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Adaptive Evolution of MERS-CoV to Species Variation in DPP4

Graphical Abstract

Highlights

- MERS-CoV infected cells expressing DPP4 from 16 bat species

- MERS-CoV spike rapidly adapted to species variation in DPP4

- Viral adaptations modified the surface charge of viral spike

- Different routes of spike adaptation enhanced entry with the same DPP4 variant

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In Brief

MERS-CoV is a zoonotic pathogen capable of infecting numerous species. However, our understanding of how this virus adapts to new species remains unclear. Letko et al. experimentally observe several different routes in the stepwise, adaptive evolution of MERS-CoV to a unique host-species variant of the viral receptor.
Adaptive Evolution of MERS-CoV to Species Variation in DPP4

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SUMMARY
Middle East Respiratory Syndrome Coronavirus (MERS-CoV) likely originated in bats and passed to humans through dromedary camels; however, the genetic mechanisms underlying cross-species adaptation remain poorly understood. Variation in the host receptor, dipeptidyl peptidase 4 (DPP4), can block the interaction with the MERS-CoV spike protein and form a species barrier to infection. To better understand the species adaptability of MERS-CoV, we identified a suboptimal species-derived variant of DPP4 to study viral adaption. Passaging virus on cells expressing this DPP4 variant led to accumulation of mutations in the viral spike which increased replication. Parallel passages revealed distinct paths of viral adaptation to the same DPP4 variant. Structural analysis and functional assays showed that these mutations enhanced viral entry with suboptimal DPP4 by altering the surface charge of spike. These findings demonstrate that MERS-CoV spike can utilize multiple paths to rapidly adapt to novel species variation in DPP4.

INTRODUCTION
Since its discovery in 2012, Middle East Respiratory Syndrome Coronavirus (MERS-CoV) has infected over 2,000 people and has a mortality rate of approximately 35% (World Health Organization, 2018WHO; Zaki et al., 2012). The ancestral host species of MERS-CoV remains elusive; however, mounting evidence suggests that the virus most likely originated in bats and passed to humans through multiple zoonotic spill-over events from dromedary camels (Azhar et al., 2014; Haagmans et al., 2014; Ithete et al., 2013; Woo et al., 2012). While species capable of supporting MERS-CoV infection have been studied, the genetic mechanisms underlying cross-species adaptation remain poorly understood.

A primary determinant of viral species-tropism is at the level of host cell entry, which is mediated by MERS-CoV spike protein binding host dipeptidyl peptidase 4 (DPP4) (Ohnuma et al., 2013; Raj et al., 2013). Structural studies have shown that this interaction relies on multiple contact points (Lu et al., 2013; Song et al., 2014; Wang et al., 2013). Our group has previously demonstrated that hamster DPP4 contains variation in these contact points, which prevents MERS-CoV replication (van Doremalen et al., 2014; van Doremalen et al., 2016). Other genetic variation responsible for the glycosylation of DPP4 in mice, hamsters, ferrets, and guinea pigs forms an additional block to the interaction with MERS-CoV spike (Cockrell et al., 2014; Peck et al., 2017). In contrast to small rodents and ferrets, MERS-CoV is capable of utilizing DPP4 from different bat species, and Artibeus jamaicensis bats have been shown to support experimental infection (Cai et al., 2014; Munster et al., 2016).

Here, we investigated the host breadth and adaptability of MERS-CoV. We demonstrate that MERS-CoV can use DPP4 from a diverse range of bat species and can rapidly adapt to variation in DPP4.

RESULTS
Identification of a Semi-Permissive DPP4 Variant
We selected 16 bat species representing a broad taxonomic and geographic distribution to screen for MERS-CoV entry (Figures 1A, S1, and S2). Plasmids encoding the bat DPP4s were transfected into Baby Hamster Kidney fibroblasts (BHK) cells, which are non-permissive to MERS-CoV infection (van Doremalen et al., 2014). All DPP4s supported viral replication to varying degrees; however, Desmodus rotundus DPP4 (dDPP4) was the least permissive to viral replication (Figure 1B). Flow cytometry showed that DPP4 surface expression varied between species but did not correlate with infectivity (Figure S3A). While this may result from variation in antibody recognition, to date, no
commercially available DPP4 antibody recognizes an epitope that is conserved across all species.

Relative to human DPP4, the bat DPP4s contained variation throughout the whole spike-binding region (Figure S3B). However, within the residues, which specifically bind MERS-CoV spike, drDPP4 only differed from human DPP4 in two amino acids at positions 295 and 317 (Figures 1C, 1D, and S3B) (Wang et al., 2013). As drDPP4 was also unique at position 317 (R317Q), we chose to focus on drDPP4. Introducing drDPP4 mutations into human DPP4 reduced viral replication, while mutating drDPP4 to match the amino acids in human DPP4 increased its ability to support MERS-CoV replication (Figure 1E). These data show that residues 295 and 317 largely determine resistance of drDPP4 to MERS-CoV replication.

Viral Adaptation of MERS-CoV to Desmodus Rotundus DPP4

As only two drDPP4 residues influenced MERS-CoV replication, we hypothesized that viral spike may be able to adapt to this variation. To test this, we generated BHK cell lines that stably expressed human DPP4, drDPP4, or blue fluorescent protein (BFP; a negative control) from a lentiviral expression cassette that also included a fluorescent reporter, mCherry, as well as a puromycin selection gene (Figure 2A). These genes were separated by 2A sequences, which allowed for all three to express under the human EF1α promoter (Letko et al., 2015). Flow cytometry for mCherry and DPP4 confirmed similar transduction efficiency and transgene expression levels, respectively, between human and drDPP4 cell lines (Figure 2B). Cells expressing drDPP4 were less susceptible than human DPP4 to MERS-CoV infection, similar to our transfection experiments (Figure 2C).

Wild-type (WT) MERS-CoV was serially passaged, in triplicate, on these DPP4 and control cell lines. While human DPP4 cells showed cytopathic effects (CPE) from the first passage, drDPP4 cells only showed CPE after eight passages, indicative of viral adaptation (Figure 2D). Sanger sequencing of the MERS-CoV spike receptor binding domain (RBD) revealed the emergence of several mutations with the drDPP4 cell cultures (Figure 2E).
By the fifth passage, spike S465F emerged in two replicates, and spike D510H emerged in one replicate (Figure 2F). By passage 8, one S465F mutant acquired a secondary D510G mutation, and on passage 12, the D510H mutant acquired a secondary S465F mutation (Figure 2F).

We sequenced the spike RBD from 14 passages, as well as full-length spike from passages 3, 6, and 9, but did not observe additional mutations. No spike mutations were observed with the human DPP4 or control cell lines, indicating that these mutations were specific for drDPP4 and not the BHK cells themselves.

Spike residues 465 and 510 are located within the RBD and interface with DPP4 (Figures 2G and 2H). Notably, spike residue 510 clusters with DPP4 residues 295 and 317 and directly interacts with DPP4 residue 317 (Figure 2H) (Wang et al., 2013). MERS spike 465S and 510D are found in the majority of published spike sequences (Figure S4A).

Characterization of MERS-CoV Spike Mutations

Human and drDPP4 are 83.8% similar at the amino acid level and both function as the receptor for MERS-CoV, suggesting the two proteins share a common structure. Therefore, we used structural data for human DPP4 to predict the structure of drDPP4. Electrostatic potential analysis showed a positive surface charge of human DPP4 at residues 295 and 317 and a negative charge at these positions in drDPP4 (Figure 3A). Complementary to human DPP4, the WT MERS-CoV spike RBD is negatively charged (Figure 3B). This analysis revealed that the spike adaptations altered the surface charge of spike from negative to positive, complementing the negative charge of drDPP4 (Figure 3C).

Because we only sequenced MERS-CoV spike, it remained possible that other mutations arose in the viral genome that increased viral replication in the drDPP4 cells. Therefore, we generated spike point mutant viruses, which failed to grow on control transduced cells but grew to similar levels as WT virus and induced CPE in the human DPP4 cells (Figures 3D and S4B). In contrast, WT virus replicated to lower titers than the spike mutants and failed to induce CPE in drDPP4 cells (Figures 3D and S4B). Next, we performed an entry assay with spike-pseudotyped vesicular stomatitis virus (VSV) particles expressing luciferase. While all spike mutations slightly increased entry over WT spike on human DPP4 cells, there was a striking
increase in entry with drDPP4 cells (Figure 3E). Collectively, these findings demonstrate that adaptation in MERS-CoV spike enhanced viral replication on drDPP4 cells through increased viral entry.

**Spike Adaptation Specificity**

We transfected cells with a subset of the bat DPP4 panel and then infected with spike mutant viruses, all of which replicated better than WT virus on cells expressing drDPP4 (Figure 4). While some mutants performed better than WT virus on cells expressing other bat DPP4s, there was not a consistent top-performing mutant. Notably, spike-510H only replicated better than WT virus with drDPP4 and not the other bat DPP4s, suggesting that this mutation is specific for drDPP4.

**DISCUSSION**

While numerous studies have shown that MERS-CoV can infect several different species, our understanding of the genetic mechanisms underlying cross species spillover remains unclear (Adney et al., 2014; Agrawal et al., 2015; Cockrell et al., 2016; de Wit et al., 2013; Falzarano et al., 2014; Munster et al., 2016). Here, we investigated species promiscuity and adaptability of MERS-CoV.

Previous studies have shown that MERS-CoV entry is less efficient with DPP4 from pipistrelle bats compared to human DPP4 (Barlan et al., 2014; Raj et al., 2013; Yang et al., 2014). This difference in receptor efficiency was used to suggest that MERS-CoV spike adapted away from using bat DPP4 as it adapted to human DPP4. While our data confirm this replication phenotype with Pipistrellus DPP4, we showed that DPP4 from several other bat species supported MERS-CoV replication comparable to or better than human DPP4 (Figure 1B). Thus, without more evidence as to the true ancestral host of MERS-CoV, conclusions regarding spike adaptation away from bats should be more reserved. This finding also highlights the importance of screening a broad selection of species when assessing host breadth.

A. planirostris and A. jamaicensis DPP4s are identical to human DPP4 at the 14 contact points with spike but differed in their ability to support MERS-CoV replication (Figure 1B). Notably, these two bat DPP4 sequences varied from human DPP4 and from each other at other positions in the receptor binding domain (Figure S3B), suggesting that additional DPP4 residues can influence the interaction with spike.
Forced adaptation of MERS-CoV spike has been a challenge in the field as DPP4 from most non-permissive species contains glycosylation that completely abrogates the interaction and blocks infection (Cockrell et al., 2014; Peck et al., 2015; Peck et al., 2017). Without viral replication, it has not been possible to experimentally demonstrate evolution of the MERS-CoV spike. We found that d\text{r}DPP4 was only semi-permissive to viral infection and, therefore, served as a tool to test MERS-CoV spike adaptation in vitro (Figures 1 and 2). Within three passages on cells expressing d\text{r}DPP4, MERS-CoV accumulated mutations in the receptor-binding domain of spike that enhanced viral entry and replication specifically with d\text{r}DPP4 and not the other bat DPP4s (Figures 2, 3, and 4). The prevalence of these adaptation mutations is low in published sequences, further suggesting that they are specific for d\text{r}DPP4 (Figure S4A). Taken together, MERS-CoV spike is likely adaptable to any DPP4 variation that does not completely ablate the interaction.

At least 14 contact points have been identified in crystal structures of human DPP4 and MERS-CoV spike (Wang et al., 2013). d\text{r}DPP4 contains variation within and around these known points (Figure S3B). Therefore, the spike-465F mutation, which is just outside of the interface, may lead to broader structural adjustments that favor interaction with d\text{r}DPP4 (Figure 2H). Surprisingly, the adaptation mutations had very little effect on the interaction with human DPP4, suggesting that other contact points

**Figure 4. Spike Adaptation Specificity**

BHKs were transfected with indicated DPP4s and infected with mutant viruses at MOI = 0.005. Viral titer of supernatants taken at 72 hr was determined by qRT-PCR. Error bars represent SDs of two replicates.
could compensate for this variation (Figure 3). For example, spike residue 510 has been shown to contact DPP4 residue 322 in addition to 317, so it may be possible that this interaction is retained with human DPP4 (Wang et al., 2013). Structural studies are needed to fully define how these spike mutations influence the interface with DPP4.

Our viral adaptation experiments revealed mutations in MERS-CoV spike, which altered the surface charge to complement the opposite charge of dR/DPP4 (Figure 3C). Interestingly, these mutations share a striking similarity to species adaptations identified in SARS-CoV spike (as reviewed in Li, 2013). The ability to rapidly adjust surface charge may be a general property of MERS-CoV spike to facilitate cross-species adaptation. However, animal studies are needed to see if the mutations we described here affect other viral phenotypes, including host immune evasion.

Previous work has assessed coronavirus spike adaptation to orthologous host receptors (Baric et al., 1999; McRoy and Baric, 2008; Roberts et al., 2007; Sheahan et al., 2008; Wu et al., 2012). These studies characterized viruses after passage in animals, long-term cell culture, or performed biochemical analysis on spike variations from different isolates. As these experiments only focused on the viral endpoint, they failed to capture the continuous evolution through viral passages. Thus, the exact speed and order in which mutations arise in response to receptors from non-cognate species remain unclear in these studies. The work we present here demonstrates that MERS-CoV rapidly adapts to DPP4 variation and can do so utilizing different mutations from non-cognate species remaining unclear in these studies. While D. rotundus is not known to be a host species for MERS-CoV, the use of its semi-permissive DPP4 allowed us to observe, with high temporal resolution, species adaptation of MERS-CoV. All together, these findings shed light on the evolutionary mechanisms underlying MERS-CoV cross-species transmission.

**EXPERIMENTAL PROCEDURES**

**BioSafety Statement**

All work with infectious MERS-CoV was approved by the Rocky Mountain Laboratories Institutional Biosafety Committee and performed under biosafety level 3 conditions.

**Sequencing of Bat DPP4**

cDNA from E. buettikoferi and R. aegyptiacus were derived from the EpoNi/22.1 and RE06 cell lines, respectively. cDNA was produced from A. planirostris, C. perspicillata, and S. bilineata primary lung tissue and H. gigas heart tissue, provided by Dr. Tony Schouzutz. Dr. Lin-Fa Wang provided cDNA extracted from R. fennuqueinum primary kidney tissue. DPP4 was amplified using the iProof High-fidelity PCR kit (Bio-Rad), following the manufacturer’s instructions and Sanger sequencing.

**Plasmids**

DPP4 coding sequences from human (NM001935.3), A. jamaicensis (KF574262), A. planirostris (MH299895), C. perspicillata (MH299896), D. rotundus (GABZ01004546.1), E. buettikoferi (MH299897), E. fuscus (XM008138769), H. gigas (MH299898), M. brandti (XM005859372.1), M. davidii (XM006766490.1), M. lucifugus (XM006083213.1), P. pipistrellus (KC249974.1), P. alecto (XM00692123.1), P. vampyrus (XM011358549), R. fennuqueinum (MH299899), R. aegyptiacus (MH299900), and S. bilineata (MH299901) were synthesized into pcDNA3.1 (Thermo-Fisher). DPP4 was cloned into the lentiviral expression vector provided by Dr. Viviana Simon. MERS-CoV spike plasmid was provided by Dr. Fang Li. Mutations were introduced into DPP4, pBAC-MERS/EMC12, or the MERS-spike plasmid by overlap PCR.

**Viruses**

MERS-CoV/EMC12 was propagated as previously described (van Doremalen et al., 2016). The WT virus stock was deep sequenced on the Illumina platform to confirm the absence of mutations described in this study. Mutant viruses were rescued as previously described (Almazán et al., 2013). Titers of all viruses used in this study were determined by endpoint titration in Vero cells as previously described (van Doremalen et al., 2014).

**Transections**

Cells were transfected, in 6-well format, with 3 μg of DNA using Lipofectamine 2000 (Life Technologies), following the manufacturer’s instructions.

**Lentiviral Vectors, Cell Transduction, and Flow Cytometry**

Lentiviral particles were produced in 293T cells. Transduced BHKs were placed under 1 μg/mL puromycin selection, as described previously (Letko et al., 2015). Flow cytometry was used to determine mCherry transduction efficiency and DPP4 expression (AF1180, R&D Systems).

**Forced Adaptation of MERS-CoV EMC 2012 on Transduced Cells**

Human DPP4, dR/DPP4, and BFP cells were infected with MERS-CoV/EMC12 at an MOI of 0.01 in triplicate. Every 72 hr, 250 μL of supernatant from each replicate was transferred to fresh cell cultures. For each passage, RNA was extracted from supernatant using the QiAamp Viral RNA kit (QiAGEN) and converted to cDNA using SuperScript III (Invitrogen). The spike RBD was amplified and Sanger sequenced. Full-length spike was sequenced for passages 3, 6, and 9.

**Structural Modeling and Electrostatic Potential Analysis**

The co-structure of MERS-CoV spike and human DPP4 (PDB ID: 4L72 [Wang et al., 2013]) was modeled in PyMol and used to predict the structure for dR/DPP4 (https://swissmodel.expasy.org). Poisson-Boltzmann analysis was performed with the PDBePOT solver (Dolinsky et al., 2004) and adaptive Poisson-Boltzmann Solver tool extension in PyMol (Baker et al., 2001).

**MERS-CoV Replication Kinetics**

Viral RNA was extracted from culture supernatants with the QiAamp viral RNA kit (QiAGEN). MERS-CoV titer of cell culture supernatants was determined by qRT-PCR as previously described (Corman et al., 2013).

**Pseudotype Entry Assay**

Pseudotyped VSV particles were produced as previously described and titrated on Vero cells (Takada et al., 1997). BHK-DPP4 cells were infected at an MOI of 1 with pseudotyped particles as previously described (Yang et al., 2014). Luciferase was measured 24 hr post-infection using a firefly luciferase detection kit (Promega) and a Synergy HTX plate reader (Biotek).

**Statistical Methods**

GraphPad (Prism) was used to analyze quantitative viral titer and viral entry data.

**DATA AND SOFTWARE AVAILABILITY**

Accession numbers for the novel DPP4 sequences reported in this paper are GenBank: MH299895-MH299901. DPP4 sequence alignment from this study is available via Mendeley Data: https://data.mendeley.com/datasets/232v2967/.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes four figures and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.07.045.
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AUTHOR CONTRIBUTIONS

M.L., R.M., N.V.D., and V.M designed experiments. M.L., R.M., K.M., S.N.S., A.C., N.V.D., and I.S. performed experiments. M.L. wrote the paper. All authors contributed to the study and provided comments on the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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