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Long-Term Tracking Mesenchymal Stem Cell Differentiation with Photostable Fluorescent Nanoparticles

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S Supporting Information

ABSTRACT: Mesenchymal stem cells (MSCs) have proved to be a promising and abundant cell source for tissue and organ repair in regenerative medicine. However, the cell fate, distribution and migration of these transplanted cells are still unclear due to the limited tracking methods. It is desirable to develop a biocompatible and photostable probe to label the MSCs for long-term tracking without affecting the cell proliferation and potency. Herein we apply a recently developed nanoprobe system, in which di(thiophene-2-yl)-diketopyrrolopyrrole (DPP) is covalently linked in the middle of polycaprolactone (PCL) forming the PCL-DPP-PCL polymer complex. Although the PCL-DPP-PCL nanoparticles uptaken by the MSCs did not affect the cell viability, it was interesting that they exhibited different effects on the multilineage potency of the MSCs in the subsequent differentiation in vitro. Specifically, we found that the PCL-DPP-PCL labeling was unfavorable to the MSC osteogenic differentiation, whereas the labeled MSCs exhibited the same adipogenic and chondrogenic differentiations compared to the unlabeled controls as verified by gene expressions and histological staining. Furthermore, the PCL-DPP-PCL nanoparticles remained strong fluorescence intensity even after 4 weeks of differentiation. This study indicated that PCL-DPP-PCL nanoparticles could be used for long-term cell tracing in MSC differentiation into adipogenic and chondrogenic lineages.

KEYWORDS: mesenchymal stem cell, differentiation, long-term cell tracking, nanoparticles, photostability

1. INTRODUCTION

Stem cell-based therapies, aiming to treat diseases using the self-renewal and multilineage differentiation capacities of the transplanted stem cells, have achieved great progress in the past decade.^{1–4} Among the various types of stem cells, mesenchymal stem cells (MSCs) are much less prone to the ethical issues and the risk of developing teratoma.⁵ MSCs are able to differentiate into multiple types of mesoderm lineages, such as chondrocytes, osteocytes, adipocytes, and some ectodermic and endodermic cells.⁵ An increasing number of MSC-based therapies, especially for tissue repair or immune disorder diseases, have been attempted since 2004.⁶ However, there are many major issues remaining to be resolved before successful clinical applications on real patients.^{5,7} One of the critical challenges is the difficulty in tracking the fate, distribution, and migration of the transplanted stem cells in the local microenvironment due to the limited tracking methods. Compared to traditional but highly invasive histological analysis, noninvasive imaging techniques are urgently demanded to track the transplanted stem cells for monitoring the therapeutic progress. The distribution and migration of the

transplanted stem cells can provide a reliable evaluation for the cell-based therapeutic effect and elucidate the optimal conditions during surgery, such as cell dosage, route of delivery and the timing of transplantation.^{8,9} Additionally, in the applications of stem cell based tissue and organ regeneration, it is essential to delineate the specific contributions of transplanted cells and host cells to the damaged tissues or organs.¹⁰

Fluorescence imaging has been widely applied in various biological fields as a noninvasive technique because of its unique advantages, such as high sensitivity, rapid acquisition, lack of radiation, low cost and easy access.^{9–14} The most popular labeling markers for fluorescence imaging are organic dyes, such as DAPI (4',6-diamidino-2-phenylindole) and Alexa Fluor family fluorophores. However, most organic dyes can only be used for short-term imaging because of photobleaching effect.¹⁰ Another emerging type of fluorescent labels are

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quantum dots (QD), which emit strong fluorescence due to quantum confinement effect and the excitation and emission spectra are highly tunable based on their unique sizes.^{10,15} However, the typical semiconductor and heavy metal-based quantum dots could release metal ions particularly under UV illumination, which are highly toxic to the cells both in vitro and in vivo.^{16–18} QDs comprising a cadmium selenide (CdSe) nanocrystal core and a zinc sulfide (ZnS) shell were developed to label human MSCs during osteogenic differentiation by liposome-mediated transportation.¹⁹ It was found that although the labeled MSCs showed normal proliferation, the gene expressions of specific osteogenic markers including osteopontin, osteocalcin and alkaline phosphatase were significantly inhibited in the presence of QDs.¹⁹ In another prior study, MSCs labeled with commercial quantum dots (QTracker 800, Thermo Fisher Scientific) were delivered to nude rats with segmental bone defects to track the cell distributions during the bone healing process after transplantation.¹⁶ Although clear fluorescence could be observed in vivo from the treated rats, the QD-labeling reduced the function of transplanted cell and thereby the bone healing responses. To address this major issue, QD probes need to be modified with biomolecules or biocompatible polymers to reduce their cytotoxicity before applications. For example, CGGGRGD (Cys-Gly-Gly-Gly-Arg-Gly-Asp) was covalently bonded with CdSe/ZnS QDs to label human MSCs for induced differentiations into adipogenic, chondrogenic, and osteogenic lineages.¹⁰ These bioconjugated QDs were shown to be able to maintain the viability as well as the multipotency of the labeled human MSCs (hMSCs). In addition to quantum dots, other nanoprobe, such as phosphonate-functionalized polystyrene nanoparticles were also used for long-term tracing of hMSC differentiation after they were labeled with the fluorescent dye N-(2,6-diisopropylphenyl)-perylene-3,4-dicarboximide.²⁰

Previously, Huang and co-workers have prepared a well-defined synthetic polymer platform based on di(thiophene-2-yl)-diketopyrrolopyrrole (DPP) covalently linked in the middle of biocompatible and bioresorbable polycaprolactone (PCL) polymer, which is abbreviated as PCL-DPP-PCL.²¹ These nanoparticles exhibited bright fluorescence and strong photostability for cancer and stem cell imaging. Although the basic material properties, photostability, and cytotoxicity to a few tumor cells and stem cells have been elaborated in detail,²¹ the potential influence of this polymeric nanoprobe on MSC multipotency and differentiation remains unclear. Herein we bring this study from cellular level to tissue level by long-term tracing MSCs labeled with PCL-DPP-PCL and elucidating the potential effect of these nanoparticles on the MSC multipotency. Specifically, we labeled hMSCs with PCL-DPP-PCL nanoparticles and then induced these labeled hMSCs into different cell lineages including adipocytes, chondrocytes, and osteocytes, respectively, which were characterized by gene expression and histological staining. Additionally, the residue fluorescence within the differentiated labeled cells were measured by flow cytometry and confocal laser scanning microscopy. We believe that this work could be interesting and inspiring to researchers in the thriving fields of stem cell-based therapies, tissue engineering, and regenerative medicine.

2. MATERIALS AND METHOD

2.1. Preparation of PCL-DPP-PCL NPs. The detailed procedure to prepare and characterize the PCL-DPP-PCL nanoparticles (NPs)

can be found in the previous report.²¹ The prepared NPs were dispersed in sterile 1× PBS before use.

2.2. Cell Culture. Human bone marrow derived mesenchymal stem cells (hMSCs) were obtained from consented patients in the National University Hospital (Singapore) following a protocol approved by Institution Review Board (IRB).²² The isolated cells were expanded in expansion medium, consisting of low glucose Dulbecco's modified Eagle medium (DMEM) supplemented with sodium pyruvate and GlutaMax (Life Technologies, Singapore), 10% fetal bovine serum (FBS) (Life Technologies, Singapore), and 100 units/mL penicillin-streptomycin (Life Technologies, Singapore), and incubated under 37 °C with 5% CO₂ and humidified atmosphere. Nonadherent cells were removed after 48 h. The adherent cells remained in culture flasks for further expansion. The hMSCs in passages 3 and 4 were used for this study.

2.3. Cell Labeling and Flow Cytometry. The hMSCs were seeded in 6-well plates in normal DMEM medium with 10% FBS and 1% penicillin-streptomycin. After cell attachment overnight, the original medium was replaced by fresh medium with dispersed PCL-DPP-PCL NPs at concentration of 0.2 mg/mL. Then the cells were grown at 37 °C with 5% CO₂ for 3 and 7 days before the excess NPs were rinsed with 1× PBS.

For confocal laser scanning microscopy, the cells were fixed by formalin and then permeabilized by 0.1% Triton-X. Then the samples were blocked by 1% BSA and stained with Alexa Fluor 633 phalloidin for F-actin and DAPI for nucleus. The 3-color laser source (405 nm, 561 and 633 nm) in a confocal microscope (Carl Zeiss, LSM 710) provided excitation for the fluorophores in DAPI, PCL-DPP-PCL and Alexa Fluor 633 phalloidin with corresponding emission windows at 410–549 nm, 566–622 nm and 638–747 nm, respectively.

For quantitative analysis by flow cytometry, the cells were trypsinized by 1 × TrypLE Express Enzyme (Life Technologies, Singapore) and then centrifuged. 1× PBS solution was added into the cell pellet to prepare monodispersed cell suspension (500 μL) for flow cytometry (LSR II, BD). The PE channel (excitation: 561 nm; emission: 567–597 nm) was used to detect the fluorescence signals from PCL-DPP-PCL NPs. Regular cells without NP treatment were used as negative control. Approximately 1 × 10⁴ cell events were recorded in each test and the data were analyzed by FlowJo software to obtain the mean fluorescence intensity (MFI) of cell populations. Then the MFI of each labeled group was normalized by the MFI of corresponding control groups, which was able to quantitatively indicate the change of fluorescence intensity before and after cell differentiations.

2.4. Cell Proliferation. The influence of PCL-DPP-PCL NPs on hMSC proliferation was evaluated using PrestoBlue assay. First, both labeled and unlabeled hMSCs were seeded on a 24-well plate at a density of 2000 cells/well and cultured at 37 °C with 5% CO₂ for 14 days. The medium was changed every 2–3 days. The absorbance was measured at day 3, 7, 10, and 14. Percentage (%) reduction that indicate the cell proliferation rate was calculated based on the formula provided by the manufacturer.

2.5. Predifferentiation Processing. The hMSCs of passage 3 or 4 were cultured in three T75 flasks. When cells became confluent after 3–4 days, PCL-DPP-PCL NPs (0.2 mg/mL) were added to the culture medium. Regular cells without NP treatment were used as control. After further incubation for 3 and 7 days, the excess NPs were removed by washing with PBS for three times before the cells were trypsinized by 1 × TrypLE Express Enzyme. The cell suspension in DMEM medium from each group was placed on 4-well plates for osteogenic and adipogenic differentiations respectively, while other cells were seeded in sterile conical tubes for chondrogenic differentiation. The DMEM medium was replaced by differentiation induction medium after 1 day.

2.6. MSC Differentiations. 6.0 × 10⁴ and 6.0 × 10³ hMSCs were seeded on 1.9 cm² Nunc 4-well plates (Thermo Fisher Scientific, Singapore) in expansion medium for adipogenic and osteogenic differentiations, respectively. Cells were first incubated overnight for effective attachment. Then the expansion medium was replaced by differentiation medium on the following day for induction of

Table 1. Primer Sequences for Specific Genes Used in RT-PCR

gene	forward primer sequence (5' – 3')	reverse primer sequence (5' – 3')
GAPDH	ACAACCTTGGTATCGTGAAGG	GCCATCACGCCACAGTTTC
PPAR- α	TACTGTCGGTTTCAGAAATGCC	GTCAGCGGACTCTGGATTGAG
leptin	TGCCTTCCAGAAACGTGATCC	CTCTGTGGAGTAGCCTGAAGC
OCN	CGCCTGGGTCTCTTCACTAC	CTCACACTCCTCGCCCTATT
ALP	ACCACCACGAGAGTGAACCA	CGTTGTCTGAGTACCAGTCCC
Col1	CAGAACGGCCTCAGGTACCA	CAGATCACGTCATCGCACAAC
Col2	CCAGATGACCTTCCTACGCC	TTCAGGGCAGTGTACGTGAAC

adipogenic or osteogenic differentiations. The adipogenic medium comprised high glucose DMEM (without L-glutamine) supplemented with 10% FBS, 1 mM sodium pyruvate (Life Technologies, Singapore), 100 units/mL penicillin-streptomycin, 0.1 μ M dexamethasone (Sigma-Aldrich, Singapore), 10 μ g/mL insulin (Life Technologies, Singapore), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich, Singapore), and 200 μ M indomethacin (Sigma-Aldrich, Singapore). The osteogenic medium included low glucose DMEM, 10% FBS, 1 $\times$ Glutamax (Life Technologies, Singapore), 100 units/mL penicillin-streptomycin, 1 mM sodium pyruvate, 50 μ g/mL ascorbic acid (Sigma-Aldrich, Singapore), 10 mM β -glycerophosphate (Sigma-Aldrich, Singapore), and 0.1 μ M dexamethasone. The cells were cultured under 37 $^{\circ}$ C under 5% CO₂ humidified conditions with the differentiation medium refreshed every 2–3 days. After adipogenic and osteogenic differentiation, the cells were washed twice by PBS and then trypsinized by 1 $\times$ TrypLE Express Enzyme. Then the cells were mixed with DMEM medium and spun down. The cell pellets were resuspended in 500 μ L of PBS for flow cytometry.

Induction of hMSC chondrogenic differentiation was performed in a three-dimensional culture condition. 2.0 $\times$ 10⁵ hMSCs were transferred to a 15 mL conical tube and then centrifuged to form a cell pellet, which was cultured in expansion medium at 37 $^{\circ}$ C and 5% CO₂ humidified conditions to allow pellet stabilization. The expansion medium was replaced by chondrogenic medium on the second day. The chondrogenic medium consisted of high glucose DMEM, 1 $\times$ Glutamax, 100 units/mL penicillin-streptomycin, 1 mM sodium pyruvate, 50 μ g/mL ascorbic acid, 0.1 μ M dexamethasone, 4 mM proline (Life Technologies, Singapore), and ITS (BioLab, Singapore) was supplemented with 10 ng/mL transforming growth factor β 3 (TGF- β 3) (R&D Systems, Singapore). The medium was changed every 2–3 days. The chondrogenic pellets after differentiation were first homogenized in collagenase type I for 2 h at 37 $^{\circ}$ C with 5% CO₂ and then spun down. Then 1 $\times$ TrypLE Express Enzyme was added to the pellet for several minutes and cells were centrifuged after adding DMEM medium. Finally, the cells were resuspended in PBS for flow cytometry.

2.7. Histological Analysis. The adipogenic and osteogenic differentiation samples were collected at day 21, while the chondrogenic pellets were harvested at day 28. The samples were washed with 1 $\times$ PBS before being fixed overnight with 10% formalin (Sigma-Aldrich, Singapore). The fixed samples were rinsed with 1 $\times$ PBS prior to histological staining.

The adipogenic lineage was stained with Oil Red to identify intracellular oil droplet accumulation that can be found in mature adipocytes.²³ The staining solution was prepared by adding 4 mL distilled water to 6 mL of 0.5% Oil Red O stock solution. After 15 min incubation, the samples were washed with 70% ethanol and distilled water to remove the excess staining solution before being counterstained with hematoxylin (Sigma-Aldrich, Singapore) for 30 s and rinsed with distilled water. For osteogenic lineage, Alizarin Red S solution (Sigma-Aldrich, Singapore) was used to stain the cells for 5 min, followed by gentle agitation with distilled water to remove nonspecific staining. The cells subjected to osteogenic differentiation presented brick-red color due to calcium deposition. The chondrogenic pellets were embedded in FSC 22 Mounting Media (Leica, Singapore) and frozen at –20 $^{\circ}$ C for about 2 h. The tissue sections of 5 μ m thick were sliced using a Leica CM1900 cryostat (Leica

Microsystems, Singapore) and were collected using Mezel-Glaser Superfrost Plus slides (Thermo Fisher Scientific, Singapore).

The collagen type II expression was analyzed by immunohistochemistry staining using UltraVision Quanto Detection System HRP DAB kit (Thermo Fisher Scientific, Singapore). The samples on the slides were first incubated with digestive enzyme containing 0.5% pepsin (Sigma-Aldrich, Singapore) for 20 min at 37 $^{\circ}$ C in humid condition, followed by treatment of hydrogen peroxide for 10 min and Ultra V Block reagent (Thermo Fisher Scientific, Singapore) for 5 min to reduce nonspecific background staining. The samples were then incubated overnight with mouse monoclonal anti-Col2 antibody (Merck Millipore, Singapore) with a dilution factor of 1:500 at 4 $^{\circ}$ C. Following the staining protocol of UltraVision Quanto Detection System HRP DAB kit, samples were incubated in Primary Antibody Amplifier Quanto for 10 min, HRP Polymer Quanto for 10 min with minimum or no light exposure, and finally a mixture of 30 μ L of DAB Quanto Chromogen and 1 mL of DAB Quanto Substrate until color development was evident (approximately 5 min). The chondrogenic sections were counterstained with hematoxylin (Sigma-Aldrich, Singapore). The coverslips were dehydrated after staining.

2.8. Gene Expression. Adipogenic and osteogenic samples were harvested at day 14, while chondrogenic pellets were only collected at day 21. Prior to RNA isolation, chondrogenic pellets were incubated in 0.25% collagenase type I for 2 h at 37 $^{\circ}$ C. The RNA was extracted using RNAeasy mini kit (Qiagen, Singapore) and quantified using NanoDrop 2000 UV–vis Spectrophotometer (Thermo Fisher Scientific, Singapore). RNA was reverse transcribed into cDNA with 5 $\times$ iScript Reverse Transcription Supermix (Biorad Laboratories, Singapore). Real-time Polymerase Chain Reactions (RT-PCRs) were performed with SYBR Green PCR Master Mix kit (Life Technologies, Singapore) using StepOnePlus Real-Time PCR System (Life Technologies, Singapore) to quantify the specific gene expression of mRNA in the samples. Samples were first denatured at 95 $^{\circ}$ C for 10 min, followed with a 40-cycle amplification consisting of 95 $^{\circ}$ C denaturation step for 15 s and 60 $^{\circ}$ C extension step for 1 min. Primers used to amplify cDNA of adipogenic cDNA includes GAPDH, PPAR- α , and leptin. Meanwhile, GAPDH, Col1, OCN, and ALP primers were used for osteogenic cDNA, and GAPDH, Col1, and Col2 primers were used for chondrogenic cDNA. The primer sequences are listed in Table 1. The expression levels of the mRNA of target genes were normalized according to the reference gene GAPDH, and then expressed as fold change with reference to their respective levels before induction of differentiation (day 0), which is calculated by the widely used comparative C_T method using the 2^{– $\Delta\Delta$ C_T} formula.²⁴

2.9. Confocal Laser Scanning Microscopy. The adipogenic and osteogenic lineages (day 21) were washed twice by PBS, fixed by formalin and then permeabilized by 0.1% Triton-X. Then the samples were blocked by 1% BSA and stained with Alexa Fluor 633 phalloidin for F-actin and DAPI for nucleus. We used three laser channels (405, 561, and 633 nm) in a confocal microscope (Carl Zeiss, LSM 710) to excite the fluorophores of DAPI, PCL-DPP-PCL NPs, and Alexa Fluor 633 phalloidin. Their fluorescence emission signals were detected in three individual channels (410–549, 566–622, and 638–747 nm).

For chondrogenic lineage, the cell pellets were washed with PBS twice and fixed with formalin. The fluorescence of the labeled samples were observed by confocal microscope (excitation, 561 nm; emission, 566–622 nm). We applied z-stack function to image different layers of

Figure 1. (A) Cellular uptake of NPs in hMSCs imaged by confocal laser scanning microscopy. Cell nuclei were counterstained with DAPI (blue), F-actin were stained by Alexa Fluor 633 phalloidin (red) and the fluorescence of NPs were colored as yellow. Scale bar: 100 μ m. (B) Quantitative analysis of cellular uptake of NPs by flow cytometry. Normalized MFI: 1143.5 for NP-LT, 691.1 for NP-ST. (C) Cell proliferation measured by PrestoBlue assays. Data presented as mean $\pm$ SD * p -value < 0.05, ** p -value < 0.01. n = 3.

the pellet. Additionally, the cryosection slides of pellets were also imaged with confocal microscopy.

2.10. Statistical Analysis. All experiments were performed at least thrice. The data were analyzed following Student's t test to determine the statistical significance between two experimental groups using One-Way ANOVA and Tukey HSD test. The difference between data were considered statistically significant if the p -value was less than 0.05.

3. RESULTS AND DISCUSSION

For abbreviation, the PCL-DPP-PCL NPs are simply referred to as NPs henceforth. The brief workflow of this study is presented in Figure S1. First, the original hMSCs were incubated with NPs for 3 days (short-term, "ST") and 7 days (long-term, "LT") with blank cells as control. Then, these labeled cells were induced into adipogenic, chondrogenic and osteogenic differentiation. To study the influence of NPs on hMSC proliferation and multipotency, we divided the MSCs

into 3 major experimental groups, i.e., unlabeled control, short-term staining for 3 days (NP-ST) and long-term staining for 7 days (NP-LT).

3.1. Cell Labeling with NPs. We first investigated the cellular uptake of NPs. The confocal fluorescence microscopy in both individual channels and ortho-views (Figure 1A and Figure S2) indicated that NPs were internalized into the cytoplasm of hMSCs, which was consistent to the finding in the previous work.²¹ Additionally, prolonged incubation time from 3 days to 7 days for cell staining enhanced the cellular intake of NPs with increased fluorescence intensity (Figure 1A), which was further verified by flow cytometry showing the peak intensity shift (Figure 1B). The staining process did not show any considerable effect on the cell viability compared with the unlabeled control (Figure S3), indicating that the NPs had minimum cytotoxicity to the hMSCs. For long-term tracking of cell differentiation, the NP labeling is desired to have minimum

Figure 2. MSC differentiation in adipogenic lineage. (A) Confocal fluorescence images of the differentiated adipocytes (day 21). Scale bar: 100 μm . (B) The retained fluorescence due to NP staining in the differentiated adipocytes (day 21) analyzed by flow cytometry. Normalized MFI: 394.7 for NP-LT, 208.8 for NP-ST. (C) The mRNA expression of leptin and PPAR- γ (day 14) quantified by RT-PCR assay. The results were normalized to the data on day 0 (before differentiation). * p -value < 0.05, ** p -value < 0.01. Data presented as mean $\pm$ SD n = 4 for leptin and n = 3 for PPAR- γ . (D) Oil Red staining for oil droplets in the differentiated adipocytes (day 21). Scale bar: 250 μm .

effect on cell proliferation to maintain the multipotency of hMSCs. We used PrestoBlue assay to measure the reduction ability of live cells for up to 2 weeks (Figure 1C), which indicated the cell proliferation rate. The results revealed that the short-term labeled group (NP-ST) and long-term labeled group (NP-LT) did not show any inhibition effect on hMSC proliferation as compared to the unlabeled control. Interestingly, the nanoparticle labeling rather enhanced cell proliferation at day 7 and day 14, whereas the underlying mechanism is still not clear and needs further investigation.

3.2. Multilineage Differentiations of Labeled hMSCs.

Figure 2 showed the long-term effect of NP labeling on hMSC-derived adipogenic differentiation after 3 weeks. The confocal microscopy indicated that differentiated adipocytes retained strong fluorescence due to NP staining (yellow, Figure 2A) for both NP-ST and NP-LT groups. The observation was further quantified by flow cytometry analysis, which also showed relatively stronger fluorescence of NP-LT than NP-ST (Figure 2B). Although the normalized MFI decreased considerably after 3 weeks of adipogenic differentiation, the NP-labeled adipocytes still exhibited high fluorescence intensity ratio

Figure 3. MSC differentiation in chondrogenic lineage. (A) The cryosections of the hMSC-derived tissue pellets (day 28). Scale bar: 100 μ m. (B) Confocal fluorescence images of the original pellets (day 28). Scale bar: 200 μ m. (C) Flow cytometry analysis of the trypsinized chondrocytes (day 28). Normalized MFI: 80.4 for NP-ST. (D–F) The mRNA expression of Col1, Col2, and aggrecan (day 21) quantified by RT-PCR assay. Data were normalized according to the results at day 0 (before differentiation). Data presented as mean $\pm$ SD $n = 5$ for Col1, Col2, and Col2/Col1, $n = 3$ for AGG. (G) Immunohistochemistry staining of Col2 in the chondrogenic pellets (day 28). Scale bar: 250 μ m.

(394.7 for NP-LT, 208.8 for NP-ST) compared to unlabeled controls indicating the strong photostability of the labeling NPs. The variation of cell morphology and fluorescence of NP labeling at intermediate stages (day 6, 13, and 20) in the adipogenic differentiation were shown in Figure S4. Figure 2D displayed the accumulation of intracellular lipids in the differentiated adipocytes by histological staining, providing the qualitative evidence of successful adipogenesis in the NP-labeled cells. Image analysis of the positive area of Oil Red staining indicated similar levels of lipid formation in the NP-labeled groups as compared with the unlabeled control (Figure S5). To evaluate the adipogenic differentiation quantitatively, we measured the gene expression of two important adipose biomarkers including leptin and peroxisome proliferator-activated receptor γ (PPAR- γ), which regulate fatty acid storage and glucose metabolism. The RT-PCR assay (Figure 2C) revealed that the time of NP staining before induction of adipogenic differentiation influenced the adipose gene expression. Specifically, the NP-ST group exhibited a similar level of leptin and an upregulation of PPAR- γ as compared to

the unlabeled control. In contrast, the gene expressions of leptin and PPAR- γ in the NP-LT group were both down-regulated significantly. These results implied that prolonged NP staining and higher concentrations of internalized NPs could interfere with the hMSC-derived adipogenic differentiation, for which the underlying mechanism of cellular transduction was still unclear and required further investigation. Nevertheless, we have demonstrated that short-term NP staining for 3 days (NP-ST) could provide strong fluorescence labeling without compromise of adipogenesis for long-term tracking of hMSC differentiation. Therefore, we applied short-term NP staining in the following chondrogenic and osteogenic differentiations.

The chondrogenic lineage was analyzed after 3–4 weeks of induced differentiation as shown in Figure 3. We observed strong fluorescence within the formed cartilage tissue pellet labeled with NPs (Figure 3B) in addition to its cryosection (Figure 3A). We further homogenized the tissue pellet, digested the matrix proteins, and obtained the suspension of chondrocytes for flow cytometry, which showed that the individual hMSC-derived chondrocytes retained strong fluo-

Figure 4. MSC differentiation in osteogenic lineage. (A) Confocal fluorescence images of the differentiated osteocytes (day 21). Scale bar: 100 μm . (B) Alizarin Red histological staining for calcium deposition after osteogenic differentiation (day 21). Scale bar: 250 μm . (C) The mRNA expression of Col1, OCN and ALP (day 14) quantified by RT-PCR assay. Data were normalized according to the results at day 0 (before differentiation). * p -value < 0.05, ** p -value < 0.01. Data presented as mean $\pm$ SD n = 4. (D) Flow cytometry analysis of the hMSC-derived osteocytes (day 21). Normalized MFI: 94.6 for NP-ST.

rescence of the NP labels even after 4 weeks of chondrogenesis (Figure 3C). Three important chondrogenic markers, including aggrecan (AGG), collagen type I (Col1) and collagen type II (Col2), were analyzed using RT-PCR assay for their gene expressions in the chondrogenic pellets at day 21. The gene expressions of Col1, Col2, and AGG in the labeled chondrogenic pellets were similar to those in the unlabeled control (Figure 3D, E, no significant difference statistically, p -value > 0.05). As the most abundant collagen in native cartilage tissues,²⁵ Col2 exhibited very high level of expression (Figure 3E) that led to Col2/Col1 ratio above 1500 (Figure 3F) in both labeled and unlabeled groups, which was a characteristic feature of hyaline-like cartilage tissue formation.^{25,26} Immunohistochemistry staining further demonstrated the similar density of Col2 in labeled and control tissue pellets after 4 weeks of chondrogenesis (Figure 3G). Therefore, the NP-labeling was also proved to be amicable for long-term tracking of chondrogenic differentiation of hMSCs.

The last differentiated group was the osteogenic lineage. Confocal fluorescence microscopy after 3 weeks of osteogenic differentiation (day 21) indicated strong fluorescence in the hMSC-derived osteocytes (Figure 4A). However, the NP-labeled osteocytes exhibited much lower intensity of Alizarin Red staining as compared to the unlabeled control (Figure 4B), suggesting that the presence of NPs suppressed the calcium deposition considerably during osteogenesis. We also quantified the gene expressions of three important osteogenic markers,

including Col1, osteocalcin (OCN) and alkaline phosphatase (ALP), using RT-PCR assay at day 14 after induction of differentiation. The RT-PCR data revealed significant down-regulation of all three osteogenic gene markers in the NP-labeled group (Figure 4C). These results implied that the NP labeling, even by short-term staining of 3 days, had inhibited the osteogenic differentiation of hMSCs. It was previously reported that TiO₂ nanoparticles exhibited adverse effects on the viability, proliferation and osteogenic differentiation of MSCs by influencing the gene expressions of ALP, OCN, and osteopontin (OPN) in a size-dependent and dose-dependent manner.²⁷ In the present study, on the other hand, we showed that the synthetic polymer-based NPs with staining concentration of 0.2 mg/ μL did not affect the cell viability and proliferation, whereas they compromised the osteogenic differentiation of MSCs.

3.3. MSC Labeling in 3D Scaffold. All above results were based on MSC labeling for conventional two-dimensional (2D) cell cultures or pellet culture. In the recent advances of tissue engineering, three-dimensional (3D) scaffolds have become more popular because of many advantages in better mimicking the native cellular environment, which consists of a 3D network with a complex extracellular environment and a highly porous nanotopography.²⁸ Cells cultured in 3D scaffolds tend to behave more like their in vivo counterparts, which may work as a better model for tissue engineering and other applications such as drug discovery and cellular signaling.²⁹ The special

Figure 5. Confocal fluorescence images of hMSCs seeded in a 3D scaffold (day 14). 3D images were reconstructed using ZEN 2012 software by taking different z-stack images. Scale bar: 100 μm .

mechanical properties of these 3D scaffolds also provide supports to the cells and newly formed tissues, which is important for weight-bearing tissues such as cartilage and bone.^{22,30} As a simple proof-of-concept study, we seeded the hMSCs on a poly(L-lactide-co-epsilon-caprolactone) (PLCL) porous scaffold (Figure S6) and cultured the cells for 14 days. The 2D and 3D images in Figure 5 obtained by confocal fluorescence microscopy demonstrated the distribution of NP-labeled MSCs scattered in the scaffold. The cell nuclei stained with DAPI was indicated as blue dots in the magnified inset of Figure 5. It was, however, difficult to distinguish the nuclei from the strong background fluorescence under excitation of 405 nm due to the autofluorescence of PLCL scaffold.³¹ Meanwhile, the bright fluorescent NPs clearly indicated the distribution of labeled hMSCs in the scaffold under excitation of 561 nm. This simple test showed that these NPs could also be used as an excellent contrast agent for cell tracking in the 3D scaffold environment, providing important information on the location, distribution, and migration of the labeled cells.

4. CONCLUSIONS

In summary, PCL-DPP-PCL nanoparticles are able to label hMSCs by internalization without affecting the cell viability and proliferation behaviors. It was found that PCL-DPP-PCL NPs labeling for 3 days at concentration of 0.2 mg/ μL did not show any negative effect on MSC adipogenic and chondrogenic differentiations, whereas the osteogenic differentiation was compromised compared to the unlabeled control. Furthermore, the labeling NPs can maintain strong fluorescence even after 4 weeks, indicating these NPs can work as efficient fluorescent probes for long-term tracing during adipogenic and chondrogenic differentiations. The PCL-DPP-PCL nanoparticles do not require any additional bioconjugation or surface modification, while showed minimum cytotoxicity and negative effects on adipogenic and chondrogenic differentiations under appropriate concentrations. Moreover, the PCL polymer skeleton of this probe has proved to be biocompatible and biodegradable, and has been approved by US Food and Drug Administration (FDA) for specific use in human body, whereas the potential toxic fluorophore DPP composes only about 1.3% in weight fraction of this probe, thereby further reducing its potential cytotoxicity. By replacing DPP with other near-infrared dye in this system, this strategy could be extended for long-term stem cell tracking applications in vivo.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.5b12371.

Cytotoxicity assay, fabrication of porous scaffold, scheme, ortho-view of cell uptake confocal microscope images, cell viability data, fluorescent images during adipogenic differentiation process and SEM images of porous scaffolds (PDF)

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Notes

The authors declare no competing financial interest.

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