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ORIGINAL ARTICLE

Optimizing Labeling of Adipose-Derived Mesenchymal Stem/Stromal Cells with Promag Superparamagnetic Iron Oxide Nanoparticles for Clinically Translatable Magnetic Particle Imaging

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What is already known on this topic?

- *Mesenchymal stem/stromal cells (MSCs) can be tracked in vivo using superparamagnetic iron oxide nanoparticles (SPIONs) and magnetic imaging techniques such as magnetic resonance imaging and magnetic particle imaging.*
- *Labeling efficiency depends on nanoparticle concentration, incubation time, and whether cells are cultured under adherent or suspension conditions. Most studies use adherent conditions, which achieve stable labeling but are less practical for clinical applications requiring rapid, non-culture-based protocols.*
- *This study identifies 20 µg/mL ProMag SPIONs as the optimal concentration for human adipose-derived MSCs, providing high labeling efficiency with acceptable cell viability. It shows that labeling can alter the expression of some non-MSC markers (CD34, CD45). It demonstrates that labeling efficiency in suspension is influenced by vessel geometry, with Petri dishes showing more favorable outcomes than Eppendorf tubes, offering practical insights for developing rapid, clinically translatable suspension-based protocols.*

Abstract

Objective: This study evaluated the labeling efficiency of human adipose-derived mesenchymal stem/stromal cells (MSCs) with ProMag superparamagnetic iron oxide nanoparticles (SPIONs) under adherent and suspension conditions, aiming to define practical parameters for translational use.

Methods: The MSCs were labeled with 3 ProMag SPION concentrations (10-40 µg/mL) under adherent culture. Labeling efficiency and viability were assessed by Prussian blue staining and an adenosine triphosphate–based viability assay. The MSC identity (CD44, CD90) and hematopoietic markers (CD34, CD45) were analyzed by flow cytometry. Nanoparticle uptake mechanisms were probed using a low-temperature endocytosis inhibition assay. Comparative experiments examined labeling efficiency between adherent cultures and suspension protocols using Eppendorf tubes and Petri dishes.

Results: A concentration of 20 µg/mL achieved an optimal balance between labeling efficiency (79%) and viability (73%) and was used in subsequent assays. The MSC identity was preserved, with >95% CD44/CD90 expression, though slight increases in CD34 (4.10%) and CD45 (8.55%) were observed after labeling. Uptake occurred predominantly via active endocytosis. Adherent conditions yielded significantly higher labeling efficiency than suspension in Eppendorf tubes ($P = .002$). Suspension in Petri dishes showed moderately improved uptake but did not reach significance ($P = .062$).

Conclusion: ProMag SPIONs effectively label adipose-derived MSCs, though efficiency is strongly influenced by the suspension environment. Optimized suspension-based strategies, considering vessel geometry and defined concentration parameters, may enable rapid labeling approaches suitable for real-time magnetic particle imaging in regenerative therapies.

Keywords: Cell labeling, cell tracking, human mesenchymal stem cells, optimization, superparamagnetic iron oxide nanoparticle

Introduction

Mesenchymal stem/stromal cells (MSCs) are widely explored for their regenerative, immunomodulatory, and trophic effects in various therapeutic applications.[1,2] To better understand the fate, distribution, and mechanism of action of MSCs post-administration, non-invasive cell tracking techniques are crucial.[3] Among these approaches, labeling with superparamagnetic iron oxide nanoparticles (SPIONs) followed by detection using magnetic imaging modalities such as magnetic resonance imaging (MRI) or magnetic particle imaging (MPI) has emerged as a cutting-edge technology, offering high sensitivity and quantitative tracking potential.[4]

Efficient labeling of MSCs with magnetic nanoparticles is a key prerequisite for successful imaging.[5] However, labeling efficiency is influenced by several factors, including the type of nanoparticle, incubation conditions, and, importantly, the mode of cell interaction with the culture substrate,

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whether the cells are adherent or in suspension during the labeling process.[6] At this point, studies in the literature have generally used adherent cell culture conditions, which provide stable nanoparticle uptake, but require additional culture time, thereby limiting their utility in clinical scenarios where cells must be applied rapidly after isolation.[5,7] A notable example is the use of freshly isolated adipose-derived regenerative cells (ADRCs), which are characterized by their high content of MSCs, and are administered without culturing.[8,9] For such applications, developing efficient and fast suspension-based labeling protocols is critical to enable pre-application labeling and subsequent in vivo tracking. Furthermore, little is known about how the physical environment of suspension labeling, such as whether labeling is performed in smaller bottom surfaced substrates like Falcon tubes or Eppendorf tubes (for smaller scale labeling), or larger bottom surface substrates like Petri dishes, affects the interaction between nanoparticles and cells. This factor may influence sedimentation behavior, surface contact dynamics, and ultimately labeling efficiency, especially in protocols that seek to avoid cell adherence.[10]

In the previous study,[11] it was demonstrated that ProMag SPIONs outperformed other commercial SPIONs (Ferumoxytol, SynoMag-D, and VivoTrax) in labeling mouse bone marrow MSCs. Building on this, the purpose of this study was to evaluate the labeling efficiency of ProMag SPIONs in human adipose-derived MSCs without significantly compromising cell viability. Additionally, it was aimed to determine whether the cells' adhesion state (adherent vs. suspension) and the physical conditions during suspension labeling (Eppendorf tube vs. Petri dish) influence ProMag uptake efficiency. By identifying optimal conditions for rapid and effective suspension labeling, this work will support the development of clinically feasible strategies for MSC tracking in regenerative therapies.

Methods

Human Adipose Tissue–Derived Mesenchymal Stem/Stromal Cells

Human MSCs (hMSCs), previously characterized by Calcat-I-Cervera et al,[12] were used in this study. As these cells represent an established cell line, no additional ethical approval or informed consent was required, in accordance with widely accepted research ethics guidelines stating that established cell lines do not constitute human subjects research (e.g., International Cell Line Authentication Committee (ICLAC) and National Institutes of Health (NIH) guidance). The hMSCs were expanded in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12, Gibco, 11320074) supplemented with 10% fetal bovine serum (FBS; Gibco, A5256701). Cells used for nanoparticle labeling and subsequent analyses were between passages 5 and 10.

Labeling of Human Mesenchymal Stem/Stromal Cells with Superparamagnetic Iron Oxide Nanoparticles

For labeling experiments, hMSCs were plated in 24-well plates at a density of 2.5×10^4 cells per well in complete growth medium and cultured overnight at 37°C in a humidified atmosphere with 5% CO_2. The following day, the medium was aspirated, and cells were rinsed twice with Ca^{++}- and Mg^{++}-free phosphate-buffered saline (PBS, Gibco, 20012027). Cells were then exposed to ProMag SPIONs (Bang Laboratories, PMC1N) at concentrations of 10, 20, or 40 µg/mL, and incubated for 2 hours under standard culture conditions (37°C, 5% CO_2). After incubation, the nanoparticle solution was discarded, and cells were washed 3 times with Hank's balanced salt solution (HBSS) to remove unbound particles. Following fixation with 4% paraformaldehyde (PFA), cells were stained with Prussian blue to visualize intracellular iron. Each concentration condition was tested in triplicate for quantitative evaluation.

Adenosine Triphosphate–Based Cell Viability Assay

Cell viability was assessed using a bioluminescent adenosine triphosphate (ATP) quantification method. The hMSCs were seeded at a density of 5×10^3 cells per well in 100 µL of medium and incubated overnight. The next day, cells were treated with ProMag SPIONs at specified concentrations, while control wells received only complete medium without nanoparticles. After 2 hours of incubation at 37°C, the labeling solution was removed, and cells were washed thoroughly. Cell viability was then measured using the CellTiter-Glo reagent (Promega, G7571), and luminescence was recorded using a Fluostar Omega plate reader. All conditions were assessed in triplicate, and viability results were normalized to control samples.

Prussian Blue Staining

For detection of iron uptake, cells were fixed in 4% PFA and stained using a solution of potassium ferrocyanide and hydrochloric acid (Sigma-Aldrich, HT20) for 30 minutes. Stained cells were imaged using a Leica DFC420C digital microscope camera at 15× magnification, with 5 randomly selected fields captured per condition. Labeling efficiency was determined by calculating the percentage of cells exhibiting blue staining.

Flow Cytometry Analysis

The hMSCs were seeded onto 100 mm Petri dishes and cultured until ~70% confluency. The growth medium was then replaced with either standard medium or ProMag labeling solution and cells were incubated for 2 hours. After labeling, cells were washed and incubated in fresh growth medium for an additional 24 hours. Adherent cells were harvested by trypsinization, resuspended in 1 mL staining buffer (0.5% BSA in PBS), and counted using a hemocytometer. Cell suspensions were centrifuged at 1000 rpm for 5 minutes, and pellets were resuspended in staining buffer at a volume of 1 µL per 1000 cells. Cells were aliquoted into 5 Eppendorf tubes and labeled with CD90-APC, CD44-FITC, CD45-APC, or CD34-APC antibodies (Miltenyi: 130-117-534, 130-098-210, 130-113-676, 130-113-738, respectively) at a 1 : 50 dilution for 10 minutes at 4°C in the dark. After incubation, 1 mL staining buffer was added to each tube, followed by centrifugation at 3000 × g for 10 minutes to remove unbound antibodies. Pellets were resuspended in at least 500 µL staining buffer and analyzed on a FACS Canto II (BD Biosciences). Raw data were processed using the online software "*Floreada.io*".

Low-Temperature Inhibition Assay

To explore the mechanism of nanoparticle internalization, a low-temperature inhibition assay was conducted.[13] Cells were either kept at 4°C or maintained at 37°C prior to and during exposure to ProMag SPIONs. After 2 hours of incubation, cells were processed for Prussian blue staining, and iron uptake was compared between temperature conditions to assess the role of endocytosis.

Labeling in Different Culture Conditions

The hMSCs were labeled either in adherent culture or in suspension. For adherent labeling, 1.5×10^5 hMSCs were seeded into a 35 mm adherent Petri dish and incubated overnight to allow cell

attachment. The following day, cells were labeled with ProMag SPIONs at the optimal concentration of 20 µg/mL (defined in the previous stage) for 2 hours. For suspension labeling, the same number of cells (1.5×10^5) was resuspended in the same concentration of ProMag SPION solution (20 µg/mL) and incubated for 2 hours in either conical-bottom Eppendorf tubes or 35 mm non-adherent Petri dishes. Following labeling, adherent cells were processed using the same procedure as described above to evaluate labeling efficiency. Suspension-labeled cells were washed 3 times with HBSS. Each wash was followed by centrifugation to pellet the cells. After the final wash, cells were cytospun onto microscope slides at 700 RPM for 7 minutes and subsequently stained using Prussian blue to assess SPION uptake. All experiments were performed in triplicate.

Statistical Analysis

All statistical analyses were carried out using SPSS software version 20.0 (SPSS Inc.; Chicago, IL, USA). Results are expressed as mean ± standard error. For comparisons among multiple groups, 1-way ANOVA followed by Tamhane's T2 post-hoc test was used. Student's *t*-test was applied for direct comparison between 2 groups. A *P*-value of less than .05 was considered statistically significant.

Results

Labeling Efficiency and Viability Analysis

Labeling efficiency analysis performed on adherent human adipose-derived MSCs revealed that the highest efficiency (91 ± 0.5%) was achieved at a ProMag concentration of 40 µg/mL. This was followed by efficiencies of 79 ± 1.1% and 32 ± 3.6% at concentrations of 20 µg/mL and 10 µg/mL, respectively (Figure 1 A-E). Under the same culture conditions in a 96-well plate format, an ATP-based viability assay was performed. The results showed a notable decrease in cell viability at the lowest concentration (10 µg/mL), with viability measured at 74 ± 2.5%. This was slightly reduced to 73 ± 1.5% at 20 µg/mL and further reduced to 67 ± 0.7% at the highest concentration (40 µg/mL) (Figure 1 F). When labeling efficiency and cell viability were considered together, 20 µg/mL was determined to be the optimal ProMag concentration, providing high labeling efficiency with a tolerable viability loss of approximately 27%. Therefore, this concentration was used for subsequent analyses comparing labeling efficiencies under adherent versus cell suspension conditions.

Immunophenotypic Characterization of Unlabeled and ProMag-labeled Human Mesenchymal Stem/Stromal Cells

To determine whether ProMag labeling affects the immunophenotypic profile of hMSCs, flow cytometry analysis was performed for both MSC-specific and non-MSC surface markers. The MSC markers CD44 and CD90 were highly expressed in both unlabeled and ProMag-labeled hMSCs, with expression levels exceeding 95%. In contrast, the non-MSC markers CD34 and CD45 showed slight differences. In unlabeled cells, the expression of CD34 and CD45 was below 1%, consistent with typical MSC profiles. However, ProMag-labeled cells exhibited a notable increase in these markers, with CD34 expression rising to 4.10% and CD45 to 8.55% (Figure 2).

Analysis of Internalization Mechanism of Superparamagnetic Iron Oxide Nanoparticle

To assess whether SPION internalization occurs via active endocytosis, a temperature-sensitive endocytosis inhibition assay was performed. The results showed a marked reduction in particle uptake at low temperature, with internalization decreasing significantly from 79.7% to 5.5% ($P < .001$; Figure 3), indicating that ProMag SPIONs are predominantly internalized through active endocytic mechanisms.

Evaluation of Effects of Different Conditions on Labeling Efficiency

Labeling efficiency was significantly higher in adherent cells compared to cells in suspension in Eppendorf tubes ($P = .002$). Although labeling efficiency of adherent cells in Petri dishes was also higher than that of cells in suspension in non-adherent Petri dishes, this difference was not statistically significant ($P = .062$). Similarly, a decrease in labeling efficiency was observed in cells suspended in Eppendorf tubes compared to those in suspension in Petri dishes, but this difference also did not reach statistical significance ($P = .056$) (Figure 4).

Discussion

In this study, the labeling efficiency of hMSCs with ProMag SPIONs under various conditions was evaluated, with the aim of identifying clinically relevant protocols for rapid, non-adherent cell labeling. The results demonstrated that ProMag SPIONs can effectively label MSCs with high efficiency, and that labeling performance is strongly influenced by both nanoparticle concentration and cell culture conditions during labeling. It was shown that 20 µg/mL ProMag offered a favorable balance between efficiency (~79%) and acceptable cell viability (~73%), making it the most suitable concentration for downstream applications. These findings are consistent with earlier studies showing that, although increasing SPION concentration improves labeling efficiency, it can negatively impact cell viability, highlighting the importance of dose optimization for translational use.[5,6,11] Trozzo et al[14] further demonstrated that 25 µg/mL ProMag achieved over 90% labeling of mouse 4T1 breast carcinoma cells after 24 hours of incubation, with less than a 5% decrease in viability. It is important to note, however, that labeling efficiency may vary between cell types, and longer incubation times could potentially improve uptake.

Interestingly, flow cytometry revealed that ProMag labeling altered the expression of non-MSC markers CD34 and CD45, which were elevated in labeled cells compared to unlabeled controls. While CD44 and CD90 remained highly expressed in both conditions (>95%), the increase in CD34 (4.1%) and CD45 (8.55%) following labeling suggests that nanoparticle internalization or surface interaction may influence cell surface marker expression. To the best of knowledge, only a limited number of studies have compared the immunophenotypic profile of SPION-labeled vs. unlabeled MSCs.[15] In 1 report, human bone marrow-derived MSCs labeled with multifunctional nanoparticles (35 nm hydrodynamic diameter, magnetic/fluorescent properties) exhibited no detectable changes in surface marker expression compared with unlabeled controls.[15] However, this may not be directly comparable to the system, as the ProMag particles used in the present study are substantially larger (>950 nm), resulting in greater cell-membrane interaction and potentially increased likelihood of modulating surface proteins. Whether such differences represent true phenotypic alterations, changes in activation state, or artifactual binding remains uncertain and should be further explored, particularly given the clinical importance of preserving MSC identity and potency.[2,16]

ProMag SPION internalization by MSCs was demonstrated to occur primarily through active endocytosis, as evidenced by a

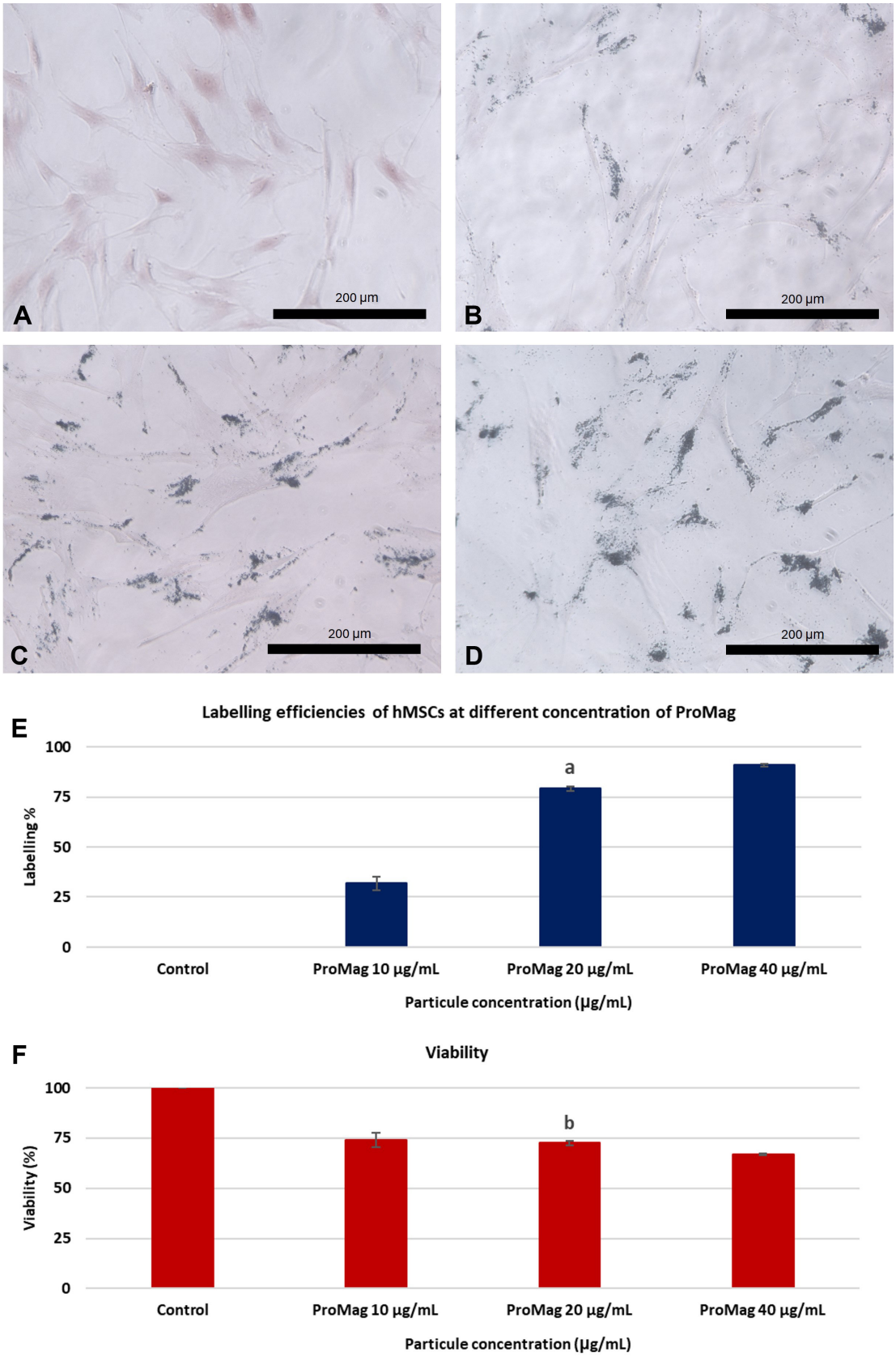

Figure 1. Representative images of adherent human MSCs incubated with increasing concentrations of ProMag nanoparticles (A–D), along with quantitative analysis of labeling efficiency (E). $P^{a} < .05$ compared to 10 and 40 µg/mL ProMag groups. (F) Graphical representation of ATP-based viability assay results. $P^{b} < .05$ compared to the 40 µg/mL ProMag group.

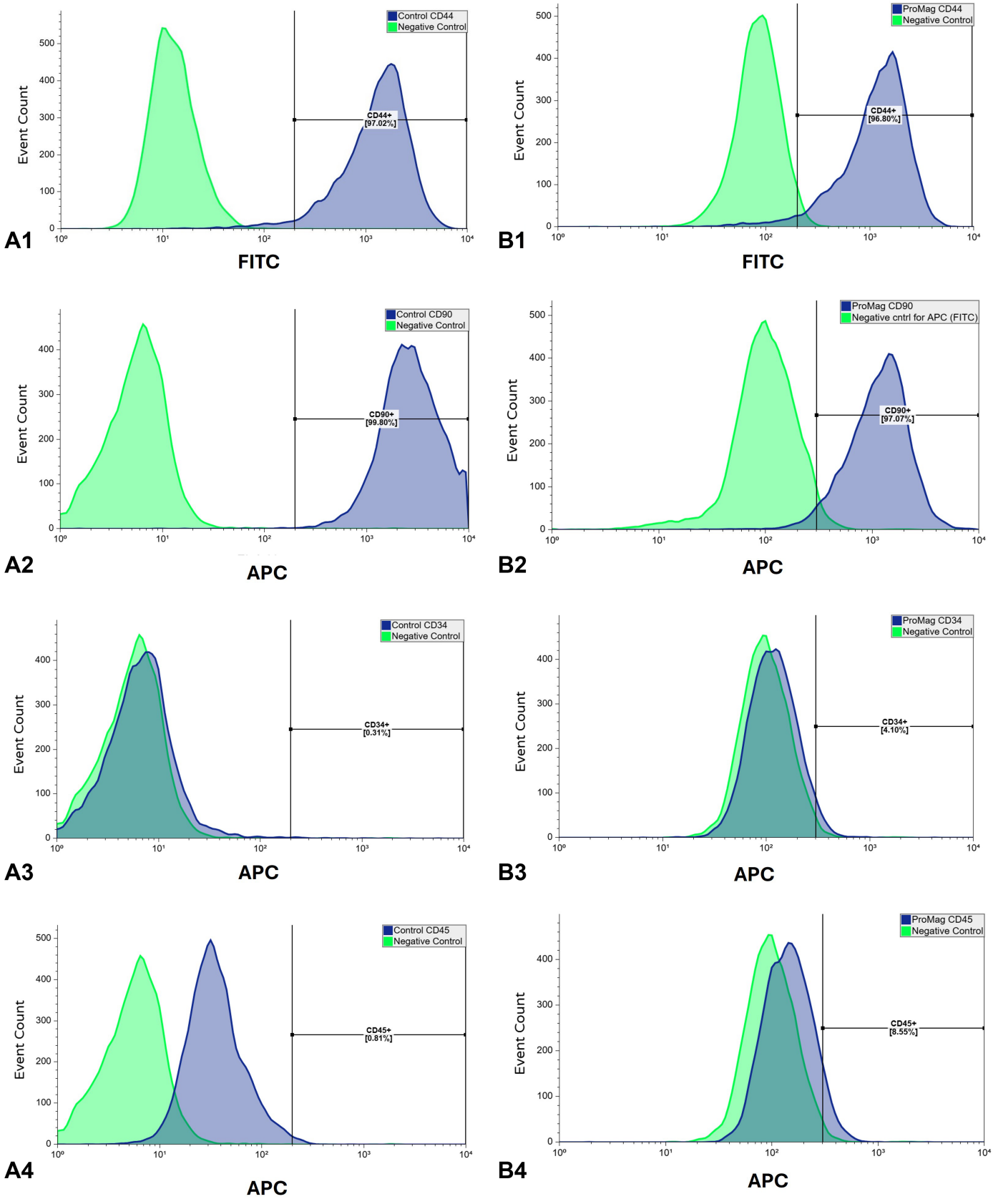

Figure 2. Flow cytometry analysis of surface marker expressions of CD44, CD90, CD34, and CD45 in unlabeled (A1: 97.02%, A2: 99.80%, A3: 0.31%, and A4: 0.81%; respectively) and ProMag-labeled hMSCs (B1: 97.07%, B2: 96.80%, B3: 4.10%, and B4: 8.55%; respectively).

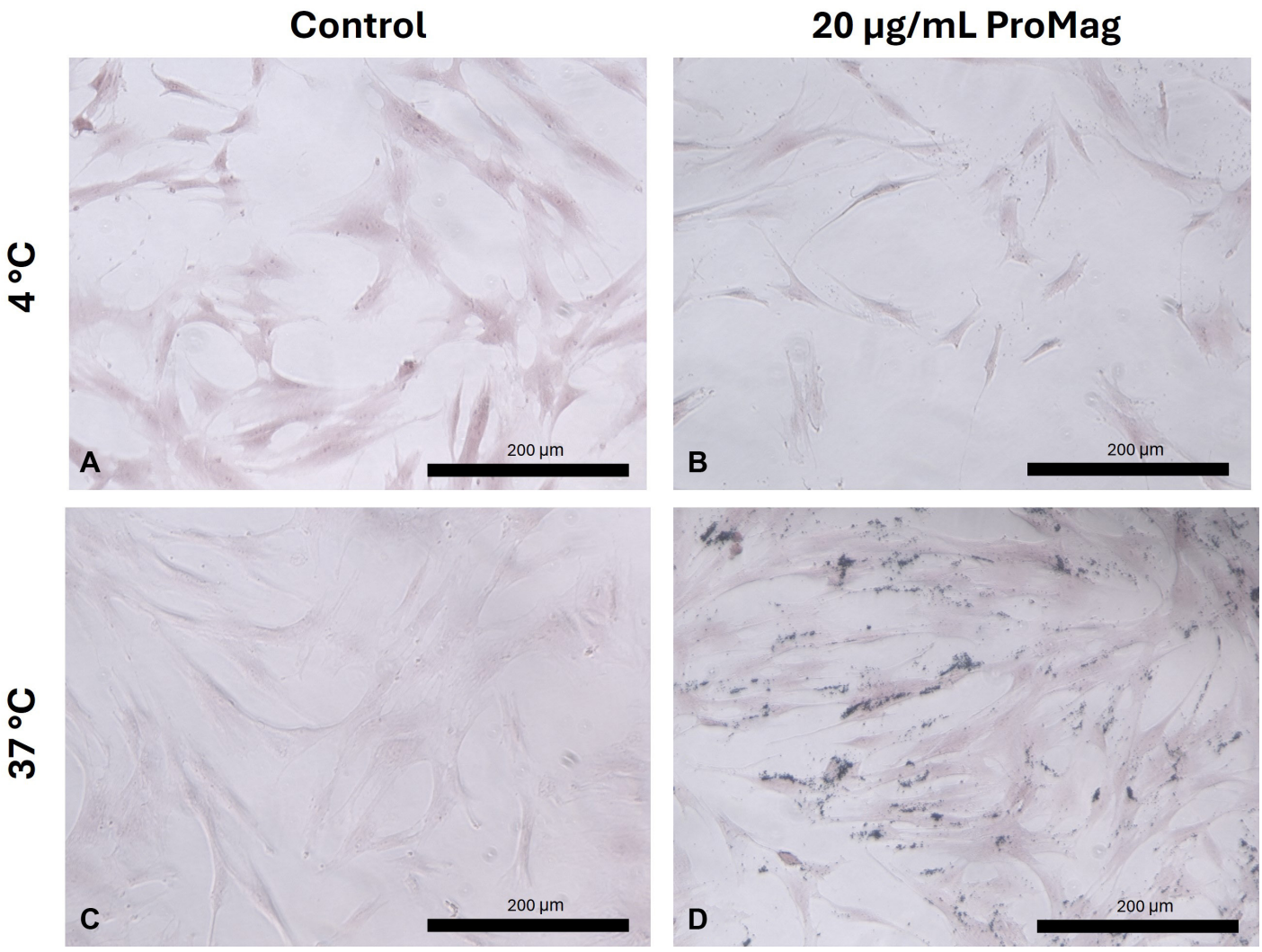

Figure 3. Representative images of Prussian blue staining showing ProMag labeling at 4°C (B) and 37°C (D), along with the corresponding control groups (A and C, respectively).

marked reduction in uptake under low-temperature conditions. This finding is consistent with previous reports indicating that nanoparticle uptake in MSCs is energy-dependent.[17] A limitation of the study, however, is that the specific endocytic pathway(s) involved were not identified. This information is critical for inferring the intracellular fate of internalized particles and the preservation of their magnetic properties, both of which are essential considerations for their further preclinical and clinical application. Based on established correlations between nanoparticle size and endocytic mechanisms, where particles <200 nm are typically internalized via clathrin-mediated endocytosis, and larger particles preferentially enter through caveolae-mediated pathways,[18,19] future studies should first evaluate whether ProMag uptake occurs through caveolae-mediated endocytosis.

Critically, the analysis of labeling under suspension vs. adherent conditions highlights the challenges of non-adherent labeling protocols. As anticipated, adherent cells achieved higher labeling efficiencies than those in suspension, particularly in Eppendorf tubes, where reduced sedimentation and limited surface contact likely hindered nanoparticle uptake.[20] Although differences between suspension labeling in Eppendorf tubes and Petri dishes did not reach statistical significance, a trend toward improved uptake in Petri dishes was observed. This suggests that physical labeling environments such as surface area-to-volume ratio and sedimentation dynamics may influence SPION-cell interactions even in non-adherent conditions. These insights are particularly relevant for protocols involving freshly isolated cells, such as ADRCs, which must be labeled in suspension prior to immediate application.

Collectively, the findings support the feasibility of using ProMag SPIONs for MSC labeling, particularly under adherent conditions. However, when rapid, non-culture-based labeling is required, careful consideration of labeling vessel geometry and nanoparticle concentration becomes essential. Future studies should explore the long-term functional consequences of marker expression changes, refine suspension labeling protocols for clinical translation, and validate in vivo tracking efficacy using MPI.

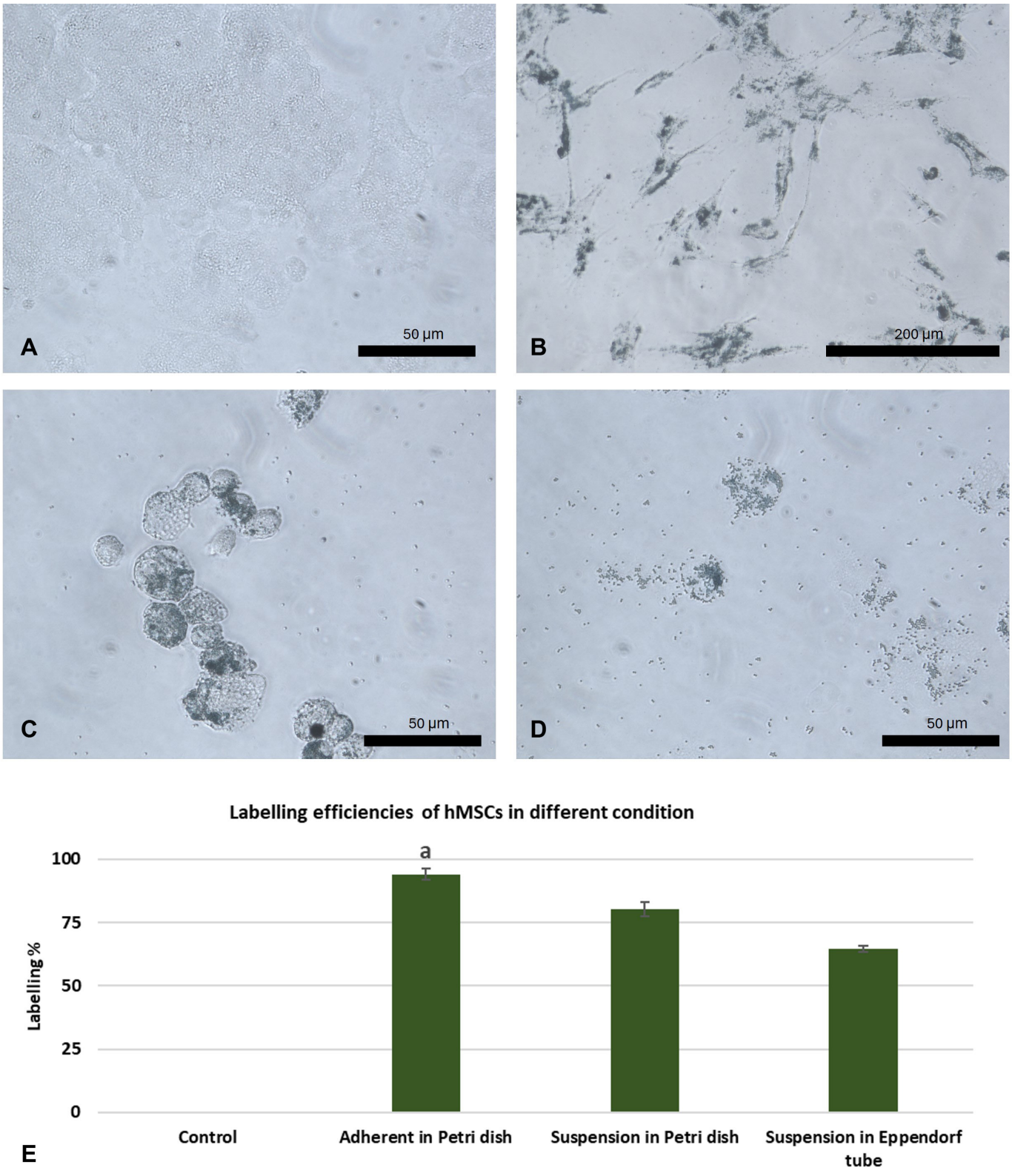

Figure 4. Representative images of adherent human MSCs incubated under different conditions using the same ProMag concentration (20 µg/mL) and cell number per unit: (A) control (unlabeled), (B) adherent cells in a Petri dish, (C) cell suspension in a Petri dish, and (D) cell suspension in an Eppendorf tube. (E) Graphical representation of labeling efficiencies under the different conditions. $P^{a} < .002$ compared to cell suspension in Eppendorf.

Data Availability Statement: The data that support the findings of this study are available on request from the corresponding author.

Ethics Committee Approval: No ethical approval was required for this study, as it did not involve human participants, identifiable human data, or live animals..

Informed Consent: No informed consent was required for this study, as it did not involve human participants or identifiable human data.y.

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Author Contributions: Concept – S.Ö.; Design – S.Ö.; Supervision – P.M.; Resources – P.M.; Materials P.M.; Data Collection and/or Processing – S.Ö., C.T.; Analysis and/or Interpretation – S.Ö., P.M., C.T.; Literature Search – S.Ö.; Writing Manuscript – S.Ö.; Critical Review – P.M.

Declaration of Interests: The authors declare that they have no competing interest.

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