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Effects of iron oxide nanoparticles on cardiac differentiation of embryonic stem cells

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ABSTRACT

The therapeutic potential of transplantation of embryonic stem cells (ESCs) in animal model of myocardial infarction has been consistently demonstrated. The development of superparamagnetic iron oxide (SPIO) nanoparticles labeling and cardiac magnetic resonance imaging (MRI) have been increasingly used to track the migration of transplanted cells *in vivo* allowing cell fate determination. However, the impact of SPIO- labeling on cell phenotype and cardiac differentiation capacity of ESCs remains unclear. In this study, we demonstrated that ESCs labeled with SPIO compared to their unlabeled counterparts had similar cardiogenic capacity, and SPIO-labeling did not affect calcium-handling property of ESC-derived cardiomyocytes. Moreover, transplantation of SPIO-labeled ESCs via direct intra-myocardial injection to infarct myocardium resulted in significant improvement in heart function. These findings demonstrated the feasibility of *in vivo* ESC tracking using SPIO-labeling and cardiac MRI without affecting the cardiac differentiation potential and functional properties of ESCs.

Cellular transplantation has been emerging as a promising therapeutic option for cardiac regeneration [1]. While various cell types including skeletal myoblasts and adult mesenchymal stem cells have been tested as donor cells, embryonic stem cell (ESC) is the cell type proven to be truly pluripotent with developmental plasticity to differentiate into functional cardiomyocytes. Numerous experimental studies demonstrated that transplantation of ESCs and/or their cardiac derivatives improves ventricular function in rodent myocardial infarct model [2–8]. However, a non-invasive *in vivo* imaging technique to track transplanted cells is required to monitor the long-term cell fate and local retention within the heart after transplantation.

Cardiac magnetic resonance imaging (MRI) is a robust diagnostic tool to provide high quality functional and morphological information and is the current reference standard technique for cardiac assessment. Employing superparamagnetic iron oxide (SPIO) nanoparticles to label cells prior to transplantation renders them MRI-visible and distinguishable from surrounding tissues [9–15]. Therefore, SPIO labeling of transplantable cells and their detection by cardiac MRI appears to the imaging technique of choice for long-term *in vivo* cell tracking within the recipient heart as well as simultaneous cardiac functional assessment. However, concerns about the potential alteration of the phenotype SPIO-labeled cells remain; in fact, it has been demonstrated that SPIO-labeling restricts the differentiation plasticity of mesenchymal stem cells [16,17]. Nonetheless, the effects of SPIO-labeling on the cardiac differentiation potential of ESC and the functionality of ESC-derived cardiomyocytes remain unclear. We therefore assessed the *in vitro* and *in vivo* cardiac differentiation capacity of SPIO-labeled ESCs.

Materials and methods

Cell culture and in vitro cardiac differentiation. Genetically modified enhanced green fluorescent protein (GFP)-expressing mouse ESC line was cultured as previously described [18]. Briefly, undifferentiated ESCs were cultured on irradiation-inactivated mouse embryonic feeders in Dulbecco's modified Eagle's medium (DMEM, GIBCO; Carlsbad, CA) supplemented with 15% fetal bovine serum (Gibco; Carlsbad, CA), 2 mM L-glutamine (Invitrogen), 1 mM sodium pyruvate (Sigma), 0.1 mM ß-mercaptoethanol (Sigma), 0.1 mM non-essential amino acids (Invitrogen), and 1000 U/ml

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leukemia inhibitory factor (Chemicon, Temecula, CA). To induce cardiac differentiation, embryoid bodies were formed with hanging drop method in the absence of leukemia inhibitory factor and feeder cells, which were then plated on 0.1% gelatin-coated tissue culture dishes after suspension for 7 days.

SPIO-labeling protocol and cell viability. Undifferentiated ESCs were incubated in serum free culture medium containing SPIO (50 μg/ml) (Center for Molecular Imaging Research, MGH, MA) and poly-L-lysine (PLL; 2.0 μg/mL) (Sigma, St. Louis, MO) at 37 °C for 24 h as previously described [19]. Prussian blue staining was used to determine labeling efficiency: samples of labeled ESCs were fixed with 4% glutaraldehyde, washed, incubated for 30 min with 2% potassium ferrocyanide (Perls reagent) in 6% hydrochloric acid. ESC viability after SPIO-labeling was assessed using Trypan blue exclusion, and the percentage of live cells was calculated by counting at least 200 cells in five randomly chosen microscopic fields.

Flow cytometry analysis. The percentage of ESC-derived cardiomyoyctes was quantified by FACS analysis. Briefly, embryoid bodies were dissociated by 0.05% Trypsin-EDTA, washed by differentiated medium and the cells were permeabilized by Cytofix/Cytoperm permeabilization kit (BD Biosciences, San Diego, CA). Cells were then stained with monoclonal anti-troponin T (NeoMarker, Fremont, CA), sacromeric actin and actinin (Sigma, St Louis, MO, USA) using anti-mouse IgG H+L-PE as a secondary antibody staining (Beckman Coulter, Fullerton, CA, USA). Analysis was performed with Beckman Coulter FC500 flow cytometer in which 50,000 events were counted.

Confocal calcium imaging. ESC-derived cardiomyocytes were loaded with 5 μM Fluo-3 AM (Sigma) for 45 min at 37 °C in Tyrode solution consisting of (mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose and 10 HEPES at pH 7.4. Calcium transient of single ESC-derived cardiomyocytes were recorded with a confocal imaging system (Olympus Fluoview System version 4.2 FV300 TIEMPO) mounted on an upright Olympus microscope (IX71) with temporal resolution of the line scan at 274 frames per second (2000 scan per 7.3 s), and were then quantified as the background subtracted fluorescence intensity changes normalized to the background subtracted baseline fluorescence using Image J.

Acute myocardial infarct and cell transplantation. Animal experiments were approved by the Committee on the Use of Live Animals in Teaching and Research at the University of Hong Kong. Adult ICR mice were anesthetized, endotracheally intubated, and mechanically ventilated. Acute myocardial infarction was created by ligation of the left anterior descending artery through a left thoracotomy. The animals were randomly allocated to control group ($n = 5$) or cell transplantation group ($n = 5$). The border zone was injected with a Hamilton syringe containing 10 μl of culture medium for control group, or 3×10^5 SPIO-labeled undifferentiated ESCs in 10 μl of culture medium (cell transplantation).

Cardiac MRI. One week to 10 days after the procedure, cardiac MRI was performed with a 7.0 Tesla MRI scanner (Bruker PharmaScan, Bruker BioSpin, Germany). Multi-slice multi-frame FALSH sequences were performed for measurement of left ventricular

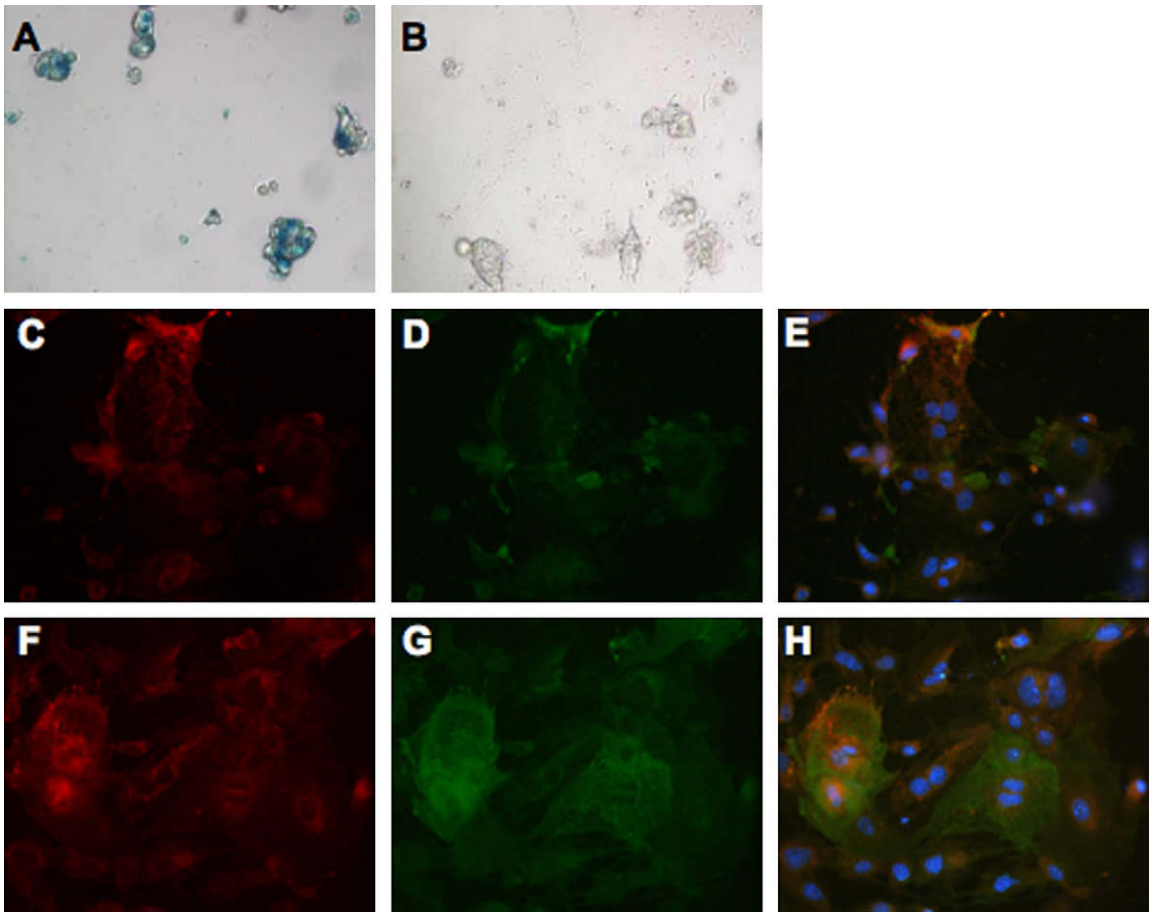

Fig. 1. Cardiac differentiation of SPIO-labeled and unlabeled cells. Prussian Blue stain of (A) clumps of SPIO-labeled undifferentiated ESCs, and (B) unlabeled undifferentiated ESCs (intracellular iron particles as blue deposits); immunostaining of SPIO-labeled ESC-derived cardiomyocytes (C–E) and unlabeled ESC-derived cardiomyocytes (F–H) with anti-troponin-T (red) (C and F), anti-α-sarcomeric-actin (green) (D and G), and overlap with DAPI nuclear stain (blue) (E and H).

ejection fraction. Four slices covering the whole heart were planed at short-axis orientation. The parameters were: TR/TE = 20.6/2 ms, flip angle = 30°, FOV = 4 cm^2, matrix size = 192 × 192, cardiac frames = 12, slice thickness = 1 mm with slice gap = 0.2 mm, NEX = 8. T2-weighed imaging, a highly sensitive sequence to detect the susceptibility artifacts (hypointensities) generated by the iron-labeled cells, was performed to visualize SPIO-labeled cells. Imaging planes were set at short-axis and 2-chamber long-axis, respectively. The parameters were: TR/TE = 80/2.5 ms, flip angle = 30°, FOV = 4 cm^2, matrix size = 256 × 256, slice thickness = 0.8 mm with 0 mm slice gap, NEX = 4.

Histological analysis. Hearts were fixed with 10% buffered formalin and embedded in paraffin, which were then sectioned to 5-μm slides and incubated with BD Living ColorsTM A.v. Peptide Antibody (BD Biosciences), followed by hematoxylin and eosin staining, anti-GFP (Santa Cruz Biotechnology, San Diego CA), and anti-troponin-T (1:100, Lab Vision, Fremont CA). ESC engraftment was confirmed by identification of GFP expression under fluorescence microscopy. Fluorescence immunostaining for troponin-T was examined and photographed under fluorescence microscopy (red). The images of heart sections were captured by Axisoplus image capturing system (Zeiss, GmbH).

Statistical analysis. Continuous variables are expressed as means ± standard deviation. Statistical comparisons were performed using Student's *t*-test. Calculations were performed with SPSS (version 14.0). A p value <0.05 was considered statistically significant.

Results

SPIO-labeling is a well-tolerated and highly efficient procedure

Consistent with previous reports with iron-labeling in mesenchymal stem cells [20], *in vitro* culture of ESCs appeared to be unaffected by SPIO-labeling procedure. Incubation of ESCs with 50 μg/ml SPIO and 2.0 μg/mL PLL at 37 °C for 24 h did not have any significant effect on ESC viability as 92 ± 4% ESCs remained viable as determined by Trypan blue exclusion assay. In addition, the labeling procedure was highly effective as more than 90% of ESCs demonstrated intracellular iron deposits by Prussian Blue

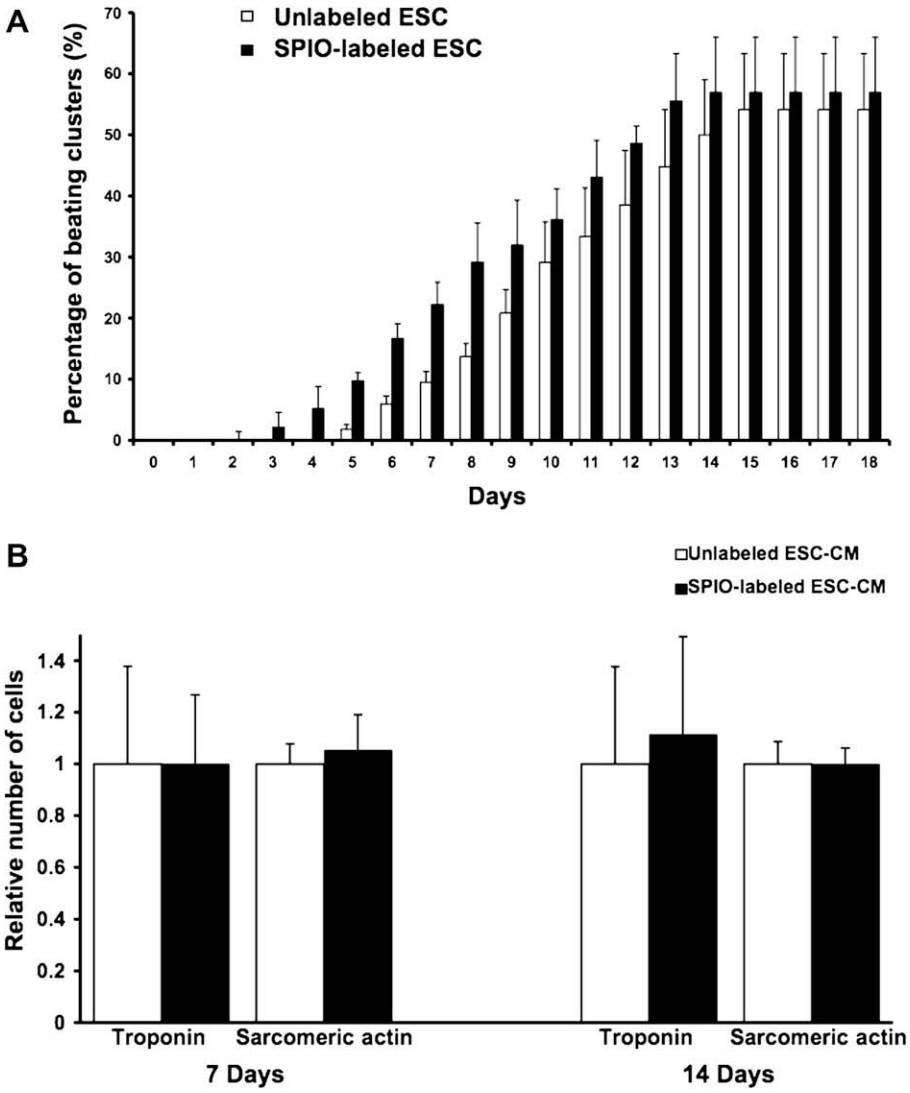

Fig. 2. Effects of SPIO labeling on *in vitro* cardiac differentiation. (A) Percentage of beating clusters, and (B) Relative number of ESC-derived cardiomyocytes (troponin +ve cells or sarcomeric actin +ve cells) on day 7 and 14 as determined by flow cytometry. Data of triplicate experiments were expressed as mean ± standard deviation, calculated as the relative number of troponin +ve cells or sarcomeric actin +ve cells of control at baseline.

staining (Fig. 1A), which was not detected in unlabeled ESCs (Fig. 1B).

SPIO-labeling did not affect the in vitro cardiac differentiation of ESCs

To determine the cardiogenic potential of labeled cells, *in vitro* cardiac differentiation from SPIO-labeled and unlabeled undifferentiated ESCs were induced with standard hanging drop method. On day 14, the presence of cardiac differentiation in both SPIO-labeled and unlabeled ESCs was confirmed with immunostaining of cardiac markers including troponin-T and α-sacromeric actin (Fig. 1C–H). In fact, the number of embryoid bodies containing spontaneously beating outgrowths of SPIO-labeled ESCs did not differ significantly with unlabeled ESCs (57.0 ± 9% vs. 54.2 ± 9% on day 18, $p > 0.05$) (Fig. 2A). Furthermore, the number of cardiomyocytes as identified as troponin positive and/or α-sacromeric actin positive cells differentiated from SPIO-labeled ESCs and unlabeled ESCs as determined by flow cytometry analysis were comparable on day 7 and 14 (Fig. 2B).

Calcium handling of ESC-derived cardiomyocytes was not affected by SPIO-labeling

To provide additional information on the effects of SPIO-labeling on ESC-derived cardiomyocytes, calcium oscillations in single SPIO-labeled and unlabeled ESC-derived cardiomyocytes with

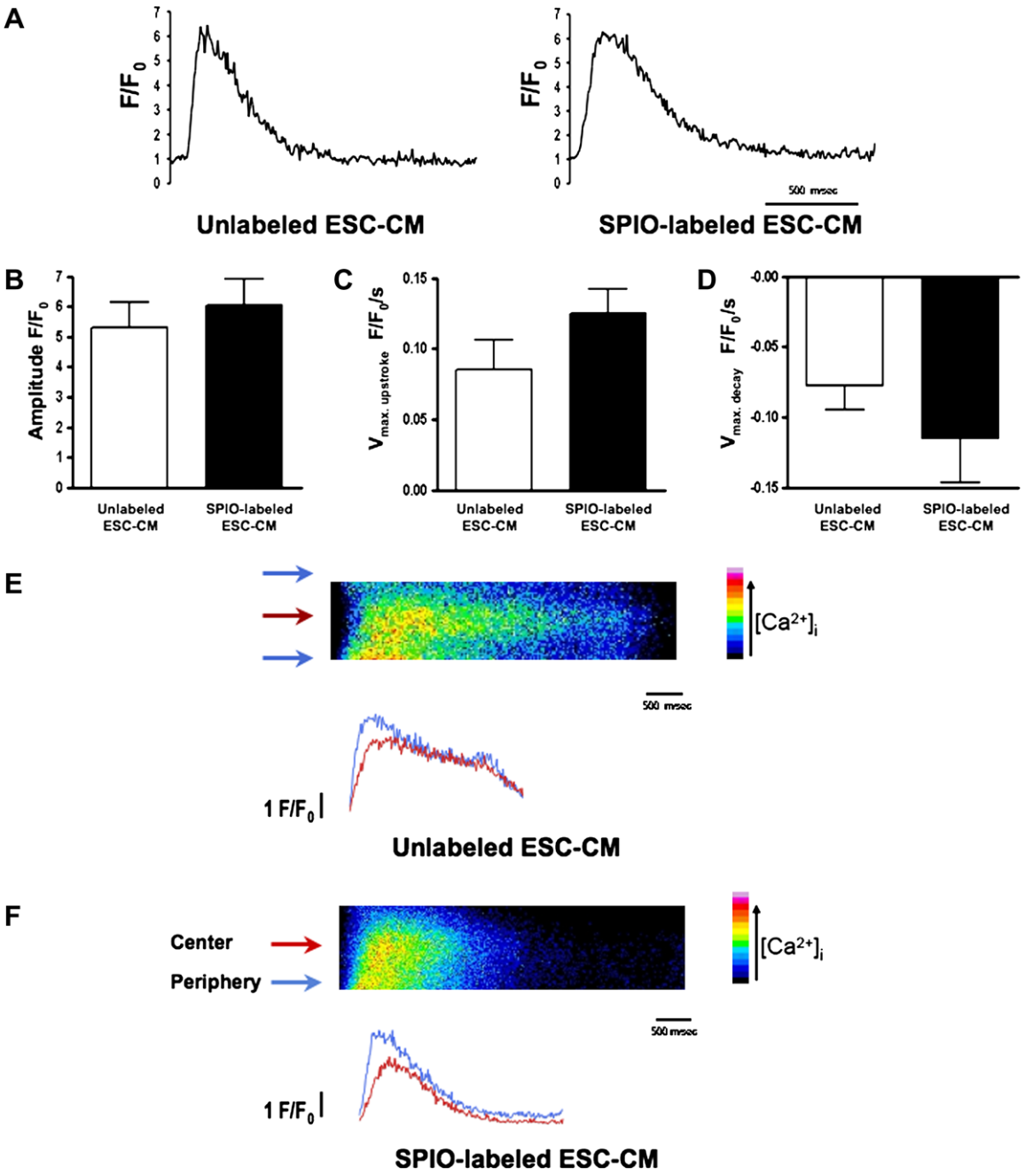

Fig. 3. (A) Representative tracings of Ca^{2+} transients in ESC-CM with and without SPIO-labeling. (B) Amplitude, (C) Maximal upstroke velocity ($V_{max\ upstroke}$), (D) Maximal decay ($V_{max\ decay}$), (E) Transverse line scan images of Ca^{2+} transients in unlabeled ESC-CM, and (F) Transverse line scan images of Ca^{2+} transients in SPIO-labeled ESC-CM. Unpaired *t*-test was performed between unlabeled and SPIO-labeled group.

spontaneous beating were recorded with confocal microscopy. Fig. 3A depicts that calcium transients from SPIO-labeled ESC-derived cardiomyocytes were not different from unlabeled counterparts in term of amplitude (Fig. 3B), maximal upstroke velocity (Fig. 3C), and maximal decay velocity (Fig. 3D) ($p > 0.05$). Furthermore, the temporal and spatial patterns of calcium transients of SPIO-labeled and the unlabeled ESC-derived cardiomyocytes were in fact similar. Specifically, the rise of calcium in the periphery of both SPIO-labeled and unlabeled ESC-derived cardiomyocytes was faster compared with their cell centers exhibiting a typical U-shape calcium wave front typical of t-tubule deficit cardiomyocytes (Fig. 3E and F) [21–23]. Taken collectively, no detectable differences in calcium handling occurred after SPIO-labeling.

Cardiac MRI and histological findings after transplantation

To test the *in vivo* functional capacity of SPIO-labeled undifferentiated ESCs for myocardial restoration, SPIO-labeled undifferentiated ESCs were transplanted to the border zone of mice with acute myocardial infarction with direct intramyocardial injection. One week after the procedure, cardiac MRI indicated that left anterior descending artery ligation resulted in markedly decreased LVEF in MI group from 63 ± 10% to 10 ± 3% ($p < 0.05$). However, transplantation of undifferentiated ESCs significantly improved the LVEF (34 ± 13%) as compared with MI group ($p < 0.05$). In addition, the LV end diastolic volume of cell transplantation group was significantly smaller (0.05 ± 0.02 ml vs. 0.09 ± 0.02 ml, $p < 0.05$) compared with MI group; likewise, the LV end systolic volume was also significantly smaller in cell transplantation group (0.03 ± 0.01 ml mm vs. 0.05 ± 0.02 ml, $p < 0.05$) compared with MI group. More importantly, areas of well-defined hypointensities were identified at the injection sites with cardiac MRI, and SPIO-labeled undifferentiated ESCs were identified in cardiac MRI as areas of well-defined hypointensities at the injection sites (Fig. 4A), whereas no such hypointensities were observed in MI group (Fig. 4B). Histological section corresponding to those well-defined hypointensities from cell transplantation group demonstrated discrete clusters of GFP positive cells (Fig. 4D). Moreover, troponin-T positive cells were also identified within these GFP positive cell clusters, suggesting *in vivo* cardiac differentiation from ESCs (Fig. 4E and F).

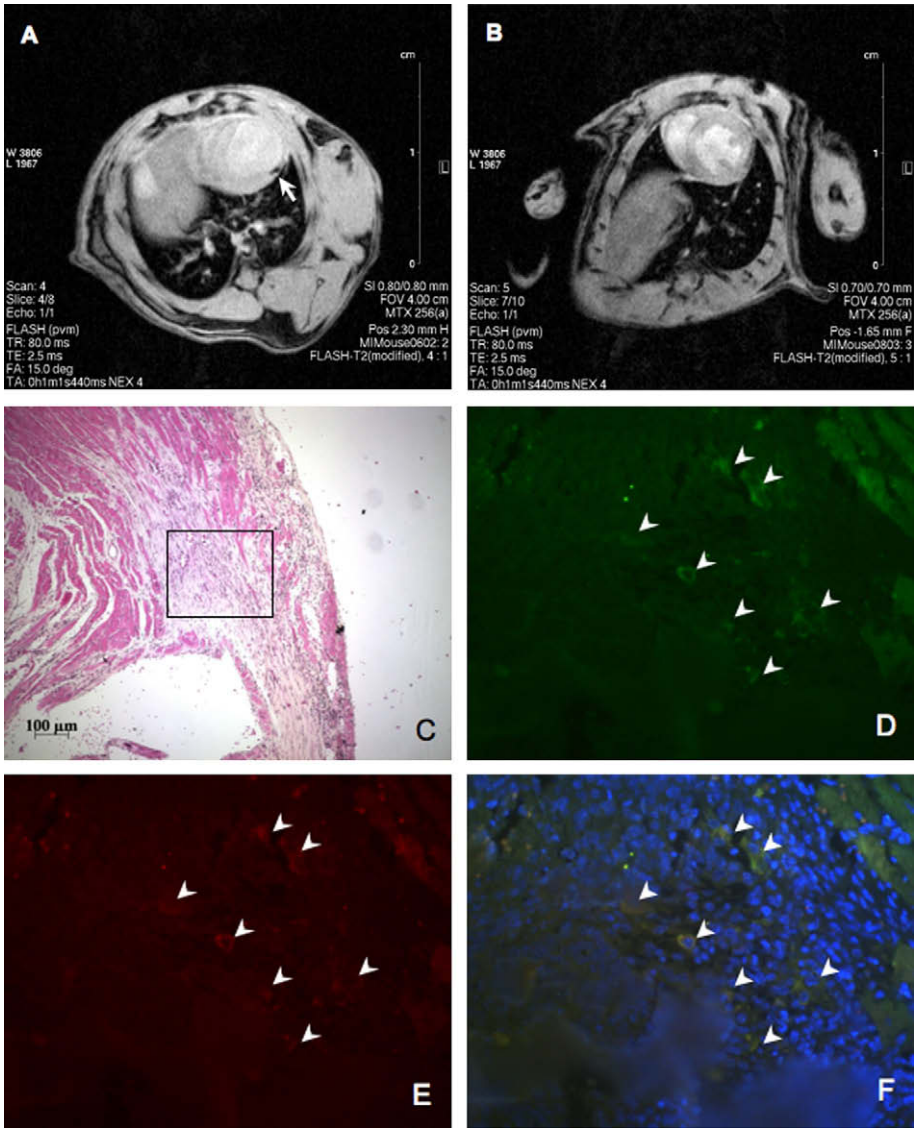

Fig. 4. Well-defined hypointensities at the injection site (arrow) were identified by cardiac MRI only in cell transplantation group (A) but not in MI group (B), suggesting the retention of SPIO-labeled undifferentiated ESCs. (C–F) MI heart with SPIO-labeled undifferentiated ESC transplantation: (C) H and E staining of the MI region, square indicated the region of higher power magnification of D to F. (D) Anti-GFP, (E) Anti-troponin-T and (F) Merge of the two staining. Asterisks indicated the surviving native cardiomyocytes, and arrows indicated cells with colocalization of GFP and troponin-T.

Discussion

Our results demonstrate that SPIO-labeling allows *in vivo* non-invasive tracking of transplanted ESCs over a time course of 1–2 weeks without any detectable effects on cardiac differentiation, calcium handling, and *in vivo* therapeutic benefits.

Advances in stem cell research have offered great promises to the field of regenerative medicine, especially to organs whose intrinsic regenerative capacity is limited, for example the heart. To date, cardiac MRI is a standard technique in cardiac imaging. It not only provides detailed anatomical and functional cardiac assessment, but also allows non-invasive *in vivo* tracking of transplanted cells at near microscopic resolution, thus facilitating cell fate determination. In order to detect transplanted cells with MRI, magnetic resonance contrast to cell labeling prior to transplantation has been developed. However, several reports have suggested that the labeling procedure may affect the differentiation potential of mesenchymal stem cells. For instance, mesenchymal stem cells labeled with SPIO demonstrated a marked inhibition of chondrogenesis despite preserved adipogenic and osteogenic differentiation [17]. More recently, Krejci and his colleagues have demonstrated that although SPIO-labeling had no effect on cell viability and self-renewal, the procedure resulted in partial inhibition of cavitation of embryoid body during ESC differentiation and enabled neuronal differentiation from ESC [24]. This deviation of ESC differentiation may undermine the potential beneficial effects of ESC transplantation for cardiac differentiation. On a contrary, as demonstrated in the present study, SPIO-labeling does not result in any observable effects on *in vitro* cardiac differentiation potential of ESCs, calcium handling properties of ESC-derived cardiomyocytes, or therapeutic effects of ESC transplantation to infarcted myocardium. These suggest that the potential adverse effects of SPIO-labeling on cardiac differentiation and its potential therapeutic use were in fact modest.

Taken collectively, SPIO-labeling when coupled with cardiac MRI appears to be a highly effective and reliable way for *in vivo* ESC tracking for cell therapy for cardiac regeneration. The lack of adverse effects of SPIO-labeling on the cardiac differentiation potential of ESC and cardiac phenotype of ESC-derived cardiomyocytes makes it an invaluable tool for research in stem cell therapy.

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