Published in final edited form as:

Magn Reson Imaging. 2011 January ; 29(1): 40–49. doi:10.1016/j.mri.2010.07.015.

Micrometer-sized Iron Oxide Particle Labeling of Mesenchymal Stem Cells for Magnetic Resonance Imaging Based Monitoring of Cartilage Tissue Engineering

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Abstract

Purpose—To examine mesenchymal stem cell (MSC) labeling with micrometer-sized iron oxide particles (MPIOs) for magnetic resonance imaging (MRI) based tracking, and its application to monitoring articular cartilage regeneration.

Methods—Rabbit MSCs were labeled using commercial MPIOs. *In vitro* MRI was performed with gradient echo (GRE) and spin echo (SE) sequences at 3 tesla, and quantitatively characterized using line profile and region of interest analysis. *Ex vivo* MRI of hydrogel- encapsulated labeled MSCs implanted within a bovine knee was performed with spoiled GRE (SPGR) and T_{1ρ} sequences. Fluorescence microscopy, labeling efficiency, and chondrogenesis of MPIO-labeled cells were also examined.

Results—MPIO-labeling results in efficient contrast uptake, and signal loss that can be visualized and quantitatively characterized via MRI. SPGR imaging of implanted cells results in *ex vivo* detection within native tissue, and T_{1ρ} imaging is unaffected by the presence of labeled cells immediately following implantation. MPIO labeling does not affect quantitative glycosaminoglycan production during chondrogenesis, but iron aggregation hinders extracellular matrix visualization. This aggregation may result from excess unincorporated particles following labeling, and is an issue that necessitates further investigation.

Conclusion—This study demonstrates the promise of MPIO labeling for monitoring cartilage regeneration, and highlights its potential in the development of cell-based tissue engineering strategies.

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Introduction

Bone marrow-derived mesenchymal stem cells (MSCs) are multi-potent cells that function as a source of undifferentiated cells for tissue rejuvenation. As part of the body's repair process, MSCs can differentiate along several specific lineages in order to replenish dying cells and regenerate tissue. Based on the ability to isolate MSCs from patients, culture them *ex vivo*, and transplant them back into the body, MSCs are currently under investigation for tissue engineering in a variety of applications. In conditions where the body is unable to regenerate or repair on its own, implanted stem cells would ideally differentiate along the appropriate pathway to restore tissue morphology and function. An important consideration in translating stem cell therapies into the clinic is the ability to effectively monitor the bio-distribution and function of cells following implantation.

Labeling MSCs with superparamagnetic iron oxides (SPIOs) prior to transplantation allows detection of cells using magnetic resonance imaging (MRI) [1,2]. This technique can facilitate longitudinal non-invasive *in vivo* bio-distribution assessment of transplanted cell populations. Labeled cells appear as signal voids on MR images due to signal intensity (SI) loss that can be visualized on iron sensitive T₂—weighted images, and detected as a characteristic magnetic susceptibility artifact on T₂*—weighted images [3]. Micrometer-sized iron oxide particles (MPIOs), a type of SPIO, have demonstrated effective labeling of MSCs for MR tracking [4]. MPIOs consist of an iron oxide core encased within an inert divinyl benzene polymer shell, and a fluorescent dye for optional co-localization. Of note, the size of MPIO particles is approximately two orders of magnitude larger than conventional SPIO nanoparticles. Hinds *et al.* [4] demonstrated efficient non-toxic labeling of MSCs via endocytosis, single cell MR detection at 11.7 tesla (T), and longitudinal detection of labeled cells. Furthermore, labeled MSCs retained the ability to undergo osteogenic and adipogenic differentiation *in vitro*.

MSCs are currently being explored for treatment of knee articular cartilage lesions, which can be generated by trauma, or a variety of diseases, most notably osteoarthritis [5]. Articular cartilage biology and the changes associated with cartilage lesions have been extensively reviewed [6,7]. Briefly, cartilage consists of chondrocytes within an extracellular matrix (ECM) consisting of collagens, proteoglycans, and other proteins, which maintain proper water content within the matrix, and allow variable load bearing through a range of motion and activities. The intricate structure and biology of cartilage is limited in its ability to repair or regenerate, due to its avascularity, and the limited ability of mature chondrocytes to move within the ECM, proliferate, or alter their synthetic activity.

Given the limited ability of cartilage to self-regenerate, MSC-based tissue engineering could provide a method to repair damaged or diseased tissues. Studies by Williams *et al.* [8] demonstrated *in vitro* chondrogenic differentiation of MSCs as evidenced by positive staining for proteoglycan and collagen II, as well as quantitative increases in DNA, glycosaminoglycan (GAG), and collagen content. *In vivo* studies using a rabbit osteochondral defect, demonstrate the survival of implanted scaffold encapsulated MSCs, and the production of immature articular cartilage containing collagen II [9]. In addition, synthetic ECM encapsulated MSCs implanted within a similar *in vivo* rabbit model resulted in the formation of articular cartilage-like tissue and integration with the surrounding native cartilage [10].

While MR-based stem cell tracking, and stem cell-based regeneration of cartilage have been active fields of study independently, no studies to date have looked at the potential of MPIO stem cell labeling to monitor cartilage regeneration. Consequently, the purpose of this study is to further examine MPIO labeling of MSCs, and investigate this technique for clinically

applicable monitoring of cartilage tissue regeneration. To this end MSCs were labeled with MPIOs, and a population of cells detected *in vitro* and *ex vivo* using a clinical MR scanner. In addition to detection, applying this technique to monitoring cells within cartilage raises questions about the effect that labeled cells will have on MR scans typically used to probe cartilage integrity. Hence, *ex vivo* T₁ ρ imaging, typically used to detect proteoglycans within cartilage [11-13] was performed in the presence of MPIO- labeled cells. Furthermore, fluorescence microscopy was used for co-validation of labeling, and to investigate the presence of extracellular particles following labeling. In addition, labeled cells were tested for labeling efficiency, cell viability, and the effect of labeling on chondrogenesis. The results of this study demonstrate the promise of this technique for monitoring cartilage regeneration, and highlight the need for future development of this method as a clinically relevant means of monitoring cell-based tissue engineering strategies for a wide variety of applications.

Methods

Cell Isolation and Expansion

Bone marrow-derived MSCs were harvested from the iliac crest of female young adult (> 5kg) New Zealand White rabbits immediately after animal sacrifice based on a technique adapted from Johnstone *et al.* [14]. Briefly, the marrow was aspirated using a 10mL syringe containing heparin (5000units). The samples were placed in 75cm² tissue culture flasks containing standard tissue culture media: high glucose DMEM (Invitrogen, Carlsbad, CA) with 10% FBS (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). After 48 hours, non-adherent cells were removed, and the remaining adherent cells were maintained in the same media (changed every 2-3 days), expanded using standard conditions (37°C and 5% CO₂), and passaged 1:4 at 80-90% confluence. Cells were sub- cultivated using 0.25% trypsin with 1mM EDTA (Invitrogen) and used for experiments between passages 6-10 to avoid cell senescence.

Labeling

Rabbit MSCs (rMSCs) were labeled with MPIOs using a technique described by Hinds *et al.* [4]. Briefly, 1.63 μ m diameter encapsulated micro-spheres (Bangs Laboratories, Fishers, IN) were added to standard tissue culture media at a concentration of 10 μ L/mL and mixed for 10 minutes. The stock solution of contrast agent particles used for cellular labeling consisted of an iron concentration of (~ 4.25mg/mL), and addition into the cell culture media resulted in a final iron concentration of 2.8 μ g/mL used for labeling. After mixing, the labeling media was added to an 80% confluent MSC monolayer in 75cm² cell culture flasks (n=3), and cell cultures incubated overnight. Unlabeled cell flasks (n=3) were also used as experimental controls. Following overnight labeling with MPIOs, samples were washed three separate times with PBS to remove excess particles not taken up by cells. Cells were then trypsinized, collected, and counted for further experiments. Cell viability was assayed during counting using Trypan Blue (Invitrogen).

Chondrogenesis

In vitro chondrogenesis via pellet culture of labeled MSCs and unlabeled MSC controls was induced using a Chondro-Bulletkit containing TGF- β 3 (Lonza, Walkersville, MD) according to the manufacturer's protocol (4 weeks). Following differentiation, labeled (n=2) and unlabeled (n=2) pellets were fixed in 10% formalin, dehydrated in ethanol, and embedded in paraffin using standard tissue processing technique. Embedded sections (5 μ m) were stained with hematoxylin and eosin (H&E), safranin-O, and fast green, visualized by light microscopy (Olympus CX41, Center Valley, PA), and photographed (Nikon Coolpix 5000, Melville, NY). For glycosaminoglycan (GAG) content, labeled (n=6) and unlabeled (n=6)

samples were digested overnight in papain, and chondroitin sulfate concentration was measured using dimethylmethylene blue dye (Biocolor, Carrickfergus, UK; DMMB). A spectrophotometer (Varian Carey 300, Walnut Creek, CA) was used to measure the absorbance of the pellet samples at 656nm, and GAG content was determined by comparing the obtained values to a known standard curve. The range of the standards was large enough to ensure that all experimental samples fell within the linear range. Differences between labeled and unlabeled pellets were compared using a student's t-test ($p < 0.05$).

Fluorescence Imaging and Analysis

To visualize the presence of MPIOs within cells following labeling, fluorescence imaging of the Dragon Green tag (Ex/Em: 480/520nm) associated with the MPIOs was performed, along with unlabeled cell controls. For studies investigating the presence of excess unincorporated particles following labeling and PBS washing, cells were seeded on Permanox Lab-Tek tissue culture slides (Fisher Scientific, Hampton, NH; $n=4$) and grown to 80% confluence before labeling. Following labeling and three PBS washes, cells were co-labeled with a Hoechst stain (Fisher; Ex/Em: 350/461nm) by adding a 1:200 dilution of the stock solution to each tissue culture well for 5 minutes. Cells labeled only with the Hoechst stain were also visualized as a control ($n=4$). Images were obtained using an Olympus BX60 microscope with fluorescent attachment, and captured using an AxioCam MRm system (Zeiss, Thornwood, NY). NIH ImageJ software (Bethesda, MD) was used for processing and image overlay.

Flow cytometry was used to quantify the mean fluorescent intensity (MFI) to analyze MPIO labeling efficiency ($n=3$ flasks). Following labeling and PBS washing, cells were collected and analyzed using a FACSCalibur Cell Counter System (BD Biosciences) set to the FL1 channel (Ex/Em: 488/530 $\pm$ 15nm). The forward and side scatter of the events was adjusted to differentiate between live and dead cells. Events within the gated live cell population were measured for MFI with at least 10,000 events per a sample. Unlabeled cells were used for calibration and multiple samples from each labeled flask were analyzed and the results averaged. All flow cytometry parameters were fixed in comparing a single analysis. All samples were run consecutively on a single machine with conserved analysis parameters.

Ex vivo animal model implantation

To investigate detection of MPIO-labeled rMSCs within native tissue, an *ex vivo* bovine osteochondral knee defect model was used. A cylindrical defect (diameter=5mm; height~9mm) was created in each condyle of a single bovine distal femur. Prior to implantation, MPIO-labeled rMSCs were encapsulated within commercially available Puramatrix hydrogel (3DM, Cambridge, MA), according to the manufacturer's instructions. Unlabeled control rMSCs were also encapsulated within a separate Puramatrix hydrogel. Following gelation, the two defects were filled as follows: (1) Puramatrix construct containing MPIO-labeled rMSCs (5×10^6 cells/mL); (2) Puramatrix construct containing unlabeled rMSCs (5×10^6 cells/mL). Care was taken to ensure that the constructs would not leak out of the defects, and following MRI each defect was visually reexamined for the presence of the construct.

MRI

All MRI was performed on a 3T GE Signa Excite system (GE Medical Systems, Waukesha, WI), using a quadrature transmit/receive knee coil (GE Medical Systems). For *in vitro* cell imaging in solution, labeled MSCs, (1×10^6 cells/mL) were suspended in tubes containing 1.0mL Ficoll (GE Healthcare Bio-Sciences, Piscataway, NJ; $n=3$) to prevent cell settling during imaging. As negative controls, unlabeled cells ($n=3$), and Ficoll alone ($n=3$) were also imaged. Tubes containing the samples were placed in a plastic container filled with

water in order to remove the air susceptibility artifact, and all imaging was carried out at room temperature (20°C). Each tube was imaged alone to prevent image distortions caused by the iron present in neighboring tubes and all tubes were imaged within the same location in the magnet to limit the effect of main field inhomogeneities. 2D Gradient echo (GRE) imaging of the tubes was performed as a single coronal slice with a (Echo Time (TE)/ Repetition Time (TR)=4-24/500ms, a 12cm field of view (FOV), flip angle=90°, matrix size=256×256, 468µm in-plane resolution, bandwidth (BW)=15.63kHz, and slice thickness=3mm. Spin-echo (SE) imaging was performed using the same parameters, except with a TE/TR=20-200/2000ms. Image analysis was performed using dedicated software written in MATLAB (Mathworks, Natick, MA) to measure the SI as a line profile passing through the center of the tube in each image, and to determine the mean SI for a defined region of interest (ROI). The line profile analysis consisted of sampling the SI value for every fifth pixel of the image passing horizontally through the center of the tube. Differences in SI between labeled and unlabeled cells were compared using a student's t-test ($p < 0.05$) for both the line profile and ROI analysis.

Ex vivo imaging of cells within the bovine knee was performed using a 3D sagittal spoiled GRE (SPGR) sequence with a TE/TR=6.7/15ms, flip angle=12°, FOV=12cm, matrix size=512×256, and slice thickness=1mm. In addition a 3D $T_{1\rho}$ -weighted sequence [15] was used to image the bovine knee sagittally, with TE/TR=3.7/9.3ms, FOV=12cm, matrix size=256×128, slice thickness=1mm, BW=31.25kHz, views per segment (VPS)=64, time of recovery (T_{rec})=1500ms, time of spin lock (TSL)=0,10,40,80ms, and frequency of spin lock (FSL)=500Hz. Image processing software written in Interactive Data Language (IDL; ITT Visual Information Solutions, Boulder, CO) was used to generate $T_{1\rho}$ maps by fitting the data on a pixel-by-pixel basis to the equation $S(TSL) \sim \exp(-TSL/T_{1\rho})$. Following segmentation using dedicated IDL software, masked $T_{1\rho}$ maps were overlaid onto the SPGR images using software written in MATLAB.

Results

MPIO labeling and signal characteristics

The *in vitro* results indicate efficient cellular uptake of MPIOs, and corresponding distinguishable SI loss of labeled cells on MR images. Figure 1a shows a single MSC cell labeled with MPIOs (fluorescence overlaid on bright field), as demonstrated by the fluorescent tag associated with the iron oxide contrast agent that is not present within the unlabeled control cell (Fig. 1b). Flow cytometry analysis following overnight labeling resulted in a labeling efficiency of $86 \pm 3\%$. Trypan blue staining to detect the presence of dead cells did not show an effect of labeling on cell viability as compared to unlabeled controls (not shown).

MPIO-labeled rMSCs appear as a distinct hypo-intense region when imaged *in vitro* using both GRE (Fig. 1c-e) and SE imaging (Fig. 1f-h). MPIO labeling has a greater effect on the GRE sequence, but is still clearly visible as a signal void on the SE image. Quantitative characterization of this SI loss based on line profile analysis and measurement of the mean SI for the ROI corresponding to each tube is depicted in Figure 2. For GRE imaging, MPIO-labeled cells (1×10^6 cells/mL) result in a significant ($p < 0.05$) loss throughout the tube in SI for labeled cells as compared to the control tubes (Fig. 2a). SE imaging results in a similar significant ($p < 0.05$) loss in SI throughout the tube containing labeled cells. The particular TE values for the GRE (TE=8ms) and SE (TE=150ms) sequence were chosen for quantitative analysis because they resulted in the greatest contrast between labeled cells and the control tubes. Figure 1c shows the mean SI for the ROI corresponding to each tube for both GRE and SE imaging. The significant loss ($p < 0.05$) of SI for MPIO-labeled rMSCs is clearly visible.

Ex vivo imaging

MPIO-labeled cells were seeded within a Puramatrix hydrogel and detected *ex vivo* within a bovine knee osteochondral defect (Fig. 3). While unlabeled cells in the hydrogel appear to have similar signal characteristics to cartilage (Fig. 3a), MPIO-labeled cells can be visualized as a distinct hypo-intense region within the osteochondral defect (Fig. 3b). This loss of SI can be distinguished from the native articular cartilage tissue as well as the underlying bone. $T_{1\rho}$ imaging of the implants within the osteochondral defects demonstrates strongly elevated $T_{1\rho}$ values for both unlabeled and labeled cells (Fig. 3c-d), as compared to native cartilage, which has values ranging from 80-100ms. For both the unlabeled and labeled cells in Puramatrix hydrogel, there also appears to be a large degree of heterogeneity in the $T_{1\rho}$ values.

Chondrogenic differentiation

In order to assess the effect of MPIO labeling on MSCs chondrogenesis, MPIO-labeled rMSCs and unlabeled rMSCs underwent *in vitro* differentiation. Safranin-O and H&E staining was used to visually assess the differentiation capacity of MPIO-labeled rMSCs. This dye combination stains GAG red-pink, nuclei blue-purple, and the cytoplasm green. Figure 4a shows a representative image of staining throughout the pellet for unlabeled control cells. The presence of pink/red staining indicates gag production, which is further demonstrated via higher magnification in Figure 4b. However in the case of MPIO-labeled cells, while pellet formation did occur during the 4-week chondro-differentiation procedure, the presence of aggregated iron throughout the pellet hinders ECM visualization (Fig. 4c). Higher magnification examination (Fig. 4d) reveals traces of positive GAG staining (arrows), but the presence of iron aggregates is clearly visible. However, quantitative assessment of proteoglycan production using a DMMB assay shows no significant difference ($p<0.05$) in GAG content per pellet (Fig. 5) between MPIO-labeled and unlabeled cell pellets following *in vitro* chondrogenesis.

Excess iron analysis

To determine whether excess unincorporated iron oxide particles were present following the labeling procedure and PBS washing, fluorescence imaging of co-labeled cells was performed (Fig. 6). A representative image of MPIO-labeled rMSCs that were Hoechst-labeled in order to stain the nucleus is shown in Figure 6b. While the image shows MPIO particles associated with cells (surrounding the nucleus), there also appears to be excess iron particles not associated with cells. The presence of excess iron oxide particles following PBS washing is further demonstrated in Figure 6c, which shows a region containing iron oxide particles without the presence of cells.

Discussion

The promise of stem cell-based tissue engineering is enhanced considerably with an effective minimally invasive method for assessment following implantation. MRI is non-invasive, capable of *in vivo* assessment, and able to allow for longitudinal imaging at successive time points. MPIO labeling of stem cells results in a hypo-intense region that can be distinguished on the corresponding MR image. While MR tracking of MPIO-labeled cells has been investigated previously, the application to regeneration of articular cartilage has not been examined to date. Application of this technique to the regeneration of cartilage is important because the ability to track implanted MSCs within the joint would be valuable as a means of non-invasively monitoring the repair and regeneration process; ultimately allowing for an assessment of the effectiveness of such treatment. As demonstrated by this study, MPIO-labeling and tracking of cells using MRI has promise as a means for studying stem cell regeneration of articular cartilage.

The *in vitro* results show efficient MSC uptake of the MPIOs, along with the associated hypo-intense region, and corresponding loss in SI using both GRE and SE imaging. The SI loss resulting from MPIO labeling of rMSCs is strongest on the GRE images (Fig. 1c; 2) which is to be expected since GRE sequences are highly sensitive to susceptibility differences between tissues. Loading of cells with MPIOs creates a large field inhomogeneity resulting in a rapid SI loss. While SE sequences are intended to minimize the effect of susceptibility differences through the use of a 180° refocusing pulse, because of water proton diffusion prior to and following refocusing, SI loss can still be clearly visualized using these sequences as well.

MPIOs have been used to label a variety of cells including, CD34⁺ cells [4], hepatocytes [16], fibroblasts [16], neural progenitors [17], macrophages [18], and T-cells [19]. Owing to their larger size, MPIOs have several advantages over conventional nano-sized iron oxide particles. First, the use of nano-sized iron oxide particles requires millions of particles per cell for MR detection necessitating highly efficient labeling techniques that often include the use of a transfection agent to label MSCs [20-21]. In this study, cells were labeled with close to 90% efficiency without the use of a transfection agent. This efficiency is similar to results by Hinds *et al.* [4] who report 100% labeling efficiency of MSCs, and 90% for non-adherent cells. One issue in labeling cells with iron oxides is determining whether the iron oxide particles are internalized by the cell, or only attached to the surface of the cell; surface attachment is undesirable because of its transient nature. When MSCs are incubated with MPIOs using the labeling technique described in this study, internalization and incorporation into cellular endosomes has been verified using the endosomal marker CM-DiI [4]. Another advantage of this methodology is that the large particle size of MPIOs also inherently reduces the number of particles per cell needed for detection while still maintaining similar iron load. This will likely facilitate clinical imaging of smaller populations of transplanted cells. While this study utilized a 3T clinical scanner for imaging which allows detection of a population of labeled cells, at ultra-high field strengths single MSCs have been detected following MPIO labeling [4]. Lastly, given that multiple cell divisions [22] cause rapid dilution of iron within each cell the ability to track fewer particles has the potential to facilitate detection for longer time periods.

MPIO-rMSCs were also detected within an *ex vivo* osteochondral defect and distinguished from the surrounding tissue. This is the first demonstration of MPIO-labeled cell detection within articular cartilage. Jing *et al.* [23] have demonstrated *in vivo* imaging of cells labeled with iron oxide nanoparticles for up to 12 weeks. In this case the labeling was performed using a Feridex-Protamine (FE-PRO) complex, and a rabbit osteochondral defect model was used for implantation. In the present study, a bovine knee was chosen based on the thickness of the cartilage, which makes visualization easier than in the rabbit knee where cartilage thickness is approximately 200µm.

Prior to implantation, labeled cells were encapsulated within a Puramatrix hydrogel scaffold. Implanted MSC populations require a scaffold to promote cell adherence, proliferation, and differentiation [24]. For MR tracking of implanted MSCs, it is important to consider the signal characteristics of the scaffold, and ensure that they do not interfere with detection of the labeled cells themselves. Among a wide variety of synthetic polymers, the use of hydrogels as 3D scaffolds for cell implantation has been well documented [25]. The Puramatrix hydrogel used in this study is commercially available, and has been shown to support growth and differentiation of MSCs [26-28]. The polymerization characteristics of Puramatrix allow implantation of the hydrogel in liquid form, and then formation of the gel upon exposure to *in vivo* physiological conditions; for *in vitro* culture, polymerization occurs upon exposure to normal cell culture media, which was used for gel formation in this study. Based on the results presented here, encapsulation within Puramatrix does not appear to

inhibit MR detection of MPIO-labeled rMSCs. Detection of MPIO-labeled cells within Puramatrix hydrogels has not been reported to date, although previous work has demonstrated MR detection of FE-Pro labeled cells within poly(ethylene glycol) hydrogels [21], as well as MR detection of iron oxide labeled cells within collagen-based gel scaffolds using an 11.7T MRI [29].

In this study, $T_{1\rho}$ imaging of labeled cells implanted within the osteochondral defect was performed. $T_{1\rho}$ describes longitudinal relaxation in the rotating frame, and is sensitive to spin-lattice energy exchange between water and large molecules such as those present within the ECM of articular cartilage. Disruption of proteoglycan content within the cartilage matrix is known to cause an elevation in $T_{1\rho}$ resulting from increased water molecule motion [11-13]. The results indicate strongly elevated $T_{1\rho}$ values for the region corresponding to the constructs for both unlabeled and MPIO-labeled cells, likely due to the extremely high water content of the hydrogels and associated water molecule motion. It is important to consider the effect that labeling will have on MRI based functional assessment techniques. As an example, ideally iron oxide labeling should allow for tracking of cell bio-distribution, but should not inhibit $T_{1\rho}$ imaging, which can be used to evaluate the integrity of the newly formed tissue and the surrounding cartilage. From this study, immediately following encapsulation and implantation, $T_{1\rho}$ imaging appears to be dominated by the high water content of the hydrogel, independent of labeling. However, over time as implanted cells differentiate and produce ECM, the effect on $T_{1\rho}$ imaging remains to be seen, including any potential effects of iron oxide labeling. While T_2 imaging is also often used to analyze cartilage [30], in this study it was not utilized because of the strong sensitivity of T_2 to the presence of iron oxides.

Previously, there has been controversy about the effect of iron oxide labeling on MSC chondrogenesis. Kostura *et al.* using poly-L-lysine (PLL)-coated Feridex reported inhibition of chondrogenesis mediated by the presence of iron oxide [31]. However, chondrogenic differentiation of FE-Pro labeled MSCs was demonstrated by Arbab *et al.* [3]. MSCs labeled with MPIOs have demonstrated osteogenic and adipogenic differentiation [4], but studies of chondrogenesis have not been performed to date. Given the considerable size differences in particles size, iron content, and transfection agent requirements of MPIOs compared to conventional iron oxide nanoparticles, this study investigated MPIO labeling effects on MSC chondrogenesis. The results indicate that MPIO-labeling does not quantitatively inhibit gag production, but extracellular iron aggregation limits visualization of the ECM. This limits the ability to use conventional staining techniques to examine proteoglycan production, and qualitatively assess chondrogenic differentiation. The source of the aggregated iron is possibly a result of the excess MPIOs present following the labeling procedure and PBS washing. During chondrogenesis, aggregation of these excess particles is likely promoted by the use of centrifugation to initiate formation of a cell pellet, and remains aggregated within the pellet throughout the chondro-differentiation procedure. As such, the aggregation of excess particles may be further exacerbated by the particular method of *in vitro* chondrogenesis performed here, and future studies are aimed at using 3-D culture [8] to promote chondro-differentiation of MPIO-labeled MSCs.

One limitation of this technique as demonstrated in this study is the presence of excess unincorporated MPIO particles following labeling and PBS washing (Fig. 6b-c). The labeling method utilized here is based on the procedure described by Hinds *et al.* used to label porcine MSCs [4], and consisted of a similar iron concentration used for labeling. In contrast to the present study, Hinds *et al.* report the ability to sufficiently remove excess particles with PBS washing following the labeling procedure. The ability to successfully remove excess unincorporated particles is important, because particles not associated with cells will result in improper localization of cells, and limit the accuracy of this technique. As

such, while MPIO labeling may provide several advantages over labeling with conventional iron oxide nanoparticles, these potential benefits should be weighed against the proclivity for residual MPIOs to remain and aggregate following the labeling procedure. Future studies should investigate alternative washing methods, and other techniques for isolating labeled cells from excess MPIO particles not taken up during the labeling procedure (e.g. FACS isolation).

This study demonstrates the promise of MPIO labeling for monitoring cartilage regeneration, and highlights its potential use in the future development of cell-based tissue engineering. Future studies aimed at ensuring the presence of only particles contained within cells, as well as longitudinal examination of MR tracking of MPIO-labeled MSCs throughout the cartilage regeneration process will aid in the development of this technique as a clinical tool for assessment of implanted stem cells.

Acknowledgments

1. The authors wish to thank Lynn DeLosSantos for assistance with histological analysis of the chondro-differentiation samples.
2. The T1 ρ -weighted sequence was implemented by Eric Han from Global Applied Sciences Laboratory, GE Healthcare.
3. This research was supported by NIHRO1-AG17762.

Grant Support: NIHRO1-AG17762.

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Figure 1. Iron oxide labeling of rabbit MSCs with MPIOs

(a) Fluorescence microscopy image of a representative cell after overnight labeling. The dragon green fluorescence indicates cellular uptake of the iron oxide contrast agent, which is not present within the (b) representative unlabeled control cell. (c) (d) & (e) GRE imaging (TE/TR=8/500ms) of labeled cells (1×10^6 cells/mL), unlabeled cells (1×10^6 cells/mL), and empty Ficoll respectively. (f) (g) & (h) SE imaging (TE/TR=150/2000ms) of labeled cells (1×10^6 cells/mL), unlabeled cells (1×10^6 cells/mL), and empty Ficoll respectively. Both GRE and SE imaging demonstrate a visible signal loss associated with iron oxide labeling that is not present within unlabeled cells or the Ficoll solution alone. (scale bar=10µm)

Figure 2. Quantitative analysis of signal loss associated with MPIO labeling of cells

Line profiles demonstrating the sharp signal drop off associated with labeled cells, detectable using both (a) GRE (TE/TR=8/500ms) imaging; & (b) SE (TE/TR=150/2000ms) imaging. Differences in SI between labeled cell and control tubes are significant ($p < 0.05$) for both GRE and SE imaging. (c) SI values for ROIs corresponding to tubes of labeled cells, unlabeled cells, and Ficoll, demonstrating significant ($p < 0.05$) SI loss that is detectable using both GRE and SE imaging. (data is presented as mean $\pm$ standard deviation for $n=3$ samples; cell concentration= 1×10^6 cells/mL)

Figure 3. *Ex vivo* MRI of implanted cells

SPGR imaging (TE/TR=6.7/15ms) of (a) unlabeled MSCs; and (b) MPIO-labeled MSCs. Labeled cells are visible as a distinct void with blooming artifact, as compared to unlabeled cells that appear to have a similar SI as native cartilage. Colormap overlaid on the SPGR image of (c) unlabeled MSCs; and (d) MPIO-labeled MSCs. The colorbar shows the associated $T_{1\rho}$ values (ms). Both unlabeled cells and labeled cells in the Puramatrix hydrogel show elevated $T_{1\rho}$ values compared to native cartilage. (5×10^6 cells/mL)

Figure 4. Chondro-differentiation of MPIO-labeled rabbit MSCs

H&E and safranin-O/fast green dye combination stains GAG red-pink, nuclei blue-purple, and the cytoplasm green. (a) Unlabeled cells showing positive GAG staining (pink/red) throughout the pellet; (b) High magnification section of pellet demonstrating positive GAG staining; (c) MPIO-labeled cells showing considerable aggregation of iron throughout the pellet which hinders visualization of the ECM; (d) While labeled sections demonstrate traces of positive GAG staining (black arrows), iron aggregates are most apparent. (scale bar=20 μ m)

Figure 5. Quantitative analysis of MPIO-labeled rabbit MSC chondrogenesis
Results of dimethylmethylene blue assay to determine GAG content in labeled and unlabeled cell pellets. No significant difference was found between labeled and unlabeled cells ($p < 0.05$). (data is presented as mean $\pm$ standard deviation for $n=6$ samples)

Figure 6. Fluorescence microscopy of MPIO labeling and Hoechst staining of rabbit MSCs (a) unlabeled cells stained with Hoechst; (b) MPIO-labeled cells stained with Hoechst as well as excess particles not associated with cells (bottom left of image; (c) region of culture slide containing MPIO particles without the presence of cells. The fluorescence associated with residual iron oxide particles indicates that PBS washing alone does not completely remove contrast agent that is not taken up by cells during the labeling procedure. (scale bar=20 μ m)