Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Cell
Leading Edge

CellPress

Letter

Evidence in favor of the essentiality of human cell membrane-bound ACE2 and against soluble ACE2 for SARS-CoV-2 infectivity

Daniel Batlle,[1,*] Vanessa Monteil,[2] Elena Garreta,[3] Luise Hassler,[1] Jan Wysocki,[1] Vasuretha Chandar,[4] Robert E. Schwartz,[4,5] Ali Mirazimi,[2,6] Nuria Montserrat,[3,7,8] Michael Bader,[9,10,11,12] and Josef M. Penninger[13,14,*]

[1]Division of Nephrology/Hypertension, Department of Medicine, Northwestern University, Feinberg School of Medicine, Chicago, IL, USA
[2]Karolinska Institute and Karolinska University Hospital, Department of Laboratory Medicine, Unit of Clinical Microbiology, 17177 Stockholm, Sweden
[3]Pluripotency for Organ Regeneration, Institute for Bioengineering of Catalonia (IBEC), The Barcelona Institute of Science and Technology (BIST), Barcelona, Spain
[4]Division of Gastroenterology and Hepatology, Department of Medicine, Weill Cornell Medicine, 1300 York Avenue, New York, NY 10065, USA
[5]Department of Physiology, Biophysics and Systems Biology, Weill Cornell Medicine, 1300 York Avenue, New York, NY 10065, USA
[6]National Veterinary Institute, 751 89 Uppsala, Sweden
[7]Catalan Institution for Research and Advanced Studies (ICREA), Barcelona, Spain
[8]Centro de Investigación Biomédica en Red en Bioingeniería, Biomateriales y Nanomedicina, Madrid, Spain
[9]Max Delbrück Center for Molecular Medicine in the Helmholtz Association (MDC), Berlin, Germany
[10]German Center for Cardiovascular Research (DZHK), Berlin, Germany
[11]Charité Universitätsmedizin Berlin, Freie Universität Berlin and Humboldt-Universität zu Berlin, Berlin, Germany
[12]University of Lübeck, Institute for Biology, Lübeck, Germany
[13]Institute of Molecular Biotechnology of the Austrian Academy of Sciences (IMBA), Vienna, Austria
[14]Department of Medical Genetics, Life Sciences Institute, University of British Columbia, Vancouver, BC, Canada
*Correspondence: d-batlle@northwestern.edu (D.B.), josef.penninger@ubc.ca (J.M.P.)
https://doi.org/10.1016/j.cell.2022.05.004

A recent paper in *Cell* entitled "Soluble ACE2-mediated cell entry of SARS-CoV-2 via interaction with proteins related to the renin-angiotensin system" by Yeung et al. (2021) reported that the secretory form of soluble angiotensin converting enzyme 2 (ACE2), can foster SARS-CoV-2 infectivity (Yeung et al., 2021). ACE2 in its cell membrane-bound form is the main receptor for SARS-CoV-2 cell entry. Yeung et al. (2021), however, reported that, in a permissive human cell line (HK2-cells), exposure to very low concentrations of soluble ACE2 promotes SARS-CoV-2 infectivity, whereas higher concentrations had a neutralizing effect. The neutralizing effect of high concentrations of soluble ACE2 on SARS-CoV-2 infectivity indeed is confirmatory of previous reports in permissive human cell lines and human kidney and vascular organoids (Monteil et al., 2020; Wysocki et al., 2021). As a consequence, studies have taken advantage of this decoy action of soluble ACE2 proteins to prevent and attenuate SARS-CoV-2 infectivity (Hassler et al., 2022). If correct, the conclusion of Yeung et al. (2021) that low concentrations of soluble ACE2 actually promote SARS-CoV-2 infectivity would have important implications for the understanding of the role of soluble versus cell membrane-bound ACE2 as a receptor essential for SARS-CoV-2 entry into the cell. Such conclusion could also have a potential negative impact on therapeutic approaches based on administration of soluble ACE2 proteins that exert a decoy effect and even for the design of vaccines targeted to the receptor binding domain of the spike protein of SARS-CoV-2 that binds to ACE2. We disagree with the conclusion of Yeung et al. (2021) that low soluble ACE2 concentrations increase SARS-CoV-2 infectivity for the theoretical reasons outlined below and based on the data that we report here (Figure S1).

ACE2 exists in two forms: a full-length form that is cell membrane bound and a shorter soluble form that is shed into body fluids and that normally circulates in the blood in a small amount. Both forms contain the same sequence used by the receptor binding domain of the SARS-CoV-2 spike protein, but soluble ACE2 lacks the transmembrane domain necessary for anchoring in the cell membrane (Tipnis et al., 2000). The current understanding of the SARS-CoV-2-ACE2 interaction is that, after binding of SARS-CoV-2 spike to the membrane-bound full-length ACE2, activation via transmembrane serine protease 2 (TMPRSS2) results in cell entry and viral replication. The full-length form of ACE2 is present in epithelial cells in the upper respiratory tract and in type 2 pulmonary cells in alveoli and also expressed in the kidney, heart, blood vessels, and intestine (Ziegler et al., 2020). It is the interaction with full-length ACE2 in cells of the upper respiratory tract that facilitates cell entry of SARS-CoV-2 particles (Ziegler et al., 2020). The question we wished to address after the Yeung et al. (2021) paper is whether there is any effect of low levels of soluble ACE2 that could promote SARS-CoV-2 infectivity.

ACE2 is a monocarboxypeptidase that cleaves angiotensin II to form angiotensin (1-7) and several other peptides (Tipnis et al., 2000). Its physiologic role is to metabolize these peptides and therefore prevent their accumulation, primarily at the local tissue level. For instance, in experimental lung injury, angiotensin II levels increase and the deficiency of ACE2 has a detrimental effect, while the administration of soluble ACE2 protein is

protective, respectively (Imai et al., 2005). Any loss of cell membrane-bound full-length ACE2 as a result of SARS-CoV-2 infection would favor angiotensin II levels to increase, which may cause inflammation and worsening of lung injury (Imai et al., 2005). It should also be noted that there are reports that soluble ACE2 in plasma is moderately increased in patients with cardiovascular disease and also in patients with COVID-19 (Ramchand and Burrell, 2020; Kragstrup et al., 2021). The significance of increased levels of soluble ACE2 in plasma in these conditions is not fully understood but likely reflects shedding of membrane-bound ACE2 or cell-death-mediated ACE2 release in these pathological conditions. The level of plasma-soluble ACE2 in humans and those with cardiovascular disease or with COVID-19 could be roughly in the range of the very low concentrations used in the study of Yeung et al. (2021). This could be erroneously interpreted to signify that the virus could utilize soluble ACE2 for entry into host cells. Binding of SARS-CoV-2 to soluble ACE2, however, should not foster infectivity *in vivo* because soluble ACE2, unlike membrane-bound ACE2, lacks the transmembrane domain essential for anchoring into the cell membrane (Tipnis et al., 2000). Therefore, there is no sound rationale for the circulating form of soluble ACE2 to foster SARS-CoV-2 infectivity at any concentration because when a complex of soluble ACE2 and SARS-CoV-2 is formed it could not be internalized to initiate replication within the cell.

Notwithstanding the forgoing considerations, it is clearly important to validate or refute the findings of Yeung et al. (2021) using low concentrations of soluble ACE2 protein. Their conclusions were derived from studies using a human kidney cell line (HK-2) that is susceptible to SARS-CoV-2 infection. The data reported in Figure S3 of their paper showed that low concentrations of 0.1, 1, and 100 ng/mL soluble ACE2 increased SARS-CoV-2 RNA levels above control. We used the same HK-2 human cell line in an effort to replicate the same protocol (details provided to us by the editors of *Cell*, since they were lacking in the original paper [Yeung et al., 2021]). HK-2 cells were pre-treated with soluble ACE2 for 24 h (Figure S1A). This was followed by infection of the HK-2 cells for 1 h with SARS-CoV-2 Wuhan strain isolated in Sweden (GenBank: MT093571). Exposure to exactly the same low concentrations of soluble ACE2 and experimental conditions as in the Yeung et al. paper (Yeung et al., 2021) did not result in any apparent change in infectivity assessed by qRT-PCR and expressed as % of mock treated control (Figure S1A). Since there were more than two groups, the data were analyzed by one-way ANOVA and, when significant, followed by post-hoc Dunnett's multiple comparisons test. This is the proper statistical analysis, and we note that this was not done by Yeung et al. (2021) or previous work by Monteil et al. (2020) who also used a paired t test. Yeung et al. (2021) used soluble ACE2 protein purchased from Sigma (Cat#SAE0064) which, like our protein (see Supplemental methods), has amino acids 18–740 and dimerizes via its collectrin-like domain (Yan et al., 2020). Thus, we could not replicate their findings using the same cell type, protocol, and equally low concentrations of soluble ACE2 (Figure S1A).

Perhaps more relevant to human disease are our findings using human lung and kidney organoids to assess the effect of soluble ACE2 concentrations in models that better replicate organ physiology and pathophysiology. The experiments reported here in these models were performed by different groups of investigators, independently of each other. We used lung organoids of human embryonic origin that closely resemble the target cells of SARS-CoV-2 entry into the body. These organoids express not only the receptor ACE2 but also the protease TMPRSS2, needed for activation and subsequent internalization (see Supplemental methods). In the lung organoids, we tested different soluble ACE2 concentrations and assessed infectivity based not only on RNA levels measured by qRT-PCR but also using plaque assay. None of the tested low soluble ACE2 concentrations, including 10 ng/mL as used by Yeung et al. (2021), had any significant effect on SARS-CoV-2 infectivity as compared to PBS controls (Figures S1B and S1C). By contrast, higher concentrations had the expected and previously reported strong neutralizing effect (Monteil et al., 2020; Wysocki et al., 2021), which was also found by Yeung et al. in HK-2 cells (Yeung et al., 2021).

In addition to these lung organoid experiments, we used different types of human kidney organoids. One was derived from wild-type pluripotent stem cells and the others from two different ACE2 knockout (KO) cell lines (Garreta et al., 2021). The kidney organoids derived from the ACE2 KO cell lines are unique in that they do not express either soluble or membrane-bound full-length ACE2. These ACE2 KO human kidney organoids, therefore, cannot be infected by SARS-CoV-2, as shown by lack of any detectable nucleoprotein (Garreta et al., 2021). Thus, this is an ideal genetic model system because there is no endogenous ACE2 expressed that could bias the experimental data; hence the proposed enhancement of SARS-CoV-2 by low-dose soluble ACE2 proteins would require the presence of a receptor(s) other than ACE2 as proposed by Yeung et al. (2021). When ACE2 KO human kidney organoids were exposed to SARS-CoV-2, however, we again did not detect enhanced infectivity with the low concentrations of soluble ACE2 (including 10 and 100 ng/mL, Figures S1D and S1E). A higher concentration of soluble ACE2 also had no effect on infectivity in these ACE2 KO human kidney organoids, whereas as expected, it inhibited infection in orthologous wild-type kidney organoids (Figures S1D–S1F). In wild-type kidney organoids, low concentrations of soluble ACE2 had no effect on infectivity, whereas high concentrations had the expected suppressive effect (Figure S1F). In the aggregate, our findings provide strong evidence for an essential requirement of full-length membrane-bound ACE2 for SARS-CoV-2 infectivity and also show a lack of effect of soluble ACE2 in the absence or presence of the membrane-bound full-length ACE2 receptor.

In summary, the findings we report here show that soluble ACE2 when applied at low concentrations does not promote a significant increase of SARS-CoV-2 infectivity in any of the cell models studied. Importantly, in the absence of membrane-bound full-length

ACE2, kidney organoids cannot be infected with SARS-CoV-2. A faithful model of severe SARS-CoV-2 infection in mice using ACE2 mutant animals, moreover, supports the evidence for the essentiality of ACE2 as the SARS-CoV-2 receptor here reported (Gawish et al., 2022). Based on our findings, including the use of the kidney organoid model of ACE2 deficiency to reduce experimental bias, we conclude that full-length membrane-bound ACE2 is the essential determinant for infectivity by SARS-CoV-2 and that the low levels of soluble ACE2 protein, that may correspond roughly to those present *in vivo*, cannot promote infectivity.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cell.2022.05.004.

ACKNOWLEDGMENTS

D.B. was supported by a gift from the Joseph and Bessie Feinberg Foundation and grant 1R21 AI166940-01. R.E.S. was supported by NCI R01CA234614, NIAID 2R01AI107301, and NIDDK R01DK121072, Department of Medicine, Weill Cornell Medicine and is an Irma Hirschl Trust Research Award Scholar. N.M. has been supported by the project COV20/00278 from Instituto de Salud Carlos III and from the Ayudas Fundación BBVA a Equipos de Investigación Científica SARS-CoV-2 years COVID-19. A.M., N.M., and J.M.P. received funding from the Innovative Medicines Initiative two Joint Undertaking (JU) under grant agreement no. 101005026, J.M.P. received funding from the T. von Zastrow foundation, the FWF Wittgenstein award (Z 271-B19), the Austrian Academy of Sciences and the Canada 150 Research Chairs Program F18-01336 as well as the Canadian Institutes of Health Research COVID-19 grants F20-02343 and F20-02015. L.H. has received a stipend from the biomedical education program (BMEP) in support of her research scholarship at Northwestern in Chicago, USA.

DECLARATION OF INTERESTS

D.B. and J.W. are coinventors of patents entitled "Active Low Molecular Weight Variants of Angiotensin Converting Enzyme 2," "Active low molecular weight variants of Angiotensin Converting Enzyme 2 (ACE2) for the treatment of diseases and conditions of the eye," and "Soluble ACE2 Variants and Uses Therefor," which includes the use of prevention and treatment of COVID-19. D.B. is founder of Angiotensin Therapeutics Inc. D.B. has received consulting fees from AstraZeneca, Relypsa, and Tricida, all unrelated to this work. J.W. reports scientific advisor capacity for Angiotensin Therapeutics Inc. R.E.S. is on the scientific advisory board of Miromatrix Inc and is a consultant and speaker for Alnylam Inc. J.M.P. is shareholder of Apeiron Biologics that is developing soluble ACE2 for COVID-19 therapy.

REFERENCES

Garreta, E., Prado, P., Stanifer, M., Monteil, V., del Pozo, C.H., Ullate-Agote, A., Vilas-Zornoza, A., Romero, J.P., Jonsson, G., Oria, R., et al. (2021). A diabetic *milieu* increases cellular susceptibility to SARS-CoV-2 infections in engineered human kidney organoids and diabetic patients. Preprint at bioRxiv. https://doi.org/10.1101/2021.08.13.456228.

Gawish, R., Starkl, P., Pimenov, L., Hladik, A., Lakovits, K., Oberndorfer, F., Cronin, S.J., Ohradanova-Repic, A., Wirnsberger, G., Agerer, B., et al. (2022). ACE2 is the critical in vivo receptor for SARS-CoV-2 in a novel COVID-19 mouse model with TNF- and IFNgamma-driven immunopathology. Elife, e74623. https://doi.org/10.7554/eLife.74623.

Hassler, L., Wysocki, J., Gelarden, I., Sharma, I., Tomatsidou, A., Ye, M., Gula, H., Nicoleascu, V., Randall, G., Pshenychnyi, S., et al. (2022). A novel soluble ACE2 protein provides lung and kidney protection in mice susceptible to Lethal SARS-CoV-2 infection. J. Am. Soc. Nephrol. https://doi.org/10.1681/asn.2021091209.

Imai, Y., Kuba, K., Rao, S., Huan, Y., Guo, F., Guan, B., Yang, P., Sarao, R., Wada, T., Leong-Poi, H., et al. (2005). Angiotensin-converting enzyme 2 protects from severe acute lung failure. Nature *436*, 112–116. https://doi.org/10.1038/nature03712.

Kragstrup, T.W., Singh, H.S., Grundberg, I., Nielsen, A.L.-L., Rivellese, F., Mehta, A., Goldberg, M.B., Filbin, M.R., Qvist, P., and Bibby, B.M. (2021). Plasma ACE2 predicts outcome of COVID-19 in hospitalized patients. PloS one *16*, e0252799. https://doi.org/10.1371/journal.pone.0252799.

Monteil, V., Kwon, H., Prado, P., Hagelkruys, A., Wimmer, R.A., Stahl, M., Leopoldi, A., Garreta, E., Hurtado Del Pozo, C., Prosper, F., et al. (2020). Inhibition of SARS-CoV-2 infections in engineered human tissues using Clinical-Grade soluble human ACE2. Cell *181*, 905–913.e7. https://doi.org/10.1016/j.cell.2020.04.004.

Ramchand, J., and Burrell, L.M. (2020). Circulating ACE2: a novel biomarker of cardiovascular risk. Lancet *396*, 937–939. https://doi.org/10.1016/s0140-6736(20)32011-0.

Tipnis, S.R., Hooper, N.M., Hyde, R., Karran, E., Christie, G., and Turner, A.J. (2000). A human homolog of angiotensin-converting enzyme: cloning and functional expression as a captopril-insensitive carboxypeptidase. J. Biol. Chem. *275*, 33238–33243. https://doi.org/10.1074/jbc.M002615200.

Wysocki, J., Ye, M., Hassler, L., Gupta, A.K., Wang, Y., Nicoleascu, V., Randall, G., Wertheim, J.A., and Batlle, D. (2021). A novel soluble ACE2 Variant with prolonged duration of action neutralizes SARS-CoV-2 infection in human kidney organoids. J. Am. Soc. Nephrol. *32*, 795–803. https://doi.org/10.1681/asn.2020101537.

Yan, R., Zhang, Y., Li, Y., Xia, L., Guo, Y., and Zhou, Q. (2020). Structural basis for the recognition of SARS-CoV-2 by full-length human ACE2. Science *367*, 1444–1448. https://doi.org/10.1126/science.abb2762.

Yeung, M.L., Teng, J.L.L., Jia, L., Zhang, C., Huang, C., Cai, J.P., Zhou, R., Chan, K.H., Zhao, H., Zhu, L., et al. (2021). Soluble ACE2-mediated cell entry of SARS-CoV-2 via interaction with proteins related to the renin-angiotensin system. Cell *184*, 2212–2228.e12. https://doi.org/10.1016/j.cell.2021.02.053.

Ziegler, C.G.K., Allon, S.J., Nyquist, S.K., Mbano, I.M., Miao, V.N., Tzouanas, C.N., Cao, Y., Yousif, A.S., Bals, J., Hauser, B.M., et al. (2020). SARS-CoV-2 receptor ACE2 is an Interferon-Stimulated Gene in human Airway epithelial cells and is detected in Specific cell Subsets across tissues. Cell *181*, 1016–1035.e19.