Magnetic Resonance Imaging of Chondrocytes Labeled with Superparamagnetic Iron Oxide Nanoparticles in Tissue-Engineered Cartilage

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The distribution of cells within tissue-engineered constructs is difficult to study through nondestructive means, such as would be required after implantation. However, cell labeling with iron-containing particles may prove to be a useful approach to this problem, because regions containing such labeled cells have been shown to be readily detectable using magnetic resonance imaging (MRI). In this study, we used the Food and Drug Administration–approved superparamagnetic iron oxide (SPIO) contrast agent Feridex in combination with transfection agents to label chondrocytes and visualize them with MRI in two different tissue-engineered cartilage constructs. Correspondence between labeled cell spatial location as determined using MRI and histology was established. The SPIO-labeling process was found not to affect the phenotype or viability of the chondrocytes or the production of major cartilage matrix constituents. We believe that this method of visualizing and tracking chondrocytes may be useful in the further development of tissue engineered cartilage therapeutics.

Introduction

STRATEGIES FOR THE repair and replacement of articular cartilage, primarily in the setting of osteoarthritis (OA), are of substantial current interest.¹ One approach to this is through cartilage tissue engineering, with the goal of providing a living cartilage substitute to replace damaged tissue.² Assessment of implant characteristics is an important component of this approach to repair, and magnetic resonance imaging (MRI) in particular has been applied to the nondestructive evaluation of cartilage.^{3–5} However, MRI has been used for matrix evaluation rather than for studies of the cellular component of cartilage. Techniques that would characterize cellular distribution within an implant would be of particular interest because cellular distribution would affect the uniformity of matrix deposition within the construct, as well as the overall viability and functionality of tissue-engineered cartilage.^{6,7} It would be valuable to determine whether cells are retained within the scaffold^{8,9} and whether there is an anisotropic distribution of cells within the construct,⁶ so it is of interest to develop methods for tracking the cells seeded within cartilage constructs.

Numerous studies have reported the use of superparamagnetic iron oxide (SPIO) nanoparticle labeling of cells to permit cell detection using MRI.^{10–15} Intracellular iron

reduces local magnetic field homogeneity, allowing the cells to be visualized as signal voids with MRI.¹⁶ The application of such methods in the context of engineered constructs may be particularly useful as a noninvasive, nondestructive method of monitoring cellular distribution. Such methods should permit longitudinal visualization over a time period on the order of at least several weeks after seeding, with the potential for clinical applicability.¹⁷

Here, we report MRI detection of SPIO-labeled chondrocytes in two tissue-engineered cartilage constructs, a hollow-fiber bioreactor system (HFBR)^{18–20} and a photopolymerizable hydrogel.^{21–23} We also investigated the effect of iron labeling on cell viability, phenotype, and function, all of which must be maintained.¹⁰ The study was designed to determine whether chondrocytes could be labeled using SPIO nanoparticles using two approaches and subsequently visualized using MRI.

Materials and Methods

Chondrocyte harvest

Chondrocytes were isolated from the patellar groove and femoral condyle of 1- to 2-week-old calves (Research 87 Inc., Boylston, MA). Chondrocytes were isolated from eight legs

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from four calves for this study. Harvested cartilage was digested for 14 h at 37°C in a 5% carbon dioxide (CO₂) atmosphere, with stirring in 0.2% (w/v) sterile collagenase solution prepared by dissolving type II collagenase powder (Worthington, Freehold, NJ) in Dulbecco's modified Eagle medium (Biofluids, Rockville, MD), supplemented with 5% v/v fetal bovine serum (Hyclone, Logan, UT), 32 nM L-ascorbic acid-2-phosphate (Sigma, St. Louis, MO), 0.46 µg/mL of amphotericin B (Fungizone, Biofluids), 18 µg/mL of gentamicin (Cambrex, Walkersville, MD), 1.85 mM L-glutamine, 0.092 mM nonessential amino acids, 92 U/mL of penicillin-G, and 92 µg/mL of streptomycin. The chondrocytes were filtered through an 80-µm nylon filter and washed several times with phosphate buffered saline (PBS) (Biofluids).

SPIO cell labeling

Chondrocyte culture medium for labeled and unlabeled chondrocytes consisted of the same medium formulation described above but without collagenase and supplemented with 10% rather than 5% fetal bovine serum. Culture was performed at 37°C and within a 95% air/5% CO₂ environment. Before SPIO labeling, primary chondrocytes were cultured in the above medium until they reached confluence (passage 1). The commercially available ferumoxides suspension Feridex IV (~80–150 nm diameter, 11.2 mg of iron (Fe)/mL; Berlex Laboratories, Wayne, NJ) was used for SPIO labeling of the chondrocytes. After SPIO labeling, cells were seeded into scaffolds (photopolymerizable hydrogels or hollow fiber bioreactors) directly, without additional expansion in two-dimensional (2D) culture.

SPIO cell labeling for the bioreactor system. Poly-L-lysine (PLL) (molecular weight 150,000–300,000, Sigma-Aldrich, St. Louis, MO) was used as a transfection agent.²⁴ The concentrations of Feridex and PLL used were selected based on preliminary studies of intracellular iron uptake using atomic absorption spectrometry (AA), as described in the Atomic Absorption subsection below.

SPIO labeling was initiated with a 1:10 dilution of Feridex with serum-free chondrocyte culture medium (diluted iron content = 1.12 mg/mL). The diluted Feridex and PLL were added to chondrocyte medium to yield final concentrations of 50 µg/mL and 3 µg/mL, respectively. The Feridex–PLL suspension was incubated for 1 h to permit formation of ferumoxide-transfection agent complexes. Finally 10 mL of this suspension was added to each 162-cm² culture flask containing confluent chondrocytes and 10 mL of medium, resulting in a final concentration of 25 µg Fe/mL SPIO nanoparticles and 1.5 µg/mL PLL. Flasks were incubated in Fe–PLL medium suspension overnight.

SPIO cell labeling for the hydrogel system. Lipofectamine was used as a transfection agent for the hydrogel experiments.²⁵ Ten µL of Lipofectamine 2000 (1 mg/mL, Invitrogen Life Technologies, Gaithersburg, MD) was diluted in 10 mL of OptiMEM (Invitrogen) serum-free medium. Based on Trypan Blue cell viability experiments (described in Cell Viability subsection below), SPIO particles were suspended to achieve an iron concentration of 67.2 µg Fe/mL (in 10 mL of OptiMEM). The diluted lipofectamine was added at room

temperature to the SPIO suspension and mixed to a final lipofectamine concentration of 0.5 µg/mL. The resulting suspension was incubated at room temperature for 15 min. For labeling of chondrocytes, the chondrocyte culture medium described above was replaced by 20 mL of the OptiMEM–SPIO–lipofectamine suspension.

After introduction of the SPIO transfection agent complexes for the bioreactor and hydrogel systems, the cell culture flasks were incubated (~24 h) at 37°C, 5% CO₂ to permit endosomal uptake of the SPIO particles. The cells were then washed several times in PBS and immersed in chondrocyte medium.

SPIO uptake by chondrocytes. Selected SPIO labeling experiments were performed directly on chondrocytes (without a scaffold) to directly evaluate SPIO uptake and subsequent extracellular matrix (ECM) production by the cells. The selected SPIO–transfection agent complex concentrations for the hydrogel (SPIO:lipofectamine = 67.2 µg Fe/mL:0.5 µg/mL) and bioreactor systems (SPIO:PLL = 25 µg Fe/mL:1.5 µg/mL) were employed, and the same labeling procedures were repeated.

After labeling cells were washed several times with PBS, recovered in pellet form, and sorted using a magnetic stand (Invitrogen, Carlsbad, CA) for 15 min to remove any cell subpopulation that did not take up iron oxide. The labeled cells were then replated on glass chamber slides (Nalge-Nunc, Naperville, IL) and cultured in chondrocyte medium (SPIO:PLL group: 20000 chondrocytes/cm² and SPIO–lipofectamine group: 31000 chondrocytes/cm²) for 6 days. The purpose of this experiment was to establish the presence of intracellular iron and to evaluate the ability of magnetically sorted SPIO-labeled chondrocytes to secrete ECM. A control group was also cultured for 6 days (SPIO:control group: 54000 chondrocytes/cm²).

Three-dimensional tissue-engineered construct preparation

Bioreactor tissue engineering system. Bioreactor construction has been described previously.^{18–20,26} Briefly, a single polyvinylidene difluoride (PVDF) fiber (inner diameter, 700 µm; outer diameter, 1300 µm; pore size, 0.1 µm; FiberCell Systems, Inc., Frederick, MD) was fixed along the long axis of a glass tube bioreactor chamber using biomedical-grade silicone (MED-1137, NuSil Silicone Technology, Carpinteria, CA). Approximately 10 million to 15 million cells were inoculated through a rubber septum covering the chamber's side port, after which the bioreactor was perfused with the medium formulation described above using a pin-compression flow pump (Cellco/Spectrum, Rancho Dominguez, CA) and maintained in an atmosphere of 5% CO₂/95% air at 37°C. The medium was changed twice weekly. Six HFBRs were seeded with SPIO-labeled chondrocytes and another two HFBRs were seeded with unlabeled cells.

Hydrogel tissue engineering system. The hydrogel used was a 15% w/v nondegradable poly(ethylene oxide) diacrylate polymer (Nektar, Huntsville, AL) in PBS.²² A photoinitiator, IrgaCure (Ciba Specialty Chemicals, Tarrytown, NY), mixed in 70% ethanol to a final concentration of 100 mg/mL,

was added to the hydrogel solution (5 $\mu\text{L}/\text{mL}$) and mixed to a final 0.05% (%w/v) photoinitiator concentration. Chondrocytes were then suspended within the polymer solution (20×10^6 cells/mL). One hundred twenty μL of the cell-seeded poly(ethylene oxide) diacrylate solution was transferred into a sterile cylindrical mold (~ 2.4 million cells/hydrogel sample), after which the solution was exposed to 365-nm light at 3 to 4 mW/cm^2 (Glowmark Systems, Upper Saddle River, NJ) for 7 min to achieve complete gelation. The seeded hydrogels were then removed from their molds, immersed in chondrocyte medium in well plates, and retained in an incubator, with medium changes performed twice weekly. Eight hydrogels were seeded with SPIO-labeled chondrocytes, and another two hydrogels were seeded with unlabeled cells.

Cell viability

The Trypan Blue dye exclusion method was used to determine the maximum SPIO concentration that could be used while maintaining cell viability of greater than 90% with a lipofectamine concentration of 0.5 $\mu\text{g}/\text{mL}$. Suspensions of SPIO-loaded chondrocytes in PBS were mixed with Trypan Blue dye (Sigma) in a 1:1 ratio, and unstained live cells and blue-stained dead cells were counted. The procedure was initiated with cells loaded with 11.2 $\mu\text{g Fe}/\text{mL}$ SPIO suspension and repeated with increasing concentrations of SPIO until cellular viability was observed to fall below 90%.

SPIO-labeled chondrocytes were also subjected to terminal deoxy-nucleotidyl transferase (TUNEL) assay (Paragon Bioservices, Baltimore, MD) and flow cytometric analysis for detection of apoptotic and necrotic cells. In brief, fluorescein isothiocyanate conjugated with annexin V (Annexin V-FITC; apoptosis assay kit, Trevigen, Inc., Gaithersburg, MD) along with propidium iodide (PI) (Sigma-Aldrich) were diluted in binding buffer (Trevigen, Inc.) in a 10:1:1000 ratio, respectively. One hundred μL of this solution was used to resuspend the chondrocytes. The cells were mixed gently, covered in aluminum foil, and incubated for 15 min at room temperature. After incubation, an additional 1 mL of binding buffer was added to each tube (3.5×10^5 cells/tube) and subjected immediately to flow cytometric analysis. This analysis also included an Annexin V-FITC-only control, a PI-only control, and a tube containing only the binding buffer. The SPIO-labeled chondrocytes were analyzed using a flow cytometer (FACSCalibur, Becton Dickinson, Palo Alto, CA) using argon laser excitation (488 nm) and detection of emission for Annexin V-FITC at 530 nm (FL1) and for PI at 650 nm (FL3).

A Live-Dead assay using calcein AM/ethidium homodimer-I (EthD-I) (Live/Dead Viability/Cytotoxicity Kit, Molecular Probes, Eugene, OR) was conducted to determine the viability of the SPIO-labeled cells within the hydrogel constructs. EthD-I stains dead cells, which have compromised plasma membranes, with a red color. Calcein AM is specific to detection of live cells through their intracellular esterase activity, resulting in a green stain. For analysis, hydrogel samples (38 days old) were cut into thin sections (<1 mm) and stained according to the manufacturer's protocol with 0.5 $\mu\text{mol}/\text{mL}$ of calcein-AM stain and 2 $\mu\text{mol}/\text{mL}$ of EthD-I stain. The samples were covered and kept in an incubator for 30 min, after which they were examined under a fluorescence microscope.

Cell proliferation

Cell proliferation experiments were performed for the SPIO-PLL, SPIO-lipofectamine, and unlabeled chondrocyte groups. SPIO-labeled groups were first magnetically sorted to ensure that all the cells were adequately labeled. For each of the groups, a single cell pellet was resuspended uniformly in chondrocyte medium. Next, an aliquot (10 μL) of the cell suspension was stained with Trypan Blue and counted. After homogeneous mixing, the cell suspension for each of the three groups was divided into equal volumes and transferred to 12.5- cm^2 flasks (Fisher Scientific, Agawam, MA) ($n=3$ flasks/group). Cells were cultured for 3 days. At the end of this period, chondrocytes were detached from each flask after a 5-min exposure to 0.25% trypsin-EDTA solution (Invitrogen). The trypsin was deactivated by dilution with chondrocyte medium, and the cell suspensions were transferred to conical tubes (Becton Dickinson, San Jose, CA), which were centrifuged (Sorvall Legend RT series, Thermo Fisher Scientific, Waltham, MA) at 3000 RPM for 10 min. A cell pellet for each flask was recovered. The supernatant was removed, and the pellet was resuspended uniformly in fresh medium. A hemacytometer-based cell count was performed for each pellet ($n=3$ /group). Cell proliferation, expressed in percentages, was assessed as the ratio of the difference in cell number between day 3 and day 0 to the initial cell count determined at day 0.

Atomic absorption

Atomic absorption spectrometry analysis for iron uptake was performed using an AA spectrometer (Thermo Fisher Scientific). Calibration was performed with standard iron concentrations prepared by serial dilution of a 1.005 mg/mL Fe AA calibration standard (Aldrich Chemical Company, St. Louis, MO) with de-ionized water. The following Fe concentrations were used: 0.50, 1.01, 2.51, 5.03, and 10.1 $\mu\text{g}/\text{mL}$.

For each sample, 150 μL of cell suspension was digested with 150 μL of concentrated hydrochloric acid immediately after labeling. The suspension was then diluted further with de-ionized water to a total volume of 2.0 mL for AA analysis.

Histology

Chamber slides with cells grown in 2D culture for 6 days were dipped in 10% neutral buffered formalin for 15 min, after which Prussian blue staining was performed. Another set of 6-day slides was stained separately with Masson's Trichrome stain to visualize any collagen secretion by the chondrocytes.

Engineered tissue derived from both the HFBR and hydrogel systems was fixed in 10% formalin for at least 24 hours. The samples were then dehydrated, paraffin-embedded and sectioned into 5- μm -thick sections (Paragon Bioservices, Baltimore, MD). For tissue grown in the HFBR, sections were cut perpendicular to the long axis of the hollow fiber while cylindrical hydrogel samples were sectioned parallel to the face of the cylinder, yielding axial sections through each tissue sample. Prussian blue staining was performed on sections of tissue grown from SPIO-labeled chondrocytes in order to visualize iron and thus SPIO nanoparticle distribution. Adjacent sections were stained with

TABLE 1. ATOMIC ABSORPTION MEASUREMENTS OF IRON UPTAKE BY CHONDROCYTES

PLL concentration ($\mu\text{g/ml}$)	SPIO concentration ($\mu\text{g Fe/ml}$)	Fe concentration measured by AA in SPIO-labeled chondrocytes (pg/cell)
1.50	12.5	30
1.50	25.0	50
0.75	50.0	16
0.00	50.0	6.9

Numbers in bold indicate poly-L-lysine and Feridex concentrations used for subsequent magnetic resonance imaging studies of tissue grown in hollow-fiber bioreactor systems from labeled chondrocytes.

Masson's trichrome for collagen or with Alcian blue or Safranin O for sulfated glycosaminoglycans (GAGs). Finally, for the cellular hydrogel constructs, selected sections matching the MR slice locations were stained with hematoxylin and eosin (HE) to indicate the distribution of chondrocytes.

Immunohistochemistry

HFBR-derived neocartilage that developed over 35 days from SPIO-labeled and unlabeled chondrocytes was subjected to immunohistochemical analysis for collagen types I and II and aggrecan. Engineered tissue, together with the hollow-fiber, was removed from the HFBR, fixed in 4% paraformaldehyde overnight at 4°C, and then washed in PBS. The hollow-fiber segment at the center of the tissue samples was removed, and the tissue was dehydrated through graded concentrations of ethanol and subsequently

embedded in paraffin. Tissue blocks were sectioned into 5- μm slices. The primary antibodies for immunohistochemistry were mouse anti-pro-collagen I (SP1-D8), anti-collagen II (II-II6B3), and anti-aggrecan link protein (9/30/8-A-4) obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). Sections were pre-digested with 2 mg/mL of hyaluronidase (Sigma) to optimize the binding of antibodies. Before applying 9/30/8-A-4, aggrecan was reduced and alkylated by incubation with dithiothreitol and iodoacetic acid. After serum blocking, primary antibody (1:5 in 1% bovine serum albumin) was added to the sections and these were incubated overnight at 4°C. Sections were then incubated with Cy3-conjugated secondary antibody (Biomedex Biotechnology, Foster City, CA) for 30 min at room temperature. Sections were counterstained with DAPI (Vector Laboratories, Burlingame, CA). Negative controls were prepared by replacing the primary antibody solution with 1% bovine serum albumin in PBS. Immunohistochemistry slides were viewed under a fluorescence microscope and images were captured using a digital camera (Olympus BX51, Olympus America, Melville, NY).

Biochemical assay of hydrogel samples for glycosaminoglycan

Hydrogel constructs were analyzed after 5 weeks incubation. Glycosaminoglycan content was determined as chondroitin sulphate using the dimethylmethylene blue spectrophotometric assay, as described in Farndale *et al.*²⁷

Magnetic resonance imaging

All MRI experiments were performed using a Bruker DMX vertical-bore MR spectrometer equipped with a 9.4 Tesla magnet and a 30-mm microimaging probe with 1000 mT/m

FIG. 2. (a) TUNEL assay of SPIO-lipofectamine labeled chondrocytes (top) and unlabeled (control) chondrocytes (middle). There is an absence of positive brownish-pink staining in both these groups. Positive control (pink stain) of skin cells showing apoptotic behavior (bottom). (b) Live-Dead assay of SPIO-labeled chondrocytes within a hydrogel construct, with green circles representing live cells and red circles representing dead cells. Color images available online at www.liebertonline.com/ten.

3-axis shielded gradients (Bruker Biospin GmbH, Rheinstetten, Germany). A mixture of 95%:5% (v/v) humidified air and CO₂ was supplied to the sample while maintaining it at 37°C during scanning.

MRI of the bioreactor system. The bioreactors were imaged at 2, 31, and 35 days after cell inoculation. Pilot scans were obtained to define and acquire imaging slices perpendicular to the hollow fiber of the bioreactor. The MRI pulse program consisted of a conventional spin echo sequence with

T₂ weighting. MRI scan parameters for these axial images were slice thickness = 500 µm, number of signal averages = 32, field of view = 1.45 cm (read) × 0.75 cm (phase), matrix size = 256 (read) × 512 (phase encode) pixels, echo time = 7.5 ms, and repetition time = 6 s. The in-plane resolution of the axial images was 57 µm in the read direction and 15 µm in the phase direction.

MRI of the hydrogel system. T₂-weighted spin-echo imaging was performed. 12 to 14 axial slices were selected in

FIG. 3. Histology of a 4-week-old hollow-fiber bioreactor (HFBR) inoculated with SPIO-labeled chondrocytes showing positive staining for (a) glycosaminoglycan (GAG; Alcian blue) and (b) collagen (Masson's Trichrome). A blue stain represents positive staining for GAG or collagen. Color images available online at www.liebertonline.com/ten.

identified regions of interest. Other imaging parameters included: slice thickness = 200 μm , number of signal averages = 40, field of view = 2 cm $\times$ 2.5 cm, matrix size = 256 $\times$ 256 pixels (in-plane resolution of 78 μm (read) and 98 μm (phase), echo time = 12.8 ms, and repetition time = 3.5 s. Samples were imaged 0 and 30 days after seeding and were maintained in an incubator between imaging time points.

Statistics

Quantitative results are presented as mean $\pm$ standard deviation unless otherwise indicated. Group comparisons were performed using student's *t*-test, with statistical significance taken as $p < 0.05$.

Results

SPIO uptake in chondrocytes

Results of AA measurements of SPIO-labeled chondrocytes are listed in Table 1. Concentrations of PLL (1.5 $\mu\text{g}/\text{mL}$) and SPIO (25 $\mu\text{g Fe}/\text{mL}$) that led to maximal iron uptake were identified and used for all subsequent experiments. Using these concentrations, positive Prussian blue staining was found to be evident intracellularly, as shown in Figure 1a.

At a constant lipofectamine concentration of 0.5 $\mu\text{g}/\text{mL}$, the highest concentration of SPIO particles consistent with $\geq 90\%$ cellular viability was 67.2 $\mu\text{g iron}/\text{mL}$. This lipofectamine:SPIO ratio was used for labeling chondrocytes before introduction into the hydrogel-based tissue engineering system. After 6 days, Prussian blue staining showed persistence of positive staining in these cells (Fig. 1a). Identically prepared samples accompanying the Prussian blue slides showed evidence of robust collagen formation by the labeled groups (SPIO-PLL and SPIO-lipofectamine) as well as the unlabeled chondrocytes (Fig. 1b). Unlabeled chondrocytes stained negative for iron after 6 days of culture (Fig. 1a).

Effect of SPIO labeling on chondrocyte viability

A TUNEL assay performed on labeled and unlabeled cells revealed lack of apoptotic activity in both groups (Fig. 2a). Flow cytometric analysis of SPIO-lipofectamine-labeled chondrocytes determined that cell viability was on average $81.0 \pm 2.0\%$, with $3.5 \pm 0.1\%$ apoptotic cells and $6.8 \pm 0.7\%$ necrotic cells. The remainder of the cells ($8.9 \pm 2.5\%$) were identified as being apoptotic or necrotic. Live-Dead assay performed on SPIO-labeled chondrocytes encapsulated in hydrogel scaffolds after 38 days of incubation showed that a majority of the cells were still viable (Fig. 2b).

Effect of SPIO labeling on cell proliferation

Cell counts with a hemacytometer provided the following chondrocyte densities for each flask at day 0 ($n = 3$ flasks/group): SPIO-PLL, 22,600 cells/ cm^2 ; SPIO-lipofectamine, 35,200 cells/ cm^2 ; and unlabeled chondrocytes, 60,800 cells/ cm^2 . The cell densities (mean $\pm$ standard error of the mean; $n = 3$) at the end of 3 days in 2D culture were as follows: SPIO-PLL,

FIG. 4. Immunohistochemistry confirming phenotypic stability of SPIO-labeled chondrocytes in the HFBR system: (a) aggrecan, (b) type II collagen, and (c) type I collagen. No Cy3 fluorescence was evident in the negative controls. Color images available online at www.liebertonline.com/ten.

FIG. 5. Histology of cellular hydrogels showing positive staining for (a) GAG (Safranin O) and (b) collagen (Masson's Trichrome) at approximately 5 weeks of growth. Hydrogels seeded with SPIO-labeled chondrocytes (top) and hydrogels seeded with unlabeled chondrocytes (bottom). Safranin O stains GAGs red and Masson's Trichrome stains collagen blue. Color images available online at www.liebertonline.com/ten.

$23,500 \pm 5,900$ cells/cm²; SPIO-lipofectamine, $43,200 \pm 900$ cells/cm²; and unlabeled chondrocytes, $62,900 \pm 3,700$ cells/cm². On average, the proliferation of chondrocytes in terms of a percentage increase in cell density was 4.0% for SPIO-PLL, 2.3% for SPIO-lipofectamine, and 3.5% for unlabeled chondrocytes.

Effect of SPIO labeling on ECM production by chondrocytes in 3D scaffolds

Bioreactor system. Typical histologic sections of engineered HFBR cartilage derived from SPIO-labeled chondrocytes are shown in Figure 3a and b. As shown, the tissue was cylindrical and radially symmetric about the fiber. The histology was that of immature cartilage, as evidenced by the high cellularity and well-defined lacunae surrounding the chondrocytes. The tissue stained positive for the presence of collagen and GAGs. The staining progressively diminished in intensity radially outward from the fiber, indicating less ECM deposition farther from the nutrition source.

Immunohistochemical staining showed that aggrecan was abundant in HFBR tissue derived from SPIO-labeled and unlabeled chondrocytes (Fig. 4a). Similarly, type II collagen was found to be abundant in the ECM (Fig. 4b), whereas type I collagen was found to be absent in both groups (Fig. 4c).

Hydrogel system. Pericellular matrix was abundant in chondrons surrounding the SPIO-labeled and unlabeled chondrocytes, with GAGs similarly abundant in both groups (Fig. 5a). The intensity of collagen staining was also found to be comparable (Fig. 5b).

GAG content as determined using the dimethylmethylene blue spectrophotometric biochemical assay was 448 ± 100 µg/mL of extract solution ($n=5$) in the hydrogels seeded with SPIO-labeled chondrocytes and 484 ± 11 µg/mL ($n=5$) in constructs derived from unlabeled cells. The difference in GAG content between the two groups was not significant ($p > 0.05$).

MRI of SPIO-labeled chondrocytes

In both systems, MRI demonstrated the presence of SPIO-labeled chondrocytes as regions of reduced signal intensity.

Bioreactor system. An axial MR image of engineered cartilage tissue scanned 2 days after chondrocyte inoculation is shown in Figure 6a. Two distinct, concentric rings of tissue, a brighter inner ring and a darker outer ring, were observed. An adjacent tissue section stained with Prussian blue (Fig. 6b) confirmed that the outer ring visualized on MRI contained SPIO nanoparticles, whereas the inner ring did not. Axial MR images of HFBRs taken after 35 days of culture (Fig. 6c) visualized the entire outer ring region and demonstrated that the dark contrast of the ring is absent when unlabeled chondrocytes are seeded onto the HFBR. This confirmed that the outer ring was a site of accumulation for SPIO-labeled cells.

Hydrogel system. SPIO-labeled chondrocytes could be visualized clearly within the hydrogel, as evidenced by signal voids. Such voids were absent in hydrogels seeded with unlabeled cells (Fig. 7a). Comparing images taken at similar slice locations in a hydrogel seeded with SPIO-labeled cells at 0 and 30 days of culture, it was evident that the spatial distribution of labeled cells had changed over time (Fig. 7b). Figure 7c shows an MR image and, as a direct representation of cell distribution, a corresponding hematoxylin-eosin-stained histological slice sectioned at approximately the same spatial location. Similar patterns of cell distribution were found using both methods.

Discussion

The development of cell-based therapeutics represents an area of intense investigation. Nondestructive *in vitro* and noninvasive *in vivo* evaluation of tissue constructs remains

FIG. 6. (a) Magnetic resonance (MR) image of a HFBR inoculated with SPIO-labeled chondrocytes demonstrating SPIO-labeled and unlabeled regions of the tissue at 2 days. (b) Corresponding Prussian blue stained histological slice confirming the presence of SPIO-labeled cells within the outer ring of the tissue and lack of SPIO-labeled cells within the inner ring. (c) Left: Axial MR image of a HFBR inoculated with SPIO-labeled chondrocytes acquired after 35 days of tissue culture. The entire outer ring showed the presence of SPIO nanoparticles, whereas these were absent in the entire inner ring. Right: Axial MR image of a HFBR inoculated with unlabeled chondrocytes showing the absence of a dark outer ring after 31 days of tissue culture. Color images available online at www.liebertonline.com/ten.

an important component of such development efforts. As part of this, there has been a great deal of recent activity in the use of iron labeling for tracking cells using MRI.^{10,11,28–35} For tissue engineering purposes, Terrovitis *et al.*³⁶ visualized SPIO-labeled mesenchymal stem cells (MSCs) on collagen scaffolds using MRI, demonstrating a method of determining the fate of the cells after the initial creation of an engineered construct. The monitoring of SPIO-labeled MSCs seeded in gelatin sponges after subcutaneous implantation in mice has also been previously investigated.³⁷

The ability to visualize cells using MRI would also be of substantial utility in tissue engineering applications. Mon-

itoring cellular distribution, in the design and construction of constructs *ex vivo* before implantation³⁶ and for noninvasive, long-term cell tracking, is made possible through this technique.³⁸ In the particular case of cartilage, the development of internal matrix anisotropy and heterogeneity within a construct would more closely mimic the structure of articular cartilage.⁶ Such tissue features would require the development of nonuniform matrix-producing cell distribution, which may potentially be brought about using physical means (e.g., centrifugation) or chemical means (e.g., application of chemotactic molecules), and MRI would aid in these efforts by visualizing the cell distributions thus generated. Moreover, iron oxide labeling of chondrocytes may have immediate applicability in current tissue repair therapies such as autologous chondrocyte implantation.^{39–43}

Heymer *et al.*⁴⁴ were the first to report on SPIO labeling specifically for the purposes of cartilage tissue engineering. Their studies showed that bone marrow-derived stem cells could still differentiate chondrogenically while retaining intracellular iron oxide, thereby permitting MRI detection within 3D collagen type I hydrogel constructs. Another recent study⁴⁵ performed detailed gene expression studies on the labeling of chondrocytes with SPIO nanoparticles. It demonstrated no adverse effects of the labeling process on the expression levels of common chondrogenic genes and also showed that chondrocyte redifferentiation capacity remained unaffected. In our study, we built on these ideas by studying two different cartilage tissue engineering protocols using SPIO-labeled chondrocytes. We also demonstrated the ability to track these cells longitudinally over multiple time points. In agreement with the two earlier studies mentioned above, we found that chondrocyte phenotype and viability were unaffected by the labeling process. In particular, we were able to uniquely show that *primary* chondrocytes could successfully be labeled with iron oxide and then utilized in a potentially clinically translatable, three-dimensional photopolymerizable hydrogel culture environment.

We found that primary chondrocytes themselves can be labeled with SPIO nanoparticles and visualized using MRI while embedded in a tissue engineered scaffold or while being cultured in a perfused bioreactor. Labeling was achieved by combining commercially available ferumoxides with established transfection agents. In 2D culture experiments, excess SPIO nanoparticles were first removed by extensive washing of the flasks, followed by magnetic sorting of the labeled cells to ensure that a subpopulation of these cells was labeled with a certain minimum SPIO load. Subsequent histology (Prussian blue and Masson's trichrome staining) of this subpopulation confirmed that chondrocytes contained intracellular iron oxide and that they were able to produce collagen comparably to unlabeled cells. We assayed the production of GAG by chondrocytes seeded in hydrogels and found no difference in its synthesis between labeled and unlabeled cells. Phenotypic stability was demonstrated. The SPIO-labeled chondrocytes were found to demonstrate minimal apoptosis and necrosis. In addition, the cells demonstrated excellent viability in the scaffold environment after more than 5 weeks. Finally, proliferation rates were different between SPIO-labeled and unlabeled chondrocytes, although given that the doubling time of bovine chondrocytes may

FIG. 7. (a) MR image of a hydrogel seeded with unlabeled chondrocytes after 30 days of growth, showing the absence of signal voids within the construct. (b) MR images (axial slice) of representative hydrogel seeded with SPIO-labeled chondrocytes at 0 days (top) and at 30 days after inoculation (bottom). (c) MR image (left) and corresponding histological section (right) of a hydrogel seeded with SPIO-labeled chondrocytes after 30 days of growth. Note the close correspondence between signal voids in the MR image and cells in the histological image.

exceed 6 days,⁴⁶ the proliferation rates we report here over a 3-day timeframe appear to be unremarkable and within the normal variability of proliferation of chondrocyte subpopulations. Therefore, SPIO labeling may be incorporated into chondrocyte-based tissue engineering studies without apparent adverse effects on cellular function.

We were able to visualize labeled chondrocytes in two different tissue engineered constructs. The HFBR system has been of great use in optimizing *in vitro* growth conditions for scaffold-free cartilage and for investigations of neocartilage

development,⁴⁷ whereas photopolymerizable hydrogels are under investigation for use in clinical cartilage repair protocols.² We observed marked changes in SPIO-labeled chondrocyte cell distribution over a 30-day period in the hydrogels (Fig. 7b), which could be attributed to intrascaffold cell movement, cell division, or metabolism of the SPIO nanoparticles. Nonetheless, we were able to demonstrate that clearly visible MRI hypointensity due to the presence of SPIO nanoparticles persisted beyond 4 weeks in both tissue engineering systems. The maintenance of observable intracellular

SPIO concentration over this duration may reflect the relatively low proliferation rate of chondrocytes since SPIO concentration is diluted by cell division. In the HFBR system, because growth of the tissue occurs radially from the hollow fibers of the HFBR, we believe that the chondrocytes tended to migrate outward along with the tissue formation. Regions of dark contrast on the periphery of engineered cartilage (Fig. 6c) scanned 35 days after initial HFBR inoculation with SPIO-labeled chondrocytes confirmed this.

Although our experiments were performed on a high-field MRI instrument, given the relatively weak effect of magnetic field strength on T_2 relaxation time, we expect that SPIO labeled chondrocytes in tissue engineered constructs will result in readily observable MR contrast at clinical field strengths as well. This is supported by the numerous published studies in which other cell types have been labeled with SPIO and visualized with clinical MRI scanners.

There has been controversy regarding the effect of SPIO labeling on chondrogenesis in MSCs,^{36,44,48,49} although we have demonstrated here that cells already possessing the chondrocyte phenotype can be successfully labeled with SPIO complexes without promoting phenotypic instability. Our results indicate that labeled chondrocytes may be successfully incorporated into cartilage tissue engineering systems. Finally, although the *in vitro* results are highly encouraging, it remains to be demonstrated whether the fate of scaffold-supported SPIO-labeled chondrocytes can be monitored *in vivo*.

In conclusion, we have demonstrated the ability to label chondrocytes with SPIO nanoparticles and to monitor their location using MRI in two different cartilage tissue engineered constructs. The ability to visualize chondrocyte distribution through SPIO labeling may contribute to the development of tissue engineering-based strategies for cartilage repair and regeneration.

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Author Disclosure Statement

No competing financial interests exist.

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