Research Report

Comparison of the labeling efficiency of BrdU, DiI and FISH labeling techniques in bone marrow stromal cells

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ABSTRACT

Cells are generally labeled during in vivo implantation studies enabling the cells to be traced. The relationship between the labeling efficiency and cellular proliferation after transplantation is critical for the interpretation of data obtained by detection of the signals on tissue sections. Here, we compare cellular labeling methods of rat marrow stromal cells that were labeled with 5-bromo-2-deoxyuridine (BrdU), 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) and fluorescence in situ hybridization (FISH). Our data show that (i) BrdU uniformly labeled the nuclei, (ii) DiI-labeled cells had many dots or stained clear and uniform when a longer exposure time was used during detection and (iii) FISH labeled the cells with dots along the edges of the nuclei. The labeling efficiency was 94.1±8.6%, 97.6±3.4% and 90.5±3.0%, in BrdU, DiI- and FISH-labeled cells, respectively. After sub-culturing of labeled cells, the percentage of BrdU-positive cells was found to be 71.9±18.0% and 18.4±6.9%, after the first and second passages, respectively. The percentage of DiI-labeled cells detected depended on the exposure time: a long exposure time (≥10 s) resulted in identification of 95.1±4.0% and 94.5±3.9% DiI-positive cells after the first and second sub-cultures, respectively. The percentage of FISH-positive cells was found to be 87.0±3.0% and 89.1±9.7%. The BrdU labeling signal quickly decreased over time. Thus, BrdU should only be used to temporarily label dividing cells. In contrast, our data indicate that DiI and FISH labeling may be used to steadily trace cells during in vivo experiments. To our knowledge, this is the first time that the effects of different labeling methods over time have been examined during a cell transplantation study.

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1. Introduction

Cellular labeling has been widely used, both in vivo and in vitro, to trace cells of interest. However, the relationship between the labeling method and cellular proliferation may affect the data obtained from cell transplantation studies, especially in long-term investigations over several weeks and months. In particular, a subdued signal detected on tissue sections may be misinterpreted. There are three commonly used methods for labeling cells: (i) 5-bromo-2-deoxyuridine (BrdU), (ii) 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) and (iii) fluorescence in situ hybridization (FISH).
tion (FISH). Here, we compare the labeling efficiency of these different methods during in vivo and in vitro labeling of rat marrow stromal cells (rMSCs).

BrdU has been widely used to label cells since the 1970s. BrdU competes with thymidine, is substituted into the DNA, and at the same time can be detected by immunohistochemistry (Rutter et al., 1973; Goz, 1978). In several studies, BrdU has been injected into animals to investigate the proliferation of cells of interest. In these experiments, the animals were usually sacrificed shortly after injection (several hours) (Andrassy et al., 2005; Sundholm-Peters et al., 2005; Hoehn et al., 2005). Other reports have described the migration and differentiation of BrdU-labeled cells in vivo after longer periods of time (Lu et al., 2001; Yang et al., 2007; Cheng et al., 2007). However, in the long-term studies, detection of the BrdU-labeled cells would presumably be affected by the concentration of BrdU within each cell, which changes as the labeled cells proliferate. To date, the relationship between amount of labeled cells and passages of proliferation is still not clear after labeling with BrdU.

DiI, a long chain dialkylcarbocyanine, has been used to label the cell membrane using cross-linkage transport processes since the 1980s (Higuchi et al., 2005). Since DiI can be detected by fluorescent microscopy, this procedure is regarded as a simple and convenient labeling method (Pitas et al., 1981; Yoon et al., 2004; Dai et al., 2005; Deng and Elberger, 2001; Yoshiba et al., 2002; McLennan and Kulesa, 2007). Although some differences in the labeling concentration and time have been reported (Pasula et al., 2002; Liu et al., 2005), labeling cells with DiI in vivo or in vitro has not been shown to affect cell viability, cell development or basic physiological properties. The effects of modality and efficiency of labeling time and cellular proliferation on the detection of DiI-labeled cells have not been reported.

FISH is a technique that uses fluorescently labeled DNA probes to detect specific genes of interest in cells while avoiding any extrinsic disturbances. Thus, this method has been used to confirm the existence of labeled cells and explore their characteristics (Coenen et al., 2005; Mahmood et al., 2001; Fang et al., 2004; Kamolz et al., 2006; Radtke et al., 2005). The rat Sry gene is located on the short arm of the Y chromosome (Kellermayer et al., 2005). The SRY protein binds to its DNA target, modulates its transcription and initiates a cascade of events that induces sexual development in the male mammal (Hildebrandt et al., 1999). The rat Sry gene cDNA probe has been used previously to be a marker for male-derived cells using Southern blotting (An et al., 1997). The Sry gene probe has also been used to trace transplanted male-derived cells in female rats (Asim et al., 2001). However, use of the FISH technique in vitro requires further examination due to the complexity of the procedure and the weaker signal obtained by this method.

Here, we have investigated the labeling efficiency of rMSCs by three different methods in vitro and in vivo. We have also examined the FISH technique in more detail and optimized the procedure to magnify the final signal. Our results show that cells labeled with BrdU become undetectable over several passages of

Fig. 1 - BrdU labeling and detection of rMSCs. (a–d) rMSCs cultured in vitro. rMSCs were spindle-like (a). Uniform staining of the entire nucleus after labeling with BrdU (green) (b). Sub-cultured rMSCs labeled with BrdU (third day of the experiment). Note that after sub-culturing only part of the nucleus was stained with BrdU (c). After continuous sub-culturing of the BrdU-labeled cells (4 days after the second passage), virtually no positive staining was observed (d). (e, f) BrdU-labeled rMSCs were transplanted into rat brains and the brains were collected 3 days after transplantation. Brain sections were prepared and stained for DAPI (blue) (e) and BrdU (f). Panels a, e, f are of the same magnification; scale bar is 100 μm; panels b–d are of the same magnification; scale bar is 50 μm.
time as the cells proliferate. Thus, this method should only be used in short-term experiments or in experiments where the cell cycle is controlled. Our data indicate that both the DiI and FISH techniques can be used to label and detect proliferating cells in vivo or in vitro and would be particularly useful during long-term cell transplantation studies in vivo.

2. Results

2.1. BrdU labeling and detection

As shown in Fig. 1a, rMSCs cultured in vitro were spindle-like. After BrdU labeling, uniform staining (green) of the entire nucleus was observed (Fig. 1b). When stained with DAPI, the number of DAPI-positive cells was almost consistent with the number of BrdU-positive cells after passage 0. However, due to the proliferation of the labeled cells, the number of BrdU-positive cells was less than the number of DAPI-positive cells in subcultures (third day in culture) of BrdU-labeled cells. Furthermore, only part of the nucleus was labeled with BrdU (green) (Fig. 1c). At this point we found that at least half of the cells were BrdU-positive. However, after sub-culturing the cells continuously (fourth day after the last sub-culture), virtually none of the cells were BrdU-positive (Fig. 1d). The percentage of BrdU-positive cells was 94.1±8.6%, 71.9±18.0% and 18.4±6.9% after passages 0, 1 and 2, respectively. Statistical analysis showed that there was a significant decrease in the percentage of positively stained cells between the first and second sub-cultures ($P<0.01$) (Fig. 4a).

During the in vivo studies, similar results were observed in the brain sections obtained 3 days after transplantation of rMSCs cultured in vitro (Figs. 1e and f).

2.2. DiI labeling and detection

Using fluorescent microscopy, punctate DiI staining (red) was observed within the DiI-labeled cells (Fig. 2a). After prolonging the exposure time (>10 s), clear and uniform staining (red) was observed throughout the whole cell (Fig. 2b). In this study, we found that the labeling efficiency depended on the length of the exposure time used during detection. When the exposure time was 5.4 s, the percentage of positively labeled cells was 91.3±5.2%, 75.7±6.0%, and 30.4±6.0%, after passages 0, 1 and 2, respectively. The decrease in the percentage of DiI-positive cells was significant after each passage ($P<0.01$). After the second sub-culture, the punctate staining within each cell was too faint to be detected (figure not shown). However, when the exposure time was extended to 10.1 s, more diffuse membrane staining became visible and increased the detection of DiI-labeled cells (Figs. 2c and d).

DAPI was used to stain the nuclei allowing easy quantification of the DiI-labeled cells (Fig. 2e). The percentages of DiI-positive cells after passages 0, 1 and 2 were very similar: 97.6±3.4%, 95.1±4.0%, and 94.5±3.9%, respectively. The difference between these values was not statistically significant (Fig. 4b). When transplanted into the rat brain, DiI-labeled cells in the frozen brain sections were easily detected by fluorescent microscopy (Fig. 2f).

Fig. 2 – DiI labeling and detection of rMSCs. (a–e) rMSCs cultured in vitro. Punctate staining of DiI-positive (red) cells (5.4 s) (a). After a long exposure time (>10 s), the cells were stained uniform (b). After passages 1 and 2, strong DiI staining was observed with an exposure time of 10.1 s (c, d). (e) The non-homogenous distribution of the DiI dye, especially when the labeled cells accumulated together, made it difficult to count the actual number of cells. (f) DiI-labeled rMSCs were transplanted into rat brains and the brains were collected 1 week after transplantation. Brain sections were prepared and stained for DiI. Panels a–e are of the same magnification; scale bar is 50 μm; the scale bar of panel f is 100 μm.
2.3. FISH labeling and detection

The Y chromosome in male-derived rMSCs was detected by FISH analysis using TSA. In addition, the nuclei were stained with DAPI (blue). As shown in Fig. 3a, red dots representing labeling with the Sry probe were observed along the edges of the nucleus (blue). When the Sry probe was used in female-derived rMSCs, virtually no signal was detected (Fig. 3b). Statistical analysis showed that the percentages of positively labeled cells of passages 3, 4 and 5 were 90.5±2.97%, 87.0±3.0% and 89.1±9.7%, respectively in the male-derived rMSCs. These values were not significantly different (Figs. 4c and d). Images show FISH staining with (Fig. 3e) or without (Fig. 3f) TSA are obviously different.

In the brain sections obtained from transplantation of labeled cells into the rat brain, the positive cells were those with green dots (Fig. 3g). When our probes were used for FISH analysis on the sections of female brains without the transplantation of male rat cells, few FISH-positive cells were detected (Fig. 3h).

2.4. Comparison of the different labeling methods

As shown in Fig. 4, we observed significant differences between the three different labeling methods (P<0.05). We found that the labeling efficiency of FISH was initially significantly less than BrdU and Dil labeling (Fig. 4d; P<0.05). We also observed significant differences between the three methods when comparing the percentage of positively labeled cells after the first and second passage (Fig. 4d). There were significantly fewer BrdU-labeled cells than Dil and FISH after the second passage compared to the first one (P<0.01). A significant difference was also observed between Dil labeling and FISH when comparing the percentage of positively labeled cells after both the first and second passages (P<0.05) (Fig. 4d).

3. Discussion

To date, there appear to be two distinct methods available for tracking cells during in vivo experiments. One method uses real-time imaging and tracing methods, such as luciferase and GFP labeling of cells, to investigate the survival, migration, and differentiation of implanted cells in the same animal over time (Zhou et al., 2006). The other method is relative to in vitro experiments. In these experiments, labeled cells, often stem cells or cells labeled with target genes, are transplanted into the animal. After several days or weeks, the animal is sacrificed and sections are prepared and examined. Labeling cells with BrdU, Dil or FISH is common during this type of experiment. In this study, we compared the labeling efficiency of cells with BrdU, Dil or FISH to determine which method provides the most accurate data (Table 1).

Although BrdU, Dil and FISH labeling are widely used, these methods have not been quantitatively evaluated. The relationship between the labeling efficiency and labeling time, cellular proliferation and detection procedure is critical for interpretation of the results obtained when detecting the signal on tissue sections. Here, we found that the labeling efficiency of the three methods was approximately 98% (Dil), 94% (BrdU) and 91% (FISH) immediately following labeling. Although no significant differences were observed between...
DiI and BrdU, these methods provided a significantly higher labeling efficiency than FISH. After sub-culturing the labeled cells, significant differences were observed between the three methods. In particular, we noted a significant decrease in the BrdU labeling efficiency. The slight decrease that was observed in the DiI- and FISH-labeled cells would still be acceptable during in vivo experiments.

BrdU labeling is rapid and simple: it can be applied directly to cells or injected into animals (Vogetseder et al., 2005). Previously, it has been hypothesized that if 100% of cells were BrdU-positive, then after the first and second passages, 50% and 25% respectively of cells would be expected to be positive (Ehmcke et al., 2005). However, in our experiments we show that approximately 72% of cells and 18% of cells are positive after the first and second passages, respectively. These observations are presumably due to partial nuclear staining of each daughter cell after proliferation (i.e., when the BrdU-labeled cells divide, the amount of BrdU assigned to each daughter cell is enough to be detected, although only part of the nuclei is labeled (Fig. 1c). However, in the next passage the BrdU in each daughter cell has decreased beyond the range of the detection. Thus, the positive labeling rate is greatly reduced (Fig. 1d).) Eventually, the amount of BrdU passed on

<table>
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<th>Labeling methods</th>
<th>Positive rate (after labeling) (%)</th>
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<tr>
<td><strong>BrdU</strong></td>
<td><strong>P0</strong> 94.1±8.64 <strong>P1</strong> 95.1±4.04 <strong>P2</strong> 94.5±3.90</td>
<td>Simple labeling</td>
<td>Signal broken down quickly</td>
</tr>
<tr>
<td><strong>DiI</strong></td>
<td><strong>P0</strong> 97.6±3.38 <strong>P1</strong> 71.9±18.02 <strong>P2</strong> 18.4±6.87</td>
<td>Simple and stable labeling, persisting time long</td>
<td>Could be absorbed leading to fake positive</td>
</tr>
<tr>
<td><strong>FISH</strong></td>
<td><strong>P0</strong> 90.5±2.97 <strong>P1</strong> 87.0±2.98 <strong>P2</strong> 89.1±9.68</td>
<td>Stable labeling</td>
<td>Complex detection procedure</td>
</tr>
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*P<0.05; **P<0.01.
to each daughter cell would be undetectable resulting in a significant reduction in BrdU-positive cells.

BrdU has been used in vivo to investigate the differentiation of cells, especially adult stem cells (Hoehn et al., 2005; Berg et al., 2005). However, the number of times the cells had divided before they were detected was not reported in these studies. Based on our observations, if the BrdU-labeled cells continue to proliferate after implantation, then the actual number of transplantation-derived cells would be very different from the number detected. Our findings indicate that it would be better to use a different labeling method for these experiments.

DiI labeling has previously been used to trace transplanted cells in vivo (Yoon et al., 2004; Dai et al., 2005; Pasula et al., 2002) and compare the relationship between cells in vivo, especially neural cells (Van Lommel et al., 1998; Konnai et al., 2000; Michel et al., 2000; Herzog et al., 2003). In our experiments, we found that after sub-culturing the Dil-labeled cells twice, the labeling efficiency was stable when detected using a suitable exposure time. These findings suggest that once the cells were labeled, the amount of Dil in each cell was sufficiently high that it would still be detected even if some had transferred to daughter cells during proliferation. Interestingly, we have detected partial Dil staining in labeled cells for as long as 7 weeks in vitro (data not shown).

During the course of these experiments, we did encounter some potential problems related to the labeling of cells with DiI. Firstly, we found that the non-homogenous distribution of the DiI dye, especially when the labeled cells accumulated together, made it difficult to count the actual number of cells (Fig. 2e). To overcome this, we simultaneously stained the nuclei with DAPI. However, we found that Dil-labeled cells should not be treated with conventional permeable reagents such as Triton® X-100. Special preparation of the samples was required. Secondly, we found that when the membranes of Dil-labeled cells were disrupted, neighboring cells absorbed the Dil that leaked out of these cells. This should also be taken into account when analyzing data obtained from Dil-labeling experiments.

The genome of every cell is passed to each of its daughter cells, making FISH a reliable and stable method to use. However, due to the complexity of this technique, for example the preparation and labeling of the probe, a lower labeling efficiency of approximately 88.6% was obtained. Furthermore, the preparation and labeling of the probe, a lower labeling efficiency is limited. Here, we enhanced the FISH signal amplification system is sensitive but its specificity and efficiency are limited. Here, we enhanced the FISH signal and increased the labeling efficiency of the cells. It is notable that TSA may sometimes amplify the background signal and result in fake positives (Fig. 3b). This may be avoided using higher intensity washing conditions. In addition, we specially prepared the cell samples without fixation in paraformaldehyde, to avoid autofluorescence caused by aldehyde.

We also optimized the conditions used for FISH. Treatment of the samples with 10 mM HCl may improve the signal-to-noise ratio that occurs during extraction of proteins and partial hydrolysis of the target sequences. Although DNA–DNA in situ hybridization could make the procedure simpler, the template and the target have to be denatured completely. In our experiments, we consulted previously described heat denaturation methods (Lindeberg and Johansen, 1990; Archard et al., 1987). We used formamide in the probes cocktail to reduce the melting temperature of the DNA–DNA duplexes. Dextran sulfate also causes an apparent increase in probe concentration and was used here. Finally, it is possible that we could improve the FISH signal and increase the reliability and ease of this technique by using RNA labeling of the probe or a chromosome Y-specific painting probe.

 Many studies have used BrdU, Dil or FISH labeling techniques to track implanted cells in vivo. Here, qualitatively examined the differences in the labeling efficiencies of Dil, BrdU and FISH labeling techniques, to determine which methods would be the most useful or tracing cells in future cell transplantation studies. In summary, we found that the BrdU labeling signal weakened quickly over time. Thus, BrdU should only be used to temporarily label dividing cells. In contrast, our data indicate that Dil and FISH labeling may be used to steadily trace cells during in vivo experiments. Consideration of the fact that Dil leaking out of cells may influence the real results, we think that FISH is the best approach to visualize and distinguish implanted cells. To our knowledge, this is the first time that the effects of different labeling methods over time have been examined during a cell transplantation study.

4. Experimental procedure

4.1. Culture of rMSCs

Primary rMSCs were isolated according to the method described by Azizi et al. (1998). Briefly, Sprague-Dawley (SD) adult rats were sacrificed, and the tibias and femurs were removed. rMSC culture media (7 ml) consisting of alpha-MEM (Gibco/BRL) supplemented with 10% fetal bovine serum (FBS) (Hyclone, ARA25685) and 100 U penicillin/100 U streptomycin (Invitrogen) was injected into the central canal of the bone to extrude the marrow. Whole marrow cells were extracted, and cultured at a density of 5–10×10^5 cells/cm² in rMSC culture media. Non-adherent cells were removed after 48 h by changing the media. The media were changed every other day. Confluent cells were split (1:2) using 0.25% trypsin and 0.02% EDTA and were passaged three times. The cells were then labeled using BrdU, Dil or FISH labeling techniques.

4.2. BrdU labeling and detection

The cells of the 3rd passage were labeled according to the method described by the 5-bromo-2’deoxy-uridine labeling
and detection kit I (Roche Cat. No. 1206736). Briefly, cultured rMSCs of the 3rd passage (50% confluency) were washed in PBS, then incubated in 20% alpha-MEM containing 10 μmol/l BrdU at 37 °C for 48 h (Lu et al., 2001). After washing the cells twice, the cells were cultured and sub-cultured in 20% alpha-MEM. Labeled cells after passages 0, 1 and 2 were washed in PBS (5 min; 3 times), and fixed in ethanol at −20 °C for 20 min, followed by incubation in 2 N HCl for 30 min. The cells were incubated with anti-BrdU working solution (1:50) for 30 min at 37 °C. After washing in washing buffer three times, the cells were incubated in the dark with anti-mouse-lg-fluorescein working solution (1: 200) for 30 min at 37 °C. The nuclei of the cells were counterstained with DAPI (4′,6-diamidino-2-phenylindole, Invitrogen (200 ng/ml)) for 10 min. The cells were counted under the fluorescent microscope (Leica, Germany). For each experiment, at least half of the cells (detected by DAPI nuclear staining) were counted (n=6).

Brain sections were obtained from rats that had been transplanted with BrdU-labeled cells of the 5th passage for 3 days. Transplantation of the cells and preparation of the brain sections is described in the FISH section. The cells were detected as described above.

4.3. Dil labeling and detection

For Dil labeling and detection, rMSCs of the 3rd passage (90% confluency) were incubated in 5.0 μg/ml DMSO solution in Dil buffer at 37 °C for 30 min (Lu et al., 2002). After washing twice in PBS, the cells were cultured in 20% alpha-MEM and samples were collected after passages 0, 1 and 2. When the cells were confluent, Dil-labeled cells were fixed with 4% paraformaldehyde, the cells were detected as described above. The nuclei were stained with DAPI (200 ng/ml). The cells were counted under the fluorescent microscope (Leica, Germany). For each experiment, at least half of the cells (detected by DAPI nuclear staining) were counted (n=6).

The brain sections were obtained from rats that had been transplanted with Dil-labeled cells of 5th passage for 1 week. Transplantation of the cells and preparation of the brain sections are described in the FISH section. After fixing in 4% paraformaldehyde, the cells were detected as described above.

4.4. FISH detection of rMSCs

4.4.1. Cloning of the rat Sry gene and generation of Sry DNA probes

We cloned the Sry gene from the Y chromosome of the SD rat and generated a Sry DNA probe. The thymus tissue of a male SD rat (obtained from the Animal Center of Capital Medical University) was cut and digested with 0.25% trypsin (GIBCO). The extracts were washed with PBS and suspended in TNE buffer (10 mM Tris-Cl (pH 7.9), 10 mM EDTA (pH 8.0), 10 mM NaCl) containing 20% SDS (10 μl) and 2 μl Proteinase K (20 mg/ml) (GIBCO). The suspension was agitated and incubated at 37 °C for 12 h. A phenol extraction–ethanol precipitation method was used to extract the genomic DNA (Dalgaard and Klar, 1999). We used a polymerase chain reaction (PCR) to obtain a 459-bp fragment of the Sry gene (An et al., 1997). After purification, the fragments were inserted into the pGEM-Teasy vector (Promega), sequenced and compared with the EMBL Nucleotide Sequence Database X89730. The DNA fragments were labeled with digoxigenin (DIG) by the random primed labeling kit (Roche Cat. No. 1093657).

4.4.2. FISH labeling and detection of rMSCs

Male rMSCs of the 3rd, 4th and 5th passage were cultured on glass cover slips and fixed in cold methanol (−20 °C) for 5 s, followed by cold acetone (4 °C) for 5 s (three times for cold acetone fixation). The samples were air dried and stored at −20 °C.

Before in situ hybridization, the samples were incubated with 1% H2O2 in the dark for 20 min, followed by Proteinase K (50 μg/ml) at 37 °C for 10 min. The samples were then washed in 10 mM HCl for 2 min, and in PBS for 5 min (twice). Samples were incubated with PBS containing 1% paraformaldehyde for 10 min at room temperature (RT), followed by washing with PBS (5 min; twice). The samples were dehydrated through a series of ethanol solutions (70%, followed by 90%, followed by 100% ethanol).

The DIG-DNA probe cocktail (1 ng/μl) consisting of 1× Denhart’s solution (5% dextran sulfate 50% (w/v); 1 μg/ml sonicated salmon sperm DNA; 2× SSC and 50% formamide) was denatured at 100 °C for 5 min, then on ice for 1 min. The probe cocktail was then applied to the samples (50 μl/cover slide with rMSCs). The whole cover slide with the probe was denatured at 94 °C (hot plate) for 5 min and on ice for 1 min, followed by hybridization at 42 °C for 16–18 h (overnight). The following day, the samples were washed in 2× SSC + 50% formamide at RT for 5 min (three times), 0.1× SSC +50% formamide at 42 °C for 10 min, and 0.1× SSC at 42 °C for 10 min.

Labeling of the cells was detected according to the method described by the Tyramide Signal Amplification (TSA) Biotin kit (NEN LIFE Science, NEL700A). The samples were rinsed in TNT buffer (0.1 M Tris–HCl (pH 7.5), 0.15 M NaCl, 0.05% Tween 20) for 5 min at RT. Nonspecific binding was blocked in freshly prepared TNB buffer (TNT buffer containing blocking reagent (NEN)) for 30 min at 37 °C. Then the samples were incubated with anti-DIG–biotin (Sigma B7405, 1:10000 in TNT) at 37 °C for 30 min. After rinsing in TNT for 5 min (three times), the cells were incubated with streptavidin–horseradish peroxidase (HRP) (1:100 in TNT) for 30 min at 37 °C. The cells were rinsed and incubated with streptavidin-Cy3 (Sigma 1:200) for 30 min at 37 °C. After washing, the nuclei were stained with DAPI (200 ng/ml). The cells were examined by fluorescent microscopy.

During the TSA procedures, the BT working solution (1:50 in 1× Amplification Diluent (NEN)) was applied for 10 min in the dark after incubation with streptavidin–HRP. Then, the samples were washed in TNT, incubated with streptavidin-Cy3, stained with DAPI and examined by fluorescent microscopy.

4.4.3. FISH detection on the brain sections

Adult female SD rats (200–300 g) were anesthetized by intraperitoneal injection with 6% Chlorali Hydras. The animals were transferred to a stereotaxic apparatus in a sterile field. A 2- to 5-mm incision was made in the scalp, 4 mm lateral to the bregma. A burr hole was made in the bone, 3 mm lateral to the bregma with a dental drill, and about 20 μl of the male rMSCs (or BrdU-labeled or Dil-labeled rMSCs) suspension was slowly injected over 30 min into the...
striatum at a depth of 4.5–5.5 mm from the surface of the brain. The wound was closed with interrupted surgical sutures. After 3 (BrdU) or 7 (DiI or FISH) days, rats were sacrificed under deep anesthesia with xylozine and ketamine and the brains were removed within 5 min without fixing in paraformaldehyde. The brains were cut into 2- to 3-cm blocks, and placed in chilled OCT and snap-frozen in liquid nitrogen. Tissue blocks were stored at −80 °C. Cryosections of 10 μm were cut at −20 °C, collected on slides, and stored desiccated at −20 °C for up to 4 weeks.

Before FISH, all sections were fixed in acetone at 4 °C for 10 min. The sections were then washed in buffer 1 (100 mM Tris–Cl, 150 mM NaCl) at RT for 5 min (twice), treated in 0.2 N HCl at RT for 10 min, and finally incubated in Proteinase K (100 μg/ml) at 37 °C for 15 min. The DIG-DNA probe cocktail (1 ng/μl) was denatured at 100 °C for 5 min. Hot probe in hybridization buffer (50 μl/section) was directly applied to dry preheated sections. Slides were incubated overnight at 42 °C in a humid chamber containing paper towels. Sections were rinsed in 2× SSC and 50% formamide at RT for 5 min (three times), 0.1× SSC+50% formamide at 42 °C for 10 min, and 0.1× SSC at 42 °C for 10 min for posthybridization washes.

After rinsing in buffer 1 (Roche; Cat. No. 1093657) for 5 min at RT, non-specific binding was blocked in freshly prepared blocking buffer (1% w/v blocking reagent in buffer 1) for 15 min. Sections were incubated with anti-digoxigenin antibody (1:5000 Roche) from sheep in blocking buffer at 37 °C for 1 h, followed by washing in buffer 1 (5 min; twice), and incubation with anti-sheep–FITC (Sigma, 1:40) in blocking buffer at 37 °C for 1 h, collected on slides, and stored desiccated at −20 °C for 4 weeks.

In order to detect the specificity of the probes, the same method was also used for FISH on cells and brain sections of female rats. All rMSCs used in these labeling methods were obtained from male rats and were transplanted into female rats.

4.5. Cell counting and statistical analysis

Quantification of the number of positively labeled cells is described below. In any given experiment, the number of positively labeled cells was expressed as a percentage of the total number of DAPI-labeled nuclei. In a typical experiment, a total of 500–1000 cells were counted for each method. Each labeling method was repeated in six independent experiments in vitro (with cells from six rats), and the mean value was used for statistical analysis. Results were presented as mean±SEM (Hildebrandt et al., 1999). The significance of differences between the three labeling methods was determined using one-way ANOVA by SPSS 13.0. A value of P<0.05 was considered to be statistically significant. If the data were not normally distributed, a non-parametric test (Kruskal–Wallis test) was used to compare the results.

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