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Magnetic Resonance Imaging of Ferumoxide-Labeled Mesenchymal Stem Cells in Cartilage Defects: In Vitro and In Vivo Investigations

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Abstract

The purpose of this study was to (1) compare three different techniques for ferumoxide labeling of mesenchymal stem cells (MSCs), (2) evaluate if ferumoxide labeling allows in vivo tracking of matrix-associated stem cell implants (MASIs) in an animal model, and (3) compare the magnetic resonance imaging (MRI) characteristics of ferumoxide-labeled viable and apoptotic MSCs. MSCs labeled with ferumoxide by simple incubation, protamine transfection, or Lipofectin transfection were evaluated with MRI and histopathology. Ferumoxide-labeled and unlabeled viable and apoptotic MSCs in osteochondral defects of rat knee joints were evaluated over 12 weeks with MRI. Signal to noise ratios (SNRs) of viable and apoptotic labeled MASIs were tested for significant differences using *t*-tests. A simple incubation labeling protocol demonstrated the best compromise between significant magnetic resonance signal effects and preserved cell viability and potential for immediate clinical translation. Labeled viable and apoptotic MASIs did not show significant differences in SNR. Labeled viable but not apoptotic MSCs demonstrated an increasing area of T₂ signal loss over time, which correlated to stem cell proliferation at the transplantation site. Histopathology confirmed successful engraftment of viable MSCs. The engraftment of iron oxide-labeled MASIs by simple incubation can be monitored over several weeks with MRI. Viable and apoptotic MASIs can be distinguished via imaging signs of cell proliferation at the transplantation site.

Osteoarthritis is a disabling joint disease affecting more than 69 million Americans¹ and causing annual health care costs of \$330,648,000,000.² Cartilage defects in arthritic joints, which are the main cause of pain and disability in affected patients, cannot be reversed or repaired by conservative treatment.³ Autologous chondrocyte-based transplants are currently investigated for restoration of trauma-induced cartilage defects.^{4,5} However, clinical

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outcomes are limited owing to the cells' inability to adapt to the unique cartilage microenvironment. Mesenchymal stem cells (MSCs) have emerged as a promising alternative for cartilage repair because they are autologous cells that can be harvested from bone marrow without further cartilage damage (chondrocyte transplants require harvesting additional cartilage from the target joint) and because of their fairly straightforward isolation and their ability to be expanded efficiently in culture.⁶ In addition, MSCs are capable of proliferating, adapting, and secreting chondrogenic matrix, which leads to improved engraftment outcomes.⁶ MSCs have been successfully implanted in human patellar defects with improvement in clinical symptoms.⁷ However, the behavior of MSCs embedded in various biomaterials in the long term and in the context of arthritic joints remains to be studied to ascertain predictable clinical outcomes.^{7–11}

An imaging method that could monitor successful MSC engraftments or diagnose a treatment failure by direct depiction of the transplanted cells would be highly desirable. Among various available imaging techniques for cell tracking,^{10–13} magnetic resonance imaging (MRI) has the following advantages (1) it is the only imaging technique that provides direct cartilage depiction, (2) it is noninvasive and is not associated with radiation exposure, and (3) stem cell labeling and tracking techniques with clinically applicable magnetic resonance contrast agents are established.^{14,15} Previous studies optimized stem cell labeling techniques with iron oxide nanoparticles toward a compromise between a cellular iron oxide load that is high enough to provide sensitive cell depiction on MRIs but also low enough to ensure an unimpaired stem cell differentiation into chondrocytes.^{16–18} In addition, our group showed previously that iron oxide–labeled stem cells can be depicted in cartilage defects with MRI^{19–21} and that iron oxide–labeled viable and nonviable stem cells demonstrate different magnetic resonance signal characteristics in ex vivo settings.^{19,21}

The purpose of our study was to translate knowledge from previous in vitro and ex vivo studies to in vivo applications by (1) comparing three different ferumoxide labeling techniques of MSCs, (2) evaluating if ferumoxide labeling allows in vivo tracking of matrix-associated stem cell implants (MASIs) in an animal model, and (3) comparing the MRI characteristics of ferumoxide-labeled viable and apoptotic human mesenchymal stem cells (hMSCs). We hypothesized that clinically applicable protamine transfection techniques improve labeling efficiencies compared to simple incubation protocols, that ferumoxide-labeled MSCs can be tracked in cartilage defects in vivo with MRI, and that iron oxide–labeled viable and apoptotic cell transplants show different magnetic resonance signal characteristics in vivo.

Materials and Methods

Contrast Agent

Ferumoxide (Endorem, Guerbet, Aulnay-sous-Bois, France) consists of superparamagnetic iron oxide (SPIO) particles with a nonstoichiometric magnetite core coated with dextran T-10.²² Ferumoxide has an r_1 relaxivity of $40.0 \text{ mM}^{-1}\text{s}^{-1}$, an r_2 relaxivity of $160 \text{ mM}^{-1}\text{s}^{-1}$ (at 37°C and 0.47 T), and a hydrodynamic diameter of 80 to 150 nm.²³ Ferumoxide is approved by the Food and Drug Administration (FDA) as a magnetic resonance contrast agent for liver imaging. Ferumoxide is taken up by cells of the reticuloendothelial system via endocytosis and stored in secondary lysosomes within the cytoplasm.²⁴

Transfection Agents

Lipofectin (Invitrogen, Carlsbad, CA) is a reagent consisting of the cationic lipids N-[1-(2,3-dioleoyloxy)propyl]-n,n,n-trimethylammonium chloride (DOTMA) and dioleoyl phosphatidylethanolamine (DOPE) in a 1:1 mixture.²⁵ The positively charged lipid

molecules form complexes with the negatively charged contrast agent. The complexes then fuse with the cell membrane and deliver the contents into the cytosol.²⁶

Protamine sulfate (American Pharmaceutical Partners, Schaumburg, IL) is a cationic peptide with a high arginine content and a molecular weight of approximately 4,000 Da.²⁷ It is FDA approved to reverse heparin anticoagulation and is used in NPH insulin preparations. It has also been investigated as a transfection agent for cell labeling with ferumoxide.¹⁶

Cell Culture

We investigated hMSCs (Lonza, Walkersville, MD), which were cultured in Dulbecco's Modified Eagle's Medium (DMEM) high-glucose medium (Invitrogen), supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT) and 1% penicillin-streptomycin in a humidified 5% CO₂ atmosphere at 37°C. The medium was changed every other day. Cells were trypsinized at 90% confluency with 0.05% trypsin (Invitrogen). To preclude the possibility of senescence, experiments were performed at cell passages 6 to 12.

Cell Labeling

MSCs were labeled with ferumoxide by simple incubation (protocol 1), protamine transfection (protocol 2), or Lipofectin transfection (protocol 3) using established methods.^{16–18,28} Briefly, cells were plated at 90% confluency and allowed to adhere overnight at standard cell culture conditions. Culture medium was replaced with labeling medium (DMEM without FBS or penicillin-streptomycin) as specified below and incubated for 1, 6, 12, or 24 hours. Cells were then trypsinized and washed two times with phosphate-buffered saline (PBS) by centrifugation (400 rcf, 5 minutes, 25°C) to remove residual contrast agent. Cell viability was determined by the trypan blue exclusion test. Inductively coupled plasma atomic emission spectroscopy (ICP-AES) analysis was performed to quantify internalized iron content. We used the following protocols:

1. *Simple incubation:* 20 mL of DMEM was mixed with ferumoxide at a concentration of 100 µg/mL and was added to the culture flasks
2. *Protamine transfection:* ferumoxide and protamine were diluted in DMEM to a concentration of 100 µg/mL and 5 µg/mL, respectively. The solution was then shaken vigorously and added to an equal amount of full culture medium (final incubation concentration = 50 µg/mL).
3. *Lipofectin transfection:* 178 µL Lipofectin reagent and 178 µL ferumoxide were dissolved in 1 mL of DMEM. Both solutions were let to sit for 30 minutes, shaken, and let to sit for another 30 minutes. The solution was then diluted in DMEM to a final volume of 20 mL (50 µg/mL) and added to the cell culture for labeling.

Fluorescence Microscopy of Ferumoxide-Labeled Cells

hMSCs were examined via confocal fluorescence microscopy for intracellular localization of contrast agent. Cells were plated onto multichamber glass slides (Nunc, Rochester, NY), allowed overnight to attach, and fixed at room temperature with Carnoy's solution. Labeled cells were stained with anti-dextran fluorescein isothiocyanate (FITC; Stem Cell Technologies, Tukwila, WA) for 1 hour at room temperature, washed three times with PBS, and counterstained with DAPI. Samples were analyzed by confocal microscopy at 40× magnification (LSM 510, Zeiss, Thornwood, NY).

Electron Microscopy of Ferumoxide-Labelled Cells

hMSCs were plated onto Thermanox coverslips (Electron Microscopy Sciences, Hatfield, PA) and allowed to adhere. Fixation with 2% glutaraldehyde in 0.1 M sodium cacodylate

buffer was performed, and cells were postfixed with 1% osmium tetroxide followed by 2% aqueous uranyl acetate. Samples were dehydrated with a graded ethanol series and embedded in epoxy resin. Ultrathin sections were stained with 2% uranyl acetate and Reynolds lead citrate and examined at 80 kV in a JEOL 100CX II (Jeol, Tokyo, Japan) transmission electron microscope.

In Vitro MRI of Ferumoxide-Labeled Cells

Samples of 150,000 hMSCs, labeled with the protocols above, were embedded in 500 μ L of 2% gelatin and underwent MRI on a 3 T clinical magnetic resonance scanner (Signa EXCITE, GE, Milwaukee, WI) using a quadrature knee coil (Clinical MR Solutions, Brookfield, WI). Cell samples were scanned in a water bath to avoid susceptibility artifacts. For determination of T_2 relaxation times, spin echo (SE) sequences were obtained with a fixed repetition time (TR) of 2,000 ms and multiple echo time (TE) (60, 45, 30, 15 ms) values. T_1 relaxation times were determined using a fixed TE of 15 ms and multiple TR (250, 500, 1,000, 4,000 ms) values. To determine T_2^* relaxation times, gradient echo images were obtained with a flip angle of 30°, a fixed TR of 500 ms, and varying TE (28.8, 14.4, 7.2, 3.7 ms) values. All sequences were acquired with a field of view (FOV) of 160 $\times$ 160 mm, a matrix of 256 $\times$ 256 pixels, a slice thickness of 3 mm, and one acquisition. These scans were performed in triplicate.

Ex Vivo MRI of MASIs in Pig Knee Specimens

Subsequently, we investigated the MRI characteristics of labeled hMSCs in Surgifoam scaffold (Johnson & Johnson, New Brunswick, NJ), an absorbable gelatin sponge. Surgifoam pads were immersed into liquid agarose (Type IX Ultra Low, 1.5% in PBS, Sigma-Aldrich, St. Louis, MO) at 37°C; 250,000 labeled cells were injected into the scaffold and cooled to 15°C to induce gelling. Scaffolds were then cut into cubic samples of 3 mm³ and implanted into artificially created full-thickness cartilage defects of pig knee joint specimens supplied by a local meat market. The following experimental groups were evaluated: scaffold only, scaffold with unlabeled hMSCs, scaffold with ferum-oxide-labeled hMSCs, scaffold with ferumoxide and Lipofectin, and scaffold with ferumoxide and protamine ($n = 6$ each). To remove trapped air, knee joints were filled with ultrasound gel (diluted 1:3 in PBS) after MASI.

MRI of pig knee specimens with MASIs was performed using a clinical 1.5 T magnetic resonance scanner (Signa EXCITE, GE) and a quadrature knee coil. T_1 -weighted SE sequences (TR 500 ms, TE 15 ms, band width [BW] 15.63 Hz, FOV 12 cm, matrix 512 $\times$ 192, two acquisitions, 3:16 minutes), moderately T_2 -weighted fat-saturated fast spin echo (FSE) sequences (4,300/25/31.25/15/512 $\times$ 256/2/4:14, echo train length 9), T_1 -weighted three-dimensional spoiled gradient recalled (SPGR) sequences (17/8.5/16/512 $\times$ 512/0.75/10:44, alpha 12), and T_2^* -weighted gradient echo sequences (500/14/15.63/12/512 $\times$ 192/2/3:16, alpha 30) were obtained with a 1 mm slice thickness.

All MRIs were analyzed using *DICOM* imaging software (OsiriX, UCLA, Los Angeles, CA). The signal intensities (SIs) of the cell suspension, chondrogenic pellet, or implanted scaffold were determined via user-defined regions of interest (ROI) and divided by the background noise to obtain the signal to noise ratio (SNR).

In Vivo MRI of MASIs in Osteochondral Defects of Rat Knee Joints

Preparation of MASI Constructs—hMSCs labeled with ferumoxide by simple incubation were used for in vivo experiments. Nonlabeled hMSCs served as controls. Subsets of labeled and unlabeled hMSCs underwent apoptosis induction via established techniques.^{21,29} In brief, hMSCs were incubated for 6 hours with mitomycin C at a

concentration of 0.5 mg/mL at standard cell culture conditions. The cells were carefully washed three times with PBS and used for in vivo experiments.

Eighty milligrams of agarose powder (Sigma) was added to 2 mL of PBS for a final concentration of 40 mg/mL. This solution was gently shaken and auto-claved. After approximately 40 minutes, the fluid agarose solution was taken out of the autoclave and refrigerated for 24 hours before use. MASI constructs were prepared by combining 60% sterile agarose solution with 40% of the hMSC solution at a concentration of 15 million cells/mL.

Animal Experiments—The study was approved by the institutional animal care and use committee. In 10 nude athymic female Harlan rats, cartilage defects were created in the distal femur of both knee joints under inhalation anesthesia with 1.5 to 2% isoflurane in oxygen. A medial patellar skin incision was made, the patella was dislocated laterally, and a circular osteochondral defect (diameter: 1.5 mm, depth: 1.5 mm) was created in the distal femoral trochlear groove with a surgical drill. Hemostasis was achieved using cotton tips. MASI constructs of 5 μ L total volume were transplanted into the defect. Six athymic rats received implantations of viable, ferumoxide-labeled hMSCs into cartilage defects of the right knee joint and mitomycin-pretreated, apoptotic ferumoxide-labeled hMSCs into the left knee joint. Four additional control animals received implantations of unlabeled viable and unlabeled apoptotic MSCs in each knee joint ($n = 2$ animals) or implantations of scaffold only in both knee joints ($n = 2$ animals). Then the patella was repositioned and the skin incision was closed by a suture.

Magnetic Resonance Imaging—All knee joints were evaluated with MRI on the day of implantation and then at weekly intervals for up to 12 weeks in rats with labeled hMSC transplants and up to 4 weeks for control rats (7 T magnetic resonance scanner, Varian, Palo Alto, CA). Animals were anesthetized with 2% isoflurane inhalation and placed supine on a custom-made animal imaging bed. Sagittal MRIs of the rat knee joints were obtained using a dedicated Helmholtz knee coil with T₂-weighted SE sequences (TR 3,000 ms, TE 30 ms, two acquisitions). All sequences were obtained with an FOV of 25.6 mm, a matrix of 128 $\times$ 128 pixels, a slice thickness of 0.75 mm, and a flip angle of 90°/80°.

MRIs were analyzed using dedicated image processing software (*ImageJ*, National Institutes of Health, Bethesda, MD). The total area of the transplant was accessed by summation of the total number of pixels with a signal void on each image covering the transplant.

The SI of the transplant (SI_{MAST}) within the cartilage defect and the SI of background noise (SI_{noise}) in front of the knee joint (phase encoding direction) were measured using dedicated ROI. The minimum size of ROI was 15 pixels and the maximum size was 30 pixels. Measured SI values were normalized to the background noise and expressed as SNR = SI_{MAST}/standard deviation of SI_{noise}.³⁰

Histopathology of Knee Joint Specimen

Following the last MRI, the animals were sacrificed, the knee joints were explanted, and specimens were placed in Cal-Ex II (a mixture of formaldehyde and formic acid; Fisher Scientific, Fair Lawn, NJ) for 48 to 72 hours. This decalcified and fixed the tissue simultaneously. Then the specimens were dissected parasagittally, dehydrated through graded alcohol washes, and embedded in paraffin. Five-micrometer sections were stained with hematoxylin and eosin (H&E), alcian blue, and Prussian blue. For immunohistochemistry, sections were deparaffinized and fixed with 4% formaldehyde followed by antigen retrieval with proteinase K and blocking of endogenous peroxidases with 1% H₂O₂ in methanol. The primary antibody used was specific for human CD44

antigen, which is a known marker for hMSCs (CD44 antibody, 1:150 dilution; Abcam, Cambridge, MA). The secondary antibody used was secondary biotinylated antimouse IgGs (Vector Laboratories, Burlingame, CA). For chromogenic staining, slides were blocked with 6% normal horse serum and developed with a chromogenic VECTASTAIN Elite ABC Kit (Vector Laboratories, Inc.) and a VIP Substrate Purple Kit (Vector Laboratories).

For fluorescent staining, slides were blocked with an avidin-biotin blocking kit (Molecular Probes, Eugene, OR) followed by blocking with 6% normal horse serum. Streptavidin-peroxidase conjugate coupled with biotinylated secondary antibody was visualized by Alexa Fluor 594 dye using tyramide amplification technique (TSA HRP-streptavidin kit, Molecular Probes).

Statistical Analysis

T₂ relaxation times of cell pellets and SNR data of MASIs in pig knee specimen were tested for significant differences between different labeling techniques using a *t*-test and a 5% level of significance. For comparison of multiple experimental groups, *p* values were adjusted with the Bonferroni correction.

For in vivo studies, SNR data and area of transplants with ferumoxide-labeled viable hMSCs and apoptotic hMSCs were tested for significant differences using a *t*-test. For all analyses, a *p* value of less than .05 was considered significant.

Results

Fluorescence and Electron Microscopy of Ferumoxide-Labeled Cells

Electron and fluorescence microscopy of samples incubated for 24 hours confirmed intracellular internalization of contrast agent regardless of transfection technique (Figure 1). MSCs labeled with transfection agent showed more intense fluorescence signal, corresponding to higher quantities of internalized contrast agent, as determined by spectrometry. Electron microscopy confirmed that contrast agent nanoparticles were incorporated into secondary lysosomes. No evidence of morphologic abnormalities of labeled cells was noted when compared to unlabeled controls.

Spectrometry

Quantification of intracellular iron uptake by ICP-AES demonstrated significant intracellular uptake of contrast agent of MSCs for all three cell labeling protocols and all incubation times (*p* < .05). There was a linear increase in iron uptake with increasing incubation times for all labeling protocols (Figure 2). Protamine transfection was significantly more efficient than simple incubation, and Lipofectin transfection was significantly more efficient than protamine transfection (*p* < .05). All cells labeled with ferumoxide by simple incubation or protamine transfection demonstrated viabilities of more than 90%, as determined by trypan blue exclusion testing. Cells labeled with ferumoxide by Lipofectin transfection showed a minor but not significant decline in viability to 87% when incubated for 24 hours.

In Vitro MRI

Imaging of test tubes with labeled and unlabeled MSCs corresponded to the results from ICP-AES analysis. MSCs labeled with three labeling protocols resulted in significant T₂ signal effects (*p* < .05; Figure 3). Longer incubation times led to increased contrast agent effect on T₂-weighted images. Given that labeling efficiency and magnetic resonance signal effects increased up to our maximal incubation time of 24 hours, we chose this time for subsequent ex vivo and in vivo studies.

Ex Vivo MRI

Review of the MRI features of MASIs revealed a marked signal effect of all ferumoxide-labeled stem cell transplants on all sequences. Corresponding quantitative SNR data showed significant differences between scaffolds with labeled and unlabeled cells on all sequences ($p < .05$). Interestingly, we found a slight but significant difference between unlabeled cells in scaffolds and scaffolds alone on FSE sequences. On T_2^* -weighted images, all ferumoxide-labeled cell transplants demonstrated a maximal signal decline without any significant differences between subgroups ($p > .05$). On T_1 , FSE and SPGR sequences, cells labeled by ferumoxide-protamine transfection, and cells labeled by ferumoxide-Lipofectin transfection demonstrated significantly stronger signal effects compared to cells labeled by simple incubation ($p < .05$). There was no significant difference in the SNR data of MASIs labeled by ferumoxide-protamine transfection and ferumoxide-Lipofectin transfection ($p > .05$). However, the ferumoxide signal corresponded approximately to the size of the MASI for MSCs labeled by simple incubation and protamine transfection, whereas MASIs labeled by ferumoxide-Lipofectin transfection showed a blooming effect, which exceeded the stem cell implant (Figure 4). We chose a simple incubation labeling protocol as the best compromise between significant magnetic resonance signal effects, preserved cell viability, and potential for immediate clinical translation.

In Vivo Studies

In Vivo MRI—Immediately after MASI, the transplanted ferumoxide-labeled hMSCs could be clearly delineated on T_2 -weighted SE images as focal hypointense areas within the distal femur (Figure 5). Both viable and apoptotic transplants demonstrated a persistent hypointense signal on T_2 -weighted MRIs up to 12 weeks following stem cell transplantation (see Figure 5). Corresponding quantitative SNR data demonstrated no significant difference between labeled viable and apoptotic transplants at any time point after stem cell transplantation ($p > .05$; see Figure 5). However, the size of the area with decreased T_2 signal demonstrated differences between the two groups: The transplants with labeled viable hMSCs demonstrated an increase in size up to 2 weeks following MASI, followed by a plateau at 4 weeks and then a gradual decrease over the next 12 weeks. The apoptotic transplants demonstrated no significant change in size over 12 weeks. The differences in the size of viable and apoptotic transplants, as measured by summation of total hypointense pixels within each implant, were statistically significant ($p < .05$).

All control groups demonstrated a homogeneous, relatively hyperintense T_2 signal (see Figure 5), without any significant difference in the SNR data or area of the transplant between the unlabeled viable, unlabeled apoptotic, and scaffold-only transplants. The hyperintense T_2 signal of control transplants in the surgically produced defect covered a smaller area than the measured area of hypointense labeled viable transplants. A hypointense rim was noted at the periphery of all control implants (see Figure 5).

Postmortem Macroscopic Evaluation of MASI—Macroscopic inspection of knee joint specimens showed persistent hMSC implants in the osteochondral defects at 4 and 12 weeks after MASI. The implants filled the defect completely, created an even joint surface, and did not show any evidence of hypertrophic growth. At 4 weeks after MASI, the treated defect sites appeared rough and the junction between the stem cell transplant and adjacent cartilage was still visible. At 12 weeks, the treated defects appeared smooth and the junction became less apparent.

Histopathology—Histopathologic evaluation of viable hMSC transplants at 12 weeks showed complete repair of the osteochondral defect with the presence of tissue and chondrocyte-like cells and matrix production on alcian blue stains (Figure 6). Prussian blue

stains revealed iron-positive cells within the repair tissue at the implantation site, in the adjacent subchondral bone and interstitium (see Figure 6). Apoptotic hMSC transplants and scaffold-only transplants (see Figure 6) demonstrated an incomplete repair with a persistent defect and the presence of scar tissue. Ferumoxide-labeled and unlabeled MASIs showed no difference in engraftment of either cell type. Sequestration of dead bone fragments and hemosiderin was noted in the subchondral area adjacent to all implants. The hMSC transplants demonstrated a population of cells that showed cytoplasmic positivity for CD44, indicating the presence of hMSCs. These cells were more abundant in viable cell transplants than apoptotic transplants and were not identified in the scaffold-only (control) transplant (see Figure 6).

Discussion

Our data showed that the engraftment process of MASI, labeled with iron oxide nanoparticles via simple incubation, could be monitored over several weeks with MRI. Iron oxide nanoparticle labeling did not alter the engraftment of hMSCs in cartilage defects when compared to unlabeled controls. Viable labeled stem cells could be differentiated from labeled apoptotic stem cells by an increasing area of T₂ signal loss on serial follow-up studies, which indicated cell proliferation.

This is apparently the first report of a direct and long-term in vivo visualization of the engraftment of MASIs in osteochondral defects by MRI. Previous attempts to visualize iron oxide-labeled cell transplants in mice were conducted on subcutaneous implants of cell-scaffold complexes.¹⁰ Other investigators visualized magnetically labeled MSCs in the knee joints of rabbits with MRI.³¹ However, these subcutaneously or intra-articularly injected cells did not integrate into joint components or participate in joint repair.³¹

Unenhanced MRI was previously used to evaluate the engraftment process of unlabeled chondrocytes in cartilage defects of articular bones of patients.³² The grafts showed gradual changes in magnetic resonance signal characteristics over a period of 1 year.³² This imaging technique would be suitable for long-term follow-up studies after autologous chondrocyte implantation, although early graft failure may not be detected.³² Other approaches used MRI after intravenous injection of gadolinium chelates for in vivo depiction of autologous chondrocyte implantation.³³ Intravenous contrast agent injection provided a better delineation of articular surfaces and better visualization of incomplete integration of the implants in cartilage defects.³³

Our technique of direct visualization of iron oxide nanoparticle-labeled cell transplants may be advantageous compared to these nonspecific MRI techniques because it delineates the transplant directly and, thus, may detect complications early after transplantation rather than diagnosing successful engraftment months after transplantation. Of note, T₂ FSE sequences demonstrated a significant difference in SNR between unlabeled cells in scaffolds and scaffolds alone. This effect is interesting as further sequence optimization may allow determining the anatomic distribution and persistence of cell transplants in cartilage defects without the use of contrast agent labels. Labeling with iron oxide nanoparticles is in principle readily clinically applicable. Ferumoxide is FDA approved for liver imaging and has been applied off-label for labeling and tracking of dendritic cells in patients with melanomas.³⁴ However, the production and distribution of ferumoxide have been discontinued by the pharmaceutical industry for commercial reasons. Thus, future studies will have to confirm our studies for alternative, second-generation iron oxide compounds, such as ferumoxylol (Feraheme), which was recently FDA approved for other indications.

In our study, ferumoxide provided long-term cell labeling and magnetic resonance detection in arthritic joints of rats over an observation period of 12 weeks. This is in accordance with previous reports of stable labeling and magnetic resonance detection of ferumoxide-labeled MSCs in the joints of rabbits for 12 weeks.³¹ Ferumoxide is slowly metabolized over time.²⁰ The maximal time interval that ferumoxide-labeled cells can be detected with MRI still needs to be defined.

Our data also showed that ferumoxide labeling did not interfere with the engraftment process of labeled hMSCs. Previous studies showed that iron oxide labels can impair the viability and differentiation capacity of stem cells when they are internalized in too high quantities into the cells.^{17,24} However, if applied in limited concentrations, iron oxides are slowly incorporated into the regular iron metabolism and do not change the physiology of the cells.^{11,16–18,35–37} Using optimized labeling protocols, a normal differentiation of hMSCs into chondrocytes^{14,16,38} and osteocytes^{14,37,38} was noted after labeling with ferumoxide^{14,16,37} or FITC-labeled iron oxide nanoparticles.³⁸ Our data confirm that hMSCs labeled with limited iron oxide concentrations show an unimpaired in vivo engraftment in cartilage defects.

To our knowledge, this is the first study to evaluate differences in engraftment outcome of viable and apoptotic hMSCs in osteochondral defects. Berman and colleagues investigated the long-term magnetic resonance signal characteristics of live and dead SPIO-labeled neural stem cells after transplantation into brain parenchyma.³⁹ They indicated that viable cell proliferation and associated label dilution may dominate contrast clearance compared to cell death and subsequent transfer and retention of iron within phagocytes and interstitium. Zhang and colleagues reported that killed neural progenitor cells labeled with SPIO and transplanted in the cisterna magna did not show iron on Prussian blue stains, indicating that iron is cleared rapidly rather than being retained in endogenous cells or interstitium.⁴⁰ In our study, proliferation of labeled viable cells could be diagnosed by an increased area of signal loss over time, whereas apoptotic implants showed a stable or decreasing area of signal loss. Comparison of SNR data between apoptotic and viable implants did not reveal a statistically significant difference. The tendency toward initial lower SNR of apoptotic implants may be related to the presence of free extracellular iron and higher superparamagnetic effects, and the slight increase in SNR at 2 weeks may be related to rapid metabolism of iron and apoptotic cells by host macrophages. Iron-positive cells were identified at the implantation site and in the adjacent marrow on Prussian blue stain in viable and apoptotic implants; these cells were more abundant in viable transplants, supporting our hypotheses that viable cells survived for a longer time than apoptotic implants.

Secondary phagocytosis of iron oxides by macrophages may be a confounding factor for interpretation of magnetic resonance signal effects. In an in vivo study by Baligand and colleagues, the iron oxide label was visible in tissues for 3 months, grossly overestimating cell survival (< 1 week).⁴¹ Chen and colleagues suggested that the role of macrophages in scavenging and concentrating iron led to prolonged magnetic resonance signal that did not reflect the viability of transplanted cells.⁴² Histopathologic correlation of our viable implants revealed engraftment with complete repair of the defect at 12 weeks, whereas apoptotic transplants showed persistence of the defect. In addition, matrix production was noticed in viable implants, suggesting that some transplanted cells survived and differentiated owing to paracrine factors. Populations of cells that showed cytoplasmic positivity for CD44 were noted at the site of transplantation, indicating the presence of hMSCs. Engraftment of unlabeled viable transplants was also noted, whereas scaffold-only transplants showed repair of the defect by fibrous or scar tissue. Previous studies have demonstrated that in addition to chondrogenic differentiation, trophic factors secreted by

MSCs help establish a regenerative microenvironment and promote healing at the site of injury.⁴³

As shown by our data, another potential confounding factor is sequestered dead bone fragments from the surgical procedure, scar tissue, and hemosiderin, which cause a hypointense signal at the periphery of unlabeled and scaffold-only implants.

Conclusion

MRI imaging and hMSC labeling with FDA-approved iron oxide nanoparticles can be readily applied for noninvasive long-term in vivo tracking of MASIs for treatment of osteoarthritis. Ferumoxide labeling does not interfere with the engraftment process. Viable and nonviable stem cells can be distinguished by distinct MRI characteristics.

A noninvasive cell-tracking technique based on MRI with an FDA-approved cell marker will lead the way to real-time in vivo tracking of transplanted stem cells and holds the potential to evolve into a new critical tool for measuring stem cell engraftment outcomes in vivo. The described magnetic resonance-based cell tracking technique could be directly applied to monitor MASIs in patients. Using expanding stem cell magnetic resonance signal as a surrogate marker for stem cell viability and engraftment would enable us to overcome the bottleneck of diagnosing transplant failures, avoid long-term and invasive follow-up studies of apoptotic or lost transplants, and help assign patients with transplant failure to early interventions or alternative treatment options.

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Figure 1.

Electron microscopy (EM) and fluorescence microscopy of labeled mesenchymal stem cells (MSCs) incubated for 24 hours with ferumoxide (Endorem) only, ferumoxide and Lipofectin, or ferumoxide and protamine. For fluorescence microscopy, cells were stained with anti-dextran fluorescein isocyanate (FITC) antibody (*green*) and nucleus was counterstained with DAPI (*blue*) ($\times 40$ original magnification). For electron microscopy, the magnification factor was approximately 2900. Fluorescence microscopy confirmed intracellular internalization of contrast agent for all labeling techniques. MSCs labeled with transfection agent showed more intense fluorescence signal, corresponding to higher quantities of internalized contrast agent, as determined by spectrometry. EM demonstrated that contrast was incorporated into secondary lysosomes and not bound to the cell surface. No evidence of morphologic abnormalities of labeled cells was noted.

Figure 2. Cellular iron content (*bar graph*), as quantified by ICP-AES spectrometry, and viability of mesenchymal stem cells (MSCs) (*plot graph*) for MSCs labeled with ferumoxide by simple incubation, protamine transfection, or Lipofectin transfection for increasing incubation intervals. Data represent means of triplicate experiments.

Figure 3.

Axial T₂*-weighted magnetic resonance images through test tubes with mesenchymal stem cells labeled with ferumoxide by simple incubation, protamine transfection, or Lipofectin transfection (*upper image*) and corresponding quantitative T₂* relaxation times (*lowergraph*) for time intervals of cell labeling. Data are displayed as means and standard deviations of triplicate experiments.

Figure 4.

Ex vivo sagittal magnetic resonance images through pig knee specimens with cartilage defects and matrix-associated stem cell implants (MASTs) containing unlabeled mesenchymal stem cells (MSCs) or MSCs labeled by simple incubation with ferumoxide (Endorem), protamine transfection, or Lipofectin transfection. The T_2 effect of cell implants labeled by simple incubation and protamine transfection corresponded approximately to the size of the implant. Cells labeled by Lipofectin transfection demonstrate a “blooming effect”; that is, the size of the magnetic resonance signal effect exceeded the size of the pellet. The lower bar graph demonstrates corresponding mean signal to noise ratios (SNRs) of ferumoxide-labeled MASIs, unlabeled MASIs and scaffold only ($n=6$ in each group) for T1-SE, T2-FSE, SPGR and T2*-GE pulse sequences. A significant difference between SNR data of labeled and unlabeled implants was noted on all sequences ($p<.05$). T2*-weighted images demonstrated a maximal signal decline in SNR data of ferumoxide-labeled MASIs compared to unlabeled controls, without any significant differences between different labeling techniques ($p>.05$).

Figure 5.

In vivo magnetic resonance imaging (MR1) of human mesenchymal stem cell implants and quantitative analysis of in vivo imaging. **A**, Sagittal T₂-weighted MRIs of rat knee transplants. Labeled viable and apoptotic matrix-associated stem cell implants are noted as a signal void on the day of implantation. At 2 weeks postimplantation, an increase in the area of the signal void of viable labeled transplants is noted. A smaller area of signal void is noted in both transplants at 12 weeks posttransplantation. **B**, Sagittal T₂-weighted MRIs of control group transplants (scaffold only, viable unlabeled, and apoptotic unlabeled) demonstrate a hyperintense T₂ signal that covered a smaller area than the hypointense signal of labeled implants. Persistent hyperintense signal is noted at the transplantation site at week 4 with a hypointense rim likely related to the presence of sequestered bone fragments from the surgical procedure, scar tissue, and hemosiderin. **C**, Graphical representation of the total number of hypointense pixels in each labeled defect over 12 weeks. Each data point represents a mean of six rat knee transplants at that particular time point. The viable transplants demonstrated an initial increase in size at 2 weeks in comparison with apoptotic implants, followed by a plateau at 4 weeks and then a gradual decrease in size over 12 weeks in both transplants. **D**, Graphical representation of the signal to noise ratio (SNR) of labeled transplants over 12 weeks. Each data point represents a mean of six rat knee transplants at that particular time point. The differences between SNR data of viable and apoptotic groups were not statistically significant.

Figure 6.

Microscopic appearance of human mesenchymal stem cell (hMSC) implants in osteochondral defects. Hematoxylin and eosin (H&E), alcian blue, and Prussian blue stains at 10 $\times$ and 20 $\times$ original magnification of viable (V; a to d) and apoptotic (A; e to h) ferumoxide-labeled transplants: complete repair of viable transplants is noted with matrix production. On Prussian blue staining, iron-positive cells (*arrows*) are noted in the transplant, which are more abundant in the viable transplants. H&E stain of a control (scaffold only) transplant (i) reveals incomplete repair with the presence of scar tissue. Anti-CD44 staining specific for hMSCs in viable transplants on fluorescent microscopy (j) and chromogenic staining (k) demonstrate cytoplasmic positivity at the transplantation site. These cells are more abundant in viable compared to apoptotic transplants (l) and are absent in control transplants (m).