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In vivo MR Imaging of Tissue-engineered Human Mesenchymal Stem Cells Transplanted to Mouse: a Preliminary Study

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Abstract—Current progress integrating stem cell biology and tissue engineering techniques has been invaluable to clinical applications. Prior to the application of celluar transplantation technique to patients, we need to establish techniques that can monitor their tissue biodistribution non-invasively. In this study, we proposed an imaging modality using MRI to not only monitor implanted scaffold *in vivo*, but also to track transplanted cells and behavior around the implant. For this purpose, human bone marrow-derived mesenchymal stem cells (hMSCs) were labeled with superparamagnetic iron oxide (Feridex) and then labeled hMSCs were cultured in a gelatin sponge used as a scaffold to support cell growth and proliferation. Histological assessment and MTT assay showed that cell labeling with MR contrast agent did not harm cell viability. Also, Feridex-labeled hMSCs showed a significant decrease in T2 signal intensity, even within the gelatin sponge *in vitro*. After implanting the sponge/cell complex *in vivo*, we could visualize cellular behavior around the implant over time using a noninvasive MRI modality and this finding was correlated with histological study, which illustrates the potential of a new approach proposed here for *in vivo* monitoring of implanted cell-based tissue-engineered product.

Keywords—MRI (magnetic resonance imaging), Scaffold, Mesenchymal stem cell, Transplantation, Cell migration.

INTRODUCTION

Current interests in regenerative medicine have prompted the development of living cells and polymeric materials (scaffold), which, when combined effectively, are crucial for the successful regeneration of tissues and organs.[14] The synthetic scaffold defines a potential space for tissue development and helps determine the gross morphology of the engineered tissue by providing a template. There are a number of different sources of living cells that could be used for tissue engineering. These include mature (not stem) cells from the patient and embryonic or adult stem cells. More recently, owing to the development of stem cell research, stem cell-based tissue engineering has attracted much attention due to its potential for use in regenerative medicine. Many approaches to stem cell-based tissue engineering cover a diverse range from epithelial surfaces to skeletal tissue, demonstrating a significant harmony of stem cell biology and tissue engineering.[2,5,20]

The survival and engraftment of implanted cells in the scaffold is critical for successful implantation of tissue-engineered product *in vivo*. Following the survival of implanted cells, the cross-talk and interaction between implants and host tissue is another important issue in the reconstitution of lost tissue. Recently, Park et al.[20] examined the interaction of implants with the recipient body in the regeneration of excessively injured central nervous system (CNS) tissue. They seeded neural stem cells (NSC) into biodegradable polymer (PGA) and then implanted the mixture into the infarction cavities of mouse brains injured by hypoxia–ischemia. In their work, PGA scaffolds were used as one tool to help bridge large cystic lesions and as templates to guide cellular organization in the injured CNS. They also paid considerable attention to interaction between the NSC–PGA complex and the injured brain. However, once implanted, such products cannot be assessed without invasive surgery. Therefore, we focused on a non-invasive imaging modality to visualize the behavior and distribution of implanted cells and scaffold as well as interactions between donor implants and the host body *in vivo*.

Several papers have addressed the topic of visualizing and tracking transplanted cells non-invasively using magnetic resonance imaging (MRI). MRI is a non-invasive technique for *in vivo* imaging and an ideal tool for fate mapping of tissue scaffolds at near cellular resolution.[1,17,18] For transplanted cells to be detectable by MRI, they need to be labeled with an MR contrast

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agent.[4] Basically, there are two kinds of MR contrast agents that perform well. The first, paramagnetic agents, are based on Gd(III) affecting the T1 rate and causing labeled cells to appear brighter (positive contrast) than non-labeled ones.[10,16,27] The second class is based on superparamagnetic iron oxide (SPIO) particles that dramatically shorten nuclear magnetic resonance T2 relaxation time. Agglomeration or intracellular clustering of iron oxide particles causes MR imaging with darker images (negative contrast) compared to cells without this agent. SPIO particles have featured prominently in work done by Weissleder et al.[9,15] and Bulte et al.[4]

In our previous papers, we developed a novel contrast agent for cancer detection *in vitro*,[12] *in vivo*[11] and for neural stem cell labeling.[25] In the current study, we used superparamagnetic iron oxide particles (Feridex) as an MR contrast agent to label cells because of the many applications of Feridex[4] and its commercial availability. Human bone marrow-derived mesenchymal cells (hMSCs) were first labeled with iron oxide. These Feridex-labeled hMSCs were cultured into gelatin sponges and implanted subcutaneously into nude mice, followed by non-invasive monitoring with MR imaging and histological study. This preliminary study utilizes one method that monitors not only the implanted tissue-engineered product, but also the behavior of magnetically labeled cells within and around the tissue-engineered product, allowing the visualization of cellular behavior over time. Even though there have been a few reports concerning MR imaging of bio-polymeric scaffold,[3,17,21,24] no papers have described non-invasive monitoring of cellular behavior around the scaffold and its distribution in the host body *in vivo*. To our knowledge, this is the first demonstration of cellular imaging in gelatin sponges as well as their tracking in the implanted mouse.

MATERIALS AND METHODS

Cell Culture

Human bone marrow-derived mesenchymal cells (hMSCs) were kindly donated by Department of Orthopedic Surgery in Yonsei University (Seoul, Korea). hMSCs were generated from bone narrow aspirations or explanted hips after obtaining informed consent. The study was approved by the ethics committee of Yonsei University, Korea. hMSCs were cultured in DMEM (Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 0.1 mg/ml streptomycin at 37°C and 5% CO_2. hMSCs were allowed to grow to 90% confluence of the surface area of the culture flask before further use. The morphology of the hMSCs obtained was not round but rather fibroblast-like spindles.

Preparation of the Feridex–PLL Complex and Cell Labeling[4]

Dextran-coated superparamagnetic iron oxide (11.2 mg/ml, Feridex IV; Berlex Laboratories, Inc., Wayne, NJ) was used as a labeling agent and Poly-L-lysine (PLL, MW 70–150 kDa; Sigma, St. Louis, MO) as a transfection agent. To examine the optimal Feridex concentration for cell labeling, cells were treated with 0, 12.5, 25, 50, 100, and 200 μg/ml of Feridex concentration. Feridex stock solution (11.2 mg/ml) was diluted with serum-free media and then PLL was added to the solution to a concentration of 1 μg/ml. The solution containing Feridex and PLL was allowed to mix for 1 h at 4°C. Next, old cell culture media was removed from hMSC culture dish and replaced with mixed Feridex-PLL complex solution. The hMSCs were treated with the complex solution at 37°C in 5% CO_2 for 18–24 h. For further cell labeling experiment, 50 μg/ml of Feridex concentration was used.

Validation of Labeling

Feridex-labeled hMSCs were washed and centrifuged (5 min, $200 \times g$) three times with phosphate buffered saline (PBS) (−) to remove excess Feridex–PLL complex. Cell labeling was validated with a histological method. Cells (3000 cells/mm^2) transferred onto a glass slide by cyto-spinning were fixed in 95% alcohol, washed, incubated for 20 min with 2% potassium ferrocyanide (Perl's reagent for Prussian blue staining) in 3.7% hydrochloric acid, washed again, and counterstained with nuclear fast red. Labeling efficiency was determined by manual counting of Prussian blue stained and unstained cells using a light microscope.

The intracellular iron content of labeled cells was measured by inductively coupled plasma-atomic emission spectroscopy (ICP-AES). In brief, sample for ICP-AES was prepared as below. Feridex-labeled hMSC were suspended and mixed with 400 μl of strong acid cocktail (sulphuric acid and hydrogen peroxide (1: 2)). After mixing, the mixture was soaked in 100°C in the oil bath for 18–24 h. Then, 5 ml of distilled water was added to the melted Feridex-labeled hMSC, followed by filtering by using a 0.22 μm syringe filter to remove undissolved impurities. And then, the obtained solution was measured by ICP-AES. The amount of iron was 4.4 pg/cell, of which value decreased during 6-day culture.

Labeled Cell Viability/Proliferation Assay (MTT Assay)

To determine cell viability/proliferation of Feridex-labeled hMSCs, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used. Ten microliter of unlabeled and Feridex-labeled cell suspension (5×10^5 cells/ml) in culture medium was loaded into each well of a 96-well microplate containing 90 μl cell media and incubated in 5% CO_2 at 37°C. After a predetermined time, cell numbers in each well were determined to examine cell proliferation on the surfaces. In brief, 10 μl of MTT dye solution (5 mg/ml in phosphate buffer pH-7.4, MTT Sigma) was added to each well. After 2-h incubation, the medium was removed and formazan crystals were solubilized with 200 μl of 2-propanol containing 0.04 M HCl with shaking for 10 min to dissolve the reacted dye. The absorbance of each well was read on a microplate reader at 562 nm.

Cell Culture in Gelatin Sponges

Unlabeled or Feridex-labeled hMSCs were cultured in SPONGOSTANs® (Absorbable Gelatin Sponge, Johnson & Johnson Medical Limited, Skipton, UK). Prior to cell seeding, gelatin sponges (two sizes: $3 \times 3 \times 3$ mm and $5 \times 5 \times 5$ mm) were incubated in PBS in culture plates overnight. PBS was removed and 30 μl of cell suspension (4×10^6 cells/ml) in culture medium was applied into each gelatin sponge. The plates were incubated in 5% CO_2 at 37°C for 2 h. After 2-h cell adhesion, gelatin sponges containing hMSCs were rinsed with culture medium to remove weakly bound cells, and then moved into a well of a 6-well culture plate containing 5 ml of medium with 10% serum and incubated in 5% CO_2 at 37°C. After a predetermined period of culture, sponge/cell complexes were stained with hematoxylin eosin (HE) and Prussian blue. Thin sections of samples with a thickness of 5 μm were prepared using a microtome and stained with HE or Prussian blue followed examination under a light microscope.

In vitro MR Imaging of Gelatin Sponges Containing hMSCs

Feridex-labeled hMSCs were suspended in a 1% agarose solution at a range of densities from 6 to 96×10^3 cells in PCR tubes and imaged at 1.5 T (Gyroscan Intera, Philips Medical Systems, The Netherlands) using a micro 47 mm surface coil.

In vitro MR imaging was conducted for unlabeled or Feridex-labeled cells in the gelatin sponges. Sponge/cell complexes cultured for 10 days *in vitro* were immersed into 0.5% agarose in a 60-mm cell culture dish and imaged at 1.5 T clinical MRI instrument. As a control, gelatin sponges without cells were also imaged. The imaging protocol consisted of gradient echo sequences with TR/TE = 115.7/11.5 ms, matrix size = 128×128, slice thickness = 1 mm, Field of view = 70 mm, averaging = 8.

Implantation of Gelatin Sponge/hMSC Complex into Nude Mice

After 6 days of culture *in vitro*, a sponge ($3 \times 3 \times 3$ mm)/cell complex was implanted subcutaneously at the right proximal thigh of a 6-week-old BALB/C-nude mouse. Procedures were approved by the Animal Care and Use Committees of our institute.

In vivo MR Imaging of Gelatin Sponge/hMSC Complex in the Nude Mouse

Three mice carrying sponge/cells complex were imaged using MRI. After 3 and 4-week implantation of sponge/cell complexes in nude mice, *in vivo* MR imaging was done using 1.5 T ((Gyroscan Intera, Philips Medical Systems, The Netherlands) using a micro 47 mm surface coil). The imaging protocol consisted of gradient echo sequences with TR/TE = 181.8/12.2, matrix size = 128×128, slice thickness = 1 mm, field of view = 60 mm, flip angle = 30°, averaging = 4.

For histological observation, specimens were removed from sacrificed mice and fixed in cryomolds. Thin sections of samples with a thickness of 5 μm were prepared with a microtome. Thin sections were stained with HE or Prussian blue and morphology observed with a light microscope.

RESULTS AND DISCUSSION

One desirable approach to optimize the repair of extensively damaged tissue involves combining implanted stem cells and scaffold. As a simple model, we used a gelatin sponge commercially available as a template (scaffold). As a cell source, hMSCs were adopted because of their application in the regeneration of heart,[19] bone,[13] and cartilage.[26] This study aimed to non-invasively monitor a gelatin sponge/hMSC complex implanted *in vivo* using MRI. To track cells and the scaffold, hMSCs were labeled with a superparamagnetic iron oxide particle (Feridex), which is a T2 MR contrast agent.

Validation of cell labeling

To examine Feridex dose-dependent cellular toxicity, several Feridex solutions with different concentration was added to cells, followed by MRI and MTT assay. Figure 1a shows the T2 value change of each cell sample by MRI. With the increase of Feridex concentration, cells treated with various concentrations represented decrease in T2 value. Also, stereoscopic pictures of cell sample and MR images showed more brownish and darker with Feridex concentration increased, indicating correlation of gradient Feridex labeling with the Feridex concentration. MTT assay results (Fig. 1b) showed that there was no cellular toxicity regardless of the increase of Feridex concentration. For further cellular labeling, 50 μg/ml of Feridex concentration was used.

Feridex-labeled hMSCs were visualized using Prussian blue staining and phase contrast microscopy. As shown in Fig. 2a, approximately 100% of Feridex-labeled hMSCs were stained by Prussian blue and iron uptake could be observed intracellularly, indicating excessive iron internalization into the cell body. In addition, MTT assay results (Fig. 2b) showed that labeled hMSCs were viable and proliferated at rates identical to those of unlabeled cells. There was no remarkable change in morphology between unlabeled and labeled hMSCs (data not shown).

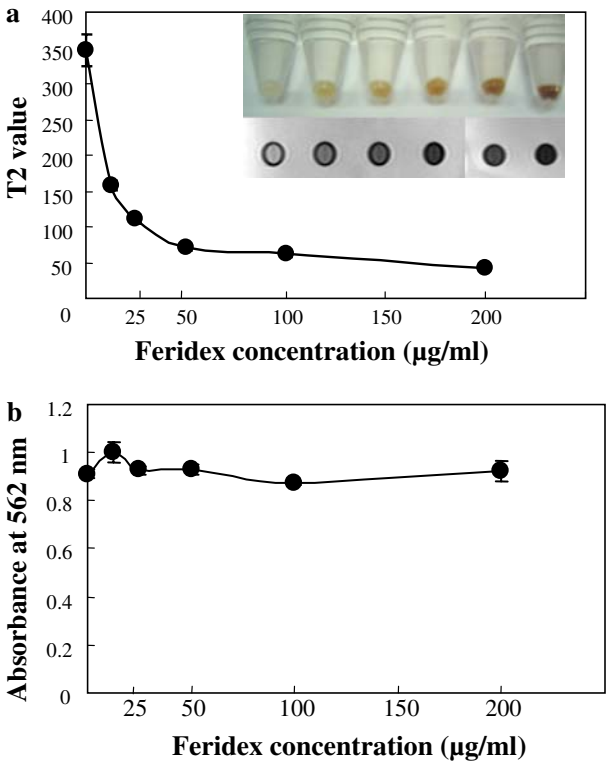

FIGURE 1. (a) The change of T2 value and (b) cellular toxicity depending on the Feridex concentration for cell labeling. In the insert in (a), upper and lower picture shows the stereoscopic imaging of labeled cells in the tube and MR images of samples in the upper picture, respectively.

To investigate the enhancement of MRI contrast properties against labeled cells, labeled hMSCs were embedded in agarose gel and those gel samples were imaged using MRI. In agarose gel, cells could be so easily suspended and homogeneous that this experimental model could give more accurate data regarding the evaluation of MR contrast properties by Feridex-labeled cells compared with using gelatin sponges. As shown in Fig. 2c, the MR signal value decreased with the increase of labeled cells, which indicates a correlation of hMSC labeling with the enhancement of MR contrast properties.

hMSC Culture in Gelatin Sponges

Unlabeled or Feridex-labeled hMSCs were seeded and cultured on gelatin sponges *in vitro* following injection of cell suspension into the sponges. After a predetermined culture period, the sponge/cell complex was imaged using MRI.

Figure 3a shows the MR images of gelatin sponges carrying unlabeled or Feridex-labeled hMSCs. Compared to the sponge carrying unlabeled hMSCs, MR imaging of gelatin sponge/Feridex-labeled hMSC complex revealed a remarkable decrease in T2 relaxivity regardless of sponge size, suggesting that Feridex-labeled hMSCs reflect T2 relaxivity decrease by iron labeling, even within the sponge. In addition, histological study (Fig. 3b and c) of the sponge/cell complex showed that most hMSCs were located in the periphery of the sponges. Unlabeled cells were unstained (data not shown), whereas labeled cells showed enough iron uptake to be detectable by Prussian blue staining. In magnified images of labeled cells shown in Fig. 3c, hMSCs attached onto the sponges with stable morphology carrying iron oxide intracellularly.

In a comparison between MRI and histological results, we found a discrepancy in that the area of MR signal loss (darkening) was substantially larger than the dimension of total cells in a sponge, which is consistent with the "blooming effect" typically observed for SPIO-labeled cells *in vitro*.[4,6,7,9,15,23] Naturally, the large magnetic susceptibility of these SPIO particles can affect an area much larger than the actual size of the particles, leading to an exaggeration of the region occupied by iron oxide. Well-designed, sophisticated labeling techniques as well as magnetic field strength and pulse sequences (such as the FIESTA imaging sequence)[7] in the MRI process would help provide better resolution at a single cell level.

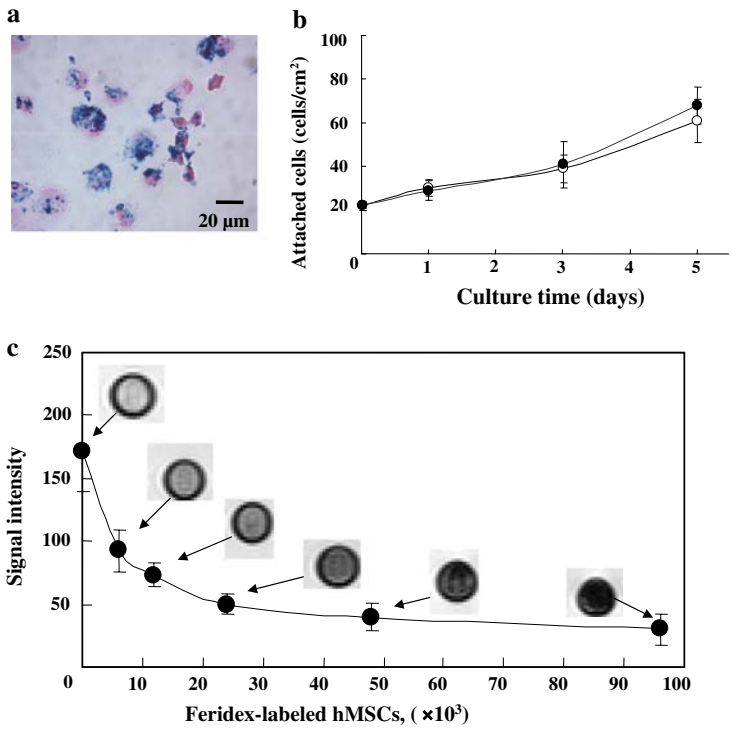

FIGURE 2. (a) Light microphotographs of Prussian blue stained human mesenchymal stem cells (hMSCs). (b) The effect of Feridex labeling on the cell viability (proliferation). Open circle and closed circles indicate unlabeled cells and Feridex-labeled cells, respectively. (c) *In vitro* relaxometry of Feridex-labeled hMSCs.

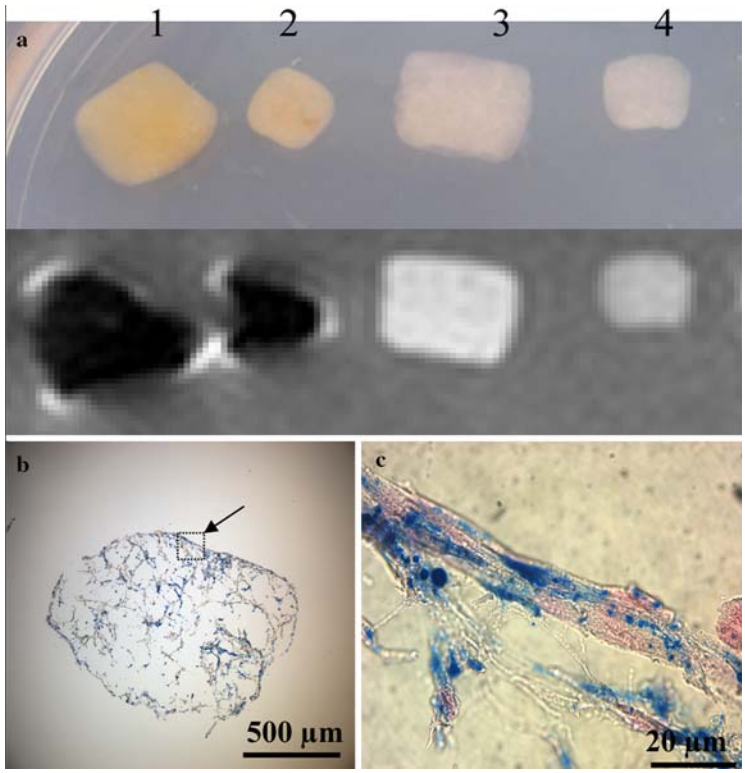

FIGURE 3. (a) Stereoscopic imaging of hMSC and sponge complexes (the upper row): Feridex-labeled hMSCs in a sponge of 5 mm^3 (1) and 3 mm^3 (2) and unlabeled cells in a sponges of 5 mm^3 (3) and 3 mm^3 (4). The lower row shows a corresponding MR image obtained with a fast spin echo sequence (details in Materials and Methods). (b) Light microscopic pictures of a sponge/hMSC complex stained with Prussian blue and (c) a magnified view of the square area indicated by an arrow in (b).

A sponge/cell complex, maintained in culture for 6 days, was implanted in the thigh of a nude mouse. Three mice carrying sponge/cells complex were imaged using MRI. All of them showed similar results in MR imaging. Figure 4a and b show mouse MR images at 3 weeks and 4 weeks against a mouse, respectively. We could easily observe the gelatin sponge containing Feridex-labeled hMSCs (arrow) and the spreading of a dark area (circle) from the original implant to the surrounding region, thus allowing the visualization of implanted scaffold and cells from the scaffold over implantation time using MRI.

To validate the existence of labeled cells *in vivo*, the sponge/cell complex and adjacent tissue was removed from sacrificed mice and stained with Prussian blue. Figure 4c shows histological results. As shown in the Prussian blue staining, implanted cells were identified within and outside of the scaffold, subcutaneous tissue, and dermal layer of the skin.

To assess the recovery of damaged tissue, non-invasive visualization of the ingrowth/outgrowth of cells (including implanted cells) that promote repair of injury is very important. In reconstituting lost tissue, reciprocal cross-talk and interaction between the implant and host tissue is crucial. Outgrowth of implanted cells to host tissue is initiated by chemical and physical factors in the microenvironment, such as the concentration gradient of chemoattractants[20] and vessel formation by host vascular cells within implants to supply oxygen and nutrients to the implant.[22] A process that follows the fate of a population of implanted cells over time would provide a new powerful way to monitor the recovery process. As we have already shown using MRI-detected evidence of cellular behavior following implantation, the visualization of implants and cellular motility around implants allows more effective evaluation of cellular functionality and distribution.

Our preliminary study has several limitations. Firstly, we can consider substantial loss of iron oxide labeled on cells with cell division. Somewhat, loss of Feridex from hMSCs was found after labeling during cell culture *in vitro*, of which observation was also reported in other studies.[4,9,15] To address this limitation, metalloprotein gene such as ferritin can be used to visualize easily by MRI with high sensitivity.[8] Secondly, we also cannot exclude the fact that tissue macrophage take up the released iron from the locally

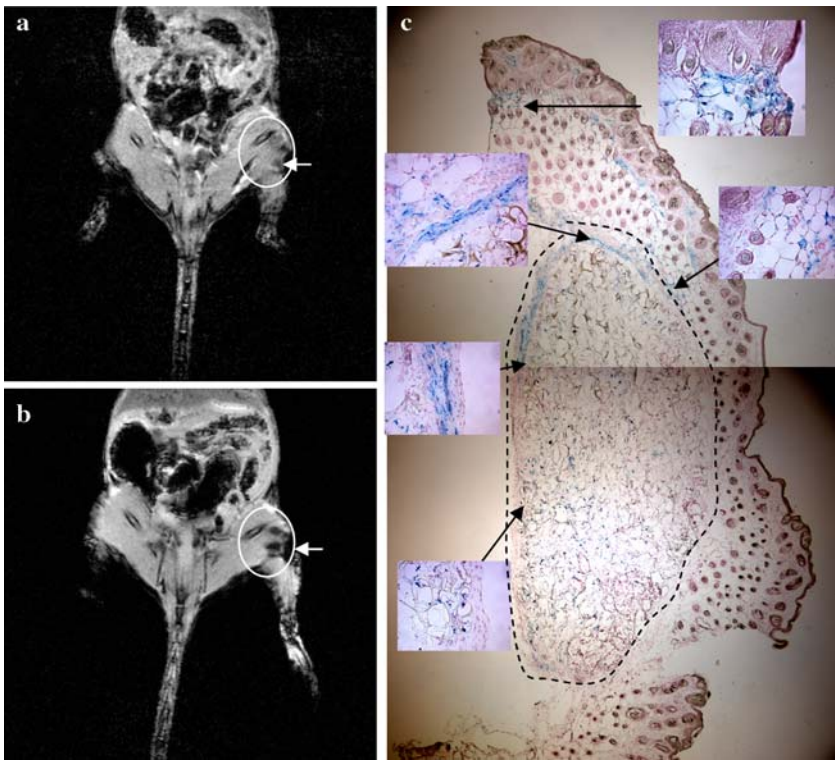

FIGURE 4. ***In vivo*** **MR imaging of a mouse at the 3rd week (a) and 4th weeks (b) after the implantation of tissue-engineered Feridex-labeled hMSCs. MRI was performed on a 1.5 T clinical magnet with a 47-mm micro-surface coil and scan parameters of TR 181 ms, TE 12 ms, flip angle 30°, matrix 128 × 128, field of view 60 mm. Arrows show the sponge/labeled cell complex and circles indicate cell migration and distribution of the implant. (c) Prussian blue staining picture of the implant and surrounding host tissue at the 4th weeks after transplantation. A dotted circle in the picture indicates an implanted scaffold.**

implanted sponge. An immunohistochemical method will be helpful to investigate the distribution of implanted hMSC.

In the current study, we used a simple quasi-tissue engineering product composed of a gelatin sponge and hMSCs to visualize cellular behavior from the implants using a noninvasive method *in vivo*. Future experiments should include the development of appropriate types of synthetic scaffolds using hMSCs for specific applications and should investigate hMSC differentiation in combination with stem cell biology.[13,19,26]

CONCLUSION

In this study, we proposed an imaging modality using MRI not only to monitor implanted scaffold *in vivo*, but also to track transplanted cells and behavior around the implant. For this study, we labeled mesenchymal cells isolated from human bone marrow with a ferrium MR contrast agent (Feridex) and then cultured Feridex-labeled hMSCs into gelatin sponges. After implanting the sponge/cell complex *in vivo*, we could visualize cellular behavior around the implant over time using a noninvasive MRI modality and this finding was correlated with histological study.

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