Retinal Pigmented Epithelium Does Not Transdifferentiate in Adult Goldfish

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SUMMARY

The neural retina of adult goldfish can regenerate from an intrinsic source of proliferative neuronal progenitor cells, but it is not known whether the retina can regenerate by transdifferentiation of the retinal pigmented epithelium (RPE), a phenomenon demonstrated in adult newts. In this study, we asked whether following surgical removal of the neural retina in adult goldfish the RPE was capable of autonomously transdifferentiating and generating new neural retina. The retina was prelabeled by injecting the fluorescent dye Fluoro-Gold (FG) into the eye prior to surgical removal; this procedure ensured that residual retina was labeled with FG and could therefore be distinguished from unlabeled, regenerated retina. To examine the time course of retinal regeneration, and to identify regenerated retinal neurons, the thymidine analogue bromodeoxyuridine was injected intraocularly, and retinas were examined up to 2 months later. We found that the RPE did not transdifferentiate; instead, retinas regenerated only when pieces of residual neural retina were left intact. Under these circumstances, newly regenerated cells derived from proliferating cells intrinsic to the residual neural retina. When retinas were completely removed, as was evident from a lack of FG labeling, there was no retinal regeneration. © 1995 John Wiley & Sons, Inc.

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INTRODUCTION

The cells of the neural retina and the retinal pigmented epithelium (RPE) have a common, embryological origin: both derive from an out-pocketing of the diencephalon known as the optic vesicle. A secondary, surface invagination of the optic vesicle produces the double-walled optic cup. The outer layer of the optic cup then thins, and these cells (the presumptive RPE) begin to synthesize melanin. Meanwhile, cells in the inner layer, facing the developing lens, proliferate to form the neuroepithelium of the presumptive retina (reviewed by Park

and Hollenberg, 1993). Although there is this clear separation of cell fates during early eye development, RPE cells have the ability to dedifferentiate and initiate production of both lens and neural retina under certain circumstances. This process is called metaplasia, or transdifferentiation (reviewed by Okada, 1980). In urodeles, like salamanders or newts (Stone, 1950; Keefe, 1973; Levine, 1975; Klein et al., 1990), the RPE can transdifferentiate throughout life and can do so autonomously, in the absence of interaction with other tissues. In all other species studied, such as anuran amphibians (Lopaschov and Sologub, 1972; Sologub, 1977; Levine, 1981; Reh and Nagy, 1987; Reh et al., 1987) and embryonic chick (Coulombre and Coulombre, 1965, 1970; Park and Hollenberg, 1989, 1991), transdifferentiation of RPE into neural retina *in vivo* has been observed only under special circumstances.

In anurans, RPE from larval animals can transdifferentiate spontaneously, but RPE from adults must be transplanted into a larval eye, where it has

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access to the retina. In chicks, only RPE from embryos can transdifferentiate, and then only when in proximity to neural retina, or after the exogenous addition of basic fibroblast growth factor (bFGF) (Park and Hollenberg, 1989, 1991). It also has been shown that chick and anuran RPE cells in culture can transdifferentiate in response to bFGF (Pittack et al., 1991; Opas and Dziak, 1994) or when grown on certain substrates (Tsunematsu and Coulombre, 1981; Reh et al., 1987).

There have been few reports on the potential of RPE to transdifferentiate in teleost fish (Dabagyan, 1959; Sologub, 1975), and these have focused on embryonic fish. In both of these studies, it was shown that presumptive RPE cells at the optic vesicle stage or earlier, when free of mesenchyme, transdifferentiate into neural retina when transplanted into a non-neural environment (the pericardial cavity). At later embryonic stages, once the RPE cells had become pigmented, transdifferentiation proceeded only under the influence of the neural retina, similar to the situation just described for embryonic chicks and anuran amphibians.

To investigate further the regenerative capacity of RPE cells in fish, we partially or completely removed the neural retina from adult goldfish. The surgical technique used was derived from Stone (1950), who demonstrated that RPE cells can transdifferentiate in adult newts. Our preliminary experiments demonstrated that new retinal neurons were produced, but the source of the regenerated cells was not clear. To identify residual fragments of retina that might have remained after the surgery, we injected the eye with Fluoro-Gold (FG; Fluorochrome, Englewood, CO) prior to surgical removal of the retina. We subsequently followed the regeneration process with single or multiple injections of the thymidine analogue 5'-bromo 3'-deoxyuridine (BrdU; Sigma, St. Louis, MO), which labels dividing cells and their progeny.

METHODS

Retinectomy

Common goldfish (*Carassius auratus*) 4 to 5 cm in body length, were obtained from Ozark Fisheries (Richland, MO), and were kept at 18 to 20 C in 10 gallon aquaria in artificial pond water (0.06 g/l calcium chloride, 0.05 g/l magnesium sulfate, 0.05 g/l potassium sulfate, and 0.05 g/l sodium nitrate in distilled water) (Allee et al., 1940).

Fish were kept in the dark for at least 12 h prior to surgery to facilitate removal of the neural retina from the RPE. Before surgery, they were deeply anesthetized in 0.1% methane tricaine sulfonate (MS 222). Retinas were removed by making an incision at the dorsal aspect of the limbus of the eye; the excision was then extended both nasally and temporally to create a hemicircular slit. The iris and cornea were peeled back, and the lens was gently removed with microscissors and fine forceps. The neural retina was then removed completely by vacuum suction with a standard, 1 ml, disposable pipette tip (Fisher Scientific) or with a gentle stream of 0.9% saline. In some cases, only part of the retina was removed. In these cases, a piece of retina was cut away with microscissors and then floated out with a stream of 0.9% saline solution. The iris flap then was sutured in place dorsally to promote healing, and the animals were returned to artificial pond water with added sodium chloride (NaCl) (5 g/l) to reduce bacterial and fungal infections. Healing progressed quickly, and was often complete by 2 weeks after surgery. Some eyes were fixed immediately after surgery to gauge the effectiveness of the retinectomy. Control eyes (unlesioned) were also fixed and processed as described later.

Intraocular Injections

After anesthetizing as already described, a small incision was made with a microknife (Tieman & Co., Plainsview, NY) at the dorsal limbus of the eye. A 10 μl syringe with a 33 gauge, fixed, blunt-tipped needle was used to inject either FG or BrdU.

For labeling retinas with FG 1 day before retinectomy, animals were injected intraocularly with 2 μl of 0.2% FG in 0.9% saline. Although FG is more commonly used for retrograde labeling of axons (Schmued and Fallon, 1986), it is also an efficient and long-lasting method for direct labeling of retinal cells used for transplantation studies (del Cerro et al., 1990). In goldfish retinas, the ganglion cell layer, inner nuclear layer, and plexiform layers were routinely labeled by FG injected intraocularly. However, in the outer nuclear layer, labeling was less homogeneous and usually only the cone inner segments and infrequently the cone nuclei were labeled. Neither rods nor the dividing rod precursors ever appeared to be labeled.

To label dividing and regenerated cells, 1.5 to 4 μl of 1 mM BrdU in 0.9% NaCl was injected intraocularly at various times after retinectomy. The amount of BrdU injected depended on the volume of the eye, which was calculated from the ocular diameter as described previously (Raymond et al., 1988a). By injecting very slowly, we took care to ensure that little if any of the BrdU solution leaked out of the eye, especially at times shortly after the initial surgery when the incision had not yet healed. The estimated intraocular concentration of BrdU in the eye was 50 μM, although injections of a 10-fold lower concentration of BrdU label dividing cells in control eyes (J. K. Knight, unpublished observations).

Immunocytochemistry

The following cell-specific monoclonal antibodies were used: RET 1, which labels an uncharacterized nuclear epitope in Müller glial cells and most retinal neurons except rods (Barthel and Raymond, 1990); FRet43, which labels double cones and a few neurons in the inner nuclear layer (Larison and BreMiller, 1990); and Rho 4D2, a bovine rhodopsin antibody (Hicks and Molday, 1986) which labels rod and green cone outer segments in goldfish (Raymond et al., 1993). These antibodies were used to identify regenerated retinal cells (defined as cells double labeled with both BrdU and a cell-specific antibody) and to evaluate the morphology of residual pieces of retina. Eyes were rinsed, cryoprotected, frozen in a 2:1 mixture of 20% sucrose and OCT (Miles, Elkhart, IN), and then cryosectioned at 3 μm as described previously (Barthel and Raymond, 1990).

All procedures were conducted at room temperature, except when indicated. Sections were blocked for 30 min with 20% normal goat serum (NGS; Sigma) diluted in phosphate-buffered saline (PBS) and then incubated overnight at 4°C with one of the monoclonal antibodies, diluted to the appropriate concentration in PBS with 1% NGS, 0.1% sodium azide, and 0.5% Triton X-100. The following day, sections were rinsed and incubated with donkey anti-mouse [immunoglobulin G (IgG)] secondary antibody preabsorbed against rat immunoglobin and conjugated to Texas Red (Jackson Immunoresearch, West Grove, PA) for 30 min. Sections were then treated with 2 nM hydrochloric acid (HCl) in PBS with 0.5% Triton X-100 for 30 min to denature the DNA in order to expose incorporated BrdU to the antibody (Schutte et al., 1987). After rinsing and another blocking step, sections were incubated with the rat monoclonal anti-BrdU (1:30; Accurate Chemical, Westbury, NY) overnight at 4°C. The secondary antibody (donkey anti-rat IgG, preabsorbed against mouse immunoglobin) was conjugated to fluorescein isothiocyanate (FITC; Jackson Immunoresearch). Coverslips were placed on slides with 60% glycerol in 0.1 M sodium carbonate buffer (pH 9.0), with 0.4 mg/ml p-phenylenediamine, and viewed with a Leitz Aristoplan epifluorescence microscope, using FITC wideband (Leitz I3, 350–490 λ) and narrowband (L3, 450–490 λ) filter cubes, a tetrarhodamine isothiocyanate (TRITC) filter cube (Leitz N2.1, 515–560 λ), and an ultraviolet (UV) filter cube (Leitz A2, 270–380 λ). FG labeling was visualized either with the UV filter cube, in which the label appeared blue, or with the I3 wideband FITC cube, in which the label appeared yellow.

RESULTS

Preliminary Results without the Fluoro-Gold Prelabel Were Ambiguous

In our initial series of experiments, 113 retinas were removed and BrdU was injected either 1 day
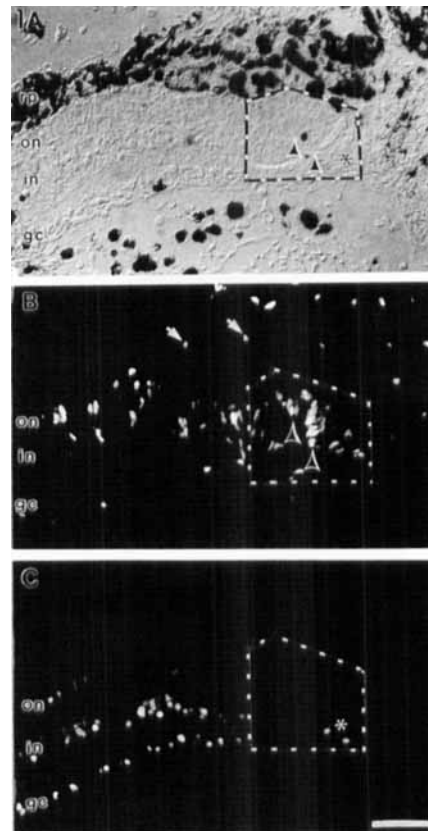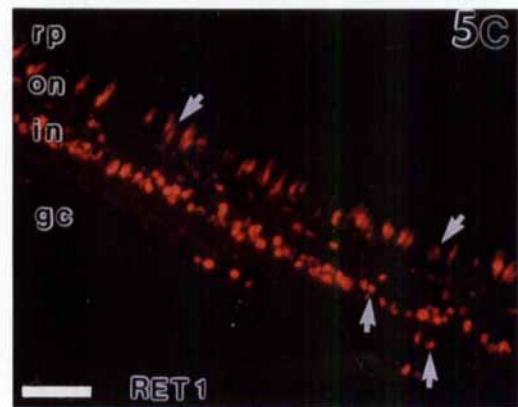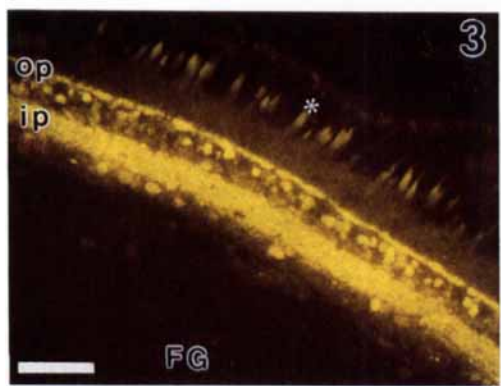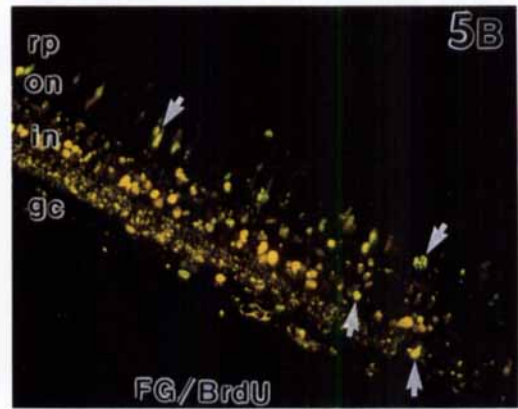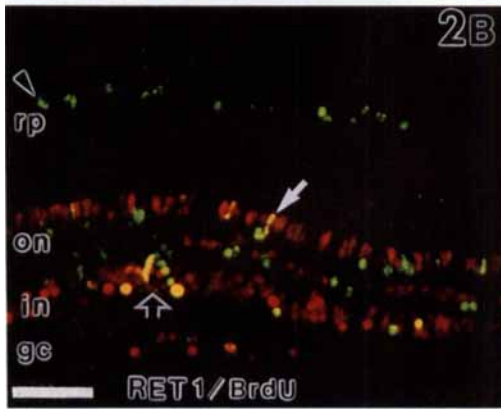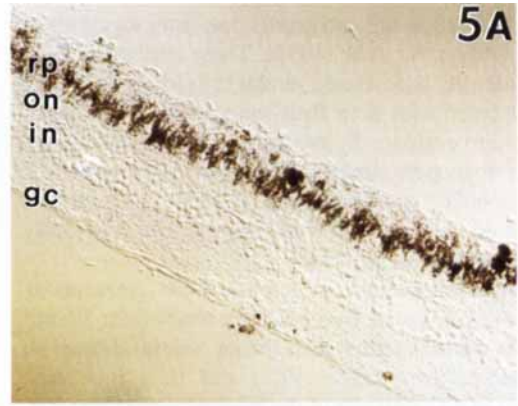

Figure 1 Cross-section of adult goldfish retina 11 days after partial retinectomy, with one BrdU injection at 10 days. This retina shows an example of a regeneration blastema, outlined by a dotted line. (A) Normarski optics. Arrowheads point to the blastema, where the laminar organization of the retina is absent and elongated neuroepithelial cells are present. (B) With FITC illumination, BrdU+ cells are seen throughout the layers of the retina, as well as in the RPE (arrows). The blastema of dividing cells is also visible (arrowheads). (C) With TRITC illumination, differentiated cells in all layers are labeled with RET 1. Note that the RET 1 labeling ends before the blastema (except for two displaced RET 1+ cells, asterisk). Abbreviations: rp = retinal pigmented epithelium; on = outer nuclear layer; in = inner nuclear layer; gc = ganglion cell layer. Scale bar = 50 μm.

before processing the tissue, in order to label dividing cells, or at various intervals up to 10 days before processing, in order to label newly generated neurons.

We observed in some of our first preparations cellular changes in the RPE that appeared similar to those described as the initial steps in transdifferentiation (Stroeva and Mitashov, 1981, 1983). For example, in some eyes the RPE was disrupted: it was detached from Bruch's membrane and was no longer organized as a simple epithelium. In addi-

tion, depigmentation of the RPE was also seen, which is thought to be a necessary prerequisite to the transdifferentiation process (Stroeva and Mitashov, 1983; Reh and Nagy, 1987). However, in most cases, the RPE did not undergo these changes, but retained its normal organization and pigmentation. With later refinements of the surgical technique, these observations became less frequent and we, therefore, attributed them to mechanical damages from the surgery.

In some eyes at early survival times (5 to 16 days), pieces of differentiated retina remained attached to the germinal zone. These fragments contained many dividing cells throughout all layers, and, in addition, what appeared to be a regeneration blastema (discussed later; also see Hitchcock et al., 1991) was seen at the cut edge of most retinas (Fig. 1). At longer survival intervals (19 to 38 days), double-labeled (BrdU+/RET 1+) and, therefore, regenerated neurons were often found scattered across the expanse of the retina, again in all nuclear layers (Fig. 2). It was clear from these results that new neurons were being produced, but it was not clear whether they derived from RPE or from the intrinsic source of proliferating cells known to participate in retinal regeneration (described previously in adult goldfish, Raymond et al., 1988a; Hitchcock and Raymond, 1992). In any given eye, one could never be sure whether the entire retina had been removed. We, therefore, modified our experimental procedure in subsequent experiments by prelabeling with FG before removal of the retina to identify residual pieces.

Regeneration of Retinal Neurons Was Always Associated with Residual Retina

In the experiments in which retinas were prelabeled with FG, some retinas were completely removed, whereas in others we intentionally left part of the retina intact. Intact, unoperated eyes injected with FG (Fig. 3) were used to compare the normal pattern of FG label with that in operated eyes injected with FG. The data are summarized in Table 1. A total of 37 eyes prelabeled with FG were examined at 5 to 82 days after retinectomy. Each eye was examined histologically and categorized as to whether it represented partial or complete retinectomy based on the presence or absence, respectively, of FG-labeled cells. In the 10 eyes that were classified as complete retinectomies (no FG labeling), there was no indication of retinal regeneration (Table 1). The lack of regeneration of neural retina in these eyes was verified by the absence of label with the retinal-specific antibodies RET 1 (which identifies most retinal neurons except rods), FRet43 (which identifies double cones), and Rho 4D2 (which identifies rods and green cones). Although not all preparations were evaluated with all three antibodies, RET 1 labeling was evaluated in all cases. Furthermore, BrdU labeling in these preparations was confined to vascular cells, cells in other non-neural ocular tissues, and, rarely, RPE cells.

The remaining 27 eyes had FG label and were therefore classified as partial retinectomies. During the first 3 weeks after retinectomy (5, 14, and 17

Figure 2   Cross-section of adult goldfish retina 33 days after partial retinectomy, BrdU injections at 9 and 12 days. (A) Nomarski optics. (B) Double exposure showing both BrdU+ (green) and RET 1+ (red) cells. Note the interspersal of new neurons, double-labeled with RET 1 and BrdU (yellow): the closed arrow indicates a new cone; the open arrow shows a cluster of new neurons in the inner nuclear layer. The arrowhead shows a line of dividing (BrdU+) RPE cells. Abbreviations as in Figure 1. Scale bar = 50 μm.

Figure 3   Cross-section of a normal adult goldfish retina, injected with 0.2% FG 1 day before processing. The FG label was visualized with FITC wideband illumination. The cell bodies in the inner nuclear and ganglion cell layers are labeled, as are cell processes in the outer plexiform (op) and inner plexiform (ip) layers. Note that in the outer nuclear layer only the cone inner segments are labeled (asterisk). Scale bar = 50 μm.

Figure 5   Cross-section of adult goldfish retina, 82 days after partial retinectomy, preinjected with FG 1 day before retinectomy, with BrdU injections at 10, 12, and 17 days. (A) Nomarski optics. This region is fully repaired, with normal lamination and retinal thickness. (B) BrdU+ cells (green), visualized with wideband FITC illumination. FG-labeled cells, also visualized with FITC illumination, are distinguished from BrdU+ cells by their yellow color. (C) RET 1+ cells, visualized with TRITC illumination. Cells double-labeled by both BrdU and RET 1 are new neurons and are indicated with white arrows in (B and C). Scale bar = 50 μm.

Table 1   Categorization of Eyes Preinjected with Fluoro-Gold (FG)

Survival after Retinectomy (days)	BrdU Injection after Retinectomy (day)	Eyes (n)	Partial Retinectomy (FG Labeled)		Complete Retinectomy (no FG Label)	
			Repair	No Repair	Repair	No Repair
5	4	6	3	0	0	3
14	13	7	4	2	0	1
17	16	3	1	2	0	0
32	5, 8, 11, 15	6	2	3	0	1
35	11, 15	5	2	0	0	3
42	11, 15	3	2	0	0	1
82	10, 12, 17	7	2	4	0	1
Total:		37	16 (43%)	11 (30%)	0	10 (27%)

days; see Table 1), eyes were examined at 1 day after BrdU injection in order to identify proliferating cells. In 4 of 12 eyes, only half (or less) of the retina had been removed, and the residual half appeared to be intact and showed no apparent increase in BrdU labeling compared to control (nonlesioned) retinas (data not shown). In the remaining eight eyes, BrdU+ (proliferating) cells were found throughout all retinal layers and also occasionally in the RPE. A mass of proliferating, elongated cells was often found at the cut edge of the retina. (Similar structures were seen in preparations not prelabeled with FG, as illustrated in Fig. 1.) Structures similar to this, which have been called regeneration blastemas, were described previously following small lesions of adult goldfish retina (Hitchcock et al., 1992). These blastemas have been shown to repair retinal wounds and are thought to originate from proliferating rod precursors endogenous to the retina (Hitchcock et al., 1992; Hitchcock and Raymond, 1992).

The other 15 eyes that contained residual pieces of retina were examined at longer survival intervals (32 to 82 days). These eyes were injected at least twice, and up to four times, with BrdU during the first to third weeks after retinectomy and were examined 10 days or more after the last BrdU injection. With this protocol, cells dividing at the time of the injections subsequently differentiated, and, therefore, regenerated neurons could be identified by double-labeling with BrdU in combination with one of the cell-specific monoclonal antibodies. Not all retinas regenerated. When at least half of the retina remained after surgery, few BrdU-labeled cells were found in the residual fragments of retina and none was double-labeled with RET 1, consistent with the lack of increased mitotic activity at short survival times. This situation applied to 7 of the 15 eyes (Table 1; 3 at 32 days and 4 at 82 days), and in all cases the residual retina appeared to be normal in all respects. When more than half of the

retina was removed (the remaining 8 of 15 eyes), the residual fragments contained regenerated neurons, double-labeled with BrdU and RET 1 (Figs. 4 and 5). Cells double-labeled with BrdU and FRet43 as well as BrdU and Rho 4D2 were also present in these retinas (data not shown).

Several characteristics typical of retinas with regenerated neurons are shown in Figure 4, at 42 days after retinectomy. First, the residual, FG-labeled retina was thinner than control (unlesioned) retinas [Fig. 4(A,D)], typically had fewer retinal cells than normal [Fig. 4(D)], and the laminar arrangement was disrupted compared to control (unlesioned) retina also labeled with FG (Fig. 3). The RET 1 labeling pattern also revealed these abnormal histological characteristics [Fig. 4(C)]. Another consistent feature was that BrdU+ cells were interspersed throughout the residual retina [Fig. 4(B)]. Finally, new retina presumably derived from a regeneration blastema was present at the edge of the FG-labeled retinal fragment. Note that the FG labeling terminated abruptly [Fig. 4(D)], and the retina adjacent to the FG-labeled fragment was heavily labeled with BrdU [Fig. 4(B)].

At the longest survival interval examined (82 days after retinectomy; Table 1), in the two (of six) retinas that had regenerated neurons, the retinas appeared to be completely laminated and of normal thickness (Fig. 5).

These data show that fragments of residual retina underwent a process of repair and regeneration. The results from both sets of experiments suggest that repair of the goldfish retina results from the regeneration of retinal neurons derived from an intrinsic source of proliferating cells, not from the RPE.

DISCUSSION

We conclude that the RPE does not contribute to retinal regeneration in adult goldfish under the
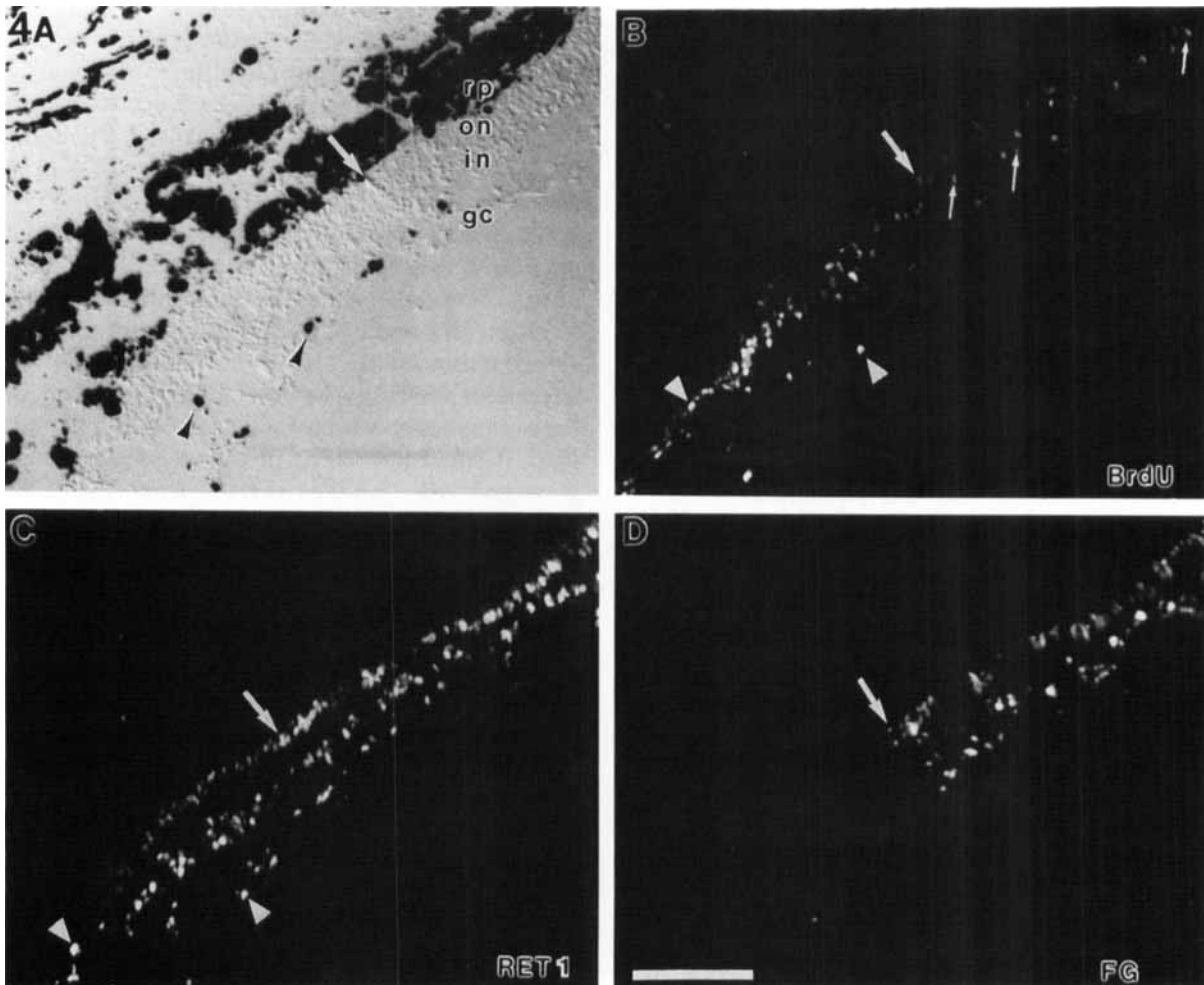
Figure 4 Cross-section of adult goldfish retina, 42 days after partial retinectomy, preinjected with FG 1 day before retinectomy, with BrdU injections at 11 and 15 days. (A) Nomarski optics. Note pigmented cells (arrowheads). The retina appears thin, and the laminae are disorganized. (B) BrdU+ cells, visualized with narrowband FITC illumination. Several BrdU+ cells are indicated with small arrows. (C) RET 1+ cells, visualized with TRITC illumination. (D) FG-labeled cells, visualized with UV illumination. On the right of each panel, a thin piece of retina to the right of the large arrow is FG-labeled (D). This region is also interspersed with BrdU+ cells (B). To the left of the large arrow in each panel is entirely new retina, since FG labeling is absent (D). This new retina is thought to be derived from the blastema-like region at the cut edge of the residual retina. New neurons are labeled with both BrdU and RET 1 (arrowheads, B and C). Scale bar = 100 μm.

present conditions. Proliferating cells intrinsic to the retina apparently initiate repair processes, including interstitial replacement of neurons in residual retinal pieces, and the addition of new cells by a regeneration blastema at the cut edge. The best candidates for a source of proliferating cells capable of producing new neurons are rod precursors (Raymond and Rivlin, 1987; Raymond et al., 1988a; Braisted and Raymond, 1992), although other possible sources (Müller glia or endogenous

quiescent neuroepithelial cells) have not been eliminated (Braisted et al., 1994).

The present study has revealed several unique features of this repair mechanism in the adult goldfish retina. It was clear from histological observations that substantial cell loss can occur in residual pieces of retina that remain after surgical manipulations. This cell loss could result from damage during the surgery or from later degenerative processes due to detachment of the neural retina from

the RPE. Although photoreceptors in cat commonly degenerate when detached from the RPE (Anderson et al., 1983, 1986), there have been no reports that neurons in the inner nuclear layer are affected by retinal detachment. However, we found that both the outer and inner nuclear layers in the residual retinas contained fewer cells up to 42 days after partial retinectomy. One possible explanation is that following the surgical procedure, the fragment of retina expanded, extending across the denuded RPE to cover areas where retina was absent. Thinning of the residual pieces of retina was also associated with blastemal and interstitial addition of new neurons. On the other hand, when residual pieces of retina were large, and appeared intact and of normal thickness, they did not contain regenerated neurons. There is evidence for retinal expansion with concomitant retinal thinning in the Black Moor, a mutant strain of goldfish with abnormally large eyes, in which one eye is often larger than the other. The retina of the larger eye is thinner, as if passively stretched, and it contains an increased number of proliferating rod precursors (Raymond et al., 1988b). The putative expansion (or stretching) of retina described here must result from a different, possibly active, mechanism in which residual retina might expand to fill the gap, as in wound repair. A similar phenomenon in which retina stretches after lesions, and new neurons are added interstitially, has also been described in sunfish (Cameron and Easter, 1994). The trigger for this expansion may depend on the extent of damage to the retina, since large, relatively intact fragments of retina did not appear to expand. By 82 days, the retinas had regained a normal thickness and lamination, suggesting that the repair process was completed successfully.

Our results are consistent with reports on another teleost fish, a cyprinid (*Leuciscus bergi*), in which the differentiated RPE phenotype was shown to be stable once the cells became pigmented (Sologub, 1975). Our results are also similar to those of Lombardo (1968, 1972), who removed a quadrant of retina in adult goldfish but did not observe evidence of RPE participation in retinal regeneration. Levine (1981) found a similar result in postmetamorphic *Xenopus*. Levine showed that after partial removal of the retina, cells at the germinal zone and at the cut edge of the retina (analogous to the blastema described here) contributed to regeneration of the retina. Levine (1981) also suggested that a population of unidentified neuroepithelial cells resides within the central retina in *Xenopus*, and that these cells are responsible for regeneration of the retinal neurons.

Although it has not been examined rigorously in *Xenopus*, these proliferative cells appear to be similar to goldfish rod precursors in their regenerative capacity, although there is no evidence that these progenitor cells produce new neurons in the intact *Xenopus* retina under normal conditions (Taylor et al., 1989).

We used a novel method to prelabel the retina prior to its surgical removal to assist in the identification of residual pieces that either intentionally or inadvertently remained after the surgery. We assumed retina with FG label was residual retina. An alternative possibility, however, is that the FG might have remained accessible for longer than 1 day, and, if so, it could have been incorporated into newly generated cells, perhaps derived from the RPE. However, if that had been the case, we would have expected to see cells double-labeled with both FG and BrdU, which we never observed at any time from 5 to 82 days after surgery. It should also be noted that, although rod precursors, which are the presumed source of the regenerated neurons, are part of the residual retina, they did not incorporate FG, consistent with our observation that FG-labeled cells were never BrdU-labeled. Other, more direct, observations also argue against this alternative interpretation. First, the FG labeling ceased abruptly at a defined border in the retina in most preparations examined at 32 days and longer [Fig. 4(D)]. Second, we repeated the experiments on newts (Stone, 1950) and prelabeled the retina with FG, just as we had done with goldfish. We predicted that if RPE transdifferentiation occurred, the regenerated BrdU-labeled cells would never be associated with FG-labeled cells. This prediction was upheld. In newts, BrdU-labeled cells were completely segregated from FG-labeled cells, when present. Furthermore, regenerated retinas were found in eyes in which the retina had been completely removed, and no FG labeling was present (J. K. Knight, unpublished observations). The lack of similar results in goldfish strengthens the argument that RPE does not transdifferentiate in this species.

Although it seems clear that regeneration does not occur in goldfish following complete removal of the retina, it is not certain whether other conditions might stimulate RPE transdifferentiation (Coulombre and Coulombre, 1965, 1970; Lopashov and Sologub, 1972). Simple access of the denuded RPE to a diffusable source of signals from differentiated retina was not sufficient in goldfish, since under conditions in which the retina was only partially removed, there was no evidence of transdifferentiation. Whenever there were regenerated

neurons or newly differentiated retina, the cells were contiguous with a residual piece of FG-labeled retina. Although these results demonstrate that the adult goldfish RPE is incapable of autonomously transdifferentiating, we cannot exclude the possibility that under more extreme conditions in which transdifferentiation of RPE was observed in other species [for example, application of exogenous growth factors (Park and Hollenberg, 1989, 1991), culturing of RPE cells (Tsunematsu and Coulombre, 1981; Pittack et al., 1991), or transplantation of differentiated RPE cells into embryonic eyes (Loposhov and Sologub, 1972; Sologub, 1975)], the RPE of adult goldfish might be capable of transdifferentiation.

REFERENCES

ALLEE, W. C., KINEL, A. J., and HOSKINS, W. H. (1940). The growth of goldfish in homotypically conditioned water: a population study in mass physiology. *J. Exp. Zool.* **84**:417-443.

ANDERSON, D. H., STERN, W. H., FISHER, S. K., ERICKSON, P. A., and BORGULA, G. A. (1983). Retinal detachment in the cat: the pigment epithelial-photoreceptor interface. *Invest. Ophthalmol. Vis. Sci.* **24**:906-926.

ANDERSON, D. H., GUERIN, C. J., ERICKSON, P. A., STERN, W. H., and FISHER, S. K. (1986). Morphological recovery in the reattached retina. *Invest. Ophthalmol. Vis. Sci.* **27**:168-183.

BARTHEL, L. B. and RAYMOND, P. A. (1990). Improved method for obtaining 3-μm cryosections for immunocytochemistry. *J. Histochem. Cytochem.* **9**:1383-1388.

BRAISTED, J. E. and RAYMOND, P. A. (1992). Regeneration of dopaminergic neurons in goldfish retina. *Development* **114**:913-919.

BRAISTED, J. E., ESSMAN, T. F., and RAYMOND, P. A. (1994). Selective regeneration of photoreceptors in goldfish retina. *Development* **120**:2409-2429.

CAMERON, D. A. and EASTER, S. S. (1994). Novel cone phenotypes in stretched fish retina. *Invest. Ophthalmol. Vis. Sci.* **35** (suppl):1728.

COULOMBRE, J. L. and COULOMBRE, A. J. (1965). Regeneration of neural retina from the pigmented epithelium in the chick embryo. *Dev. Biol.* **12**:79-92.

COULOMBRE, J. L. and COULOMBRE, A. J. (1970). Influence of mouse neural retina on regeneration of chick neural retina from chick embryonic pigmented epithelium. *Nature* **228**:559-560.

DABAGYAN, N. V. (1959). [Regulatory properties of the embryonic eyes of sturgeons]. *Dokl. Akad. Nauk SSSR* **125**:938-941.

DEL CERRO, M., QI JIANG, L., WIEGAND, S. J., and LAZAR, E. (1990). In vivo staining of the mammalian ret-

ina by means of a simple fluorescent method. *J. Neurosci. Methods* **32**:81-86.

HICKS, D. and MOLDAY, R. S. (1986). Differential immunogold-dextran labeling of bovine and frog rod and cone cells using monoclonal antibodies against bovine rhodopsin. *Exp. Eye Res.* **42**:55-71.

HITCHCOCK, P. F. and RAYMOND, P. A. (1992). Retinal regeneration. *Trends Neurosci.* **15**:103-108.

HITCHCOCK, P. F., LINDSEY MYHR, K. J., EASTER, S. S. JR., MANGIONE-SMITH, R., and JONES, D. D. (1992). Local regeneration in the retina of the goldfish. *J. Neurobiol.* **23**:187-203.

KEEFE, J. R. (1973). An analysis of urodelian retinal regeneration: IV. Studies of the cellular source of retinal regeneration in Triturus cristatus carnifex using 3H-thymidine. *J. Exp. Zool.* **184**:239-258.

KLEIN, L. R., MACLEISH, P. R., and WIESEL, T. N. (1990). Immunolabelling by a newt retinal pigment epithelium antibody during retinal development and regeneration. *J. Comp. Neurol.* **293**:331-339.

LARISON, K. D. and BREMILLER, R. (1990). Early onset of phenotype and cell patterning in the embryonic zebrafish retina. *Development* **109**:567-576.

LEVINE, R. L. (1975). Regeneration of the retina in the adult newt, Triturus cristatus, following surgical division of the eye by a limbal incision. *J. Zool.* **192**:363-379.

LEVINE, R. L. (1981). La regenerescence de la retine chez Xenopus laevis. *Rev. Can. Biol.* **40**:19-27.

LOMBARDO, F. (1968). La rigenerazione della retina negli adulti di un Teleosteo. *Accad. Lincei-Rend. Sci. Fis. Mat. Nat.* **45**:631-635.

LOMBARDO, F. (1972). Andamento e localizzazione delle mitosi durante la rigenerazione della retina di un Teleosteo adulto. *Accad. Lincei-Rend. Sci. Fis. Mat. Nat.* **53**:323-327.

LOPASHOV, G. V. and SOLOGUB, A. A. (1972). Artificial metaplasia of pigmented epithelium into retina in tadpoles and adult frogs. *J. Embryol. Exp. Morphol.* **28**:521-546.

OKADA, T. S. (1980). Cellular metaplasia or transdifferentiation as a model for retinal cell differentiation. *Curr. Top. Dev. Biol.* **16**:349-380.

OPAS, M. and DZIAK, E. (1994). bFGF-induced transdifferentiation of RPE to neuronal progenitors is regulated by the mechanical properties of the substratum. *Dev. Biol.* **161**:440-454.

PARK, C. M. and HOLLENBERG, M. J. (1989). Basic fibroblast growth factor induces retinal regeneration in vivo. *Dev. Biol.* **134**:201-205.

PARK, C. M. and HOLLENBERG, M. J. (1991). Induction of retinal regeneration in vivo by growth factors. *Dev. Biol.* **148**:322-333.

PARK, C. M. and HOLLENBERG, M. J. (1993). Growth factor-induced retinal regeneration in vivo. *Int. Rev. Cytol.* **146**:49-74.

PITTACK, C., JONES, M., and REH, T. A. (1991). Basic fibroblast growth factor induces retinal pigment epi-

thelium to generate neural retina in vitro. *Development* **113**:577–588.

RAYMOND, P. A. and RIVLIN, P. K. (1987). Germinal cells in the goldfish retina that produce rod photoreceptors. *Dev. Biol.* **122**:120–138.

RAYMOND, P. A., REIFFLER, M. J., and RIVLIN, P. K. (1988a). Regeneration of goldfish retina: rod precursors are a likely source of regenerated cells. *J. Neurobiol.* **19**:431–463.

RAYMOND, P. A., HITCHCOCK, P. F., and PALOPOLI, M. F. (1988b). Neuronal cell proliferation and ocular enlargement in Black Moor goldfish. *J. Comp. Neurol.* **276**:231–238.

RAYMOND, P. A., BARTHEL, L. K., ROUNSIFER, M. E., SULLIVAN, S. A., and KNIGHT, J. K. (1993). Expression of rod and cone visual pigments in goldfish and zebrafish: a rhodopsin-like gene is expressed in cones. *Neuron* **10**:1161–1174.

REH, T. A. and NAGY, T. (1987). A possible role for the vascular membrane in retinal regeneration in Rana catesbienna tadpoles. *Dev. Biol.* **122**:471–482.

REH, T. A., NAGY, T., and GRETTON, H. (1987). Retinal pigmented epithelial cells induced to transdifferentiate to neurons by laminin. *Nature* **330**:68–71.

SCHMUED, L. C. and FALLON, J. H. (1986). Fluoro-Gold: a new fluorescent retrograde axonal tracer with numerous unique properties. *Brain Res.* **377**:147–154.

SCHUTTE, B., REYNDERS, M. J., BOSMAN, E. T., and BLIJHAM, G. H. (1987). Studies with anti-bromodeoxyuridine antibodies: II. Simultaneous immunocyto-chemical detection of antigen expression and DNA synthesis by in vivo labeling of mouse intestinal mucosa. *J. Histochem. Cytochem.* **35**:371–374.

SOLOGUB, A. (1975). Differentiation of the pigmented epithelium and stimulation of its metaplasia in teleost. *Ontogenez* **6**:39–46.

SOLOGUB, A. (1977). Mechanisms of repression and derepression of artificial transformation of pigmented epithelium into retina in Xenopus laevis. *Roux's Arch. Dev. Biol.* **182**:277–291.

STONE, L. S. (1950). The role of retinal pigment cells in regenerating neural retinae of adult salamander eyes. *J. Exp. Zool.* **113**:9–31.

STROEVA, O. G. and MITASHOV, V. I. (1981). Developmental potential of vertebrate eye tissues: regeneration of retina and lens. In: *Problems of Developmental Biology*, N. Khrushcov, Ed. MIR Publishers, Moscow, pp. 168–206.

STROEVA, O. G. and MITASHOV, V. I. (1983). Retinal pigment epithelium: proliferation and differentiation during development and regeneration. *Int. Rev. Cytol.* **83**:221–293.

TAYLOR, J. S. H., JACK, J. L., and EASTER, S. S., JR. (1989). Is the capacity for optic nerve regeneration related to continued retinal ganglion cell production in the frog? A test of the hypothesis that neurogenesis and axon regeneration are obligatorily linked. *Eur. J. Neurosci.* **1**:626–638.

TSUNEMATSU, Y. and COULOMBRE, A. J. (1981). Demonstration of transdifferentiation of neural retina from pigmented retina in culture. *Dev. Growth Differ.* **23**:297–311.