Amine-Surface-Modified Superparamagnetic Iron Oxide Nanoparticles Interfere with Differentiation of Human Mesenchymal Stem Cells

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ABSTRACT: Superparamagnetic iron oxide (SPIO) nanoparticles have been widely used for stem cell labeling and tracking. Surface modification has been known to improve biocompatibility, biodistribution, and labeling efficiency of SPIO nanoparticles. However, the effects of amine (NH_3^+)-surface-modified SPIO nanoparticles on proliferation and differentiation of human mesenchymal stem cells (hMSCs) remain unclear. The purpose of this study is to investigate how amine-surface-modified SPIO nanoparticles affected hMSCs. In this study, intracellular uptake and the contiguous presence of amine-surface-modified SPIO nanoparticles in hMSCs were demonstrated by Prussian blue staining, transmission electron microscopy and magnetic resonance imaging. Moreover, accelerated cell proliferation was found to be associated with cellular internalization of amine-surface-modified SPIO nanoparticles. The osteogenic and chondrogenic differentiation potentials of hMSCs were impaired after treating with SPIO, while adipogenic potential was relatively unaffected. Altered cytokine production profile in hMSCs caused by amine-surface-modified SPIO nanoparticles may account for the increased proliferation and impaired differentiation potentials; concentrations of the growth factors in the SPIO-labeled condition medium including amphiregulin, glial cell-derived neurotrophic factor, heparin-binding EGF-like growth factor and vascular endothelial growth factor, as well as soluble form of macrophage colony-stimulating factor receptor and SCF receptor, were higher than in the unlabeled-condition medium. In summary, although amine-surface-modified SPIO labeling is effective for cell tracking, properties of hMSCs may alter as a consequence and this needs to be taken into account when evaluating therapeutic efficacies of SPIO-labeled stem cells in vivo. © 2012 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 30:1499–1506, 2012

Keywords: mesenchymal stem cells; superparamagnetic iron oxide; nanoparticles; multilineage differentiation

In the past, superparamagnetic iron oxide (SPIO) nanoparticles have been shown to be biocompatible, capable of being metabolized, non-toxic to various stem cells, and having no effects on stem cell phenotypes or differentiation potential.[1–7] Therefore, SPIO nanoparticles have been used for stem cell labeling and tracking in vivo in magnetic resonance imaging (MRI),[8,9] as well as clinical diagnosis of focal liver lesions and colorectal liver metastasis.[10,11] However, it was recently reported that ferumoxide labeling of human mesenchymal stem cells (hMSCs) inhibits chondrogenesis and affects osteogenesis.[12,13] Ferucarbotran has been reported to magnetically label hMSCs more efficiently than does ferumoxide, without using a transfection agent and without cytotoxicity.[14] However, the use of ferucarbotran for the labeling of hMSCs aggravates clinical symptoms in experimentally induced autoimmune encephalomyelitis.[15] Ferucarbotran stimulates hMSC proliferation significantly,[16] and the inhibitory effect of ferucarbotran on osteogenic differentiation in hMSCs was reported in a follow-up study.[17] Recently, ferucarbotran has been identified as an independent factor that affect the gene expression and functional biology of labeled hMSCs.[18] These findings have raised toxicity and other safety concerns in regard to using newly developed SPIO nanoparticles for stem cell labeling and tracking.

The superparamagnetic cores of ferumoxide and ferucarbotran are embedded in biocompatible polymers and in dextran and carboxydextran, respectively, to enhance cellular uptake.[19] However, the effects of amine (NH_3^+)-surface-modified SPIO nanoparticles, without polymer coating, on hMSCs remain unclear.[20] Moreover, the previous investigation of the differentiation potential is usually limited to a few hMSC differentiation markers or to qualitative research only.[12,13,16–18,21] To further evaluate the possible toxicity of amine modification, this study seeks to elucidate how amine-surface-modified SPIO nanoparticles affected the proliferation and differentiation of hMSCs and the possible molecular mechanisms involved.

MATERIALS AND METHODS

Cell Culture and Reagents

Isolation of hMSCs from bone marrow was performed using a previously reported protocol.[22] An approval from the Institutional Review Board of the Taipei Veterans General Hospital was obtained prior to commencement of the study. hMSCs were seeded at a density of 3×10^5 cells/75T flask and grown in a MesenPRO RS^{TM} medium (Invitrogen, Carlsbad, CA).

Additional supporting information may be found in the online version of this article.

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hMSCs at passages 10–15 were used for the experiments. Poly-L-lysine, MTT reagents, Alizarin-red S and other reagents were purchased from Sigma–Aldrich (St. Louis, MO). All reagents were prepared as per manufacturers' instructions.

SPIO Labeling of hMSCs

Amine-surface-modified SPIO nanoparticles were prepared as reported previously.[20] The detailed experimental methods were described in the Supplementary Information.

- Prussian Blue Staining and Intracellular Iron Content Measurement
- In vitro MR Imaging and Transmission Electron Microscopy (TEM)
- Effects of SPIO Labeling on hMSC Proliferation

The detailed experimental methods were described in the Supplementary Information.

In Vitro Differentiation of hMSCs

hMSCs were incubated with 0 and 50 μg/ml of SPIO for 24 h. Differentiation induction into mesodermal lineage progenies was carried out with our previously reported protocols.[23] The detailed experimental methods were described in the Supplementary Information.

- Total RNA Isolation and RT-PCR
- Antibody-Based Growth Factor Array

The detailed experimental methods were described in the Supplementary Information.

Statistical Analysis

Data were presented as the mean ± standard error of mean (SEM) for at least three rounds of independent experiments. Results were compared with one-way ANOVA, Student's t tests and chi-square tests. Statistical significance was determined at 95% confidence intervals.

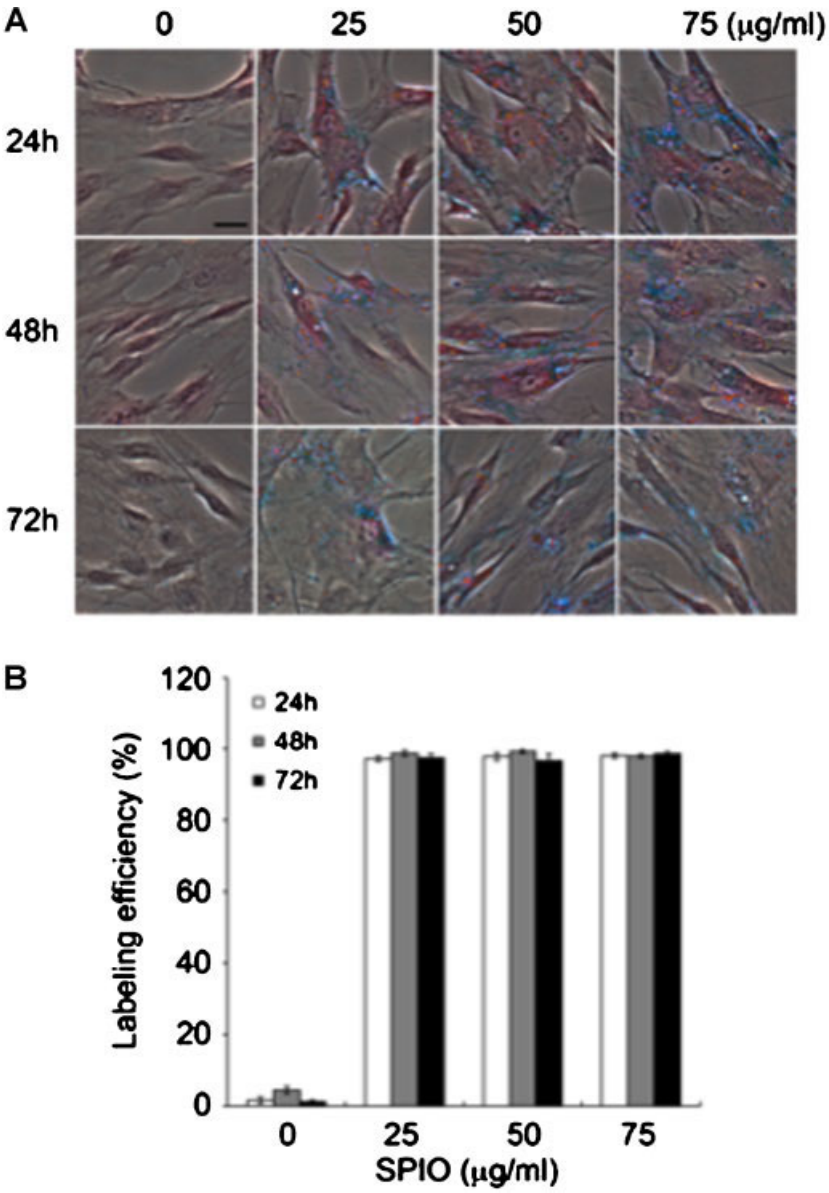


Figure 1. Labeling efficiency of SPIO on human mesenchymal stem cells. Cells were labeled with 0, 25, 50, and 75 μg/ml of SPIO for 24, 48, and 72 h. (A) Prussian blue stain showed increase of iron accumulation in the hMSCs by elevation of SPIO concentrations. Scale bar = 25 μm. (B) Quantitative analysis showed over 95% of hMSCs were efficiently labeled at each concentration for different labeling time periods.

RESULTS

Optimization of SPIO-Labeling Efficiency of hMSCs

Morphology of hMSCs was not changed by SPIO labeling (Fig. 1A). Over 95% of hMSCs were efficiently labeled by SPIO nanoparticles at each concentration (Fig. 1B). Escalation of SPIO concentrations slightly increased iron content in the cell lysate (Fig. 2A). The iron content in the medium was the lowest at 50 μg/ml of SPIO, which suggested the uptake of SPIO nanoparticles was most significant at this concentration (Fig. 2B). In a fixed SPIO concentration, prolonged incubation of hMSCs with SPIO decreased the normalized iron content of each single cell (Fig. 2C). The iron content of a single cell was decreased significantly in a cell number-dependent manner (Fig. 2D) ($p < 0.05$, one-way ANOVA).

In Vitro MRI of SPIO-Labeled hMSCs

Spin echo T1-weighted (T1WI) and T2-weighted images (T2WI) revealed the presence of a hypointense signal (Fig. 3A and C). A good linear correlation between cell density and relaxation values of T1WI (r1, $R^2 = 0.9922$) and T2WI (r2, $R^2 = 0.9982$) was obtained (Fig. 3B and D).

Uptake of SPIO by hMSCs as Demonstrated by TEM

The TEM examination demonstrated that SPIO nanoparticles were present in the endosomal vesicles in cytoplasma (Fig. 4A). SPIO nanoparticles also were found in the endocytotic cups on the cell surface, suggesting the initiation of endocytosis (i, arrow). It was also found that endosomal vesicles were surrounded by coated pit structures (ii and iii). Similar to the results from Prussian blue staining, SPIO nanoparticles were not observed in the nucleus. The addition of colchicine, which terminated endocytosis by inducing depolymerization of microtubule, significantly reduced intracellular iron content in hMSCs (one-way ANOVA, $p < 0.05$; Fig. 4B).

SPIO Labeling Promoted Proliferation of hMSCs

hMSCs incubated with 50, 75, and 100 μg/ml of SPIO showed significantly increased cell viability compared with unlabeled controls (one-way ANOVA, $p < 0.05$; Fig. 5A). Analysis of cumulative PD levels confirmed the promotion of cell proliferation in SPIO-labeled cultures (slope = 0.54 and 0.35 for labeled and unlabeled groups; Fig. 5B). Doubling time for labeled and unlabeled hMSCs was 28 and 49 h, respectively. A significant increase in cell numbers in the S and G2/M phases was observed in SPIO-labeled hMSCs ($p < 0.05$, chi-square test; Fig. 5C).

Differentiation Abilities of MSCs Were Impaired after SPIO Labeling

After osteogenic induction, unlabeled and SPIO-labeled hMSCs exhibited an osteoblast-like flattened morphology. Positive staining of alkaline phosphatase was powerless in the SPIO-labeled group. When normalized by cell number, the SPIO-labeled group showed a significant decrease in the amount of calcium mineral at the third week of induction (Fig. 6A). Expression levels of osteogenic lineage marker genes, RUNX2, osteocalcin, and type I collagen were lower in

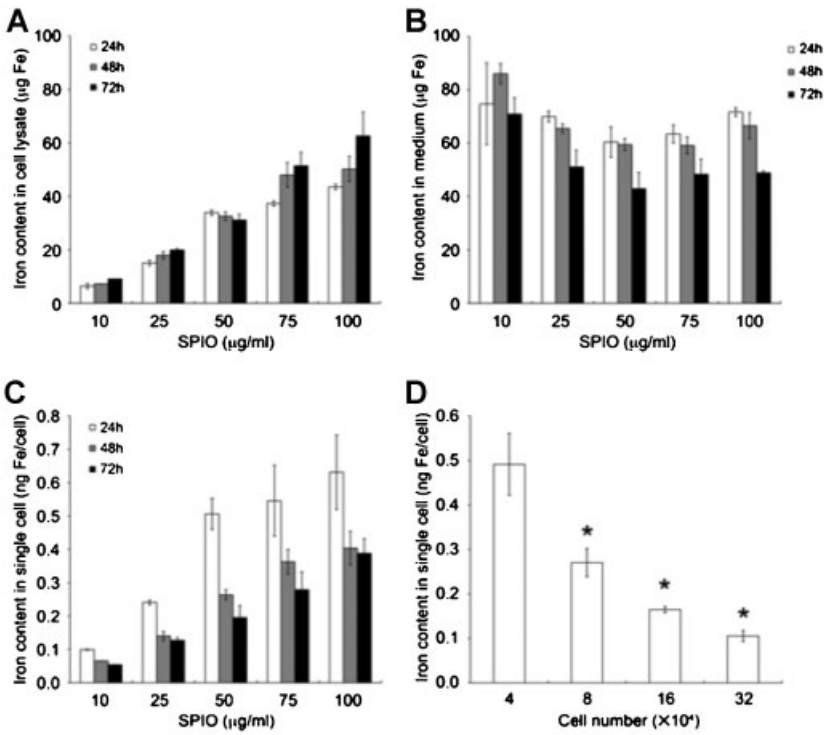


Figure 2. Iron content in SPIO-labeled human mesenchymal stem cells. Cells were labeled with 0, 10, 25, 50, 75, and 100 μg/ml of SPIO for 24 h. Iron content in the cell lysate (A) and in the condition medium (B) of SPIO-labeled hMSCs was shown. (C) In a fixed SPIO-loading concentration, prolonged incubation of hMSCs with SPIO decreased the normalized iron content of each single cell. (D) The iron content of a single cell was decreased significantly in a cell number-dependent manner ($^*p < 0.05$, one-way ANOVA).

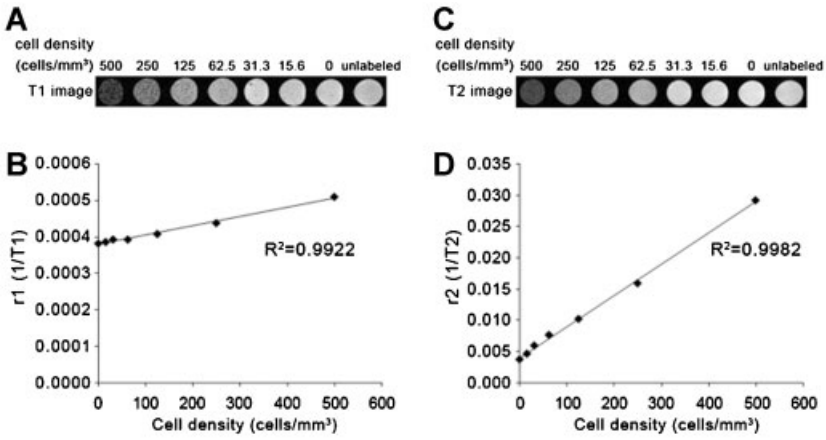


Figure 3. In vitro MR imaging for SPIO-labeled human mesenchymal stem cells. Cells were labeled with 50 μg/ml of SPIO for 24 h. Spin echo T1-weighted images (A) and T2-weighted images (C) were obtained by using a 7T MR imaging system. Spin echo T1-weighted and T2-weighted images revealed the presence of a hypointense signal. The values of r1 (B) and r2 (D) relaxation were plotted in correlation with the cell density.

the SPIO-labeled group as compared to the control group during the induction, except expression of RUNX2 and osteocalcin at the third week (Fig. 6B). These results suggested an impaired osteogenic commitment after SPIO labeling. For adipogenic differentiation ability, fat droplet deposition was found in both groups (Fig. 6C). Expression of adipogenic lineage marker genes, PPAR-γ, fatty acid binding protein, and fatty acid synthase was similar in both groups during the induction, suggesting a preserved adipogenic commitment (Fig. 6D). Formation of the glycosaminoglycan of chondrocytes in the control group was found by alcian blue staining. SPIO-labeled hMSCs failed to form a pelleted micromass, aggregated into sheet-like structures with loose attachment (Fig. 6E). Expression of aggrecan and type II collagen expression were not detectable in the SPIO-labeled group, while expression of cartilage oligomeric matrix protein (COMP) was similar to the gene expression in the control group (Fig. 6F).

SPIO Labeling Induced Growth Factor Release

The relative amount of the six growth factors in the SPIO-labeled condition medium was higher than in the unlabeled condition medium and included amphiregulin (AR), glial cell-derived neurotrophic factor (GDNF), heparin-binding EGF-like growth factor (HB-EGF), soluble form of macrophage colony-stimulating

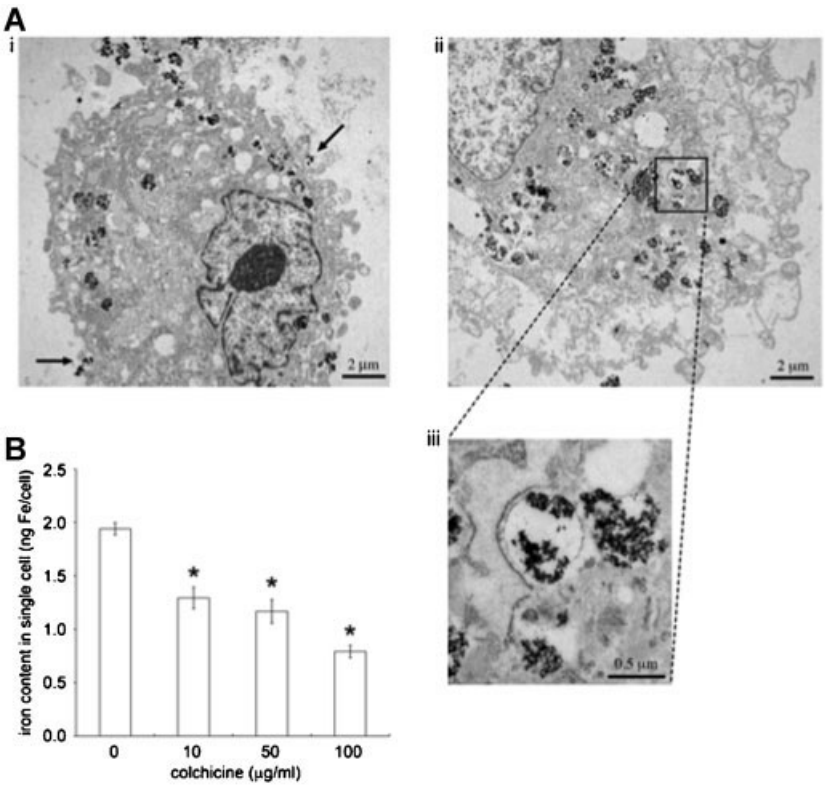
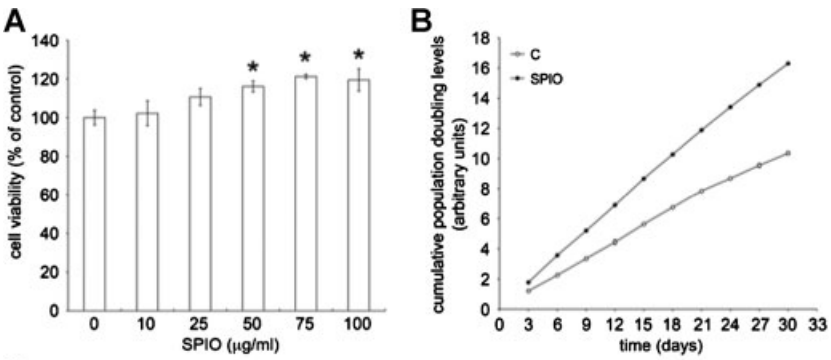


Figure 4. Intracellular distribution of SPIO in hMSCs and effect of colchicine on SPIO uptake. Cells were labeled with 50 μg/ml of SPIO for 24 h. (A) Intracellular distribution of SPIO in hMSCs was examined by TEM. SPIO nanoparticles were accumulated in the endosomes, as well as in the endocytotic cup on the surface of hMSCs (arrow) (i). Aggregation of SPIO nanoparticles was also found in the coated-pit vesicle (ii). (iii) Higher magnification image showed coated pit structures, seen as dark staining, surrounded the endosomal vesicles. Scale bar = 2 μm in (i) and (ii), 0.5 μm in (iii). (B) Iron content in single cell was reduced in colchicine-treated hMSCs. Data were presented as mean ± SE of three independent experiments ($^{*}p < 0.05$, one-way ANOVA).



C

	% of Gated		
	G1	S	G2/M
control	93.52 ± 4.21	5.70 ± 0.67	1.10 ± 0.14
SPIO	82.34 ± 2.43	10.95 ± 0.84	5.50 ± 0.28

Figure 5. Promotion of cell proliferation by SPIO labeling. Cells were labeled with 50 μg/ml of SPIO for 24 h. (A) MTT assay indicated the increase of cell viability when the hMSCs were labeled by SPIO ($^{*}p < 0.05$, one-way ANOVA). (B) Cells were serially subcultured from passage 10 to 20, up to 30 days. Analysis of cumulative population doubling levels confirmed the promotion of cell proliferation in SPIO-labeled cultures. (C) A significant increase of cell number in S and G2/M phases was observed in SPIO-labeled hMSCs after 24 h by flow cytometry analysis ($^{*}p < 0.05$, chi-square test).

factor receptor (M-CSFR), a soluble form of SCF receptor (SCFR), and vascular endothelial growth factor (VEGF) (Fig. 7).

DISCUSSION

Amine-surface-modified SPIO nanoparticles without polymer coating exhibit well-dispersed morphology with a small diameter of 6 nm,[20] and the diameter of ferumoxide and ferucarbotran is larger (60 and 80–100 nm, respectively). Moreover, like most transfection agents, the cationic surface of amine-surface-modified SPIO nanoparticles may facilitate cellular uptake, while the surface of ferucarbotran is anionic due to deprotonated carboxydextran under physiological conditions.[14] There is also evidence to indicate that polymer coatings on ferumoxide and ferucarbotran are not strongly bound and are therefore more prone to detachment leading to aggregation and precipitation.[24] Reports show that SPIO nanoparticles are non-toxic to various stem cells, and having no effects on stem cell phenotypes or differentiation potential.[1–7] However, our findings in this study and more recent evidence[12–18,25] show that the biological effects of various SPIO nanoparticles, with or without different surface coating, on stem cells should not be neglected.

In the present study, intracellular incorporation of amine-surface-modified SPIO nanoparticles in hMSCs is demonstrated by Prussian blue staining, and labeling efficiency is high (Fig. 1). In a fixed SPIO-loading concentration, prolonged incubation of hMSCs with SPIO decreases the iron content of single cells in a cell number-dependent manner (Fig. 2). This finding may result from the proliferation of hMSCs during long-term incubation. T2WI imaging shows greater T2 relaxation levels than does T1WI imaging for SPIO-labeled hMSCs (Fig. 3). Thus, T2-weighted MR images should be adopted to visualize SPIO-labeled stem cells for cell tracking. The presence of SPIO nanoparticles in the endosomal vesicles in cytoplasma is demonstrated by TEM (Fig. 4).

Significantly increased cell viability, shortened doubling time, and accelerates cell cycle progression of hMSCs was demonstrated after labeling with amine-surface-modified SPIO nanoparticles (Fig. 5). Similar effects of ferucarbotran on hMSCs have been reported, and osteogenesis was inhibited.[16,17] Several recent studies reported that the differentiation capacity was not inhibited by SPIO labeling.[4,5,7] However, osteogenesis and chondrogenesis are impaired when hMSCs are labeled with amine-surface-modified SPIO nanoparticles in this study (Fig. 6). Interestingly, SPIO-labeled hMSCs aggregated into sheet-like structures and overexpressed COMP during chondrogenic induction (Fig. 6F), as COMP supports chondrocyte attachment[26] and protects chondrocytes from cell death.[27] Previous studies have indicated that hMSC commitment depends on cell density.[28] In this study, SPIO-labeling accelerates cell proliferation and leads hMSCs to a higher cell density, which may contribute to preserved adipogenesis and impaired osteogenesis.

Alteration of the growth factor release from hMSC by SPIO labeling (Fig. 7) has not been reported in the literature before. Among the six increased growth factors, amphiregulin, a member of the epidermal growth factor (EGF) family, plays a critical role in regulating the MSC pool in the bone marrow and inhibiting further differentiation of preosteoblasts.[29] HB-EGF, which also belongs to the EGF family, had been suggested to induce cell expansion of MSCs and to prevent multilineage differentiation.[30] Finally, addition of VEGF in the culture medium increases MSC proliferation.[31] As a result, the observed biological effects of amine-surface-modified SPIO nanoparticles on hMSCs may result from modulation of released growth factors, especially amphiregulin, HB-EGF, and VEGF.

In conclusion, cellular internalization of SPIO nanoparticles promotes proliferation of hMSCs and impairs their osteogenic and chondrogenic differentiation, which may result from the altered cytokine and soluble growth factor receptor production. Although SPIO

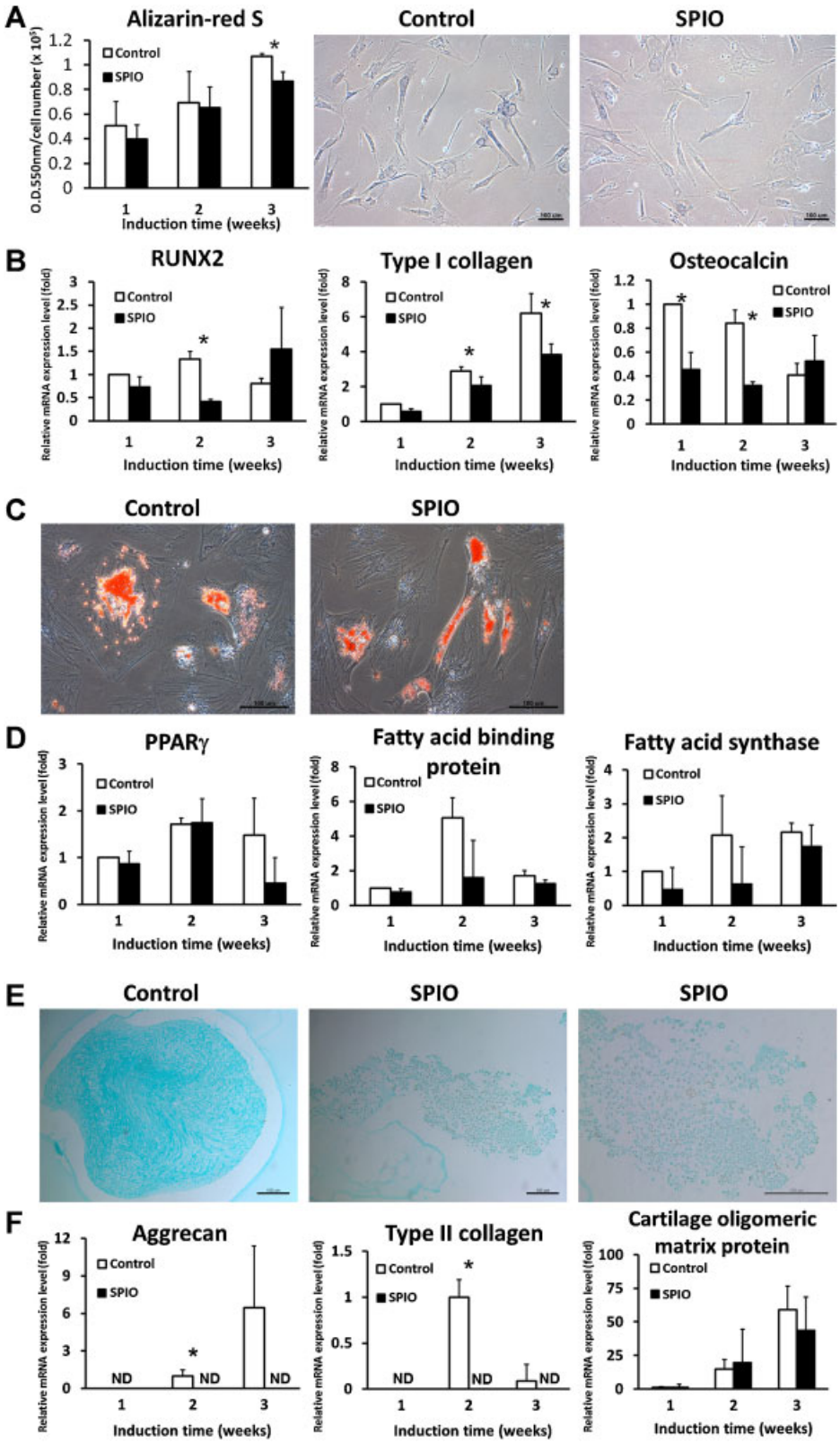
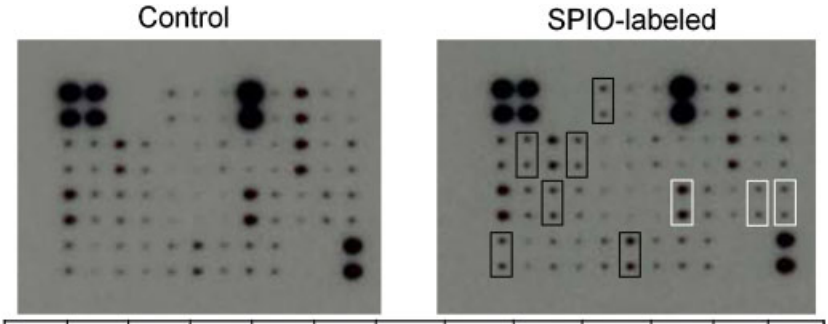


Figure 6. Multilineage differentiation of human mesenchymal stem cells was affected by SPIO treatment. Cells were labeled with 50 μg/ml of SPIO for 24 h. (A) Results of quantitative Alizarin-red S staining procedure (normalized by cell number) during osteogenic induction and alkaline phosphatase staining after osteogenic induction for 2 weeks, and (B) Expression of osteogenic lineage marker genes of hMSCs from unlabeled (Control) and SPIO-labeled hMSCs (SPIO) followed by osteogenic induction for 3 weeks. (C) Oil red O staining after adipogenic induction for 3 weeks and (D) Expression of adipogenic lineage marker genes followed by adipogenic induction for 3 weeks. (E) Alcian blue staining after chondrogenic induction for 3 weeks and (F) Expression of chondrogenic lineage marker genes followed by chondrogenic induction for 3 weeks. Data of gene expression were presented as fold changes compared with GAPDH mRNA levels. Scale bar represented 100 μm ($^*p < 0.05$, t test).



	A	B	C	D	E	F	G	H	I	J	K	L
1	POS	POS	NEG	NEG	AR	bFGF	b-NGF	EGF	EGF-R	FGF-4	FGF-6	FGF-7
2	POS	POS	NEG	NEG	AR	bFGF	b-NGF	EGF	EGF-R	FGF-4	FGF-6	FGF-7
3	GCSF	GDNF	GM-CSF	HB-EGF	HGF	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-6	IGF-I	IGF-ISR
4	GCSF	GDNF	GM-CSF	HB-EGF	HGF	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-6	IGF-I	IGF-ISR
5	IGF-II	M-CSF	M-CSF R	NT-3	NT-4	PDGF Rα	PDGF Rβ	PDGF-AA	PDGF-AB	PDGF-BB	PlGF	SCF
6	IGF-II	M-CSF	M-CSF R	NT-3	NT-4	PDGF Rα	PDGF Rβ	PDGF-AA	PDGF-AB	PDGF-BB	PlGF	SCF
7	SCF R	TGF-α	TGF-β	TGF-β2	TGF-β3	VEGF	VEGF-R2	VEGF-R3	VEGF-D	BLANK	BLANK	POS
8	SCF R	TGF-α	TGF-β	TGF-β2	TGF-β3	VEGF	VEGF-R2	VEGF-R3	VEGF-D	BLANK	BLANK	POS

Figure 7. Effect of SPIO on growth factor release of human mesenchymal stem cells. After labeling with 0 and 50 μg/ml of SPIO for 24 h, the culture was washed thoroughly with PBS and incubated in a fresh medium for another 3 days. At the end of incubation, culture mediums were collected and subjected to Bioray® Human Growth Factor Antibody Array I. Upper panel was result of an antibody-based human growth factor array. Black open box marked the factors which increased release, and white open box marked the factors which decreased release in SPIO-labeled hMSC condition medium. The symbol of each growth factor corresponded to the position of each spot in the array image (lower panel).

labeling is effective for cell tracking, the properties of hMSCs may alter as a consequence and this needs to be taken into account when evaluating therapeutic efficacies of SPIO-labeled stem cells in vivo.

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