Molecules and Cells

Stability of Retroviral Vectors Against Ultracentrifugation Is Determined by the Viral Internal Core and Envelope Proteins Used for Pseudotyping

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Retroviral and lentiviral vectors are mostly pseudotyped and often purified and concentrated via ultracentrifugation. In this study, we quantified and compared the stabilities of retroviral [murine leukemia virus (MLV)-based] and lentiviral [human immunodeficiency virus (HIV)-1-based] vectors pseudotyped with relatively mechanically stable envelope proteins, vesicular stomatitis virus glycoproteins (VSVGs), and the influenza virus WSN strain envelope proteins against ultracentrifugation. Lentiviral genomic and functional particles were more stable than the corresponding retroviral particles against ultracentrifugation when pseudotyped with VSVGs. However, both retroviral and lentiviral particles were unstable when pseudotyped with the influenza virus WSN strain envelope proteins. Therefore, the stabilities of pseudotyped retroviral and lentiviral vectors against ultracentrifugation process are a function of not only the type of envelope proteins, but also the type of viral internal core (MLV or HIV-1 core). In addition, the fraction of functional viral particles among genomic viral particles greatly varied at times during packaging, depending on the type of envelope proteins used for pseudotyping and the viral internal core.

Keywords: envelope proteins, genomic particles, pseudotyping, retroviral vectors, stability, transducing particles, ultracentrifugation

INTRODUCTION

Viruses are fatal pathogens to cause various infectious human diseases, including acquired immunodeficiency syndrome (AIDS), respiratory syndrome, hemorrhagic fever, and cancer. However, viruses can be also used to improve human health. For example, viruses are physically, chemically, and/or genetically attenuated to develop vaccines against viral infections (Lim et al., 2006; Sanders et al., 2016). Viruses have been recently engineered to kill specific cancer cells as oncolytic agents (Peng et al., 2002). Most of all, viruses have been often converted into gene delivery vectors by modifying their genomes to remove genes encoding viral proteins and insert therapeutic genes instead (Hossain et al., 2014; Naldini et al., 1996; Schaffer et al., 2008).

Among different types of viral vectors, retroviral vectors (including lentiviral vectors) can be easily modified to target specific cell types by the process of pseudotyping. In this process, the envelope proteins (surface proteins) of retroviruses are replaced with the envelope proteins of other viruses to confer the target cell entry capability of the donor viruses. For example, retroviral vectors pseudotyped with Lyssavirus surface proteins were used to deliver genes to neurons (Cronin et al., 2005) and those pseudotyped with hepatitis B virus surface proteins could be used to transduce primary human hepatocytes (Chai et al., 2007).

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Retroviral and lentiviral vectors are mostly pseudotyped and often purified and concentrated via ultracentrifugation before being used to transduce cells (Galipeau et al., 1999; Lim et al., 2010). Especially, *in vivo* cell transduction studies and gene therapy clinical trials always require purification and concentration steps for retroviral vectors to remove impurities, including cell debris and serum, from the supernatant of virus-packaging cells and to minimize the volume of vector required per dose. However, ultracentrifugation may decrease the yield and thereby the titer of retroviral particles by exerting shear stress on these particles. Retroviral vectors pseudotyped with different envelope proteins can show different levels of stability against ultracentrifugation process.

In this study, we quantified the stabilities of retroviral and lentiviral vectors pseudotyped with influenza virus envelope proteins, hemagglutinin (HA) and neuraminidase (NA), against ultracentrifugation. Retroviral and lentiviral vectors with the influenza virus surface proteins can be used in studies to advance influenza virus vaccines (Tao et al., 2013), develop novel detection systems for newly emerging influenza viruses (Lim et al., 2015), and deliver genes to cells of airway tissues. The stabilities of retroviral and lentiviral vectors pseudotyped with HA and NA were compared with those of vectors displaying VSVGs, which are known to be relatively stable against ultracentrifugation. In addition, the effect of the viral internal core on vector stability was also investigated by comparing the stabilities of retroviral and lentiviral vectors pseudotyped with the same surface proteins.

MATERIALS AND METHODS

Cell culture

Human embryonic kidney (HEK) 293T cells were cultured in Iscove's modified Dulbecco's medium (Life Technologies) supplemented with 10% (v/v) fetal bovine serum at 37℃ and 5% CO_2.

Vector packaging

Retroviral and lentiviral vectors were pseudotyped with the influenza virus WSN strain (A/WSN/33 H1N1) HA (WSNHA) and NA (WSNNA) proteins or VSVGs. To package retroviral vectors plasmids encoding the murine leukemia virus (MLV) genome (pCLPIT GFP, 10 μg), Gag-Pol polyprotein (pCMV gag-pol, 6 μg), and envelope proteins (pcDNA IVS VSVG, 4 μg or pcDNA IVS WSNHA and pcDNA IVS WSNNA, 4 μg each) were introduced into each 10-cm dish containing HEK 293T cells via the calcium phosphate-based transfection method. To package lentiviral vectors, plasmids encoding the human immunodeficiency virus (HIV)-1 genome (pFUGW, 10 μg), Gag-Pol polyprotein (pMDLg/pRRE, 5 μg), Rev protein (pRSV-Rev, 1.5 μg), and envelope proteins (pcDNA IVS VSVG, 3.5 μg or pcDNA IVS WSNHA and pcDNA IVS WSNNA, 3.5 μg each), were also introduced into each 10-cm dish of HEK 293T cells by the same method as for retroviral vectors. The cell supernatant containing packaged viral particles was harvested twice (1.5 and 2.5 days post-transfection).

Ultracentrifugation

The harvested cell supernatant containing viral particles was first filtered through a 0.45-μm syringe filter and then purified and concentrated in a 20% (w/v) sucrose cushion by ultracentrifugation (Optima™ Ultracentrifuge LE-80K, Beckman Coulter) at 4℃. The ultracentrifugation was performed with an SW28 rotor at 24,000 rpm (76,221 g, on the average) for 2 h and an SW41 rotor at 25,000 rpm (77,175 g, on the average) for 1.5 h. The obtained virus particle pellet was resuspended with cooled phosphate-buffered saline (PBS).

Titration of retroviral genomic particles

Titration of viral genomic particles (considered as physical virus particles) was done by real-time qPCR, following the same methods as in our recent study (Jang et al., 2016), using a CFX Connect™ real-time PCR detection system (Bio-Rad) and SYBR® Green I (Enzynomics). Each vector sample was analyzed with the primers 5′-ATTGACTGAGTCGCCCGG GTAC-3′ and 5′-TCCCAAGGAACAGCGAGACCAC-3′ for retroviral vectors, and 5′-AGCTTGCCTTGAGTGCTTCAAGTA GTG-3′ and 5′-TGACTAAAAGGGTCTGAGGGATCTCTAG-3′ for lentiviral vectors.

Titration of retroviral transducing particles

To quantify the titer of transducing (functional) virus particles, the gene encoding enhanced green fluorescent protein (eGFP) was introduced into the viral genomes. Titration of transducing viral particles in the cell supernatant and the concentrated samples was performed by transducing HEK 293T cells at a multiplicity of transduction less than 1 and then counting eGFP-positive cells. Cellular expression of eGFP was measured by flow cytometry on a FacsCanto™ II flow cytometer (BD Biosciences) three days post-transduction for retroviral vectors and four days post-transduction for lentiviral vectors. Retroviral and lentiviral vectors pseudotyped with the influenza virus surface proteins especially needed to be activated for cell transduction by protease treatment, since this is also a requirement for live influenza viruses (Lim et al., 2015). This activation step was performed by incubating the viral samples with TPCK-treated trypsin at 0.1 μg/μl and 37℃ for 1.5 h during transduction. All the experiments with viral vectors were performed in the biosafety level 2 (BSL-2) laboratory. Analysis of differences in the shape and density of viral particles by electron microscopy and sucrose density gradient separation may be helpful to distinguish physical and functional viral particles. However, a considerable level of natural heterogeneity of particle morphology for enveloped viruses (Akpinar and Yin, 2015) can limit the use of these methods for the given purpose. Therefore, we instead applied more direct and more commonly used methods to quantify physical and functional viral particles as above.

RESULTS AND DISCUSSION

VSVGs-pseudotyped retroviral genomic particles assembled at later time points post-transfection contain a higher proportion of transducing particles

Retroviral (MLV-based) and lentiviral (HIV-1-based) vectors were harvested once at 1.5 days and again at 2.5 days post-

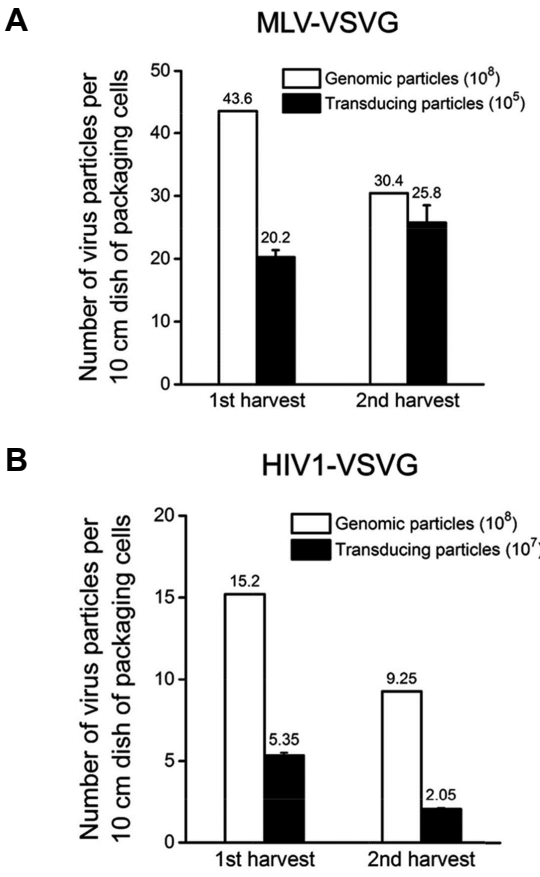

Fig. 1. The numbers of genomic and transducing vector particles produced per 10-cm dish of packaging cells. The first and second harvests of viral particles were made from the supernatant of packaging cells 1.5 days and 2.5 days post-transfection, respectively. Standard error bars for each linear regression of diluted samples are shown for the genomic titer data (Jang et al., 2016). Standard error bars for three independent flow cytometry measurements are shown for the transduction titer data. (A) Retroviral vectors pseudotyped with VSVGs. (B) Lentiviral vectors pseudotyped with VSVGs.

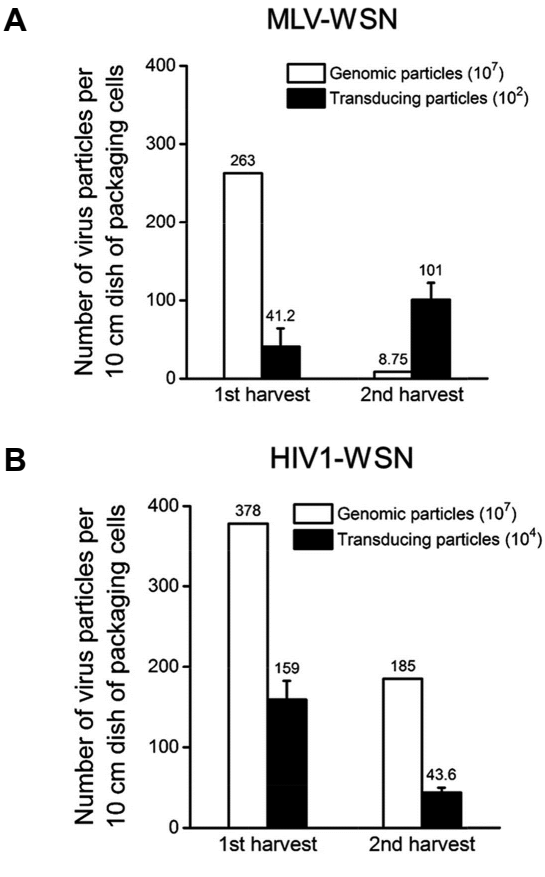

Fig. 2. The numbers of genomic and transducing vector particles produced per 10-cm dish of packaging cells. The first and second harvests of viral particles were made from the supernatant of packaging cells 1.5 and 2.5 days post-transfection, respectively. Standard error bars for each linear regression of diluted samples are shown for the genomic titer data (Jang et al., 2016). Standard error bars for three independent flow cytometry measurements are shown for the transduction titer data. (A) Retroviral vectors pseudotyped with WSN HA and NA. (B) Lentiviral vectors pseudotyped with WSN HA and NA.

transfection. Retroviral genomic particles pseudotyped with VSVGs (gMLV-VSVG) were produced at a higher titer during the first harvest than during the second harvest (Fig. 1A). However, more retroviral transducing particles (functional ones, tMLV-VSVG) were produced during the second harvest than during the first harvest. The number of gMLV-VSVG particles required for the transduction of a single cell (genomic to transducing particle ratio) can be calculated to be 2,200 for the first harvest, but 1,200 for the second harvest. This result indicates that gMLV-VSVG particles assembled at later time points post-transfection are more infectious than the ones assembled earlier.

In contrast, both lentiviral genomic and transducing particles pseudotyped with VSVGs (gHIV1-VSVG and tHIV1-VSVG, respectively) were produced at a higher titer during the first harvest than during the second harvest (Fig. 1B). The genomic to transducing particle ratio for the first harvest (28:1) was lower than that for the second harvest (45:1), indicating that gHIV1-VSVG particles assembled at earlier time points post-transfection are more infectious than the ones assembled later. Not all genomic or physical vector particles have the ability to transduce cells because the composition of proteins and genomic molecules in virus particles may be incomplete and there are multiple barriers during the transduction process, including diffusion to the cell surface, binding to cellular receptors, internalization into the cytoplasm, uncoating, reverse transcription, entry into the nucleus, and gene expression (Gruter et al., 2005).

Most retroviral physical particles that were assembled with WSN HA and NA at early time points post-transfection were not functional

Genomic MLV vector particles pseudotyped with WSN HA

and NA (gMLV-WSN) were mostly produced during the first harvest, compared with the second harvest (almost 30 fold more than during the second harvest, Fig. 2A). However, more transducing MLV vector particles with the WSN HA and NA (tMLV-WSN) were produced during the second harvest. Hence, the genomic to transducing particle ratio for the first harvest was much higher (640,000:1) than that for the second harvest (8,700:1). This finding indicates that most gMLV-WSN particles assembled at early times are not functional for the transduction of cells.

On the other hand, both genomic and transducing HIV-1 vector particles pseudotyped with WSN HA and NA (gHIV1-WSN and tHIV1-WSN, respectively) were produced at a higher titer during the first harvest than during the second harvest (Fig. 2B). The genomic to transducing particle ratio for the first harvest (2,400:1) was lower than that for the second harvest (4,200:1), showing that gHIV1-WSN particles assembled earlier are more functional than the ones assembled later.

At the first harvest, a significantly smaller proportion of functional particles among genomic particles was observed for the MLV vector pseudotyped with WSN surface proteins than for the HIV-1 vector pseudotyped similarly (1:640,000 *vs.* 1:2,400 functional:genomic particles, respectively). This result suggests that interactions between the surface proteins and internal core of vector particles containing multiple structural proteins and genomic molecules are important for vector particles to be functional. Compared with the HIV-1 core, the MLV core is not a good match for the WSN surface proteins to generate functional vector particles at early assembly times.

MLV-VSVG vectors are not highly resistant to shear force from ultracentrifugation

Retroviral vectors are often used after concentration via ultracentrifugation. This process exerts shear force on viral particles, thus limiting the use of mechanically unstable envelope proteins for pseudotyping. VSVG has been known to be relatively resistant to such shear force, allowing the preparation of highly concentrated retroviral vector samples via ultracentrifugation (Naldini et al., 1996). In contrast to this broadly accepted knowledge, we found that only 24.2% and 13.4% of MLV-VSVG genomic particles (gMLV-VSVG) recovered from the first and second harvests survived (75.8% and 86.6% losses, respectively) an ultracentrifugation process (24,000 rpm for 2 h, Fig. 3A). In addition, only 23.7% and 24.6% of MLV-VSVG transducing particles (tMLV-VSVG) recovered from the first and second harvests survived ultracentrifugation under the same condition (76.3% and 75.4% losses, respectively) (Fig. 3B).

To further assess the stability of MLV-VSVG vectors we performed additional ultracentrifugation for the previously ultracentrifuged samples (2 h spin at 24,000 rpm followed by 1.5 h spin at 25,000 rpm). After this second round of ultracentrifugation, 58.9% of the remaining gMLV-VSVG particles and 20.6% of the remaining tMLV-VSVG particles were further lost (Fig. 3). Therefore, a significant fraction of viral particles in the samples can be lost during ultracentrifugation even after being pseudotyped with relatively mechanically stable envelope proteins.

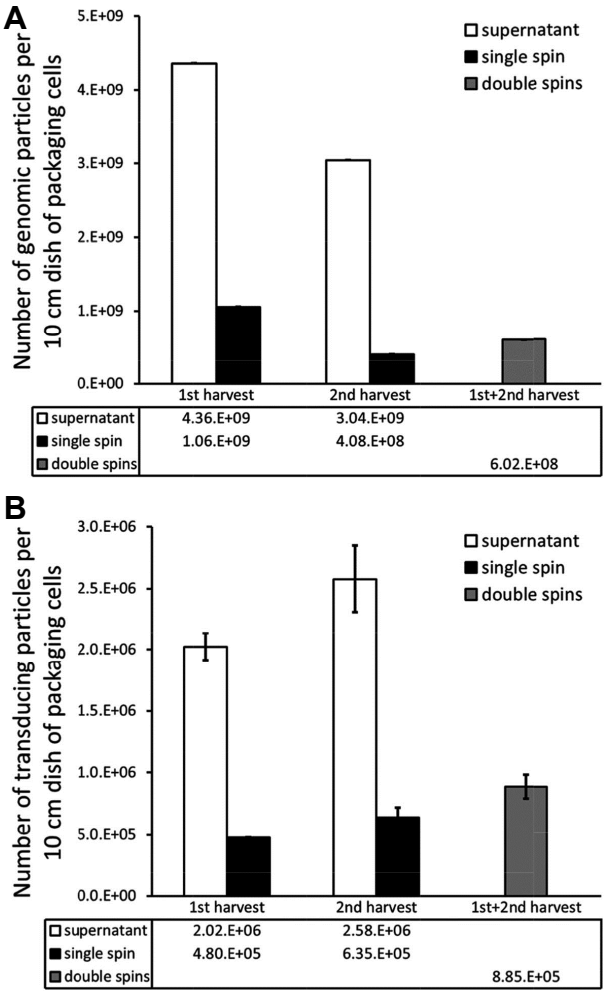

Fig. 3. The numbers of remaining MLV-VSVG vector particles after harvesting and ultracentrifugation. Standard error bars for each linear regression of diluted samples are shown for the genomic titer data. Standard error bars for three independent flow cytometry measurements are shown for the transduction titer data. (A) For genomic particles. (B) For transducing particles.

HIV1-VSVG vectors are more stable than MLV-VSVG vectors against ultracentrifugation

Unexpectedly, ultracentrifugation destroyed significant amounts of retroviral genomic and functional particles that were pseudotyped with VSVGs (Fig. 3). In contrast, 70.0% and 70.8% of gHIV1-VSVG particles in the first and second harvests, respectively, survived ultracentrifugation (2 h at 24,000 rpm, Fig. 4A). The surviving fractions of these genomic particles were much higher than those of gMLV-VSVG particles, with an increase in survival of up to 5.3 fold (Figs. 3 and 4). In addition, 54.4% and 53.9% of tHIV1-VSVG particles in the first and second harvests, respectively, survived ultracentrifugation under the same condition (Fig. 4B). These surviving fractions were also higher than those for tMLV-VSVG particles by up to 2.3 fold (Figs. 3 and 4). The higher stabilities of genomic and transducing lentiviral

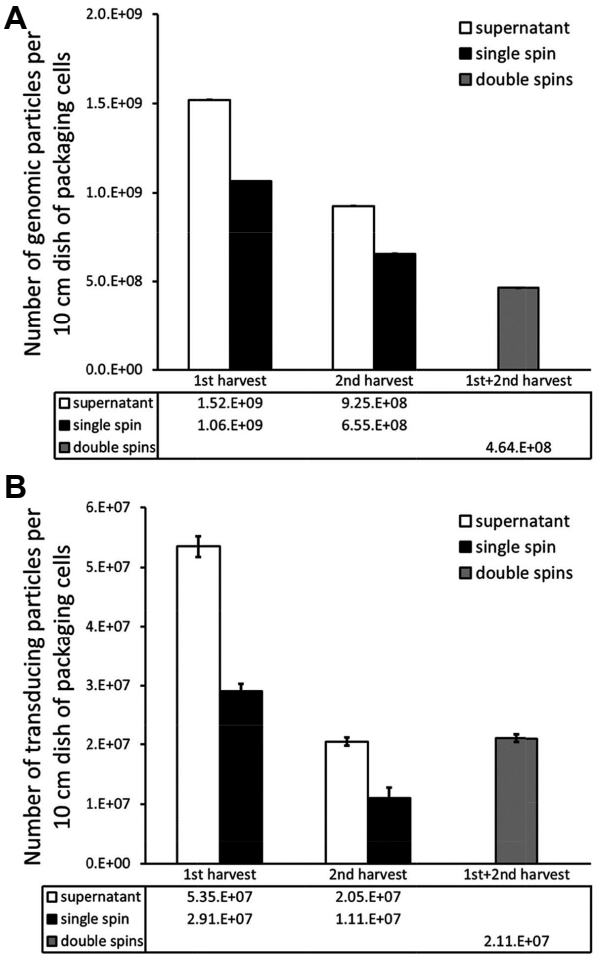

Fig. 4. The numbers of remaining HIV1-VSVG vector particles after harvesting and ultracentrifugation. Standard error bars for each linear regression of diluted samples are shown for the genomic titer data. Standard error bars for three independent flow cytometry measurements are shown for the transduction titer data. (A) For genomic particles. (B) For transducing particles.

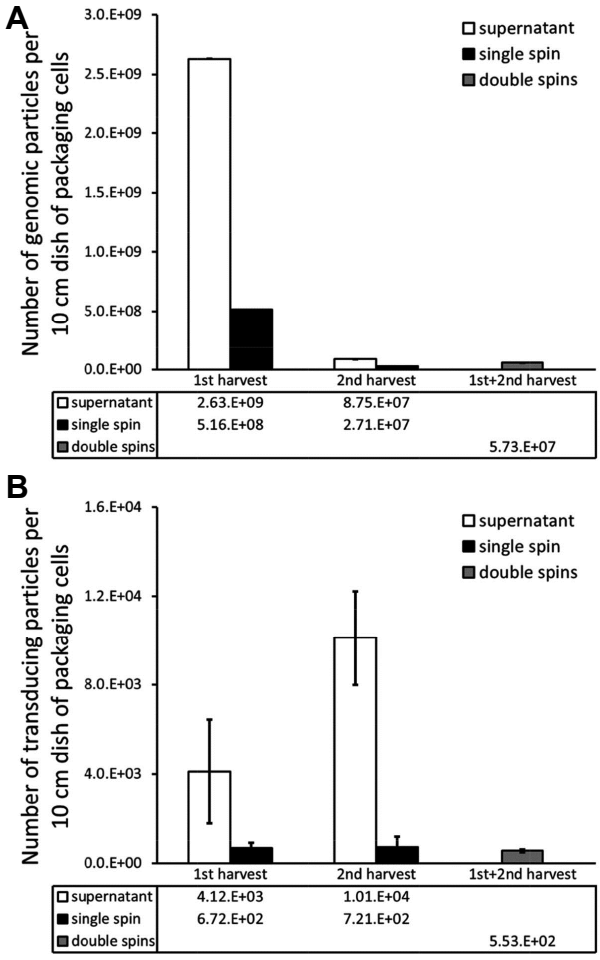

Fig. 5. The numbers of remaining MLV-WSN vector particles after harvesting and ultracentrifugation. Standard error bars for each linear regression of diluted samples are shown for the genomic titer data. Standard error bars for three independent flow cytometry measurements are shown for the transduction titer data. (a) For genomic particles. (b) For transducing particles.

vector particles against ultracentrifugation indicate that the HIV-1 internal core may interact with VSVGs better than the MLV internal core to generate stable vector particles.

However, HIV-1 vectors pseudotyped with VSVGs that had shown a significant level of stability against the first round of ultracentrifugation eventually lost 73.0% of genomic particles and 47.5% of functional particles after the second round of spin (1.5 h at 25,000 rpm, Fig. 4). This quantitative analysis of vector stabilities highlights that viral vector particles cannot robustly resist prolonged or consecutive ultracentrifugation steps, even when the vectors are pseudotyped with relatively mechanically stable envelope proteins.

MLV-WSN vectors were much less stable against ultracentrifugation than MLV-VSVG vectors

Retroviral vectors have been pseudotyped with influenza virus envelope proteins in studies and practical applications (Lim et al., 2015; Lin and Cannon, 2002; Patel et al., 2013). Here, we quantified the stabilities of retroviral vectors pseudotyped with the WSN HA and NA proteins against ultracentrifugation to concentrate the vector particles. Only 19.6% and 31.0% of MLV-WSN genomic particles (gMLV-WSN) in the first and second harvests survived ultracentrifugation (80.4% and 69.0% losses, respectively, after 2 h spin at 24,000 rpm) (Fig. 5A). Furthermore, only 16.3% and 7.1% of MLV-WSN transducing particles (tMLV-WSN) in the first and second harvests survived ultracentrifugation under the same condition (83.7% and 92.9% losses, respectively) (Fig. 5B). From this result it was noted that MLV transducing vector particles became even less stable when pseudotyped with the influenza virus WSN strain envelope proteins than when pseudotyped with VSVGs.

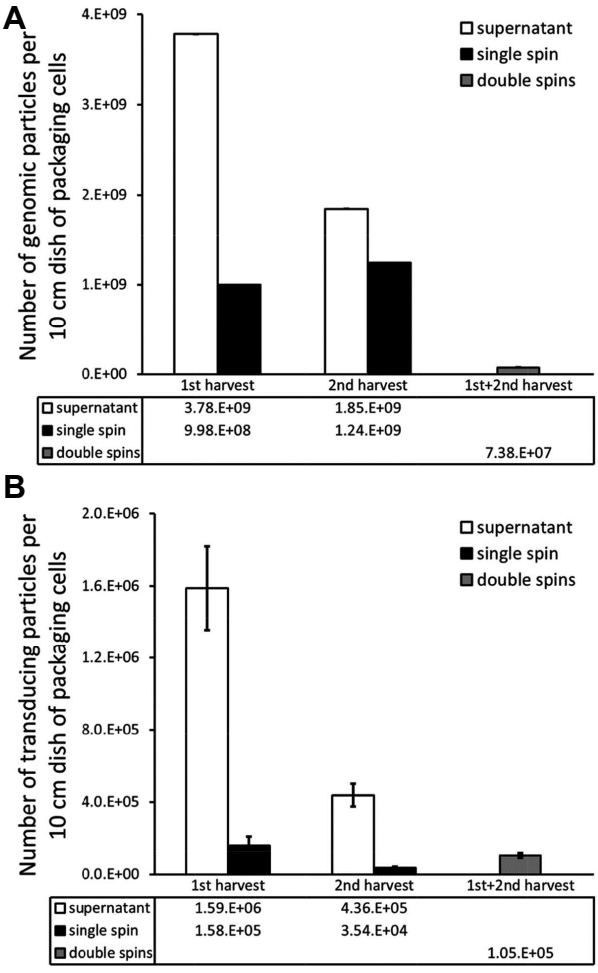

Fig. 6. The numbers of remaining HIV1-WSN vector particles after harvesting and ultracentrifugation. Standard error bars for each linear regression of diluted samples are shown for the genomic titer data. Standard error bars for three independent flow cytometry measurements are shown for the transduction titer data. (A) For genomic particles. (B) For transducing particles.

MLV-WSN vectors also suffered from significant additional damage during the second round of ultracentrifugation (Fig. 5). After this second round, a staggering 89.4% of the remaining gMLV-WSN particles and 60.3% of the remaining tMLV-WSN particles were further lost (Fig. 5). Influenza virus surface proteins can provide opportunities to construct novel vectors and model influenza viruses via pseudotyping for use in various studies and applications. However, this strategy will not be applicable to studies and applications that require virus samples highly concentrated via ultracentrifugation.

HIV1-WSN vectors also showed only modest stability against ultracentrifugation as observed for MLV-WSN vectors

Functional HIV1-VSVG vector particles were more stable against ultracentrifugation than the corresponding MLV-VSVG vector particles (Figs. 3B and 4B). However, this advantage of using the HIV-1 core rather than the MLV core to achieve higher stability of functional viral particles, as observed for pseudotyping with VSVGs, did not apply to pseudotyping with the WSN surface proteins. First, 26.4% and 67.3% of HIV1-WSN genomic particles (gHIV1-WSN) in the first and second harvests survived the first round of ultracentrifugation (73.6% and 32.7% losses, respectively) (Fig. 6A). These stabilities were higher than those observed for gMLV-WSN particles (Figs. 5A and 6A). In contrast, only 9.9% and 8.1% of HIV1-WSN transducing particles (tHIV1-WSN) in the first and second harvest survived ultracentrifugation under the same condition (90.1% and 91.9% losses, respectively) (Fig. 6B). Furthermore, as observed for the MLV-WSN vector particles, the HIV1-WSN particles also sustained considerable damage during the second round of ultracentrifugation. After this second round, a staggering 96.7% of the remaining gHIV1-WSN particles and 45.7% of the remaining tHIV1-WSN particles were further lost (Fig. 6). These results highlight that the influenza virus WSN strain surface proteins are not a promising candidate for use in the pseudotyping of retroviral and lentiviral vectors that need to be concentrated for subsequent applications.

CONCLUSION

Pseudotyping of viruses with foreign envelope proteins is a valuable technique to advance virological and genetic studies and extend biomedical applications based on the use of viral particles. However, an insufficient stability of pseudotyped viral particles against ultracentrifugation may limit their use. The physical and chemical properties of a viral vector, such as lipid composition of shell and outer and internal protein compositions and their arrangement, can affect its stability against stress (Beer et al., 2003; Segura et al., 2006). However, the complete understanding of how stabilities of retroviral vectors are determined is still elusive. In this study, we found that the stability of pseudotyped viruses against ultracentrifugation is a function of not only the mechanical stability of envelope proteins, but also the type of viral internal core. Additionally, the conventionally obtainable degree to which pseudotyped viral particles are concentrated by ultracentrifugation, i.e., up to 150 fold, cannot be reached for particles pseudotyped with certain envelope proteins, as observed for the vectors pseudotyped with the influenza virus WSN strain envelope proteins, owing to the severe damage of viral particles by shear force during this process. Such technical hurdles may be overcome by using more stable envelope proteins from closely related virus strains or genetic library screening. For genetic library screening or directed evolution of viral protein variants to enhance the stabilities of retroviral vectors, all the viral protein components can be genetically varied and selected against various physical and chemical stresses (Vu et al., 2008).

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