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Letter

A natural five-amino-acid insert at the S2' cleavage site of MERS-CoV spike enhances viral membrane fusion

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Dear Editor,

Coronaviruses (CoVs), a large family of single-stranded RNA viruses, cause severe diseases in animals and humans. Among them, severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV), and SARS-CoV-2 have posed great threats to humans. MERS-CoV was firstly isolated from a patient with acute pneumonia in Saudi Arabia (Zaki et al., 2012). Since its discovery in 2012, MERS-CoV has caused over 2600 confirmed cases across 27 countries, with more than 900 deaths, most of them in Saudi Arabia (https://www.emro.who.int/health-topics/mers-cov/mers-outbreaks.html). Nowadays, MERS-CoV is still circulating in the Arabian Peninsula and Africa (Hassan et al., 2025). Two new imported MERS cases with travel history to the Arabian Peninsula were reported in France in 2025 (Casassus, 2025). Recently, a new MERS-like coronavirus from Malayan pangolins, named *Manis javanica* HKU4-related coronavirus (MjHKU4r-CoV), was reported, which uses human dipeptidyl peptidase 4 (DPP4) as its receptor (Chen et al., 2023; Xia et al., 2025).

The spike protein of MERS-CoV consists of two subunits, a receptor binding subunit (S1) and a membrane fusion subunit (S2), which are responsible for binding to the receptor and fusing with the plasma or endosome membrane, respectively (Raj et al., 2013). There are two different cleavage sites in the spike protein of MERS-CoV: the S1/S2 and S2' cleavage sites. The cleavage event at the S1/S2 site occurs first, facilitating the opening of the S2' cleavage site, a site located upstream of the fusion peptide, for further proteolytic cleavage. The S2 subunit can be cleaved at the S2' site by cell surface proteases, such as TMPRSS2, or by cathepsins in the endosome, resulting in the exposure of the fusion peptide for membrane fusion (Millet and Whittaker, 2014; Kleine-Weber et al., 2018). Despite this, it is reported that cleavage of the S1/S2 site is not a prerequisite for the spike of HCoV-229E, and the fusion activation is highly reliant on the S2' region (Bonnin et al., 2018).

An amino-acid insert or point mutation at the cleavage site of coronavirus spike protein usually alters viral tropism, virulence or transmission. A distinct four-amino-acid insert sequence PRRA has been identified at the S1/S2 site in the spike protein of SARS-CoV-2, resulting in a putative functional polybasic cleavage site (Hoffmann et al., 2020). A natural insertion at the S1/S2 cleavage site of the spike protein was also identified in a bat coronavirus closely related to SARS-CoV-2 (Zhou et al., 2020). A camel-derived MERS-CoV with a substitution at the S2' cleavage site of the spike exhibited attenuated spike-mediated fusion and viral entry activity (Millet et al., 2016).

MERS-CoV circulates in dromedary camels across the Arabian Peninsula and Africa and is undergoing evolution (Hassan et al., 2025). We focused on the genetic mutations of the spike proteins from different MERS-CoV variants and found retrospectively that a unique five-amino-acid insert, TSGVF, at the S2' cleavage site of the MERS-CoV/dromedary camel/Egypt/NC270-P9/2015 spike protein (GenBank: UYV90088.1). MERS-CoV/dromedary camel/Egypt/NC270-P9/2015 was isolated from dromedary camels in Egypt from nasal swab samples by a local research group, which is described along with the genome sequence (GenBank: OP654179.1). Phylogenetic analysis based on the sequences of full-length spike proteins revealed that this strain belongs to the clade C, which is dominant in camels in Africa (Fig. 1A). With the exception of the TSGVF insert, the spike protein sequence of MERS-CoV/dromedary camel/NC270-P9 (referred to as MERS-CoV/NC270-P9) is identical to that of MERS-CoV/dromedary camel/Egypt/NC283 (referred to as MERS-CoV/NC283) (GenBank: UYR00344.1), which has no TSGVF insert (Fig. 1B and Supplementary Table S1). Besides, the ORF5 protein sequences from these two variants are different (Supplementary Table S1). Structural prediction of the MERS-CoV/NC270-P9 spike protein revealed that the extruded loop region with the S2' cleavage site is five amino acids longer, making it possibly more exposed and accessible to cellular proteases (Fig. 1C).

We synthesized the coding sequence of the spike protein of MERS-CoV/NC270-P9 and generated an expression vector for the MERS-CoV/NC283 spike protein via subcloning. The expression vector of the MERS-CoV/EMC/2012 spike was used as control. Each spike expression vector

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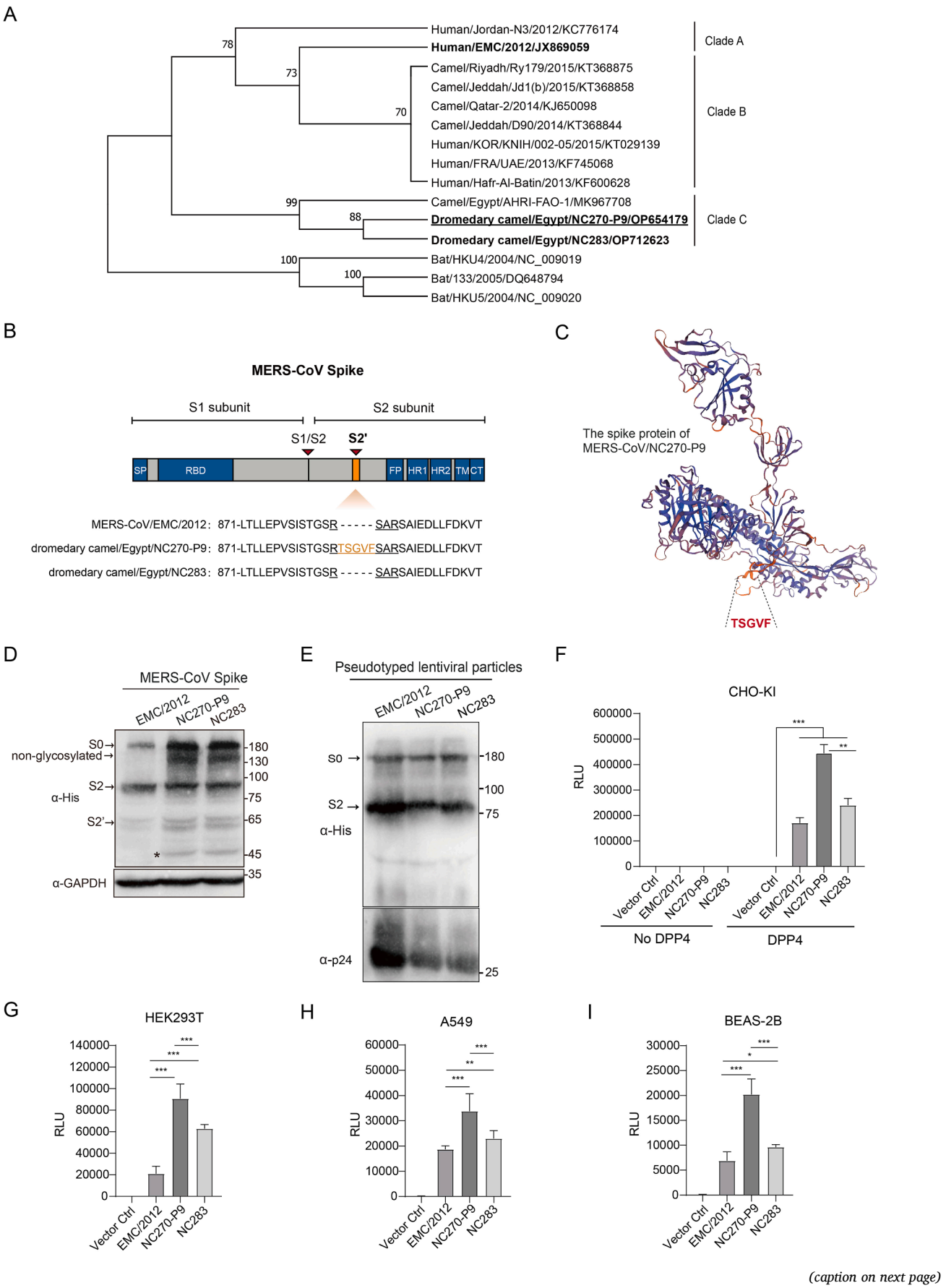

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was transfected into HEK293T cells. In addition to the full-length spike protein (S0), S2 subunit and S2' fragment, an additional cleaved fragment, close to 45 kDa, was observed for the spike proteins of MERS-CoV/NC270-P9 and MERS-CoV/NC283 (Fig. 1D), but not for the spike protein of MERS-CoV/EMC/2012. These results indicate that the spike proteins of MERS-CoV/NC270-P9 and MERS-CoV/NC283 undergo different proteolytic processing during biogenesis.

We further investigated whether the TSGVF insert at the S2' cleavage site affects viral entry. The receptor-binding motif (RBM) of the spike protein from MERS-CoV/NC270-P9 and MERS-CoV/EMC is highly conserved, although a few amino acid substitutions were present across the full spike protein (Supplementary Fig. S1A, S1B), which suggests that MERS-CoV/NC270-P9 also utilizes DPP4 as a receptor. We generated pseudotyped lentiviral particles bearing the MERS-CoV/NC270-P9 spike protein and performed the pseudovirus-based entry assays. The production of pseudotyped lentiviral particles was verified by Western blotting with a p24-specific antibody (Fig. 1E). Exogenous human DPP4 is expressed in Chinese hamster ovary (CHO) cells. Indeed, all of them entered CHO-hDPP4 cells (Fig. 1F), which confirms that MERS-CoV/NC270-P9 utilizes DPP4 as an entry receptor. Moreover, MERS-CoV/NC270-P9 pseudoviruses exhibited a higher entry efficiency, compared with MERS-CoV/NC283, which suggests that the TSGVF insert at the S2' cleavage site of the spike protein increases viral entry. We further performed the pseudovirus-based entry assays in different human cells, including HEK293T, A549, and the immortalized human bronchial epithelial cell line BEAS-2B. Consistently, MERS-CoV/NC270-P9 pseudovirus exhibited higher entry efficiency in all these cells (Fig. 1G–I). These results confirm that the TSGVF insert at the S2' cleavage site of the MERS-CoV spike protein increases viral entry.

The transmembrane serine protease TMPRSS2 is known to cleave the S2' site for MERS-CoV spike activation. We further investigated whether the TSGVF insert at the S2' cleavage site of the spike protein affects TMPRSS2 cleavage. The TMPRSS2 inhibitor Camostat inhibited cell entry of MERS-CoV/NC270-P9 and MERS-CoV/NC283 pseudoviruses in both A549 and HEK293T cells (Fig. 2A, B, Supplementary Fig. S2A, S2B). Moreover, compared with MERS-CoV/NC283, MERS-CoV/NC270-P9 pseudovirus was still able to enter A549 cells in the presence of a high concentration of Camostat, such as 2, 5, or 10 μM, though the inhibition efficiency is comparable at each concentration (Fig. 2A, B, Supplementary Fig. S2A, S2B). We performed the same pseudovirus-based entry assays with the treatment of Camostat in TMPRSS2 overexpressing A549 cells and similar findings were observed (Supplementary Fig. S2C, S2D). MERS-CoV can also utilize a cathepsin-dependent endocytic pathway for entry. Indeed, the cathepsin inhibitor E-64d also inhibited the entry of both MERS-CoV/NC283 and MERS-CoV/NC270-P9 pseudoviruses. MERS-CoV/NC270-P9, but not MERS-CoV/NC283, retained robust entry activity in A549 cells at concentrations of 2 and 5 μM of E-64d, although the inhibition efficiencies on both pseudoviruses are comparable (Fig. 2C,D, Supplementary Fig. S2E–H). These results suggest that the presence of the TSGVF insert at the S2' cleavage site of MERS-CoV/NC270-P9 spike increases virus entry via promoting membrane fusion.

The spike protein of MERS-CoV is known to induce syncytia formation in the presence of its receptor DPP4. We further performed the syncytia formation assays to analyse whether the TSGVF insert at the S2' cleavage site of the MERS-CoV/NC270-P9 spike affects its membrane fusion activity. DPP4, spike and GFP expression vectors were cotransfected into HEK293T cells (Fig. 2E). As shown in Fig. 2F–G, compared with the MERS-CoV/NC283 spike protein, the MERS-CoV/NC270-P9 spike protein more efficiently induced syncytia formation. These results demonstrate that the TSGVF insert at the S2' cleavage site of the MERS-CoV spike enhances its fusogenicity.

An insert at the S1/S2 or S2' cleavage site within the coronavirus spike protein is rare. Previously, a novel genotype of human coronavirus OC43 (HCoV-OC43) with a four-amino-acid insertion in the S2 region just downstream of the S1/S2 cleavage site was believed to be associated with a fatal case of pneumonia (Lau et al., 2022). It's interesting to further investigate experimentally whether such an insert in the S2 region of the HCoV-OC43 spike contributes to the potential hypervirulence. Compared to other beta coronaviruses, the spike protein of MHV-A549 appears to harbor several additional amino-acid residues just upstream of the fusion peptide. This may lengthen the loop region containing the S2' cleavage site and increase its accessibility to cellular proteases (Burkard et al., 2014). In this study, we report that MERS-CoV/dromedary camel/-Egypt/NC270-P9/2015 harbors a TSGVF insert at the S2' cleavage site of the spike protein. The TSGVF insert at the S2' cleavage site of the MERS-CoV spike protein increased membrane fusion activity. From a structural point of view, the loop with this TSGVF insert is five amino acids longer than those of other MERS-CoV spikes, making it more exposed and accessible to cellular proteases, such as TMPRSS2, which may lead to its higher membrane fusion activity. It's reported that a minimal furin cleavage site (FCS) was present just upstream of the fusion peptide, though its functionality is largely unknown (Burkard et al., 2014). The TSGVF insert is present just before the S2' cleavage site, which may disrupt the FCS. However, the S2' cleavage site (RS) in the spike protein of MERS-CoV/NC270-P9 is still intact. The spike protein of MERS-CoV is capable of mediating both cell-free virus infection and cell-cell transmission. The enhanced membrane fusion activity of MERS-CoV/NC270-P9 spike may also promote viral transmissibility. The cleavage pattern differed among MERS-CoV/NC270-P9, MERS-CoV/NC283 and MERS-CoV/EMC/2012 in HEK293T cells, as an additional cleaved fragment of approximately 45 kDa, was observed for the spike proteins of MERS-CoV/NC270-P9 and MERS-CoV/NC283 (Fig. 1D). However, such a difference is not due to the TSGVF insert at the S2' cleavage site, as the MERS-CoV/NC283 spike protein without such an insert also showed this shorter fragment. An additional cleavage site is likely present in the spike proteins of MERS-CoV/NC270-P9 and MERS-CoV/NC283, but not that of MERS-CoV/EMC/2012.

Currently, the origin of this insert at the S2' cleavage site is unknown. Recombination events often occur in coronaviruses, and recombination is believed to be an evolutionary driver of MERS-like coronavirus emergence (Su et al., 2016). The recombination frequency in coronaviruses is high, which occurs largely due to the exchange of functional motif sequences or even entire genes. Though the TSGVF insert at the S2' cleavage site of the MERS-CoV spike protein enhances membrane fusion and entry efficiency, it does not mean that this variant will become a dominant strain or cause more infections in camels. We

Fig. 1. A natural TSGVF insert is present at the S2' cleavage site of the MERS-CoV spike protein. **A** Phylogenetic analysis based on multiple sequence alignment of spike protein sequences from representative MERS-CoV strains (clades A, B, and C) and related bat β-coronaviruses (GenBank accession numbers provided). MERS-CoV/NC270-P9, NC283 and EMC/2012 strains are indicated in bold. **B** Schematic of different MERS-CoV spike proteins. The TSGVF insert within the spike of MERS-CoV/NC270-P9 is highlighted by orange. **C** Structural prediction of the MERS-CoV/NC270-P9 spike protein using the SWISS-MODEL server (https://swissmodel.expasy.org/). The structure of the MERS-CoV spike (SMTL ID: 7x29.1) was used as a model template. **D** Western blotting analysis of His-tagged spike proteins in HEK293T cells with anti-His antibody. The arrows indicate full-length spike (S0), the S2 subunit and S2', respectively. The asterisk indicates the additional cleaved fragment for the spike proteins of MERS-CoV/NC270-P9 and MERS-CoV/NC283. **E** Western blotting analysis of pseudotyped lentiviral particles bearing different spike proteins with anti-p24 and anti-His antibodies. **F** Entry efficiency of different pseudoviruses in CHO–K1 cells expressing human DPP4. CHO-K1 cells transfected with DPP4 or vector control were transduced with pseudotyped lentiviral particles bearing the spike protein of MERS-CoV/NC270-P9, NC283, EMC/2012 or vector control. The cells were lysed for measurement of luciferase activity 48 hours post-transduction. Three technical replicates are included for each treatment. Data represent mean ± SEM. Statistical significance was determined by Student's *t*-test (**$P < 0.01$, ***$P < 0.001$). **G–I** Pseudovirus-based entry assays in HEK293T, A549 and BEAS-2B cells. Forty-eight hours post-transduction, luciferase activity was measured. The pseudovirus-based entry assays were performed in biological triplicates in HEK293T cells and repeated twice in A549 and BEAS-2B cells. Bars show mean ± SEM in triplicate. Statistical significance was determined by Student's *t*-test: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

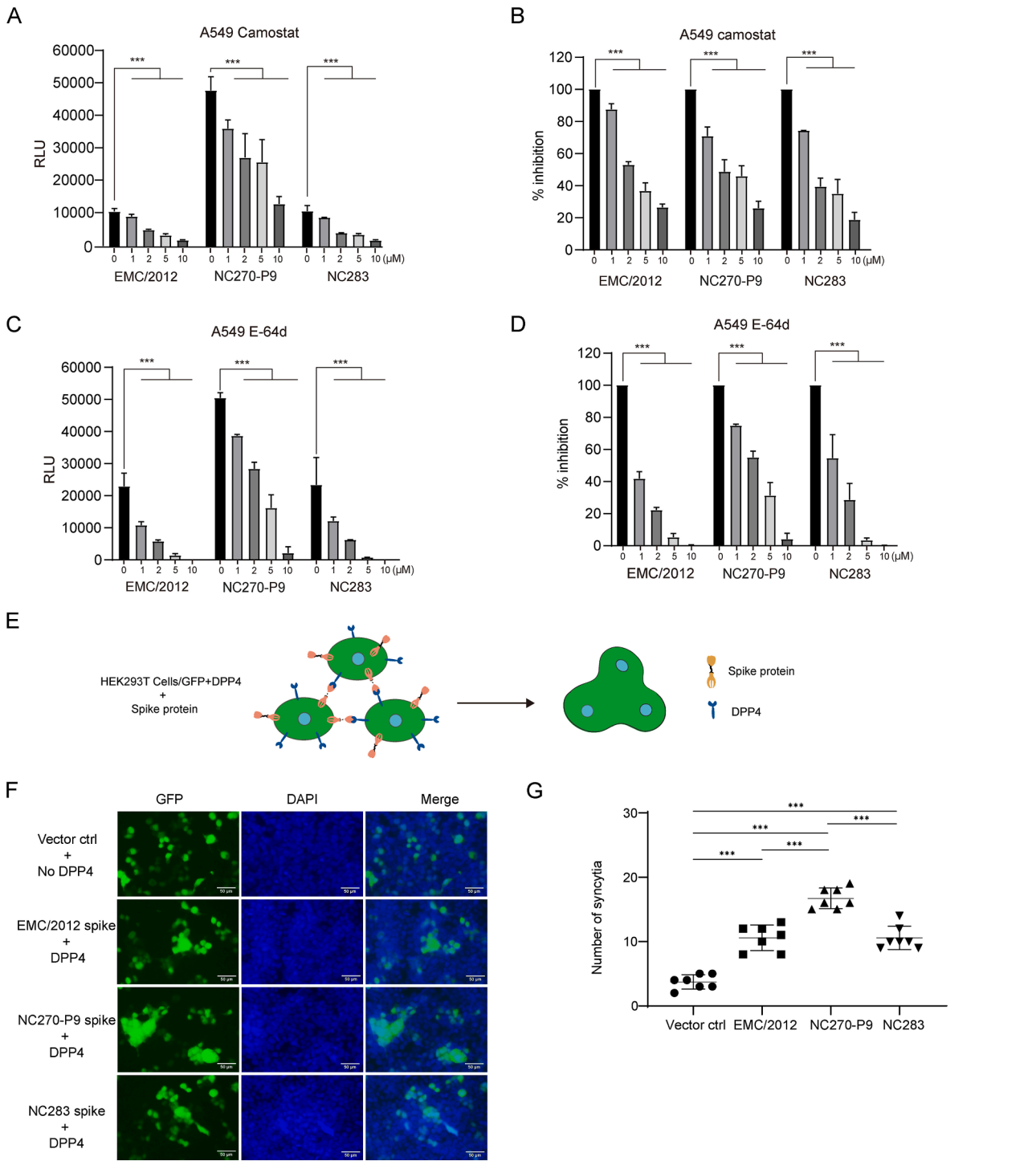

Fig. 2. The TSGVF insert at the S2' cleavage site of the MERS-CoV/NC270-P9 spike enhances membrane fusion. **A, B** A549 cells were pre-treated with Camostat at different concentrations for 1 h, followed by incubation with MERS-CoV pseudoviruses for 5 h. The cells were then cultured in fresh medium containing Camostat. Forty-eight hours later, the cells were lysed for measurement of luciferase activity and normalized to untreated control (set as 100 %). **C, D** Similarly, A549 cells were treated with E-64d (0, 1, 2, 5, and 10 μM), and luciferase activity was measured. The inhibitor treatment and pseudovirus-based entry assays were performed in biological triplicates. The bars show the mean ± SEM of triplicate samples. Data were analyzed by Student's *t*-test (***$P < 0.001$). **E** Schematic diagram illustrating spike protein-mediated syncytium formation. **F** HEK293T cells co-transfected with GFP and DPP4 expression vectors were subsequently transfected with spike protein vectors. Forty-eight hours after transfection, the cells were fixed, stained with DAPI, and imaged by fluorescence microscopy. Scale bar = 50 μm. **G** Quantification of syncytia from seven randomly selected microscopic fields per group. Data were analyzed by Student's *t*-test (***$P < 0.001$).

did not find more MERS-CoV variants harboring such an insert in the database. Usually, for the purpose of virus surveillance, the camels are sampled for detection of the nucleocapsid or RdRp (RNA-dependent RNA polymerase) gene, instead of the spike gene. Therefore, few spike protein sequences from MERS-CoV variants were obtained and stored in the database. Currently, three MERS-CoV clades are identified: clade A, B, and C. The MERS-CoV/dromedary camel/Egypt/NC270-P9/2015 strain belongs to the clade C. In Africa, the clade C is dominant in

camels, and human cases are rare. The absence of zoonotic MERS-CoV in Africa is still enigmatic, though more than 70% of MERS-CoV infected dromedaries are found in Africa (Karani et al., 2025). As there is no more epidemiological data regarding the MERS-CoV/NC270-P9/2015 variant, we are unable to establish a correlation between the spike insert and MERS-CoV zoonoses. One of the limitations of this study is that we have not verified the role of the TSGVF insert at the S2' cleavage site in enhancing membrane fusion with authentic virus. Generation of isogenic MERS-CoVs via reverse genetics is largely not permitted. In this study, we focus on the role of the 5-aa insert in spike protein-mediated cell entry. The pseudovirus platform is widely used as a surrogate for authentic virus to mimic the initial stage of virus infection, although it cannot complete a full replication cycle. It's valuable to systematically investigate the replication fitness and transmission of MERS-CoV/NC270-P9/2015 with live virus both *in vitro* and *in vivo*.

In summary, our study reports a natural occurrence of TSGVF insert at the spike S2' cleavage site, which enhances viral membrane fusion and pseudovirus entry efficiency. This study also highlights the importance of surveillance for MERS-CoV variants in camels both in the Arabian Peninsula and Africa.

FOOTNOTES

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All data relevant to the study are included in the article or uploaded as supplementary information. Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2026.04.006.

REFERENCES

Bonnin, A., Danneels, A., Dubuisson, J., Goffard, A., Belouzard, S., 2018. HCoV-229E spike protein fusion activation by trypsin-like serine proteases is mediated by proteolytic processing in the S2' region. J. Gen. Virol. 99, 908–912.

Burkard, C., Verheije, M.H., Wicht, O., van Kasteren, S.I., van Kuppeveld, F.J., Haagmans, B.L., Pelkmans, L., Rottier, P.J.M., Bosch, B.J., de Haan, C.A.M., 2014. Coronavirus cell entry occurs through the Endo-/Lysosomal pathway in a proteolysis-dependent manner. PLoS Pathog. 10, e1004502.

Casassus, B., 2025. MERS: france confirms first cases in 12 years. BMJ Br. Med. J. (Clin. Res. Ed.) 391, r2577.

Chen, J., Yang, X.L., Si, H.R., Gong, Q.C., Que, T.C., Li, J., Li, Y., Wu, C.G., Zhang, W., Chen, Y., Luo, Y., Zhu, Y., Li, B., Luo, D.S., Hu, B., Lin, H.F., Jiang, R.D., Jiang, T.T., Li, Q., Liu, M.Q., Xie, S.Z., Su, J., Zheng, X.S., Li, A., Yao, Y.L., Yang, Y., Chen, P.Y., Wu, A.Q., He, M.H., Lin, X.H., Tong, Y.G., Hu, Y.L., Shi, Z.L., Zhou, P., 2023. A bat MERS-like coronavirus circulates in pangolins and utilizes human DPP4 and host proteases for cell entry. Cell 186, 850–863.

Hassan, A.M., Mühlemann, B., Al-Subhi, T.L., Rodon, J., El-Kafrawy, S.A., Memish, Z., Melchert, J., Bleicker, T., Mauno, T., Perlman, S., Zumla, A., Jones, T.C., Müller, M. A., Corman, V.M., Drosten, C., Azhar, E.I., 2025. Ongoing evolution of Middle East respiratory syndrome Coronavirus, Saudi Arabia, 2023-2024. Emerg. Infect. Dis. 31, 57–65.

Hoffmann, M., Kleine-Weber, H., Schroeder, S., Krüger, N., Herrler, T., Erichsen, S., Schiergens, T.S., Herrler, G., Wu, N.H., Nitsche, A., Müller, M.A., Drosten, C., Pöhlmann, S., 2020. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. Cell 181, 271–280.

Karani, A., Ombok, C., Situma, S., Breiman, R., Mureithi, M., Jaoko, W., Njenga, M.K., Ngere, I., 2025. Low-level zoonotic transmission of clade C MERS-CoV in Africa: insights from scoping review and cohort studies in hospital and community settings. Viruses-Basel 17, 125.

Kleine-Weber, H., Elzayat, M.T., Hoffmann, M., Pöhlmann, S., 2018. Functional analysis of potential cleavage sites in the MERS-coronavirus spike protein. Sci. Rep. 8, 16597.

Lau, S.K.P., Li, K.S.M., Li, X., Tsang, K.Y., Sridhar, S., Woo, P.C.Y., 2022. Fatal pneumonia associated with a novel genotype of human coronavirus OC43. Front. Microbiol. 12, 795449.

Millet, J.K., Goldstein, M.E., Labitt, R.N., Hsu, H.L., Daniel, S., Whittaker, G.R., 2016. A camel-derived MERS-CoV with a variant spike protein cleavage site and distinct fusion activation properties. Emerg. Microb. Infect. 5, e126.

Millet, J.K., Whittaker, G.R., 2014. Host cell entry of Middle East respiratory syndrome coronavirus after two-step, furin-mediated activation of the spike protein. Proc. Natl. Acad. Sci. USA. 111, 15214–15219.

Raj, V.S., Mou, H.H., Smits, S.L., Dekkers, D.H.W., Müller, M.A., Dijkman, R., Muth, D., Demmers, J.A.A., Zaki, A., Fouchier, R.A.M., Thiel, V., Drosten, C., Rottier, P.J.M., Osterhaus, A.D.M.E., Bosch, B.J., Haagmans, B.L., 2013. Dipeptidyl peptidase 4 is a functional receptor for the emerging human coronavirus-EMC. Nature 495, 251–254.

Su, S., Wong, G., Shi, W.F., Liu, J., Lai, A.C.K., Zhou, J.Y., Liu, W.J., Bi, Y.H., Gao, G.F., 2016. Epidemiology, genetic recombination, and pathogenesis of coronaviruses. Trends Microbiol. 24, 490–502.

Xia, S., Jiao, F.K., Chen, J., Wang, L.J., Lu, T.Y., Wang, Q., Xu, W., Wang, X.L., Sun, F., Zhi, Y., Zhou, P., Jiang, S.B., Lu, L., 2025. MERS-related coronavirus circulating in pangolins exhibits strong fusogenicity in human cells and high sensitivity to fusion inhibitors. Cell Rep. Med. 6, 102277.

Zaki, A.M., van Boheemen, S., Bestebroer, T.M., Osterhaus, A.D.M.E., Fouchier, R.A.M., 2012. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. N. Engl. J. Med. 367, 1814–1820.

Zhou, H., Chen, X., Hu, T., Li, J., Song, H., Liu, Y.R., Wang, P.H., Liu, D., Yang, J., Holmes, E.C., Hughes, A.C., Bi, Y.H., Shi, W.F., 2020. A novel bat coronavirus closely related to SARS-CoV-2 contains natural insertions at the S1/S2 cleavage site of the spike protein (vol 30, 2196.e1, 2020). Curr. Biol. 30, 3896, 3896.