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REVIEW

Current status on the development of pseudoviruses for enveloped viruses

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Summary

Emerging and reemerging infectious diseases have a strong negative impact on public health. However, because many of these pathogens must be handled in biosafety level, 3 or 4 containment laboratories, research and development of antivirals or vaccines against these diseases are often impeded. Alternative approaches to address this issue have been vigorously pursued, particularly the use of pseudoviruses in place of wild-type viruses. As pseudoviruses have been deprived of certain gene sequences of the virulent virus, they can be handled in biosafety level 2 laboratories. Importantly, the envelopes of these viral particles may have similar conformational structures to those of the wild-type viruses, making it feasible to conduct mechanistic investigation on viral entry and to evaluate potential neutralizing antibodies. However, a variety of challenging issues remain, including the production of a sufficient pseudovirus yield and the inability to produce an appropriate pseudotype of certain viruses. This review discusses current progress in the development of pseudoviruses and dissects the factors that contribute to low viral yields.

KEYWORDS

HIV, lentiviral vector, MLV, packaging system, pseudovirus, VSV

1 | INTRODUCTION

A pseudovirus is a recombinant viral particle with its core/backbone and envelope proteins derived from different viruses[1]; moreover, the genes inside the pseudovirus are usually altered or modified so that they are unable to produce the surface protein on their own. As such, an additional plasmid or stable cell line expressing the surface proteins is needed to make the pseudovirus.[2]

Pseudoviruses are capable of infecting susceptible cells, but they only replicate for 1 round in the infected host cells.[3] Compared with wild-type (WT) viruses, pseudoviruses can be safely handled in biosafety level (BSL)-2 laboratories[4] and are usually easier to manipulate experimentally.[5] Nevertheless, the conformational structure of pseudoviral surface proteins bears high similarity to that of the native viral proteins, and these surface proteins can effectively mediate viral entry into host cells. Therefore, pseudoviruses are widely used for the study of cellular tropism,[6] receptor recognition,[7] and virus inhibition,[8] as well as for developing and evaluating antibodies[9] and vaccines.[10] In addition, data from in vitro pseudovirus-based antiviral assays and in vivo biodistribution analyses have been found to correlate very well with the results generated by using live WT viruses.[11,12]

As pseudoviruses have usually been engineered to carry reporter genes, it is much easier to perform quantitative analyses on these viruses than on WT viruses,[13] and the number of pseudovirus-infected cells has been shown to be directly proportional to reporter gene expression. The reporter genes usually encode either an enzyme or a fluorescent protein, with each option having its particular strengths and weaknesses. Specifically, chemiluminescence assays usually have lower background and are more sensitive, but the data acquisition and analyses for these assays are time-consuming and more expensive. In contrast, assays using a florescence protein, such as green fluorescent protein, are cheaper and easier to operate in both in vitro and in vivo systems; however, they are less sensitive and may have higher background.[14-16] In this review, we provide an update on the development and application of pseudoviral systems and discuss some challenging technical issues.

Abbreviations used: EBOV, Ebola virus; MARV, Marburg virus; LASV, Lassa fever virus; HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; FIV, feline immunodeficiency virus; MERS-CoV, Middle East respiratory syndrome-coronavirus; RV, rabies virus; CHIKV, chikungunya virus; NiV, nipah virus; VSV, vesicular stomatitis virus; MLV, murine leukemia virus; GALV, gibbon ape leukemia virus; RRV, Ross River virus; DENV, dengue virus; WNV, West Nile virus; JEV, Japanese encephalitis virus; SFV, Semliki Forest virus; VEEV, Venezuelan equine encephalitis virus; RSV, respiratory syncytial virus; ADCC, antibody-dependent cell-mediated cytotoxicity

2 | CLASSIFICATIONS OF THE PACKAGING SYSTEMS FOR ENVELOPE-PSEUDOTYPED VIRUSES

Several packaging systems to create envelope-pseudotyped viruses have been explored for use with emerging viruses, such as Ebola virus (EBOV), Marburg virus (MARV), and Lassa fever virus (LASV).[17,18]

2.1 | Lentiviral vector packaging systems

Because of their high efficiency, lentiviral vectors are often the first choice as packaging systems to make envelope-pseudotyped viruses. These vectors are mainly derived from human immunodeficiency virus (HIV-1), simian immunodeficiency virus (SIV), or feline immunodeficiency virus (FIV). The vectors retain all of the genetic sequences needed for viral transcription, packaging, and integration, except for those encoding the envelope proteins.

2.1.1 | The human immunodeficiency virus packaging system

The HIV-1 packaging system is the most widely used pseudovirus packaging system. To make this packaging system, HIV genes are selectively cloned into DNA vectors. Specifically, 2 to 4 plasmids are used as the vectors, a strategy that aims to minimize viral gene recombination and thereby reduce the possibility of reversion to the WT virus. Table 1 lists the currently used HIV-1-based systems.

The original 2-plasmid system comprises 1 envelope plasmid and 1 HIV-1 backbone plasmid, ie, pSG3Δenv and pNL4-3 (the *env* gene sequence in pSG3Δenv is destroyed).[51] However, this system is not ideal, as its viral yield is usually very low. Improvements have been made through the addition of other sequences for better reporter gene expression. Specifically, our research group inserted the reporting gene, firefly luciferase (Fluc), into pSG3Δenv between *env* and *nef* to produce pSG3Δenv.Fluc.Δnef. In addition, we also generated pSG3Δenv.CMVFluc, which carries a functional *nef* and CMV promoter to drive the reporter gene.[10] By using these optimized backbone and envelope protein expression plasmids, our group succeeded in producing several HIV pseudoviruses carrying the envelope proteins of EBOV, MARV, LASV, Middle East respiratory syndrome-coronavirus, rabies virus (RV), chikungunya virus, and nipah virus (NiV). The yields of pseudoviruses constructed with this optimized system were improved by 100 to 1000-folds as compared with those of pseudoviruses constructed with pNL4-3.Luc.R-E.[52]

The HIV 3-plasmid system is usually comprised of a packaging plasmid, a transfer plasmid containing the reporter gene, and an envelope-expressing plasmid. Specifically, this system is made by splitting the HIV-1 backbone into separate packing and transfer plasmids. The packaging plasmid expresses the gag and pol proteins, while the transfer plasmid contains the cis-regulatory elements needed for HIV reverse transcription, integration, and packaging as well as multiple cloning sites and a reporter gene under the control of a heterogeneous promoter.[48-50] The envelope-expressing plasmid is made of a vector carrying the envelope gene driven by a CMV promoter.

The HIV 4-plasmid system is based on the 3-plasmid system, with the Rev protein being expressed by an additional, separate plasmid. Specifically, this system comprises 1 packaging plasmid expressing the gag and pol proteins, a second packaging plasmid encoding Rev, 1 plasmid producing the WT envelope protein, and a transfer plasmid with cis-regulatory elements.[53]

These 3 HIV-based systems were reported by different groups, and, as yet, no comparison has been made among the different systems in safety and efficiency. Our group is able to drastically improve the viral yield with 2-plasmid system, while no safety issue of this HIV pseudoviral systems was observed in animals.[52]

2.1.2 | The simian immunodeficiency virus packaging system

As HIV is the causative agent of AIDS, which could raise some safety concerns, some investigators employed SIV vectors in the development of similar 3-plasmid systems and explored their potential for use in gene therapy.

The SIV packaging system was constructed based on the viral genome of SIVmac251. Sandrin et al cotransfected 293 T cells with the pSIV-12 packaging vector, pSIV-T1$^+$ transfer vector, and RD114/TR expressing envelope protein. The SIV-based pseudoviruses demonstrated high transduction efficiency in primary blood lymphocytes and CD34$^+$ cells derived from either humans or macaques.[54] These investigators also used a pR4SA-green fluorescent protein vector and a pSIV-3$^+$ packaging vector to determine the factors that contribute to the intracellular and cell surface formation of various target viruses and the yield of pseudoviruses.[55] Moore et al used an SIV packaging system to construct a pseudotyped severe acute respiratory syndrome coronavirus (SARS-CoV) to screen anti-SARS-CoV compounds.[56] In theory, the SIV-based pseudovirus may be safer than its HIV counterpart as well as resistant to preexisting neutralizing antibodies against the vesicular stomatitis virus (VSV), murine leukemia virus (MLV), or gibbon ape leukemia virus; when pseudotyped with the RD114 (feline endogenous virus) envelope glycoprotein, the SIV-based pseudovirus demonstrated augmented transduction efficiency in blood cells from both humans and primates. These reports indicate that these pseudoviruses have facilitated preclinical studies in antiviral screening and gene therapy studies.

2.1.3 | The feline immunodeficiency virus packaging system

Feline immunodeficiency virus-based systems have also been found to be promising methods of pseudovirus production. Medina et al used the transfer vector pVC-LacZwP and packaging plasmid pCFIVΔorf2Δvif of FIV to construct a pseudotyped EBOV. When compared with the EBOV pseudovirus packaged by the HIV system, the EBOV pseudovirus packaged by the FIV system had a higher titer. Apparently, the mutation of the GP proteins contributed to higher pseudoviral titers and better safety of the resulting pseudoviruses.[57] By using the FIV packaging system as a backbone, other investigators have developed various pseudotyped viruses for SARS-CoV, VSV, RV, and Ross River virus. These viral particles have been found to be valuable in studying viral receptor recognition, gene transduction, and therapy.[58,59]

TABLE 1 Pseudoviruses packaged by HIV-1 system

Virus Protein	Research Area and Application	Packaging System	Reporting Gene	Reference
CHIKV C/E3/E2/6 K/E1	Impact of amino acid mutation of envelope proteins on the cellular tropism, neutralization antibody assay, and function of envelope proteins	pNL-luc, pNL-GFP, pNL-HSA; pLenti CMV Puro LUC, psPAX2; pDR8.2 (HIV gag-pol), pTrip luciferase	Luciferase	19-21
EBOV GP	Gene therapy, cellular tropism, mechanism of virus entry, and antivirus inhibitor	pCMVΔR8.2, pHR′LacZ (β-gal), pHR′EGFP; pR8ΔEnv, BlaM-Vpr, pcREV; pNL4-3.Luc.R-E-	β-Galactosidase, EGFP, luciferase	22,23
MARV GP	Cellular tropism, mechanism of virus entry, and evaluation on antivirus activity	HIV-1 JR-FL, HIV-1 NL4-3; pNL4-3.Luc.R-E-	Luciferase	24,25
HIV Env	Cellular tropism, neutralization antibody assay, impact of Env amino acid mutation, and glycosylation on the neutralization epitope, drug screening and validation, and receptor recognition	pSG3ΔEnv; pNL4-3.Luc.R-E-	Luciferase	26-29
LASV GPC	Effect of conservative property of amino acid sequence of glycoprotein on viral fusion and infection and neutralization antibody assay	pJK2 (HIV-1-LTR-b-gal); pSG3ΔEnv	β-Galactosidase, luciferase	9,30
MERS-CoV spike	DPP4 receptor validation, neutralization, antibody evaluation, research on amino acids of binding domain of viral receptor, screening inhibitor of viral entry, and evolution and host	pNL4-3.Luc.R-E-; pCSFLW, p8.91	Luciferase	31-35
SARS-CoV spike	Cellular tropism, ACE2 receptor recognition, cellular entry mechanism, neutralization antibody epitope, and gene transduction	pNL4-3.Luc.R-E-; pCMVΔR8.2, pHxLacZWP/pHR′-CMVLuc	Luciferase, β-galactosidase	36-39
NiV G/F	Gene transduction	pCSCGW (pCSC-SP-PW-GFP), pMDL, pRSV-REV; pCMVΔR8.9, FG12/FUhLucW	EGFP, luciferase	40,41
FIV RD114 env	Gene transduction	pSIN-18-PGK-GFP, pCMV R8.74; pCMVΔR8.2, TRIP GFP/HPV-402	GFP, β-galactosidase	42,43
RRV E2E1A	Cellular tropism	pRRLsin-CMV-GFP/pcDNA-HIV-CS-CGW, pMDLg, pRSV/Rev	EGFP	44,45
hRSV SH/G/F	Mechanism of virus entry, antiviral mechanism, neutralization antibody assay, screening antivirus inhibitor, and combination of envelope proteins	pCMV-ΔR8.74, pWPI_F-Luc_BLR/pWPI_Venus-GFP_BLR	Luciferase, GFP	46
RV GP	Neutralization activity of antibody, gene transduction, and vaccine evaluation in vivo and in vitro	pRRLsincppt-CMV-LacZ WPRE, pMD2-LgpRRE, pRSV-Rev; pHR9SIN-cPPT-SGW/pCSFLW, pCMV-Δ8.91 pSG3ΔEnv.sCMV.fluc	Luciferase, GFP, β-galactosidase	10,11,47
Influenza virus HA/NA/M2	Mechanism of virus entry, molecular screening of inhibitor, and antibody neutralization activity	pNL4-3.Luc.R-E-; pCMVΔR8.2, pHxLacZWP/pHR′-CMVLuc	EGFP, β-galactosidase, luciferase	48-50

Abbreviation: HIV indicates human immunodeficiency virus; Env, envelope; CHIKV, chikungunya virus; EBOV, Ebola virus; GP, glycoprotein; EGFP, enhanced green fluorescence protein; MARV, Marburg virus; LASV, Lassa fever virus; GPC, glycoprotein precursor; MERS-CoV, Middle East respiratory syndrome-coronavirus; DPP4, dipeptidyl peptidase-4; SARS-CoV, severe acute respiratory syndrome-coronavirus; ACE2, angiotensin converting enzyme 2; NiV, nipah virus; FIV, feline immunodeficiency virus; RRV, Ross River virus; hRSV, human respiratory syncytial virus; RV, rabies virus; G, attachment protein; F, fusion protein; SH, small hydrophobic protein; NA, neuraminidase; HA, hemagglutinin.

2.2 | The vesicular stomatitis virus packaging system

The VSV packaging system is a versatile tool for making pseudotyped viruses; this system is advantageous in that it has no stringent selectivity for the envelope proteins, and the resulting virus may be manipulated in a BSL-2 laboratory. Early studies jointly employed VSV and a second virus to coinfect cells, resulting in the pseudotyped virus carrying the core of VSV with envelope proteins derived from the other virus.[60] Stillman et al were the first to clone the VSV genome into a plasmid to make stable VSV,[61] which was subsequently used to generate pseudoviruses carrying heterogeneous glycoprotein.[62,63] Various reporter genes were successively inserted into this plasmid to facilitate its easy detection.[64,65] Some examples of VSV-based pseudovirus system are listed in Table 2.

Notably, when the VSV packaging system is used to make a pseudovirus, there may be residual VSV virus mixed with the pseudovirus, thereby complicating the neutralization assay in which it is used or producing false-positive results. Preferably, the amount of VSV should be minimized; however, if excess VSV is believed to be interfering with a pseudovirus-based assay, treatment of the pseudovirus preparation with a VSV neutralizing antibody could be considered before its use in future assays.

2.3 | The murine leukemia virus packaging system

The MLV packaging system, also called the retroviral system, is commonly used to make pseudoviruses. Table 3 lists the pseudoviruses packaged by the MLV system that have been reported in the literature. Early work by Witte and colleagues showed that when they used VSV to infect the cells in which MLV is packaged, they were able to harvest pseudovirus for use in neutralization antibody assays.[98] Since then, the genome of MLV had been split into 2 parts: one encoding *gag-pol* and the other containing the reporter gene. The 2 gene sets were further cloned into plasmids to generate highly efficient MLV packaging systems.[99]

To improve the stability of this system, investigators established several cell lines that were confirmed to be stable in transfection and expression. Murine leukemia virus may actually be a better choice than HIV as a packaging system in some cases. For example, in studying LASV-mediated entry into cells, Cosset et al compared the MLV and HIV systems and found that the former is 5-fold more efficient than the latter.[100]

2.4 | Other packaging system

The aforementioned pseudovirus packaging systems have not always been successful in generating certain types of pseudoviruses. In those cases, other alternatives such as reverse genetics have been reported. For example, Hu et al prepared a pseudotyped dengue virus (DENV) types 1 to 4 by using the HIV system, but its titer was insufficiently high.[101] However, by using reverse genetics, reporter genes were inserted into the viral genome to construct a plasmid-dependent, self-assembled, pseudotyped flavivirus. Successful examples using reverse genetics include DENV, West Nile virus (WNV), and Japanese encephalitis virus, all of which have been subsequently used to develop neutralization antibody assays. Specifically, Pierson et al cotransfected cells with 3 plasmids containing a subreplicon of a reporting gene, capsid, and prME to generate pseudotyped WNV,[102] while Sobrinho et al developed pseudotyped DENV-1 and WNV-DENV-1-CprME based on the method used by Pierson and colleagues.[17] Moreover, Qing et al constructed pseudotyped DENV1, Semliki Forest virus-DENV-1-CprME, and Venezuelan equine encephalitis virus-PAC-2A-DENV-1-CprME by 2-step electroporation; these pseudoviruses proved to be useful in screening antiviral compounds.[103]

TABLE 2 Pseudoviruses packaged by VSV system

Virus Protein	Research Area and Application	Reporting Gene	Reference
EBOV GP	Cellular tropism, function of glycoprotein, inhibitor of viral entry	GFP	66,67
HCV E1/E2	Function of envelope protein in viral attachment and entry	GFP	68
Hantavirus G1/G2	Neutralization antibody assay, mechanism underlying virus entry (effect of PH, receptor), cellular tropism, vaccine	GFP/RFP, luciferase	69-72
SFTSV G1/G2	Infection diagnosis, drug screening, mechanism of virus entry	GFP, luciferase	73
Arenavirus GPC	Receptor recognition, mechanism of virus entry	GFP Luciferase	73-75
NiV G/F	Receptor recognition, neutralization antibody assay	GFP/RFP Luciferase SEAP	65,76-78
JEV PrM/E	Factors affecting viral infection and reproduction	Luciferase	79
CHIKV E2/E1	Neutralization antibody assay, mechanism of virus entry	GFP, luciferase	80
Measles virus H/F	Neutralization antibody assay	Luciferase	81
Lyssavirus GP	Neutralization antibody assay	GFP, luciferase	64
Avian influenza viruses HA/NA	Neutralization antibody assay, antiviral screening	GFP, luciferase	82,83
CCHFV GP	Mechanism of virus entry	GFP, luciferase	84

Abbreviation: VSV indicates vesicular stomatitis virus; EBOV, Ebola virus; GFP, green fluorescence protein; HCV, hepatitis C virus; RFP, red fluorescence protein; SFTSV, severe fever with thrombocytopenia syndrome virus; GPC, glycoprotein precursor; NiV, nipah virus; JEV, Japanese encephalitis virus; CHIKV, chikungunya virus; CCHFV, Crimean-Congo hemorrhagic fever virus; H, hemagglutinin; F, fusion protein; prM, precursor membrane; E, envelope; NA, neuraminidase; HA, hemagglutinin; SEAP, secreted alkaline phosphatase.

TABLE 3 Pseudoviruses packaged by MLV system

Virus Protein	Research Area and Application	Packaging System	Reporting Gene	Reference
EBOV GP	Neutralization antibody assay, analysis on the function of glycoprotein, cell receptor recognition	pHIT60 (MLV gag-pol); pHIT111	β-Galactosidase	85
La Crosse virus (G1/G2)	Cellular tropism, neutralization antibody assay	pcGP (MLV gag-pol) pcnbG (MLV LTR)	β-Galactosidase	86
Hantavirus (G1/G2)	Cellular tropism, neutralization antibody assay	pcGP (MLV gag-pol) pcnbG (MLV LTR)	β-Galactosidase	86
HIV-1 Env	Neutralization antibody assay, screening inhibitor of viral entry	TELCeB6 cells; pTMgp140; FLY-HIV-87-GFP; GP2-293luc cells	β-Galactosidase, GFP, luciferase	14,87,88
HIV-2 Env	Interaction of virus and cell	Anjou65-LacZ cells	β-Galactosidase	87
Visna virus Env	Cellular tropism	pCgp (MuLV gag-pol); pMX-GFP	GFP	89
RRV GP	Cellular tropism, mechanism of virus entry	gpnlslacZ cells; pJ6Vpuro	β-Galactosidase	90
SIVagm Env	Vector candidate for gene therapy in vivo	pHIT60 (MLV gag-pol); pMFG-nlsLacZ; MgEGFP-LNGFR	β-Galactosidase, EGFP	91
HCV GP (E1/E2)	Mechanism of virus entry	MLV CMV-gag-pol; MLV-GFP	GFP	6
SARS-CoVs Spike	Mechanism of virus entry, neutralization antibody assay, receptor ACE2 validation	TELCeB6 cells; GP2-293 cell line; pQCXIX (GFP)	β-Galactosidase, GFP	56,92
MERS-CoVs spike	Cellular tropism	pCMV-MLV gag-pol; pTG-Luc	Luciferase	93
VSV GP	Transgenic study, RNAi gene expression interference	GP2-293 cell line; pLNHX	EGFPluciferase	94,95
Arenavirus GP	Validate the receptor of new world arenavirus TfR1	MLV gag/pol; pQCXIX (EGFP)	EGFP	7
Influenza virus HA	Screening specific antibody	MLV gag/pol; pkat2βgal; MLV luc constructs	β-Galactosidase, luciferase	96,97

Abbreviation: MLV indicates murine leukemia virus; EBOV, Ebola virus; GP, glycoprotein; HIV, human immunodeficiency virus; GFP, green fluorescence protein; RRV, Ross River virus; SIV, simian immunodeficiency virus; EGFP, enhanced green fluorescence protein; SARS-CoV, severe acute respiratory syndrome-coronavirus; MERS-CoV, Middle East respiratory syndrome-coronavirus; VSV, vesicular stomatitis virus; HA, hemagglutinin; G, glycoprotein.

3 | FACTORS CONTRIBUTING TO THE PSEUDOVIRUS YIELD

A high yield is needed for practical applications of the pseudoviruses. There are several factors that can critically influence their yield/titer.

3.1 | Effect of envelope protein localization on pseudovirus formation and titer

In general, the subcellular localization for viral packaging and maturation may largely dictate the pseudoviral titer. Specially, if the envelope proteins are localized on the surface of cell membrane, it is relatively easy to generate high-yield pseudoviruses by using either HIV or VSV packaging systems as reported for filovirus and rhabdovirus.[55,104] In contrast, if the envelope protein is localized in the membrane of organelles (eg, endoplasmic reticulum or Golgi complex), as in bunyavirus and yellow fever virus, it is more difficult to obtain a pseudovirus; furthermore, when the envelope protein is localized in a cholesterol-rich section of the membrane, it is nearly impossible to form a pseudovirus.[105]

Previous studies suggest that the localization of envelope proteins is closely related to their structures, particularly that of the transmembrane portion of the envelope protein. Therefore, efforts have been made to modify the envelope protein to improve the pseudovirus yield. For example, by truncating the cytoplasmic region of envelope protein, it can be possible to reduce the intracellular accumulation of envelope protein, thereby facilitating the assembly of envelope proteins with the vector core proteins.[40,57,106,107] Additionally, replacing the cytoplasmic tail of an envelope protein with the envelope tail of HIV-1, VSV, or MLV can also improve the pseudovirus yield. Furthermore, retargeting the envelope protein intracellularly is another method of potentially improving the pseudoviral titer.[54,56] However, it should be noted that truncating the cytoplasmic region of an envelope protein may have unwanted consequences, such as altering the native structure of the surface domain, which could impact the functional and antigenic phenotype of the pseudovirus.

3.2 | The effects of envelope protein expression

The expression level of envelope protein could significantly affect the efficiency of pseudovirus assembly. Apparently, increased expression of the envelope protein is able to substantially improve the pseudovirus titer. Nie et al reported that the expression level of WT S protein in 293 T cell was very low in pseudotyped SARS-CoV; however, codon optimization of the S gene drastically improved the yield of pseudotyped SARS-CoV.[31,36] These data indicate that the expression of envelope protein by a highly efficient expression vector could increase the pseudoviral titer.

3.3 | The effects of the packaging system and corresponding cell lines

The choice of packaging system can significantly affect the viral yield and may need to be optimized on a case-by-case basis. Temperton et al compared the MLV and HIV systems and found that the pseudovirus prepared by MLV was better than the one prepared by HIV when these packaging systems were used in 293 T cells; they

successfully used the MLV packaging system to generate a pseudotyped influenza virus.[108] Cosset et al reached the same conclusion for pseudotyped LASV preparations.[100] Moreover, in our experiences, pseudotyped LASV packaged by the VSV system had a higher titer than that produced by using the HIV system. Furthermore, the VSV system was able to incorporate hantavirus glycoprotein that had failed to be packaged by HIV. Nonetheless, our group developed a modification of the HIV packaging system that could improve the pseudoviral yield by 100-fold; specifically, the backbone plasmid pSG3ΔEnv.CMVFluc that was developed in our lab was superior to pNL4-3.Luc.R-E-.[10]

3.4 | The effect of packaging conditions

The packaging conditions can also drastically influence the pseudovirus yield. In the HIV packaging system, the pseudovirus titer could be increased by optimizing the packaging cells, transfection reagents, and the ratio and absolute amounts of plasmid DNAs used for transfection[52]; in VSV packaging system, the pseudovirus titer could be improved by optimizing the harvesting time.[63] For pseudotyped human respiratory syncytial virus made by using the HIV packaging system, Haid et al cotransfected 5 plasmids into 293 T cells by using the transfection reagent PEI; they then optimized the transfection conditions by adding sodium butyrate 21 hours posttransfection to induce gene expression.[46,109,110] Some pseudoviruses need special enzymes/reagents during production to optimize titer. Scott et al optimized the production of equine influenza-HA-pseudotyped viruses via the addition of exogenous neuraminidase from *Clostridium perfringens* to allow the release of nascent pseudovirus particles.[111] These observations indicate that the packaging conditions should be optimized to improve the pseudovirus yield on a case-by-case basis.

4 | APPLICATION OF PSEUDOVIRUS

4.1 | Mechanistic study of viral infection

Pseudoviruses have been widely used for conducting in vitro studies on the interaction between the virus and the host cells.[112] They have also proven to be very useful for in vivo studies, particularly studies on the mechanism of viral infection as well as on the biodistribution.[113] Our lab used a pseudotyped RV carrying reporter genes to establish an in vivo imaging model in mice. This mouse model was used to study viral tissue tropism and its dynamic change over time.[10] We also established a pseudotyped EBOV mouse model; the EBOV pseudoviruses were mainly detected in the thymus and spleen following viral infection, revealing that the pseudotyped EBOV and WT EBOV have the same tissue tropism.[52] Other groups have also used pseudotyped HSV-1 and MARV in small animal models to investigate viral infections.[114]

4.2 | Application of pseudoviral systems to neutralization antibody and antibody-dependent cell-mediated cytotoxicity assay

Antibody neutralization assay based on pseudoviruses has been widely used, particularly for the analyses of some virulent viruses that would otherwise need to be handled in BSL-3 or BSL-4 laboratories. Compared with the traditional assays, the reported pseudovirus-based assays have demonstrated a good correlation with the WT virus-based assay; the pseudovirus-based assays are usually high-throughput procedures with fewer amounts of serum samples needed.[1,104,115,116] Wilkinson et al compared 22 platform technologies for assaying antibody against EBOV with neutralization assays by using the WT virus and found that the 5 best assays included methods based on WT and VSV pseudotype neutralization and ELISA, but the lentiviral and other platforms were problematic.[117] Notably, Duehr J et al indicated that some anti-EBOV glycoprotein monoclonal antibodies could neutralize and protect against pseudotyped VSV-EBOV but were nonneutralizing against WT EBOV.[118]

Recently, pseudoviral systems have also been explored for the analysis of antibody-dependent cell-mediated cytotoxicity (ADCC) activity, which might be a better choice than the use of target cells transfected with a DNA plasmid. The success in employing pseudovirus in ADCC assays may be because the surface proteins of pseudovirus largely retain their native conformation and, following infection, a portion of these proteins remains on the surface of the target cells. In the pseudovirus-based ADCC assay, target cells are first infected with the pseudovirus, followed by the addition of monoclonal antibody and modified Jurkat cells (effector cells). If the monoclonal antibody has ADCC activity, the Fab portion will bind to the target cell, while its Fc will bind and activate the modified Jurkat cells, which have been engineered to stably express the Fc receptor, V158 (high affinity) variant in addition to luciferase under the control of an NFAT response element.[52]

4.3 | Drug screening

Various studies have been carried out by using pseudoviruses to screen small-molecule compounds including those against LASV, EBOV, MARV, SARS-CoV, NiV, and influenza H5N1.[119,120] Wang et al used pseudotyped MARV, influenza H5N1, and LASV to screen 1200 small-molecule compounds that have been approved by the US Food and Drug Administration to treat other medical conditions. It was found that some of these compounds have a broad range of antiviral activities against MARV, influenza H5N1, and LASV.[8] By using pseudovirus, Elshabrawy et al were able to select a small molecule compound that had a broad spectrum of antiviral activity against SARS-CoV, MARV, Hendra virus, and NiV. Further investigation by these authors revealed that 1 compound prevents viral entry into the cells by blocking cathepsin L-mediated enzyme digestion.[121] Besides small molecules, pseudoviruses have also been used to screen herbal medicines for their potential antiviral activities.[122,123]

5 | LIMITATIONS AND CONSIDERATIONS OF PSEUDOVIRAL SYSTEMS

While some of the technical challenges associated with pseudoviral systems have been described above, there are several remaining issues that are also worth mentioning. By definition, pseudoviruses only contain the membrane/envelope proteins of the WT virus. While the

membrane/envelope protein can largely mediate viral entry in a fashion like that of the WT virus, these viruses can only replicate for 1 round or may not always induce pathogenesis as their WT counterparts do. Therefore, results from assays using pseudotyped viruses should be compared and validated against the WT virus-based assay, which remains the golden standard.[69,76] Additionally, the virus shape may influence its suitability for constructing a corresponding pseudotyping virus. For example, MLV, HIV, and SIV are spherical viruses, whereas VSV is bullet-shaped. Therefore, the pattern of glycoprotein distribution/conformation/density on a pseudotyped virus may not reflect the "natural" state of envelope proteins on the WT viruses (eg, as in filoviruses, which are thread-like). It is also a good practice to compare various packaging systems. Indeed, investigators sometimes used 2 packaging systems simultaneously to prepare pseudoviruses or compared their resulting pseudovirus with one packaged by the VSV glycoprotein.[15,19,93,124,125] Notably, Bilska et al showed that some Env-pseudotyped virus preparations give rise to low levels of replication-competent virus, which highlights the need to perform rigorous testing for replication-competent virus before initiating work on any pseudovirus preparations at a lower BSL.[126] While the successful generation of pseudoviral particles is mainly limited to enveloped viruses, efforts should be made to determine whether nonenveloped viruses could also be effectively pseudotyped.

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CONFLICT OF INTEREST

The authors have no competing interest.

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