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Intracellular labeling of mouse embryonic stem cell–derived neural progenitor aggregates with micron-sized particles of iron oxide

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Abstract

Background aims. Pluripotent stem cell (PSC)-derived neural progenitor cells (NPCs) represent an unlimited source for the treatment of various neurological disorders. NPCs are usually derived from PSCs through the formation of embryoid body (EB), an aggregate structure mimicking embryonic development. This study investigated the effect of labeling multicellular EB-NPC aggregates with micron-sized particles of iron oxide (MPIO) for cell tracking using magnetic resonance imaging (MRI). *Methods.* Intact and dissociated EB-NPC aggregates were labeled with various concentrations of MPIOs (0, 2.5, 5 and 10 μg Fe/mL). The labeled cells were analyzed by fluorescent imaging, flow cytometry and *in vitro* MRI for labeling efficiency and detectability. Moreover, the biological effects of intracellular MPIO on cell viability, cytotoxicity, proliferation and neural differentiation were evaluated. *Results.* Intact EB-NPC aggregates showed higher cell proliferation and viability compared with the dissociated cells. Despite diffusion limitation at low MPIO concentration, higher concentration of MPIO (i.e., 10 μg Fe/mL) was able to label EB-NPC aggregates at similar efficiency to the single cells. *In vitro* MRI showed concentration-dependent MPIO detection in EB-NPCs over 2.0–2.6 population doublings. More important, MPIO incorporation did not affect the proliferation and neural differentiation of EB-NPCs. *Conclusions.* Multicellular EB-NPC aggregates can be efficiently labeled and tracked with MPIO while maintaining cell proliferation, phenotype and neural differentiation potential. This study demonstrated the feasibility of labeling EB-NPC aggregates with MPIO for cellular monitoring of *in vitro* cultures and *in vivo* transplantation.

Key Words: *cell tracking, neural progenitor aggregates, iron oxide particles, pluripotent stem cells*

Introduction

Magnetic resonance imaging (MRI) provides high spatial resolution to monitor stem cell migration and differentiation *in vivo* and has emerged as a frontline technique to track the transplanted stem cells in the injured nervous system [1–3]. Among various types of contrast agents for MRI, iron oxides have demonstrated suitability because of their biocompatibility and the enhanced relaxivity and hypointensity compared with other molecules such as gadolinium chelates for cell detection [4,5]. Moreover, iron oxide particles can be modified for easy tracking by other modalities, particularly with fluorescent agents for immunohistochemical study, and can be encapsulated in polymers to reduce the cytotoxicity of iron oxide [6] while enhancing cytocompatibility [7]. Various types of iron oxide-based particles are currently tested as contrast agents for MRI tracking of stem cells, including superparamagnetic iron oxide (SPIO; 50–120 nm), ultrasmall iron oxide (10–50 nm) and micron-sized particles of iron oxide (MPIO; ≥0.75 μm) [8–10]. For the same iron concentration, MPIOs are reported to display enhanced detectability, magnetic susceptibility and relaxivities compared with nanosized particles [11–13]. Although labeling and tracking single cells have been demonstrated for MPIO, the ability to label multicellular aggregates

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and the consequent cellular effects have not been well characterized.

Neural progenitor cells (NPCs) derived from pluripotent stem cells (PSCs), including induced PSCs and embryonic stem cells (ESCs), represent unlimited sources for neural cells [14–16]. Although NPCs could be isolated from adult and fetal tissues, issues remain in terms of accessible cell number and ethical concerns [17]. PSC-derived NPCs have been shown to ameliorate the functions of injured or disordered neural tissues, such as stroke, Parkinson disease, Huntington disease and spinal cord injury [18–21]. Recently, brain organoids derived from induced PSCs have also been shown to provide neural disease models for pathological study [22]. For these applications, *in vitro* and *in vivo* tracking of PSC-derived NPCs using MRI can provide valuable information toward the clinical translations [3].

NPCs are usually generated from PSCs through the formation of aggregate structure known as embryoid bodies (EBs) [23]. Maintaining three-dimensional (3D) multicellular structures during differentiation, replating and maturation has been shown to enhance the function of PSC-derived neural cells [15,24]. The multicellular microenvironment is composed of cell-cell contacts, growth factors and extracellular matrix (ECM), mimicking the local stem cell niche [25]. Compared with NPCs derived in monolayer culture, 3D NPC aggregates generated a dense neurite network and displayed *in vivo*–like neuronal electrophysiology [24]. The endogenous ECMs inside NPC aggregates may also regulate neural differentiation due to the important role of ECMs in neurogenesis [26,27], and disruption of cell-cell contacts by dissociation has been shown to affect NPC proliferation, survival and differentiation [28–30]. Hence, labeling multicellular EB-NPC aggregates may better support neural tissue development and functional differentiation during MRI tracking.

To date, most studies have investigated *in vitro* labeling of single cells of PSCs, although the formation of aggregates or EBs from the SPIO-labeled undifferentiated PSCs has been demonstrated [31–34]. In addition, MPIOs have only been investigated for limited cell types, such as fetal and adult NPCs [8,11]. To understand the impact of MPIO on neural tissue development during cell tracking, this study investigates the intracellular labeling of PSC-derived NPC aggregates with MPIO at various concentrations. Labeling efficiency was assessed for both the intact and dissociated NPC aggregates. More importantly, the effects of MPIO incorporation on cell proliferation and neural differentiation were evaluated. MRI analysis was also performed to demonstrate the distinct contrast effect of MPIO after the labeling. Taken together, this study demonstrates the feasibility of labeling multicellular EB-NPC aggregates with MPIOs while maintaining cellular function, aiming at *in vitro* and *in vivo* cell tracking by MRI for treating and modeling neurological diseases using PSC-derived neural cells.

Methods

Undifferentiated ESC culture and generation of NPC aggregates

Murine ES-D3 line (American Type Culture Collection, Manassas, VA, USA) was maintained on 0.1% gelatin-coated six-well plates in a standard 5% CO_2 incubator. The expansion medium was composed of Dulbecco's Modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% ESC-screened fetal bovine serum (FBS, Hyclone, Logan, UT, USA), 1 mmol/L sodium pyruvate, 0.1 mmol/L β-mercaptoethanol, penicillin (100 U/mL), streptomycin (100 μg/mL) (all from Invitrogen), and 1000 U/mL leukemia inhibitory factor (Millipore, Billerica, MA, USA). The cells were seeded at $2–4 \times 10^4$ cells/cm^2 and sub-cultured every 2–3 days.

To derive NPCs, ESCs were seeded at 1×10^6 cells into Ultra-Low Attachment six-well plates (Corning Incorporated, Corning, NY, USA) in 3 mL of DMEM-F12 plus 2% B-27 serum-free supplement (Invitrogen) through the formation of EBs as previously reported [27,35]. At day 4, all-trans retinoic acid (Sigma-Aldrich, St Louis, MO) was added at 1 μmol/L in the differentiation medium. After another 4 days, the aggregates (referred as EB-NPCs) contained 60–70% nestin$^+$ and 80–90% Musashi 1$^+$ cells [27]. The EB-NPCs were used for MPIO labeling experiments either as intact aggregates or as dissociated single cells. The dissociation was performed by trypsinization using 0.05% Trypsin/EDTA (Invitrogen).

Intracellular labeling of EB-NPCs with MPIO

Approximately 5×10^5 EB-NPCs were replated as intact aggregates or single cells after dissociation into 24-well plates coated with Geltrex (Invitrogen) in DMEM-F12 plus 2% B-27. After 24 h, cell outgrowth was observed around the attached aggregates. The single EB-NPCs also attached to the surface. Both the attached aggregates and single cells were washed with phosphate buffered saline (PBS) and incubated with 1 mL of fresh DMEM-F12 plus 2% B-27 containing 0.25, 0.5 or 1×10^8 fluorescent (flash red, 660/690 nm) MPIOs/mL (Bangs Laboratories, Fishers, IN, USA, part no. ME03F/9772; Table I),