• Basic Research •

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

Hui Shi[1], *Xin-Rui Wang*[2], *Ming-Chao Bi*[1], *Wei Yang*[1], *Dan Wang*[1], *Hai-Le Liu*[1], *Ling-Ling Liang*[1], *Xiao-Hong Li*[1], *Qian Hao*[1], *Zhi-Hua Cui*[1], *E Song*[3,1]

[1]Department of Ophthalmology, First Hospital, Jilin University, Changchun 130021, Jilin Province, China
[2]Key Laboratory of Zoonosis Research, Ministry of Education, Institute of Zoonosis, College of Veterinary Medicine, Jilin University, Changchun 130062, Jilin Province, China
[3]Department of Ophthalmology, Lixiang Eye Hospital, Soochow University, Suzhou 215021, Jiangsu Province, China
Correspondence to: E Song. Department of Ophthalmology, Lixiang Eye Hospital, Soochow University, Suzhou 215021, Jiangsu Province, China. songe23@163.com; Zhi-Hua Cui. Department of Ophthalmology, First Hospital, Jilin University, Changchun 130021, Jilin Province, China. cui3201@sina.com
Received: 2017-06-04 Accepted: 2017-08-18

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 (Dil-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 Dil-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 florescent 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 Dil-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 Dil-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 photocoagulation

DOI:10.18240/ijo.2018.04.07

Citation: Shi H, Wang XR, Bi MC, Yang W, Wang D, Liu HL, Liang LL, Li XH, Hao Q, Cui ZH, Song E. Comparison of three fluorescence labeling and tracking methods of endothelial progenitor cells in laser-injured retina. *Int J Ophthalmol* 2018;11(4):580-588

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 endothelial 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[4]. 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 neovasculature at ischemic sites. Subsequent studies by other groups also demonstrated the existence of circulating EPCs[5-8]. 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[1]. 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′,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[1,13]. In brief, human umbilical cord blood was collected in blood bags (Negale, 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℃ 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 2×10^6 cells/cm^2 on human fibronectin-coated plates (Chemicon Inc., USA), and then incubated at 37℃ in an atmosphere of 5% CO_2. 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 DiI-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℃ for 15min or 10 μg/mL DiI-AcLDL (Molecular Probes Biotec, USA) at 37℃ 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℃ in a 5% CO_2

incubator for 5h, and then 400 μL DMEM in 20% FBS was added for 24h. The supernatant of 293T cells was collected at 48 and 72h, and virus solution obtained by 0.45 μm filtration. EPCs were mixed with virus solution and incubated at 37℃ in 5% CO_2.

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 1×10^5 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 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 florescence intensity at the beginning, which dropped gradually in a time-dependent

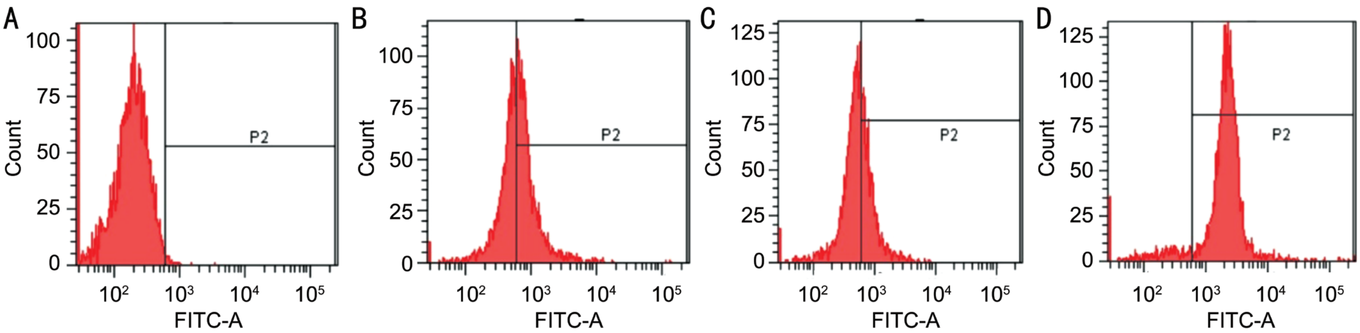

Figure 1 Expressions of EPC-specific markers, CD34, CD133, and VEGFR-2 detected by FACS A: Negative control; B: CD34; C: CD133; D: VEGFR-2.

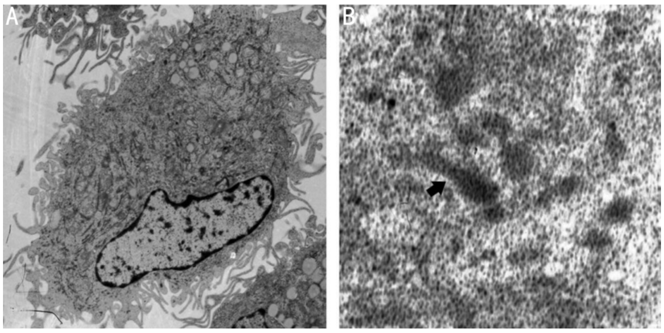

Figure 2 EPCs and Weibel-Palade bodies observed under electron microscopy A: One single cell with tiny microvilli on the surface, heterochromatin in oblong nucleus, and various cytoplasmic organelles (4000×); B: Part of a cell with several elongated Weibel-Palade bodies, which are the storage granules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

Table 1 Characteristics of EPCs labeled with three different fluorescent agents

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

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

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% florescence 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.

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).

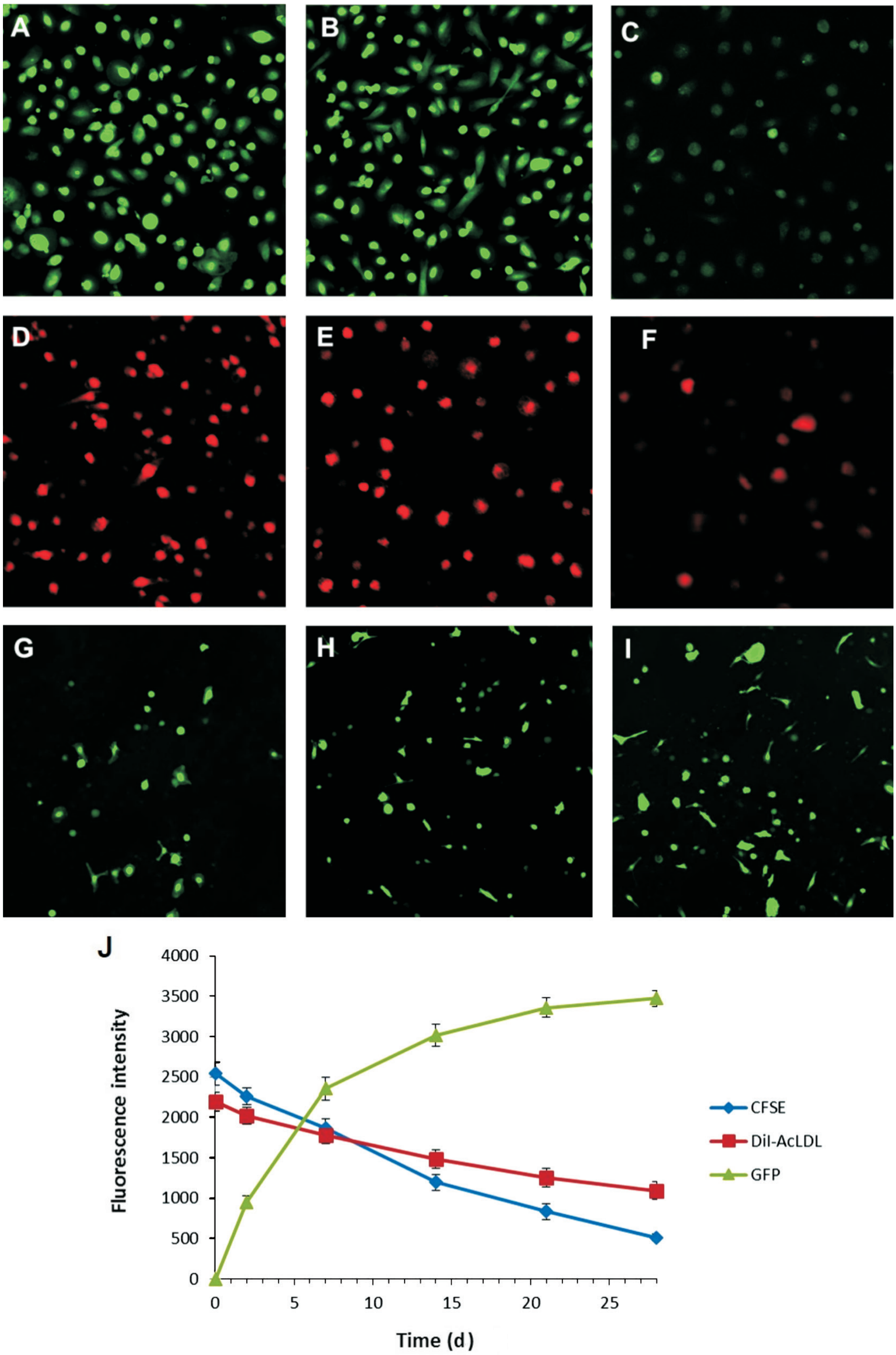

Figure 3 Stability of fluorescence intensity of labeled EPCs A-I: Fluorescent intensity of EPCs labeled with CFSE (A: 7d; B: 14d; C: 28d), DiI-AcLDL (D: 7d; E: 14d; F: 28d) and GFP (G: 7d; H: 14d; I: 28d) was examined by confocal laser scanning microscopy (magnification ×400). J: Statistical analysis of the fluorescent intensity of EPCs labeled by CFSE, DiI-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[19]. We showed

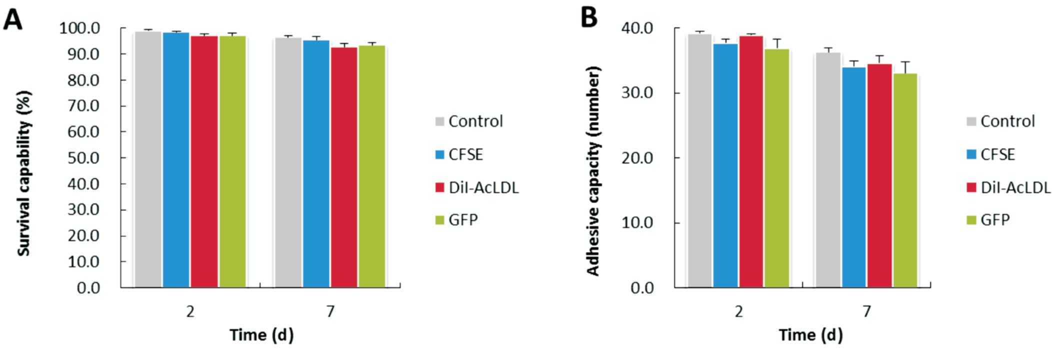

Figure 4 Survival and adhesive capability of EPCs labeled with CFSE, DiI-AcLDL, and GFP after 2 and 7d A: Survival capability of labeled EPCs; B: Adhesive capability of labeled EPCs.

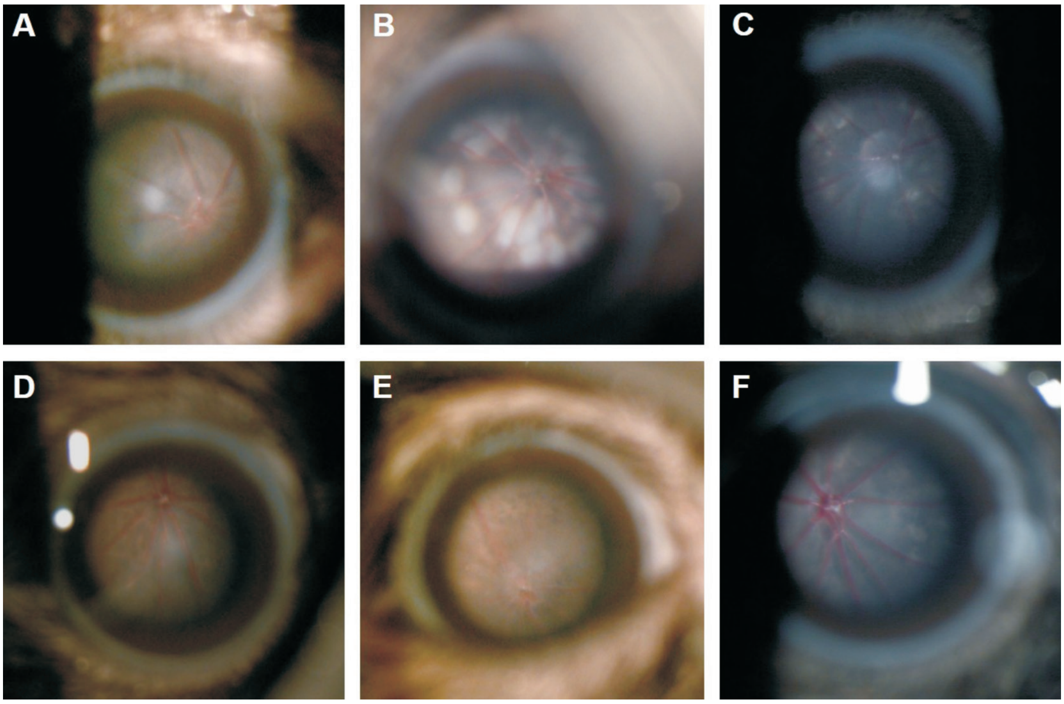

Figure 5 Fundus photography before and after transplantation of EPCs Retinal injury was induced in C57BL/6N mice by laser photocoagulation. A: The optic disc and retinal vessels in normal fundus of C57BL/6N mice; B: Laser photocoagulation induced injury of retinal veins and capillaries around the optic disc; C: Fundus of mice injured by laser photocoagulation at 28d; D-F: Fundus of mice injured by laser photocoagulation 28d after transplantation of EPCs labeled with CFSE (D), DiI-AcLDL (E) and GFP (F).

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 *et al*[9] demonstrated that healthy EPCs could effectively repair ischemic vascular damage in neonatal animal models of oxygen-induced retinopathy. Medina *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

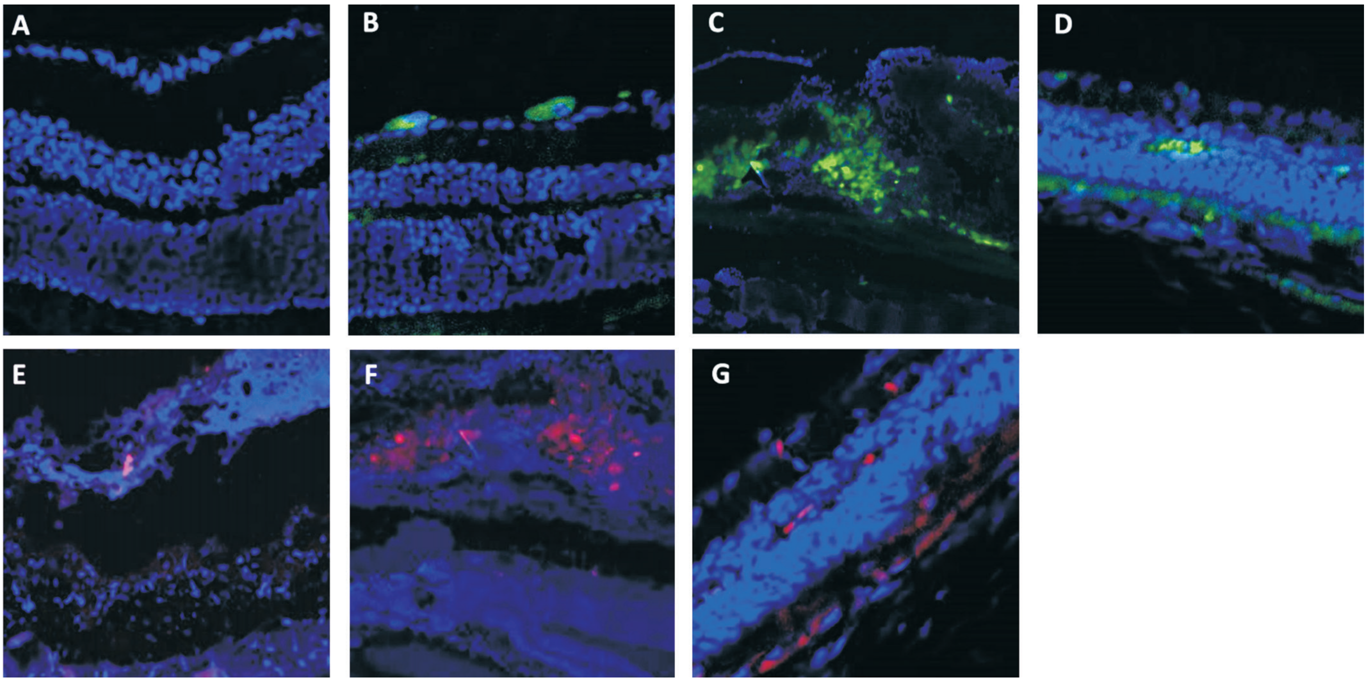

Figure 6 Tracking of EPCs in retina by frozen section before and after EPCs transplantation Mice were sacrificed at 2, 7, 28d after laser photocoagulation. Frozen sections of the eye balls were stained by 1:200 Hoechst and examined by confocal microscopy. A: Normal retina; B-D: Injured retina treated with CFSE-labeled EPCs (B: 2d; C: 7d; D: 28d); E-G: Injured retina treated with DiI-AcLDL-labeled EPCs (E: 2d; F: 7d; G: 28d) (magnification ×400).

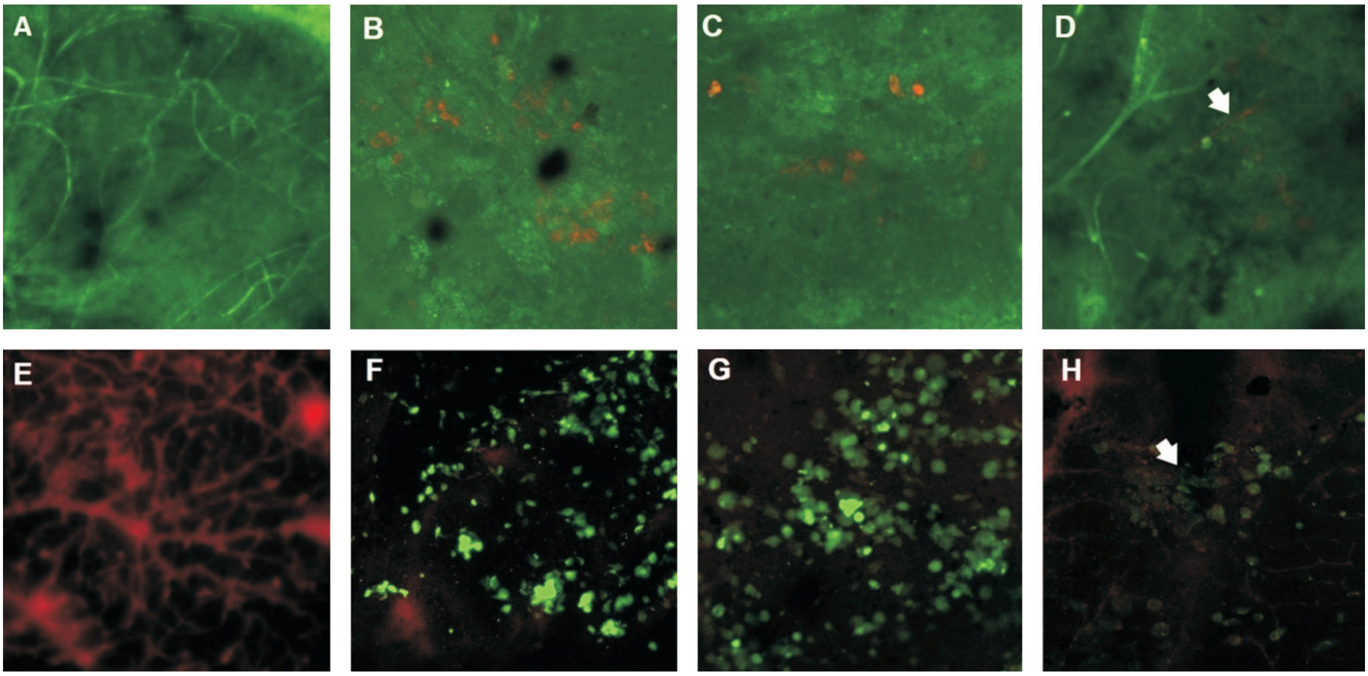

Figure 7 Fluorescein angiography of flat-mounted retinal specimens before and after transplantation of EPCs A-D: CFSE-labeled EPCs before and after transplantation by Evans blue angiography; A: Normal retinal vascular structure; B: Green fluorescent cells in the retina 2d after intravitreal injection; C: EPCs aggregated at the sites of photocoagulation at 7d; D: Green cells formed tube-like structures (arrow) in the retina at 28d; E-H: DiI-AcLDL-labled EPCs before and after transplantation by fluorescent sodium angiography; E: Normal retinal vascular structure; F, G: A population of red fluorescent cells can be seen spread in the retina at 2 and 7d after intravitreal injection; H: Labeled red cells have formed networks (arrow) at 28d. Fluorescence was observed by confocal laser microscopy (magnification ×200).

to yield highly fluorescent, amine reactive fluorophores[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[28].

In this study, we further employed two other agents for fluorescence, *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. DiI 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 DiI-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 DiI-AcLDL[12-13].

DiI-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 DiI-AcLDL-labeled EPCs seemed lower than that of EPCs in the CFSE group; however, the decay rate of DiI-AcLDL was also lower than that of CFSE. After 28d, EPCs labeled by DiI-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 DiI-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 DiI-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, DiI-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, DiI-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 DiI-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 DiI-AcLDL fluorescence at each time-point. Twenty eight days later, fluorescent cells in the CFSE and DiI-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 DiI-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.

ACKNOWLEDGEMENTS

Foundations: Supported by the National Natural Science Foundation of China (No.81400403); the International Science and Technology Cooperation Program of Jilin Province (No.20110733); the Technology Program of Soochow City (No.SYS201375).

Conflicts of Interest: Shi H, None; **Wang XR,** None; **Bi MC,** None; **Yang W,** None; **Wang D,** None; **Liu HL,** None; **Liang LL,** None; **Li XH,** None; **Hao Q**, None; **Cui ZH,** None; **Song E,** None.

REFERENCES

1 Shi H, Yang W, Cui ZH, Lu CW, Li XH, Liang LL, Song E. Tracking of CFSE-labeled endothelial progenitor cells in laser-injured mouse retina. *Chin Med J (Engl)* 2011;124(5):751-757.

2 Dorrell M, Uusitalo-Jarvinen H, Aguilar E, Friedlander M. Ocular neovascularization: basic mechanisms and therapeutic advances. *Surv Ophthalmol* 2007;52 Suppl 1:S3-S19.

3 Delles C, Michelson G, Harazny J, Oehmer S, Hilgers KF, Schmieder RE. Impaired endothelial function of the retinal vasculature in hypertensive patients. *Stroke* 2004;35(6):1289-1293.

4 Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 1997;275(5302):964-967.

5 Hur J, Yoon CH, Kim HS, Choi JH, Kang HJ, Hwang KK, Oh BH, Lee MM, Park YB. Characterization of two types of endothelial progenitor cells and their different contributions to neovasculogenesis. *Arterioscler Thromb Vasc Biol* 2004;24(2):288-293.

6 Rehman J, Li J, Orschell CM, March KL. Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors. *Circulation* 2003;107(8):1164-1169.

7 Reinisch A, Hofmann NA, Obenauf AC, Kashofer K, Rohde E, Schallmoser K, Flicker K, Lanzer G, Linkesch W, Speicher MR, Strunk D. Humanized large-scale expanded endothelial colony-forming cells function in vitro and in vivo. *Blood* 2009;113(26):6716-6725.

8 Ingram DA, Mead LE, Tanaka H, Meade V, Fenoglio A, Mortell K, Pollok K, Ferkowicz MJ, Gilley D, Yoder MC. Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. *Blood* 2004;104(9):2752-2760.

9 Caballero S, Sengupta N, Afzal A, Chang KH, Li Calzi S, Guberski DL, Kern TS, Grant MB. Ischemic vascular damage can be repaired by healthy, but not diabetic, endothelial progenitor cells. *Diabetes* 2007;56(4):960-967.

10 Goldenberg-Cohen N, Avraham-Lubin BC, Sadikov T, Askenasy N. Effect of coadministration of neuronal growth factors on neuroglial differentiation of bone marrow-derived stem cells in the ischemic retina. *Invest Ophthalmol Vis Sci* 2014;55(1):502-512.

11 Li X, Dancausse H, Grijalva I, Oliveira M, Levi AD. Labeling Schwann cells with CFSE-an in vitro and in vivo study. *J Neurosci Methods* 2003;125(1-2):83-91.

12 Barnett JM, Penn JS, Jayagopal A. Imaging of endothelial progenitor cell subpopulations in angiogenesis using quantum dot nanocrystals. *Methods Mol Biol* 2013;1026:45-56.

13 Song E, Lu CW, Fang LJ, Yang W. Culture and identification of endothelial progenitor cells from human umbilical cord blood. *Int J Ophthalmol* 2010;3(1):49-53.

14 Tsien RY. The green fluorescent protein. *Annu Rev Biochem* 1998;67:509-544.

15 Gubin AN, Reddy B, Njoroge JM, Miller JL. Long-term, stable expression of green fluorescent protein in mammalian cells. *Biochem Biophys Res Commun* 1997;236(2):347-350.

16 Nakagawa Y, Masuda H, Ito R, Kobori M, Wada M, Shizuno T, Sato A, Suzuki T, Kawai K, Asahara T. Aberrant kinetics of bone marrow-derived endothelial progenitor cells in the murine oxygen-induced retinopathy model. *Invest Ophthalmol Vis Sci* 2011;52(11):7835-7841.

17 Chang KH, Chan-Ling T, McFarland EL, Afzal A, Pan H, Baxter LC, Shaw LC, Caballero S, Sengupta N, Li Calzi S, Sullivan SM, Grant MB. IGF binding protein-3 regulates hematopoietic stem cell and endothelial precursor cell function during vascular development. *Proc Natl Acad Sci U S A* 2007;104(25):10595-10600.

18 Guthrie SM, Curtis LM, Mames RN, Simon GG, Grant MB, Scott EW. The nitric oxide pathway modulates hemangioblast activity of adult hematopoietic stem cells. *Blood* 2005;105(5):1916-1922.

19 Rui G, Wei W, Yuliang W, Kai L, Xiaobing C, Changle Z, Longshu S. Protective effects of Radix Pseudostellariae extract against retinal laser injury. *Cel Physiol Biochem* 2014;33(6):1643-1653.

20 Scotti F, Maestroni A, Palini A, Introini U, Setaccioli M, Lorenzi M, Zerbini G. Endothelial progenitor cells and response to ranibizumab in age-related macular degeneration. *Retina* 2014;34(9):1802-1810.

21 Stitt AW, O'Neill CL, O'Doherty MT, Archer DB, Gardiner TA, Medina RJ. Vascular stem cells and ischaemic retinopathies. *Prog Retin Eye Res* 2011;30(3):149-166.

22 Liu X, Li Y, Liu Y, Luo Y, Wang D, Annex BH, Goldschmidt-Clermont PJ. Endothelial progenitor cells (EPCs) mobilized and activated by neurotrophic factors may contribute to pathologic neovascularization in diabetic retinopathy. *Am J Pathol* 2010;176(1):504-515.

23 Lee IG, Chae SL, Kim JC. Involvement of circulating endothelial progenitor cells and vasculogenic factors in the pathogenesis of diabetic retinopathy. *Eye (Lond)* 2006;20(5):546-552.

24 Medina RJ, O'Neill CL, Humphreys MW, Gardiner TA, Stitt AW. Outgrowth endothelial cells: characterization and their potential for reversing ischemic retinopathy. *Invest Ophthalmol Vis Sci* 2010;51(11):5906-5913.

25 Thebaud NB, Aussel A, Siadous R, Toutain J, Bareille R, Montembault A, David L, Bordenave L. Labeling and qualification of endothelial progenitor cells for tracking in tissue engineering: an in vitro study. *Int J Artif Organs* 2015;38(4):224-232.

26 Parish CR, Warren HS. Use of the intracellular fluorescent dye CFSE to monitor lymphocyte migration and proliferation. *Curr Protoc Immunol* 2002;Chapter 4:Unit 4.9.

27 Dumitriu IE, Mohr W, Kolowos W, Kern P, Kalden JR, Herrmann M. 5,6-carboxyfluorescein diacetate succinimidyl ester-labeled apoptotic and necrotic as well as detergent-treated cells can be traced in composite cell samples. *Anal Biochem* 2001;299(2):247-252.

28 Lyons AB. Analysing cell division in vivo and in vitro using flow cytometric measurement of CFSE dye dilution. *J Immunol Methods* 2000;243(1-2):147-154.

29 Grant MB, May WS, Caballero S, Brown GA, Guthrie SM, Mames RN, Byrne BJ, Vaught T, Spoerri PE, Peck AB, Scott EW. Adult hematopoietic stem cells provide functional hemangioblast activity during retinal neovascularization. *Nat Med* 2002;8(6):607-612.