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Evaluation of parameters for efficient purification and long-term storage of herpes simplex virus-based vectors

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Replication competent oncolytic herpes simplex virus (HSV) vectors have been used extensively to treat solid tumors with promising results. However, highly defective HSV vectors will be needed for applications that require sustained therapeutic gene expression in the absence of vector-related toxicity or inflammation. These vectors require complementing cell lines for their manufacture, creating significant challenges to achieve high yields of infectious virus particles. We recently described an improved upstream process for the production of a non-cytotoxic HSV vector for gene therapy applications. Here, we sought to optimize the downstream conditions for purification and long-term storage of the same vector, JΔNI5. We compared different methods to remove cellular impurities and concentrate the vector by monitoring both physical and biological titers, resulting in the establishment of optimal conditions for vector production. To optimize the long-term storage parameters for non-cytotoxic HSV vectors, we evaluated vector stability at low temperature and sensitivity to freeze-thaw cycles. We report that suboptimal purification and storage methods resulted in loss of vector viability. Our results describe effective and reproducible protocols for purification and storage of HSV vectors for pre-clinical studies.

INTRODUCTION

Genetically engineered herpes simplex virus (HSV) vectors are emerging as promising oncolytic and gene therapy agents.[1–13] Two genetically engineered oncolytic HSV vectors have already been approved for treatment of metastatic melanoma[14,15] and malignant glioma[16] by the U.S. Food and Drug Administration and the Japan Ministry of Health, Labor, and Welfare, respectively, and replication-defective HSV vectors have been generated for therapeutic gene delivery to target tissues.[17,18] Replication-defective HSV vectors are deleted for single or multiple immediate-early (IE) genes to block lytic infection and limit IE gene-mediated cytotoxicity, and replication-defective HSV vectors encoding therapeutic genes have been clinically tested for chronic pain and dystrophic epidermolysis bullosa (NCT00804076, NCT03536143). To further improve the safety of these vectors, our group has developed fully non-cytotoxic HSV vectors that are devoid of all IE gene expression, yet are capable of robust transgene expression *in vitro* and *in vivo*.[19–22] Furthermore, these vectors provide space for additional transgene insertions.[23] The key difference between these new and previous replication-defective HSV vectors is the complete elimination of both copies of the viral gene encoding the highly toxic ICP0 protein. However, the growth of these vectors on complementing cells is less efficient than that of less debilitated vectors,[24] presenting an obstacle to thorough pre-clinical testing.

Previously, our group evaluated multiple growth parameters to improve the production of a series of replication-defective HSV vectors[24] and established protocols for their efficient generation and production.[25,26] More recently, we have optimized the upstream process for the production of our novel non-cytotoxic HSV vectors using a prototype, JΔNI5, as a model and described the importance of monitoring the genome copy number (gc)/plaque-forming units (pfu) ratio to estimate the quality of HSV vector stocks.[27] Our optimized protocol greatly improved the yield and quality of the non-toxic JΔNI5 vector. Additionally, JΔNI5 produced by the optimized protocol showed elevated transgene expression in rat dorsal root ganglion neurons.[27]

In this study, we further expanded our research to evaluate the downstream process for high-yield purification of highly defective HSV vectors. Downstream processing significantly influences both vector yield and quality.[28,29] While several purification protocols have been published for HSV vectors, the effects of different combinations

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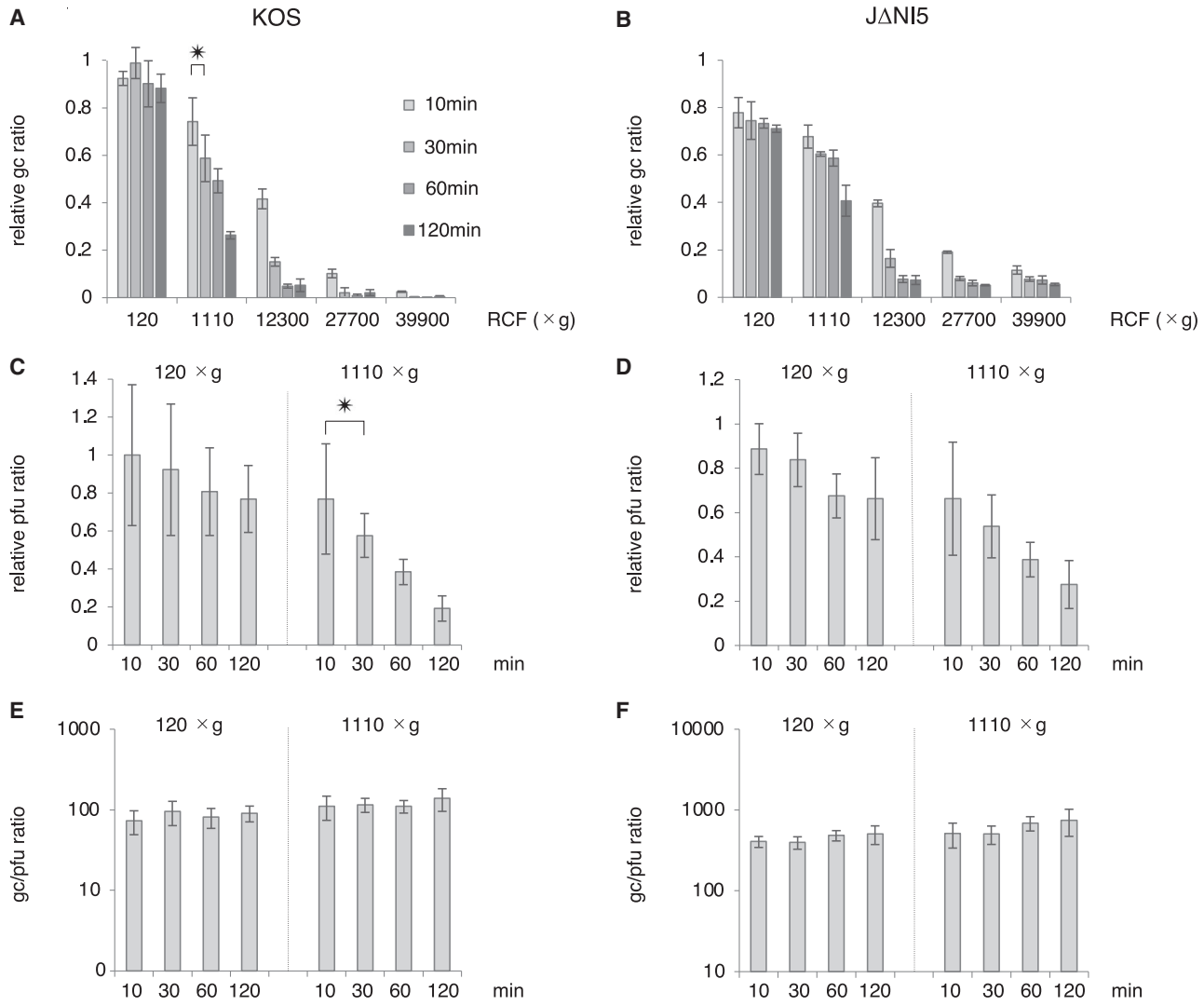

Figure 1. Optimization of clarification of HSV supernatants by low-speed centrifugation

(A, B) KOS and JΔNI5 viruses were produced as described in Materials and methods. The viral supernatants were centrifuged at 120–39,900 × *g* for 10–120 min for clarification. The viral gc in the supernatant after centrifugation were measured by qPCR and expressed as the relative ratios with the gc before centrifugation (A, B). (C, D) The biological titers (pfu) for samples after centrifugation at low speed (120 or 1,110 × *g*) were determined by standard plaque assay and expressed as the relative ratios with the pfu before centrifugation (C, D). (E, F) The gc/pfu ratios were calculated based on the results of (A, B) and (C, D). Differences between pairs were analyzed by the Student t-test. Data are presented as means ± SD (n = 3; *p < 0.05).

of genetic alterations in the viral genome on the efficiency of infectious virus purification remain largely unknown. We undertook the current study in an effort to enhance the production of the non-cytotoxic HSV vector JΔNI5,[19] since this vector serves as our principal backbone vector for the incorporation of therapeutic transgenes and JΔNI5 derivatives have been shown to provide long term transgene expression in the central nervous system of rodents.[20–22] We also examined the stability of JΔNI5 under different storage conditions by monitoring both physical and biological titers. Together, the results offer guidance for the effective production of new, highly defective HSV vectors for gene therapy.

RESULTS

Optimization of clarification method of HSV supernatants by centrifugation

We first explored the optimal centrifugal speed for the removal of cell debris from supernatants of wild-type (wt) HSV strain KOS-infected Vero cells and defective vector JΔNI5-infected U2OS-ICP4/27 cells. The supernatants harvested on the ninth day post virus infection were centrifuged at 120–39,900 × *g* (1,000–18,000 rpm) for 10–120 min (Figure 1). For comparison, a conventional method of clarification of virus supernatants uses centrifugation at 1,110 × *g* (3,000 rpm) for 10 min to remove cellular debris.[12,30] In our analysis,

Table 1. Filter membrane materials

	Material property	Throughput	Protein binding
CN	Hydrophobic	Middle	Strong
NYL	Hydrophilic	High	Strong
SFCA	Hydrophilic	Low	Weak
aPES	Hydrophilic	High	Weak

the genomic titers dramatically decreased when the rotation speed was higher than that of the conventional method (Figures 1A and 1B). Notably, KOS mostly remained in the supernatants at 120 × *g* whereas JΔNI5 showed increased sedimentation and the genomic titer for JΔNI5 significantly decreased to 78%–71%, even at 120 × *g*. To further explore whether genetic modifications affect the sedimentation of HSV vectors at low speed, we performed the same experiments with a previous replication-defective HSV vector, QOZHG,[31] which preserves ICP0 expression (Figure S1). QOZHG showed greater loss than KOS in the supernatant at 120 × *g*, whereas more virus remained compared with JΔNI5 vector, suggesting that HSV genetic modification can increase the sedimentation by low speed centrifugation.

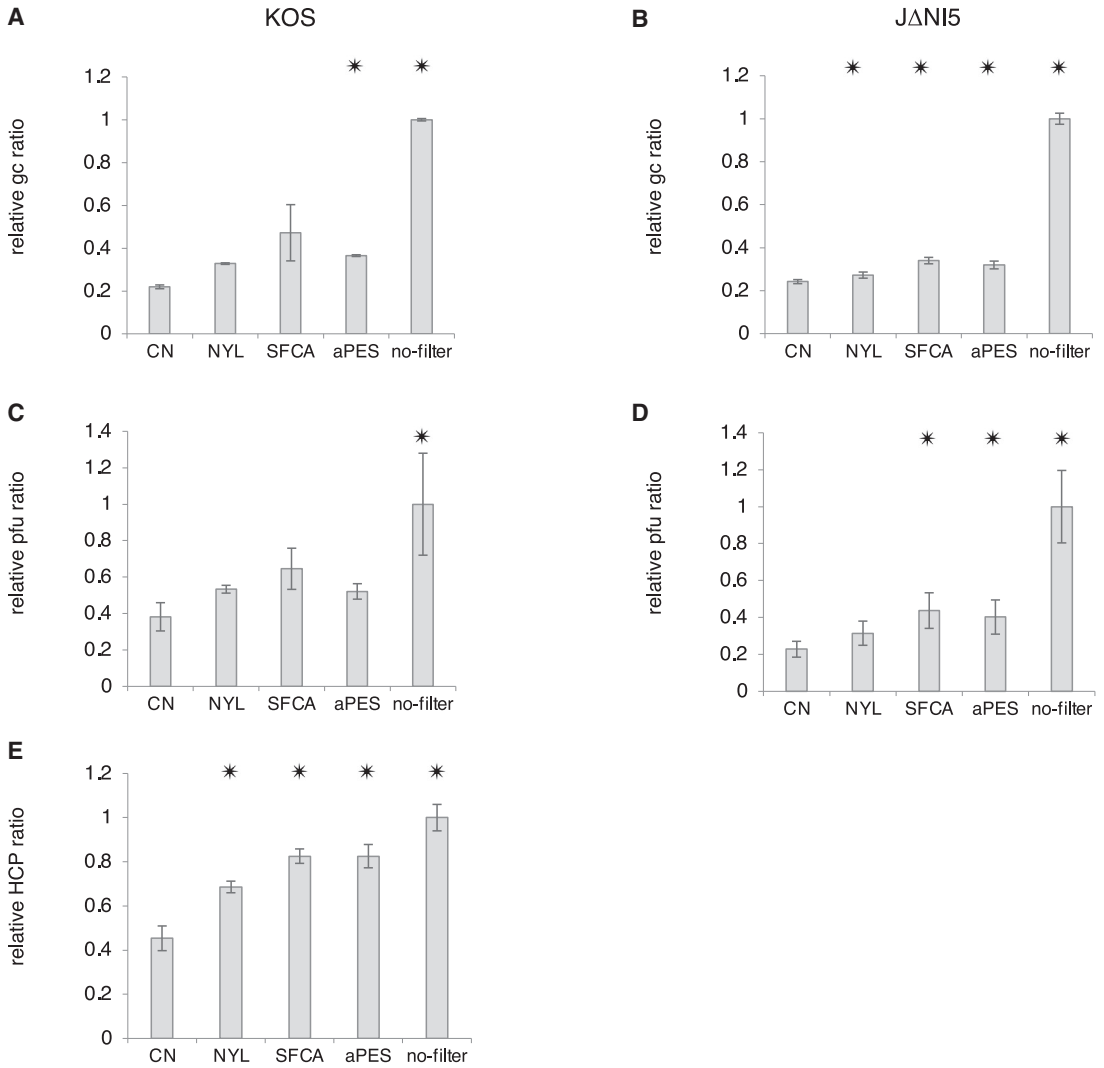

Figure 2. Effect of filter-membrane materials for clarification of HSV supernatants on viral titer

(A–D) KOS and JΔNI5 viruses were produced and clarified by centrifugation at 1,110 × *g* for 10 min. The supernatants were clarified by four types of filters (CN, NYL, SFCA, and aPES). The viral recovery after filtration was quantified by qPCR for gc and standard plaque assay for biological titer, and expressed as the relative ratios with the gc before filtration (A,B) and the pfu before centrifugation (C, D). (E) The level of Vero cell derived host protein impurity in viral supernatant after filtration was quantified by Vero HCP ELISA and expressed as the relative ratios with the HCP score before filtration. Group comparisons were performed with one-way ANOVA with post hoc Dunnett's multiple comparison test. Data are presented as means ± SD (n = 3; *$p < 0.05$).

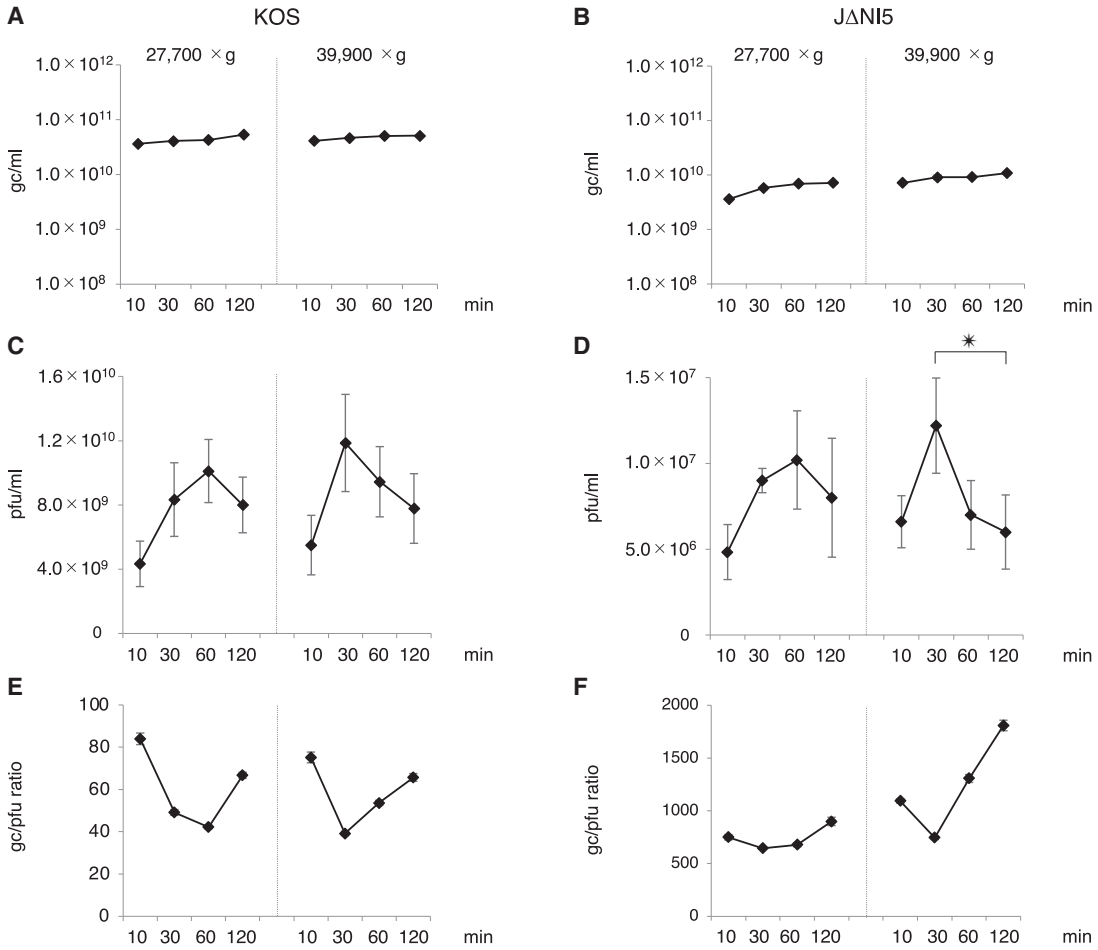

Figure 3. Optimization of HSV concentration by high-speed centrifugation

(A–D) KOS and JΔNI5 viruses were produced and clarified by centrifugation at 1,110 × *g* for 10 min. The viruses were concentrated by centrifugation at 27,700 or 39,900 × *g* for 10–120 min. The viral titers in pellet were determined by qPCR for gc titer (A, B) and standard plaque assay for biological titer (C, D). (E, F) The gc/pfu ratios were calculated based on the results of (A, B) and (C, D). Differences between pairs were analyzed by the Student t-test. Data are presented as means ± SD (n = 3; *p < 0.05).

We further explored the biological titer of the supernatants centrifuged at 120 or 1,100 × *g* for 10–120 min (Figures 1C and 1D). The biological titer was inversely proportional to the rotation speed and time (Figures 1C and 1D). There was no significant difference in gc/pfu ratio for either wt or mutant virus, indicating that the low speed centrifugation did not influence the quality of HSV vectors (Figures 1E and 1F).

Evaluation of filter membranes for clarification of HSV supernatants

To evaluate the effect of material of filter unit for clarification of HSV supernatants, four different types of filter materials—nitrocellulose (CN), Nylon (NYL), surfactant-free cellulose acetate (SFCA), and asymmetric polyethersulfone (aPES) were tested (Table 1).[32–36] The physical and biological titers of both HSVs were significantly decreased after filtration, with CN filtration having the most substantial effect (decrease by approximately 80%) (Figures 2A–2D). To determine the contamination of host cell proteins (HCPs) in the supernatants after filtration, the amounts of HCPs in KOS supernatants were examined by ELISA (Figure 2E). The data showed that CN filter was superior in eliminating Vero-derived HCPs compared with other filter materials.

Optimization of HSV vector concentration by centrifugation

We sought to determine the optimal high-speed centrifugal speed for vector concentration of HSVs (Figure 3). Supernatant virus was concentrated by centrifugation at the indicated speeds. The viral pellets were dissolved in PBS, and the physical and biological titers were determined. The physical titers of both HSVs increased slightly as the rotation speed and the time increased (Figures 3A and 3B), whereas the biological titers peaked at 27,700 × *g* (15,000 rpm) for 60 min and at 39,900 × *g* (18,000 rpm) for 30 min (Figures 3C and 3D); the gc/pfu ratios showed the inverse tendency (Figures 3E and 3F), suggesting that prolonged high-speed centrifugation can result in particle damage. To further confirm the negative effect of extended high-

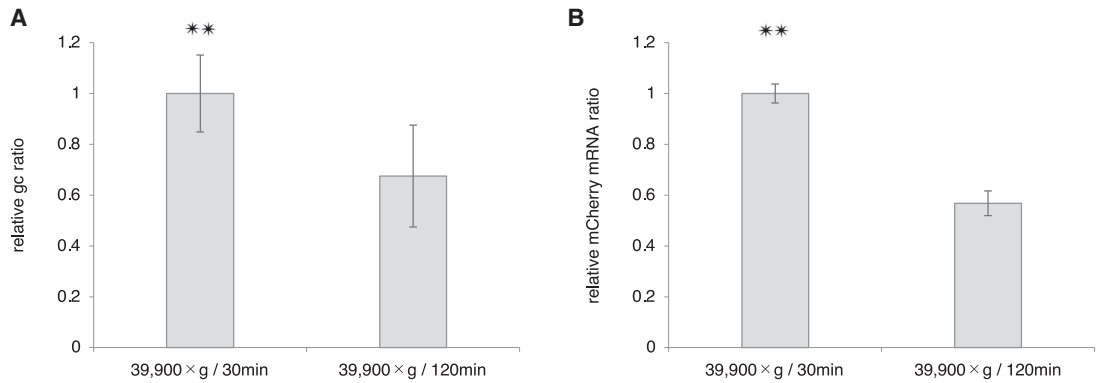

Figure 4. Comparison of the functionality of HSV vectors purified by different centrifugation conditions

(A) Transduction of rat hippocampal cells with JΔNI5 virus purified by centrifugation at 39,900 × *g* for 30 min or 120 min. Rat hippocampal cells were infected with each JΔNI5 virus preparation at 3,000 gc/cell. (B) The viral genome and the mCherry mRNA level at 7 days after infection were determined by qPCR and qRT-PCR, respectively. Intracellular viral genome numbers and mCherry mRNA levels were respectively normalized to cellular 18S rRNA genes and RNA levels and expressed as the relative ratios with the scores at 39,900 × *g*/30 min. Differences between pairs were analyzed by the Student t-test. Data are presented as means ± SD (n = 3; $^{*}p < 0.05$, $^{**}p < 0.01$).

speed centrifugation on the transduction activity of JΔNI5, we compared the transduction efficiency of two different preparations of JΔNI5 on rat hippocampal cells (Figure 4). Rat hippocampal cells were infected with JΔNI5 vectors at 3,000 gc/cell, and the intracellular viral DNA levels and the transgene mRNA levels were analyzed by qPCR at 7 days after infection. As expected, JΔNI5 purified by longer centrifugation showed less viral entry and transgene expression in rat hippocampal cells than the preparation purified by shorter centrifugation, indicating that enhanced vector concentration by longer centrifugation reduces virus infectivity.

Evaluation of long-term stability of HSVs at low temperature

We next asked whether the long-term storage of HSV supernatants and purified HSVs at low temperature could cause virus instability. HSVs were produced and harvested as described above, the supernatants were stored at 4°C, and the viral titers in the stored supernatants were monitored over a period of 28 d (Figure 5). Neither the physical nor the biological titers of the supernatants changed over this period. The physical titers of purified HSVs also remained essentially unaltered through 28 d of storage at 4°C (Figures 6A and 6B). However, the biological titers of the purified viruses declined gradually over 14 d and decreased dramatically over the next 7 d (Figures 6C and 6D). The results suggested that the stability of HSVs at low temperature depended on the storage buffer used.

Evaluation of tolerance of HSVs to freeze-thawing cycles

Last, the sensitivity of HSVs to frequent freeze-thaw operations was evaluated. HSV supernatants or purified HSVs were freeze-thawed repeatedly from −20°C or −80°C, and the viral titers at each thaw cycle were determined (Figures 7 and 8). Repeated freeze-thawing did not significantly affect the physical titers of the supernatants, but did result in gradual decreases in biological titers (Figure 7). In contrast, repeat freeze-thawing of purified viruses rapidly and extensively decreased the biological titers of these preparations, although their physical titers were stable (Figures 8C and 8D), again suggesting that the solvent may be an important factor in determining the freeze-thaw tolerance of HSVs. To examine whether medium components and/or cellular proteins secreted from host cells can contribute to the stability of HSV vectors, we resuspended the purified JΔNI5 in media or conditioned media derived from the U2OS-ICP4/27 host cells, and analyzed the vector stability during increasing numbers of freeze-thaw cycles (Figure S2). The results revealed that the purified JΔNI5 resuspended in media and conditioned media was more stable than in PBS, while the vector stability in conditioned media was significantly superior to that in media (Figures 8D and S2), suggesting that both medium and cellular proteins can include components to protect the biological activity of HSV vectors. Interestingly, the repeated freeze-thawing affected the biological titer of JΔNI5 and QOZHG more dramatically than that of KOS, while QOZHG was more stable than JΔNI5 (Figure S3), indicating that replication defects requiring complementation may decrease the tolerance of HSVs to cycles of freezing and thawing. We examined the effect of repeated freeze-thaw cycles on the efficiency of purified JΔNI5 transduction of rat hippocampal cells (Figure 9). As expected, JΔNI5 exposed to multiple freeze-thaw cycles displayed less viral entry and reduced transgene expression compared with virus exposed to a single cycle (Figure 9). The results clearly demonstrated that repeated freeze-thaw cycles adversely affect the biological activity of purified HSV preparations.

DISCUSSION

Preclinical studies to evaluate the performance of highly defective HSV vectors for a variety of gene therapy applications following introduction into different tissues requires that vector production methods and lot release criteria be harmonized to compare experimental findings. Methods for vector growth, harvest, and purification require strict development of a production process. The production process completely depends on vector features such as replication competence and the cell line used for efficient vector production. For the production of highly defective vectors, it is essential to develop cell lines that effectively complement essential viral genes that are

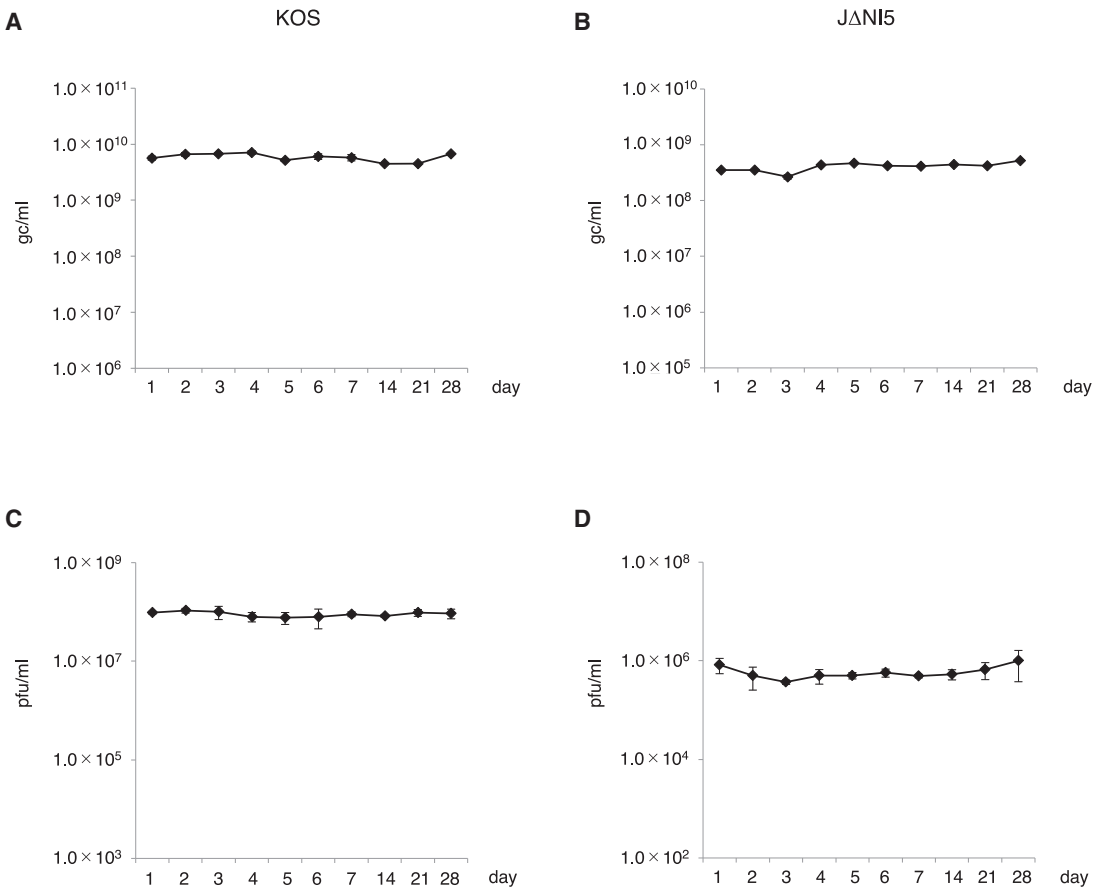

Figure 5. Effect of long-term storage of viral supernatants at 4°C on viral titer

(A–D) KOS and JΔNI5 supernatants produced as described in Materials and methods were stored at 4°C for 28 days. The viral titers were measured daily until 7 days after infection and at 14, 21, and 28 days after infection by qPCR (A, B) and standard plaque assay (C, D).

removed from the vector genome. The released virus particles can have different properties based on completeness of particle formation and this will in turn impact the purification process. These properties can affect vector release from infected cells, sensitivity to centrifugation, interaction with filter membranes, and stability to freeze-thaw and storage temperature. To evaluate these properties, we compared the purification, storage, and handling steps using wt virus and a highly defective HSV-1 mutant, JΔNI5, that is our principal backbone vector to derive gene transfer variants suitable for gene therapy applications. Our findings indicate that, although the stability of JΔNI5 was similar to that of wt virus under the pressures of concentration by centrifugation and filtration, JΔNI5 was more sensitive to freeze-thaw and storage at cold temperature.

The first step of the viral vector purification process is low-speed centrifugation to clarify the viral supernatants of large cellular debris. As mentioned, the conventional condition of centrifugation-based clarification for HSV vectors is in the range of 1,000–2,060 × *g* for 10 min.[12,37] Consistent with these previous reports, our results showed that centrifugation exceeding 2,060 × *g* or for longer than 10 min resulted in significant sedimentation of the viruses tested in this study (Figure 1). Therefore, the use of centrifugation to remove cellular debris should be limited to less than 2,060 × *g* for up to 10 min. JΔNI5 and QOZHG showed greater virus loss than wt virus even at low-speed centrifugation, and JΔNI5 was more precipitated than KOS and QOZHG at low-speed centrifugation (Figures 1A, 1B, and S1 120 × *g*), indicating that different genetic modifications can differentially affect the sedimentation of HSV vectors. There may be unpackaged viral genomes and incomplete virus particles in the supernatants of replication defective HSV owing to its replication defects and potentially associated packaging dysfunction. For example, JΔNI5 is functionally deleted for the IE genes ICP4, 22, 27, and 47 and has a complete deletion of the Joint region, which includes multiple HSV miRNA genes. Although the essential ICP4 and ICP27 proteins were provided by the producer U2OS-ICP4/27 cells during virus growth,[19] these cells do not provide the ICP0 gene product or HSV miRNAs. ICP0 and ICP4 are reported to be components of the mature virion.[38] In addition, the levels of other viral and host proteins incorporated into virions may be less than with wt virus owing to the gene alterations. The potential lack or decreased

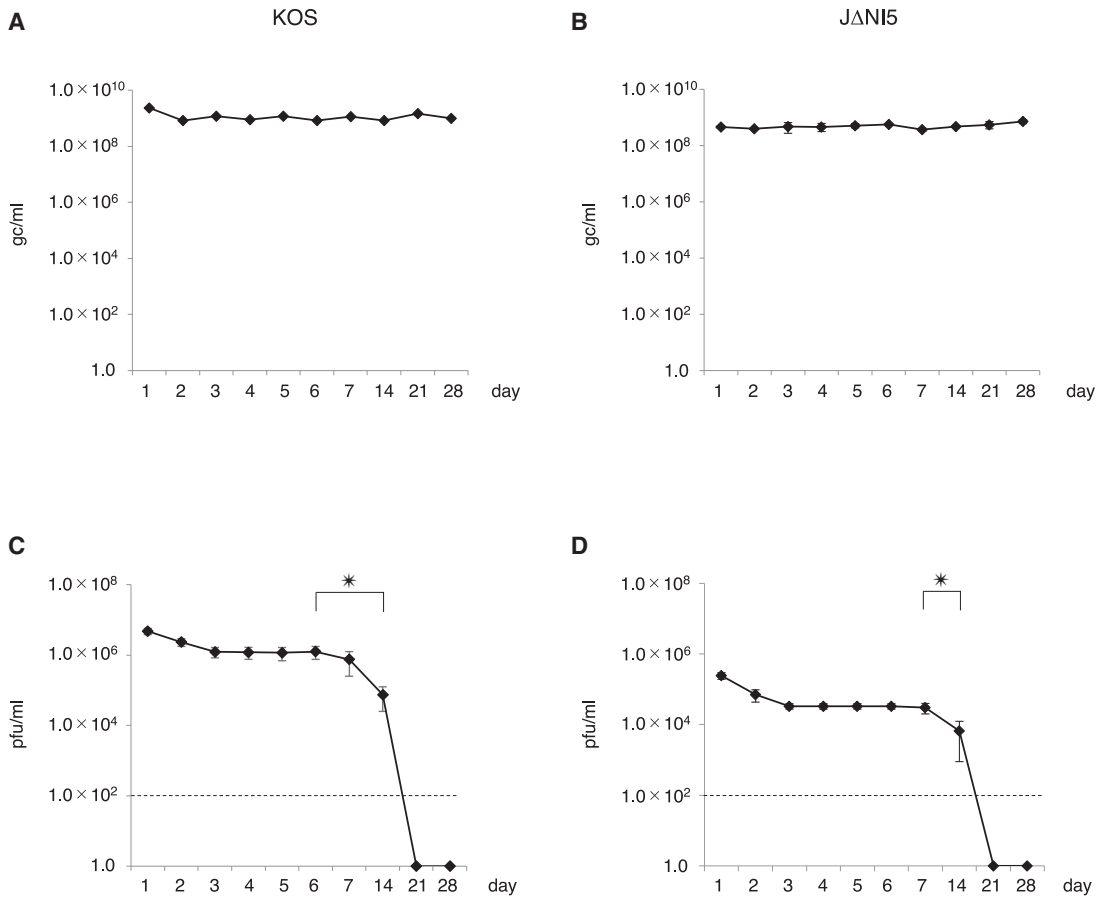

Figure 6. Effect of long-term storage of purified virus at 4°C on viral titer

(A–D) Purified KOS and JΔNI5 viruses were stored at 4°C for 28 days. The viral titers were measured daily until 7 days after infection and at 14, 21, and 28 days after infection by qPCR (A, B) and standard plaque assay (C, D). Dotted lines indicate the limit of detection. Differences between pairs were analyzed by the Student t-test. Data are presented as means ± SD (n = 3; *$p < 0.05$).

incorporation of structural proteins into virions could lead to elevated levels of defective or incomplete particles and vector instability in certain situations, such as low temperature storage and thawing.

Conventional purification methods using sucrose gradients and Optiprep have been reported.[26,39] However, these methods are laborious and time consuming for laboratory-grade HSV preparation. The procedures described in this study are designed to purify and store adequate amounts of laboratory-grade HSV vector stock for pre-clinical studies. Highly defective non-toxic HSV vectors purified and stored by our protocol showed high transduction efficiency for primary rat hippocampal cells without any evidence of vector-mediated toxicity (Figures 4 and 9), supporting the likelihood that our protocol provides HSV vectors of sufficient quality for different types of *in vivo* studies. A limitation of our purification protocol is the low recovery of HSV vector at filtration steps to separate HSV from cellular debris. Evaluation of filtration-based clarification revealed that all HSVs were trapped to similar degrees by the different filter membranes tested (approximately 60%–80%) (Figure 2). The results indicated that the genetic alteration of JΔNI5 did not change the affinity to filter membranes. CN filtration was superior to other methods for removal of HCPs from the HSV supernatants (Figure 2E), but this method did not provide the best active virus recovery (Figure 2A). These observations suggest that there remains room for improvement in the filtration conditions. Virus recovery might be improved by optimizing the pore size of the membrane filter (Figure S4).

Based on measurement of physical titers, the centrifugation time did not influence the precipitation efficiency after secondary high-speed centrifugation. However, it did significantly affect the precipitation efficiency measured as biological titers (Figure 3). The functional analyses of JΔNI5 concentrated with different conditions validated these influences (Figure 4). Previous reports showed that prolonged high-speed centrifugation might cause overcompaction of HSV-1.[40]

We characterized the potential impact of several downstream methods for virus handling and storage on the maintenance of virus titer. Purified virus was comparatively stable at low temperature,

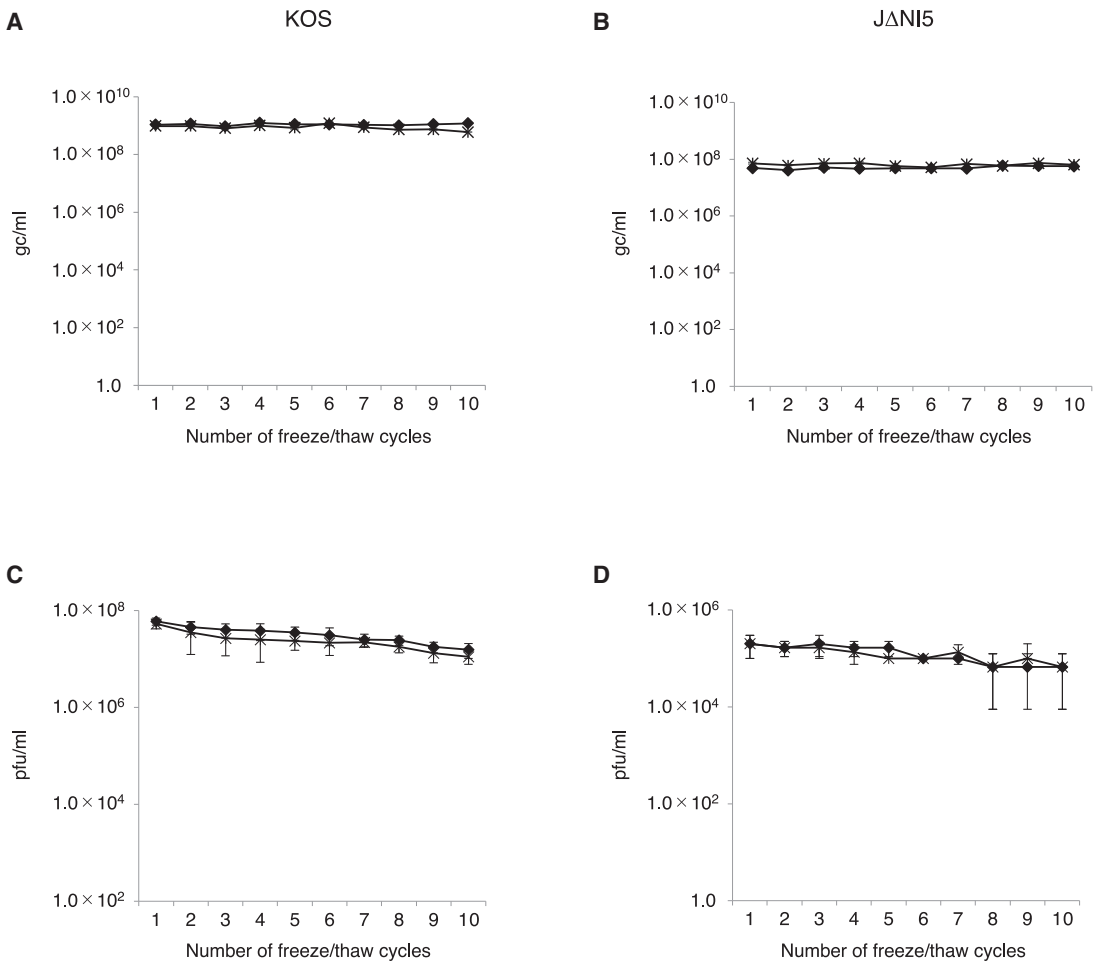

Figure 7. Effect of freeze-thawing of viral supernatants on viral titer

(A–D) KOS and JΔNI5 supernatants were produced as described in Materials and methods. Freezing and thawing of the viral supernatants was repeated 10 times at −80°C (diamond) or −20°C (asterisk). Viral titers were measured daily by qPCR (A, B) and standard plaque assay (C, D). Data are presented as means ± SD (n = 3).

whereas cycles of freeze-thaw were highly damaging, especially after vector purification (Figures 5–8). In addition, our findings revealed that both medium and cellular proteins can play an important role in the protection of the biological activity of HSV vector (Figure S2). Several groups have also reported that the viral vector formulation is a critical determinant for expiration.[41] Therefore, the storage conditions, including the length of time and temperature, should be optimized depending on the HSV vector formulation.

Our results offer guidance for the optimal production of an advanced, highly defective HSV vector for gene therapy applications (Figure 10). We monitored both physical and biological titers to assess trade-offs between yield and biological activity and compared several parameters with the production of wt virus. We showed that HSV can be stored in medium for a relatively long period of time, but it must be used immediately after purification, freezing, and thawing. Importantly, our studies demonstrate that genetic alterations can influence the efficiency of purification and long-term storage. Our results, along with previous reports,[27] may contribute to the establishment of a cost-effective and reproducible protocol for efficient production of HSV vectors for pre-clinical and clinical evaluation.

MATERIALS AND METHODS

Cells and viruses

Vero (African green monkey kidney) cell was obtained from the American Type Culture Collection (ATCC), and they were grown in DMEM (ThermoFisher Scientific) with 10% (vol/vol) FBS (ThermoFisher Scientific) and 1% penicillin-streptomycin (P/S; Sigma). U2OS-ICP4/27 cells were as described previously[19] and were grown in DMEM with 10% FBS, 1% P/S, puromycin (1 μg/mL), and blasticidin (5 μg/mL). An HSV-1 wt strain KOS was purchased from ATCC and produced in Vero cells. JΔNI5 vector was an engineered non-toxic HSV vector as described previously.[20,27,42] JΔNI5 and a replication-defective HSV vector QOZHG[31] were propagated in U2OS-ICP4/27 cells. Confluent Vero cells in T-225 culture flasks were infected with KOS at MOI 10^{-4}, and confluent U2OS-

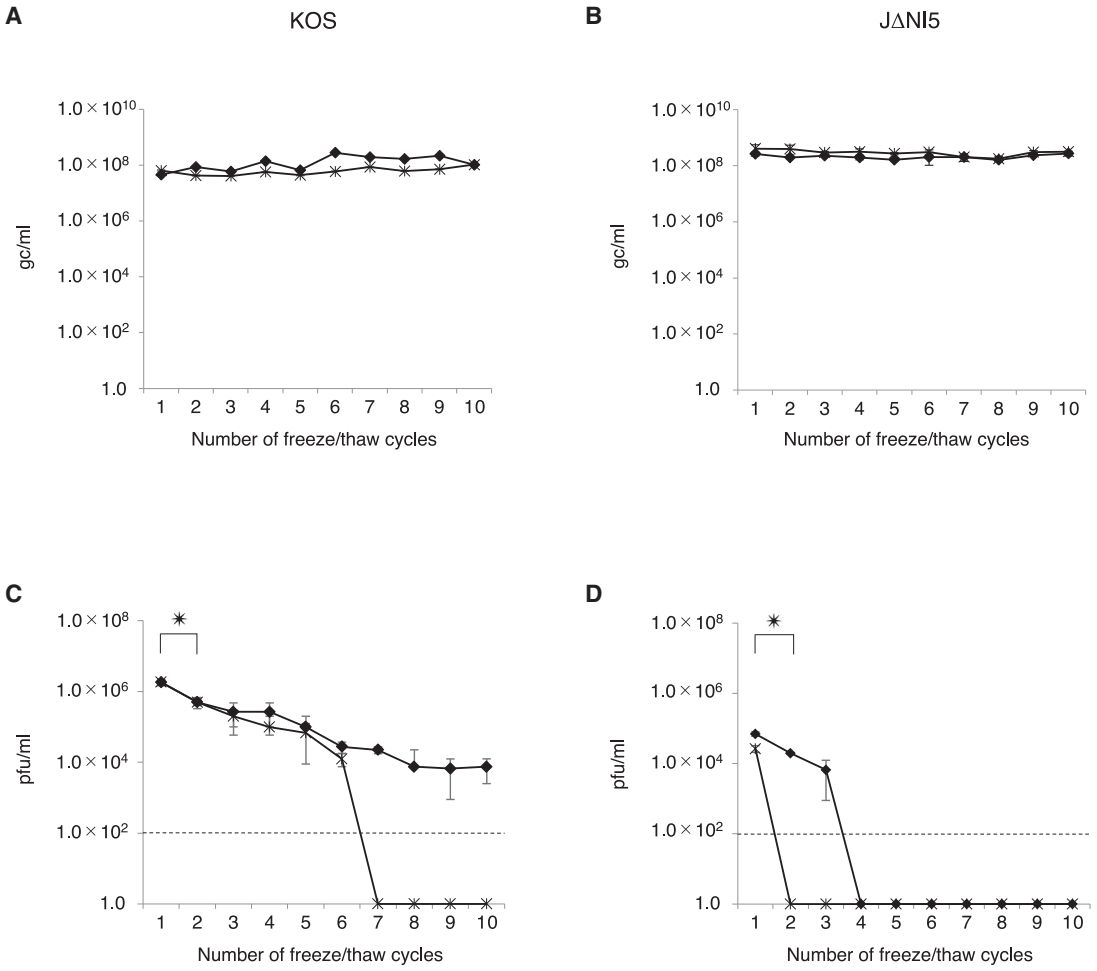

Figure 8. Effect of freeze-thawing of purified virus on viral titer

(A–D) KOS and JΔNI5 viruses were produced and purified as described above. Freezing and thawing of the purified virus was repeated 10 times at −80°C (diamond) or −20°C (asterisk). Viral titers were measured daily by qPCR (A, B) and standard plaque assay (C, D). Dotted lines indicate the limit of detection. Differences between pairs were analyzed by the Student t-test. Data are presented as means ± SD (n = 3; $^{*}p < 0.05$).

ICP4/27 cells in T-225 culture flasks were infected with JΔNI5 at MOI 10^{-5} and QOZHG at MOI 10^{-4}. The cells were cultured in a 5% CO_2 incubator at 33°C, and the supernatants were harvested on dpi 8–9. Primary fetal rat hippocampal cells were microdissected from day 21 rat embryos, dissociated with 10 mg/mL papain (Sigma), 0.1 mg/mL DNaseI (Sigma) in Hank's balanced salt solution (HBSS) for 30 min at 37°C. After dissociation, hippocampal cells were washed by HBSS twice and plated on poly-D-lysine/laminin-coated coverslips (BD Biosciences) at 5×10^4 cells per well in 24-well plates in 500 μL of rat hippocampal culture media: neurobasal medium (ThermoFisher Scientific) with 2% B-27 Supplement (ThermoFisher Scientific), 10% FBS, 25 μg/mL pyruvic acid, 1% GlutaMax (ThermoFisher Scientific), 6.25 mM monosodium L-glutamate (Wako), and P/S. At 1 d post plating, the media were replaced with rat hippocampal culture media without FBS and monosodium L-glutamate. At 2 d post plating, the media were replaced with rat hippocampal culture media with arabinofuranoside hydrochloride (Sigma) in the above media for 7 days to remove the dividing cells. Cells were then incubated with rat hippocampal culture media as above.

Conventional purification method

At first, to remove the cell debris the viral supernatants were centrifuged at 1,110 × *g* (3,000 rpm) for 10 min. Followed by, they were filtered with 0.8 and 0.45 μm CN membrane filter. Finally, secondary high-speed centrifugation was performed at 39,900 × *g* (18,000 rpm) for 40 min for the purpose of recovering the virus.

Clarification and concentration of viral supernatants by centrifugation and filtration

For clarification of HSV viral supernatants by centrifugation, 30-mL viral supernatants were centrifuged at 120–39,900 × *g* (1,000–18,000 rpm) for 10–120 min by high gradient magnetic affinity chromatography (himac) CR21N high-speed refrigerated centrifuge. For

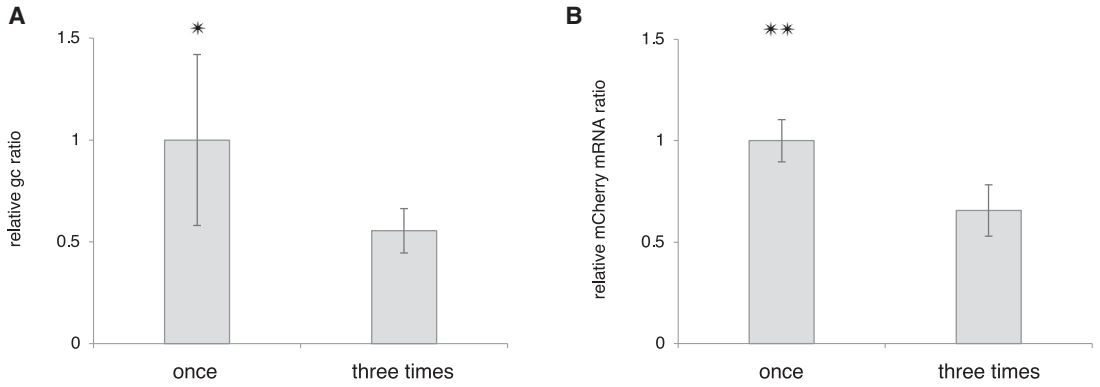

Figure 9. Comparisons of the functionality of HSV vectors after different numbers of freeze-thaw cycles

(A) Transduction of rat hippocampal cells with JΔNI5 virus treated with different numbers of freeze-thaw cycles at −80°C (once and three times). Rat hippocampal cells were infected with each JΔNI5 virus preparation at 3,000 gc/cell. Rat hippocampal cells were infected with each JΔNI5 virus preparation at 3,000 gc/cell. (B) The relative viral genome and the mCherry mRNA level at 7 days after infection was determined by qPCR and qRT-PCR, respectively. Intracellular viral genome numbers and mCherry mRNA levels were respectively normalized to cellular 18S rRNA genes and RNA levels, and expressed as the relative ratios with the scores at one freeze-thaw. Differences between pairs were analyzed by the Student t-test. Data are presented as means ± SD (n = 3; $^{*}p < 0.05$, $^{**}p < 0.01$).

clarification of HSV viral supernatants by filtration, the filter units with four different membrane materials (CN, NYL, SFCA, and aPES; 0.45-μm pore sizes, 75-mm diameter; ThermoFisher Scientific) were used. For centrifugal concentration of HSV, HSV viral supernatants were centrifuged at 27,700–39,900 × *g* for 10–120 min by himac CR21N high-speed refrigerated centrifuge. The viral pellets were resuspended in PBS. The physical viral titers of viral supernatants and pellets were determined as described.[27] The biological titers were determined by standard plaque assay on Vero cells (KOS) or U2OS-ICP4/27 cells (JΔNI5 and QOZHG).

Genomic qPCR

Quantification of HSV genomic titer was carried out as described[27] by real-time qPCR in triplicate using the 7500 Fast Real-Time PCR System (Applied Biosystems). PCR primers used in this study were as described.[19] For qRT-PCR, five wells of rat hippocampal cells (n = 3) were infected with JΔNI5 vector at 5,000 gc/cell. Seven days after transduction, the cells were pooled and the genomic DNA/total RNA extraction and reverse-transcription were performed by RNeasy Mini kit (Qiagen) and SuperScript IV Reverse Transcriptase (ThermoFisher Scientific). Real-time qPCR was performed in triplicate using 7500 Fast Real-Time PCR System (Applied Biosystems). The data were normalized to cellular 18S rRNA. PCR primers used in this study are listed in Kuroda et al.[27]

Quantification of HCP contamination by ELISA

Immunoenzymetric Assay Kit for the Measurement of Vero Cell Host Cell Proteins (Vero HCP ELISA, Cygnus Technologies) was used for quantitative assessment of Vero cell-derived HCP in media.

Statistical analyses

All values are presented as the mean ± SD. Differences between pairs were analyzed by the Student *t*-test or one-way ANOVA with post hoc Dunnett's multiple comparison test using Microsoft Excel 14.7.7 or IBM SPSS statistics version 25.0. A p value of less than 0.05 was considered statistically significant.

DATA AVAILABILITY

On reasonable request, derived data supporting the findings of this study are available from the corresponding author.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2022.06.007.

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AUTHOR CONTRIBUTIONS

S.K. and Y.M. conceived and designed the research. S.K., Y.M., M.S., T.T., K.A., M.Y., and Y.S. performed the experiments. S.K. and Y.M. analyzed the data. S.K. and Y.M. wrote the manuscript. Y.M., G.V., W.F.G., J.B.C., J.C.G., and T.O. supervised the research. All authors reviewed and edited the manuscript.

DECLARATION OF INTERESTS

Y.M., J.B.C., and J.C.G. are co-inventors of intellectual property licensed to Replay Therapeutics, Inc. J.B.C., and J.C.G. are co-inventors of intellectual property licensed to Oncorus, Inc. J.C.G. is a founder and consultant of Coda Biotherapeutics and Oncorus, Inc. W.F.G. is a consultant of Oncorus, Inc.

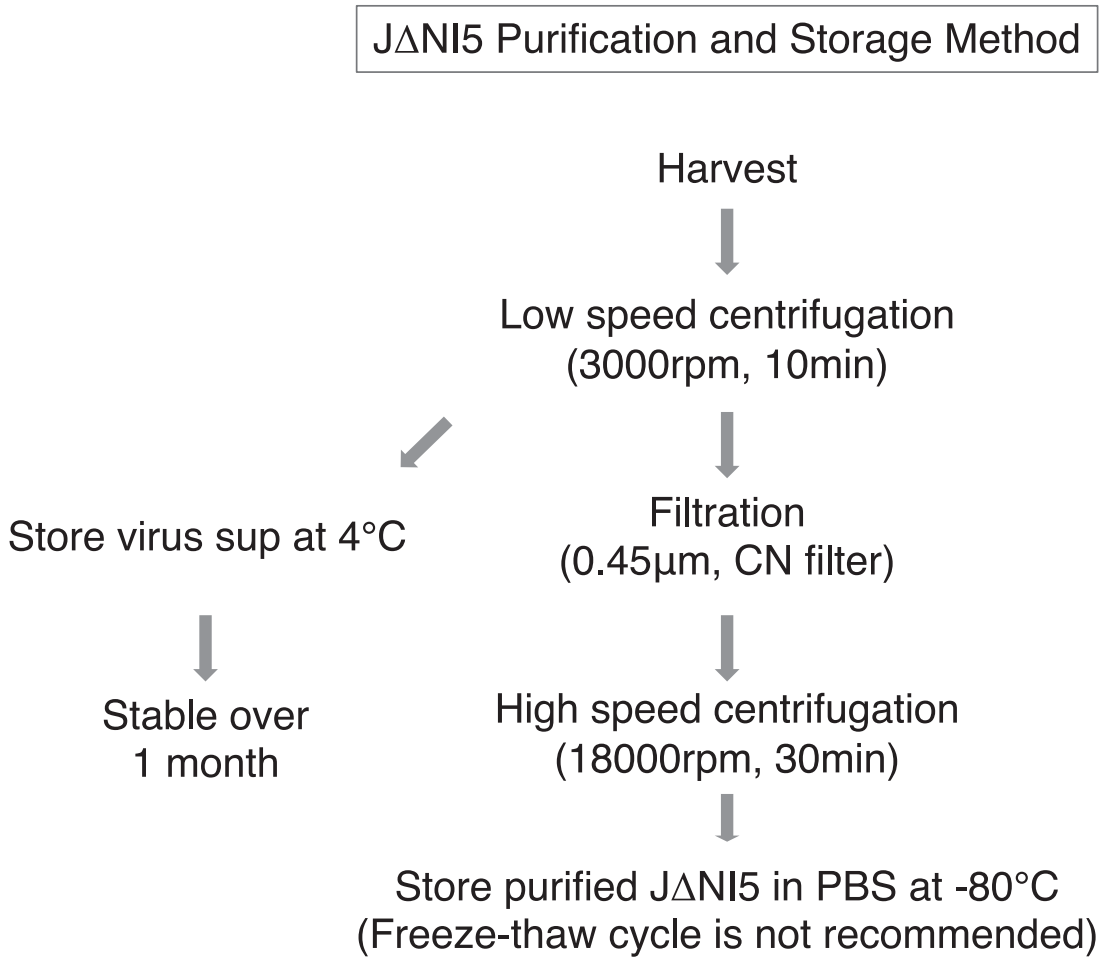

Figure 10. New virus purification method algorithm

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