# Supplementary Materials and Methods

## Cell lines

The baby hamster kidney cell line BHK-21 (ATCC-CCL-10) used for SFV rescue and propagation were cultured in Glasgow minimal essential medium supplemented with 10% foetal bovine serum (FBS), 20mM HEPES, 10% tryptose phosphate broth (BD Biosciences). Murine glioma cells GL261 (kind gift from Dr. Geza, Safrany, National Research Institute for Radiobiology and Radiohygiene, Hungary), GL261-luc ([1](#_ENREF_1)) and murine neuroblastoma cells NXS2 (kind gift from Dr. Lode, University of Greifswald, Germany) were cultured in DMEM supplemented with 10% FBS and 1% PEST. Murine glioma cells CT-2A and CT-2A-fLuc (kind gift form Dr. Markus Vähä-Koskela, Ottawa Hospital Research Institute, Canada) were cultured in RPMI-1640 medium supplemented with 10% FBS, 1% L-glutamine, 1% PEST. All patient-derived primary glioblastoma cells lines were obtained from Uppsala University Human Glioblastoma Cell cultures (HGCC). HGCC cells were cultured in serum-free defined Neurobasal and DMEM F:12 Glutamax medium (1:1 v/v) supplemented with B27, N2, 10ng/ml EGF (Peprotech), 10 ng/ml FGF (Peprotech) and 1% PEST on laminin coated petri dishes (BDPrimaria, BD Biosciences). Human neuroblastoma cell lines SHEP, SK-N-FI (ATCC-CRL-2142), SH-SY5Y (ATCC-CRL-2266), SK-N-SH (ATCC-HTB-11) and SK-N-BE(2) (ATCC-CRL-2271) were cultured in MEM with Earl’s salt and L-glutamine, supplemented with 10% FBS, 1% PEST and 1mM sodium pyruvate. HeLa and Mel526 (kind gift from T Boon, Ludwig Institute for Cancer Research, Brussels, Belgium) cells were cultured in DMEM supplemented with 10% FBS and 1% PEST. Murine neural stem cells (NSCs) isolated from new born mice were cultured in culture dishes coated with extracellular matrix (Sigma). NSCs were cultured in NSC basic medium containing DMEM/F12 Glutamax medium supplemented with 1% HEPES, 1% PEST, B27, 1mg/ml insulin (Sigma), supplemented with growth factors 20ng/ml EGF (Peprotech) and 20ng/ml FGF (Peprotech). Neurons were differentiated from NSCs using neuron differentiation medium (NSC basic medium without growth factors EGF and FGF) for 4 days. Astrocytes were differentiated from NSCs using astrocyte differentiation medium (DMEM Glutamax supplemented with 1% N2 supplement and 2% FBS) for 4 days. Oligodendrocytes were differentiated from NSCs using oligodendrocyte differentiation medium (NSC basic medium supplemented with 30ng/ml 3,3′,5-Triiodo-L-thyronine sodium salt (Sigma)) for 4 days. All cells were cultured at 37⁰C, 5% CO2/95% air in a humidified incubator. All medium and supplements were purchased from Thermo Fisher Scientific, except when stated otherwise. All cell lines were tested for mycoplasma contamination (Lonza). Human neuroblastoma cell lines were authenticated by short tandem repeat (STR)-profiling using AmpFℓSTR® Identifiler™ PCR amplification kit (Applied Biosystems) which amplifies 15 STR loci and the Amelogenin locus and compared to reference loci used by ATCC. STR profiles for the HGCC lines were recently published by Xie *et. al.*  ([2](#_ENREF_2)).

## Virus construction and production

All cloning was performed in the infectious plasmid of SFV, pCMV-SFV4 and pCMV-A7(74)wt ([3](#_ENREF_3)). The sequence corresponding to the subgenomic (SG) promoter of SFV4 (from position -37 to +17 with respect to the transcription start site) was inserted immediately downstream of the termination codon of the structural open reading frame (ORF) ([4](#_ENREF_4)). The enhanced green fluorescent protein (eGFP) or nano-luciferase (nLuc) reporter gene were cloned behind the second SG promoter to construct pCMV-SFV4-2SG-GFP, pCMV-A7/74-2SG-GFP, pCMV-SFV4-2SG-nLuc and pCMV-A7/74-2SG-nLuc. Two copies of complementary sequences to miRT124, miRT125 and miRT134 were inserted at the 3’ untranslated region (UTR) of the modified SFV genomes in the order described in Figure 1B.

Infectious plasmids of SFV, pCMV-SFV4 and pCMV-A7/74 with or without reporter and miRT were used to transfect BHK-21 cells by electroporation. Briefly, 5μg of infectious plasmid was mixed with 50μg salmon sperm DNA (Invitrogen) and 5x106 BHK-21 cells; electroporation was performed using Gene-Pulser II (BioRad, Hercules, CA) and the following conditions: 180-220V, 975µF. Electroporated cells were seeded on 10cm tissue culture dishes and overlaid with fresh medium. Recombinant virus stocks were collected 24 hours post-transfection, concentrated by layering virus-containing supernatant on a 20% sucrose cushion and ultracentrifugation for 3 hours at 25,000 rpm. Virus pellet was resuspended in PBS and titrated using a plaque forming assay as described earlier ([5](#_ENREF_5)).

## Validation of SFVmiRT attenuation

Murine NSCs and *in vitro*-differentiated murine neurons, astrocytes and oligodendrocytes cultured in a 24-well plate were infected with 2000 PFU/cell SFV4-GFP or SFV4-GFPmiRT for 16 hours. GFP-positive cells were quantified 16 hours post-infection using flow cytometer (FACS Canto II, BD Biosciences). Furthermore, cells were stained for cell specific markers using mouse anti-NESTIN, mouse anti-MAP2, mouse anti-GFAP and mouse anti-CNPase primary antibodies. They were then probed with goat anti-mouse A680 secondary antibody (Thermo Fisher Scientific) and imaged in Leica SP-8 confocal microscope (Leica microsystems). BHK-21 cells were plated in 24-well plates at density 50,000 cells/well. Cells were transfected with 40nM miRNAs (miR124, miR125, miR134 or mix of all three) or unrelated miRNA (miRneg) using INTERFERIN transfection reagent (PolyPlus, Illkirch, France). After 4 hours of incubation, cells were washed and infected with SFV4-GFP, SFV4-GFPmiRT, A7/74-GFP or A7/74-GFPmiRT viruses at 0.1 plaque-forming units (PFU) per cell. GFP-positive cells were quantified 48 hours post infection using flow cytometer (FACS Canto II, BD Biosciences).

## *In vitro* cell killing assay using human neuroblastoma cell lines

Human neuroblastoma cell lines SHEP, SK-N-FI, SK-N-BE(2), SH-SY5Y, SK-N-SH were seeded at densities 20,000 cells/well (96 well plate) and infected with SFV4, SFV4miRT, A7/74 or A7/74miRT at a multiplicity of infection (MOI) range of 0.001-10 PFU/cell. Cell viability was analyzed 2 days post infection using 20µl MTS aqueous cell titer reagent (Promega).

# Supplementary references

1. Zhang L, Kundu S, Feenstra T, Li X, Jin C, Laaniste L, et al. Pleiotrophin promotes vascular abnormalization in gliomas and correlates with poor survival in patients with astrocytomas. Sci Signal. 2015;8:ra125.

2. Xie Y, Bergstrom T, Jiang Y, Johansson P, Marinescu VD, Lindberg N, et al. The Human Glioblastoma Cell Culture Resource: Validated Cell Models Representing All Molecular Subtypes. EBioMedicine. 2015;2:1351-63.

3. Ulper L, Sarand I, Rausalu K, Merits A. Construction, properties, and potential application of infectious plasmids containing Semliki Forest virus full-length cDNA with an inserted intron. J Virol Methods. 2008;148:265-70.

4. Rausalu K, Iofik A, Ulper L, Karo-Astover L, Lulla V, Merits A. Properties and use of novel replication-competent vectors based on Semliki Forest virus. Virol J. 2009;6:33.

5. Ratnik K, Viru L, Merits A. Control of the rescue and replication of Semliki Forest virus recombinants by the insertion of miRNA target sequences. PLoS One. 2013;8:e75802.

**Supplementary Tables**

## Supplementary Table 1: miR124, miR125 and miR134 expression relative to human or mouse U6 snRNA in normal mouse CNS tissues, mouse neural stem cells, *in vitro*-differentiated mouse brain cells, mouse neuroblastoma, human neuroblastoma and in human glioblastoma cell culture (HGCC) lines.

|  |  |  |  |
| --- | --- | --- | --- |
| **Tissue/Cell lines** | **miR124** | **miR125** | **miR134** |
| **Mouse brain** | 0.5425 | 10.9614 | 1.0391 |
| **Mouse spine** | 0.2522 | 10.0576 | 0.0892 |
| **Mouse dorsal root ganglion (DRG)** | 0.5300 | 6.0640 | 0.1358 |
| **Mouse Neural Stem cells** | 0.0120 | 0.1400 | 0.0553 |
| **Mouse Neurons** | 0.6664 | 0.3716 | 0.1146 |
| **Mouse Astrocytes** | 0.0122 | 0.6259 | 0.1878 |
| **Mouse Oligodendrocytes** | 0.0098 | 0.4582 | 0.6482 |
| **NXS2 (mouse neuroblastoma)** | 0.0042 | 0.4435 | 0.0069 |
| **GL261 (mouse glioblastoma)** | 0.0031 | 0.1249 | 0.0079 |
| **CT-2A (mouse glioblastoma)** | 0.0104 | 0.4630 | 0.0063 |
| **SH-SY5Y (human neuroblastoma)** | 0.0246 | 0.4688 | 0.0137 |
| **SK-N-BE(2) (human neuroblastoma)** | 0.0026 | 0.0343 | 0.1454 |
| **SK-N-SH (human neuroblastoma)** | 0.0172 | 0.1534 | 0.1791 |
| **SK-N-FI (human neuroblastoma)** | 0.0877 | 0.1660 | 0.0621 |
| **U3034 (human glioblastoma)** | 0.2387 | 2.5529 | 0.3988 |
| **U3031 (human glioblastoma)** | 0.0300 | 0.6541 | 0.2171 |
| **U3028 (human glioblastoma)** | 0.0255 | 0.9765 | 0.2261 |
| **U3024 (human glioblastoma)** | 0.0653 | 2.2590 | 0.9052 |
| **U3021 (human glioblastoma)** | 0.0513 | 1.4561 | 0.2667 |
| **U3053 (human glioblastoma)** | 0.0088 | 0.5241 | 0.0509 |
| **U3016 (human glioblastoma)** | 0.0077 | 0.3049 | 0.0436 |
| **U3065 (human glioblastoma)** | 0.0413 | 2.2268 | 0.1795 |
| **U3101 (human glioblastoma)** | 0.0910 | 0.7083 | 0.3344 |
| **U3054 (human glioblastoma)** | 0.0143 | 0.1313 | 0.0870 |
| **U3013 (human glioblastoma)** | 0.0026 | 0.2596 | 0.0208 |
| **Human Astrocytes** | 0.0117 | 0.2048 | 0.0407 |
| **Hela (human cervical cancer)** | 0.0191 | 0.8111 | 0.0155 |
| **Mel526 (human melanoma)** | 0.0436 | 0.1607 | 0.0298 |

## Supplementary Table 2: Comprehensive information of HGCC lines used in the experiments

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Cell line | GBM Subtype | Sex | Age | Passage of cells when used in experiment |
| U3013 | Proneural | F | 78 | 17 |
| U3016 | Proneural | M | 70 | 16 |
| U3021 | Neural | M | 50 | 17/20 |
| U3024 | Mesenchymal | F | 73 | 11/12 |
| U3028 | Classical | F | 72 | 16/17 |
| U3034 | Mesenchymal | M | 73 | 20 |
| U3053 | Mesenchymal | M | 64 | 10/16 |
| U3054 | Mesenchymal | F | 60 | 19/20 |
| U3065 | Mesenchymal | M | 77 | 13 |
| U3101 | Mesenchymal | M | 73 | 14/15 |
| U3213 | Mesenchymal | F | 77 | 18 |

# Supplementary Figures



Supplementary Figure S1. **SFV4-GFPmiRT and A7/74-GFPmiRT are inhibited in the presence of exogenous miR124, miR125 or miR134.** BHK-21 cells were transfected with 40nM neural-specific miRNAs (miRNA124, miR125, miR134 or mix of all three miRNAs), or as controls were transfected with unrelated miRNA (miRneg). At 4 hours post transfection media was removed, cells were washed and infected with SFV4-GFP, SFV4-GFPmiRT, A7/74GFP or A7/74-GFPmiRT at 0.1 PFU/cell. GFP-positive cells were quantified 48 hours post-infection using flow cytometry. Data are presented as %mean±SD GFP+ cells of total (n=3). Statistical comparison of means was assessed using Two-way ANOVA, with Tukey post-test for correcting multiple comparisons (\*\*\*\* = p<0.0001, \*\*=p<0.01, n.s =p>0.05).



Supplementary Figure S2. **SFV infection induces killing of human neuroblastoma cell lines.** *In vitro* killing ability of SFV4, SFV4miRT, A7/74 and A7/74miRT on human neuroblastoma cell lines SK-N-BE(2) **(A)**, SK-N-FI **(B)**, SH-SY5Y **(C)**, and SK-N-SH **(D)** were analyzed at MOI 0.001-10. Cell viability was measured using MTS at 48 hours after infection and values represent viability normalized to that of mock-infected cells. Data are presented as mean±SD (n=2, with 3 internal replicates).



Supplementary Figure S3. **Oncolytic efficiency of A7/74miRT in murine tumor models *in vitro* and *in vivo*.** **(A-C)** *In vitro* killing ability of A7/74 and A7/74miRT on murine neuroblastoma cells NXS2 **(A)**, murine glioma cells GL261 **(B)** and murine glioma cells CT-2A **(C)** at MOIs 0.001-10. Cell viability was measured using MTS at 72 hours post-infection and values represent viability normalized to that of un-infected cells. Data are presented as mean±SD (n=2, with 3 internal replicates). **(D-G)** NXS2 neuroblastoma cells were injected s.c. in the right hind flank of female A/J mice. Mice were treated when they had palpable tumors 7 days after tumor inoculation. **(D)** Tumor size of individual mice and **(E)** Kaplan-Meier survival curves for mice treated i.t. either with 50µl PBS (n=10) or A7/74miRT (n=7, 5×107 PFU). **(F)** Tumor size of individual mice and **(G)** Kaplan-Meier survival curves for mice treated i.v. with either with 100µl PBS (n=10) or A7/74miRT (n=8, 1×107 PFU). The survival curves were compared to PBS-treated mice by performing a Log-rank (Mantel-Cox) test (\*\*\*\* = p< 0.0001).



Supplementary Figure S4**. SFV4miRT-mediated killing of HGCC lines *in vitro* in the presence and absence of IFN-β.** *In vitro* cell-killing ability of SFV4miRT was assayed on eleven HGCC lines: U3013, U3016, U3021, U3024, U3028, U3034, U3053, U3054, U3065, U3101 and U3213 in the presence of exogenous human IFN-β (10ng/ml, 1ng/ml, 0.01ng/ml or no IFN-β) at MOIs 0.01-1. Cell viability was measured using AlamarBlue at 72 hours post-infection; values of graphs represent viability of infected cells normalized to that of mock-infected cells cultured in medium without human IFN-β. Data are presented as mean±SD (n =2, with 3 internal replicates).