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A comparative study of PKH67, DiI, and BrdU labeling techniques for tracing rat mesenchymal stem cells

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Abstract Mesenchymal stem cells (MSCs) have generated a great deal of promise as a potential source of cells for cell-based therapies. Various labeling techniques have been developed to trace MSC survival, migration, and behavior in vitro or in vivo. In the present study, we labeled MSCs derived from rat bone marrow (rMSCs) with florescent membrane dyes PKH67 and DiI, and with nuclear labeling using 5 μM BrdU and 10 μM BrdU. The cells were then cultured for 6 d or passaged (1–3 passages). The viability of rMSCs, efficacy of fluorescent expression, and transfer of the dyes were assessed. Intense fluorescence in rMSCs was found immediately after membrane labeling (99.3±1.6% PKH67+ and 98.4±1.7% DiI+) or after 2 d when tracing of nuclei was applied (91.2±4.6% 10 μM BrdU+ and 77.6±4.6% 5 μM BrdU+), which remained high for 6 d. Viability of labeled cells was 91±3.8% PKH67+, 90±1.5% DiI+, 91±0.8% 5 μM BrdU+, and 76.9±0.9% 10 μM BrdU+. The number of labeled rMSCs gradually decreased during the passages, with almost no BrdU+ nuclei left at final passage 3. Direct cocultures of labeled rMSCs (PKH67+ or DiI+) with unlabeled rMSCs revealed almost no dye transfer from donor to unlabeled recipient cells. Our results confirm that labeling of rMSCs with PKH67 or DiI represents a non-toxic, highly stable, and efficient method suitable for steady tracing of cells, while BrdU tracing is more appropriate for temporary labeling due to decreasing signal over time.

Keywords Mesenchymal stem cells · PKH67 · DiI · BrdU · Cell labeling

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Introduction

Mesenchymal stem cells (MSCs) are adult multipotent cells with the potential to differentiate into various tissues (Pittenger et al. 1999). MSCs derived from bone marrow play a crucial role in supporting the physiological functions of hematopoiesis (Dazzi et al. 2006). However, these stem cells can also be generated from adipose tissue; cord blood; placenta; or organs such as the lung, heart, and kidney (Askenasy et al. 2007). MSCs are known for their immunosuppressive, anti-inflammatory, and neurotrophic properties, and this has initiated interest in their use for regenerative therapy of various degenerative or malignant diseases (Horwitz et al. 1999; Nasef et al. 2007). However, in the process of cell-based therapy evaluation, tracing the transplant inside the host tissue is considered essential and can be achieved by appropriate labeling of the MSCs with a variety of markers.

Many previous studies have used different labeling techniques, including membrane dyes (e.g., PKH, DiI, DiO, whose aliphatic portion binds to the cell membrane lipid bilayer), magnetic resonance imaging (MRI) labeling (nanoparticles Fe) (Sykova and Jendelova 2007), DNA dyes (5-bromo-2′deoxy-uridine, BrdU, DNA intercalating dye), or labeling via gene transfer (adenovirus-mediated expression of enhanced green fluorescence protein (EGFP) and β-galactosidase (LacZ)) (Cizkova et al. 2006; Leiker et al. 2008), to visualize MSCs after transplantation. Each of these methods represents a powerful tool for stem cell identification

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and phenotype characterization; however, some restrictions have to be taken into account, such as low efficacy of labeling or toxicity. The labeling should therefore not interfere with MSC attachment, proliferation, or their expression of growth factors, cytokines, or immunologic properties.

One of the first dyes used for cellular labeling was PKH67, which is commercially available in kit form together with PKH2 and PKH26, and is routinely used in various cell proliferation studies (Yamamura et al. 1995; Ladd et al. 1997; Pricop et al. 1997; Allsopp et al. 1998; Traycoff et al. 1998; Quade and Roth 1999). The PKH67-GL cell linker kit uses membrane labeling technology to stably incorporate a fluorescent dye with long aliphatic tails (PKH67) into lipid regions of the cell membrane without affecting cell growth (Horan and Slezak 1989). PKH67 has been found to be useful for in vitro cell labeling and cell proliferation studies (Boutonnat et al. 1998; Rousselle et al. 2001) as well as for in vivo cell tracking applications (Jenne et al. 2000; Askenasy and Farkas 2002; Cizkova et al. 2011).

Other widely used dyes are Vybrant™ DiI cell-labeling solutions (DiI). They are structurally related to PKH67 dye and have been developed and optimized for extensive applications (Quade and Roth 1999). The lipophilic carbocyanine dye DiI is weakly fluorescent in water but highly fluorescent and quite photostable when incorporated into lipid regions of the cell membrane. DiI solutions are mostly used as tracer in cell-cell fusion (Spotl et al. 1995; Blumenthal et al. 1996), cellular adhesion (Kreft et al. 1997; Malhotra et al. 1998), and migration (Kuriyama et al. 1998), and have high resistance to intercellular transfer (Gant et al. 1992).

PKH67 and DiI labeling procedure can be used for in vitro or ex vivo labeling of stem cells, lymphocytes, monocytes, endothelial cells, or any other type where partitioning of dye into lipid regions of the cell membrane is desired (Maus et al. 2001).

However, PKH67 dye labeling protocols require suspension of cells in an isoosmotic mannitol loading medium, while DiI can be added directly to normal culture media to uniformly label suspended or attached culture cells. When labeled cells divide, the resulting daughter cells receive half the label, reducing the fluorescence intensity to one half of the parent cells (Boutonnat et al. 1998). As a consequence, the proliferation of labeled cells leads to decrease in fluorescence.

In contrast to the membrane-bound dyes, nuclear dye BrdU is a synthetic thymidine analog that gets incorporated into a cell's DNA when the cell divides (during the S phase of the cell cycle). Antibodies against BrdU that are conjugated to fluorescent markers can be used to label these cells, thereby providing visual evidence of cell division (Cizkova et al. 2006). BrdU labeling has been used to track stem or non-stem cells that are labeled in vitro and subsequently transplanted in vivo (Gage et al. 1995; Kopen et al. 1999).

One of the main problems when using both PKH and DiI membrane dyes is their possible release into the extracellular space and phagocytosis by the vesicles of other cells. In this relation, previous analyses have confirmed that non-specific membrane labeling in naïve neighboring cells is most prominent when the labeled cells are dead (Li et al. 2013). Optimizing the labeling protocol in vitro, therefore, should limit the dye-induced toxicity and partially attenuate dye transfer in vivo.

The objective of the present study was to compare in vitro labeling procedures of rat MSCs (rMSCs) with PKH67, DiI, and BrdU, and to evaluate the following parameters: the viability of rMSCs, efficacy of fluorescence expression, and stability/transfer of the dyes, and correlate these with conditions of increasing passages of rMSCs under in vitro conditions.

Materials and methods

Isolation and culture of rMSCs. Bone marrow was isolated from the long bones (femur, tibia) of adult male Wistar rats (300 g, n=3) which were terminally anesthetized (thiopental, 50 mg/kg, i.p.). Whole bone marrow was flushed with ice-cold physiological saline solution, dissected into small pieces on ice, homogenized, and centrifuged at 400×g for 10 min. The obtained cell pellet (containing both hematopoietic cells and marrow mesenchymal cells) was resuspended; plated on a 75-cm^2 flask; cultured in 13 ml of culture medium (CM) containing Minimum Essential Medium (MEM) (Biowest, Nuaillé, France), 15% fetal bovine serum (FBS) (Biowest), and 1% penicillin-streptomycin (Biochrom AG, Berlin, DE); and incubated at 37°C in a humidified atmosphere with 5% CO_2. Non-adherent cells were removed after 48 h by changing the medium. Upon reaching 90% confluence, the cells were passaged using 0.05% trypsin-EDTA (Gibco; Invitrogen, Carlsbad, CA) and plated on culture flasks at a density of 0.7×10^6/75 cm^2. To evaluate the parameters of individual dyes (PKH67, DiI, and BrdU), we used the rMSCs from passage number 3, which formed a homogenous population of rMSCs.

Flow cytometry. To confirm the rMSC phenotypic characteristics at passage 3, the surface markers (CD29, CD90, CD45) were analyzed. For flow cytometry, the following antibodies were employed according to the supplier's recommendations: PE anti-mouse/rat CD29 (clone: HMβ1-1; BioLegend, San Diego, CA), PE anti-rat CD45 (clone: OX-1, BioLegend), and PE anti-rat CD90 (clone: OX-7, BioLegend) and their isotype controls: PE Armenian Hamster IgG (CD29) and PE Mouse IgG1 (CD45, CD90) from BioLegend. Samples were analyzed using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA) operated with CellQuest software, and at least 20,000 events were collected per sample. Data were analyzed using WinMDI software (version 2.8).

Membrane labeling of rMSCs with PKH67and DiI. The rMSCs from passage 3 were labeled with green fluorescent cell linker PKH67 according to the previously described

protocol (Wallace et al. 2008). Briefly, immediately prior to staining, PKH67 dye was prepared and added to 1 ml of resuspended rMSCs (2×10^6 cells). After incubation at 25°C for 20 min, the staining reaction was stopped by adding an equal volume of α-MEM with 1% FBS.

Similarly, the rMSCs were labeled with DiI according to the Vybrant™ DiI cell-labeling protocol (Molecular Probes, Invitrogen). Five hundred microliters of rMSC suspension (7×10^5 cells) with 5 μl of cell labeling solution DiI was mixed well by gentle pipetting and incubated for 20 min at 37°C followed by a 20-min incubation at room temperature and appropriate washing to remove residual dye.

Labeled rMSCs (PKH67+ and DiI+) were replanted into 4-well culture plates in CM ($n=8$) (seeding density=5×10^5 cells). After 1, 2, 3, and 6 d, PKH67+ and DiI+ cells ($n=4$) were fixed with 4% paraformaldehyde (PHA) and stained with nuclear 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen). Simultaneously, a second set of 4-well culture plates ($n=4$) with a confluent layer of PKH67+ and DiI+ cells was further passaged (passages 1–3), fixed with PHA, and stained with DAPI. Efficacy of staining with PKH67+ and DiI+ cells during days 1, 2, 3, and 6 or after passages 1–3 was evaluated by counting the number of positively labeled cells compared to the total number of DAPI-labeled nuclei using fluorescent microscopy (Nikon ECLIPSE Ti) within identical fields.

Nuclear labeling of rMSCs with BrdU and immunohistochemistry. The rMSCs at a density of 5×10^5 cells per well in 4-well plate were maintained in CM containing BrdU at a final concentration of 5 or 10 μmol/l (5 μM BrdU+ and 10 μM BrdU+) for 1, 2, and 3 d and incubated at 37°C, 5% CO_2. After the 3 d, 5 μM BrdU+ and 10 μM BrdU+ cells were incubated in BrdU-free CM for an additional 3 d. Afterwards, the cells were fixed with 4% PHA for 10 min and washed with 0.1 M phosphate-buffered saline (PBS). For DNA denaturation, 5 μM BrdU+ and 10 μM BrdU+ cells were pretreated with 2 N HCl for 20 min at room temperature, followed by incubation in Holmes Borat buffer 20 min, then washed with 0.1 M PBS and processed for routine immunohistochemistry technique (IHC). Briefly, rMSCs were blocked with 10% normal goat serum for 1 h at room temperature and then incubated overnight at 4°C with the primary antibody rat anti-BrdU (1:250, Sigma-Aldrich, Inc., St. Louis, MO). Next, cell cultures were washed with 0.1 M PBS and incubated with fluorescent secondary antibody anti-rat (Alexa Fluor 594, 1:500; Molecular Probes, Eugene, OR) and with DAPI-specific nuclear staining for 1 h at room temperature. Under an inverted Nikon ECLIPSE Ti fluorescence microscope, we examined the percentage of 5 μM BrdU+ and 10 μM BrdU+ cells calculated from the total number of DAPI-positive rMSCs. After 1 wk, 5 μM BrdU+ and 10 μM BrdU+ cells were passaged three times and labeling efficacy was evaluated as labeling efficacy after 1, 2, 3, and 6 d.

Viability of cells. Cell viability was evaluated by counting viable cells in a hemocytometer using the standard trypan blue exclusion test (0.4% trypan blue, Invitrogen). Cell suspension (10 μl) of labeled rMSCs was mixed with trypan blue (90 μl) (1:10) in a clean Eppendorf tube and incubated at room temperature for 1–2 min, and then, 10 μl of the cell suspension was added to the hemocytometer, which counted the viable and dead (blue) cells in 25 medium-sized squares. The total number of viable cells was calculated as equals the number of live cells×dilution factor×10^4×total volume (ml), and % viability equals the number of live cells:(number of live cells+number of dead cells)×100.

Dye transfer from labeled rMSCs. To analyze transfer of lipophilic membrane dyes between cells, we performed a coculture system experiment (Lassailly et al. 2010; Li et al. 2013). Labeled rMSCs with PKH67+ or DiI+ (2.5×10^5 cells per well in a 4-well plate) were cocultured with controls/unlabeled rMSCs at the ratio 1:1 in the CM for 3 d. Subsequently, cocultured cells were fixed with 4% PHA and stained with DAPI, and the percentage of labeled/unlabeled cells was analyzed. The ratio of fluorescent/unstained cells refers to the labeling stability or the spread to neighboring rMSCs in time.

Quantitative analysis. The number of positive-membrane and nuclear-labeled rMSCs was counted as the percentage of the total number of cells stained with DAPI in 10 random visual fields (600 μm×600 μm) of cells. Data are presented as mean ±SEM. Statistical differences between groups were evaluated with Student's t test and one-way analysis of variance (ANOVA). A value of $p<0.05$ was considered to be statistically significant.

Results

In the first part of our study, we examined the dye-induced toxicity of the membrane PKH67, DiI, and nuclear BrdU (10 μM BrdU and 5 μM BrdU) cell tracking techniques and the efficacy of fluorescence expression and labeling stability during the 6 d of rMSCs cultivation. In the second part, we evaluated the consequences of rMSC passaging (1–3) on the above-mentioned parameters.

The rMSCs at passage 3 under an inverted phase-contrast microscope showed uniform spindle-shaped morphology (Fig. 3*A*). The immunophenotype characteristics of the rMSCs were analyzed using flow cytometry. The rMSCs expressed non-hematopoietic surface markers, CD90=99% (Fig. 1*A*), CD29=99% (Fig. 1*B*), and hematopoietic marker CD45=4% (Fig. 1*C*).

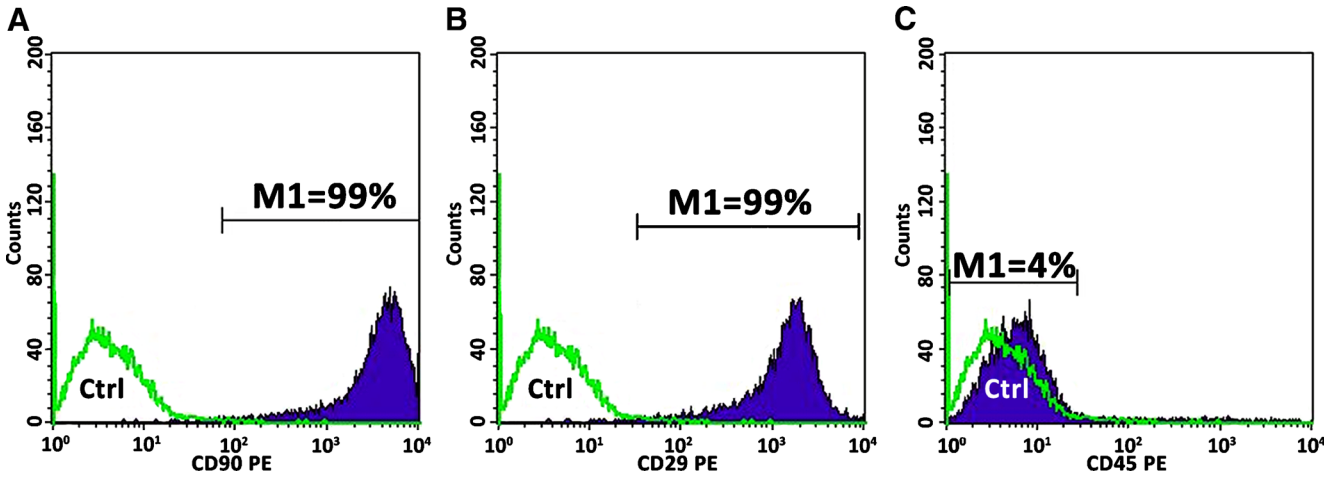

Figure 1. Representative flow cytometry analysis of cell surface markers CD90 (*A*), CD29 (*B*), and CD45 (*C*) in rMSCs at passage 3.

Viability of labeling procedures. Viability of PKH67+ and DiI+ cells did not show significant differences in comparison with the control after 6 d (91±3.8% PKH67+ and 90±1.5% DiI+) but reflected some changes after 1 d (DiI+ cells) and 3 d (PKH67+ cells) after labeling procedure (87.4±1.7% DiI+ and 88.8±1.2% PKH67+; $p<0.05$) (Fig. 2*A*). The higher concentration of BrdU (10 μM BrdU) significantly reduced the number of viable BrdU+ cells (85.7±1.8, 80.7±2.3, 88.9±1.7, and 76.9±0.9% after 1, 2, 3, and 6 d, respectively) when compared to the control ($p<0.01$, $p<0.001$) (Fig. 2*B*). There were no significant differences in viability of 5 μM BrdU+ cells during the whole time of incubation.

Efficacy of labeling procedures: membrane vs. nuclear dyes. When cells were stained with membrane markers (PKH67 or DiI), a bright signal could be visualized directly with the fluorescent microscope. Typical punctate PKH67 (green) or homogeneously dispersed DiI (red) fluorescence staining throughout the whole plasma membrane of cells could be detected throughout the 6 d (Fig. 3*B*, *C*). However, the area of nuclei showed almost no fluorescence staining. The labeling efficacy was high for both PKH67 and DiI fluorophores for 1, 2, and 3 d, but the percentage of labeling efficacy of DiI+ cells in comparison with PKH67+ cells significantly decreased on the sixth day of incubation (85.7±6.6% DiI+ vs. 93.2±4.3% PKH67+; $p<0.01$) (Fig. 4*A*).

In contrast to direct detection of membrane dyes, incorporation of BrdU in rMSCs was evaluated using routine IHC. The nuclei of rMSCs labeled with BrdU showed specific diffuse fluorescence that colocalized with DAPI counterstaining. However, differences in fluorescence intensities were observed among the nuclei after 6 d of in vitro culture. Intensely, moderately, or lightly BrdU-stained nuclei were observed at each time point, after 1, 2, 3, and 6 d (Fig. 3*D*). The labeling rate was evaluated based on the ratio of rMSCs double-stained with BrdU and DAPI and cells single-stained with DAPI. Here, we took into account all labeled BrdU nuclei independently of the fluorescence intensities. The highest labeling efficacy of 10 μM BrdU+ cells was observed after 2 d (91.2±4.6% 10 μM BrdU+) and of 5 μM BrdU+ cells after 3 d (88.7±3.1% 5 μM BrdU+), with the effective fluorescence pattern retained during the monitored time (Fig. 4*B*). For the first 2 d, there were significant differences between 5 μM BrdU and 10 μM BrdU labeling efficacy ($p<0.01$).

Stability of labeling procedures. In our next experiments, we analyzed whether fluorescence dyes incorporated in a cell membrane can spread to neighboring cells. Our results

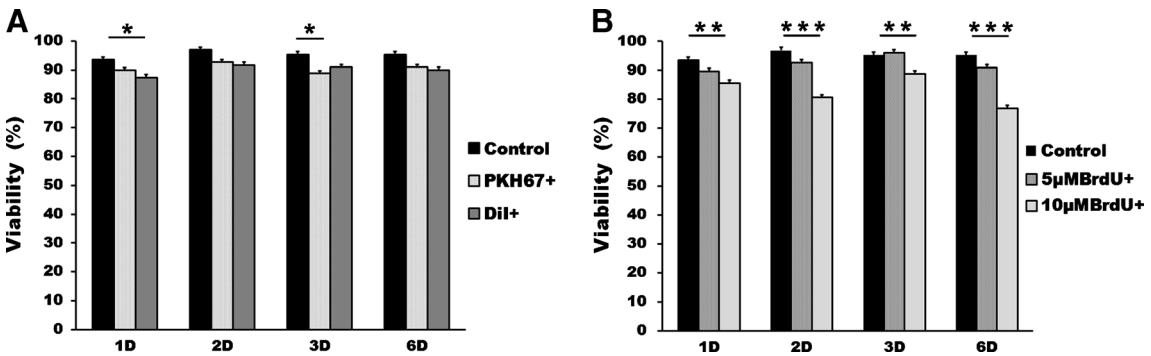

Figure 2. Viability of the labeled rMSCs. Comparison between the viability of *A* PKH67+ and DiI+ cells and *B* 5 μM BrdU+ and 10 μM BrdU+ cells and unlabeled control cells after 1, 2, 3, and 6 d. Data are presented as mean±SEM (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

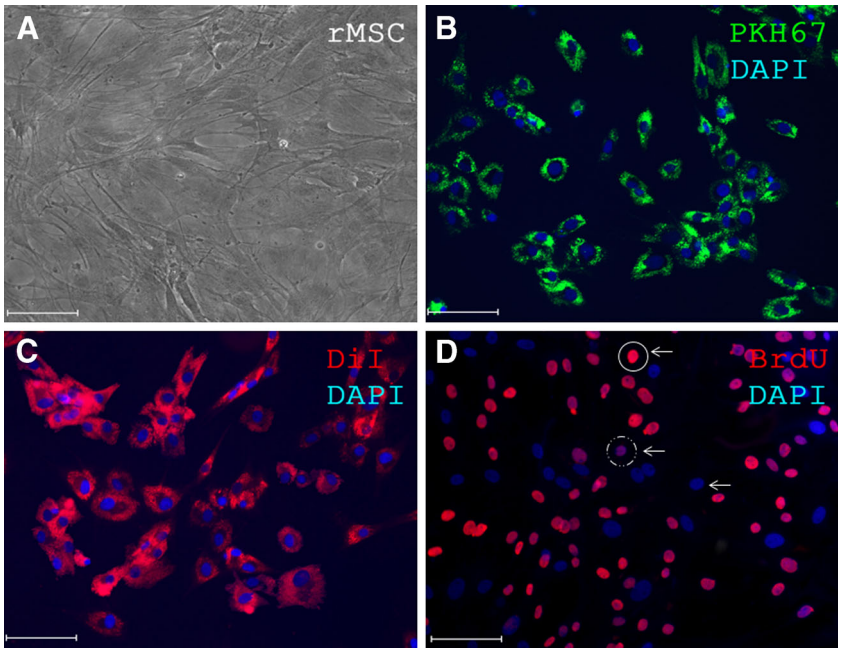

Figure 3. Morphology and labeling pattern of rMSCs cultured in vitro. (*A*) rMSCs maintained typical spindle shape after passage 3, light microscopy. Intense punctate fluorescence staining was uniformly distributed throughout the entire membrane of (*B*) PKH67+ (*green*) rMSCs and (*C*) DiI+ (*red*) rMSCs. (*D*) Image showing different fluorescent signal for nuclear labeling of rMSCs with 10 μM BrdU. *Note* intensely stained nuclei (*arrow pointing to full circle*), moderately stained nuclei (*arrow pointing to intermittent circle*), and unlabeled nuclei (*arrow pointing to DAPI-counterstained nuclei*, used to identify labeled and unlabeled cells). *Scale bar*=100 μm.

confirmed that the number of PKH67+ or DiI+ cells did not increase in the cocultured system containing unlabeled controls (PKH67− or DiI−) at the ratio 1:1. The labeling efficacy after 3 d in the cocultured system represented 46.1±7.6% PKH67+ and 51.4±15.1% DiI+ cells, which suggests good membrane dye stability for both dyes (Fig. 4*C*).

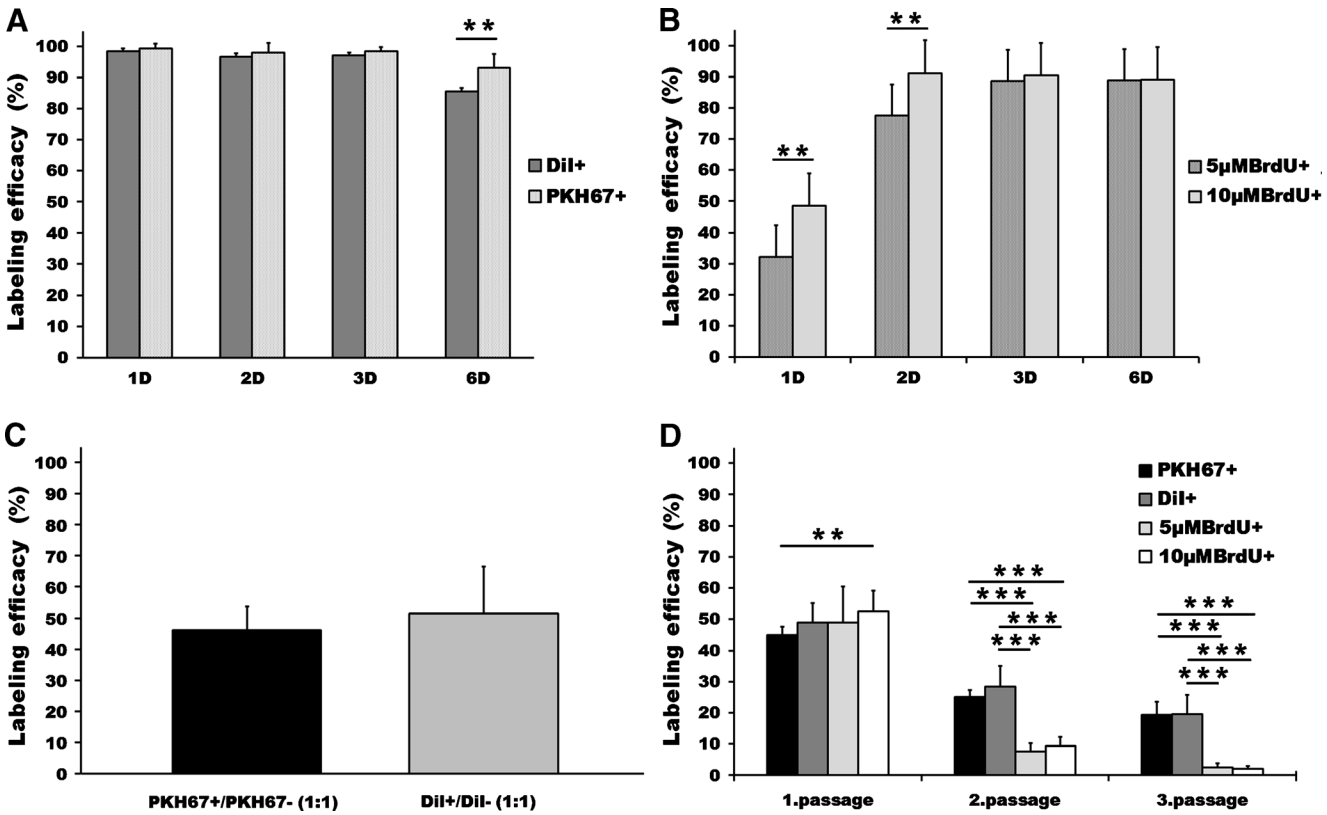

Figure 4. Comparison of the labeling efficacy of rMSCs marked with (*A*) DiI+ and PKH67+ and (*B*) 5 μM BrdU+ or 10 μM BrdU+ after 1, 2, 3, and 6 d. (*C*) Coculture of membrane-labeling cells with unlabeled rMSCs (DiI+/DiI−, PKH67+/PKH67−) (1:1) reveal good membrane dye stability. (*D*) Labeling efficacy of PKH67+ and DiI+ or 5 μM BrdU+ and 10 μM BrdU+ rMSCs significantly decreased after passages 1, 2, and 3. Data are presented as mean±SEM (**p<0.01; ***p<0.001).

Evaluation of cell division-dependent dye dilution. Our next aim was to investigate the impact of rMSC passaging on the fluorescence expression. In fact, here we confirmed the dilution of dye labeling due to rMSC division. Thus, the percentage of membrane-positive cells gradually decreased with the increased number of passages (PKH67+=45.4±2.7, 25.1±2.2, and 15. 3±4.1% and DiI+=48.9±4.9, 28.4±6.6, and 12.4±6.3%, passage 1, 2, 3, respectively). Similarly, in the case of BrdU labeling, the number of labeled nuclei after the first passage was halved (5 μM BrdU+=49±11.4% and 10 μM BrdU+=52.6±6.6%), which was followed by abrupt decrease at passage 2 (5 μM BrdU+=7.6±2.7% and 10 μM BrdU+=9.4±2.9%) and almost complete lack of fluorescence signal at passage 3 (5 μM BrdU+=2.6±1.3% and 10 μM BrdU+=2±1%) (Fig. 4*D*).

Discussion

Here, we have studied three types of gene transfer-free labeling dyes, two of them membrane-type and one nuclear. They were used for fluorescent tracing of rMSCs in two different culture conditions: (a) during 6 d of expansion and (b) after three subsequent passages, where at each new passage a new progeny of parent cells appeared. The main aim was to determine the most powerful dye, the one which has the highest fluorescent labeling potency but the least cytotoxic effect and good incorporation stability into the membrane with no penetration into the neighboring cells, thereby producing no false positivity.

During the past two decades, various fluorophores have been developed for widespread use in labeling cells or tissue structures. They could be roughly categorized into (a) endogenous reporters, i.e., fluorescent proteins constitutively produced by the specific cells and (b) exogenous fluorophores that interact with cellular or tissue components. Furthermore, the development of promising in vivo tracing methods leads to the possibility of monitoring biological processes in real time. All available dyes present a variety of different advantages or limitations, but in general, they need to be non-toxic and sufficiently bright to generate a signal detectable through living tissues. In order to recognize mesenchymal stem cells used either in cell coculture systems with direct cell-to-cell contact or in transplantation animal studies, it is essential to label cells with optimal markers (Li et al. 2008).

Furthermore, after transplantation of rMSCs into injured CNS, grafts are most likely exposed to hostile, growth factor-free environment of recipient tissue, unfavorable for further proliferation and vital processes, which may significantly decrease their survival (Menasche 2008). Thus, to prevent loss of transplanted cells, it is necessary to use dyes with low cytotoxicity. Here, we demonstrate that tracing rMSCs with PKH67, DiI, and with 5 μM BrdU over 6 d in vitro is non-toxic. We recorded a decrease in the viability of DiI on day 1 ($p<0.05$), with a tendency to increase the cell viability during the next cultivation. In contrast, 10 μM BrdU labeling ($p<0.01$) significantly decreased the cell viability during the whole time of cultivation (Fig. 2*B*), which is most likely related to the negative side effects of BrdU (Nowakowski and Hayes 2001). In addition, BrdU labeling is currently the most frequent technique used for studying adult neurogenesis in situ, regardless of its toxic and mutagenic effects triggering cell death, formation of teratomas, altering DNA stability, and lengthening the cell cycle, and has mitogenic, transcriptional, and translational effects on labeled cells (Taupin 2007). However, to minimize the negative effects of BrdU labeling, it is necessary to optimize the BrdU concentration, the desired number of labeled cells, and the time frame required for optimal DNA incorporation (Li et al. 2008). Based on our findings, we suggest that cultivating rMSCs in CM with 5 μM BrdU is suitable for labeling rMSCs, without affecting the cell viability with any significant differences.

Our second aim was to evaluate the labeling efficacy represented by sufficiently bright fluorescence signal. Membrane labeling of rMSCs with PKH67 and DiI showed intense fluorescence with punctuate-like or homogeneous patterns in the majority of cells during 6 d in culture. In contrast to membrane-binding dyes, BrdU is a synthetic thymidine analog capable of incorporating into DNA during the S phase (Nowakowski et al. 1989). In our in vitro settings, cultured rMSCs remained in different stages of the cell cycle. Therefore, depending on the specific S phase of rMSC division, incorporation of BrdU into DNA varied, resulting in intensely, moderately, or lightly stained nuclei fluorescence. Thus, in comparison to membrane dyes, BrdU showed higher variability in nuclei fluorescence signal expression. Increasing the concentration of BrdU during the labeling procedure did not enhance the number of intensely stained nuclei. On the other hand, the efficacy of BrdU labeling in rMSCs was time dependent. Thus, for BrdU labeling, a minimum 2–3-d BrdU incubation period is required, revealing the highest number of BrdU-labeled rMSCs. The present data correlate with similar findings showing 98% efficacy labeling of rMSCs with 10 μM BrdU (Feng et al. 2005), but in contrast, in our experiments, this concentration was more toxic to cells.

The ability to perform long-term tracking depends on the cell growth rate of stem cells. Here, we simulated conditions inducing proliferation of rMSCs with a routine passaging procedure. However, after passaging the cells, a new set of cells arose, in which the equal partitioning of PKH67, DiI, and BrdU dyes between daughter cells decreased for the particular cell marker. This was confirmed also in our study, while the number of labeled rMSCs with PKH67, DiI after passaging decreased to approximately half after passage 1, followed by gradual decrease, but around 20% of the membrane rMSCs could be still

visualized with a dot-like or homogenous fluorescence pattern at final passage 3. In contrast, BrdU labeling could be detected only after passage 1, while almost no BrdU+ cells (5 μM BrdU+ and 10 μM BrdU+) occurred after passages 2 and 3.

Our data show that fluorescence labeling efficacy during the first 3 d achieved high values in both membrane dyes, with significant decrease in DiI staining on day 6. In contrast, the passaging procedure which yielded a new set of daughter rMSC population showed significant reduction of fluorescence labeling pattern. These results are in line with other studies demonstrating that PKH/DiI and BrdU labeling is significantly decreased through cell division (Boutonnat et al. 2000). In the present study, therefore, we used adult stem cells which in contrast to the embryonic or neural stem cells exhibit a low proliferation cycle (Cizkova et al. 2006).

Conclusions

Our results suggest that rMSCs labeled with PKH67 and DiI show maximum labeling rate, no toxicity, and high efficacy and stability with a tracing capacity from 6 d up to 1–2 passages. Similarly, labeling with 5 μM BrdU, but not with 10 μM BrdU, shows an effective fluorescence pattern over 6 d, although it was inappropriate following the passaging procedure. Thus, we assume that cultured prelabeled rMSCs with membrane markers rather than with BrdU will retain their primary fluorescence pattern longer and may be more appropriate for cell tracing and tissue engineering studies.

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