Novel MRI Contrast from Magnetotactic Bacteria to Evaluate In Vivo Stem Cell Engraftment

Ji-Hye Jung, Yuko Tada and Phillip C. Yang

1 The Limited Stem Cell Engraftment Reduces Therapeutic Efficacy

The tremendous potential of human pluripotent stem cells (hPSCs) for regenerative medicine today is a topic of intense investigation to scientists worldwide. Since the first report of human embryonic stem cells (hESCs) in 1998, steady progress has been made as the cornerstone of stem cell research [1]. In 1998, Thomson and colleagues succeeded in isolating the hESCs from the inner cell mass of blastocysts. The pluripotency of the hESCs are considered to be a promising source to achieve any cell replacement therapy. They exhibit unlimited self-renewal and are enable to differentiate into every cell type in the adult body. The hESCs offer the possibility of cell therapy for many incurable and degenerative diseases. They hold great promise for tissue engineering and drug discovery applications. However, a major limitation of hESC research is the constant dispute regarding immune rejection, tumorigenesis and ethical concerns. However, in 2007, Shinya Yamanaka of Japan reported a landmark discovery, the human induced pluripotent stem cells (iPSCs), which has broadened the horizons of regenerative medicine. iPSCs are generated when the Yamanaka Factors (sex determining region Y-box 2 (Sox2), Krüppel-Like Factor 4 (Klf4), V-Myc Avian Myelocytomatosis Viral Oncogene Homolog (cMyc) and POU Class 5 Homeobox 1 (Oct3/4)) are transfected into adult somatic cells.

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The cells are reprogrammed to embryonic stage with pluripotent properties [2]. The discovery of the iPSCs led to revolutionary changes in stem cell research. This breakthrough not only addressed the ethical limitations of hESCs but also advanced the development of personalized medicine. Astounding progress has been made, leading to a variety of pharmacogenomics approaches. Disease-specific iPSCs have also offered great promise with profound insights into the pathophysiology of previously incurable diseases [3–6].

Although the challenges of the immune rejection and ethical issues of the hESCs were obviated by the advent of somatic cell reprogramming, significant challenges remain in clinical translation. One of the major problems is to establish safe and effective methods to differentiate the hPSCs into a pure population of specific lineage in vitro. The proper manipulation of hPSCs is not completely understood even though several essential differentiation factors have been identified. Another major hurdle is the limited cell engraftment in vivo following delivery. Although researchers today are able to generate a specific cell lineage, it is difficult to reach therapeutic benefit when the injected cells do not engraft into the recipient's organ of interest [7].

Stem cell therapy should prioritize patient safety and tolerance. Stem cells are associated with number of risks and require successful integration of the transplanted cells in the desired microenvironment of the target tissue. In this chapter, we will focus on the challenges of stem cell engraftment in the heart and discuss a novel in vivo MRI contrast agent based on magnetotactic bacteria, which enables high live cell specificity.

2 Technological Development Is Required to Assess Stem Cell Engraftment

Myocardial infarction (MI) usually results in irreversible myocardial cell loss and heart failure due to the disruption of blood supply. Despite recent advances in the standard of care of MI, injured myocardium and scar tissue still cannot be restored [8]. It has been shown that stem cell therapy in the acute phase of MI attenuates cardiomyocyte apoptosis and local inflammatory response while promoting local neoangiogenesis and myocardial perfusion [9, 10]. In the late phase of MI, cell therapy may replace the dead myocardium with viable cardiomyocytes, smooth muscle cells, and endothelial cells to reduce scar formation [11]. These discoveries have been followed by clinical trials with transplantation of adult somatic stem cells in patients with acute MI [12].

In one of the first reported pre-clinical studies of human pluripotent stem cell-derived cardiomyocytes (CMs), Kehat and colleagues demonstrated that the hESC-derived CMs (hCMs) transplanted into pigs have shown their potential to function as biological pacemakers in electrophysiologically silenced or atrioventricular (AV) blocked hearts [13]. One of the technical challenges in the

implementation of hCMs was the purity and yield of the differentiated hCMs. Efficient and reproducible methods of mice and hESC differentiation have been introduced by manipulating the cardiac-specific signaling pathways in embryonic development [14]. Various differentiation methods including, genetic modifications, cytokines, and small molecules have been conducted to obtain a homogeneous and functional hCMs. Recently, hCMs, successfully generated on a large scale, demonstrated reliable engraftment and restoration of damaged heart tissue in a primate MI model [15]. These results seemed promising; however, clinical translation of is still limited by tumorigenesis, immune rejection, genetic instability, ventricular arrhythmia, and ethical considerations.

Similar to the hESCs, iPSCs are pluripotent and generate all three germ layers. The cardiogenic potential of the iPSC population has been studied in both mice [16] and human derivatives [17]. The iPSCs have been differentiated into CMs (iCMs) and other cardiovascular cells such as smooth muscle, fibroblast, and endothelial cells.

Since the advent of stem cell transplantation, intense efforts have been made to track the transplanted stem cells. However, the inability to localize and assess the viability of the cells delivered into the heart is recognized as one of the major limitations for clinical translation of cell therapy [8, 18]. Such information is important to evaluate the engraftment of iCMs and their therapeutic efficacy in vivo. These challenges necessitate novel imaging technologies to conduct reliable translational investigation of any hPSC derivatives. An ideal non-invasive platform will enable high sensitivity, optimal spatial and temporal resolution, and exquisite tissue contrast. Longitudinal visualization of live cell specificity to confirm the engraftment of the delivered cells within the host myocardium is necessary.

3 Imaging Technology Monitors In Vivo Stem Cell Engraftment

3.1 Imaging Modalities Used for In Vivo Stem Cell Tracking

At present, no available imaging technology satisfy the ideal properties of in vivo stem cell tracking [19]. Multiple techniques are used currently for in vivo monitoring of labeled cells, including magnetic resonance imaging (MRI), single photon emission computed tomography (SPECT), positron emission tomography (PET), and optical imaging such as fluorescence imaging (FLI) and bioluminescence imaging (BLI). Each modality has individual advantages and drawbacks related to spatial resolution, sensitivity, clinical availability, safety, and labeling method. Cell labeling methods are largely divided into direct and indirect labeling. In direct labeling, tracers are bound to the cell surface or transported inside the cells by diffusion, endocytosis, phagocytosis, or active transport. While direct labeling

needs no gene editing, one of the disadvantages is that the produced signals can be diluted by cell division or proliferation. Another critical problem is that the signal may persist regardless of the cell viability because of the uptake of dead cells by phagocytizing cells [20]. In contrast, in indirect labeling, reporter genes such as firefly luciferase (Fluc) and/or herpes simplex virus thymidine kinase (HSV-tk)) are transfected into the target cells to express specific proteins that react with the administered substrates, leading to the emission of signals [21]. Thus, the detected signal strength correlates with cell viability [22]. However, genetic modifications are not desirable for clinical translation because of the problems including inflammatory toxicity and carcinogenesis of the virus vectors.

In BLI, bioluminescent gene, Fluc, are inserted into the genome of the target cell. Expressed enzyme catalyzes the conversion from administered luciferin into oxiluciferin, releasing photon energy. In FLI, fluorescence signals from the fluorescent protein such as the enhanced green fluorescent protein (eGFP) are detected. Although their sensitivity is excellent and long-term cell monitoring is possible, the application of BLI and FLI is limited to the small animals because of the limited signal penetration. PET/SPECT generally utilize direct radioscintigraphic cell labeling of high-energy gamma-emitting radiotracers. Radionucleotides such as 111In-oxine, which passively diffuse into the cytosol, are used for SPECT cell tracking. PET achieves better contrast and spatial resolution than SPECT. The advantage of PET/SPECT is the exquisite signal sensitivity and transmission in addition to the ease of clinical translation. Although tracers with different half-life and decay profiles such as FDG, Cu, Zr, and Mn are used, the short half-life and the radioactive tracers makes PET unsuitable for long-term cellular tracking [23]. On the other hand, MRI has an exquisite spatial resolution compared to PET/SPECT or BLI/FLI; however, the sensitivity of MRI is relatively low. Superparamagnetic iron oxide particles (SPIONs), improves the capability of MRI to detect stem cells in the myocardium longitudinally and offers the only suitable agent for clinical translation of stem cell tracking [24, 25]. Higher sensitivity makes SPIONs feasible for molecular MRI [26] while also combining with precise evaluation of cardiac function and myocardial tissue characterization [27]. This capability enables precise cell localization and assessment of their specific regional effects in the different areas of myocardial injury to correlate cell engraftment with therapeutic efficacy [28, 29]. Stem cell labeling with magnetic particles can also be combined with magnetically-targeted stem cell delivery, which utilize external magnet devices manipulate cells to guide cells to the target lesion sites [30, 31].

3.2 Superparamagnetic Nanoparticle Labeled Cells Enable In Vivo Monitoring with MRI

The SPIONs are the magnetic nanoparticles most commonly used to label cells for MRI tracking. These nanoparticles have been widely used to detect inflammation

since they are taken up by phagocytizing cells after their administration into the body [32] (Fig. 1). Supraparamagnetic property of SPIONs accelerates transverse relaxation of magnetized protons by producing local field inhomogeneity, called T2* relaxation effect. SPION containing tissues are shown as signal defect on T2*-weighted gradient echo sequence consisting of long TR/TE and low flip angle [33]. In general, exact cell quantification using MRI is difficult [20]. However, accumulation of the SPION-labeled cells in the tissue can be quantified by acquiring several gradient echo sequences at different TEs and calculating T2* decrease from the T2* decay curve [34].

The structure of SPIONs consists of the functional core, coating, and surface properties, affecting the efficiency of cellular uptake, distribution, metabolism and potential toxicity. The functional core with superparamagnetic property is a single-domain iron oxide molecule (<10 nm) consisting generally of Fe_3O_4 (magnetite), gamma-Fe_2O_3 (maghematite), or alpha-Fe_2O_3 (hematite) [35]. SPIONs are coated with a biocompatible polymer such as dextran and carboxydetran polymer to prevent aggregations, structural changes, and degradation when exposed to the biological system [36, 37]. SPIONs usually label cells in a direct manner. Clathrin mediated endocytosis serves as the main mechanism for the most cell types including stem cells without phygocytizing capacity, which is facilitated by cationic compounds such as poly-L-Lysine and protamine [38–40]. Magnetoeloctroporation and magnetosonoporation are alternatively used to allow rapid incorporation into freshly isolated cells [41, 42]. In certain culture conditions, mesenchymal stem cells (MSCs) are known to regain its innate phagocytic capacity and take up SPIONs without facilitators [43, 44]. Antibody mediated targeting such as anti-CD34 antibody coated magnetic particles for hematopoietic stem cells could be another promising method to label target stem cells specifically in vivo [45, 46].

SPIONs are classified into standard SPION (SSPIO, 50–150 nm), ultra-small SPION (USPIO, 10–50 nm), and very-small SPION (VSPIO, <10 nm) based on

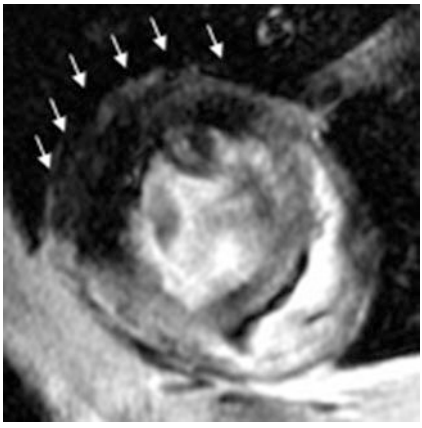

Fig. 1 Myocarditis detected by ferumoxytol. The short axis cardiac MRI was taken from the rat induced with autoimmune myocarditis. Myocardial inflammation where ferumoxytol accumulates is clearly detected as signal void on GRE image (arrows)

Table 1 Examples of clinical SPION

Generic name	Brand name	Classification	Coating	Diameter (nm)	Status
Ferumoxide	Feridex/ Endorem	SSPIO	Dextran	120–180	Discontinued
Ferumoxtran-10	Combidex/ Sinerem	USPIO	Dextran	15–30	Discontinued
Ferucarbotran	Resovist/ Cliavist	SSPIO	Carbodextran	60	Discontinued
Ferucarbotran	Supravist	USPIO	Carbodextran	21	
Feruglose	Clariscan	USPIO	Pegylated starch	20	Discontinued
Ferumoxytol	Faraheme	USPIO	Carboxymethyl dextran	30	FDA approved

their hydrodynamic diameters [47]. Optimal particle size of SPIONs for intravenous administration is 10–100 nm [48]. SPIONs are clinically used as contrast agents for evaluating blood volume fraction, perfusion, and cancer metastasis (Table 1). Unfortunately, most of them were discontinued due to the safety reason or infrequent use. Ferumoxytol is the only clinically available SPION approved by FDA as an iron replacement for the patients with renal anemia. In addition, various non-clinical SPIONs are available for experimental use. For instance, Molday (BioPAL, Inc) is an USPIO MRI contrast agent with a magnetic core and hydrodynamic sizes of around 8 and 35 nm, respectively, which is designed for cell labeling and requires no transfection reagents.

The major limitation of SPIO-labeling is the maintenance of in vivo signal after cell death. There is no reliable MRI viability signal from the transplanted cells [49, 50]. They generate false positive signals even after transplanted cell death. Persistence of significant MRI signal derived from ferumoxide-containing macrophages is seen despite only few viable stem cells 3 weeks after transplantation, indicating that the MRI of ferumoxide-labeled stem cells does not demonstrate long-term stem cell engraftment signal in the heart [51]. Another limitation is the decrease in the SPIO signal as the transplanted stem cells divide, presenting difficulty in tracking the cells in longitudinal studies [52]. SPION has generally low toxicity to the cells [40]. Nevertheless, some in vitro experiments suggested the possibility that iron oxide or transfection facilitators could cause cellular stress (mitochondrial dysfunction, ROS generation), alterations in gene expression and cellular differentiation (promoting chondrogenic, osteogenic, and adipogenic differentiation of mesenchymal stem cells), decrease in cell proliferation, and promotion of pro-inflammatory environment [25].

4 Magnetotactic Bacteria Evaluate Live Cell Specificitiy of Engrafted Stem Cells

It is important to evaluate stem cell engraftment for clinical translation of stem cell therapy. The yield of the cell engraftment in the myocardium characterizes the most essential process of post-transplantation biology. With the potential emergence of cell therapy for heart failure, accurate in vivo evaluation of stem cell engraftment and the resultant effects on the regional myocardial viability and function may represent a critical measure of therapeutic efficacy [53, 54]. Thus, live cell specific technology is necessary to advance regenerative medicine.

4.1 Development of Magnetotactic Bacteria

Magnetotactic bacteria (MTB) are a polyphyletic group of gram-negative prokaryotes. Members of this group are characterized by their ability to align themselves and exist along a magnetic field. They owe this ability to the magnetosome, a specific intracellular structure that contains crystals of magnetic metals (usually magnetite or greigite) bounded by a membrane [55].

The magnetosome, the most important and defining intracellular component of the MTB, is a membrane-bound inorganic magnetic iron crystal. Since its discovery, researchers have recognized it as a true prokaryotic organelle because of its distinct complexity that rivals that of eukaryotic counterpart. Despite the universal presence of the magnetosomes within the MTB, there are inter-species difference in their size, composition and morphology. Morphologically, a few different forms of magnetosomes have been observed across species by electron microscopy (Fig. 2). In most strains of MTB, the magnetosomes are fixed in several chains that are in turn bound to the cell membrane in order to limit magnetostatic energy. The most commonly used minerals in the magnetosomes are, as mentioned previously, magnetite or greigite. However, many other iron sulfides have also been found within sulfide producing MTBs, such as cubic FeS and tetragonal FeS [57]. The researchers recognized that the magnetosome formation has few different processes. The most recent proposed model lists three discrete steps. The first step consists of the creation of magnetosome membrane, which invaginates out of the cytoplasmic membrane (primed by GTPase), as well as the lining up of these vesicles along cytoskeletal filaments in order to form a chain structure. Second, iron is transported into the cell. This transport is strictly regulated by an oxidation-reduction system because of the potentially dangerous effects of having too much intracellular iron. In the third and final step, magnetosome proteins regulate morphology and trigger magnetite crystal nucleation (Fig. 3) [58].

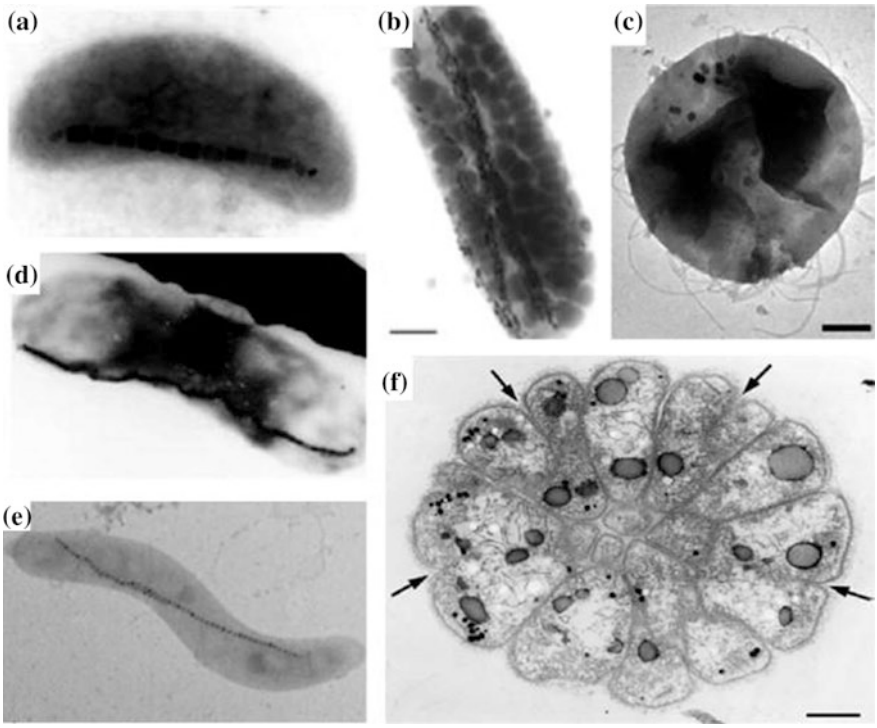

Fig. 2 Various morphology of MTB. **a** vibrios; **b** and **d** rods (Bar = 1.0 μm) and **c** coccoid (Bar = 200 nm); **e** spirilla; and **f** multicellular organism (Bar = 1.0 μm). Reprinted with permission from Yan et al. [56]

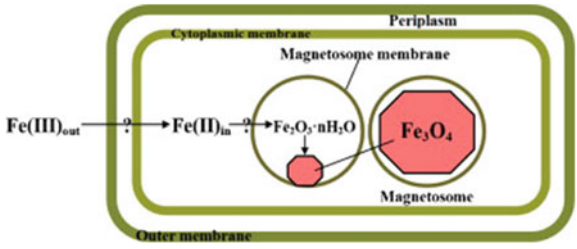

Fig. 3 A simplified proposed model of magnetite biomineralization. Reprinted with permission from Yan et al. [56]

4.2 Development of Novel MRI Agent for In Vivo Tracking

Since the discovery of the magnetatotic bacteria, the biomedical application such as the evaluation of cell engraftment, employing this technology has been attempted. However, the major hurdles to the widespread application of magnetosomes involve the fastidiousness of MTB growth, which makes them difficult to culture on a large scale and requires full investigation of the genetic and environmental control over

magnetosome synthesis. These limitations are constantly being addressed by numerous scientists [59]. To address these issues, the novel magneto-endosymbionts (MEs) contrast agent was developed by biotech company (Magnelle® reagents Bell Biosystems, Inc., CA), which are derived from the magnetotactic bacteria Magnetospirillum magneticum strain AMB-1 (AMB-1) (Fig. 4a). AMB-1 coordinates over 100 genes to synthesize membrane bound magnetic nanoparticles in magnetosomes, which are highly effective MRI contrast agents [61]. Importantly, MEs are removed by macrophages and cleared from the tissue, eliminating any confounding MRI contrast as evidenced by the absence of non-specific dephasing signal. Hence, the MEs are maintained only in the viable cells to provide accurate longitudinal engraftment signal of the transplanted stem cells. The transfection of MEs into any recipient cells have no effects on the cell viability and cytotoxicity in vitro. Propidium iodide (PI) is a membrane

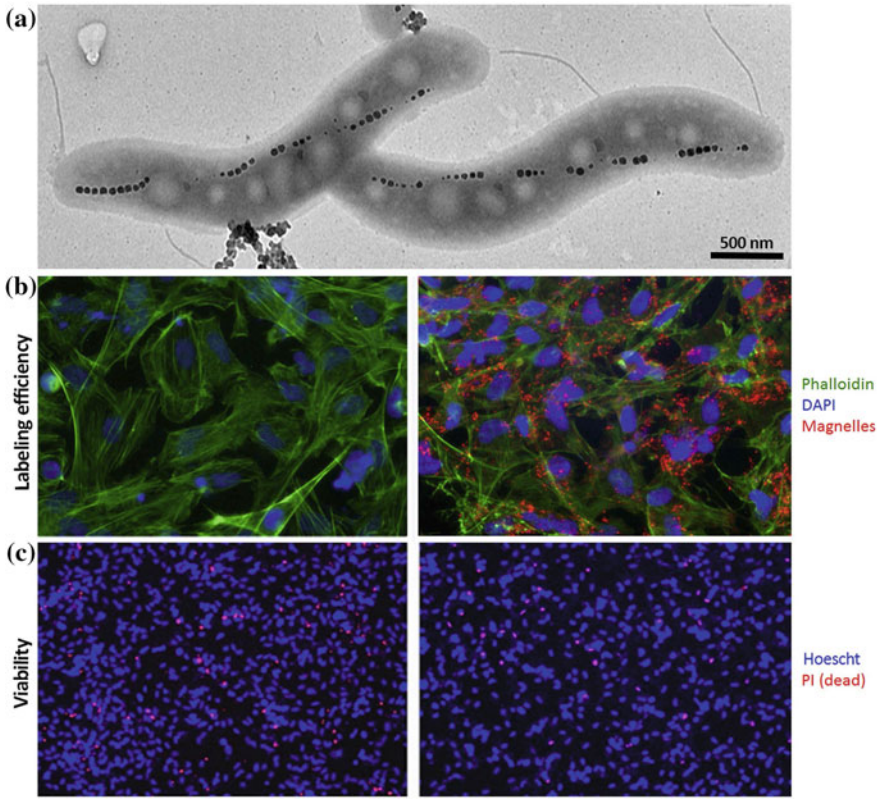

Fig. 4 **a** TEM image showing the magnetosome structure within MEs; **b** fluorescent images of the unlabeled (left panel) and ME labeled (right panel) iCMs stained with ME anti-body in red, phalloidin in green and DAPI in blue, showing internalization of MEs in the iCMs; and **c** viability assessment of unlabeled (left panel) and ME labeled (right panel) iCMs using propidium iodide (PI) assay, dead cells are shown with a red signal while blue Hoescht staining corresponds to the total cell population Reprinted with permission from Mahmoudi et al. [60]

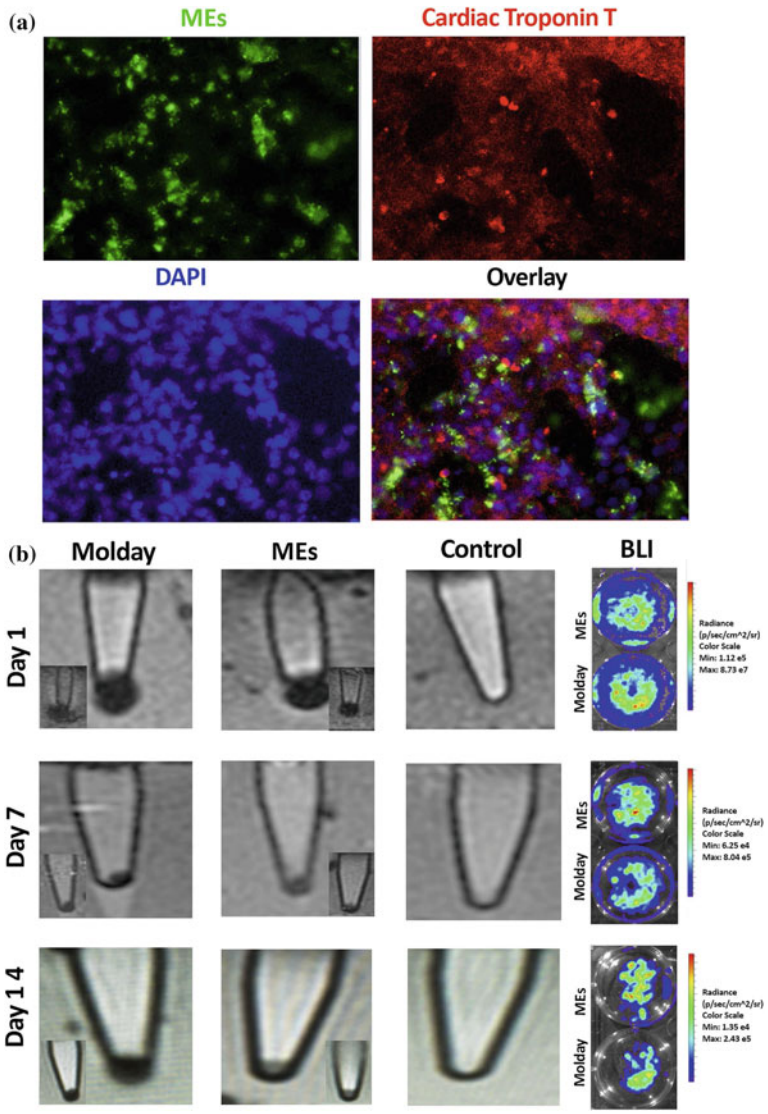

Fig. 5 **a** Fluorescent images of the ME-labeled iCMs used for the evaluation of potential ME effects on cardiac marker. MEs are stained in green and cardiac troponin T in red. Nuclei are counterstained with DAPI in blue and **b** MRI images (T2*) of the bottom of Eppendorf tubes of Molday-labeled iCMs, ME-labeled iCMs, and PBS only control sample at different time points (days 1, 7, and 14 after labeling); the bottom panel shows the replicate MRI images for Molday- and ME-labeled cells. Right panel shows the corresponding BLI signal, indicating viability of the Molday- and ME-labeled iCMs at days 1, 7, and 14 post labeling (BLI experiments were performed in triplicate). Reprinted with permission from Mahmoudi et al. [60]

impermeable dye and does not enter viable cells with intact membranes. As demonstrated by the PI staining (Fig. 4c), there was no significant difference between control unlabeled cells, which had a viability of 88% ± 2, and ME-labeled cells which had 93% ±3 cell viability ($p > 0.05$). No significant decrease in cell viability was seen after labeling with MEs (Fig. 4b, c). Moreover, we have demonstrated that the ME-labeled iCMs maintain their unique character such as contractility and strong expression of the sarcomeric protein (i.e., cardiac troponin T). This experimental demonstration indicates that the MEs do not affect the cardiac cell properties after magnetic labeling (Fig. 5a). The signal generated from the in vitro and in vivo bioluminescence imaging (BLI) and MRI studies showed corresponding evidence of robust contrast to track the iCMs based on our in vitro MRI signal on days 1, 7, and 14 when compared to the Molday ION labeled iCMs. The phantom experiment showed that the strong T2* signal of the labeled cells (with both Molday IONs and MEs) on days 7 and 14 after labeling are seen by the dark dephasing signal. The viability of the cells at different time points was monitored by BLI (Fig. 5b). After the injection of ME-labeled iCMs into a live mouse heart, both BLI and MRI signal were detected seven days after cell injection. Mice #2 and #3 show BLI signal clearance after a week while mouse #1 retains BLI signal through 2 wks. As expected, the Molday-labeled cells indicated no BLI signal by day 14; however, the cells continued to generate dephasing MRI signal consistent with the persistence of the nanoparticle signal in the mouse heart. In contrast to the Molday-labeled iCMs, the ME-labeled cells demonstrated significant correlation between BLI and MRI signals in vivo (Fig. 6). From these experiments, the mice exhibited strong MRI signal in both ME- and Molday-labeled iCMs. This finding, indicates the major limitation of synthetic iron oxide nanoparticles for in vivo monitoring of live cells in the heart as shown previously while the ME-labeled iCMs demonstrate live cell specificity [49, 50].

5 Future Perspectives

Stem cell technology represents an attractive approach to realize regenerative medicine. iPSC technology represents a landmark discovery to deliver this promise. Adult stem cells, such as MSCs, may also represent an alternative cell source to treat degenerative diseases as there are no ethical concerns or tumorigenicity potential. The development of novel stem cell therapy will continue to evolve. Various stem cell types from specific patients with unique disease process, novel gene-editing/modification, or stem cell secretomes such as the exosomes may replace damaged tissue and prevent organ failure. Thus, reliable and precise in vivo stem cell tracking represents one of the key essential technology in advancing the field of stem cell therapy. However, there is no reliable in vivo imaging method to confirm the therapeutic contribution of the transplanted stem cells. In order to obtain high resolution and reliable signal of the transplanted cells, sensitive detection of the cell engraftment signals is necessary.

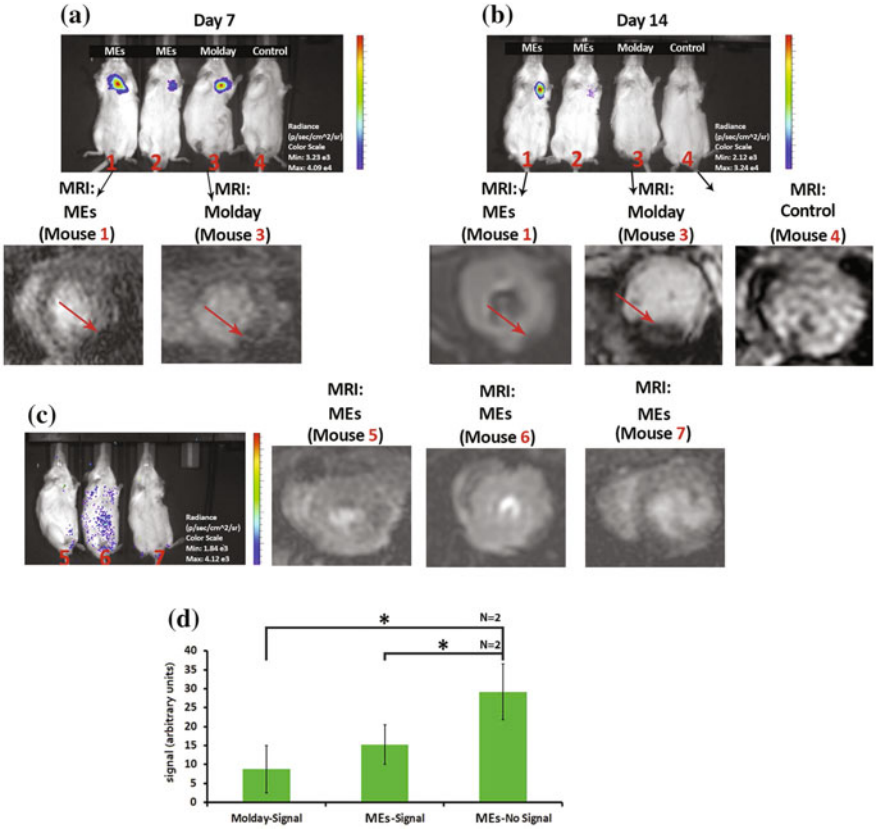

Fig. 6 Representative BLI images of the mice injected with ME- and Molday-labeled reporter gene-transduced iCMs or PBS control after **a** 7 days; **b** 14 days following cell injections (top) and their corresponding in vivo MRI images of the murine hearts (below). Red arrows show the signal from the injected labeled cells; **c** representative in vivo BLI images of the selected mice with dead cardiomyocytes at day 14 of ME labeled cells and the corresponding in vivo MRI images; **d** signal intensity (SI) for Molday and ME (dephasing signal) and ME (no dephasing signal). A significant absence of signal of ME-labeled dead cells compared to other samples with positive signal ($p < 0.05$). Reprinted with permission from Mahmoudi et al. [60]

High spatial and temporal resolution imaging of the organ function and morphology is imperative for in vivo cell tracking. MRI represents a promising imaging modality as it combines the chemical sensitivity of nuclear magnetic resonance with exquisite tissue contrast and high spatial and temporal resolution. These specifications provide optimal technical features to assess organ physiology. However, MRI lacks the sensitivity to detect the transplanted cells. The high sensitivity provided by the magnetic nanoparticles for cell tracking applications addresses this issue; however, in vivo monitoring of the cell viability has not been feasible due to the confounding signal of the nanoparticles in the tissue or the macrophages after

the death of transplanted stem cells. The novel ME approach reviewed here leverages the rapid response of the immune system, which may detect and eliminate the biological component of the ME derived from MTB. Existence of biological impurities (particularly proteins, nucleic acids, and polysaccharides) of the MEs exposed by dead stem cells allow rapid clearance by the immune system.

Recent advances in regenerative medicine demonstrate the vast potential of cell therapies for a wide range of therapeutic needs in neurodegenerative disease, cardiomyopathy, oncology, and immunology. However, a robust method to track these cells after delivery had been a limiting factor for rapid clinical translation. Our group demonstrated the first use of MEs to track the iCMs in vivo by MRI. The study showed the use of ME as a novel contrast agent for MRI-based cell tracking, which allowed longitudinal visualization of cell survival in a murine model. The use of the ME enabled live cell sensitivity (LCS), which may have significant advantage over commercially available IONs, such as the Molday IONs. This novel technology will allow researchers to effectively track live cells in vivo to monitor their engraftment and biological distribution. Several issues, including the sensitivity of MRI and dosing of ME, should be addressed to optimize the reliability and precision in cell tracking applications for future cell-based investigation. The highly novel MTB technology represents one of the viable stem cell tracking method to advance regenerative medicine.

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