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Original Article

Assessing the application of a pseudovirus system for emerging SARS-CoV-2 and re-emerging avian influenza virus H5 subtypes in vaccine development

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ABSTRACT

Background: Highly pathogenic emerging and re-emerging viruses continuously threaten lives worldwide. In order to provide prophylactic prevention from the emerging and re-emerging viruses, vaccine is suggested as the most efficient way to prevent individuals from the threat of viral infection. Nonetheless, the highly pathogenic viruses need to be handled in a high level of biosafety containment, which hinders vaccine development. To shorten the timeframe of vaccine development, the pseudovirus system has been widely applied to examine vaccine efficacy or immunogenicity in the emerging and re-emerging viruses.

Methods: We developed pseudovirus systems for emerging SARS coronavirus 2 (SARS-CoV-2) and re-emerging avian influenza virus H5 subtypes which can be handled in the biosafety level 2 facility. Through the generated pseudovirus of SARS-CoV-2 and avian influenza virus H5 subtypes, we successfully established a neutralization assay to quantify the neutralizing activity of antisera against the viruses.

Results: The result of re-emerging avian influenza virus H5Nx pseudoviruses provided valuable information for antigenic evolution and immunogenicity analysis in vaccine candidate selection. Together, our study assessed the potency of pseudovirus systems in vaccine efficacy, antigenic analysis, and immunogenicity in the vaccine development of emerging and re-emerging viruses.

Conclusion: Instead of handling live highly pathogenic viruses in a high biosafety level facility, using pseudovirus systems would speed up the process of vaccine development to provide community protection against emerging and re-emerging viral diseases with high pathogenicity.

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At a glance of commentary

Scientific background on the subject

Highly pathogenic emerging and re-emerging viruses continuously threaten lives worldwide. To shorten the time-frame of vaccine development of the high risk viruses, the pseudovirus system, which can be handled in the biosafety level 2 facility, has been widely applied to examine vaccine efficacy, antigenic analysis, or immunogenicity.

What this study adds to the field

We successfully established a neutralization assay to quantify the neutralizing activity of antisera against the SARS-CoV-2 and avian influenza virus H5 subtypes. We assessed the utilization potency of pseudovirus systems to accelerate the process of vaccine development, which would provide rapid community protection against highly pathogenic viruses.

Throughout the years, many viruses have emerged and re-emerged. An emerging virus may be defined as a newly discovered virus that has recently appeared within the population. Re-emerging viruses on the other hand may be defined as viruses that continuously reappear, thus causing many epidemics/outbreaks. Viruses such as enteroviruses (EV), dengue virus (DENV), and influenza viruses (e.g. H1N1, H5N1, H7N9) to name a few, are considered re-emerging viruses due to their constant reappearance in the population [1]. Viral adaptation mechanisms such as mutation (e.g. RNA viruses) [2], reassortment (e.g. influenza virus) [3], and recombination (e.g. enteroviruses) [4] aid in the evolution of the virus, allowing it to cause new outbreaks, and potential pandemics, with each re-emergence [1]. Emerging viruses such as severe acute respiratory syndrome-associated coronavirus (SARS-CoV), Middle East respiratory syndrome – coronavirus (MERS-CoV), Ebola virus, Zika virus (ZIKV), Chikungunya virus (CHIKV), Nipah virus (NiV), and the most recent SARS-CoV-2 have also resulted in large epidemics and pandemics [1]. In order to combat both emerging and re-emerging viruses, effective drugs and vaccines are needed to treat and prevent the viral infections. However, in order to do so, certain bio-safety level facilities are required for the handling of viruses that are considered to be highly pathogenic agents, such as bio-safety level 3 (BSL-3) facilities for SARS-CoV-2, MERS-CoV, CHIKV, and avian influenza, and BSL-4 for Ebola, Marburg virus (MARV), Lassa virus (LASV), and NiV. This poses a major limitation in the development of vaccines and drugs against the viruses.

On the other hand, the use of a pseudovirus system can greatly aid in avoiding the handling of highly contagious viruses. The pseudovirus system is a generating platform which utilizes vesicular stomatitis virus (VSV) or lentivirus vectors, among others [5]. The advantages of using such a system is its rapid production, it is quantifiable, and is relatively safe since progeny viruses can only undergo a single replication cycle [6,7]. In the lentivirus-vector system, although most of the vectors are derived from human immunodeficiency virus type 1 (HIV-1), modified versions of the lentivirus vector are non-pathogenic [8]. This is also the case in the VSV-ΔG pseudovirus system. In this system, the VSV-ΔG vector lacks the G envelope protein of VSV, thus allowing for recombination with a foreign envelope protein and/or reporter gene. Establishment of pseudoviruses using the lentivirus-vector system have been previously reported for emerging and re-emerging viruses that require BSL-3/4 facilities such as NiV [9], MARV [10], CHIKV [11], SARS-CoV-2 [12], and certain influenza viruses [6,7]. In addition, MERS-CoV [13,14], SARS-CoV [15,16], Ebola [17], and LASV [18,19] pseudoviruses have produced using both lentivirus and VSV-ΔG pseudovirus systems. In cases such as influenza and coronaviruses (MERS-CoV, SARS-CoV, SARS-CoV-2) which their surface proteins mediate entry into host cells, hemagglutinin (HA) and spike (S) protein, respectively, are combined with the pseudovirus vector. Cleavage of HA0 to HA1 and HA2 has been reported to be necessary for virus infectivity and determines the pathogenicity of the virus [20–23]. Similarly, cleavage of the S protein to S1 and S2 of coronavirus enhances cell–cell fusion, increases viral entry efficiency, and determines virus infectivity [12,13,24–27].

With the safer use of pseudoviruses in lower biosafety level facilities, it has opened up greater possibilities for antibody detection, vaccine research, and drug selection to better combat both emerging and re-emerging viruses. These viruses could be screened for the antigenicity/immunogenicity of antigen or antibody response by using neutralization tests and would be a useful tool for vaccine studies. Several studies have used pseudoviruses to detect and test neutralizing antibodies against viruses that pose a threat to the population and their response to viral infection [7,14,28–32]. The use of pseudoviruses serve as an alternative to test vaccines, thus eliminating one of the limitations once placed on vaccine development [9,33–35]. Furthermore, better evaluation on the efficacy of drugs and selection of new drugs has been advantageous in combatting viral infections [36,37]. Here we established pseudovirus systems for emerging SARS-CoV/SARS-CoV-2 viruses and re-emerging avian influenza viruses including H5N2, H5N6, and H5N8 (H5Nx). We assessed the feasibility of the pseudovirus system for neutralization assay and found a broad spectrum of applications such as vaccine candidate selection and vaccine efficacy, including antigenic evolution and immunogenicity, which provide valuable information in vaccine development, especially for emerging and re-emerging viruses.

Materials and methods

Production of SARS-CoV/SARS-CoV-2 and avian influenza viruses H5Nx pseudoviruses

To generate SARS-CoV or SARS-CoV-2 and avian influenza virus pseudovirus, we applied the lentiviral vector system provided by National RNAi Core of Academic Sinica Taiwan to produce the pseudoviruses expressing full-length S protein

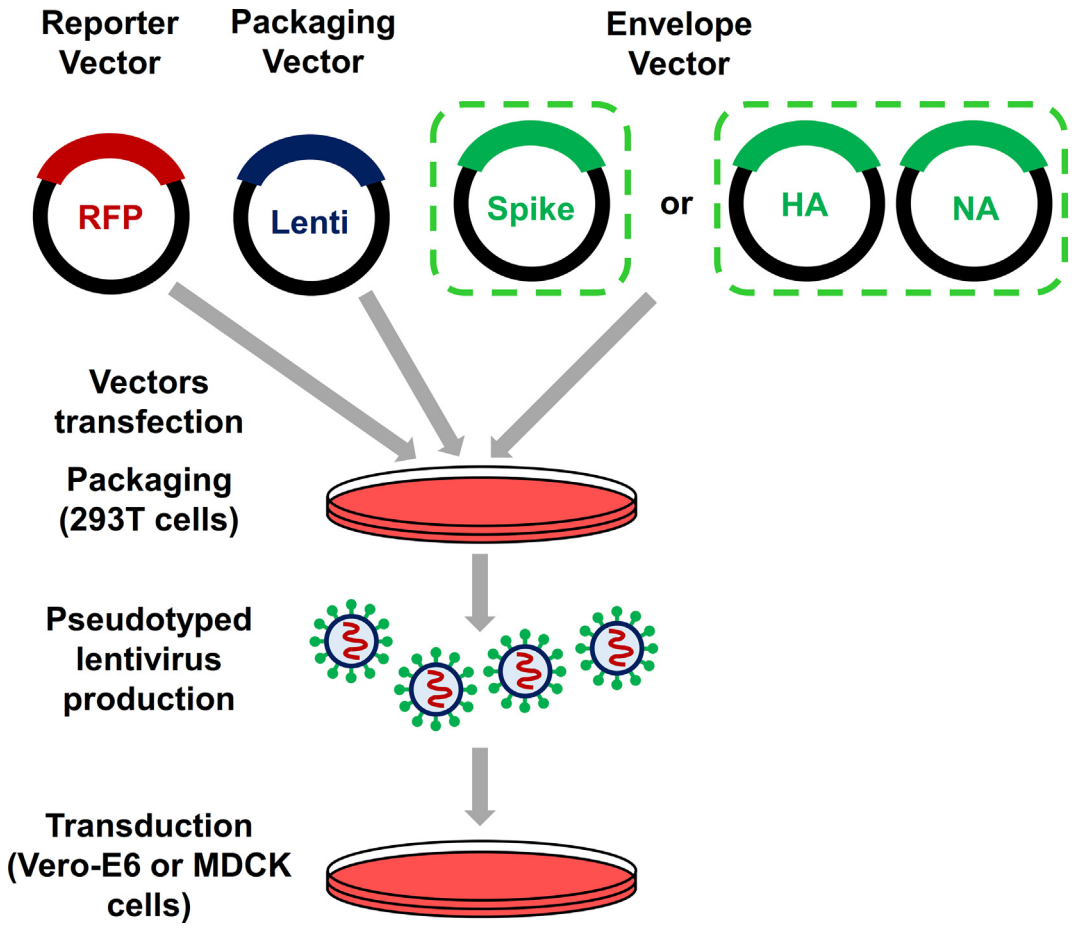

Fig. 1 Lentiviral pseudovirus system of SARS-CoV or SARS-CoV-2 and avian influenza H5. Structural protein genes, including S protein of SARS-CoV or SARS-CoV-2 and HA/NA protein of avian influenza H5, were subcloned into envelope expression plasmid derived from pMD.G vector. To generate SARS-CoV or SARS-CoV-2 and avian influenza H5Nx pseudoviruses, we co-transfected the structural protein expressing either S protein or HA and NA vectors, a package vector, and a reporter vector into HEK-293T cells. Generated SARS-CoV or SARS-CoV-2 and avian influenza H5Nx pseudoviruses were harvested and transduced into Vero-E6 or MDCK cells, respectively.

and HA/NA proteins, respectively. For SARS-CoV or SARS-CoV-2 pseudovirus, the sequences of S protein were *de novo* synthesized. Synthesized genes were cloned into pMD.G plasmid to express SARS-CoV or SARS-CoV-2 pseudoviruses. For avian influenza virus pseudovirus, HA and NA sequences from different avian influenza viruses H5N2, H5N6, and H5N8 (H5Nx) were synthesized and replaced the VSV-G envelope glycoprotein in pMD.G plasmid. Cloned plasmids were transformed into One Shot Stbl3 Chemically Competent cell (Invitrogen) and were amplified in Lysogeny Broth with 100 μg/ml ampicillin. Plasmids were extracted by Zymo Research midi kit. The 293T cells were seeded at the concentration of 2×10^6 cells in 6-well plates at 37 °C with 5% CO_2 for 24 h, and then the cells were transfected with 1 μg of pCMVdeltaR8.91, pLAS2w.RFP-C.Pneo and pMD.G plasmids (pMD.G with S gene for SARS-CoV or SARS-CoV-2 tagged by HA on the C-terminus and pMD.G with indicated HA and NA gene pairs for avian influenza virus, respectively) by PolyJet reagent according to manufacturer's instructions [Fig. 1]. In the following of 24 h post-transfection, and the culture medium was displaced by FreeStyle™ 293 expression medium (Gibco) and then cultured for an additional 24 h. The total cell lysates collected were centrifuged to remove the cell debris then filtered through 0.45 μm filters for immunoblot and further experiments. For the SARS-CoV and SARS-CoV-2 pseudovirus, we utilized the rabbit polyclonal antibody against SARS-CoV S protein (ARG54885, arigo Biolaboratories) and the mouse anti-HA tag monoclonal antibody (C05012-100UG, Croyez Bio.) against C terminal tag of SARS-CoV-2 S protein to detect S protein expression with 1:1000 dilution, respectively. For avian influenza virus H5Nx pseudovirus, we utilized mouse monoclonal H5N1 HA antibody (11048-MM06, Sino Biological) with 1:1000 dilution. The HRP-labeled secondary antibodies (474–1802, KPL) with 1:1000 dilution were used for all immunoblot assays.

Quantification and neutralization assay of pseudoviruses

Vero-E6 (for SARS-CoV or SARS-CoV-2 pseudovirions) and MDCK cells (for avian influenza virus H5Nx pseudovirions) were seeded in 24-well plates with 1.5×10^5 cells/well. After 24 h of culture, cells were infected with 200 μL of two-fold diluted viruses, adsorbed for 1 h and cultured at 37 °C (for SARS-CoV or SARS-CoV-2 pseudovirions) or 35 °C (for avian influenza virus H5Nx pseudovirions). Mouse antisera were

complement inactivated at 56 °C for 30 min before neutralization assay. The pseudoviruses were incubated with serially diluted antisera at 37 °C for 30 min. The mixtures were added into Vero-E6 at 37 °C or MDCK cells at 35 °C for 1 h incubation. The assays were performed in duplicates. Cell medium were then refreshed with Vero-E6 medium (Eagle's MEM with 1 mM sodium pyruvate, 100 U/ml penicillin, and 0.1 mg/ml streptomycin) or MDCK medium (Eagle's MEM with 1 μg/ml trypsin, 1 mM sodium pyruvate, 100 U/ml penicillin, and 0.1 mg/ml streptomycin). Four days post-infection, infected cells with fluorescence were observed and fixed with 1% paraformaldehyde. Cells were resuspended with PBS for calculating the percentage of fluorescent positive cells through flow cytometer. Virus transduction unit was calculated with the formula: titer = N × Cn × DF/V (F: The frequency of RFP-positive cells determined through flow cytometry; Cn: The total number of target cells infected; V: The volume of the inoculum; DF: The virus dilution factor) [38]. To quantify the neutralization titers for both SARS-CoV-2 and avian influenza pseudoviruses, the neutralization titers were defined by 50% reduction of the transduction unit (TU) in both duplication of diluted antisera concentration compared with the average transduction units. Neutralization results of avian influenza virus subtypes were further visualized by antigenic cartography analysis, which revealed the antigenic diversity of antigens. In brief, the short distance of antigens on antigenic map indicated similar antigenic properties between two antigens. Clustering scenario of antigens revealed the degree of diversity among tested antigen.

Mice immunization

For SARS-CoV-2 antisera, 6–8 weeks old BALB/c mice were subcutaneously immunized twice with 50 μg of recombinant SARS-CoV-2 spike protein (S1+S2 ECD) (Sino Biological) and emulsified in Complete Freund's Adjuvant (CFA, Sigma) for priming and Incomplete Freund's Adjuvant (IFA, Sigma) for the boost in a total of 100 μl at a 3-week interval. Recombinant protein solutions in PBS were mixed 1 to 1 with the respective adjuvant. Blood samples were collected by submandibular blood sampling at week 5 after the first immunization. For the avian influenza virus H5Nx pseudovirus, 20 ml of pseudoviruses were concentrated by Lenti-X™ Concentrator (Clontech) then resuspended in PBS. Pseudoviruses were inactivated by UV light for 30 min and then used in mice immunization. The pseudoviruses were used to immunize 6–8 weeks old BALB/c mice with the addition of adjuvant (Freund's Complete Adjuvant and Freund's Incomplete Adjuvant) which were performed by LEADGENE, Inc. Antisera were collected at 14, 28, 45 and 60-days post-immunization and pooled for neutralization assays.

Results

Develop pseudovirus system for emerging viruses by applying lentivirus vectors

Due to the high infectivity and high pathogenicity of emerging virus SARS-CoV or SARS-CoV-2 and re-emerging virus avian influenza virus H5Nx, handling of the viruses in the biosafety level 3 facility are necessary but uneasily accessible to evaluate the vaccine efficacy by neutralization assay. Here, we thus established two pseudovirus systems for emerging virus SARS-CoV or SARS-CoV-2 and re-emerging virus avian influenza virus H5Nx including H5N2, H5N6, and H5N8. In order to generate pseudoviruses expressing emerging viruses structure proteins (Spike protein of SARS-CoV or SARS-CoV-2 and HA/NA of avian influenza virus H5Nx), we replaced the VSV-G protein gene in pMD.G plasmid by either the S gene of SARS-CoV or SARS-CoV-2 (pMD.SARS-CoV-S/pMD.SARS-CoV-2-S) or HA genes (pMD.H5Nx-HA) and NA genes (pMD.H5Nx-NA) of avian influenza viruses. For avian influenza viruses, HA and NA genes from one to two strains of each subtypes were selected to develop pseudovirus systems, including a WHO available or pending vaccine strain and a circulating strain. We applied the lentiviral system which employed to express the structural proteins on the surface of pseudovirus and to package lentiviral RNA with red fluorescence protein (RFP) reporter gene to get pseudoviruses with S or HA/NA proteins expressed on the surface of virions, respectively [Fig. 1]. The amount of generated pseudoviruses can thus be quantified according to the number of the cells expressing RFP following transduction by using the produced pseudovirus.

To verify whether the S or HA protein were incorporated into SARS-CoV or SARS-CoV-2 and avian influenza virus H5Nx pseudovirus, we used Western blot to detect the structural proteins on the virions. In SARS-CoV or SARS-CoV-2 pseudovirus particles, complete S proteins (approximately 190 kDa and 220 kDa) of SARS-CoV and SARS-CoV-2 were detected by anti-SARS-CoV S protein antibody and anti-HA tag antibody, respectively [Fig. 2A]. In contrast to the complete S protein of SARS-CoV/SARS-CoV-2, HA protein of avian influenza virus including HA0 (approximately 80 kDa) and HA2 protein (approximately 20 kDa) were observed in influenza virus pseudovirus by blotting with anti-influenza virus H5 HA2 protein antibody, which indicated the partial cleavage of HA0 protein to HA1 and HA2 [Fig. 2B]. The different cleavage manner between S and HA proteins in the pseudovirions might result from the diverse host protease requirement to digest the two structural proteins. Both S protein of coronavirus and HA protein of influenza virus harbor cleavage sites for different proteases in the boundary of S1/S2 subunit and HA1/HA2 subunit, respectively [12,13,20–27]. As complete HA0 and cleaved HA2 coexisted in the influenza virus pseudovirions, the host cell HEK293T supporting pseudovirus production might provide limited host proteases specific for the cleavage of HA protein.

Optimization of SARS-CoV or SARS-CoV-2 pseudovirus transduction and neutralization assay

In following the generation of the pseudoviruses, we first focused on investigating the transduction efficiency by titrating the SARS-CoV or SARS-CoV-2 pseudovirus. We first examined whether SARS-CoV or SARS-CoV-2 pseudovirus can transduce into Vero-E6 cells. Abundant RFP fluorescence was observed in the SARS-CoV and SARS-CoV-2 pseudovirus-transduced Vero-E6 cells, suggesting the successful transduction of the pseudovirus [Fig. 3A]. SARS-CoV and SARS-CoV-

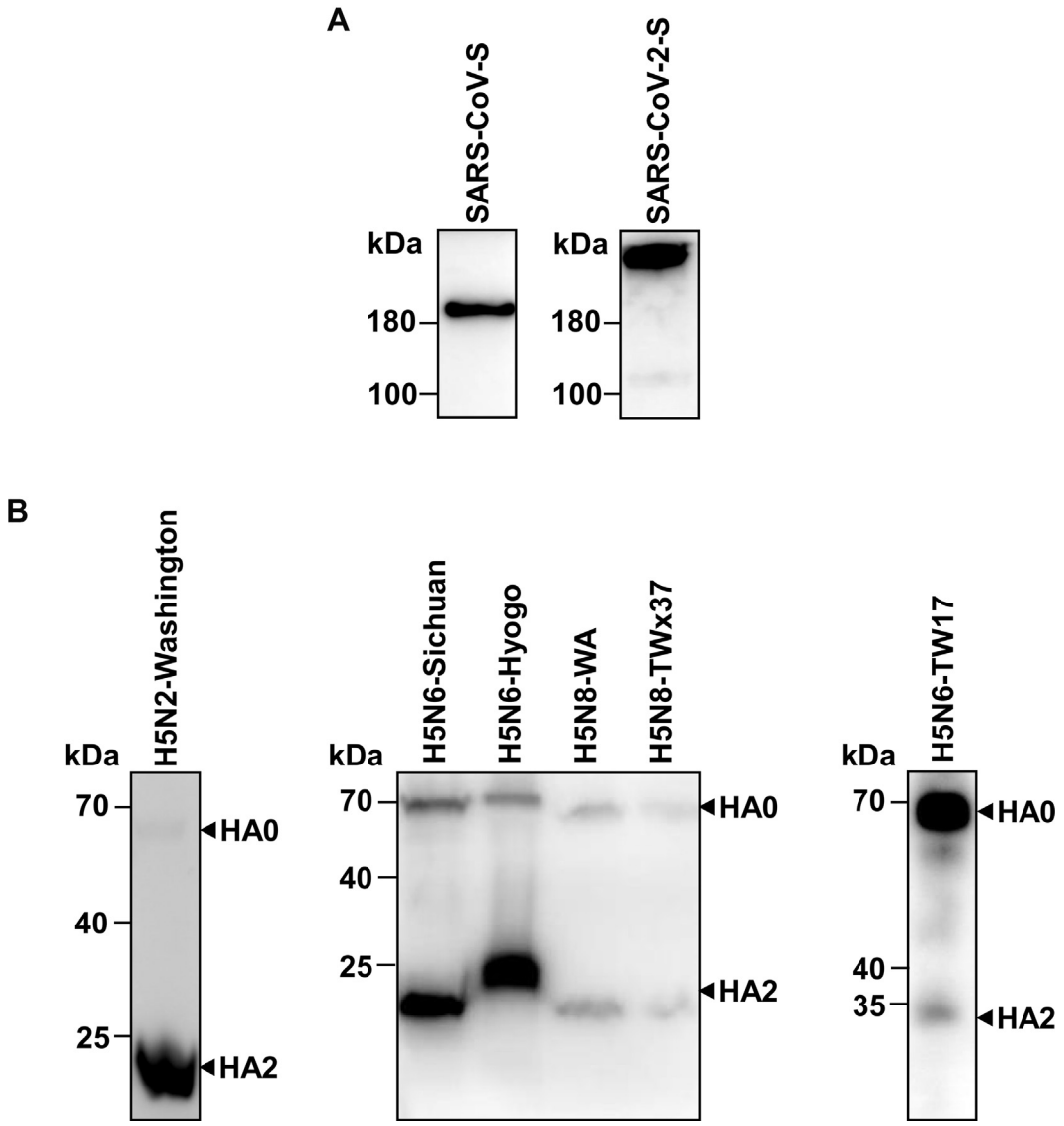

Fig. 2 Immunoblotting of S protein of SARS-CoV or SARS-CoV-2 and HA protein of avian influenza H5. (A) S proteins of SARS-CoV and SARS-CoV-2 were immunoblotted with mouse anti-SARS-CoV S protein antibody and mouse anti-HA tag protein antibody, respectively. (B) HA proteins of avian influenza H5 were immunoblotted with mouse anti-influenza virus H5 HA protein antibody. As the antibody recognized the HA2 epitope, both of HA0 and HA2 protein were detected by the immunoblotting.

2 pseudoviruses were serially diluted and then transducted into Vero E6 cells, respectively [Fig. 4A]. Transduction unit (TU) of the virions were determined according to the percentage of RFP positive cells (%RFP) in 48 h post-transduction. Maximum % RFP reached 19.9% and 22.6%, and % RFP increased in dose-dependent manners for SARS-CoV and SARS-CoV-2 pseudoviruses [Figs. 5 and 6], respectively. As we quantified the transduction units according to the absolute number of RFP positive cells, SARS-CoV-2 pseudovirion (2.36×10^6 TU/ml) [Fig. 5] had similar TU with SARS-CoV (2.33×10^5 TU/ml) [Fig. 6] according to the results of % RFP. Titer was also performed by using VSV-G pseudovirus as control group, and maximum % RFP achieved 31.05% referring to 3.85×10^6 TU/ml. Taken together, the TU result implied similar pseudovirus production efficiency between SARS-CoV and SARS-CoV-2 in our lentiviral pseudovirus system [Fig. 7].

Early SARS coronavirus reports have shown that S1–S2 cleavage can be enhanced by exogenous proteases, such as trypsin, thermolysin, and elastase [16,39]. Next, we examined whether exogenous trypsin increases the transduction efficiency of our generated SARS-CoV and SARS-CoV-2 pseudovirus. We added exogenous trypsin during SARS-CoV and SARS-CoV-2 pseudovirus absorption to Vero-E6 cells, until we harvested cells for cytometry analysis. Unexpectedly, we observed a reduction in virion transduction with trypsin presence, especially for SARS-CoV virions. With trypsin treatment, the TU of SARS-CoV virions decreased 93% from 9.5×10^5 to 6.5×10^4 TU/ml. In contrast

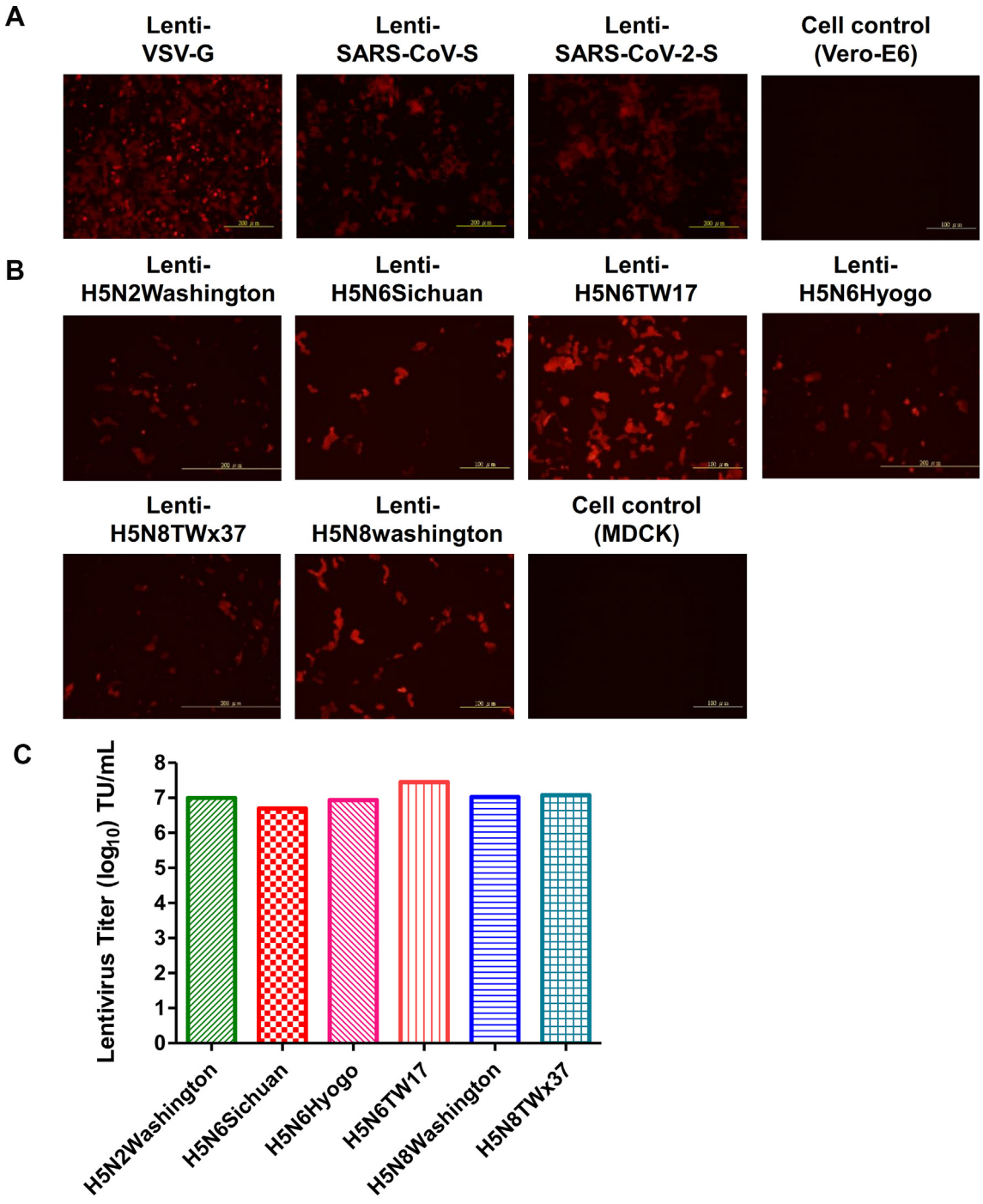

Fig. 3 Pseudovirus transduction of SARS-CoV or SARS-CoV-2 and avian influenza H5Nx. Generated (A) SARS-CoV or SARS-CoV-2 and (B) avian influenza H5Nx pseudoviruses were transduced into Vero-E6 or MDCK cells, respectively. Red fluorescence indicated the cells transduced by the indicated pseudoviruses with RFP reporter gene. (C) Transduction titers of avian influenza H5Nx pseudoviruses were determined according to the numbers of cells expressing red fluorescence.

to dramatic TU reduction of SARS-CoV virions, the TU of SARS-CoV-2 moderately reduced 43% from 1.09×10^6 to 6.2×10^5 TU/ml [Fig. 4B]. The transduction reduction effects of exogenous trypsin presence since virion absorption might have resulted from the trypsin cleavage of partial S proteins on the unbound virions being cleaved into S1/S2 subunits by trypsin in advance of virion fusion in the Vero-E6 cells; however, only moderate effects on SARS-CoV-2 virion fusion in contrast with SARS-CoV might imply that the S protein of SARS-CoV-2 were more resistant to trypsin cleavage. In addition, SARS-CoV-2 virion might mainly utilize proteases other than trypsin, such as furin [40], as fusing with Vero-E6 cells. Whether trypsin or furin treatment after virion binding to cells enhances SARS-CoV or

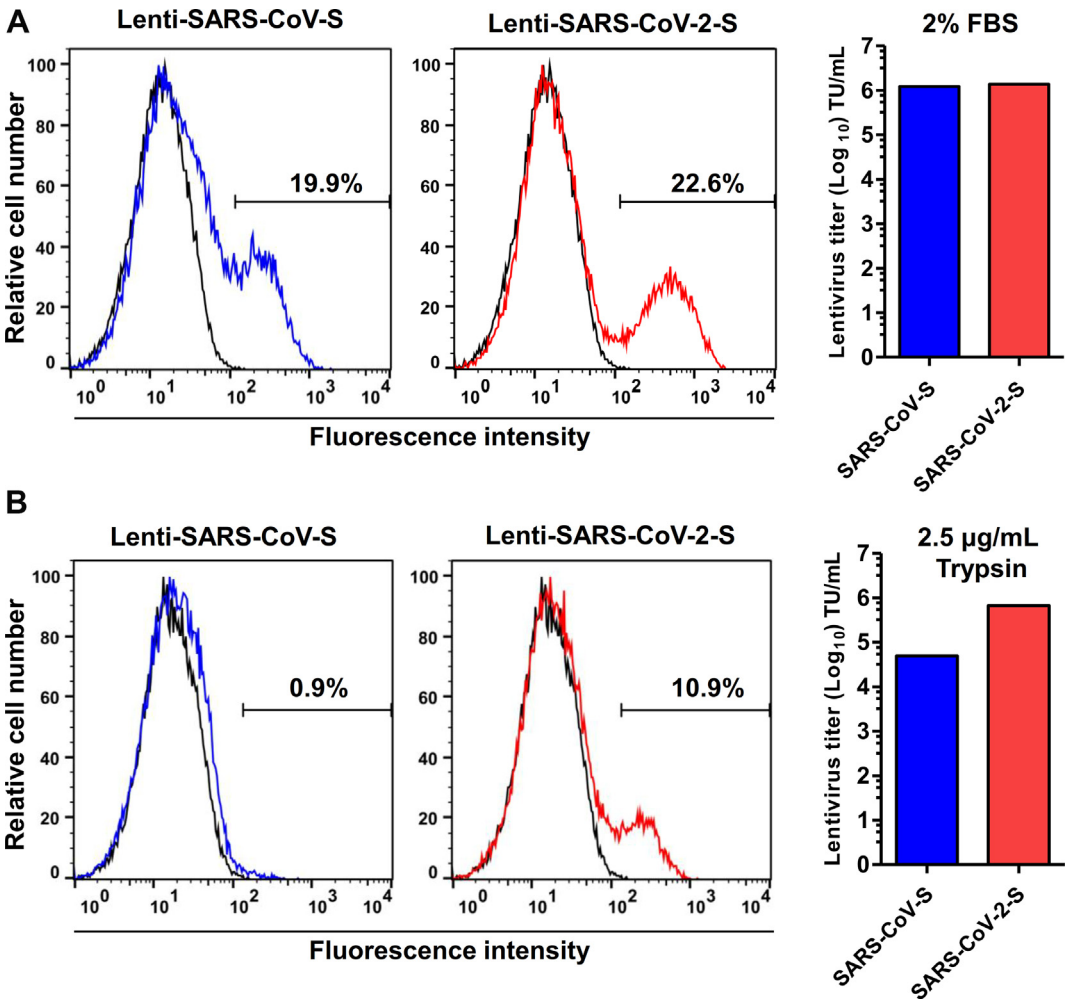

Fig. 4 Transduction optimization of SARS-CoV and SARS-CoV-2 pseudoviruses. Generated SARS-CoV and SARS-CoV-2 pseudoviruses were transduced into Vero-E6 cells. Different transduction medium with (A) 2% FBS or (B) 2.5 μg/ml trypsin. Using transduction medium with 2% FBS showed higher transduction rate for SARS-CoV and SARS-CoV-2 pseudoviruses. Using transduction medium with 2.5 μg/ml trypsin obviously reduced transduction rate, especially for SARS-CoV pseudoviruses.

SARS-CoV-2 virions entry into Vero-E6 cells will be further investigated in the future.

In facing the emerging SARS-CoV-2 virus outbreak, evaluation of the vaccine efficacy as the vaccine development of SARS-CoV-2 becomes urgently needed to reduce the threat of the virus. In order to evaluate vaccine efficacy against such highly contagious virus more efficiently, we applied the generated SARS-CoV-2 pseudovirus for evaluation of the neutralization antibody by using neutralization assay. To examine whether the lentivirus-based pseudovirus is capable to be applied in the neutralization assay, we retrieved two mouse antisera from the National Health Research Institutes, whereby the antisera were obtained from mice were immunized by SARS-CoV-2 S recombinant protein. Antisera were serially diluted in two-folds to neutralize a total amount of 6.4×10^5 TU of SARS-CoV-2 pseudovirus with duplication. We determined the antisera titers according to the antisera dilution exhibiting >50% reduction of virus transduction in both duplicated neutralization experiments [Table 1]. Antisera neutralization results demonstrated that the lentivirus-based pseudovirus successfully applied in determining neutralization antibody titrations of antisera. We successfully defined the titers of two different mouse antisera. Neutralization result indicated that 1:50 to 1:400 diluted antiserum MS-1 neutralized the SARS-CoV-2 spike pseudovirus in both duplication experiments, although 1:800 diluted antiserum MS-1 still neutralized over 50% SARS-CoV-2 pseudovirus transduction in one of the duplicates. In contrast to MS-1 antiserum, no dilutions of MS-2 antiserum neutralized the SARS-CoV-2 spike pseudovirus in both duplication experiments. According to the result, antiserum MS-1 exhibited higher titers (1:400) against SARS-CoV-2 pseudovirus than antiserum MS-2 (≦1:50). In summary, the results demonstrated that we successfully generated SARS-CoV and SARS-CoV-2 lentivirus-based pseudovirus that is able to be applied in determining neutralization antibody of the antisera within *in vitro* neutralization assay. Producing neutralization antibody against the immunodominant viral protein is one of the important factors to evaluate vaccine efficacy. Our

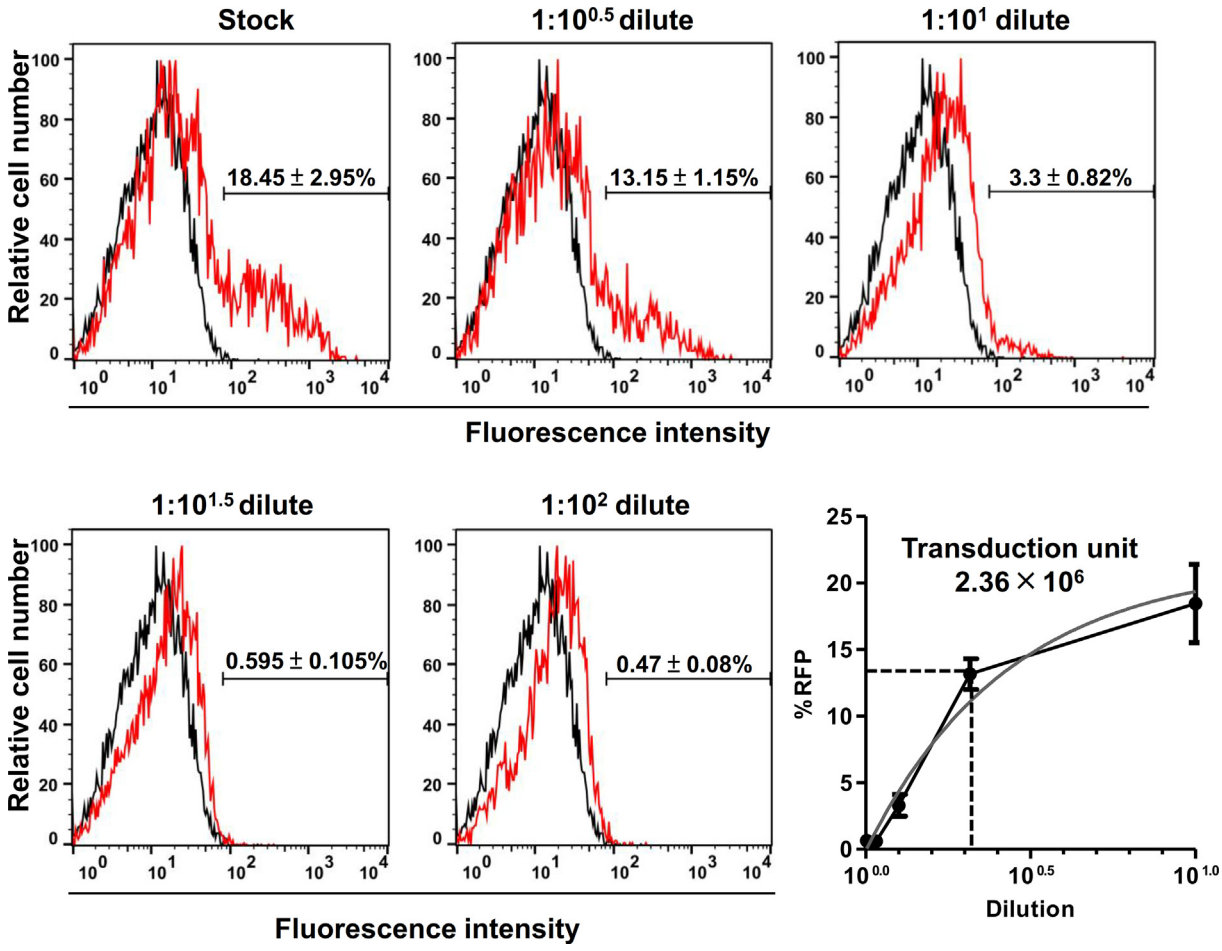

Fig. 5 Dose-dependent transduction rates of SARS-CoV-2 pseudoviruses. Generated SARS-CoV-2 pseudoviruses were serially diluted and then transduced into Vero-E6 cells. Transduction rate of SARS-CoV-2 was gradually reduced in a dose-dependent manner. According to the transduction rate curve, the titer of SARS-CoV-2 pseudovirus was quantified as 2.36×10^6 transduction unit.

pseudovirus neutralization assay will become a feasible tool in the SARS-CoV-2 vaccine development.

Optimize the influenza virus H5Nx pseudovirus transduction and neutralization assay

After successfully establishing emerging virus SARS-CoV-2 pseudovirus transduction and neutralization assays, we next investigated the generated pseudovirions of re-emerging virus avian influenza virus H5Nx. In contrast to using Vero-E6 by SARS-CoV or SARS-CoV-2 pseudovirus, we examined whether avian influenza virus H5Nx pseudovirus can be transduced into MDCK cells as host cells. Alike to SARS-CoV or SARS-CoV-2 virions, we observed abundant RFP fluorescence pseudovirus-transduced MDCK cells, indicating the successful transduction of the avian influenza virus H5Nx pseudovirus [Fig. 3B]. According to the percentage of RFP positive cells (%RFP) in 48 h post-transduction, different influenza H5Nx subtypes showed similar transduction unit (TU) within 10^6 to 10^8 TU/ml in MDCK cells [Fig. 3C] although the diverse TUs were seen among the subtypes. We thus successfully transduced pseudovirions of re-emerging avian influenza virus H5Nx into MDCK cells as host cells.

We next examined the applicability of avian influenza virus H5Nx pseudovirus in neutralization assay. Contrast to single type of SARS-CoV-2 due to only one dominant strain, various avian influenza virus subtypes co-circulated in worldwide. Our study selected three recently circulating subtypes of avian influenza virus in Taiwan and worldwide, including H5N2, H5N6, and H5N8 subtypes to determine the neutralization antibody by using H5Nx pseudovirus. Instead of raising antibody by structural protein expressing recombinant protein subunit vaccine in SARS-CoV-2 virus, we concentrated the inactivated avian influenza pseudovirus as immunogens to raise antisera against avian influenza HA and NA structural proteins in mice. We determined neutralization antibody titers of raised antisera against different subtypes of influenza virus and generated an antigenic map to analyze the similarity of antigenicity according to neutralization antibody titers. Mouse antisera were respectively generated by each selected strain and examined neutralization activities against all selected avian influenza pseudovirions. Neutralization titers demonstrated that avian influenza virus exhibited partial cross-reactivity among H5N2, H5N6, and H5N8 subtypes. High neutralization titers of antisera between 1:160 and ≧ 1:5120 were observed among examined subtypes [Table 2], implied

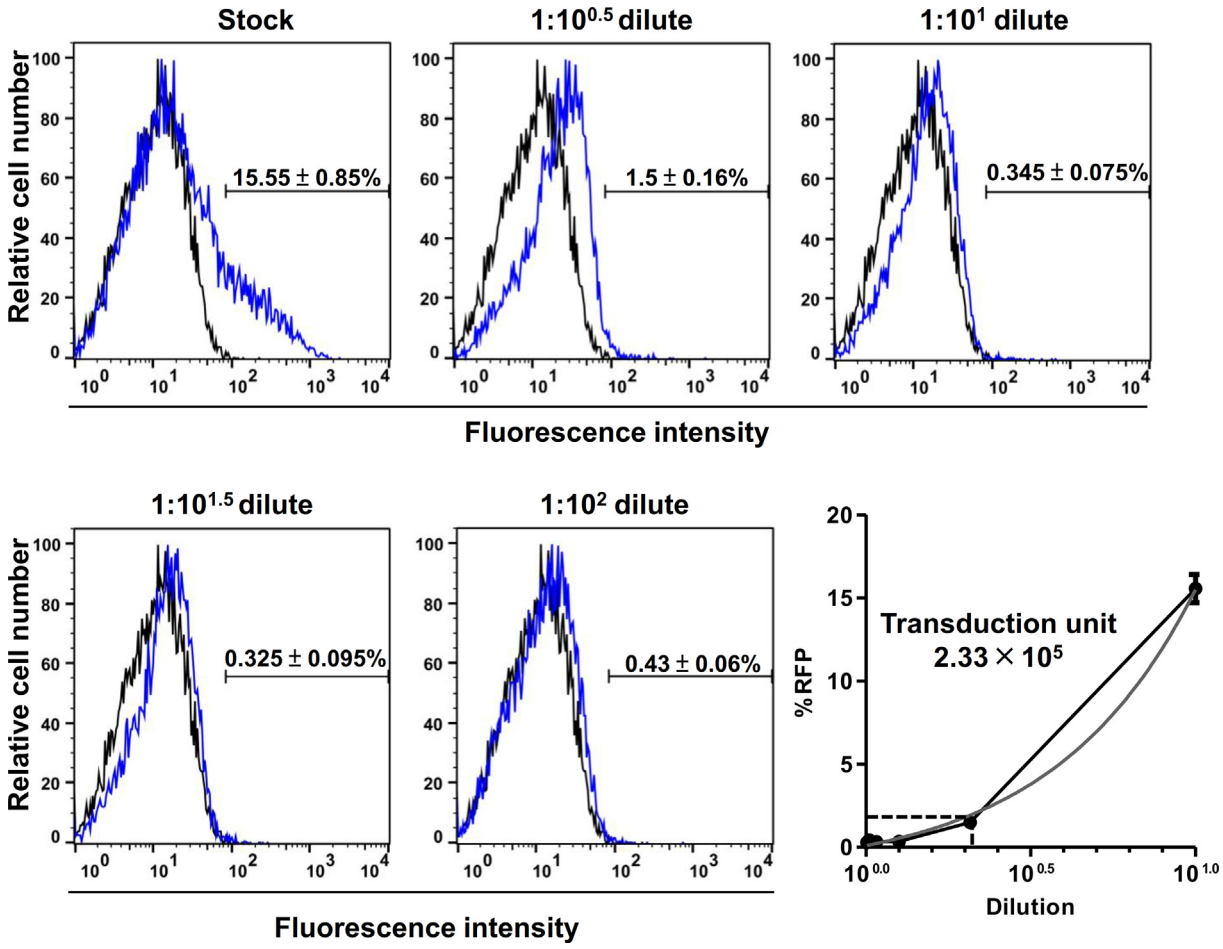

Fig. 6 Dose-dependent transduction rates of SARS-CoV pseudoviruses. Generated SARS-CoV pseudoviruses were serially diluted and then transduced into Vero-E6 cells. Transduction rate of SARS-CoV was gradually reduced in a dose-dependent manner. According to the transduction rate curve, the titer of SARS-CoV pseudovirus was quantified as 2.33×10^5 transduction unit.

the immunogens combining HA and NA proteins of H5N2, H5N6, or H5N8 can induce antisera with high neutralization activities. Among the examined pseudovirions as immunogens, antiserum which was raised by H5N8 TWX37 virus showed consistently high titers (1:1280 to 1: 2560) against all pseudovirions, suggesting that H5N8 TWX37 virus can be a suitable vaccine candidate for broad spectrum avian influenza virus vaccine. Although on average high neutralization titers were generally seen among the antisera against diverse subtype pseudovirus, we still observed the 2 to 8-folds of different titers among examined pseudovirions as which were neutralized by each antiserum. Anti-H5N2 Washington antiserum exhibited high neutralization titers (≧1:1280) against all tested pseudovirions except for H5N6-TW17 strain (1:320) with 4 folds of titer reduction. In contrast, anti-H5N6 Hyogo antiserum showed the highest titers against itself but lower titers against other pseudovirus with 2–8 folds of reduction. Also, antigenic cartography indicated that H5N2 Washington pseudovirus exhibited diverse antigenic properties in comparing with other virions, suggesting antigenic diversity occurrence among avian influenza H5Nx subtypes [Fig. 8]. Together, we developed lentiviral pseudovirus systems of emerging SARS-CoV2 virus and re-emerging avian influenza virus as biologically safer efficacy evaluation assays than conventional neutralization assays needed to perform within Biosafety Level 3 containment when we developed a vaccine. The neutralization assay results not only reflected neutralization activities of antisera but also revealed the immunogenicity and antigenic property diversity of re-emerging viral subtypes, which is another important issue for vaccine seed selection in re-emerging virus vaccine development.

Discussion

Vaccine development of emerging and re-emerging viruses are always urgent as we continuously face their strong threats for our lives. To examine the efficacy of developed vaccines, neutralization activity of immunized animals and even human individuals was one of the most important indicators for vaccine evaluation; however, the highly pathogenic viruses needed to be operated in a high level of biosafety containment to protect not only laboratory personnel, as well as the surrounding environment and community, which increased the accessability difficulty for *in vitro* viral infection experiment including neutralization assay. Pseudovirus

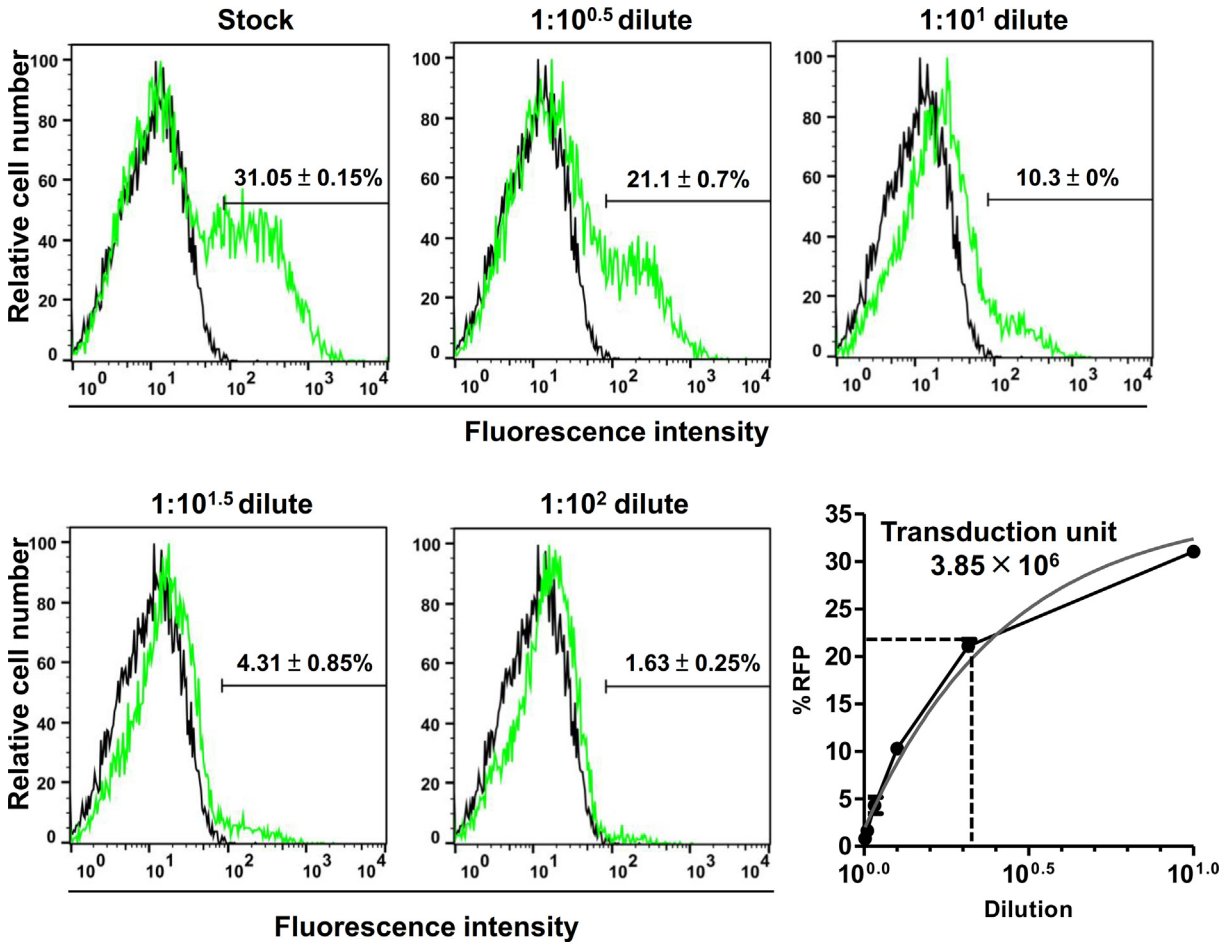

Fig. 7 Dose-dependent transduction rates of VSV-G pseudoviruses. Generated VSV-G pseudoviruses were serially diluted and then transduced into Vero-E6 cells. Transduction rate of VSV-G was gradually reduced in a dose-dependent manner. According to the transduction rate curve, the titer of VSV-G pseudovirus was quantified as 3.85×10^6 transduction unit.

system have been widely applied in the studies of highly pathogenic viruses, including SARS-CoV/SARS-CoV-2 and high pathogenic avian influenza virus, which reduced the safety requirement and increase the accessability of neutralization assay against these viruses [9,10,14,29,30,32,36,37,40]. We here developed two series of pseudoviruses for emerging coronavirus SARS-CoV or SARS-CoV-2 and re-emerging virus avian influenza virus H5Nx by using lentiviral packaging system. The pseudoviruses stimulated the viral entry of studied viruses through their structural proteins and were applied to examine whether antisera had neutralization activities to block viral fusion into the target cells. Indeed, our results demonstrated that the viral entry of the SARS-CoV-2 and avian influenza H5Nx pseudoviruses can be blocked by the antisera raised by SARS-CoV-2 S protein and inactivated avian influenza H5Nx pseudovirus, respectively. The successful neutralization indicated that the assay can be applied in vaccine development of either emerging or re-emerging viruses and the assay can be operated in a more available biosafety facility.

In addition to evaluate the neutralization activity, vaccine development needs to evaluate the antigenicity and

Table 1 Neutralization assay result of SARS-CoV-2 mouse antisera by pseudovirus system.

Serum samples	Transduction unit per ml (TU/ml)						
	Dilution					Virus control	50% of virus control (mean)
	1:50	1:100	1:200	1:400	1:800		
MS-1	**2.07×10^5**[a]	**2.24×10^5**	**2.18×10^5**	**3.03×10^5**	3.39×10^5	6.62×10^5	3.18×10^5
	1.78×10^5	**2.45×10^5**	**2.91×10^5**	**1.64×10^5**	**2.12×10^5**	6.10×10^5	
MS-2	3.90×10^5	6.41×10^5	6.62×10^5	5.74×10^5	8.29×10^5		
	4.17×10^5	5.71×10^5	5.01×10^5	4.71×10^5	5.75×10^5		

[a] Bold numbers indicate the individual experiment which had transduction unit lower than 50% of transduction unit of virus control.

Table 2 Cross-neutralization assay result of avian influenza virus H5Nx by pseudovirus system.

Virus tested	Neutralizing antibody titers of antiserum raised by					
	Lenti-H5N2 Washington	Lenti-H5N6 Sichuan	Lenti-H5N6 Hyogo	Lenti-H5N6 TW17	Lenti-H5N8 TWX37	Lenti-H5N8 WA
Lenti-H5N2Washington	1:1280	1:1280	1:160	1:640	1:1280	1:1280
Lenti-H5N6Sichuan	1:1280	≥1:5120	1:160	1:1280	1:2560	1:2560
Lenti-H5N6Hyogo	1:2560	1:2560	1:1280	1:2560	1:2560	1:2560
Lenti-H5N6TW17	1:320	1:1280	1:320	1:640	1:1280	1:1280
Lenti-H5N8 TWx37	1:1280	1:1280	1:320	1:1280	1:1280	1:640
Lenti-H5N8 WA	≥1:5120	≥1:5120	1:640	1:2560	1:2560	1:1280

immunogenicity for vaccine seed strain selection, especially for re-emerging viruses such as avian influenza virus H5 containing various subtypes. Although recent emerging SARS-CoV-2 has only one dominant strain with genetic variants, we suspected that the emerging virus might steadily evolve with "antigenic drift" like influenza virus under strong herd immunity in the near future due to the high transmission rate with millions of confirmed reported cases around the world. If the recent emerging SARS-CoV-2 continuously circulates among the community and becomes another 're-emerging virus', SARS-CoV-2 will also face a strong positive selection pressure from herd immunity which drives the virus to exhibit diverse antigenic properties and causes antigenic property changes. Constant surveying of the antigenic evolution of SARS-CoV-2 virus and updating the vaccine antigen for inactivated vaccine, recombinant S protein antigen for subunit vaccine, or even delivered S gene for DNA/RNA vaccine are necessary in the process of vaccine development. In the following of surveying antigenic property of re-emerging virus, the immunogenicity of re-emerging S protein/gene will be next examined. The immunogenicity analysis of vaccine antigen provides valuable information for vaccine selection, indicating the true scenario of raised neutralizing antibody response against target virus. According to the immunogenicity results, we will assess the feasibility of utilizing selected antigens as immunogens to induce proper neutralizing antibody response. Here we used avian influenza virus H5 as an example to demonstrate the feasibility of our pseudovirus system applying in not only vaccine efficacy evaluation by using neutralization assay but also antigenicity and immunogenicity assessments by utilizing pseudovirus of re-emerging viruses. As we used various H5Nx concentrated pseudovirus as antigens to immunize mice, we revealed minor diversity of antigenic property and immunogenicity of each examined H5Nx pseudovirus by antigenic cartography. In previous studies, antigenic cartography was successfully applied using influenza, enterovirus A71, and dengue viruses to access the antigenic variation [41–46]. Combining with cross-neutralization results and antigenic cartography, we visualized the antigenic properties of influenza virus H5Nx subtypes. Results demonstrated that the avian influenza virus H5Nx localized in a short distance on the antigenic map, which shows the close antigenic property among examined H5Nx subtypes. Nonetheless, the cross-neutralization results revealed the different feasibility of H5Nx antigens to raise neutralizing antisera. We found that antisera raised from H5N8 TWX37 immunization showed the generally broad spectrum of neutralization against all examined subtypes pseudovirus, suggesting its good immunogenicity against avian influenza viruses H5N2, H5N6, and H5N8.

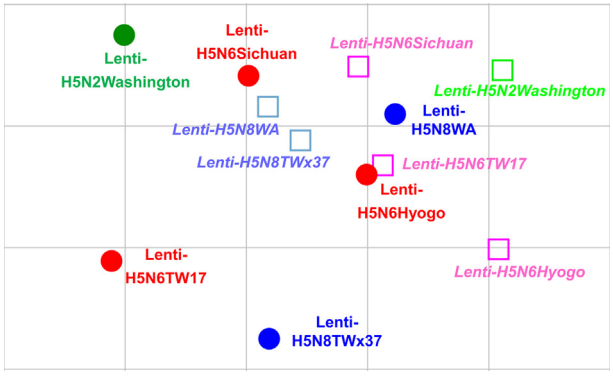

Fig. 8 Antigenic cartography of avian influenza virus H5Nx pseudoviruses. Antigenic cartography displays the antigenic properties of avian influenza virus H5Nx pseudoviruses. The viruses are shown in color and the antisera as open shapes. Distances between each subtype and antiserum on the map represent the corresponding neutralization assay titers. Both the vertical and horizontal dimensions represent antigenic distance; only the relative positions of antigens and antisera can be determined, i.e., the map can be freely rotated. Each grid line represents a unit of antigenic distance, corresponding to a 2-fold dilution of antiserum in the neutralization table.

Taken together, our investigations successfully established lentiviral pseudovirus systems for emerging SARS-CoV-2 and re-emerging avian influenza virus H5Nx. We assessed the feasibility of pseudovirions in the process of vaccine development. Pseudovirus system showed convincing results for vaccine efficacy by applying pseudovirions in neutralization assay for both SARS-CoV-2 and avian influenza viruses. In addition, we showed the potency of pseudovirions to be applied in antigenic property analysis and immunogenicity analysis, which is especially important for antigen candidate selection. Most important of all, the pseudovirus system exhibited a lower biosafety risk than wild-type high risk viruses such as SARS-CoV/SARS-CoV-2 and highly pathogenic avian influenza virus H5 that can be handled in a biosafety level 2 laboratory. Through this system, we might shorten the time frame and provide more suitable vaccine candidate in the process of vaccine development.

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Conflicts of Interest

The authors have declared that there is no conflict of interest.

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