

Efficient *In Vitro* Labeling Rabbit Bone Marrow-Derived Mesenchymal Stem Cells with SPIO and Differentiating into Neural-Like Cells

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Mesenchymal stem cells (MSCs) can differentiate into neural cells to treat nervous system diseases. Magnetic resonance is an ideal means for cell tracking through labeling cells with superparamagnetic iron oxide (SPIO). However, no studies have described the neural differentiation ability of SPIO-labeled MSCs, which is the foundation for cell therapy and cell tracking *in vivo*. Our results showed that bone marrow-derived mesenchymal stem cells (BM-MSCs) labeled *in vitro* with SPIO can be induced into neural-like cells without affecting the viability and labeling efficiency. The cellular uptake of SPIO was maintained after labeled BM-MSCs differentiated into neural-like cells, which were the basis for transplanted cells that can be dynamically and non-invasively tracked *in vivo* by MRI. Moreover, the SPIO-labeled induced neural-like cells showed neural cell morphology and expressed related markers such as NSE, MAP-2. Furthermore, whole-cell patch clamp recording demonstrated that these neural-like cells exhibited electrophysiological properties of neurons. More importantly, there was no significant difference in the cellular viability and $[Ca^{2+}]_i$ between the induced labeled and unlabeled neural-like cells. In this study, we show for the first time that SPIO-labeled MSCs retained their differentiation capacity and could differentiate into neural-like cells with high cell viability and a good cellular state *in vitro*.

INTRODUCTION

Mesenchymal stem cell (MSC) transplantation is one promising approach to treat various central nervous system (CNS) diseases,

such as spinal cord injury (Eftekharpour et al., 2008), stroke (Gutiérrez-Fernández et al., 2013), and neurodegenerative diseases (Joyce et al., 2010). Many studies based on animal experiments have demonstrated that MSCs can produce cytokines or growth factors that could lead to neural cell rescue and promote regeneration through neuroprotection (Hsieh et al., 2013) or that they can differentiate into neural stem cells and ultimately develop into neural cells (Wang et al., 2013) and replace a lost population of cells to promote regeneration *in vivo*. Accordingly, MSCs and MSC-derived neural cells are considered to be ideal seed cells for replacement therapy in nervous system disorders due to the advantages of a plentiful source of cells, low immunogenicity, and fewer ethical concerns during autotransplantation applications (Harris et al., 2012; Van Velthoven et al., 2013).

To evaluate the safety and efficacy of stem cell-based therapy, there is a need for suitable imaging techniques that can ideally monitor the global cellular distribution, migration, differentiation, and homing of transplanted cells *in vivo*. Moreover, the fates of transplanted cells within the host should be effective and specific as well as non-invasive and non-toxic, especially in clinical medicine patients who require non-invasive techniques that can repeatedly monitor the tissue biodistribution of these cells.

For these points, cell labeling with contrast agents for magnetic resonance (MR) imaging provides the ability to monitor the *in vivo* behavior of the transplanted MSCs non-invasively. Previously, MR imaging has been successfully used to localize stem cells and to track their persistence and migration over time in animal models of stroke (Lu et al., 2013), injury (Li et al., 2013), degenerative diseases (Moraes et al., 2012) of CNS. Peldschus (Peldschus et al., 2007) detected single superparamagnetic iron oxide nanoparticle (SPIO)-labeled MSCs using 3-T MR and proposed that the quantitative analysis of labeled stem cells was feasible. MR is an ideal means of detecting and tracking stem cells (Reddy et al., 2010). Resovist is a class of dextran-coated SPIO nanoparticles that has been approved by the Food and Drug Administration (FDA) as a MR imaging contrast agent. Previous studies have shown that transplanted MSCs and NSCs (Daadi et al., 2013) labeled with SPIO can be detected by MR imaging.

However, no studies have described whether SPIO-labeled MSCs could differentiate into neural-like cells, which is the basis for the treatment and tracking of SPIO-labeled MSCs *in vivo*.

In this study, BM-MSCs were labeled with SPIO and induced into neural-like cells. Moreover, the effect of labeling on the cellular viability, proliferation, and differentiation was determined. The

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results of this research may provide useful evidence for the future clinical application of SPIO-labeled MSCs for the treatment of nervous system disorders.

MATERIALS AND METHODS

Animals

Two healthy New Zealand white rabbits were purchased from the animal experiment center of our university and housed in a standard animal facility with 12-h on/off light conditions, and they were allowed standard food and water ad libitum. The rabbits were aged 2 months and weighed 600–700 g. All procedures were approved by the Institutional Animal Care and Use Committee at our institution.

Isolation, induction, and identification of BM-MSCs

Approximately 1–3 ml bone marrow was obtained from each rabbit by puncture of the spinallia posterior superior thighbone under sterile conditions after being anesthetized by an intravenous injection of 3% pentobarbital sodium (30 mg/kg). Details of the isolation, induction, and identification of BM-MSCs are provided as supplementary data.

Cellular labeling and neural-like cell induction

Rabbit BM-MSCs were obtained according to the previous methods. The basic medium was DMEM supplemented with 10% fetal bovine serum (FBS). Passage 3 BM-MSCs were cultured in basic medium supplemented with Resovist containing 25 µg/ml superparamagnetic iron oxide nanoparticle (Schering Co., Ltd., Germany) at 37°C, in 5% CO₂ and saturated humidity for 24 h (Jing et al., 2008).

For neural-like cell differentiation, the SPIO-labeled BM-MSCs were pre-induced with 1 mmol/L β-mercaptoethanol for 24 h, washed three times with PBS, and cultured with DMEM supplemented with 40 ng/ml basic fibroblast growth factor (bFGF) for another 24 h (Suzuki et al., 2004), the cells were subjected to immunocytochemical analyses. To acquire the functional neural-like cells, the cells were then incubated in the presence of 100 ng/ml bFGF and over the next seven days, the cells were subjected to electrophysiological analyses (Janget al., 2010).

Validation of labeling

After incubation with SPIO, Prussian blue staining was used to detect iron within the cell cultures. This process induces a reduction of the ferric iron to the ferrous state with the formation of a blue precipitate. SPIO-labeled cells were washed twice with D-Hank's solution and fixed in 4% paraformaldehyde for 10 min. After washing with distilled water, the cells were incubated for 30 min with 2% potassium ferrocyanide in 2% hydrochloric acid, and the cells were incubated for 10 min by 0.5% neutral red. The labeling efficiency was determined by the manual counting of Prussian blue-stained cells.

RNA isolation and quantitative real-time polymerase chain reaction

Total RNA was extracted using TRIZOL (Invitrogen) according to the manufacturer's instructions. Two micrograms of total RNA from each sample were reverse transcribed using M-MLV (Takara) in a final volume of 30 µl. The polymerase chain reaction (PCR) amplification was carried out using the IQ5 System (Bio-Rad) with SYBR Green Mastermix (Takara). All quantitative RT-PCR tests were carried out in duplicate and normalized to β-actin. The primer sequences used for qRT-PCR are listed in Supplementary Table S1.

Immunocytochemistry

The cultured cells were fixed for 10 min at 4°C in ice cold methanol, washed several times in phosphate-buffered saline (PBS), blocked in 3% H₂O₂ for 30 min at room temperature, and then blocked for 30 min in 4% normal goat serum in 0.1M PBS with 1% PVP, 1% BSA, and 0.3% Triton X-100. The primary antibodies (NSE, MAP-2) were incubated overnight at 4°C, washed with PBS, and then incubated with the secondary antibodies at room temperature for 20 min. The incubated cells were washed in PBS, and DABC was incubated for 30 min at temperature. The cells were incubated in the DAB/H₂O₂ solution for approximately 10 minutes and were then checked under a microscope for an optimal signal-to-noise ratio.

Electrophysiological detection

SPIO labeled BM-MSCs and SPIO labeled induced cells with neural-like morphology were chosen for whole-cell patch clamp recording. Plastic cover slips (Nunc) containing a monolayer cells were transferred to a recording chamber on the stage of an inverted microscope. The culture medium was replaced with extracellular solution containing: 140 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 10 mM HEPES (pH = 7.3); pipettes were filled with an intracellular-like solution containing 140 mM KCl, 5 mM NaCl, 1 mM CaCl₂, 10 mM HEPES, 5 mM EGTA, 2 mM Mg-ATP. The resistance of fire-polished pipettes was 5–10 MΩ. All experiments were performed at room temperature. Ionic currents were recorded using the patch-clamp whole-cell configuration with Axopatch-200B amplifier (Axon Instruments, USA) and digitized using a digidata 1322A A/D converter. Data were analyzed using pClamp10.1 and Originpro 8.0 software.

Assessment of cell viability

Calcein-AM/PI staining was used to evaluate the viability of labeled neural-like cells, and the cells were observed by confocal microscope (excitation 490 nm, emission 617 nm). The unlabeled and labeled neural-like cells were cultured in neurogenic medium, and the cells were stained with Calcein-AM and PI for 20 min at 1, 3, and 6 days of induction. Three fields were selected randomly, the number of live cells was counted, and the percentage was calculated.

Determination of [Ca²⁺]_i

The unlabeled and labeled induced cells were loaded with 5 µM fluo-3 AM (Invitrogen) for 45 min at 37°C. After they were rinsed, the cells were viewed using a Zeiss confocal microscope (400× oil immersion objective; Leica Microsystems, Germany). Fluo-3 fluorescence was produced by excitation from a 75-W xenon arc lamp with the appropriate filter sets (excitation 488 nm; emission 526 nm; Sutter Instruments, USA). After baseline images were acquired, the cells were stimulated with 90 mM KCl. Image acquisition continued for 20 min, and the intensities of the intracellular fluorescence were measured at 1600 s, 2000 s, and 2400 s using software. Briefly, regions of interest were defined by drawing an outline around each cell body, and the mean fluorescence was extracted across the time-lapse sequence of the images to obtain fluorescence versus time plots for each cell. Background fluorescence was obtained from a region with no cells for every field examined and subtracted from the mean fluorescence. The mean fluorescence was also corrected by the mean baseline fluorescence, which was determined before cell stimulation. For each treatment condition, 35–40 cells within a single field of view were analyzed.

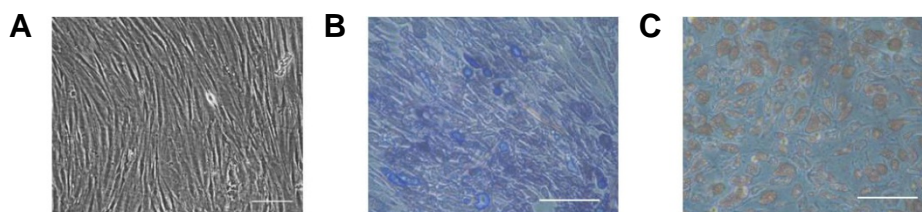


Fig. 1. (A) The morphology of rabbit BM-MSCs. (B) Alkaline phosphatase staining. (C) Oil red O staining of BM-MSCs. Scale bar, 100 μ m.

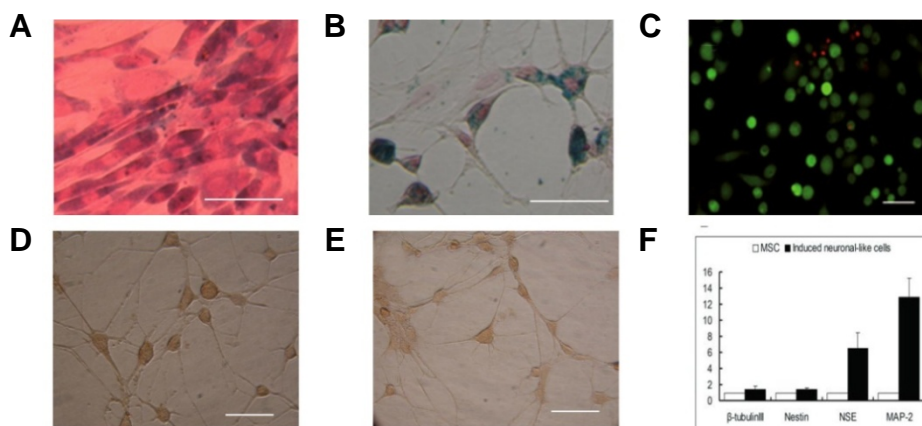


Fig. 2. Characterization of SPIO-labeled BM-MSC derived neural-like cells by stepwise differentiation. (A) Prussian blue staining of SPIO labeled BM-MSCs, with an efficiency up to 100%, Scale bar, 50 μ m. (B) Prussian blue staining of neural-like cells derived from labeled BM-MSCs, scale bar, 50 μ m. (C) The average viability of induced SPIO-labeled neural-like cells by Calcein-AM/PI staining. Green indicates living cells, and red indicates dead cells. Scale bar, 50 μ m. (D, E) Immunocytochemical staining of NSE (D) and MAP-2 (E) of SPIO-labeled neural-like cells. Scale bar, 50 μ m. (F) Passage 3 BM-MSCs were stimulated with neurogenic medium for 24 h; the expression levels of neural-related genes β -tubulinIII, Nestin, NSE, and MAP-2 were analyzed by qRT-PCR.

BM-MSCs after exposure to neurogenic medium. Scale bar, 50 μ m. (F) Passage 3 BM-MSCs were stimulated with neurogenic medium for 24 h; the expression levels of neural-related genes β -tubulinIII, Nestin, NSE, and MAP-2 were analyzed by qRT-PCR.

Statistical analysis

Data were expressed as the mean \pm S.D, unless stated otherwise. Prussian blue staining and cell viability were expressed as a percentage of the corresponding unlabeled control cells, and analysis of variance (ANOVA) was performed to test for statistical significance. Comparisons of signal intensity between unlabeled and labeled neural-like cells were performed using the unpaired Student's *t*-test. Statistical analysis was performed using the SPSS 13.0 software for Windows. A *P* value of <0.05 was considered to indicate a statistically significant difference.

RESULTS

Morphology and phenotypes of cultured BM-MSCs

After isolation from rabbit bone marrow, BM-MSCs were cultured in growth medium. By 14 days of culturing, the cell morphology was characterized as being spindle-shaped under a light microscope, and these BM-MSCs retained an active proliferation capacity *in vitro* (Fig. 1A). When BM-MSCs were cultured under osteogenic medium for 14 days, these cells could differentiate into osteogenic lineage, as demonstrated by the alkaline phosphatase (ALP) staining (Fig. 1B). In addition, when BM-MSCs were cultured under adipogenic medium for 21 days, these cells could differentiate into adipocytes with lipid droplets stained with Oil red O (Fig. 1C). The FACS analysis (Supplementary Fig. S1) showed that these cultured cells were positive for CD29 and CD44 and negative for CD34 (endothelial cell marker) and CD45 (pan-leukocyte marker); this phenotype is similar to that reported by Dominici M et al. (Kuhn et al., 2010).

BM-MSCs are labeled efficiently by SPIO

SPIO particles are easily internalized by cells such as macrophages and stem cells, and dextran-coated SPIO are more easily internalized by mesenchymal stem cells (Reddy et al., 2010), so

optimal (and often toxic) concentrations are needed for efficient cell labeling. In this study, we chose 25 μ g/ml SPIO as the labeling concentration. BM-MSCs were incubated with SPIO for 24 h, and at the end of the stage, SPIO particles were clearly visible inside the cytoplasm. A Prussian blue iron stain demonstrated that the labeling efficiency of SPIO was nearly 100% (Fig. 2A).

In vitro viability of induced SPIO-labeled neural-like cells

Over 99% of induced neural-like cells were labeled with SPIO after 24 h of culture in neurogenic medium (Fig. 2B). Calcein-AM/PI staining (Fig. 2C) showed that the average viabilities of induced SPIO-labeled neural-like cells and induced unlabeled neural-like cells were 93.5% and 94.1%, respectively ($P > 0.05$). They suggested that the SPIO-labeled cells have the same viability as do the unlabeled cells.

Induction of labeled BM-MSCs into neural-like cells

SPIO-labeled BM-MSCs were cultured in neurogenic medium. By 24 h, the labeled BM-MSCs showed a neuron-like morphology, which exhibited distinct bipolar or multipolar morphologies with branched processes. Immunocytochemistry staining revealed that the induced cells were NSE- and MAP-2-positive (Figs. 2D and 2E), suggesting that the induced cells expressed the markers of neural lineage cells. The RT-PCR results showed that the expressions of β -tubulinIII, Nestin, NSE, and MAP-2 (Yan et al., 2013) were up-regulated in induced cells compared with undifferentiated BM-MSCs (Fig. 2F). To evaluate whether these neural-like cells possess function, the inward sodium currents of neural-like cells differentiated from BM-MSCs were evoked by depolarizing voltage commands. Induced 8 days neural-like cells displayed a voltage-dependent sodium current, which showed a feature of fast activation and fast inactivation (Figs. 3B and 3C). On the contrary, undifferentiated BM-MSCs were quiescent, and no inward sodium current was detected (Fig. 3A).

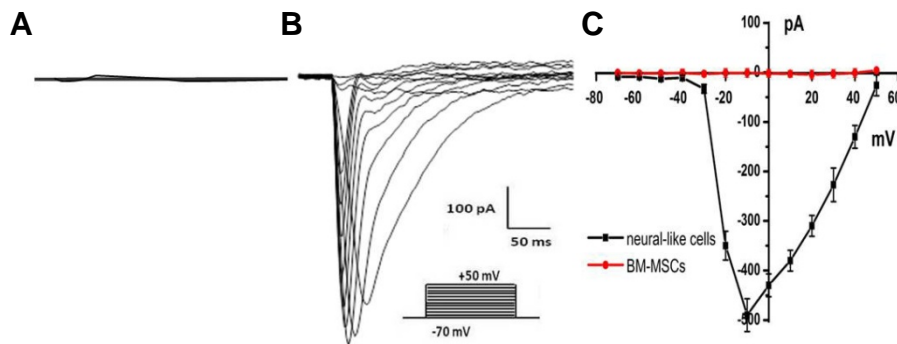


Fig. 3. Electrophysiological analysis for inward sodium current. (A) No inward sodium current was detected in BM-MSCs. (B) Voltage-dependent sodium current was detected in BM-MSCs derived neural-like cells. (C) The peak current-voltage relationship was plotted against the voltages.

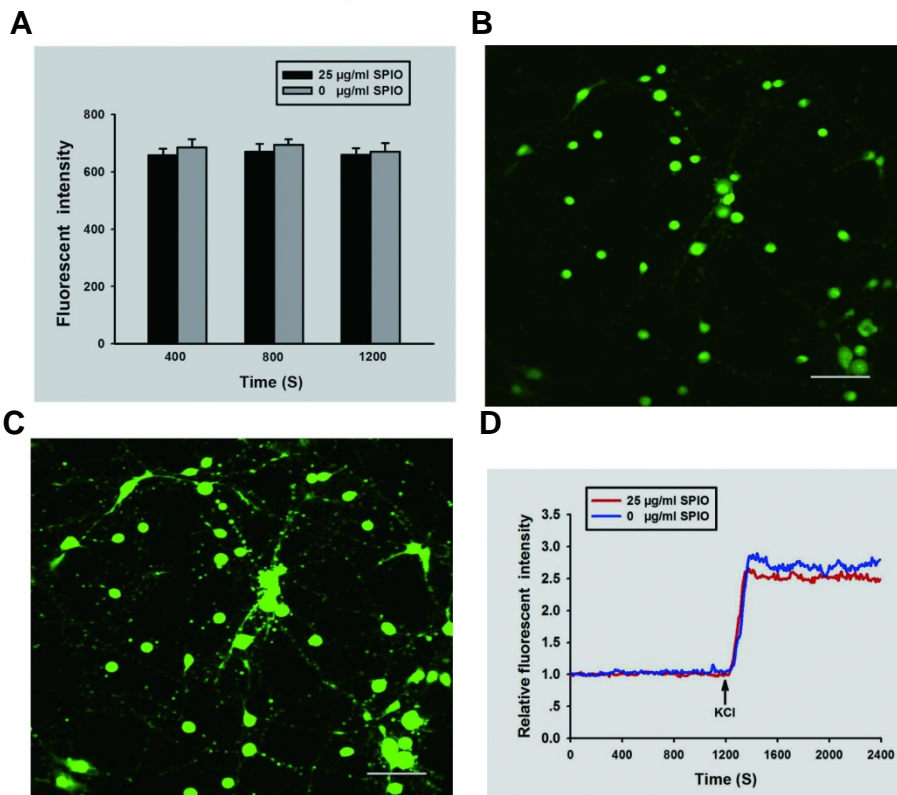


Fig. 4. The induced SPIO-labeled neural-like cell state. (A) The resting $[Ca^{2+}]_i$ concentration tests of induced unlabeled cells and induced SPIO-labeled cells by the fluorescence intensity. (B) The fluorescence staining of induced SPIO-labeled neural-like cells with Flu0-3/AM, stimulation of the cells with 90 mM KCL. Scale bar, 50 μ m. (C) The fluorescence staining of induced SPIO-labeled neural-like cells showed neural-like cell morphology. Scale bar, 50 μ m. (D) The relative fluorescence intensity of labeled and unlabeled neural-like cells.

In vitro cell state of induced SPIO-labeled neural-like cells

The resting $[Ca^{2+}]_i$ was not significantly different between induced unlabeled cells and induced SPIO-labeled cells (Fig. 4A). The $[Ca^{2+}]_i$ can significantly increase when the extracellular $[K^+]$ is high in normal cells, so stimulating cells with KCL can be used to evaluate the cell state. After stimulating cells with 90 mM KCL, the fluorescence intensity of cells was increased significantly (Fig. 4B), and the morphologies of these neural-like cells are shown in Fig. 4C. Stimulation with 90 mM KCL caused an increase in $[Ca^{2+}]_i$ that reached a peak up to 260% (Fig. 4D). The relative fluorescence intensities of labeled and unlabeled neural-like cells were not significantly different (Fig. 4D). These results suggest that induced SPIO-labeled cells and unlabeled cells have the same differentiation ability.

DISCUSSION

Replacement treatment by neural-like cell transplant is a developing and promising neurological treatment strategy for CNS diseases. In many animal models of CNS diseases, such as

spinal cord injury, Parkinson's disease, Alzheimer's disease, and ischemic stroke, cellular replacement therapy using exogenous neuron cells has achieved initial success. For cell transplantation, MR imaging, as a non-invasive method with excellent resolution and without radiation, has been widely used to follow the migration and fate of implanted stem cells (Walczak et al., 2007). Farrell et al. (2008) have demonstrated that long term SPIO labeling of BM-MSCs uninhibited multi-lineage BM-MSCs differentiation (osteogenic, chondrogenic and adipogenic) and could be traced by MRI. Ke et al. (2009) also demonstrated that neural stem cells from BM-MSCs could be labeled with SPIO efficiently. In this study, we show for the first time that BM-MSCs from rabbits labeled *in vitro* with the superparamagnetic iron oxide (SPIO) contrast agent Reviost can be induced into neural-like cells without affecting the cells' viability and labeling efficiency.

In MR imaging tracking, the contrast agent labeling of stem cells or progenitor cells is a necessary step. Arbab et al. (2003) showed that cells could be labeled with 25-50 μ g/ml SPIO efficiently, thus allowing the maintenance of stable functional properties. Walczak et al. (2007) showed the same results, namely

that labeled cells could be tracked with MRI *in vitro* for extended periods of time following labeling. Other studies have also demonstrated that the SPIO labeling of BM-MSCs caused no short- or long-term toxic effects (Neriet al., 2008). In this study, we labeled BM-MSCs with 25 µg/ml SPIO and found that the efficiency of labeling was nearly 100%. These results suggest that BM-MSCs from rabbits could be successfully labeled *in vitro* using commercially and clinically approved Revoist.

However, our primary goal was to investigate whether SPIO-labeled BM-MSCs retained their differentiation capacity and could differentiate into neural-like cells *in vitro*. In this study, we showed that a combination of β-mercaptoethanol and FGF2 (40 ng/ml) may generate neural-like cells from SPIO-labeled BM-MSCs. According to previous studies (Woodbury et al., 2000), β-mercaptoethanol is capable of inducing the differentiation of neural-like cells from BM-MSCs. The induced cells may express neuron-specific enolase (NSE). FGF2 has been reported to benefit neural-lineage development (Murdoch and Roskams, 2013) and has been used extensively in differentiation protocols to promote a neuron cells and glial cells fate (Maricet al., 2007), which may be involved in the start of differentiation from BM-MSCs into neuron cells as promote differentiation factor (Pincus et al., 1998). Based on these studies, we acquired the NSE-positive and MAP2-positive neural-like cells from unlabeled and SPIO-labeled BM-MSCs, which showed the morphology of neural-like cells. Most importantly, neural-like cells derived from BM-MSCs exhibited electrophysiological characteristics of neurons *in vitro*. The results suggest that the SPIO labeling of BM-MSCs can differentiate into neural-like cells *in vitro*. After induction, the SPIO is still observed by Prussian blue staining. However, the induction time is only 24 h, and SPIO particles in the intracellular environment need to be observed for a longer time in future.

With regard to cell viability, some reports (Sun et al., 2012) have suggested that the SPIO labeling of BM-MSCs does not affect cell viability, and our results are consistent with these previous studies. Moreover, the viability of induced SPIO-labeled neural-like cells is as high as that of induced unlabeled cells, and it is very important to use induced SPIO-labeled neural-like cells for cell transplantation and tracking. We also evaluated the induced SPIO-labeled neural-like cells' states by testing the concentration of $[Ca^{2+}]_i$ using a confocal microscope. The abnormality of $[Ca^{2+}]_i$ is considered to be an important factor for intracellular calcium homeostasis and cell death in neuron cells (Hidalgo and Núñez, 2012). In induced unlabeled and labeled cells, the concentrations of $[Ca^{2+}]_i$ are at normal levels. After stimulating the cells with KCL, the $[Ca^{2+}]_i$ concentrations increased significantly and rapidly, suggesting that the cells were in a good state. The induced SPIO-labeled neural-like cells in a good state might be a potential candidate for seed cells for tissue engineering in nerve regeneration or for replacement therapy in CNS diseases. Moreover, we speculated that the neural-like cells derived from SPIO-labeled BM-MSCs might be in a good state *in vivo*.

In conclusion, our study showed that BM-MSCs from rabbits labeled with SPIO could be induced into neural-like cells with high cell viability and a good cell state and could be reliably tracked with *in vitro* MRI for extended periods of time. The results of this research may provide useful evidence for treating nervous system disorders and the future clinical applications of labeling BM-MSCs with SPIO. In addition, the neural-like cells derived from SPIO-labeled BM-MSCs represent a good cell source for regenerative medicine. In summary, the present study demonstrates that BM-MSCs from rabbits labeled with SPIO could be induced into neural-like cells. This strategy does not affect the induced efficiency and cell viability; therefore, it will

provide researchers with a simple and straightforward method of magnetically labeling stem cells to track the extent of differentiation *in vivo* following implantation.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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