Construction of SARS-CoV-2 spike-pseudotyped retroviral vector inducing syncytia formation

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Abstract
Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is handled in biosafety level 3 (BSL-3) facilities, whereas the antiviral screening of pseudotype virus is conducted in BSL-2 facilities. In this study, we developed a SARS-CoV-2 spike-pseudotyped virus based on a semi-replication-competent retroviral (s-RCR) vector system. The s-RCR vector system was divided into two packageable vectors, each with gag-pol and env genes. For env vector construction, SARS-CoV-2 SA19 env was inserted into the pCLXSN-IRES-EGFP retroviral vector to generate pCLXSN-SA19 env-EGFP. When pCLXSN-gag-pol and pCLXSN-SΔ19 env-EGFP were co-transfected into HEK293 T cells to generate an s-RCR virus, titers of the s-RCR virus were consistently low in this transient transfection system (1 × 10⁴ TU/mL). However, a three-fold higher amounts of MLV-based SARS-CoV-2 pseudotyped viruses (3 × 10⁴ TU/mL) were released from stable producer cells, and the spike proteins induced syncytia formation in HEK293-hACE2 cells. Furthermore, s-RCR stocks collected from stable producer cells induced more substantial syncytia formation in the Vero E6-TMPRSS2 cell line than in the Vero E6 cell line. Therefore, a combination of the s-RCR vector and the two cell lines (HEK293-hACE2 or Vero E6-TMPRSS2) that induce syncytia formation can be useful for the rapid screening of novel fusion inhibitor drugs.

Keywords COVID-19 · Neutralization assay · Pseudovirus · SARS-CoV-2 · s-RCR vector

Introduction
Pandemic of coronavirus disease 2019 (COVID-19) was caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Coronaviruses are classified into four genera: alpha-CoV, beta-CoV, gamma-CoV, and delta-CoV. SARS-CoV-2, an enveloped, single-stranded, positive-sense RNA virus, was identified as a new member of the genus Betacoronavirus [1]. This virus binds to the cognate receptor–angiotensin-converting enzyme 2 (ACE2) using the spike glycoprotein (S), and the spike is proteolytically activated for entering the host cell by a type II transmembrane serine protease (TMPRSS2) [2, 3]. The ACE2 protein is localized on the apical plasma membrane of respiratory epithelial cells [4]. The colon carcinoma cell line (Caco-2), a lung carcinoma cell line (Calu-3), and a monkey kidney cell line (Vero E6) express ACE2 on the apical membrane domain. In SARS-CoV-2 infection, TMPRSS2 may play an important role. TMPRSS2 cleaves at single arginine or lysine residues (R/K↓) and TMPRSS2 inhibitor camostat mesylate can block the SARS-CoV-2 entry into cells [2]. Vero E6 cells constitutively expressing TMPRSS2 (Vero E6/TMPRSS2) are highly susceptible to SARS-CoV-2 infection [5]. HEK293T cells showed only modest viral replication because HEK293T cells did not express endogenous hACE2 [6]. However, HEK293T-hACE2 cells stably express human ACE2 receptor most widely used in SARS-CoV-2 infection.

Cells infected with SARS-CoV-2 express the spike protein and form syncytia by fusing with ACE2 receptor-positive neighboring cells [7]. Expression of the spike protein alone, even in the absence of other proteins (M; membrane, E; envelope, and N; nucleocapsid), induces receptor-dependent syncytial formation [8, 9]. The spike protein, which contains two subunits, the S1 receptor-binding and the S2 fusion subunits, is cleaved by furin at the S1/S2 site and by TMPRSS2 at the S2’ site [10, 11]. Its high transmissibility results from a unique furin-like cleavage site...
Envelope proteins from nonrelated viruses can be “pseudo-
dotyped” into safer nonreplicative viral particles. These
proteins facilitate the entry of pseudotype virus into cells
[15–18]. The production of pseudotyped viral particles is a
powerful tool for studying viral tropism and immunogenic-
ity. Although SARS-CoV-2 requires handling in biosafety
level 3 (BSL-3) facilities, the pseudotype virus allows anti-
viral screening to be conducted in BSL-2 facilities [19–21].
Recently, a SARS-CoV-2 pseudotyped virus was developed
based on human immunodeficiency virus (HIV), murine leu-
kemia virus (MLV), and vesicular stomatitis virus (VSV)
[22]. Although the titers of the VSV-based SARS-CoV-2
pseudotyped virus are about 100-fold higher than those of
HIV-based pseudotyped lentiviral particles, the former is
not as practical as HIV-1-based SARS-CoV-2 pseudotyped
virus [21, 23, 24]. An HIV-1-based SARS-CoV-2 pseudo-

typed virus can assess the neutralization efficiency and
entry inhibition. In the spike protein, 19 amino acids at the
C-terminal were cleaved to overcome the low yield of the
pseudotyped virus [25]. Previous studies have shown that
C-terminal truncation of the spike protein and the D614G
mutation enhances pseudoviral titers [26–28].

Murine leukemia virus (MLV) is a small RNA virus and
contains three genes (gag, pol, and env). Replication-defec-
tive retroviral (RDR) vectors have insufficient gene transfer
efficiency (10^1–10^6 transduction units/mL) and the inability to
transfer genes into nondividing cells. To improve the low-
level transduction efficiency of these vectors, MLV-based
replication-competent retroviral (RCR) vectors have been
developed [29]. RCR vectors have demonstrated improved
efficacy of gene delivery, however, they still present the risk of
accidental spread. To decrease this risk, semi-replication-
competent retroviral (s-RCR) vectors were developed. They
propagate transgenes as efficiently as RCR vectors and have
an insert capacity of up to 7.3 kb of transgene. In a semi-
replication-competent retroviral(s-RCR) system, the gag-pol
and env genes were split into two vectors [30–32]. Our novel
chimeric replication-competent Mo-MLV-10A1-EGFP vec-
tor was used as backbone plasmid to construct s-RCR [29].

In this study, incorporation of SARS-CoV-2 S protein
into MLV particles was studied to construct an s-RCR vec-

tor that induces syncytial formation. The enhanced green
fluorescent protein (EGFP) reporter gene was expressed in a
gag-pol vector or an env vector to facilitate the detection of
syncytial formation after transduction of target cells. In addi-
tion, an s-RCR vector released from stable producer cells
was developed, as MLV-based SARS-CoV-2 pseudotyped
virus do not exhibit high titers through transient transfec-
tion [23].

When we compared the transient transfection system with
stable producer cells to obtain high-titer s-RCR vectors that
induce syncytia formation, titers of s-RCR virus—obtained
from stable producer cells—were higher than those of the
virus obtained from transient transfection. This study also
shows that the s-RCR virus progressively replicates and
induces syncytia formation in HEK293-hACE2 and Vero
E6-TMPRSS2 cell cultures.

Materials and methods

Cell lines

The human embryonic kidney cell line HEK293 (ATCC,
CRL-1573), HEK293 T cells expressing the SV40 T-antigen
(ATCC, CRL-3216), and the African green monkey kidney
cell line Vero E6 (ATCC, CRL-1586) were maintained in
DMEM with 10% fetal bovine serum, 100 U/mL penicillin,
and 100 µg/mL streptomycin.

Creation of HEK293-hACE2 and Vero E6-TMPRSS2
cells

From Hyeryun Choe [4] (Addgene plasmid #1786) and
Roger Reeves [33] (Addgene plasmid # 53,887), pcDNA3.1-
hACE2 and pCSDestTMPRSS2 were obtained, respectively.
To create HEK293-hACE2 cells, pCLXSN-hACE2 was gen-
erated by cloning hACE2 from pcDNA3.1-hACE2 into the
EcoRI and BamHI sites of pCLXSN retroviral vector. Next,
VSV-G-pseudotyped retrovirus packaging human ACE2 was
generated by co-transfecting 3.5 × 10^5 HEK293 T cells in
6-well plates with pCLXSN-hACE2 and packaging plasmids
(2 µg of pVPack-GP and 2 µg of pCMV-VSV-G). The result-
ing retrovirus was used to infect HEK293 cells in the pres-
ence of 8 µg/ml polybrene. Transduced cells were selected
in the growth medium containing G418 (800 µg/mL) for a
period of 14 days.

The TMPRSS2 was cloned into the EcoRI and NotI
sites of the retroviral vector pLPCX-IRES-EGFP (pLPCX-
TMPRSS2). A VSV-G-pseudotyped retrovirus packag-
ing human TMPRSS2 was constructed in the same way as
described above. Vero E6 cells expressing TMPRSS2 were

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Construction of C-terminal 19 aa-truncated SARS-CoV-2 S protein

The full-length SARS-CoV-2 S protein was codon-optimized and synthesized using GenScript (Piscataway, NJ, USA: https://www.molecularcloud.org/plasmid/pUC57-2019-nCoV-SHuman/MC-0101081.html). The SARS-CoV-2 S gene was subcloned into the EcoRI and NotI sites of the pcDNA3.1 vector (pcDNA3.1-S env). Hemagglutinin (HA)-tagged and C-terminal-truncated SARS-CoV-2 S expression constructs were generated by amplifying a fragment of approximately 760 bp using the forward primer 5'-GTA TGG GTA ACA GCA GGA GCC ACA GCT ACA-3’ and the reverse primer 5'-GGA TCC TTA AGC GTA ATC TGG AAC ATC GTA TGG GTA ACA GCA GGA GCC ACA GCT ACA-3’ (BamHI restriction sites are underlined). Products of polymerase chain reaction (PCR) of C-terminal truncated SARS-CoV-2 S protein vectors were ligated into the pGEM-T Easy Vector System (Promega, Madison, WI, USA). Using the restriction enzymes DraIII and BamHI, pGEM-T-SARS-CoV-2 Δ19 was digested, and the fragments were ligated into pcDNA3.1-S env to generate pcDNA3.1-Δ19 env that encodes for the C-terminal 19 aa-truncated SARS-CoV-2 S and HA tag.

Construction of s-RCR vectors

A previously developed s-RCR vector system was used for the development of high-titer retroviral vectors [30]. To generate the s-RCR vector, pCLXSN-S env was constructed by cloning the EcoRI/BamHI fragment of SARS-CoV-2 into the retroviral vector pCLXSN. Construction of the pCLXSN-gag-pol-IRES-EGFP plasmid has been described previously [29, 30]. To investigate the complementation of gag-pol and env vectors, 3 × 10^5 Vero E6 cells in 6-well plates were transiently co-transfected with 3 µg of pCLXSN-gag-pol-IRES-EGFP and 3 µg of pCLXSN-S env using the CalPhos Mammalian Transfection Kit (TaKaRa Bio, Shiga, Japan). The replication of the s-RCR virus was measured by assessing the spread of syncytia and increase in EGFP-positive cells. To obtain s-RCR viruses from a stable producer cell line, 7 × 10^5 HEK293 T cells in 6-well plates were transiently co-transfected with 3 µg of pCLXSN-gag-pol, 3 µg of pCMV- VS-V-G, and 3 µg of pCLXSN-S env-EGFP or 3 µg of pCLXSN-S19 env-EGFP. Next, 6 × 10^5 HEK293 T cells in 6-well plates were infected with 1 mL of the s-RCR virus obtained from transfected HEK293 T cells. After two days, HEK293 cells were selected in G418 for 14 days (800 µg/mL). To produce s-RCR virus in transient transfection, 7 × 10^5 HEK293 T cells in 6-well plates were co-transfected with 3 µg of envelope expression plasmids (pCLXSN-S env or pCLXSN-VSV-G) and 3 µg of pCLXSN-gag-pol-IRES-EGFP. To investigate the release of virions from stable producer cells, viral lysates and cell lysates were detected with an anti-HA antibody (Sigma-Aldrich, MO, USA), a mouse monoclonal antibody anti-MLV p30 (1:1,000; Abcam, Cambridge, UK) and a rabbit polyclonal antibody anti-β-actin (1:10,000; AbClon, Seoul, Korea). To determine the viral titers, EGFP-positive cells were analyzed using a FACScalibur flow cytometer (Becton, Dickinson and Company, NJ, USA). Vector titers were calculated using the following equation (N x P)/(V x D). N = cell number in each well used for infection; P = percentage of GFP-positive cells; V = viral volume used for infection; and D = dilution factor.

Infectivity assay

Receptor-dependent syncytia formation was induced via SARS-CoV-2 S expression. To examine syncytia formation induced by s-RCR viruses, 6 × 10^5 HEK293-hACE2 cells in 12-well plates and 6 × 10^5 Vero E6-TMPRSS2 cells in 12-well plates were infected with 0.5 mL of this virus obtained from stable producer cells. Both EGFP-positive and syncytia-forming cells were analyzed using fluorescence microscopy. Virus titers (syncytial-forming units [sfu]/mL) were determined by infecting Vero E6-TMPRSS2 target cells with a tenfold serial dilution of the viral supernatant obtained from stable producer cells.

Results

Construction of s-RCR vectors

The s-RCR vector system was constructed wherein two replication-defective vectors could generate infectious viruses from a cell infected by both vectors (Fig. 1). The two complementary replication-defective vectors are based on the Moloney-MLV (Mo-MLV) vector. To create the env vector (pCLXSN-S env-EGFP or pCLXSN-S19 env-EGFP), the spike protein gene was inserted into the retroviral vector pCLXSN, with IRES-EGFP positioned directly downstream of the gene. To generate the gag-pol vector, pCLXSN-gag-pol was constructed by deleting the EGFP gene from the previously developed pCLXSN-gag-pol-IRES-EGFP recombinant vector [29, 34]. The 19 amino acids at the C-terminal, which codes for the ER/Golgi retention signal, are absent in S19 env.

Production of s-RCR viruses

To confirm the propagation of complementary defective vectors, the gag-pol vector (pCLXSN-gag-pol-IRES-EGFP) and env vector (pCLXSN-S env) were co-transfected into Vero E6 cells. As shown in Fig. 2, a progressive
increase in EGFP-positive cells and syncytia formation was detected, whereas syncytia formation was not observed on Vero E6 cells transfected by pCLXSN-gag-pol-IRES-EGFP alone. This result suggests that s-RCR can spread through transfected Vero E6 cells, and that this viral propagation is accompanied by progressive syncytial formation.

**Fig. 1** Structure of s-RCR vectors. Vectors derived from Moloney-Murine Leukemia virus (Mo-MLV). The plasmid pCLXSN-gag-pol contains the entire Mo-MLV gag-pol coding sequence and a neomycin resistance gene. An IRES-EGFP construct was inserted downstream of the gag-pol sequence of pCLXSN-gag-pol to construct pCLXSN-gag-pol-IRES-EGFP. The plasmid pCLXSN-senv contains the codon-optimized spike protein gene. C-terminal HA-tagged SΔ19 was inserted to pCLXSN-IRES-EGFP to produce pCLXSN-SΔ19 env-EGFP.

**Fig. 2** Generation of s-RCR viruses encoding spike proteins. Vero E6 cells were transfected with 3 µg pCLXSN-senv and 3 µg pCLXSN-gag-pol-IRES-EGFP in 6-well plates to produce s-RCR viruses. Transfection of pCLXSN-gag-pol-IRES-EGFP alone in Vero E6 cells used as negative control. Syncytia formation was detected using fluorescence microscopy at 3, 6, 7, and 8 days post transfection.

s-RCR viruses are efficiently released from stable producer cells

Roy et al. showed that stable HEK293GP cells (HEK293 cells that express MLV gag-pol) expressing SΔ19 are more useful than cells transiently transfected for generating SARS-CoV-2 pseudotyped viruses [35]. Stable HEK293
cells expressing MLV gag-pol and S or SΔ19 were generated via transduction. The presence of SARS-CoV-2 SΔ19 pseudotyped viruses released in the supernatant of stable HEK293 cells was analyzed using western blotting. The presence of spike protein at the stable HEK293 cells and the MLV viral particles—produced from stable producers—was detected using an antibody against HA (Fig. 3). These results show that SΔ19 was efficiently incorporated into MLV viral particles. It has been reported that SARS-CoV-2 spike-pseudotyped viruses are inefficiently released by transient transfection [21–24, 35]. In agreement with the previous results, titers of $5 \times 10^5$ transducing units (TU)/mL and $1 \times 10^4$ transducing units (TU)/mL were obtained for VSV-G and SARS-CoV-2 spike-pseudotyped viruses, respectively (Fig. 4a). A titer of SARS-CoV-2 spike-pseudotyped viruses was 50-fold lower than VSV-G-pseudotyped positive control vector. As shown in Fig. 4b, the SARS-CoV-2 SΔ19 env pseudovirus titer in the culture supernatant of stable producer cells was $3 \times 10^4$ transducing units (TU)/mL—detected by fluorescence-activated cell sorting (FACS), whereas the viral titer of SARS-CoV-2 S was $2 \times 10^4$ transducing units (TU)/mL. Both GFP-positive cells and syncytia formation were detected.

![Western blot analysis of SARS-CoV-2 SΔ19 in viral lysates and cell lysates.](image1)

![Comparison of the yields of pseudotyped virus generated from transient transfection or stable producer cells.](image2)
s-RCR viruses induced syncytia formation in Vero E6-TMPRSS2

To investigate the effect of TMPRSS2 on syncytia formation, VeroE6-TMPRSS2 cells were infected with s-RCR viruses. As shown in Fig. 5, the syncytia formed in VeroE6-TMPRSS2 cells were larger than those in Vero E6 cells. The SARS-CoV-2 $\Delta 19$ env pseudovirus titer was $2 \times 10^3$ sfu/mL. In addition, syncytia formation increased over time as the s-RCR virus spread throughout the culture. These results suggest that TMPRSS2 enhances syncytial formation.

Discussion

Retroviral vectors pseudotyped with the SARS-CoV-2 spike protein are a powerful tool for screening SARS-CoV-2 entry inhibitors and neutralization assays [19–21, 24]. However, Mo-MLV cannot efficiently pseudotype the full-length SARS-CoV-2 spike protein in transient transfection systems [35]. We also obtained very low titers of the Mo-MLV-based SARS-CoV-2 pseudotyped virus using a transient transfection system (Fig. 4a). Recent studies reported that MLV pseudotyped with the C-terminal 19 aa-truncated SARS-CoV-2 S proteins obtained from stable producer cells infect HEK293-hACE2 cells more efficiently than with wild-type SARS-CoV-2 S protein [35]. In this study, we developed an s-RCR vector system that induces syncytia formation for large-scale production of s-RCR viruses in stable producer cells.

To investigate the generation of s-RCR virus through the transcomplementation of two defective retroviral vectors, the gag-pol vector carrying an EGFP reporter gene and an env vector were co-transfected into Vero E6 cells. As shown in Fig. 2, a progressive increase in the number of EGFP-positive cells and syncytium formation was observed, suggesting a successful generation of s-RCR viruses. Syncytia showing green fluorescence suggest co-expression of SARS-CoV-2 S and EGFP.

To generate stable producer cells, pCLXSN-gag-pol and pCLXSN-S env-EGFP or pCLXSN- SΔ19 env-EGFP were used to transduce HEK293 cells. A clone that was both EGFP-positive and resistant to G418 was selected. The presence of SΔ19 env in the supernatant of stable producer cells was confirmed via western blotting. Western blots of s-RCR viruses showed that most spike proteins were cleaved in the S1/S2 conformation (Fig. 3). In agreement with the results of previous studies [35], MLV pseudotyped with the C-terminal 19 aa-truncated SARS-CoV-2 S infected HEK293-hACE2 cells more efficiently than with SARS-CoV-2 S (Fig. 4). Interestingly, MLV pseudotyped with full-length spike protein also induced syncytium formation. These data suggest that two replication-deficient packageable vectors complement each other to create a semi-replication-competent virus.

Cells infected with SARS-CoV-2 viruses express the spike protein in the plasma membrane and fuse with hACE2-positive neighboring cells. In addition, the expression of SARS-CoV-2 spike protein alone (in the absence of other viral proteins) is known to induce receptor-dependent syncytia formation [8]. TMPRSS2 is involved in the fusion between spike proteins and receptors, increasing syncytia formation [7]. To examine the impact of TMPRSS2 on syncytia formation, Vero E6-TMPRSS2 cells were infected with s-RCR viruses. As shown in Fig. 5, larger syncytia were formed in Vero E6-TMPRSS2 cells than in Vero cells. These results suggest that SΔ19 env was better incorporated into s-RCR than S env and that TMPRSS2 was involved in SΔ19 env-receptor fusion. Vero E6 cells are known to highly express African green monkey ACE2 but expression level of TMPRSS2 is quite low in this clone. TMPRSS2-over-expressing Vero E6 cells are widely used to replicate and
isolate SARS-CoV-2 [5]. To determine whether expression levels of hACE2 or TMPRSS2 contribute to spike-mediated syncytia formation, the construction of the 293 T-hACE2-TMPRSS2 stable cell line is required for further studies. ImageJ software can be used to measure the size and number of syncytial formations during drug screening [36].

In conclusion, an s-RCR vector that induces syncytium formation was constructed. Syncytia formation was readily detected upon infection of HEK293-hACE2 and Vero E6-TMPRSS2 cells with s-RCR viruses obtained from stable producer cells. Thus, the screening of novel fusion inhibitor drugs is possible using an s-RCR vector system and the two cell lines used in this study.

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Author contributions YTJ designed research and finalized the manuscript; SYL and DWK performed research and analyzed data.

Declarations

Conflict of interest The author of this study has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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