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Revisiting nestin expression in retinal progenitor cells *in vitro* and after transplantation *in vivo*

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Abstract

The purpose of this study is to characterize the co-expression of nestin—a neuroectodermal stem cell and a reactive glial marker—with various mature retinal cell markers in retinal progenitor cells (RPCs) expanded *in vitro*, followed either by *in vitro* induction or subretinal transplantation. Rat RPCs derived from embryonic day (E) 17 rat retina were expanded in serum free defined culture, and induced to differentiate by all-*trans* retinoic acid (RA). Following induction, cells were stained for nestin in combination with retinal neuronal and glial markers. Cultured cells were collected for quantitative RT-PCR gene expression analysis prior to and after induction. In a second series, passage 2 RPCs were transplanted into the subretinal space of S334ter-3 retinal degeneration rats at postnatal day 28. After 1–4 weeks, sections through the transplant were double immunostained for nestin and various retinal specific neuronal markers. The cultured RPCs treated with RA exhibited nestin co-expression with various retinal specific markers, including protein kinase C α (PKC), neurofilament 200 (NF200), cellular retinaldehyde binding protein (CRALBP), and rhodopsin. Following RA induction, quantitative RT-PCR analysis demonstrated downregulation of nestin, PAX-6, thyl1.1, and PKC α , and upregulation of rhodopsin, glial fibrillary acidic protein (GFAP), and CrX. No nestin coexpression was observed with any of the retinal specific neuronal markers in RPC transplants *in vivo* except for some nestin-immunoreactivity overlapping with GFAP positive cells in the host retina. The role of nestin as a unique neural stem/progenitor cell marker should be reconsidered. Nestin expression during RPC maturation appears to be different *in vitro* versus *in vivo*.

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Keywords: retina; nestin; retinal progenitor cells; RT-PCR; transplantation; retina

1. Introduction

Stem cell research and the potential stem cell-based replacement therapies both depend critically on the identification of specific markers that can be used to identify and select stem and progenitor cells. The intermediate filament

protein nestin, originally detected by the Rat-401 monoclonal antibody, was first identified in neuroepithelial stem cells (Lendahl et al., 1990; Hockfield and McKay, 1985). While nestin protein is robustly expressed in the developing CNS—in which it appears during the time of CNS stem and progenitor cell proliferation and neuronal migration—it subsequently decreases as the brain develops (Lendahl et al., 1990; Tohyama et al., 1992; Dahlstrand et al., 1995; Kojima et al., 2004). Postnatal expression of nestin is limited to the brain subventricular (SVZ) zone and endothelial cells (Treutelaar et al., 2003; Mokry et al., 2004; Romanko et al., 2004; Ernst and Christie, 2005; Tonchev et al., 2005). Re-expression of nestin has recently been observed in activated astrocytes

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following various pathological conditions both *in vitro* and *in vivo* (Schmidt-Kastner and Humpel, 2002; Wei et al., 2002; Lang et al., 2004; Namba et al., 2005).

During mouse eye development, the presence of nestin protein has been shown by immunohistochemistry in the optic stalk as early as E9.5, and its expression has been found to increase over time. By E17.5, it is prominently expressed in the outer layer of the neural retina, the developing ciliary body and the optic disc, but it disappears at postnatal day one (Yang et al., 2000). Nestin re-expression was found in epiretinal membranes in adult human eyes under pathological conditions (Mayer et al., 2003).

However, even in normal development, there is emerging evidence that nestin is not exclusively restricted to neural progenitor/stem cells, as its presence has been reported in multiple lineage-committed cell types, including cardiac muscle, lens-epithelial cells, multipotent pancreatic stem cells, Schwann cells, microglia cells and oligodendrocytes (Zimmerman et al., 1994; Yang et al., 2000; Vanderwinden et al., 2002; Treutelaar et al., 2003; Yokoyama et al., 2004; Amoh et al., 2005). In addition, nestin co-expression with early or late stage neuronal phenotypic markers, such as beta-tubulin-III and MAP-2, has also been reported at a certain stage of neuronal development (Rosser et al., 1997). Co-expression of nestin with late stage neuronal phenotypic markers in retinal progenitor cells has not been well described. Based on previous studies of embryonic development (Yang et al., 2000), we hypothesize that down-regulation of nestin expression coincides with increased expression of mature retinal specific neural markers, such as opsin and rhodopsin.

With the advances in stem cell technology and in developmental biology, the traditional definitions of stem cells and the concept of nestin as a neuro-ectodermal marker are being challenged.

Retinal progenitor cells (RPC) may be a potential cell source for the repair of diseased retinæ, but nestin expression has not been reexamined in light of the recent findings. Previously, we established a reliable and efficient culture system for the isolation and expansion of rat retinal progenitor cells, and developed a subretinal injection methodology, which allowed us to achieve a high proportion of cells that expressed photoreceptor markers both *in vivo* and *in vitro* (Qiu et al., 2004, 2005). Given the recent nestin controversy, reevaluating nestin expression in RPCs appears warranted and may provide insights into the differentiation pathway of the retinal stem cell lineage. Therefore, the aim of this study is to investigate whether nestin is co-expressed with retinal specific neuronal markers in cultured RPCs following retinoic acid induction and in grafted RPCs following subretinal transplantation in retinal degenerate rats.

2. Materials and methods

2.1. Experimental animals

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. Donor cells for this study were derived from transgenic rats expressing human alkaline phosphatase (hPAP) in all cells (Kisseberth et al., 1999), which were bred with ACI rats (Harlan, Indianapolis, IN) to produce a pigmented strain. A total of 18 timed-pregnant hPAP rats were used for this study. Recipients of RPC transplants were ten pigmented S334ter-line-3 transgenic rats (28 days old) expressing a mutated human rhodopsin protein. The rats were produced by Xenogen Biosciences (formerly Chrysalis DNX Transgenic Sciences, Princeton, NJ, USA) and were developed and supplied with the support of the National Eye Institute by Dr. Matthew LaVail, University of California San Francisco, CA, USA (<http://www.ucsfeye.net/mlavailRDratmodels.shtml>). The F1 generation, of a cross between homozygous line 3 and pigmented Copenhagen rats (Harlan, Indianapolis, IN), was used for the experiments. Rats of both sexes were used.

2.2. Retinal progenitor cell isolation and *in vitro* induction

The methods of *in vitro* isolation and expansion of rat RPCs in serum free culture have been described previously (Qiu et al., 2004; Qiu et al., 2005). In brief, RPCs were isolated from embryonic day 17 hPAP(+) rat retina. To avoid glial and epithelium cell contamination, the retinal pigment epithelium layer, optic disc and ciliary margin zone were excluded. The isolation procedure was performed on a cold stand (4 °C) using Hibernation™ medium (Brainbits, US patent No. 6180404) plus B27 supplement (Invitrogen, CA, USA) to maintain the embryonic tissue. Isolated RPCs were cultured and expanded in serum-free defined culture medium supplemented with both EGF (200 ng/ml) and bFGF (10 ng/ml) (Invitrogen, CA). Cells reaching 85% confluence were passaged, using 0.25% trypsin (Invitrogen, CA, USA) and manual trituration. *In vitro* expanded RPCs at passage 2 and passage 6 were seeded either on 4-chamber slides (for immunocytochemical study) or on 6-well culture plates (RT-PCR) and subjected to an 8 day induction protocol with all-*trans* retinoic acid (Sigma-Aldrich, MO, USA). To initiate the induction, EGF and bFGF were removed from the culture media and 1% fetal bovine serum (FBS) (Invitrogen, CA, USA) along with 10^{-6} M all-*trans* retinoic acid (RA) was added. After adding RA, the dishes were protected from light by an aluminum foil cover. The induction culture media was changed every other day. During the 8 day RA treatments, the cultured cells were examined twice daily to observe the morphological changes using an inverted light microscope.

After the 8 day induction, the cultured cells were prepared for both immunocytochemical assays and RT-PCR analysis. For immunocytochemical assays, cultured cells derived from passages 2 and 6 were fixed with 4% paraformaldehyde for 20 min at room temperature followed by three washes with PBS. For gene expression profile analysis, only passage 2 RPCs were used. All the induction experiments were repeated at least three times.

2.3. *In vitro* differentiation profile of RPCs

2.3.1. Immunocytochemical assays

To analyze the phenotypic expression profile of RPCs following *in vitro* induction, double immunocytochemical assays were conducted on the fixed adherent differentiated cells, which had been derived from passages 2 and 6 RPCs. The protocol of immunocytochemical assays has been described previously (Qiu et al., 2004). Briefly, following fixation, the cultured cells were blocked in 20% goat serum for 30 min at room temperature. Various primary antibody combinations were prepared and added onto the same batch of cell samples. All related antibodies used in this study are listed in Table 1. The cultured cells were incubated with primary antibodies at 4 °C overnight, followed by three PBS washes. The next day, the cells were incubated with goat anti-mouse and goat anti-rabbit fluorescence-conjugated secondary antibodies (Table 1) for 1 h at room temperature followed by three PBS washes. Cell nuclei were stained with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) by using DAPI containing mounting media (Vector Labs, Burlingame, CA, USA). The stained slides were studied with a Zeiss LSM 510 confocal microscope (Carl Zeiss, Germany). Positive stained cells were counted using 40× magnification. From each sample, counts were averaged from six randomly selected microscopic fields.

2.3.2. Real time quantitative RT-PCR

To compare the gene expression of RPCs before and after induction with all-*trans* retinoic acid (RA) treatment,

quantitative RT-PCR was performed on the cultured passage 2 cells. Various genes representing early and late retinal development stages or different retinal cell phenotypes were selected for gene-expression analysis. For example, Pax and CrX were expressed in RPCs during the early stage of retinal development. Opsin and thy1.1 genes were expressed in mature photoreceptors and ganglion cells, respectively. PKC and GFAP represented bipolar cell and Muller glia cell markers respectively. The total RNA of the cultured RPC cells was extracted from cells both before and after the induction by using the RNA extraction reagent, RNeasy (Ambion™, USA, Cat: #9736). Quantitative RT-PCR was performed for end-stage mature retinal specific makers such as Thy1.1 and opsin as well as the glial cell marker GFAP. The RT-PCR primer sequences are shown in Table 2.

Real time quantitative RT-PCR was carried out using a commercial kit “LightCycler-FastStart DNA Master SYBR Green I” (Roche Diagnostics GmbH, Germany, Cat: #3003230) as described previously (Larramendy et al., 2002). Four dilutions of beta-globulin gene (DNA Control Kit, Roche), from 0.0015 to 15 ng/ml, were used to acquire the standard curve analysis option. Data analysis was performed using the Light-Cycler Software Version 3.5. The housekeeping gene beta-actin was used as the reference against which the expression levels of the transcript genes of interest were normalized. For every sample, the target mRNA copy number was divided by the beta-actin mRNA copy number to obtain a normalized target/beta-actin value (Fig. 4).

Table 1
Antibody list

	Species	Specific for	Dilution	Supplier
<i>Primary antibodies</i>				
Recoverin	Rabbit	Photoreceptors (cones and rods); cone bipolar cells	500	McGinnis (McGinnis et al., 1997)
Rhodopsin	Mouse	Photoreceptor(rods)	50	Gift from Molday (Molday and MacKenzie, 1983)
Rhodopsin	Rabbit	Photoreceptors (rods)	1000	Plantner (Plantner et al., 1982)
Protein kinase C (PKC)	Rabbit	Rod bipolar cells	200	Oxford Biomed, (Oxford, MI, USA)
Nestin (Rat-401)	Mouse	Undifferentiated stem cells	1:50	NICHD, (IO, USA)
GFAP	Rabbit	Astrocytes, glial cells	1000	Chemicon (Temecula, CA, USA)
(glial fibrillary acidic protein)				
GFAP	Mouse	Astrocytes, glial cells	10,000	Sigma, (St Louis, MO, USA)
Calbindin	Rabbit	Horizontal and amacrine cells	2000	Calbiochem, (San Diego, CA, USA)
Calretinin	Rabbit	Amacrine cells	2000	Chemicon, (Temecula, CA, USA)
MAP-2	Rabbit	Neuronal cells	200	Sigma, (St Louis, MO, USA)
NF (neurofilament) 200 kDa	Mouse	Ganglion cells, horizontal cells	200	ICN, Costa (Mesa, CA, USA)
hPAP	Mouse	Cells containing hPAP protein	600	Sigma, (St Louis, MO, USA)
(human alkaline phosphatase)				
CRALBP	Rabbit	Müller's cells, RPE	1000	Saari, (Saari et al., 1984)
(cellular retinaldehyde binding protein) (IG 83)				
<i>Secondary antibodies</i>				
AF488 anti-rabbit IgG	Goat	All rabbit antibodies	200	Molecular Probes Inc. (CA, USA)
AF546 anti-mouse IgG	Goat	All mouse antibodies	200	Molecular Probes Inc. (CA, USA)
AF488 anti-mouse IgG	Goat	All mouse antibodies	200	Molecular Probes, (Eugene, OR, USA)
AF546 anti-rabbit IgG	Goat	All rabbit antibodies	200	Molecular Probes Inc. (CA, USA)

Table 2
PCR primer sequence

Oligo name	Sequence (written 5'–3')	Size (bp)
Nestin (forward)	TGG CAC ACC TCA AGA TGT CCC TTA	118
Nestin (reverse)	AGA AAG CCA AGA GAA GCC TGG GAA	118
PKC α (forward)	GCT GAG AAG AAT TCG GAT GTG CCT	118
PKC α (reverse)	ACT CAG AAC ACA CTT CCA CAG GCT	118
CRX (forward)	ATG CAC CAG GCTGTC CCA TAC TCA	126
CRX (reverse)	ATA CAC ATC CGG GTA CTG GGT CTT	126
PAX 6 (forward)	TTC CCG AAT TCT GCA GGT GTC CAA	108
PAX6 (reverse)	ACT CTT GGC TTA CTG CCT CCG ATT	108
Rhodopsin (forward)	AAC CTT GAG GGC TTC TTT GCC A	107
Rhodopsin (reverse)	AAG TTG CTC ATG GGC TTG GAG A	107
Thy 1.1 (forward)	AGC CAA CTT CAC CAC CAA GGA T	132
Thy 1.1 (reverse)	AGG CTT ATG CCA CCA CAC TTG A	132
GFAP (forward)	CTG AAA GTG TCC CCT GAG TT	35
GFAP (reverse)	ACA GTA CTG CTC TGA AGG TTA G	35
β -Actin (forward)	AGC CAT GTA CGT AGC CAT CCA	
β -Actin (reverse)	TCT CCG GAG TCC ATC ACA ATG	

2.4. Immunohistochemical analysis of retinal progenitor cells *in vivo*

2.4.1. *In vivo* RPC transplants

Passage 2 RPCs derived from E17 hPAP-labeled fetal retina were transplanted into the subretinal space of S334ter-3 (also termed “line 3”) retinal degenerate rats at the age of postnatal day 28. This S334ter-3 rat is a fast retinal degeneration rat model characterized by photoreceptor cell loss starting at postnatal day 14, with only a few photoreceptors sparsely distributed in the remaining neural retina by postnatal day 45.

Initially, a total of 10 recipient rats including 3 PBS injection controls were used for this study. Surgery was performed on one eye per animal. Under a Carl Zeiss surgical microscope (Carl Zeiss, Germany), *in vitro* expanded passage 2 hPAP(+) RPCs (or PBS alone in the case of controls) were slowly delivered to the subretinal space by a 32G Hamilton syringe through a trans-scleral approach. All the surgery rats including controls were sacrificed at 4 weeks after surgery.

In addition, a time course study of 12 recipients was also performed to investigate the nestin expression in grafted RPCs at various earlier time points: 8 line-3 rats received a subretinal RPC transplant and 4 received a subretinal injection of PBS. Following transplantation, the rats were sacrificed at week 1, 2, 3, or 4 (two experimental rats and one PBS control rat were assigned to each time point). Eye cups were immersion fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 2 h on ice, washed with 0.1 M Na-phosphate buffer, infiltrated with 30% sucrose overnight and embedded in Tissue-Tek (SAKURA, USA) for serial cryosections. Every fifth slide was stained histochemically for hPAP to assist in the identification of graft and the selection of sections for immunohistochemical assays. The hPAP histochemical assay has been described previously (Qiu et al., 2005).

2.4.2. Immunohistochemical assays

On selected sections, double immunohistochemical assays were performed to study whether or not grafted RPCs co-expressed nestin with end-stage-retinal specific neuronal cell markers. Prior to the staining procedure, the slides were dried on a slide warmer (37 °C) and washed with PBS to remove OCT. The protocol of immunohistochemical assays was similar to that described for the cultured cells in this study (Qiu et al., 2004, 2005). The primary antibody combinations for double staining included the following: nestin with rhodopsin, nestin with GFAP, nestin with PKC, and nestin with calbindin. The concentrations and sources of primary antibodies, as well as the secondary antibodies used in these experiments, are listed in Table 1. Following staining, the slides were evaluated with a Zeiss LSM 510 Confocal microscope (Carl Zeiss, Germany) focusing on nestin distribution in both host and graft cells.

3. Results

3.1. *In vitro* characteristics of cultured retinal progenitor cells

RPCs are, like brain-derived neural progenitor cells, characterized by their potential to proliferate, self-renew and differentiate into neurons and glial cells. With our previous established serum free culture system (Qiu et al., 2004), E17-derived rat RPCs can maintain their multipotency and self-renewal capacity through various passages. Both passage 2 and 6 RPCs can generate neurospheres under the current culture conditions stimulated by EGF and bFGF. After removal of EGF and bFGF from the culture media and supplementation with a low concentration of fetal bovine serum, the adherent cultured neurospheres responded to RA treatment. By the second day of RA induction, the cells expanded neurites around the edge of the neurospheres (Fig. 1), and gradually elongated their cell processes. By the sixth day of induction, the majority of the cells had spread out, migrated away from their original neurospheres, and differentiated into several cell types, which varied in morphology and the presence of neural processes (Fig. 1). At the end of induction (day 8), the original neurospheres had flattened out, but the differentiated cells tended to grow in cell clusters in the vicinity of the original neurosphere location.

3.2. *In vitro* phenotypic differentiation profile of RPCs

Our experiments confirmed our previously published data, which had shown that E17 derived rat RPCs can maintain nestin-immunoreactivity over various passages in serum free culture-media prior to induction with RA (Qiu et al., 2004). No co-expression of nestin with any of the retinal specific neuronal cell markers had been observed in cultured RPCs prior to induction (Qiu et al., 2004, 2005). Following *in vitro* induction, immunofluorescent double staining showed that the RA-treated cells co-expressed nestin simultaneously with both retinal specific neuronal and glial cell markers. Nestin

Fig. 1. Retinal progenitor cell differentiation *in vitro*. E17 derived RPCs were treated with all-*trans* retinoic acid for 8 days to induce differentiation. Phase-contrast photographs demonstrate migration of cultured cells away from their original neurospheres, with formation of neural process at day 2 of RA induction (A, 20 $\times$; bar 100 μ m). At day 6 of RA induction, most of the cultured cells have spread out and developed into cells with various shapes and processes (B, 40 $\times$; bar 50 μ m).

co-expression with PKC- α (rod bipolar cell marker), CRALBP (Müller cell marker), MAP-2 (mature neuronal marker), and rhodopsin (photoreceptor marker) was observed in both passage 2 or passage 6 RPCs after the 8-day induction period with all-*trans* retinoic acid (Figs. 2 and 3). There appeared to be no significant differences in nestin co-expression in the cultured cell samples from passage 2 and passage 6. In addition, co-expression of MAP-2 with rhodopsin and co-expression of NF-200 with Calbindin (horizontal and amacrine cells) was also observed in the cultured cells following induction (Figs. 2 and 3). Nestin-immunoreactivity did not appear to overlap completely with retinal specific neuronal markers, although both markers appeared to be located in the same cells.

Quantitative evaluation of the cells under the confocal microscope indicated that 85% of RPCs expressed nestin before the induction, which was consistent with our previous published data (Qiu et al., 2004). Following the induction, the co-expression of nestin was as follows: nestin and PKC was 50% of the nestin-positive cells; nestin and NF200 was 32% of the nestin-positive cells; nestin and CRALBP was 10% of the nestin-positive cells; nestin-GFAP was 10% of the nestin-positive cells; nestin-rhodopsin was 1% of the nestin-positive cells.

3.3. Real time qRT-PCR analysis on cultured RPCs

Based on the *in vitro* phenotypic differentiation profile of RPCs, a quantitative RT-PCR was designed to evaluate mRNA expression of various retinal markers in passage 2 RPCs before and after RA induction. Rhodopsin, CrX and GFAP were found to be up-regulated in the cells after RA treatment, whereas nestin, PAX, Thy1.1 and PKC α were down-regulated (compared to pre-RA treatment levels) (Fig. 4). Fig. 4 illustrates the relative amount of mRNA in the RA(+) samples compared to the RA(-) samples. After RA induction, Thy-1 was reduced to 26% (of the pre-induction level), PAX-6 to 30%, PKC α to 82% and Nestin to 13%.

Rhodopsin increased by 18.6%, CrX by 24.2%, and GFAP by 30%.

3.4. In vivo evaluation of nestin expression in grafted retinal degenerate rats

This experiment confirmed our previously published *in vivo* data, which showed that E17-derived RPCs can differentiate into retinal specific neurons, which predominantly express the photoreceptor cell marker rhodopsin, following subretinal transplantation in retinal degenerate rats, with minimal GFAP expression among grafted cells (Qiu et al., 2005). In sections containing grafts, the double staining of nestin with various retinal specific markers, including rhodopsin, recoverin, calretinin, and calbindin, did not demonstrate co-localization of nestin expression with any of the retinal specific neuronal markers (Fig. 5), but did show some co-expression with GFAP and CRALBP (Figs. 5 and 6) within the graft area. In the remaining host retinas of these late stage retinal degenerate rats, the nestin immunostaining pattern also showed overlap with glial processes expressing GFAP and CRALBP (Figs. 5 and 6).

The time course study of grafted RPCs in line 3 rats revealed no co-expression of nestin with any retinal specific neuronal markers in either the graft- or the remaining host retina area at any of the earlier time points. However, some nestin co-expression with GFAP was found in the graft and host retina at all time points. In addition, the differentiation profile of RPCs at weeks 1, 2, 3 were similar to that observed at week 4. The nestin immunostaining assay, however, suggested a trend for greater nestin expression at earlier time points (such as weeks 1 and 2) than in late time points (weeks 3 and 4). The week 1 results are shown in Fig. 7.

4. Discussion

In this study, E17-derived rat RPCs were found to co-express nestin with end-stage retinal specific neuronal

Fig. 2. Passage 2 RPC differentiation profile *in vitro*. Following 8 days of *in vitro* RA induction, confocal immunofluorescent analysis shows cultured cells co-expressed nestin with rhodopsin (A–D), CRALBP (E–H), MAP-2 (I–L), and PKC (M–P); Rhodopsin co-expression with MAP-2 (Q–T); and calbindin co-expression with NF-200 (U–X) can be observed in the cultured cells treated with RA. Bar 20 μm.

Fig. 3. Passage 6 RPC differentiation profile *in vitro*. Following 8 days of *in vitro* RA induction, confocal immunofluorescent analysis shows cultured cells co-expressed nestin with rhodopsin (A–D), PKC (M–P) and CRALBP (Q–T). Rhodopsin co-expression with MAP-2 (E–H); and calbindin co-expression with NF-200 (I–L) can be observed in the cultured cells treated with RA. Bar 20 μ m.

markers, including rhodopsin, PKC, and calbindin, as well as with Müller glial cell markers after 8 days of *in vitro* induction with retinoic acid. Quantitative RT-PCR indicated down-regulation of nestin and Pax-6, and up-regulation of CrX and rhodopsin in cultured RPCs following RA treatment. Four weeks following subretinal transplantation (without prior RA treatment) of cultured RPCs in line 3 retinal degenerate rats, however, nestin co-expression was not found in the cells that were immunoreactive with retinal specific neuronal markers such as

rhodopsin and recoverin. GFAP up-regulation that overlapped with nestin-re-expression was observed in both graft and host retinas.

4.1. Revisiting nestin expression in retinal progenitor cells

Various molecular markers are used to characterize early developmental stages of neurons or astrocytes. The

Fig. 4. Relative amount of mRNA in the RA+ samples compared to the RA– samples. Quantitative RT-PCR raw data (before and after RA induction) was normalized with respect to the β -actin (value of 1) in each sample. The difference between the RA– and RA+ samples normalized with respect to the β -actin represents the relative amount of mRNA in the RA(+) samples compared to the RA(–) samples (value of 1 for β -actin). Any value in the graph above 1 is an increase and below 1 is a decrease. After RA induction, Thy-1 is reduced to 26%, PAX is reduced to 30%, PKC α is reduced to 82%, Nestin is reduced to 13%; while opsin increased to 18.6%, Crx increased to 24.2%, GFAP increased to 30% etc. RA(+) = after RA treatment; RA(–) = before RA treatment.

intermediate filament protein nestin shows early and widespread expression in the developing CNS, as well as in the retina, and is one of the best-characterized protein markers for immature neural cells (Dahlstrand et al., 1995; Yang et al., 2000; Krugliakova et al., 2004;). Although, nestin co-expression with several neuronal cell markers, including beta-tubulin-III and MAP-2, and glial cell markers GFAP, has been recently reported in adult CNS (Rosser et al., 1997; Blumcke et al., 2001; Piper et al., 2001), it is interesting that we also found E17 derived RPCs co-expressed nestin with various end-stage retinal specific neuronal and glial markers *in vitro* after RA(+) treatment. Rhodopsin co-expression with MAP-2 and NF200 was also observed in our *in vitro* induction experiment, which may suggest that retinal specific neurons share some properties with CNS neurons.

Nestin co-expression may indicate a transitional stage in the RPC maturation process during which nestin expression is a temporary developmental event. In that case, it is unknown whether these cells are restricted to differentiate along the lineage of the co-expressed marker (for example, a cell that expresses both nestin and rhodopsin may be committed along the rod cell lineage), or if these cells still retain the multipotency typical of RPCs. Regardless, this observation raises the concern that nestin may not be a specific marker for RPCs. These findings suggest that other developmental markers such as Ki67 should also be considered, especially for the identification and purification of RPCs *in vitro*.

4.2. Nestin expression in retinal progenitor cells *in vitro*

During embryonic development, RPC maturation and migration occurs in a restricted temporal and spatial order, in which retinal ganglion cells (RGCs) are the first and Müller cells are the last cells to develop (Cepko, 1993, 1999; Turner and Cepko, 1987; Lillien and Wancio, 1998). It is unlikely that

any *in vitro* induction system will create an environment that allows all the cultured cells to complete their cell fate determination simultaneously. E17 derived RPCs are heterogeneous and each neurosphere in the same culture system may comprise different lineage retinal precursors with varying potential for proliferation and differentiation. During the 8-day RA induction process, some progenitor cells may begin to differentiate immediately, whereas others may respond to the *in vitro* induction signals at a later time and may continue to proliferate throughout the induction period. At the end of induction, some cultured cells may have also retained their stem cell properties, which may partially explain why the nestin protein was still present in the cultured RPCs at the end of the induction period. Previous studies have shown that *in vitro* culture systems appear to shorten the time course of stem cell differentiation and maturation (Bain et al., 1995, 1996; Tang et al., 2000). Although the microenvironment and time course of RPC induction *in vitro* is different from that *in vivo*, the differences in nestin expression may also be due to differences in signaling pathways involved in RPC cell fate determination between the *in vitro* and *in vivo* environments.

Retinoic acid (RA) plays a critical role in the development of the eye, the maturation of visual function, as well as for photoreception in the functional retina (Luo et al., 2006). All-trans retinoic acid is commonly used *in vitro* to differentiate stem cell populations including adult neural stem cells and embryonic stem cell into neurons (Bain et al., 1995, 1996; Fraichard et al., 1995). Our previous studies have showed RA could efficiently induce cultured RPCs differentiation *in vitro* (Qiu et al., 2004, 2005). However, it should be noted that the current RA induction protocol for RPCs may not be the optimal choice. The co-expression of nestin with retinal neuronal markers may be a transitional event that occurs during the *in vitro* induction process.

4.3. Nestin down-regulation in cultured RPCs following *in vitro* induction

Previous studies showed that cells exiting the cell cycle at the beginning of differentiation, down-regulate nestin and subsequently up-regulate alternative intermediate filaments during both neuronal (Cattaneo and McKay, 1990) and glial differentiation (Reynolds and Weiss, 1992; Vescovi et al., 1993). In our study, down-regulation of nestin and up-regulation of rhodopsin and GFAP were found in cultured RPCs after RA treatment *in vitro*, which is consistent with the *in vitro* differentiation profile of RPCs illustrated by the immunocytochemical assays. Previous studies showed that nestin down-regulation during neural maturation correlates with several signaling pathways (Bao and Cepko, 1997; Nacher et al., 2005). Using organotypic hippocampal slice-co-cultures with the neural stem cell line MHP36, it was found that the loss of nestin expression in MHP36 was very rapid, indicating an early step in the differentiation process *in vitro* (Hugnot et al., 2001). Their study suggested that the notch signaling pathway was involved in the nestin down regulation. In our study, decreased mRNA expression of both nestin and Pax-6

Fig. 5. Immunoreactive pattern of nestin and retinal markers in the grafted RPCs in S334ter-3 rats. Passage-2 hPAP(+) RPCs were transplanted into the subretinal space in postnatal day 28 S334ter-3 rats and followed for 4 weeks. Double immunostaining of retinal specific markers with hPAP or nestin was performed on adjacent sections. Confocal immunohistochemical analysis shows that the majority of grafted hPAP(+) cells express rhodopsin (A–D) and recoverin (I–L). A few grafted cells were immunoreactive for the Müller cell marker, CRALBP (Q–T). No coexpression of nestin with rhodopsin (E–F) or recoverin (M–P) within the graft area was observed, although the nestin staining pattern appears to resemble that of Müller's glial cell process (F, U–V) in both the host retina and the grafts (details see Fig. 6). Rho, rhodopsin; INL, inner nuclei layer; RGC, retinal ganglion cell. Bar 20 μ m. Pictures are oriented with the ganglion cell layer down.

Fig. 6. Overlap of nestin and GFAP expression in host retina and RPC grafts. Passage-2 hPAP(+) RPCs were transplanted into the subretinal space of postnatal day 28 S334ter-3 rats and followed for 4 weeks. Immunostaining for hPAP (A1–A4), and double staining for nestin and GFAP (B1–B4) was performed on adjacent sections. The nestin staining patterns showed overlap with some of the GFAP+ glia cell processes within the grafted cells (arrowheads in A and B column). In the transplant area, GFAP upregulation and nestin re-expression were not observed in the host retina (C1–C4). A few nestin-positive cells show overlap with GFAP-positive cells within the grafts (arrows in C column). Outside of the transplant area, the host retina also shows some cells with overlap between GFAP upregulation and nestin re-expression (D1–D4, E1–E4). Bar 50 μ m. Pictures are oriented with the ganglion cell layer up.

was found in cultured RPCs after RA treatment, which is consistent with the hypothesis that the PAX signaling pathway may be involved in the nestin down regulation during RPC *in vitro* differentiation. Pax-6 is known to be widely expressed in RPCs during early eye development. The significant down-regulation of Pax 6 (reduced to 30% of pre-induction level) suggests that RPCs are differentiated into more late stage lineage restricted progenitors. Characterization of the relative level of down-regulation of the various Pax-6 isoforms will be performed in subsequent studies.

The Otx-like homeobox gene CrX was originally isolated from the mouse retina. Crx expression is restricted to

developing and mature photoreceptor cells, and is important in regulation of photoreceptor differentiation *in vivo* (Furukawa et al., 1997). Quantitative RT-PCR analysis showed that up-regulation of rhodopsin and CrX mRNA expression was detected in cultured RPCs after RA treatment, suggesting that the *in vitro* photoreceptor differentiation pathway may involve the same gene (CrX) as that involved *in vivo*.

4.4. Nestin expression in adult rats with RPC transplants

In contrast with the *in vitro* phenotypic differentiation profile, nestin co-expression with retinal specific neuronal cell

Fig. 7. Nestin co-expression with GFAP *in vivo* at an earlier time point. S334-line 3 retinal degenerate rats, received passage-2 RPCs subretinal transplants and were sacrificed 1 week after implantation, for double immunohistochemical assays of nestin and GFAP. Significant nestin expression can be observed in the graft area (A and D), with very weak expression in the remaining host retina. GFAP expression (B and E) is found in both the graft and remaining host retina. Some overlap of nestin and GFAP expression can be seen in the graft (C and F). Bar 100 μ m. Orientation was with the ganglion cell layer up.

markers such as rhodopsin, recoverin, and PKC, was not found in the grafted RPCs 4 weeks after subretinal transplantation in retinal degenerate rats, although the majority of the graft-derived cells expressed photoreceptor cell markers such as rhodopsin (as previously published by Qiu et al., 2005). Only a few grafted cells co-expressed nestin and GFAP in the subretinal space during the first week post-transplantation. The subretinal microenvironment may have induced further RPC differentiation towards retinal specific neurons, and could have possibly provided a better developmental “niche” for the survival of newly formed retinal neuronal cells, especially rhodopsin-immunopositive cells. It also should be noted that any cellular event and developmental stage may take a shorter time *in vitro* than that *in vivo* (Bain et al, 1995, 1996), which makes it difficult to compare *in vitro* and *in vivo* data over the same time course.

Re-expression of nestin has been found in reactive astrocytes of the adult rodent brain after brain seizure, trauma and ischemic injuries (Kuroda et al., 2002; Nakamura et al., 2003; Jang et al., 2004). In this study, we used S334ter line 3 retinal degenerate rats as hosts to receive RPC transplants. Late-stage (P56 by the time of sacrifice) degenerate retina undergoes extensive retinal remodeling, glial proliferation and migration, as well as glial scar formation (Marc et al., 2003; Marc and Jones, 2003). Accordingly, abundant GFAP immunoreactivity was found in the remaining host retina. The overlapping pattern of GFAP and nestin expression in the host retina *in vivo* most likely reflects this active retinal remodeling process. Co-expression of nestin and GFAP was also observed at some sites of surgical intervention. Regardless, nestin re-expression may reflect the reversal of glia/astrocytes to a more immature phenotype during the reactive state, and may reflect a higher plasticity in these cells.

In summary, nestin can be co-expressed with mature retinal neuronal markers *in vitro*, but not *in vivo*, after a longer time

period. Nestin expression may not be a pathognomonic marker for RPCs and its role in retinal neuronal development warrants further study.

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