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In Vivo Magnetic Resonance Imaging Tracking of SPIO-Labeled Human Umbilical Cord Mesenchymal Stem Cells

Sheng-Li Hu, Pei-Gang Lu, Li-Jun Zhang, Fei Li, Zhi Chen, Nan Wu, Hui Meng, Jiang-Kai Lin, and Hua Feng*

Department of Neurosurgery, Southwest Hospital, Third Military Medical University, No. 29, GaoTanYan Street, Chong-Qing 400038, PR China

ABSTRACT

Human umbilical cord mesenchymal stem cells (hUC-MSCs) can be efficiently labeled by superparamagnetic iron oxide (SPIO) nanoparticles, which produces low signal intensity on magnetic resonance imaging (MRI) in vitro. This study was to evaluate the feasibility of in vivo tracking for hUC-MSCs labeled by SPIO with noninvasive MRI. SPIO was added to cultures at concentrations equivalent to 0, 7, 14, 28, and 56 μg Fe/ml (diluted with DMEM/F12) and incubated for 16 h. Prussian Blue staining was used to determinate the labeling efficiency. Rats were randomly divided into three groups, control group, hUC-MSCs group, and SPIO-labeled hUC-MSCs group. All groups were subjected to spinal cord injury (SCI) by weight drop device. Rats were examined for neurological function. In vivo MRI was used to track SPIO-labeled hUC-MSCs transplanted in rats spinal cord. Survival and migration of hUC-MSCs were also explored using immunofluorescence. Significant improvements in locomotion were observed in the hUC-MSCs groups. There was statistical significance compared with control group. In vivo MRI 1 and 3 weeks after injection showed a large reduction in signal intensity in the region transplanted with SPIO-labeled hUC-MSCs. The images from unlabeled hUC-MSCs showed a smaller reduction in signal intensity. Transplanted hUC-MSCs engrafted within the injured rats spinal cord and survived for at least 8 weeks. In conclusion, hUC-MSCs can survive and migrate in the host spinal cord after transplantation, which promote functional recovery after SCI. Noninvasive imaging of transplanted SPIO-labeled hUC-MSCs is feasible. J. Cell. Biochem. 113: 1005–1012, 2012.

KEY WORDS: HUMAN UMBILICAL CORD MESENCHYMAL STEM CELL; SPINAL CORD INJURY; MAGNETIC RESONANCE IMAGING; SUPERPARAMAGNETIC IRON OXIDE NANOPARTICLE; FUNCTIONAL RECOVERY

Human umbilical cord mesenchymal stem cells (hUC-MSCs), an easily attainable source of potentially multipotent stem cells from Wharton's jelly of umbilical cord, were firstly identified in 2003 [Mitchell et al., 2003]. Recent evidences show that they have characteristics of fast proliferation, low immunogenicity, and great ex vivo expansion [Troyer and Weiss, 2008; Weiss et al., 2008]. Moreover, hUC-MSCs are able to differentiate to different blastoderm cells including endothelium cells [Wu et al., 2007], skeletal muscle [Conconi et al., 2006], heart [Wang et al., 2004], bone [Sarugaser et al., 2005], and neural cells [Mitchell et al., 2003; Fu et al., 2004, 2006; Ma et al., 2005]. In addition, accumulated studies have shown that UC-MSCs transplantation can promote neuroprotection and locomotion recovery in experimental models of brain diseases [Fu et al., 2006; Weiss et al., 2006; Jomura et al., 2007].

Therefore, hUC-MSCs have a potential therapeutic role for treating patients with injuries or degenerative diseases of central nervous system. However, it is important to follow the in vivo fates of grafted cells in order to advance such strategy into clinical medicine practice. Hence, design of noninvasive methods for in vivo tracking donor cells in host tissues is needed imminently.

Previous studies have demonstrated that different stem cells can be labeled by superparamagnetic iron oxide (SPIO) particles and be tracked by magnetic resonance imaging (MRI) [Stroh et al., 2005; Politi et al., 2007]. Our study also reveals that hUC-MSCs can be

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Sheng-Li Hu and Pei-Gang Lu contributed equally to the article.

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*Correspondence to: Prof. Hua Feng, MD, PhD, Department of Neurosurgery, Southwest Hospital, Third Military Medical University, No. 29, Gaotanyan Street, Chong-Qing 400038, PR China. E-mail: fenghua8888@yahoo.com.cn

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efficiently labeled by SPIO and show low signal intensity on MRI in vitro [Hu et al., 2009]. Thus, the purpose of this study was to evaluate the feasibility of in vivo tracking for hUC-MSCs labeled by SPIO with noninvasive MRI.

MATERIALS AND METHODS

CELL CULTURE

All parts of this study, especially the isolation of the hUC, were performed according to the Declaration of Helsinki. Ethical approval was obtained from Third Military Medical University (Chong-Qing, 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 [Hu et al., 2009, 2010]. Briefly, each UC was collected from full-term cesarian 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) 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% CO_2. They were re-fed and passaged as necessary.

LABELING OF hUC-MSCs WITH SPIO

Cultures of hUC-MSCs were passaged at least four times before labeling. To determine the optimal labeling concentration of SPIO, the label (80 nm, a generous gift of FaBao Gao, M.D., Ph.D., Molecular Imaging Center, West China Hospital, Cheng-Du, Si-Chuan Province, China) was added to the cultures at concentrations equivalent to 7, 14, 28, and 56 μg Fe/ml and incubated for 16 h. Control cultures were incubated without SPIO. SPIO solutions were prepared at a 1× concentration in growth medium. When necessary, SPIO solutions were added to 24-well plates or culture flasks with plated hUC-MSCs. At the end of the incubation period, cells were collected for determination of the labeling efficiency [by Prussian Blue (PB) staining; number of PB-labeled cells/total number of cells in the sample] and transplantation studies.

PRUSSIAN BLUE STAINING

After incubation with SPIO, a modification of the Perls' PB method was used for the detection of iron within the cell cultures. This induces a reaction of ferric iron to the ferrous state with the formation of a blue precipitate. After washing in PBS to remove any free SPIO, hUC-MSCs were fixed for 40 min using 4% paraformaldehyde (PFA). 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. The fixed cells were then stored in the dark at 4°C to prevent photobleaching. In some experiments nuclei were counterstained with Nuclear Fast Red (BoShiDe Corp, WuHan, China).

ANIMAL PREPARATION AND EXPERIMENTAL PROTOCOL

The animal study protocol used in this research was approved by the ethics committee for animal experimentation and was conducted according to the Guidelines for Animal Experimentation of our institute. Female Sprague–Dawley rats (from Experimental Animal Center of Third Military Medical University) weighing between 230 and 270 g were used. Rats were allowed free access to food and water before and after the surgical procedure. They were randomly divided into three groups, control group (n = 12), hUC-MSCs group (n = 14), and SPIO-labeled hUC-MSCs group (n = 2). All groups were subjected to spinal cord injury (SCI) by weight drop device based on the technique described by Wamil et al. [1998]. Control group received DMEM/F12 injections, while the hUC-MSCs group and SPIO-labeled hUC-MSCs group undertook unlabeled and labeled cells transplantation respectively.

SURGICAL PROCEDURES

Animals were anesthetized with an intraperitoneal (i.p.) injection of pentobarbital (40 mg/kg). A 4 cm long skin incision was made in the midline and a laminectomy was carried out at the T9–T11 level to expose the T9–T11 spinal segment, leaving the dura intact. An area of T10 spinal cord was contused by delivery of a 12.5 g/cm force, produced by dropping a 10 g weight a distance of 1.25 cm through a stainless steel guide tube onto a 2.3-mm diameter steel cylinder. After impact, the skin and muscles incision was closed using sutures.

TRANSPLANTATION OF hUC-MSCs

One day after SCI, rats were anesthetized with pentobarbital (40 mg/kg, i.p.). The spinal cord was reopened at the injury area. To prepare the cell suspension, passage 5 hUC-MSCs were collected and resuspended in the appropriate volume of DMEM/F12 to produce the final concentration ($4 \times 10^4/\mu l$). Cell viability was assessed by trypan blue. The cells were then loaded into a 5 μl microsyringe mounted on a stereotactic apparatus for transplantation. Cells were injected into the dorsal spinal cord 2 mm rostrally and 2 mm caudally to the injury site at a depth of 1.2 and 0.75 mm laterally from midline at a rate of 1 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. A total volume of 10 μl of cell suspension was injected (total 4×10^5 cells). After operation, incision was closed using sutures and animals were allowed to recover. Control group rats received 10 μl DMEM/F12 injections and SPIO-labeled hUC-MSCs group undertook SPIO-labeled cells graft.

BASSO, BEATTIE, AND BRESNAHAN OPEN FIELD LOCOMOTION SCORE

The Locomotor Rating Scale was used to assess locomotor recovery in an open field [Basso et al., 1996]. Before testing, bladders were expressed, because spontaneous bladder contraction often accompanies hindlimb activity. For examination, the rats were placed individually in an open field with a nonslippery surface. The 22-point (0–21) Basso, Beattie, and Bresnahan (BBB) scale was used to assess hindlimb locomotor recovery including joint movements, stepping ability, coordination, and trunk stability. A score of 21 indicates unimpaired locomotion as observed in uninjured rats. The duration of each session was 5 min per rat. One BBB tests were performed at 1 day

after injury, and after transplantation of hUC-MSCs the rats were tested at 1, 3, 5, 7, and 8 weeks after transplantation.

MRI OF TRANSPLANTED hUC-MSCs IN VIVO

In vivo MR studies were performed using a 7.0 T MR scanner (BioSpec70/30 USR, Bruker, Germany), 1 and 3 weeks after transplantation. T2-weighted images [repetition time (TR), 3,000 ms; echo time (TE), 45 ms; FOV, 125.6; slice thickness, 1 mm] were acquired from sagittal and horizontal slices. The animals were then removed from the magnet and allowed to recover.

HISTOLOGY

Eight weeks after transplantation, animals were perfused via the aorta under deep pentobarbitone anesthesia with 0.9% NaCl followed by 4% PFA in phosphate buffer. A length of fixed spinal cord was removed (with the injection as center). After dehydration in graded concentrations of ethanol and butanol, the tissue was embedded in paraffin. Sections of spinal cord tissue were cut and stained with HE.

IMMUNOFLUORESCENCE

Animals were killed with an overdose of pentobarbital and perfused transcardially with 4% PFA. The spinal cords were subsequently postfixed in the perfusing solution for 24 h at 4°C. Then, the tissues were cryoprotected in 30% sucrose in PBS for 24 h at 4°C. A 1.5 cm length of the spinal cord centered at the injury site was separated and embedded in tissue embedding medium on dry ice. Cryostat sections (20 μm) were cut and mounted onto poly-L-lysine (pll)-coated slides and stored at −70°C. For immunostaining, the frozen slides were air-dried at room temperature for 30 min and washed three times with PBS. Then they were blocked using 5% normal goat serum, and 0.3% Triton X-100 in PBS for 1 h at room temperature, and mouse anti-hNu (to label cells of human origin; 1:30; Chemicon) was applied for 1.5 h at room temperature or overnight at 4°C. The slides were washed in PBS three times and incubated with Cy3-conjugated goat anti-mouse secondary antibody (1:400; Bi-Yun-Tian Corp., Peking, China). The images were taken using a Zeiss (Thornwood, NY, Germany) 510META laser confocal microscope.

STATISTICAL ANALYSIS

Data are expressed as means ± standard deviation. BBB scores were analyzed by repeated measure ANOVA. A P-value of ≤ 0.05 was considered significant.

RESULTS

hUC-MSCs ARE LABELED EFFICIENTLY BY SPIO

Three days after the last subculturing passage, hUC-MSCs were incubated in growth medium in the presence of increasing concentrations of SPIO particles (equivalent to 0, 7, 14, 28, and 56 μg Fe/ml) for 16 h. We observed a good correlation between SPIO uptake, as assessed by PB staining, and the iron concentration in the

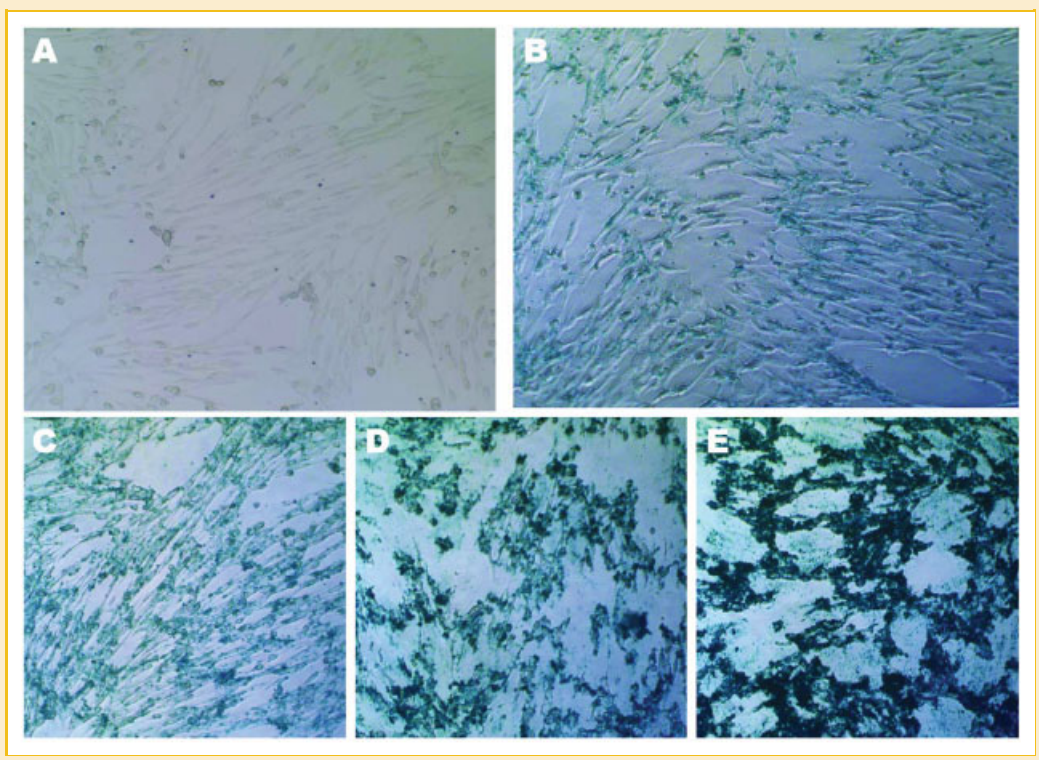

Fig. 1. hUC-MSCs are labeled efficiently by SPIO. Representative pictures of hUC-MSCs treated with 0 (A), 7 (B), 14 (C), 28 (D), and 56 μg Fe/ml (E) for 16 h showed increased intracellular iron accumulation, as visualized by Prussian Blue staining. As the concentration of SPIO increased, the amount of intracellular iron increased, resulting in heavy labeling in almost all the cells. Treatment with doses 7 μg Fe/ml, which resulted in 99% labeling efficiency (B), did not significantly affect hUC-MSCs survival. However, treatment with 28 and 56 μg Fe/ml impaired cell survival significantly (D,E). A–E: original magnification, ×100. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

culture medium (Fig. 1A–E). Blue cytoplasmatic inclusions were present only in cells incubated with the SPIO (Fig. 1B–E). At the lowest doses, we could recognize different intensities of staining among the cell population. As the concentration of SPIO increased, the amount of intracellular iron increased, resulting in heavy labeling in almost all the cells. Treatment with doses 7 μg Fe/ml, which resulted in 99% labeling efficiency (Fig. 1B), did not significantly affect hUC-MSCs survival. However, treatment with 28 and 56 μg Fe/ml impaired cell survival significantly (Fig. 1D,E). On the basis of these results, we decided to use SPIO at 7 μg Fe/ml for a 16 h incubation time.

SPIO-LABELED hUC-MSCs CAN BE TRACKED IN VIVO

Having established a protocol allowing efficient labeling of hUC-MSCs (in terms of number of labeled cells and intensity of labeling) using low dosages of SPIO without overall cell impairment, we sought to address whether SPIO-labeled cells could be localized by MRI after transplantation into the adult rats spinal cord. Rats receiving transplants of SPIO-labeled or unlabeled hUC-MSCs were scanned by using 7.0 T MRI. MRI in vivo 1 week (Fig. 2D,E) after injection showed that typical regions of hypointense signal attributable to SPIO-labeled cells. Three weeks later, hypointense signal still existed and spreaded towards the injury site (Fig. 2F,G). MRI showed a smaller reduction in signal intensity when similar numbers of unlabeled cells were injected (Fig. 2A–C).

hUC-MSCs SURVIVE AND ENGRAFT WITHIN THE INJURED SPINAL CORD

Anti-human nuclei (hNu) antigen immunostaining was used to trace the survival and migration pattern of the hUC-MSCs. Rats receiving hUC-MSCs grafts were euthanized at 8 weeks post-transplantation. Immunofluorescence for hNu antigen revealed extensive human cells survival and engraftment within the injured rat spinal cord. A series of sections showed that large numbers of hUC-MSCs survived at least for 8 weeks after transplantation (Fig. 3A–D). By 8 weeks, many human cells had migrated away from the implantation site (Fig. 3A,B) for about 5 mm along the rostrocaudal axis. In sagittal sections, migration into the rim of spared tissue in the contused cord, even at the epicenter (Fig. 3C,D), was frequently observed.

hUC-MSCs REDUCE SCI AND PROMOTE LOCOMOTOR RECOVERY

Spinal cord morphology and structure 8 weeks after transplantation as shown by light microscopy was greatly diminished in control group, with dramatic decreases in cells and formation of big cavity

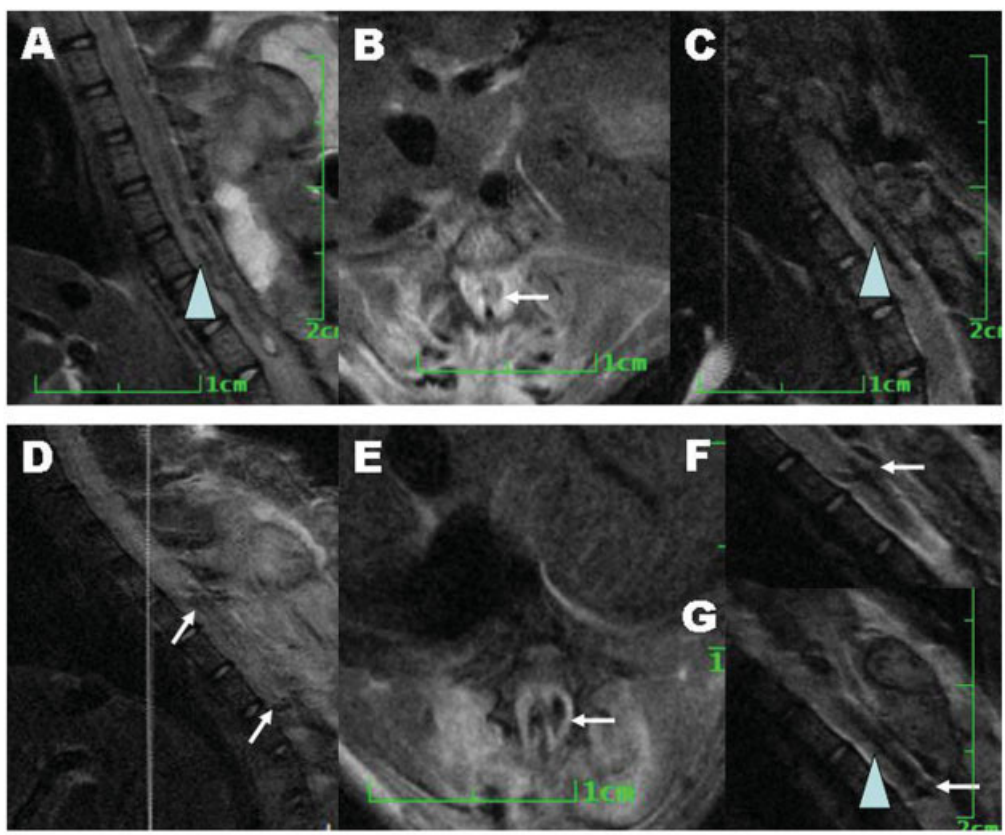

Fig. 2. SPIO-labeled hUC-MSCs can be tracked in vivo. Sagittal (D) and horizontal (E) slices of MRI in vivo 1 week and after injection showed that typical regions of hypointense signal attributable to SPIO-labeled cells. Three weeks later, hypointense signal still existed and spreaded towards the injury site (F, rostral; G, caudal). MRI showed a smaller reduction in signal intensity when similar numbers of unlabeled cells were injected (A: sagittal position, 1 week after injection; B: horizontal position, 1 week after injection; C: sagittal position, 3 weeks after injection). Arrow: transplantation site; arrowhead: injury epicenter. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

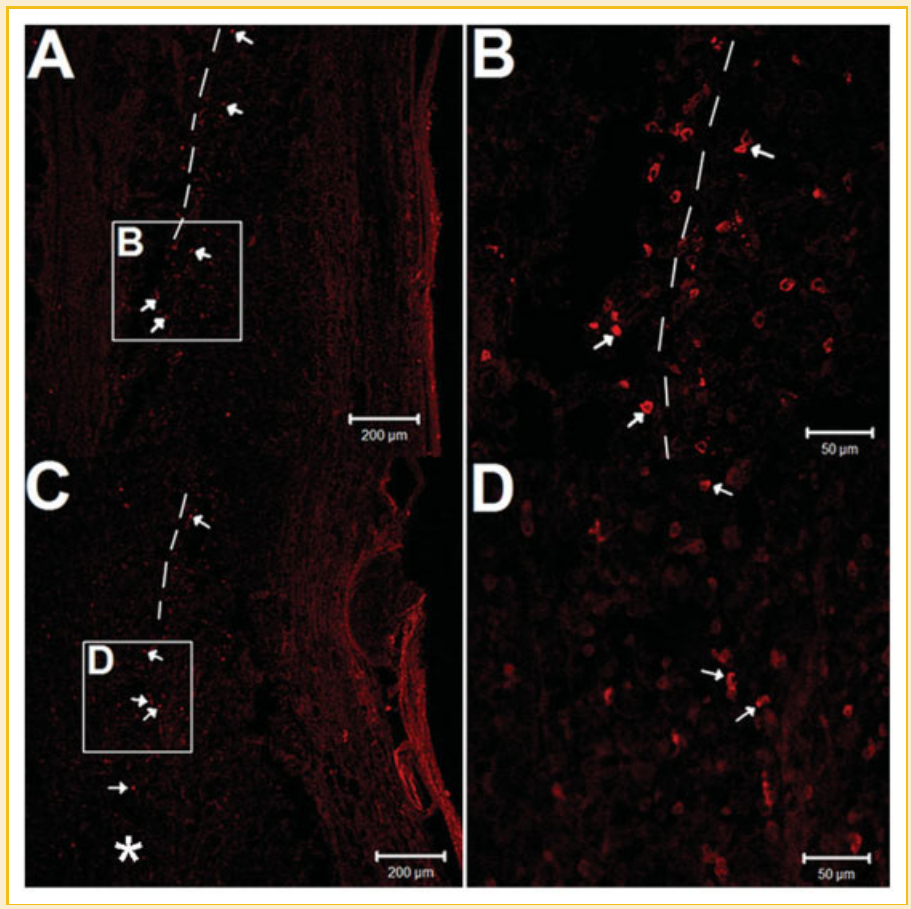

Fig. 3. hUC-MSCs survive and engraft within the injured spinal cord. A series of sections showed that large numbers of hUC-MSCs survived at least for 8 weeks after transplantation (A–D). By 8 weeks, many human cells had migrated away from the implantation site (A,B) for about 5 mm along the rostrocaudal axis. In sagittal sections, migration into the rim of spared tissue in the contused cord, even at the epicenter, was frequently observed (C,D). Arrow: hUC-MSCs. Asterisk: injury site. B,D: enlarging picture of the inbox in A and C, respectively. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

(Fig. 4B), while smaller pathological injuries occurred in hUC-MSCs group (Fig. 4A), There were better recovery in this group than the control. The BBB scores showed that the hindlimb locomotion of injured animals dramatically decreased on the 1st day after SCI but recovered over time in the hUC-MSCs group and control group. Scores were significantly higher at weeks 3, 5, 7, and 8 than at week 1 after injury. More importantly, BBB scores of hUC-MSCs group were significantly higher than those of the control group (Fig. 4C).

DISCUSSION

The major findings of this study are that hUC-MSCs can be labeled efficiently by SPIO. After transplantation into the rats spinal cord, they can survive and migrate in the host tissue, promote function recovery and produce specific hypointense signal on MRI.

Mitchell et al. [2003] firstly identified MSCs from Wharton's jelly of hUC in 2003. Further research find that the hUC-MSCs can be isolated from perivacular regions [Baksh et al., 2007], subamnion [Troyer and Weiss, 2008], and vein subendothelium [Panepucci et al., 2004; Kaltz et al., 2008] of the umbilical cord. hUC-MSCs express markers similar to MSCs derived from umbilical cord blood, bone marrow, and placenta. Recent studies show that OCT-4 is also expressed by hUC-MSCs [Weiss et al., 2006; Jomura et al., 2007; Zhang et al., 2009], which indicates hUC-MSCs may be an intermediate hierarchy of stem cells between embryonic stem cells (ESCs) and adult stem cells (ASCs). Therefore, hUC-MSCs may be superior to bone marrow-MSCs and ESCs in a clinical setting because they possess properties of low immunogenicity, easy availability, and great expansion capabilities [Troyer and Weiss, 2008; Weiss et al., 2008; Cao and Feng, 2009].

Past studies have shown that hUC-MSCs can differentiate into neurons, glial cells [Mitchell et al., 2003; Fu et al., 2004; Ma et al., 2005], and especially dopaminergic neurons [Fu et al., 2006]. Moreover, obtained data have demonstrated that hUC-MSCs transplantation can induce neuroprotection in experimental models of Parkinson's disease [Fu et al., 2006; Weiss et al., 2006], retinal degenerative diseases [Lund et al., 2007], and cerebral global ischemia [Ding et al., 2007; Jomura et al., 2007]. The present study also revealed that significant improvements in locomotion and reduced damage were observed in the hUC-MSCs groups

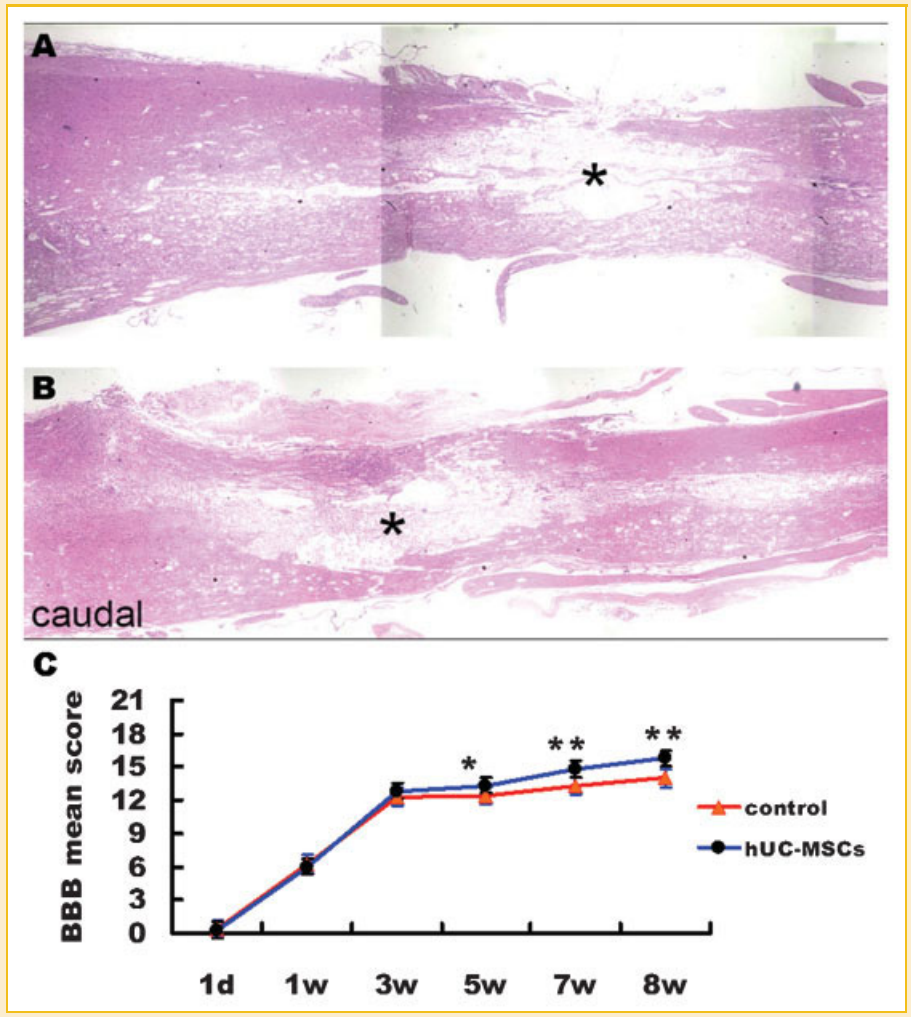

Fig. 4. hUC-MSCs reduce SCI and promote locomotor recovery. Spinal cord morphology and structure as shown by light microscopy was greatly diminished in control group, with dramatic decreases in cells and formation of big cavity (B, asterisk, injury epicenter), while fewer pathological injuries occurred in hUC-MSCs group (A, asterisk, injury epicenter). The BBB scores showed that the hindlimb locomotion of injured animals dramatically decreased on the 1st day after SCI but recovered over time in the hUC-MSCs group ($n = 8$) and control group ($n = 8$). More importantly, BBB scores of hUC-MSCs group were significantly higher than those of the control group at weeks 5, 7, and 8 (C). $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the control group. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

after SCI. There was statistical significance compared with control group, which was consistent with a previous report by Yang et al. [2008].

Nevertheless, it is important to follow the in vivo fates of grafted cells for translational medicine. In the past, histopathological methods are usually applied to assess engraftment, migration, and differentiation of exogenous transplanted stem cells. There are always three methods to label exogenous cells: (1) cells transfected with exogenous gene, such as green fluorescent protein (GFP) gene and β2-galactosidase gene by using transgenic technique; (2) cells DNA labeled with 5-bromodeoxyuridine (BrdU); (3) cells nuclei stained with fluorescence dye including DAPI and Hoechst. However, these approaches can be performed only using histological sections after animals are killed. So noninvasive methods for in vivo tracking live transplanted cells in host tissues is needed.

Weissleder [1999] firstly introduce the concept of molecular imaging that means real-time in vivo imaging of physiological or pathological phenomenon on molecular level using modern image technique. MRI is good method for this purpose with high spatial and temporal resolution, especially with the development of magnetic resonance contrast agents.

SPIO is a new kind of magnetic resonance contrast agents, which produces negative contrast effects on T2-weighted sequences. To date, two iron oxide-based agents have been developed clinically and approved for MRI. Those particle size are >50 nm are referred to as SPIO agents while those particle size are <50 nm are the ultrasmall SPIO (USPIO) agents [Kirchin and Runge, 2003]. In this study, SPIO of 80 nm in size was used, which had enhanced MR susceptibility [Hinds et al., 2003]. Our study shows a dose-dependent uptake of SPIO in hUC-MSCs, in line with previous studies on olfactory ensheathing cells, Schwann 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 needs to establish protocols obtaining high label efficiency while preserving

the viability of labeled cells. Our data showed that survival potential was not impaired in dealing with 7 μg Fe/ml, which resulted in 99% labeling efficiency. However, treatment with 28 and 56 μg Fe/ml impaired cell survival significantly, which indicated that SPIO concentration should be carefully taken into accounts when using SPIO-based cell tracking techniques in clinical practice.

Our results also showed that typical regions of hypointense signal was observed in the SPIO-labeled hUC-MSCs group and the signal loss persisted at least 3 weeks. More important, 3 weeks later, hypointense signal seemed to spread towards the injury site, which indicated that the hUC-MSCs survived and migrated towards injury epicenter. This was further confirmed by using immunofluorescence, which showed numerous hUC-MSCs at the injury site. Guzman et al. [2007] even found that MRI can track the SPIO-labeled human neural stem cells transplanted in the host tissue for more than 18 weeks. Although a clear signal was obtained from SPIO-labeled hUC-MSCs grafted spinal cord. Unlabeled cells transplanted rats also demonstrated a smaller hypointense signal. The most likely explanation for the smaller reduction in signal intensity is the presence of iron-containing hemosiderin and deoxyhemoglobin decomposed from small hematoma induced by injection procedure.

In summary, this study reveals that hUC-MSCs can survive and migrate in the host spinal cord after transplantation, which promote functional recovery after SCI. MRI can track transplanted SPIO-labeled hUC-MSCs in vivo. These results may be beneficial to cell-based therapies for clinical implementation. A limitation of this study is that we do not examine the differentiation ability of SPIO-labeled hUC-MSCs in vitro and in vivo. Further research is needed to be explored in future work.

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