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Glioma Gene Therapy Using Induced Pluripotent Stem Cell Derived Neural Stem Cells

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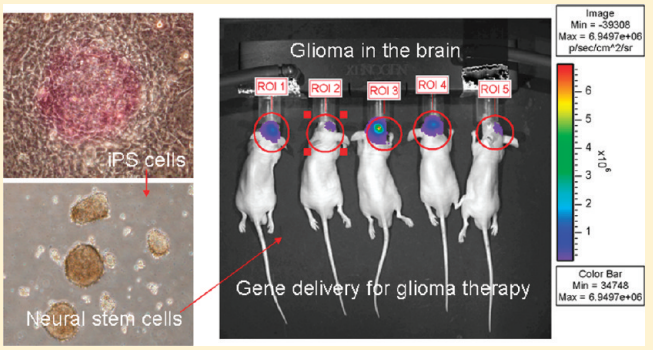
Supporting Information

ABSTRACT: Using neural stem cells (NSCs) with tumor tropic migratory capacity to deliver therapeutic genes is an attractive strategy in eliminating metastatic or disseminated tumors. While different methods have been developed to isolate or generate NSCs, it has not been assessed whether induced pluripotent stem (iPS) cells, a type of pluripotent stem cells that hold great potential for regenerative medicine, can be used as a source for derivation of NSCs with tumor tropism. In this study, we used a conventional lentivirus transduction method to derive iPS cells from primary mouse embryonic fibroblasts and then generated NSCs from the iPS cells. To investigate whether the iPS cell derived NSCs can be used in the treatment of disseminated brain tumors, the cells were transduced with a baculoviral vector containing the herpes simplex virus thymidine kinase suicide gene and injected into the cerebral hemisphere contralateral to a tumor inoculation site in a mouse intracranial human glioma xenograft model. We observed that NSCs expressing the suicide gene were, in the presence of ganciclovir, effective in inhibiting the growth of the glioma xenografts and prolonging survival of tumor-bearing mice. Our findings provide evidence for the feasibility of using iPS cell derived NSCs as cellular vehicles for targeted anticancer gene therapy.



KEYWORDS: induced pluripotent stem cells, neural stem cells, glioma, gene therapy

INTRODUCTION

For more than three decades now, stem cells have been used in replenishment of blood and immune cells damaged by cancer chemotherapy and radiotherapy. Recently, stem cells have been exploited as vehicles to deliver therapeutic genes for cancer treatment. Especially, neural stem/progenitor cells (NSCs) are found to be able to migrate through brain parenchyma and home in on tumor foci.[1] In animal tumor models, the tropism of NSCs for tumors has been utilized to target not only major tumor mass but also distant tumor outgrowths, with successful treatments reported for glioma, nonglial brain tumors and solid tumors of a non-neural origin.[1−12]

Due to the availability of valid murine tumor models, murine NSC lines or murine primary NSCs are often used to investigate the migratory capacity of tumor tropic NSCs and predict their preclinical therapeutic efficacy.[1−3,6,9,12] Pluripotent stem cells, such as embryonic stem (ES) cells and induced pluripotent stem (iPS) cells, can be expanded indefinitely in culture and have the potential to generate all types of cells *in vitro* in virtually unlimited numbers. Hence these cells are attractive cell sources to derive differentiated cells, including NSCs, for drug screening and regenerative medicine.[13,14] Although ES cells and iPS cells are functionally equivalent in many aspects, iPS cells could be a more powerful option for animal experimentation since they can be relatively easily generated from somatic cells. The differentiated progeny of iPS cells can be fully syngeneic in immunocompetent hosts, thus circumventing problems of immunological incompatibility. The method based on retroviral or lentiviral transduction of four transcription factors, Oct4, Sox2, c-Myc and Klf4, has been demonstrated to be highly effective in generating iPS cells.[15]

In this proof of principle study, we examined whether tumor tropic NSCs can be derived from iPS cells and used for cancer

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therapy. We first generated iPS cells from mouse embryonic fibroblasts and then used a monoculture method to generate NSCs from the iPS cells. After introducing a therapeutic gene into the NSCs using a baculoviral vector, we tested the tumor killing effects of the transduced NSCs *in vitro* and *in vivo*. Our results provide evidence for the feasibility of generating competent NSCs from iPS cells with tumor tropism. This approach could be extended to generation of human NSCs for cancer therapy in patients.

EXPERIMENTAL SECTION

Generation of Mouse iPS Cells. STEMCCA-EF1α, a lentiviral vector that uses the constitutive EF1α promoter to drive the expression of a single stem cell cassette composed of four transcription factors, Oct4, Sox2, Klf4 and c-Myc, was used for reprogramming.[16] The cassette plasmid was kindly provided by Gustavo Mostoslavsky, Department of Medicine, Boston University School of Medicine. Lentiviruses were produced using 293FT packaging cells with ViraPower Lentiviral Expression Systems (Invitrogen, Carlsbad, CA). Supernatants were collected every 12 h over two consecutive days starting 48 h after transfection. Viral particles were concentrated by centrifugation at 16,500 rpm at 4 °C. Lentiviral titering was measured by transducing HT1080 cells with 10-fold serial dilutions of STEMCCA-EF1α lentivirus. After 10−12 days of antibiotic selection, antibiotic-resistant colonies can be detected using crystal violet staining. Blue-stained colonies were counted and used to determine lentiviral titer.

Primary mouse embryonic fibroblasts (MEFs, CF-01) were obtained from forebrain of embryonic mice strain CF1 (Biopolis Shared Facilities, Singapore). CF01 MEFs (1×10^5) were seeded in 35 mm dishes and transduced with 10 μL of concentrated STEMCCA-EF1α at multiplicity of infection (MOI) 5 in the presence of Polybrene (5 μg/mL) in MEF medium composed of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% of 200 mM L-glutamine and 1% of pen/strep (Invitrogen). After 16 h, MEF medium was replaced with mouse ES cell medium composed of DMEM supplemented with 15% FBS, L-glutamine, NEAA, β-mercaptoethanol (Invitrogen) and 1000 U/mL leukemia inhibitory factor (LIF, Peprotech, Rocky Hill, NJ). The ES medium was changed every 2 to 3 days. Colonies were picked 14 days post-transduction based on morphology and clonally expanded on a feeder layer of MEFs treated with mitomycin C (Sigma-Aldrich, St Louis, MO) in mouse ES cell medium. Derived mouse iPS cells were cultured in mouse ES culture medium and routinely expanded in 1:5−1:8 ratio. The cells were assessed using alkaline phosphatase (AP) staining with the AP substrate kit (Sigma-Aldrich) in accordance with the manufacturer's instruction. For *in vitro* differentiation into embryoid bodies, iPS cells were trypsinized, transferred to bacterial culture dishes, and cultured in the ES cell medium without LIF.

Derivation of NSCs from Mouse iPS Cells. We used a monoculture method for differentiation of mouse iPS cells into neural cells.[17,18] Prior to initiating differentiation, mouse iPS cells were cultured under feeder-free conditions in mouse ES cell medium for 7 days. These mouse iPS cells were then resuspended in NDiff N2B27 medium (StemCells Sciences, Palo Alto, CA) and plated onto a 0.1% gelatin-coated 100 mm dish at a density of 1.2×10^6 per dish. Culture medium was changed every day to remove dead or detached cells. For monoculture differentiation of mouse iPS cells, the cells were dissociated to single cells using trypsin and plated onto 0.1% gelatin-coated 6-well cell culture plates at a density of 10^6 per well and cultured in NSC expansion medium consisting of DMEM/F12 (1:1 mixture) medium (Invitrogen) supplemented with 2% B27 (Invitrogen), 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/mL streptomycin, 20 ng/mL basic fibroblast growth factor (bFGF, Sigma-Aldrich) and 20 ng/mL epidermal growth factor (EGF, Invitrogen). Half of the cell-culture medium was changed every 2 days, and cells were split at 1:2 when they reached 90% confluence using TrypLE Express Dissociation Enzyme (Invitrogen). After 1 month of passaging, a homogeneous cell population with characteristic bipolar NSC morphology was achieved.

For passaging established NSCs, cells were treated with TrypLE and subcultured twice weekly at a split ratio of 1:2. To induce neural differentiation, NSCs were transferred to uncoated, low cell binding 6-well plates (Nalge Nunc International, Rochester, NY) in the above NSC culture medium. After culturing for 1 to 3 weeks, round neurospheres were formed. The spheres were then plated into poly-D-lysine (Sigma-Aldrich) and laminin (Sigma-Aldrich) coated dishes. Neuronal and glial differentiation was induced by the withdrawal of EGF and bFGF from the culture medium.

Cell Characterization. For RT-PCR analysis, RNA was isolated with TRIzol (Invitrogen) and cleaned up with Turbo DNase (Applied Biosystems, Foster City, CA). RNA was used to produce cDNAs by reverse transcription with oligo(T) priming in Superscript III First Strand Synthesis System (Invitrogen). PCR amplification of cDNAs was carried out using Taq polymerase. RT-PCR primer sequences are listed in Supplementary Table 1 in the Supporting Information. The mouse embryonic stem cell array PAMM-081A purchased from QIAGEN (Valencia, CA) was also used to compare the expression of embryonic stem cell markers between MEFs and mouse iPS cells. RT^2 First Strand Kit (QIAGEN) and 2X RT^2 SYBR Green/Fluorescein qPCR Master Mix (QIAGEN) were used to prepare first strand cDNA for real-time PCR. Real-time PCR was performed with an iCycler (Bio-Rad, Hercules, CA) using the following thermal cycling condition: at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min for fluorescence detection. Three independent replicates per cell group were used for analysis. The threshold cycle (C_t) values were then exported to an Excel spreadsheet for analysis with SABiosciences PCR Array Data Analysis Web Portal (http://www.SABiosciences.com/pcrarraydataanalysis.php).

For immunostaining and Western blot analysis, the following primary antibodies were used: anti-Sox2, anti-Oct3/4, anti-KLF4, anti-Nanog (R&D systems, Minneapolis, MN), anti-c-Myc, anti-Nestin, anti-GFAP, anti-thymidine kinase (TK), anti-SSEA1, anti-beta-III-tubulin (Tuj1, Santa Cruz, Santa Cruz, CA) and anti-actin (Sigma-Aldrich). Antibodies were diluted at 1:200 for immunostaining and 1:2000 in Western blot analysis. Secondary horseradish peroxidase-linked antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Secondary fluorescent Alexa488, Alexa546 conjugated anti-mouse and anti-rabbit antibodies were purchased from Invitrogen. The samples were counterstained with DAPI (Invitrogen) before observation.

In vitro migration of NSCs toward glioma cells was examined using Boyden chamber assays. A migration kit with 24-well cell culture plates was purchased from BD Biosciences (San Diego, CA). Plates were separated into 2 chambers by an insert membrane

with 8 μm pore. One day before assay, 2.5×10^5 MEFs, rat C6 glioma cells purchased from American Type Culture Collection (ATCC, Manassas, VA) or human U87 glioma cells (ATCC) were seeded into lower chambers. NSCs were labeled with calcein (Invitrogen) and serum starved overnight. The next day cell culture medium in the lower chamber was replaced with fresh OptiMEM and calcein labeled NSCs (0.8×10^5 per well diluted in fresh OptiMEM) were seeded into the upper chamber. Cell migration toward optiMEM alone, without tumor cells in the lower chamber, was included for measurement of basal migration rate. After 24 or 48 h incubation at 37 °C, migrating cells found at insert bottoms were quantified by measuring fluorescence intensity with a fluorescence plate reader. Values are expressed as the mean ± SD in percentage of NSC migration toward optiMEM alone.

Genetic Modified iPS-NSCs for Glioma Gene Therapy. Recombinant baculovirus vectors containing the EGFP gene or herpes simplex virus thymidine kinase (HSVtk) gene expression cassette were constructed previously.[19] Viruses were produced and propagated in *Spodoptera frugiperda* (Sf9) insect cells pre-adapted to Sf-900 II serum-free medium (Invitrogen). Subsequent baculovirus preparation steps and virus titer measurements were conducted according to the Bac-to-Bac Baculovirus Expression system manual (Invitrogen)

For *in vitro* tests, mouse iPS derived NSCs were seeded at a density of 5×10^4 cells per well in a 6-well plate and transduced overnight with baculoviral vectors at an MOI of 100 in NSC expansion medium. Fluorescence-activated cell sorting (FACS) analysis was used to quantify the transduction efficiency of the baculoviral vector with the EGFP gene. After trypLE dissociation, the cells were collected, washed in PBS twice and analyzed with FACSCalibur flow cytometer (BD Biosciences). To evaluate the sensitivity of HSVtk-expresssing cells to GCV, cells were transduced with the baculoviral vector containing the HSVtk gene. Cells were then seeded in a 96-well cell culture plate at a density of 1,000 cells per well and treated with GCV at concentrations of 0 to 100 μM in NSC expansion media as specified in the text. GCV containing medium was changed every 2 days. Cell viability assay was performed 5 days later using CellTiter-96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI).

For *in vivo* experiments, adult female Balb/c nude mice aged 6−8 weeks were used. An intracranial glioma model was generated by injecting 5×10^4 U87MG-luciferase cells (U87-*luc*, Caliper Life Sciences, Hopkinton, MA) in 5 μL of PBS into right hemisphere of mouse brain at a speed of 1×10^4 cells/min using a Hamilton syringe connected with 30G needle. On day 7, baculovirus-transduced, HSVtk-expressing NSCs (10^6 in 5 μL PBS) or 5 μL of PBS was injected into the contralateral hemisphere. Animals were intraperitoneally administered with 200 μL of 5 mg/mL GCV (50 mg/kg body weight) or PBS daily from day 14 to day 35 post tumor inoculation. All together, 4 groups (n = 10 per group) were tested: brain injection of PBS followed by PBS intraperitoneal (ip) injection, brain injection of PBS followed by GCV ip injection, brain injection of NSCs followed by PBS ip injection, and brain injection of NSCs followed by GCV ip injection. To monitor bioluminescence signals of U87-*luc* cells, isoflurane gas-anesthetized animals were ip injected with D-luciferin (Promega) at 100 mg/kg 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). 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.

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 represented as mean ± SD. The statistical significance of differences was determined by ANOVA with replication followed by Fisher's least significant difference post hoc analysis. The statistical analysis of survival data was performed using the log rank test followed by the Holm−Sidak method for pairwise multiple comparison tests. A p value of <0.05 was considered to be statistically significant.

RESULTS

Reprogramming of Mouse Embryonic Fibroblasts to Induced Pluripotent Stem Cells and the Derivation of Neural Stem Cells. We used a conventional protocol based on lentivirus-mediated expression of four transcription factors, Oct4, Sox2, Klf4 and c-Myc, to reprogram mouse embryonic fibroblasts (MEFs) to generate iPS cells. Already evident in post infection day 7, MEFs showed a significant change in morphology and formed colonies (Figure 1A). The formed colonies could be clonally expanded and displayed the typical morphology of ES cell colonies. In contrast with fibroblasts prior to reprogramming, cells in these colonies were alkaline phosphatase (AP) positive, displayed strong expression of classic ES cell marker genes, and expressed the Oct3/4 and SSEA1 proteins (Figure 1A−C). Moreover, PCR superarray analysis using PAMM-081A Mouse Embryonic Stem Cells Array showed significant upregulation of embryonic stem cell specific genes, especially those genes encoding the transcription factors that are involved in maintaining "stemness" (Table 1). These genes include *Nanog*, *Nr5a2*, *Pou5f1*, *Sox2*, *Tcfcp2l1*, *Utf1* and *Zfp42*, which were upregulated 273-, 151-, 2018-, 218-, 229-, 1195-, and 1107-fold, respectively, relative to gene expression in MEFs. Among total 44 embryonic stem cell specific genes tested, 22 genes were upregulated for more then 5-fold, with half of them being massively (>100-fold) upregulated (Table 1; Supplementary Table 2 in the Supporting Information). In contrast, 11 out of 40 embryonic stem cell differentiation and lineage markers were upregulated and many of them had a fold change less than 10 (Table 1; Supplementary Table 2 in the Supporting Information). When the cells isolated from the clones were propagated in ES medium without LIF, spherical aggregates, termed embryoid body (EB), were formed spontaneously, which expressed markers for three germ layers (Figure 1D). These results indicate that the cells derived from MEFs are indeed iPS cells.

To further examine the differentiation potential of the mouse iPS cells, we used an adherent monoculture method[17,18] to generate NSCs in a defined culture condition containing basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF). The generated adherent NSCs were able to form neurospheres after culturing in a low cell binding plate (Figure 2A). The doubling time of the NSCs was approximately 3−4 days. The NSCs could be continuously cultured for at least 6 months. Furthermore, these iPS cell derived NSCs could be cryopreserved in an NSC medium containing 10% DMSO and remain viable after liquid nitrogen storage.

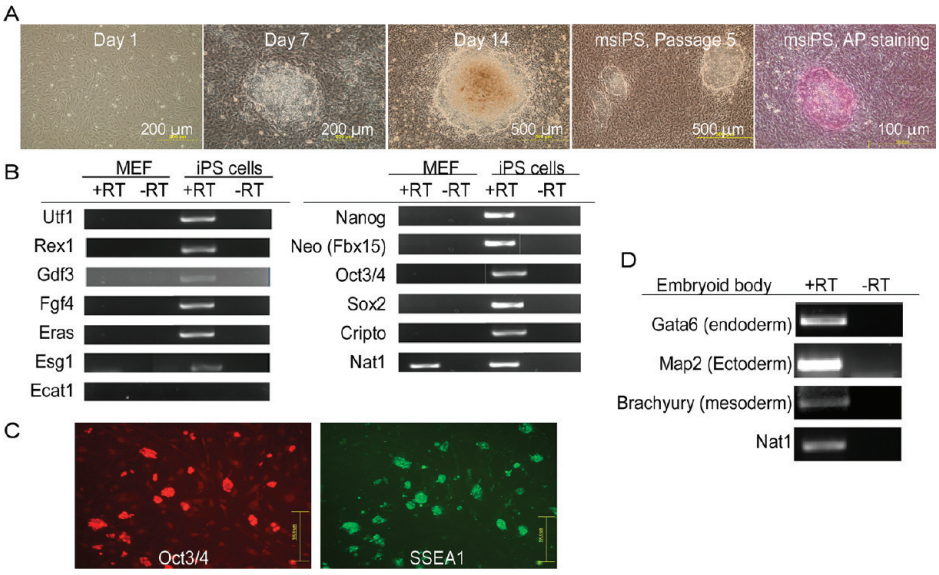

Figure 1. Generation of iPS cells from mouse embryonic fibroblasts (MEFs). (A) Mouse iPS colony formation after MEF transduction with a lentiviral vector expressing transcription factors OCT4, KLF4, SOX2 and c-MYC. Generated colonies can be maintained and expanded on MEF feeder layer. An alkaline phosphatase (AP) positive colony is shown. (B) RT-PCR with Nat1 as loading control confirms the expression of known "stemness" genes in generated iPS cells, but their expressions are absent in MEFs. +RT and −RT: With and without reverse transcriptase. (C) Immunocytochemical staining to show protein expression of embryonic stem cells markers SSEA1 and Oct3/4 in generated iPS cells. (D) RT-PCR analysis to show the expression of markers for three germ layers. Nat1 was included as a loading control. +RT and −RT: With and without reverse transcriptase.

Table 1. Changes in Expression of Embryonic Stem Cell Specific Marker Genes and Embryonic Stem Cell Differentiation/Lineage Marker Genes between Mouse iPS Cells and Mouse Embryonic Fibroblasts (MEFs) as Assessed by Quantitative PCR Array[a]

functional gene grouping[b]	upregulated genes/tested genes
Embryonic Stem Cell-Specific Genes	
transcription factors maintaining "stemness": Foxd3 (15), Gata6, Gbx2 (51), Nanog (273), Nr5a2 (151), Nr6a1 (15), Pou5f1 (2018), Sox2 (218), Tcfcp2l1 (229), Utf1 (1195), Zfp42 (1107)	10/11
signaling molecules required for pluripotency and self-renewal: Commd3, Crabp2, Ednrb, Fgf4 (347), Fgf5, Gabrb3, Gal, Grb7 (27), Hck, Ifitm1 (27), Il6st, Kit (36), Lefty1 (213), Lefty2 (288), Lifr, Nodal (41), Nog, Numb, Pten, Sfrp2, Tdgf1 (897)	8/21
other embryonic stem cell specific genes: Bxdc2, Cd9, Diap2, Dnmt3b (16), Ifitm2, Igf2bp2, Lin28 (101), Podxl (35), Rest, Sema3a, Tert, Gdf3 (48)	4/12
total (%)	22/44 (50%)
Embryonic Stem Cell Differentiation/Lineage Markers	
extraembryonic endoderm markers: Foxa2, Gata4, Ptf1a	0/3
trophoblast markers: Cdx2 (7), Eomes (26), Gcm1 (9), Krt1 (8)	4/4
visceral endoderm markers: Afp, Serpina1a	0/2
parietal endoderm markers: Fn1, Lama1 (21), Lamb1−1, Lamc1, Sox17	1/5
mesoderm markers: T (97), Wt1	1/2
muscle: Des, Myf5, Myod1	0/3
blood: Hba-x, Hbb-y	0/2
bone: Col1a1, Runx2	0/2
neural: Nes, Neurod1 (27), Pax6 (6)	2/3
endothelial: Cd34, Cdh5, Flt1, Pecam1 (79)	1/4
germ cell: Ddx4 (8), Sycp3 (140)	2/2
pancreas: Gcg, Iapp, Ins2, Pax4, Pdx1, Sst	0/6
other embryonic stem cell differentiation/lineage markers: Olig2, Tat	0/2
total (%)	11/40 (27.5%)

[a] Mouse embryonic stem cell array PAMM-081A from QIAGEN was used. [b] Fold change values are shown in parentheses if they displayed a >5-fold upregulation relative to MEFs.

To characterize the iPS cell derived NSCs, we examined the expression of neural progenitor markers and neural multipotency of these cells. RT-PCR analysis confirmed the expression of markers associated with neural precursor cells, including NCAM,

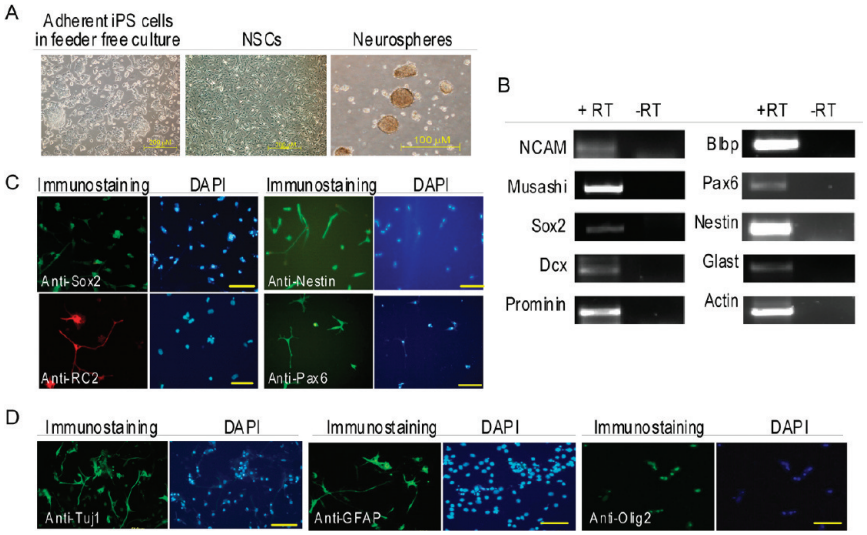

Figure 2. Generation and characterization of neural stem cells derived from mouse iPS cells. (A) Neural stem cell induction. iPS cells were first grown on feeder-free, gelatin-coated plates. Neurospheres were formed and expanded in the absence of LIF and serum in the N2B27 medium. Neurospheres were then trypsinized to single cells and maintained on Matrigel-coated plates for expansion. (B) RT-PCR analysis to show the expression of NSC markers in iPS cell derived NSCs. +RT and −RT: With and without reverse transcriptase. (C) Immunocytochemical staining to show protein expression of NSC markers, nestin, RC2 and Pax6, in the generated NSCs. Sox2 protein was also detectable. (D) Immunocytochemical staining to show protein expression of NSC differentiation markers. The NSCs differentiate into Tuj1-positive neurons, GFAP-positive astrocytes, as well as Oligo2-positive oligodendrocytes.

musashi, Sox2, dcx, prominin, BLBP, Pax6, nestin and Glast (Figure 2B). Immunocytochemistry analysis showed that NSCs were positive for Sox2, nestin, RC2 and Pax6 (Figure 2C). To obtain a rich population of neurons, NSCs were harvested and replated on laminin-coated plates in the presence of EGF and bFGF for 7 days. After a further 7 days of culturing without EGF and bFGF, Tuj1-positive neuronal cells were observable (Figure 2D). For glial differentiation, NSCs were seeded on gelatin coated dishes and differentiated into GFAP-positive astrocytes when cultured in NSC expansion medium supplemented with 1% FCS and N2 supplement but without EGF and bFGF (Figure 2D). Oligodendrocyte differentiation was obtained by culturing NSCs in Neurobasal medium supplemented with 2% B27 and 30 ng/mL T3 for 3 to 4 weeks (Figure 2D).

Glioma-Specific Migration Ability of Mouse iPS Cell Derived NSCs. The glioma tropism of primary NSCs and NSC lines has been well documented. To examine the selective tropism of iPS cell derived NSCs for glioma, we seeded C6 rat glioma cells, U87 human glioma cells, and nontumor MEFs, respectively, into the lower Boyden chamber as attractants and determined the migration of NSCs labeled with a green fluorescent dye. As quantified using a fluorescence microplate reader, iPS cell derived NSCs displayed high migration capacities toward the glioma cells, but not to MEFs (Figure 3A). To examine the *in vivo* glioma tropism of iPS cell derived NSCs, an intracranial glioma model was generated by injection of the U87 human glioma cells into the right striatum of BALB/c nude mice. To facilitate histological examination, we labeled iPS cell derived NSCs with the red fluorescent dye CM-DiI and injected the labeled cells into the striatum contralateral to the tumor inoculation site 7 days later. In the sections of the brains collected on day 14 post NSC injection, we observed extensive colocalization of iPS cell derived NSCs with the tumor mass. Many NSC1 cells had penetrated several hundreds of micrometers into the tumor mass by then (Figure 3B). The strong tropism of iPS cell derived NSCs toward glioma cells suggests that these cells are well suited as delivery vectors for glioma therapy.

HSVtk-Expressing NSCs Generated by Baculoviral Transduction Display Bystander Killing Effects on Glioma Cells. Therapeutic applications of NSCs may benefit from loading a functional gene into the cells before transplantation. To test whether baculoviral transduction is suitable for effectively loading iPS cell derived NSCs with transgenes and maintaining transgene expression in the transduced cells long enough to achieve therapeutic effects, we examined the transduction efficiency and transgene expression duration of baculoviral vectors in NSCs. Using eGFP as a reporter, we observed many transduced cells 48 h post-transduction, although the number decreased obviously by day 7 (Figure 4A). FACS analysis demonstrated that approximately 80% NSCs were successfully transduced by baculovirus at an MOI of 100 on day 2 post-transduction and there was a significant decline in percentage of transduced cells to approximately 25% by 1 week post-transduction (Figure 4B). Baculoviral transduction caused no significant cell death nor morphological changes or proliferation defects in transduced NSCs. The transduced cells retained the expression of the essential NSC markers nestin, Glast, Pax6 and BLBP, as revealed by RT-PCR analysis (Figure 4C).

The herpes simplex virus thymidine kinase (HSVtk)/ganciclovir (GCV) system is commonly used in suicide gene therapy for cancer. In this regime, the HSVtk gene is delivered via either direct transduction or stem cell targeting of tumors. Within the tumor, HSVtk will phosphorylate GCV and the phosphorylated GCV exhibits strong bystander effect to interfere with DNA replication in tumor cells, thus resulting in their eventual death.[20,21] To test whether iPS cell derived NSCs can be used for targeted glioma therapy, we used a baculovirus vector containing the *HSVtk* gene to transduce NSCs and confirmed *HSVtk* gene expression upon baculovirus transduction in the cells with Western blotting (Figure 4D). The HSVtk protein expression

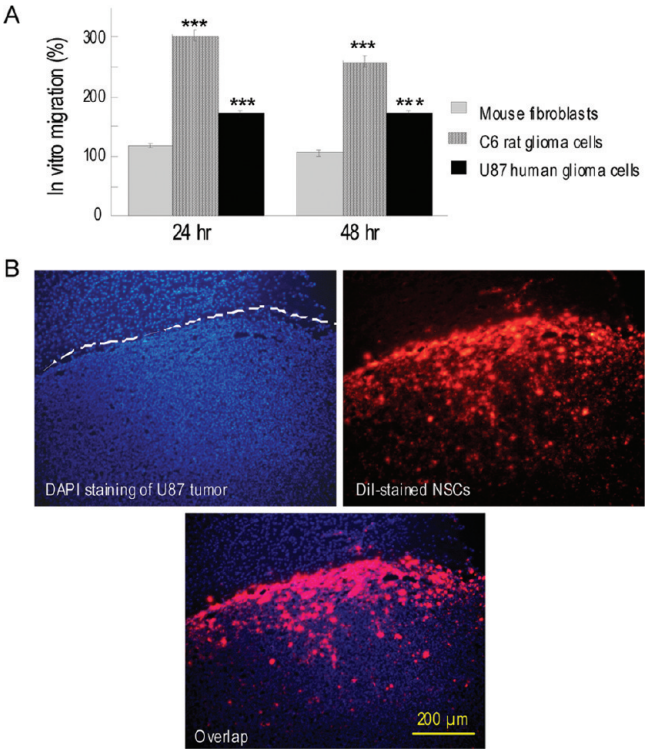

Figure 3. Glioma-specific migration of mouse iPS cell derived NSCs. (A) *In vitro* migration. Boyden chamber assays demonstrate the NSCs displayed significantly higher migration capacity toward C6 rat glioma cells and U87 human glioma cells as compared to migration toward mouse fibroblasts. $n = 4$ per group. bars: SD *** $P < 0.001$ by ANOVA. (B) *In vivo* glioma tropism in the brain. U87 cells were injected into the right striatum. CM-DiI-labeled NSCs (red) were injected into the hemisphere contralateral to the tumor inoculation site 7 days later. Brain sections were collected on day 21 post tumor inoculation (14 days after NSC injection). Red NSCs were observed in a region with a U87 tumor.

level at 7 days post-transduction appeared comparable to that of 2 days post-transduction. We used an image processing program, ImageJ (www.rsb.info.nig.gov/ij), to measure staining intensities of bands in the Western blot and compared the intensities of HSVtk bands after being adjusted with the band intensity of β-actin. The optical density mean gray values were 102.3 and 104.5 at days 2 and 7 post-transduction respectively, suggesting that HSVtk expression was stable for at least 7 days. These results indicate that in contrast with baculovirus-mediated eGFP expression, which declined quickly within 7 days (Figure 4A,B), the HSVtk protein is relatively more stable in the transduced cells.

To examine the *in vitro* bystander effect of GCV, cell viability tests were conducted in a coculture system by mixing NSC cells with glioma cells. The mixed cells were cultured in medium containing GCV for 5 days before performing a cell viability assay. Without phosphorylation, GCV did not display any significant toxicity on either untransduced NSCs or glioma cells (Figure 4E). When NSC cells expressing HSVtk were used, GCV was cytotoxic at a concentration as low as 5 μM and the cell killing effect of GCV increased with increasing concentration of GCV (Figure 4E). The cell death rates could be up to 60% of a mixed cell population when glioma cells and the NSCs were mixed at a ratio of 90:10, providing clear evidence of bystander effect. These results also indicate that only a small number of HSVtk-expressing NSCs would be enough to convert sufficient amounts of GCV to kill large numbers of nearby tumor cells through a bystander effect. Since the HSVtk protein was still clearly detectable by Western blotting on day 7 post-transduction, we mixed NSCs and glioma cells on day 8 and cultured the mixed cells in medium containing GCV for 5 days. We observed cell killing effects again, which were close to those obtained when NSCs and glioma cells were mixed on day 3 under most testing conditions (Figure 4F). Thus, there is a therapeutic window of at least 8 days after baculoviral transduction in NSCs.

Glioma Gene Therapy Using HSVtk-Expressing NSCs in a Mouse Model. We next examined the *in vivo* efficacy of iPS-derived NSCs in cancer gene therapy. We established U87 glioma xenografts in nude mice by inoculating U87-*luc* cells into the right striatum. Seven days later, the tumor inoculated mice were divided into 4 groups ($n = 10$ per group). The experimental group received injection of HSVtk-expressing NSCs into the left striatum contralateral to the tumor inoculation site followed by daily intraperitoneal (ip) administration of GCV from day 14 to 35 post tumor inoculation. Three control groups received intracranial injection of NSCs with ip injection of phosphate-buffered saline (PBS), intracranial PBS injection followed by ip PBS injection, or intracranial PBS injection followed by ip injection of GCV. Seven-day delay in GCV injection allows NSCs to migrate from the injection site to the tumor site, thus allowing us to test tumor targeting effects of NSCs. Tumor growth was monitored by bioluminescent imaging of U87-*luc* cells with the IVIS Imaging System. Figure 5A shows brain images of representative U87-*luc* cell bearing mice from the 4 groups at 3 different time points. The bioluminescence intensities, indicative of the tumor volume, demonstrate that injection of HSVtk-expressing NSCs followed by ip GCV treatment produced more pronounced inhibitory effects on tumor growth compared to other treatments (Figure 5B). Attributed to the inhibitory effect on tumor growth provided by the HSVtk/GCV regime, the survival of the tumor-inoculated mice in this group was significantly prolonged (Figure 5C). By day 33, while all animals in the 3 control groups had died, only 50% of animals (5 out of 10 mice) died in the group injected with HSVtk-expressing NSCs followed by ip GCV treatment. Statistical analysis revealed significant difference between the experimental group and other control groups ($p < 0.05$), but no significant difference among the three control groups. Histological examination (Figure 5D) confirmed tumor shrinkage in the experimental group and more rapid tumor growth in the control groups of animals. Therefore, the use of HSVtk-expressing NSCs coupled with GCV treatment was powerful enough to slow down *in vivo* growth of glioma cells at a tumor site distant from the NSC injection site and extend the lifespan of tumor-bearing mice.

DISCUSSION

Several types of murine and human NSCs have been tested for cancer gene therapy in animal tumor models.[1−12] We have furthered the previous observations by deriving glioma tropic NSCs from iPS cells and for the first time reported the use of these *in vitro* derived NSCs in inhibiting glioma growth after injection into a site distant from the tumor inoculation site.

iPS cells generated in this study have a gene expression profile similar but not identical to that of ES cells (Table 1; Supplementary Table 2 in the Supporting Information;[15,22]). This is consistent with previous reports showing that mouse and human

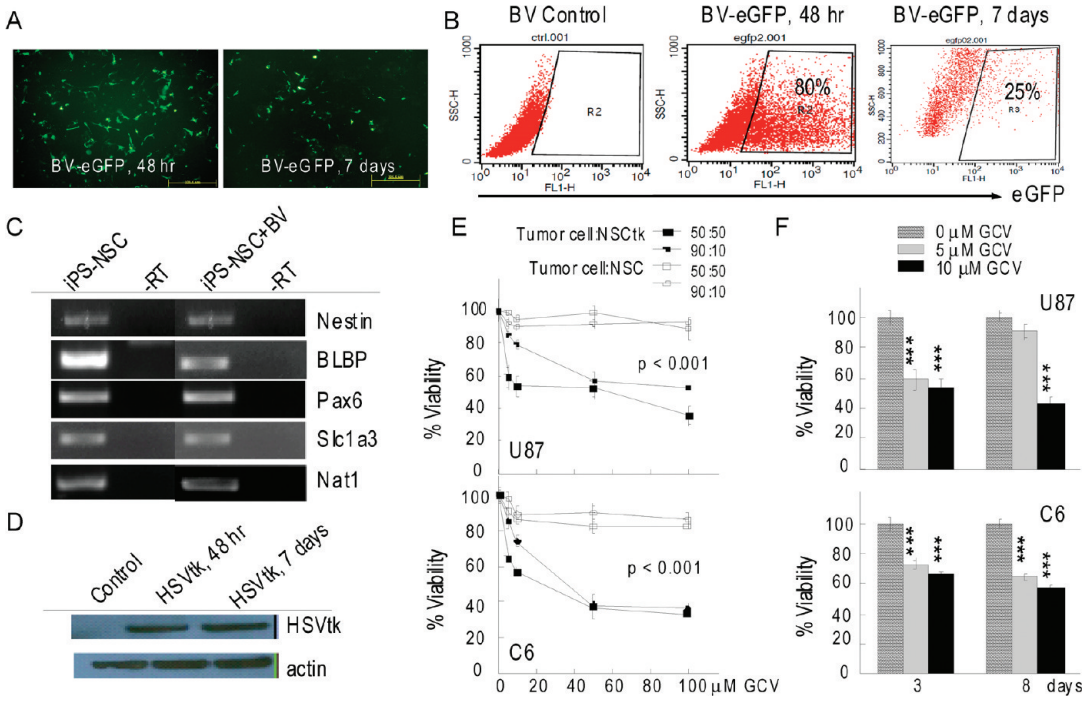

Figure 4. Mouse iPS cell derived NSCs can be used to deliver the HSVtk suicide gene. (A) Baculoviral transduction of NSCs. Fluorescence images of eGFP-expressing NSCs are shown. The images were taken 48 h and 7 days, respectively, after transduction with a baculoviral vector containing the eGFP gene at an MOI of 100. (B) FACS analysis to determine the percentage of eGFP positive NSCs. (C) RT-PCR analysis to show that NSCs retain the expression of essential NSC markers after baculovirus transduction (BV). Nat1 was used as a loading control. −RT: Without reverse transcriptase. (D) Western blot analysis to demonstrate HSVtk suicide gene expression in NSCs after transduction with a baculoviral vector containing the HSVtk gene. (E) Dose-dependent *in vitro* bystander killing effect on U87 and C6 glioma cells assessed in a coculture system. Three days after transduction with the HSVtk baculoviral vector, transduced NSCs (NSCtk) were mixed with U87 or C6 glioma cells at indicated ratios. Mixed cells were treated with GCV at varying concentrations for 5 days before cell viability assay. Each point corresponds to the mean + SD of three independent experiments. ANOVA analysis revealed that the differences between the presence of NSCtk and the presence of nontransduced NSCs were significant at all tested GCV concentrations ($p < 0.001$), except the lowest tested dose (5 μM) at a glioma cell/NSC mixing ratio of 90:10. (F) Time-dependent *in vitro* bystander killing effect. HSVtk-expressing NSCs were mixed with glioma cells 3 or 8 days after baculoviral transduction at a mixing ratio of 50:50. Mixed cells were treated with 5 or 10 μM GCV for 5 days before cell viability assay. Each point corresponds to the mean + SD of three independent experiments. ***, $p <$ 0.001 versus the control group without GCV treatment.

iPS cells and ES cells have overlapping but still distinct gene expression signatures, regardless of where the cell lines were generated, the methods by which they were derived or the species from which they were isolated, possibly due to differential promoter binding by the reprogramming factors used to generate iPS cells.[23,24] Nevertheless, our iPS cells can form EBs with all three germ layers and differentiate into NSC-like cells that may further differentiate into neurons and astrocytes.

Recent years have seen the development of a variety of techniques enabling the differentiation of NSCs from embryonic stem cells.[17,18,25−28] Most of the methods have used the formation of neurospheres or EBs. These ES cell derived NSCs are similar to adult NSCs in their gene expression profile, surface marker profile and potential in differentiating into neurons, astrocytes, and oligodendrocytes.[29] They exhibit much greater proliferative capacity than their adult counterparts, and could be consistently derived in large amounts. iPS cells, like ES cells, have the potential to become all of the tissues in the body, including neural tissue.[30] In this study, long-term proliferating NSCs were derived from mouse iPS cells using a monolayer culture condition that does not require cell-aggregate formation. NSCs derived from iPS cells can be propagated under monolayer culture condition and cryopreserved without losing their neural stem cell properties. The NSCs proliferated for at least 6 months under standard cell-culture conditions. This sustained proliferative capability is necessary to produce sufficient amounts of cell-based therapeutics in a reproducible manner. Long-term proliferation is also useful for *ex vivo* genetic modification and selection of the modified cells. The NSCs can be expanded in a chemically defined medium, allowing greater control over cell quality.

Using an adherent monoculture method similar to what we used, a recent publication reported the deviation of NSCs from mouse iPS cells.[31] However, the iPS cells used in the study were generated using multiple individual viral vectors, which may result in multiple viral integrations across the genome in the transduced host cells. This will make it almost impossible to remove the integrated transgenes in generated iPS cells when the production of safer cells for medical applications is required. We used a Cre-excisable polycistronic lentiviral vector to generate our mouse iPS cells and confirmed that the integrated reprogramming transcriptional factors can be removed in generated iPS cells with a baculoviral vector expressing Cre-recombinase (unpublished observation). Since the excision of reprogramming transcription factors can improve the differentiation potential of iPS cells and eliminate the potential risks associated with reactivation or sustained expression of these factors,[32] NSCs generated in our study will have great potential in medical research.

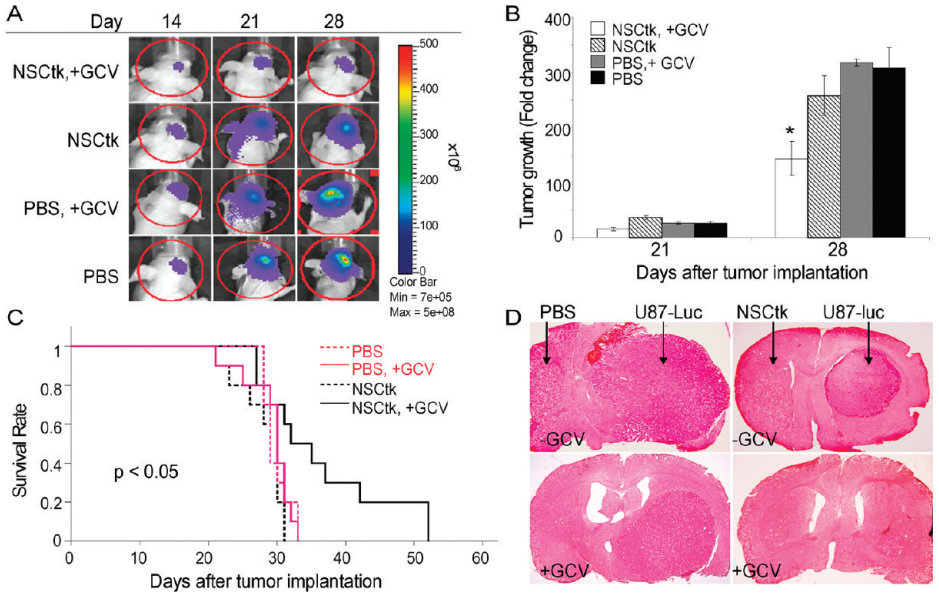

Figure 5. HSVtk/GCV gene therapy for glioma mediated by iPS cell derived NSCs. U87-*luc* cells were inoculated into the right striatum of the mouse brain, and NSCtk cells or PBS was injected contralaterally into the left striatum. GCV or PBS was intraperitoneally administered daily from day 14 to day 35 after tumor implantation. $n = 10$ per group. (A) Bioluminescence images of tumor growth in representative animals from different groups. Heat map represents the tumor area, and color represents the intensity. Images collected at three time points, days 14, 21, and 28 after tumor implantation, are shown. (B) Tumor growth as quantified by bioluminescence signal measurement. The results are expressed as fold changes over the measurements at day 0 post tumor inoculation. bars: SD * $p < 0.05$ versus the PBS group by ANOVA. (C) Brain injection of NSCtk cells coupled with GCV treatment prolongs life of U87-bearing mice. Survival curves are shown. Statistical analysis reveals the significant difference between the NSCtk + GCV group and the 3 control groups ($p < 0.05$). (D) Representative H&E staining pictures of brain sections show tumor shrinkage in the NSCtk + GCV group, but not in other groups.

A recent study has demonstrated direct conversion of mouse fibroblasts to functional neurons by combinatorial expression of three neural lineage-specific transcription factors, *Ascl1*, *Brn2* and *Myt1l*.[33] One of the interesting findings reported in the paper was the observation of Tuj1-, TauEGFP-positive cells exhibiting a simple mono- or bipolar morphology after individual *Ascl1* expression. Whether or not these cells can be used for targeted cancer therapy would be worthy of investigation. Fibroblast-derived iPS cells can be used to generate various types of functional cells, providing rich opportunities for therapeutic applications. Thus, in addition to the potential for generating a continuous supply of therapeutic cells, iPS cells can be used to generate different types of functional cells to treat one disease. For example, murine iPS cells can be derived from and differentiate into natural killer T (NKT) cells.[34] These iPS cell derived NKT cells recapitulate the adjuvant effects of natural NKT cells and are capable of suppressing tumor growth *in vivo*. One may envisage that NKT cells and NSCs derived from the same iPS cells can be used together to improve the efficacy of tumor therapy.

To use NSCs to deliver therapeutic products for cancer therapy, a safe and effective method is required to load stem cells with therapeutic payloads of interest and for the cells to carry them over the period of time crucial to the therapy. We demonstrated in the present study that baculoviral vectors can be used for genetic modification of iPS cell derived NSCs to provide a window of transgene expression suitable for cancer therapy. During baculoviral transduction, transgenes are usually not integrated into the genome of host mammalian cells. Only with antibiotic selection could stable cells expressing GFP be isolated following baculovirus transduction,[35,36] suggesting that the frequency of spontaneous chromosomal integration of delivered DNA sequences without positive selection, if any, would be very low in transduced cells. Thus, baculoviral vectors appear not to disturb host genome and should pose much less risk of insertional mutagenesis. However, the transient gene expression mediated by baculovirus may limit its efficacy in the studies that require long-term transgene expression. This limitation is not unique to baculovirus, but is also faced by adenovirus, which can be overcome to some extent by optimization of viral expression cassette.[37] As an important advantage of using baculovirus for genetic modification of NSCs that are going to be transplanted into the body, there is no pre-existing antivirus immunity against baculoviruses in humans.[36] Pre-existing host immune response against adenoviral vectors, a type of vector commonly used for transient transgene expression, is a concern that restricts the use of these vectors.[38] Insect baculovirus also 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.[38]

Glioma gene therapy clinical trials over the past 10 years have tested adenovirus, retrovirus, HSV-1, and liposome vectors.[39] The results of most of these clinical studies have been poor, possibly because transfection efficiency of nonviral vectors was low *in vivo* and/or because of immune reactions against viral vectors. The limited distribution of transgene and vectors within the tumor mass is also a possible reason. With the demonstrated tumor inhibitory effects of tumor-infiltrating feature of iPS cell derived NSCs after being injected into a remote site, these cells can be used to overcome the problems associated with current viral and nonviral vector-based gene therapy approaches by delivering therapeutic genes to disseminated glioma satellites distant from the main tumor mass.

While the results reported here are encouraging, there are two issues that are however worth mentioning. A recent study has demonstrated that immunologically matched iPS cells are more immunogenic than matched ES cells and cells derived from iPS cells trigger T-cell-dependent immune response in syngeneic recipients.[40] This was unexpected as iPS cells would not be attacked by the immune system given that their DNA is identical to that of the animal population whose somatic cells were used to generate the iPS cells. As noted in the study, iPS cells generated using an episomal approach were less immunogenic than those generated using viral vectors, suggesting that alternative iPS generating methods may reduce and prevent immunogenicity. It should also be noted that the study was conducted with iPS cells. Whether the immunogenicity will be the same in therapeutically relevant, differentiated cell types, like NSCs, remains unclear. Previous studies have shown that hematopoietic progenitors derived from iPS cells and matured *in vitro* can successfully engraft in genetically matched mice, though the recipient mice had to be irradiated first.[41]

C6 rat glioma cells and U87 human glioma cells have traditionally been used for glioma studies *in vivo* due to reproducible tumor occurrence rate, easy generation of large cohorts of tumors, and narrow survival window. These features are vital for preclinical cancer treatment tests designed for target validation, toxicity evaluation, and efficacy assessment. But it is widely acknowledged that these cell lines do not recapitulate many histological and developmental features characteristic of human gliomas. Due to these limitations, orthotopic xenograft models by transplanting tumor pieces from glioma patients directly into the mouse brain have been explored. These intracranial transplants resemble human glioma disease more closely, but studies on their implementation and validation for preclinical testing of potentially useful glioma therapy agents are still limited.[42,43]

In conclusion, we demonstrated in a human glioma xenograft mouse model that NSCs derived from iPS cells could be used for targeted cancer therapy after coupling these NSCs with HSVtk suicide gene expression and GCV treatment. Although the cellular system was derived from mouse cells, the protocol can probably be modified for generation of NSCs from human iPS cells, an approach that may help bypass the ethical and allogenic issues posed by the use of many other types of human NSCs.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Two spread sheets comprising tables of PCR primers used in this study and the gene expression profile of mouse iPS cells relative to mouse embryonic fibroblasts. This material is available free of charge via the Internet at http://pubs.acs.org.

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