Supplementary methods

Cell Culture and Establishment of Fluorescent-Labeled Cell Lines. The U87MG, U118MG, U251 and A172 human glioma cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained according to vendor recommendations. U87MG and U118MG cells were cultured in MEM (minimum essential medium) (HyClone, Thermo Fisher Scientific, Waltham, MA, USA), while U251MG and A172 cells were cultured in DMEM (Dulbecco's modified Eagle's medium) containing 2% penicillin-streptomycin antibiotic (Cellgro; Mediatech, Manassas, VA) and 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA). All cells were grown in a humidified atmosphere, with 5% CO_2 and 37^{o}C conditions. All cell lines were sub-cultured for experimentation using 1ml/10^6 cells 0.25% trypsin/2.21mmol/1 EDTA solution (cat. No. 25-053-CI; Mediatech). Trypsin activity was quenched using the appropriate media for each cell type. Cells were then washed at 300 relative centrifugal forces (rcf) and plated at the indicated densities.

Human primary brain tumor specimens (GBM43 and GBM12) were obtained from Dr. David James (UCSF) in accordance to a protocol approved the by IRB at UCSF. Tumor specimens were confirmed as World Health Organization grade IV malignant glioma by an attending neuropathologist. All human tissue specimens were treated with 1% hyaluronidase (Sigma-Aldrich, St. Louis, MO, USA) and 2% collagenase (Sigma-Aldrich) enzymes and subsequently minced through 70 μm strainers. After several washings in phosphate-buffered saline (PBS) solution, cells were then cultured in flasks containing neural basal media (NBM) (Invitrogen) supplemented with 100μg mL^{-1} ampicillin/streptomycin and 20ng mL^{-1} each of EGF (epidermal growth factor)

(Millipore, Billerica, MA, USA) and bFGF (basic fibroblast growth factor) (Millopore). Cells were maintained in a humidified atmosphere containing 5% CO_2 at 37^{o}C.

Following previously described protocols (12,30), we established and maintained a human GBM xenograft panel using the above depicted cell lines (GBM43 and GBM12). Shortly, to maintain these cell lines exclusively in animals, patient tumor specimens were implanted into the flank of nude mice and then serially passaged as heterotopic tumors. For primary cell culture and *in vitro* analyses, flank glioblastoma tissues were minced through 70 μm strainers, mechanically disaggregated and cultured in flasks coated with growth factor reduced Matrigel (Fisher Scientific, Hampton, NH, USA). After several washings in phosphate-buffered saline (PBS) solution, cells were cultured in DMEM 1% FBS containing 2% penicillin-streptomycin antibiotic (Cellgro; Mediatech, Manassas, VA).

Human ReNcells (NSCs) were obtained from Millipore (Temecula, CA) and maintained according the manufacturer's protocol. Briefly, these NSCs were isolated from the cortical region of 14-week-old fetal tissue and immortalized by retroviral transduction and insertion of the *c-myc* gene. Cells were characterized according to the expression of nestin, SOX-2, CD133, and CD44 (data not shown) stem cell markers. Subcultures of human NSCs for experimentation were conducted as follows: tissue culture plastic dishes were coated with laminin (Sigma-Aldrich, St Louis, MO) at a concentration of 20μg/ml in serum-free DMEM in 37°C and 5% CO_2 atmospheric conditions 4 hours before NSC plating. NSCs were detached from plastic dishes using 1ml/10^6 cells of Accutase (Millipore), centrifuged at 300 rcf for 5 min, resuspended in ReNcell NSC Maintenance Medium (Millipore), supplemented with 20ng/ml bFGF

(Millipore) and 20ng/ml EGF (Millipore), and seeded at the indicated cell densities.

HB1.F3-CD, a v-myc immortalized human NSC line that constitutively expresses cytosine deaminase (CD), was extracted from human fetal brain (31). These NSCs were maintained in adherent cultures in DMEM supplemented with 10% FBS (Atlanta Biologicals, Lawrenceville, GA), 2mmol l^{-1} L-glutamine, 100 units ml^{-1} penicillin, 100μg ml^{-1} streptomycin (Invitrogen, Carlsbad, CA).

To detect the distribution of NSCs *in vivo*, we generated both GFP labeled ReNcell and HB1.F3-CD cell lines. In short, cells were seeded at a density of 5 x 10^4 cells/well (or 50-60% confluence) in six-well plastic culture dishes (Becton Dickinson, Franklin Lakes, NJ). One day after plating, cells were incubated for 24h with replication-deficient lentiviral vectors containing GFP expression cassettes. For the establishment of stable clonal populations, 48 hours post-transduction, medium was replaced with fresh DMEM 10% FBS containing 4μg ml^{-1} puromycin (Sigma-Aldrich). Following selection, FACS was performed to verify GFP expression in HB1.F3-CD NSCs.

To detect tumor location and track tumor volume post-therapy *in vivo*, stable and fluorescently labeled glioma cell lines were established as follows. Using the same protocol depicted above, U87MG and GBM43 glioma cell lines were incubated for 24 hours with replication-deficient lentiviral vectors containing F-luciferase (Fluc) expression cassettes. After 48 hours, media was replaced with fresh culture media appropriate for each cell type containing 1 μg/ml puromycin (Sigma-Aldrich) for the establishment of stable clonal populations.

To detect NSCs migration *in vivo* via MRI (Magnetic Resonance Imaging),

HB1.F3-CD NSCs were labeled with MPIO contrast reagent. Succinctly, MPIO reagent from Bangs Laboratories (Fisher, IN, US) (1 mm diameter) was transfected overnight into HB1.F3-CD NSCs, using Fugene transfection reagent (Roche) and Opti-MEM reduced serum medium (Life Technologies) in 37°C and 5% CO_2 at in a ratio of 17 particles per cell. Cells were then returned to their recommended medium and posteriorly used for intracranial injection in U87MG tumor-bearing mice.

Analysis of the Optimal *Ex-vivo* Loading. The total number of cells to be injected *in vivo* was based on our previous studies, where we reported that infection with 50 I.U. per cell of CRAd-S-pk7 viruses resulted in maximum progeny released over time with minimum toxicity to carrier cells and proved superior survival benefit to glioma-bearing mice (8). To optimize the *ex-vivo* loading protocol, we first examined infection efficiency of CRAd-S-pk7 virus. For this, cell suspension and monolayer of HB1.F3.CD cells were incubated with DMEM 10% FBS containing 50 I.U. per cell of CRAd-S-pk7 viruses for 1, 2 and 4 h. Infected cells were washed and cultured for 24 h. Cells were then harvested and subjected to fluorescence-activated cell sorting analysis with goat anti-hexon FITC-conjugated antibody (Millipore), and the measurement of viral DNA replication was done by the PCR method as described previously.

Evaluation of Relative Gene Expression by qRT-PCR. Total cellular RNA was isolated using an RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol and in each instance 1μg of purified mRNA was reverse transcribed to complementary DNA using the iScript cDNA conversion kit (Bio-Rad). Quantitative PCR was conducted using the SYBR Green quantitative PCR kit (Invitrogen, Carlsbad, CA) for all experiments. Optimization of annealing temperatures for each transcript was

first conducted. Each transcript of interest was amplified in triplicates at its proper annealing temperature and products were analyzed using the Opticon 2 software (Bio Rad, Hercules, CA). Amplification of the correct product, together with confirmation of product size was verified by running samples on 2% agarose gel electrophoresis. Relative expression was evaluated using the ΔCT method (ΔCT = *CT* gene of interest − *CT* GAPDH) where a ΔCT of 3.33 is equivalent to one magnitude change in gene expression. This logarithmic dependence was verified for each gene studied by conducting quantitative PCRs on serial complementary DNA dilutions. Expression data are presented as fold change of the linearized ΔCT (2−ΔCT) over control expression levels.

Flow Cytometry Analysis of Protein Expression. Cells were labeled and analyzed for both surface and intracellular markers as previously described (7). Briefly, cells were permeabilized, fixed and stained on ice using the Cytofix/Cytoperm buffer (BD Biosciences, San Jose, California) according to manufacturer's instructions. The following antibodies were used: rabbit polyclonal anti-GFAP (Abcam, Cambridge, MA), Data was acquired and analyzed using Canto with CellQuest (Becton Dickinson) and FlowJo (TreeStar, Ashland, OR) software.

Evaluation of NSC Migration and Viral Delivery *in Vitro*. To analyze the migratory capacity and oncolytic adenovirus delivery characteristics of HB1.F3-CD and ReNcells *in vitro*, we used a similar system to that described earlier (33), with a slight modification. To characterize the specificity of each stem cell carrier migration in response to glioma, we used a BD Biocoat Tumor Invasion System (BD Biosciences, http://www.bdbiosciences.com) containing BD Falcon Fluoroblock 24-Multiwell inserts (8-μm pore size; PET membrane) in accordance with the manufacturer's protocol. To aid

in quantification of stem cell migration, fluorescently labeled HB1.F3-CD and ReNcells (described above) were used. The migration was characterized with respect to four different conditioned media and a negative control. Conditioned medium was obtained by culturing 1 x 10^5 cells of each cell type (GBM43, GBM12, U118MG, U87MG, U251MG and A172) in serum-free/growth factor-free medium for 24 h, after which equal amounts of each conditioned medium was aliquoted in the bottom wells of the migration chamber to serve as a chemo attractant. For migration studies without adenovirus, HB1.F3-CD and ReNcells were plated in Serum-free MEM at a density of 5 x 10^4 cells/well. Twenty-four hours after plating HB1.F3-CD and ReNcells into the top insert, the number of migrating cells/field view was determined using an Olympus IX81 inverted microscope and MetaMorph software (Olympus, Tokyo, Japan). Cells were counted in three random field views/ well (original objective: 10x). A total of 4 wells were used for each experimental condition (i.e., U118MG = 4 wells).

For studies involving HB1.F3-CD and ReNcells-mediated delivery of adenoviruses, the same migration apparatus was used; however, HB1.F3-CD and ReNcells were loaded with different I.U. of CRAd-S-pk7 virus prior to being plated in the top chamber of the migration apparatus (5 x 10^4 cells/well). Instead of conditioned medium, U87MG cells were plated in the bottom wells of the migration chamber in serum-free MEM supplemented with 20 ng/mL of bFGF and EGF at a density of 5 x 10^4 cells/well two days before NSCs were plated in the top well of the migration chamber. The number of migrating cells was assessed as described above. Non-loaded HB1.F3-CD and ReNcells were plated in chambers immersed in serum-free MEM as a reference control. Nine days after plating loaded NSCs in the top inserts, we quantified the number

of infectious units in each of the bottom wells (4 wells/experimental condition) using the Adeno-X Rapid Titer Kit as described above. Cytotoxicity resulting from stem cell release of viral progeny was quantified by counting the number U87MG glioma spheroids/field view (4 original objective) using the same Olympus IX81 inverted microscope. Three random field views per well were captured. There were a total of 4 wells per experimental condition.

Animal Studies. Intracranial glioma xenograft implantation: U87MG glioma cells were implanted *via* cranial guide screws as described previously. Briefly, mice were anesthetized with a ketamine/xylazine mixture (115/17 mg/kg), and a burr hole was made. Stereotactic injection was carried out by using a 10 µl Hamilton syringe (Hamilton, Reno, NV) with a 30-gauge needle, which was inserted through the burr hole to a depth of 3mm mounted on a mice-exclusive stereotactic apparatus (Harvard Apparatus, Holliston, MA). Male athymic/nude mice were obtained from Charles River Laboratory (Wilmington, MA). Animals were cared for according to a study-specific animal protocol approved by the University of Chicago Institutional Animal Care and Use Committee.

To detect loaded HB1.F3-CD and ReNcells *in vivo*, mice were injected with 3 x 10^5 U87MG cells in 2.5µL of PBS/ mouse. Five days later, mice were randomly divided into 6 groups (n = 6-9 mice/group) that received the following injections: one group received an injection of 2.5µL of PBS/mouse (Mock); one group received an injection of 1 x 10^5 HB1.F3-CD NSCs in 2.5µL of PBS/mouse; one group received an injection of 1 x 10^5 ReNcells in 2.5µL of PBS/mouse; one group received 2.5µL injections of 1 x 10^5 HB1.F3-CD NSCs infected with 5 I.U. of CRAd-S-pk7/ mouse (NSC + Virus); and one group received 2.5µL injections of 1 x 10^5 ReNcells infected with 5 I.U. of CRAd-S-

pk7/mouse (NSC + Virus). U87MG glioma cells were detected via luciferase expression, and HB1.F3-CD and ReNcells were detected by GFP expression.

Three mice from each group were sacrificed, and their brains were flash frozen in OCT solution at days six, nine, and twelve after the second round of injections (thirteen, sixteen, and nineteen days after U87MG injection). Brains underwent serial coronal sectioning (6μm/section) for a total of 20-25 slices per tissue, altogether spanning approximately 3 mm of brain tissue. Slices were fixed (4% paraformaldehyde, 10 min) and mounted on glass slides using Prolong Gold Antifade Reagent (Invitrogen). Fluorescent microscope analysis was performed using a Zeiss 200 M Axiovert inverted microscope (Carl Zeiss, Inc., Oberkochen, Germany). U87MG tumors (Luciferase) were detected by using anti-CD44 antibody and HB1.F3-CD and ReNcells (GFP) were detected by using a GFP optical band-pass filter. Fluorescent images were analyzed and rendered for publication using Openlab v5.0 (Improvision, Coventry, England) and Adobe Photoshop CS2 (Adobe Systems, Inc., San Jose, CA).

To evaluate the therapeutic efficacy of NSCs loaded with CRAd-S-pk7 oncolytic virus (OV), six groups of seven nude mice were implanted with U87MG cells (5×10^5 cells in 2.5μL of PBS/mouse into the right hemisphere as previously described). Five days post-tumor implantation, mice received an intracranial, intratumoral injection of 5×10^5 NSCs loaded with OV (50 I.U./cell) or an equal dose of naked virus. Both the stem cells were incubated with the oncolytic adenovirus for 2 h at room temperature, washed 3 times with PBS, resuspended in PBS ($5\ 10^5$ stem cells in 2.5μL/mouse) and injected intratumorally in the right hemisphere. Animals losing ≥30% of their body weight or having trouble ambulating, feeding, or grooming were euthanized by CO_2 followed by

cervical dislocation.

Immunohistochemistry Staining for *In Vivo* Evaluation of Cell Migration. For immunohistochemistry, brains were sectioned in 10-mm thick sections. After thawing, sections underwent fixation/permeabilization with a solution of 50/50 acetone-methanol, at -20^{o}C for 5 min. The slides were washed with ice-cold PBS and blocked with 10% bovine serum albumin for 30 min. They were then incubated overnight at 4^{o}C with primary antibodies, followed by 1h incubation at room temperature with the secondary antibody. After washing the excess antibody, slides were mounted with Prolong Gold antifade reagent with 4,6-diamidino-2-phenyl indole (Invitrogen). Fluorescent images were documented with an inverted Axiovert200 Zeiss microscope (Carl Zeiss Microscopy, Thornwood, NY). In this process, the following antibodies were used: FITC-conjugated anti-GFP antibody, biotin-conjugated anti-hexon and FITC-conjugated immunoglobulin controls were purchased from Abcam; human CD44 rabbit monoclonal antibody purchased from Epitomics (Burlingame, CA); AlexaFluor555-streptavidin and Alexafluor350 donkey anti-rabbit were purchased from Invitrogen.

***In Vivo* Bioluminescence Imaging.** Mice were imaged for Fluc (Luciferase) activity by intraperitoneal injection of D-luciferin (4.5 mg/ animal in 150μL of saline), and photon counts were recorded 10 min after D-luciferin administration by using a cryogenically cooled high efficiency charged-coupled device camera system (Xenogene).

NSC labeling with Microparticles of Iron Oxide (MPIO). The properties of NSC's that we evaluated were the loading efficiency and retention time, cell viability, the effect on differentiation status, and the loading effect on tumor tropic migration; all in relation

to varying doses of MPIO's, introduced into the NSC's using a Lipofectamin (Fugene, Roche) based method.

First we wished to evaluate the relationship between cell number increase and MPIO marked cells (MPIO loading efficiency), to see if the cells were receiving the MPIOs and dividing with them still embedded, or if the cell divisions caused a total loss of MPIO's. A total loss of MPIOs would indicate that any divisions of the NSCs before they reached the disseminated tumor burden would cause a loss of signaling, hampering tracking. Loading efficiency was tested using FACS (fluorescence activated cell sorting), with APC-conjugated nanoparticles. The NSCs were transfected 16 hours in advance, and then labeled with violet crystal staining, a dye which becomes more diluted as the cell divides more (as measured by the PacBlue %).

We found there to be a loading threshold between 17 nanoparticles per cell and 34 nanoparticles per cell. Literature seems to indicate that about 20 nanoparticles per cell corresponds with a high percentage of nanoparticle uptakes, which explains the threshold. There is a visible increase in cell division over the two-day period, as well as a corresponding decrease in number of APC-conjugated cells over the same timeframe. This would indicate some relationship between the decreases in the percentage of nanoparticle labeled cells with the cell divisions, as measured by PacBlue levels. We plan to repeat the findings, to optimize the APC levels.

We also evaluated cell viability using the Trypan Blue exclusion method. Different dosages were evaluated, using 0 nanoparticles per cell as a control, with 8.5, 17 and 34 nanoparticles per cell as the corresponding dosages. Looking at the data, the take

home message is that there was no observable decrease in cell viability over the two-day period post MPIO loading. There were even significant increases in cell number for some dosages. Room remains for the cell viability to be evaluated in a longer timeframe.

Loading effect on migration was evaluated using qRT-PCR (quantitative real time polymerase chain reaction. Due to difficulties with the machinery, we were not able to evaluate many markers, however, the results for Nestin (stem cell marker), Oct4 (neuro progenitor cell marker), Tuj1 (neuron marker) and VEGFR (marker for migrations) showed no dramatic difference between the control and various dosages of nanoparticles. We can see that VEGFR expressed highly at the RNA level, which indicates no decline in the tumor tropic migrations, meaning that nanoparticles do not inhibit migration. Further validation of protein expression will follow.

***In Vivo* MRI Imaging.** MPIO is an important functional imaging tool known to create a potent hypointense contrast effect on MRI imaging (34). In our study, HB1.F3-CD NSCs previously labeled with MPIOs were tracked by magnetic resonance imaging *in vivo*. Nude mice previously injected or not with U87MG glioma cells on the right brain hemisphere and MPIO-labeled HB1.F3-CD NSCs on the contralateral (left) brain hemisphere were studied two days post-NSC injection on a 33 cm horizontal bore Bruker 9.4 T small animal scanner with a Bruker console (Bruker-Biospin, Billerica, MA). The machine was equipped with a 12 cm shielded gradient set with a maximum strength of 600 mT/m and was available through the University of Chicago Core Facility. Prior to MRI study, animals were anesthetized by 2% isoflurane in oxygen and fixed in a prone position during scanning. In order to achieve sufficient resolution to visualize labeled NSCs within the mouse brain, a multi-slice axial and coronal T1/T2-weighted A Fast

Low Angle Shot (FLASH) gradient echo sequence was acquired using the following parameters: flip angle 30^{o}; echo time (TE), 4.9 ms; time of repetition (TR), 200ms; NEX, 4; slice thickness, 0.5 mm; matrix size, 256 x 256; field of view (FOV), 2.56 x 2.56 cm. Same level slices were used to track and compare NSCs migration towards U87MG in tumor bearing mice.

SUPPLEMENTARY RESULTS:

Initial characterization of the NSC lines and their permissiveness for oncolytic adenovirus

i) Phenotypic characteristics of the NSC lines: To identify an optimal NSC-based carrier, we first characterized the two neural stem cell lines HB1.F3.CD and ReNcell. The HB1.F3 cells were isolated from fetal human telencephalon cells (at 15 weeks gestation) and subsequently immortalized by retrovirus mediated stable integration of the v-myc gene to create a multipotential neural stem cell line (35). This cell line was further modified by retrovirus mediated insertion of the E. coli cytosine deaminase (CD) gene as a suicide gene therapeutic system and is currently being evaluated in a human phase I clinical trial in patients with recurrent high-grade glioma (2). ReNcells were purchased from Millipore and isolated from the ventral mesencephalon region of human fetal brain. This cell line was also immortalized by retroviral transduction with the v-myc oncogene.

Both cell lines expressed high levels of the pluripotency genes Sox-2, Oct4 and the neural progenitor marker nestin at the mRNA and the protein level (Supplementary figure SF 1A, 1B and 1C). However, the ReNcell line expressed significantly elevated levels of nestin protein per cell bases as indicated by the mean fluorescent intensity (MFI; SF 1B),

CD133 mRNA (SF 1A) and protein (data not shown) levels. Bars represent the mean values from three independent experiments, error bars refer to 95% confidence intervals Almost 92.8% of ReNcells were positive for GFAP, which is indicative of an astrocytic lineage. On the other hand, HB1.F3.CD cells expressed high levels of Musashi 1 and the neural marker beta-III tubulin transcripts and expressed no CD133 on the transcription (SF 1A) or protein level or (data not shown). The immortalized cell lines also retained their functional neural stem cell characteristics such as the ability to form neurosphere like structures and differentiate into neurons, astrocytes or oligodendrocytes in the presence of the appropriate growth factor conditioned media.

ii) Permissiveness to oncolytic virus: In order to function as an effective cell carrier for anti-cancer oncolytic virotherapy, a cell system must not only be susceptible to therapeutic viral infection, but also capable of supporting viral replication and amplifying the therapeutic payload at the target site. To identify an optimal NSC-based carrier, we first compared the cell lines HB1.F3.CD and ReNcell for their permissiveness to the glioma tropic oncolytic adenovirus CRAd-S-pk7, and assessed their ability to support viral replication *in vitro*. Fluorescent Activated Cell Sorting (FACS) analysis was conducted to assess the permissiveness to CRAd-S-pk7 infection by examining the expression of adenovirus binding and internalization receptors on the NSCs. The ReNcells expressed significantly higher levels of initial virus attachment Coxsackie Adenovirus Receptor (CAR) (ReN vs. HB1.F3.CD, 97.5% vs. 13.4% difference=84.1%, 95% CI=83.4%-84.8%, P<0.001) and the cell entry receptor integrin $\alpha_v\beta_3$ (ReN vs. HB1.F3.CD, 42.5% vs. 16.6% difference=25.9%, 95% CI=24.2%-27.6%, P<0.001), as well as Syndecan-1 (ReN vs. HB1.F3.CD, 69.8% vs. 5.0% difference=64.8%, 95% CI=63.7%-65.9%, P<0.001) but expressed almost identical

levels of integrin $\alpha_v\beta_5$ and Perlecan as compared to HB1.F3.CD cells (SF 2A & 2B). Next, we examined the expression of heparan sulfate proteoglycan (HSPG) (the pk7 retargeted attachment receptors for CRAd-S-pk7) expression on the NSCs by FACS or reverse transcriptase polymerase chain reaction (RT-PCR) with results shown in SF 2A, 2B, & 2C From this data we concluded that even though ReNcells expressed a significantly higher level of the primary attachment receptor CAR for adenovirus entry, HSPGs were expressed by both of the tested NSC lines and should allow the entry of CRAd-S-pk7.

Replication kinetics of CRAd-S-pk7 in NSC carriers

The glioma restricted oncolytic virus CRAd-S-pk7, consists of two genetic modifications: (i) a fiber modification containing polylysine that binds with high affinity to HSPGs and (ii) E1A transcription under the control of the survivin promoter (11,36). Thus, replication initiation of CRAd-S-pk7 in the host cell population is dependent on the activity of the survivin promoter.As shown in SF 3A, survivin mRNA levels is significantly elevated in HB1.F3.CD cells as compared to ReNcells. Moreover, we observed higher survivin transcript levels in the three tested glioma cell lines than the two NSC lines. Next, to evaluate the replication kinetics of CRAd-S-pk7 in NSCs, we first examined the mRNA level of replicative essential adenovirus genes by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). We observed that HB1.F3.CD cells exhibit a higher mRNA transcript level of E1B (SF 3B-II, HB1.F3.CD vs. ReN, 60.43 vs. 6.16 difference=54.27, 95% CI=3.52-105, P=0.04) as well as the fiber protein (SF 3B-IV) (HB1.F3.CD vs. ReN, 6.87 vs. 0.24 difference=6.63, 95% CI=-6.32-

19.57, P=0.16) at 4 days post infection (d.p.i.) compared to ReNcells. Additionally, we saw an elevated level of adenovirus EIA copies (data not shown).

Next, we established an optimal *ex vivo* OV loading dose by infecting NSCs with differing doses of CRAd-S-pk7 (0.1 I.U./cell-100.0 I.U./cell) and cells were harvested and subjected to total titer evaluation at 3 d.p.i. HB1.F3.CD cells produced a similar total viral progeny from the doses of 1 I.U./cell to 100 I.U./cell whereas ReNcells produced a maximum total viral progeny at the loading dose of 50 I.U./cell, which was over a third of a fold lower than the progeny produced by HB1.F3.CD cells at that loading dose (ReN vs. HB1.F3.CD, 7.7 vs. 8 difference=0.37, 95% CI=0.30-0.43, P<0.001 log scale) (SF 3C). Based on this data as well as our previous published data (16), we choose to use 50 I.U./cell as the loading dose for our NSCs in the subsequent studies. Next, to evaluate virus replication and release over time, we infected HB1.F3.CD cells and ReNcells with 50 I.U./cell of CRAd-S-pk7 and harvested the cells and supernatant separately at 2, 3, 4 and 5 d.p.i. At both 4 and 5 d.p.i. HB1.F3.CD cells had a significantly higher cell associated viral titer compared to ReNcells (SF 3D-I) (4 d.p.i: HB1.F3.CD vs. ReN, 7.67 vs. 6.92 difference=0.75, 95% CI=0.60-0.88, P<0.001; 5 d.p.i: HB1.F3.CD vs. ReN, 7.66 vs. 6.0 difference=1.66, 95% CI=1.54-1.79, P<0.001; log scale). More importantly as compared to ReNcells, HB1.F3.CD cells released significantly higher levels of CRAd-S-pk7 progeny at 3 (ReN vs. HB1.F3.CD, 5.7 vs. 6.2 difference=0.55, 95% CI=0.41-0.68, P<0.001 log scale), 4 (ReN vs. HB1.F3.CD, 5.8 vs. 6.9 difference=1.04, 95% CI=0.92-1.16, P<0.001 log scale) and 5 (ReN vs. HB1.F3.CD, 6.7 vs. 7.0 difference=0.38, 95% CI=0.23-0.54, P<0.001 log scale) d.p.i.(SF 3D-II).

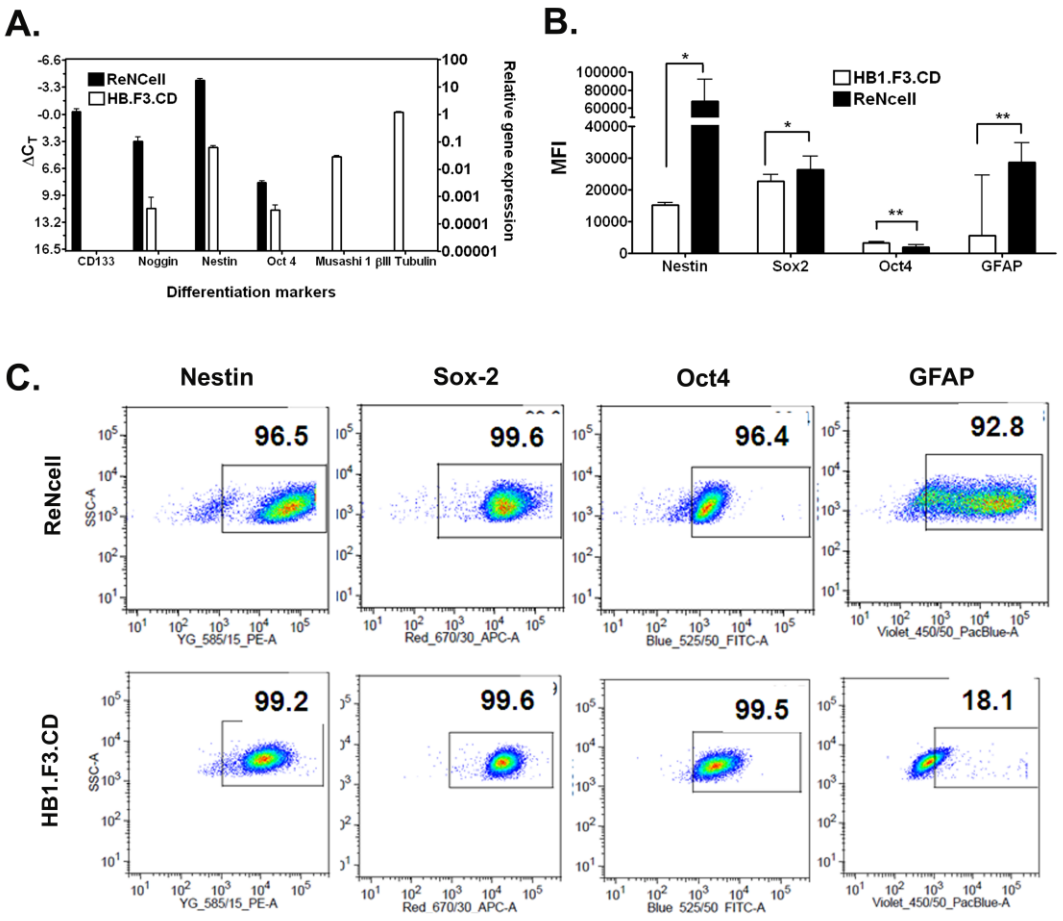
The inherent tumor pathotropism of NSCs is central to their utility as a reliable cell carrier for cancer gene therapy. As such we next evaluated the glioma tropic migratory capacity of NSC lines by using a previously described transwell migration assay (8). As shown in SF 3E, the HB1.F3.CD line showed more robust migration towards several established glioma cell lines as compared to ReNcells, which showed greater migration in response to the U118 glioma line (HB1.F3.CD vs. ReNcell, 888.9 vs. 12220 difference=-11330, 95% CI=-14780 to -7885, P<0.001; 6778 vs. 2000 difference=4778, 95% CI=426.6-9129, P=0.04; 10110 vs. 2111 difference=8000, 95% CI=5754-10250, P<0.001 for U118, U87, U373 respectively).

Loading MPIO into NSCs

To evaluate whether OV-loaded HB1.F3.CD cells migrate to distant tumor foci in animal brains, we employed Magnetic Resonance Imaging (MRI) to non-invasively monitor the migratory behavior of the implanted HB1.F3.CD-loaded with OV in the orthotropic glioma xenograft model. It has been previously reported that MRI-based cell tracking can be achieved by labeling cells with microparticles of iron oxide (MPIO) pre-implantation (13). Based on this, we set to establish an *in vitro* protocol to label HB1.F3.CD cells with fluorescent-tagged MPIOs (purchased Bang's Laboratory MC03F). The loading of MPIOs into NSCs was achieved by using FuGene6-based transfection reagent according to the manufactures protocol (Roche).

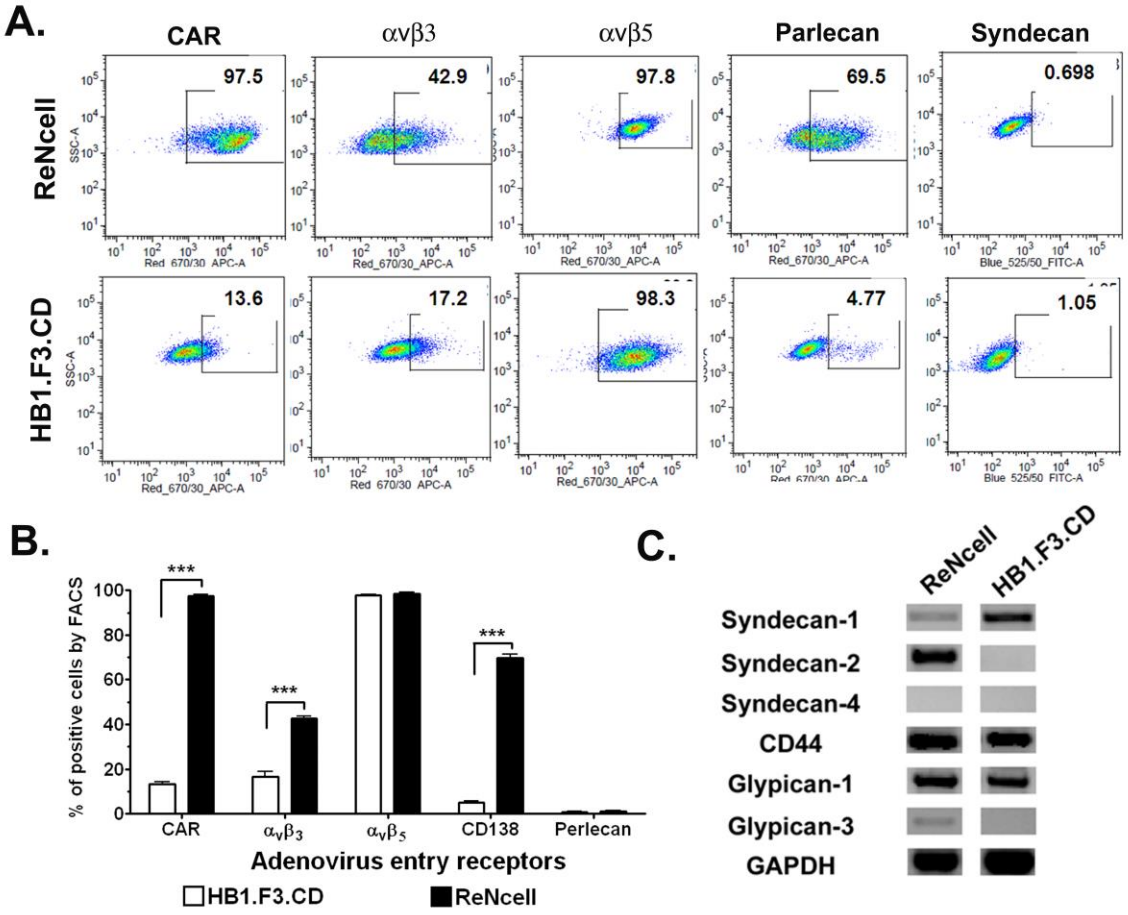
First, we evaluated the relationship between cell number increase and MPIO marked cells (MPIO loading efficiency) in order to evaluate whether cells were receiving MPIO's and then dividing with them still embedded within the cell, or if cell division

caused a total loss of MPIO's. A total loss of MPIOs would indicate that the division of NSCs before reaching the disseminated tumor burden would cause a loss of signaling, and therefore hamper tracking. Loading efficiency was tested using FACS, with APC-conjugated nanoparticles. The NSC's were transfected 16 hours in advance, and then labeled with violet crystal staining, a dye which becomes more diluted as cellular divisions increase (as measured by the PacBlue %). We found the loading threshold to be between 17 nanoparticles per cell and 34 nanoparticles per cell (SF 5A). The published literature seems to indicate that about 20 nanoparticles per cell corresponds with the highest percentage of nanoparticle uptake, which explains the threshold (37). At this dose, the viability of HB1.F3.CD cells after loading was unchanged for up to 3 days (SF 5B). We observed a significant increase in the proliferation rate of the MPIO-loaded HB1.F3.CD cells at day 2 post loading. However, within 24h of loading the difference between loaded and non-loaded groups was resolved. Finally, we examined the differentiation status of the MPIO-loaded HBI.F3.CD cells at 3 days post loading and observed only a decreased level in Oct4 mRNA at the loading dose of 17 MPIOs/cell. Also the chemoattractant receptor VEGFR transcript level was unchanged at this loading dose (SF 5C). Based on this data, we proposed to use a loading dose of 17 MPIOs/cell for all of our future experiments.



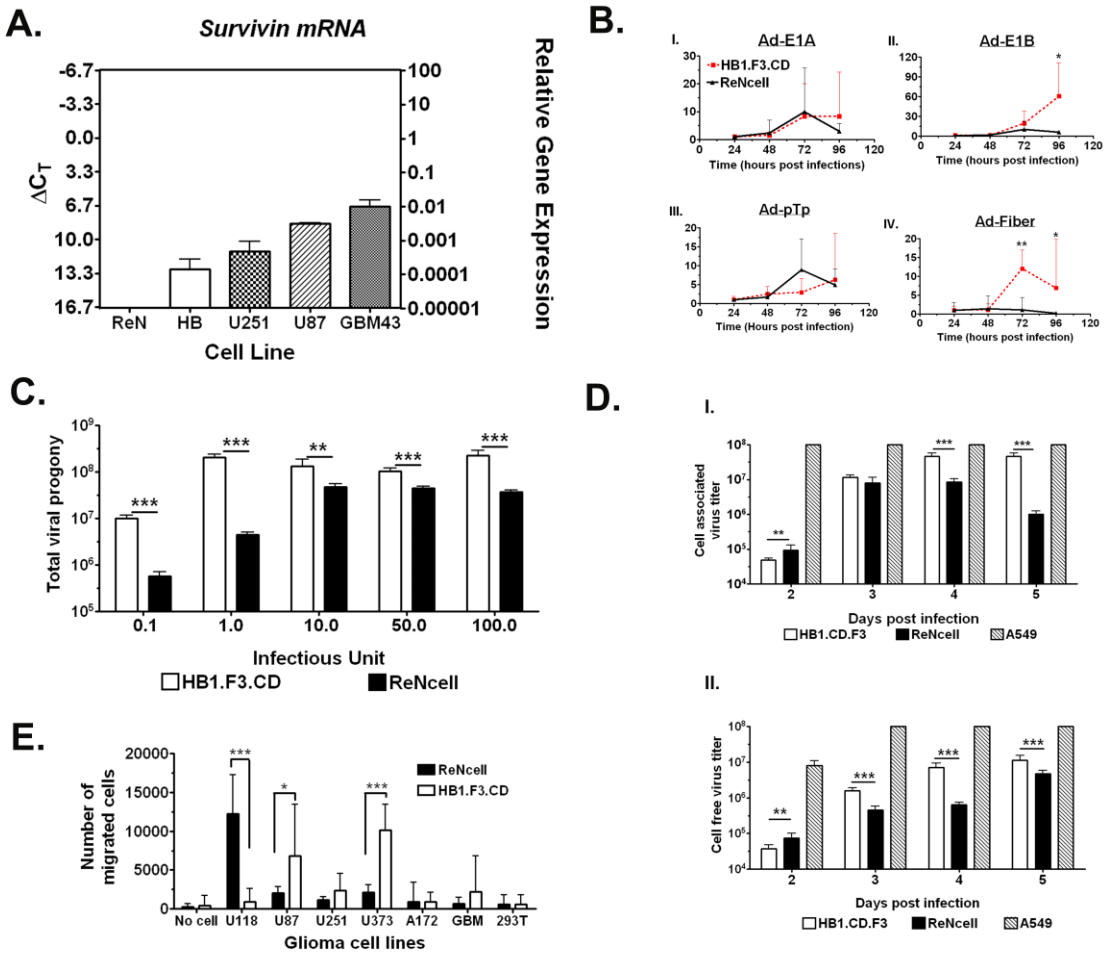
Supplementary Figure 1. Phenotypic characterization of NSCs (A) Relative gene expression of a panel of stem cell and differentiation marker mRNA was tested with qRT-PCR in neural stem cell-base cell carrier. **(B)** To compare the differentiation status of both NSC lines, ReNcells and HB1.F3.CD cells were plated for 3 days and stained with antibodies against the stem cell markers Nestin, Sox-2, and Oct4 as well as the astrocytic lineage marker, GFAP. Mean fluorescence intensity of the representative FACS plots. Bars represents means from three independent experiments, error bars refer to 95% confidence intervals. **(C)** Representative FACS plots shown the expression

profile of differentiated markers. All statistical tests were two-sided. *** indicates a p-value <0.001; ** indicates a p-value <0.01; * indicates a p-value <0.05.



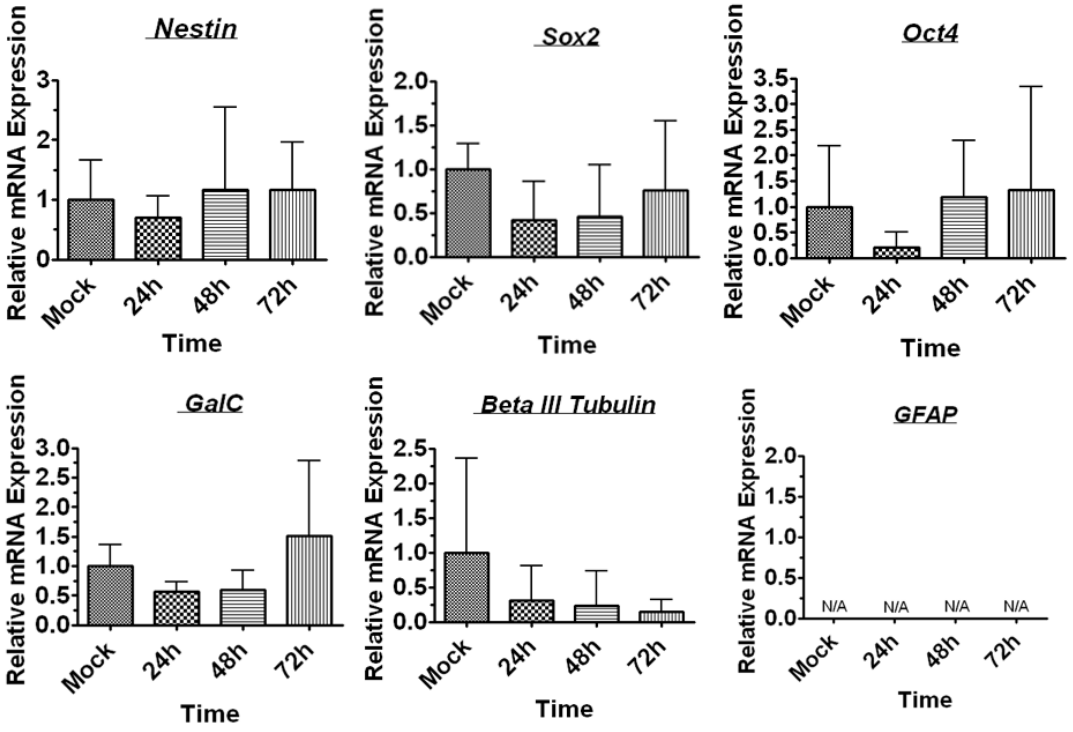
Supplementary Figure 2. Adenovirus entry receptors expression in the NSCs (A) The receptors for adenoviral entry into NSCs were evaluated by FACS. Representative FACS plots of the adenoviral entry receptors expressed on ReNcells and HB1.F3.CD cells as quantified. 10^5 HB1.F3.CD and ReNcells were plated and after 48 hours cells were stained with antibodies against human adenoviral entry receptors expressed on the surface of HB1.F3.CD and ReNcells. **(B)** FACS analysis from three independent experiments was added and represented in the bar graph. ReNcells expressed higher levels of CAR, $\alpha_v\beta_3$, and syndecan-1 compared with HB1.F3.CD cells using student's t test ($P<0.001$). The entry receptor $\alpha_v\beta_5$ and perlecan was expressed at comparable levels.

Bars represents means from three independent experiments, error bars refer to 95% confidence intervals. **(C)** qRT-PCR was used to validate the expression of adenoviral entry receptor and HSPG protein expression on ReNcells and HB1.F3.CD cells. Analysis revealed that both NSC cell lines expressed CD44 and Glypican-1 at comparable levels, while ReNcells expressed Syndecan-2 and HB1.F3.CD cells expressed Syndecan-1. All statistical tests were two-sided. *** indicates a p-value <0.001; ** indicates a p-value <0.01; * indicates a p-value <0.05.

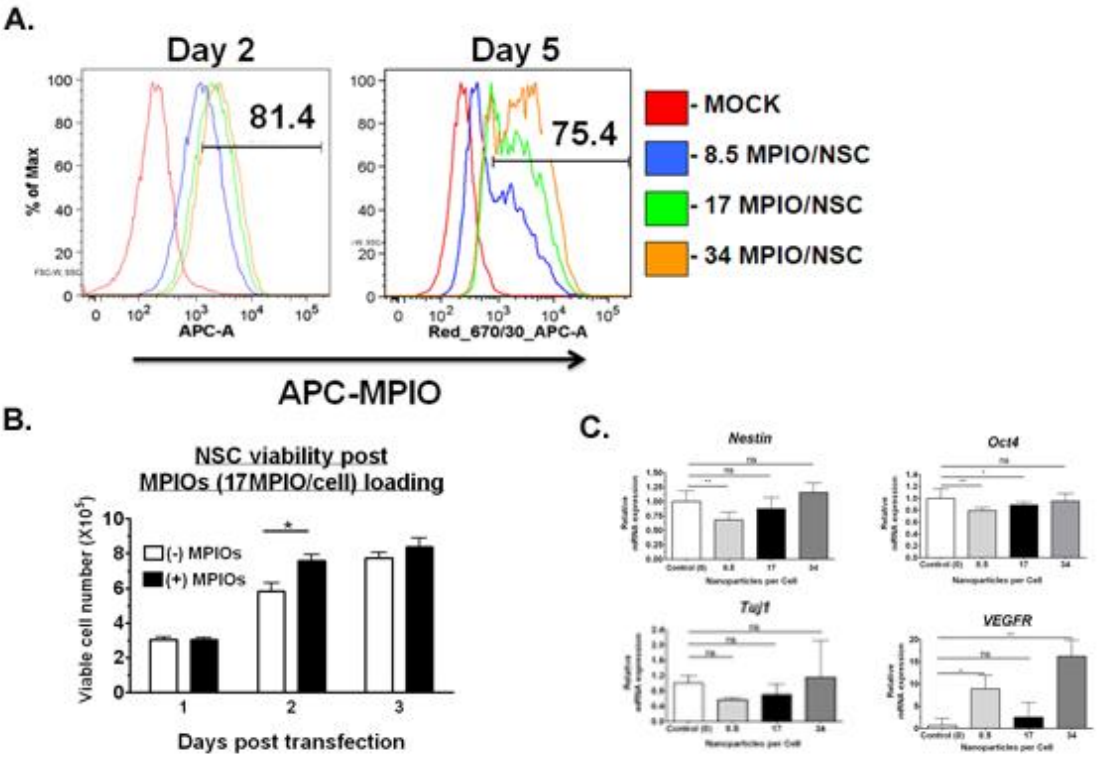


Supplementary Figure 3. Permissiveness of NSC lines to adenovirus infection. **(A)** Relative gene expression of survivin mRNA of ReNcells and HB1.F3.CD cells as well as various glioma cell lines was tested with qRT-PCR after 3 days of incubation. Bars represents means from three independent experiments, error bars refer to 95% confidence intervals. **(B)** CRAd-S-pk7 replication kinetics in NSCs was assessed by measuring relative mRNA expression with quantitative real-time PCR (qRT-PCR). Cells were infected with 50 infectious units (I.U.) /cell. At 4 d.p.i adenovirus E1A, E1B, pTp, and fiber transcripts were expressed at higher levels in HB1.F3.CD cells compared with ReN cells, with the statistical significance observed for E1B using student's t test with welch's

correction (P=0.16). Dots represents means from three independent experiments, error bars refer to 95% confidence intervals. **(C)** Viral permissiveness of HB1.F3.CD and ReNcells at different infectious units (0.1-100 I.U./cell). Total viral progeny was measured at 3 d.p.i. by using Adeno-X Rapid Titer Kit (Clontech, Mountain View, CA) according to the manufacturer's protocol. HB1.F3.CD cells expressed significantly higher viral progeny than ReNcells, compared using student's t test. Bars represents means from six independent experiments, error bars refer to 95% confidence intervals. **(D)** To assess viral permissiveness over time (2-5 d.p.i) HB1.F3.CD and ReN cells were plated and infected with CRAd-S-pk7 at an infectious dose of 50 I.U./cell. D-I. Cell associated viral titer (intracellular virus titer) was analyzed with a titer assay. HB1.F3.CD cells showed significantly higher titer levels at 2, 4, and 5 d.p.i., compared to ReN cells using student's t test. Bars represents means from five independent experiments, error bars refer to 95% confidence intervals.**D-II.** Cell free virus titer was significantly higher at 2, 3, 4 and 5 d.p.i. compared to ReNcells using student's t. Bars represents means from five independent experiments, error bars refer to 95% confidence intervals. **(E)** NSC migration in response to different glioma cell lines was evaluated by a transwell migration chamber assay, and quantified. U87 and U373 cells significantly stimulated HB1.F3.CD migration over ReNcells, while U118 cells stimulated more ReNcells migration. Comparison between groups was performed using student's t test. Bars represents means from three independent experiments, error bars refer to 95% confidence intervals. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Data shown are Mean±SEM.



Supplementary Figure 4. Differentiation status of HB1.F3.CD cell after OV infection. Expression of genes associated with neural stem cell neural stem cell "stemness" and pluripotency post OV loading. Cells were infected with 50 infectious units (I.U.) /cell. Relative mRNA expression of stem cell markers nestin, *Sox2*, *Oct4* and *Galc* (oligodendrocyte marker), GFAP (astrocyte), beta-III tubulin (neuronal marker) was measure with quantitative real-time PCR (qRT-PCR). Comparison between groups was performed using student's t test. Bars represents means from three independent experiments, error bars refer to 95% confidence intervals.* P<0.05, **P<0.01, ***P<0.001. Data shown are Mean±SEM.



Supplementary Figure 5. Fluorescent labeling of HB1.F3.CD cells with MPIOs. (A) HB1.F3.CD cells were transfected with a liposome-based method with either 0, 2, 4, or 6 ug of MPIOs. FACS analysis was performed in order to test the effectiveness of MPIO-Flash Red transfection. Minimal loss of fluorescents was detected up to 5 days. **(B)** Trypan blue exclusion was used to detect the toxicity of MPIO transfection to the HB1.F3.CD carrier cell. At 17 MPIOs/cell NSCs there was significant viability differences compared with non-transfected HB1.F3.CD cells, compared by student's t test. Bars represents means from four independent experiments, error bars refer to 95% confidence intervals. **(C)** The differentiation status of HB1.F3.CD cells was tested 4 days post transfection with varying concentration of MPIOs. mRNA levels were measured by

qRT-PCR and their relative mRNA expression is expressed compared with non-transfected HB1.F3.CD cells.