

Human Embryonic Stem Cell-derived Mesenchymal Stem Cells as Cellular Delivery Vehicles for Prodrug Gene Therapy of Glioblastoma

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Abstract

Mesenchymal stem cells (MSCs) possess tumor-tropic properties and have been consequently utilized to deliver therapeutic agents for cancer treatment. Their potential in cancer therapy highlights the need for a consistent and renewable source for the production of uniform human MSCs suitable for clinical applications. In this study, we seek to investigate whether human embryonic stem cells can be used as a cell source to fulfill this goal. We generated MSC-like cells from two human embryonic stem cell lines, Hues9 and H1, and observed that MSC-like cells derived from human embryonic stem cells were able to migrate into human glioma intracranial xenografts after being injected into the cerebral hemisphere contralateral to the tumor inoculation site. We engineered these cells using baculoviral and lentiviral vectors respectively for transient and stable expression of the herpes simplex virus thymidine kinase gene. The engineered MSC-like cells were capable of inhibiting tumor growth and prolonging survival of tumor-bearing mice in the presence of ganciclovir after they were injected either directly into the xenografts or into the opposite hemisphere. Our findings suggest human embryonic stem cell-derived MSCs as a viable and attractive alternative for large scale derivation of targeting vehicles for cancer therapy.

Key words: Mesenchymal stem cells, human embryonic stem cells, cancer gene therapy

Introduction

Human mesenchymal stem cells (MSCs) are a group of multipotent stem cells with differential potential for osteogenic, chondrogenic, adipogenic and even neuronal lineages (Pittenger *et al.*, 1999; Sadan *et al.*, 2009). Its function in wound healing has long attracted its employment in applications such as regeneration of damaged tissues and regulation of cardiac function in cardiovascular disease. Interestingly, research has shown that MSCs migrate *in vitro* and *in vivo* towards tumors, abnormalities in the body commonly perceived to be wounds that never heal. MSCs derived from different sources, such as adult bone marrow, adipose tissues and umbilical cord blood, have been shown to harbor this tumor-tropic ability and can be used in the 'Trojan horse' approach as cellular vectors for targeted delivery of toxic agents to tumor sites (Lee *et al.*, 2009; Loebinger *et al.*, 2009; Bexell *et al.*, 2010). The tested toxic agents include tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), IFN-beta, herpes simplex virus thymidine kinase (HSVtk)/ganciclovir system, and cytosine deaminase/5-Fluorocytosine system (CD/5-Fc) (Nakamizo *et al.*, 2005; Kucerova *et al.*, 2007; Miletic *et al.*, 2007; Loebinger *et al.*, 2009). These previous studies consistently demonstrated the ability of MSC vectors loaded with toxic genes to either slow down tumour growth or to eliminate the tumors.

The vast therapeutic potential of MSCs highlights the need to identify an accessible and reliable source of these cells. While adult MSCs could be reliably isolated from bone marrow and adipose tissues, the isolation procedure is invasive, with the isolated MSC population declining in proliferation capacity and differential potential with increasing age in culture (Kern *et al.*, 2006). Another source of MSCs, the umbilical cord blood, allows the derivation of MSCs with relatively greater proliferation capacity in culture. However, it still remains controversial whether cord blood can generate sufficient MSCs for subsequent studies and clinical applications (Perdikogianni *et al.*, 2008). Human embryonic stem cells (hESCs), on the other hand, are an alternative source that enables standardization and large-scale production of downstream differentiated cells. This offers the potential for the manufacture of single batches of cell therapy products sufficient for repeated treatments in multiple patients, thus eliminating variability in the quality of cell therapeutics and facilitating reliable comparative analysis of clinical outcomes. The use of hESC-derived cells for cell

therapy may also increase cost-effectiveness by reducing the laboriousness in collecting and expanding cells from individual patients and simplify the logistics by employing cryopreserved cells in ready-to-go format.

Recent years have seen the development of techniques enabling the differentiation of MSCs from human embryonic stem cells (Lian *et al.*, 2007; Arpornmaeklong *et al.*, 2009; Liu *et al.*, 2009). These hESC-derived MSCs are similar to adult MSCs in their gene expression profile, surface marker profile and differential potential. They exhibit much greater proliferative capacity than their adult counterparts, and could be consistently derived in large amounts. hESC-MSCs have also been shown to possess the same immune suppressive effects found in adult MSCs, and could potentially be tolerated by the immune systems of allogenic recipients (Trivedi and Hematti, 2008; Yen *et al.*, 2009). Most importantly, they are functional in *in vivo* disease models, conferring neuroprotective effects in rat transient focal cerebral ischemia, cardioprotective effects during porcine myocardial ischemia/ reperfusion injury, as well as in repairing defective rat cartilage (Timmers *et al.*, 2007; Hwang *et al.*, 2008; Liu *et al.*, 2009; Lai *et al.*, 2010).

In view of these advantages, we set out to investigate the feasibility of using hESC-derived MSCs as prodrug delivery vectors for cancer gene therapy. We are interested in whether these cells could exhibit the tumor tropism property demonstrated in adult MSCs and whether they could give efficient tumor killing effect when coupled with the herpes simplex virus thymidine kinase gene (HSVtk)/ganciclovir system. We loaded the cells with the HSVtk transgene using either baculovirus or lentivirus gene transfer methods. Baculovirus, or *Autographa californica multiple nucleopolyhedrovirus*, is an insect virus that is emerging as a potentially safe class of gene delivery vectors because of its inability to replicate or to cause toxicity in mammalian cells (Wang and Balasundaram, 2010). Due to its non-integrating nature, it mediates a transient transgene expression in cells. Lentivirus, on the other hand, is an animal virus well known to preferentially integrate into transcriptionally active regions of host genomes, raising concerns over insertional mutagenesis, oncogene activation and cellular transformation (Schroder *et al.*, 2002; Hacein-Bey-Abina *et al.*, 2003; Liu *et al.*, 2006). Yet, despite the risks involved in its usage, the stable transgene expression instigated by this virus has proven useful for many applications. Our study seeks to

reveal the differences in the tumor killing efficacy that arise from the use of these two gene transfer systems mediating different durations of transgene expression, and most importantly, highlights the potential of hESC-MSCs in cancer therapy.

Materials and Methods

Cells

Generation and characterization of Hues9 human embryonic stem cell (hESC) derived-mesenchymal stem cells (MSCs) were published previously (Lian *et al.*, 2007). These cells have been sorted to obtain a homogenous CD105+ and CD24- population, and tested for purity by flow cytometry and for their ability to differentiate into osteogenic, chondrogenic and adipogenic lineages (Lian *et al.*, 2007). Cells are positive for CD105, CD166, CD29, and CD44 and negative for CD14, CD34 and CD45. They were obtained at passage 13 and used not later than passage 20 for the current study. Human embryonic stem cell line H1 derived-MSCs were generated using the method described by Hwang *et al.* (2008). H1 embryonic stem cells were obtained from WiCell Research Institute (Madison, WI) and cultured in mTeSR-1 medium (Stem Cell Technologies, Inc.) on matrigel (BD Biosciences, USA). H1 cells were incubated for 7 minutes with 1 mg/ml dispase, followed by two washes in unsupplemented DMEM-F12 (Gibco). Cells were scraped and cultured in suspension in DMEM-F12 supplemented with 20% Knockout Serum Replacer, 1mM L-glutamine with trace amounts of 2-mercaptoethanol solution, 0.1mM nonessential amino acids, and 1% penicillin-streptomycin (Gibco) for the generation of embryoid bodies (EBs). Ten-day old EBs were seeded on gelatin-coated 100 mm cell culture dishes in the EB generation medium. Outgrowths of cells with fibroblastic morphology were selected, mechanically-scraped, and expanded in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 2mM L-glutamine, 0.1mM nonessential amino acids and 1% penicillin-streptomycin. Human glioblastoma U87MG cell line was purchased from American Type Culture Collection (Manassas, VA, USA). The stable U87 cell clone expressing luciferase gene (U87-*luc*) was generated previously (Balani *et al.*, 2009)

MSC migration in vitro and in vivo

For the *in vitro* migration assay, hESC-MSCs were serum-starved overnight in reduced serum Opti-MEM medium and collected for use. Fifteen thousand MSCs were seeded into each 96-well Corning cell culture insert with a PET membrane of 8 μ M pore size. Inserts were placed into a Corning 96 well receiver plate containing Opti-MEM, U87 seeded in Opti-MEM, or Opti-MEM supplemented with 15% FBS. U87 cells were seeded at a density of 50,000 cells per well. The plates were incubated for 24 hours at 37°C in 5% CO₂, after which the inserts were transferred to a new receiver plate. Migrated cells on the bottom side of the membrane were treated with a cell dissociation and staining solution made up 4x diluted trypsin containing 2.4 μ l of Calcein-AM (50 μ g resuspended in 30 μ l DMSO) per ml. Fluorescence from the detached cells was measured using a microplate reader (GENios™ Pro, Tecan) and the relative fluorescent units plotted against a standard curve to estimate the actual number of cells that had migrated. All experiments were done with at least 6 replicates and values expressed as mean \pm s.d.

The *in vivo* migration assay was performed with 3 adult male Balb/c athymic, immunoincompetent nude mice (weight 20 g; aged 6-8 weeks). U87 and hESC-MSCs were labeled with CM-DiO and CM-Dil (Molecular Probes, Invitrogen) respectively according to manufacturer's protocol. The mice were inoculated with 5×10^5 CM-DiO-labeled U87 cells at the right striatum of anesthetized animals (designated as day 0). On day 7, 1 million CM-Dil-labeled hESC-derived MSCs were injected into a site contralateral to the tumor inoculation site. The brains were collected 7 days later, fixed in 4% paraformaldehyde overnight, and sectioned.

Viral vector preparation and cell transduction

Baculoviral vectors with the eGFP reporter gene were constructed in our previous studies (Zeng *et al.*, 2007, 2009; Du *et al.*, 2010). The transfer plasmid pFastBac1 (Invitrogen) was used to generate recombinant baculoviruses with different expression cassettes. To generate the recombinant baculovirus vector containing the herpes simplex virus thymidine kinase (HSVtk) gene (Balani *et al.*, 2009), the HSVtk gene with flanking EcoRI and XhoI sites was obtained through PCR amplification from the pORF-HSVtk plasmid (InvivoGen, San Diego, CA, USA) using forward primer 5' –

GTGAACCGTCAGATCGAATTCCTGAGATCACCGGCGAAGGA – 3' and reverse primer 5' – CCAGAGGTTGATTATCGCTCGAGTCAGTTAGCCTCCCCATCT – 3' and used to replace the eGFP gene in our CMV-W vector. BacPAK6, a baculovirus vector without a mammalian gene expression cassette, was purchased from Clontech (Mountain View, CA, USA). Viruses were produced and propagated in *Sf9* insect cells according to the manual of Bac-to-Bac Baculovirus Expression System (Invitrogen). Budded viruses in the insect cell culture medium were centrifuged at 2000 g for 10 min to remove cell debris, and concentrated by a second round of centrifugation at 28,000 g for 60 min. Viral pellets were re-suspended in 0.1 M phosphate-buffered saline (PBS) and their infectious titers (pfu) were determined by plaque assay on *Sf9* cells.

For baculovirus transduction, cells were transduced in minimal volume of medium at MOI 100 and left to incubate overnight at 37°C, 5% CO₂. This is followed by a top up in medium the next day, and a full change of the medium on day 2 post-transduction if transduced cells were cultivated for longer than 2 days.

Lentivirus with the CMV-W expression cassettes containing either eGFP or HSVtk transgenes were constructed in our previous study (Zhao and Wang, 2010). Briefly, expression cassettes were cloned into the pLenti6/v5-TOPO expression plasmid (Invitrogen) from Invitrogen's ViraPower™ Lentiviral Directional TOPO Expression Kit. The pLenti6/v5-TOPO expression plasmid containing either the expression cassette for eGFP or HSVTK were co-transfected with pLP1, pLP2 and pLP/VSVG packaging plasmids into 293FT cells. Transfection was performed overnight, followed by a full medium change. Lentiviruses were collected 48 hours post transfection and concentrated at 75,000 g, 4°C for 1 hour. Concentrated virus particles were resuspended in PBS and stored at -80°C or used immediately for transduction.

For lentivirus transduction, 3 million cells were suspended in 5 ml of medium and seeded into each 100 mm dish; 40 µl of concentrated virus were added to each dish while cells were still in suspension (MOI < 1). Transduction was allowed to take place overnight at 37 °C and medium was topped up to 10 ml the next day. On the second day post lentivirus transduction, the virus containing medium was completely removed and cells were washed 3x with copious amounts of PBS. This is followed by a 2 weeks antibiotic selection for transduced cells using medium

containing 5 $\mu\text{g/ml}$ blasticidin (Invitrogen). Selected cells were expanded in normal medium and used for subsequent experiments.

FACS analysis and Western blotting

When fluorescent-activated cell sorting (FACS) analysis was used to evaluate transduction efficiency, baculovirus-transduced cells were trypsinized to single cells and collected in PBS before analysis with the FACSCalibur flow cytometer (BD Biosciences, San Diego, CA, USA). To examine effects of virus transduction on surface marker expression, the cell pellet was re-suspended in 1% BSA/PBS and incubated for an hour with phycoerythrin (PE)-conjugated primary monoclonal antibodies, including anti-CD34, anti-CD44, anti-CD73, anti-CD90, anti-CD105 and anti-CD166, allophycocyanin (APC)-conjugated anti-CD45, and their respective isotypes from BD Pharmingen (San Diego, CA, USA). After two washes with PBS, samples were re-suspended in PBS for FACS analysis.

Western blotting was used to verify HSVtk gene expression after baculovirus or lentivirus transduction. MSCs transduced with the HSVtk baculoviral vector were lysed on day 2 post-transduction. Cell lysates were centrifuged at 10,000 g for 10 min at 4°C. Samples were mixed with one quarter volume of NuPAGE LDS Sample Buffer and one tenth volume of NuPAGE Reducing Agent (Invitrogen), heated at 70°C for 10 min, and separated on 4-12% Bis-Tris Gel with MES running buffer. Electroblothing was performed using the iBlot Dry Blotting System (Invitrogen). The membrane was blocked in 5% non fat milk in TBST for an hour, and incubated overnight with goat polyclonal anti-HSVtk primary antibody (1:800; Santa Cruz Biotechnology Inc., USA), and for 1 hour with mouse monoclonal beta-actin primary antibody (1:1000; Sigma, St Louis, MO, USA). After 3 washes of 5 min each with TBST, the membrane was incubated with HRP-conjugated anti-goat IgG or anti-mouse IgG (Abcam plc, Cambridge, UK) for 1 hour, followed by a second round of washing. Protein bands were visualized on an X-Ray film after a 10 seconds exposure.

In vitro cytotoxicity assay

Baculovirus or lentivirus transduced hESC-MSCs were trypsinized and mixed with U87 glioblastoma cells at different ratios. The mixed cells were seeded on 96-well plates (1000 cells per well) and cultivated in MSC medium with ganciclovir. After 5 days, cell viability was examined using CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega).

In vivo animal study

Adult male Balb/c athymic, immuno-incompetent nude mice (weight 20 g; aged 6-8 weeks) were used. For tumor inoculation, 0.1×10^6 human glioma U87-*luc* cells were injected into the right side of the striatum of anesthetized animals (designated as day 0).

To test baculovirus transduced hESC-MSCs, the tumor-inoculated animals were divided into 3 groups ($n = 8$ per group) on day 8 post-tumor inoculation and received an intratumor injection of sample solutions: phosphate buffered saline (PBS), PBS suspension of 2.5×10^5 MSCs transduced with a baculoviral vector containing the *eGFP* gene, and PBS suspension of 2.5×10^5 MSCs transduced with a baculoviral vector containing the *HSVtk* gene. Five μ l of sample solutions was injected into each tumor xenograft, followed by daily intraperitoneal injection of ganciclovir (50 mg/kg body weight) for 2 weeks starting 1 day after MSC injection.

To test lentivirus transduced hESC-MSCs, the tumor-inoculated animals are similarly divided into 3 groups ($n = 10$ per group) on day 5 post-tumor inoculation. Sample solutions of PBS, PBS suspension of 1×10^6 MSCs transduced with a lentiviral vector containing the *eGFP* gene, or PBS suspension of 1×10^6 MSCs transduced with a lentiviral vector containing the *HSVtk* gene were injected into the left striatum at a site contralateral to the tumor inoculation site. Ten μ l of sample solutions was injected into the brain, followed by daily intraperitoneal injection of ganciclovir (50 mg/kg body weight) for 2 weeks starting 7 days after MSC injection.

To monitor bioluminescent signals of U87-*luc* cells, isoflurane gas-anesthetized animals were injected intraperitoneally with 200 μ l of D-luciferin (5mg/ml, Promega) in PBS and placed on a warmed stage inside the camera box of the IVIS imaging system coupled with cool CCD camera (Xenogen, Alameda, CA, USA). Luminescent images were taken 20 min after luciferin injection as a 60-second acquisition. The detected light emitted from U87-*luc* cells was digitized and electronically

displayed as a pseudocolor overlay onto a grayscale image of the animal. Images and measurements of luminescent signals were acquired and analyzed with the Xenogen living imaging software v2.5 and quantified as photons per second.

Animal survival was monitored until all the animals are dead. All handling and care of animals was carried out according to the Guidelines on the Care and Use of Animals for Scientific Purposes issued by the National Advisory Committee for Laboratory Animal Research, Singapore.

Statistical analysis

All data are presented as mean \pm s.d. The statistical significance of differences was determined by the unpaired Student's *t*-test, or one-factor or two-factor analysis of variance (ANOVA) with replication followed by Holm-Sidak method. The statistical analysis of survival data was performed using the log rank test followed by Holm-Sidak method for pairwise multiple comparison tests. A *P* value of < 0.05 was considered to be statistically significant.

Results

We used human MSCs generated from two hES cell lines, Hues9 and H1. Generation and characterization of Hues9 derived-MSCs (Hues9-MSCs) were published previously (Lian *et al.*, 2007) while H1 derived MSC-like cells (H1-MSCs) were generated in a feeder-free condition using the method reported by Hwang *et al.* (2008). H1 embryoid bodies were derived and seeded onto gelatin coated plates, after which fibroblastic outgrowth from the embryoid bodies were isolated after 10 days and expanded. These cells displayed MSC-like morphology, exhibited a surface marker profile characteristic of MSCs, expressing CD44, CD73, CD90, CD105 and CD166 but not CD34 and CD45 (**Figure 1**). To determine whether H1-MSCs are pluripotent and hold differentiation potential, the cells were cultured in selection media to undergo adipogenesis, osteogenesis or chondrogenesis. Adipogenic differentiation was induced in medium containing insulin, dexamethasone, phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine, indomethacin, osteogenic differentiation was induced in medium containing dexamethasone, ascorbate, and beta-glycerophosphate, and chondrogenic differentiation was induced in medium containing TGF-beta.

While H1-MSCs did not spontaneously differentiate during cell expansion, their differentiation into various cell types was observed in the selection media and confirmed by RT-PCR analysis of mesodermal lineage markers and specific staining (**Figure 2**).

To examine the feasibility of using hESC-MSCs for targeted glioma gene therapy, we investigated whether Hues9-MSCs and H1-MSCs are similar to adult bone marrow MSCs in displaying tropism for tumors. Our results from *in vitro* transwell cell migration assays using Boyden chambers showed that compared to the migration towards plain Opti-MEM medium (the blank) there was significantly increased migration of these MSC-like cells towards U87 glioma cells, increasing from 12% migrated cells towards the blank to 25% towards U87 cells in Hues9-MSCs and from 7% towards the blank to 12% towards U87 cells in H1-MSCs (**Figure 3a, b**). The U87 cell-directed migration of H1-MSCs was comparable to that directed by Opti-MEM with 15% fetal bovine serum, a positive control for cell migration (**Figure 3a**). These results from *in vitro* assays clearly suggest the tropism of hESC-MSCs towards some chemo-attractants secreted by the U87 glioma cells.

To investigate if this tumor tropism can be observed in an *in vivo* setting, Hues9-MSCs were labeled with the red fluorescent dye CM-Dil and injected into the left hemisphere of mouse brains inoculated with green fluorescent dye CM-DiO labeled U87 tumors in the right hemisphere. One week after injection, the mice were sacrificed and their brains collected for histological sectioning. Red fluorescent dye labeled Hues9-MSCs were either observed to be en route towards the right brain hemisphere (**Figure 3c**), or found located within the tumor (**Figure 3d**). The migration tract in the brain appeared specific as the Hues9-MSCs in all 3 tested mice were observed to stream towards the right hemisphere.

Our previous study demonstrated that insect baculovirus-transduced bone marrow MSCs can be used as targeting vehicles for gene delivery into tumors (Bak *et al.*, 2010). Baculoviral vectors typically mediate transient transduction with transgene expression lasting for days to weeks. To select a proper baculoviral vector that can sufficiently prolong transgene expression in hESC-MSCs for cancer gene therapy, we tested 6 baculoviral vectors containing different expression cassettes for the enhanced green fluorescent protein (eGFP) reporter gene in Hues9-MSCs. These

vectors were developed previously in the lab and contained either the EF1 α promoter, the EF1 α promoter with a CMV enhancer element or the CMV promoter, with or without the woodchuck hepatitis virus post-transcriptional regulatory element, also known as WPRE (**Figure 4a**). We observed that the vector containing the CMV promoter in combination with the WPRE gave the highest efficiency with more than 90% of eGFP-positive cells (**Figure 4b**). Transgene expression from this baculoviral vector could be maintained at more than 80% in the first week after transduction, followed by a decline to less than 10% at the end of the second week, hence providing a transgene expression window of approximately 2 weeks (**Figure 4c**).

In view of the above findings, we used a baculoviral vector with the CMV promoter and WPRE element to drive the expression of our suicide gene of interest, the HSVtk gene, in hESC-MSCs (**Figure 4d**). A lentiviral vector with the CMV promoter and WPRE element was also constructed and tested for stable HSVtk expression in these cells (**Figure 4d**). We checked if virus transduction had any adverse effect on the stem cell identity through antibody staining and FACS analysis and observed no significant differences between the transduced and mock-transduced cells in the surface marker profile, which includes the expression of CD44, CD73, CD90, CD105 and CD166 and no expression of CD34 and CD45 (**Supplemental Figure 1**). This finding suggests that baculovirus or lentivirus transduction and HSVtk expression do not affect the characteristics of these hESC-derived MSC-like cells.

The tumor tropism property of hESC-MSCs, along with their capacity to be efficiently loaded with the HSVtk transgene, prompted us to test if these loaded cells could efficiently kill cancer cells when coupled with the HSVtk/ganciclovir system. This system is notable for exhibiting strong bystander effect by transferring the toxic phosphorylated form of ganciclovir from HSVtk-expressing cells to nearby unmodified tumor cells, thus resulting in the inhibition of DNA replication in these cells and their eventual death (Mesnil and Yamasaki, 2000; Asklund *et al.*, 2003). To test the bystander effect *in vitro*, we investigated the cytotoxicity effect mediated by mixing U87 glioma cells with HSVtk-expressing, hESC-MSCs at different ratios. While 10 μ M ganciclovir caused negligible killing effect on U87 cells in the absence of HSVtk-expressing hESC-MSCs or H1-MSCs (100:0 ratio), this concentration of ganciclovir induced obvious cell death in U87 cells in the

presence of HSVtk-expressing cells, regardless of whether the HSVtk gene was introduced by a baculoviral or lentiviral vector. The tumor cell killing effect increased when the cell mixing ratio between U87 and HSVtk-expressing cells was altered from 90:10 to 50:50 (**Figure 5a, c, e**). Cell death in the 0:100 groups provided direct evidence that HSVtk-expressing MSCs were susceptible to the cytotoxic effects of ganciclovir. When baculoviral vectors were used to introduce the HSVtk gene into hESC-MSCs, we noted tumor cell killing effect even when HSVtk-expressing cells transduced 8 days ago were mixed with U87 cells (**Figure 5a, b, c, d**), providing evidence for the presence of HSVtk in baculovirus transduced hESC-MSCs for at least 8 days. To test the dose-dependent cytotoxic effects of ganciclovir, U87 cells were mixed with HSVtk-expressing cells at a cell mixing ratio of 50:50 and treated with ganciclovir at concentrations ranging from 0 to 100 μM . The drug brought about pronounced cytotoxic effect at a concentration as low as 1 μM , and gave rise to an increased killing effect at higher concentrations, particularly when HSVtk-expressing cells that have been transduced with baculovirus 8 days ago or with lentivirus were used (**Figure 5b, d, f**). There was no obvious cytotoxicity to untransduced hESC-MSCs at all ganciclovir concentrations employed (**Figure 5b, d, f**).

We next tested the tumor killing efficacy of these HSVtk-expressing cells in *in vivo* settings. U87 cells stably expressing luciferase (*U87-luc*) were injected into the right striatum of nude mice to establish tumor xenografts. In our first *in vivo* experiment, baculovirus-transduced, HSVtk-expressing Hues9-MSCs (BVTk-MSC) were injected directly into the tumors. Hues9-MSCs transduced with a baculoviral vector expressing eGFP (BVeGFP-MSC) and PBS were respectively injected into the tumors as controls. Ganciclovir was administered to the mice through daily intraperitoneal injection. Our previous study (Zhao and Wang, 2010) has demonstrated that ganciclovir alone, at the tested dose and in the tested mouse species, has no effects on glioma growth. When the IVIS Imaging System was employed for bioluminescent imaging of *U87-luc* cells in living animals, we observed a slow tumor growth rate in the BVTk-MSC group (**Figure 6a**). Bioluminescent photos taken on day 9 and 25 after tumor implantation revealed tumor shrinkage in 3 of 8 animals in this group and a slight increase in tumor bioluminescence for the other 5 animals, as apposed to the great increase in tumor bioluminescence in the 2 control groups (**Figure 6a**).

Quantitative analysis of the bioluminescence signals indicated that mice injected with BVTK-MSC had 10-fold lower tumor luciferase expression than mice in the 2 control groups on day 25 after tumor implantation (**Figure 6b**). This slowed tumor growth prolonged animal survival by approximately 10 days (**Figure 6c**), increasing the maximum survival time of 36 days in the PBS group and 38 days in the BVeGFP-MSC group to 48 days in the BVTK-MSC group ($P = 0.0000925$ and 0.00222 respectively).

We then investigated the *in vivo* targeted delivery after the injection of HSVtk-expressing hESC-MSCs into the left striatum contralateral to the tumor inoculation site on day 5 post-tumor inoculation. Ganciclovir injection started 7 days after MSC injection, providing the cells ample time to migrate from the contralateral side to the tumor side. We used lentivirus-transduced Hues9-MSCs (LVTK-MSC) in this experiment as they provide a broadened HSVtk expression window. Histological examination of the mice brain collected 30 days after tumor inoculation revealed significant tumor tissue necrosis in the group of mice that received LVTK-MSC treatment and extensive tumor growth in the control groups of mice that were injected with either eGFP-expressing MSCs or PBS (**Figure 7a**). While mice in the control groups mostly died around day 30 days, the maximum survival time of the LVTK-MSC group was significantly prolonged to 40 days, ($P = 0.00174$ vs. the PBS control and 0.00088 vs. the LVeGFP-MSC control, **Figure 7b**).

Discussion

In this study, we successfully demonstrated the use of hESC-MSCs as therapeutic gene delivery vectors in cancer treatment. hESC-MSCs can be easily differentiated and isolated from embryonic stem cells, and also have greater life span *in vitro* as well as a faster proliferation rate than adult MSCs. The Hues9 embryonic stem cells-derived MSCs, for instance, can be passaged up to at least 35 population doublings with a fast population doubling time of 72 hours, enabling large scale derivation of cells within a short period of time (Lian *et al.*, 2007). Interestingly, consistent with reports describing adult MSC migration, the hESC-MSCs we tested in this study also possess the ability to migrate towards tumors both *in vitro* and *in vivo*. hESC-MSCs injected in the left brain hemisphere of mice were found streaming towards, or located within the tumor inoculated in the

right brain hemisphere one week after injection. Adult MSCs are well known to home into sites of injury to facilitate wound healing and tissue regeneration. It has been hypothesized that the tumor-tropic property of adult MSCs could be attributed to the tumors secreting factors that mimic those from damaged tissues (Hall *et al.*, 2007). PDGF-BB, EGF, SDF-1alpha and VEGF-alpha have been identified as some possible chemo-attractants that could have triggered adult MSC tropism for tumors (Nakamizo *et al.*, 2005; Schichor *et al.*, 2006; Menon *et al.*, 2007). The up-regulation of matrix metalloproteinase 2, tissue inhibitor of metalloproteinase 2, and the Wnt target genes such as membrane type 1 MMP and cyclin D1 is also responsible for enhancing adult MSC invasiveness (Neth *et al.*, 2006; Ries *et al.*, 2007). We postulate that some of these factors might be responsible for the hESC-MSC tumor tropism. Further evaluation will need to be done to confirm the exact migratory mechanism.

Our *in vitro* killing data suggest that both Hues9 and H1 embryonic stem cells-derived MSCs (Hues9-MSCs and H1-MSCs respectively) exhibit good bystander killing effect on U87 glioma cells when they were used 3 days after transduction with baculovirus containing the HSVtk expression cassette. This is similar to the results we obtained in our previous paper where bone marrow-derived MSCs were transduced with the same baculovirus vector and tested for their bystander effect on U87 glioma cells (Bak *et al.*, 2010). However, while the bystander effect mediated by baculovirus-transduced BM-MSCs was enhanced when the cells were used 8 days after transduction, the bystander effect for baculovirus-transduced Hues9-MSCs and H1-MSCs diminished instead. We hypothesized that the faster proliferation rate of Hues9-MSCs and H1-MSCs could have led to a rapid dilution of the baculovirus genome, HSVtk transcripts and/or HSVtk protein in the cells, causing the drop in killing effect. In view of the small window of time for effective bystander effect, we injected baculovirus-transduced Hues9-MSCs into tumors directly to assess their *in vivo* tumor killing efficacy. Our results from this experiment are comparable to those achieved by other groups using adenovirus to confer IFN-beta or IL-2 expression in MSC for intra-glioma gene delivery (Nakamura *et al.* 2004; Nakamizo *et al.*, 2005), suggesting that baculovirus provides efficiency similar to adenovirus as a transient gene delivery vector.

Compared to baculovirus-transduced cells, lentivirus-transduced Hues9-MSCs appear to give a weaker tumor killing effect in our *in vitro* studies. We hypothesized that this is due to the combined effect of the much lower multiplicity of infection (less than 1) used during lentivirus transduction that caused a lower level HSVtk protein expression in the lentivirus-transduced cells, and the short period of 5 days over which the *in vitro* killing effect was assessed. As lentivirus-transduced Hues9-MSCs give long term HSVtk expression, we decided to inject them at a site contralateral to the U87 tumor inoculation site to test their *in vivo* tumor targeting capacity. The results from this experiment clearly indicate that the lentivirus-transduced, HSVtk-expressing Hues9-MSCs were capable of migrating to a remote tumor site to give a tumor killing effect there when coupled with the HSVtk/ GCV system.

Despite the long-term expression of HSVtk, our lentivirus-transduced hESC-MSCs were unable to completely terminate tumor growth or eliminate tumor volume in this study. Drawing inference from our migration experiment in which not all hESC-MSCs were found located within the tumor 7 days after injection into a contralateral site, we propose that the amount of HSVtk-expressing MSCs that eventually reached the tumor could be too little to give a killing effect that can overcome the rate of tumor growth. Nakamizo *et al.* (2005) estimated that there was about 25% MSC engraftment into gliomas inoculated in the mice brains after injection of IFN-beta-expressing BM-MSCs into the ipsilateral carotid artery. In a contralateral injection model, it is hence reasonable to infer that the long distance over which the MSCs had to migrate would likely lead to an even lower percentage of MSC engraftment into the glioma. One way to improve the killing prowess of this method would be to enhance hESC-MSC migration towards tumors. Clinical low-dose irradiation of murine tumors has been reported to enhance the engraftment of circulating murine MSCs (Klopp *et al.*, 2007), and is one avenue that can be explored and considered. Another way could be to shorten the migratory route of MSCs to the tumor masses. Bexell *et al.* (2009) reported in 2009 that a single intratumoral injection of MSCs is enough to track down $72 \pm 14\%$ of glioma extensions, and $32 \pm 6\%$ of distant microsatellites, suggesting that intratumoral injection is an effective way of stem cell delivery for tumor infiltration.

The natural ability of lentivirus to stably integrate transgenes into the genomes of dividing and non-dividing cells makes it a preferred vector for studies requiring long term transgene expression. It has been used to successfully introduce anti-tumor agents in other stem cell-coupled studies, such as TNF-alpha against prostate cancer (Zhang *et al.*, 2009), pigment epithelium-derived factor against hepatocellular carcinoma (Gao *et al.*, 2010), and S-TRAIL against glioblastoma (Menon *et al.*, 2009). The use of lentivirus is however not without concerns. Derived from the HIV-1 vector, its use poses the risk of subsequent virus replication and viral infection in host cells. Safety features incorporated into the design of the replication-defective lentivirus vector has sought to address these issues. They include a multi-plasmid packaging system to minimize the chance of generating replication-competent lentiviruses through spontaneous homologous recombination between the plasmids, the removal of accessory genes crucial to lentiviral pathogenicity, and the deletion of promoter and enhancer elements at the U3 region of the lentivirus transfer vector, which addresses the issues of homologous recombination, insertional oncogenesis and mobilization of the integrated virus upon host infection with wild type virus (Pauwels *et al.*, 2009). Yet, despite the safety features, the preferential integration of the lentivirus vector into parts of the host genome containing transcriptionally active genes could still potentially give rise to loss or enhancement of host gene function, causing adverse effects when critical genes such as growth-controlling genes, tumor suppressor genes or oncogenic genes are affected (Schroder *et al.*, 2002; Hacein-Bey-Abina *et al.*, 2003; Liu *et al.*, 2006).

Insect baculovirus bypasses the risk of virus replication and potential viral infection in host cells, risks borne by conventionally-used animal viruses such as adenovirus, retrovirus and adeno-associated virus. A large cloning capacity, ease of virus production and low cytotoxicity to transduced cells are other advantages associated with the emergence of baculovirus in the gene delivery field (Hu, 2008). The transient gene expression mediated by baculovirus of non-integrating nature, however, limits its efficacy in studies that necessitate long-term transgene expression. This limitation is not unique to baculovirus, but is also faced by adenovirus, another transient gene delivery vector. In recent years, oncolytic adenoviruses have emerged as a possible alternative to prolong transgene expression from a single round of adenoviral infection. Pre-existing host immune

response against adenoviral vectors and cytotoxicity inflicted by secondary adenovirus infection on the carrier however restricts the efficacy of this approach (Sonabend *et al.*, 2008; Tyler *et al.*, 2009; Ahmed *et al.*, 2010). Unlike adenovirus, baculovirus is not targeted by pre-existing immunity in humans (Strauss *et al.*, 2007). Baculovirus-transduced MSCs has also been shown to not illicit any immune response in immuno-competent recipients (Chuang *et al.*, 2009). To overcome the limitation of transient gene delivery for long term studies, ongoing research with baculovirus has seen the recent development of a hybrid baculovirus vector system that can prolong baculovirus-mediated transgene expression beyond 63 days (Lo *et al.*, 2009). We postulate that incorporation of this baculovirus vector design into future work can help extend the period of HSVtk expression in the hESC-MSC delivery vectors for an improved tumor killing effect.

More research will also need to be done on defining the properties of hESC-MSCs. One particular issue would be the culture purity as the presence of rogue undifferentiated embryonic stem cells in the culture could potentially cause tumorigenesis in downstream applications. Immunogenicity is another issue. While hESC-MSCs have been shown to exhibit the same immunosuppressive effects as adult MSCs (Trivedi and Hematti, 2008; Yen *et al.*, 2009), the eventual fate of hESC-MSCs after tumor targeting and tumor treatment remains unclear. There may be cells that escape the toxic effects of the HSVtk/ganciclovir system by staying quiescent or differentiating in the brain. The latter of these prospects may bring about the generation of terminally differentiated cells that are not immunosuppressive, and which may be targeted by the host immune system. Also, in the field of using MSCs for glioma therapy, controversy has risen over the years over the effects of adult MSCs on tumor growth. In one study, native rat BM-MSCs were reported to suppress the growth of rat glioma (Nakamura *et al.*, 2004). In another, human adipose-derived MSCs were reported to promote the cell viability of human glioma *in vivo* (Yu *et al.*, 2008). To further confuse matters, Bexell *et al.* (2009) reported that adult MSCs, in fact, have no effects on tumor growth. Thorough investigation into the effects of hESC-MSCs on glioma can shed more light on the actual effect of MSCs on these tumors, and more importantly, validate the suitability of these cells as delivery vectors to tumors.

In conclusion, we have shown that MSCs derived through different methods from two human embryonic stem cell lines could be recruited to tumours, and could bring about a killing effect when coupled with the HSVtk/ganciclovir prodrug cancer gene therapy system. The ease with which large amounts of these cells could be derived and genetically modified makes them an attractive option for stem cell-based gene therapy. The potential of this approach is not limited to hESC-MSCs but can likely be extended to MSCs derived from induced pluripotent stem cell lines, an exciting venture that does not just provide a source for large scale derivation of MSCs, but also bypasses the ethical and allogenic issues posed by hESC-MSCs.

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Figure legends

Figure 1. Characterization of H1 hESC-derived MSC-like cells. **(a)** Comparable fibroblast-like morphology of H1-MSCs to that of bone marrow derived MSCs (BM-MSC) as observed under microscope. **(b)** Expression of characteristic MSC surface markers in H1-MSCs. Cells were stained with phycoerythrin (PE)- or allophycocyanin (APC)- conjugated antibodies against the indicated surface markers. Surface marker expression was analyzed through FACS.

Figure 2. Differentiation of H1 hESC-derived MSC-like cells. **(a)** RT-PCR analysis of mesodermal lineage markers. Adipogenic markers used are adipocyte fatty acid binding protein 2 (α P2), peroxisome proliferator-activated receptor γ (PPAR- γ), and lipoprotein lipase (LPL). Osteogenic markers used are osteonectin (OSN), alkaline phosphatase (ALP), Col I, collagen type 1 (Col I), and core binding factor alpha 1 (Cbfa-1). Chondrogenic markers used are link protein (Link), aggrecan (AGN), and collagen type II (Col II). +, with reverse transcriptase; -, minus reverse transcriptase control. **(b)** Specific staining to confirm adipogenic and osteogenic differentiation of H1-MSCs. After 6 weeks of adipogenic culture, adipogenically-differentiated cells with lipid vesicles are shown in b1 and those stained with Oil Red O are shown in b2. Osteogenically-differentiated cells were labeled for calcium deposition using Von Kossa staining and neutral red after 1 month of osteogenic culture and are shown in b3.

Figure 3. Migration of hESC-MSCs towards glioma cells. **(a)** Migration of Hues9-MSCs and H1-MSCs in Boyden chamber towards plain Opti-MEM medium alone (Blank), U87 human glioblastoma cells (U87), or medium with 15% fetal bovine serum (FBS, as the positive control). *Columns*, percentage of migrated cells; *bars*, SD. Statistical comparisons to the blank samples are calculated using ANOVA. +, $P < 0.05$; ++, $P < 0.01$. **(b)** The migration of Calcein AM-labeled Hues9-MSCs on the bottom side of the upper chamber membrane of Boyden chamber. **(c)** Migration of Hues9-MSCs towards glioma in the brain. Green fluorescent dye CM-DiO-labeled U87 glioma cells were inoculated in right hemisphere of the mouse. Seven days after the tumor inoculation, red fluorescent dye CM-Dil-labeled MSCs were inoculated in the left hemisphere. The brains were sectioned 7 days after MSC inoculation. Arrows indicate migrating MSCs. The brain midline and the borders between inoculated MSCs or glioma cells and normal parenchyma are shown. **(d)** High-magnification view of CM-Dil-labeled Hues9-MSCs inside a glioma.

Figure 4. Viral vectors for transduction of hESC-MSCs. **(a)** Schematic drawing of expression cassettes of baculoviral vectors. CMV, the human cytomegalovirus immediateearly gene promoter and enhancer; CMV-W, the CMV promoter and enhancer plus the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) in 3' untranslated region; EF1a, the human elongation factor-1a promoter; EF1a-W, EF1a promoter plus WPRE in 3' untranslated region; CMVe, the human cytomegalovirus immediate-early gene enhancer; C-EF1a, CMVe plus EF1a promoter; C-EF1a-W, C-EF1a plus WPRE;

eGFP, enhanced green fluorescent protein gene; HSVtk, the herpes simplex virus thymidine kinase; pA, polyA tail. **(b)** Transduction efficiency of baculoviral vectors with various expression cassettes containing the *eGFP* gene. Flow cytometry analysis was performed on transduced Hues9-MSCs day 2 posttransduction. **(c)** Time course analysis of *eGFP* gene expression in Hues9-MSCs after CMV-W baculovirus transduction. Flow cytometry analysis was performed on the indicated days. The data represent the mean \pm s.d. of three independent experiments. **(d)** The HSVtk expression after virus transduction in hESC-MSCs. Western blotting analysis was performed using proteins extracted from MSCs collected 2 days after baculovirus transduction or MSCs subjected to lentivirus transduction followed by antibiotic selection for 2 weeks.

Figure 5. *In vitro* tumor cell-killing effects of suicide gene expressing hESC-MSCs. Bystander killing effects were assessed in a co-culture system by mixing U87 glioblastoma cells with Hues9- or H1-MSCs. A baculoviral vector expressing HSVtk (BVTk) was used for transduction of Hues9-MSCs or H1-MSCs in **a-d** and a lentiviral vector expressing HSVtk (LVTK) was used for transduction of Hues9-MSCs in **e** and **f**. The cell mixing ratios are indicated in **a**, **c**, and **e** and a ratio of 50:50 was used in **b**, **d**, and **f**. The baculovirus-transduced cells were mixed with MSCs on day 3 or 8 post-transduction in **a-d**. The lentivirus-transduced cells were used after antibiotic selection. Mixed cells were treated for 5 days with 10 μ M ganciclovir in **a**, **c**, and **e** or the indicated concentrations of ganciclovir in **b**, **d**, and **f** and then collected for cell viability assay. Data are presented as percentages of mixed viable cells without ganciclovir treatment. Each point corresponds to the mean \pm s.d. of 6 data values. ANOVA analysis revealed statistically significant differences between the 100:0 group and the other groups ($P < 0.01$) in **a**, **c**, and **d** and between the untransduced MSC control and transduced MSC groups at all tested ganciclovir concentrations ($P < 0.001$) in **b**, **d**, and **f**.

Figure 6. *In vivo* glioma therapy using hESC-MSCs transduced with baculoviral vectors and injected directly into an established human glioma. Mice were inoculated with U87-*luc* glioma cells at their right striatum. Hues9-MSCs transduced with a baculoviral vector expressing HSVtk (BVTk-MSC) were injected into the established tumor xenografts. Mice injected intratumorally with Hues9-MSCs transduced with a baculoviral vector expressing *eGFP* (BVeGFP-MSC) or with PBS served as controls. Ganciclovir was administered to the mice through intraperitoneal injection right after intratumor injection of MSCs or PBS. **(a)** Bioluminescence images of tumor growth in animals from each group ($n = 8$), 9 days and 25 days after tumor implantation. **(b)** Quantitative analysis of bioluminescence signals from U87-*luc* cells. ANOVA analysis revealed statistically significant differences between the BVTk-MSC group and the two control groups. $P < 0.01$ versus the PBS or BVeGFP-MSC group. **(c)** A prolonged survival in the BVTk-MSC group. Statistical analysis of survival curves was performed using the log-rank test. $P < 0.01$ versus the PBS or BVeGFP-MSC group.

Figure 7. *In vivo* glioma therapy using hESC-MSCs transduced with lentiviral vectors and injected into the cerebral hemisphere opposite an established human glioma. Mice were inoculated with U87-*luc* glioma cells at their right striatum. Hues9-MSCs transduced with a lentiviral vector expressing HSVtk (LVTK-MSC) were injected into the cerebral hemisphere opposite the established tumor xenografts. Mice injected with a lentiviral vector expressing eGFP (LVeGFP-MSC) or with PBS in the same way served as controls. Ganciclovir was administered to the mice through intraperitoneal injection right after brain injection of MSCs or PBS. **(a)** Representative brain sections showing tumor size in animals from each group on day 30 after tumor implantation. **(b)** A prolonged survival in the LVTK-MSC group. Statistical analysis of survival curves was performed using the log-rank test. $P < 0.01$ versus the PBS or LVeGFP-MSC group.

Supplementary Figures

Supplementary Figure 1. Effects of virus transduction on surface marker profile in human embryonic stem cell-derived mesenchymal stem cells. Hues9-MSCs were transduced with a baculoviral vector containing the HSVtk expression cassette and the cells were stained 2 days after transduction with phycoerythrin (PE)- or allophycocyanin (APC)- conjugated antibodies against the indicated surface markers. To perform lentivirus transduction, a lentiviral vector containing the HSVtk expression cassette and a blasticidin antibiotic marker for selection of cells with stably integrated *HSVtk* gene was used. After transduction and two weeks of antibiotic selection, the selected cells were similarly stained with PE- or APC- conjugated antibodies against indicated surface markers. Surface marker expression was analyzed through FACS.

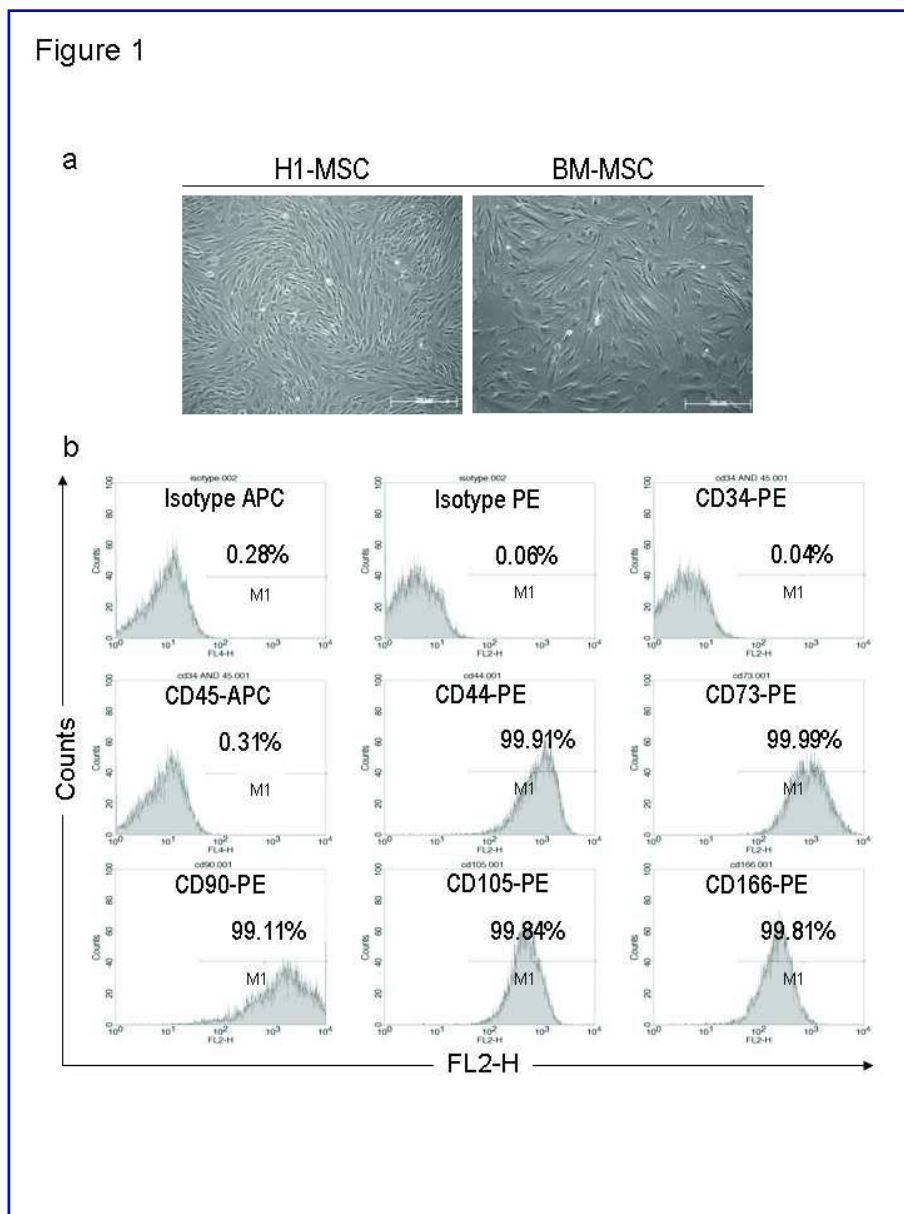


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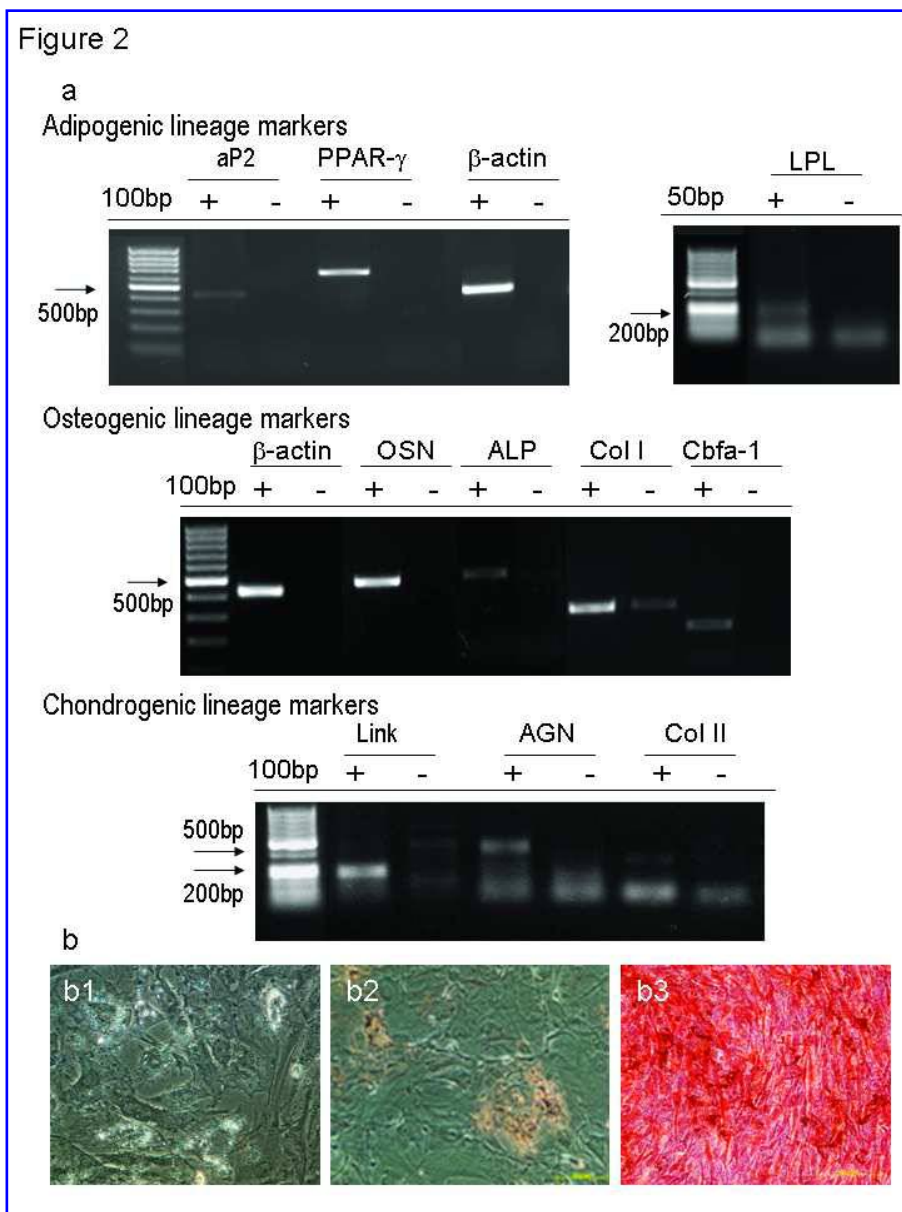


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Human Embryonic Stem Cell-derived Mesenchymal Stem Cells as Cellular Delivery Vehicles for Prodrug Gene Therapy of Glioblastoma (doi: 10.1089/hum.2010.212)
This article has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

Human Gene Therapy

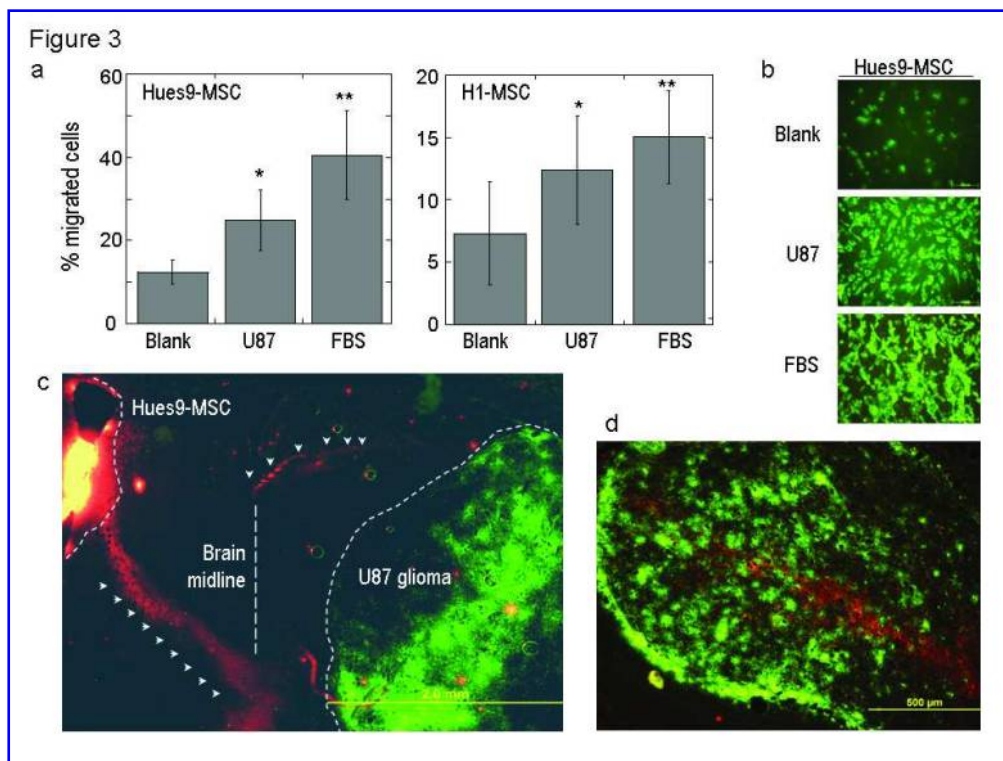


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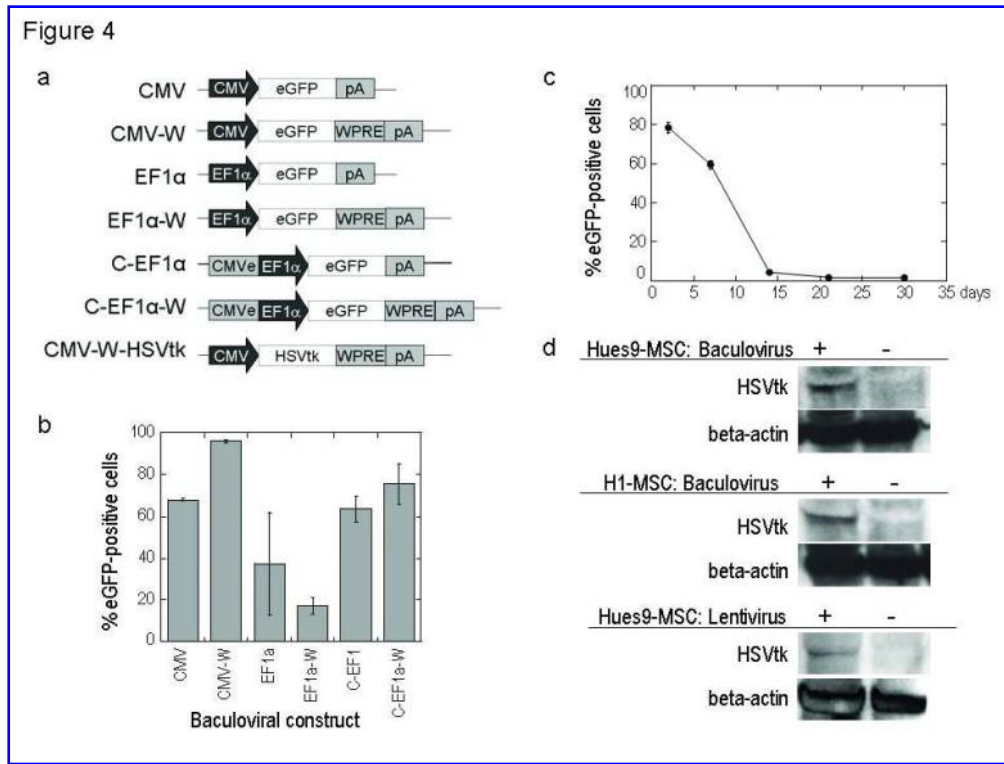


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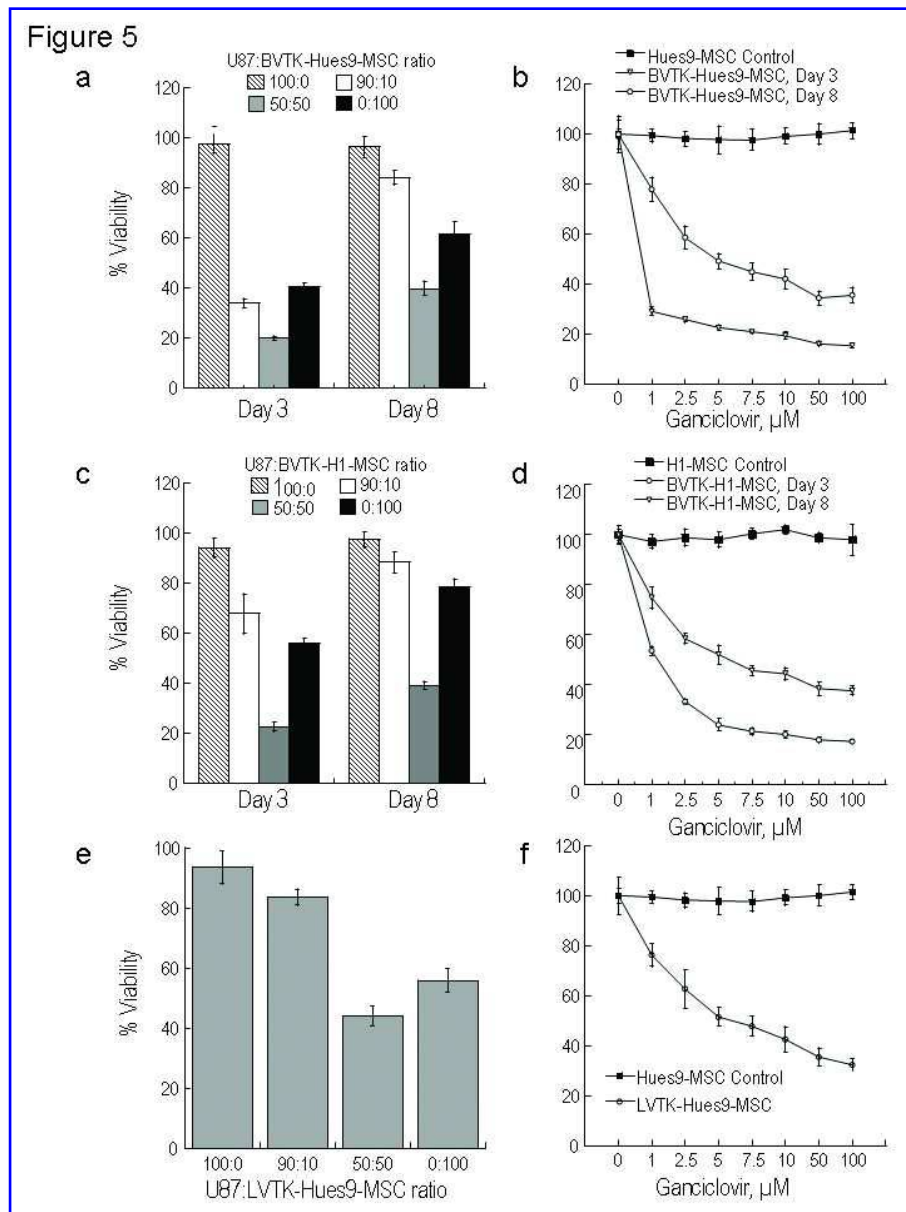


Figure 5. In vitro tumor cell-killing effects of suicide gene expressing hESC-MSCs. Bystander killing effects were assessed in a co-culture system by mixing U87 glioblastoma cells with Hues9- or H1-MSCs. A baculoviral vector expressing HSVtk (BVTk) was used for transduction of Hues9-MSCs or H1-MSCs in a-d and a lentiviral vector expressing HSVtk (LVTK) was used for transduction of Hues9-MSCs in e and f. The cell mixing ratios are indicated in a, c, and e and a ratio of 50:50 was used in b, d, and f. The baculovirus-transduced cells were mixed with MSCs on day 3 or 8 post-transduction in a-d. The lentivirus-transduced cells were used after antibiotic selection. Mixed cells were treated for 5 days with 10 μ M ganciclovir in a, c, and e or the indicated concentrations of ganciclovir in b, d, and f and then collected for cell viability assay. Data are presented as percentages of mixed viable cells without ganciclovir treatment. Each point corresponds to the mean \pm s.d. of 6 data values. ANOVA analysis revealed statistically significant differences between the 100:0 group and the other groups ($P < 0.01$) in a, c, and d and between the untransduced MSC control and transduced MSC groups at all tested ganciclovir concentrations ($P < 0.001$) in b, d, and

f.
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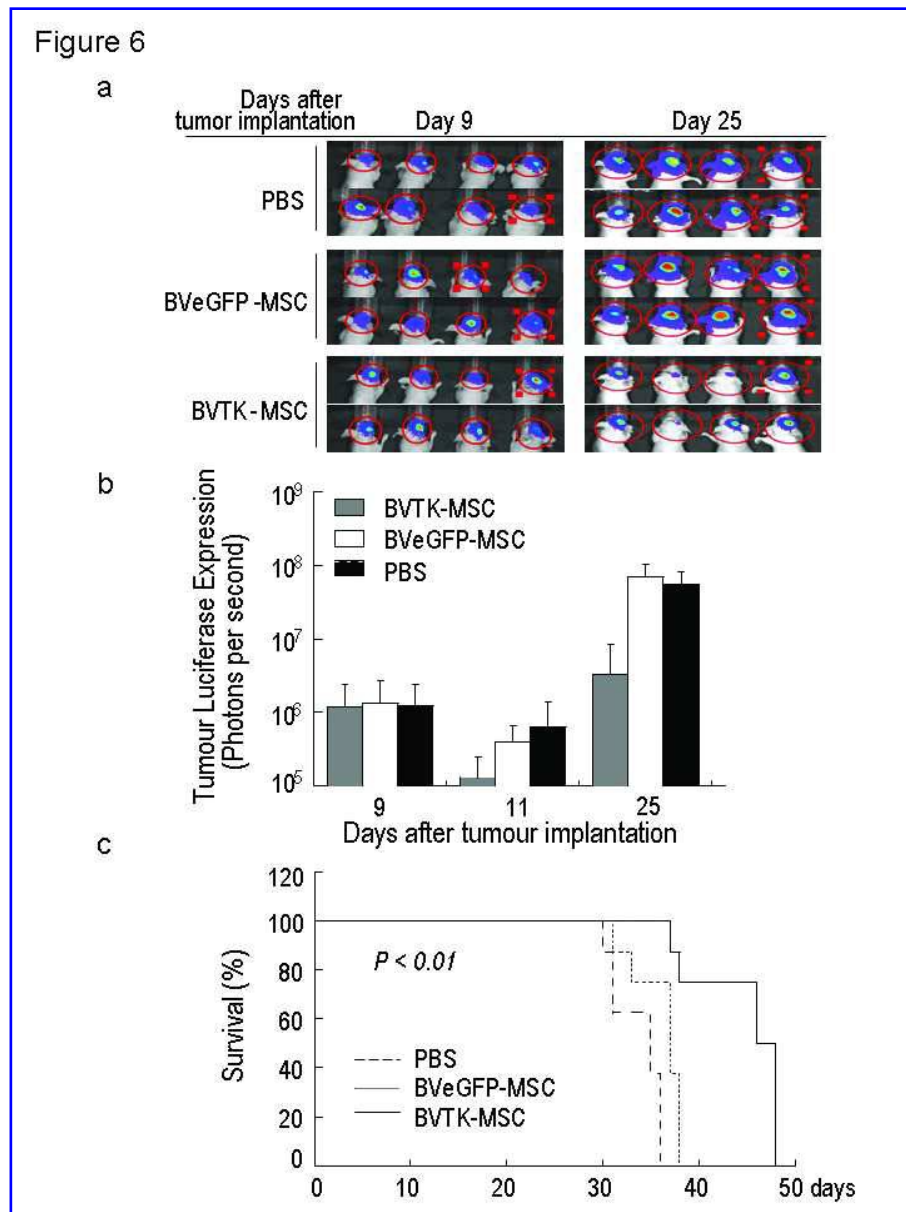


Figure 6. In vivo glioma therapy using hESC-MSCs transduced with baculoviral vectors and injected directly into an established human glioma. Mice were inoculated with U87-luc glioma cells at their right striatum. Hues9-MSCs transduced with a baculoviral vector expressing HSVtk (BVTK-MSC) were injected into the established tumor xenografts. Mice injected intratumorally with Hues9-MSCs transduced with a baculoviral vector expressing eGFP (BVeGFP-MSC) or with PBS served as controls. Ganciclovir was administered to the mice through intraperitoneal injection right after intratumor injection of MSCs or PBS. (a) Bioluminescence images of tumor growth in animals from each group (n = 8), 9 days and 25 days after tumor implantation. (b) Quantitative analysis of bioluminescence signals from U87-luc cells. ANOVA analysis revealed statistically significant differences between the BVTK-MSC group and the two control groups. $P < 0.01$ versus the PBS or BVeGFP-MSC group. (c) A prolonged survival in the BVTK-MSC group. Statistical analysis of survival curves was performed using the log-rank test. $P < 0.01$ versus the PBS or BVeGFP-MSC group.

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Human Embryonic Stem Cell-derived Mesenchymal Stem Cells as Cellular Delivery Vehicles for Prodrug Gene Therapy of Glioblastoma (doi: 10.1089/hum.2010.212)
This article has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

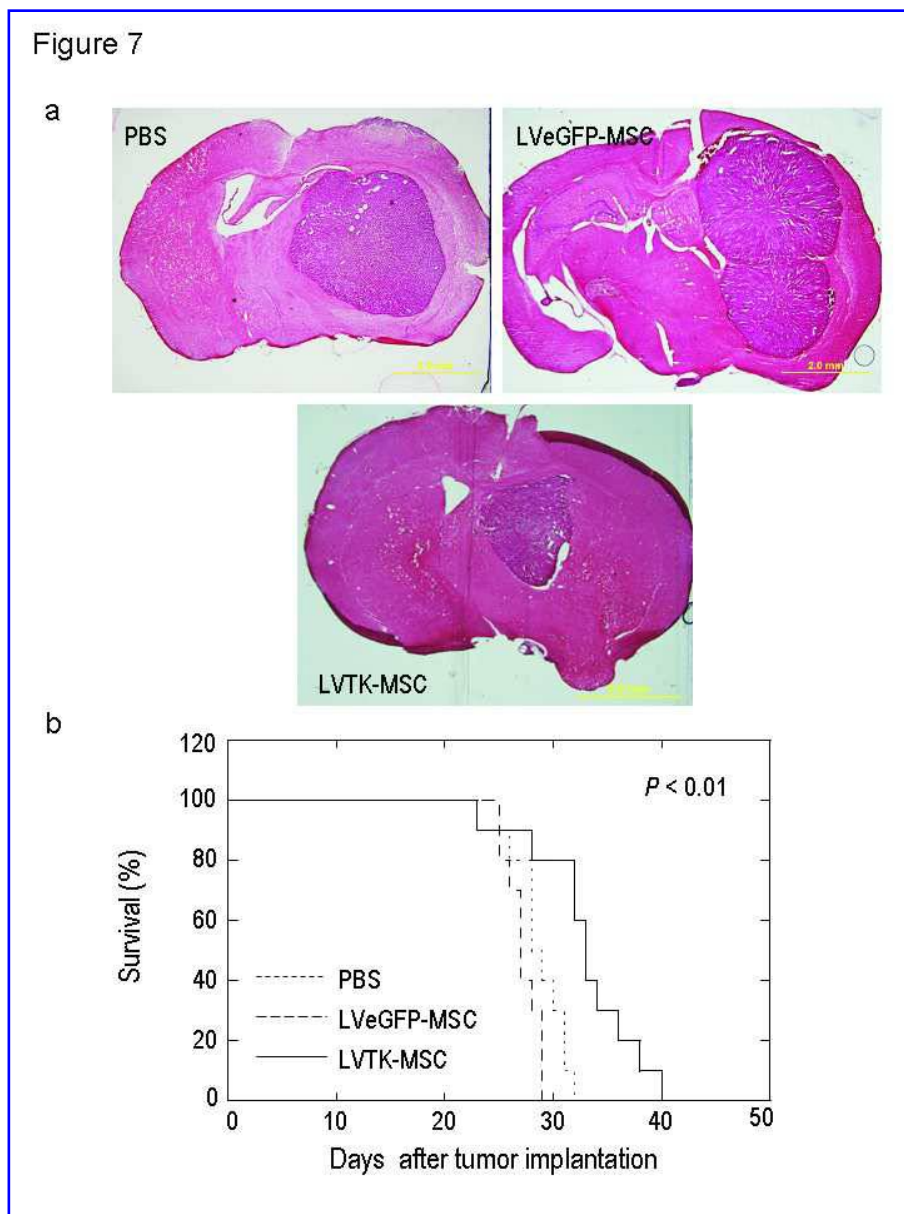
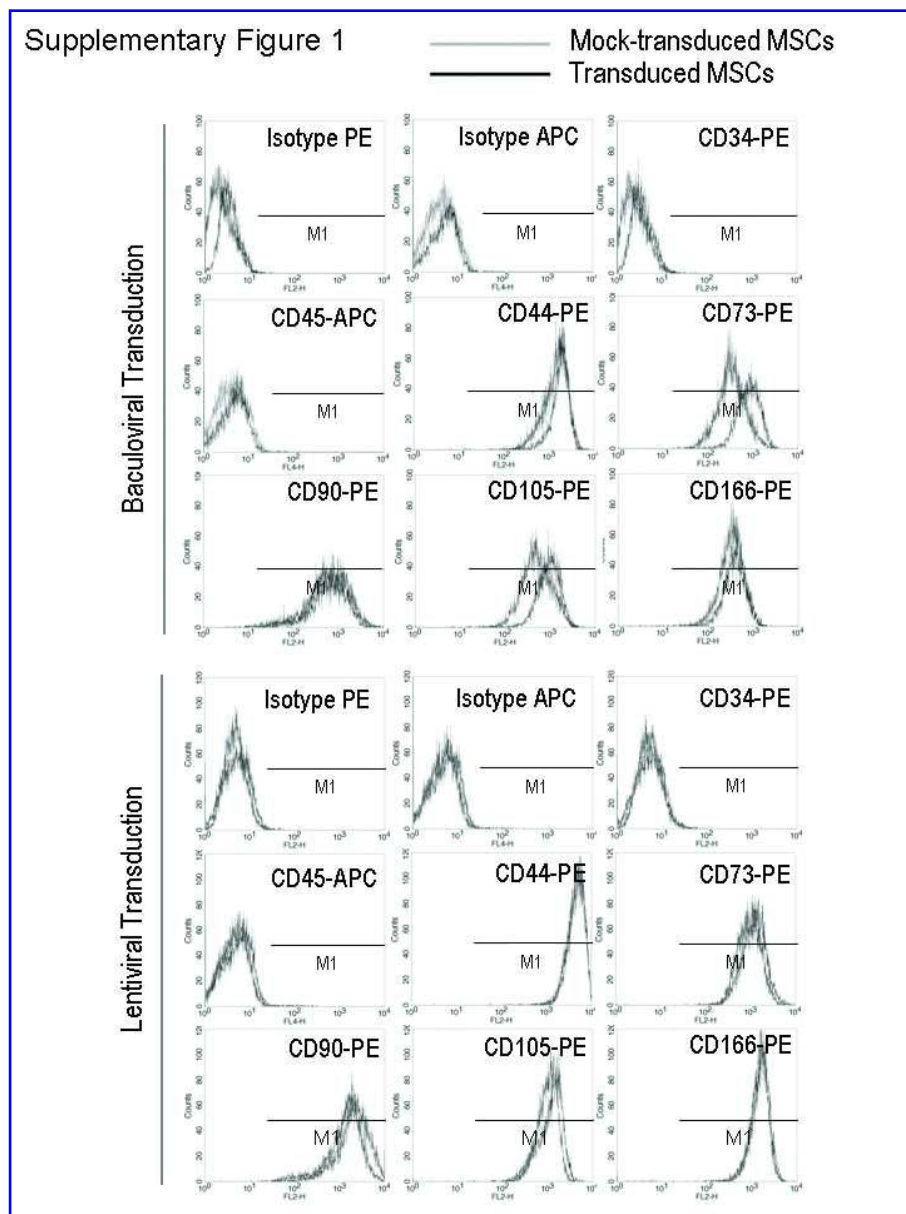


Figure 7. In vivo glioma therapy using hESC-MSCs transduced with lentiviral vectors and injected into the cerebral hemisphere opposite an established human glioma. Mice were inoculated with U87-luc glioma cells at their right striatum. Hues9-MSCs transduced with a lentiviral vector expressing HSVtk (LVTK-MSC) were injected into the cerebral hemisphere opposite the established tumor xenografts. Mice injected with a lentiviral vector expressing eGFP (LVeGFP-MSC) or with PBS in the same way served as controls. Ganciclovir was administered to the mice through intraperitoneal injection right after brain injection of MSCs or PBS. (a) Representative brain sections showing tumor size in animals from each group on day 30 after tumor implantation. (b) A prolonged survival in the LVTK-MSC group. Statistical analysis of survival curves was performed using the log-rank test. $P < 0.01$ versus the PBS or LVeGFP-MSC group.

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Supplementary Figure 1. Effects of virus transduction on surface marker profile in human embryonic stem cell-derived mesenchymal stem cells. Hues9-MSCs were transduced with a baculoviral vector containing the HSVtk expression cassette and the cells were stained 2 days after transduction with phycoerythrin (PE)- or allophycocyanin (APC)- conjugated antibodies against the indicated surface markers. To perform lentivirus transduction, a lentiviral vector containing the HSVtk expression cassette and a blasticidin antibiotic marker for selection of cells with stably integrated HSVtk gene was used. After transduction and two weeks of antibiotic selection, the selected cells were similarly stained with PE- or APC- conjugated antibodies against indicated surface markers. Surface marker expression was analyzed through FACS.

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