Transplanted olfactory ensheathing cells reduce retinal degeneration in Royal College of Surgeons rats

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Short running title: OECs/ONFs transplanted in RCS rats

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Abstract

Purpose of the study: Retinitis pigmentosa (RP) is a group of genetic disorders and a slow loss of vision is caused due to a cascade of retinal degenerative events. We examined whether these retinal degenerative events were relieved after the mixtures of cultured adult olfactory ensheathing cells (OECs) and olfactory nerve fibroblasts (ONFs) were transplanted into the subretinal space of one month old RCS rat, a classic model of RP.

Materials and Methods: After cell transplantation, the changes in retinal photoreceptors and Müller cells of RCS rats were tested respectively by recoverin expression, PNA-positive cones counting, Caspase-positive apoptotic figures and GFAP expression by immunohistochemistry, and retinal function was detected by Flash ERG. For further investigation into the mechanisms of OECs/ONFs playing important roles in retinas, NGF, BDNF, and bFGF secretion of the cultured cells were analyzed through ELISA. The ability of OECs/ONFs to ingest porcine retinal outer segments and the amount of phagocytosis were compared with retinal pigment epithelium (RPE) cells.

Results: Our research showed that retinal degenerative events were relieved including recoverin expression, protected retinal outer segments, increased PNA-positive cones and reduced Caspase-positive apoptotic figures, downregulated GFAP, and the maintained b-wave of the ERG after OECs/ONFs transplantation. Cultured OECs/ONFs expressed and secreted NGF, BDNF, and bFGF which were sufficient to assist survival of the photoreceptors. In vitro Phagocytosis Assay, we showed the OECs, but not the ONFs, can phagocytose porcine retinal outer segments, and the phagocytic ability of OECs was even stronger than that of RPE cells.

Conclusions: These findings clearly demonstrated that transplantation of OECs/ONFs cleaned up the accumulated debris in subretinal space, and provided an intrinsic continuous supply of

neurotrophic factors. It suggested that transplantation of OECs/ONFs might be a possible future route for protection of the retina and reducing retinal degeneration in RP.

Key words: retinitis pigmentosa, photoreceptor, neurotrophic factors, phagocytosis, OECs/ONFs.

Introduction

Retinitis pigmentosa (RP) is a group of genetic disorders and a slow loss of vision is caused due to a cascade of retinal degenerative events. The Royal College of Surgeons (RCS) rat is a classic animal model of RP with Mer tyrosine kinase (MerTK) gene expression defects, which results in a failure of the retinal pigmented epithelium (RPE) to internalize the retinal outer segments (ROS). [1] The circadian shedding of the tips of ROS, and their engulfment by the adjacent RPE is vital to the survival of the retina. The resultant accumulation of debris in the subretinal space [2] led to loss of photoreceptors, increased glial fibrillary acidic protein (GFAP) expression in the retinal Müller cells [3] and glial scar formation, and large-scale retinal remodeling [4]. Until now, there is no cure for RP.

More recent studies have shown that transplantation of olfactory ensheathing cells (OECs) has become one of the most promising therapies for damage to the nervous system by protecting neurons [5]. OECs are thought to open up the glial scar, and provide a pathway for the successful axon regeneration throughout the life of adult mammals [6-9], which is positively influenced by the presence of olfactory nerve fibroblasts (ONFs) [10]. However, the possibility of their application in pathology and trauma of the retina and optic nerve has only recently been explored [11-15].

Here we report morphological and visual function analysis of RCS rats transplanted with mixture of OECs/ONFs. Compared with unoperated and sham operated groups at the same age, we noted that RCS rats at 4, 8 and 12 weeks after OECs/ONFs transplantation had an increased survival

of photoreceptors by detecting recoverin, retinal outer segments, and PNA-positive cones. Caspase-positive apoptotic figures in the outer nuclear layer were decreased. The upregulation of GFAP by the Müller glia was reduced, and the b-wave of electroretinography (ERG) was maintained. Little is known about the mechanisms of transplanted OECs/ONFs playing important roles in retinas. Given that the transplanted OECs/ONFs have protecting effect on degenerative retinas, we examined the expression of neurotrophic factors and identified the cultured OECs/ONFs mixtures expressed and secreted NGF, BDNF, and bFGF which were sufficient to assist survival of the photoreceptors. Furthermore, in a culture assay, we surprisingly noted that the OECs, but not the ONFs, can phagocytose porcine ROS, and the phagocytic ability of OECs was even stronger than that of RPE cells.

In summary, this study reveals the important functions of OECs/ONFs transplanted into subretinal space. It also suggests transplantation of OECs/ONFs might be a possible future route for protection of the retina and reducing retinal degeneration in RP.

Materials and Methods

Cell preparation

OECs/ONFs culture Primary cultures of OECs and their accompanying ONFs were obtained from adult (about 250g) RCS-rdy^{+}-P^{+} rats. All experimental procedures were performed according to the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 85-23). Details of the procedures are as the publication before [16]. Briefly, the outer nerve and glomerular layers of olfactory bulbs were dissected out, dissociated in 0.1% trypsin for 15 min at 37 °C, plated on to 35mm dishes coated with poly-L-lysine, and cultured for 14 days in DMEM/F-12 culture medium and 10% fetal bovine serum (FBS) (Gibco, U.S.A). On the 14th day, there were about

3×10^5 cells per dish, of which 50% were S100-positive OECs and 50% were fibronectin-positive ONFs.

RPE culture Fresh porcine eyes were obtained from the slaughter house and transported in cold phosphate buffered saline (PBS), pH 7.2. The RPE cells were isolated according to the method of Grisanti and Guidry [17]. Eyeballs were dissected, and the anterior parts including neural retina were removed. The RPE cells were dissociated from the eyecups by 30min incubation with 0.25% trypsin and 0.02% EDTA at 37°C. The enzyme reaction was terminated by DMEM containing 10% FBS. The cells were plated on to 35mm dishes coated with poly-L-lysine after centrifugation. The medium was changed every 2–3 days. Once the cells formed a monolayer, they were passaged. The 4th passage was used for the phagocytosis experiment in the same number cells with OECs/ONFs.

Transplantation

At day 9 the cultured OECs/ONFs were transfected with a lentivirus stably expressing EGFP [18], and the cells were harvested at day 14. Flow cytometry based on EGFP expression confirmed that about 80% of the cultured cells were labeled (Fig.1C). Cells were dissociated into single-cell suspension, washed with PBS, and kept on ice during surgery.

30 days old RCS-P$^+$ rats (n=9) were anesthetized with intraperitoneal chloral hydrate (0.4 ml/100 g). The superior temporal pole of the sclera exposed. The sclera and choroid were penetrated by a 30g syringe needle, and 3µl of a suspension containing around 100,000 cultured OECs/ONFs injected into the subretinal space (Fig.1 A); controls were provided by injections of 3µl 0.01 M PBS (n=9). The scleral aperture was closed by 10-0 sutures. Further controls were provided by 9 age matched unoperated rats.

ERG recording

The magnitude of the b-wave of the ERG was used as a surrogate indicator of retinal function [19]. ERG responses were recorded at 4, 8 and 12 weeks after transplantation. Following overnight dark adaptation, animals were prepared for recording under dim red light. Under intraperitoneal anesthesia with chloral hydrate, pupils were dilated using Compound Tropicamide Eye Drops. A drop of Carboxymethylcellulose Sodium Lubricant was applied on the cornea before recording to prevent dehydration and allow electrical contact with the recording electrode. A 25-gauge needle inserted under the scalp served as the reference electrode and with a ground electrode under the tail skin. Amplification was at 0.1–300 Hz bandpass, without notch filtering, stimulus presentation and data acquisition were provided by Retiport 32 4.3.8 software of the Roland Electrophysiological Systems (Germany). 27 eyeballs were tested, consisting of n=3 at each of 4, 8 and 12 weeks of age in three groups: (a) with OECs/ONFs, (b) with injection of PBS only, and (c) unoperated controls. For the quantification of dark-adapted b-waves, recordings consisted of single flash presentations (duration <5ms), repeated 3 times to verify the response reliability and improve the signal-to-noise ratio, and duration inter-stimuli-intervals was longer than 2 seconds.

Immunostaining

Immediately following ERG recording, the rats were anesthetized with intraperitoneal urethane and transcardially perfused with normal saline. Eyes were enucleated. The eyecups were immersed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at pH 7.4 at 4°C for 2 h, transferred to 10%, then 30% sucrose in 0.1 M PB, and stored at 4°C overnight. Next day the eyecups were embedded in optimum cutting temperature (O.C.T.) compound and 10μm thick sections cut in the mid-sagittal plane (Fig.1 A) on a cryostat (Leica, Germany) at −20°C. The sections were treated for 5 min at room temperature with 0.5% Triton X-100 followed by 1h in 5% goat serum in PBS, immunostained for

recoverin (Pro tech,1:500 diluted), caspase-3 (Bioworlde, USA, 1:400 diluted; a marker of apoptosis) and GFAP (Boster, China, 1:400 diluted) overnight at 4°C. Next day sections were washed three times (15 min each) with PBS, incubated for 2h at room temperature in the dark in secondary antibodies, either goat anti-rabbit IgG-cy3 (Beyotime, China) or goat anti-mouse IgG-cy3 (Beyotime, China). All secondary antibodies were used at a 1:500 dilution in 0.01M PBS. The sections were washed and incubated for 5min at room temperature with DAPI (Beyotime, China), and coverslipped with water soluble mounting liquid (Boster, China), examined in the confocal microscope. For immunostaining with peanut agglutinin (PNA)-cy3 (1:400; a marker of the extracellular matrix domain surrounding cone photoreceptor outer segments) [20], the primary antibody was incubated at room temperature for one hour, followed by 5min DAPI counterstain, and the sections washed and mounted as above. The thickness of recoverin-positive photoreceptors, the depth of the outer segment layer, and the caspase-3 positive area were measured from confocal images with image-analysis software Image-Pro Plus 6.0 (IPP 6.0). The numbers of PNA positive cones were counted manually.

Enzyme linked immunosorbent assay (ELISA) of culture media

The OECs/ONFs culture supernatant of the last change before transplantation was filtered through a 0.22μm syringe filter. The concentrations of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and basic fibroblast growth factor (bFGF) in the OECs/ONFs culture supernatants were quantified using ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Culture medium without OECs/ONFs was used as control. The optical density was read at 450nm wavelength within 30min. The standard curve for each factor diluted in assay diluents (block and sample buffer) provided a linear curve on a plot of absorbance versus concentration.

In vitro assay of phagocytosis (n=32)

Retinal outer segments were isolated from fresh porcine eyes obtained from the slaughter house by modification of the method of Molday et al [21]. The excised retinae were homogenized by gently shaking in a solution consisting of 20% sucrose, 65mM NaCl, 1mM $MgCl_2$, 10mM glucose and 5 mM taurine in 20mM Tris-HCl at pH 7.4. The resultant large pieces of neural retina were pelleted by centrifugation (Eppendorf 5471R) at 1,000 rpm for 5min, re-suspended in the same buffer, centrifuged for 2min at 15,000 rpm at 4°C, then re-suspended in the buffer again and centrifuged at 15,000 rpm at 4°C for 30min. The pellets of ROS discs were stored suspended in 10mM Na-phosphate (pH 7.2), 0.1M NaCl, and 2.5% sucrose at -80°C. Before use, ROS were labeled in 1 mg/ml fluorescein isothiocyanate (FITC; Invitrogen) in 0.1M Na-bicarbonate (pH 9.0), for 1h at room temperature, washed and re-suspended in cell culture medium. Culture wells of OECs/ONFs and control culture wells of RPE were prepared. Fluorescent ROS were added at 0.5×10^5 for each well. Phagocytosis was allowed for 1, 2, 4 and 6 hours under the culture conditions. To quench external FITC fluorescence, samples were incubated with 0.2% trypan blue in PBS-CM for 10min prior to fixation [22]. The cultures were fixed in 4% paraformaldehyde in PBS for 20 min at 4°C. Rat anti-S-100 (Dakocytomation, Denmark, 1:500 diluted) labels OECs, mouse anti-fibronectin (Millipore, USA, 1:1000 diluted) labels ONFs. Goat anti-mouse IgG-FITC and goat anti-rabbit IgG-cy3 (Boster, China) were separately used as second antibodies. The FITC-fluorescence positive area of the ROS was measured with IPP 6.0 as index of ingested ROS.

Statistical analysis

A one-way ANOVA was used to detect differences in the thickness of recoverin positive ONL, ratio of Caspase 3 positive area to ONL thickness and b-wave of ERG recording of the transplanted

eyes, and PBS injected and unoperated control eyes at each timepoint. An independent sample t-test was used to detect total area of FITC positive ROS ingested by culture cells between OECs and RPE, and neurotrophic factors secreted by OECs/ONFs compared with which contained in culture medium without OECs/ONFs. In all cases, significance was taken as $P < 0.01$ or $p < 0.05$.

Results

Transplanted OECs/ONFs spread within subretinal space and entered inner retinas

As previously reported [16], the transplanted OECs/ONFs spread within the host subretinal space (Fig.1B). Twelve weeks after surgery, the transplanted cells had become dispersed through the subretinal space over the entire retina from the temporal injection point as far as the nasal side (Fig.1 D). Within the retinal layers, most of the transplanted cells were in the inner and outer plexiform layers (IPL and OPL), and to a lesser extent in the ganglion cell layer (GCL) and the outer and inner nuclear layers (ONL and INL). The cells generally adopted bipolar or multipolar morphologies.

Transplanted OECs/ONFs offered effective neuroprotective effects for degenerative retinas

To test the effects of the transplanted OECs/ONFs on degenerative retinas, immunofluorescence staining for recoverin and PNA was carried out. Recoverin-positive photoreceptor cells were observed in all transplanted eyes, and in the PBS injected and unoperated control eyes (Fig.2). The thickness of ONL of the host retina stained by recoverin in the sham surgery and unoperated control groups decreased over the age matched period of the transplanted rats (as in [1]). The thickness of the ONL in the OECs/ONFs transplanted rats at 4, 8 and 12 weeks after surgery also decreased, but was significantly greater at all time points than the control groups (Fig.7A). In the transplant groups at 4, 8 and 12 weeks after surgery the numbers of PNA-positivity cones were

2920±378.07, 1920±130.64 and 960±184.75/mm^2 respectively, and the mean lengths of ROS were 35.05±1.06, 25.56±1.82 and 24.84±2.55μm respectively. However, in the PBS injected and unoperated control groups the numbers of PNA^+ cones were 2000±92.38 and 1520±206.56/mm^2 and lengths of ROS were 19.57±5.22 and 22.09±3.76μm at 4 weeks. By 8 and 12 weeks, there were nearly no remaining PNA^+ cones or ROS in the control groups (Fig.3). These data suggested that transplanted OECs/ONFs offered effective neuroprotective effects for degenerative retinas.

To confirm the protective effects of transplanted OECs/ONFs, immunofluorescence staining for Cleaved Caspase-3 was also performed. Meanwhile, to accurate evaluate the degree of apoptosis before and after transplantation, we used the ratio of caspase-3 positive area to thickness of ONL as apoptotic index instead of absolute value of apoptotic cells. Immunofluorescence staining showed that there were more photoreceptor neurons surviving in the transplant groups when compared with PBS injected and unoperated control groups, and the apoptotic cells and cell debris were mainly located in the ONL and subretinal space (SRS) layers. The apoptotic index (Fig.7B) in the transplanted eyes was significantly less at all 3 survival times than in the PBS injected and unoperated control groups (Fig.4).

Comparison of the GFAP positive area in each visual field indicated that Müller cell gliosis in the OECs/ONFs transplanted eyes was markedly less at all three measured times points than in the two control groups (Figures 5 and 7C). In particular, the presence of OECs/ONFs reduced the marked gliosis seen in the SRS of the control groups (e.g. Figure 5E).

Visual Function was significantly improved in the Dystrophic RCS Rat after OECs/ONFs transplantation

To test the effects of the transplanted OECs/ONFs on visual function of dystrophic RCS rats, ERG recording was performed. Over the three age-matched time points, there was a marked decrease

in b-wave of the ERG in the two control groups. In contrast, there was a sustained preservation of the b-wave in the OECs/ONFs transplanted eyes at 4, 8, and 12 weeks after surgery (Figures 7D and 8). Together, the sustained b-wave reflected retinal function improvement, but stillmuch lower than the amplitude of the b-wave of RCSrdy-P+ rats, which were 362.67 ± 11.02, 375.00 ± 2.65, and 454.67 ± 22.01 μV at each timepoint.

Transplanted OECs/ONFs secrets neurotrophic factors

Neurotrophic factors have been proven to exert survival-promoting and trophic actions on neurons. Therefore, we tested whether some neurotrophic factors were secreted by cultured OECs/ONFs through ELISA. The results showed that the concentrations of NGF, BDNF, and bFGF in medium conditioned by 3×10^5 OECs/ONFs for a period of 3 days were NGF 27.01±5.31, BDNF 65.28±12.33, and bFGF 74.65±11.72 (mean ±SD) pg/ml, statistically higher than the control culture medium (n=18) (Fig.7E). These data revealed that transplanted OECs/ONFs can provide an intrinsic continuous supply of neurotrophic factors for degenerative retinas.

Phagocytic ability of the transplanted cells

Immunofluorescence staining showed that the accumulated debris in the subretinal space of RCS rats was reduced after OECs/ONFs transplantation. We guessed the accumulated debris in the subretinal space might be phagocytosed by transplanted OECs/ONFs because some studies have shown that OECs exhibit unique phagocytic properties. To confirm the hypothesis, in vitro phagocytosis assay was carried out. The comparison of total areas of FITC positive ingested ROS at 1, 2, 4, 6 hours after fluorescent ROS were added to the cultured cells are shown in Fig.7D. With increasing time the area of FITC positive ROS increased in the S-100 positive OECs but not in the fibronectin positive ONFs (Fig.6A, B). The accumulation in the OECs/ONFs was much greater than in

the RPE (Fig.6C, D). These results showed the OECs, but not the ONFs, can phagocytose porcine retinal outer segments, and the phagocytic ability of OECs was even stronger than that of RPE cells.

Discussion

Currently, there is no effective treatment for retinitis pigmentosa. Considering that OECs/ONFs hold some important properties such as opening up the glial scar, promoting robust axonal growth, secreting neurotrophic growth factors and so on, we explored the effects of transplantation of OECs/ONFs mixutures as a means of ameliorating retinal degeneration initiation in the RCS rat retinitis pigmentosa model.

The defect in RCS rats results from an inherited mutation in the MerTK gene, which encodes receptor tyrosine kinase and normally expresses in the RPE. Mertk protein involves in the binding of photoreceptor debris and is absent from RCS. Accordingly, a progressive, postnatal failure of RPE to phagocytose shed rod ROS that are continually shed from photoreceptors is one of major characteristics. The resulting accumulation of debris in the subretinal space leads to a progressive loss of photoreceptors. After this, the secondary injuries appear one after another including accumulation of debris in the subretinal space, death of rod and later cone-photoreceptor cells, and a cascade of secondary neuronal death in the remaining retinal layers. Similarly, mutations in MerTK have also been described in patients with RP.

We demonstrated that transplantation of OECs/ONFs mixtures into the subretinal space of 1-month-old RCS rats reduced the damage and loss of photoreceptors, preserves the outer segments, reduced accumulated debris in subretinal space, reduced Müller glial activation and preserved the b-wave of the ERG.

There is previous evidence that OECs exhibit unique migratory and phagocytic properties.23,

24 We can suppose that the transplanted OECs/ONFs can exhibit phagocytic function and compensate the failure of RPE of RCS rats to internalize the ROS. The guess was confirmed by our followed In vitro Phagocytosis Assay experiments. Our results showed that the OECs (but not ONFs) phagocytose porcine retinal outer segments. OECs had even stronger phagocytosis ability than RPE, suggesting that the beneficial effects of the transplants may be due to phagocytosis of outer segment debris.

ELISA analysis of the culture supernatants confirmed previous reports (for NGF,[25, 26] BDNF,27 and bFGF [28)] that the OECs/ONFs mixtures secrete an array of neurotrophic factors which may be significant in maintaining the survival of the photoreceptors.

After death of rod- and cone-photoreceptor cells, the Müller cells respond by forming a dense fibrotic layer in the subretinal space during retinal remodeling. This layer invests the surviving retina and impairs access to the choroidal vasculature.[29] Consonant with the decreased degeneration, GFAP immunostaining was reduced in the OECs/ONFs transplanted eyes in the present study. This may also reflect an opening up of the glial scar.[16,30,31] We propose a working model in which transplanted OECs/ONFs secreted neurotrophic factors that promoted photoreceptor cells survival in degenerative retinas, and cleaned up the accumulated debris in subretinal space. Consequently, debris-stimulated Müller cells activation and glial scar formation were reduced.

There has been considerable research into the use of cell transplantation in retinal degeneration.[1,32,33] Our present observations show good survival of transplanted adult OECs/ONFs mixtures over 12 weeks in the subretinal space of RCS rats. Since human OECs can be isolated from biopsies of the adult olfactory mucosa without adverse effects,[34] autologous transplantation is an attractive choice to avoid immune rejection, and the ethical concerns of using fetal tissue. The use of OECs/ONFs mixtures was suggested by reports that OECs and ONFs had synergistic effects in

promoting axon regeneration.[10,11] The ratio of OECs to ONFs mixture, as controlled by S100 and fibronectin staining in this study, was around 50/50. We have no evidence as to how long these allografts will be tolerated because the degree of immune privilege of the subretinal space is neither absolute nor permanent[35]; However, the current demonstration of an acute protective effects of OECs/ONFs transplantation opens up new horizons in researches of RP therapeutic strategies.

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Illustrations

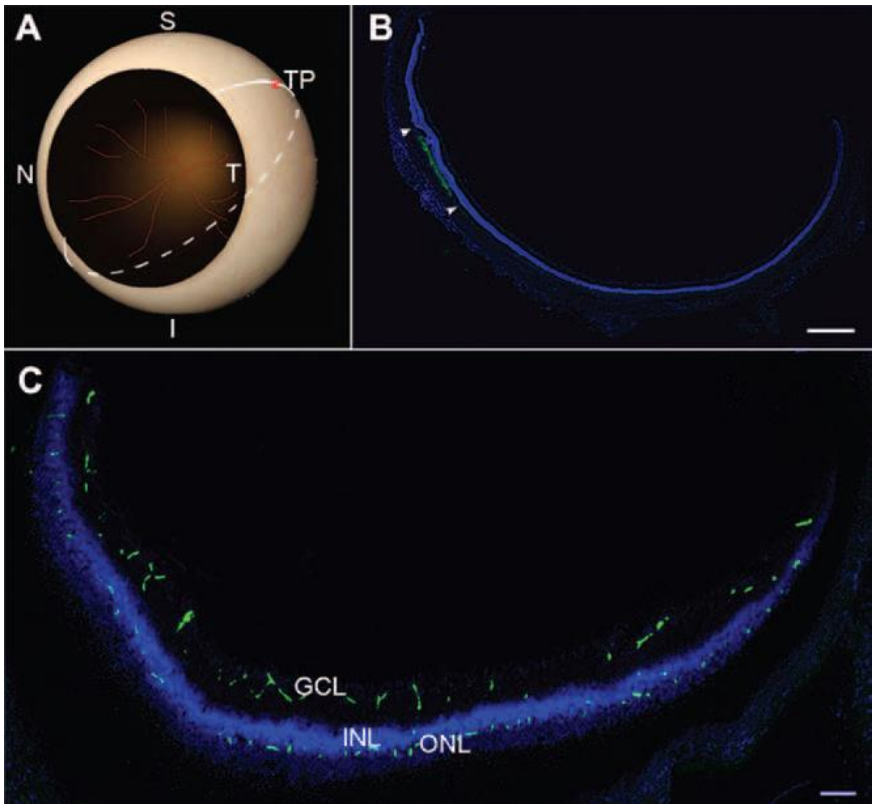

FIGURE 1 Subretinal space transplantation. (A) Schematic diagram of the point of transplantation. S, superior; I, inferior; N, nasal; T, temporal; TP, transplanted point. (B) Spread of the transplanted cells (arrowhead, green) in the subretinal space around the injection site. (C) Spread of the transplanted cells into all the retinal layers. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; Counterstain with DAPI, blue; scale bars, 500 μm (B), 100 μm (C).

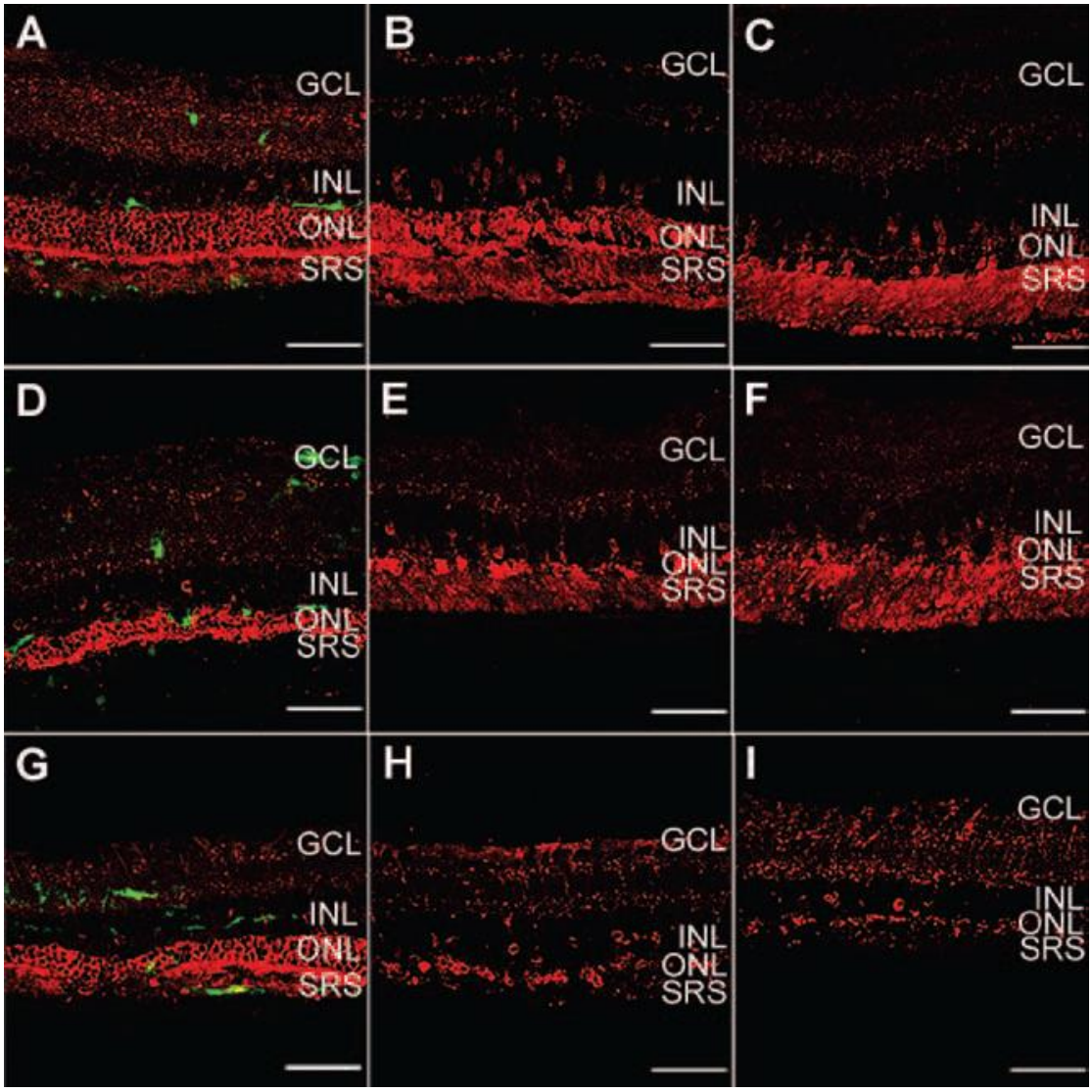

FIGURE 2 Immunofluorescence staining of retinal photoreceptors for recoverin (red). A, D, G: Retinae with transplanted OECs at 4 weeks (A), 8 weeks (D) and 12 weeks (G); B, E, H: With PBS injection at 4 weeks (B), 8 weeks (E) and 12 weeks (H); C, F, I: Unoperated RCS rat at 4 weeks (C), 8 weeks (F) and 12 weeks (I). The recoverin-positive photoreceptors in transplanted groups were much more than the PBS injected control and unoperated control at all time points. SRS, subretinal space. Transplanted cells, green. Scale bars 50 μm.

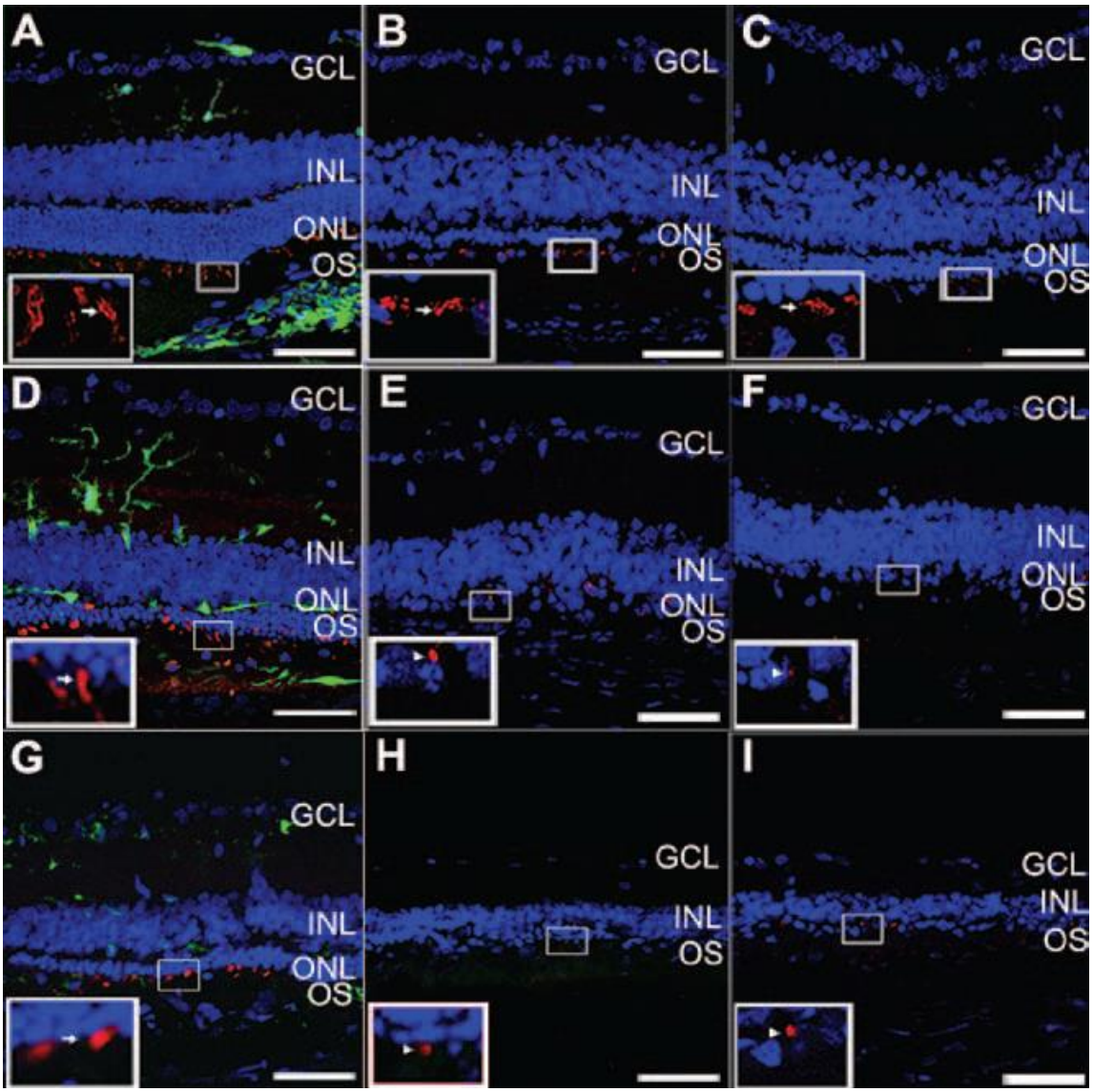

FIGURE 3 Immunofluorescence staining of cone outer segments for PNA (red). A, D, G: Retinae with transplanted OECs at 4 weeks (A), 8 weeks (D) and 12 weeks (G); B, E, H: With PBS injection at 4 weeks (B), 8 weeks (E), and 12 weeks (H); C, F, I: Unoperated RCS rat at 4 weeks (C), 8 weeks (F) and 12 weeks (I). OS, outer segments layer. Counterstain with DAPI, blue; transplanted cells, green; white arrows, outer segments of cone photoreceptor; arrowhead, cone-photoreceptor in OPL. Pictures on the lower left are magnified from the small white frames in centers. Scale bars 50 μm.

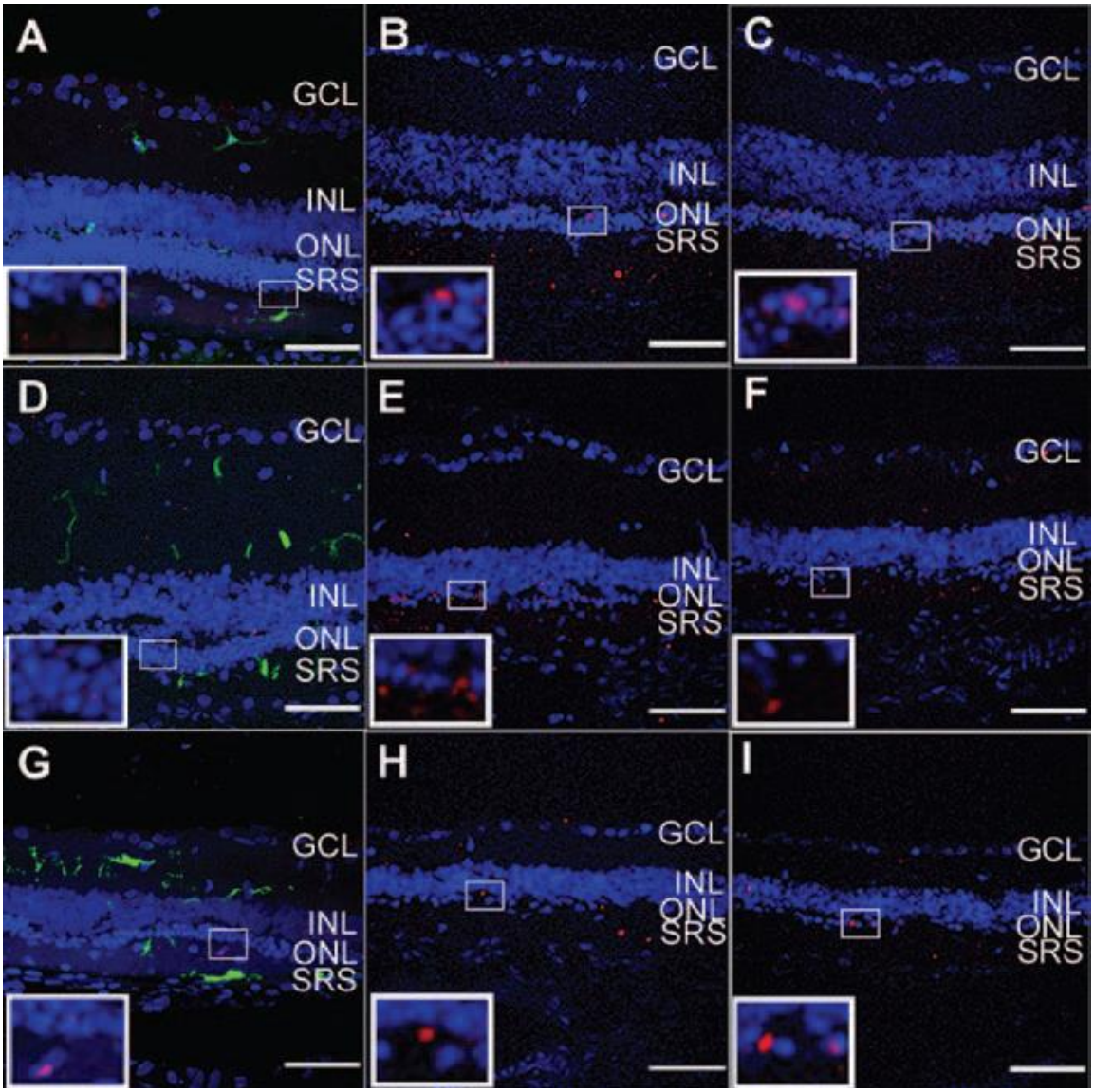

FIGURE 4 Immunofluorescence staining of apoptotic cells for cleaved caspase-3 (red). A, D, G: OECs transplanted retinae at 4 weeks (A), 8 weeks (D) and 12 weeks (G); B, E, H: With PBS injection at 4 weeks (B), 8 weeks (E) and 12 weeks (H); C, F, I: Unoperated RCS rat at 4 weeks (C), 8 weeks (F), and 12 weeks (I). Counterstain with DAPI, blue; transplanted cells, green. Pictures on the lower left are the local magnification of the small white frames in centers. Scale bars 50 μm.

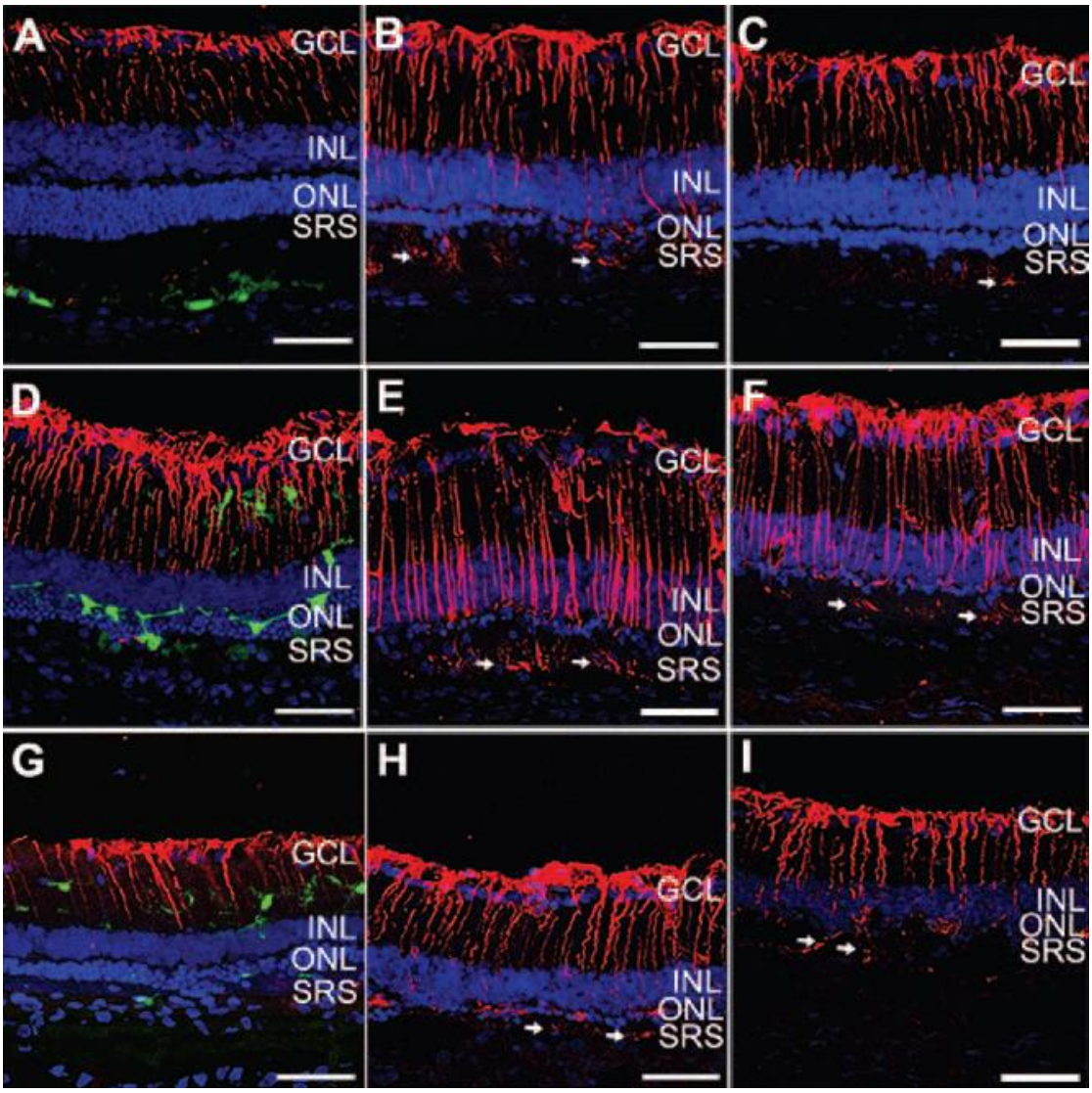

FIGURE 5 Immunofluorescence staining of Müller cells for GFAP (red). A, D, G: OECs transplanted retinae at 4 weeks (A), 8 weeks (D) and 12 weeks (G); B, E, H: PBS injection at 4 weeks (B), 8 weeks (E) and 12 weeks (H); C, F, I: Unoperated RCS rat at 4 weeks (C), 8 weeks (F) and 12 weeks (I). Counterstain with DAPI, blue; transplanted cells, green; white arrows, gliosis in the subretinal space; scale bars 50 μm.

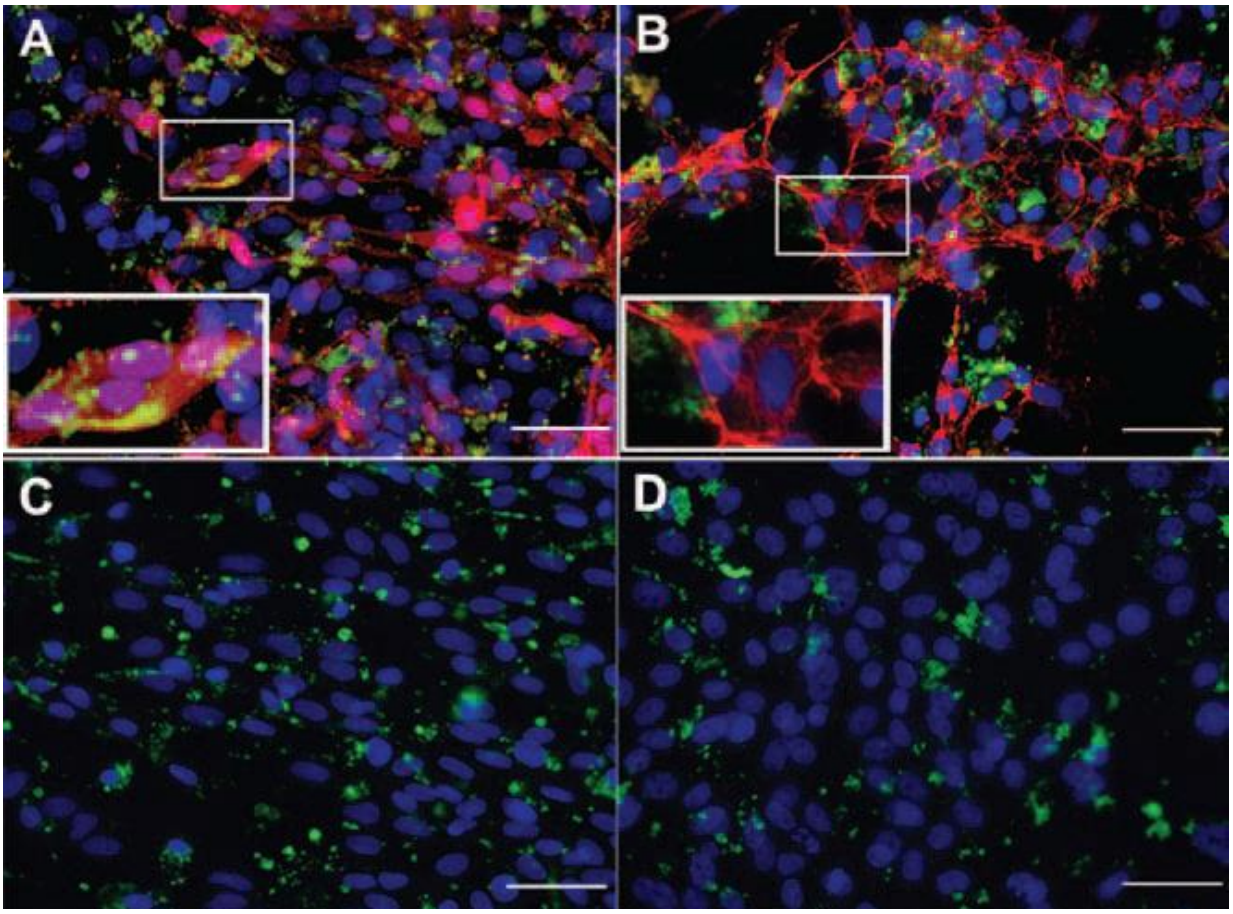

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FIGURE 6 Phagocytosis of ROS (green) by OECs/ONFs cultures (A, B, C) and RPE cultures (D). (A) OECs (red; S100 immunostain). (B) ONFs (red; fibronectin immunostain). Blue, DAPI counterstain in all; scale bars 50 μm.

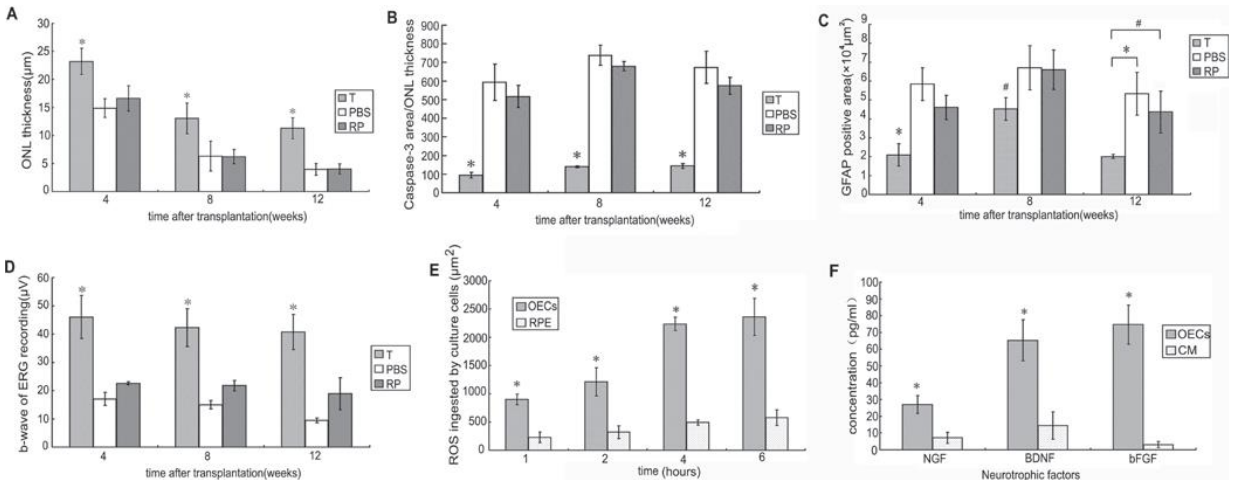

FIGURE 7 (A) Thickness of recoverinpositive ONL. (B) Ratio of caspase-3 positive area to ONL thickness. (C) GFAP-positive area. (D) b-wave of ERG recording. (E) Total area of FITC-positive ROS ingested by culture cells. (F) Concentration of neurotrophic factors. (T) Cells transplantation group; PBS, PBS injection group; RP, Unoperated group; CM, control media. *p < 0.01; #p < 0.05.

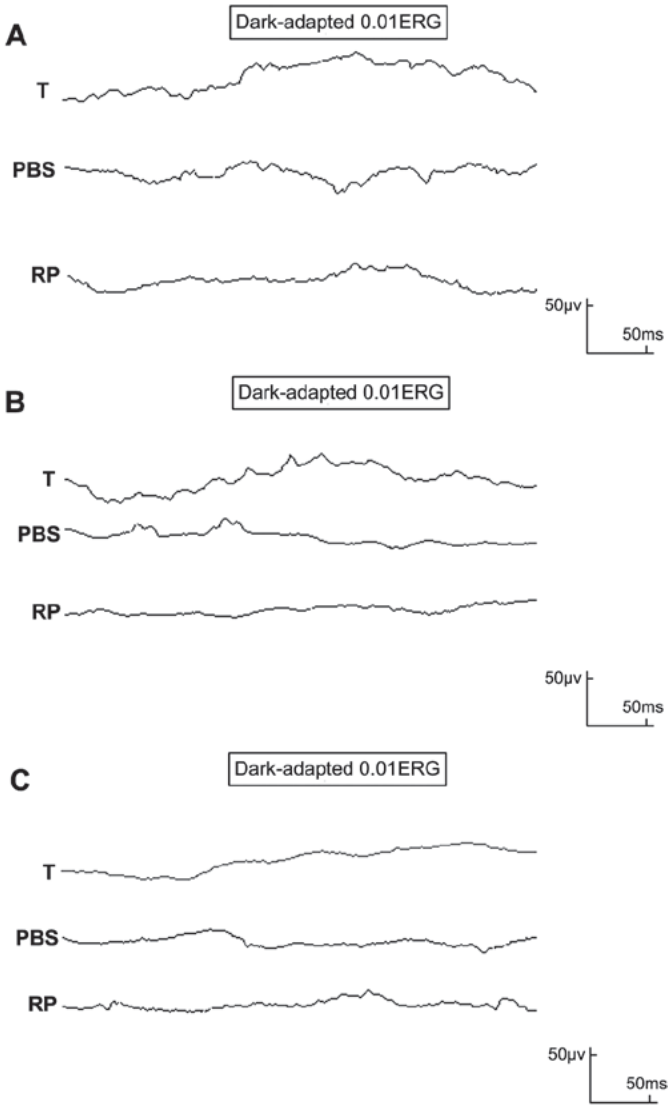

FIGURE 8 ERG traces at 4 (A), 8 (B), and 12 (C) weeks after transplantation.