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Original Article

Dual inhibition of TMPRSS2 and Cathepsin B prevents SARS-CoV-2 infection in iPS cells

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It has been reported that many receptors and proteases are required for severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection. Although angiotensin-converting enzyme 2 (ACE2) is the most important of these receptors, little is known about the contribution of other genes. In this study, we examined the roles of neuropilin-1, basigin, transmembrane serine proteases (TMPRSSs), and cathepsins (CTSs) in SARS-CoV-2 infection using the CRISPR interference system and ACE2-expressing human induced pluripotent stem (iPS) cells. Double knockdown of TMPRSS2 and cathepsin B (CTSB) reduced the viral load to 0.036% ± 0.021%. Consistently, the combination of the CTPB inhibitor CA-074 methyl ester and the TMPRSS2 inhibitor camostat reduced the viral load to 0.0078% ± 0.0057%. This result was confirmed using four SARS-CoV-2 variants (B.1.3, B.1.1.7, B.1.351, and B.1.1.248). The simultaneous use of these two drugs reduced viral load to less than 0.01% in both female and male iPS cells. These findings suggest that compounds targeting TMPRSS2 and CTSB exhibit highly efficient antiviral effects independent of gender and SARS-CoV-2 variant.

INTRODUCTION

To develop a therapeutic agent for coronavirus disease 2019 (COVID-19), it is necessary to elucidate the infection mechanism of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). During infection, SARS-CoV-2 Spike (S) protein binds to angiotensin-converting enzyme 2 (ACE2) expressed on the cell surface and is cleaved by proteases such as transmembrane protease serine 2 (TMPRSS2),[1] causing S protein to split into S1 and S2 domains. SARS-CoV-2 invades into a cell by fusing cell and viral membrane using the S2 domain.[2] Therefore, SARS-CoV-2 infection is thought to depend on the expression of ACE2 and TMPRSS2 in host cells.[1] However, recently it has become evident that molecular factors besides ACE2 and TMPRSS2 play important roles in SARS-CoV-2 infection.

For example, neuropilin-1 (NRP1) and basigin (BSG; known as CD147) act as SARS-CoV-2 receptors. Previous reports showed that SARS-CoV-2 infects neurons and T cells via NRP1[3,4] and BSG,[5] respectively. However, few reports have compared the contribution rates of NRP1 and BSG in the viral infection. With regard to the processing of S protein, it has been reported that several cellular type II transmembrane serine proteases (TTSPs) and cathepsins (CTSs) contribute to the cleavage of S protein. TTSPs are expressed on the cell surface, while CTSs are expressed in the endosome. TMPRSS11s (e.g., TMPRSS11D, 11E, and 11F) and TMPRSS13 are two examples,[6] while TMPRSS2 and TMPRSS4 contribute to the viral infection in intestinal organoids.[7] In addition, cathepsin L (CTSL) affects the infection efficiency of pseudotyped SARS-CoV-2 in Huh7 cells.[8] These reports suggest that both TTSPs and CTSs play an important role in processing of S protein, but few reports have compared the contribution rate of TTSPs and CTSs. By comparing the functions of receptors and proteases involved in SARS-CoV-2 infection, it will be possible to clarify which receptors and proteases are the promising targets for effective COVID-19 treatment.

In this study, we used the CRISPR interference (CRISPRi) system[9] and induced pluripotent stem (iPS) cells to compare the functions of these receptors and proteases in SARS-CoV-2 infection. The CRISPRi system can selectively suppress the expression of a target gene using catalytically dead Cas9 (dCas9) fused with krüppel-associated box (KRAB) suppressor domain and short guide RNA (sgRNA).[10] We have previously reported that ACE2 is essential for SARS-CoV-2 infection in undifferentiated iPS cells.[11] The combination of CRISPRi and iPS cells can suppress the expression of the target gene to about 1% or less.[10,12] Moreover, the expression stays low in somatic cells induced from the iPS cells.[12] So far, SARS-CoV-2 research using iPS cell-derived somatic cells has been widely performed.[13,14] If iPS cells with reduced expression levels of receptors and proteases involved in SARS-CoV-2 can be established, it will be useful for SARS-CoV-2 research using various iPS cell-derived somatic cells.

RESULTS

Expression of SARS-CoV-2-related receptors and proteases in undifferentiated iPS cells

The gene expression levels of *ACE2*, *BSG*, *CTSL*, *CTSB*, *TMPRSS2*, *4*, *11E*, *13*, *NRP1*, and *IFNAR2* in undifferentiated iPS cells were examined by quantitative PCR (qPCR) (Figure 1A). Among receptors, the gene expression level of *BSG* was highest, and, among proteases, *CTSL*

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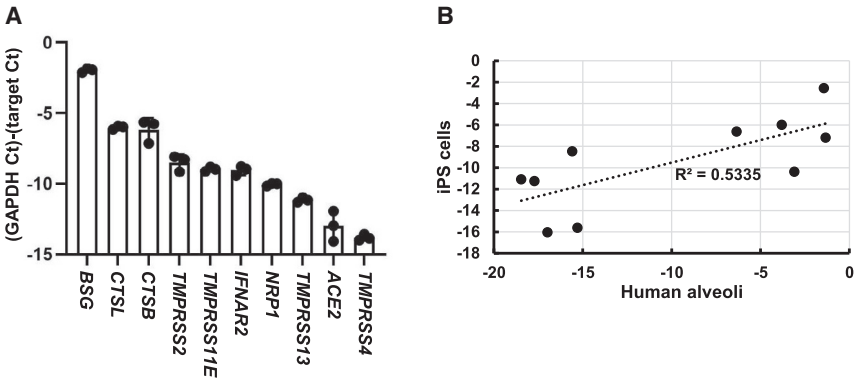

Figure 1. Gene expression profiles of receptors and proteases in undifferentiated human iPS cells

(A) The gene expression levels of receptors and proteases (*ACE2*, *BSG*, *CTSL*, *CTSB*, *TMPRSS2*, *4*, *11E*, *13*, *NRP1*, and *IFNAR2*) in undifferentiated iPS cells (WTB CRISPRi Gen1 cells) were examined by qPCR. Data are shown as means ± SD (n = 3). (B) The gene expression levels of receptors and proteases in undifferentiated human iPS cells and human alveoli (total RNA) were compared.

and *CTSB* were highest. The expression profiles of the receptors and proteases in undifferentiated iPS cells and in human alveoli showed a mild correlation (R^2 = 0.5335) (Figure 1B). The expression levels of the receptors and proteases were also examined in HEK293 (embryonic kidney), Calu-3 (lung cancer), and Caco-2 (colorectal adenocarcinoma) cell lines (Figure S1). The gene expression profile in these cell lines was also correlated with the gene expression profile in human alveoli (Figure S2). Therefore, in the following experiments, we analyzed the functions of the receptors and proteases in SARS-CoV-2 infection using human iPS cells.

Functions of receptors and proteases in SARS-CoV-2 infection

To elucidate the functions of receptors and proteases other than ACE2 in SARS-CoV-2 infection, ACE2-overexpressing iPS cells (ACE2-iPS cells) and the CRISPRi system were used. ACE2-expressing adenovirus (Ad) vectors (Ad-ACE2) were used to overexpress ACE2. To decrease the expression of the target gene, WTB CRISPRi Gen1 cells, which express the fusion protein of KRAB and dCas9 under control of doxycycline (DOX)-inducible promoter,[10] were used (Figure 2A). To suppress the expression levels of *BSG*, *CTSL*, *CTSB*, *TMPRSS2*, *4*, *11E*, *13*, *NRP1*, and *IFNAR2*, we designed sgRNAs that target these genes. The iPS cells were transfected with plasmids expressing sgRNAs, mKate2, and blasticidin S resistance genes (Figure 2A). By performing blasticidin S selection and single-cell cloning based on the mKate2 brightness, the iPS cell clone that most strongly expressed the sgRNAs was selected (Figure 2B). From this clone, we established nine iPS cell lines that express sgRNAs targeting the nine genes, respectively. After suppressing the expression levels of the target gene in ACE2-iPS cells by DOX-mediated dCas9-KRAB expression (Figure S3), SARS-CoV-2 infection was performed (Figure 2C). The suppression of *CTSL*, *CTSB*, and *TMPRSS2* reduced the viral RNA copy numbers in ACE2-iPS cells to 11.7% ± 3.77%, 0.84% ± 0.062%, and 20.6% ± 10.4%, respectively. Consistently, the suppression of *TMRPSS2* decreased the number of ACE2-iPS cells expressing the nucleocapsid (N) protein (Figure 2D). We also confirmed that the expression levels of the target gene can be suppressed by DOX treatment in the presence of SARS-CoV-2 (Figure S4). These results suggest that *CTSB*, *CTSL*, and *TMPRSS2* are involved in the SARS-CoV-2 infection of ACE2-iPS cells.

Inhibition of SARS-CoV-2 infection using cathepsin and TMPRSS2 inhibitors

Based on the above findings, we next investigated whether CTSB, CTSL, and TMPRSS2 inhibitors could inhibit SARS-CoV-2 infection in ACE2-iPS cells. Ad-ACE2 were used to overexpress ACE2 for the data in Figures 1 and 2, but iPS cells that stably express ACE2 (Figures S5 and S6) were used for the data in Figure 3. Because there are gender differences in TMPRSS2 expression levels,[11] female iPS cells (WTB CRISPRi Gen1) (Figure 3A) and male iPS cells (1383D6) (Figure 3B) were examined. After infecting ACE2-expressing iPS cells with SARS-CoV-2, the cells were cultured in the presence of CA-074 methyl ester (CA-074 Me, CTSB inhibitor), Z-Phe-Tyr (tBu)-diazomethylketone (CTSL inhibitor), camostat (TMPRSS2 inhibitor), or nafamostat (TMPRSS2 inhibitor). In ACE2-expressing WTB CRISPRi Gen1 cells, the copy number of viral RNA was reduced to 0.62% ± 0.25% by CA-074 Me treatment. In ACE2-expressing 1383D6 cells, the virus RNA copy number was reduced to 0.48% ± 0.023% and 0.23% ± 0.14% by camostat and nafamostat treatment, respectively. Among the four compounds, nafamostat caused cytotoxicity in ACE2-iPS cells (Figure 3C). These results suggested that the CA-074 Me or camostat treatment can reduce the SARS-CoV-2 infection efficiency in ACE2-iPS cells.

Dual inhibition of TMPRSS2 and CTSB prevent SARS-CoV-2 infection in ACE2-iPS cells

Lastly, we examined whether a dual inhibition of TMRPSS2 and CTSB synergistically inhibit SARS-CoV-2 infection in ACE2-iPS cells. The viral RNA copy number in the infected CTSB and TMPRSS2 double-knockdown cells was lower than that in the infected single-gene knockdown cells (Figure 4A). In addition, we investigated whether the combination of CA-074 Me and camostat treatments improved the inhibition efficacy of the viral infection. In three female iPS and embryonic stem (ES) cell lines, CA-074 Me treatment reduced the viral RNA copy number to 0.87% ± 0.26% (Figure 4B). On the other hand, in three male iPS/ES cell lines, the viral RNA copy number was decreased to 0.74% ± 0.24% by camostat treatment (Figure 4C). The CTSB inhibitor had a stronger infection inhibitory effect than the TMPRSS2 inhibitors in female iPS/ES cells, but the opposite was true for male iPS/ES cells. This gender difference may be due to differences in the TMPRSS2 expression levels. Importantly, the combination of CA-074 Me and camostat reduced the viral RNA copy number to 0.0078% ± 0.0057% with almost no cytotoxicity

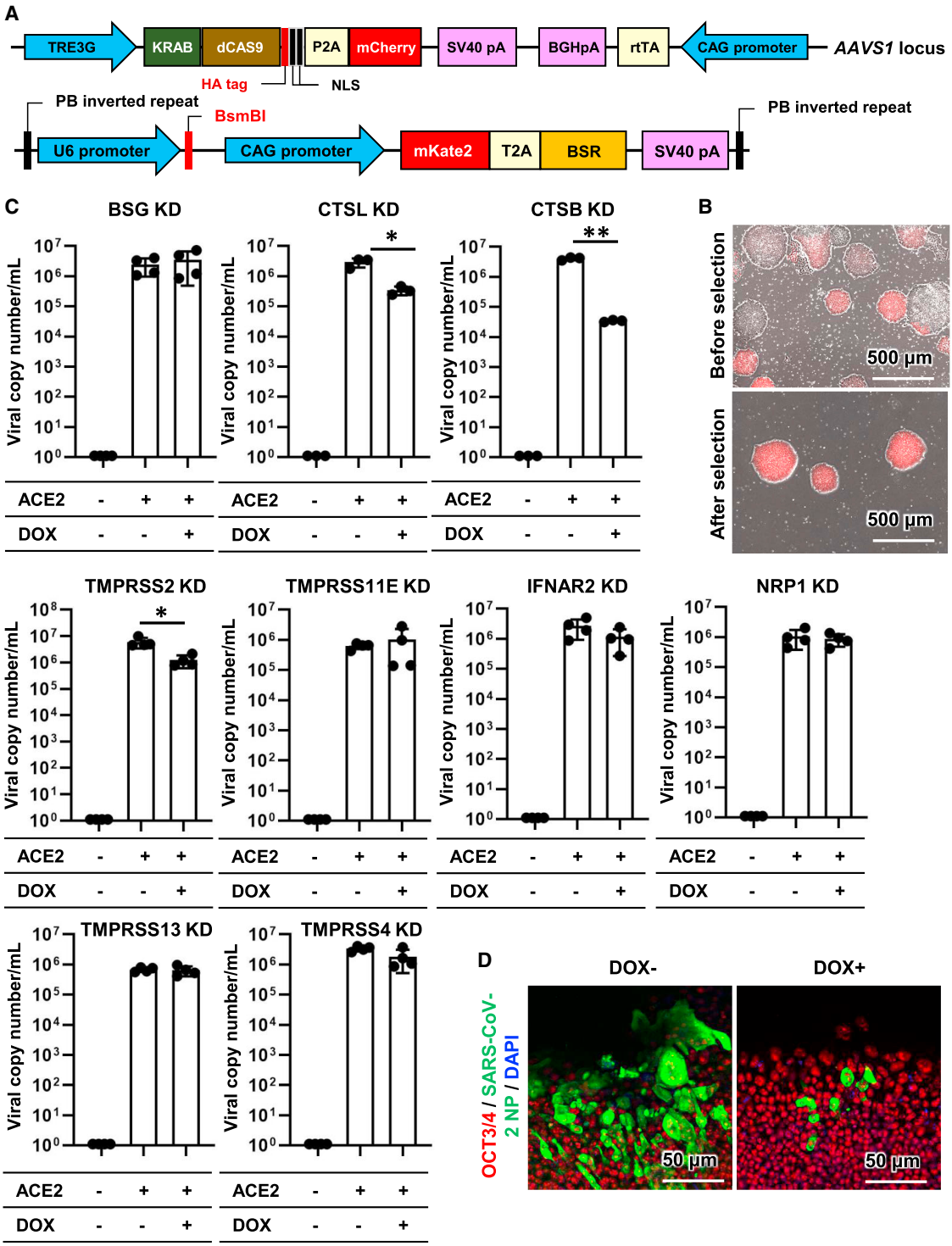

Figure 2. Knockdown of CTSB, CTSL, or TMPRSS2 expression decreased the SARS-CoV-2 infection efficiency in undifferentiated human iPS cells

(A) To perform CRISPRi experiments, WTB CRISPRi Gen1 iPS cells that have a DOX-inducible KRAB-dCas9 cassette in *AAVS1* locus were used. For sgRNA expression, we inserted an sgRNA into the BsmBI site (indicated in red) of the *piggyBac* vector that expresses mKate2 and blasticidin S resistance genes under CAG promoter. Tet response element (TRE), KRAB, catalytically dead Cas9 (dCas9), 2A peptide derived from porcine teschovirus-1 (P2A), simian virus 40 (SV40), bovine growth hormone (BGH), tetracycline-dependent transactivator (rtTA), CMV early enhancer/chicken β actin (CAG), *piggyBac* (PB), 2A peptide derived from Thosea asigna virus (T2A), blasticidin S

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(Figure S7). Consistently, the combination of CA-074 Me and camostat treatments synergistically improved the morphology of iPS cell colony (Figure 4D) and decreased the number of ACE2-iPS cells expressing the nucleocapsid (N) protein (Figure 4E). Therefore, CA-074 Me and camostat synergistically inhibit the SARS-CoV-2 infection of iPS cells. Similar results were observed for three other SARS-CoV-2 variants: B.1.1.7, B.1.351, and B.1.1.248 (Figure 4F).

DISCUSSION

We found that CTSB and TMPRSS2 are required for SARS-CoV-2 to infect ACE2-iPS cells. It is known that TMPRSS2 is present in the cell membrane and CTSB in endosomes,[15] suggesting TMPRSS2 and CTSB play important roles in endocytosis-independent and endocytosis-dependent infection, respectively. Notably, the combination of CA-074 Me and camostat treatment showed a marked decrease in virus infection efficiency, but a complete inhibitory effect was not observed. Therefore, other unidentified genes probably contribute to the infection too.

Using a genome-wide sgRNA library, it may be possible to find more genes related to SARS-CoV-2 infection and replication. For example, the detection of enriched sgRNAs in SARS-CoV-2-resistant iPS cells could reveal genes that play an important role in inhibiting viral infection. Recently, genome-wide association studies (GWAS) reported novel genes that are thought to contribute to COVID-19 aggravation.[16,17] The combination of iPS cells and CRISPRi presented in the current study should assist in elucidating the functions of these genes.

In this study, we elucidated the functions of receptors and proteases in undifferentiated iPS cells, but similar experiments using somatic cells derived from these iPS cells should also be done. Because the expression profiles of SARS-CoV-2-related genes differ among somatic cell types, the contribution rate of these genes will vary. Because iPS cells are easier to genome edit than primary somatic cells, the differentiation of genome-edited iPS cells to different cell types will provide a human model to search for more SARS-CoV-2-related genes that regulate infection. We hope that iPS cells and the CRISPRi system will accelerate the discovery of genes that can prevent SARS-CoV-2 infection and replication.

MATERIALS AND METHODS

Human ES/iPS cells

The human ES/iPS cell lines WTB CRISPRi Gen1 (provided by Dr. Bruce R. Conklin, Gladstone Institutes), 1383D6[18] (provided by Dr. Masato Nakagawa, Kyoto University), 201B7[19], Tic (JCRB1331, JCRB Cell Bank), H1 (WA01), and H9 (WA09) (WiCell Research Institute) were maintained on 0.5 μg/cm^2 recombinant human laminin 511 × 10^8 fragments (iMatrix-511, Nippi) with StemFit AK02N medium (Ajinomoto) containing 10 μM Y-27632 (from day 0 to day 1, FUJIFILM Wako Pure Chemical). To passage the cells, ES/iPS cell colonies were treated with TrypLE Select Enzyme (Thermo Fisher Scientific) for 8 min at 37°C. After centrifugation, the cells were seeded at an appropriate density (1.3×10^4 cells/9 cm^2) onto iMatrix-511 and cultured every 6 or 7 days. Human ES cells were used following the Guidelines for Derivation and Utilization of Human Embryonic Stem Cells of the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the study was approved by an independent ethics committee. Except for Figures 3, 4, and S5, WTB CRISPRi Gen1 was used for all experiments.

Cell lines

HEK293 cells (JCRB cell bank, JCRB9068) were cultured with Dulbecco's Modified Eagle's Medium (DMEM; FUJIFILM Wako Pure Chemical) containing 10% fetal bovine serum (FBS), 1× GlutaMAX (Thermo Fisher Scientific), and penicillin-streptomycin. Caco-2 cells (RIKEN BRC, RCB0988) and Calu-3 cells (American Type Culture Collection [ATCC], HTB-55) were cultured with Medium Minimum Essential Medium (MEM; Sigma-Aldrich) containing 10% FBS, 1× GlutaMAX (Thermo Fisher Scientific), and penicillin-streptomycin.

SARS-CoV-2 preparation

The SARS-CoV-2 strain B.1.3 (SARS-CoV-2, Isolate New York-PV09197/2020) was purchased from BEI Resources (https://www.beiresources.org/). The SARS-CoV-2 strain B.1.3 was used except for Figure 4F. Three SARS-CoV-2 strains used in this study (B.1.1.7, B.1.351, and B.1.1.248) were provided from the National Institute of Infectious Diseases. The virus was proliferated in TMPRSS2/Vero cells (JCRB1818, JCRB Cell Bank)[20] and stored at −80°C. TMPRSS2/Vero cells were cultured with MEM (Sigma-Aldrich) supplemented with 5% FBS and 1% penicillin-streptomycin. All experiments including virus infections were done in a biosafety level 3 facility at Kyoto University strictly following regulations.

Viral titration of SARS-CoV-2

Viral titers were measured by median tissue culture infectious dose (TCID50) assay. TMPRSS2/Vero cells (JCRB1818, JCRB Cell Bank)[20] were cultured with MEM (Sigma-Aldrich) supplemented with 5% FBS, and 1% penicillin-streptomycin, and seeded into 96-well cell culture plates (Thermo Fisher Scientific). The samples were serially diluted 10-fold from 10^{-1} to 10^{-8} in the cell culture medium. Dilutions were placed onto the TMPRSS2/Vero cells in

resistance (BSR). (B) All sgRNA-expressing WTB CRISPRi Gen1 cells were mKate2 positive. After blasticidin S treatment and single-cell cloning, all iPS cell colonies were mKate2-positive. (C) sgRNA-expressing WTB CRISPRi Gen1 cells were transduced with 600 VP/cell of beta-galactosidase (LacZ)- or ACE2-expressing Ad vectors (Ad-LacZ or Ad-ACE2, respectively) for 2 h and cultured with AK02 medium (with or without 1 micromolar per milliter (μM/mL) DOX) for 2 days. The cells were then infected with SARS-CoV-2 (0.1 multiplicity of infection [MOI]) for 2 h and cultured with AK02 medium (with or without 1 μM/mL DOX). Four days after the SARS-CoV-2 infection, the viral RNA copy number in the cell culture supernatant was measured by qPCR. One-way ANOVA followed by Tukey's post hoc test (*$p < 0.05$, **$p < 0.01$). Data are shown as means ± SD ($n = 3$). (D) Immunofluorescence analysis of SARS-CoV-2 N protein (green) and OCT3/4 (red) in sgRNA-TMPRSS2-expressing WTB CRISPRi Gen1 cells in the presence or absence of 1 μM/mL DOX. Nuclei were counterstained with DAPI (blue).

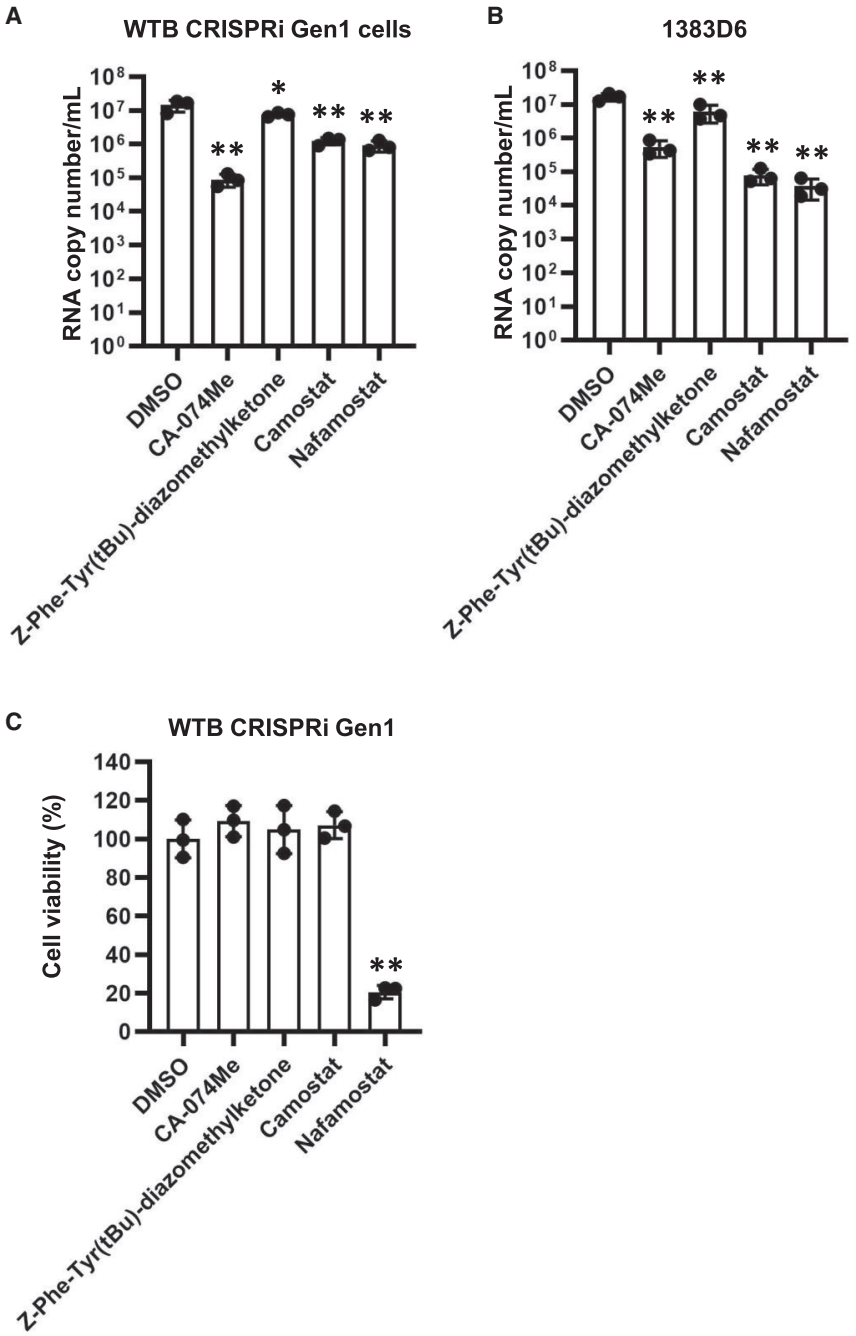


Figure 3. Effect of CTSB, CTSL, and TMPRSS2 inhibitors on SARS-CoV-2 infection efficiency

(A and B) ACE2-expressing WTB CRISPRi Gen1 cells (A) or 1383D6 cells (B) were infected with SARS-CoV-2 (0.1 MOI) for 2 h and cultured with AK02 medium containing 10 μM/mL CTSB, CTSL, or TMPRSS2 inhibitor for 4 days. Then, the viral RNA copy number in the cell culture supernatant was measured by qPCR. (C) ACE2-expressing WTB CRISPRi Gen1 cells were cultured with AK02 medium containing 10 μM/mL CTSB, CTSL, or TMPRSS2 inhibitors for 4 days. The cell viability was measured using the WST-8 assay. The viability of DMSO-treated cells was taken as 100. To compare drug-treated cells with DMSO-treated cells, one-way ANOVA followed by Tukey's post hoc test (*$p < 0.05$, **$p < 0.01$) was performed. Data are shown as means ± SD ($n = 3$). CA-074 Me (CTSB inhibitor), Z-Phe-Tyr(tBu)-diazomethylketone (CTSL inhibitor), camostat (TMPRSS2 inhibitor), and nafamostat (TMPRSS2 inhibitor).

triplicate and incubated at 37°C for 96 h. Cytopathic effects were evaluated under a microscope. TCID50/mL was calculated using the Reed-Muench method.

Ad vectors

ACE2-expressing Ad vectors (Ad-ACE2) were generated in our previous report.[11] Briefly, the vectors were constructed using Adeno-X Adenoviral System 3 (Takara Bio). The ACE2 gene was inserted into Adeno-X Adenoviral System 3, resulting in pAdX-ACE2. Ad-ACE2 was propagated in HEK293 cells (JCRB9068, JCRB Cell Bank). Beta-galactosidase (LacZ)-expressing Ad vectors were purchased from Vector Biolabs. The vector particle (VP) titer was determined by spectrophotometry.[21]

ACE2-expressing iPS cells

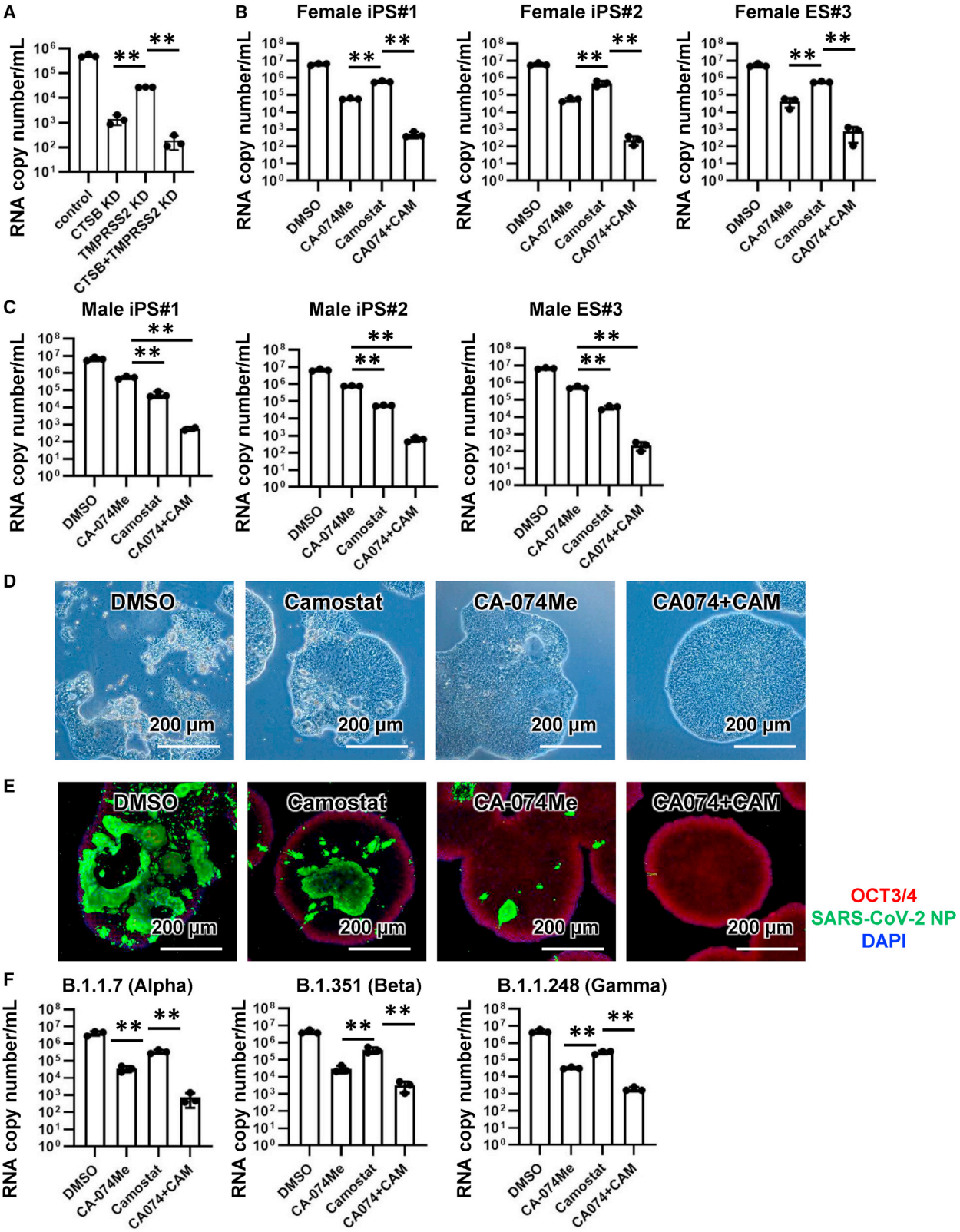
1383D6 and WTB CRISPRi Gen1 were electroporated with pPV-EF1α-ACE2-A and pHL-EF1a-hcPBase-A[22] vectors using NEPA21 electroporator (Nepa Gene), and then selected with 1 μg/mL puromycin (InvivoGen). The *piggyBac*-based ACE2-expressing plasmid, pPV-EF1α-ACE2-A, was constructed by replacing the EGFP gene of pPV-EF1a-EiP-A with ACE2 gene. pPV-EF1a-EiP-A is a *piggyBac*-based EGFP-expressing plasmid.

SARS-CoV-2 infection and drug treatment

ACE2-iPS cells cultured in a 96-well plate (2.0×10^4 cells/well) were infected with 2.0×10^3 TCID50/well of SARS-CoV-2 for 2 h and then cultured with StemFit AK02N medium containing drugs. This medium was replaced with fresh medium every day. Four days after the infection, the viral RNA copy number in the cell culture supernatant was measured by qPCR. The cell viability was examined using a WST-8 assay and a Cell Counting Kit-8 purchased from Dojindo Laboratories. Drugs used in the infection experiments are summarized in Table S1.

Quantification of viral RNA copy number

The cell culture supernatant was mixed with an equal volume of 2× RNA lysis buffer (distilled water containing 0.4 U/μL SUPERase I



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RNase Inhibitor [Thermo Fisher Scientific], 2% Triton X-100, 50 mM KCl, 100 mM Tris-HCl [pH 7.4], and 40% glycerol) and incubated at room temperature for 10 min. The mixture was diluted 10 times with distilled water. Viral RNA was quantified using a One Step TB Green PrimeScript PLUS RT-PCR Kit (Perfect Real Time) (Takara Bio) on a StepOnePlus real-time PCR system (Thermo Fisher Scientific). The primers used in this experiment are as follows: (forward) AGCC TCTTCTCGTTCCTCATCAC and (reverse) CCGCCATTGCCAGC CATTC. Standard curves were prepared using SARS-CoV-2 RNA (10^5 copies/μL) purchased from Nihon Gene Research Laboratories.

qPCR

Total RNA was isolated from human iPS cells using ISOGENE (NIPPON GENE). In Figures 1, S1, and S2, total RNA from pulmonary alveolar epithelial cells, bronchial epithelial cells (ScienCell Research Laboratories), and human heart (Clontech) were used as the control. cDNA was synthesized using 500 ng of total RNA with a Superscript VILO cDNA Synthesis Kit (Thermo Fisher Scientific). Real-time RT-PCR was performed with SYBR Green PCR Master Mix (Thermo Fisher Scientific) using a StepOnePlus real-time PCR system or QuantStudio 1 (Thermo Fisher Scientific). The relative quantitation of target mRNA levels was performed using the $2^{-\Delta\Delta CT}$ method. The values were normalized to the housekeeping gene *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*). The PCR primer sequences are shown in Table S2.

Immunofluorescence staining

For the immunofluorescence staining of human iPS cells, the cells were fixed with 4% paraformaldehyde in PBS at 4°C. After blocking the cells with PBS containing 2% BSA and 0.2% Triton X-100 at room temperature for 45 min, the cells were incubated with a primary antibody at 4°C overnight and then with a secondary antibody at room temperature for 1 h. All antibodies used in this report are described in Table S3.

CRISPRi experiments in human iPS cells

To perform CRISPRi experiments, we used a human iPS cell line that carries a DOX-inducible KRAB domain-fused dCas9 (KRAB-dCas9) gene expression cassette in the *adeno-associated virus integration site 1* (*AAVS1*) locus (WTB CRISPRi Gen1).[10] The sgRNA sequences were selected from a reported sgRNA sequence list.[23] A mixture of five sgRNAs was used to knock down the expression of each gene. The sgRNA sequences used in this study are summarized in Table S4. To establish sgRNA-expressing WTB CRISPRi Gen1 cells, the iPS cells (1×10^6 cells) were transfected with 7.5 μg of PB-U6-CNKB[24] encoding each sgRNA sequence and 2.5 μg of pCW-hyPBase using the NEPA21 electroporator (Nepa Gene). After 10 μg/mL-blasticidin S (InvivoGen) selection and single-cell cloning, mKate2-positive WTB CRISPRi Gen1 cells were cultured with or without 1 μg/mL DOX to induce the expression of KRAB-dCas9.

Statistical analyses

Statistical significance was evaluated using unpaired two-tailed Student's t test or one-way analysis of variance (ANOVA) followed by Tukey's post hoc tests. Statistical analyses were performed using GraphPad Prism8 and 9. Data are representative of three independent experiments. Details are described in the figure legends.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2021.10.016.

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AUTHOR CONTRIBUTIONS

R.H. performed SARS-CoV-2 infection experiments, CRISPRi experiments, qPCR analyses, data analyses, and statistical analysis. A.S. performed iPS cell culture and qPCR analyses. S.D. performed CRISPRi experiments and generated ACE2-expressing iPS cells. R.X. performed CRISPRi experiments. E.S. performed immunostaining analyses. A.H.

Figure 4. Dual inhibition of TMPRSS2 and CTSB prevents SARS-CoV-2 infection in ACE2-iPS cells

(A) sgRNA-TMPRSS2 and/or CTSB-expressing WTB CRISPRi Gen1 cells were cultured with AK02N medium containing 1 μM/mL DOX, and then transduced with 600 VP/cell of Ad-ACE2 for 2 h and cultured with AK02 medium for 2 days. The cells were then infected with SARS-CoV-2 (0.1 MOI) for 2 h and cultured with AK02 medium for 4 days. The viral RNA copy number in the cell culture supernatant was measured by qPCR. (B and C) Three female iPS/ES cell lines (B) and three male iPS/ES cell lines (C) were transduced with 600 VP/cell of Ad-ACE2 for 2 h and cultured with AK02 medium for 2 days. The cells were then infected with SARS-CoV-2 (0.1 MOI) for 2 h and cultured with AK02 medium containing 10 μM/mL CA-074Me or camostat. Four days after the SARS-CoV-2 infection, the viral RNA copy number in the cell culture supernatant was measured by qPCR for each cell line. (D) Phase images of drug-treated ACE2-expressing WTB CRISPRi Gen1 cells. (E) Immunofluorescence analysis of SARS-CoV-2 N protein (green) and OCT3/4 (red) in drug-treated ACE2-expressing WTB CRISPRi Gen1 cells. Nuclei were counterstained with DAPI (blue). (F) ACE2-expressing WTB CRISPRi Gen1 cells were infected with SARS-CoV-2 (0.1 MOI) for 2 h and cultured with AK02 medium containing 10 μM/mL CA-074Me or camostat for 4 days. Four days after the SARS-CoV-2 (B.1.1.7 (Alpha), B.1.351 (Beta), and B.1.1.248 (Gamma)) infection, the viral RNA copy number in the cell culture supernatant was measured by qPCR for each cell line. To compare drug-treated cells with each other, one-way ANOVA followed by Tukey's post hoc test (**$p < 0.01$) was performed. Data are shown as means ± SD (n = 3). Female iPS#1 (WTB CRISPRi Gen1 cells), female iPS#2 (201B7 cells), female ES#3 (H9 cells), male iPS#1 (1383D6 cells), male iPS#2 (Tic), male ES#3 (H1 cells).

performed construction of piggyBac vector and data analyses. K. Takahashi performed CRISPRi experiments and data analyses. S.Y. performed research design and data analyses. K. Takayama performed research design, SARS-CoV-2 infection experiments, data analyses, writing paper, funding acquirement, and gave final approval.

DECLARATION OF INTERESTS

K. Takahashi is on the scientific advisory board of I Peace, without salary. S.Y. is a scientific advisor, without salary, to iPS Academia Japan and Altos Labs. The other authors declare no competing financial interests.

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