µg/ml of streptomycin, 2.5 µg/ml
121 Fungizone and 5 µg/ml human transferrin (Sigma Aldrich) and cells were seeded at a density of $5 \times 10^4$
122 cells/$cm^2$. The ferret primary kidney cell line was maintained in DMEM/F12 + GlutaMAX supplemented
123 with 10% FBS, 50 U/ml penicillin, 50 µg/ml of streptomycin, 2.5 µg/ml Fungizone and 50 µg/ml human
124 transferrin. All cell lines were maintained at 37°C in 5% $CO_2$. All reagents were purchased from Gibco,
125 unless otherwise specified. MERS-CoV (strain HCoV-EMC/2012) was propagated on VeroE6 cells
126 using DMEM supplemented with 2% FBS, 2 mM L-Glutamine, 50 U/ml penicillin and 50 µg/ml of
127 streptomycin (complete DMEM). MERS-CoV was titrated by end-point titration in quadruplicate in
128 VeroE6 cells cultured in complete DMEM as follows: cells were inoculated with ten-fold serial dilutions
129 of virus, and scored for cytopathic effect 5 days later. $TCID_{50}$ was calculated by the method of Spearman-
130 Karber.

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DPP4 western blot analysis

Cells were washed in phosphate-buffered saline (PBS) and lysed in radioimmunoprecipitation (RIPA) buffer (50 mM Tris-HCL, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium-deoxycholate, 0.1% SDS and protease inhibitor cocktail tablets (Sigma)). Lysates were treated with TURBO™ DNase (Life Technologies). Protein concentrations were determined with the bicinchoninic assay (Thermo Scientific). Cellular lysates were separated on 10% SDS-PAGE gels and transferred to PVDF membranes (Life Technologies). After blocking in 5% non-fat milk powder in PBS-0.1% Tween (Fisher Scientific), membranes were incubated overnight with α-DPP4 rabbit polyclonal antibody (1:700, AbCam, ab28340) or an α-actin antibody (1:5000, Sigma Aldrich, A5441). Membranes were then incubated with a horseradish peroxidase conjugated α-rabbit or α-mouse IgG respectively (1:12,500, Jackson Immunoresearch). Signals were detected with Pierce ECL 2 Western Blotting Substrate (Thermo Scientific) and developed on blue autoradiography film (GeneMate).

Immunohistochemistry

Immunohistochemistry was performed as described previously (24) using an α-DPP4 rabbit polyclonal antibody (Abcam, ab28340) at a 1:400 dilution for rhesus macaque, mouse, ferret, sheep, goat and cow or a 1:800 dilution for hamster, and biotinylated anti-rabbit SS link (undiluted, Biogenex, HK336-9R) as a secondary antibody. Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin and processed for immunohistochemistry using the Discovery XT automated processor (Ventana Medical Systems) with a DapMap kit (Ventana Medical Systems).

Sequencing and cloning of DPP4 sequences

Total RNA from lung and kidney samples from different species was extracted using the RNeasy Mini Kit (Qiagen) and cDNAs were synthesized using random hexamers and SuperScript III Reverse

Transcriptase (Applied Biosystems). Complete DPP4 genes were amplified using iProof High-Fidelity DNA Polymerase (BioRad) and in-house designed primers (sequence available upon request).

Plasmids

DPP4 amplicons were sequenced by Sanger sequencing and sequences were aligned using the MEGA5.2 software package. DPP4 gene sequences for each species were synthesized in expression plasmid pcDNA3.1(+) (GeneArt). All newly generated DPP4 nucleotide sequences are available from GenBank under accession numbers KF574262–8. Mutagenized DPP4 expression plasmids were generated by synthesizing hamster DPP4 containing five human-specific amino acid residues (Ala291, Ile295, Arg336, Val341 and Ile346, humanized hamster) and human DPP4 containing five hamster-specific amino acid residues (Glu291, Thr295, Thr336, Leu341 and Val346, hamsterized human), flanked by restriction sites BamHI and BsgI (human DPP4) or BamHI and EcoRV (hamster DPP4). Human and hamster DPP4 in pcDNA3.1(+) were restriction digested, purified and ligated with the humanized hamster or hamsterized human DPP4 fragments respectively using T4 DNA ligase (New England Biolabs). Modified DPP4 sequences were confirmed by Sanger sequencing.

Transfection of cells

BHK and primary ferret cells were transfected with 3 µg pcDNA3.1(+) containing the DPP4 genes from different species or pCAGGS-GFP using 8 µl of Lipofectamine 2000 (Life Technologies). DPP4 expression was confirmed by qRT-PCR and flow cytometry.

Replication kinetics

Multistep replication kinetics were determined by inoculating cells in triplicate with MERS-CoV with a multiplicity of infection (MOI) of 0.01 (normal cell lines) or 1 (transfected cell lines) 50% tissue culture infectious dose (TCID50) per cell. The lower MOI of 0.01 was chosen for experiments performed to determine the ability of cell lines to support multiple replication cycles of MERS-CoV, whereas the

higher MOI of 1 was chosen for cells naturally non-susceptible for MERS-CoV but with the various DPP4s transiently expressed to maximize the likelihood of the transfected cell to encounter MERS-CoV. One hour after inoculation, cells were washed once with DMEM and fresh media was placed on the cells. Supernatants were sampled at 0, 24, 48 and 72 h after inoculation, and virus titers in these supernatants were determined as described.

Flow Cytometry

Cells were washed with PBS and removed with 5 mM EDTA (BHKs and 3T3s) or spun down from suspension (primary ferret cells, huh7 cells, vero cells, and MEF) and then washed twice, resuspended in PBS with 2% FBS and stained at 4°C using α-human DPP4 antibody (R&D, AF1180), followed by staining with FITC-tagged donkey anti-goat antibody (Life technologies, A11055). As a control, samples of cells were stained with secondary antibody only. After staining, cells were washed, resuspended in PBS with 2% FBS, stained with 7-amino actinomysin-D (Life Technologies) and analyzed immediately. Samples were collected using a LSRII flow cytometer (BD Biosciences). Analysis gates were set on viable cells and 10,000 gated events were analyzed for each sample. Data were analyzed using FlowJo software (Treestar) comparing transfected cells against untransfected cells.

Binding energy modeling

The DPP4 homology models were constructed using the human DPP4 structure (PDB ID: 4KR0, Chain A) as template. The sequence alignment was generated using CLUSTALW2 (31) and the initial model was built using Nest (32) based on the alignment and the human DPP4 structure. The resulting structural model was briefly optimized using the TINKER minimization program "minimize.x" with OPLS all-atom force field and L-BFGS quasi-Newton optimization algorithm (33). For each species, the RBD/DPP4 complex model was generated by merging the RBD domain (PDB ID: 4KR0, Chain B) with the DPP4 model, which was then subjected to the binding energy calculation using an all-atom distance-dependent

pairwise statistical potential, DFIRE (34). The energy difference between the complex and two individual structures - DPP4 and RBD - was taken as the binding energy.

qRT-PCR of DPP4 mRNA expression

Expression of DPP4 mRNA was measured via qRT-PCR. Total RNA was extracted from transfected homogenized cells using the standard TRIzol-chloroform procedure (Life technologies), followed by further extraction using the RNeasy mini kit (Qiagen) combined with a 30 minute on-column DNase I (Qiagen) digestion according to manufacturer's instructions. mRNA was purified from total RNA via the NucleoTrap mRNA mini kit (Macherey-Nagel). One-step qRT-PCR was performed in three separate experiments on the Rotor-GeneQ (Qiagen) for the detection of DPP4 and HPRT using the Quantifast Probe PCR Master Mix (Qiagen) according to manufacturer's instructions. Probes for DPP4 (FAM-AGCTTTGATGGCAGAGGAAGTGGT-BHQ1) and HPRT (FAM-ACTTTGTTGGATTTGAAATTCCAGACAAGTTTG-BHQ1) were designed using a cross-species high conservancy region in the gene. Forward and reverse primer sets were species specific (Sequence available upon request). Relative fold increase was calculated by the comparative $C_T$ method (35), where DPP4 expression is normalized to HPRT.

## Results

Replication kinetics of MERS-CoV in different cell lines

The replication kinetics of MERS-CoV was studied in cells of different mammalian origin: Huh-7 (human), Vero (African green monkey), BHK (hamster), MEFC57Bl6 and 3T3 (mouse) and ferret primary kidney cells. MERS-CoV replicated efficiently in Huh-7 and Vero cells. In contrast, MERS-CoV did not replicate in BHK, MEFC57Bl6, 3T3 and ferret primary kidney cells (*Figure 1A*). These data correspond with the current information on the ability of MERS-CoV to infect humans and non-human

primates (rhesus macaques (23, 25)) and the inability of MERS-CoV to infect mice, hamsters and ferrets (24, 26, 27). The presence of the MERS-CoV receptor, DPP4, is essential in the initiation of infection. To investigate whether the lack of infection by MERS-CoV of non-susceptible cell lines was due to a lack of expression of the DPP4 receptor we performed western blot analyses. DPP4 protein was detected in cell lines both permissive and non-permissive for MERS-CoV infection although not uniformly found to be expressed on the cell surface (*Figure 1B, C*).

Detection of DPP4 in tissues

To determine the cell types in which DPP4 was expressed in the lungs and kidney of rhesus macaque, hamster, mouse and ferret, immunohistochemistry (IHC) was performed using an α-DPP4 antibody. In both the lungs and kidneys of the investigated species, DPP4 was found to be present. In the lungs, DPP4 was abundantly present on bronchiolar epithelium cells and occasionally present on alveolar interstitium, or absent in the case of ferrets (*Figure 2, Table 1*). The intensity of the α-DPP4 staining of bronchiolar epithelium ranged from weak in ferrets, to moderate in the macaque and hamster and very intense in the mouse. All species tested, except the ferret, also demonstrated weak α-DPP4 immunoreactivity at the level of alveoli. The kidney was similar to lung tissue in that all species demonstrated α-DPP4 immunoreactivity to epithelial cells. The intensity of staining was again variable, with weak staining in the hamster, moderate staining in the macaque and mouse and intense staining in ferret. DPP4 was present on kidney vascular smooth muscle cells, with weak staining in the macaque, mouse, ferret and moderate staining in the hamster. All species except the mouse displayed presence of DPP4 on either glomerular or vascular endothelium with the strongest staining seen in the glomeruli of hamsters and ferrets.

The presence of DPP4 in cell lines non-susceptible to MERS-CoV and on cells in the respiratory tract of non-permissive species (mouse, hamster and ferret) suggests that the inability of MERS-CoV to replicate in these species is either due to an inability of the MERS-CoV spike protein to bind to the respective DPP4s or an incompatibility of MERS-CoV with the cellular machinery of these respective species.

Specificity of MERS-CoV spike protein for DPP4

The DPP4 coding sequences of human, hamster and ferret, obtained from GenBank or by sequencing, were cloned into expression vector pcDNA3.1(+) and transfected into cell lines non-susceptible to MERS-CoV replication. The expression of DPP4 on transfected cells was determined by qRT-PCR and flow cytometry (*Figure 3B, C*). Transient expression of human DPP4 in BHK and primary ferret kidney cells allowed these previously non-susceptible cells to support MERS-CoV replication, whereas transient expression of hamster DPP4 in BHK cells, ferret DPP4 in ferret primary cells or GFP in either cell type did not render these cells susceptible to MERS-CoV replication (*Figure 3A*). As surface expression of human DPP4, but not hamster or ferret DPP4, allowed MERS-CoV replication in previously non-susceptible cell lines, the observed MERS-CoV species tropism is most likely a result of the inability of its spike protein to bind to hamster or ferret DPP4 rather than the incompatibility of MERS-CoV with the hamster or ferret cellular machinery.

Structural modeling of MERS-CoV receptor binding domain with multispecies DPP4

Recent co-crystallization studies of the MERS-CoV spike protein and the human DPP4 identified 14 amino acids in DPP4 important in binding to the MERS-CoV spike protein (36, 37). Alignment of the DPP4 amino acid sequences of human and hamster origin revealed a total of five differences within these 14 amino acids (*Table 2)*. To investigate the binding potential of the different DPP4s to MERS-CoV spike protein, DPP4 homology models were built using the human DPP4 structure (PDB ID: 4KR0, Chain A) as a template (36). Of the five amino acid residues at the RBD interface that differ between human and hamster DPP4, the residues at positions 291 and 336 appear to be most critical for the species specificity. In the human DPP4:RBD crystal structure, the small methyl side chain of Ala291 in DPP4 nestles into a small pocket in the RBD, which cannot accommodate the size and charge of the corresponding glutamic acid residue found in the hamster DPP4 molecule. This steric clash alone is likely sufficient to abrogate binding. In addition, the side chain of Arg336 in human DPP4 forms hydrogen bonds with RBD residue Tyr499 and a salt bridge to RBD residue Asp455. These interactions would not

be formed by the corresponding threonine side chain in hamster DPP4. The remaining three DPP4 residues at the RBD interface that differ in humans and hamsters are conserved substitutions and are not predicted to greatly impact binding to the RBD (*Figure 4A*). Furthermore, these analyses showed that hamster DPP4 has significantly higher binding energy (less favorable interactions) than human DPP4 to MERS-CoV (*Figure 4B*). When mutant DPP4s were designed *in silico* by introducing the five human-specific amino acid residues (Ala291, Ile295, Arg336, Val341 and Ile346) into the hamster DPP4 (humanized hamster DPP4), and the five hamster-specific amino acid residues (Glu291, Thr295, Thr336, Leu341 and Val346 into the human DPP4 (hamsterized human DPP4), a reversion of the binding energies was found; the binding energy associated with the humanized hamster DPP4 and MERS-CoV spike protein complex was lower than that of hamsterized human DPP4 and MERS-CoV spike protein (*Figure 4B*). This suggests that the five DPP4 human-specific amino acid residues are responsible for the ability of MERS-CoV spike protein to bind to DPP4.

In vitro characterization of mutagenized DPP4s

The modeling data suggested that introduction of the five human-specific amino acid residues in hamster DPP4 would allow recognition of this DPP4 by the MERS-CoV spike protein. To test this hypothesis, expression plasmids were synthesized with human DPP4 containing the five hamster-specific amino acid residues (hamsterized human DPP4) and hamster DPP4 containing the five human-specific amino acid residues (humanized hamster DPP4). Inoculation of BHK cells transiently expressing humanized hamster DPP4 with MERS-CoV resulted in virus replication, whereas inoculation of BHK cells transiently expressing hamsterized human DPP4 did not (*Figure 5A*). Expression of DPP4 on BHK cells was confirmed via flow cytometry (*Figure 5B*).

Modeling and in vitro characterization of the DPP4 of putative intermediate host species

We determined the binding energy of DPP4s to MERS-CoV spike protein of known binders (rhesus macaque) and non-binders (ferret and mouse). Like hamster DPP4, the binding energy of ferret and

mouse DPP4 to MERS-CoV spike protein was found to be relatively high. In contrast, rhesus macaque DPP4 was found to have binding energy levels similar to human DPP4 (*Figure 4B*). These results were confirmed *in vitro* by transfecting BHK cells with DPP4 from rhesus macaque and subsequently inoculating these cells with MERS-CoV, resulting in virus replication. In contrast, transfection of the DPP4 of ferret or mouse origin into DPP4 did not render these cells susceptible to MERS-CoV replication (*Figure 6*). With the identification of dromedary camels as a potential intermediate host species for MERS-CoV in mind, we determined the ability of DPP4 from putative intermediate host species (dromedary camel, cow, sheep and goat) to bind to MERS-CoV spike protein. Like DPP4 from human and rhesus macaque, the binding energy associated with dromedary camel, goat, sheep and cow DPP4 was found to be relatively low, suggesting these proteins can function as a receptor for MERS-CoV (*Figure 4B*). Furthermore, expression of dromedary camel, goat, sheep and cow DPP4 on BHK cells supported replication of MERS-CoV (*Figure 6A*). Expression of DPP4 on BHK cells was confirmed via flow cytometry and qRT-PCR (*Figure 6B, C*). DPP4 was detected on cells in lung and kidney tissue of camel, goat, cow, and sheep by IHC. Interestingly, DPP4 was more abundantly expressed on alveolar interstitium in cow, goat and sheep lungs compared to dromedary camel, rhesus macaque, hamster, mouse and ferret lungs (*Figure 7, Table 1*). Finally, we carried out full-length and partial (DPP4 binding domain, amino acids 220-350) protein sequence alignments between DPP4 of camel, goat, cow and sheep. Camel DPP4 diverged from goat, cow, and sheep DPP4, in particular when comparing partial DPP4 protein sequences (*Table 3*).

## Discussion

Surface receptors play an essential role in initiating virus entry into the host cell, thereby playing a major role in the tissue- as well as host species-tropism of viruses. DPP4 was recently identified as the cellular receptor for MERS-CoV (16). Based on the ability of MERS-CoV to replicate in cell lines originating from a wide variety of mammalian species (bats, non-human primates, pigs, and humans), it was

speculated to have a broad host tropism (19, 21). However, it is currently unclear whether *in vitro* results correlate directly with *in vivo* susceptibility (38). MERS-CoV was found to be unable to infect some of the major respiratory animal models (Syrian hamster, mouse and ferrets (24, 26, 27)) in contrast to the ability of MERS-CoV to replicate efficiently in rhesus macaques (23). This suggests the existence of a host-barrier restriction of MERS-CoV for some species. Using *in vitro* growth kinetics of MERS-CoV we were able to demonstrate this host-species restriction in cell lines from different mammalian origins. MERS-CoV replicated efficiently in cells of human and non-human primate origin, but was not able to replicate in cells of mouse, hamster or ferret origin, despite the presence of DPP4 (*Figure 1*). Analysis of the presence of DPP4 in Syrian hamster, mouse and ferret lung and kidney tissues as well as the rhesus macaque lung and kidney tissues suggested that the inability of MERS-CoV to infect Syrian hamster, mouse and ferret is not due to a lack of expression of the DPP4 receptor (*Figure 2*). Our hypothesis that the host species restriction of MERS-CoV lies on the receptor-binding level was supported by the lack of replication in hamster and ferret cells upon exogenous expression of the hamster or ferret DPP4 on the surface of these cells, whereas expression of the human DPP4 receptor rendered these previously non-permissive cell lines permissive for MERS-CoV (*Figure 3*). We observed a >2 log difference in growth of MERS-CoV between hamster and ferret cells, which may be explained by the difference in transfection efficiency (BHKs, 93.0%; primary ferret cells, 65.8%; *Figure 3B*). The co-crystallization between the human DPP4 and the MERS-CoV spike protein revealed the receptor binding domain of MERS-CoV and the amino acid residues of human DPP4 interacting with this domain (36, 37). Alignment of DPP4 amino acid residues identified as interacting with the MERS-CoV spike protein between human and rhesus DPP4s (binders) and hamster DPP4 (non-binder) revealed a minimal subset of five amino acid changes between the human and the hamster DPP4 (*Table 2*). These five differential human amino acid residues were introduced into the hamster DPP4 (humanized hamster DPP4) and the five differential hamster amino acid residues were introduced into the human DPP4 (hamsterized human DPP4). Expression of the humanized hamster DPP4 in BHK cells rendered these cells permissive for MERS-CoV, whereas expression of hamsterized human DPP4 did not change the non-permissiveness of the cells (*Figure 5*).

For ferret DPP4 it was recently shown that exchanging the 246 to 505 amino acid region with that of human DPP4 resulted into the ability of MERS-CoV to utilize this chimeric DPP4 (28), as was the case for mouse DPP4 when exchanging amino acids 279 to 346 with the human DPP4 counterpart (29). In addition, both of these studies found amino acids to be important in spike binding that were identified in this study (295, 336 and 346 for mouse and 295, 336 and 341 for ferret) (28, 29). Interestingly, although the humanized hamster DPP4 was able to facilitate MERS-CoV infection, MERS-CoV titers were considerably lower. This could indicate that although substitution of five human amino acids into hamster DPP4 is sufficient for spike binding, additional amino acid residues in the interface might be required for optimal binding and infectivity.

Currently the only available animal disease model for MERS-CoV is the rhesus macaque. Research into therapeutic and prophylactic countermeasures is severely restricted by the absence of a small animal model allowing high-throughput *in-vivo* screening of antivirals with *in-vitro* efficacy or candidate vaccines (24, 25, 39-42). Our data indicate that transgenic mice expressing the human DPP4 will likely be susceptible to MERS-CoV and would allow the establishment of a much-needed small animal model. This is supported by recent experimental evidence which showed that transient expression of human DPP4 in the lower respiratory tract of mice supported MERS-CoV replication (43).

Subsequent modeling between DPP4s of species known to be able to bind MERS-CoV spike protein (human and rhesus macaque) and species known not to be able to bind MERS-CoV spike protein (hamster, mouse and ferret) displayed a stark difference in binding energies between binders and non-binders. Utilizing the same model, DPP4s of species implicated in the emergence of MERS-CoV (dromedary camel, sheep, goat and cow) were predicted to be able to bind to the spike protein of MERS-CoV (*Figure 4B*). DPP4 modeling data was supported by experimental work showing that indeed the DPP4s of dromedary camel, sheep, goat and cow were able to support MERS-CoV replication (*Figure 6*). MERS-CoV-like antibodies have been found in dromedary camels, but not yet in goat, sheep or cow species (7, 44). However, DPP4 from goat, cow and sheep can function as a receptor for MERS-CoV, although with lesser efficiency (*Figure 6*). Protein sequence alignments of full-length and partial DPP4

388 revealed that camel DPP4 differs from goat, cow and sheep DPP4, in particular when amino acids 220-
389 350 of DPP4, which are of importance in spike binding, were investigated (*Table 3*). It is possible that
390 subtle differences in binding of spike and DPP4 account for the apparent lack of MERS-CoV circulation
391 in the goat, cow and sheep population. The close evolutionary relationship between MERS-CoV and bat
392 CoVs as well as the detection of a short fragment of viral RNA in a bat in Saudi Arabia suggest that
393 MERS-CoV originates from bats (1, 3). The ability of MERS-CoV to utilize the DPP4 from an
394 insectivorous bat underlines the broad host range (16). Future receptor binding and experimental infection
395 studies are needed to investigate the suitability of different bat species to function as a host for MERS-
396 CoV.

397 MERS-CoV was found to replicate predominantly in type I and II pneumocytes in the lower respiratory
398 tract of experimentally infected rhesus macaques. The replication of MERS-CoV correlates with DPP4
399 expression on type I and II pneumocytes in the lungs of rhesus macaques (*Figure 2*) (25). The presence of
400 DPP4 in the lower respiratory tract of dromedary camel, sheep, goat and cow (*Table 1*) suggest that the
401 tropism of MERS-CoV for these species could be similar to the respiratory tropism observed in rhesus
402 macaques and humans (13, 14, 25). Since the first emergence of MERS-CoV in 2012, the epidemiology
403 has remained unclear. The recent identification of the circulation of MERS-CoV in dromedary camels (6-
404 9) together with the multiple introductions of MERS-CoV into the human population (11, 12) suggests
405 that both zoonotic transmission from an intermediate host and human-to-human transmission occur
406 simultaneously. Our data supports the potential for the existence of one or multiple natural reservoirs for
407 MERS-CoV. Although our data do not provide any formal proof for the existence of such a reservoir, the
408 ability of DPP4s of cows, sheep, goats and dromedary camels (the major Middle East livestock species)
409 to function as a MERS-CoV receptor and the abundant DPP4 presence in the respiratory tract of these
410 species suggest that they would be susceptible for MERS-CoV infection. The combination of modeling
411 the binding energies of the MERS-CoV spike with the DPP4s of different species and a molecular
412 experimental approach could guide field programs focused at identifying the existence of an intermediate

host. With the potential susceptibility of major livestock species for MERS-CoV, renewed focus should be aimed at elucidating the existence of an intermediate host and thereby preventing further spread of MERS-CoV.

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585

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591 Infectious Diseases (NIAID), National Institutes of Health (NIH).

592 Table 1. DPP4 expression in cells lung and kidney tissue of different mammalian species.

| **Lung** | Rhesus macaque | Hamster | Mouse | Ferret | Camel | Sheep | Goat | Cow |
|---|---|---|---|---|---|---|---|---|
| apical bronchial/bronchiolar epithelium | II | III | IIII | I | III | III | II | III |
| bronchiolar smooth muscle | I | II | III | III | I | I | I | I |
| vascular smooth muscle | I | III | III | II | II | I | II | II |
| endothelial cells | I | I | I | 0 | 0 | I | II | I |
| axonal cells | ND | IIII | IIII | III | ND | ND | ND | 0 |
| alveolar macrophages | II | I | II | 0 | I | ND | II | 0 |
| alveolar interstitium | I | II | I | 0 | 0 | IIII | III | III |
| mesothelium | I | I | 0 | 0 | 0 | III | IIII | III |
| **Kidney** | | | | | | | | |
| cortical apical proximal tubular epithelium | II | I | II | IIII | II | II | II | I |
| arteriolar smooth muscle | I | II | I | I | III | II | II | I |
| endothelial cells | I | II | 0 | 0 | 0 | II | 0 | II |
| glomerular endothelial cells | I | III | 0 | III | 0 | I | II | I |
| axonal cells | 0 | IIII | II | IIII | ND | I | III | III |

593

594 The lungs and kidneys of the various species were examined by IHC using an α-DPP4 antibody. The
595 tissues were evaluated using a scale of 0 to IIII based on the intensity of the IHC signal and/or the
596 distribution of antigen throughout the tissue. A score of 0 indicates that no anti-DPP4 staining was
597 detected. I was used when the signal was very weak and/or was found in only a few, scattered cells. II
598 demonstrated a moderate IHC signal in multifocal to diffuse areas within the tissue. III was used to score
599 cells that stained in a moderate to intense fashion in coalescing to diffuse areas. IIII indicates intense and
600 diffuse IHC staining in the cells of interest. ND = not detected.

601

602 Table 2. Alignment of DPP4 amino acid residues of different mammalian species interacting with the

603 MERS-CoV spike protein.

604

| | Amino acid residues (Human DPP4 numbering) | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Species** | **229** | **267** | **286** | **288** | **291** | **294** | **295** | **298** | **317** | **322** | **336** | **341** | **344** | **346** |
| Human | N | K | Q | T | A | L | I | H | R | Y | R | V | Q | I |
| Hamster | . | . | . | . | E | . | T | . | . | . | T | L | . | V |
| Rhesus Macaque | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Mouse | . | . | . | P | . | A | R | . | . | . | T | S | . | V |
| Ferret | . | . | E | . | D | S | T | Y | . | . | S | E | E | T |
| Camel | . | . | . | V | . | . | . | . | . | . | . | . | . | . |
| Sheep | . | . | . | V | G | . | . | . | . | . | . | . | . | . |
| Cow | . | . | . | V | G | . | . | . | . | . | . | . | . | . |
| Goat | . | . | . | V | G | . | . | . | . | . | . | . | . | . |

605

606 Full DPP4 protein sequences were compared using MegAlign software.

607

608 Table 3. Percent identity between DPP4 protein sequences

| | | **Full DPP4** | | | |
|---|---|---|---|---|---|
| | | Camel | Goat | Cow | Sheep |
| **Partial DPP4** | Camel | | 91.6 | 91.6 | 91.5 |
| | Goat | 88.5 | | 98.4 | 99.3 |
| | Cow | 88.5 | 100 | | 98.2 |
| | Sheep | 88.5 | 100 | 100 | |

609

610 Full DPP4 and partial (amino acid 220-350) DPP4 protein sequences were compared and percent identity
611 were analyzed using MegAlign software.

612 Figure 1. Replication kinetics of MERS-CoV in cell lines of human, non-human primate, hamster, mouse
613 and ferret origin.
614 A.) Huh7 (red, ●), Vero (red, ■), BHK (blue, ●), 3T3 (blue, ■), MEFC57Bl6 (blue, ▲) and primary ferret
615 (blue, ▼) cell lines were inoculated with MERS-CoV using an MOI of 0.01 $TCID_{50}$/cell. Supernatants
616 were harvested at 0, 24, 48 and 72 hours post inoculation (hpi) and viral titers were determined by end-
617 point titration in quadruplicate in VeroE6 cells. Red lines indicate cell lines originating from species
618 known to be susceptible to MERS-CoV infection; blue lines indicate cell lines originating from species
619 non-susceptible to MERS-CoV infection. B.) Western blots of cellular lysates of Huh7, Vero, BHK,
620 primary ferret, 3T3 and MEFC57Bl6 cells probed with α-DPP4 or α-actin antibodies. C.) Cells were
621 stained using α-DPP4 (R&D) and a FITC-conjugated secondary antibody (Life Technologies). Samples
622 were collected using a LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo and GraphPad
623 software. Mean titers were calculated from three independent experiments. Error bars indicate standard
624 deviations.
625
626 Figure 2. DPP4 in rhesus macaque, hamster, mouse and ferret lung and kidney tissue.
627 IHC was performed on lung and kidney tissues from rhesus macaque, hamster, mouse and ferret tissue
628 using an α-DPP4 antibody. Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin.
629 IHC images lung: closed arrow = bronchiolar epithelium; open arrow = smooth muscle; asterisk =
630 alveolar macrophage; closed arrowhead = alveolar interstitium. IHC images kidney: closed arrow = renal
631 tubular epithelium; open arrow = glomerular endothelium (magnification, 200x).
632
633 Figure 3. Replication kinetics of MERS-CoV on hamster and ferret cell lines expressing human, hamster
634 or ferret DPP4.
635 A.) Human DPP4 (red), hamster DPP4 (blue), ferret DPP4 (blue,) and GFP (green) were expressed in
636 BHK (●) or primary ferret (■) cells. Twenty-four hours post transfection, cells were inoculated with
637 MERS-CoV using a MOI of 1 $TCID_{50}$/cell. Supernatants were harvested at 0, 24, 48 and 72 hpi and viral

titers were determined by end-point titration in quadruplicate in VeroE6 cells. Geometric mean titers were calculated from three independent experiments. Error bars indicate standard deviations. B.) BHK or primary ferret cells were left untransfected (red) or transfected with DPP4 (blue) and stained 24 hours post-transfection using α-DPP4 (R&D) and a FITC-conjugated secondary antibody (Life Technologies). Samples were collected using a LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software. C.) Expression of DPP4 mRNA was measured via qRT-PCR. Relative fold increase was calculated by the comparative $C_T$ method (35), where DPP4 expression is normalized to HPRT.

Figure 4. Interaction between MERS-CoV spike protein and DPP4s of different mammalian species
A. Cartoon representing the binding between human DPP4 or hamster DPP4 and the spike protein of MERS-CoV. DPP4 is depicted in white; the receptor binding domain (RBD) of the spike protein of MERS-CoV is depicted in magenta and cyan. The far right panel is obtained by clockwise rotation of the middle panel along a longitudinal axis. B. Binding energies between spike protein of MERS-CoV and DPP4 of different species as well as humanized hamster DPP4 and hamsterized human DPP4. Red bars indicate the binding energies of known binders (human and rhesus macaque DPP4), blue bars indicate the binding energies of non-binders (hamster, mouse and ferret DPP4), green bars indicate the binding energies of unknown binders (dromedary camel, goat, cow and sheep) and purple bars indicate the binding energies of the *in-silico* mutagenized hamster and human DPP4s. The DPP4 homology models were constructed using the human DPP4 structure (PDB ID: 4KR0, Chain A) as a template and subjected to the binding energy calculation using an all-atom distance-dependent pairwise statistical potential, DFIRE.

Figure 5. Replication kinetics of MERS-CoV on BHK cells expressing mutagenized DPP4s.
A.) Humanized hamster DPP4 (blue ●) or hamsterized human DPP4 (red ■) were expressed on BHK cells. As a control, human DPP4 (red ●) was expressed on BHK cells. Twenty-four post transfection, cells were inoculated with MERS-CoV using an MOI of 1 $TCID_{50}$/cell. Supernatants were harvested at 0, 24,

664 48 and 72 hpi and viral titers were determined by end-point titration in quadruplicate in VeroE6 cells.
665 Geometric mean titers were calculated from three independent experiments. Error bars indicate standard
666 deviations. B.) BHK cells were left untransfected (red) or transfected with DPP4 (blue) and stained 24
667 hours post-transfection using α-DPP4 (R&D) and a FITC-conjugated secondary antibody (Life
668 Technologies). Samples were collected using a LSRII flow cytometer (BD Biosciences) and analyzed
669 using FlowJo software.
670
671 Figure 6. Replication kinetics of MERS-CoV on BHK cells expressing DPP4 of livestock species.
672 Camel (●), cow (■), goat (▲) or sheep (▼) DPP4 (green) as well as rhesus macaque (■, red), ferret (■,
673 blue), or mouse (▲, blue) DPP4 were expressed on BHK cells. As a control, human (red) or hamster
674 (blue) DPP4 were expressed on BHK cells. Twenty-four hours post transfection, cells were inoculated
675 with MERS-CoV using an MOI of 1 $TCID_{50}$/cell. Supernatants were harvested at 0, 24, 48 and 72 hpi and
676 viral titers were determined by end-point titration in quadruplicate in VeroE6 cells. Geometric mean titers
677 were calculated from three independent experiments. Error bars indicate standard deviations. B.) BHK
678 cells were left untransfected (red) or transfected with DPP4 (blue) and stained 24 hours post-transfection
679 using α-DPP4 (R&D) and a FITC-conjugated secondary antibody (Life Technologies). Samples were
680 collected using a LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software. C.)
681 Expression of DPP4 mRNA was measured via qRT-PCR. Relative fold increase was calculated by the
682 comparative $C_T$ method (35), where DPP4 expression is normalized to HPRT.
683
684 Figure 7. DPP4 in camel, goat, cow and sheep lung and kidney tissue.
685 IHC was performed on lung and kidney tissues from camel, goat, cow and sheep using an α-DPP4
686 antibody. Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin. IHC images lung:
687 closed arrow = bronchiolar epithelium; open arrow = smooth muscle; asterisk = alveolar macrophage;
688 closed arrowhead = alveolar interstitium. IHC images kidney: closed arrow = renal tubular epithelium;
689 open arrow = glomerular endothelium (magnification, 200x).