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Original Article

Transplanted retinal progenitor cells exhibit long-term survival and function in a murine model of laser photocoagulation

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Abstract: Purpose: Research endeavors over the last decade has shown the potential of retinal progenitor cell transplantation, both autologous and analogous, to have potential in ameliorating retinal degeneration. However, such studies were largely performed on a time period of one month and the long-term survival and functionality of the transplanted retinal progenitor cells have not been investigated, which was the objective of the current study. Methods: Retinal progenitor cells were harvested from the neural retinas of postnatal day 1 enhanced green fluorescent protein (GFP) mice and transplanted in host mice induced to have retinal photocoagulation using a diode laser. Cell morphometry and immunofluorescence analyses were conducted 4 and 12 weeks post-transplantation to assess the integration of donor cells. Results: There was numerous retinal progenitor cells that migrated into the recipient outer nuclear layer at both 4 and 12 weeks and there was no observed significant difference between the observed integration numbers at these two times. The majority of the cells developed morphological features associated with mature photoreceptors, expressed and maintained photoreceptor specific proteins. Furthermore, they formed and maintained synaptic connections with bipolar neurons as assessed by expression of bassson and protein kinase C alpha in the transplanted retinal progenitor cells at 12 weeks. Conclusions: Cumulatively our findings indicate that given their long term survival and functionality, autologous donor retinal progenitor cells are optimal for therapeutic approaches to repair retinal degeneration.

Keywords: Retinal degeneration, cell transplantation, mice, survival and functionality

Introduction

The largest cause of untreatable blindness in the developed world is retinal degeneration [1]. Retinal degeneration encompasses an irreversible loss of the sensory photoreceptor cells. Most available therapies aim at slowing the disease progression and research is still ongoing to find permanent cures or reversible strategies for retinal degeneration. On this regard, photoreceptor cell transplantation hold great potential and many studies using laboratory animal models have looked at the potential of retinal progenitor cell transplantation to augment retinal degeneration [2-5].

It has been previously shown that rod receptor precursors can integrate within the outer nuclear layer (ONL) of recipient retinae and display unambiguous rod morphology inclusive of correct orientation of inner (IS) and outer segments (OS) and spherule synapses [1-4]. Furthermore, some of these studies have additionally provided evidence that the transplanted photoreceptor cells have intact phototransdcution signaling mechanism and synaptic transmission [1, 2] and can restore the pupil reflex [2]. However, most of these studies have evaluated the survival of the transplanted photoreceptors up to 4 weeks post-transplantation, thus severely impending their translation potential to treatment modalities. It is imperative to determine if the transplanted cells survive and are functional over extended periods of time post-transplantation.

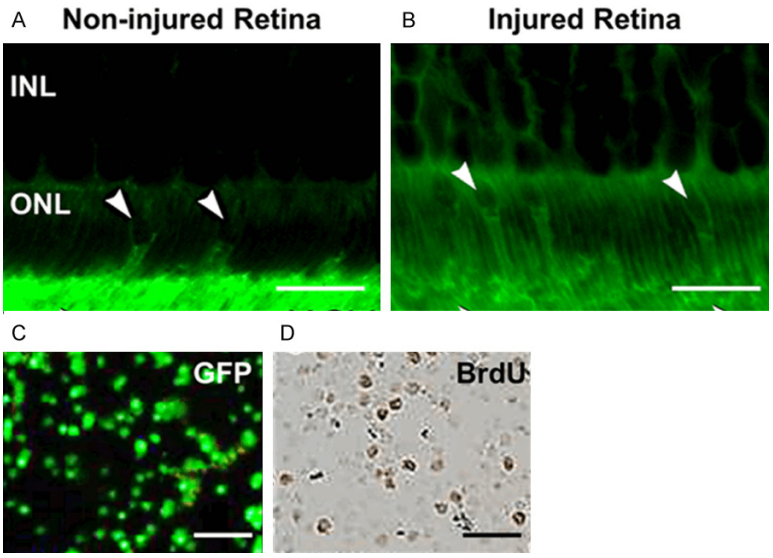

Figure 1. Donor rod cells migrate into ONL of recipients and survive post-transplantation. (A, B) GFP-labeled transplanted retinal cells substantially migrate into the ONL of the host retina more promiscuously (B), in comparison to non-inured retina. (A) (white arrows). Scale bar represents 100 μm. Shown are representative images at 12 weeks post-transplantation. (C, D) BrdU positive staining (D) confirms survivability of the donor rod cells in ONL (green, C). ON*L*, outer nuclear layer; *INL*, inner nuclear layer. Scale bar: 20 μm.

Some studies have indicated that sub retinal space receiving neonatal retinal allografts can induces immune deviation [6]. Others have shown that transplanted retinal sheets can survive in the sub retinal space for several months, although older grafts presented a loss of retinal lamination and structure, and functional loss of synaptic connectivity [7-9]. It has been previously shown that laser-induced damage primarily involves the outer layers of the retina [4] and that transplanted retinal progenitor cells (RPCs) can integrate as well as differentiate in the sub retinal space in these animals [4].

Hence, the aim of the current work was to determine the ability of transplanted photoreceptors to integrate and survive within a recipient retina post laser induce damage. We show that transplanted photoreceptor cells integrated and differentiated within the adult mouse retina and survived for extended periods of time.

Materials and methods

Animals

C57BL/6J (B6) mice (8 weeks, Shanghai Laboratory Animal Center, Shanghai, China) and enhances green fluorescent protein (eGFP) mice (C57BL/6 background) (Shanghai Laboratory Animal Center, Shanghai, China) were housed in the animal facility at General Hospital of People's Liberation Army in a 12 hours light-dark cycle with water and food provided ad libitum. All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the Institute Animal Care and Use Committee of General Hospital of People's Liberation Army.

Dissociation of retinal cells, culture, and transplantation

Neural retinal cells from post-natal day 1 eGFP mice were isolated and maintained in culture as described previously [2] and resuspended at a concentration of 400,000 cells/μl. Retinal photocoagulation and transplantation of retinal progenitor cells. Retinal photocoagulation was done as described previously [4]. Animals (8 weeks) received cell transplants (1 μl) via a transcleral injection into the sub retinal space. The intraocular pressure was continuously monitored and intentionally lowered during the procedure by making a small puncture through the cornea.

Cryosections and immunofluorescence

Tissue fixation, sectioning, and immunohistochemistry were performed as described previously [10]. Mice were sacrificed 3 months post-transplantation by CO_2 inhalation and eyes were fixed immediately in 3.7% PFA in PBS, cryoprotected in serial sucrose solutions, frozen in optimal cutting temperature compound (Tissue-Tek, Miles Diagnostic Division, Elkhart, IN) prior to cryosectioning (18 μm sections). All sections were collected for subsequent analysis. For immunofluorescence, sections were air dried, rinsed in TBS, and blocked (5% NGS, 1% BSA in TBS) for 2 hours before being incubated with primary antibody overnight at 4°C. After washing, sections were incubated with second-

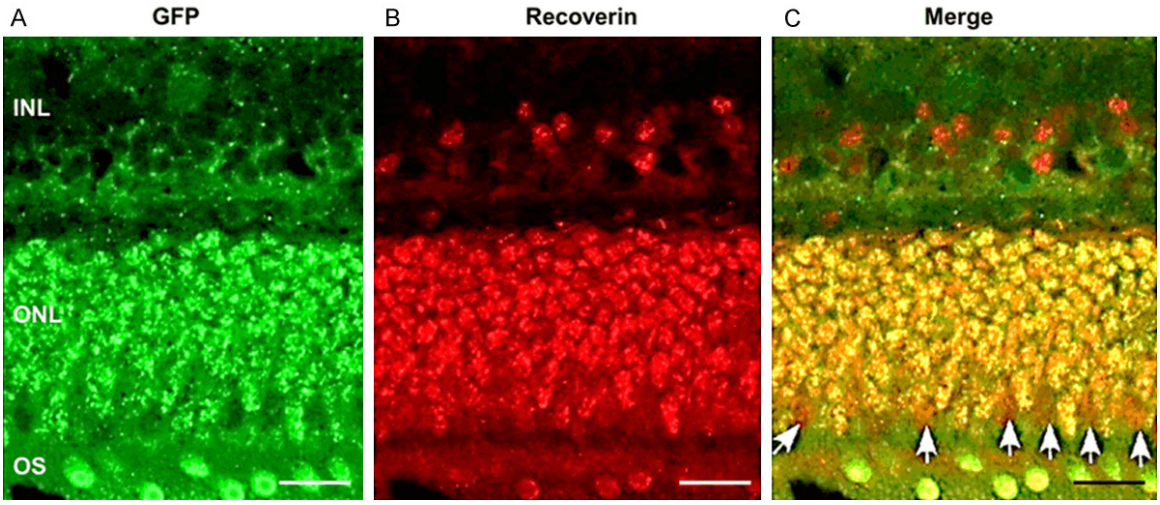

Figure 2. Co-localization of rhodopsin and recoverin in the GFP$^+$ donor photoreceptors. The integrated GFP$^+$ donor photoreceptors (green) (A) expressed the photoreceptor marker recoverin (red) exclusively in the cell bodies (B). The merged panel is shown on the right (C) (*white* arrows pointing to the cell bodies). *OS*, outer segments; *ONL*, outer nuclear layer; *INL*, inner nuclear layer. Scale bar: 30 μm.

ary antibody for 2 hours at room temperature, washed. Negative controls omitted the primary antibody. Antibodies used: rabbit anti-GFP (1:100; Clontech), mouse anti-rhodopsin (1:100; Chemicon Temecula), rabbit anti-recoverin (1:1,000; Chemicon), mouse anti-bassoon (Stressgen), and goat anti-protein kinase C alpha (PKCα; 1:100; Santa Cruz). Sections were imaged using a Zeiss LSM510 confocal microscope. XY optical sections, ~0.5-μm apart, were taken throughout the depth of the section and built into a stack to give an XY projection image. Cell proliferation levels were detected by bromodeoxyuridine (BrdU). BrdU was administered intraperitoneally at 0.1 mg/g of bodyweight into host mice 10 weeks post-transplantation (two weeks before they were euthanized). BrdU-incorporated cells were subsequently detected with a BrdU Staining Kit (Life Technologies, Carlsbad, California, USA).

Cell counts

Cell count was done as described previously [2, 4] three months after transplantation. Transplanted GFP$^+$ photoreceptors were counted as integrated if the whole cell body was correctly located within the ONL and at least one of spherule synapse, inner/outer processes and/or inner segments was visible [2]. The average number of integrated cells per section was determined by counting all the integrated GFP-positive cells in every 1 in 4 serial sections through the site of injection in each eye. This was multiplied by the total number of sections that encompassed the injection site to give an estimate of the mean number of integrated cells per eye (six eyes were sampled per group). Cell counts for individual eyes were only excluded if there were cells in the vitreous, in turn indicating accidental intravitreal transplantation of the cells.

Statistical analysis

Data are presented as means ± SEM (standard error of the mean), unless otherwise stated; N, number of animals; n, number of eyes or sections examined, where appropriate. A p value of <0.05 was considered statistically significant.

Results

Our aim was to confirm that laser-induced retinal damage can promote migration and successful integration of transplanted retinal progenitor cells (RPCs) in to the outer retina, which has been previously established [4], and if the integrated RPCs can survive over 12 weeks. For the same expanded passage five donor RPCs from post natal day 1 GFP transgenic were transplanted to the sub retinal space of recipient host adult B6 mice. The total number of integrated GFP$^+$ cells were quantified at 4 weeks (data not shown) and 12 weeks and the increased cell migration and integration was observed in all animals with induced laser photocoagulation; however, we did not observe significant integration of RPCs into ONL of unin-

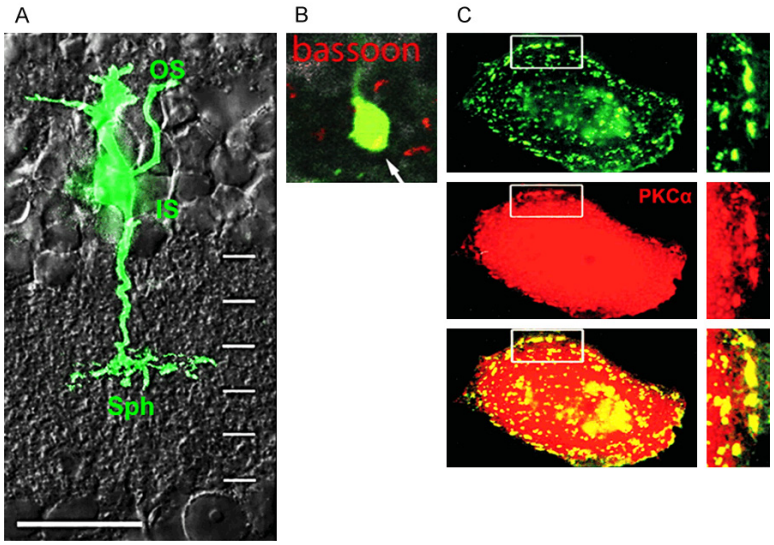

Figure 3. Integrated mature rod photoreceptors elaborate complete morphology and formation of morphological synaptic connections between bassoon protein and PKCα-labeled bipolar cells. Transplanted GFP$^+$, shown in green throughout, that integrated into the ONL make inner (*IS*) and outer segments (*OS*), an axon, and a spherule synapse (*Sph*) (A, Z-stack) Scale bar: 200 µm. The spherule synapse of integrated cells has a single ribbon that labels for bassoon (B, arrow; 1-µm optical section). Synaptic terminals appose directly to rod bipolar processes immunolabeled for PKCα (C, Z-stack; 1-µm optical section).

jured retina at either 4 or 12 weeks post-transplantation (102 ± 12 vs. 83 ± 3 cells per eye; P = 4.2138, ANOVA) (**Figure 1A**, **1B** and data not shown). Importantly, the integrated RPCs survived at 12 weeks as assessed by BrdU staining, which showed mitotic activity reminiscent of RPCs (**Figure 1C**, **1D**).

Even though only a portion of integrated cells developed morphological features reminiscent of mature photoreceptors at 4 weeks, their numbers increased significantly at 12 weeks. Almost the entire population (93 ± 4.2%) of surviving integrated cells expressed photoreceptor associated markers at 12 weeks (**Figure 2A-C** and Supplementary Figure 1). Rhodopsin expression was exclusively seen on the outer segments of adult mice retina (Supplementary Figure 1).

Finally, to assess if functional synaptic connectivity was maintained in the surviving RPCs at 12 weeks, immunofluorescence analysis was performed. As shown in **Figure 3A**, transplanted RPCs elaborated complete morphology as evident by distinct migration of GFP$^+$ cells in the IS and OS, axon, and spherule synapse. Furthermore, the integrated cells showed robust expression of the ribbon synapse protein, bassoon (**Figure 3B**) [4]. Finally, the synaptic contact was intact with bipolar neurons as evident by colocalization of protein kinase C alpha (PKCα) (**Figure 3C**).

Discussion

Photoreceptor transplantation presents a prospective strategy of treatment for retinal degeneration [2, 5]. However, the survivality of RPCs has not been previously established. Our experiments show that the integrated rod photoreceptors can survive for several months following sub retinal transplantation. These cells maintain normal rod morphology, including inner/outer segments and spherule synapses and express components of the synaptic machinery.

Furthermore, our results show that the transplanted RPCs are functionally and anatomically rightly positioned within the recipient retina, similar to previous observations [1-5]. Cumulatively, our results indicate that appropriately integrated photoreceptors from autologous sources can survive for extended periods of time, at least up to 12 weeks as tested here, in the adult host retina.

In conclusion, we have shown here that transplanted rod precursor cells can survive for extended periods of time in the adult murine retina and display all the morphological characteristics of correctly integrated rod photoreceptors. Even though our findings suggest that autologous donor cells are likely to be optimal for therapeutic approaches to repair the neural retina, it would be interesting and important to determine if non-autologous cells may also be effective.

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Disclosure of conflict of interest

None.

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References

[1] West EL, Pearson RA, Barker SE, Luhmann UF, Maclaren RE, Barber AC, Duran Y, Smith AJ, Sowden JC, Ali RR. Long-term survival of photoreceptors transplanted into the adult murine neural retina requires immune modulation. Stem Cells 2010; 28: 1997-2007.

[2] MacLaren RE, Pearson RA, MacNeil A, Douglas RH, Salt TE, Akimoto M, Swaroop A, Sowden JC, Ali RR. Retinal repair by transplantation of photoreceptor precursors. Nature 2006; 444: 203-7.

[3] Klassen H, Kiilgaard JF, Zahir T, Ziaeian B, Kirov I, Scherfig E, Warfvinge K, Young MJ. Progenitor cells from the porcine neural retina express photoreceptor markers after transplantation to the subretinal space of allorecipients. Stem Cells 2007; 25: 1222-30.

[4] Jiang C, Klassen H, Zhang X, Young M. Laser injury promotes migration and integration of retinal progenitor cells into host retina. Mol Vis 2010; 16: 983-90.

[5] Pearson RA, Hippert C, Graca AB, Barber AC. Photoreceptor replacement therapy: challenges presented by the diseased recipient retinal environment. Vis Neurosci 2014; 31: 333-44.

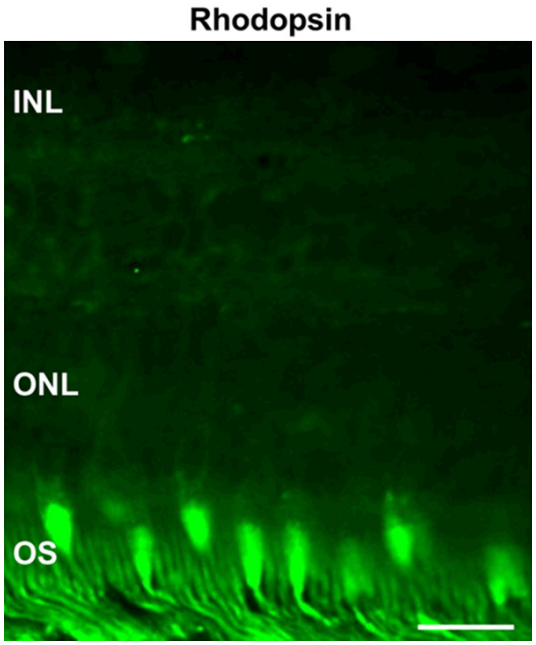
[6] Jiang LQ, Jorquera M, Streilein JW. Subretinal space and vitreous cavity as immunologically privileged sites for retinal allografts. Invest Ophthalmol Vis Sci 1993; 34: 3347-3354.

[7] Ghosh F, Arner K. Transplantation of full-thickness retina in the normal porcine eye: Surgical and morphologic aspects. Retina 2002; 22: 478-486.

[8] Ghosh F, Bruun A, Ehinger B. Graft-host connections in long-term full-thickness embryonic rabbit retinal transplants. Invest Ophthalmol Vis Sci 1999; 40: 126-132.

[9] Ghosh F, Engelsberg K, English RV, Petters RM. Long-term neuroretinal full-thickness transplants in a large animal model of severe retinitis pigmentosa. Graefes Arch Clin Exp Ophthalmol 2007; 245: 835-846.

[10] Jiang C, Moore MJ, Zhang X, Klassen H, Langer R, Young M. Intravitreal injections of GDNF-loaded biodegradable microspheres are neuroprotective in a rat model of glaucoma. Mol Vis 2007; 13: 1783-92.



Supplementary Figure 1. Exclusive expression of the photoreceptor marker rhodopsin (green) in the outer segments of the integrated GFP^+ donor photoreceptors. The GFP staining is not shown here. Scale bar: 30 µm.