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Retinal Stem Cells Transplanted into Models of Late Stages of Retinitis Pigmentosa Preferentially Adopt a Glial or a Retinal Ganglion Cell Fate

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Abstract

Purpose—To characterize the potential of newborn retinal stem cells (RSCs) isolated from the radial glia population to integrate the retina, this study was conducted to investigate the fate of in vitro expanded RSCs transplanted into retinas devoid of photoreceptors (adult *rd1* and old VPP mice and rhodopsin-mutated transgenic mice) or partially degenerated retina (adult VPP mice) retinas.

Methods—Populations of RSCs and progenitor cells were isolated either from DBA2J newborn mice and labeled with the red lipophilic fluorescent dye (PKH26) or from GFP (green fluorescent protein) transgenic mice. After expansion in EGF+FGF2 (epidermal growth factor+fibroblast growth factor), cells were transplanted intravitreally or subretinally into the eyes of adult wild-type, transgenic mice undergoing slow (VPP strain) or rapid (*rd1* strain) retinal degeneration.

Results—Only limited migration and differentiation of the cells were observed in normal mice injected subretinally or in VPP and *rd1* mice injected intravitreally. After subretinal injection in old VPP mice, transplanted cells massively migrated into the ganglion cell layer and, at 1 and 4 weeks after injection, harbored neuronal and glial markers expressed locally, such as β -tubulin-III, NeuN, Brn3b, or glial fibrillary acidic protein (GFAP), with a marked preference for the glial phenotype. In adult VPP retinas, the grafted cells behaved similarly. Few grafted cells stayed in the degenerating outer nuclear layer (ONL). These cells were, in rare cases, positive for rhodopsin or recoverin, markers specific for photoreceptors and some bipolar cells.

Conclusions—These results show that the grafted cells preferentially integrate into the GCL and IPL and express ganglion cell or glial markers, thus exhibiting migratory and differentiation preferences when injected subretinally. It also appears that the retina, whether partially degenerated or already degenerated, does not provide signals to induce massive differentiation of RSCs into photoreceptors. This observation suggests that a predifferentiation of RSCs into photoreceptors before transplantation may be necessary to obtain graft integration in the ONL.

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Disclosure: **K. Canola**, None; **B. Angénioux**, None; **M. Tekaya**, None; **A. Quiambao**, None; **M.I. Naash**, None; **F.L. Munier**, None; **D.F. Schorderet**, None; **Y. Arsenijevic**, None

Neuroretinal degenerative diseases such as retinitis pigmentosa and age-related macular degeneration are the major causes of blindness in the Western world. Such disorders are the consequence of various intrinsic and extrinsic factors (reviewed in Ref. 1) and generally result in the complete loss of visual function as a consequence of photoreceptor degeneration.

Cellular transplantation has been proposed as a potential treatment of these retinal-degenerative diseases (reviewed in Ref. 2). The donor cells and the host recipient are two major key aspects of the procedure. Various injection studies have been undertaken with different cellular types to replace lost cells, but have not so far been unequivocal. Hippocampal,^{3–5} embryonic (ES),⁶ and bone marrow⁷ stem cells have been tested in models of retinal degenerative disorders. These cells show high migratory and differentiation capacity, as they can differentiate into neurons, astrocytes, and oligodendrocytes. Of note, cells derived from ES cells can form retinal neurons.^{8,9}

Conversely, the use of retinal stem/progenitor cells in suspension or as neurospheres exhibit poor migration but are successful in expressing retina-specific markers after transplantation.^{10–12} Moreover, we recently showed that adult human retinal stem cells (RSCs) can generate retinal cells when grafted into a developing retina.¹³ It has been described that cell integration occurs in the adult rodent eye primarily in mechanically³ or genetically injured retinas¹⁴ but not in the normal adult one.¹⁴ The types of the retinas investigated can be subdivided into two groups: partially degenerated retinas and fully degenerated retinas. From a clinical point of view, the latter is of prime importance to investigate,^{15–18} to assess whether these retinas, completely devoid of any photoreceptors, would allow RSC incorporation.

To address whether newborn mouse RSCs would populate a normal or diseased retina, we used characterized cells contained in the radial glia cell population, showing a high proliferation potential that generates RSCs and progenitor and precursor cells in culture (previously described in Ref. 19). These cells are contained in the RC2-positive population (radial glia marker), they express transcription factors usually found in radial glia (Mash1, Pax6), and ~3% to 5% of the cultured cells meet the criteria of stem cells: high capacity for expansion, maintenance of an undifferentiated state, and multipotency demonstrated by clonal analysis.¹⁹ These cells are different from the retinal stem cells and progenitor cells previously isolated. Of note, another group has also isolated central nervous system (CNS) stem cells in the radial glia cell population of different regions of the mouse brain.²⁰ Moreover, these RSCs can give rise in vitro to cells committed to a retinal neuron fate including photoreceptors and retinal ganglion cells.^{19,21} In the current study, we transplanted them intravitreally into the rapidly degenerating eyes of *rd1* mice or into slow-degenerating eyes of VPP transgenic mice,²² as Young et al.⁵ showed a widespread incorporation of adult rat hippocampal progenitor cells into the retina of dystrophic rats when injected intravitreally. As results in the intravitreal transplantation were not satisfying, we transplanted the RSCs subretinally, to bring the cells into the vicinity of the remaining inner nuclear layer (INL). For this purpose, VPP mice received subretinal transplantation and wild-type animals were used to assess the feasibility and reproducibility of the subretinal injection. We showed that RSCs retain the capacity to differentiate into retinal cells, either at the morphologic level or both morphologically and at the level of protein expression, in certain layers of the retina (GCL, INL) although the cells failed to differentiate toward the photoreceptor fate, except in rare cases of partially degenerated retinas. Depending on the model and grafting procedure used, the cells extensively migrated toward the innermost layers of the retina (i.e., the GCL, where some cells expressed RGC markers). This demonstrates that RSCs can respond to cues in the natural microenvironment of a diseased retina, but newly generated photoreceptors would be needed to support photoreceptor replacement.

Materials and Methods

Animals

Animals were handled according to the guidelines on care and use of experimental animals set by the cantonal veterinary of Vaud and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Donor strains were DBA2J mice (The Jackson Laboratory, Bar Harbor ME) and eGFP-3' UTR mice (gift from Masaru Okabe, Osaka University). C57Bl/6J (The Jackson Laboratory), VPP transgenic mice,²² and FVB/NJ *rdl* mutant mice (gift from Marten van Lohuizen, The Netherlands Cancer Institute, Amsterdam) were used as recipient animals in our experiments and maintained at 21 °C with a dark–light cycle of 12 hours and fed ad libitum with standard laboratory food and water. eGFP-3'UTR mice contain an enhanced green fluorescent protein (eGFP).²³ FVB/NJ inbred mice are homozygous for the *Pde6b^{rd1}* allele (*rdl* mutation) located in exon 7 of the rod cGMP-phosphodiesterase- β , which causes an early and severe onset of retinal degeneration.²⁴ VPP mice are transgenic mice heterozygous for a mutated form of the rhodopsin gene. The VPP transgene consists of three amino acid substitutions near the N terminus of rhodopsin, Val-20→Gly (V20G), Pro-23→His (P23H) and Pro-27→Leu (P27L) and causes slow photoreceptor degeneration.²² A total of 66 animals received cellular transplants in one or both eyes. Grafted cells were found in each of the grafted eyes, and no graft recipient died. The distribution of the animals in the different experimental groups is summarized in Table 1.

Preparation of RSCs

RSCs were isolated either from the retina of DBA2J or eGFP-3'UTR newborn mice (between P0 and P2) as we previously described.¹⁹ In brief, the radial glia population was selected by medium condition, plastic, and cell density used. The eyes were dissected and the optic nerve, the retinal pigmented epithelium, as well as the central retina were discarded. The tissue was then digested 10 minutes in an enzyme solution containing Mg^{2+} (3.2 mM $MgCl_2$) and low Ca^{2+} (0.1 mM $CaCl_2$), 1.33 mg/mL trypsin, 0.67 mg/mL hyaluronidase, and 0.2 mg/mL kynurenic acid (all products were obtained from Sigma-Aldrich, St. Louis, MO), then triturated with a fire-polished pipette and centrifuged at 1800 rpm. The pellet was resuspended in a serum-free medium²⁵ containing 20 ng/mL EGF and 20 ng/mL basic FGF-2 (Peprotech, Rocky Hill, NJ). The cells were plated in a 75-cm² flask (Nunc, Naperville, IL) at a density of 3 or 5 \times 10⁵ cells/mL. Cells were trypsinized when they reached 100% confluence and were either expanded or harvested for transplantation. Because RSCs retain a robust capacity to differentiate into retinal neurons from early to late cell passages,²¹ we used cells harvested at passages 4 to 20. Before transplantation, RSCs from DBA2J were labeled with PKH26 (Sigma-Aldrich) a lipophilic red dye, at a concentration of 2 μ M and washed with PBS or HBSS (Sigma-Aldrich). Stained cells or GFP-expressing cells were counted with a hemocytometer and the suspension to be transplanted was diluted to the appropriate cell density, 50,000 cells/ μ L. We used mainly PKH26-labeled cells, because they show almost no cell death after transplantation. We spent much time trying to optimize the handling of GFP-expressing cells to prevent cell aggregation resulting in massive cell death after the passage of the needle. Indeed, after dissociation the cells showed a pronounced tendency to stick to each other, rapidly forming clusters when concentrated, thus most of the results were obtained with PKH26-labeled cells.

Transplantation Procedure

Surgical procedures were performed under volatile anesthesia (Isoflurane; Baxter, Deerfield, IL). In addition, tetracaine (Tetracaine 1% SDU Faure; Novartis, Basel, Switzerland) was applied on the surface of the eye as a local anesthesia. A small area of the surface of the sclera was removed in the superior half of the eye, and a 23-gauge needle (BD Biosciences, Franklin Lakes, NJ) was used to open the choroid gently. A 5- μ L syringe (Hamilton, Reno, NV) with a 34-gauge needle was used to inject 1.5 μ L of cell suspension (~75,000 cells) slowly, into the

subretinal space (SS) or the vitreous. Control retinas were of three types: noninjected eyes, sham surgery, or dead cell injections. Sham surgery was performed by injection of PBS or HBSS, depending on the last wash of the cell suspension, whereas dead cells were obtained by successive immersions of the cell suspensions in liquid nitrogen before grafting.

Tissue Preparation

Animals were killed by cervical dislocation at different time points after transplantation. Eyes were removed and fixed in a 4% paraformaldehyde solution for at least 1 hour and then transferred into 25% sucrose in PBS for at least 2 hours before cryosectioning. Retinal sections (14 μ m) were mounted on APTES (3-aminopropyltriethoxysilane)-treated slides and stored at room temperature until further processing.

Immunohistochemical Analysis

Sections were blocked in PBS containing 10% normal goat serum and 0.3% Triton for 1 hour at room temperature and incubated with primary antibodies overnight at 4°C. Primary antibodies used for immunohistochemical analysis included mouse monoclonal anti-neuronal nuclei (NeuN, 1:100; Chemicon, Temecula, CA); mouse monoclonal anti- β -tubulin isotype III (1:1000; Sigma-Aldrich); rabbit polyclonal anti-Brn3b (1:500; gift from Eric E. Turner, University of California, San Diego); rabbit polyclonal anti-recoverin (1:1000; Chemicon); rabbit polyclonal anti-gial fibrillary acidic protein (GFAP; 1:400; Dako, Carpinteria, CA). After incubation with the primary antibody, sections were successively washed in PBS, incubated with a secondary antibody for 1 hour at room temperature (goat anti-mouse or goat anti-rabbit conjugated to FITC [1:100] or Cy3 [1:1000] from Jackson ImmunoResearch, West Grove, PA), washed with PBS and nuclei were counter-stained with DAPI (4',6-diamidino-2-phenylindole, dilactate; Invitrogen-Molecular Probes, Eugene, OR). Immunofluorescence was observed with an epifluorescence microscope (BX60; Olympus, Tokyo, Japan) and a confocal microscope (LSM 510 Meta; Carl Zeiss Meditec, Dublin, CA).

Cell Survival Analysis

Cells were passed through the syringe needle used for transplantation, and colored with trypan blue solution 0.4% (Sigma-Aldrich). The percentage of dead cells was then counted on a hemocytometer.

TUNEL (terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick-end labeling) was performed on cryostat sections (Dead-End Colorimetric TUNEL System; Promega, Madison, WI) after the manufacturer's directions. The number of dead cells was then evaluated in bright-field images.

Results

Grafted Cell Survival

As the passage through the needle during the graft procedure may be detrimental to the cells, we evaluated cell death *in vitro* before and after injection. Cultures from five different mice were tested with three different 34-gauge needles. We counted 2% of dead cells in the cell suspension after harvesting and 6% after the passage through the different needles, illustrating the harmlessness of the procedure for the cells ($n = 5$; passages 10–20).

We then conducted a TUNEL analysis on six retinas of wild-type C57/Bl6 mice at 1, 2, and 5 days after subretinal injection (short-term survival) to investigate cell death occurring in the transplanted cells and in the retina. We evaluated both host- and graft-labeled cells, but we did not observe any increase in cell apoptosis compared with retinas of noninjected wild-type mice ($n = 3$ per group; data not shown). Finally, we counted the number of surviving grafted cells

on bright-field images on C57Bl/6 retinas ($n = 3$) at 3 months after surgery. We obtained a mean of 328 ± 104 surviving cells in three different experiments—a long-term cell survival of 0.44% of the number of injected cells in a normal retina.

These results indicate that the procedure and cells used allow a satisfactory cell survival in the normal retina during the first days after injection and that no tumors are formed, even after extensive cell passaging.²⁶

Distribution of Retinal Stem Cells Intravitreally Transplanted into Fully Degenerated Retinas

To reveal further the potential of RSCs to differentiate and incorporate into the retina, we transplanted RSCs into degenerated retinas that had lost all their photoreceptors. In VPP mice, culture of RSCs (containing also progenitors and precursors) were injected intravitreally at 10 months of age and eyes were collected 1 and 4 weeks after surgery. By 1 week after injection, grafted cells were observed mainly in a cluster shape that did not infiltrate the neuroretina but stayed in the vicinity of the GCL along the inner limiting membrane (ILM; Fig. 1A) or near the lens (data not shown) with only a few isolated cells ($n = 3$ eyes). In comparison, at 4 weeks after injection ($n = 7$ eyes) part of the grafted cells migrated as single scattered elements into the GCL (Fig. 1B), and a few of them reached the IPL.

To test whether the absence of cell migration in the VPP retina was due to the genetic background of the mice, we injected the cells into the vitreous of *rd/rd* mice. The grafted cells behaved much as they did in VPP mice (Figs. 1C, 1D, $n = 6$ and 7, respectively, for 1 week and 4 weeks after transplantation). In addition, RSCs were observed to reach as far as the anterior chamber (Fig. 1E) and the ciliary body (CB; Fig. 1F).

The contralateral eye of injected animals was used as control, consisting of noninjected eye, sham surgery, or dead cell-injected retinas. We observed no PKH26- or GFP-expressing cells in these untreated or sham-injected retinas. In sham-surgery eyes only some retinal detachment due to the surgical procedure was observed. In the dead cell-injected retinas, we observed no fluorescent labeling (GFP or PKH26) within the neuroretina, and we mainly found the remaining PKH26 dye concentrated in the RPE layer. In some cases, we observed a recruitment of PKH26-positive pigmented cells in various layers of the retina up to the GCL. Four weeks after injection, the pigmented cells seemed to have cleared out all the fluorescent cell debris, which can be observed only in rare cases in other layers of the retina in the form of small isolated fluorescent spots (data not shown). These observations ruled out any occurrence of dye reabsorption by host cells and appearance of false-positive cells within the retina.

Migration and Differentiation of Retinal Stem Cells in Normal Retinas

As intravitreal injections of RSCs did not show wide incorporation into the host retina that Young et al.⁵ demonstrated with the adult rat hippocampal progenitor cells, we investigated cell behavior after subretinal injections. To evaluate the feasibility and reproducibility of subretinal injections, we first tested transplantation into normal retinas. We observed that the grafted cells formed a homogeneous layer between the RPE and the neuroretina (Fig. 2A). The entire surface of the retina covered by the graft was evaluated by adding the successive graft sizes measured on all histologic samples multiplied by the width of the tissue cut and the distance separating each slice. These experiments, performed on six retinas from 1 to 7 days after transplantation, resulted in a mean size of $10\% \pm 2.9\%$ of the total size of the retina (Fig. 2B) without any influence of the time elapsed after injection. Migration of the grafted cells toward the neuroretina was observed only occasionally and seemed to be facilitated by a mechanical lesion (data not shown). A few of these migrating cells reached the RGC layer. This migration was occasionally accompanied by differentiation toward the ganglion cell phenotype, as suggested by the presence of PKH26-positive grafted cells colabeled with NeuN,

a marker specific for RGCs in the retina (data not shown). In summary, the transplanted RSCs survived transiently in the SS after the injection, but migration was observed only in regions showing lesions due to the grafting procedure.

Distribution of Retinal Stem Cells Subretinally Transplanted into Fully Degenerated Retinas

We then tested RSC fate after transplantation into the SS of 10 months old VPP mice, in the place of the lost photoreceptors. One week after injection ($n = 3$), most of the donor cells were in the SS between the remaining INL and the RPE, but contrary to the intravitreal injection depicting an almost static situation, a good proportion of the cells injected subretinally were found within all the layers constituting the retina, with some cells having reached the GCL (Figs. 3A, 3B). It seemed that the donor cells were attracted by the innermost layers of the retina instead of staying at the place of injection. To verify this, we examined retinas 4 weeks after injection ($n = 7$). At this time point, the grafted cells were mainly found in the GCL (Fig. 3C), because of a massive migration toward the inner layers of the retina, with only a few remaining cells present at the site of injection, or elsewhere in the SS (Fig. 3B). PKH26- and GFP-positive cells behaved similarly. Moreover, subretinal injections allowed migration of the RSCs only into the retina and not into other structures of the eye. Taken together, these observations show that the RSCs have migratory preferences and cannot adopt a photoreceptor phenotype in our different experimental paradigms (C57Bl/6 normal retina, VPP, or *rd1* degenerated retinas). Nevertheless, the architectural organization of the host retina is not disrupted by the transplantation procedure or cell migration and RSCs can morphologically incorporate into the retina.

Transplantation and Cell Death

Previous studies have shown that neural progenitors can migrate toward the location of neuronal degeneration. We investigated whether the VPP retina undergoes cell death in the ganglion cell layer at 10 months of age and if the injection procedure induces apoptosis. Seven days after transplantation, we used TUNEL labeling to determine the cell death rate in the GCL. At this period, cells were still migrating, suggesting that some signals were delivered by the GCL. These results showed no differences in the number of dying RGCs in fully degenerated compared with wild-type retinas (data not shown). Surprisingly, the INL demonstrated a slight increase of TUNEL-positive cells in degenerated compared with wild-type retinas (data not shown). Few transplanted cells were dying. These data suggest that, in this case, the site of migration is not directly linked to neuronal degeneration. Concerning the survival of the transplanted cells, thousands of cells were still present in the degenerated and the partially degenerated retina 1 month after the injection, showing a good survival in a diseased retina.

Homotopic Differentiation of RSCs Transplanted into the Degenerated Retina

After the characterization of cell migration and morphology transformation, we investigated cell fate acquisition in all the cell transplantation paradigms tested. After subretinal injection, because we observed that several transplanted cells were located in the GCL, the IPL, or the INL, we investigated whether they had differentiated into cell phenotypes consistent with the layer they occupied. We observed that transplanted RSCs differentiated along neuronal and glial phenotypes. In the GCL we observed that several transplanted cells expressed the pan-neuronal marker β -tubulin-III (Figs. 4A1–A3). To better assess the neuronal phenotype of the donor cells located in the GCL, we used anti-NeuN and anti-Brn3b antibodies. In the retina, Brn3b is a transcription factor expressed only by a subpopulation of RGCs²⁷ and NeuN is expressed only in the RGC layer²⁸ (Figs. 4B1–B3, 4C1–C3, respectively). Strikingly, all grafted cells positive for these markers were located in the GCL, or in the close vicinity to it only, indicating that the cells responded to the specific region of the ganglion cell layer. On

the one hand, this suggests that RSCs expanded in vitro can differentiate toward the RGC phenotype as we previously observed in vitro.¹⁹ On the other hand, RSCs failed to express recoverin, a specific marker for photoreceptors and rare bipolar cells,²⁹ and calbindin, a horizontal cell specific marker, despite the fact that some of them had a morphology resembling this cell type (Figs. 4D1, 4D2). It seemed that in the present transplantation paradigm, the acquisition of morphologic characteristics occurred before the expression of specific markers. Indeed, after subretinal injections, we observed grafted cells with a typical bipolar shape (Fig. 4E) located in the INL and possessing two large neurites ending in the two adjacent plexiform layers. Of interest, we observed in rare cases grafted cells in the SS expressing rhodopsin (Rho1D4; Figs. 4F1–4F3). This occurred 1 week after injection; such cells were never found 1 month after surgery.

Moreover, in the IPL, normally devoid of any cell bodies, we observed multistratified cells with dendrites terminating at the two sublaminae of the IPL (data not shown). Nevertheless, most grafted cells did not express specific neuronal markers but many expressed GFAP, a specific glial marker (Figs. 5A1, 5A2, 5A3). After intravitreal injections, we observed clusters of grafted cells labeled by GFAP near the GCL (Fig. 5B), the lens (Fig. 5B), the iris (data not shown), and the CB (data not shown), regions that are normally devoid of any immunoreactivity to the GFAP marker. Moreover, the subretinal transplantation was accompanied by increased glial activity in the region in which grafted cells were located as well as at the injection site (Fig. 5D) compared with noninjected retinas. These observations clearly indicate that the RSCs have a marked preference to differentiate along the glial phenotype in this microenvironment.

Distribution and Retinal Phenotypes of RSCs Transplanted into Partially Degenerated Retinas

Because we concluded that delivered cells did not adopt a photoreceptor fate in the completely degenerated retina, we wanted to know whether the remaining photoreceptors in the younger partially degenerated VPP retina could provide support for correct localization and photoreceptor differentiation of the grafted cells. We injected RSCs in 7-month-old VPP mice in which three to six rows of photoreceptors were still present (Figs. 6A1, A2, B). Intravitreal injections gave results almost identical with the older, fully degenerated retina (compare Figs. 6B, 6C to Figs. 1E, 1F; ($n = 9$)). Some differences appeared only after subretinal injections. Four weeks after transplantation, a few cells had morphologically incorporated into the remaining ONL. The massive migration that was observed in the fully degenerated retina was thus slightly less extended in this earlier form of the disease. We also identified grafted cells in the ONL that expressed recoverin, a specific marker for photoreceptors, but only in very rare cases (Figs. 6E1–6E3), which suggests that the RSCs may retain the capacity to differentiate into the photoreceptor lineage, but the ONL was clearly not the principal migratory target of the RSCs grafted in the partially degenerated VPP retina. Indeed, RSCs, when injected subretinally, behaved similarly to previous experiments and most of the cells migrated toward the GCL and acquired a glial phenotype expressing GFAP (see above; Figs. 6D1–6D3).

Discussion

To investigate the ability of the allogeneic retinal stem and progenitor cells derived from the radial glia to incorporate into the retina, we examined their fate after transplantation within normal and diseased retinas. Our observations showed that RSCs transplanted into the fully degenerated retina either stay in the vicinity of the GCL when transplanted into the vitreous or massively migrate toward the GCL when transplanted subretinally. Moreover, several grafted cells differentiated into retinal cells in accordance with the cell layer in which they had integrated. However, they failed to give rise to photoreceptors in a model in which the latter degenerate.

After transplantation into the vitreous of two mouse models of retinal degeneration (VPP mice and *rd1* mice), grafted cells were widely distributed in a clusterlike form along the inner limiting membrane 1 week after transplantation. The lack of migration across the retina indicated that the ILM may constitute a barrier preventing further migration of the grafted cells as previously described in other models.^{6,30} However, after subretinal injections into the completely degenerated as well as the younger but partially degenerated VPP retinas, massive migration of the grafted cells was observed toward the GCL. Cells transplanted into the SS—close to the degenerating photoreceptors—were expected to diffuse laterally and not migrate to the retinal innermost layers. This extensive migration implies that certain intrinsic or extrinsic signals act on the grafted cells and that these cells can actually respond to these signals in a seemingly rapid and active process, as the migration is almost complete 4 weeks after surgery. This migration potential of RSCs appears to be different from the behavior of the RPCs tested in other studies,^{11,12} which remained at the injection site. Radial glia are known to show a marked mobility during development, with a permanent movement of the cell body between the surface of the ventricle and the proliferating zone. Moreover, radial glia migrate to different layers of the brain to generate neurons and astrocytes (reviewed in Ref. 31). It appears that the RSCs of the radial glia maintained their capacity to migrate even after extensive amplification *in vitro*.

In addition, we observed that this migration is accompanied by the sprouting of neurites from the grafted cells into other layers of the retina, further suggesting the fact that these cells respond to signals.

Although donor cells massively incorporated into the GCL when transplanted subretinally, they failed to integrate the ONL and to differentiate along the photoreceptor lineage. Several hypotheses emerge from these observations. It has been shown that neural progenitors can migrate toward the location of neuronal degeneration.^{5,32} In the present study, however, by the time of the transplantation experiment, the process of degeneration had reached the INL, but was not followed by incorporation of grafted cells into this layer. It is possible that the death of INL cells induces a metabolic change in one of their targets, the RGCs. After reorganization of the retinal cells during the degeneration, the VPP retina may generate signals to establish new contacts during the degeneration process, as previously observed in the *rd1* mice.^{33,34} Such signals are usually constituted of neurotrophic factors and neurotransmitter secretion, which allow neurite sprouting, outgrowth, and synaptic remodelling (reviewed in Ref. 35,36). Similar signals are also required during cell differentiation and maturation. As a consequence, it is possible that the transplanted RSCs respond to these signals and differentiate into neurons and glia in the RGC. In fact, the major differentiation into RGCs after transplantation was observed in the fully degenerated retina and not during degeneration.

Finally, during the expansion period, the EGF used in our culture medium may prime the cells preferentially toward the RGC phenotype.¹⁹ Indeed, we have shown that EGF in the presence of laminin is sufficient to commit RSCs to the RGC fate. This priming may occur during the expansion period *in vitro* and, in the natural microenvironment, some cells may respond to other environmental cues (such as laminin) to enhance RGC differentiation. Transplantation studies have shown very different results in animal models of retinal degeneration. It has been shown that hippocampus-derived neural progenitor cells transplanted into the retina can differentiate into neurons and glial cells and incorporate into the developing^{13,37–39} and adult retina if it is mechanically or genetically injured.^{3,14,40} However, these cells constantly failed to undergo differentiation into specific retinal neurons.^{4,5,32,41–43} Conversely, retinal cells successfully differentiated into retinal neurons and glia, but were not accompanied by extended migration into the retina.^{10–12} In the present study, subretinally transplanted cells showed a robust migration into the GCL accompanied by a moderate differentiation toward the ganglion cell phenotype. We have shown that stem cells isolated at a late stage of neurogenesis can give

rise in vitro to early born neurons.¹⁹ This indicates that the state of the RSCs is permissive for differentiation into RGCs and may suggest that they will also be able to give rise to the other retinal cells if appropriate stimulations are found, as we previously observed in vitro, for the generation of RSCs committed to the photoreceptor fate.²¹ This notion is further supported by the acquisition at the morphologic level of phenotypes resembling bipolar cells and horizontal cells located in the correct retinal compartment.

However, RSCs have a marked preference for the glial phenotype. Throughout the whole retina, Müller cells are the main glial cell type present, whereas astrocytes are exclusively found in the GCL. Activation of the glial cells by surgical procedures has been largely demonstrated (reviewed in Ref. 44). It is thus possible that after transplantation the grafted cells respond preferentially to signals that induce glial activation instead of signals that promote neuronal differentiation. We have recently shown that during the expansion period in culture medium containing EGF and FGF-2, the cells express markers not only present in stem cells such as nestin45 and Bmi146–49 but also RC2,⁵⁰ Mash1, and Pax6, factors that have been shown to be expressed by radial glia.^{19, 47, 51} RSCs injected into the retina may thus retain glial characteristics and undergo glial differentiation, especially in the absence of signals driving the cells toward the neuronal lineage. On the opposite side, RSCs may give rise to glial cells in response to signals promoting glial differentiation present in the microenvironment, as has been suggested with NSCs transplanted into the CNS due to reactive gliosis after injury. Lundberg et al. and others^{52–54} have shown that stem/progenitor cells transplanted into the brain preferentially acquire glial characteristics.

Finally, in rare cases we obtained expression of the recoverin and rhodopsin markers in grafted cells. Such expression is not satisfactorily reliable because of the scattered occurrence of this observation but clearly indicates that RSCs can indeed undergo differentiation in vivo into another retinal fate than RGCs. It is thus important to determine why, in our experimental paradigm, RSCs failed to repopulate the ONL and what can be done to achieve photoreceptor differentiation and integration in vivo.

First, cell contact between grafted cells and remaining photoreceptors seems insufficient to drive photoreceptor differentiation. Only scattered cells were found in the ONL after transplantation into partially degenerated retinas and expression of recoverin and rhodopsin was sparse. In our hands, in vitro culture conditions allowed the differentiation of numerous cells into the photoreceptor cell type, expressing several photoreceptor proteins or genes.²¹ This shows that the RSCs have the ability to undergo photoreceptor differentiation, but that the degenerated retina of the VPP mice do not provide signals supporting differentiation of this kind.

Second, during degeneration, remaining host photoreceptors may release apoptotic signals, or stop releasing survival factors,⁵⁵ which could be harmful for RSCs. This is a potential explanation for the small number of donor cells that survived in the ONL. However, because donor cells migrated and did not die, this hypothesis is unlikely.

Our results contrast with those describing that RPCs are capable of generating cells expressing photoreceptor markers after transplantation. Of note, it seems that fetal RPCs have a better capacity to generate a large number of cells committed to the photoreceptor pathway than newborn RPCs¹¹ and RSCs, respectively. In fact, newborn RPCs showed a potential to generate photoreceptor-like cells in a degenerating retina, but to a lesser extent than fetal RPCs. However, the proliferation and differentiation potentials of the fetal RPCs are not known, and it is difficult to evaluate their primitiveness. Moreover, the cell type, the animal model, or the culture conditions used in these studies are all different and it is difficult to identify what favors the appearance of the photoreceptor phenotype in vivo.

In conclusion, we have shown that RSCs can differentiate into retinal phenotypes and are able to migrate extensively toward inner structures of the retina responding to cues not yet determined. Also, to obtain robust differentiation or integration into the ONL, it seems likely that the grafted cells must be driven, before transplantation, through the neuronal lineage instead of the glial one. These findings suggest that (1) more specialized cells may be needed to integrate the ONL, like restricted progenitors primed to become photoreceptors, or fully differentiated photoreceptors. Indeed, cells derived from mouse ES cells previously induced toward the neuronal lineage in vitro were able to incorporate the retina and expressed retinal cell-specific markers,^{8,9} whereas transplantation of mouse ES cells is accompanied by tumor formation.⁵⁶ (2) An artificial substrate to enhance cell survival or cell differentiation could be useful.⁵⁷

Another possibility is to couple the injection of the cell suspension with growth or proneural factors or to engineer the cells genetically to commit them to the desired fate.⁵⁸

Furthermore, we have shown that in our transplantation model, RSCs can incorporate into a diseased retina when subretinally transplanted but exhibit a migration and a differentiation preference. Last, the acquisition of the retinal phenotype, either morphologically or at the level of protein expression, seems to be region-specific for the ganglion cell layer and indicates that the cells respond to specific signals suggesting that manipulation of these signals may help to replace lost cells at the right place.

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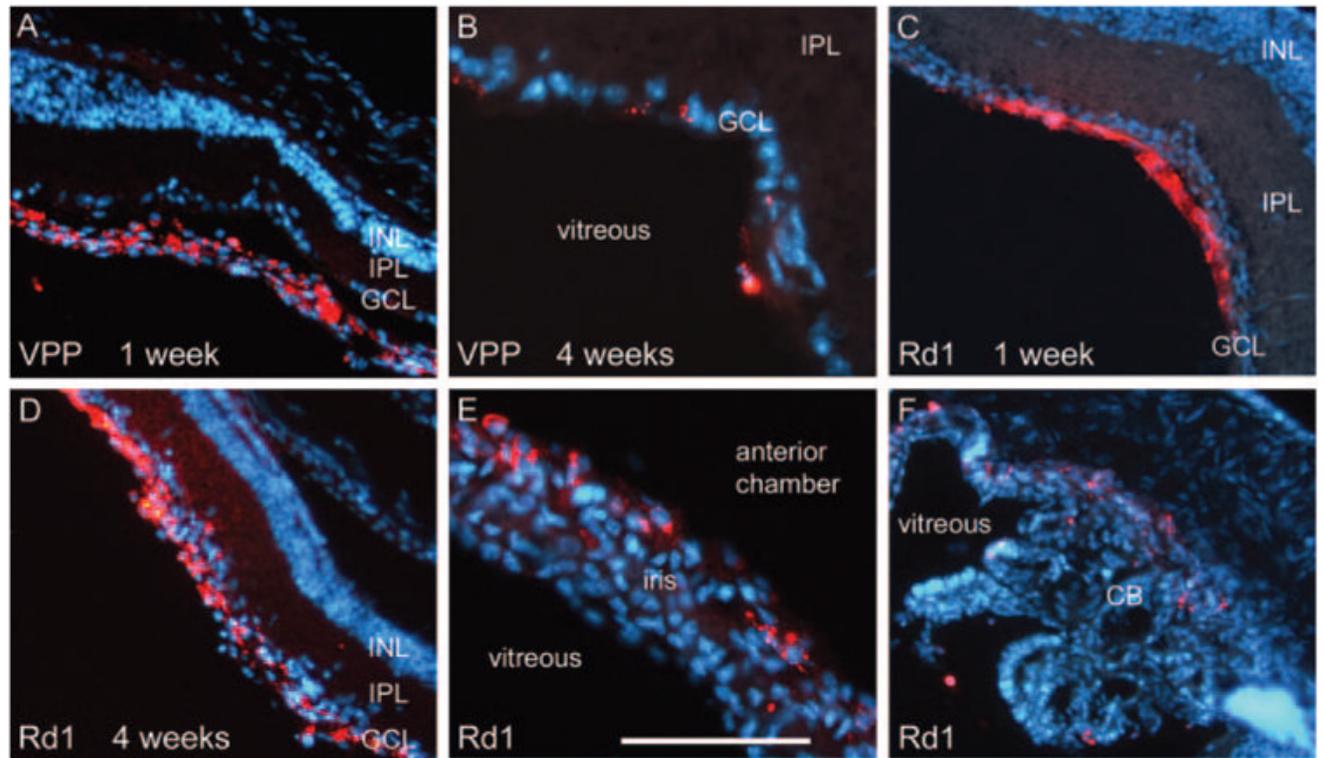


Figure 1.

Cell localization after transplantation in retinas devoid of photoreceptors showed only slight migration when injected intravitreally in VPP as well as in *rd1* mice. (A) By 1 week after injection, RSCs intravitreally injected into the VPP mouse retina formed elongated clusters located in close proximity to the GCL but not within. (B) By 4 weeks after surgical intervention, some grafted cells had migrated within the GCL. In the *rd1* mice, transplanted cells showed similar localization equally close to the GCL during the week after injection (C) and slight migration within it 4 weeks after transplantation (D). Moreover, intravitreal injection allowed the cells to migrate into various structures of the *rd1* eye such as the iris (E) and the CB (F), which is not the case with subretinal injections (not shown).

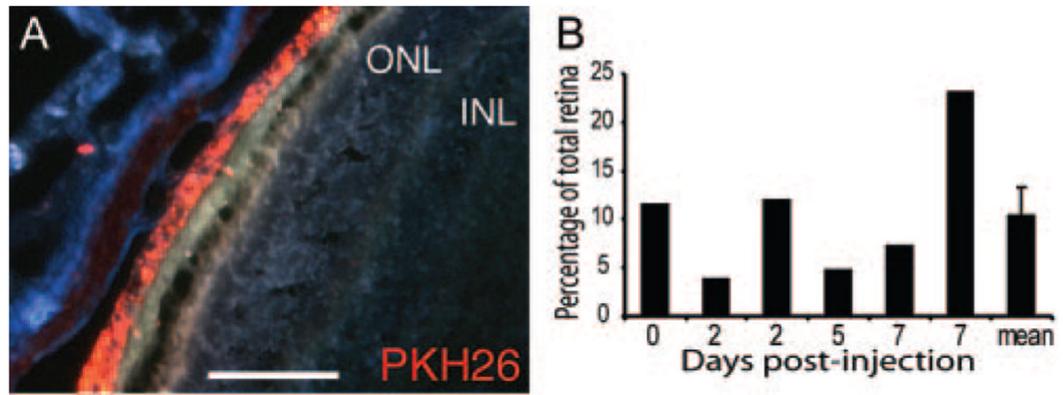


Figure 2.

Assessment of the subretinal injection in the normal retina. The size of the graft is expressed as a percentage of the total surface of the retina in six retinas from 0 to 7 days after injection (A). Subretinal injection of RSCs into the eye of C57Bl/6 mice shows that the cells labeled with the PKH26 were restricted to the SS and were not scattered in other layers of the retina 2 days after the surgery (B). Scale bar, 50 μ m.

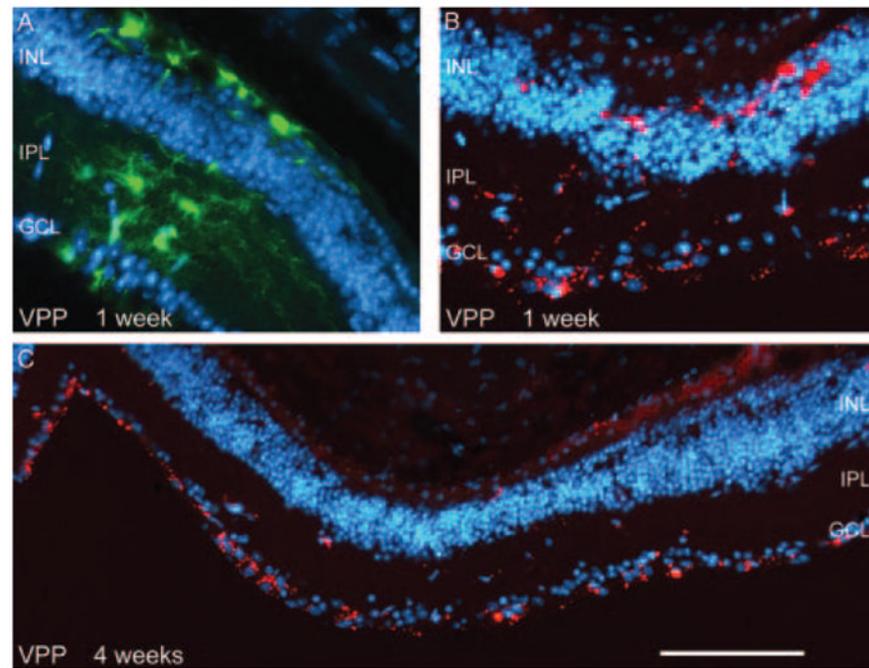


Figure 3. RSCs massively migrated toward the innermost layer of the retina after subretinal injection into fully degenerated VPP retinas. Compared with the poor migration of intravitreally injected cells (Fig. 1), subretinal injections exhibited a massive migration of the RSCs toward the GCL of VPP retinas. This migration was already observable by 1 week after surgery, with cells present in the subretinal space (the site of injection) as well as in all the other layers of the retina up to the GCL with GFP-expressing RSCs (**A**) and PKH26-labeled RSCs (**B**). The migration into the GCL was completed by 4 weeks after injection (**C**). Scale bars: (**A**, **B**) 50 μm ; (**C**) 100 μm .

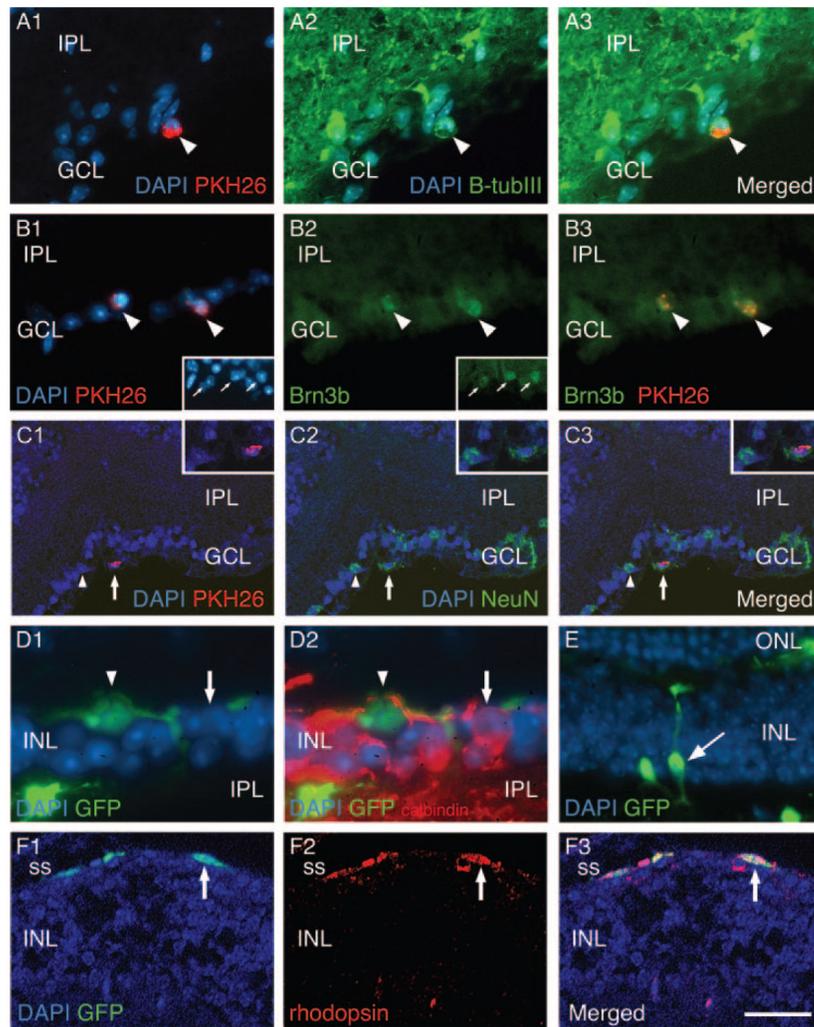


Figure 4.

RSCs expressed neuronal and neuroretinal markers after transplantation in a fully degenerated retina. (**A1, B1, C1, F1**) Fluorescence images of degenerated retinal sections showing DAPI staining (*blue*) of the nuclei and PKH26-labeled RSCs (*red*) within the host GCL; (**A2, B2, C2, F2**) grafted cells and host retina labeled with specific antibodies revealed by secondary antibodies coupled to FITC (*green*) and nuclei labeled with DAPI; (**A3, B3, C3, F3**) merged images showing colocalization of the PKH26 (*red*) with the antibody labeling (*green*) and the DAPI staining (*blue*). RSCs injected subretinally incorporated into the GCL and expressed the early neuronal marker β -tubulin-III (**A**). The RSCs that incorporated into the GCL expressed Brn3b (**B**) a marker for mature ganglion cells (*inset*: positive control for Brn3b labeling, note that Brn3b labels a subpopulation of RGC). Integrated RSCs also express NeuN (**C**) a specific marker for ganglion cell lineage in the retina as shown in confocal images (**C**). Colocalization of these antibodies (Brn3b and NeuN) and the PKH26 among the host retina was observed exclusively in the GCL, denoting a region of specific immunoreactivity. *Insets*: higher magnification of the area defined by the *arrows* (**D, E**). Fluorescence images of GFP-expressing RSCs subretinally injected and exhibiting morphologies resembling horizontal cells (**D1, D2, arrowheads**) close to calbindin-positive cells (a specific marker for horizontal cells, **D2, red, arrow**) but negative for the latter, even if exhibiting a similar morphology, and with bipolar cells (**E, arrow**) correctly located and oriented in the INL. Occasionally, RSCs that did

not migrate toward the GCL but stayed close to the INL, where few remaining photoreceptors were located, expressed rhodopsin, a specific marker for photoreceptors (**F**, confocal image). Scale bar: (**A**, **B**) 25 μm ; (**C**, **F**) 30 μm ; (**D**) 10 μm ; (**E**) 100 μm .

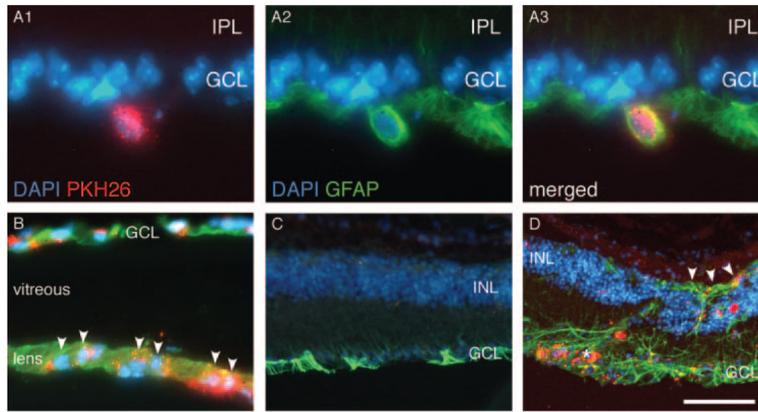


Figure 5.

Glial cell differentiation after transplantation. (A1) Fluorescence images of retinal sections showing PKH26-labeled RSCs (*red*) within the host degenerated retina and DAPI staining (*blue*) of the nuclei; (A2) grafted cells and host retina labeled with anti-GFAP revealed by a secondary antibody coupled to FITC (*green*) and nuclei labeled with DAPI; (A3) merged image showing colocalization of the PKH26 (*red*) with the antibody labeling (*green*) and the DAPI staining (*blue*). Colocalization depicts a grafted cell positive for the glial marker GFAP within the host GCL after subretinal injection (A). The preference for the glial phenotype is supported by the colocalization of PKH26 and GFAP in cells integrated after intravitreal injections. This preference is further supported by the high degree of GFAP expression (*green*) by grafted (*red*) cells that have migrated close to the lens (B) after intravitreal delivery of RSCs. Endogenous glia revealed by GFAP immunostaining (*green*) in a control retina, labels only end feet of Müller cells and the astrocytes contained in the GCL (C). In comparison, in transplanted retina, 4 weeks after surgery, extensive gliosis related to increased GFAP labeling (*green*) is observed (D) in the site of injection (*arrowheads*) up to the site where grafted cells (*red*) incorporate (*). DAPI staining to visualize nuclei was performed on each section (*blue*). Scale bar, (A) 25 μm ; (B) 50 μm ; (C, D) 100 μm .

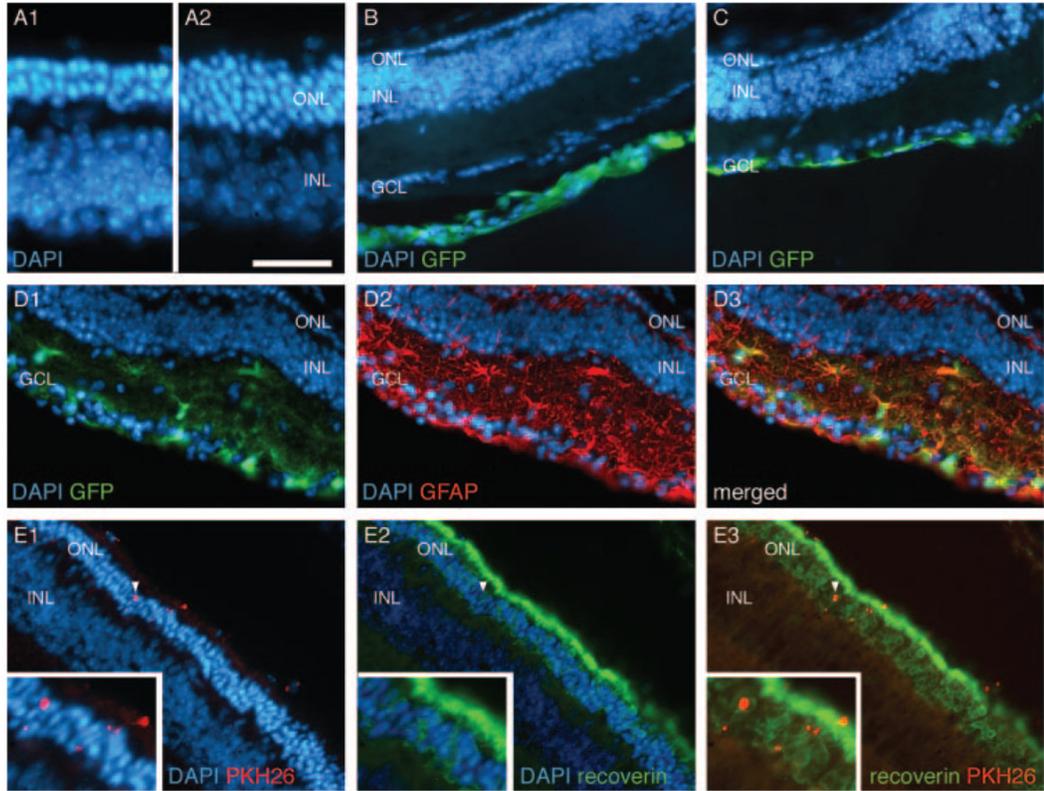


Figure 6.

RSCs transplanted to partially degenerated retina (7-month-old VPP) did not integrate better into the ONL and showed a higher tendency to acquire glial characteristics than cells transplanted into fully degenerated retina. The ONL of 7-month retinas presented one to six rows of photoreceptors, illustrating the heterogeneity of the degeneration as shown in retinal sections from the same retinal area of two animals of the same age (**A1**, **A2**). Intravitreal injection in the partially degenerated retina was rigorously similar to the fully degenerated retina by 1 week and 4 weeks after injection (**B**, **C**, respectively). After subretinal injections, grafted RSCs had massively migrated toward the GCL and expressed the glial marker, GFAP (**D1**, **D2**, **D3**). Indeed, GFP expressing RSCs (*green*, **D1**) and GFAP immunostaining (*red*, **D2**) colocalized, shown in a merged image (*orange*, **D3**). Thus, RSCs transplanted into the partially degenerated 7-month-old VPP mouse retina behaved almost like those in their counterparts at 10 months of age. Nevertheless, 4 weeks after transplantation, some subretinally injected RSCs stayed in the remaining ONL (**E1**, *arrowhead*) and in rare cases express the recoverin marker (**E2**, **E3**, *arrowhead*). DAPI staining to visualize nuclei was performed on each section. Scale bar: (**A**) 25 μm ; (**B–E**) 50 μm .

Table 1

Distribution of the Animals in the Different Experimental Groups

Mouse Strain	Age at Injection Time	Type of Injection	Time after Injection	<i>n</i>
C57B1/6	Adult	Subretinal	0–30 d	28
<i>rd1</i> mouse strain	1 mo	Intravitreal	1 wk	6
			1 mo	7
	1 mo	Subretinal	1 wk	—
			1 mo	—
VPP mouse strain	10 mo	Intravitreal	1 wk	3
			1 mo	7
	10 mo	Subretinal	1 wk	3
			1 mo	7
	7 mo	Intravitreal	1 wk	2
			1 mo	7
7 mo	Subretinal	1 wk	3	
		1 mo	3	