



Human pluripotent stem cell-derived functional sympathetic neurons express ACE2 and RAAS components: a framework for studying the effect of COVID-19 on sympathetic responsiveness

Hsueh-Fu Wu^{1,2} · Chia-Wei Huang^{2,3} · Kanupriya R. Daga^{4,5} · Ross A. Marklein^{4,5} · Natalia Ivanova^{1,6} · Nadja Zeltner^{1,2,7} 

Received: 23 September 2021 / Accepted: 30 December 2021 / Published online: 29 January 2022
© The Author(s), under exclusive licence to Springer-Verlag GmbH Germany 2022

Since its onset in 2019, SARS-CoV-2/COVID-19 has had a devastating impact on the entire world. Respiratory and cardiovascular symptoms, such as dyspnea and arrhythmias, are the most common life-threatening outcomes after COVID-19 infection. These symptoms can be amplified by comorbidities, including chronic obstructive pulmonary disease (COPD), heart failure, renal damage, obesity, and metabolic diseases. It has been suggested that COVID-19 symptoms, comorbidities, and mortality are associated with dysregulation and overactivity of the sympathetic nervous system (SNS) [1]. Additionally, patients with so-called long-COVID (post-acute COVID-19) suffer from palpitations and orthostatic intolerance syndrome [2] that could be caused by a disruption of the autonomic nervous system. SARS-CoV-2 infection may thus trigger additional SNS discharge, perpetuating these problems. However, it has not yet been shown whether and how the virus impacts sympathetic neuron (symN) functions. Here, we use human pluripotent stem

cell (hPSC)-derived symNs [3] to demonstrate that the cellular components that are necessary to trigger SARS-CoV-2 infection are present on symNs and contribute to imbalance of the renin–angiotensin–aldosterone system (RAAS), thus potentially exacerbating COVID-19 symptoms.

The main entrance for SARS-CoV-2 into cells is the angiotensin-converting enzyme 2 (ACE2, Supp. Fig. 1a), which is part of the RAAS that is responsible for regulating blood volume and systemic vascular resistance. The RAAS consists of two antagonistic pathways: (1) ACE1/angiotensin II (Ang II)/angiotensin receptor 1/2 (AGTR_{1/2}) activation, which leads to inflammation, vasoconstriction, and cell death and triggers hypertension and cardiopulmonary disease, and (2) ACE2/angiotensin (1–7) (Ang 1–7)/Mas receptor (MasR), which activates cell protective and vasodilatory effects and protects against hypertension and cardiopulmonary disease (Supp. Fig. 1b). The balance of these pathways within the RAAS is crucial, and its disruption has been associated with hypertension, inflammation, cardiovascular disease, and hyperactivity of the SNS [4]. SARS-CoV-2 generally infects cells by binding and internalizing ACE2, which reduces available receptor levels on the cell surface. Thus, it negatively skews the RAAS balance, resulting in increased Ang II levels and inflammatory signaling, which triggers SNS hyperactivation [4] and hypertension, both of which worsen COVID-19 symptoms [2].

Expression levels of ACE2 have been assessed in various tissues, including lung, heart, intestine, and brain, and have been found to be correlated with SARS-CoV-2 infection-related tissue damage [5]. Interestingly, the role of the SNS in SARS-CoV-2 infection and symptom perpetuation has not been investigated to date, despite its role as a regulator of organ homeostasis. Sympathetic hyperactivation has been reported in the aging population and in people with pre-existing comorbidities such as chronic lung disease and hypertension, as well as in SARS-CoV-2 infected patients

✉ Nadja Zeltner
nadja.zeltner@uga.edu

¹ Center for Molecular Medicine, University of Georgia, 325 Riverbend Road, Athens, GA 30602, USA

² Department of Biochemistry and Molecular Biology, Franklin College of Arts and Sciences, University of Georgia, Athens, GA, USA

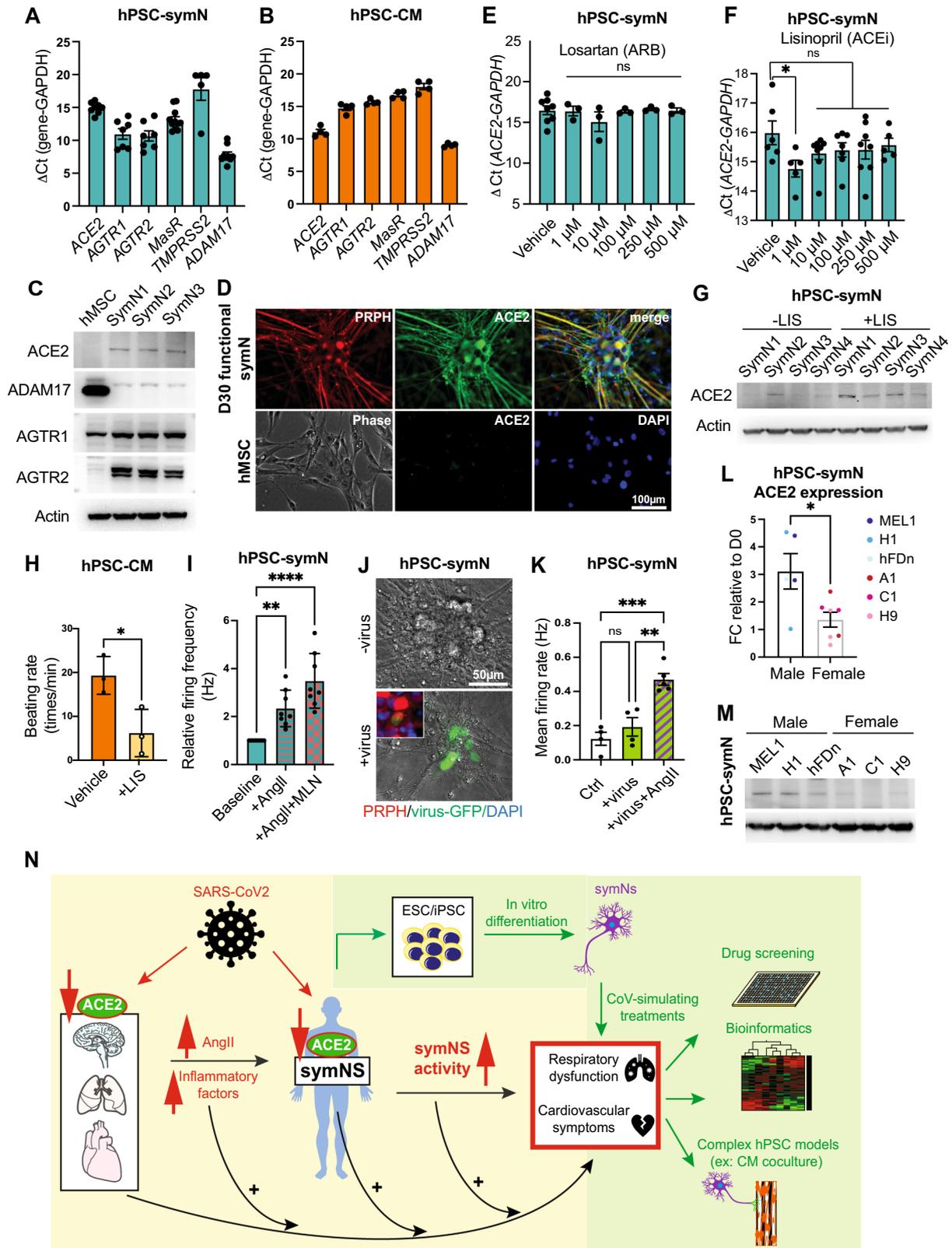
³ Complex Carbohydrate Research Center, University of Georgia, Athens, GA, USA

⁴ School of Chemical, Materials and Biomedical Engineering, University of Georgia, Athens, GA, USA

⁵ Regenerative Bioscience Center, Rhodes Center for Animal Dairy Science, University of Georgia, Athens, GA, USA

⁶ Department of Genetics, Franklin College of Arts and Sciences, University of Georgia, Athens, GA, USA

⁷ Department of Cellular Biology, Franklin College of Arts and Sciences, University of Georgia, Athens, GA, USA



[1]. ACE2 and RAAS components have been detected in human and rodent stellate ganglia [6] and in human dorsal root ganglia [7]. However, to our knowledge, it has not yet

been shown whether SARS-CoV-2 infects human symNs. Furthermore, it remains unclear how the SNS and its regulatory abilities are affected by SARS-CoV-2 infection [2] and

Fig. 1 The presence of ACE2 and RAAS component expression in hPSC-derived sympathetic neurons (symNs) suggests them as a novel modeling system to study COVID-19-related complications. **a** RAAS component gene expression in hPSC-symNs and **b** cardiomyocytes (CM) by RT-qPCR. **c** RAAS component gene expression in day 30 hPSC-symNs and adipose-derived hMSCs by western blot. **d** ACE2 and peripherin (PRPH) immunofluorescence staining in day 30 symNs and hMSCs. Changes in ACE2 expression levels in day 35 symNs under treatments with losartan (**e**) and lisinopril (**f**) for 24 h. **g** Western blot analysis supports the increased ACE2 expression after 1 μ M lisinopril treatment. **h** Beating rate in hPSC-CMs treated with 1 μ M LIS for 24 h. **i** SymN activity under treatment for 1 h with Ang II (1 nM) and MLN measured by MEA. **j** Pseudovirus-infected GFP⁺ cells are shown in phase-contrast live image and co-stained with PRPH (inset). **k** SymN activity under treatment for 1 h with Ang II and 24 h with pseudovirus measured by MEA. **l, m** Comparison of ACE2 expression levels between male and female hPSC-derived symNs performed by RT-qPCR (**l**) and western blot (**m**). **n** Summary of symN-mediated pathology and potential future applications of this model system. *FC* fold change. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, *t* test for (**h, l**), One-way ANOVA for (**e, f, i, k**). Error bars are shown as SEM

its downstream effects on the RAAS. One reason for this gap in knowledge is the lack of a human modeling system to assess symN response for research. SNS functional tests in patients mainly rely on indirect indexes, such as sweating, blood pressure, and heart rate measurements [8]. Direct measurements of neural activity in human symNs are not feasible, since such cells cannot be easily obtained from subjects. Rodent models have naturally low susceptibility to SARS-CoV-2 since the mouse ACE2 receptors do not effectively bind the virus. Artificial modifications such as human ACE2 (hACE2)-expressing mice or mouse-sensitized virus are therefore used [9]. Unpublished data (SfN 2021, poster # P322.06) further suggest that symNs in hACE2 transgenic mice are infected with SARS-CoV-2 at low levels. Here, we employ the powerful hPSC technology [10] that allows us to derive untransformed, functional human symNs. These hPSC-derived symNs provide a modeling system to study the cellular components necessary for SARS-CoV-2 infection and its potential downstream effects. This model system can be used to assess symN pathologies and it may provide a framework for studying the effects of COVID-19 on sympathetic responsiveness.

We previously established an efficient, fast, and easy-to-use differentiation protocol to generate functional symNs from hPSCs [3]. This protocol and further improvements from our lab (as yet unpublished) yield a highly pure (~80%) population of neurons with a high specificity of symNs (>90% of neurons are *TH⁺/PHOX2B⁺*) [3]. We have applied this and its precursor protocols for disease modeling, for example of the genetic, sympathetic disorder familial dysautonomia [3, 11]. Here, we first tested whether the cellular components that would allow SARS-CoV-2 entry were preserved in our symN model system. We differentiated symNs from the hPSC line H9/WA09 for 30 days, a

time point when the neurons are functionally active (i.e., fire action potentials) [3]. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis showed that human symNs express ACE2, MasR, AGTR1, and AGTR2 (Fig. 1a). We also confirmed that the ACE2 levels were not affected by cryopreservation during symN differentiation (Supp. Fig. 1c). In addition, low expression of TMPRSS2 (transmembrane serine protease 2), which mediates ACE2 priming and viral entry [12], and ADAM17 (A disintegrin and metalloproteinase 17), which regulates ACE2 shedding [13], were also observed (Fig. 1a). As a parallel assessment, we used hPSC-derived cardiomyocytes (hPSC-CMs) [14], since ACE2 expression in this cell type is well documented [15]. CMs expressed higher mRNA levels of ACE2 compared to symNs (Fig. 1b), consistent with findings of higher ACE2 levels in human heart tissues compared to the nervous system [5]. However, expression levels of MasR, TMPRSS2, AT₁R, AT₂R, and ADAM17 were comparable or higher in symNs, which might indicate SNS sensitivity to an unbalanced RAAS axis during SARS-CoV-2 infection. The expression of ACE2 and RAAS components was also validated by western blot (Fig. 1c) and immunofluorescence (IF, Fig. 1d). To ensure antibody specificity, we compared hPSC-derived symNs with human mesenchymal stromal cells (hMSCs), which are negative for ACE2 [16]. We observed consistent expression levels of ACE2, ADAM17, and AGTR1/2 in six unrelated symN batches (Fig. 1c, Supp. Fig. 1d). Interestingly, hMSCs expressed a very high level of ADAM17, which may explain why hMSC is ACE2-null (Fig. 1c). IF analysis confirmed solid ACE2 expression in symNs, co-stained with peripheral neuron marker peripherin (Fig. 1d), and again showed the absence of ACE2 in hMSCs.

A RAAS that is unbalanced towards ACE1/Ang II/AGTRs is associated with disease, including hypertension and cardiovascular disease [2]. ACE1 inhibitors (ACEi) and Ang II receptor blockers (ARB) are common treatments for high blood pressure and are known to increase ACE2 expression [17], fueling some worries that patients who are taking these drugs might be more susceptible to SARS-CoV-2 infection [18]. Accordingly, assessment of ACE2 levels in response to treatment with ACEi/ARBs is a critical clinical evaluation. We, therefore, tested whether ACE2 levels change in response to ARB and ACEi in symNs using our model system. We treated hPSC-derived symNs with losartan (an ARB) and lisinopril (an ACE1i) and analyzed ACE2 expression changes after 24 h. We found that, while losartan showed no significant effect on ACE2 expression levels in symNs (Fig. 1e), lisinopril significantly increased ACE2 levels in these cells (Fig. 1f, g). As a comparison/control, we also assessed ACE2 expression in hPSC-derived CMs. Lisinopril treatment of hPSC-derived CMs did not increase ACE2 expression significantly (Supp. Fig. 1e). It remains somewhat unclear how to interpret these data in

the context of the current literature, as some reports in CMs of animal models have shown an increase in ACE2 mRNA levels, whereas others have shown no change in ACE2 activity after lisinopril treatment [18, 19]. Additionally, a recent report showed that pretreatment of hPSC-derived CMs with lisinopril or losartan did not alter susceptibility to SARS-CoV-2 infection [15]. Nevertheless, lisinopril is known to exert cardioprotective effects [20, 21] by modulating cardiac contractility/membrane ionic currents [20, 21]. Thus, we also checked CM beating rate, and found that in our system, lisinopril lowered the beating rate of CMs (Fig. 1h). We also confirmed that lisinopril did not cause toxicity in symNs (not shown) or CMs (Supp. Fig. 1f). Together, these data support the possibility that the use of lisinopril during the COVID-19 pandemic might increase patients' susceptibility to infection of symNs, due to its effects of increasing ACE2 expression and providing more entry points for the virus.

Considering the fact that the SNS innervates most organs, including the lungs, and given the current pathology of SARS-CoV-2, we hypothesized that the SNS may be one of the secondary tissues that are infected and affected by the virus. SARS-CoV-2 infection of primary tissue, such as the lungs, triggers a release of Ang II by the infected tissue, which further triggers a systemic proinflammatory response and vasoconstriction. Viral entry into the cell results in downregulation of ACE2, which leads to further accumulation of Ang II. To evaluate the response of the SNS to these events, we first treated symNs with Ang II. Using a multi-electrode array (MEA) system to measure symN firing in live cells, we found that Ang II treatment significantly increased symN activity (Fig. 1i, Supp. Fig. 1g). To further mimic ACE2 downregulation after SARS-CoV-2 infection, we treated symNs with Ang II and MLN-4760, an ACE2 inhibitor, which further increased symN activity (Fig. 1i).

ACE2 expression in human dorsal root ganglia, the sensory branch of the peripheral nervous system (PNS), has been reported recently, and the implications of SARS-CoV-2 infection for PNS neurons have been discussed [7]. However, to our knowledge, there have been no reports showing whether SARS-CoV-2 infects human symNs. Thus, we next sought to investigate and mimic the stage when symNs are directly infected by the virus. We used a commercially available SARS-CoV-2 pseudovirus, i.e. BSL-1 baculovirus pseudotyped with the original SARS-CoV-2 spike protein, found in Wuhan, China, which also expresses the mNeonGreen fluorescent reporter. After treating hPSC-derived symNs with the pseudovirus for 24 h, we detected infected symNs (green fluorescent protein [GFP]⁺/PRPH⁺) within the typical self-organized neural clusters (Fig. 1j). We quantified the infection rate by counting clusters with a GFP signal. Approximately 20% of neurons within clusters derived from four independent differentiations were GFP⁺, thus infected. Additionally, using enzyme-linked immunosorbent assay

(ELISA) quantification of our pseudovirus-infected symN cultures, we observed a viral dose-dependent increase in the GFP signal (Supp. Fig. 1h). Of note, approximately 20% of the cells in our symN cultures of unknown character [3] also were infected at a similar rate, which is a confounding factor when interpreting the ELISA data. We next asked whether pseudovirus-treated symNs fired action potentials at an increased rate. MEA measurements showed that viral infection alone did not increase the firing rate (Fig. 1k), likely due to the lack of infection-associated Ang II increase and/or proinflammatory signals. Thus, we added Ang II to the infected cultures. Indeed, co-treatment with pseudovirus and Ang II significantly increased the firing rate of symNs (Fig. 1k). In sum, our data suggest that symN activity might increase in SARS-CoV-2 infected patients and that SNS hyperactivation might be an important factor in the pathophysiology of COVID-19, as it may lead to exacerbation of symptoms. The model we propose here will be instrumental in further assessing these and other hypotheses.

The hPSC-based symN model that we use here appears to be a promising tool for assessing both the pathophysiology and emerging treatments for COVID-19 with respect to the responsiveness of symNs. To further test the usefulness of our model system, we focused on a reported finding that males have a higher risk for SARS-CoV-2 infection and complications than females [22]. To test whether there is a gender difference in ACE2 expression in symNs, we differentiated symNs from three female [H9/WA09, C1 (Coriell #AG02602) and A1 (Coriell #GM04895)] and three male [MEL1, H1/WA01, and hFDn-iPSC (in house)] hPSC lines. We found that female symNs had significantly lower ACE2 expression compared to male symNs (Fig. 1l, m). This result highlights the usefulness of our symN model system.

Here, we propose an hPSC-based model of symNs that may be useful for studying SNS-associated effects of SARS-CoV-2 infection and could provide a platform for future drug testing and screening. Using this model, we confirmed previous reports [6] that ACE2/RAAS components are expressed in human symNs. We further showed that their electrical activity is sensitive to Ang II, ACE2 downregulation, and SARS-CoV-2 pseudovirus infection. Thus, SARS-CoV-2-mediated ACE2 downregulation in symNs (and other organs) in combination with Ang II upregulation and inflammatory factors may all lead to increased SNS activation and may exacerbate respiratory dysfunction and cardiovascular symptoms (Fig. 1n, yellow shading). This human symN-based modeling system may serve as a crucial tool for predicting and testing the sympathetic outcome for future SARS-CoV-2 therapeutics (Fig. 1n, green shading).

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10286-021-00850-1>.

Acknowledgements We want to thank Dr. V. Kenyi Saito-Diaz for critical reading of the manuscript.

Funding This work was funded by faculty start-up funds from the University of Georgia to NZ and NIH/NINDS 1R01NS114567 to NZ.

Declarations

Conflict of interest The authors have no conflicts to disclose.

Ethical approval This work does not include human or animal data.

References

1. Porzionato A et al (2020) Sympathetic activation: a potential link between comorbidities and COVID-19. *FEBS J* 287(17):3681–3688
2. Dani M et al (2021) Autonomic dysfunction in ‘long COVID’: rationale, physiology and management strategies. *Clin Med (Lond)* 21(1):e63–e67
3. Wu HF, Zeltner N (2020) Efficient differentiation of postganglionic sympathetic neurons using human pluripotent stem cells under feeder-free and chemically defined culture conditions. *J Vis Exp*. <https://doi.org/10.3791/60843>
4. Huang BS, Leenen FH (2009) The brain renin-angiotensin-aldosterone system: a major mechanism for sympathetic hyperactivity and left ventricular remodeling and dysfunction after myocardial infarction. *Curr Heart Fail Rep* 6(2):81–88
5. Li MY et al (2020) Expression of the SARS-CoV-2 cell receptor gene ACE2 in a wide variety of human tissues. *Infect Dis Poverty* 9(1):45
6. Bardsley EN, Neely OC, Paterson DJ (2020) Angiotensin peptide synthesis and cyclic nucleotide modulation in sympathetic stellate ganglia. *J Mol Cell Cardiol* 138:234–243
7. Shiers S et al (2020) ACE2 and SCARF expression in human dorsal root ganglion nociceptors: implications for SARS-CoV-2 virus neurological effects. *Pain* 161(11):2494–2501
8. Zygmunt A, Stanczyk J (2010) Methods of evaluation of autonomic nervous system function. *Arch Med Sci* 6(1):11–18
9. Munoz-Fontela C et al (2020) Animal models for COVID-19. *Nature* 586(7830):509–515
10. Saito-Diaz K, Zeltner N (2019) Induced pluripotent stem cells for disease modeling, cell therapy and drug discovery in genetic autonomic disorders: a review. *Clin Auton Res* 29(4):367–384
11. Zeltner N et al (2016) Capturing the biology of disease severity in a PSC-based model of familial dysautonomia. *Nat Med* 22(12):1421–1427
12. Hoffmann M et al (2020) SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cell* 181(2):271–280e8
13. Schreiber B, Patel A, Verma A (2020) Shedding light on COVID-19: ADAM17 the missing link? *Am J Ther* 28(3):e358–e360. <https://doi.org/10.1097/MJT.0000000000001226>
14. Lin Y, Zou J (2020) Differentiation of cardiomyocytes from human pluripotent stem cells in fully chemically defined conditions. *STAR Protoc* 1(1):100015. <https://doi.org/10.1016/j.xpro.2020.100015>
15. Iwanski J et al (2021) Antihypertensive drug treatment and susceptibility to SARS-CoV-2 infection in human PSC-derived cardiomyocytes and primary endothelial cells. *Stem Cell Reports* 16(10):2459–2472
16. Avanzini MA et al (2021) Human mesenchymal stromal cells do not express ACE2 and TMPRSS2 and are not permissive to SARS-CoV-2 infection. *Stem Cells Transl Med* 10(4):636–642
17. Sharma RK et al (2020) ACE2 (angiotensin-converting enzyme 2) in cardiopulmonary diseases: ramifications for the control of SARS-CoV-2. *Hypertension* 76(3):651–661
18. South AM, Diz DI, Chappell MC (2020) COVID-19, ACE2, and the cardiovascular consequences. *Am J Physiol Heart Circ Physiol* 318(5):H1084–H1090
19. Ferrario CM et al (2005) Effect of angiotensin-converting enzyme inhibition and angiotensin II receptor blockers on cardiac angiotensin-converting enzyme 2. *Circulation* 111(20):2605–2610
20. Juggi JS, Koenig-Berard E, Van Gilst WH (1993) Cardioprotection by angiotensin-converting enzyme (ACE) inhibitors. *Can J Cardiol* 9(4):336–352
21. Valenzuela C et al (1994) Effects of lisinopril on cardiac contractility and ionic currents. *Gen Pharmacol* 25(5):825–832
22. Samuel RM et al (2020) Androgen signaling regulates SARS-CoV-2 receptor levels and is associated with severe COVID-19 symptoms in men. *Cell Stem Cell* 27(6):876–889e12