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Magnetic resonance imaging detects differences in migration between primary and immortalized neural stem cells

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Abstract

Rationale and Objectives—The study was performed to evaluate the effect of MRI contrast agent (super paramagnetic iron oxide (SPIO)) on differentiation and migration of primary murine neural stem cells (NSCs) in comparison to a neural stem cell line (C17.2). Since detection of labeled cells depends on the concentration of SPIO particles per imaging voxel, the study was performed at various concentrations of SPIO particles to determine the concentration that could be used for *in vivo* detection of small clusters of grafted cells.

Materials and Methods—Murine primary NSCs or C17.2 cells were labeled with different concentrations of SPIO particles (0, 25, 100 and 250 µg Fe/ml) and *in vitro* assays were performed to assess cell differentiation. *In vivo* MRI was performed seven weeks after neonatal transplantation of labeled cells to evaluate the difference in migration capability of the two cell populations.

Results—Both the primary NSC's and the C17.2 cells differentiated to similar number of neurons (Map2ab positive cells). Similar patterns of engraftment of C17.2 cells were seen in transplanted mice regardless of the SPIO concentration used. *In vivo* MRI detection of grafted primary and C17.2 cells was only possible when cells were incubated with 100 µg/ml or higher concentration of SPIO. Extensive migration of C17.2 cells throughout the brain was observed, while the migration of the primary NSCs was more restricted.

Conclusion—Engraftment of primary NSC's can be detected non-invasively by *in vivo* MRI and the presence of SPIO particles do not affect the viability, differentiation or engraftment pattern of the donor cells.

Keywords

C17.2 cells; murine primary neural stem cells; neural stem cell differentiation; neural stem cell transplantation; stem cell tracking; MRI; iron oxide particles

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Introduction

Stem cells have the ability to differentiate into specific cell types. They have been used in several pre-clinical models of disease (1–3) and are currently being used in phase I–III clinical trials (4–7). Neural stem cells (NSCs) can differentiate into neuronal or glial cells and express trophic factors to rescue dysfunctional brain tissue (8–12). These properties of NSCs provide an opportunity to use them as delivery vehicles for therapeutic molecules into specific regions or into entire brain (9,10,13,14). Stem cell lines are generally used for investigation of basic properties of stem cells. One of the commonly used NSC lines, known as C17.2, was originally derived from neonatal mouse cerebellum and immortalized by the introduction of a *v-myc* oncogene (15). When transplanted into the developing brain, this cell line consistently results in robust and stable engraftment throughout the brain. Since this line does not usually lead to formation of tumors, it has been extensively used in transplantation biology studies including treatment of mouse models of lysosomal storage diseases (9,14,16–19). However, use of neural stem cell lines for treatment of diseases in humans is potentially problematic due to their immortalized nature. An earlier study suggested that the biology of C17.2 cells differs significantly from primary neural stem cells (20). Therefore, it is important to assess the transplantation and migration properties of primary stem cells in experimental models.

Stem cell engraftment is generally determined post-mortem by histological, immunological, or fluorescence assays. However, to evaluate the efficacy of stem cell migration and survival over time, it is necessary to use non-invasive techniques. Magnetic resonance imaging (MRI) methods have been applied to monitor implanted stem cells by loading the cells with iron-oxide particles as a contrast agent (21,22,23,24). The particles exhibit strong magnetic moments when placed in a magnetic field and create a hypointense (dark) signal on MR images. Based on their size, these agents can be broadly classified into three categories; ultra small paramagnetic iron oxide particles (USPIO, 10–30 nm), super paramagnetic iron oxide particles (SPIO, 30–150 nm) and micron sized paramagnetic iron oxide particles (MPIO, 0.5–2 μ m). The SPIO particles are more commonly used (22,25,26) since they are commercially available and have been used in the clinic for the detection of liver tumors (27) and have recently been shown to be efficient in the detection of implanted cells in humans (28).

As transplanted cells proliferate, the concentration of the contrast agent decreases, eventually falling below the sensitivity threshold of the existing MRI methods. In addition, migration of transplanted cells reduces the number of labeled cells within an imaging voxel which further reduces the sensitivity of MRI in detecting these cells. We have previously shown that limited migration of labeled stem cells (1–2 mm from transplanted site) can be detected *in vivo* by MRI using a low concentration of iron oxide particles (25 μ g Fe/ml) to label the cells (14,29). However, when more extensive migration take place and formation of dispersed small clusters of cells occurs, this concentration of the contrast agent is not sufficient for *in vivo* detection of the transplanted cells (29). To overcome this problem, higher concentrations of the SPIO particles have been used (23,30), with increased toxicity (31,32), limiting the applicability of iron oxide particles for detection of stem cell migration. While cell viability (determined by Trypan blue exclusion, MTT assay or by bio-luminescence) has typically been used to evaluate toxicity due to contrast agent (33), only few studies have been performed to assess the differentiation abilities of stem cells in the presence of iron oxide particles (34,35). Furthermore, since the host tissue may affect the engraftment, migration and differentiation of transplanted cells, it is important to assess whether iron labeled cells can integrate into the brain tissue.

The present study was thus designed to compare the migration and differentiation patterns of immortalized NSC cell line C17.2 with primary murine NSCs in the presence of SPIO particles. We determined a concentration of SPIO particles that would permit detection of engrafted stem

cells by *in vivo* MRI without significantly altering the viability or the engraftment and migration potential of these cells.

Methods

Experimental animals

Animal procedures were conducted in strict accordance with the NIH *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee of the Children's Hospital of Philadelphia. Normal C3H/SCID and transgenic mutant mice (TgGUSB/TgGUSB, gus^{mps}/gus^{mps}) were maintained in a breeding colony at the Children's Hospital of Philadelphia. These mice have the MPS VII genotype gus^{mps}/gus^{mps} and are transgenic for the human β -glucuronidase gene (36).

Isolation of mouse neural precursor cells

Brains from three postnatal day 1 transgenic mutant mouse pups (TgGUSB/TgGUSB, gus^{mps}/gus^{mps}) were collected after humane euthanasia *via* cervical dislocation using cryoanesthesia. The brains were removed, placed into a balanced salt solution, and then dissected grossly. The rostral brain was transversely sectioned caudal to the olfactory bulbs and rostral to the hippocampus. The tissues were pooled, minced, and then digested in 0.25% trypsin (Worthington) in a 37°C water bath for 15–20 minutes. The enzymatic digestion was stopped with addition of fetal bovine serum (FBS; Hyclone). The tissues were then incubated with DNase I (Sigma) for fifteen minutes in a 37°C water bath and triturated to a single cell suspension with successively smaller diameter pipettes, ending at a flame-polished Pasteur pipette. The cell suspensions were centrifuged at 700 rpm at 4°C for 8 minutes, re-suspended in 10% FBS plating medium and triturated. The total number of viable cells was determined by manual count on a hemacytometer; cell viability was assessed using trypan blue exclusion (0.4%; Sigma).

Primary mouse neural precursor cell culture

The neural precursor cells (NPCs) were plated at a concentration of $4 \times 10^4/\text{cm}^2$ in 10% serum-containing plating medium consisting of DMEM:F12 (1:1 ratio; GibcoBRL) supplemented with 10% FBS, 1% N2 supplement (GibcoBRL), 1% antibiotic-antimycotic (PSF) (100 U/mL penicillin; 100 $\mu\text{g}/\text{mL}$ streptomycin; 0.25 $\mu\text{g}/\text{mL}$ amphotericin B; GibcoBRL), and 1% L-glutamine (2mM; GibcoBRL). After 24–48 hours, the medium was changed to a serum-free feeding medium consisting of DMEM:F12 supplemented with 1% N2 supplement, 1% PSF, and 1% L-glutamine. The standard combination of growth factors consisted of 20 ng/mL epidermal growth factor (EGF) (recombinant murine; Promega), 20 ng/mL basic fibroblast growth factor (bFGF) (recombinant human; Promega), and heparin (5 $\mu\text{g}/\text{mL}$; Sigma). The NPC cultures were maintained at 37°C in humidified 5% CO_2 tissue culture incubators. Cultures were fed every 3–5 days by changing half of the medium and adding fresh growth factors. The NPCs were plated into 25 cm^2 tissue culture flasks (Corning) coated with 10 $\mu\text{g}/\text{mL}$ poly-D-lysine (Sigma). Cultures were passaged at approximately 90% confluence by trypsinizing (0.05% trypsin-EDTA; GibcoBRL) and re-plating at a concentration of $4 \times 10^4/\text{cm}^2$.

Cell culture of C17.2 cells

C17.2 cells were maintained in uncoated 75 cm^2 tissue culture flasks and split at a ratio of 1:10, twice a week by gentle trypsinization and low-speed centrifugation at 700 rpm for 5 min (9). The growth medium comprised of DMEM (CellGro) containing 4.5 g/L glucose and 1 mM sodium pyruvate supplemented with 10% fetal bovine serum, 5% horse serum, 2 mM L-

glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin sulfate, 0.25 mg/ml fungizone and 1.85 mg/ml bicarbonate.

Labeling of cells with iron oxide particles

Super paramagnetic iron oxide particles (Feridex, Berlex Imaging) and poly-D-lysine (Sigma, PDL, $M_r(\text{PDL}) = 120 \text{ KDa}$) were mixed in the cell culture medium at four different concentrations: 0, 25, 100, 250 $\mu\text{g Fe/ml}$ and 0, 1.2, 4.8, 12 $\mu\text{g/ml}$ respectively. The mixtures were incubated at room temperature for 60 min with occasional stirring. The growth medium of C17.2 cells, when it reached 75% confluence was replaced with Fe/PDL medium (at various concentrations listed above) and incubated for 24 or 48 hr with a maximum volume of 74 μl of the contrast agent per ml of medium (37). To remove excess iron oxide particles, trypsinized cells were washed three times with phosphate buffered saline (PBS) and concentrated by centrifugation, re-suspended in PBS and kept on ice.

The iron concentration per cell was determined using inductively coupled plasma mass spectroscopy on separate batches of labeled cells. The trypsinized labeled cells were re-suspended in 1 ml of PBS and counted. After counting, the cells were digested with equal amount of nitric acid overnight and the resulting solution was used for standard inductively coupled plasma mass spectroscopy measurement. The concentration of iron per cell was calculated by dividing the total iron content in the sample by the total number of cells. These experiments were repeated thrice and average iron concentrations were calculated.

Differentiation assay of NSCs

The regular incubation medium was replaced with serum free DMEM containing 4.5 g/L glutamate, 1 mM sodium pyruvate supplemented with 1.85 mg/ml bicarbonate and 1% antibiotic-antimycotic (penicillin 100 U/mL; streptomycin 100 $\mu\text{g/mL}$; 0.25 $\mu\text{g/mL}$ amphotericin B; GibcoBRL). Cells were allowed to differentiate for 5 days at 37°C. For immunofluorescent staining of intracellular markers, cells were rinsed in TBS (50 mM Tris-base, 0.15M NaCl; pH 7.6), fixed for 10 minutes in 4% paraformaldehyde (Sigma), rinsed three times with TBS, blocked in 5% goat serum (GibcoBRL) with 0.1% Triton X-100 (Sigma) for 40 minutes, and then incubated with primary antibody in 1% goat serum with 0.02% Triton X-100 overnight at 4°C. After three TBS washes, the secondary antibody was applied for 1 hour at room temperature. All slides were washed three times with TBS before mounting in Vectashield containing 4', 6 diamidino-2-phenylindole (DAPI; Vector Laboratories). Primary antibodies used were mouse anti-Map2ab, 1:200 dilution (IgG; Chemicon). Secondary antibodies used were goat anti-mouse IgG/IgM FITC, 1:300 dilution (Chemicon). For primary NPCs, the cultures were differentiated by plating in the absence of growth factors in an 8-well chamber slide (Lab-Tek II; Nalge Nunc) at a concentration of 4×10^4 cells/well in feeding medium supplemented with 1% FBS. Differentiated mouse NPC cultures were processed for immunocytochemistry after 10–12 days. Fluorescent images of the cell cultures were taken with a digital camera at 40 $\times$ magnification. The total number of cells and number of differentiated cells was determined by manual counting in five random fields and the mean value of the percentage of differentiated cells was calculated. The differentiation assay was repeated three times.

Intracranial cell implantation

Neonatal C3H/SCID mice ($n = 15$ were injected with C17.2 cells, and $n = 11$ were injected with primary cells) were cryo-anaesthetized and injected on the day of birth with SPIO-labeled C17.2 or primary NSC's using trans-illumination to guide the injection as described previously (9,14). Two micro-liters of the labeled cell suspension (4.9×10^4 cells/ μL), labeled with different concentrations of SPIO (25, 100 or 250 $\mu\text{g/ml}$), were injected into each lateral

ventricle using a glass pipette to minimize the damage to the tissue. Control animals (n = 4) were injected with the same number and volume of unlabelled C17.2 cells.

Magnetic resonance imaging experiments

In vivo imaging—*In vivo* imaging of mouse brain was performed using a 4.7 T horizontal bore magnet equipped with a 12 cm, 25 G/cm gradient insert (50 cm horizontal bore magnet with a Varian imaging console (Varian Inc)). Mice were anesthetized with 1% isoflurane in air during the MRI experiment. A home built circularly polarized 1-inch inner diameter birdcage coil was used for imaging. Core body temperature and ECG were monitored during the exam using an MRI-compatible unit (SA instruments Inc). The body temperature of animals was maintained at $37 \pm 1^\circ\text{C}$ by blowing warm air through the magnet bore, which was regulated by a feedback loop. *In vivo* three-dimensional (3D) gradient echo imaging was performed with the following parameters: repetition time (TR) 100 ms, echo time (TE) 4.5 ms, 4 scans, field of view (FOV) of 2 cm^3 , $128 \times 128 \times 128$ matrix, leading to an isotropic resolution of $156\text{ }\mu\text{m}$ and an acquisition time of about 2 hours.

At the end of the *in vivo* experiments, mice were sacrificed under deep anesthesia, fixed by transcardial perfusion with 10 ml of cold Dulbecco's PBS followed by 30 ml of 4% paraformaldehyde. Brains were removed and post-fixed for 4 – 5 hours in 4% paraformaldehyde, before *ex vivo* imaging.

Ex vivo imaging—For high-resolution imaging, the perfusion fixed-brains were imaged using a 9.4 T vertical bore magnet interfaced to a Varian console. Mouse brains (n = 15) were placed in a 20 mm NMR tube and immersed in 4 ml Fomblin (Ausimount) to avoid any background signal. A 20 mm high resolution Helmholtz probe (Naolrak) was used for imaging. A 3D gradient echo imaging was performed with the following acquisition parameters: TR/TE 100/10 ms, 28 scans, FOV $\sim 2 \times 1 \times 1\text{ cm}^3$, $256 \times 128 \times 128$ matrix, leading to an isotropic resolution of approximately $78\text{ }\mu\text{m}$ and an acquisition time of 12 hours.

Image analysis—The acquired 3D images were displayed using the “ImageJ” (NIH image, <http://rsb.info.nih.gov/nih-image/>) program and reformatted into a stack of 2D slices. A 3D mask of the brain was created by slight dilation of 2D images in order to remove the borders from the ventricular system and the hypointense regions due to labeled cells, and depicted in a transparent blue color. Hypointense areas due to labeled cells were manually highlighted with different color (red) on each 2D slice and the stack of highlighted areas was saved as a separate 3D file. 3D image of brain mask and 3D image of hypointense regions were then superimposed using 3D-slicer program (www.slicer.org).

Histology—At the end of the *ex vivo* MRI experiments, brains were removed from the NMR tube, rinsed with PBS solution, cryo-protected in 30% sucrose, embedded in OCT and frozen on a bed of crushed dry ice. Twenty μm -thick cryosections were made and alternate sections were stained with Prussian blue and X-gal or human GUSB as described below.

Prussian blue staining for detection of iron—Slides were placed in a Coplin jar containing a 2:1 mixture of 2% potassium ferrocyanide and 2% hydrochloric acid for 30 minutes in a 37°C water bath. The slides were then rinsed with distilled water and counterstained with nuclear fast red (Vector Laboratories) for 20 minutes.

β -galactosidase staining—The cryosections were reacted with X-gal using standard methods to detect β -galactosidase activity and counterstained with nuclear fast red as described previously (29). Briefly, after fixing in 0.5% glutaraldehyde, sections were washed in PBS containing 1 mM magnesium chloride and reacted with X-gal solution at 37°C for 3 – 5 hours.

GUSB staining—Frozen tissue sections were assayed for enzymatic activity by staining with a naphthol-AS-BI- β -D-glucuronide substrate as reported previously (38). Briefly, slides were thawed for 5 min at room temperature and treated with chloral-formal-acetone fixative for 30 min at 4°C. The slides were washed three times with 0.05 M sodium acetate buffer (pH 4.5). Next, 0.25 mM naphthol-AS-BI- β -D-glucuronide (pH 4.5) was added and the slides incubated at 4°C for 4 h, at which point the solution was removed. Equal volumes of 4% pararosaniline solution in 2 N hydrochloric acid and 4% sodium nitrite solution in deionized water were mixed. This solution was diluted 1 to 500 in 0.25 mM naphthol-AS-BI β -dglucuronide (pH 5.2), placed on slides, and incubated at 37°C overnight. The third wash in 0.05 M sodium acetate was performed at 65°C for all sections. Heat-inactivation serves to distinguish between the human and murine β -glucuronidase (GUSB) proteins. Murine GUSB is inactivated at 65°C, whereas the human protein is stable.

CD11b staining—Frozen sections (20 μ m thick) from the cerebellum were stained for the presence of microglia using the CD11b antibody then stained for the presence of iron within the microglia using Prussian blue as above. The sections were incubated in 0.3% H₂O₂ for 5 minutes to quench endogenous peroxidases, blocked with 2% goat serum for 20 minutes, and incubated overnight at 4°C with a monoclonal rat anti-mouse CD11b (clone M1/70, BD Pharmingen, San Diego, CA) diluted 1:100. A negative control consisted of adjacent sections stained without the primary antibody. After multiple rinses in PBS, the sections were incubated for 1 hour at room temperature with a secondary biotinylated rabbit anti-rat IgG (Vector Laboratories, Burlingame, CA) at a 1:500 dilution. After PBS rinses, the sections were treated with avidin-biotin-peroxidase complex (Vectastain® Elite ABC kit, Vector Laboratories) for 30 minutes at room temperature, followed by incubation with diaminobenzidine (DAB) and peroxidase (DAB substrate kit for peroxidase, Vector Laboratories) for 10 minutes at room temperature.

Results

Iron uptake of cells

Prussian blue staining of C17.2 and primary mouse NSCs labeled with different concentrations of SPIO particles demonstrated increased internalization of iron oxide particles (Figure 1A, B). The uptake of iron oxide particles by C17.2 cell was notably higher than by primary cells as evident by the higher iron concentration in C17.2 cells in comparison to the primary mouse NSCs (Table 1).

Cell differentiation in the presence of SPIO particles

In vitro neuronal differentiation of C17.2 and primary NSCs is depicted in Figure 1 C and D (green). The total number of cells and Map2ab positive cells were counted from five different fields. The percentage of Map2ab positive C17.2 cells ranged between 6–18% while the percentage of primary Map2ab-positive cells varied between 2 – 10% (Figure 1, Table 2). There was no significant difference between the number of Map2ab positive cells from unlabeled or SPIO labeled C17.2 or primary mouse NSC's (Table 2), indicating that SPIO particles did not affect the neuronal differentiation at the concentrations used.

Engraftment pattern

In order to detect the effect of SPIO particles on the engraftment potency of transplanted stem cells, distribution of β -galactosidase positive cells was tested after intra-cranial transplantation of C17.2 cells labeled with different concentrations of SPIO particles. The X-gal staining (for the lacZ gene expressed in transplanted C17.2 cells) demonstrated a similar engraftment pattern in mice injected with SPIO labeled C17.2 cells to that of control animals indicating that the SPIO particles do not have any adverse effects on the engraftment potency of these cells (Figure

2 A). Figure 2 B depicts iron positive cells from the cerebellar region exhibiting processes (insets). Iron positive cells with differentiated morphology were detected in brains injected with cell labeled with 25, 100 or 250 $\mu\text{g Fe/ml}$ of SPIO, indicating they can differentiate both *in vitro* and *in vivo*.

MRI detection of labeled C17.2 cells

Figure 3 shows *in vivo* images of a mouse brain injected with C17.2 and murine primary cells labeled with SPIO particles at concentrations of 25, 100 or 250 $\mu\text{g Fe/ml}$. Unlabeled control cells were not detectable by MRI (data not shown). Cells labeled with 25 $\mu\text{g Fe/ml}$ were not detectable *in vivo* (A) but were visible by *ex vivo* MRI, similar to the results published earlier (29). At iron concentration of 100 (B) and 250 $\mu\text{g Fe/ml}$ (C), transplanted cells were detectable *in vivo* (Figure 3, arrows). The hypo-intense areas due to labeled cells were more prominent at an iron concentration of 250 $\mu\text{g Fe/ml}$ compared to 100 $\mu\text{g Fe/ml}$ of SPIO (Figure 3 B, C).

MRI detection of murine primary stem cells

Similar to the C17.2 cells, it was not possible to detect transplanted mouse primary NSCs when labeled with 25 $\mu\text{g/ml}$ of SPIO particles (Figure 3D), but were detectable at higher SPIO concentrations (Figure 3E, F). The primary NSCs were primarily detected in the vicinity of the ventricular system (arrows in Figure 3) indicating that they did not migrate as far into the parenchyma as C17.2 cells. The MRI results were confirmed with Prussian blue and HPR staining that detects the human GUSB positive transplanted cells. Figure 4 depicts an *in vivo* MR image, Prussian blue and HPR staining of mouse brain injected with murine primary NSC's labeled with 100 $\mu\text{g/ml}$ of SPIO particles. The MRI signal hypo-intensity correlated well with Prussian blue positive areas and with the GUSB positive cells (Figure 4B, C). The inset in Figure 4C depicts a magnified region containing a GUSB positive cell exhibiting morphological features of a differentiated cell. These results suggest that the presence of SPIO particles did not alter the engraftment and differentiation pattern of primary NSCs.

Detection of iron-positive grafted cells and microglia

The hypo-intense signal on MRI arises from iron positive cells. While most of the signal is from the transplanted cells or their progeny, it is possible that some signal is present in microglial cells that have taken up the iron oxide particles from dead cells. To assess this, sections from the cerebellum of mice injected with 250 $\mu\text{g/ml}$ of SPIO particles were co-stained with CD11b, a marker for macrophages and microglia, and Prussian blue. Figure 5 shows cells that were Prussian blue positive, but were negative for the CD11b staining (A, B) indicating that these cells are transplanted cells or their progenies. Some cells stained positively for CD11b but were negative for Prussian blue (C) indicating the presence of microglia in these sections. Some cells were positive for both CD11b and Prussian blue (D), indicating that some of the hypo-intense MRI signal may have originated from iron-positive microglial cells.

In order to further elucidate the extent of cell migration that can be detected by MRI, we performed *ex vivo* MRI experiments on post-mortem brains, as these studies can be performed with much higher resolution and provide higher sensitivity for detection of grafted cells. Three-dimensional (3D) reconstruction of brain images were used to visually estimate the extent and differences in migration patterns (Figure 6). C17.2 cells were broadly distributed throughout the brain (left panel) while primary cells were detected primarily in the center of the brain around the ventricles (right panel). However, some primary cells migrated from the lateral ventricles into the septum, fimbria, hippocampus, and thalamus. Representative transaxial MRI sections of the brains injected with each cell type are shown in the Figure 6 illustrating the difference in the migration pattern between the C17.2 and primary mouse NSCs.

Discussion

Noninvasive observation of implanted cells may assist in selection of a place, time and cell type for transplantation experiments. Neural stem cells migrate and divide after transplantation, which leads to reduction of contrast agent concentration and reducing the intensity of label cells detection. Thus, enhancement of MRI sensitivity for the detection of grafted cells is an important step for the development of stem cells and gene therapy. We found that labeling cells with SPIO particles at concentrations of 100 µg Fe/ml resulted in minimum impact on murine primary NSCs, allowed detection of implanted cells by *in vivo* MRI, and provided an opportunity to compare migration ability of different cells after neonatal transplantation.

In vivo detection of SPIO labeled cells has been reported by several groups (21,23,29,39,40). However, there is a lack of consensus about the optimal/minimal concentration of SPIO particles that should be used for *in vivo* detection of implanted cells. While the use of a high concentration of SPIO particles is advantageous for increasing the sensitivity for detection, the higher amount of intracellular iron might have adverse effect on cell viability, as seen with immune cells (31). The effect of iron-oxide particles on stem cell differentiation has been studied in mesenchymal and hematopoietic stem cells (32,33,41,42). One of these studies reported an adverse affect of SPIO particles on mesenchymal cell differentiation into chondrocytes (32), while another found no differences in the differentiation pattern of hematopoietic or mesenchymal stem cells (41).

Under appropriate conditions, NSCs can differentiate into neurons, astrocytes and glia after intracranial transplantation (35,43,44,45). In order to detect the effect of SPIO particles on stem cell viability and differentiation, we labeled two types of NSC's with different concentrations of iron oxide particles and measured the differentiation, migration and engraftment potential of these cells using *in vitro* and *in vivo* assays. As a rigorous and reliable measure of differentiation, we evaluated Map2ab expression of labeled cells which is a marker of mature neurons (46). We did not observe any adverse effect of SPIO particles on *in vitro* neuronal differentiation with either C17.2 or primary cells (Figure 1). Approximately 10% of unlabeled C17.2 cells differentiated into neurons, which is in agreement with a previously published study (47) and a similar number of neuronal cells were observed in cell cultures labeled with iron oxide particles up to 250 µg Fe/ml (Table 2). Since the experimental conditions for assaying cell differentiation *in vitro* are much different than the physiological conditions *in vivo*, where tissue microenvironment can play a critical role in the way the transplanted cells engraft and differentiate, we further analyzed the tissue sections histologically after seven weeks of intra-cranial transplantation in the neonatal brain. We were able to identify iron positive cells exhibiting morphology of differentiated neurons or astrocytes (Figure 2B). The presence of iron positive cells with neuronal morphology suggest that C17.2 cells labeled with SPIO particles up to 250 µg Fe/ml can differentiate not only *in vitro* but *in vivo* as well.

The ability of MRI to detect transplanted cells depends on several factors including the concentration of the contrast agent, degree of migration of the transplanted cells, magnetic field strength, and the imaging parameters used. We observed that a SPIO concentration of 25 µg/ml was not sufficient to detect extensively migrated cells *in vivo*, but this could be used to detect cells in *ex vivo* brain tissue (Figure 3A, D and (29)). In our experimental conditions, a minimum SPIO concentration of 100 µg/ml was necessary to consistently detect C17.2 cells and murine primary stem cells *in vivo*. It is to be noted that occasional detection of mouse primary cells labeled with 25 µg/ml of SPIO in the ventricular area was also possible (data not shown). This was probably due to the fact that primary cells exhibit more limited migration ability (see below) and frequently formed large cell aggregates in the sub-ventricular zone. In a previous study, we have shown that we can detect about 500 cells or more by *in vivo* MRI at

SPIO loading concentrations of 25 µg/ml (29). However, in order to consistently and reliably detect smaller cluster of cells, it may be necessary to increase the SPIO concentration to 100 µg/ml.

It is possible to substantially increase the sensitivity of detection labeled cells by increasing the magnetic field strength. Detection of smaller cluster of cells (~100 cells) has been reported using field strength of 17.6 T (48). However, such high field strength magnets are not easily available and such studies may not be translated into the clinic since the currently approved FDA limits for magnet field strengths is 3T. In contrast, our studies were performed with a more readily accessible 4.7T magnet, which is closer to the clinically available magnetic field strength. It is to be noted that clinical magnets have also been used for detection of approximately 2000 dendritic cells per imaging voxel in humans (28) and for detection of single cells in mice using dedicated small animal imaging gradients (49). Thus the sensitivity of detection of iron positive cells depends on the available hardware and software with a lower sensitivity for human studies than small animals. Radio-nucleotide techniques, such as positron emission tomography (PET), scintigraphy as well as optical imaging methods have also been used for tracking cells *in vivo* (50–53). While the sensitivity of these techniques is higher than MRI, they are limited with lower spatial resolution (51,54–58). In a recent study, de Vries et al compared MRI with scintigraphy in detecting migration of dendritic cells to the metastatic nodes in patients with melanoma (28). These authors demonstrated that even with a lower sensitivity of detecting iron labeled cells, MRI was superior as it was able to accurately demonstrate the number of nodes in which the cells migrated due to the inherent higher resolution of MRI over scintigraphic methods.

Magnetic resonance imaging techniques have also been used for infiltration of macrophages in response to immune reactions (59,60). In the brain, the microglia is responsible for clearing up cell debris. Presence of hypo-intense areas on MR images is indicative of the presence of iron oxide particles. However, it is not possible to distinguish *in vivo* whether these SPIO particles are located inside viable stem cells or have been engulfed by microglia. When a dual labeling for CD11b and Prussian blue was performed from the cerebellum, most cells were only positive for iron or CD11b, however, some double-positive cells were seen suggesting that some grafted cells died and the contrast agent was picked up by microglial cells. It is interesting to note that the microglia were detected seven weeks after neonatal transplantation at a distance from the original transplantation site (ventricular). This observation, coupled with the fact that iron positive cells were always observed in the vicinity of LacZ or GUSB positive cells indicate that a majority of transplanted cells migrated and differentiated to different regions of the brain after transplantation. Since hypo-intensity on MRI was also observed from these regions, the results indicate that MRI can identify the difference in the engraftment pattern of the immortalized cell line from primary NSCs.

The C17.2 cells provide a useful model for testing the efficacy of MRI in detection of neural stem cell migration *in vivo*. These cells are very easy to grow and after neonatal implantation, exhibit exceptionally good migration (9). We used this cell line to validate that 100 µg/ml of SPIO particles is necessary for the robust *in vivo* MRI detection of widely distributed cells and observed that this concentration of contrast agent did not adversely affect the differentiation and engraftment potential of this NSC line. The *in vivo* MR imaging experiments demonstrated the presence of SPIO labeled C17.2 cells in olfactory bulbs, corpus callosum and parenchyma. In contrast to our findings, another study has reported very limited migration of SPIO labeled C17.2 cells after neonatal implantation into shiverer (shi/shi) mouse brain (35), where the iron-positive cells were found only in ventricular system. A possible explanation of differences in migration of iron positive cells may be due to differences in microenvironment of recipient, since the shiverer mice have a severe myelin defect. However, stem cell-based gene therapy should be modeled on primary stem cells isolated from the tissue since a potential goal of cell

based therapies is to use autologous cells. Thus the optimized imaging protocols from C17.2 cells were implemented for the primary NPCs experiments. We observed that the SPIO concentrations needed to detect the cells were not toxic to the primary cells and did not affect the differentiation and engraftment potency of these cells. As expected, the extent of migration of these cells after neonatal transplantation was more limited than the C17.2 cell line. Since the C17.2 line was originally selected from among many clones for its exceptional engraftment ability (8), they are not representative of the heterogeneous population of NSCs after growth factor-mediated expansion from primary brain cell cultures. We found that both the C17.2 and primary cells were viable. The primary cells were found mostly in per-ventricular areas, including septum, fimbria, hippocampus, and thalamus. In comparison to the C17.2 cells, the SPIO uptake by primary cells was much lower. The difference in the endocytotic affinity of cells to SPIO particles may reflect the biologic differences in the cells. The decreased uptake of SPIO particles by primary cells may have affected our ability in detecting smaller cell grafts at a distance from periventricular areas due to lower iron content. However, as limited migration was also confirmed by histological studies, we believe that the limited degree of migration of primary cells is more likely to be representative of what may be expected *in vivo* cell therapy transplants.

In conclusion, we have shown that an SPIO concentration of 100 µg/ml allows for *in vivo* detection of smaller cell grafts and does not appear to alter the viability, differentiation, or migration capability of NSC's. The difference in migration pattern of the primary NSC's from an immortalized neural stem cell line can be detected *in vivo* by MRI. These studies may facilitate development and non-invasive monitoring of stem cell based therapies.

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Figure 1.

Prussian blue (rows A and B) and Map2ab staining (rows B and C) of NSC's labeled with different concentrations of SPIO particles. The results from C17.2 cells are shown in rows A and C, while that from primary NSC's are shown in rows B and D. A magnification factor of 40x is used for the Map2ab sections. In comparison to the C17.2 cells, the primary cells exhibited limited uptake of SPIO particles. The presence of neuronal differentiation is depicted in green (rows C and D).

Figure 2.

Engraftment (top row, A) and differentiation (bottom row, B) pattern of transplanted C17.2 cells in the presence of SPIO particles. Images in the top row are LacZ stained sections of the cerebellar region after 7 weeks of cell transplantation in the neonatal brain. The images are depicted at a magnification of 5x. Presence of β -galactoside positive cells (blue) indicate no adverse effect on engraftment potency of cells labeled with SPIO particles. The images in the bottom row are Prussian blue positive sections from similar regions of the brain as above. These images are shown at a magnification of 40x. Iron positive transplanted C17.2 cells are depicted in blue. The insets show a larger magnification of some of these cells exhibiting processes and morphology similar to that of differentiating neuronal or astrocytic cells indicating that the presence of SPIO particles does not alter the differentiation capability of NSCs.

Figure 3. In vivo detection of SPIO labeled NSCs after seven weeks of transplantation in the neonatal mouse. Representative transverse sections from mice injected with C17.2 cells incubated with 25 (A), 100 (B), 250 µg Fe/ml (C) and mice injected with primary mouse NSCs with 25 (D), 100 (E), 250 µg Fe/ml (F). Labeled cells were not detectable at low iron concentration (25 µg/ml of Fe). Arrows indicate hypo-intense areas due to the presence of cells labeled with high concentration of SPIO (100 and 250 µg Fe/ml).

Figure 4.

Detection of transplanted mouse primary cells by *in vivo* MRI (A) as confirmed by Prussian blue staining (B) and HPR staining for detection of human GUSB positive cells (C). The murine primary NSC's were labeled with 100 μ g Fe/ml of SPIO particles. Panels B and C are shown at 10x magnification, while panels D and E are shown at a 40x magnification for the rectangular regions indicated in panels B and C. Arrows in panel D indicate presence of iron positive primary NSCs. The inset in panel E shows a GUSB positive cell with processes similar to a neuronal cell indicating *in vivo* differentiation.

Figure 5. Detection of iron-positive grafted NSCs and microglia cells in the cerebellum region of the brain after seven weeks of neonatal transplantation of labeled C17.2 cells. Representative sections showing CD11b-negative and Prussian blue positive cells (A, B) indicating presence of iron positive transplanted cells; CD11b-positive and Prussian blue negative cells (C) indicating presence of reactive microglia; and CD11b-positive, Prussian blue positive cells (D) indicating the uptake of iron oxide particles by microglia. The horizontal bar in D reflects a distance of 25 μ m.

Figure 6. Ex vivo

MR images of mouse brains transplanted with SPIO (100 $\mu\text{g Fe/ml}$) labeled NSCs and imaged seven weeks post transplantation. Representative axial images from a 3D data set are displayed for an animal injected with C17.2 cells (left panel) and murine primary NSC's (right panel). The 3D reconstruction of the images is displayed in the central panel with the brain mask displayed in blue and iron positive cells displayed in red. In comparison to the C17.2 cells, which exhibited extensive migration throughout the brain, the primary murine NSCs were primarily located near the ventricular region of the brain.

Table 1

Net iron concentration per cell as measured by inductively coupled plasma mass spectroscopy after incubation of cells with a solution of ferumoxide-poly-D-lysine. Values are reported as mean $\pm$ SD. * - The higher SD is probably due to the difficulty in washing out the free iron particles at this concentration.

Iron concentration per cell (pg/cell)	Iron concentration in incubation medium (mg/ml)			
	0	25	100	250
C17.2	<0.15	10.6 $\pm$ 1.3	46.5 $\pm$ 27.5	152.9 $\pm$ 112*
Primary NSCs	<0.15	5.5 $\pm$ 2.2	4.5 $\pm$ 2.1	8.3 $\pm$ 9.0*

Table 2

Percentage of Map2ab (neuronal) positive C17.2 and murine primary stem cells. C17.2 cells were differentiated for five days while primary cells were differentiated for approximately 10 days. Values are reported as the average number of positive cells with respect to the total number of cells $\pm$ SD (from five random fields and three separate experiments).

% Map2ab Positive	Iron concentration in incubation medium (mg/ml)			
	0	25	100	250
C17.2 cells	9.08 $\pm$ 3.9	10.9 $\pm$ 4.7	11.6 $\pm$ 7.9	11.3 $\pm$ 5.3
Primary NSCs	5.4 $\pm$ 4.7	1.7 $\pm$ 1.9	6.3 $\pm$ 5.9	10.1 $\pm$ 6.0