Comparison of three fluorescence labeling and tracking methods of endothelial progenitor cells in laser-injured retina

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Abstract
• AIM: To compare three kinds of fluorescent probes for in vitro labeling and in vivo tracking of endothelial progenitor cells (EPCs) in a mouse model of laser-induced retinal injury.
• METHODS: EPCs were isolated from human umbilical cord blood mononuclear cells and labeled with three different fluorescent probes: 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE), 1,1′-dilinoleyl-3,3,3′,3′-tetramethylindo-carbocyanine perchlorate linked acetylated low-density lipoprotein (DiI-AcLDL), and green fluorescent protein (GFP). The fluorescent intensity of EPCs was examined by confocal microscopy. Survival rate of labeled EPCs was calculated with trypan blue staining, and their adhesive capability was assessed. A mouse model of retinal injury was induced by laser, and EPCs were injected into the vitreous cavity. Frozen section and fluorescein angiography on flat-mounted retinal samples was employed to track the labeled EPCs in vivo.
• RESULTS: EPCs labeled with CFSE and DiI-AcLDL exhibited an intense green and red fluorescence at the beginning; the fluorescence intensity decreased gradually to 20.23% and 49.99% respectively, after 28d. On the contrary, the fluorescent intensity of GFP-labeled EPCs increased in a time-dependent manner. All labeled EPCs showed normal morphology and no significant change in survival and adhesive capability. In the mouse model, transplantation of EPCs showed a protective effect against retinal injury. EPCs labeled with CFSE and DiI-AcLDL were successfully tracked in mice during the development of retinal injury and repair; however, GFP-labeled EPCs were not detected in the laser-injured mouse retina.

• CONCLUSION: The three fluorescent markers used in this study have their own set of advantages and disadvantages. CFSE and DiI-AcLDL are suitable for short-term EPC-labeling, while GFP should be used for long-term labeling. The choice of fluorescent markers should be guided by the purpose of the study.
• KEYWORDS: endothelial progenitor cells; cell tracking; 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester; 1,1′-dilinoleyl-3,3,3′,3′-tetramethylindo-carbocyanine perchlorate linked acetylated low-density lipoprotein; green fluorescent protein; retinal laser photoagulation

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Introduction
Most eye diseases which lead to loss of vision involve retinal ischemia and neovascularization. Retinal neovascularization is a common pathological change in retinal vascular disorders such as retinal vein occlusion (RVO), diabetic retinopathy (DR), and retinopathy of prematurity (ROP)[1]. Neovascularization may result in complications such as retinal edema, vitreous hemorrhage, fibrovascular proliferation, and retinal detachment, which often culminate in irreversible loss of vision[2]. Currently, there is no effective therapy to restore vision loss caused by ischemic retinopathy. Previous studies have demonstrated impaired retinal function in the retinal vasculature in patients with retinal ischemic diseases. Repair of damaged vascular endothelium in the retina may play a key role in the control of retinal neovascularization[3].
Endothelial progenitor cells (EPCs), a population of circulating cells at low concentrations, are involved in tissue regeneration through microvascular repair and facilitate re-perfusion of ischemic areas[6]. Circulating EPCs were first identified in 1997 by Asahara et al[4] as CD34+ VEGFR2+ mononuclear cells. These cells were shown to differentiate into an endothelial phenotype, express endothelial markers, and incorporate into neovascularization at ischemic sites. Subsequent studies by other groups also demonstrated the existence of circulating EPCs[5-4]. The use of EPCs as potential therapy for retinal disease has been explored in different animal models[9]. Intravenous or intravitreal injection of human CD34+ EPCs sourced from peripheral blood or bone marrow was shown to lead to their aggregation in the damaged retinal vasculature. Moreover, an apparent normalization of the damaged retinal vasculature was observed, which indicated a potential therapeutic effect[10].

Study of the distribution and function of EPCs in the setting of retinal damage and repair requires an efficient and stable method for labeling and tracking of EPCs transplanted in the retina. Fluorescence has been widely used in the last few decades. We have used 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) to label and track EPCs in a mouse model of laser-induced retinal injury[11]. CFSE-labeled EPCs showed normal morphology; further, no significant change in survival or apoptosis rate both in vitro as well as in the retinal vascular networks was observed for at least 28d after transplantation. Nevertheless, CFSE was found to be toxic to cells at high concentrations, and the fluorescence intensity of cells rapidly decreased within 4wk[11].

We further investigated other methods for labeling and tracking of EPCs using 1,1'-dilinoleyl-3,3',3'-tetramethylindocarbocyanine perchlorate linked acetylated low-density lipoprotein (DiI-AcLDL), and green fluorescent protein (GFP). DiI-AcLDL is a lipophilic fluorescent dye commonly used for labeling of EPCs and shows an intensive expression of red florescence[12-13]. GFP is also widely used for labeling and tracking of EPCs in animal models of retinal diseases[14-18]. GFP signal can be detected easily and rapidly, and the expressed fusion proteins are generally not toxic to cells. However, GFP signal cannot be amplified in a controlled manner, and thus may not be amenable to detection at low expression levels.

In this study, we compared these three fluorescent markers for labeling EPCs in vitro and tracked the labeled EPCs in vivo. Our purpose is to establish a reliable and simple method for labeling and tracking of EPCs in animal models, in order to further explore therapeutic application of EPCs in retinal diseases.

MATERIALS AND METHODS

Isolation of Endothelial Progenitor Cells from Human Umbilical Cord Blood EPCs were isolated using a previously described method at our laboratory[11-13]. In brief, human umbilical cord blood was collected in blood bags (Nagale, China) using citrate-dextrose as an anticoagulant. The mixture of blood with 6% hydroxyethyl starch (HES; Sigma Co., USA) (4:1 ratio) was left undisturbed at 4°C for 2h. The supernatant was collected and subjected to centrifugation. The precipitate was resuspended, loaded on to 60% Percoll (1.077 g/mL), and centrifuged at 500×g for 20min. The cells were then collected and resuspended in medium 199 (M199, Gibco, USA) which comprised of 20% fetal bovine serum (FBS; Hyclone Co., USA), 10 ng/mL vascular endothelial growth factor (VEGF; Peprotech, USA), 20 ng/mL basic fibroblast growth factor (bFGF; Peprotech, USA), and 15 μg/mL bovine pituitary extract (BPE; ScienCell Lab, USA). The cells were seeded at a density of 2×10⁶ cells/cm² on human fibronectin-coated plates (Chemicon Inc., USA), and then incubated at 37°C in an atmosphere of 5% CO₂. The study was approved by the Institutional Ethics Committee of the First Hospital of Jilin University, China. Written informed consent was obtained from parents of newborns.

Characterization of Endothelial Progenitor Cells Cells were isolated and cultured as above. On day 10, the cells were collected and the expressions of EPC surface markers were determined by flow cytometry (from Becton Dickinson, USA). The following antibodies were used: mouse monoclonal anti-human CD34 conjugated with fluorescein isothiocyanate (FITC; Becton Dickinson, USA); mouse monoclonal anti-human CD133 conjugated with phycoerythrin (Miltenyi Biotec, Germany); and mouse monoclonal anti-human VEGFR-2 conjugated with FITC (R&D, USA). The morphology of EPCs was further examined under electron microscope (JEOL, Japan).

Labeling of Endothelial Progenitor Cells with CFSE and Dil-AcLDL Ten days after their seeding, the cells were found to have acquired the shape of elongated cobblestones. EPCs were collected, washed thrice with PBS, and then stained with 5 μmol/L CFSE (Molecular Probes Biotec, USA) at 37°C for 15min or 10 μg/mL Dil-AcLDL (Molecular Probes Biotec, USA) at 37°C for 4h. The labeling procedure was completed after addition of an equal volume of heat-inactivated FBS for 1min. Subsequently, the cells were washed twice with PBS for further analysis.

Lentivirus-mediated Green Fluorescent Protein Transfection into Endothelial Progenitor Cells A 4-plasmid derived lentiviral vector system was used in this study for EPCs transfection. Briefly, 293T cells were cultured in 24-well plates. Twenty-four hours prior to transfection, the culture medium was removed and replaced with DMEM in 10% FBS. Of 200 μL Opti-MEM (Invitrogen, USA) which contained 0.4 μg plasmid (4 plasmids: pEGFP, pMD.G, pMDL g/p and pRSV-Rev in the ratio 18:15:10:6) and 5 μL lipofectamine reagents (Invitrogen, USA) were added into DMEM as transfection medium. The 293T cells were cultured at 37°C in a 5% CO₂
Characterization of Labeled Endothelial Progenitor Cells

Morphology of EPCs was examined, and the fluorescence intensity was recorded by confocal laser microscopy (Olympus, Japan). The data were analyzed with Olympus software program FV10-ASW.

The survival capability of labeled EPCs was measured by trypan blue staining. At 2 and 7d, EPCs labeled with CFSE, DiI-AcLDL, and GFP were incubated with 0.4% trypan blue (ScienCell Lab, USA) for 5min at room temperature and then observed under an inverted optical microscope (Olympus, Japan). The survival rate was calculated.

For assessment of adhesive capability, labeled EPCs were trypsinized, seeded at a concentration of $1 \times 10^5$ in 24-well plates, cultured for 30min, washed by PBS, and observed under an inverted optical microscope. The attached cells were counted.

Retinal Injury by Laser Photocoagulation and Transplantation of Endothelial Progenitor Cells

C57BL/6N mice (male, 18-20 g, ageing 8-10wk) were obtained from the Center of Laboratory Animals, School of Basic Medical Sciences, Jilin University. The animal procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals from the Ministry of Science and Technology of China and the Principles of Laboratory Animal Care from NIH (publication No.86-23), and approved by the Institutional Animal Use and Care Committee of Jilin University.

Mice were anesthetized with ethyl carbamate (1 g/kg, Sinopharm Chemical Reagent Co. Ltd., China) administered by intraperitoneal injection. This was followed by instillation of 0.5% tropicamide (Santen Inc., Japan) to induce papillary dilatation. Injury of retinal veins and capillaries around the optic disc was induced by laser photocoagulation with krypton laser (20 burns per eye; power: 75 mW; duration: 100ms; spot size: 50 μm) operated by an experienced retinal specialist.

Totally 1 μL of EPCs labeled with CFSE, DiI-AcLDL or GFP were injected immediately after photocoagulation into the vitreous cavity under an ophthalmic surgical microscope (66 VISION, China). One eye from each animal was injured, while the contralateral eye served as the control. Mice were randomly divided into 4 groups: retinal injury without treatment and retinal injury with injection of EPCs labeled with CFSE, DiI-AcLDL or GFP. Each group comprised of 12 mice.

Examination of Retinal Injury and Endothelial Progenitor Cells Transplantation by Fundus Photography

Fundal photographs were obtained under a slit lamp at 0, 2, 7 and 28d after laser photocoagulation. Retinal injury and repair were observed and recorded.

Tracking of Labeled Endothelial Progenitor Cells in Retina by Frozen Section and Angiography

Mice were sacrificed by cervical dislocation at 2, 7, 28d after laser photocoagulation. The eye balls were dissected out and subjected to frozen sections. Sections were then stained with 1:200 Hoechst (Sigma, USA) for 5min and washed twice with PBS. The stained slides were examined under confocal microscope.

For angiography, 2% Evans blue (Sigma, USA) and 10% fluorescent sodium was dissolved in normal saline. Mice were administered deep anesthesia, and the chest cavity cut open. Evans blue or fluorescent sodium was perfused through the left ventricle. The mice turned visibly blue or yellow immediately, which confirmed the uptake and distribution of Evans blue and fluorescent sodium in the mouse. Eyes were enucleated, and fixed in 4% paraformaldehyde for 1.5h at 4℃. Retinas were then dissected, flat-mounted with glycerol gelatin, and photographed under confocal laser microscope.

Statistical Analysis

Data are presented as mean±standard error of mean (SEM) and evaluated by one-way ANOVA followed by Student-Newman-Keuls test. All statistical analyses were performed with SPSS 11.0 (USA). A $P$ value $<$0.05 was considered as statistically significant.

RESULTS

Characterization of Isolated Endothelial Progenitor Cells

Isolated EPCs were identified by morphology and expression of cell surface markers. Attached cells exhibited linear cord-like structures at 10-14d after culture and cobblestone morphology after 14-28d. On day 10, expressions of EPC-specific markers, CD34, CD133, and VEGFR-2 were assessed by flow cytometric analysis. It was found that 50.8%±4.3%, 36.2%±3.9%, and 90.5%±4.6% of adherent cells showed expression of CD34, CD133, and VEGFR-2 (Figure 1).

The morphology of EPCs was examined by electron microscopy, which showed a single cell with tiny microvilli on the surface, heterochromatin in oblong nucleus, and various organelles in the cytoplasm (Figure 2A). Weibel-Palade bodies, which are the storage granules of endothelial cells, were also observed in EPCs (Figure 2B).

Characterization of Endothelial Progenitor Cells Labeled by CFSE, DiI-AcLDL, and Green Fluorescent Protein

CFSE-labeled EPCs exhibited bright green fluorescence within the cytoplasm and the nucleus. In a previous study, we demonstrated that the fluorescence intensity of EPCs increased in a dose-dependent manner up to 40 μmol/L CFSE[1], CFSE at $>$40 μmol/L showed toxic effects to EPCs. Of 5 μmol/L CFSE was finally selected for the in vitro and in vivo experiments.

EPCs labelled with 10 μg/mL DiI-AcLDL exhibited red fluorescence and showed similar characteristics as those observed with CFSE-labeled EPCs. Both CFSE- and DiI-AcLDL-labeled EPCs exhibited strong fluorescence intensity at the beginning, which dropped gradually in a time-dependent
manner (Figure 3). EPCs labeled with CFSE showed higher intensity than those labeled with DiI-AcLDL initially; however, the intensity declined at a faster rate. After 28d, EPCs labeled with CFSE and DiI-AcLDL maintained about 20.23% and 49.99% fluorescence intensity, respectively. On the contrary, GFP-labeled EPCs, which exhibited green fluorescence, were only detectable after 2d; however, the intensity increased dramatically up to 7d and continued to rise in a time-dependent manner. This implies that CFSE and DiI-AcLDL are suitable for short-term labeling, while GFP should be used for long-term labeling of EPCs.

Two and seven days after labeling with CFSE, DiI-AcLDL and GFP, EPCs showed minor decline in survival and adhesive capability as compared to that in control (Figure 4); however, the between-group difference was not statistically significant (P>0.05). The adhesive capability of DiI-AcLDL-labeled EPCs was comparable to that in control (Figure 4). This suggests that CFSE-, DiI-AcLDL-, and GFP-labeling did not affect the function of EPCs. The characteristics of 3 labeling methods are summarized in Table 1.

### Table 1: Characteristics of EPCs labeled with three different fluorescent agents

<table>
<thead>
<tr>
<th>Groups</th>
<th>CFSE</th>
<th>DiI-AcLDL</th>
<th>GFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Excitation/Emission (nm)</td>
<td>492/517</td>
<td>514/560</td>
<td>488/507</td>
</tr>
<tr>
<td>Fluorescence color</td>
<td>Green</td>
<td>Red</td>
<td>Green</td>
</tr>
<tr>
<td>Positive labeling rate (%)</td>
<td>95</td>
<td>80</td>
<td>30 (in 28d)</td>
</tr>
<tr>
<td>Initial fluorescence intensity</td>
<td>+++</td>
<td>++±</td>
<td>−, + (in 4d)</td>
</tr>
<tr>
<td>Fluorescence lasting time</td>
<td>28d±</td>
<td>28d+</td>
<td>28d+++</td>
</tr>
<tr>
<td>Effect on cell morphology</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Effect on cell survival</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Effect on cell adhesion</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Operation difficulty</td>
<td>+</td>
<td>++</td>
<td>+++±</td>
</tr>
<tr>
<td>Experimental cost</td>
<td>+</td>
<td>+++</td>
<td>+±±</td>
</tr>
</tbody>
</table>

+: Positive or strong; ±: Unstable or uncertain; -: Negative.

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**Figure 1** Expressions of EPC-specific markers, CD34, CD133, and VEGFR-2 detected by FACS  
A: Negative control; B: CD34; C: CD133; D: VEGFR-2.

**Figure 2** EPCs and Weibel-Palade bodies observed under electron microscopy  
A: One single cell with tiny microvilli on the surface, heterochromatin in oblong nucleus, and various cytoplasmic organelles (4000×); B: Part of a cell with several elongated Weibel-Palade bodies, which are the storage granules of endothelial cells (25 000×).

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**Protective Effect of Endothelial Progenitor Cells transplantation Against Retinal Injury Induced by Laser Photocoagulation**

Mouse model of retinal injury was induced by laser photocoagulation (Figure 5) and appeared as scattered white laser spots with edematous halo on fundus photography (Figure 5B); after 28d, the edematous halo had disappeared and pigmentation and scar formation was observed (Figure 5C). In the CFSE-, DiI-AcLDL- and GFP-labeled EPCs groups, alleviation of pigmentation and scar formation with some retinal blood supply was observed (Figure 5D-5F).

**Tracking of Labeled Endothelial Progenitor Cells in Injured Mouse Retina**

Fluorescent cells were observed on the surface of the retina 2d after the transplantation of EPCs labeled with CFSE (Figure 6B) and DiI-AcLDL (Figure 6E). At 7d, EPCs are seen aggregated at the sites of injury (Figures 6C, 6F). Twenty-eight days later, fluorescent EPCs are seen distributed among retinal layers, apically in retinal nerve fiber layer and inner nuclear layer (Figure 6D, 6G). The intensity of green fluorescence emitted by CFSE-labeled EPCs looks stronger than that of red DiI-AcLDL fluorescence at each time-point. However, we were unable to detect GFP-labeled EPCs although they did show a protective effect against laser damage.

Similar results were observed on fluorescein angiography. Evans blue exhibiting red fluorescence was employed to track the distribution of green CFSE-labeled EPCs, while green fluorescent sodium was used to track red DiI-AcLDL in the transplanted EPCs (Figure 7).
A mouse model of retinal injury induced by laser photocoagulation was employed in this study. Scattered white laser spots surrounded by edematous halo were observed after laser photocoagulation, pigmentation and scar formation were perceived at 28d. Laser photocoagulation-induced animal models of retinal injury have been widely used to evaluate the efficacy of retinoprotective therapies. For example, the extract of radix pseudostellariae was found to exhibit a protective effect against retinal laser injury in rabbits\cite{19}. We showed

**Figure 3 Stability of fluorescence intensity of labeled EPCs**  
A-I: Fluorescent intensity of EPCs labeled with CFSE (A: 7d; B: 14d; C: 28d), Dil-AcLDL (D: 7d; E: 14d; F: 28d) and GFP (G: 7d; H: 14d; I: 28d) was examined by confocal laser scanning microscopy (magnification ×400). J: Statistical analysis of the fluorescent intensity of EPCs labeled by CFSE, Dil-AcLDL and GFP over time. Data were presented as mean±SEM.

**DISCUSSION**

A mouse model of retinal injury induced by laser photocoagulation was employed in this study. Scattered white laser spots surrounded by edematous halo were observed after laser photocoagulation, pigmentation and scar formation were perceived at 28d. Laser photocoagulation-induced animal models of retinal injury have been widely used to evaluate the efficacy of retinoprotective therapies. For example, the extract of radix pseudostellariae was found to exhibit a protective effect against retinal laser injury in rabbits\cite{19}. We showed
that transplantation of EPCs alleviated pigmentation and scar formation accompanied by some retinal blood supply, which suggests a protective effect of EPCs against retinal injury. EPCs transplantation is a promising treatment for retinal injury induced by ischemia and hypoxia. Many studies have demonstrated the therapeutic potential of EPCs in retinal diseases, such as age-related macular degeneration \[20\], ischemic retinopathies \[21\], and DR \[22-23\], by promoting vascular repair and reversing ischemic injury. Caballero \textit{et al} \[9\] demonstrated that healthy EPCs could effectively repair ischemic vascular damage in neonatal animal models of oxygen-induced retinopathy. Medina \textit{et al} \[24\] reported that direct incorporation of EPCs in the resident vasculature led to a significant decrease in avascular areas and an increase in normovascular areas, and prevented pathological preretinal neovascularization.

However, identification and localization of engrafted EPCs are key challenges in this therapeutic strategy. In order to monitor the migration and differentiation of EPCs following transplantation, several non-invasive in vivo tracking imaging techniques, such as nuclear medicine and fluorescence imaging, have been investigated. Owing to their simplicity and relative safety, fluorescence labeling methods have been frequently used to identify and track EPCs in preclinical and biological studies. Moreover, fluorescence labeling represents a faster and more accurate modality to obtain experimental data \[25\]. In our previous study, we labeled EPCs by CFSE in vitro and tracked them successfully in vivo in a mouse model of retinal injury \[1\]. CFSE is a lipophilic molecule which displays minimal fluorescence until it enters cells by passive diffusion, where intracellular esterases cleave the acetyl groups.
to yield highly fluorescent, amine reactive fluorophores\cite{26-27}. The fluorescence intensity of CFSE in labeled EPCs was found to decrease rapidly in a time-dependent manner. The reason is that fluorescence is inherited by daughter cells after either cell division or cell fusion\cite{28}.

In this study, we further employed two other agents for fluorescence, \textit{i.e.} DiI-AcLDL and GFP, and compared their relative advantages and disadvantages. Low-density
lipoprotein (LDL) delivers cholesterol via receptor-mediated endocytosis by binding to a specific receptor on the cell surface. The labeled LDL, such as that with an acetylated (Ac) apoprotein, can be used to study cell types that express this Ac type receptors, e.g. endothelial and microglial cells. Dil is a lipophilic membrane stain which diffuses laterally into and stains the entire cell. It exhibits weak fluorescence until its incorporation into the membrane. The unique properties of Dil-AcLDL make it a particular suitable and specific fluorescent dye for labeling of EPCs, endothelial cells, and macrophages; other cell types, such as fibroblasts, smooth muscle cells, and epithelial cells, cannot be labeled by Dil-AcLDL.[12-13]. Dil-AcLDL-labeled EPCs displayed intensive expression of red florescence, and the florescence lasted for several weeks even though the intensity showed a gradual decline. The fluorescent intensity of Dil-AcLDL-labeled EPCs seemed lower than that of EPCs in the CFSE group; however, the decay rate of Dil-AcLDL was also lower than that of CFSE. After 28d, EPCs labeled by Dil-AcLDL maintained about 49.99% fluorescence intensity, while those in the CFSE group exhibited only 20.23% intensity. The rapid decrease in fluorescence intensity of CFSE and Dil-AcLDL over time is a disadvantage, which limits their use for long-term imaging. In such cases, another kind of fluorescence GFP was investigated. GFP can be transfected into cells, typically by plasmid or virus as vectors, or into transgenic mice.[29]. We have applied liposomal and nonliposomal transfection reagents to transfect GFP into EPCs; however, we found it difficult to get stable transfected cells due to low transfection rate and short expression time of the transient transfection (data not shown). Thus, in this experiment, we employed lentivirus to mediate GFP transfection in EPCs. EPCs transfected with GFP using lentivirus showed a time-dependent increase in florescence intensity which reached up to a 30% positive labeling rate; the associated fluorescence was stable and lasted longer than that associated with use of CFSE and Dil-AcLDL. However, folding of GFP into its active, fluorescent form is quite slow and occurs over hours, which makes it unsuitable for study of fast transcriptional activation processes. The weak light emitted by GFP, poor resistance to photobleaching, and low protein expression in certain environments limit its application for labeling of EPCs. Moreover, lentivirus-mediated GFP transfection in EPCs requires a complicated and time-consuming operative procedure. The potential risks of viral replication and carcinogenicity associated with the use of lentivirus is also a safety issue in pre-clinical and clinical application.

Three kinds of fluorescent markers showed different outcomes, advantages and disadvantages. Cell labeling did not affect cell morphology, survival, or adhesion at 2 and 7d, which suggests that cell labeling did not influence the physiological functioning of EPCs. The positive labeling rate with use of CFSE, Dil-AcLDL, and GFP was 95%, 80% and 30%, respectively; a similar trend was observed with respect to the initial fluorescence intensity (high to low, respectively). Duration of fluorescence was lowest with CFSE and highest with GFP; the latter was associated with the highest operational difficulty and experimental cost.

The characteristics of CFSE, Dil-AcLDL, and GFP were also evaluated in vivo in a mouse model of retinal injury. Fluorescent cells were observed on the surface of retina for 2d after transplantation of EPCs labeled with CFSE and Dil-AcLDL. After 7d, a lot of CFSE-labeled EPCs were found to have aggregated at the sites of injury, and the intensity of green fluorescence emitted by CFSE-labeled EPCs looked stronger than the red Dil-AcLDL fluorescence at each time-point. Twenty eight days later, fluorescent cells in the CFSE and Dil-AcLDL groups were found distributed among the retinal layers, and especially in the retinal nerve fiber layer and the inner nuclear layer. Although the grafted EPCs still expressed visible fluorescence at 28d, the intensity declined thereafter. More intense cell staining may be required for a longer term study in order to overcome the fluorescence decay due to cell division. Results of angiography were similar to those of frozen section. However, fluorescent sodium caused obvious leakage due to its small molecular weight, and thus could not exhibit clear retinal vascular structure. Evans blue angiography of the retina clearly displayed retinal microvasculature. GFP-labeled EPCs were not detected in vivo although they did show a protective effect against laser damage. This could be due to: 1) loss of GFP owing to its water-solubility, or deformation during sample preparation; 2) GFP-labeled EPCs did not express GFP or the expression of GFP was too low to be detected; 3) photobleaching was observed in GFP-labeled EPCs. We are still working to improve the experimental protocol.

In summary, the three kinds of fluorescent markers used in this study have their own inherent advantages and disadvantages. CFSE and Dil-AcLDL are suitable for short-term EPC-labeling, while GFP should be used for long-term labeling. The choice of fluorescent agents should be based on the purpose of the study.

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