BMB
*Reports*

**BMB Rep. 2022; 55(12): 615-620**
www.bmbreports.org

# Split genome-based retroviral replicating vectors achieve efficient gene delivery and therapeutic effect in a human glioblastoma xenograft model

*Moonkyung Kang[1,#], Ayoung Song[2,#,†], Jiyoung Kim[1,#], Se Hun Kang[3], Sang-Jin Lee[3] & Yeon-Soo Kim[1,*]*

[1]Graduate School of New Drug Discovery and Development, Chungnam National University, Daejeon 34134, [2]Institue of Molecular Biology, Inje University, Seoul 04551, [3]Research Institute and Hospital, National Cancer Center of Korea, Goyang 10408, Korea

**The murine leukemia virus-based semi-retroviral replicating vectors (MuLV-based sRRV) had been developed to improve safety and transgene capacity for cancer gene therapy. However, despite the apparent advantages of the sRRV, improvements in the *in vivo* transduction efficiency are still required to deliver therapeutic genes efficiently for clinical use. In this study, we established a gibbon ape leukemia virus (GaLV) envelope-pseudotyped semi-replication-competent retrovirus vector system (spRRV) which is composed of two transcomplementing replication-defective retroviral vectors termed MuLV-Gag-Pol and GaLV-Env. We found that the spRRV shows considerable improvement in efficiencies of gene transfer and spreading in both human glioblastoma cells and pre-established human glioblastoma mouse model compared with an sRRV system. When treated with ganciclovir after intratumoral injection of each vector system into pre-established U-87 MG glioblastomas, the group of mice injected with spRRV expressing the herpes simplex virus type 1-thymidine kinase (HSV1-tk) gene showed a survival rate of 100% for more than 150 days, but all control groups of mice (HSV1-tk/PBS-treated and GFP/GCV-treated gruops) died within 45 days after tumor injection. In conclusion, these findings suggest that intratumoral delivery of the HSV1-*tk* gene by the spRRV system is worthy of development in clinical trials for the treatment of malignant solid tumors. [BMB Reports 2022; 55(12): 615-620]**

*Corresponding author. Tel: +82-42-821-8631; Fax: +82-42-821-8923; E-mail: kimys58@cnu.ac.kr
†Current address: SL Bigen, 85 Songdogwahak-ro, Incheon 21983, Korea
#These authors contributed equally to this work.

https://doi.org/10.5483/BMBRep.2022.55.12.136

**Received 5 September 2022, Revised 26 September 2022, Accepted 4 October 2022**

**Keywords:** Cancer, Gene delivery, Gene therapy, Glioma, Replicating retroviral vector

## INTRODUCTION

Murine leukemia virus (MuLV)-based retroviral replicating vectors (RRVs) have been developed for cancer gene therapy due to their tumor selectivity, high transduction efficiency, and low viral clearance (1, 2). However, there are limitations in the size of therapeutic genes that can be delivered using these vectors due to the highly compact nature of the MuLV genome (3, 4). The size of the therapeutic gene correlates with increased deletion of the introduced sequence, resulting in attenuated kinetics (3). Therefore, only relatively small genes such as the cytosine deaminase (CD) gene can be used for cancer gene therapy studies with the RRV system (5, 6).

To overcome the packaging size limitation, the sRRV system was developed (7, 8). In this system, the *gag-pol* and *envelope* (*env*) genes were inserted separately into two complementary replication-defective vectors. New progeny sRRV can be produced in cells infected with both complementary replication-defective vectors. However, viral propagation in the first-generation sRRV system was low in both *in vitro* and *in vivo* experimental systems, so further development for clinical gene therapy studies has not progressed.

MuLV envelope glycoprotein interacts with Pit2 and infects both rodent and human cells, while GaLV infects heterologous cells but not rodent cells through an interaction with Pit1 (9-13). Host cells can therefore be co-infected with MuLV and GaLV without superinfection interference (14). To improve the infectivity of retroviruses in diverse cells, recombinant chimeric retroviruses have been engineered in which the envelope protein of the amphotropic virus is replaced with that of another virus type. It has been shown that GaLV-pseudotyped recombinant retroviruses produced high-titer viral vectors (15-17) and also showed improved gene transfer efficiency in human progenitor cells and in baboons (18-20).

In the present study, we constructed MuLV-based, GaLV-pseudotyped semi-retroviral vectors (spRRV system) with improved transduction efficiency. The spRRV vectors propagated more quickly than sRRV system (MuLV-based semi-retroviral vectors) *in vitro* and also an *in vivo* intracerebral xenograft model. Furthermore, when spRRV harboring the *tk* gene of

ISSN: 1976-670X (electronic edition)

herpesvirus type 1 (HSV1-tk) was intracerebrally injected into glioblastoma-bearing nude mice, GCV (ganciclovir) administration resulted in the eradication of tumors and the survival of all experimental group animals. These results indicate that the spRRV system achieved significantly effective tumor-specific propagation. Therefore, the spRRV system allows specific and superior delivery of therapeutic genes to tumors.

## RESULTS

### Design and construction of spRRV system

We developed a semi-MuLV-based, GaLV-pseudotyped RRV (spRRV). This spRRV system is composed of two complementary replication-defective vectors, MuLV-Gag-Pol (sRRVgp) and GALV-Env (spRRVe), which express the *gag-pol* genes of MuLV and the *env* gene of GaLV, respectively (Fig. 1). The *rfp* gene was encoded in the sRRVgp vector and was used to monitor the virus transduction. The *gfp* gene was encoded in either sRRVe or the spRRVe vector, generating sRRVe-GFP or spRRVe-GFP virus. HSV1-TK gene was used as a therapeutic gene and was encoded in the sRRVe or the spRRVe vector (sRRVe-TK or spRRVe-TK, respectively).

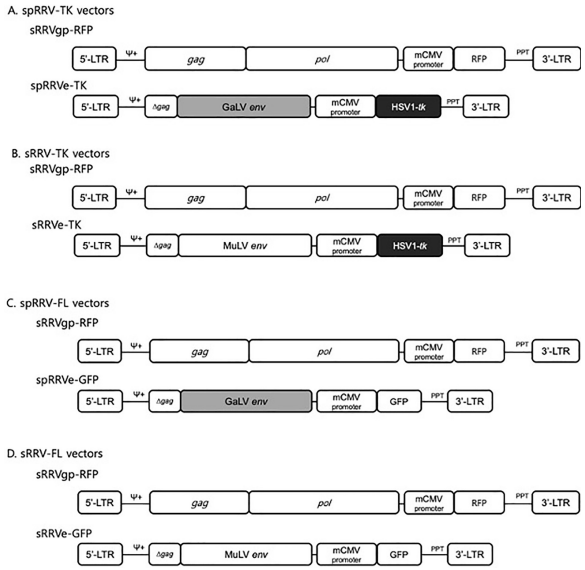


**Fig. 1.** Structures of the sRRV and spRRV vectors used in this study. (A) spRRV-TK vectors consist of the common element vector (sRRVgp-RFP) and spRRVe-TK vector. The sRRVgp-RFP vector contains the murine leukemia virus *gag-pol* coding sequence and RFP gene. The spRRVe-TK vector contains the gibbon ape leukemia virus env coding sequence and HSV1-TK gene. (B) sRRV-TK vectors consist of the common element vector (sRRVgp-RFP) and sRRVe-TK vector. (C) spRRV-FL vectors consist of the common element vector (sRRVgp-RFP) and spRRVe-GFP vector. (D) sRRV-FL vectors consist of the common element vector (sRRVgp-RFP) and sRRVe-TK vector.

### spRRV system provides more efficient transgene delivery to human glioblastoma cells than sRRV system

To determine whether semi-RRVs were capable of replicating efficiently in cultured cells, human glioblastoma U-87 MG cells were transduced with the same number of genomic copies (gc) of the sRRV or spRRV. sRRVgp-RFP + sRRVe-GFP (sRRV-FL) and sRRVgp-RFP + spRRVe-GFP (spRRV-FL) were used to examine the spread efficiency of each gene delivery system. At 20 days post-infection, the percentage of GFP-expressing spRRV-FL-transduced U-87 MG cells was nearly 100%, but only approximately 30% of sRRV-FL-transduced cells expressed GFP (Fig. 2A, B). We, as expected, observed the superinfection resistance phenomenon showing a plateau state in the proportion of RFP-positive cells at a low ratio as compared to GFP-positive cells (Fig. 2B). As with the experimental results for cell lines, both the GFP and RFP signals were strong and restricted to the tumor area in spRRV-FL-injected mice, whereas the GFP and RFP signals in sRRV-FL-injected tumors were weak (Fig. 2C). Tumor cells from sRRV-FL injected mice showed relatively modest transduction efficiency and 53.433 ± 7.4% of cells

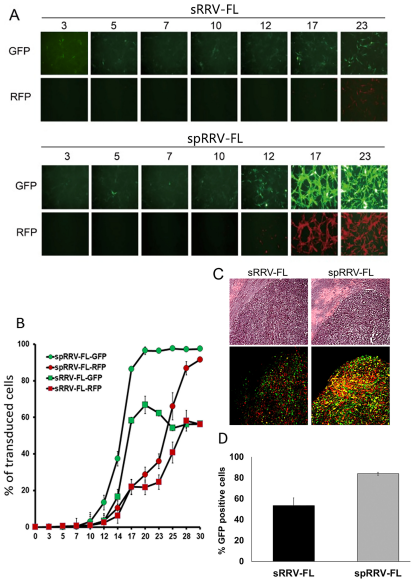


**Fig. 2.** spRRV provides more efficient transgene delivery to human glioblastoma cells than sRRV. (A, B) The replication kinetics of sRRV-FL (sRRVgp-RFP + sRRVe-GFP) and spRRV-FL (sRRVgp-RFP + spRRVe-GFP). U-87 MG cells transduced with sRRV-FL or spRRV-FL. (B) The percentages of sRRV-FL- and spRRV-FL-transduced cells that expressed GFP or RFP. (C) *In vivo* spread of sRRV-FL and spRRV-FL vectors. Each semi-RRV-FL vector combination was injected into the U-87 MG tumor xenograft (see Materials and Methods for details). Merged images for RFP and GFP expression patterns are shown for sRRV-FL and spRRV-FL transduced gliomas (lower panel). The same brain sections were then subjected to H&E staining (upper panel). (D) FACS analysis of viral vector spread. Intratumoral tissue was also collected from three mice 14 days after virus injection and analyzed by flow cytometry. Data are represented as mean ± SEM.

were GFP-positive. On the other hand, the percentage of cells expressing GFP in spRRV-FL-injected animals was 84 ± 1.48% (Fig. 2D), 1.5-fold higher than that of sRRV-FL ($P = 0.0046$). These data indicate that the transduction efficiency of spRRV-FL is significantly improved compared to sRRV-FL.

### spRRV-TK vectors propagate and express therapeutic gene efficiently

We next evaluated the level of the HSV1-*tk* gene expression encoded in the sRRV-TK (sRRVgpRFP + sRRVeTK), and spRRV-TK (sRRVgpRFP + spRRVeTK) systems was examined by immunoblotting (Fig. 3A). To examine the suicide effect of the TK, U-87 MG cells transduced with spRRV-FL, sRRV-TK, or spRRV-TK were treated daily with 2 μg/ml GCV from 10 days after the transduction and then were evaluated by MTT assay. Although the proportion of infected cells after 10 days of virus treatment was not thought to be high, treatment of GCV induced cytotoxicity in U-87 MG cells infected with all retroviral replicating vectors encoding the HSV1-TK gene (Fig. 3B). As shown in Fig. 2B (in the revised manuscript), there is little difference between sRRV and spRRV in the infection rates on the 7 days or 10 days after the initial viral infection. In addition, since apoptosis induced by the TK-mediated GCV modification is accompanied by bystander effect, it is a well-known phenomenon that even if only some cells are infected and express the TK gene, it induces the death of most cells cultured in the same culture dish. We interpret these experimental conditions caused little difference in the amount of TK protein and cytotoxicity. We also observed that the spRRV-TK vectors were efficiently spread in U-87 MG xenograft tumors established in the mouse brain with PET-CT (Positron emission tomography-computerized tomography). Seven days after stereotaxic injection of U-87 MG cells into the striatum of the mouse brain, spRRV-TK vectors or replication-defective spRRVe-TK viruses were injected into the pre-established tumor site. As expected, efficient uptake of [$^{18}$F]FHBG (21) was observed in mouse brains injected with the spRRV-TK vectors, but not in mouse brains injected with only replication-defective spRRVe-TK throughout the study period (Fig. 3C).

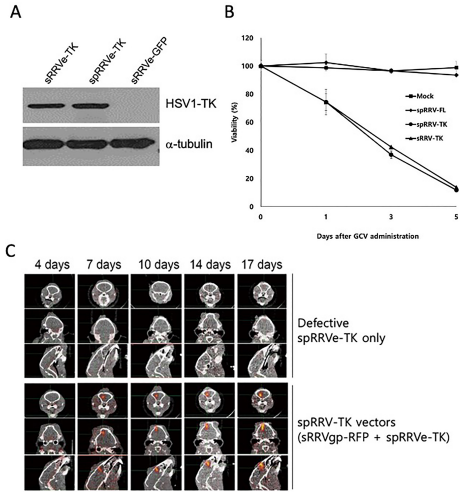


**Fig. 3.** spRRV-TK vectors propagate efficiently and highly expresses transgene. (A) HSV-TK expression profiles in sRRVe-TK- or spRRVe-TK-transduced U-87 MG cells. Cell extracts were analyzed in 10% SDS-PAGE and the expression of TK was analyzed by immunoblotting with an anti-HSV-TK antibody. (B) *In vitro* cytotoxicity in U-87 MG cells transduced with $1 \times 10^7$ gc of spRRV-FL, sRRV-TK, or spRRV-TK. The cells were cultured for 10 days and then seeded onto 96-well plates in triplicate (2,000 cells/well). After 24 h, the transduced cells were exposed to 2 μg/ml of GCV for 5 days. The number of viable cells was measured daily by MTT assay. (C) *In vivo* spread of spRRV-TK in U-87 MG xenograft.

### spRRV-TK vectors eradicated human glioblastoma established in xenograft model mice

The anti-tumor activity of *tk* gene expression was evaluated in spRRV-TK-injected mouse xenograft model. Seven days after stereotaxic injection of U-87 MG cells into the striatum of the mouse brain, $4.5 \times 10^7$ gc of spRRV-TK or spRRV-FL vectors were injected intratumorally. Propagation of the virus was confirmed by examining the spRRV-TK vector-injected brain sections under a fluorescence microscope because the spRRV-TK vectors include sRRVgp-RFP as well spRRVe-TK. The sRRVgpRFP was detectable throughout the tumor but not in the normal mouse brain region as early as day 7 (data not shown). Mice were then given daily intraperitoneal injections of PBS or GCV for spRRV-TK injected mice and GCV for spRRV-FL injected mice, starting 14 days after injection of the vector and lasting for 30 consecutive days. Mice injected with spRRV-TK vectors and

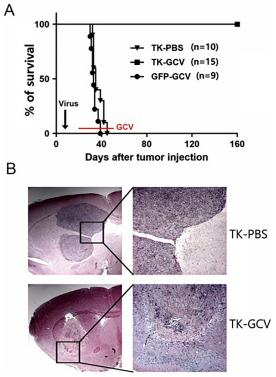


**Fig. 4.** Survival and histological analyses of glioblastoma-bearing mice after spRRV-TK gene therapy. (A) Survival of xenograft mice after spRRV-TK gene therapy. U-87 MG cells and viral vectors were stereotactically injected into the striata of BALB/c nude mice ($n = 34$). Survival curves were prepared using the Kaplan-Meier method. $P < 0.0001$. (B) Brain sections from the control group (spRRV-TK/PBS, 24 days after tumor establishment) and the GCV-treated group (spRRV-TK/GCV, 150 days after tumor establishment) were histologically analyzed by H&E staining.

treated with PBS showed a median survival time of approximately 35 days following tumor inoculation, and mice injected with spRRV-FL and treated with GCV showed a median survival time of 33 days. Whereas the GCV-treated spRRV-TK group (n = 15) showed tumor-free survival for a follow-up period of more than 150 days ($P < 0.0001$) (Fig. 4A). In this experimental mouse group, extensive necrosis was observed in the tumor lesions of the mouse brain, and as expected (22), the brains of the control group stained numerous mitotic tumor cells (Fig. 4B).

## DISCUSSION

Here, we report the construction of a pseudotyped, semi-replicating retroviral vector that largely enhanced the transduction efficiency of sRRV and thereby prolonged the survival of the glioblastoma model mouse. To improve the transduction efficiency of the retroviral vector, the previously reported sRRV was modified in two ways. First, one of the two retroviral vectors was altered to encode the GaLV envelope glycoprotein instead of the MuLV envelope glycoprotein. Second, the internal promoter (mCMV MIEP) for the therapeutic gene expression was inserted instead of IRES sequences. Splitting the RRV viral genome into two vectors and pseudotyping one viral vector with GaLV envelope glycoprotein would provide several advantages as compared with conventional RRV vectors. First, it can deliver multiple therapeutic genes at the same time into the target organs. Tumor tissue is a collection of heterogeneous cells with differences in cell markers, genetic abnormality, growth rate, and apoptosis (23). These characteristics of tumor tissue make it hard to treat cancer because some cells would escape from certain kinds of therapy and provide resistance. Delivery of multiple therapeutic genes at the same time would provide advantages on tumor treatment through the synergistic effect of each therapeutic gene (24, 25). Second, the relatively small size of the retroviral packaging vector enables to encode of large size of the therapeutic gene. Despite the efficient propagation of viral vectors with a stable viral genome, conventional RRVs bear limitations in the size of the therapeutic gene because of the compact nature of the MuLV genome. Only less than 1.2 Kb transgene including the internal promoter can be encoded in RRVs because the larger size of the therapeutic gene would reduce the propagation of the virus. In the case of the spRRV system, however, therapeutic genes of approximately 3 kb and 4.5 kb including a promoter are able to be inserted both in our sRRVgp and spRRVe vectors, respectively.

As in the *in vitro* culture models (Fig. 2A, B), brain tumor cells in the animal xenograft model continue to survive and produce viruses containing therapeutic genes efficiently until prodrugs such as GCV is treated, so it can be interpreted that the transmission of therapeutic genes can occur sufficiently to effectively kill cancer tissues. Consistently, FACS analysis using spRRV-FL showed that 84% of the cells in U-87 xenograft were transduced with spRRVe-GFP after 14 days of injection (Fig. 2C, D), and spRRV-TK vectors eradicated human glioblastoma established in xenograft model mice (Fig. 4A, B). Taken together, our study demonstrated for the first time that xenografted tumors can be efficiently eradicated even in animal models by semi-RRV vectors. In addition, our results suggested that the application area of cancer gene therapy can be expanded by using the spRRV system that delivers the HSV1-TK gene together with a cytokine gene or a CAR target antigen gene instead of the RFP gene.

## MATERIALS AND METHODS

### Animals

This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Korea Food and Drug Administration. All animal studies were reviewed and approved by the Animal Care and Use Committee of the Chungnam National University Animal Resource Center (permit number CNU-00822).

### Cell lines

U87-MG and 293T cells were obtained from American Type Culture Collection (ATCC, Bethesda, MD, USA) where cell line authentication and species identification were performed. Cells were grown in Dulbecco's modified Eagle medium with 10% fetal bovine serum and antibiotics. All cells were maintained in a humidified atmosphere with 5% $CO_2$ at 37°C.

### Retroviral vector production

The sRRV vectors (spRRV-FL, sRRV-FL, sRRV-TK and spRRV-TK) were produced by transient transfection of 293T-cells with the sRRV plasmid vectors using Lipofectamine Plus. After 48 h, the supernatant was harvested, filtered through 0.2 μm syringe filters, and stored at −80°C. Real-time PCR was performed with a Retrovirus Titer Set (TAKARA) to determine viral titers.

### Replication kinetics of RRV *in vitro*

Replication kinetics were analyzed *in vitro* by infecting human glioblastoma U-87 MG cells at 20% confluency in a 6-well plate with 8 μg/ml polybrene and $1.5 \times 10^6$ gc of sRRV-FL or spRRV-FL for 24 h. Cells were then split 1:5 at serial time points and aliquots were collected. GFP and RFP expression were analyzed by flow cytometry on a BD FACS Calibur.

### Replication kinetics of RRV in an intracerebral human glioblastoma model and FACS analysis

For the *in vivo* experiments, $2 \times 10^5$ U-87 MG cells were stereotactically injected into the left frontal lobe of 6-week-old female BALB/c nude mice (Orient Bio Inc, Korea). The propagation of sRRV-FL- and spRRV-FL-containing marker genes was analyzed by stereotactically injecting 10 μl of sRRV-FL or spRRV-FL into the brain tumor 7 days after tumor inoculation. The mice were then sacrificed and perfused 16 days after vector inoculation. The brains were dissected, embedded in

optimal cutting temperature (OCT) compound, and then frozen at −80°C for cryosection. Frozen blocks were sectioned at a thickness of 5 μm with a cryostat. Each slide was assessed by laser scanning confocal microscopy (Zeiss). Part of brain tumors dissected from these mice were chopped into small pieces with sterilized blades and were digested with 0.2% collagenase in PBS at 37°C for 2 h. Cells were harvested by centrifugation at 1,000 rpm for 3 min, resuspended in PBS, and then passed through a 100 μm cell strainer (BD Bioscience). The samples were analyzed by flow cytometry on a BD FACS Calibur for GFP fluorescence.

### Western blotting of RRV-TK-, sRRV-TK-, and spRRV-TK-transduced U-87 MG cells

To assess HSV-TK gene expression in RRV-infected cells, U-87 MG were transduced with $1 \times 10^7$ gc of sRRV-TK, spRRV-TK, or sRRV-FL. Cells were harvested 7 days after infection and were resuspended in lysis buffer composed of 150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 8.0), and a protease inhibitor cocktail (Gen Depot). Each sample was boiled in SDS loading dye and resolved by 10% SDS-PAGE. The proteins were then transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Immunoblotting was performed with a goat polyclonal anti-HSV-TK primary antibody for 2 h at room temperature (1:1000, Santa Cruz Biotechnology) and a peroxidase-conjugated donkey anti-goat secondary antibody for 1 h at room temperature (1: 5000, Santa Cruz Biotechnology). Bound antibodies were detected using the ECL Plus system (GE Healthcare Life Sciences, Amersham, UK). An α-tubulin antibody was used as an internal loading control (1:1000, Santa Cruz Biotechnology).

### *In vitro* cytotoxicity assay

The toxicity of GCV (Sigma) was determined *in vitro* by pre-transducing U-87 MG cells with spRRV-FL, sRRV-TK, or spRRV-TK at $1 \times 10^7$ gc. The cells were cultured for 10 days and then seeded onto 96-well plates in triplicate (2,000 cells/well). After 24 h, the transduced cells were exposed to 2 μg/ml of GCV for 5 days. The numbers of viable cells were measured daily by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.

### Animal PET-CT imaging of spRRV propagation

spRRV-TK was administered intratumorally at $4.5 \times 10^7$ gc 7 days after injection of U-87 MG cells into the mouse brain. On days 4, 7, 10, 14, and 17, animals were injected with 18.5 MBq $^{18}$F-FHBG via the tail vein. An hour after substrate uptake, $^{18}$F-FHBG PET-CT scans were performed in three-dimensional (3-D) acquisition mode (eXplore VistaCT, GE). For CT scans, the X-ray conditions were 250 μA and 40 kV for 6 min. The CT resolution was 200 μm, and the number of acquired projections was 360. For PET scans, normalization was applied, while scatter corrections and attenuation corrections were not. PET scans were acquired for 10 min per bed position for all studies. All images were reconstructed with iterative reconstruction (OSEM 2-D, 32 subsets, two interactions). Images were normalized as standardized uptake values (SUV) using the formula: SUV = decay-corrected mean tissue activity concentration (in Bq/ml)/[injected dose (in Bq) × body weight (in g)]. MR images were obtained by an animal MRI system (7 T Biospin, Bruker). Animal PET-CT and MRI systems shared one bed for PET-MR image fusion. All PET/CT/MR data analyses, including multimodal images and 3-D images, were compiled using OsiriX imaging software (www.osirix-viewer.com).

### Survival studies using the intracerebral glioblastoma model and histological analyses

Intracerebral U-87 MG glioblastomas were established as described above (n = 34). One week after the tumor inoculation, the animals were stereotaxically injected with spRRV-FL or spRRV-TK ($4.5 \times 10^7$ gc/10 μl). Two weeks after the vector inoculation, the spRRV-TK-injected animals were split into two groups. One group (n = 15) received daily intraperitoneal injections of GCV (100 mg/kg) for 30 days, while the other group (n = 10) received daily intraperitoneal injections of PBS for 30 days. spRRV-FL-injected mice (n = 9) received daily intraperitoneal injection of GCV (100 mg/kg) for 30 days. Experimental mice that survived for 150 days and control mice that survived for more than 30 days after tumor establishment were perfused. The brains were then dissected and embedded in paraffin. Paraffin blocks were sectioned at a thickness of 5 μm and the sections were stained with H&E.

### Statistical analyses

Student's t-tests were used for the cell viability and FACS experiments. Survival percentages were assessed using Kaplan-Meier survival analyses. Prism 5 statistical software was used for all analyses (GraphPad Software). P-values that were $< 0.05$ were considered to be statistically significant.

## ACKNOWLEDGEMENTS

This research was funded by grants from the Bio & Medical Technology Development Program of the National Research Foundation (NRF), funded by the Korean government (MSIT) (NRF-2018M3A9B5059544).

## CONFLICTS OF INTEREST

The authors have no conflicting interests.

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