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SHORT REPORT

Human neural progenitor cells retain viability, phenotype, proliferation, and lineage differentiation when labeled with a novel iron oxide nanoparticle, Molday ION Rhodamine B

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Abstract: Ultrasmall superparamagnetic iron-oxide particles (USPIOs) loaded into stem cells have been suggested as a way to track stem cell transplantation with magnetic resonance imaging, but the labeling, and post-labeling proliferation, viability, differentiation, and retention of USPIOs within the stem cells have yet to be determined for each type of stem cell and for each type of USPIO. Molday ION Rhodamine B™ (BioPAL, Worcester, MA, USA) (MIRB) has been shown to be a USPIO labeling agent for mesenchymal stem cells, glial progenitor cells, and stem cell lines. In this study, we have evaluated MIRB labeling in human neuroprogenitor cells and found that human neuroprogenitor cells are effectively labeled with MIRB without use of transfection reagents. Viability, proliferation, and differentiation properties are unchanged between MIRB-labeled neuroprogenitors cells and unlabeled cells. Moreover, MIRB-labeled human neuroprogenitor cells can be frozen, thawed, and replated without loss of MIRB or even without loss of their intrinsic biology. Overall, those results show that MIRB has advantageous properties that can be used for cell-based therapy.

Keywords: ferumoxides, USPIO, MION, neural stem cells, SC121 antibody, human, toxicology

Introduction

Transplantation of stem cells has great potential as restorative therapeutics for both neurodegenerative diseases and central nervous system damage caused by stroke and trauma. Clinical trials have already begun with patients with amyotrophic lateral sclerosis, multiple sclerosis, and stroke, with an initial focus on safety.[1–5] Several different types of stem cells have been proposed as therapies, with the basis of their potential benefit ranging from providing nutritive support for injured host tissue, as a reservoir for growth factors, and as replacement neurons for host cells lost to injury or disease. An underlying prerequisite for all of these goals is survival and appropriate localization of the transplanted stem cells. Unfortunately, no human studies have been able to address these issues with quantitative and validated methods, making it very difficult to address issues of cell "dose" and means of administration as these trials move toward developing stem cells as effective therapies.

One form of stem cell that has been extensively studied in animal models and has been utilized in recent clinical trials as a therapeutic is the human neural progenitor cell.[6–8] These cells have been expanded from fetal human brain tissue and are widely available. Human neural progenitor cells have several favorable properties including their spontaneous differentiation into neurons and glial cells as well as their lack

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of proliferation in vivo, lessening the risk of teratomas formation following transplantation. Recombinant fluorescent proteins (eg, green fluorescent protein) have been used to unequivocally distinguish transplanted human neural progenitor cells from host brain cells.[9] Unfortunately, this approach is less suitable for human studies because of the added risk posed by genetic alteration of the cellular therapy. Antibodies against human cellular antigens, while useful for histological identification of transplanted neuroprogenitor cells in animal studies, are clearly not useful for human brain transplantation.[10] Thus, for human use, the label for stem cells in in-vivo studies utilizes dyes or markers internalized by the cells in vitro. These include fluorescent dyes useful for histological analysis of experimental animals, and iron conjugated dyes detectable not only by histochemical methods but with magnetic resonance imaging (MRI) in both animals and humans.[11]

With superior resolution and localization, MRI is the preferred imaging modality for tracking transplanted stem cells in both living experimental animals and clinical studies with minimal risk.[12] At the present time, the best MRI contrast agents suitable for use in cellular imaging is a colloidal iron oxide generically referred to as an ultrasmall superparamagnetic iron-oxide nanoparticle (USPIO). These nanoparticles display dramatic signal loss in T2*/susceptibility weighted MRI images and have been used to image transplanted stem cells in the lungs[13] and brain[14] of small animals. USPIOs contain an iron-oxide core surrounded by a biocompatible, solubilizing coating of dextran, albumin, or chitosan.[15,16] The major limitation of this method is the possibility that the marker will be extravasated by the stem cell after transplantation (particularly in the setting of stem cell death) and re-internalized by neighboring host cells such as microglia, macrophages, or astrocytes resulting in misinterpretation as surviving transplanted stem cells.[17]

To validate a stem cell marker for both experimental animals and eventual clinical use, several properties must be demonstrated. The marker must: 1) not alter the intrinsic biology of the stem cell with regard to cell replication, migration, and differentiation; 2) have uniform labeling and long term retention within the labeled cell; and 3) dissipate into the tissue without the spurious labeling of neighboring cells if the marker is extravasated. Retention of the label after cell storage by freezing is also a useful property to allow rapid access to labeled stem cells.

MIRB (Molday ION Rhodamine B™; BioPAL, Worcester, MA, USA) is a commercial product with many favorable properties, and has been utilized in labeling primary stem cells, mesenchymal stem cells, and various stem cell lines. MIRB is specifically formulated for cell labeling, and is readily internalized by non-phagocytic cells and phagocytic cells without the use of adjuvant transfection agents. MIRB conjugated with rhodamine allows for straightforward detection in histological specimens, while its iron moiety makes it detectable in tissue with histochemical methods and by MRI in vivo. In cynomolgus monkey, untreated and MIRB-treated mesenchymal stem cells show no difference with respect to viability, proliferation, mitogen suppression, gene expression, phenotype, and differentiation.[18,19] Here, we report on a labeling procedure of human neuroprogenitor cells with MIRB and the demonstration of retention, and no perturbation of cell proliferation, migration, survival, or differentiation.

Methods

Cell culture

Human neural precursor cells were purchased from Lonza Inc (Walkersville, MD, USA). They are derived from human fetal cortex (15 weeks gestation) and were maintained as neurospheres in growth medium (Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 [DMEM/F-12] containing 2% B27, 20 ng/mL basic fibroblast growth factor [bFGF], and 20 ng/mL epidermal growth factor [EGF]; Invitrogen Life Technologies, Carlsbad, CA, USA). To differentiate the human neuroprogenitor cells, cultures were placed in differentiation medium (DMEM/F-12 containing 2% B27, without bFGF and EGF). Medium was changed every 3 days. For analysis of MIRB-labeling of the human neuroprogenitor cells, neurospheres were plated on coated cell cultureware or dissociated with Accutase® (Sigma-Aldrich, St Louis, MO, USA) and replated as single cells.

MIRB nanoparticles

MIRB is a commercial product sold by BioPhysics Assay Laboratory (BioPAL, Inc) CL-50Q02-6A-50. MIRB is a homogeneous, fluorescent iron-oxide-based superparamagnetic (USPIO) contrast reagent designed to label cells efficiently and simply. It is synthesized in a three-step procedure and is an unmodified USPIO, similar in size, zeta potential, and magnetic properties to Molday ION (MION). MIRB can be visualized by both MRI and fluorescence. MIRB is stable for 4 days at 37°C in phosphate-buffered saline or saline. MIRB preparations show no change in size over a period of 2 years when stored at 4°C. It has an effective diameter of 31.4 nm and a zeta potential of +31 mV. The R1 and R2 values are 30.42 s^{-1} mM^{-1} and 75.85 s^{-1} mM^{-1}, respectively.

It is prepared by reaction of its parent compound Molday ION C6 Amine with rhodamine B isothiocyanate. It is a crosslinked dextran-coated iron-oxide nanoparticle.[20] Transmission electron microscopy of cells loaded with MIRB is shown in Figure S1.

MIRB labeling of human neuroprogenitor cells

MIRB optimal labeling of human neuroprogenitors occurs in a concentration-dependent manner during undifferentiated conditions. The optimal concentration of MIRB for labeling is 20 μg Fe/mL in culture medium without any added transfection agent necessary (Figure 1A and J; 48 hours). Detectable labeling occurs as rapidly as 6 hours in MIRB, and maximal labeling occurs at 48–72 hours. To detect MIRB labeling, direct rhodamine fluorescence visualization or Perl's iron staining (2% HCl, 2% potassium ferrocyanide) was performed.

Immunocytochemistry

For immunocytochemistry analysis, human neuroprogenitor cells were fixed with 4% paraformaldehyde (pH 7.4) and immunolabeled as previously described.[21] Antibodies include: SC121 (human specific cytoplasmic antibody, 1:500; Stem Cells Inc, Cambridge, UK), BrdU (5-bromo-2-deoxyuridine, 1:400; Accurate Chemical and Scientific Corporation, Westbury, NY, USA), SOX2 (sex determining region Y)-box 2, 1:500; EMD Millipore, Billerica, MA, USA), Tuj1 (neuron-specific class III beta-tubulin, 1:500; Covance), GFAP (glial fibrillary acidic protein, 1:1000; DakoCytomation, Glostrup, Denmark), and LAMP1 (lysosomal-associated membrane protein 1, 1:1000; BD Biosciences, San Jose, CA, USA).

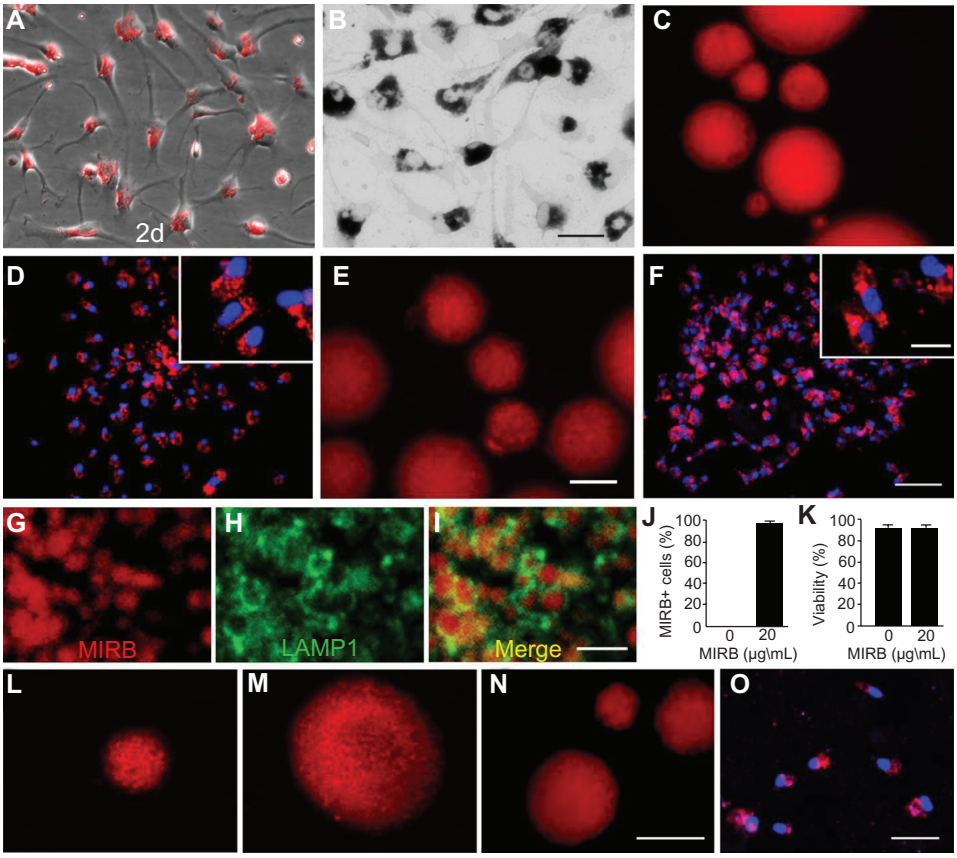

Figure 1 Labeling of human neuroprogenitor cells with MIRB.

Notes: Human neuroprogenitor cells were effectively labeled with MIRB in vitro. Dissociated human neuroprogenitor cells were incubated with MIRB (20 μg Fe/mL) for 2 days (**A**, red). Almost every cell contains MIRB. MIRB uptake was confirmed by Perl's staining (**B**). In addition to labeling in monolayer cultures, neurospheres were effectively loaded with MIRB (**C**, 2 days). Labeled human neuroprogenitor neurospheres were grown on poly-lysine-coated chamber slides; their outgrowth cells contained MIRB (**D**). Cell counting showed that 98.2% of the outgrowth cells were MIRB labeled (**J**). MIRB-labeled human neuroprogenitor neurospheres were frozen and stored in liquid nitrogen for 3 days. After thawing and replating, human neuroprogenitor neurospheres retained MIRB (**E** and **F**). LAMP1+ lysosomes contained MIRB (**G**–**I**, 3 days incubation). Viability of human neuroprogenitor cells was measured by Trypan blue exclusion. MIRB did not affect cell viability (**K**, 2 days). (Panels **L**, **M**, and **N**) show MIRB labeling of different sizes of neurospheres. A 40 μm cryostat section was taken from the middle of different sized neurospheres (diameter 150 μm, **L**; and 400 μm, **M**) following fixation. No loss of MIRB labeling across different sized spheres was observed. After 3 weeks in vitro, MIRB-labeled neurospheres (**N**) were dissociated and replated. Panel **O** shows that the dissociated neuroprogenitor cells replated still held MIRB. Scale bars: **A**, **B**, and **O**=50 μm; **C**, **E**, and **L**–**N**=200 μm; **D** and **F**=25 μm; **G**–**I**=2 μm.

Abbreviations: LAMP1, lysosomal-associated membrane protein 1; MIRB, Molday ION Rhodamine B™ (BioPAL, Worcester, MA, USA).

Cell counting and statistical analysis

Data are expressed as mean ± standard deviation. To assess the effects of MIRB labeling on proliferation, viability, and differentiation, human neuroprogenitor cells were incubated with MIRB for 2 days; a total of 1000–2000 outgrowth cells from five random fields from nine separated neurospheres were counted. Viability: for Trypan blue exclusion analysis, human neuroprogenitor cells were incubated with 0.2% Trypan blue for 1 minute, rinsed with PBS (phosphate buffered saline, pH 7.4), then fixed with paraformaldehyde (pH 7.4) and counted. In quantitative analysis, data are presented as the mean ± standard deviation. Student's *t*-test (two-tailed, unpaired) was used to compare MIRB-labeled human neuroprogenitor cells to control cells. *P*-values were calculated using Prism 4.0 software (GraphPad Software, Inc, La Jolla, CA, USA); *P*-values less than 0.05 were considered significant.

Results and discussion

Traditionally, labeling with USPIOs requires the addition of transfection reagents to the culture in order to improve uptake; however, this may introduce perturbations to the stem cells. Here we tested the ability of MIRB to label human neuroprogenitor cells when grown either as dissociated cells or as neurospheres. We have found that incubation with MIRB in the absence of transfection reagents effectively labeled both neurospheres and dissociated human neuroprogenitor cells with sufficient intensity to be detected by the fluorescence of the MIRB rhodamine conjugation (Figure 1A and C). The iron-oxide particle could also be detected using a Perl's iron stain (Figure 1B). MIRB was stored in lysosomes, which were identified by co-localization of MIRB and LAMP1 (Figure 1G–I). Those results indicated that MIRB does not require permeabilization of the cells for an effective uptake, and human neuroprogenitor cells display uniform MIRB labeling.

Human neuroprogenitor cells, while surviving and proliferating as dissociated cells, retain stem cell properties and also grow when maintained as neurospheres. Reagents added to the culture media of human neuroprogenitor neurospheres have to penetrate from the periphery of the sphere into the core in order to label all of the cells. Low efficiency labeling of cells may indicate penetration of the label only to the outermost cells. When human neuroprogenitor neurospheres were labeled with 20 μg Fe/mL of MIRB, robust fluorescence of the neurospheres was observed (Figure 1C). When these spheres were allowed to settle and adhere to the culture dish, migratory cells exiting the human neuroprogenitor neurospheres were all labeled MIRB (Figure 1D). To quantify the efficiency of MIRB labeling in neurospheres, the human neuroprogenitor neurospheres were dissociated, and the percentage of MIRB-positive human neuroprogenitor cells counted. Following a 48 hour labeling of human neuroprogenitor neurospheres, 98.2%±1.8% of human neuroprogenitor cells were positive for MIRB (Figure 1J). Viability of human neuroprogenitor cells was measured by Trypan blue exclusion following 2 days of MIRB labeling. No difference in viability between MIRB-labeled and -unlabeled human neuroprogenitor cells was seen (Figure 1K). These data suggest that MIRB effectively penetrates throughout human neuroprogenitor neurospheres (Figure 1L and M), allowing for labeling in a neurosphere culture condition that best preserves neural precursor cell properties. We tested MIRB labeling over a 3-week cell culture period (longest time tested) and found that long-term viability of human neuroprogenitor neurospheres was also not affected by MIRB labeling (Figure 1N). After 3 weeks in vitro, when the largest neurospheres were dissociated and replated, neuroprogenitor cells still held MIRB (Figure 1O).

Commercialization of human neuroprogenitor cells for clinical intervention would best be served by packaging the cells as pre-labeled frozen aliquots that could be thawed for immediate use. Interaction between MIRB inside the cell and ice crystal formation during freezing could reduce the viability of pre-frozen human neuroprogenitor cells. To assess this, human neuroprogenitor neurospheres were labeled with MIRB, frozen, then thawed and plated (Figure 1C–F). The number of surviving cells, measured by exclusion of Trypan blue, was identical whether the human neuroprogenitor neurospheres were labeled or unlabeled (Figure 1K). The ability to pre-label human neuroprogenitor cells with unaltered viability is a critical property of MIRB.

Human neuroprogenitor cell self-renewal properties, "stem-cell-ness," relies upon expression of the transcription factor SOX2 (sex determining region Y-box 2). Exogenous labels for these cells, particularly genetic modification, might perturb transcription factor expression and alter their self-renewal properties. To test whether MIRB labeling affected phenotype and proliferation, human neuroprogenitor neurospheres were labeled with MIRB for 48 hours and allowed to settle. The proportion of all cells that expressed SOX2 was identified in control (SOX2+ cells: 95.0%±1.8%) versus MIRB-labeled human neuroprogenitor cells (SOX2+ cells: 95.7%±4.1%; Figure 2A, B, and L). SOX2+ human neuroprogenitor cells continued to express the human-specific antigen SC121 (99.1%±0.6% SOX2+ in control human

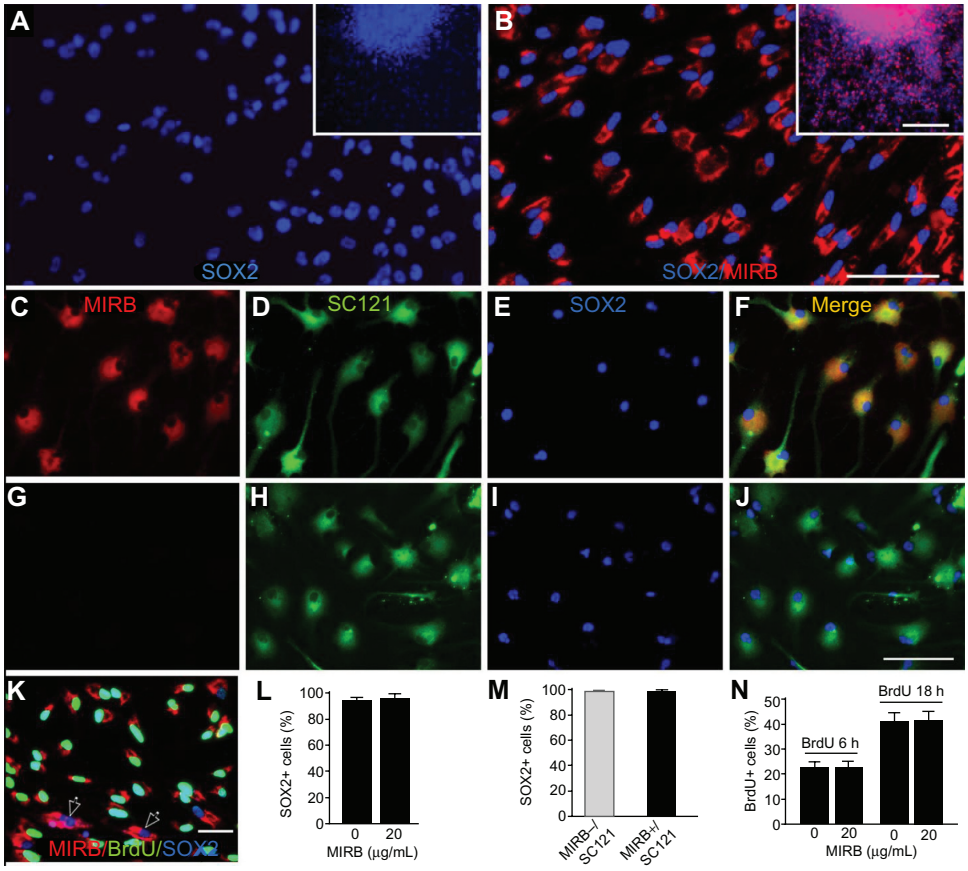

Figure 2 MIRB labeling of human neuroprogenitor neurospheres.

Notes: MIRB labeling of human neuroprogenitor neurospheres did not affect migration, phenotype, or cell proliferation. It also did not change the migratory ability of SOX2+ outgrowth cells. Human neuroprogenitor neurospheres were incubated with or without MIRB (20 μg Fe/mL) for 2 days, fixed, and labeled with SOX2 antibody. There was no difference in SOX2+ labeling in the outgrowth cells of the control (**A**) or MIRB-labeled cells (**B**); MIRB labeling did not change the phenotype of neuroprogenitor neurosphere outgrowth cells. Triple labeling of MIRB labeled neurospheres and outgrowth cells with anti-SC121 and anti-SOX2 indicated that MIRB does not affect the phenotype of outgrowth human neuroprogenitor cells which are both SOX2+ and SC121+, with or without MIRB labeling (**C–F**, **G–J**, **L**, and **M**). Outgrowth cells (SOX2+) are proliferative; incubation with MIRB and BrdU (4 μM) together for 2 days showed that almost all of the outgrowth cells (87.5%±3.7%) were BrdU+ (**K**, black arrows point to a few BrdU–/SOX2+ cells). To test whether prior MIRB labeling affected cell proliferation, human neuroprogenitor cells were incubated with MIRB for 2 days, then pulse labeled with BrdU for 6 or 18 hours. No difference in proliferation between MIRB-labeled and unlabeled human neuroprogenitor cells at either time point was found (**N**). Scale bars: **A–J**=100 μm; inset **A** and **B**=200 μm; **K**=50 μm.

Abbreviations: BrdU, 5-bromo-2-deoxyuridine; MIRB, Molday ION Rhodamine B™ (BioPAL, Worcester, MA, USA); SC121, human specific cytoplasmic antibody; SOX2, (sex determining region Y)-box 2.

neuroprogenitor cells and 99.2%±0.1% SOX2+ human neuroprogenitor cells following MIRB labeling; Figure 2C–F and M). The shape/size of cells that migrated out from the neurosphere, determined by measuring randomly selected outgrowth cells (n=700) that were SOX2+/SC121+, were identical with or without MIRB (Figure 2C–F, MIRB; and Figure 2G–J, control).

Expression of SOX2 in MIRB-labeled outgrowth cells suggested that these cells were still capable of self-renewal, but did not directly demonstrate that the division rate of MIRB-labeled cells matched that of control. To test proliferative rate in MIRB-labeled human neuroprogenitor cells, cultures were incubated with BrdU, which incorporates into replicating cell DNA during S-phase of the mitotic cycle, for either 6 or 18 hours, and the percentage of labeled cells was assessed. The proportion of BrdU-labeled human neuroprogenitor cells with short or long BrdU incubation was identical between control and MIRB-labeled cells (Figure 2N). These BrdU+/MIRB+ cells continued to express SOX2 (Figure 2K). Taken together, these data indicate that MIRB labeling does not alter expression of critical transcription factors or the proliferative rate of human neuroprogenitor cells. It should be noted that similar to studies with other stem cells, after successive generations of proliferation of SOX2+/MIRB-labeled cells, there was a decrease in the amount of MIRB labeling per cell.[19]

The differentiation of stem cells into selected lineages is highly dependent on both the cell state and the differentiating media to which cells are exposed. Human neuroprogenitor cells, as a neural precursor lineage, should differentiate into

a mix of glia and neurons. To test whether MIRB altered the normal differentiation rate or ratio of lineage types, human neuroprogenitor cells were labeled with MIRB for 2 days and then either fixed (this time point referred to as d0, undifferentiated) or cultured in differentiation medium for an additional 8 days (this time point referred to as d8, differentiated). Control cultures were treated similarly except not labeled with MIRB. Glial lineage was identified by GFAP expression (Figure 3A–D), and the neural lineage was identified by Tuj1 expression (neuron-specific class III beta-tubulin, Figure 3F–I).

Undifferentiated outgrowth cells (d0) showed either no GFAP or weak GFAP labeling with short processes There was no difference in the percentage of GFAP+ cells labeled with or without MIRB at d0 (Figure 3A, 24.6%±9.4%; Figure 3B, 22.9%±8.3%). After differentiation (d8), glial cells displayed intensive GFAP labeling with radial-glia like long processes (Figure 3C and D). The percentage of glial cells was the same in both cultures after d8 (control 35.7%±7.5%, MIRB 38.1%±12.6%). MIRB does not affect Tuj1+ cell populations in the undifferentiated or differentiated state. In the undifferentiated state, Tuj1+ cells were seen with a similar percentage in both cultures (Figure 3E, 17.2%±3.1%; Figure 3F, 19.0%±6.0%). After differentiation, MIRB labeling does not alter the percentage of neurons (control 32.3%±10.8%, MIRB+ 31.3%±3.0%). Thus, MIRB labeling does not change GFAP+ and Tuj1+ cell populations in either undifferentiated or differentiation conditions. In addition, human neuroprogenitor cells were labeled with both MIRB and BrdU. After 8 days differentiation, GFAP+ glia cells or Tuj1+ neurons, which are both MIRB+ and BrdU+, were observed by immunocytochemistry staining, indicating that human neuroprogenitor cells do not lose MIRB when differentiation into mature cells (data not shown). These results show that MIRB labeling does not affect the intrinsic biology of the human neuroprogenitor cells. It allows them to proliferate and to differentiate like in the control conditions. Once the human neuroprogenitor cells differentiated into either neurons or glia, MIRB labeling was still present, indicative that human neuroprogenitor cells do not lose MIRB during differentiation.

We have shown that labeling human neuroprogenitor cells with MIRB is unlike labeling human neuroprogenitor cells with other USPIOs such as Feridex.[22] MIRB labeling can occur without use of transfection reagents that might induce cell damage.[11,23,24] Once labeled, MIRB resides in LAMP1+ lysosomes. MIRB labeling was dose dependent; we used 20 μg Fe/mL for all of the studies shown here. The labeling is stable, and we followed human neuroprogenitor cells labeled with MIRB for over 3 weeks in vitro; there was no effect on cell viability. MIRB labeled cells can be frozen and thawed without loss of label and without affecting viability which is an important property for commercialization of labeled human neuroprogenitor cells for clinical intervention.

Comparison of the properties of MIRB labeled human neuroprogenitor cells as regards to proliferation, differentiation, and maintenance of phenotype with control human neuroprogenitor cells showed no differences. There was no loss of the transcription factor expression (SOX2) or human antigen expression (SC121), and the proliferation rate of SOX2+ progenitors labeled with MIRB was the same as unlabeled progenitors. MIRB labeling did not affect the differentiation process of human neuroprogenitor cells. The

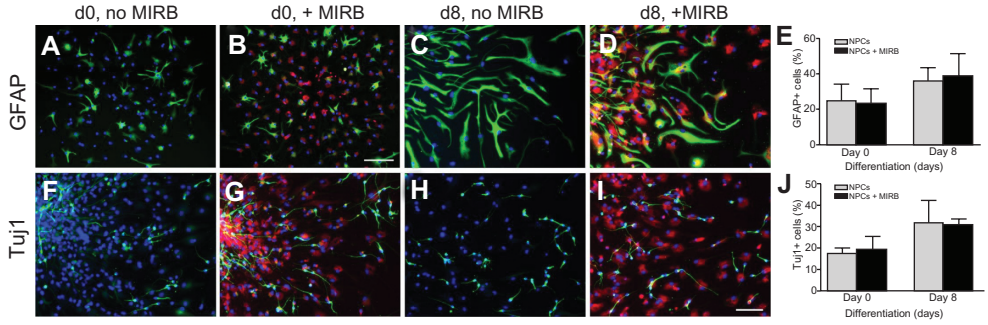

Figure 3 MIRB labeling does not affect differentiation in vitro.

Notes: MIRB labeling did not affect human neuroprogenitor neurosphere outgrowth cells' differentiation in vitro. Human neuroprogenitor neurosphere outgrowth cells grown on chamber slides with or without MIRB labeling were either fixed (undifferentiated, designated 0 day [d0]) or cultured in differentiation medium for an additional 8 days (d8). Cell differentiation was analyzed with GFAP or Tuj1 labeling. In undifferentiated neurosphere cultures, outgrowth cells with or without MIRB labeling showed weak GFAP+ labeling and had short processes (**A** and **B**). After differentiation, outgrowth cells displayed intensive GFAP+ labeling with radial glia-like processes and remained MIRB labeled (**C** and **D**). In undifferentiated cultures, there were Tuj1+ cells at the periphery of the neurosphere with or without MIRB labeling (**F** and **G**). After differentiation, there were Tuj1+ cells in outgrowth cells throughout the culture (**H** and **I**). Hoechst counterstained. Quantitative analysis indicated no differences between the percentage of total cells that were either GFAP+ or Tuj1+ in differentiated cultures with or without MIRB labeling (**E** and **J**). Scale bars: **A** and **B**=200 μm; **C**, **D**, and **F–I**=100 μm.

Abbreviations: GFAP, glial fibrillary acidic protein; MIRB, Molday ION Rhodamine B™ (BioPAL, Worcester, MA, USA); NPC, neuroprogenitor cell; Tuj1, neuron-specific class III beta-tubulin.

percentages of astrocytes and neurons that occurred following 8 days of differentiation were similar between control and MIRB labeled cells. In addition, the percentages of SOX2+/BrdU+ progenitors that differentiate into GFAP+ astrocytes or Tuj1+ neurons were similar between MIRB labeled and control human neuroprogenitor cells (Shen et al, unpublished observation, 2013). Similar findings were also made with another neuronal transcription factor such as doublecortin, a marker of immature neurons (Shen et al, unpublished observation, 2013). In addition, it was found that once MIRB-labeled progenitor cells differentiated into astrocytes or neurons, they did not lose their MIRB label.

In summary, MIRB displayed the necessary potentials as a stem cell labeling agent since: 1) it did not alter the intrinsic biology of the stem cell with regard to cell replication and differentiation; 2) it had uniform labeling and long-term retention within the labeled cell; and 3) MIRB was retained by the stem cells after cell storage by freezing. MIRB labeling has advantages in preparing human neuroprogenitor cells for in-vivo transplantation where a minimum of additional steps should be used in order to make cells more compatible for the environment they are placed in. These properties of the USPIO-MIRB encourage further examination of MIRB-labeled human neuroprogenitor cells for in vivo transplant studies.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

Transmission electron microscopy (TEM) images

TEM preparations of 3T3 cells loaded with MIRB (Molday ION Rhodamine B™ [BioPAL, Worcester, MA, USA]) were prepared by fixation with glutaraldehyde and formalin. Dehydration and infiltration with Epon was performed following standard protocols. The small dark spheres within the vesicles are the iron oxide core of MIRB nanoparticles.

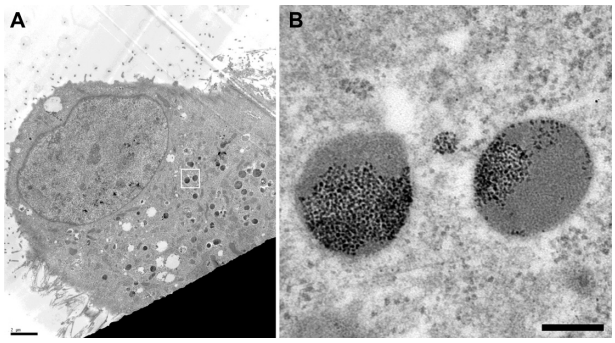

Figure S1 (**A**) and (**B**) are transmission electron microscopy images of MIRB located within lysosomal vesicles. The size distribution of the iron oxide crystals was between 6 and 9 nm. Image **B** represents two vesicles loaded with MIRB selected from **A**.

Notes: The magnification of image **B** is 36000×. The bar in image **A** is 2 μm; in **B** it represents 200 nm.

Abbreviation: MIRB, Molday ION Rhodamine B™ (BioPAL, Worcester, MA, USA).