SARS-CoV-2 pseudovirus infectivity and expression of viral entry-related factors ACE2, TMPRSS2, Kim-1, and NRP-1 in human cells from the respiratory, urinary, digestive, reproductive, and immune systems

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Abstract

Infection by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes a wide spectrum of syndromes involving multiple organ systems and is primarily mediated by viral spike (S) glycoprotein through the receptor-binding domain (RBD) and numerous cellular proteins including ACE2, transmembrane serine protease 2 (TMPRSS2), kidney injury molecule-1 (Kim-1), and neuropilin-1 (NRP-1). In this study, we examined the entry tropism of SARS-CoV-2 and SARS-CoV using S protein-based pseudoviruses to infect 22 cell lines and 3 types of primary cells isolated from respiratory, urinary, digestive, reproductive, and immune systems. At least one cell line or type of primary cell from each organ system was infected by both pseudoviruses. Infection by pseudoviruses is effectively blocked by S1, RBD, and ACE2 recombinant proteins, and more weakly by Kim-1 and NRP-1 recombinant proteins. Furthermore, cells with robust SARS-CoV-2 pseudovirus infection had strong expression of either ACE2 or Kim-1 and NRP-1 proteins. ACE2 glycosylation appeared to be critical for the infections of both viruses as there was a positive correlation between infectivity of either SARS-CoV-2 or SARS-CoV pseudovirus with the level of glycosylated ACE2 (gly-ACE2). These results reveal that SARS-CoV-2 cell entry could be mediated by either an ACE2-dependent or -independent mechanism, thus providing a likely molecular basis for its broad tropism for a wide variety of cell types.

KEYWORDS

Angiotensin-converting enzyme 2 (ACE2), and glycosylation, Kim-1, NRP-1, pseudovirus, receptor binding domain (RBD), SARS-CoV, SARS-CoV-2, spike protein, TMPRSS2, viral entry tropism, virus receptor

INTRODUCTION

During the coronavirus disease 2019 (COVID-19) pandemic, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection has manifested high transmission efficiency and complex clinical features affecting multiple human organ systems.1,2 Apart from the respiratory system, increasing evidence shows that SARS-CoV-2 infects urinary, digestive, reproductive, neurological, and immune system organs, causing a wide range of clinical symptoms such as dry cough, diarrhea, acute kidney injury, neurological complications, and various degrees of liver damage.3 Indeed, RNA-sequencing (RNAseq), reverse-transcription PCR (RT-PCR), and immunohistochemistry (IHC) have shown SARS-CoV-2 to be present...
in cells from different organ systems during natural infection. Besides lung and kidney cells, SARS-CoV-2 RNA or proteins have been detected in the liver, placenta, intestine, and immune cells. In lung tissues from severe COVID-19 patients, SARS-CoV-2 infection has been detected in an extensive range of parenchymal cells including type II pneumocytes, ciliated, goblet, club-like, and endothelial cells (ECs) as well as immune cells including macrophages, monocytes, neutrophils, and natural killer (NK), B and T cells with up to 90% of them positive for viral proteins, providing additional evidence for a broad SARS-CoV-2 cell tropism. The numbers of cells infected are associated with the extent of tissue damage.

TABLE 1 Cell lines used in the present study

| Cell name | Origin | Source of cell | Culture medium |
|-----------|--------|---------------|----------------|
| **Respiratory system** | | | |
| JHU-029 | Laryngeal squamous cell carcinoma | CVCL_5993 | RPMI-1640 complete medium |
| NCL H460 | Large cell lung cancer | ATCC HTB-177 | RPMI-1640 complete medium |
| NCL H322 | Large cell lung cancer | ATCC CRL5806 | DMEM complete medium |
| NCL H520 | Large cell lung cancer | ATCC HTB-182 | DMEM complete medium |
| A549 | Lung adenocarcinoma | ATCC CRM-CCL-185 | DMEM complete medium |
| Primary human lobar bronchial epithelial cells (HLBEC) | Lobar bronchial tissue | Lifeline® Cell Technology | Lifeline® BronchiaLife™ Medium |
| Primary human small airway epithelial cells (HSAEC) | Small airway tissue | Lifeline® Cell Technology | Lifeline® BronchiaLife™ Medium |
| **Urinary system** | | | |
| 769-P | Renal cell adenocarcinoma | ATCC CRL-1933 | RPMI-1640 complete medium |
| 768-O | Renal cell adenocarcinoma | ATCC CRL-1932 | RPMI-1640 complete medium |
| A498 | Renal cell adenocarcinoma | ATCC HTB-44 | RPMI-1640 complete medium |
| Caki-1 | Renal cell carcinoma | ATCC HTB-46 | DMEM complete medium |
| ACHN | Renal cell adenocarcinoma | ATCC CRL-1611 | DMEM complete medium |
| HRC45 | Renal cell carcinoma | CVCL_IS24 | DMEM complete medium |
| HRC63 | Renal cell carcinoma | CVCL IS25 | DMEM complete medium |
| HRC59 | Renal cell carcinoma | FCCC | DMEM complete medium |
| **Immune system** | | | |
| BCP-1 | Primary effusion lymphoma | ATCC CRL-2294 | RPMI-1640 complete medium |
| BC-3 | Primary effusion lymphoma | ATCC CRL-2277 | RPMI-1640 complete medium |
| BJAB | Primary effusion lymphoma | CVCL_5711 | RPMI-1640 complete medium |
| THP-1 | Acute monocytic leukemia | ATCC TIB-202 | RPMI-1640 complete medium |
| **Digestive system** | | | |
| Huh-7 | Hepatocellular carcinoma cell | JCRB0403 | DMEM complete medium |
| PCI-13 | Oral cavity squamous cell carcinoma | CVCL_C182 | RPMI-1640 complete medium |
| UD-SCC-2 | Hypopharyngeal squamous cell carcinoma | CVCL_E325 | RPMI-1640 complete medium |
| **Reproductive system** | | | |
| HUVEC | Umbilical Vein Endothelial Cells | ATCC PCS-100-010 | ECBM(Cell applications, 210-490) |
| T47D | Ductal carcinoma | ATCC HTB-133 | RPMI-1640 complete medium |
| MCF-7 | Breast adenocarcinoma | ATCC HTB-22 | RPMI-1640 complete medium |

Note: DMEM or RPMI-1640 complete medium is made of DMEM or RPMI-1640 basal medium with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin 100× Solution (25-512, GenClone).

Abbreviations: DMEM, Dulbecco’s modified Eagle medium; HUVEC, human umbilical vein endothelial cells.
observed in a broad range of parenchymal and immune cells in agreement with the detection of SARS-CoV-2 proteins in these cells. Interestingly, ACE2-negative SARS-CoV-2-infected cells have been detected in infected tissues, suggesting the likely presence of an ACE2-independent infection pathway. In this study, we investigated the infectivity of SARS-CoV-2 and SARS-CoV pseudoviruses in 22 cell lines and 3 primary cell types from 5 human organ systems including respiratory, urinary, digestive, reproductive, and immune systems, and demonstrated that at least 1 cell line or cell type from each of these organ systems is permissive for both SARS-CoV-2 and SARS-CoV pseudoviruses. We showed the expression of ACE2, TMPRSS2, Kim-1, and NRP-1 in a broad range of cells from the five human organ systems, and that cells infected by SARS-CoV-2 pseudovirus either expressed ACE2 or Kim-1 and NRP-1 proteins. Finally, we found a high degree of correlation between glycosylated ACE2 (gly-ACE2) and SARS-CoV-2 pseudovirus infectivity suggesting that posttranslational modification of ACE2 is critical for SARS-CoV-2 patency.

### 2.1 | Cell lines and cell culture

Cell lines and primary cells, sources, the organs and tissues where they are isolated, and culture conditions are listed in Table 1. Cells were maintained in their respective medium with 10% heat-inactivated fetal bovine serum (FBS) and 1% Penicillin-Streptomycin 100× Solution (25-512, Genesee). HEK293T cells and HEK293 ACE2 stable cells (CVD19-200A-1, SBI System Biosciences) were cultured in Dulbecco’s Modified Eagle Medium (DMEM) with 10% FBS and 1% Penicillin-Streptomycin 100× Solution. HEK293T cells were used for pseudovirus packaging while HEK293 ACE2 stable cells were used for titration of pseudoviruses.

### 2.2 | Pseudovirus packaging

The plasmid sets used for packaging of pseudoviruses included the reporter plasmid pNL4-3. Luci.R.-E-expressing the firefly gene (3418, AIDS Reagent Program, National Institute of Health), pcDNA3.1-SARS-CoV-2-S expressing the codon-optimized spike protein gene from Wuhan-Hu-1 strain SARS-CoV-2, kindly provided by Dr. Wei Cun, and pcDNA3.1-SARS-CoV-S expressing the spike protein gene from BJ302 strain SARS-CoV. pcDNA3.1(+) was used to generate a control "naked pseudovirus." A pseudovirus generated with pMD2.G expressing vesicular stomatitis virus (VSV) G envelope protein (VSV-G) was used as an additional control. To generate a pseudovirus, HEK293T cells were cotransfected with pNL4-3. Luci.R.-E- and pcDNA3.1-SARS-CoV-2-S, pcDNA3.1-SARS-CoV-S, pMD2.G, or pcDNA3.1(+) using a jetOPTIMUS transfection reagent (117-15, Polyplus). The supernatant was harvested at 48 h
posttransfection, centrifuged at 3,000 rpm for 10 min, aliquoted, and stored at −80°C for later use.

2.3 | Infectivity assay and blocking assay

To investigate the susceptibility of various cells to pseudoviruses, cells were seeded at $10^4$ cells per well in a 96-well plate 1 day before infection. The medium was removed, and 100 µl of supernatant containing SARS-CoV-2 or SARS-CoV pseudovirus was added to the cells and incubated overnight. The medium was changed with fresh medium and cells were incubated for another 48 h and analyzed for luciferase activity using the Luciferase Assay System (E1501, Promega). HEK293 ACE2 stable cells were used to titrate and normalize the viral titers of different viral preparations.

We used Huh-7 cells to examine the effects of different recombinant proteins on the infectivity of SARS-CoV-2 or SARS-CoV pseudovirus. Recombinant proteins used in this study included SARS-CoV-2 spike S1-hFc (40591-V02H, Sino biological), SARS-CoV-2 spike receptor-binding domain (RBD) RBD-mFc (40592-V05H1, Sino biological), human ACE2-His (10108-H08H, Sino biological), human KIM-1/TIM-1-His&Fc (11051-H16H, Sino biological), human neuropilin (NRP)-1-hFc (10011-H02H, Sino biological), and human Fc protein (hFc, AG100, Sigma). The supernatant containing the pseudovirus was mixed with an equal volume of a specified recombinant protein diluted in medium to achieve the specified concentration at 37°C for 1 h, and used for the infection assay.

2.4 | ACE2 protein deglycosylation

Protein lysates of Huh7 were prepared in NP40 Cell Lysis Buffer (FNN0021, Thermo Fisher Scientific) according to the manufacturer’s instructions. Deglycosylation was performed using PNGase F Glycan Cleavage Kit (A39245, Thermo Fisher Scientific). The deglycosylated and nondeglycosylated samples were analyzed by Western blotting.
| Cell name     | Luciferase activity SARS-CoV | SARS-CoV-2 | Mock | ACE2 Total | Gly | Un-gly | mRNA | TMPRSS2 Protein | mRNA | Kim-1 Protein | mRNA | NRP-1 Protein | mRNA |
|--------------|-----------------------------|-----------|------|------------|-----|--------|------|----------------|------|---------------|------|---------------|------|
| **Respiratory system** |                             |           |      |            |     |        |      |                |      |               |      |               |      |
| JHU029       | 22.33 ± 0.67                | 433.3 ± 93.54* | 27.00 ± 1.16 | 8279 | 1131 | 7148 | 30.42 | 2087 | 26.92 | 3827 | 28.38 | 0 | 21.29 |
| NCL H460     | 37.67 ± 2.85                | 22.33 ± 1.45  | 26.00 ± 3.06  | 5170 | 1246 | 3924 | 29.04 | 6720 | 28.49 | 2040 | 22.96 | 2996 | 20.19 |
| NCL H322     | 35.33 ± 6.17                | 36.00 ± 3.22  | 28.67 ± 1.86  | 15387 | 9539 | 5848 | 23.42 | 2328 | 24.32 | 0 | 28.69 | 0 | 21.82 |
| NCL H520     | 28.00 ± 4.58                | 57.33 ± 27.43* | 37.00 ± 2.52  | 448 | 448 | 0 | 22.24 | 3987 | 21.92 | 0 | 23.05 | 0 | 19.85 |
| A549         | 34.33 ± 6.12                | 160.0 ± 57.10* | 24.67 ± 6.49  | 4601 | 271 | 4330 | 21.03 | 0 | 21.59 | 19544 | 15.30 | 16783 | 19.22 |
| HLBEC        | 408.67 ± 208.99*            | 378.67 ± 172.20* | 33.00 ± 1.16  | 13544 | 423 | 13121 | 25.50 | 0 | 28.50 | 879 | 19.88 |
| HSAEC        | 185.33 ± 61.39*             | 204.33 ± 99.11* | 32.67 ± 2.33  | 13724 | 624 | 13100 | 28.53 | 0 | 29.78 | 3011 | 19.75 |
| **Urinary system** |                             |           |      |            |     |        |      |                |      |               |      |               |      |
| 769-P        | 211471 ± 8974*              | 1555 ± 215.90* | 26.00 ± 1.73  | 20436 | 14305 | 6131 | 20.30 | 1147 | 27.71 | 17436 | 15.45 | 7816 | 18.73 |
| 768-O        | 9823 ± 131.4*               | 617.3 ± 304.00* | 36.33 ± 5.93  | 11533 | 9520 | 2018 | 28.60 | 0 | 27.36 | 2622 | 18.40 | 11387 | 19.22 |
| A498         | 2873 ± 110.90*              | 906.3 ± 374.10* | 25.00 ± 3.46  | 20476 | 15492 | 4984 | 23.97 | 2768 | 27.32 | 19337 | 15.58 | 20782 | 17.40 |
| Caki-1       | 22.00 ± 2.65                | 43.33 ± 16.37  | 32.67 ± 4.67  | 3146 | 888 | 2258 | 25.50 | 822 | 24.28 | 21953 | 15.51 | 13374 | 20.12 |
| ACHN         | 622.3 ± 225.7*              | 24.67 ± 2.19  | 20.00 ± 2.65  | 5050 | 4905 | 145 | 26.80 | 16760 | 27.17 | 2903 | 16.83 | 16385 | 20.23 |
| HRC-45       | 25.67 ± 5.24                | 607.7 ± 281.60* | 27.33 ± 4.37  | 944 | 401 | 543 | 27.00 | 3951 | 25.83 | 15533 | 15.46 | 14228 | 20.13 |
| HRC-63       | 25.33 ± 3.28                | 19.00 ± 2.89  | 32.00 ± 1.53  | 3812 | 571 | 3241 | 28.12 | 2340 | 27.31 | 9618 | 16.07 | 7017 | 19.36 |
| HRC-59       | 26.33 ± 2.60                | 39.67 ± 10.73  | 38.00 ± 4.62  | 965 | 800 | 165 | 27.16 | 0 | 25.11 | 23.95 | 13139 | 18.35 |
| **Immune system** |                             |           |      |            |     |        |      |                |      |               |      |               |      |
| BJAB         | 31.00±2.08                  | 76.00±1.00*  | 31.67 ± 6.69  | 0 | 0 | 0 | 21.43 | 961 | 16.72 | 0 | 25.31 | 0 | 23.14 |
| BCP-1        | 30.67±1.20                  | 72.67±2.60*  | 33.00 ± 1.16  | 0 | 0 | 0 | 26.67 | 3627 | 21.73 | 0 | 28.89 | 4700 | 23.84 |
| BC-3         | 26.67±4.98                  | 38.00±1.73*  | 27.33 ± 1.33  | 0 | 0 | 0 | 26.06 | 0 | 25.78 | 0 | 31.17 | 0 | 29.15 |
| THP-1        | 33.33±3.71*                 | 51.00±7.51*  | 25.00 ± 1.16  | 1946 | 0 | 1946 | 32.67 | 2323 | 26.31 | 0 | 28.99 | 11857 | 22.68 |
| **Digestive system** |                             |           |      |            |     |        |      |                |      |               |      |               |      |
| HuH-7        | 114797±7622*                | 151463±10774* | 28.67 ± 6.17  | 12768 | 12045 | 723 | 19.52 | 0 | 24.47 | 11532 | 16.42 | 20991 | 20.77 |
| PCI-13       | 276.3±47.35*                | 41.67±1.67*  | 27.33 ± 2.03  | 9431 | 4897 | 4534 | 22.02 | 0 | 22.64 | 0 | 32.26 | 0 | 21.66 |
| UDSCC2       | 30.00±2.08                  | 30.00±4.04   | 30.33 ± 4.63  | 8306 | 1069 | 7237 | 26.60 | 1930 | 26.92 | 2429 | 26.34 | 0 | 20.80 |

(Continues)
2.5 | Western blotting

Protein lysates were prepared in sample buffer and loaded onto a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel. The proteins were transferred onto a nitrocellulose membrane (A13420267, GE Healthcare Life Science). The membranes were blocked with 5% nonfat milk at room temperature for 1 h and then incubated with primary antibodies diluted at 1:1000 in Tris-buffered saline with 0.1% Tween-20 (TBS-T) buffer containing 1% bovine serum albumin (BSA, Sigma) overnight at 4°C. The primary antibodies used were a rabbit polyclonal antibody to ACE2 (ab15348, Abcam), rabbit monoclonal antibodies to TMPRSS2 (EPR3861) (ab92323, Abcam), Kim-1 (E1R9N) (14971 S, CST) and NRP-1 (EPR3113) (ab81321, Abcam), and a mouse monoclonal antibody to β-tubulin. Following washing, the membranes were incubated with their respective secondary antibodies for 1 h at room temperature. Goat anti-rabbit horseradish peroxidase (HRP) conjugated IgG (7074 V, CST) and horse antimouse HRP conjugated antibody (7076 V, CST) were diluted at 1:5,000 in TBS-T with 1% BSA. After wash, the signal was detected with SuperSignal™ West Femto Maximum Sensitivity Substrate (34096, Thermo Fisher Scientific) and visualized with the ChemiDoc™ MP Imaging System (Bio-Rad). The intensity of the protein bands was quantified with the ImageJ Software.

2.6 | mRNA expression analysis

Reverse transcription-quantitative real-time PCR (RT-qPCR) was performed to analyze levels of transcripts. Briefly, total RNA was extracted using TRI Reagent (T9424-200ML, Sigma) according to the manufacturer's instructions. Total RNA was converted to complementary DNA (cDNA) using a High-Capacity cDNA Reverse Transcription Kit (4368814, Thermo Fisher Scientific). Quantitative real-time PCR (qPCR) was performed using SsoAdvanced™ Universal SYBR® Green Supermix (172-5272, Bio-Rad) in the CFX Connect Real-Time System (Bio-Rad). The specific primers were listed in Table 2. β-actin gene was used for normalization, and the relative expression levels were shown as Ct values.

2.7 | Immunofluorescence assay (IFA)

The expression levels and cellular localization of ACE2, Kim-1, and NRP-1 were analyzed by IFA in Huh-7, 769-P, HRC45, ACHN, H520, MCF-7, H322, HSAEC, and HLBEC cells. Cells seeded overnight on slides were treated with precooled methanol for 10 min at −20°C. Following incubation with primary antibodies of NRP-1, TMPRSS2, Kim-1, and ACE2 diluted at 1:200 in PBS with 3% BSA overnight at 4°C, and then a fluorescein isothiocyanate (FITC)-labeled secondary antibody for 1 h at room temperature,
the slides were stained with 4',6-diamidino-2-phenylindole (DAPI) for 10 min at room temperature. The slides were sealed with MilliporeSigma™ Calbiochem FluorSave™ Reagent (34-578-920 ML, Thermo Fisher Scientific) and visualized with an Olympus IX83 Microscope.

2.8 Statistical analysis

All the experiments were independently performed at least three times, each with at least three repeats. GraphPad Prism 6 was used for statistical analysis. Results were presented as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) was performed if multiple samples were involved. p-value was calculated by unpaired two-tailed Student’s t test. p < 0.05 was considered statistically significant. Correlation analysis was performed by Pearson correlation analysis using Graphpad Prism 6, and the p and r values were presented.

3 RESULTS

3.1 SARS-CoV-2 pseudovirus can infect a wide range of cell types

To investigate the cell tropism, we used 22 cell lines and 3 types of primary cells isolated from five human organ systems for infection of SARS-CoV-2 and SARS-CoV pseudoviruses. Cells infected with pseudoviruses for 60 h were examined for luciferase activity. Cells infected with the “naked pseudovirus” were used as negative controls, which produced almost no luciferase signal similar to the mock-infected cells. We considered an infection as “positive” when the luciferase signal was higher than the mean of the mock-infected cells plus 5 SEMs (Figure 1A and Table 3). At least one cell line or cell type from each of the five organ systems gave positive signals after infection by SARS-CoV-2 pseudovirus. Strong signals were detected for both SARS-CoV-2 and SARS-CoV pseudoviruses in primary human lobar bronchial epithelial cells (HLBEC) and primary human small airway epithelial cells (HSAEC) from the respiratory system, 769-P, 786-O, and A498 cells from the urinary system, and Huh-7 cells from the digestive system.

Variations between SARS-CoV-2 and SARS-CoV pseudoviruses were observed for several cell lines. Strong signals were observed in JHU029 and A549 cells from the respiratory system, and HRC45 cells from the urinary system for SARS-CoV-2 but not SARS-CoV pseudovirus. In contrast, robust infection was observed for urinary ACHN cells, digestive system PCI-13 cells, and breast tumor MCF-7 cells for SARS-CoV but not SARS-CoV-2 pseudovirus. Weak but significant pseudovirus infections were also observed in H520 cells from the respiratory system, BCP-1 and BJAB cells from the immune system, and primary human umbilical vein endothelial cells (HUVEC) from the reproductive system for SARS-CoV-2 but not SARS-CoV pseudoviruses. In contrast to SARS-CoV and SARS-CoV-2 pseudoviruses, all cell lines infected by VSV-G pseudovirus uniformly produced strong luciferase signals across all six cell lines examined (Figure 1B). These results indicated that SARS-CoV and SARS-CoV-2 pseudoviruses had tropism for specific types of cells.

3.2 Entry-related recombinant proteins interfere with infection of SARS-CoV-2 and SARS-CoV pseudoviruses

Entry of SARS-CoV-2 into cells is mediated by the S protein through RBD interaction with cellular receptor(s). To demonstrate the specificity of the pseudovirus infections, we performed blocking assays using S1 and RBD fusion recombinant proteins in Huh-7 cells, which had the highest pseudovirus infection levels (Figure 1A). Both S1 and RBD recombinant proteins effectively blocked the infection of SARS-CoV-2 pseudovirus in a dose-dependent manner, reducing luciferase signals by >60 to >95% at the highest dose of 25 µg/ml, respectively (Figure 2A,B).

In contrast, a control hFc recombinant protein did not block any infectivity of both SARS-CoV-2 and SARS-CoV pseudoviruses (Figure S1). Recombinant ACE2 protein also effectively blocked the infection of SARS-CoV-2 in a dose-dependent manner, reducing infection by >95% at the highest dose of 25 µg/ml (Figure 2C). Interestingly, only marginal reduction of infectivity was observed when Kim-1 and NRP-1 recombinant proteins were used in the blocking assay. Although the inhibitory effects were statistically significant in one-way ANOVA analyses (p = 0.0023 for Kim-1 and p = 0.0136 for the NRP-1), no statistical difference was detected between individual blocking groups at any concentrations and the unblocked group (Figure 2D,E), suggesting weak roles of Kim-1 and NRP-1 proteins in SARS-CoV-2
infection. Similar results were observed for SARS-CoV pseudovirus (Figure 2A–E).

3.3 | mRNA and protein expression levels of SARS-CoV-2 entry-related factors ACE2, TMPRSS2, Kim-1 and NRP-1

To examine cellular receptor abundance as contributing factors to pseudovirus infectivity (Figure 1A), we performed RT-qPCR to detect the mRNA levels of ACE2, TMPRSS2, Kim-1, and NRP-1 genes and presented the results as C_t values (Figure 3). There was no obvious correlation of SARS-CoV-2 pseudovirus infectivity with the mRNA levels of these cellular genes. Among cells with high SARS-CoV-2 pseudovirus infectivity, A549, 769-P, A498, and Huh-7 cells had relatively higher ACE2 expression levels (C_t values lower than 25) while JHU029, HLBE, HSAEC, 786-O, and HRC45 cells had relatively lower ACE2 expression levels; and only A549 and Huh-7 cells had relatively higher TMPRSS2 expression levels (C_t values lower than 25) (Figure 3). Numerous cell lines had extremely high Kim-1 expression levels (C_t values lower than 18); however, among cells with high SARS-CoV-2 pseudovirus infectivity, A549, 769-P, A498, Huh-7, 786-O, and HRC45 cells had relatively higher Kim-1 expression levels (C_t values lower than 20) while JHU029, HLBE, and HSAEC cells had relatively lower Kim-1 expression levels (Figure 3). Most of the cell lines and primary cells had robust NRP-1 expression levels (C_t values lower than 25 C_t) except HUVEC and BC-3 cells (Figure 3).

We then examined expression levels of ACE2, TMPRSS2, Kim-1, and NRP-1 proteins by Western blotting (Figure 4A). Despite some discrepancies, the protein levels were in general in agreement with the mRNA levels of genes encoding these proteins. For ACE2 protein, we detected two bands representing the glycosylated and unglycosylated forms (gly-ACE2 and ungly-ACE2) with the expected molecular mass sizes of ~85 kD and ~120 kD, respectively (Figure 4A). Treatment of cell lysate with
We examined the correlation between SARS-CoV-2 pseudovirus infectivity and expression levels of ACE2, TMPRSS2, Kim-1, and NRP-1 proteins (Figure 1A). SARS-CoV-2 pseudovirus infectivity had a positive correlation with the gly-ACE2 protein level, approaching statistical significance ($r = 0.3840$, $p = 0.0581$), but not with unglycosylated ACE2 ($r = 0.1651$, $p = 0.4303$) (Figure 6A,B). Similarly, SARS-CoV pseudovirus infectivity had a strong positive correlation with gly-ACE2 ($r = 0.6140$, $p = 0.0011$) but not ungly-ACE2 ($r = 0.03706$, $p = 0.8604$) (Figure 6C,D). These results confirmed the critical role of ACE2 in SARS-CoV-2 and SARS-CoV infection, and that glycosylation may be essential for entry, particularly for SARS-CoV. There was no correlation between SARS-CoV-2 pseudovirus infectivity and the expression of TMPRSS2 or...

**FIGURE 4** Expression levels of ACE2, TMPRSS2, Kim-1, and NRP-1 proteins in different cell lines/types, and examination of ACE glycosylation in Huh-7 cells analyzed by Western blot analysis. (A) Expression levels of ACE2, TMPRSS2, Kim-1, and NRP-1 proteins in different cell lines/types. Results showed that there are different expression profiles of ACE2, TMPRSS2, Kim-1, and NRP-1 in different cell lines/types. (B) Examination of ACE glycosylation in Huh-7 cells. Untreated and PNGase F-treated cell lysates were examined. β-tubulin was used for loading normalization. Two bands of ~85 kD and ~120 kD were detected for ACE2 protein representing the unglycosylated and glycosylated forms of ACE2 (ungly-ACE2 and gly-ACE2), respectively. Kim-1, kidney injury molecule-1; NRP-1, neuropilin-1; TMPRSS2, transmembrane serine protease 2

PNGase F, which removed the carbohydrate, indeed shifted all the gly-ACE2 to ungly-ACE2 in Huh-7 cells (Figure 4B). For cells that had high SARS-CoV-2 pseudovirus infectivity, 769-P, 786-O, A498, JHU029, HLHBC, HSAEC, and Huh-7 cells had overall relatively higher levels of ACE2 protein, of which 769-P, 786-O, A498, and Huh-7 cells had relatively higher levels of gly-ACE2, while A549 cells had a relatively low expression level and HRC45 cells had an almost undetectable level of ACE2 protein (Figure 4A). The expression levels of TMPSS2 protein were in general low in most of these cell lines and primary cells except T47D and H460 cells (Figure 4A). Among cells that had relatively higher levels of SARS-CoV-2 pseudovirus infectivity, 769-P, A498, HRC45, A549, and Huh-7 cells had relatively higher levels of Kim-1 protein, while 786-O, JHU029, HLHBC, and HSAEC cells had relatively lower or undetectable levels of Kim-1 protein (Figure 4A). For cells that had relatively higher levels of SARS-CoV-2 pseudovirus infectivity, relatively higher levels of NRP-1 protein were detected in 769-P, 786-O, A498, HRC45, A549, and Huh-7 cells while JHU029, HLHBC, and HSAEC cells had relatively lower or undetectable NRP-1 protein levels (Figure 4A). Overall, cells with high SARS-CoV-2 pseudovirus infectivity had relatively higher levels of ACE2 and gly-ACE2 proteins (769-P, 786-O, A498, JHU029, HLHBC, HSAEC, and Huh-7) or Kim-1 and NRP-1 proteins (A549 and HRC45).

We further examined the expression of ACE2, TMPRSS2, Kim-1, and NRP-1 proteins by IFA in selected types of cells including 769-P, HRC45, HLHBC, HSAEC, and Huh-7 cells with relatively higher levels of SARS-CoV-2 pseudovirus infectivity, and ACHN, MCF-7, H322, and H520 cells with relatively lower levels of SARS-CoV-2 pseudovirus infectivity (Figure 5). Overall, the staining intensities of all four proteins in different types of cells was consistent with their levels detected by Western blotting. No difference in the staining pattern of the four proteins in different types of cells was observed (Figure 5).
Kim-1 protein (Figure 6E,G). However, there was a weak correlation between SARS-CoV-2 pseudovirus infectivity and the expression of NRP-1 protein approaching statistical significance ($r = 0.3455, p = 0.0907$) (Figure 6I). There was no correlation between SARS-CoV pseudovirus infectivity and the expression of TMPRSS2 or NRP-1 protein (Figure 6F,J). However, there was a correlation between SARS-CoV pseudovirus infectivity and the expression of Kim-1 protein, approaching statistical significance ($r = 0.3896, p = 0.0542$) (Figure 6H). Hence, in addition to gly-ACE2 protein, NRP-1 protein expression might be important for SARS-CoV-2 infection while Kim-1 expression might be important for SARS-CoV infection.

4 | DISCUSSION

Infection by SARS-CoV-2 has been detected in diverse types of cells and tissues such as AECs in alveoli, nasopharyngeal region, kidney, pneumocytes, alveolar macrophages, and lymph nodes, and so on. We have recently examined lung tissues from patients with severe COVID-19 and found extensive expression of SARS-CoV-2 proteins in a wide range of parenchymal and immune cells. Results of these studies suggest that SARS-CoV-2 could infect a wide range of cell types.

In the current study, we investigated the cell tropism of SARS-CoV-2 using a pseudovirus to infect 22 cell lines and 3 types of primary cells isolated from respiratory, urinary, immune, digestive, and reproductive systems (Figure 1A and Table 3). At least one cell line or one type of primary cell from each of five body systems was infected by SARS-CoV-2 pseudovirus. These results confirm the broad tropism of SARS-CoV-2, which likely contributes to the complex pathological syndromes in COVID-19 patients. Interestingly, broad infection by SARS-CoV pseudovirus in at least one cell line or cell type of these body systems except the immune system was also observed. It is known that SARS-CoV pathology is mostly localized to the respiratory system, and to a lesser extent with other organ systems. This could be due to its more rapid progression and higher case fatality rate than SARS-CoV-2 infection, which might mask
FIGURE 6  (See caption on next page)
nonrespiratory pathologies that would otherwise occur with SARS-CoV infection.

Close to half of the cell lines examined in this study were refractory to SARS-CoV-2 pseudovirus infection. It is possible that complex factors might be involved in regulating SARS-CoV-2 infection in vivo. Indeed, numerous cytokines including type 1 and 2 interferons have been shown to upregulate the expression of ACE2 and promote SARS-CoV-2 infection.37,38 Further studies to identify factors that might contribute to SARS-CoV-2 infection and spread are warranted.

Entry of SARS-CoV-2 and SARS-CoV is primarily mediated by viral S1 protein through its interaction with cellular receptors.39 Our results show an efficient inhibitory effect of S1 and RBD recombinant proteins on the infection of both SARS-CoV-2 and SARS-CoV pseudoviruses, and that RBD recombinant protein had a more potent inhibitory effect, confirming the essential role of S1 protein in mediating the infection of both SARS-CoV-2 and SARS-CoV through the RBD domain.

Numerous cell types have been shown to have roles in SARS-CoV-2 entry, including ACE2, TMPRSS2, Kim-1, and NRP-1 proteins.11,24,40-42 RNAseq has demonstrated high ACE2 expression in diverse cell types such as pulmonary AT2, respiratory epithelial, myocardial, digestive tract epithelial, nasal goblet secretory and kidney cells, ileal absorptive enterocytes, and salivary glands.37,43,44 IFA staining has revealed widespread expression of ACE2 protein in different parenchymal and immune cell types.10 ACE2 is present in a different part of the kidney and has a high expression level,15,46 explaining why SARS-CoV-2 kidney infection had a significantly higher risk of inhospital death.47

Although most of the SARS-CoV-2-infected cells are ACE2-positive in lung tissues from COVID-19 patients, we have also observed ACE2-negative SARS-CoV-2-infected cells, suggesting possible SARS-CoV-2 infection through an ACE2-independent mechanism.10 Indeed, among the cell lines and primary cell types that were positively infected by SARS-CoV-2 pseudovirus, we also found that A549 and HRC45 cells had extremely weak levels of ACE2 protein expression (Figure 4A). Nevertheless, we found a trend of positive correlation of SARS-CoV-2 pseudovirus infectivity with the level of gly-ACE2 protein but not ungly-ACE2 protein (Figure 6A, B).

It has been reported that in engineered human tissue, SARS-CoV-2 infection was inhibited by soluble human ACE2.48 Our results showed that recombinant ACE2 protein effectively blocked the infectivity of SARS-CoV-2 pseudovirus in Huh-7 cells and that the ACE2 inhibitory effect occurred in a lower concentration than that reported in a previous study48 (Figure 2C). These results support an important role of ACE2 protein, particularly gly-ACE2 protein, in SARS-CoV-2 infection of ACE2-dependent cells. On the other hand, we found a statistically significant positive correlation of SARS-CoV pseudovirus infectivity with the levels of gly-ACE2 protein but not ungly-ACE2 protein. Hence, compared with SARS-CoV, SARS-CoV-2 infection is less dependent on gly-ACE2, which might explain its promiscuous broad tropism and more infectious nature.10 In fact, the dual nature of human ACE2 glycosylation in binding to SARS-CoV-2 spike has been reported.49,50 Specifically, the glycans at the N90 and N322 glycosylation sites had opposite effects on S protein binding.49

Interestingly, A549 and HRC45 cells had a robust SARS-CoV-2 pseudovirus infection but weak levels of ACE2 protein and both expressed strong levels of Kim-1 and NRP-1 proteins (Figure 4). We found a trend of positive correlation of SARS-CoV-2 pseudovirus infectivity with the level of NRP-1 protein. Furthermore, recombinant NRP-1 protein blocked SARS-CoV-2 pseudovirus in a dose-dependent fashion albeit no significant difference was detected when individual recombinant protein treated groups were compared with the untreated infected group. These results suggest that NRP-1 might mediate SARS-CoV-2 infection in ACE2-negative cells though this role is less obvious in ACE2-dependent SARS-CoV-2 infection. On the other hand, we did not find any correlation of SARS-CoV pseudovirus infectivity with the level of NRP-1 protein level. In contrast, the Kim-1 protein level was not correlated with SARS-CoV-2 pseudovirus infectivity, however, it was weakly correlated with the SARS-CoV pseudovirus infectivity. Similar to NRP-1, recombinant Kim-1 protein blocked infection of both SARS-CoV-2 and SARS-CoV pseudoviruses in a dose-dependent fashion in one-way ANOVA analysis albeit no significant difference was detected when the treated groups were compared with the untreated infected group. Hence, the roles of NRP-1 and Kim-1 proteins in infection of SARS-CoV-2 or SARS-CoV are likely minor, if any, in ACE2-dependent SARS-CoV-2 infection.

Taken together, SARS-CoV-2 can infect a wide range of cells from different human body systems. As factors that mediate SARS-CoV-2 infection, ACE2, TMPRSS2, Kim-1, and NRP-1 are broadly distributed in cells from different body systems. In cells that SARS-CoV-2 infection depends on ACE2 protein, TMPRSS2, Kim-1, and NRP-1 proteins are likely not critical for SARS-CoV-2 infection. However, in cells that SARS-CoV-2 infection does not depend on

**FIGURE 6** Analyses of the correlation between the infectivity of SARS-CoV-2 or SARS-CoV and the protein expression levels of entry-related factors. (A–J) Correlation of the infectivity of SARS-CoV-2 (A, C, E, G, and I) or SARS-CoV (B, D, F, H, and J) with glycosylated ACE2 (gly-ACE2) (A and B), unglycosylated ACE2 (ungly-ACE2) (C and D), TMPRSS2 (E and F), Kim-1 (G and H) and NRP-1 (I and J). The "red" dots represent cell lines/types that were not infected by the pseudoviruses. The "green" dots represent cell lines/types that were infected by the pseudoviruses. Kim-1, kidney injury molecule-1; NRP-1, neuropilin-1; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SARS-CoV, severe acute respiratory syndrome coronavirus; TMPRSS2, transmembrane serine protease 2
ACE2 proteins, Kim-1 and NRP-1 proteins are likely to mediate SARS-CoV-2 infection. The usage of these diverse factors in cell entry might endow the promiscuity of SARS-CoV-2 infection, and hence broad cell tropism.

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CONFLICT OF INTERESTS
The authors declare that there are no conflict of interests.

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DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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