Proceedings of the 28th IEEE
EMBS Annual International Conference
New York City, USA, Aug 30-Sept 3, 2006

Magneto-Optical Labeling of Fetal Neural Stem Cells for *in vivo* MRI Tracking

J. A. Flexman[1], S. Minoshima[1,2], Y. Kim[1,2], D. J. Cross[2]
[1]Department of Bioengineering, University of Washington, Seattle, WA, USA
[2]Department of Radiology, University of Washington, Seattle, WA, USA

Abstract—**Neural stem cell therapy for neurological pathologies, such as Alzheimer's and Parkinson's disease, may delay the onset of symptoms, replace damaged neurons and/or support the survival of endogenous cells. Magnetic resonance imaging (MRI) can be used to track magnetically labeled cells *in vivo* to observe migration. Prior to transplantation, labeled cells must be characterized to show that they retain their intrinsic properties, such as cell proliferation into neurospheres in a supplemented environment. *In vivo* images must also be correlated to sensitive, histological markers. In this study, we show that fetus-derived neural stem cells can be co-labeled with superparamagnetic iron oxide and PKH26, a fluorescent dye. Labeled cells retain the ability to proliferate into neurospheres in culture, but labeling prevents neurospheres from merging in a non-adherent culture environment. After labeled NSCs were transplantation into the rat brain, their location and subsequent migration along the corpus callosum was detected using MRI. This study demonstrates an imaging paradigm with which to develop an *in vivo* assay for quantitatively evaluating fetal neural stem cell migration.**

Keywords— Imaging in drug discovery, development and therapy; Neural Imaging; Magnetic Resonance Imaging

I. Introduction

Stem cells are a promising source of neuro-regeneration for devastating brain diseases such as Alzheimer's disease and Parkinson's disease. Researchers have already shown that donor stem cells can integrate into a damaged neuronal network and respond to injury; a replacement of as few as 10% of damaged neurons from stroke may be needed to restore functionality [1]. Stem cells have been labeled with a contrast agent prior to transplantation and tracked non-invasively in the living brain using magnetic resonance imaging (MRI) as they migrate toward a contra-hemispheric stroke after stereotaxic injection [2]. MRI tracking of stem cells shows cellular migratory patterns, and represents an *in vivo* assay with which to either: 1) evaluate the effects of drugs or genetic modifications designed to enhance or inhibit migration; or 2) uncover intrinsic biological information about stem cell migration in various disease pathologies.

The hemagglutinating virus of Japan envelope (HVJ-E) viral vector is an effective transfection vehicle because of its affinity for sialic acid, which is abundantly found on the nerve growth factor (NGF) receptors of neural progenitor cells [3]. We have previously shown that HVJ-Es can efficiently introduce SPIO particles within a neural progenitor cell line with no effect on cell differentiation or viability [4]. However, this cell line is limited in its progenitor fate and immortalization presents a greater risk of tumor formation after transplantation. The work presented here demonstrates the transfection of non-immortalized cells, and the integration of optical labeling for histological verification. In addition, the *in vivo* behavior of labeled stem cells after transplantation is demonstrated using MRI, which was not investigated in our previous study.

Primary neural stem cells (NSCs) can be isolated from the brain of the developing fetus or from select regions of the adult brain where neurogenesis persists and cultured to form neurospheres [5, 6]. NSCs may be more suitable for transplantation that other, more differentiated cell lines due to their proliferative behavior, which is arrested in a non-supplemented environment, and their multipotency. The effect of SPIO labeling on the formation of neurospheres has not been investigated, but remains an important characterization prior to MRI tracking.

The inclusion of iron-containing SPIO particles within the cell membrane enables histological confirmation of *in vivo* cell localization by Prussian blue staining, but remains a non-specific marker and susceptible to artifacts formed during the staining process. A non-endogenous fluorescence marker is desirable for its specificity and the lack of required staining procedure. PHK26 is a red fluorescent dye established for use in many cell types that is highly aliphatic and incorporates stably into the lipid bilayer of cellular membranes [7]. The dye has been used *in vivo* to track various cell types, including human NSCs [8], and persists for months [9].

In this study, we have characterized the SPIO labeling of primary fetal NSCs using HVJ-Es in terms of transfection efficiency and neurosphere formation. In addition, we have incorporated the fluorescent labeling of NSCs with the transfection of SPIO particles using HVJ-Es. Finally, we demonstrate that MRI can detect NSC migration after transplantation into the rat brain.

II. Methodology

A. Cell Culture and Labeling

NSCs were isolated from rat fetal striatum on embryonic day 16 and used for labeling with SPIO (Feridex IV; Berlex Laboratories; Wayne, NJ) after expansion in 5% CO_2 and at 37°C. Cells were mechanically dissociated into a single cell suspension after 3-4 days of neurosphere-formation in proliferative medium (Neurocult; StemCell Technologies; Vancouver, BC). HVJ-Es exposed to 10 μL of SPIO were added to 6-well plates containing approximately 10^6 NSCs/well and cultured for three or six

hours. After washing, cells were mounted on slides, fixed and stained with Prussian blue for iron content. Representative photographs were taken using a microscope equipped with a digital camera.

The PKH26 cell membrane embedding dye (Sigma Aldrich; St. Louis, MO) was used to incorporate the dye into NSC membranes. Briefly, after SPIO transfection, cells were suspended in the diluent C provided, mixed with an equal volume of 4 μM solution of PHK26 cell linker, and incubated for 4 min. PKH26 is excited at a wavelength of 551 nm and emits at a wavelength of 567 nm. Labeling was stopped with an equal volume of 1% bovine serum albumin in phosphate buffered saline (PBS) for 1 min. Cells were washed three times, mounted on slides, fixed and observed under fluorescence microscopy using a Texas Red-HYQ filter (excites wavelengths from 530 – 580 nm and detects wavelengths from 600 – 680 nm).

B. Cell Characterization

Transfection efficiency of labeled NSCs was determined using atomic absorption spectrophotometry (AAS). Briefly, labeled NSCs were initially digested using an solution of 3:1 perchloric to nitric acid and heated for at least 3 hours at 60°C to ensure a uniform solution matrix. The iron content was then determined using AAS with autosampling (PE-1500; Perkin-Elmer; Shelten, CT). Two trials of 10 μL each were measured for each labeling characteristic (method and volume of Feridex), of which three independent samples were prepared.

A neurosphere assay was performed to assess the effect of SPIO labeling on neurosphere formation. NSCs were labeled as described in the previous section, and then approximately 10^4 cells/well were cultured in a non-adherent 24-well plate to prevent cell attachment and differentiation for 7 d. Five samples were prepared for each of labeled cells and control (unlabeled) cells. Culture dishes were observed under phase contrast microscopy using an inverted microscope and digital photographs were taken of each neurosphere. Neurosphere diameter was measured from images using Neuron Morpho plugin for ImageJ (National Institutes of Health; Bethesda, MD).

C. Intracerebral Injection of Neural Stem Cells in the Rat

The University of Washington Animal Care Committee approved all animal experiments. Animals were kept on a normal 12 h light/dark cycle and had free access to chow and water. A Sprague Dawley male rat was anesthetized with 2% isofluorane gas and restrained in a stereotaxic holder (Kopf Instruments; Tujunga, CA). The skin was retracted to expose the skull and a 1.5 mm diameter hold was be drilled using a manual pin vise. Approximately 10^4 labeled cells in 2 μL were injected into the corpus callosum of the brain (approximately Bregma +2.5 mm, 3.0 mm lateral on the right side and 4.0 mm deep from the surface of the skull) using a picoliter syringe pump (Pico Plus Syringe Pump; Harvard Apparatus; Holliston, MA) over 20 min. After injection, the needle was left in place for 5 min and then retracted. The brain was harvested after a perfusion with PBS and 4% paraformaldehyde and processed for Prussian blue staining and microscopy. Stained tissue sections were photographed using a microscope equipped with a digital camera.

D. Magnetic Resonance Imaging

Two percent isofluorane gas was used for MR scans the day of injection and 2, 3 and 8 d after. T2*-weighted (3-D gradient echo) images were acquired using a custom saddle coil with TE=23 ms/TR=50 ms/FA=10° in a 1.5 T MRI system (GE Signa; General Electric; Milwaukee, WI). In-plane pixel resolution was 0.23 mm and scan thickness was 0.5 mm. Images were trimmed, co-registered and normalized using NEUROSTAT (University of Washington).

III. Results and Discussion

A. Cell Labeling Efficiency

HVJ-Es containing SPIO particles were able to transfect primary NSCs, as demonstrated by Prussian blue staining (Fig. 1). The incubation time of NSCs with HVJ-Es for 3 or 6 h did not result in a significantly different transfection efficiency (Figs. 1 and 2). The average iron content per cell was 3.04±0.26 and 2.97±0.39 pg/cell for an incubation of 3 and 6 h, respectively. Reducing the incubation time for trasnsfection while maintaining the transfection efficiency is important for the practical translation of this technique to a clinical setting.

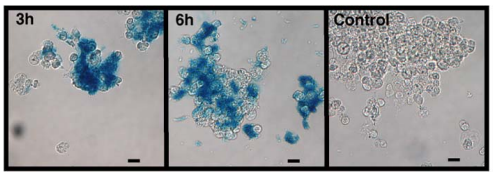

Fig. 1. Prussian blue staining for iron of fetal neural stem cells after a 3 h (left) or 6 h (middle) incubation with HVJ-Es containing iron oxide particles or control (unlabelled) cells. Scale bars are 10 μm.

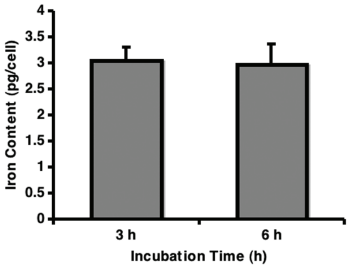

Fig. 2. Average iron content per cell based on atomic absorption spectrophotometry for HVJ-E transfection of fetal neural stem cells for an incubation time of 3 h or 6 h.

B. Fluorescent Co-labeling

The fluorescent dye PHK26 efficiently labeled NSCs after SPIO transfection (Fig. 3A-B). Approximately 100% of cells were labeled based on microscopic images. Dual labeling with iron oxide, which can be detected with Prussian blue staining, and PKH26, which can be detected by fluorescence, will enable more sensitive and specific histological correlation of MRI signal intensities with stem cell grafts *in vivo*.

NSCs dually tagged with SPIO and PKH26 retained the ability to proliferate and form neurospheres (Fig. 3C). After 7 days of proliferation, fluorescence was detected in neurospheres, but was not uniformly distributed within the sphere (Fig. 3D). Since PKH26 is embedded in the cell membrane, the fluorescence signal is diluted during cell division. However, transplanted, dissociated NSCs are expected to differentiate into glial or neuronal cells after only 2-3 cell divisions in the *in vivo* environment due to the reduction of proliferative mitogens [10, 11].

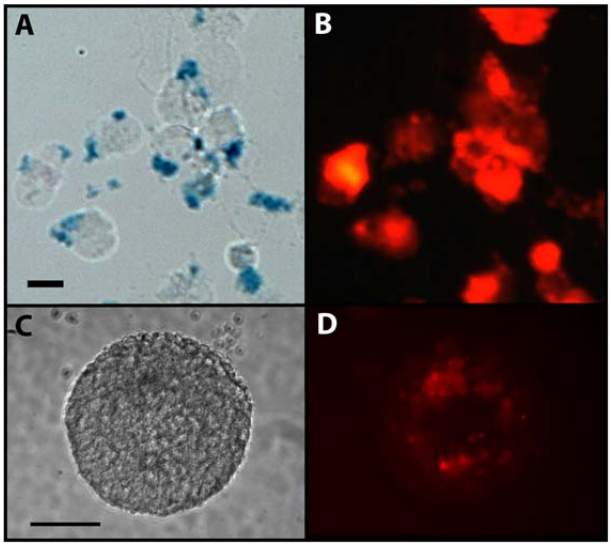

Fig. 3. Representative microscopic images of fetal neural stem cells transfected with SPIO particles and dually tagged with PHK26, a red emitting fluorescent dye. SPIO transfection is demonstrated by Prussian blue staining (A) and fluorescence is shown in cell membranes (B). Cells transfected with SPIO and tagged with PHK26 were able to form neurospheres (C), and cells within the sphere demonstrate a persistent fluorescence (D). Scale bars in A and C are 10 and 100 μm, respectively.

C. Neurosphere Assay

Figure 4A shows that SPIO-labeled NSCs were able to form neurospheres in culture, and formed significantly more neurospheres (16.4±5.0) than unlabeled control cells (5.2±1.9). Control cells resulted in neurospheres with a significantly greater diameter (261.9±40.3) than SPIO-labeled cells (116.5±20.5). These results are reflective of the fact that, in the non-adherent plate, control neurospheres merged together while labeled neurospheres did not.

The effect of SPIO labeling by HVJ-Es on cell merging may stem from the viral proteins present on the cell membranes of transfected cells, which interfere with cell-cell interactions, or from clumps of viral particles, which physically hinder the complete merging of spheres. Further investigation is needed to confirm whether the number of neurospheres formed is affected by labeling prior to merging, and if the same effect is observed in the absence of a non-adherent plate.

Because single cells are unlikely to form large neurospheres and merge *in vivo*, this result may not affect dissociated cell transplantation studies. However, transplantation of intact neurospheres has shown that these grafts form all three neural lineages *in vivo* and that the sphere environment may encourage survival and proliferation [12]. If intact, labeled neurospheres are transplanted into the brain, this effect may be of greater importance.

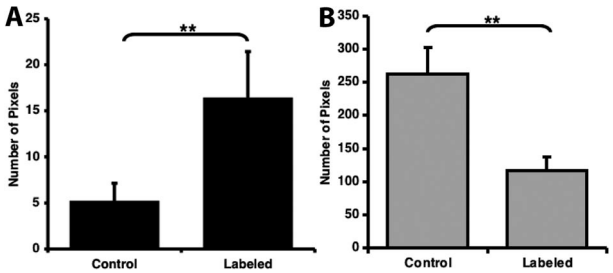

Fig. 4. Fetal neural stem cells (NSCs) labeled with SPIO formed neurospheres. The total number of neurospheres formed per well was significantly higher for labeled cells (A, **=p<0.01). However, the average diameter of neurospheres formed by SPIO labeled NSCs was significantly lower than that of control cells (B, **=p<0.01).

D. MRI of Neural Stem Cells in the Rat Brain

Labeled NSCs, once transplanted into the rat brain, can be observed on MR images from a clinical scanner as a reduction in signal intensity (Fig. 5). The migration of cells is shown as a decrease of intensity over time in certain directions from the site of transplantation. Within one week, the injected cells have moved along the corpus callosum lateral to the injection site and dorsal along the needle track. The corpus callosum is a white matter structure bridging the two cerebral hemispheres, and can serve as a migratory pathway in the brain for both endogenous and transplanted stem cells [2, 13]. Movement along the needle track may have occurred because of the reduction of physical hindrance in this direction, and/or because of factors secreted from damaged cells along the track.

Histological analysis confirmed the presence of labeled cells within the brain and that NSCs were migrating away from the site of transplantation (Fig. 6). Co-labeling with fluorescence increased the sensitivity of microscopy of histological sections for detecting labeled cells. However, Prussian blue staining may obscure fluorescent emissions in some parts (top of Fig. 6A-B) and the fixation process may

result in the leakage of the fluorescent dye from the cell membrane.

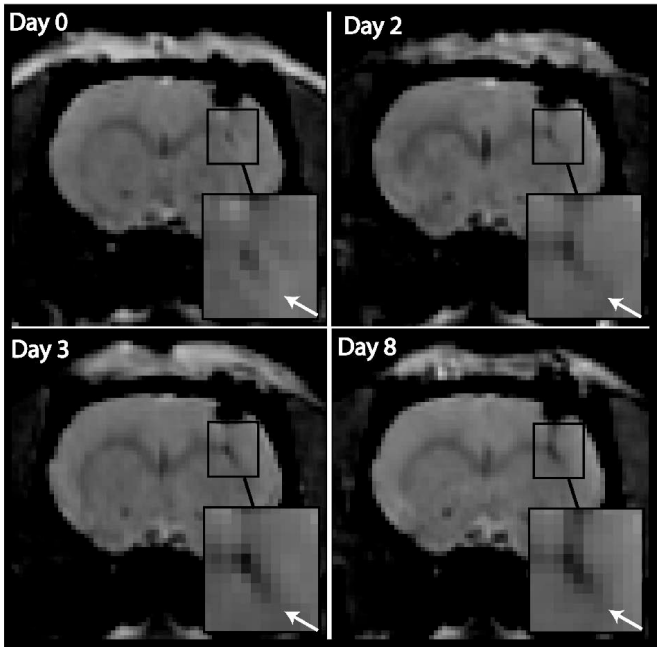

Fig. 5. T2*-weighted MR images of the rat brain after an injection of labeled neural stem cells (inset images are magnified at 2.3X). Initial injection site (white arrow in A) is shown in the top left corner (day 0) as a reduction in signal intensity. Subsequent movement of cells from the injection site is seen 2, 3 and 8 days after laterally along the corpus callosum and dorsally in the needle track.

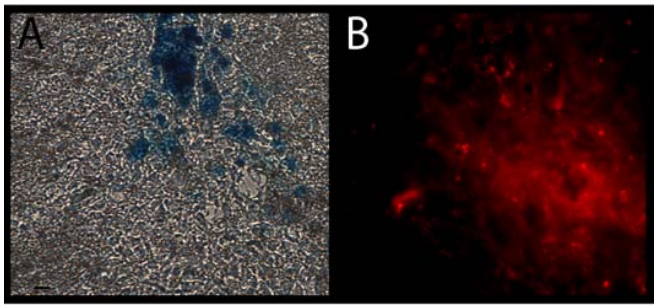

Fig. 6. Histological confirmation of labeled cells using Prussian blue staining for iron (A) and red fluorescence (B). Fluorescence imaging shows greater contrast from the background than Prussian blue staining. Scale bar is 10 µm.

IV. Conclusions

This study demonstrates that neural stem cells can be co-labeled by a magnetic contrast agent for *in vivo* imaging and a fluorescent dye for histological confirmation. These cells retain the ability to form neurospheres, but SPIO transfection with a viral vector prevents neurospheres from merging in a non-adherent plate.

The ability to track neural stem cells *in vivo* will enable the study of these cells in different disease pathologies and when confronted with artificial, biochemical stimulus. A de facto *in vivo* assay of cell migration is needed that is quantitative, user independent and dynamic. The imaging of tagged stem cells can address this need to create a standard evaluation tool for drug development and the translation of cell therapies to a clinical reality.

References

[1] K. I. Park and E. Y. Synder, "Neural stem cell biology provides insights into new therapeutic strategies for hypoxic-ischemic brain injury," in *New Concepts in Cerebral Ischemia*, R. C. S. Lin, Ed. Boca Raton: CRC Press LLC, 2002, pp. 61-77.

[2] M. Hoehn, E. Kustermann, J. Blunk, D. Wiedermann, T. Trapp, S. Wecker, M. Focking, H. Arnold, J. Hescheler, B. K. Fleischmann, W. Schwindt, and C. Buhrle, "Monitoring of implanted stem cell migration in vivo: a highly resolved in vivo magnetic resonance imaging investigation of experimental stroke in rat," *Proc Natl Acad Sci U S A*, vol. 99, pp. 16267-72, 2002.

[3] R. D. Vale, M. Hosang, and E. M. Shooter, "Sialic acid residues on NGF receptors on PC12 cells," *Dev Neurosci*, vol. 7, pp. 55-64, 1985.

[4] S. Miyoshi, J. A. Flexman, D. J. Cross, K. R. Maravilla, Y. Kim, Y. Anzai, J. Oshima, and S. Minoshima, "Transfection of neuroprogenitor cells with iron nanoparticles for magnetic resonance imaging tracking: cell viability, differentiation, and intracellular localization," *Mol Imaging Biol*, vol. 7, pp. 286-95, 2005.

[5] A. Arvidsson, T. Collin, D. Kirik, Z. Kokaia, and O. Lindvall, "Neuronal replacement from endogenous precursors in the adult brain after stroke," *Nat Med*, vol. 8, pp. 963-70, 2002.

[6] B. A. Reynolds and R. L. Rietze, "Neural stem cells and neurospheres--re-evaluating the relationship," *Nat Methods*, vol. 2, pp. 333-6, 2005.

[7] P. K. Horan and S. E. Slezak, "Stable cell membrane labelling," *Nature*, vol. 340, pp. 167-8, 1989.

[8] K. Chu, K. I. Park, S. T. Lee, K. H. Jung, S. Y. Ko, L. Kang, D. I. Sinn, Y. S. Lee, S. U. Kim, M. Kim, and J. K. Roh, "Combined treatment of vascular endothelial growth factor and human neural stem cells in experimental focal cerebral ischemia," *Neurosci Res*, vol. 53, pp. 384-90, 2005.

[9] C. R. Parish, "Fluorescent dyes for lymphocyte migration and proliferation studies," *Immunol Cell Biol*, vol. 77, pp. 499-508, 1999.

[10] Y. H. An, H. Y. Wang, Z. X. Gao, and Z. C. Wang, "Differentiation of rat neural stem cells and its relationship with environment," *Biomed Environ Sci*, vol. 17, pp. 1-7, 2004.

[11] C. Lundberg, A. Martinez-Serrano, E. Cattaneo, R. D. McKay, and A. Bjorklund, "Survival, integration, and differentiation of neural stem cell lines after transplantation to the adult rat striatum," *Exp Neurol*, vol. 145, pp. 342-60, 1997.

[12] J. Karbanova, J. Mokry, and L. Kotingova, "Neural stem cells transplanted into intact brains as neurospheres form solid grafts composed of neurons, astrocytes and oligodendrocyte precursors," *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub*, vol. 148, pp. 217-20, 2004.

[13] G. E. Goings, V. Sahni, and F. G. Szele, "Migration patterns of subventricular zone cells in adult mice change after cerebral cortex injury," *Brain Res*, vol. 996, pp. 213-26, 2004.