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Photoreceptor differentiation and integration of retinal progenitor cells transplanted into transgenic rats

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Abstract

Previous studies evaluating neural stem cells transplanted into the mature retina have demonstrated limited levels of graft–host integration and photoreceptor differentiation. The purpose of this investigation is to enhance photoreceptor cell differentiation and integration of retinal progenitor cells (RPC) following subretinal transplantation into retinal degenerate rats by optimization of isolation, expansion, and transplantation procedures.

RPCs were isolated from human placental alkaline phosphatase (hPAP)-positive embryonic day 17 (E17) rat retina and expanded in serum-free defined media. RPCs at passage 2 underwent *in vitro* induction with *all trans* retinoic acid or were transplanted into the subretinal space of post-natal day (P) 17 S334ter-3 and S334ter-5 transgenic rats. Animals were examined post-operatively by ophthalmoscopy and optical coherence tomography (OCT) at weeks 1 and 4. Differentiation profiles of RPCs, both *in vitro* and *in vivo* were analysed microscopically by immunohistochemistry for various retinal cell specific markers. Our results demonstrated that the majority of passage 2 RPCs differentiated into retina-specific neurons expressing rhodopsin after *in vitro* induction. Following subretinal transplantation, grafted cells formed a multi-layer cellular sheet in the subretinal space in both S334ter-3 and S334ter-5 rats. Prominent retina-specific neuronal differentiation was observed in both rat lines as evidenced by recoverin or rhodopsin staining in 80% of grafted cells. Less than 5% of the grafted cells expressed glial fibrillary acidic protein. Synapsin-1 (label for nerve terminals) positive neural processes were present at the graft–host interface. Expression profiles of the grafted RPCs were similar to those of RPCs induced to differentiate *in vitro* using *all-trans* retinoic acid.

In contrast to our previous study, grafted RPCs can demonstrate extensive rhodopsin expression, organize into layers, and show some features of apparent integration with the host retina following subretinal transplantation in slow and fast retinal degenerate rats. The similarity of the *in vitro* and *in vivo* RPC differentiation profiles suggests that intrinsic signals may have a significant contribution to RPC cell fate determination.

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Keywords: retinal progenitor cell; stem cell; transplant; integration; transgenic rat; hPAP; subretinal injection

1. Introduction

The concept that the nervous system loses the capacity for regeneration after birth has been the central dogma of developmental biology for decades. Despite pioneering work beginning in the 1960s (Davis and Temple, 1994; Gage et al., 1995; Johansson et al., 1999a,b; Reh and Levine, 1998; Snyder et al., 1997; Weiss et al., 1996)

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providing substantial evidence to the contrary, the existence of adult neurogenesis was not extensively accepted until recent fundamental advances in developmental biology in the 1990s (Davis et al., 1994; Gage et al., 1995; Johansson et al., 1999a,b; Reh and Levine, 1998; Snyder et al., 1997; Weiss et al., 1996). Indeed, the discovery of neural stem cells in the adult has raised the possibility for the development of potential powerful new therapeutic strategies to restore function in degenerative neurological diseases including Parkinson's disease (PD), Huntington's disease (HD), Alzheimer's disease (AD), retinitis pigmentosa (RP) and age-related macular degeneration (AMD) (Armstrong et al., 2003; Baetge, 1993; Bjorklund, 2000; Drucker-Colin and Verdugo-Diaz, 2004; Freeman et al., 2000; Martinez-Serrano and Bjorklund, 1997; Redmond, 2002; Sayles et al., 2004; Storch and Schwarz, 2002).

The first evidence that stem cells could potentially replace lost retinal cells emerged with the observation that adult hippocampus derived neural stem cells transplanted into the vitreous of neonatal or adult normal rats survived and integrated into the host retina (Takahashi et al., 1998). In order to study whether certain pathological conditions could improve graft integration and promote retinal specific neuron differentiation of transplanted brain-derived neural progenitor cells, investigators performed transplants in normal rats following ischemic or mechanical injury, and in retinal degenerate rats (Chacko et al., 2003; Guo et al., 2003; Kurimoto et al., 2001; Mizumoto et al., 2003; Nishida et al., 2000; Young et al., 2000). However, in all of these studies, despite morphological similarities to various retinal cell types and extensive incorporation within the host retina, the transplanted hippocampus-derived cells failed to differentiate into retinal-specific lineages.

Retinal progenitor cells (RPCs) recently have been successfully isolated from embryonic and adult eyes in rodents and humans (Ahmad et al., 1999, 2000; Tropepe et al., 2000; Yang et al., 2002a,b). Studies have shown that retinal progenitor cells can display stem cell properties both in vitro and in vivo (Ahmad et al., 1999; Akagi et al., 2003; Qiu et al., 2004a). RPCs can proliferate and be expanded in vitro, and upon removal of mitogens and the addition of serum, they can differentiate along neuronal or glial pathways. Following in vivo transplantation, cultured rat retinal progenitors (cell suspension) can also differentiate into retinal specific neurons (as demonstrated by immunohistochemistry), however, normal morphologic development (e.g. elaboration of photoreceptor outer segments), graft lamination, and extensive integration with the host retina has not been observed (Chacko et al., 2000; Yang et al., 2002a,b). For example, RPCs that were transplanted as retinal spheres, formed cell clumps in the subretinal space (Chacko et al., 2000). When RPCs were instead transplanted as dissociated cells, rosettes and cell clumps were not observed, but grafted cells did not organize into layers and did not integrate well with the host retina. Moreover, with

both dissociated cell and neurosphere transplants, retinal specific neuronal differentiation of the RPCs was still quite limited (Chacko et al., 2000). In a study by Yang et al., the majority of the grafted RPCs differentiated into glia cell lineages expressing GFAP (Yang et al., 2002a,b). Ahmad and co-workers observed expression of retinal specific makers such as RET-P1 (opsin marker) in some grafted cells, but the number of positive cells appeared limited (Chacko et al., 2000).

The purpose of the present study was to optimize the differentiation of transplanted RPCs into the desired retinal neuronal lineages, and to enhance the integration of these grafted cells with the host retina in two rat models of retinal degeneration, S334ter-3 (early degeneration) (Sagdullaev et al., 2003) and S334ter-5 (late degeneration) rats (Thomas et al., 2004). Experimental techniques used to improve outcomes focused on careful attention to the selection of culture media for in vitro manipulation, cell density for transplantation, cell passage selection, and use of gentle surgical procedures.

2. Materials and methods

2.1. Animals

Three different transgenic rat strains, pigmented hPAP transgenic rats, albino S334ter-5 and pigmented S334ter-3 transgenic rats were obtained from the colonies maintained at the Doheny Eye Institute. All animal procedures used for this study adhered to the provisions of the ARVO statement for the use of Animals in Ophthalmic and Vision Research, and were approved by University of Southern California IACUC.

hPAP transgenic rats (Kisseberth et al., 1999; Mujtaba et al., 2002) stably express human placental alkaline phosphatase (hPAP) in the cytoplasm of all cells, which is easily identified by immunohistochemistry or a standard hPAP histochemistry assay. As a result, hPAP-labelled animals are a useful source of donor tissue which can be readily identified following transplantation. Founder breeders were generously provided by Dr Sandgren of the University of Wisconsin. Importantly, hPAP-derived RPC's (Qiu et al., 2004b, ARVO) appear to have the same stem cell properties (neurosphere formation, self-renewal, multipotency) at those of RPC's derived from normal Long Evans rats in our previous studies (Qiu et al., 2004a).

S334ter-5 and S334ter-3 transgenic rats (generous gift from Matthew LaVail, University of California, San Francisco) express a mutated rhodopsin gene. S334ter-3 pigmented rats exhibit an early retinal degeneration, with only one row of photoreceptors remaining at the age of 1 month, and *complete loss* of rod photoreceptors by post-natal (P) day 45 (Steinberg et al., 1996; Liu et al., 1999; Sagdullaev et al., 2003). In S334ter-5 albino rats, the degeneration occurs later. Photoreceptors only *begin* to

degenerate by 5 weeks of age, and rod degeneration is nearly complete by the age of 2 months (Thomas et al., 2004; Lee et al., 2003; Steinberg et al., 1996). For the present study, P17 rats (in which neural retinal development is essentially complete but the photoreceptor degeneration is still at an early stage) were used as recipients. It is important to note, however, that the P17 microenvironments of these two lines are still likely very different, as they represent early vs late stages of the prelude to degeneration.

2.2. hPAP RPC isolation and in vitro expansion

Retinal progenitor cells (RPC) were isolated from the normal retinas of E17 hPAP-positive transgenic rats which express the human placental alkaline phosphatase protein (hPAP). The method of RPC isolation and in vitro expansion has been previously described (Qiu et al., 2004a). Briefly, the eyes were carefully enucleated from E17 hPAP (+) fetuses and maintained in Hibernation TM media (Brainbits, US patent No. 6180404) in 35 mm petri dishes on ice. The retina was dissected free of retinal pigment epithelium (RPE) and mesenchymal tissue. In addition, a 1.5 mm wide zone was removed in the ciliary margin (to remove ciliary epithelium) and around the optic disc (to remove optic nerve/glial cells).

After dissection, retinal fragments were incubated with 0.25% trypsin without EDTA-Ca (Invitrogen, Carlsbad, CA) for 15–20 min at room temperature and neutralized by adding 10% fetal bovine serum and phosphate-buffered saline (PBS). Cells were centrifuged at 5000/rpm for 5 min, and the supernatant was aspirated. Cell pellets were resuspended in the serum-free defined culture media

supplemented with EGF (200 ng ml⁻¹) and bFGF (10 ng ml⁻¹) and seeded on poly-D-lysine/laminin (Sigma) coated 6-well culture plates at a cell density of 4–5 × 10⁴/cm². The culture medium was changed every other day and the cells were passage when they reached 85% confluence. RPC passaging was performed using 0.25% trypsin combined with mechanical trituration. Cells at passage 2 were selected for transplantation.

2.3. RPC cell fate determination: in vitro

Passage 2 hPAP (+) retinal progenitor cells underwent 8 days of in vitro induction to study and characterize their differentiation potential. In order to initiate differentiation, EGF and bFGF were removed from serum-free defined media, and the media was supplemented with 1% fetal bovine serum and 10⁻⁷ M *all-trans* retinoic acid (RA) on the first day of induction. Passage 2 RPCs were plated on PDL-laminin coated four-chamber slides at a cell density of 3–4 × 10⁴/cm². Cells were allowed to remain under induction conditions for a period of 8 days. The induction culture medium was changed every day. Control cells not subject to induction were only cultured for 4 hr to allow cell adherence, and then fixed for immunocytochemistry.

After 8 days of induction, cells cultured in chamber slides were processed for immunocytochemistry according to standard protocols. Briefly, cells were first rinsed in PBS buffer and fixed in 4% paraformaldehyde in PBS, and rinsed. Cultured cells were incubated with 20% goat serum in PBS, and then incubated with different combinations of primary antibodies (see Table 1) overnight at 4°C followed by a PBS wash. Cells were then incubated for 90 min with

Table 1
Antibody information listings

	Species	Specific for	Dilution	Supplier
<i>Primary antibodies</i>				
Rhodopsin	Rabbit	Photoreceptors (rods)	1000	Plantner et al. (1982)
Rhodopsin	Mouse	Photoreceptor (rods)	1:50	Gift from Hicks and Molday (1986)
Protein kinase C (PKC)	Rabbit	Rod bipolar cells	200	Oxford Biomed, Oxford, MI
Recoverin	Rabbit	Photoreceptors (cones and rods); Cone bipolar cells	500	McGinnis et al. (1997)
hPAP (human alkaline phosphatase)	Mouse	Cells containing hPAP protein	600	Sigma, St Louis, MO
GFAP (glial fibrillary acidic protein)	Rabbit	Astrocytes, glial cells	1000	Chemicon (Temecula, CA)
GFAP	Mouse	Astrocytes, glial cells	10 000	Sigma (St Louis, MO)
Synapsin-1	Rabbit	Synaptic layers	2000	StressGen, San Diego, CA
Calbindin	Rabbit	Horizontal and amacrine cells	2000	Calbiochem, San Diego, CA
Calretinin	Rabbit	Amacrine cells	2000	Chemicon, Temecula, CA
MAP-2	Rabbit	Neuronal cells	200	Sigma, St Louis, MO
NF (neurofilament), 200 kDa	Mouse	Ganglion cells, horizontal cells	200	ICN, Costa Mesa, CA
Nestin (Rat-401)	Mouse	Undifferentiated stem cells	1:50	NICH, Iowa
CRALBP (cellular retinaldehyde binding protein) (IG 83)	Rabbit	Muller's cells, RPE	1000	Saari et al., 1984
<i>Secondary antibodies</i>				
AF488 anti-mouse IgG	Goat	All mouse antibodies	200	Molecular Probes, Eugene, OR
AF546 anti-mouse IgG	Goat	All mouse antibodies	200	Molecular Probes
AF488 anti-rabbit IgG	Goat	All rabbit antibodies	200	Molecular Probes
AF546 anti-rabbit IgG	Goat	All rabbit antibodies	200	Molecular Probes

a fluorescent conjugated secondary antibody followed by PBS wash. The slides were coverslipped with 4',6'-diamidino-2-phenylindole hydrochloride (DAPI) mounting medium and analysed with a Zeiss LS 510 Confocal microscope (Zeiss, Germany) or a Nikon FXA fluorescence microscope (Nikon, Japan). Quantification of cells was achieved by counting DAPI stained nuclei and specific immunoreactive markers in 5–6 randomly selected fields. The induction experiments were repeated three times.

2.4. Subretinal transplantation of RPCs into retinal degenerate rats

Twelve (two litters of 7 and 5 rats each) S334ter-5 and seven (one litter) S334ter-3 rats at the age of P17 were used as recipients. hPAP-positive retinal progenitor cells at passage 2 were dissociated into cell aggregates consisting of 2–3 cells (confirmed by microscopy) and immediately transplanted into the subretinal space using a transscleral approach. PBS injection (using an identical surgical procedure) was used as a sham control in six eyes (three in S334ter-3 rats and 3 in S334ter-5 rats). The surgical procedures were performed under an ophthalmic surgical microscope. Briefly, a temporal conjunctival incision near the limbus was made to expose the anterior sclera. After the sclera exposure, a temporal sclera incision was made at 2 mm beyond the limbus using a 30 G needle. A 32 G blunt needle attached to 10 μ l Hamilton syringe was carefully introduced into the sclera incision site and advanced into the subretinal space and then slowly directed posteriorly. A 1.5 μ l cell suspension containing approximately 100 000 cells was slowly delivered to the subretinal space through the 32 G needle with the Hamilton syringe. The duration of the injection was approximately 40–50 sec. After withdrawing the needle, very little outflow was observed around the injection site.

2.5. Post-operative examination

Post-operative examinations including fundus examination and optical coherence tomography (OCT-3, Carl Zeiss Meditec Germany) were performed at week 2 and week 4 following surgery. Fundus examination was performed under the ophthalmic microscope by using a coverslip placed over the cornea. OCT was performed only in pigmented S334ter-3 transgenic rats, as increased reflections degraded the image quality in albino animals.

2.6. Tissue processing and immunohistochemistry

Four weeks after surgery, animals were sacrificed by intraperitoneal injection with sodium phenobarbital (300 mg kg⁻¹, Abbott Laboratories, North Chicago, IL). The eyes were immediately removed and immersion fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer for 15 min, and a corneal window was created to permit more

efficient and complete fixation. Tissues were then fixed for an additional 3–4 hr on ice, rinsed with 0.1 M phosphate buffer, immersed in 30% sucrose in 0.1 M phosphate buffer overnight, and embedded in OCT compound (Tissue-Tek, VWR International, West Chester, PA) on dry ice for cryosectioning. Serial sections (8 μ m thickness) were performed starting from the dissection edge. For each eye, about 50 slices were acquired on slides for further processing.

To identify grafted cells, every 5th slide was stained for hPAP (histochemical staining). Briefly, slides with cryosections were air dried, washed with PBS, and incubated with hPAP buffer (0.1 M Tri-HCl, 0.1 M NaCl, 5 mM MgCl₂, pH 9.5) for 1 hr at 65°C. BCIP/NBT substrate (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) liquid substrate system Cat: B1911 Sigma, St Louis, MO) was added onto the sections and incubated for about 5 min at room temperature to observe the chemical reaction (positive staining was indicated by a blue coloration). The sections adjacent to sections showing hPAP (+) staining were selected for further immunohistochemical analysis to assess the survival, differentiation and integration of the grafted cells. Different combinations of primary antibodies were used to detect the expression of retinal specific neuronal and glial cell markers. The primary and secondary antibodies used in this study are illustrated in Table 1. The procedures were performed according to standard immunohistochemistry protocols, which were similar to the immunocytochemistry assay on cultured cells already described. The slides were examined under a Zeiss LSM Confocal microscope (Zeiss, Germany) or a Nikon FXA Fluorescence microscope (Nikon, Japan). Quantification of the features of grafted cells was achieved by counting DAPI stained nuclei and specific immunoreactive markers in 5–6 randomly selected graft slides in each animal. To depict the relative proportions of cells expressing specific markers, the positive cells were graded as a percentage of total hPAP-labelled cells into four categories: >70% (+++); 30–70% (+++); 5–30% (++); <5% (+).

3. Results

3.1. Cell fate determination of hPAP-positive retinal progenitor cells in vitro

Retinal progenitor cells isolated from E17 hPAP-positive rat retina continued to proliferate and form neurospheres while maintained in serum-free defined culture medium supplemented with EGF and bFGF. The cell morphology and proliferative properties of hPAP-positive RPCs were similar to those of RPCs isolated from age-matched normal Long-Evan rats (Qiu et al., 2004a). Similar to our previous study (Qiu et al., 2004a), RPCs cultured in these conditions maintained a characteristic property of stem cells

Fig. 1. Embryonic day 17 derived retinal progenitor cells at passage 2 expressed nestin (green) before in vitro induction (A). After in vitro induction with *all-trans* retinoic acid, the treated cells showed evidence of neurite outgrowth (D). Immunocytochemistry indicated that the differentiated cells expressed rhodopsin (red) (B), and calbindin (red) (C). DAPI stained as blue. (D) A brightfield image demonstrates the network-like pattern created by the processes interlinking the cells. An arrow illustrates one of the cells within the network elaborating numerous thin processes consistent with a neuronal morphology. A, C and D: 40 $\times$; bar: 50 μ m. B: 20 $\times$, bar: 100 μ m.

(a capacity for self-renewal) and expressed nestin (Fig. 1A). Following in vitro induction in the presence of 1% serum and 1×10^7 M RA, the RPCs extended long processes, often contacting other cells, forming apparent networks. Many cells adopted a variety of neuronal cell morphologies, and a few cells also displayed a flattened morphology similar to that of astrocytes (Fig. 1D).

Specific antibodies were used to assess cell phenotypes of RPCs at passage 2 following induction. Cells were immunoreactive for a variety of end-stage retinal neuronal markers, including Calbindin (a marker for amacrine and bipolar cells) (Fig. 1B), rhodopsin (a marker for rod photoreceptor cells) (Fig. 1C), protein kinase C (a marker for rod bipolar cells, data not shown). A few cells were immunoreactive for GFAP (glial fibrillary acidic protein, a marker for glial cells) (data not shown). hPAP expression

was present in all cells and did not change over different cell passages and following in vitro cell differentiation, suggesting that hPAP was a reliable label for tracking RPCs.

3.2. Post-operative examination

At post-operative weeks 2 and 4, fundus examination revealed no retinal haemorrhages, retinal detachment, vitreous opacity, or other signs of intraocular inflammation or clinical toxicity. OCT examination on S334ter-3 rats at week 2, however, demonstrated a shallow retinal detachment in one (of seven) transplanted rats (1/7) (data not shown). A linear band (presumably corresponding to the graft) was noted between the host retinal layers and the RPE (Fig. 2) in three (3/7) animals.

Fig. 2. OCT images in transplant and non-transplant areas. S334ter-3 fast degenerate rat, 2 weeks following subretinal transplantation of retinal progenitor cells in S334ter-3 fast degenerate rats at age of postnatal day 17. OCT examination shows a hyper-reflective band (arrow) indicating the host neural retina in a region outside of the transplant area (A). In the transplant area (B), an additional hyper-reflective band (red/yellow) indicating the graft (arrow) was observed between the host retinal band (arrow head) and the host choroidal band.

Fig. 3. Retinal progenitor cell (RPC) differentiation profiles following transplantation into the subretinal space of a S334ter-3 fast retinal degenerate rat. Passage 2 E17 retinal progenitor cells grafted into the subretinal space of a post-natal day 17 S334ter-3 fast retinal degenerate rat. Immunohistochemistry and confocal microscopic examination were used to evaluate the grafted cell differentiation characteristics. At the age of 7 weeks, an area (A–C) distant from the transplant, shows only a few rhodopsin-positive cells (B). In the transplant area (D–F), the grafted cells formed a layered sheet (E, arrow) in the subretinal space with the majority of the cells expressing rhodopsin (E, F, G and N) and recoverin (P). Synapsin-1 positive processes were observed between the grafted cells and the remaining host retina (J and K, arrow heads), as well as within the graft (J and K, arrows). Only few cells were immunoreactive with GFAP (M). Grafted cells continued to express hPAP, allowing their rapid identification (H, K, and Q). A–D: PKC; B, E, G, and N: rhodopsin; J: Synapsin-1; H, K and Q: hPAP; P: recoverin; M: GFAP; C, F, I, L, O, and R: merged images, yellow indicates co-expression; Figure orientation: ganglion cell layer up. Scale bar: 50 μ m.

Fig. 4. Retinal progenitor cell (RPC) differentiation profiles following transplantation into the subretinal space of a S334ter-5 slow retinal degenerate rat. Transplanted progenitor cells expressed recoverin (C), calbindin (K), protein kinase C (PKC, O). Synapsin-1 positive processes were often seen within the graft (arrow, F–H) and also at the interface between the graft and host retina (arrow heads, F–H). In some cases, cell processes from the graft extended into remaining neural retina (arrows, J and L). A, E, I and M: DAPI nuclei staining; B, F, J and N: hPAP labelled for graft cells; D, H, L, P: merged images. Yellow indicates co-expression. Note in (B), the recoverin staining of C is bleeding through the hPAP staining. The staining of host cone bipolar cells is a confocal artefact. Blue: DAPI nuclear staining. Figure orientation: ganglion cell layer up. All the images were taken at the same magnification: $\times 40$. Scale bar: 50 μm .

3.3. Evaluation of survival, differentiation and integration of cultured RPCs following grafting

One month following subretinal transplantation into P17 retinal degenerate rats (S334ter-5 and S334ter-3), passaged RPCs could be easily identified by hPAP staining (histochemistry) and immunohistochemistry. The hPAP (+) cells were detected as multiple rows of cells in the subretinal space in most of the eyes (15/19) with apparently little disturbance or disruption of the host retina. In S334ter-3 fast degenerate rats, the majority of the hPAP-positive cells also expressed rhodopsin and appeared to organize into a laminated sheet (defined as separation of plexiform and cellular layers) in the subretinal space in the transplant area (Fig. 3E, F, G and N). In contrast, only a few residual

rhodopsin-positive cells were detected in the non-transplanted control eye at the age of P47 (Fig. 3B and C). Overall, about 80% of grafted cells in both S334ter-3 (Fig. 3) and S334ter-5 rats (Fig. 4) were observed to express photoreceptor cell markers, including rhodopsin (rods) or recoverin (label for rods and cones, but also cone bipolar cells). It should be pointed that a few non-hPAP-positive cells also expressed rhodopsin, consistent with rescued host photoreceptors. Other retina-specific neuronal markers such as calbindin and PKC were also observed (Table 2, Figs. 3 and 4). Less than 5% of grafted cells expressed glial fibrillary acidic protein (GFAP) (Fig. 3M) or cellular retinaldehyde binding protein (CRALBP). There appeared to be no significant difference in the RPC differentiation profiles between fast and slow degenerate rats.

Table 2
In vivo RPC differentiation profiles

Recipients	Graft position	Rhodopsin	Recoverin	PKC	Calbindin	GFAP	CRALBP	PKC-Rhod
Line 3	Subretinal	++++	++++	++	++	+	+	+
Line 5	Subretinal	+++	++++	++	++	+	+	+
Line 5	Vitreous	++++	++++	+++	++	+	+	+

++++, >70%; +++, 30–70%; ++, 5–30%; +, <5%; PKC-Rhod, double staining with PKC and rhodopsin.

Fig. 5. Retinal progenitor cell (RPC) differentiation profiles in epiretinal vitreous space in S334ter-3 rat (fast degeneration). In this animal, grafted cells were found in an epiretinal location. Immunohistochemistry and confocal microscopy demonstrated that majority of the grafted progenitor cells expressed recoverin (A) and rhodopsin (G and S), the others were immunoreactive with protein kinase C (PK14) (K), calbindin (O), and GFAP (R). Grafted cells continued to show hPAP expression (A, E, I, M and Q) 4 weeks after transplantation. D, H, L, P and T: merged images, yellow indicates co-expression. Blue: DAPI nuclear staining. Figure orientation: ganglion cell layer up. All the images were taken at the same magnification: $\times 40$. Scale bar: 50 μm .

Differentiation profiles of the grafted RPCs in vivo were similar to those of RPCs induced to differentiate in vitro using *all-trans* retinoic acid.

Passaged RPCs showed some evidence of possible anatomic integration with the remaining host retina 4 weeks after transplantation depicted as synapsin-1 immunoreactivity in fast retinal degenerate rats (Fig. 3K and L). In all eyes containing RPC transplants, neural synapsin-1 positive processes (simulating a 'plexiform' layer) were found at the interface between the graft and the host, as well as within the graft (Fig. 3K, L and Fig. 4G, H). In some cases, transplanted cells also projected long neuritic processes into the host inner plexiform layer (Fig. 4J and L).

The neural processes at the graft–host interface appeared qualitatively more extensive in early degenerate rats

compared to late degenerate rats (Fig. 3J and Fig. 4G), although this could not be quantified precisely. Extensive synapsin-1 expression, however, was seen *within the grafts* in both degenerate rats (Fig. 3K and Fig. 4G).

In five of the 19 rats, some graft cells were also found in an epiretinal location, forming a thin contiguous cellular layer in intimate contact with the host nerve fiber layer (Fig. 5). No discontinuities were observed in the host retina in these eyes to suggest possible inadvertent direct implantation into the epiretinal space. The cell differentiation profiles of the epiretinal graft cells were similar to those of cells residing in the subretinal space, with the majority of cells showing positive staining for rhodopsin and recoverin (Fig. 5C, G and S and Table 2), but no synapsin-1 immunoreactivity was found at the interface of graft and host retina (data not shown).

4. Discussion

Previous studies evaluating neural stem cells transplanted into the mature retina have demonstrated limited levels of graft–host integration and photoreceptor differentiation (Chacko et al., 2000; Yang et al., 2002a,b; Young et al., 2000). In the present study, we describe a methodology for preparation and transplantation of RPCs which increases the percentage of grafted cells showing features of photoreceptor differentiation and apparently improves integration of these cells with the host retina.

Indeed, we observed that the vast majority of cells expressed markers suggestive of photoreceptor differentiation, including rhodopsin (rods), and recoverin (rods and cones, but also cone bipolar cells). The next most frequently expressed marker was that of rod bipolar cells (protein kinase C), followed by amacrine cells (Calbindin). Interestingly, marker expression for Muller and glial cell lineages was quite low. Previously, Yang and co-workers used *serum-containing culture media* for RPC isolation and expansion, and observed that the majority of E17 derived passage 2 RPCs differentiated into glial phenotypic lineages following subretinal transplantation into RCS rats (Yang et al., 2002a,b). Ahmad et al. cultured retinal progenitor cells in serum-free defined media *without EGF* and transplanted them into 2-week old *normal rats*. They observed that grafted cells expressed the photoreceptor-specific marker (RET-P1). However, no other retinal neuronal specific markers were reported and layering of graft cells and extensive integration with the host retina were not observed (Chacko et al., 2000). Young et al., demonstrated morphological integration of hippocampal progenitor cells transplanted to the retina of immature and mature dystrophic rats, however, transplanted cells failed to express any retina-specific markers (Young et al., 2000).

Of note, in our study, similar to that of Yang and co-workers, we chose RPCs which were isolated from donor eyes at embryonic day 17. During normal retinal development in rodents, there is a temporal and spatial order of histogenesis for each retinal cell type. Embryonic day 17 represents the beginning of the late-stage retinal development. Based on retroviral marking studies in the normal retina, RPCs at E17 predominantly differentiate into rod, bipolar and Muller's cell in sequence (Cepko et al., 1996; Lillien and Cepko, 1992). In attempting to explain our observed results in which extensive photoreceptor cell but limited Muller cell differentiation was observed following transplantation, one could hypothesize that RPCs were captured from the developing retina at a precise point in which most of the cells had been determined or destined to become photoreceptors, but just before a transition occurred at which point most RPCs would be shuttled down a Muller cell development pathway. This hypothesis, however, implies that RPC fate is determined by intrinsic cues that are acquired during development. An alternate hypothesis, however, is that extrinsic cues present in the local

environment (e.g. the subretinal space) are the critical elements that influence the fate of transplanted RPCs. Two lines of evidence would argue in favour of the former hypothesis over the latter. First, RPCs isolated in the same conditions used for transplantation, but induced *in vitro*, also preferentially developed along a photoreceptor lineage. Second, preferential photoreceptor differentiation was observed irrespective of whether the cells were located in a subretinal or epiretinal location. Moreover, the percentage of cells exhibiting photoreceptor-specific differentiation was unaffected by the stage of host retinal degeneration (early vs late).

With respect to the inadvertent epiretinal grafts, one could argue that the cells were originally transplanted into the subretinal space, acquired features of photoreceptor differentiation, and then migrated through the host retina to an epiretinal location. If this was the case, however, one might expect to see some evidence of grafted cells at various levels of migration into the host retina in at least a few sections; this however, was not observed in this study. Instead, we suspect that a few RPCs may have gained access to the vitreous cavity and epiretinal space via the site of the scleral incision. Occasionally, during creation of the scleral incision (which is remote from the site of ultimate implantation of the graft), the retina may also be transected allowing an opening into the vitreous space. Although the cannula is then inserted into the subretinal space and advanced posteriorly prior to injection, it is certainly possible that some cells could reflux back anteriorly and enter into the vitreous. An alternative mechanism of access into the epiretinal space is direct transection of retina at the implantation site, but retinal discontinuities or other signs of host retinal injury were not observed at the implantation site or in adjacent sections.

The predominance of grafted cells with features of photoreceptor differentiation, organization of graft cells into a layered configuration, and the formation of a layer of neuritic processes at the graft–host interface, appear to be departures from the predominant results obtained in prior studies including those previously published by our group. Several alterations in our methodology may have contributed to this apparently improved result, although the present study is not designed to determine which of these factors were most important. These alterations include: the age of the donor tissue (E17), the recipients (normal vs degenerate), use of a serum-free defined culture media with both bFGF and EGF to maintain and expand cells following isolation, cell culture density ($4\text{--}5 \times 10^4/\text{cm}^2$), the technique of cell isolation and dissociation, and improved surgical procedures with slower and less traumatic delivery of cells.

As discussed previously, the age of the donor may be relevant as we chose a donor age for isolation of RPCs which reflected the peak time for yielding a photoreceptor lineage during retinal development. In addition, the use of a serum-free defined culture media containing EGF and bFGF may have an important factor as well. We have described

(Qiu et al., 2004a) that this media is highly effective in maintaining the stem cell properties of these cells. Perhaps this isolation and expansion media was also able to maintain the intrinsic cues in these cells which we have postulated have destined these cells to evolve into photoreceptors following in vitro (by retinoic acid) or in vivo induction (by subretinal transplantation into diseased retina).

The procedure for isolation and dissociation of passage 2 RPCs for transplantation may also be important. Previous studies have shown that RPCs transplanted as neurospheres resulted in cell clumps in the subretinal space instead of an organized layer of cells. In the present study, we used 0.25% trypsin combined with gentle mechanical trituration to dissociate cultured RPCs into 2–3 cell aggregates. Fortuitously, this cell aggregate size may prove to be the optimal nidus to promote favourable interactions among grafted cells as well with the host retina, promoting formation of the laminated morphology observed in many of the grafts in this series. The relatively atraumatic delivery technique with a very slow injection rate may also be an important factor in the good morphology observed in many of the grafts. Certainly, numerous previous studies of transplantation of intact fetal retinal sheets has demonstrated that minimization of surgical trauma is a critical factor in the achievement of laminated transplants and reducing rosette formation (Aramant et al., 1999; Ghosh et al., 1998). In our study, no rosettes were observed following subretinal transplantation of RPCs.

The grafted cell differentiation profiles in both degenerate rats was similar in that the vast majority of graft cells were positive for recoverin or rhodopsin. The organization and integration in fast degenerate rats, however, appeared qualitatively better compared to slow degenerate rats. This observation, if it can be confirmed in future studies, may suggest that the host subretinal environment can have a significant influence on transplant cell organization and integration.

A limitation of the present study is that it was not designed to determine the relative importance of these factors. Future studies can certainly explore each of these issues in detail. The purpose of this study, however, was to determine whether any method of RPC cell transplantation could produce evidence of preferential photoreceptor differentiation and extensive anatomic integration with the host. Although these objectives were achieved, it is important to note that while photoreceptor marker expression was observed, the development of photoreceptor outer segments within the graft was not observed (though not evaluated for by electron microscopy). Furthermore, although anatomic integration was observed, the presence of functional connections between grafted cells and the host retina was not evaluated. These areas, however, will certainly be the subjects of future studies.

In summary, this study demonstrates that passage 2 retinal progenitor cells derived from normal E17 hPAP (+) could yield cells expressing a variety of retinal neuronal

markers both in vitro and following transplantation into the eyes of retinal degenerate rats. Grafted cells appeared to predominantly differentiate along a photoreceptor lineage, organized into a layered configuration, and showed some evidence of anatomic integration with the host retina. Although the observations are promising, considerable additional research is necessary to improve our understanding of the biology of retinal progenitor cells during normal retinal development, during in vitro differentiation, and following exposure to a variety of in vivo environments.

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