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Optimization of *in vitro* cell labeling methods for human umbilical cord-derived mesenchymal stem cells

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Abstract. – BACKGROUND AND OBJECTIVES: Human umbilical cord-derived mesenchymal stem cells (hUCMSCs) are a novel source of seed cells for cell therapy and tissue engineering. However, *in vitro* labeling methods for hUCMSCs need to be optimized for better detection of transplanted cells.

AIM OF THE STUDY: To identify the most stable and efficient method for labeling hUCMSCs *in vitro*.

MATERIALS AND METHODS: hUCMSCs were isolated using a modified enzymatic digestion procedure and cultured. hUCMSCs of passage three (P3) were then labeled with BrdU, PKH26, or lentivirus-GFP and passaged further. Cells from the first labeled passage (LP1), the fourth labeled passage (LP4) and later passages were observed using a fluorescence microscope. The differentiation potential of LP4 cells was assessed by induction with adipogenic and osteogenic medium. Flow cytometry was used to measure the percentage of labeled cells and the percentage of apoptotic or dead cells. The labeling efficiencies of the three hUCMSC-labeling methods were compared *in vitro*.

RESULTS: BrdU, PKH26, and lentivirus-GFP all labeled LP1 cells with high intensity and clarity. However, the BrdU labeling of the LP4 cells was vague and not localized to the cell nuclei; LP9 cells were not detected under a fluorescence microscope. There was also a significant decrease in the fluorescence intensity of PKH26-labeled LP4 cells, and LP11 cells were not detected under a fluorescence microscope. However, the fluorescence of LP4 cells labeled with lentivirus-GFP remained strong, and cells labeled with lentivirus-GFP were detected up to LP14 under a fluorescence microscope. Statistical analyses indicated that percentages of LP1 cells labeled with PKH26 and lentivirus-GFP were significantly higher than that of cells labeled with BrdU ($p < 0.05$), and that the LP4 cells were more efficiently labeled with lentivirus-GFP than with PKH26 or BrdU ($p < 0.05$). BrdU-, PKH26-, and lentivirus-GFP labeled LP4 cells were all differentiated to adipocytes or osteoblasts with adipogenic and osteogenic medium. No statistical significance ($p > 0.05$) was observed between the death rates of labeled and unlabeled cells.

CONCLUSIONS: Lentivirus-GFP is a valid method for long-term *in vitro* labeling, and it may be used as a long-term hUCMSC tracker following transplantation *in vivo*.

Key Words:
Lentivirus, Green fluorescent protein, 5-bromo-2-deoxyuridine, Fluorescent dye, PKH26, Mesenchymal stem cells, Cell labeling.

Introduction

Mesenchymal stem cells (MSCs) are a group of multipotent somatic stem cells that were first discovered in bone marrow. Umbilical cord-derived mesenchymal stem cells (UCMSCs) are an alternative stem cell source that offers several advantages over bone marrow MSCs. UCMSCs are abundant in supply, easily acquired, and have low antigenicity. In addition, use of UCMSCs may circumvent the ethical concerns associated with stem cell usage. Moreover, UCMSCs posses features similar to fetal cells and have been shown to enhance wound healing better than mature cells[1,2]. MSCs that are derived from umbilical tissue not only possess weak immunogenicity, but

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have also been shown to inhibit alloantigen immune responses. Furthermore, UCMSC may inhibit local inflammatory reactions by secreting anti-inflammatory cytokines[3]. Thus, UCMSCs are poised to become the new source of seed cells for cell therapy and tissue engineering.

Successful labeling and *in vivo* tracking methods for UCMSCs are very important tools for cell biology research. Currently, a variety of seed cell labeling and tracking methods exist, each with its own set of advantages and disadvantages. In the present study, we compareds three commonly used methods for labeling UCMSCs *in vitro*, including 5-bromo-2-deoxyuridine (BrdU), red fluorescent dye (PKH26) and lentivirus-green fluorescent protein (GFP), to discern an optimizsed long-term, stable cell labeling method that does not affect the differentiation and growth, or metabolism of human UCMSCs (hUCMSCs).

Materials and Methods

Main Reagents and Instruments

The following reagents and instruments were used in this study: BrdU (Sigma-Aldrich, St. Louis, MO, USA); mouse α-BrdUu antibody (Abcam, Cambridge, UK); goat α-mouse-FITC second antibody (Santa Cruz, CA, USA); PKH26 kit (Sigma-Aldrich); lentivirus-GFP vector (pGC FU-RNAi-NC-LV) (Genechem Co. Ltd., Shanghai, China); Dulbecco's modified eagles medium (DMEM) (Gibco, Carlsbad, CA, USA); fetal bovine serum (FBS) (Gibco); trypsin (Gibco); ethylenediaminetetraacetic acid (EDTA) (Gibco); Annexin-V kit (Sigma-Aldrich); an inverted phase contrast microscope (Leica, Solms, Germany); and FACSsort Flow Cytometer (BD Co. Ltd., Mill Creek, WA, USA).

Isolation, Culturing, and Identification of hUCMSCs

Human UCMSCs were isolated from the umbilical cords of normal full-term healthy newborns by enzyme digestion with 0.2% collagenase Type II for 16 to 20 h. The cells were then cultured. The hUCMSC morphology was evaluated using inverted phase contrast microscopy and hematoxylin and eosin (H&E) staining. Immunofluorescence staining was used to identify cell- surface markers. Adipogenic, and osteogenic induction were performed to verify the multi-lineage differentiation potential of the hUCMSCs. Detailed procedures of these experiments were reported in previous publications[4].

Labeling hUCMSCs with BrdU

When hUCMSCs of passage 3 (P3) reached 50% confluence, the culture medium was discarded, and the hUCMSCs were then cultured in a medium containing 10 μM BrdU for 48 hours. Cells from the first labeled passage (LP1) were collected when they reached 90% confluency, washed with phosphate-buffered saline (PBS), and fixed with 40 g/L polyoxymethylene for 30 min. They were then washed 3 more times with PBS and once with 10 g/L bovine serum albumin. Next, the cells were incubated with anti-BrdU antibodies in a wet box at 37°C for 4 hours. After being washed with PBS an additional 3 times, the cells were incubated with goat-anti-mouse FITC fluorescent antibodies in the dark for 1 hour. The cells were washed with PBS 3 final times, after which the labeling of the LP1 cells was observed under a fluorescent microscope.

Labeling hUCMSCs with PKH26

Human UCMSCs of passage 3 were collected and labeled with the red fluorescent dye, PKH26. The experimental procedures were conducted according to the protocol included in the dye kit. All procedures were performed at room temperature. The cells were trypsinized to form a single-cell suspension, and then, 2×10^7 cells were collected and rinsed once with serum-free DMEM. Cells were then mixed with 1 mL of dilution C and resuspended to ensure a complete separation. Sample materials were cautiously handled by researchers during all procedures to avoid shaking them. Prior to the fluorescent labeling of the cells, PKH26 dye at a concentration of 4×10^{-6} mol/L (contained in dilution C) was prepared in a conical tube. Then, 1 mL of 2× cells was added to the PKH26 and immediately mixed with 1 mL of 2× dye using a dropper. This solution was incubated for 2 min in a conical tube and was gently and regularly inverted to ensure thorough mixing. An equal volume of FBS was added to stop the reaction, and the resulting mixture was incubated for 1 min. The solution was diluted with an equal volume of serum-containing DMEM and centrifuged at 400×*g* for 10 min, and the resulting supernatant was removed. The precipitated cells were washed 3 times with an equal volume of serum-containing DMEM and resuspended at a density of 1×10^5 cells/mL of com-

plete cell medium. The resuspended cells were then cultured to confluency to obtain cell line LP1. The labeling of the LP1 cells was observed under a fluorescence microscope.

Labeling hUCMSCs with Lentivirus-GFP

Based on a multiplicity of infection (MOI) of 20, an appropriate amount of pGC FU-RNAi-NC-LV was used to infect hUCMSCs growing in log phase. At the same time, 5 μg/mL polybrene was added to the culture medium. The cells were then returned to an incubator set at 37°C with 5% CO_2 and saturated humidity. All cell media were replenished after 12 hours. When the cells reached 90% confluence the morphology and levels of GFP expression were observed. Some of the cells were further passaged and cultured to reach line LP4 and later passages so that the *in vitro* GFP expressions of those descendants could be observed.

Passaging of Labeled Cells

The cells treated with BrdU, PKH26, or lentivirus-GFP were cultured to 90% confluency to obtain the labeled LP1 cell lines. They were then digested with trypsin and passaged further to obtain LP4 lines and post-LP4 lines using regular methods. The labeling of LP4 lines and post-LP4 lines was observed under a fluorescence microscope.

Evaluation of Differentiation Potential of Labeled Cells

The differentiation potential of the LP4 lines, which were cultured using a modified enzyme digestion method, was assessed. The cells were cultured in a complete medium that contained either osteogenic (0.1 μM dexamethasone, 10 μM β-glycerophosphate, and 50 μM ascorbate-phosphate) or adipogenic (0.5 μM isobutyl-methylxanthine, 1μM dexamethasone, 10 μM insulin, and 200 μM indomethacin) stimuli. As a negative control, cells were cultured in a complete medium deprived of the differentiation factors. All regents were from Sigma-Aldrich (St. Louis, MO, USA). Two weeks later, osteogenic differentiation was assessed with von Kossa staining, and intracellular lipid accumulation was visualized using Oil-Red-O staining.

Determination of Cell Labeling and Cell Death Using Flow Cytometry

Cells labeled with BrdU, PKH26, or lentivirus-GFP were resuspended into a single- cell suspension and fixed with 20 g/L paraformaldehyde for 10 min. The percentage of cells labeled by PKH26 and lentivirus-GFP was directly detected by flow cytometry. The detection of BrdU-labeled cells required the addition of a mouse anti-BrdU antibody and a FITC-conjugated goat anti-mouse secondary antibody, as well as fixation, before the results could be read with the flow cytometer. Cell death was measured as a percentage using the annexin-V method.

Statistical Analysis

The results in this study are reported as the means ± the standard deviation (SD). The data were analyzed using SPSS10.0 statistical software (SPSS Inc., Chicago, IL, USA). A *t*-test was used, and a value of $p < 0.05$ was considered statistically significant.

Results

Morphological Characteristics of hUCMSCs

Human UCMSCs at P3 exhibited a typical fibroblast-like morphology and were evenly distributed (Figure 1). These cells expressed CD44 and CD90 but not CD31or CD45. In addition, they were able to differentiate into both bone and adipose tissues upon induction[4]. Throughout cultivation, the hUCMSCs maintained their fibroblast-like morphology and potent proliferative capacity.

Fluorescent Microscopic Observations of UCMSCs Labeled by Each Method

The LP1 cells labeled with BrdU displayed green fluorescence in their nuclei when viewed under the fluorescent microscope. However, when passaged to LP4, only the cytoplasm remained fluorescent. Green fluorescence was weekly expressed by cells passaged to LP8, not detected at all in LP9 cells. In the PKH26-labeled LP1 cells, the red fluorescent dye was evenly distributed across the cell membranes. Under an inverted fluorescent microscope, these cells displayed clear cellular profiles with red fluorescence. In the PKH26-labeled LP4 cells, the red fluorescence intensity was significantly reduced, and the cellular profiles were no longer clear. Red fluorescence was weekly expressed by cells passaged to LP10, not detected at all in LP11 cells. The green fluorescence in of the lentivirus-GFP-labeled cells was primarily dis-

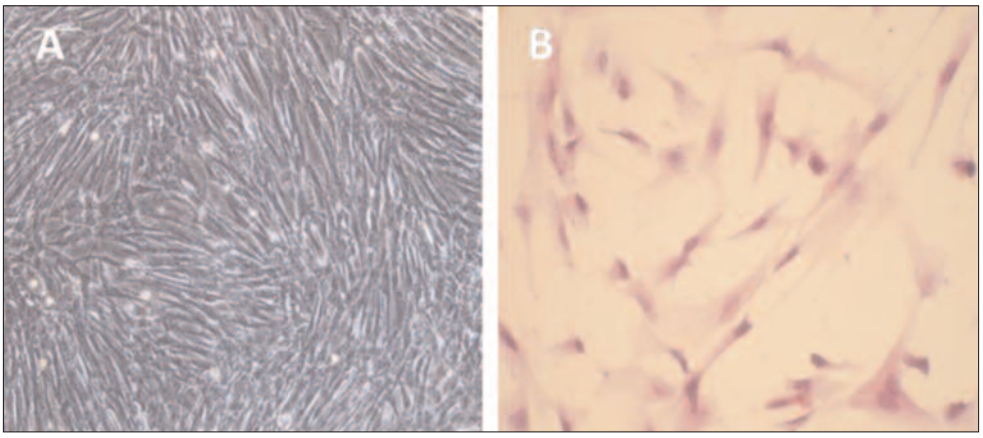

Figure 1. Morphological characteristics of hUCMSCs isolated using a modified enzymatic digestion method, then cultured. ***A,*** The morphology of hUCMSCs at passage three under an inverted phase contrast microscope. ***B,*** The morphology of hUCMSCs at passage three stained with H&E under a microscope. A: × 100, B: × 200. Abbreviation: hUCMSCs, Human umbilical cord-derived mesenchymal stem cells

tributed in the cytoplasm surrounding the nuclei; the green fluorescence was expressed with high intensity and clarity and in both the LP1 and LP4 cells. Green fluorescence was weekly expressed by cells passaged to LP13, and, not detected in LP14 cells (Figure 2).

Cell Labeling Efficiency of Each Method

The percentage of cells labeled by each of the three different methods was determined by flow cytometry, and is summarized in Table I. Statistical analyses indicated that the LP1 labeling efficiency of lentivirus-GFP was significantly higher than the efficiency of BrdU ($p < 0.05$). Similarly, the percentage of the descendant LP4 cells that remained successfully labeled by lentivirus-GFP was significantly higher when compared to PKH26 and BrdU ($p < 0.05$).

Differentiation of LP4 cells to Adipocytes and Osteocytes

At the end of the second week of culture, BrdU, PKH26, and lentivirus-GFP labeled LP4 cells were all positive for von Kossa staining following induction with osteogenic medium (Figure 3A, B, C). Similarly, at the end of the second week of culture, numerous Oil-Red-O-positive lipid droplets were observed in BrdU, PKH26, and lentivirus-GFP labeled LP4 cells supplemented with adipogenic medium (Figure 3D, E, F). Non-treated control cultures did not exhibit spontaneous adipocyte or osteoblast formation at the end of the second week.

Quantification of Cell Apoptosis Induced by Each Method

The amount of apoptosis induced by each method was determined by measuring the percentage of dead cells using flow cytometry, as summarized in Table II. No statistical difference was found between the apoptosis observed in unlabeled cells and that observed in cells labeled by any of the methods employed in this study ($p > 0.05$).

Discussion

Human UCMSCs have been recognizsed as an optimal substitute for bone marrow mesenchymal stem cells[5,6]. In the present study, these fibroblast-like cells were isolated from the umbilical cord tissue of newborns via a modified enzymatic digestion procedure and cultured[4]. The hUCMSCs readily adhered to the cell culture plates and highly expressed CD44 and CD90, characteristic MSC surface markers. However, neither CD31 nor CD45 were expressed by these cells. In addition, they hUCMSCs could be induced to differentiate into either bone or adipose tissue. Therefore, based on these characteristics, the fibroblast-like cells isolated from umbilical cord tissue were determined to meet the criteria used for identifying MSCs[7].

One of the primary problems encountered with the use of MSCs in tissue engineering and gene therapy has been the difficulty of labeling the cells after transplantation; such labeling would

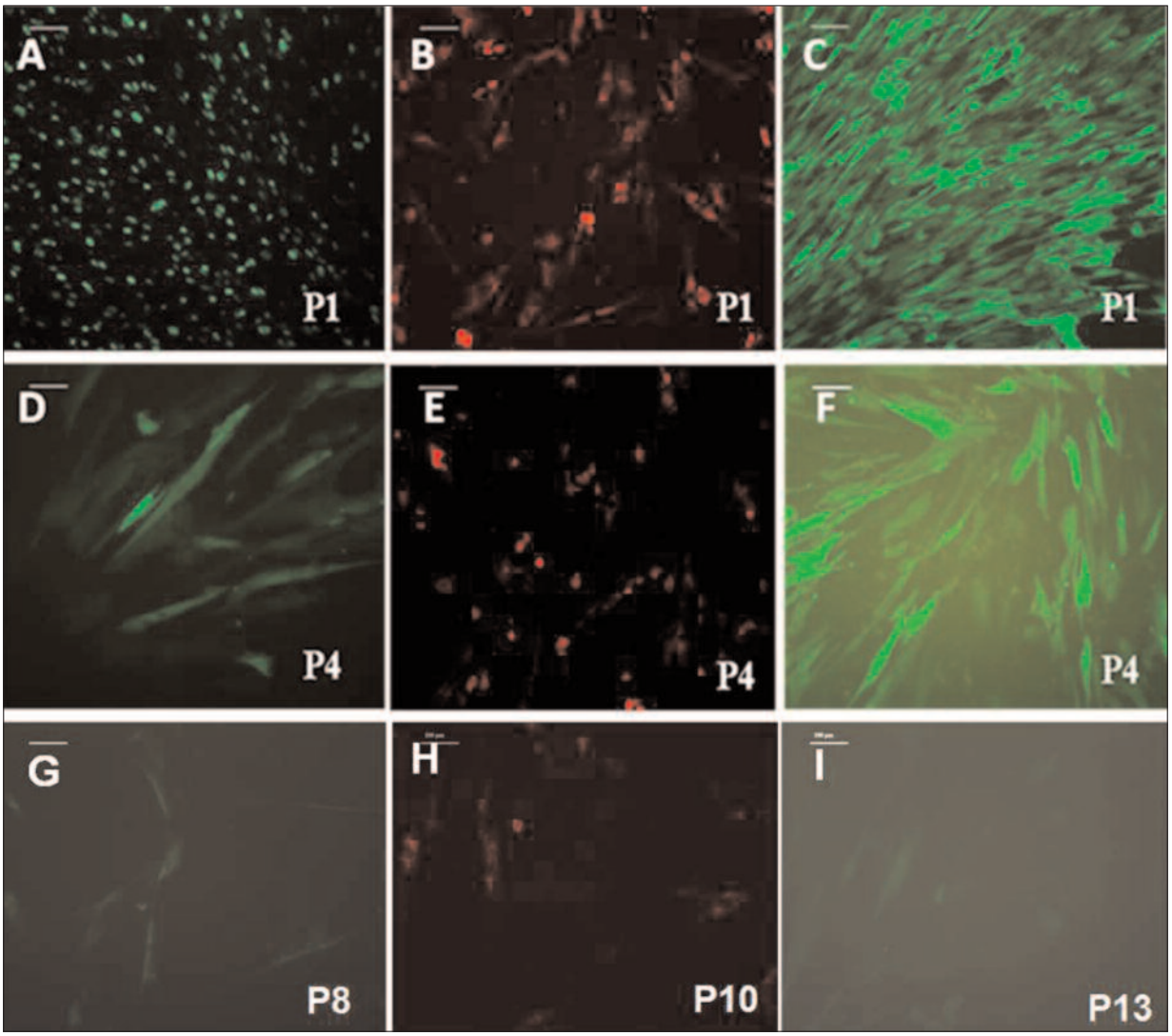

Figure 2. Fluorescent microscopic observations of hUCMSCs Labeled with each evaluated method. ***A,*** D, and G) LP1, LP4, and LP8 cells labeled with BrdU. ***B,*** E, and H) LP1, LP4, and LP10 cells labelled with PKH26. ***C,*** F, and I, LP1, LP4, and LP13 cells labeled with lentivirus-GFP. A-F: ×100. Abbreviation: hUCMSCs, Human umbilical cord-derived mesenchymal stem cells.

allow researchers to track the cells' survival, growth and differentiation[8-11]. Numerous methods for cell labeling have been established, including BrdU labeling, DAPI labeling, GFP labeling, Y chromosomal labeling, magnetic tagging, and several others. Nevertheless, a number of problems are associated with these methods. In DAPI-labeled cells, DAPI is often quenched during cell division, such that the compound can only track cells for a short period of time. Y chromosomal labeling requires expensive reagents[12], magnetic tagging has strict requirements for reagents and detection equipment[13]. Thus, it is necessary to find an effective, stable and practical cell labeling method for UCMSC tracking.

Table I. Comparison of cell labeling efficiency.

Labeling method	LP1 (%)	LP4 (%)
BrdU	78 ± 1.9*	50.2 ± 1.3*
PKH26	95.6 ± 0.5	64.3 ± 1.5*
Lentivirus-GFP	91.2 ± 2.1	83.5 ± 1.7

Data are expressed as mean ± SEM (n=6), *Denotes $p <$ 0.05 versus Lentivirus-GFP.

BrdU labeling is an easy, efficient and non-toxic cell labeling method. In the present study, 78% of LP1 cells were successfully labeled with BrdU. The percentage of apoptotic BrdU-labeled

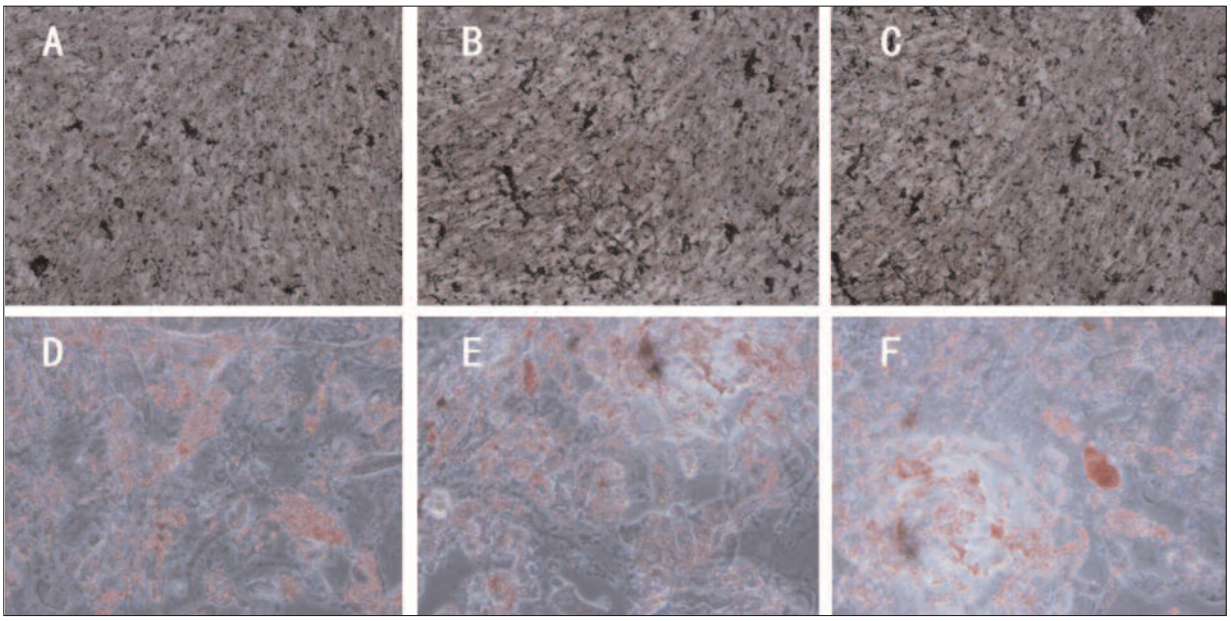

Figure 3. Multi-lineage differentiation potential of BrdU-, PKH26-, and lentivirus-GFP- labeled LP4 hUCMSCs. ***A, B,*** and ***C,*** Results of von Kossa staining in BrdU-labeled LP4, PKH26-labelled LP4, and lentivirus-GFP-labeled LP4 cell cultures grown for 2 weeks in osteogenic medium. Part of the hUCMSCs were positive for von Kossa staining. ***D, E,*** and ***F,*** Results of Oil-Red-O staining in BrdU-labeled LP4, PKH26-labeled LP4, and lentivirus-GFP-labeled LP4 cell cultures grown for 2 weeks in adipogenic medium. Part of the cells contained numerous Oil-Red-O-positive lipid droplets.

cells was not statistically different compared to that of the unlabeled group. In BrdU-labeled LP4 cells, the labeling efficiency was reduced to 50.2%. However, BrdU-labeled of LP9 and later passages were not detected. In additon, the BrdU-labeled LP4 cells differentiated to adipocytes and osteocytes in the presence of adipogenic and osteogenic medium, and no statistical significance in cell death was found between these labeled cells and unlabeled cells, suggesting that BrdU does not influence cell differentiation, growth, or metabolism. The percentage of labeled cells was significantly decreased after cell passaging, indicating that during cell mitosis, the intensity of the BrdU dye was reduced or the dye was lost. Therefore, BrdU is only appropriate for the short-term labeling of MSCs. Some previous studies have reported that BrdU labeling was both simple to use and highly accurate and efficient for initial cell labeling, although. After 1 to 2 weeks, however, the BrdU labels began to disperse and shift, and were difficult to measure[14]. Our results were consistent with those reported in the literature; after four passages, the nuclei of BrdU-labeled cells did not fluoresce after immunofluorescent staining. Rather, the fluorescence appeared distant from the nuclei and its intensity was markedly reduced, making it difficult to observe. Moreover, it has been shown that BrdU may be released from transplanted cells upon their death and may be absorbed by neighboring receptor cells, generating false positives[15].

Table II. Comparison of the death rates of labeled cells.

Labeling method	LP1 (%)	LP4 (%)
BrdU	3.5 ± 0.4*	4.3 ± 0.7*
PKH26	3.4 ± 0.3*	4.1 ± 0.5*
Lentivirus-GFP	3.7 ± 0.6*	4.4 ± 0.8*
No labeling	3.3 ± 0.5	3.9 ± 0.6

Data are expressed as mean ± SEM (n=6), *Denotes $p >$ 0.05 versus No labeling.

PKH26 is a red fluorescent dye that can be used to identify cultured cells and track their distribution within tissue *in vivo* under visible light. PKH26 generally does not influence cell growth or metabolism when used at appropriate concentrations[16,17], and it maintains a stable intensity with good repeatability. We labeled hUCMSCs with PKH26 and used a fluorescent microscope to observe that the dye was evenly distributed across the cell membranes. Analysis using flow cytometry showed that 95.6% of LP1 cells were

labeled with PKH26. When the PKH26-labeled cells were passaged to obtain LP4 cells, we observed a significant decrease in the fluorescence intensity, and only 64.3% of the cells remained labeled. PKH26-labeled cells of LP11 and later passages were not detected. The death rate of PKH26- LP4 cells that were induced into adipocytes and osteocytes with adipogenic and osteogenic medium, was not statistically different from that of the unlabeled group, suggesting that PKH26 does not influence cell differentiation, growth, or metabolism. These experimental results indicate that a gradual decrease in PKH26 labeling intensity and efficiency occurred as the cells underwent passaging and propagation, suggesting that PKH26 is also only suited for short-term labeling.

Gene transfer via a lentiviral vector is advantageous of its high efficiency and safety, as well as its long-term stability in the expression of the exogenous genes[18]. In the present study 91.2% of LP1 hUCMSCs infected with lentivirus-GFP, and the percentage of labeled cells remained as high as 83.5% at LP4. This percentage of labeled cells at LP4 is significantly higher than those of the same passage labeled with the BrdU or PKH26 ($p < 0.05$). Both the LP1 and LP4 cells displayed strong green fluorescence. however, lentivirus-GFP-labeled cells of LP14 and later passages were not detected. The lentivirus-GFP labeled cells of LP4 were induced into adipocytes and osteocytes with adipogenic and osteogenic medium, and the percentage of apoptotic cells was not statistically different than that of the unlabeled group, suggesting that lentivirus-GFP does not influence cell differentiation, growth, or metabolism. This study suggests that using lentivirus-GFP to label hUCMSCs provide a high infectivity and potent fluorescence intensity that would allow for stable and sustained expression of the label to aid in the detection of cells *in vitro*.

Conclusions

BrdU-labeled cells had a low labeling efficiency and were difficult to measure after passaging. While PKH26-labeled hUCMSCs were easy to observe, they displayed both a gradual decay of the red fluorescent dye and a significant reduction in labeling efficiency after a few generations. The use of lentivirus-GFP, however, resulted in high labeling efficiency, stable label expression, strong fluorescence intensity, and easy label observation, indicating lentivirus-GFP is a valid option for long-term labeling and tracking of hUCMSCs *in vitro*. This study establishes a basis for future studies to evaluate long-term *in vivo* hUCMSC labeling.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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