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In Vitro Labeling of Human Umbilical Cord Mesenchymal Stem Cells With Superparamagnetic Iron Oxide Nanoparticles

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ABSTRACT

Human umbilical cord mesenchymal stem cells (hUC-MSCs) transplantation has been shown to promote regeneration and neuroprotection in central nervous system (CNS) injuries and neurodegenerative diseases. To develop this approach into a clinical setting it is important to be able to follow the fates of transplanted cells by noninvasive imaging. Neural precursor cells and hematopoietic stem cells can be efficiently labeled by superparamagnetic iron oxide (SPIO) nanoparticle. The purpose of our study was to prospectively evaluate the influence of SPIO on hUC-MSCs and the feasibility of tracking for hUC-MSCs by noninvasive imaging. In vitro studies demonstrated that magnetic resonance imaging (MRI) can efficiently detect low numbers of SPIO-labeled hUC-MSCs and that the intensity of the signal was proportional to the number of labeled cells. After transplantation into focal areas in adult rat spinal cord transplanted SPIO-labeled hUC-MSCs produced a hypointense signal using T2-weighted MRI in rats that persisted for up to 2 weeks. This study demonstrated the feasibility of noninvasive imaging of transplanted hUC-MSCs. J. Cell. Biochem. 108: 529–535, 2009.

KEY WORDS: HUMAN; MAGNETIC RESONANCE IMAGING; MESENCHYMAL STEM CELLS; SUPERPARAMAGNETIC IRON OXIDE; TRANSPLANTATION; UMBILICAL CORD

Bone marrow derived mesenchymal stem cells (BM-MSCs) have been functionally defined as non-hematopoietic, multipotential cells that can differentiate into cells of various tissues. However, the use of BM-MSCs has its own limitations due to the disadvantage that they drop significantly in cell number and proliferative/differentiation capacity with age [Romanov et al., 2003]. Mitchell et al. [2003] firstly identified an conveniently attainable source of mesenchymal stem cells from human umbilical cord-Wharton's jelly. Human umbilical cord mesenchymal stem cells (hUC-MSCs) have faster proliferation, greater ex vivo expansion capabilities and may be better tolerated following transplantation because of less incidence of graft versus host disease than BM-MSCs [Troyer and Weiss, 2008]. Moreover, hUC-MSCs have been induced to form bone, cartilage, adipose [Sarugaser et al., 2005; Karahuseyinoglu et al., 2007], heart [Wang et al., 2004], skeletal muscle [Conconi et al., 2006], endothelium [Wu et al., 2007], and especially neural cells [Mitchell et al., 2003; Fu et al., 2004, 2006; Ma et al., 2005]. In addition, recent studies have shown that transplantation of hUC-MSCs can promote regeneration, neuroprotection, and locomotion recovery in experimental models of traumatic spinal cord injury [Wang et al., 2006; Yang et al., 2008], cerebral global ischemia [Jomura et al., 2007], and Parkinson's disease [Fu et al., 2006; Weiss et al., 2006].

For these reasons transplantation of the cells has a potential therapeutic role for treating patients with central nervous system (CNS) injuries and neurodegenerative diseases. However, it is important to follow the in vivo fates of transplanted cells by noninvasive imaging in order to advance this approach into a clinical setting. Standard histopathological methods are usually used to evaluate engraftment, migration, and differentiation of

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exogenous stem cells in physiological conditions [Consiglio et al., 2004] or after mobilization to site of damage [Cooper and Isacson, 2004; Mitchell et al., 2004]. Design and improvement of sensitive, noninvasive techniques for tracking donor cells transplanted into host tissues will enable more accurate interpretation of the functional consequences of transplantation.

Recently, stem and progenitor cells of neural and non-neural origin have been labeled using superparamagnetic iron oxide (SPIO) particles to track them by magnetic resonance imaging (MRI) in cell therapy approaches for various diseases [Ben-Hur et al., 2007; Politi et al., 2007; Heymer et al., 2008]. However, the validation of SPIO-based protocols to label hUC-MSCs has not been extensively addressed. Thus, the purpose of this study is to prospectively evaluate the influence of SPIO on hUC-MSCs and the feasibility of tracking for hUC-MSCs by noninvasive imaging. This study describes a simple protocol to label UC-MSCs of human origin using SPIO at optimized low dosages and demonstrates the feasibility of noninvasive imaging of labeled cells after transplantation into the spinal cord.

MATERIALS AND METHODS

CELL CULTURE

All parts of this study, especially the isolation of the human umbilical cord (UC), were performed according to the Declaration of Helsinki. Ethical approval was obtained from the Third Military Medical University (ChongQing, China), and written informed consent was obtained from donors of UC. The isolation and culture of hUC-MSCs were carried out according to the modified method previously described [Mitchell et al., 2003]. Briefly, each UC was collected from full-term Caesarian section births and processed within 3–6 h. Umbilical arteries and vein were removed, and the remaining tissue was transferred to a sterile container in Dulbecco's modified essential media: Nutrient Mixture F-12 (DMEM/F12; Hyclone, America) and was diced into small fragments. The explants were transferred to 50 ml culture flasks containing the DMEM/F12 along with 10% fetal bovine serum (FBS; PAA, Austria). They were left undisturbed for 4–6 days to allow migration of cells from the explants, at which point the media was replaced. Cultures were maintained at 37°C in a incubator containing 5% CO2. They were re-fed and passaged as necessary.

FLOW CYTOMETRY

The mesenchymal cells were trypsinized and suspended in DMEM/F12 at a concentration of 3×10^6/ml. A 100 μl sample was incubated for 35 min at 4°C with 15 μl of various mouse antihuman antibodies against following surface markers: CD14, CD34, CD45, CD90, CD73, CD105, HLA-DR (all obtained from BD Pharmingen, San Diego, CA). Finally, they were washed, centrifuged, and fixed in 1.5 ml of 4% paraformaldehyde. A FACScan machine (Becton Dickinson, USA) was used to analyze antibody binding. BDDiva software was used for analysis of data.

LABELING OF hUC-MSCs WITH SPIO

The label (Feridex, Advanced Magnetic corp.; particle size, 80 nm; stock solution, 11.2 mg Fe/ml) was added to the cultures at concentrations equivalent to 5.6, 11.2, 22.4, and 44.8 μg Fe/ml (diluted with DMEM/F12) and incubated for 12 or 24 h. Control cultures were incubated without SPIO. At the end of the incubation period, cells were collected for determination of the labeling efficiency (by Prussian Blue staining; number of Prussian Blue-labeled cells/total number of cells in the sample) and other studies.

PRUSSIAN BLUE (PB) STAINING

After washing in PBS to remove any free SPIO, hUC-MSCs were fixed for 40 min using 4% paraformaldehyde. Cultures were then washed three times with PBS and incubated with Perls' reagent (4% potassium ferrocyanide/12% HCl, 50:50, v/v) for 30 min. The cells were washed in PBS and observed using phase microscopy.

ASSESSMENT OF CELL VIABILITY AND GROWTH RATE

For cell viability assay, cells were plated on 96-well plate (5×10^3 cells/well; six replicates for each condition). Twenty-four hours after plating they underwent treatments with SPIO (control, 5.6, 11.2, 22.4, and 44.8 μg Fe/ml; incubated for 24 h). Cultures were then washed with PBS to remove residual SPIO. Twenty microliter tetrazolium dye 3-(4,5-dimethylthiazol-2)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT; Amersco, USA; 5 mg/ml) was added to the medium (final dilution of 500 μg/ml). Four hours later, the supernatant was removed and pellet was lysed by adding 150 μl of DMSO (Amersco). MTT reduction was measured spectrophotometrically (wavelength: 490 nm).

For growth curves, cells were plated on five 96-well plates [5×10^3 cells/well; six replicates for each condition, five condition (control, 5.6, 11.2, 22.4, and 44.8 μg Fe/ml) for each plate]. The plates were examined using MTT assay on the second, fourth, sixth, eighth, and tenth day, respectively.

ELECTRON MICROSCOPY (EM) ANALYSIS OF hUC-MSCs AFTER LABELING WITH SPIO

After incubation in DMEM/F12 containing SPIO equivalent to 22.4 μg Fe/ml for 24 h, hUC-MSCs were processed for EM analysis. Ultrathin sections were cut and stained, using uranyl acetate and lead citrate, for analysis with a transmission electron microscope (Phillips TECNAI10, The Netherlands).

IN VITRO MRI OF SPIO-LABELED hUC-MSCs

hUC-MSCs were incubated with SPIO at 22.4 μg Fe/ml for 24 h. They were washed thoroughly with PBS to ensure complete removal of unbound SPIO. For magnetic resonance (MR) detection, 500 μl of SPIO-labeled and unlabeled control cell suspensions at the desired density (3×10^4, 5×10^4, 7.5×10^4, 10×10^4 cells/500 μl) were immobilized in agar (1%, w/v) in 10 mm plastic tubes. Gradient echo T2-weighted (GRE T2*WI) images [repetition time (TR) 3,200 ms; echo time (TE) 354 ms; FOV = 172.5 × 172.5; slice thickness = 3 mm] and spin echo T2-weighted (SE T2WI) images (TR 6,000 ms; TE 98 ms; FOV = 220 × 220; slice thickness = 4 mm) were acquired from coronal slices using a 3.0 T MR scanner (Siemens Trio Tim, German). Optical density of MR images at each concentration was examined by software Image Pro Plus 5.1.

TRANSPLANTATION OF SPIO-LABELED hUC-MSCs

Adult female Sprague–Dawley rats weighing 250–300 g were used. All animal experiments were approved by Third Military Medical University Committee on Ethics for the Care and Use of Laboratory Animals. After anesthetization with sodium pentobarbital (40 mg/kg, i.p.), the spinal cord was exposed by laminectomy. After operation, skin incision was closed using sutures and animals were allowed to recover.

hUC-MSCs were incubated with SPIO at 22.4 μg Fe/ml for 24 h and resuspended in the appropriate volume of DMEM/F12 to produce the final concentration (5×10^4 SPIO-labeled or unlabeled cells in 5 μl). The cells were then loaded into a 5 μl microsyringe mounted on a stereotactic apparatus for transplantation. Cells were injected into the T_9 (labeled) or T_{10} (unlabeled) dorsal spinal cord at a depth of 1.3 mm and 0.6 mm laterally from midline at a rate of 0.5 μl/min. At each site, 2.5 μl of cell suspension was injected. The needle was left in position for a further 2 min before being slowly withdrawn.

IN VIVO MRI OF TRANSPLANTED SPIO-LABELED CELLS

For in vivo MRI studies, animals were anesthetized by intraperitoneal injection of sodium pentobarbital (40 mg/kg) 3 days and 14 days after transplantation. In vivo MR studies were performed using a 1.5 T MR scanner (Siemens Avanto Tim, German). In order to raise the signal to noise ratio, a commercially available circular surface coil (CG-MUC18-H150-AS, ChenGuang Corp., Shanghai, China) of 50 mm in diameter was used. T2-weighted Turbo Spin Echo (TSE) images (TR 2,370 ms; TE 79 ms; FOV 100 × 65.6; slice thickness 2 mm) were acquired from sagittal slices. The animals were then removed from the magnet and allowed to recover.

HISTOLOGY

Animals were sacrificed by perfusion via the aorta under deep pentobarbitone anesthesia with 4% paraformaldehyde in PBS. A length of spinal cord was removed (with the injection as center). Sections of spinal cord tissue were cut at a thickness of 5 μm and stained with PB and nuclear fast red.

STATISTICAL ANALYSIS

Statistical analysis was performed using two-tailed unpaired t test and one-way ANOVA followed by Tukey's multiple comparison test. A P value of ≤ 0.05 was considered significant. Coefficient of determination (R^2) was calculated with linear regression analysis to characterize the relationship between number of SPIO-labeled cells and optical density of MR images (in vitro MRI studies).

RESULTS

CHARACTERIZATION OF MSCs IN hUC

hUC- MSCs were successfully isolated from umbilical cord explants and expanded as primary cultures. The cells demonstrated a fibroblast-like or spindle-shaped morphology in confluent wave-like layers in culture. Images of cells at the second (Fig. 1A) and fourth (Fig. 1B) passage are shown in Figure 1A,B. Flow cytometry showed that the cells expressed high levels of matrix markers (CD90)

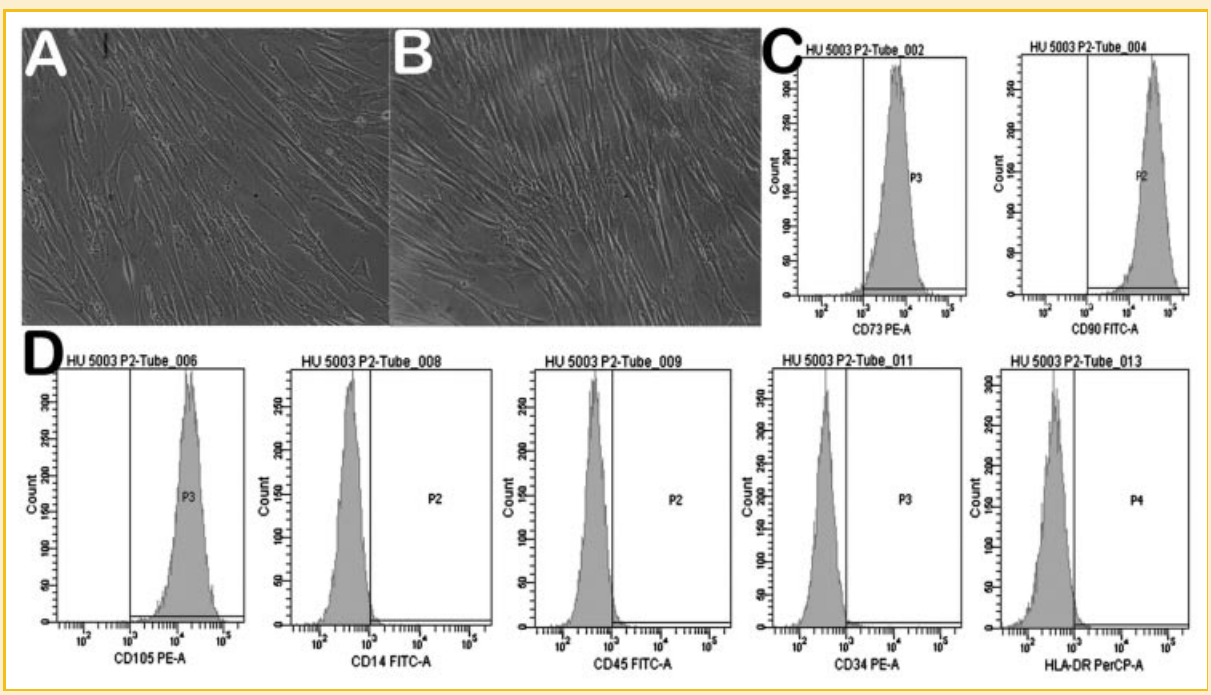

Fig. 1. Characteristics of mesenchymal stem cells from human umbilical cord. The cells on the second (A) and fourth (B) passage had a fibroblast-like or spindle-shaped morphology. Flow cytometric analysis showed that the cells expressed high levels of CD90, CD73, and CD105 but did not express CD34, CD45, CD14, and HLA-DR (C,D).

and mesenchymal stem cells (MSCs) markers (CD73, CD105) but did not express hematopoietic lineage markers (CD34, CD45, CD14) and HLA-DR (Fig. 1C,D).

INCORPORATION OF SPIO BY hUC-MSCs IS DOSE- AND TIME-DEPENDENT

We observed a good correlation between SPIO uptake and the iron concentration in the culture medium (Fig. 2A–E). As the concentration of SPIO and the incubation time increased, the amount of intracellular iron increased. Treatment with doses up to 22.4 μg Fe/ml and incubation times up to 24 h, which resulted in 94.1% labeling efficiency (Fig. 2C), did not significantly affect hUC-MSCs survival (Fig. 3A). Furthermore, labeled cells proliferated normally (Fig. 3B). However, treatment with 44.8 μg Fe/ml for 24 h impaired cell survival and proliferation significantly (Fig. 3A,B). The higher amounts of iron deposits were visualized by EM analysis (Fig. 2F,G). On the basis of these results, we decided to use SPIO at 22.4 μg Fe/ml for a 24 h incubation time.

IN VITRO MRI OF SPIO-LABELED hUC-MSCs

GRE T2*WI and SE T2WI images revealed the presence of a hypointense signal (Fig. 4A,B). A good linear correlation between the number of labeled cells and optical density of the GRE T2*WI images was observed (Fig. 4D; $R^2 = 0.9694$). Similar result was obtained in the SE T2WI images (Fig. 4E; $R^2 = 0.9085$). Unlabeled cells showed hyperintense signal in MR images (Fig. 4A,B).

SPIO-LABELED hUC-MSCs CAN BE TRACKED IN VIVO

In vivo MRI 3 days (Fig. 5A) and 14 days (Fig. 5B) after injection showed a large reduction in signal intensity in the region transplanted with SPIO-labeled hUC-MSCs. The images from

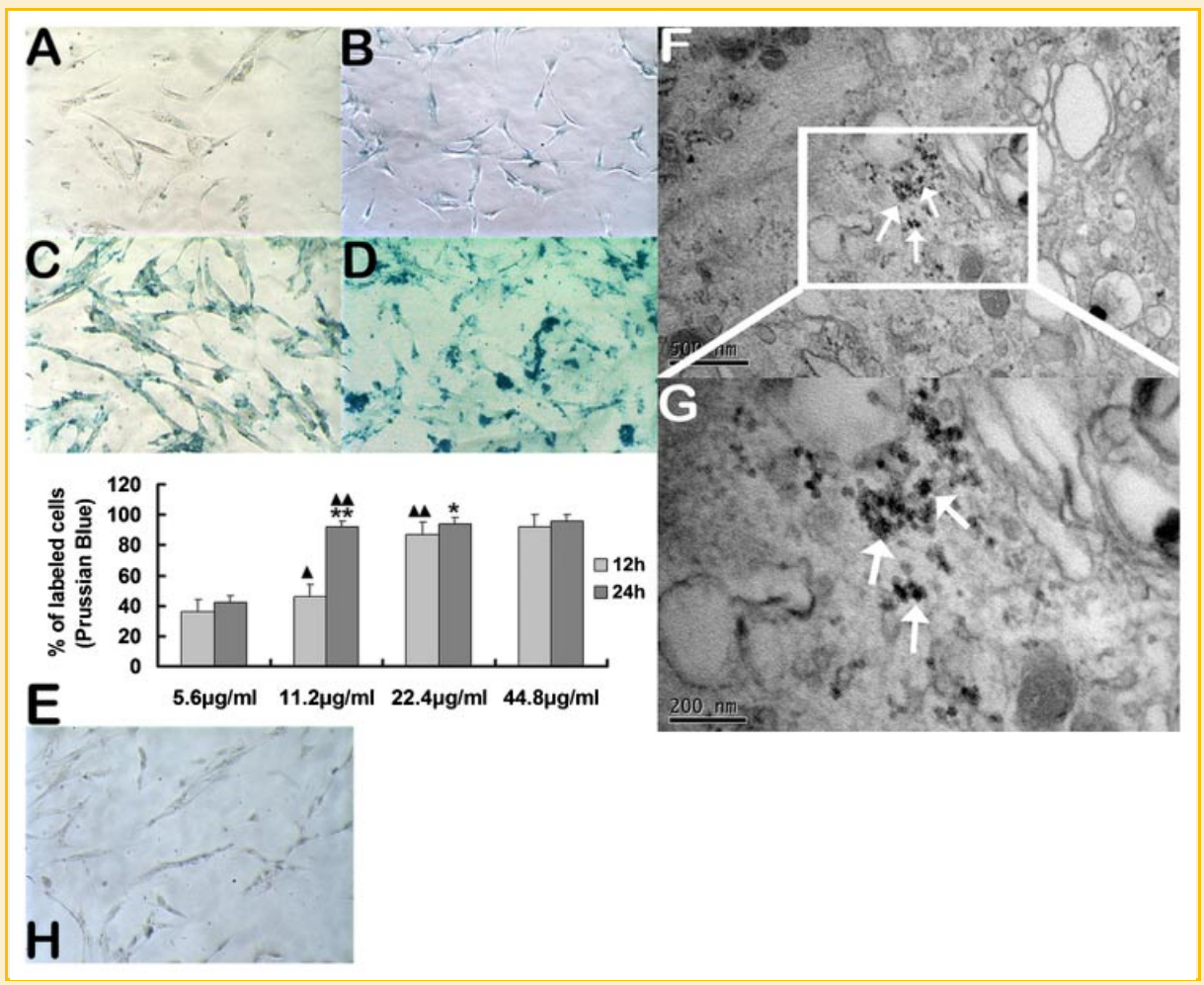

Fig. 2. Human umbilical cord mesenchymal stem cells (hUC-MSCs) labeled with superparamagnetic iron oxide (SPIO). Prussian blue staining showed increased iron accumulation in hUC-MSCs treated with 0 μg Fe/ml (H), 5.6 μg Fe/ml (A), 11.2 μg Fe/ml (B), 22.4 μg Fe/ml (C), and 44.8 μg Fe/ml (D; original magnification, 100×) for 24 h. The incorporation of SPIO was proportional to both the incubation time and the iron concentration in the culture medium (E). The higher amounts of iron deposits (arrow) were visualized by electron microscopy analysis (bar: F, 0.5 μm; G, 0.2 μm). $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the same concentration group (12 h); $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$, compared with the same time group (previous concentration). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

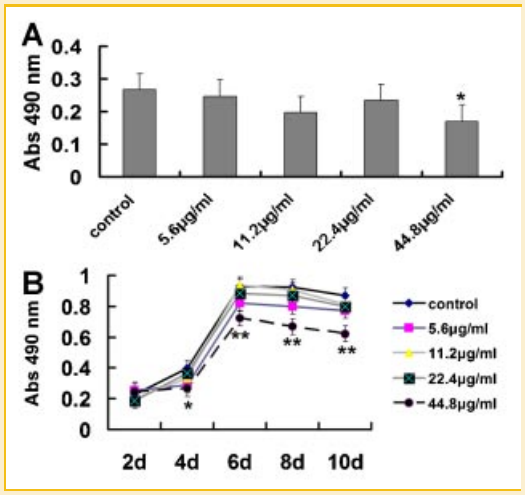

Fig. 3. Cells survival and proliferation labeled with superparamagnetic iron oxide. Doses up to 22.4 μg Fe/ml and incubation times up to 24 h resulted in 94.1% labeling efficiency and did not significantly affect human umbilical cord mesenchymal stem cells survival and proliferation as evaluated by MTT assay. However, treatment with 44.8 μg Fe/ml and for 24 h impaired cell survival and proliferation significantly. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the control group. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

unlabelled hUC-MSCs showed a smaller reduction in signal intensity. PB co-stained with nuclear fast red confirmed the presence of SPIO-labeled cells (Fig. 5D) in the region of transplant site with SPIO-labeled hUC-MSCs.

DISCUSSION

During embryogenesis, totipotent cells, such as primordial germ cells and hematopoetic stem cells, migrate from the yolk sac through Wharton's jelly to populate target tissues in the embryo and fetus. Therefore, Wharton's jelly of umbilical cord is a rich source of primitive cell types and may be an easily attainable source of multipotent stem cells [Mitchell et al., 2003]. The results obtained suggest that MSCs are present in the human umbilical cord and can be successfully isolated, cultured, and expanded using routine technical approaches. They express matrix markers (CD90) and MSCs markers (CD73, CD105) but do not express hematopoietic lineage markers (CD34, CD45, CD14) and HLA-DR marker, which fulfill the criteria for MSCs. Recent studies have shown that hUC-MSCs share most of their immunophenotype with BM-MSCs, including a cluster of differentiating makers, neural markers, and extracellular adhesion molecules. Moreover, they have genes of proteins shown to have a neurotrophic effect and may be superior to BM-MSCs for translational medicine [Bailey et al., 2007; Cao and Feng, 2009].

However, it is important to follow the in vivo fates of transplanted cells. The validation of SPIO-based protocols to label neural precursor cells and hematopoietic stem cells has been extensively addressed. Here, we report SPIO-based protocols to label hUC-MSCs. Our studies show a dose- and time-dependent uptake of SPIO by hUC-MSCs, in line with previous studies on rodent olfactory ensheathing cells [Dunning et al., 2004] and human neural precursor cells [Neri et al., 2008]. In the perspective of using SPIO-labeled cells for CNS disorders, it is essential to establish protocols yielding high

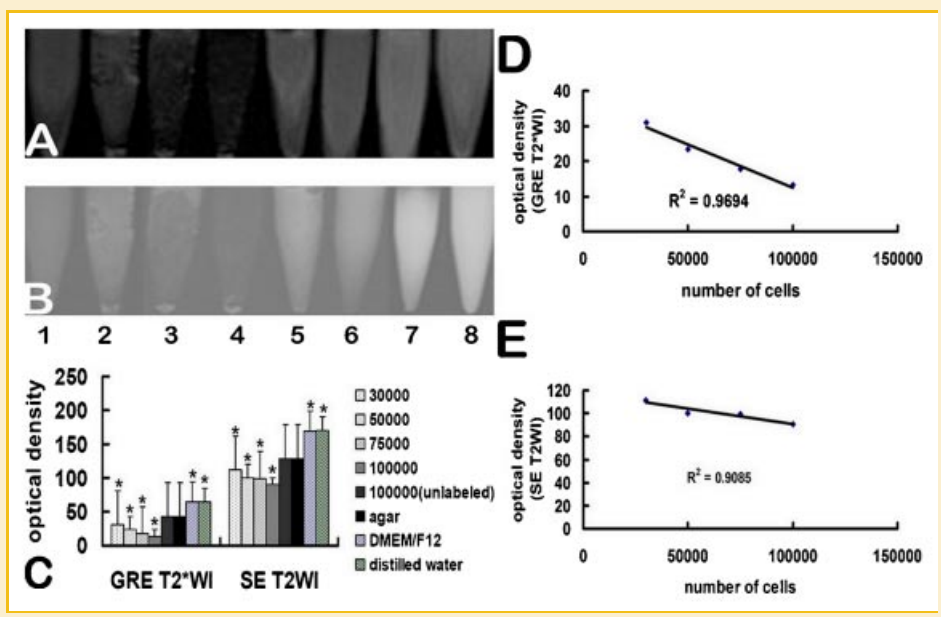

Fig. 4. In vitro MR images of human umbilical cord mesenchymal stem cells (hUC-MSCs) labeled with superparamagnetic iron oxide (SPIO). Gradient echo T2-weighted (GRE T2*WI; A) and spin echo T2-weighted (SE T2WI; B) images of SPIO-labeled hUC-MSCs showed increasingly hypointensity as the number of cells was increased. The optical density decreased significantly as compared to that of unlabeled cells (C). A good linear correlation between the number of labeled cells and optical density in the GRE T2*WI (D) and SE T2WI (E) images was observed. $^{*}P < 0.01$, compared with the unlabeled cells group: (1) 30,000 cells; (2) 50,000 cells; (3) 75,000 cells; (4) 100,000 cells; (5) 100,000 cells, unlabeled; (6) agar; (7) DMEM/F12; (8) distilled water.

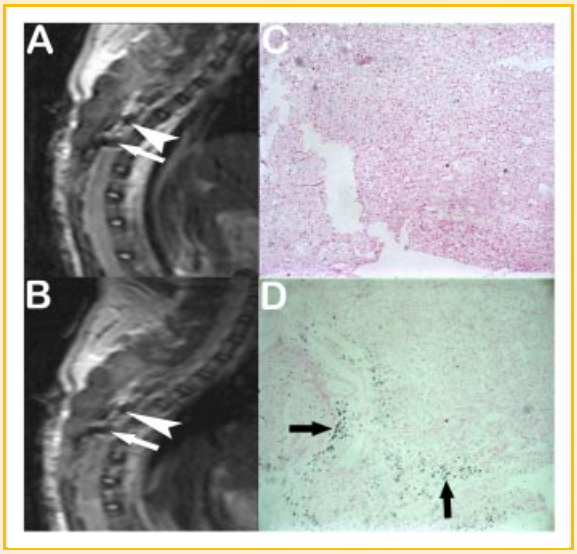

Fig. 5. In vivo MR images after transplantation of superparamagnetic iron oxide (SPIO) labeled human umbilical cord mesenchymal stem cells (hUC-MSCs) into focal areas of the spinal cord. A (arrowhead, 3 days after transplantation) and B (arrowhead, 14 days after transplantation) showed a large reduction in signal intensity in the region transplanted with SPIO-labeled hUC-MSCs. The images from unlabeled hUC-MSCs (arrow) showed a smaller reduction in signal intensity. Prussian blue co-stained with nuclear fast red (D, original magnification, 100×) confirmed the presence of SPIO-labeled cells (arrow) at transplant site with labeled hUC-MSCs and C showed few SPIO-labeled cells at the control site. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

label efficiency (in terms of both number of labeled cells and intensity of labeling) while preserving the functional properties of the labeled cells. Our data show that neither survival nor proliferation potential is severely impaired in dealing with 22.4 μg Fe/ml for 24 h. However, treatment with 44.8 μg Fe/ml for 24 h impairs cell survival and proliferation significantly. This indicates that SPIO concentration and incubation time should be carefully taken into account when developing preclinical strategies relying on SPIO-based cell tracking techniques. We could solve the problem by reducing both the SPIO concentration and the incubation time. Doses of 22.4 μg Fe/ml for 12 or 24 h, or doses of 11.2 μg Fe/ml for 24 h, results in efficient (>85%) cell labeling without impairment of cell survival and proliferation potential.

Our in vitro data optimize the conditions to label hUC-MSCs using Feridex. This condition results in robust SPIO uptake and detectability by MRI. A good linear correlation between the number of labeled cells and optical density of the GRE T2*WI and SE T2WI images is observed. These data are supported by MRI studies performed in living animals. In vivo MR images show that low numbers of SPIO labeled hUC-MSCs (5×10^4 cells) can be efficiently detected 14 days following transplantation into the rat spinal cord.

Although a clear signal is obtained from spinal cord into which labeled hUC-MSCs have been transplanted, a smaller signal is also obtained after transplantation of unlabelled cells. The injection procedure induces small focal areas of hemorrhage. The most likely explanation for the smaller reduction in signal intensity is the presence of iron-containing hemosiderin and deoxyhaemoglobin, which are associated with hemorrhage and are both paramagnetic [Lee et al., 2004]. Our results reveal the possibility of hemorrhage that confounds the interpretation of SPIO-labeled cell therapies. It is an issue that need to be considered for further developments of such approach.

Although SPIO-based cell therapies is a feasible noninvasive approach, it also has potential limitations that have to be carefully considered. Firstly, Intracellular SPIO is progressively diluted due to cell proliferation [Sun et al., 2005]. Secondly, some time after transplantation, partial cells will be dead and the released iron may result in loss of MR signal. Thirdly, the injection procedure, even with fine glass micropipettes, may induce small focal areas of hemorrhage which lead to the presence of hemosiderin and deoxyhaemoglobin, resulting in hypointensity in T2WI MR imaging. Therefore, further research is needed to explore all these problems in future study.

In summary, this study describes for the first time a simple in vitro protocol to label MSCs of human umbilical cord origin using SPIO (Feridex) at optimal low dosages that allows maintaining stable biological features without toxicity and shows the feasibility of noninvasive imaging of low numbers of SPIO-labeled cells after transplantation in the spinal cord. These results may be beneficial to cell-based therapies for clinical implementation.

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