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Labeling of cells with ferumoxides–protamine sulfate complexes does not inhibit function or differentiation capacity of hematopoietic or mesenchymal stem cells

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ABSTRACT: Two FDA-approved agents, ferumoxides (Feridex®), a suspension of superparamagnetic iron oxide (SPIO) nanoparticles and protamine sulfate, a drug used to reverse heparin anticoagulation, can be complexed and used to label cells magnetically *ex vivo*. Labeling stem cells with ferumoxides–protamine sulfate (FePro) complexes allows for non-invasive monitoring by MRI. However, in order for stem cell trials or therapies to be effective, this labeling technique must not inhibit the ability of cells to differentiate. In this study, we examined the effect of FePro labeling on stem cell differentiation. Viability, phenotypic expression and differential capacity of FePro labeled CD34 + hematopoietic stem cells (HSC) and mesenchymal stem cells (MSC) were compared with unlabeled control cells. Colony-forming unit (CFU) assays showed that the capacity to differentiate was equivalent for labeled and unlabeled HSC. Furthermore, labeling did not alter expression of surface phenotypic markers (CD34, CD31, CXCR4, CD20, CD3 and CD14) on HSC, as measured by flow cytometry. SDF-1-induced HSC migration and HSC differentiation to dendritic cells were also unaffected by FePro labeling. Both FePro-labeled and unlabeled MSC were cultured in chondrogenesis-inducing conditions. Alcian blue staining for proteoglycans revealed similar chondrogenic differentiation for both FePro-labeled and unlabeled cells. Furthermore, collagen X proteins, indicators of cartilage formation, were detected at similar levels in both labeled and unlabeled cell pellets. Prussian blue staining confirmed that cells in labeled pellets contained iron oxide, whereas cells in unlabeled pellets did not. It is concluded that FePro labeling does not alter the function or differentiation capacity of HSC and MSC. These data increase confidence that MRI studies of FePro-labeled HSC or MSC will provide an accurate representation of *in vivo* trafficking of unlabeled cells. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: ferumoxides; protamine sulfate; hematopoietic stem cells; mesenchymal stem cells; dendritic cells; colony-forming unit; chondrogenesis

INTRODUCTION

Complexing polycationic transfection agents (TA) [e.g. poly-L-lysine (PLL)] to ferumoxides occurs through electrostatic interactions and is an efficient and effective technique for incorporating SPIO nanoparticles within endosomes, thereby labeling cells that can be detected by MRI [1–6]. Iron oxide-labeled cells appear as hypointense areas in tissues with an associated susceptibility artifact or amplification of the decreased signal intensity on iron-sensitive *T*2-weighted and *T*2*-weighted gradient echo images [2,3,5–14]. Most transfection agents are toxic to cells when used alone and, for example, PLL has a relatively small toxic/efficacious ratio, with concentrations as low as 10 μg/ml in media causing significant cell death [15,16]. Ferumoxides complexed to PLL effectively label cells, but residual Fe–PLL complexes may remain on the surface of the cells or clump cells together in the final cell preparation prior to infusion. Moreover, PLL is combined with ferumoxides, large complexes can form if the mixture is not monitored. A

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Abbreviations used: FePro, ferumoxides–protamine sulfate complexes; aHSC, adult hematopoietic stem cells; cbHSC, cord blood hematopoietic stem cells; MSC, mesenchymal stem cells; TA, transfection agent; PLL, poly-L-lysine; CFU, colony-forming unit; MACS, magnetic activated cell sorter; SCF, stem cell factor; TPO, thrombopoietin; GM-CSF, granulocyte macrophage colony-stimulating factor; IL-4, interleukin 4; SDF1, stromal derived factor 1; PB, Prussian blue.

recent report indicated that labeling mesenchymal stem cells (MSC) with ferumoxides–PLL complexes inhibited the chondrogenic differentiation capacity of MSC [17,18]. It is important to note that most polycationic transfection agents are not approved by the US Food and Drug Administration (FDA) for clinical use.

Protamine sulfate is a low molecular weight ($\sim$4000 Da), natural product polycationic peptide that is approved by the FDA as an antidote to heparin anticoagulation [19,20]. Protamine sulfate (Pro) is well tolerated by cells, with a high therapeutic window of $>$ 50 mg/ml, and when complexed to DNA is about 100 times more efficient than PLL in transfecting cells [21]. Recently, we developed a technique to label cells by combining ferumoxides and protamine sulfate [22]. Preliminary results showed that labeling cells with ferumoxides–protamine sulfate (FePro) complexes did not alter the viability and functional capacity of a variety of cell types.

The purposes of this study were to extend the findings of previous studies with FePro and to explore further the effects of FePro labeling on the functional properties and differentiation capacity of stem cells.

MATERIALS AND METHODS

Cells

Both freshly collected and cryopreserved mammalian cells were labeled with FePro complexes.

Adult hematopoietic stem cells

CD34 + adult hematopoietic stem cells (aHSC) were obtained by immunomagnetic selection (Miltenyi Biotec, Auburn, CA, USA) of peripheral blood leukapheresis collections from G-CSF mobilized healthy normal volunteers who had signed informed consent for an IRB-approved protocol. aHSC were cultured in serum-free Stemspan medium (Stem Cell Technologies, Vancouver, BC, Canada), containing stem cell factor (SCF, 40 ng/ml), thrombopoietin (Tpo, 10 ng/ml) and Flt3 ligand (40 ng/ml) (R&D Systems, Minneapolis, MN, USA). After 3–5 days in culture, aHSCs were harvested for further study.

Cord blood hematopoietic stem cells

Cord blood CD34 + hematopoietic stem cells (cbHSC) were obtained from the umbilical cord blood of full-term neonates under an IRB-approved protocol. With all aseptic precautions, cord blood was collected directly into 50 ml tubes containing phosphate-buffered saline (PBS) mixed with 2 mmol/liter EDTA. After collection, blood was diluted twice with PBS mixed with EDTA. Mononuclear cells were separated using the standard Ficoll technique. Remaining red blood cells (RBC) were removed by using ACK lysis buffer (Quality Biological, Gaithersburg, MD, USA). CD34 + cells were separated by positive selection using a CD34 + cell magnetic beads separation kit (Miltenyi Biotec, Auburn, CA, USA) and a magnetic activated cell sorter (MACS) column. Collected cbHSC were cultured in serum-free stem cell medium (Stemline, Sigma, St Louis, MO, USA) containing SCF (40 ng/ml), Tpo (10 ng/ml) and Flt3 ligand (40 ng/ml). The cells were cultured for 7–10 days with change of medium every alternate day and then harvested for further study.

Human mesenchymal stem cells

Human mesenchymal stem cells (MSC, Cambrex Bio-Sciences, Walkersville, MD, USA) were cultured in a 75 cm^3 flask with standard growth media at 37 °C with 5% CO2 to 80–90% confluence. At the time of labeling, old medium was discarded and replaced by 10 ml of fresh medium.

Preparation of Ferumoxides–protamine sulfate (FePro) complex and cell labeling

The commercially available ferumoxides suspension (Feridex IV®, Berlex Laboratories, Wayne, NJ, USA) contains particles $\sim$80–150 nm in size and has a total iron content of 11.2 mg/ml (11.2 μg of iron /μl). Protamine sulfate (American Pharmaceutical Partners, Schaumburg, IL, USA), supplied at 10 mg/ml, was prepared as a fresh stock solution of 1 mg/ml in distilled water at the time of use. Ferumoxides at a concentration of 100 μg/ml was placed in a mixing flask or tube containing serum-free RPMI 1640 medium (Biosource, Camarillo, CA, USA) containing 25 mmol/liter HEPES, MEM non-essential amino acids, sodium pyruvate and L-glutamine. Protamine sulfate was then added to the solution at a concentration of 5 μg/ml. The solution containing ferumoxides and protamine sulfate was mixed for 3–5 min with intermittent manual shaking. After 3–5 min, an equal volume of the solution containing FePro complexes was added to the existing medium in MSC culture. For aHSC and cbHSC, harvested cells were centrifuged and resuspended in the FePro labeling suspension at 4×10^6 cells/ml, incubated for 2–3 h and an equal volume of the respective complete medium was added to the cells, for a final concentration of 50 μg ferumoxides/ml of medium. The cell suspension was then incubated overnight. After labeling, all cells were washed twice with sterile phosphate-buffered saline (PBS), with the last wash containing heparin (10 U/ml), to dissolve extracellular FePro complexes if present, and then

collected. The viability of labeled and unlabeled control cells was determined by trypan blue dye exclusion.

Functional and differentiation capacity of FePro-labeled cells

Both labeled and unlabeled aHSC were evaluated for migration to the chemo-attractant SDF-1. To measure differentiation capacity, hematopoietic colonies were quantitated after both FePro-labeled and unlabeled aHSC were cultured in semisolid medium (StemCell Technologies). To measure the differentiation capacity of cbHSC to dendritic cells, cbHSC were cultured in dendritic cell media containing IL-4 and GM-CSF (6 days, for immature dendritic cells) or IL-4, GM-CSF and TNF-α (another 2 days, for mature dendritic cells). Cell surface marker expression of cbHSC cells was determined by flow cytometry (FACSCaliber, BD Bioscience, San Jose, CA, USA) during differentiation to dendritic cells.

Both FePro-labeled and unlabeled MSC were cultured in chondrogenesis-inducing conditions for 20 days using materials and methods obtained from the MSC supplier (Cambrex, Walkersville, MD, USA). Frozen sections of labeled and unlabeled cell pellets underwent histological and immuno-histochemical analyses for chondrogenesis. Prussian blue staining was used to detect iron oxide and Alcian blue (Sigma) was used to stain for glycosaminoglycans. Pellets were also stained for the presence of collagen X proteins (primary antibodies from Sigma) using appropriate horseradish peroxidase (HPR)-conjugated secondary antibodies and a DAB substrate kit (DAKO, Carpinteria, CA, USA). Three sets of labeled and unlabeled MSCs were subjected to differentiation for each lineage. Different phenotypic markers of human hematopoietic stem cells and dendritic cells (such as CD34, CD31, CD14, CD83) were analyzed, using fluorescent conjugated antibodies (BD Bioscience, San Diego, CA) for the labeled and unlabeled cells at different time points. Collagen X positive sections were also stained for iron using Perl's reagent [Prussian blue (PB) staining].

Histology

After incubation with FePro, cells were washed three times with PBS and heparin sulfate (10 U/ml) to remove excess FePro, trypsinized to release adherent cells and transferred to cytospin slides. Cells were fixed with 4% glutaraldehyde, washed, incubated for 20–30 min with 2% potassium ferric-ferrocyanide (Perl's reagent for PB staining) in 3.7% hydrochloric acid, washed again and counterstained with nuclear fast red. For diaminobenzide (DAB) enhanced PB staining, slides after PB staining were immersed in hydrogen peroxide-activated DAB solution for 5–10 min, washed with PBS and counterstained with nuclear fast red.

Data analysis

The percentage viability and number of CFU are expressed as mean $\pm$ standard deviation. Student's t-test was utilized to analyze the differences between two conditions.

RESULTS

There was no significant difference in viability between the labeled and unlabeled cells ($P > 0.05$) and nearly 100% cells labeled with FePro (Fig. 1). Labeled and unlabeled aHSC had comparable numbers of hematopoietic colony forming units (Fig. 2, $P > 0.05$). Both labeled and unlabeled aHSC showed equivalent migration in response to SDF-1 (Fig. 3, $P > 0.05$), with both showing the greatest migration with 100 μg/ml of SDF-1 stimulation.

Both labeled and unlabeled cbHSC showed similar differentiation to dendritic cells. Morphological changes from day 0 to maturation were comparable with round cells becoming elongated and developing multiple dendritic projections on the cell surface (Fig. 4). In addition, surface marker expression patterns were comparable. Soon after differentiation, cbHSCs were positive for CD34 and CD14, but CD83 was negative or negligible. During the maturation process, cbHSCs became CD34 negative and CD14 expression decreased. After addition of TNF-α, a marked increase in CD83 expression, with negligible CD14 expression was observed (Table 1).

Chondrogenesis in MSC was also unaffected by FePro labeling. Alcian blue staining shows that glycosaminoglycans are expressed similarly in labeled and unlabeled cells (Fig. 5). Immunohistochemical staining shows collagen X, an indicator of cartilage formation, expressed equally in labeled and unlabeled cell pellets. It is also important to note that labeled and unlabeled cell pellets grew at similar rates and are of similar size (data not shown). This may indicate similar levels of proliferation and differentiation, as pellets grow in size during chondrogenesis. PB-stained section showed positive collagen X and iron in same area [Fig. 5(D)] within the labeled pellet.

DISCUSSION

The results indicate that FePro labeling does not alter the function or differentiation capacity of hematopoietic stem cells from either cord blood or peripheral blood or of marrow-derived mesenchymal stem cells. Our previous reports, and also those of others, have demonstrated no alteration in the function or differentiation capacity of stem cells after labeling with ferumoxides [22–25]. Colony-forming unit assays indicated that labeled

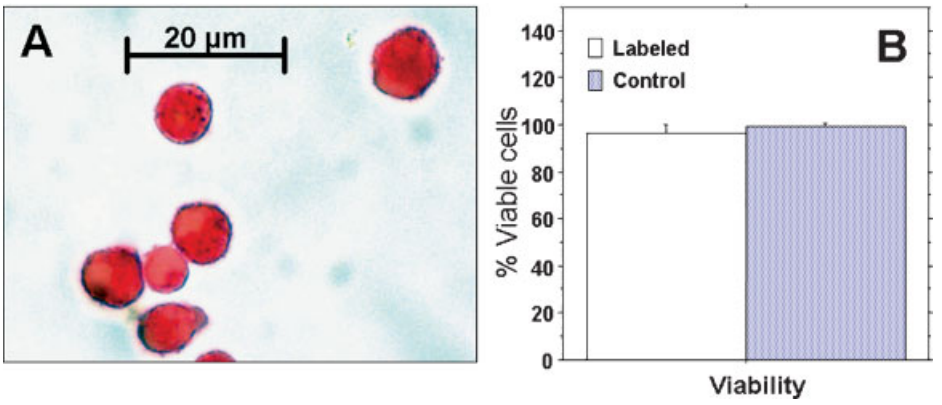

Figure 1. (A) Prussian blue-stained labeled HSC; (B) viability of labeled and unlabeled HSC as determined by Trypan blue dye exclusion (mean ± SD). Staining revealed that cells were iron positive. The viability of cells under the two conditions was not significantly different ($P > 0.05$)

aHSC differentiate into erythrocyte and granulocyte/macrophage lineages in a manner similar to unlabeled aHSC. Furthermore, no adverse effect from FePro labeling could be demonstrated on cell proliferation, viability, cell surface marker expression or SDF-1 induced migration assays.

The culture conditions required to differentiate the peripheral blood monocytes (CD14 positive) and HSC (CD34 positive) from adult and cord blood into dendritic cells are well established [26,27]. GM-CSF and IL-4 added to culture media results in the transformation of monocytes and HSC to immature dendritic cells and the addition of TNF-α stimulates immature dendritic cells to mature. Using cbHSC as the cell source, it was found that immature and mature dendritic cells from labeled and unlabeled cbHSC were generated in a comparable

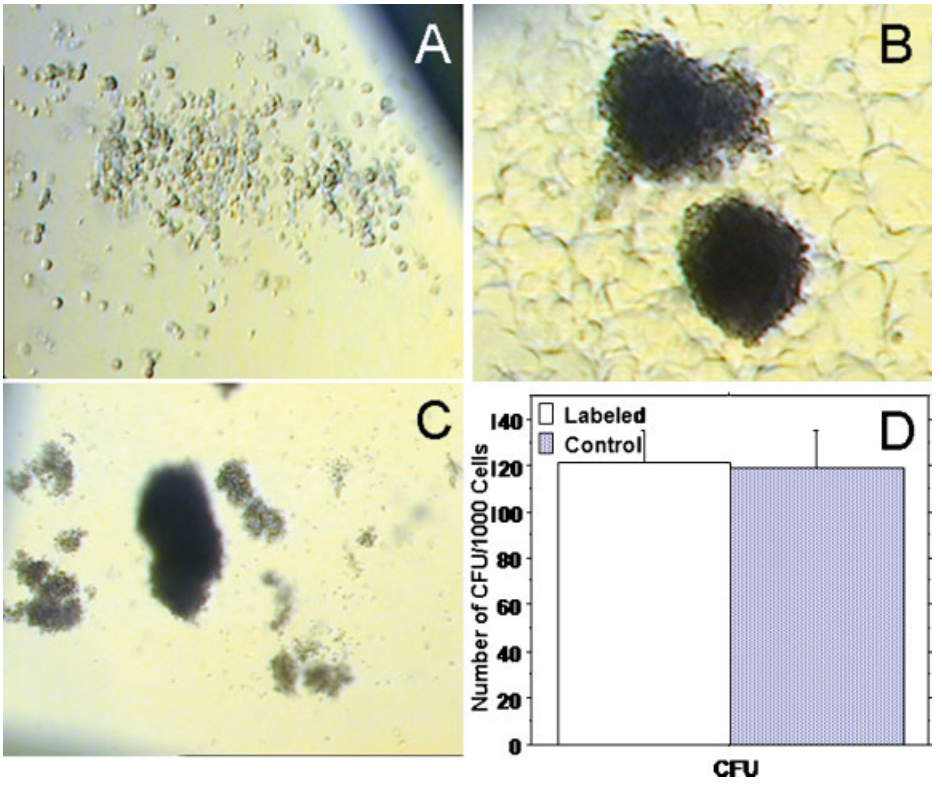

Figure 2. (A) Colony-forming unit-granulocyte macrophage (CFU-GM)-differentiated labeled HSC; (B) blast forming unit-erythroid (BFU-E)-differentiated labeled HSC; (C) BFU-E- and CFU-GM-differentiated unlabeled HSC; (D) CFU count (mean ± SD) for labeled and unlabeled HSC. Total CFU for the two conditions were not significantly different ($P > 0.05$)

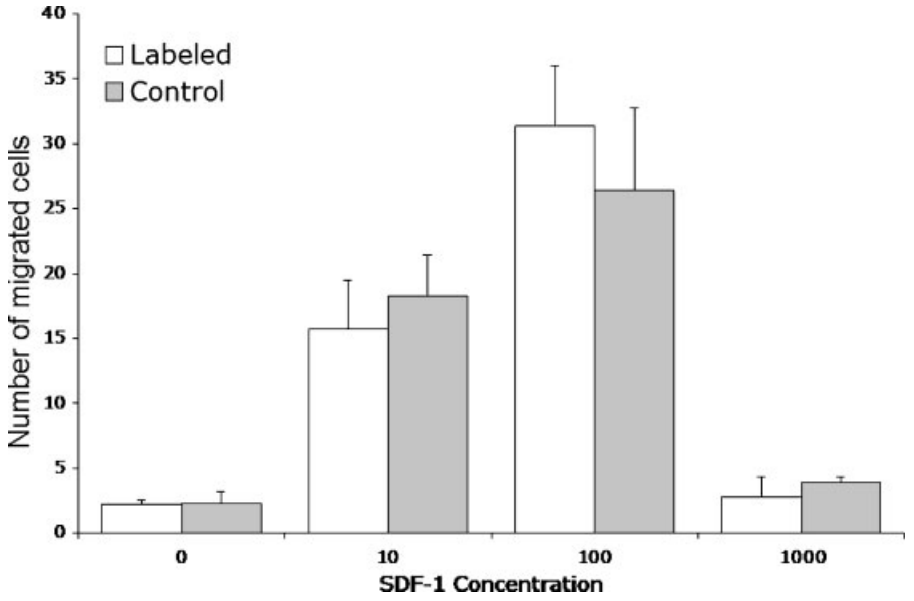

Figure 3. Results (mean ± SD) of SDF-1-induced migration of labeled and control HSC. Values for the two conditions are not significantly different at each SDF-1 concentration ($P > 0.05$)

fashion and that FePro did not interfere with this process of differentiation and maturation.

Chondrogenesis in MSC was also unaffected by FePro labeling. Safranin-O and Alcian blue staining showed that glycosaminoglycans are expressed similarly in labeled and unlabeled cells [22,23]. Immunohistochemical staining showed collagen X, an indicator of cartilage formation, expressed at equal levels in labeled and unlabeled cell pellets. It is also important to note that labeled and unlabeled cell pellets are of similar size (data not shown). This indicated similar levels of differentiation, as pellets grow in size during chondrogenesis. In a previous report, we indicated local inhibition of glycosaminoglycan and collagen X expression in a few cells within the labeled pellet that were near large, extracellular clumps of FePro complexes [23]. FePro labeling generally leads to intracellular iron particles and the addition of heparin sulfate to the wash results in the disassociation of the FePro complexes, leaving few, if any, extracellular iron oxide nanoparticles. This may explain why FePro labeling does not inhibit chondrogenesis, unlike some other reported labeling methods where ferumoxides–poly-L-lysine complexes (FePLL) were used [17,18].

Table 1. Expression of different markers during transformation of CD34+ cells to mature dendritic cells[a]

	Unlabeled/labeled cells (%)			
	CD14	CD34	CD31	CD83
Day 1	39.82/36.89	14.51/14.15	99.08/99.01	ND
Day 4	24.21/23.59	3.23/3.81	95.89/94.80	3.53/3.85
Day 6	18.37/19.01	4.15/3.96	96.07/95.73	3.81/3.64
TNF(+)	1.10/0.87	ND	ND	39.92/56.62

[a] Both labeled and unlabeled cells were cultured in dendritic cell differentiating media. The number of both CD34+ and CD14+ cells decreased over 6 days. However, the number of CD31+ cells remained unchanged in both labeled and unlabeled cell populations. After washing and reincubation of immature dendritic cells in media containing TNF-α, there was a dramatic increase in the number of CD83+ cells, indicating maturation of dendritic cells. Both labeled and unlabeled CD34+ cells showed similar differentiation to immature and mature dendritic cells.

The differences observed in the FePLL and FePro labeling techniques may be responsible for conflicting results obtained by Kostura *et al.* [17] and the results of this study. It has been demonstrated that labeling stem cells and other cells with FePro is more efficient and results in a cleaner preparation compared with FePLL, especially if heparin sulfate (3–10 U/ml) is included in the PBS washes after labeling. Heparin sulfate competes with the dextran coating of the SPIO complexes for protamine sulfate, dissolving the extracellular FePro complexes. Although Kostura *et al.* presented convincing evidence of FePLL interfering with differentiation of MSCs along chondrocytic lineages, [17] we believe that the interpretation of their results needs to be clarified. A more appropriate interpretation of Kostura *et al*.'s results might be due to the presence of extracellular FePLL complexes in surface contact with MSC, which might interfere with chondrogenesis. Moreover, these authors did not prove that intracellular ferumoxides in MSC endosomes inhibited their differentiation into chondrocytes. Hinds *et al*. also reported that excess quantities of BANG superparamagnetic particles (divinyl-coated SPIO), which have a size of close to one micrometer, inhibited proliferation of MSCs [28]. Although complexes of ferumoxides with transfection agents are smaller than BANG particles, they appear to interfere with the

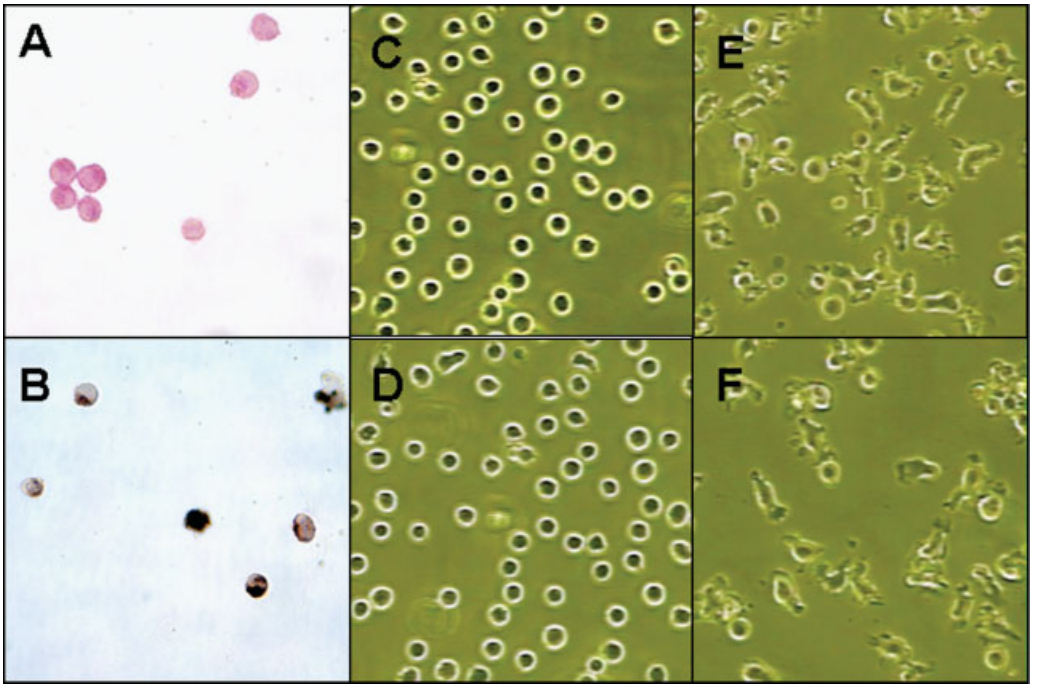

Figure 4. Upper panels (A, C, E) are from unlabeled CD34 and lower panels (B, D, F) from labeled CD34 cells. Prussian blue staining with DAB enhancement (left column, A, B) shows iron in labeled cells only (B). On day 0 (middle column, C and D), both labeled and unlabeled CD34 showed similar round morphology, which changed to an elongated shape with multiple dendritic projections from the surface of the cell membrane on day 6 (E, F) following incubation in dendritic cell media containing GM-CSF and IL-4. (Magnification $\times$ 40.)

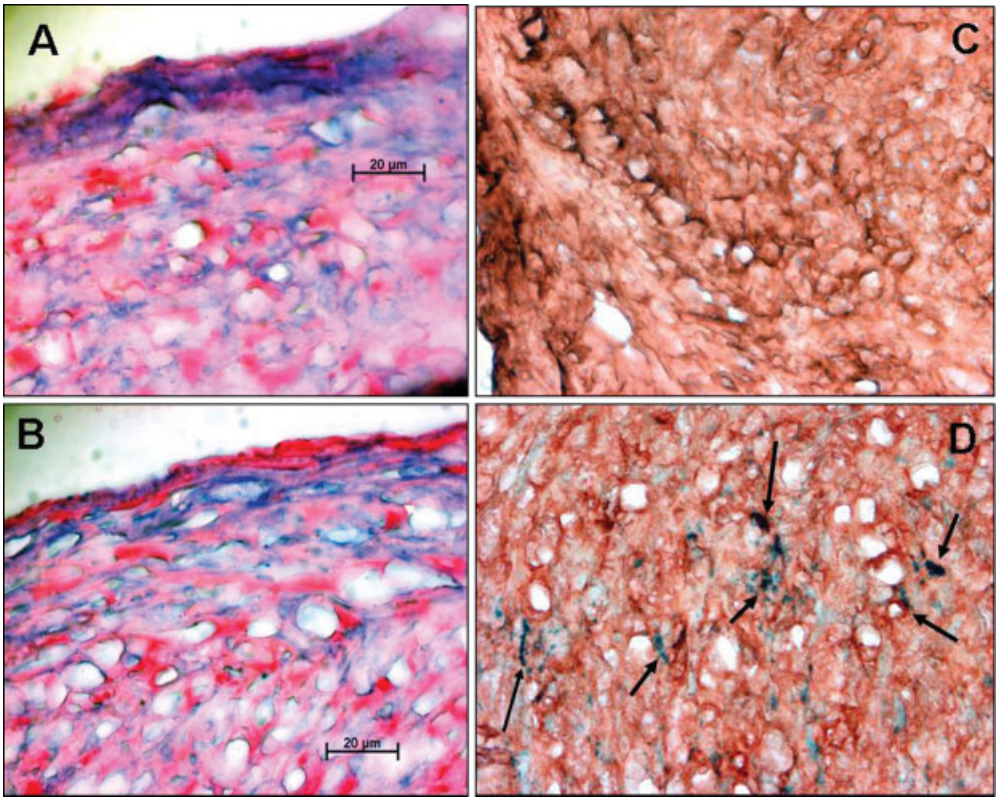

Figure 5. Alcian blue staining (nuclear fast red counterstain) of glycosaminoglycan distribution in (A) unlabeled and (B) labeled MSCs pellets. Collagen X followed by Prussian blue staining of both (C) unlabeled and (D) labeled MSC pellets. Arrows indicate collagen X expression in iron-positive cells (D). The bars represent 20 μm on A–B and C–D images are $\times$ 50 magnification. Glycosaminoglycan and collagen X expression were similar in labeled and unlabeled pellets. Sections of collagen X were not counterstained with nuclear fast red

differentiation of MSCs when left in contact with the cell surface.

In conclusion, labeling of HSC and MSC with FePro using the methods described is not toxic to cells and does not interfere with their ability to differentiate. FePro labeling of HSC also did not impair cell surface marker expression or migration induced by SDF-1. These results strongly suggest that MRI studies of FePro labeled HSC or MSC in clinical trials will provide an accurate representation of *in vivo* trafficking of unlabeled cells.

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