Published in final edited form as:

Cell Transplant. 2013 ; 22(5): 881–892. doi:10.3727/096368912X656144.

Imaging Neural Stem Cell Graft-Induced Structural Repair in Stroke

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Abstract

Stem cell therapy ameliorates motor deficits in experimental stroke model. Multimodal molecular imaging enables real-time longitudinal monitoring of infarct location, size, and transplant survival. In the present study, we used magnetic resonance imaging (MRI) and positron emission tomography (PET) to track the infarct evolution, tissue repair, and the fate of grafted cells. We genetically engineered embryonic stem cell-derived neural stem cells (NSCs) with a triple fusion reporter gene to express monomeric red fluorescence protein and herpes simplex virus-truncated thymidine kinase for multimodal molecular imaging and SPIO labeled for MRI. The infarct size as well as fate and function of grafted cells were tracked in real time for 3 months using MRI and PET. We report that grafted NSCs reduced the infarct size in animals with less than 0.1 cm³ initial infarct in a dose-dependent manner, while larger stroke was not amenable to such beneficial effects. PET imaging revealed increased metabolic activity in grafted animals and visualized functioning grafted cells in vivo. Immunohistopathological analysis demonstrated that, after a 3-month survival period, grafted NSCs dispersed in the stroke-lesioned parenchyma and differentiated into neurons, astrocytes, and oligodendrocytes. Longitudinal multimodal imaging provides insights into time course dose-dependent interactions between NSC grafts and structural changes in infarcted tissue.

Keywords

Human neural stem cells (NSCs); Molecular imaging; Position emission tomography (PET); Magnetic resonance imaging (MRI); Cell therapy

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The authors declare no conflict of interest.

INTRODUCTION

Neural plasticity after stroke is aimed to compensate for neural loss by restructuring connectivity in the brain and restoring homeostasis and function was first reported in early 1990s (13). However, this endogenous process is inherently limited, and long-term disabilities in stroke patients prevail. Currently, no therapeutic intervention offers repair of stroke-damaged tissue. Cell transplantation therapy may be one approach to fulfill this need (28). Numerous ongoing and planned clinical trials hold great potential to bring this therapeutic approach to patients. (Examples of ongoing trials may be found at ClinicalTrials.gov Identifier: NCT01151124, NCT01287936, NCT01436487.)

Human pluripotent stem cells offer a wide array and unlimited supply of specialized cells that can be used for brain repair (36). Multipotent neural stem cells (NSCs) have the ability to differentiate into the three central nervous system (CNS) cell types in stroke-damaged brain, including neurons, astrocytes, and oligodendrocytes (8). The efficacy of the grafted NSCs depends not only on their properties but also on the extent of the damage caused by stroke. This study investigates dose-dependent effects, time course of graft survival, and its relationship with infarct size using a real-time multimodal longitudinal imaging approach.

MATERIALS AND METHODS

Lentiviral Production and Generation of a Stable hESC Line

Production of the triple fusion lentiviruses has been previously reported (29). Polymerase chain reaction amplification and standard cloning techniques were used to insert and fuse monomeric red fluorescence protein (mRFP), firefly luciferase (Fluc), and herpes simplex virus truncated thymidine kinase (HSV-ttk) genes from pCDNA plasmids as previously described (29). Self-inactivating lentivirus was prepared by transient transfection of HEK293FT (Invitrogen, Carlsbad, CA, USA). Two days after transfection, vector particles of the LV-pUB-fLuc-mRFP-HSV-ttk triple fusion (TF) reporter gene were harvested from the medium, concentrated by ultracentrifugation at 25,000 rpm for 2 h at 4°C. To estimate the concentration of transducing units/ml, 293T-cells were transduced with serial dilutions of each vector preparation. After 48 h, cells were harvested and subjected to flow cytometry analysis of RFP expression. Based on these data, the H7 human embryonic stem cells (hESCs; Wicell, Madison, WI, USA) were transduced with the LV-pUB-fLuc-mRFP-HSV-ttk TF reporter gene at a multiplicity of infection of 10. The infectivity was determined by mRFP expression as analyzed on FACScan (BD Bioscience, San Jose, CA, USA). The mRFP- positive cell populations were isolated by fluorescence-activated cell sorting (FACS) Vantage SE Cell Sorter (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) and expanded in feeder-free cell culture condition according to Wicell's protocol.

Derivation of hNSCs From hESCs

Human neural stem cells (hNSCs) were isolated from the H7 human embryonic stem cell lines (WiCell), as we previously described (7). The serum-free culture medium was composed of Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1) including glucose (0.6%), glutamine (2 mM), sodium bicarbonate (3 mM), and HEPES buffer (5 mM) (all

from Sigma, St. Louis, MO, USA; except glutamine from Invitrogen). A defined hormone mix and salt mixture (Sigma) that included insulin (25 µg/ml), transferrin (100 µg/ml), progesterone (20 nM), putrescine (60 µM), and selenium chloride (30 nM) was used in place of serum. Growth factors including 20 ng/ml mouse epidermal growth factor (EGF; Upstate Cell Signaling, Lake Placid, NY, USA), 10 ng/ml human recombinant basic fibroblast growth factor (bFGF), and 10 ng/ml of human recombinant leukemia inhibitory factor (LIF; R&D Systems, Minneapolis, MN, USA) were added to the culture 2 h after plating. Cells were incubated at 37°C in a 95% air/5% CO₂ humidified atmosphere.

Induction of Focal Ischemia and Cell Transplantation

All animal experiments were conducted according to the National Institute of Health (NIH) guidelines including the use of anesthetics and analgesics for surgery and sacrifice and animal welfare conditions for housing and care and approved by the Stanford University's IACUC. Sprague–Dawley (SD) adult male rats (275–310 g, Charles River Laboratories, Wilmington, MA, USA) were subjected to a transient 1.5-h suture occlusion of the middle cerebral artery (MCAO) as we previously described (5,7). SD rats were immunosuppressed 2 days before cell transplantation and daily thereafter with IP injections of cyclosporine A (20 mg/kg, Sandimmune, Novartis Pharmaceuticals, East Hanover, NJ, USA). Undifferentiated hNSCs from passages between P9 and P15 were single cells dissociated using trypsin-EDTA (Invitrogen) in preparation for cell transplantation. One week after the stroke lesion, animals were randomized based on their MRI-measured infarct size. Based on T2-weighted serial scans, stroked animals were separated into a moderate infarct size group ($n = 8$) with infarct size less than 0.1 cm³ (100 mm³) and into large infarct size group ($n = 8$) with infarct size more than 0.1 cm³. Volume of 1–2 µl of the hNSCs, at a concentration either 50,000 cells/µl for low dose or 100,000 cells/µl for high dose, were stereotactically transplanted into one site for the 50,000 cell dose or into two sites for the 500,000 and 1 million cell doses, within the lesioned striatum at the following coordinates: AP: +0.5 mm, ML: +3.0 mm, DV: –5.0; AP: –0.5 mm, ML: +3.0 mm, DV: –5.0 with the incisor bar set at 3.4 mm (6).

MR Imaging

Transduced hNSCs were labeled with SPIO (super-paramagnetic iron oxide; Feridex IV, Berlex Laboratories, Wayne, NJ, USA) using the poly-D-lysine method (14) and grafted at increasing cell doses into the striatum of stroked rats ($n = 15$ /group). Magnetic resonance (MR) imaging was performed on a “microSigna 7.0” T-MR scanner (7.0T/310/AS System; Varion, Inc., Palo Alto, CA, USA) starting 2 days after transplantation and for up to 2 months on all animals. The scanner consists of a 310-mm (horizontal) bore scanner with gradient drivers, EXCITE2 electronics, eight-channel multicore radio frequency (RF) and multinuclear capabilities and volume RF coils, and the supporting LX11 platform. The Resonance Research Instruments BFG-150/90-S shielded gradient insert (Billerica, MA, USA; 770 mT/m, SR-2500 T/m/s) has a bore size of 9 cm. Animals were anesthetized with isoflurane, the respiratory and heart rates were monitored and the temperature was autocontrolled and kept at 37°C. The imaging protocol consisted of imaging in 2D multi-slice followed by a spin-echo sequence using the following parameters: echo time (TE) =

82.5 ms, repetition time (TR) = 4,000 ms, number of excitations (NEX) = 10, 5 cm × 5 cm field of view (FOV), matrix = 256 × 256, slice thickness = 0.6 mm, gap = 0.

Serial T2-weighted coronal MRI scans 600 µm spaced were used for transplant volume analysis using the DICOM viewer software Osirix v.3.1 (<http://www.osirix-viewer.com>) as we previously reported (6). Briefly, serial T2-weighted coronal MRI scans were used for transplant infarct size analysis using the DICOM viewer software Osirix v.3.1 (<http://www.osirix-viewer.com>). Region of interest (ROI) that is the hypointense areas of the SPIO-labeled grafted hNSCs are drawn by an investigator blinded to treatments and the surface area is measured in mm² automatically by the software. The infarct volume was then expressed in mm³ by factoring the total thickness of brain slices observed to the surface area.

Histopathology, Immunocytochemistry, and Microscopic Analysis

Cultures were fixed with 4% paraformaldehyde (Sigma) for 15 min. Both cells and brain sections were rinsed in PBS for 3 × 5 min then incubated for 2 h (cultures) or overnight (brain sections) with the appropriate primary antibodies for multiple labeling. Secondary antibodies raised in the appropriate hosts and conjugated to fluorescein isothiocyanate (FITC), rhodamine isothiocyanate (RITC), amino-methyl-coumarin-acetate (AMCA), cyanine 3 (CY3), or CY5 chromophores (Jackson Immuno Research; West Grove, PA, USA) were used. Cells and sections were counterstained with the nuclear marker 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI; Sigma). Positive and negative controls were included in each run. Immunostained sections were cover-slipped using fluorsave (Calbiochem, San Diego, CA, USA) as the mounting medium. The following antibodies were used: anti-vimentin (monoclonal 1:500, Calbiochem); anti-neuron specific class III β-tubulin (TuJ1; monoclonal 1:100, Covance, Princeton, NJ, USA; Polyclonal 1:200, Aves Labs, Tigard, OR, USA); anti-glial fibrillary acidic protein (GFAP, monoclonal 1:1,000, Chemicon; polyclonal 1:200, Aves Labs); anti-nestin (polyclonal 1:1,000, Chemicon); anti-2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase; 1:200, Aves Labs); anti-RFP (polyclonal 1:200, MBL International, Woburn, MA, USA). Fluorescence was detected, analyzed, and photographed with a Zeiss LSM550 laser scanning confocal photomicroscope (Thornwood, NY, USA). Double labeling was determined using the confocal laser scanning microscope by random sampling of 100 or more cells per marker for each animal, scoring first for human nuclei (hNUC⁺; RFP), followed by DAPI⁺ nuclei and then the marker of choice. The double labeling was always confirmed in *x-z* and *y-z* cross-sections produced by the orthogonal projections of *z* series. For Prussian blue staining, sections were fixed using 2% glutaraldehyde (Sigma), washed in PBS, and incubated with Pearls reagent (Sigma; 4% potassium ferrocyanide/12% HCL, 50:50 vol) for 40 min under agitation. Sections were then washed in PBS and twice in water, dehydrated through graded alcohols, and counterstained with nuclear fast red (Sigma) (6,7).

Small Animal PET Imaging

Positron emission tomography (PET) imaging was acquired using the Vista system (GE Healthcare, Chalfont St. Giles, UK). Animals (*n* = 5) underwent intravenous injection of the metabolic probe fluorine-18-fluorodeoxyglucose ([¹⁸F]-FDG; Stanford University Medical

Center, Cyclotron Radiochemistry, Stanford, CA, USA) on weeks 1 and 5 posttransplant or the reporter probe 9-(4-[^{18}F]fluoro-3 hydroxymethylbutyl) guanine ([^{18}F] FHBG; Stanford University Medical Center) at 4 mCi/kg on weeks 4 and 8 posttransplant. At 45–70 min after injection, animals were anesthetized with 2% isoflurane. Images were performed, reconstructed by filtered back projection, and analyzed using image software Amide (Amide's Medical Image Data Examiner, SourceForge, Inc., Mountain View, CA, USA). Three-dimensional ROIs were drawn of the stroke or the NSC graft area. For each ROI, time activity curves were expressed as the percentage of injected dose per cubic centimeter (%ID/cc). [^{18}F]FDG and -FHBG PET images were assembled into polar maps, and activity measured was then normalized to activity in a control region, the cerebellum.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from NSCs cultured under different conditions using RNAeasy kit (Qiagen, Valencia, CA, USA). Aliquots (1 μg) of total RNA from the cells were reverse transcribed (RT) as previously described (9) in the presence of 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl_2 , 10 mM DTT, 0.5 μM dNTPs, and 0.5 μg oligo-dT(12–18) with 200 U Superscript RNase H-Reverse Transcriptase (Invitrogen).

Quantitative real-time polymerase chain reaction (Q-PCR), using Applied Biosystems TaqMan Gene Expression Assays (Foster City, CA, USA), was performed in the CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) equipped with software for gene expression analysis. The TaqMan Gene Expression Assay ID/Gene Symbol used were Hs03003631_g1/Eukaryotic 18S rRNA; Hs02387400_g1/Nanog homeobox; Hs00909236_m1/GFAP; Hs03929064_g1/ β -tubulin; Hs00185584_m1/Vimentin; Hs00232764_m1/forkhead box A2 (FoxA2); Hs00999632_g1/POU class 5 homeobox 1 (octamer binding transcription factor 4 [Oct4]); Hs00941821_m1/Neural Cell Adhesion Molecule 1 (NCAM1); Hs01057642_s1/SRY (sex determining region Y)-box 1 (Sox1); Hs01053049_s1/Sox2. The expression of the gene of interest was determined in triplicate for each culture condition. Expression of the reference gene, 18S, was determined for each sample in triplicate. Quantification was performed at a threshold detection line ("threshold cycles," Ct value). The Ct of each target gene product was normalized against that of the reference gene 18S, which was run simultaneously for each marker. Data were expressed as mean $\pm$ SEM. The Ct for each candidate was calculated as Ct of [Ct (target gene) – Ct (18S)] and the Ct was the difference between the Ct of treated sample and the Ct of control sample. The relative expression was calculated as the $2^{-\text{Ct}}$ according to the methods (24) and plotted as relative levels of gene expression.

Statistics

Outcome measurement for each experiment was reported as mean $\pm$ SEM. All data were analyzed using SPSS 11 for Mac OS X (SPSS, Inc., IBM, Armonk, NY, USA). Significance of intergroup differences was performed by applying Student's *t* test where appropriate. The one-way ANOVA analysis was used to compare group differences for the infarct size as the dependent variable and groups as the single independent factor variable. Differences between the groups were determined using Bonferroni's post hoc test. A value of $p < 0.05$ was considered to be statistically significant.

RESULTS

Isolation and Characterization of hNSCs

The TF reporter gene construct consisted of Fluc, mRFP, and HSV-ttk reporter genes linked by a 14-amino acid-long linker (Fig. 1A) inserted into a self-inactivating lentiviral vector. hESCs were stably transduced with the lenti-TF reporter gene construct and selected based on mRFP expression using fluorescence activated cell sorting. Multipotent and self-renewable NSCs were isolated from these hESCs and perpetuated in vitro using serum-free media supplemented with EGF, bFGF, and LIF (7). The NSCs grew as an adherent monolayer culture. They were fully neuralized and uniformly expressed the NSC markers vimentin (Fig. 1B, C) and nestin (data not shown). Nuclei are counterstained with DAPI. qRT-PCR analysis demonstrated that these hNSCs did not express the pluripotency marker Oct4 or Nanog and did express neural markers, such as Sox1, NCAM, GFAP, and β -tubulin class III (Fig. 1D).

MRI Analysis of Dose and Infarct Size-Dependent Efficacy of NSC Grafts

To visualize grafts with MRI, the undifferentiated NSCs were first labeled with the MR contrast agent SPIO using the poly-D-lysine technique, as we previously reported (6). The NSCs were transplanted into stroke lesioned rats at increasing cell densities (50,000, 500,000, or 1,000,000 cells) and followed for up to 12 weeks.

In T2-weighted MR images the stroke zone appeared as a hyperintense region in the striatum and cortex (Fig. 2), and the NSC graft appeared as hypointense areas in the striatum (arrows in Fig. 2C, D). Three-dimensional reconstructions of the grafts and stroke by surface rendering from the serial MR scans allowed for an accurate representation of both the graft and stroke sizes and visualized the overtime changes in stroke size in vehicle- and NSC-treated animals. In the T2-weighted serial scans spanning week 1 to week 12 (Fig. 2A, B, right inset) the increased T2 intensity in the stroke region of vehicle treated animals and the increased size by approximately 10 mm³ suggested that stroke evolved to the pannecrosis phase. In contrast, stroke volume from 3-D representations of animals receiving the hNSC grafts showed a reduction in stroke size with less hyperintensity T2 (Fig. 2C, D, right inset).

Effects of the Ischemic Infarct Size on the Efficacy of the NSC Grafts

To investigate the relationship between the efficacy of NSCs and the infarct size, we separated animals with moderate infarct size (less than 0.1 cm³) from animals with large infarct size (more than 0.1 cm³) and performed volumetric analysis of the stroke and the NSC grafts. Animals with moderate stroke demonstrated a significant reduction over 10 weeks by up to 73.2 $\pm$ 11.7% of the infarct volume in comparison to the large infarct animals and to vehicle animals with moderate stroke (Fig. 3). Large infarcts increased in volume by 2.4% over 10 weeks. The postmortem analysis of infarct volumes in brain sections stained with cresyl violet confirmed that the moderate group infarcts were significantly smaller than the large group infarcts defined by the MRI analysis. Both MRI and histological measurements of the infarct size showed a strong correlation between infarct size and cell dose (Fig. 4A, B). These results validated the infarct volume group analysis as defined by the MRI T2 measurements. Quantitative analysis of the transplants in the serial MRI scans

(Fig. 4C–E) demonstrated the graft size significantly increased with the dose of cells injected ($p < 0.05$) within each of the moderate and the large infarct groups (Fig. 4C–E).

PET Imaging of the Metabolic Activity in the Stroke Region and of the Ttk Reporter Gene in the Grafted hNSCs

To assess metabolic alterations, PET imaging with [^{18}F] FDG was used to measure the rate of regional glucose utilization in the brain in response to stroke and hNSC treatment. Analysis of the stroke and grafted NSCs of the transplanted animals demonstrated an approximate 40% increase in metabolic activity in the lesioned hemisphere ($p < 0.05$) from week 1 to week 5 (Fig. 5A). The NSC-expressing HSV-ttk reporter gene enabled us to directly and specifically image the grafts in the brain (Fig. 5B) based on detecting phosphorylation and entrapment in the cells of the radiolabeled reporter probe [^{18}F]fluoro-hydroxymethylbutyl-guanine ([^{18}F]FHBG). The stability and efficiency of this reporter gene has been previously described (3). Imaging signals were stable from week 4 to week 8 after transplantation and correlated with the graft size measured by MRI analysis (Fig. 5D). The marked difference in PET activities was consistent with the expression of the HSV-ttk reporter gene by the NSCs and with the integrity of brain tissue in the stroke lesion.

Differentiation of hNSCs in the Stroke-Damaged Tissue

Histological analysis with Prussian blue staining of SPIO-labeled grafts confirmed the MRI data and demonstrated the survival of the grafts, as well as migration towards the stroke-damaged areas. Grafted NSCs exhibited typical neuronal morphologies (Fig. 6A–C). To investigate the fate of transplanted cells, we used double immunofluorescence labeling with an anti-RFP antibody specific to the grafted hNSCs and various human neural specific markers. Immunocytochemistry for RFP and the neuronal markers β -tubulin class III and NeuN (Fig. 6D) confirmed the differentiation into neurons. The grafted NSCs elaborated oligodendroglial lineages as demonstrated by the expression of CNPase (Fig. 6D) and astrocytic lineage as determined by the coexpression of RFP and GFAP (Fig. 6D).

DISCUSSION

Neural stem cell therapy is a promising approach to restore brain function due to neural loss after stroke. However, for successful stem cell product development, several critical steps need to meet a minimum standard (28). These steps include first the properties of the cells, preclinical safety and efficacy, delivery route, timing of transplantation, imaging, biodistribution, and mechanism of actions (8,19,26). Noninvasive molecular imaging may offer a valuable means to assess prognosis, define patient-specific neurosurgical intervention, measure the outcome, and guide the success of the approach.

We report that genetically engineered NSCs can be monitored efficiently in an experimental stroke model with molecular imaging of PET reporter gene expression for up to 3 months after transplantation. Using real-time longitudinal MRI, we demonstrated that a single suspension of grafted NSCs reduced the size of moderate stroke in a dose-dependent manner, while larger stroke was not amenable to such improvement in tissue repair. The histopathological infarct measurement using cresyl violet staining confirmed the group

segregation based on T2 MRI measurements into moderate and large infarct groups. Immunocytochemical analysis demonstrated that grafted NSCs dispersed in the stroke-lesioned parenchyma with typical neural morphologies and differentiated into neurons, astrocytes, and oligodendrocytes.

Our study demonstrated that, in a rat experimental stroke model, vehicle animals exhibited worsening of the infarct region, which extended for approximately 10 mm³ over 3 months and became hyperintense, suggesting necrosis. Indeed, previous MR imaging studies, including T1- and T2-weighted, diffusion-weighted and perfusion MRI, have reported dynamic changes poststroke in tissue parameters around the infarct zone over time that correlated with tissue necrosis and loss and worsening behavioral outcome (2,10,12,15,21,22,27,30,35). Immortalized mouse hippocampal (MHP36) NSCs labeled with the contrast agent gadolinium rhodamine dextran (GRID) or with PKH26 were investigated for their structural repair up to 1 year posttransplant survival time (25). Using T2-hyperintensity, this study showed a 25% increase in lesion volume in MCAO animals while those treated with PKH26-labeled NSCs showed an increase in lesion volume of 18% by 4 weeks, suggesting a therapeutic effect of the grafted cells. Small changes in lesion volume occurred between 4 and 12 weeks followed by a steady decrease by 35% in lesion volume after 1 year. The GRID-labeled cells did induce the same lesion reduction and exhibited a slight worsening of the stroke. This study showed that the GRID contrast agent interfered with the stem cell-induced recovery process (25). The current evidence suggests that SPIO does not alter the physiological properties of the NSCs in vitro or in vivo (6,18,20,33,37).

Our findings are in line with previous studies reporting that absolute T1 and T2 relaxation times increased within the first 3 months and correlated with neural death (22,35). The correlation of MRI parameters measuring infarct evolution suggests that functional recovery is associated with the preservation or restoration of normal ipsilesional activation (11,12,34) and contra-lesional connectivity (31). We report the reduction of MRI infarct area size in animals receiving NSCs with an initial stroke size of less than 0.1 cm³. We have also previously demonstrated that NSC grafts improve sensory motor behavior (4,5,7) and induce ipsilateral changes in microglial response (4), synaptogenesis, and contralateral axonal sprouting (1,4,5). Further longitudinal studies combining cell therapy and poststroke temporal evolution of MRI parameters, such as T2, T1, and apparent diffusion coefficient are necessary and may prove reliable to noninvasively image in real time the therapeutic benefit of cell therapy.

In addition to MRI, PET imaging is now routinely used for diagnosis and posttherapeutic monitoring of patients. FDG-PET is approved by the U.S. Food and Drug Administration and is currently the most commonly performed imaging modality in the clinical arena. Our study demonstrated a significant increase in FDG uptake, suggesting an improvement in functional integrity of the stroke lesioned area of the forebrain in NSC-injected animals. In line with our findings is a clinical study testing the efficacy of transplanting a purified population of neurons into stroke patients (23). PET scans performed 6 months after surgery revealed a 15% increase in [¹⁸F] FDG uptake at the transplant site or in the ipsilateral adjacent parenchyma. This was observed in 6 of 11 patients and was relative to pretransplant scans.

Our PET reporter-probe imaging approach is based on using the HSV1-ttk gene (16) to phosphorylate the radiolabeled substrate transported into the cells. As a result, the phosphorylated substrate is trapped and imaged only in cells carrying the HSV1-ttk gene. HSV-sr139tk, a mutated form of the thymidine kinase gene, was more efficient than the wild type in converting the radiotracer (17). This approach has been successfully used for imaging human embryonic stem-based therapy for the heart (29). One study used the HSV1-ttk reporter gene approach to image the C17.2 murine neural progenitors after implantation in an intracranial glioma animal model (32). The reporter probe ($[^{18}\text{F}]\text{FHBG}$) was used to visualize the migratory pattern of the C17.2 cells. However, PET imaging was possible only in regions where the blood–brain barrier was disrupted. Additionally, an aberrant migratory pattern of the cells was detected. Given the triple imaging modality, complexity of the model, and route of cell delivery, further studies are necessary to reconcile discrepancies with previous reports on the pattern of cell migration. We have previously used this vector to efficiently monitor pluripotent mouse embryonic stem cells for tumor formation after subcutaneous injections (3). The study demonstrated that HSV-ttk enables reliable noninvasive imaging and tracking of the marked increase in signal activity-associated subcutaneous tumor formation (3).

In summary, we report that genetically engineered NSCs can be accurately imaged with MRI and PET over a 3-month posttransplant period. The NSCs reduced infarct size in moderate stroke, survived for 3 months, and differentiated into neuron, astrocytes, and oligodendrocytes. This therapeutic effect was measurable by MRI and FDG PET. HSV-ttk PET reporter gene enabled the monitoring and specific visualization of the grafted cells in the forebrain. Thus, by addressing critical questions in preclinical development, such as cell delivery, monitoring and outcome measurement, molecular imaging is playing a central role in the development of cell-based therapies.

Acknowledgments

The authors thank Beth Hoyte for preparation of the figures. This work was supported in part by Russell and Elizabeth Siegelman, Bernard Lacroute, Ronni Lacroute, the William Randolph Hearst Foundation, CIRM DR1-01480, Edward G. Hills Fund, NIH NINDS grants RO1 NS27292, P01 NS37520 and R01 NS058784 (G.K.S.), and CIRM RS1-00322 (J.C.W.).

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Figure 1.

Generation and characterization of human embryonic stem cell (hESC)-derived neural stem cells (NSCs) genetically engineered for multimodal imaging. (A) Schematic representation of the triple fusion (TF) reporter gene containing firefly luciferase (fLuc), monomeric red fluorescence protein (mRFP), and Herpes simplex virus truncated thymidine kinase (HSV-ttk) driven by the human ubiquitin promotor (pUbiquitin) in a self-inactivating long-term repeat lentiviral vector (5' LVLTR-SINLTR3'). (B, C) Derived cell line expression of the NSC marker vimentin at two different magnifications. Scale bars: 20 μ m (B) and 10 μ m (C). (D) qRT-PCR for target genes expressed by the NSCs. Expression was normalized to the expression level of the same genes by the pluripotent hESCs. β -Tub, neuronal β III tubulin; FoxA2, forkhead box A2; GFAP, glial fibrillary acidic protein; NCAM, neural cell adhesion molecule; Oct4, octamer binding transcription factor 4; Sox1, sex-determining region Y box 1.

Figure 2.

Magnetic resonance imaging (MRI) analysis of stroke and NSC grafts. MRI horizontal serial scans show superparamagnetic iron oxide (SPIO)-labeled NSC grafts as hypointense areas in the striatum (arrow in C, D, left panel) and stroke as hyperintense areas on T2-weighted MR images. Three-dimensional surface rendering reconstruction of grafted rat brain from high resolution T2-MRI illustrates the grafts (bright green, arrow in C, D) and stroke (red) in a representative control (A, B) and transplanted animals (C, D).

Figure 3.

MRI analysis of stroke size. The stroke area distinguished as hyperintense areas on serial T2-weighted images was measured in consecutive horizontal MRI scans, 600 μ m spaced (see Materials and Methods) from all animal groups in vehicle (A, $n = 6$) and NSC-treated animals (B–D). Cell doses were 50,000 (B, $n = 9$), 500,000 (C, $n = 9$), and 1,000,000 (D, $n = 6$). * $p < 0.001$.

Figure 4.

Correlation between stroke size and cell dose. Strong correlation exists between cell dose actions on the infarct size measured either by MRI and histological approaches (A, B). Quantitative analysis of graft size (C–E) appearing as hypointense areas on T2-weighted images in consecutive horizontal MRI scans, 600 μ m spaced (see Materials and Methods) at 2, 4, 6, 8, and 10 weeks. Measurements were by DICOM viewer software Osirix v.3.1 in three animal groups ($n = 10$) with moderate or large strokes transplanted with 50,000, 500,000, or 1,000,000 NSCs.

Figure 5.

Positron emission tomography (PET) imaging analysis of stroke and NSC grafts. (A) Increase of uptake of the metabolic probe [^{18}F]fluorodeoxyglucose (FDG) in the stroke region of hNSC-grafted rats imaged in horizontal and coronal planes at 1 and 5 weeks posttransplant. (B) 9-(4-[^{18}F]fluoro-3 hydroxymethylbutyl) guanine ([^{18}F]FHBG) PET activity visualizing the grafted cells in the striatal region of the forebrain imaged in horizontal and coronal planes at 4 and 8 weeks posttransplant. (C) Representation of the [^{18}F]FDG uptake measured at 1 and 5 weeks after transplantation of NSCs in five animals showing the significant increase in FDG activity. ID/cc, injected dose per cubic centimeter of tissue. (D) Correlation between MRI and PET activity in grafted NSCs. Grafts were measured in T2-weighted MRI. Uptake of the PET reporter probe [^{18}F]FHBG by the HSV-ttk-expressing hNSCs was represented at 4 and 8 weeks posttransplant.

Figure 6.

Transplanted NSCs migrate and differentiate into neural lineages. (A) Histological analysis at 13 weeks poststroke (12 weeks post-NSC transplant) using Prussian blue staining for supraparamagnetic iron oxide (SPIO) particles demonstrate cytosolic deposition of blue crystals in the grafted NSCs and migration towards stroke area. (B,C) High power magnification of the grafted NSCs in the parenchyma showing typical neuronal morphologies (arrows). (D) Immunocytochemistry and confocal image analysis of the grafted hNSCs. Grafted NSCs express the RFP transgene and thus can be easily tracked. The

panels of markers represent confocal photomicrographs showing double immunostaining for the RFP (red) and the three CNS lineages (green): β -tubulin class III for neurons, 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) for oligodendrocytes and GFAP for astrocytes. Arrows show the positive staining for these markers. Scale bars: 50 μ m (A), 25 μ m (B, C), 20 μ m (D) (top), and 50 μ m (for the two bottom panels).