Molecular Therapy
Methods & Clinical Development
Original Article

Comprehensive and systemic optimization for improving the yield of SARS-CoV-2 spike pseudotyped virus

Xinping Fu,[1] Lihua Tao,[1] and Xiaoliu Zhang[1]

[1]Department of Biology and Biochemistry and Center for Nuclear Receptor and Cell Signaling, University of Houston, Houston, TX 77204, USA

Virus neutralization assay is principally conducted by measuring the ability of the antibodies in patient sera to prevent the infection of susceptible cells by the virus. As SARS-CoV-2 is classified as a risk group 3 pathogen, neutralization assay using a live virus needs to be handled in a biosafety level 3 laboratory. To overcome this limitation, pseudotyped viruses have been developed as an alternative for the live SARS-CoV-2. However, one of the issues that we and others have encountered during the production of pseudotyped virus with SARS-CoV-2 spike protein was the low virus yield. In our own experience, we were only able initially to produce a stock with a virus titer that is more than two orders of magnitude lower than what we usually get with a vesicular stomatitis virus glycoprotein (VSV-G) pseudotyped lentiviral vector. We have conducted a series of improvements, including using a C-terminal truncated form of spike protein and a D614G mutated spike. Together, these have led to a significant improvement in the yield of the pseudotyped virus. Finally, our data show that using a high-affinity ACE2-expressing cell line resulted in a reduction in detection sensitivity of the neutralization assay.

INTRODUCTION

The recent emergence and rapid global spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and the resulting coronavirus disease 2019 (COVID-19) has created an unprecedented health crisis.[1] There is an urgent need to develop efficient, reliable, and cost-effective diagnostics to fight this global pandemic.[2] Serological detection of virus-specific antibodies (Abs) is one of the most important diagnostic tests in monitoring patients' immune responses for viral infections such as SARS-CoV-2 and for surveilling the magnitude of virus spread in the population. Additionally, Ab detection also plays a pivotal role in evaluating and facilitating vaccine development. Qualitative and quantitative measurement of Abs are primarily based on two major detection procedures: enzyme-linked immunosorbent assay (ELISA) and neutralization assay.[3] The former is used mainly for measuring the total serum Abs, and the latter is used specifically for quantifying neutralization Abs (nAbs), which are considered to be the key Ab fraction in preventing virus infection.

In principle, virus neutralization assay is conducted by measuring the ability of the Abs in patient sera to prevent the infection of susceptible cells by the virus. For SARS-CoV-2, the nAbs act mainly by binding to the receptor-binding domain (RBD) within the spike protein to prevent the virus from attaching to the ACE2 receptor to gain entry.[4] As SARS-CoV-2 is classified as a risk group 3 pathogen, neutralization assay using a live virus must be handled in a biosafety level 3 (BSL-3) laboratory.[5] To overcome this limitation, pseudotyped viruses, based on either a lentiviral vector or vesicular stomatitis virus (VSV), have been developed as an alternative for the live SARS-CoV-2.[6] The pseudotyping is carried out by providing the SARS-CoV-2 spike protein during the viral vector package so that it can be assembled to the viral envelope of the pseudotyped virus, which renders it with the same infection mechanism as SARS-CoV-2. Consequently, there is a good correlation between the experimental results obtained between the live SARS-CoV-2 and the pseudotyped viruses. Moreover, pseudotyped viruses have two distinct advantages over the live SARS-CoV-2 for nAb assays. First, the pseudotyped virus allows the assay to be done in a regular BSL-2 lab. Second, the pseudotyped virus often contains a marker gene (e.g., luciferase or GFP), which allows for easier and more accurate quantification than if the assay were performed with live SARS-CoV-2.

However, one of the issues that we and others have encountered during the production of a pseudotyped virus with SARS-CoV-2 spike protein was that the virus yield was extremely low. In our own experience, we were only able to initially produce a stock with a titer of around 1×104 infectious units (IU) per milliliter, which is more than two orders of magnitude lower than what we usually get with a VSV glycoprotein (VSV-G) pseudotyped lentiviral vector. We have since conducted a series of improvements, including using a C-terminal truncated form of spike protein and the D614G mutated spike, and tested the packaging efficiency in a series of cell lines. Together, these have led to over two orders of magnitude of improvement in the yield of the pseudotyped virus. Finally, our data show that using a high-affinity ACE2-expressing

Received 5 November 2020; accepted 21 December 2020;
https://doi.org/10.1016/j.omtm.2020.12.007.

Correspondence: Xiaoliu Zhang, Department of Biology and Biochemistry and Center for Nuclear Receptor and Cell Signaling, University of Houston, Houston, TX 77204, USA.
E-mail: xzhang5@central.uh.edu

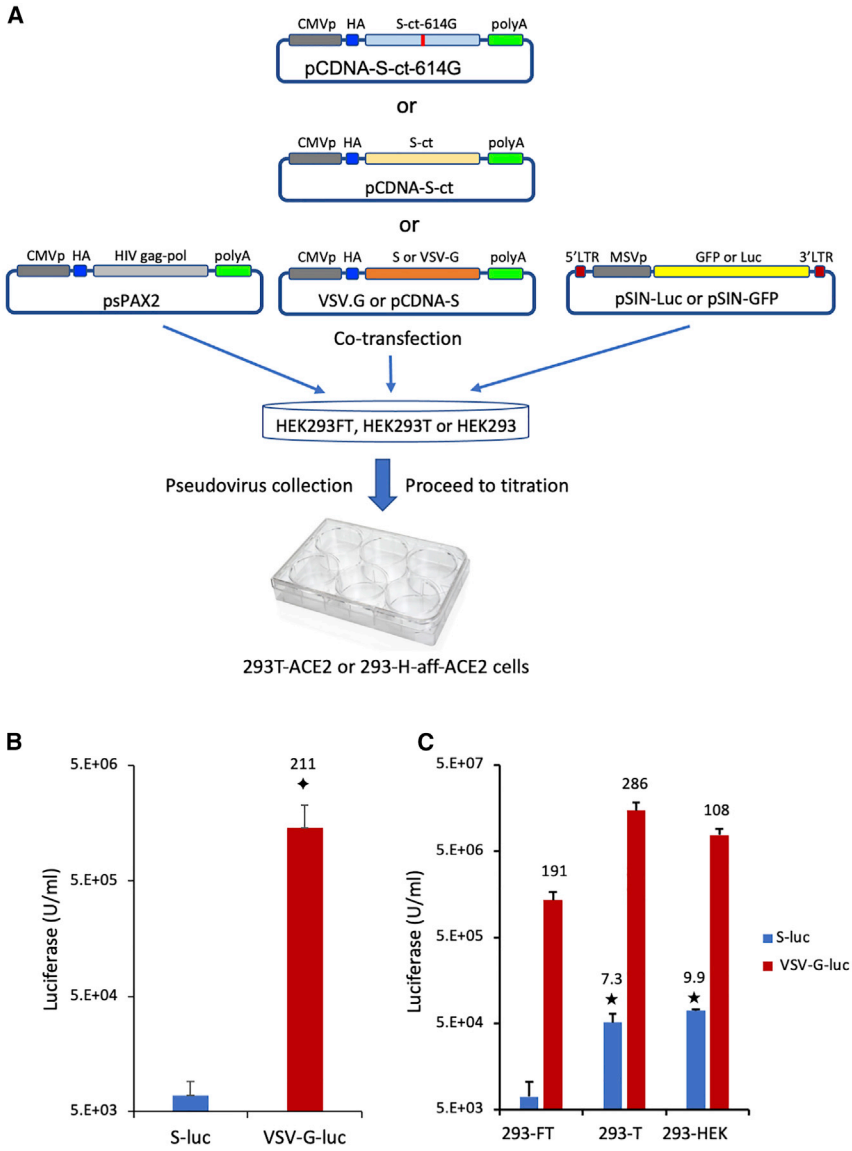

Figure 1. Comparison of lentiviral vector pseudotyping efficiency between SARS-CoV-2 spike and VSV-G in different derivatives of 293 cells

(A) Procedures of pseudovirus production and titration. The plasmids used for the preparation of either S pseudotyping or VSV-G pseudotyping are depicted at the top. They are co-transfected into different derivatives of 293 cells, and the generated pseudovirus is subsequently used for titration on ACE2-expressing 293T cells. The modified versions of S used in the experiments shown in the following figures are also depicted. (B) Comparison of pseudotyping efficiency between SARS-CoV-2 S and VSV-G in 293FT cells. The number on the top of the red bar indicates the fold of difference of the luciferase reading over those from pSIN-Luc vector. (C) Comparison of pseudotyping efficiency between S and VSV-G pseudotyping in different 293 cell derivatives. The numbers on the top of the red bars indicate the fold of increase of the luciferase reading over those from S. The numbers on the top of the blue bars indicate the fold of increase of the luciferase reading over those in 293FT cells. ◆$p < 0.01$ as compared to S-luc; ★$p < 0.01$ as compared to that in 293FT cells.

cell line resulted in a reduction in detection sensitivity of the neutralization assay.

RESULTS

Unmodified process led to low-titer pseudotyped virus production

We synthesized the full-length spike gene once the sequence of SARS-CoV-2 was published.[7] We then used it to pseudotype the lentiviral vectors pSIN-Luc and pSIN-GFP, which carry either the *luciferase* or the *green fluorescent protein* (*GFP*) as the marker gene for detection and are routinely used in our laboratory via packaging with the VSV-G protein.[8] We initially focused on the pSIN-Luc vector by co-transfecting it with VSV-G, which was included in this experiment as a positive control. We chose to conduct the initial experiment in 293FT cells, as they are frequently used for generating lentiviral vectors.[9] The supernatants were collected at 48 h after plasmid transfection, and the pseudotyped lentiviral vectors were titrated on 293-ACE2 cells, which were established by us by stably transducing 293FT cells with the human ACE2 gene. The results in Figure 1 showed that, despite the successful pseudotyping, the titer of the viral vector generated from the SARS-CoV-2 spike (S-luc), as represented by the luciferase activity expressed by the viral vector, was more than 200-fold lower than that from the VSV-G pseudotyped virus.

Next, we conducted another pseudotyping for vector production with either S or VSV-G in two other 293 cell lines. One of them was the parental HEK293 cells and the other one was 293T, which is similar to 293FT and is also derived from HEK293. The result of the luciferase assay shown in Figure 1C indicated that both HEK293 and 293T cells gave a significantly higher yield of S pseudotyped virus than that from 293FT cells (7.3- and 9.9-fold increase, respectively). However, these two cells also support the yield of the VSV-G pseudotyped virus better. Consequently, the difference between the large gap in the yield of these pseudotypings remains largely unchanged. Together, these data suggest that S is significantly less efficient than VSV-G in pseudotyping lentiviral vectors for gene transduction.

These results showed that SARS-CoV-2 spike protein in its natural form is significantly less efficient than VSV-G, which is frequently used in pseudotyping a lentiviral vector. These results also

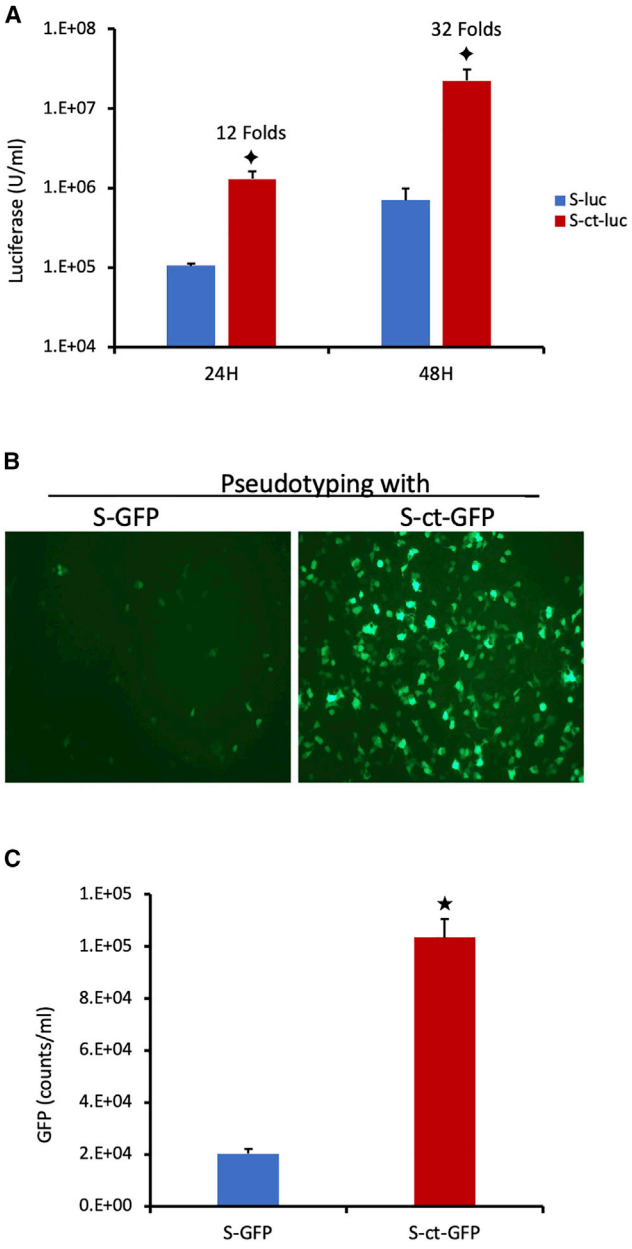

Figure 2. C-terminal 19 aa truncation of S can significantly enhance the pseudotyping efficiency

pSIN-GFP or pSIN-Luc were co-transfected with psPAX2 and pCDNA-S or pCDNA-S-ct to 293T cells. The supernatants were harvested 48 h later and used to transduce 293T-ACE2 cells. (A) For quantification of pSIN-Luc pseudovirus transduction efficiency, cells were harvested 72 h later for quantification of luciferase activity. (B and C) The cells transduced by pSIN-GFP were visualized for GFP expression 72 h later (B), before they were harvested for quantitative measurement by flow cytometry (C). ◆$p < 0.01$ as compared to S-luc; ★$p < 0.01$ as compared to S-GFP.

demonstrated that unlike VSV-G, the pseudotyping efficiency with SARS-CoV-2 spike protein varied widely in cells that have been derived from the same parental origin. In certain cases, a cell efficiently supporting VSV-G pseudotyping (e.g., 293FT) shows the opposite for SARS-CoV-2 spike protein pseudotyping effectiveness.

Truncation of 19 aa from the C terminus of S can significantly increase pseudotyped virus yield

It has been reported in the literature that truncation of 19 amino acids (aa) at the C terminus of the SARS-CoV-1 spike protein can significantly increase the pseudotyping efficiency on both lentiviral and VSV-based viral vectors.[10,11] To determine if the same truncation can also enhance the pseudotyping efficiency on SARS-CoV-2, we truncated the C-terminal 19 aa of S to generate S-ct. We then compared S-ct with the undeleted S for pseudotyping efficiency in 293T cells. In this experiment, we collected cells at two different time points, 24 h and 48 h after plasmid transfection. Again, the generated viral vectors in harvested supernatants were titrated on 293T-ACE2 cells. For this experiment, we measured the luciferase activity at two time points (i.e., at 24 and 48 h). The assay on both time points showed that S-ct is significantly more effective at pseudotyping the lentiviral vector than S, with 12- and 32-fold increases, respectively (Figure 2A). The result also showed that the luciferase activity was more than ten times higher in the cells harvested at 48 h than in the cells collected at 24 h, indicating that a longer transduction time may be helpful if sensitive detection is needed (e.g., for samples with low levels of nAbs).

Next, we compared S-ct with S for packaging efficiency on the same lentiviral vector but containing GFP instead of luciferase as the detection marker. This would allow the cells transduced by the pseudotyped virus to be visualized and the virus titer to be quantitated as IUs by flow cytometry detection for GFP expression. The pSIN-GFP was co-transfected in the same way as in Figure 1, and the subsequent transduction was also carried out in a similar way. The results in Figure 2B show that both the number and intensity of GFPs from cells transduced with S-ct pseudotyped virus exceed those of the same lentiviral vector pseudotyped with S. The enumeration of GFP^+ cells by flow cytometry showed that the C-terminal truncation led to more than a 5-fold increase in the actual transduction units of the pseudotyped virus (Figure 2C).

Together, these results showed that a 19 aa truncation at the C terminus of the SARS-CoV-2 spike protein led to a significant improvement in the pseudotyping efficiency. This significant improvement can be demonstrated by both luciferase measurement and GFP detection, albeit the former showed a larger magnitude of a difference than the latter.

D614G mutation led to a two-fold increase in pseudotyped virus production with the C-terminal truncated spike protein

Among the naturally occurring SARS-CoV-2 S mutants, D614G amino acid substitution was rare in the origin of COVID-19 but has increased in frequency as the pandemic has spread to the rest of the world, appearing in over 74% of all published sequences.[12] Subsequent studies show that pseudotyped lentiviruses with 614G have an increased infectivity over 614D on cells bearing ACE2 orthologs

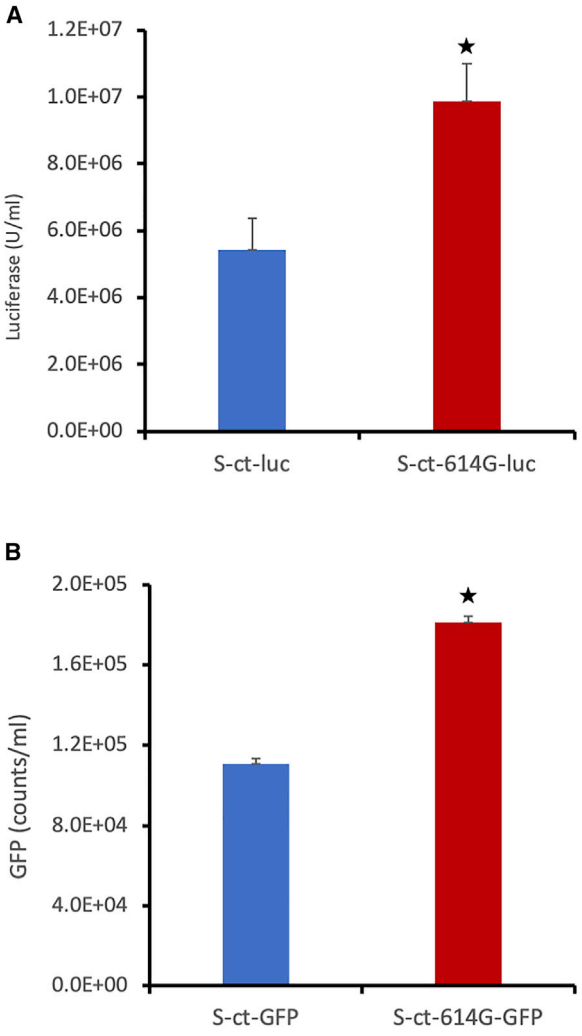

Figure 3. Incorporation of D614G into S-ct moderately increases the pseudotyping efficiency

pSIN-GFP or pSIN-Luc were co-transfected with psPAX2 and pCDNA-S-ct or pCDNA-S-ct-614G to 293T cells as described in Figure 2. The subsequent cell transduction and quantitative measurement of luciferase and GFP were also performed in the same manner as in Figure 2. (A) Luciferase activity as determined at 48 h after pseudovirus transduction. (B) GFP+ cell count by flow cytometry measured 72 h later after pseudovirus transduction. ★$p < 0.05$ as compared to S-ct pseudotyping.

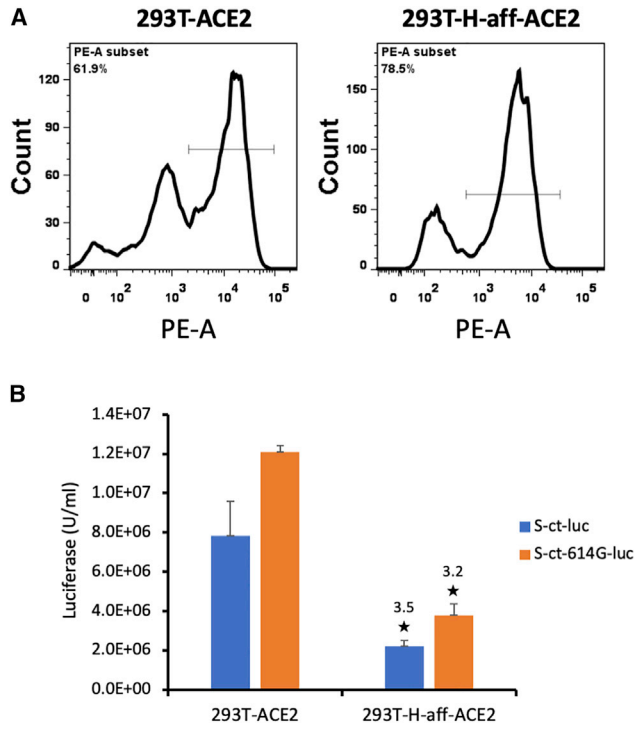

Figure 4. Comparison of pseudovirus transduction efficiency between 293T-ACE2 and 293T-H-aff-ACE2 cells

(A) Comparison of RBD binding between 293T-ACE2 and 293T-H-aff-ACE2 cells. The cells were first incubated with His-tagged RBD (in culture supernatants). This was followed by incubation with PE-conjugated mouse anti-His IgG, and, finally, by analysis by flow cytometry. The difference in the percentage of positive RBD between these two cells is significant ($p < 0.05$). (B) Both cells were seeded into 96-well plate in triplicate and were transduced with an equal amount of either S-ct or S-ct-614G pseudotyped pSIN-Luc virus. The cells were harvested 48 h later for quantification of luciferase activity. The numbers on the top of the bars in the 293T-H-aff-ACE2 group indicate the fold of reduction in luciferase activity. ★$p < 0.01$ as compared to the result in 293T-ACE2 cells.

from multiple species.[13,14] To investigate if the D614G could further enhance the pseudotyping efficiency of the C-terminal truncated spike, we introduced this mutation to S-ct to generate S-ct-614G. We then co-transfected pSIN-Luc or pSIN-GFP with psPAX2 and pCDNA-S-ct-614G to 293T cells, and the generated viruses were then used to transduce 293T-ACE2 cells for quantitative measurement for luciferase and GFP, respectively. The results in Figure 3 show that incorporation of D614G into S-ct improved the pseudotyping efficiency by approximately two-fold for both vectors. These results thus confirm the recent reports from other groups that this D614G mutation in SARS-CoV-2 spike can improve the pseudotyping efficiency; however, in the context of S-ct, the magnitude of pseudotyping enhancement seems to be less than if this mutation was in the context of a spike without the C-terminal truncation.

A cell line expressing a high-affinity ACE2 can increase the nAb detection sensitivity

Recently, Chan et al.[15] used deep mutagenesis to generate several mutations on ACE2 that show increased binding to the SARS-CoV-2 spike. Some variants containing a combination of these mutations show a high binding affinity that rivals those of monoclonal Abs.[15] To determine if these variants with the enhanced binding affinity to S could help improve the pseudovirus infectivity, we synthesized one of such variants and then transduced it into 293T cells, generating the 293T-H-aff-ACE2 cell line. We initially conducted a flow cytometry analysis on the binding of RBD to these two cell lines. The result in Figure 4A showed that RBD could bind to both cells efficiently, with 293T-H-aff-ACE2 showing a significantly higher percentage of

cells with RBD binding than in 293T-ACE2 cells. We then compared the transduction efficiency of the pseudoviruses generated from S-ct and Sct-614G forms of spike protein (shown in Figure 3 on 293T-ACE2 and 293T-H-aff-ACE2 cells). The data in Figure 4B showed an opposite result to that we had anticipated (i.e., the transduction efficiency for both S-ct and S-ct-614G pseudotyped viruses was more than 3-fold lower in 293T-H-aff-ACE2 cells than in the regular ACE2-expressing 293T-ACE2 cells). A repeated assay with varying amounts of pseudoviruses showed a similar reduction in transduction efficiency in these high-affinity ACE2-expressing cells (data not shown).

DISCUSSION

As the COVID-19 pandemic is still rampant around the globe, there is an increasing demand on using the S-pseudotyped virus for detecting anti-SARS-CoV-2 nAbs and other studies for both clinical settings and laboratory research. Hence, production of high-titer pseudotyped virus is needed to meet this increasing demand. In our initial effort in producing a pseudotyped lentiviral vector with an unmodified SARS-CoV-2 spike gene and in 293FT cells, the resultant virus yield was extremely low. We subsequently conducted a series of tests, as detailed in this article, which have led to a significant improvement in the pseudotyping efficiency with the SARS-CoV-2 S. Currently, both lentiviral vector and VSV-based vector are used for pseudotyping with SARS-CoV-2 S. Although we only conducted the tests on lentiviral vector, we believe our studies will offer valuable insight for VSV-based vector preparation with SARS-CoV-2 S pseudotyping.

One of the major factors that affects the SARS-CoV-2 S pseudotyping efficiency is the different derivatives of HEK293 cells. We initially chose to use 293FT cells for the vector production, as they are commonly chosen in many laboratories for lentiviral production.[9] However, they were found to be significantly less supportive than the other two 293 cells (the parental HEK293 and 293T cells) in producing the SARS-CoV-2 S pseudotyped lentiviral vector. The 293T cells were established from the HEK293 parental line by stably transfecting the cells with a plasmid encoding a temperature-sensitive mutant of the SV40 large T antigen.[9] The 293FT cell line is derived from a fast-growing, highly transfectable clonal isolate (293F) of HEK293 and stably expresses the SV40 large T antigen from the human cytomegalovirus (CMV) promoter. The underlying reason for this different pseudovirus yield in these three 293 cells is not clear. Transfection efficiency is an unlikely cause for this apparent difference, as all three cells seem to be equally and efficiently transfected by the combination of the three plasmids used for the vector generation and pseudotyping. SV40 large T antigen expression status is not considered as the likely cause either, as both 293FT and 293T express it. One plausible explanation is that the rapid cell cycling of 293FT might have created a disturbance and/or discordance in the assembly and/or budding of the pseudotyped virus. Another major factor that affects the SARS-CoV S pseudotyping efficiency is the C-terminal 19 aa truncation. Previous studies on SARS-CoV-1 and more recent studies on SARS-CoV-2 have shown that truncation of a short sequence of 19 aa in the C terminus of S spanning this retention signal can significantly improve the pseudotyping efficiency.[10,16] Our data showed a similar magnitude of enhancement in the pseudovirus yield with SARS-CoV-2 S containing a similar C-terminal truncation. The C terminus of the spike protein in the coronavirus family, including SARS-CoV-2, contains an endoplasmic reticulum retrieval signal that allows its retention in the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) for virus assembly and/or budding. Mutation or deletion of this retention signal changes the transportation of S to the plasma membrane instead,[16] which may have contributed to the higher pseudotyping efficiency, as lentiviruses are primarily assembled/released through plasma membrane.[17,18]

One of the frequent mutations in the clinical isolates of SARS-CoV-2 on the spike is the D614G, which emerged early during the pandemic as the subordinate and has since become a dominant variant in many places around the world.[19] Subsequent studies have shown that SARS-CoV-2 isolates containing 614G are more infectious than those with 614D.[20–22] Our results showed that incorporating D614G into the S-ct construct can further increase the yield of pseudovirus by approximately 2-fold even after the procedure has been vastly enhanced with the multiple steps of improvement. So, for maximum efficiency in producing a high-titer SARS-CoV-2 pseudotyped virus, the proposed minimum combination will be the following: (1) to use a spike with the C-terminal 19 aa truncation and with the incorporation of D614G, and (2) to conduct the procedure in 293T cells, not in 293FT cells that are currently widely used in many laboratories. One potential concern about condition 1 is that the introduced modifications on S may change the spike protein's tertiary structure and hence impact the accuracy of the neutralizing assay. However, the recently published data in the literature do not seem to show that either of these modifications significantly impacts the integrity and/or the antigenicity of the assembled pseudovirus (C-terminal 19 aa truncation) or the wild-type virus (D614G).[10,11,23]

ACE2 is the cellular receptor for both SARS-CoV-1 and SARS-CoV-2. It has been reported that the spike from SARS-CoV-2 has a stronger binding affinity to ACE2 than that from SARS-CoV-1.[24–26] This enhanced binding affinity is believed to contribute to the highly contagious transmission rate of COVID-19, possibly by enhancing ACE2 carboxypeptidase activity.[27] A recent study by Chan et al.[15] has identified several mutations on ACE2 that can significantly increase the binding affinity to the SARS-CoV-2 spike. To determine if the cell line expressing a high-affinity ACE2 could further improve the detection sensitivity of SARS-CoV-2 pseudovirus, we have established a stable 293T cell line that expresses a mutant ACE2 containing a combination of these mutations. Our subsequent testing showed that, although the cells could be transduced by the pseudovirus, the transduction efficiency is over three-fold lower than in 293T cells expressing the regular ACE2. It has been reported that there is an affinity threshold for membrane fusion triggering by viral glycoproteins.[28] For a given cell surface receptor density, cell-cell fusion proceeds efficiently at or above such an affinity threshold, and suprathreshold affinities do not further enhance membrane fusion efficiency.[28] However, it seems that a suprathreshold affinity for SARS-CoV-2 can

impede the virus infectivity. In combination with the concern that using a mutant form of ACE2 with a higher affinity for S for neutralization assay may not truly reflect the neutralization profile of Abs, an ACE2 with a suprathreshold affinity for SARS-CoV-2 is thus not recommended for incorporation into any neutralization assay.

In summary, we have comprehensively and systemically tested a series of conditions to improve the packaging efficiency of SARS-CoV-2 spike protein. While each of the individual improvements may not be particularly novel, the identified combination has led to a significant improvement in the yield of the pseudotyped virus. With the widespread usage of the pseudotyped virus for many COVID-19 assays, such as the detection of anti-SARS-CoV-2 nAbs, this improved procedure will be useful for many laboratories currently using the pseudotyped virus as a tool for their research.

MATERIALS AND METHODS

Cells

HEK293, 293T (American Type Culture Collection, Rockville, MD, USA) and 293FT cells (Thermo Fisher) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1× glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). All cells were incubated at 37°C in a humidified atmosphere saturated with 5% CO_2.

Plasmids

All the SARS-CoV-2-related sequences were optimized for human expression and synthesized by GenScript (Piscataway, NJ, USA), and a short tag sequence (either HA or Myc) was added to each of the synthesized genes for the purpose of easiness in detection. The SARS-CoV-2 spike coding sequences were cloned into pCDNA 3.1 vector (Invitrogen) to generate pCDNA-S (containing the full-length spike sequence, designated as S), pCDNA-S-ct (containing the full-length S with truncation of 19 aa at the C terminus, designated as S-ct), pCDNA-S-ct-614G (a truncation of 19 aa at the end of C terminus plus D614G mutation, designated as S-ct-614G), and pCDNA-Spike-RBD (containing the SARS-CoV-2 S receptor-binding domain [aa 253–640, designated as RBD]). The wild-type human ACE2 and a high-affinity ACE2 (containing three mutations of T27Y, L79T, and N330Y) were also synthesized by GenScript (Piscataway, NJ, USA) and were cloned into self-inactivating (SIN) lentiviral vector,[29] generating pSIN-ACE2 and pSIN-H-aff-ACE2, respectively. The lentivector pSIN-GFP (containing GFP as the marker gene) and pSIN-Luc (containing luciferase as the marker gene) were constructed in our lab using pSIN as the parental vector. Lentiviral vector packaging plasmid psPAX2 (containing HIV *gag* and *pol* genes) and plasmid VSV.G (containing the gene encoding VSV-G) were obtained from Addgene (Watertown, MA, USA).

Pseudovirus virus production

Initially, pSIN-GFP or pSIN-Luc was mixed with psPAX2 and VSV.G or one of the spike-containing plasmids (pCDNA-S, pCDNA-S-ct, or pCDNA-S-ct-614G) at a ratio of 4:3:1. The mixed plasmid DNA was then transfected into cells using polyethylenimine (MilliporeSigma, St. Louis, MO, USA). The virus supernatant was harvested at either 24 or 48 h after transfection. After cell debris was removed by filtration with a 0.45 μM filter, the virus-containing supernatants were stored at −80°C until titration.

Pseudovirus transduction assay

For titration with luciferase assay, 2 × 104 293T-ACE2 or 293T-H-aff-ACE2 cells were seeded in 96-well plates. Serially diluted pseudovirus supernatants were mixed with polybrene in DMEM at a concentration of 10 μg/mL in a total volume of 100 μL. The mixture was then added to each well. After 24, 48, or 72 h incubation at 37°C, the medium was removed. Luciferase activity was assayed by using the Bright-Glo Luciferase Assay System (Promega, Madison, WI, USA) and measured with the Spectramax 5 plate reader (Molecular Devices, San Jose, CA, USA). IU per mL was calculated by this formula: (infected cell readings − non-infected cell readings) × dilution factor.

For titration with GFP count, 1 × 105 293T-ACE2 cells were seeded in 24-well plates. When cells were seeded, serially diluted pseudovirus supernatants were mixed with polybrene in DMEM at a concentration of 10 μg/mL in a total volume of 1 mL. The mixture was then added to each well. After 72 h incubation at 37°C, the cells were imaged under Nikon inverted fluorescent microscopy (Melville, NY, USA), and the number of GFP^+ cells was then quantified by flow cytometry (BD Biosciences, San Jose, CA, USA).

Establishment of cell lines stably expressing ACE2

To establish cell lines for stably expressing ACE2, 93T cells in 6-well plates were transduced with packaged pSIN-Ace2 or pSIN-H-aff-Ace2 viruses. The transduced cells were sorted by flow cytometry (BD Biosciences, San Jose, CA, USA) after they were labeled with 30 ng of phycoerythrin (PE)-conjugated mouse anti-HA tag immunoglobulin G (IgG) (BioLegend, San Diego, CA, USA) followed by washing with PBS. Repeated sorting was applied until ACE2 expression on the cell surface reached near homogeneous (over 90%). The obtained cells were designed 293T-ACE2 and 293T-H-aff-ACE2 cells, respectively.

Flow cytometry analysis on RBD binding

Supernatants were collected from cells transfected with pCDNA-Spike-RBD. The supernatants were collected 48 h after transfection or infection and were then filtered to remove cell debris. Supernatants of 100 μL were added to 1 × 106 293T-ACE2 or 293T-H-aff-ACE2 cells and incubated for 30 min at room temperature. After washing three times with PBS, 5 μL of PE-conjugated mouse anti-His tag IgG (BioLegend, San Diego, CA, USA) in 2% FBS-PBS was incubated for another 30 min at 4°C. After three times washing with 2% FBS-PBS, the cells were subjected to an analysis by flow cytometry (BD Biosciences, San Jose, CA, USA).

Statistical analysis

All experiments were performed in triplicate, and all data are expressed as the mean ± SD. Student's t test (two-tailed) or one-way ANOVA was used to determine the statistical significance ($p < 0.05$) of various comparisons.

ACKNOWLEDGMENTS

This work was partly supported by the William and Ella Owens Medical Research Foundation (to X.Z.). We thank Dr. Tho Tran for the careful reading of the manuscript.

AUTHOR CONTRIBUTIONS

L.T. and X.F. conducted the experiments. X.F and X.Z. designed the experiments and wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Blumenthal, D., Fowler, E.J., Abrams, M., and Collins, S.R. (2020). Covid-19 - Implications for the Health Care System. N. Engl. J. Med. *383*, 1483–1488.
2. Pascarella, G., Strumia, A., Piliego, C., Bruno, F., Del Buono, R., Costa, F., Scarlata, S., and Agrò, F.E. (2020). COVID-19 diagnosis and management: a comprehensive review. J. Intern. Med. *288*, 192–206.
3. Grzelak, L., Temmam, S., Planchais, C., Demeret, C., Tondeur, L., Huon, C., Guivel-Benhassine, F., Staropoli, I., Chazal, M., Dufloo, J., et al. (2020). A comparison of four serological assays for detecting anti-SARS-CoV-2 antibodies in human serum samples from different populations. Sci. Transl. Med. *12*, eabc3103.
4. Robbiani, D.F., Gaebler, C., Muecksch, F., Lorenzi, J.C.C., Wang, Z., Cho, A., Agudelo, M., Barnes, C.O., Gazumyan, A., Finkin, S., et al. (2020). Convergent antibody responses to SARS-CoV-2 in convalescent individuals. Nature *584*, 437–442.
5. World Health Organization (2020). Laboratory Biosafety Guidance Related to Coronavirus Disease (COVID-19): Interim Guidance (World Health Organization).
6. Schmidt, F., Weisblum, Y., Muecksch, F., Hoffmann, H.H., Michailidis, E., Lorenzi, J.C.C., Mendoza, P., Rutkowska, M., Bednarski, E., Gaebler, C., et al. (2020). Measuring SARS-CoV-2 neutralizing antibody activity using pseudotyped and chimeric viruses. J. Exp. Med. *217*, e20201181.
7. Zhu, N., Zhang, D., Wang, W., Li, X., Yang, B., Song, J., Zhao, X., Huang, B., Shi, W., Lu, R., et al.; China Novel Coronavirus Investigating and Research Team (2020). A Novel Coronavirus from Patients with Pneumonia in China, 2019. N. Engl. J. Med. *382*, 727–733.
8. Rivera, A., Fu, X., Tao, L., and Zhang, X. (2015). Expression of mouse CD47 on human cancer cells profoundly increases tumor metastasis in murine models. BMC Cancer *15*, 964.
9. Shoji, T., Higuchi, H., Zaitsu, Y., Nishijima, K., and Iijima, S. (2015). Enhanced lentiviral vector production in 293FT cells expressing Siglec-9. Cytotechnology *67*, 593–600.
10. Giroglou, T., Cinatl, J., Jr., Rabenau, H., Drosten, C., Schwalbe, H., Doerr, H.W., and von Laer, D. (2004). Retroviral vectors pseudotyped with severe acute respiratory syndrome coronavirus S protein. J. Virol. *78*, 9007–9015.
11. Fukushi, S., Watanabe, R., and Taguchi, F. (2008). Pseudotyped vesicular stomatitis virus for analysis of virus entry mediated by SARS coronavirus spike proteins. Methods Mol. Biol. *454*, 331–338.
12. Korber, B., Fischer, W.M., Gnanakaran, S., Yoon, H., Theiler, J., Abfalterer, W., Hengartner, N., Giorgi, E.E., Bhattacharya, T., Foley, B., et al.; Sheffield COVID-19 Genomics Group (2020). Tracking Changes in SARS-CoV-2 Spike: Evidence that D614G Increases Infectivity of the COVID-19 Virus. Cell *182*, 812–827.e19.
13. Yurkovetskiy, L., Wang, X., Pascal, K.E., Tomkins-Tinch, C., Nyalile, T.P., Wang, Y., Baum, A., Diehl, W.E., Dauphin, A., Carbone, C., et al. (2020). Structural and Functional Analysis of the D614G SARS-CoV-2 Spike Protein Variant. Cell *183*, 739–751.e8.
14. Johnson, M.C., Lyddon, T.D., Suarez, R., Salcedo, B., LePique, M., Graham, M., Ricana, C., Robinson, C., and Ritter, D.G. (2020). Optimized pseudotyping conditions for the SARS COV-2 Spike glycoprotein. J. Virol *94*, e01062, 20.
15. Chan, K.K., Dorosky, D., Sharma, P., Abbasi, S.A., Dye, J.M., Kranz, D.M., Herbert, A.S., and Procko, E. (2020). Engineering human ACE2 to optimize binding to the spike protein of SARS coronavirus 2. Science *369*, 1261–1265.
16. Hu, J., Gao, Q., He, C., Huang, A., Tang, N., and Wang, K. (2020). Development of cell-based pseudovirus entry assay to identify potential viral entry inhibitors and neutralizing antibodies against SARS-CoV-2. Genes Dis *7*, 551–557.
17. Ono, A., and Freed, E.O. (2001). Plasma membrane rafts play a critical role in HIV-1 assembly and release. Proc. Natl. Acad. Sci. USA *98*, 13925–13930.
18. Welsch, S., Keppler, O.T., Habermann, A., Allespach, I., Krijnse-Locker, J., and Kräusslich, H.-G. (2007). HIV-1 buds predominantly at the plasma membrane of primary human macrophages. PLoS Pathog. *3*, e36.
19. Grubaugh, N.D., Hanage, W.P., and Rasmussen, A.L. (2020). Making Sense of Mutation: What D614G Means for the COVID-19 Pandemic Remains Unclear. Cell *182*, 794–795.
20. Daniloski, Z., Guo, X., and Sanjana, N.E. (2020). The D614G mutation in SARS-CoV-2 Spike increases transduction of multiple human cell types. bioRxiv. https://doi.org/10.1101/2020.06.14.151357.
21. Zhang, L., Jackson, C.B., Mou, H., Ojha, A., Rangarajan, E.S., Izard, T., Farzan, M., and Choe, H. (2020). The D614G mutation in the SARS-CoV-2 spike protein reduces S1 shedding and increases infectivity. bioRxiv. https://doi.org/10.1101/2020.06.12.148726.
22. Ogawa, J., Zhu, W., Tonnu, N., Singer, O., Hunter, T., Ryan, A.L., and Pao, G.M. (2020). The D614G mutation in the SARS-CoV2 Spike protein increases infectivity in an ACE2 receptor dependent manner. bioRxiv. https://doi.org/10.1101/2020.07.21.214932.
23. Li, Q., Wu, J., Nie, J., Zhang, L., Hao, H., Liu, S., Zhao, C., Zhang, Q., Liu, H., Nie, L., et al. (2020). The Impact of Mutations in SARS-CoV-2 Spike on Viral Infectivity and Antigenicity. Cell *182*, 1284–1294.e9.
24. Lan, J., Ge, J., Yu, J., Shan, S., Zhou, H., Fan, S., Zhang, Q., Shi, X., Wang, Q., Zhang, L., and Wang, X. (2020). Structure of the SARS-CoV-2 spike receptor-binding domain bound to the ACE2 receptor. Nature *581*, 215–220.
25. Wang, Q., Zhang, Y., Wu, L., Niu, S., Song, C., Zhang, Z., Lu, G., Qiao, C., Hu, Y., Yuen, K.Y., et al. (2020). Structural and Functional Basis of SARS-CoV-2 Entry by Using Human ACE2. Cell *181*, 894–904.e9.
26. Wang, Y., Liu, M., and Gao, J. (2020). Enhanced receptor binding of SARS-CoV-2 through networks of hydrogen-bonding and hydrophobic interactions. Proc. Natl. Acad. Sci. USA *117*, 13967–13974.
27. Lu, J., and Sun, P.D. (2020). High affinity binding of SARS-CoV-2 spike protein enhances ACE2 carboxypeptidase activity. bioRxiv. https://doi.org/10.1101/2020.07.01.182659.
28. Hasegawa, K., Hu, C., Nakamura, T., Marks, J.D., Russell, S.J., and Peng, K.-W. (2007). Affinity thresholds for membrane fusion triggering by viral glycoproteins. J. Virol. *81*, 13149–13157.
29. Miyoshi, H., Blömer, U., Takahashi, M., Gage, F.H., and Verma, I.M. (1998). Development of a self-inactivating lentivirus vector. J. Virol. *72*, 8150–8157.