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Neural retinal regeneration in the anuran amphibian *Xenopus laevis* post-metamorphosis: Transdifferentiation of retinal pigmented epithelium regenerates the neural retina

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Abstract

In urodele amphibians like the newt, complete retina and lens regeneration occurs throughout their lives. In contrast, anuran amphibians retain this capacity only in the larval stage and quickly lose it during metamorphosis. It is believed that they are unable to regenerate these tissues after metamorphosis. However, contrary to this generally accepted notion, here we report that both the neural retina (NR) and lens regenerate following the surgical removal of these tissues in the anuran amphibian, *Xenopus laevis*, even in the mature animal. The NR regenerated both from the retinal pigment epithelial (RPE) cells by transdifferentiation and from the stem cells in the ciliary marginal zone (CMZ) by differentiation. In the early stage of NR regeneration (5–10 days post operation), RPE cells appeared to delaminate from the RPE layer and adhere to the remaining retinal vascular membrane. Thereafter, they underwent transdifferentiation to regenerate the NR layer. An *in vitro* culture study also revealed that RPE cells differentiated into neurons and that this was accelerated by the presence of FGF-2 and IGF-1. The source of the regenerating lens appeared to be remaining lens epithelium, suggesting that this is a kind of repair process rather than regeneration. Thus, we show for the first time that anuran amphibians retain the capacity for retinal regeneration after metamorphosis, similarly to urodeles, but that the mode of regeneration differs between the two orders. Our study provides a new tool for the molecular analysis of regulatory mechanisms involved in retinal and lens regeneration by providing an alternative animal model to the newt, the only other experimental model.

Keywords: Regeneration; Retina; Lens; *Xenopus laevis*; Organ culture; RPE65; Pax6; BrdU

Introduction

Urodele amphibians like the newt show a remarkable capacity for regenerating organs such as the tail, limb, jaw, lens, and retina, and the newt has been a useful animal model, most intensively studied for its regenerative capacity. Neural retinal regeneration in urodele amphibians has been studied since the 19th century (Colluci, 1891), and they are the sole animal species that can regenerate the neural retina (NR) in the adult stage; following removal of the neural retina, an initial dedifferentiation of retinal pigmented epithelium (RPE) cells occurs, which then begin to proliferate and finally give rise to a newly formed NR (Stone, 1950; Hasegawa, 1958; Reyer, 1971). These events are called transdifferentiation, the transformation of one differentiated cell type into another (Okada, 1991). In addition, cells from the ciliary marginal zone also contribute to the regenerating NR (Reyer, 1971, 1977). The cellular and molecular mechanisms involved in newt retinal regeneration are as yet poorly understood. Recent organ culture studies have shown that the choroid which underlies the RPE plays an essential role in newt retinal regeneration and that the RPE-choroid interaction is mediated by diffusible substances like FGF-2 and IGF-1 (Ikegami et al., 2002; Mitusda et al., 2005).

In anuran amphibians, the regenerative capacities of the lens and NR have also been studied intensively and are present only in larval stages (Bosco, 1988; Hitchcock et al., 2004); in *Xenopus laevis*, the lens and retinal regeneration capacity decreases

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during larval development and finally disappears after metamorphosis (Freeman, 1963), and a similar reduction in regenerative potency has also been observed both in limb and central nervous system regeneration (Wallace, 1981; Filoni, 1992; Cannata et al., 2001). Retinal regeneration in anuran larvae was precisely described *in vivo* in *Rana catesbieanna* tadpoles, in which RPE transdifferentiation appears to play a substantial role in the regenerating retina (Reh and Nagy, 1987). RPE cell transdifferentiation into the NR was also described in *X. laevis* tadpoles under a special *in vitro* condition (Sakaguchi et al., 1997). In intraocular transplantation, the dorsal iris undergoes a process of transdifferentiation in *X. laevis* larva, and the new retina regenerates when the iris is isolated from its surrounding tissue and implanted in the vitreous chamber without the lens (Sologub, 1977; Cioni et al., 1986).

Lens regeneration in anuran amphibians has been fully demonstrated in larval stages (Freeman, 1963; Bosco et al., 1979; Henry and Elkins, 2001; Cannata et al., 2003). Freeman (1963) showed that the lens regenerates from the inner layer of the outer cornea after its removal during larval stages, a different tissue source from that of the newt, in which the dorsal iris pigmented epithelium regenerates the lens (Eguchi, 1998; Tsonis, 2002). It has been argued as to whether the lens and NR can still regenerate in anurans after metamorphosis, and here we report our new findings on this subject after careful examination of the regeneration processes in *X. laevis*, indicating that *X. laevis* can regenerate a lost retina even after metamorphosis and that the lens can also be repaired. Since accumulated information on gene expression patterns and molecular biology technologies are available in *X. laevis*, the present findings will afford a new experimental model for the molecular regulatory mechanisms involved in amphibian retinal regeneration. The present results indicate the possibility that some animals may possess a wider potential for retinal regeneration than has been previously recognized, and stimulate our efforts to identify mechanisms of retinal regeneration in higher vertebrate species.

Materials and methods

Animals

X. laevis were obtained from a local supplier (Xenopus Company, Ibaragi, Japan) and fed in the laboratory. Animals used in the present study were at stages between 3 and 9 months after metamorphosis. They were kept in tap water at 20 ±2 °C and fed three times a week with mixed feed, originally prepared for fish cultures (Taiyou Siryo Co., Ltd.).

Surgical removal of the lens and NR

Animals were deeply anesthetized in 0.15% MS222 solution (Sigma) for 20 min. The dorsal boundary area between the cornea and sclera was incised with a sharp razor and cut open with scissors without injuring the cornea tissue. The lens was then pulled out through the incision with forceps. The retina was separated from the RPE by pouring distilled water into the eye chamber several times using a fine tip pipette through the incision. Subsequently, the whole retina was separated from the eye cavity. Normally, the retina was detached from the retinal vascular membrane (RVM). Accordingly, the RVM was left in the cavity and was put back again to the ocular chamber (Fig. 1). The remaining retinal tissues adhering to the iris portion were carefully removed with fine forceps.

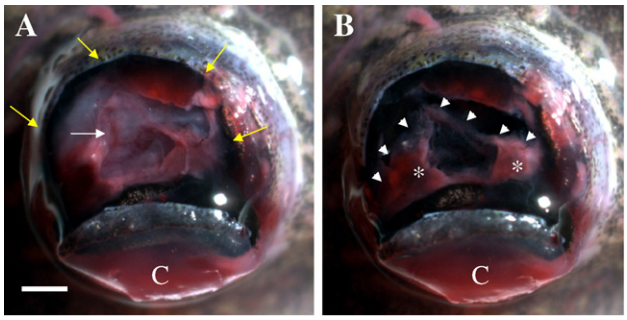


Fig. 1. Surgical operation for retinectomy of *Xenopus* eye. (A) An incision was made along the dorsal boundary of the iris (yellow arrows). The retinal vascular membrane (RVM) is visible on the whitish retinal tissue. White arrow indicates blood vessels of the RVM. C: cornea. (B) For surgical retinectomy, RVM was turned out towards the lens. Immediately after removal of the retinal tissue, the RVM was put back again to the ocular cavity as shown in B. Arrowheads deliminate the peripheral edge of RVM and asterisks indicate blood cell clusters. Scale bar is 500 μm.

Operated on animals were kept on wet absorbent cotton until they awoke from anesthesia.

Histological preparation

For histological observations, tissues were fixed with a Bouin fixative and embedded in paraffin. Sections of 6 μm thickness were cut and stained with hematoxylin and eosin.

Tissue culture

The procedure for tissue culture was largely the same as described previously (Ikegami et al., 2002; Mitusda et al., 2005). Animals were deeply anesthetized with 0.15% MS222, and the heads were decapitated and immersed twice in 70% ethanol for sterilization, each time for 30 s, followed by washing in newt–Hanks' balanced salt solution. The eyeballs were enucleated carefully, and then adherent muscles and fat tissues were cleanly removed. The anterior parts of the eyeballs including the irido-corneal complex and lens were discarded, and posterior eyecups were kept in Ca^{2+},Mg^{2+}-free newt–Hanks' solution. This treatment caused the neural retina tissue to detach easily from the RPE. The sclera was then removed and the remaining tissues, consisting of the RPE and choroid, were placed flat on a filter cup membrane (Millicell-CM, pore size 0.4 μm, Millipore), with the choroid facing the filter membrane. The membrane was pre-coated with type I collagen (Cellmatrix type I-C, Nitta Gelatin). Each filter cup was placed in one well of a 6-well culture plate. The medium was Livowitz L15 (GIBCO BRL) (diluted to 66% of the prescribed concentration for mammalian cell cultures) supplemented with 8% fetal bovine serum (FBS) (Hyclone Laboratories Inc.) and kanamysin sulfate (8 mg/dl, Sigma). Cultures were maintained in a humidified dark incubator at 25 °C, and fed with fresh medium every 5 days. Growth factors, such as FGF-2 (50 ng/ml, Boehringer Mannheim Biochemica) and IGF-1 (70 ng/ml, GroPeg), were added to the culture medium together with 7.5 μg/ml heparin sulfate (Wako Pure Chem.) 24 h after the culture was initiated.

To obtain a single RPE sheet without any connective tissue, the RPE sheet was detached from the choroid after the tissue was incubated in dispase solution (50 units/ml, Godo Shusei Ltd., Japan) for 40 h at 25 °C. Usually, a whole RPE sheet from each eye was obtained with minimal damage to the peripheral region. Histological preparation of the isolated RPE sheet confirmed that the choroids had been cleanly removed.

Immunocytochemistry

Enucleated eyes were fixed with an ice-chilled mixture of 2% paraformaldehyde and 0.5% glutaraldehyde in 50 mM phosphate-buffered saline (PBS, pH 7.4) for 10 min, followed by a second fixation for 3–5 h with 2%

paraformaldehyde in PBS. Cryostat sections were processed for immunocytochemistry with fluorescence-labeled secondary antibodies. Firstly, they were treated for 1 h with PBS containing 2% FBS and 0.02% Triton X-100, and then incubated in a primary antibody for 1 h at room temperature after being incubated overnight at 4 °C. They were then washed three times (each time for 10 min) with PBS containing 0.02% Triton X-100, followed by incubation with the secondary antibody for 1 h at room temperature. The secondary antibodies used were anti-mouse IgG conjugated with either Alexa-488 or Alexa-594 Fluorescent (Molecular Probes). The primary antibodies used were anti-acetylated tubulin (Sigma) (Piperno and Fuller, 1985), anti-neurofilament 200 (Sigma), anti-laminin (Sigma), anti-BrdU (Becton Dickinson), anti-PCNA (Boehringer Mannheim), anti-Pax6 (a gift of Dr. Kondoh) and RPE65 (Chemicon), a monoclonal antibody specific to RPE. They were diluted 1000–2000 times.

In some cases, paraffin sections were deparaffinized and used for immunocytochemistry using the methods described above. Primary and secondary antibodies were used in the following dilutions: guinea pig anti-newt whole lens, 1:300 and anti-guinea pig IgG conjugated with Alexa-488 Fluorescent, 1:400. The anti-newt whole lens antibody has been reported to react with newt lens fiber cells (Okamoto et al., 1998).

BrdU labeling

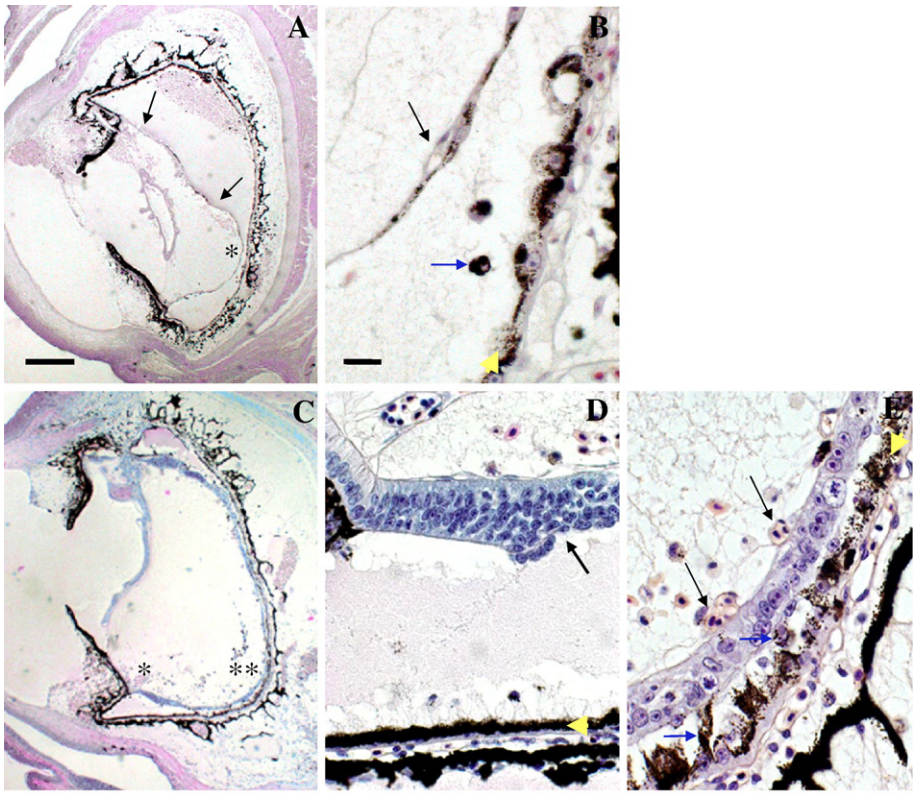
To detect proliferating cells under culture conditions, BrdU (5-bromo-2′-deoxyuridine; Sigma) was added to the medium at 5 μg/ml. Cultures were fixed as described above, and treated with 2 N HCl for 1 h before being subjected to immunocytochemistry.

For BrdU labeling of *in vivo* regenerating retina, retinectomized animals were given an injection of BrdU (5 mg/100 g body weight) in the dorsal lymphatic sac on post-operative day 10 (Gagliardino et al., 1993). The animals were fixed on the following day and subjected to BrdU immunostaining.

Results

Retinal regeneration in retinectomized eyes

X. laevis, 120 animals in total, between 3 and 9 months after metamorphosis, were surgically operated on to remove both the



A, B: day 7
C, D, E: day 10

B, E: central portion. B corresponds to the area shown by an asterisk in A, and E to the area shown by double asterisks in C.
D: marginal portion.

Fig. 2. An early stage of retinal regeneration in *X. laevis* eyes (3 months after metamorphosis). All images in this figure and in Figs. 3–10 are illustrated and placed in the same direction: the anterior (corneal) of the eye at the left and the posterior (scleral) at the right. The dorsal is at the upper. (A, B) Day 7 and (C, D, E) Day 10 after retinectomy. The area indicated by asterisk in (A) is shown in (B) at a higher magnification. Arrows indicate RVM (retinal vascular membrane). (B) Black arrow indicates RVM where pigmented cells form an epithelial monolayer sheet. Blue arrow indicates single pigmented cell located between the retinal pigmented epithelium (RPE) (indicated by yellow arrowhead) and the RVM. (C) The areas indicated by a single and by double asterisks in (C) are shown in (D) and (E) at a higher magnification, respectively. (D) shows regenerating retina (arrow) at the marginal zone, continuous to the iris epithelium. Yellow-colored arrowhead indicates the RPE layer. (E) shows regenerating retina on the RVM located more posteriorly. Black arrows indicate capillaries on RVM and blue arrows indicate pigmented cells located in a narrow space between the newly formed epithelium and RPE layer (yellow arrowhead). A pigmented cell (indicated by the lower left blue arrow) extends from RPE layer to the regenerating epithelium. Scale bar in A is 200 μm and is applied to C. Scale bar in B is 10 μm and is applied to D and E.

lens and NR. At around post-operative day 30 (PD 30), the neural retinal layer and lens were found to have regenerated. Retinal regeneration was observed in approximately 70% of operated animals (45 out of 65 animals operated on and fixed after PD 15). In many cases with successful retinal regeneration, the lens had also regenerated. No profound difference was observed in the regeneration process, regardless of the animal stages (Figs. 2 and 4). In the rest of the animals (20 out of 65), neither the lens nor NR could be seen, and in these unsuccessful cases, the vitreous cavity was usually occluded by incision closure (Fig. 4E). No retinal regeneration was observed when the retinal vascular membrane was intentionally removed (8 cases).

We examined histological preparations of surgically operated on eyes fixed on PD 7, 10, 15, 20, 30 and 40. By PD 7, a pigmented cell layer became apparent in the vitreous chamber, facing the original RPE layer (Figs. 2B and 3E). As a result, two epithelial layers were observed, separating each other, and during the subsequent period, this newly formed epithelial layer (inner layer) appeared to undergo transdifferentiation into the neural retina. The inner layer was found to have been formed on the retinal vascular membrane. The retinal vascular membrane normally covers the vitreous surface of the retina and constitutes the inner limiting membrane of the retina (Fig. 3A), and was intensely stained for laminin, often much more intensely than the RPE basement membrane (Bruch's membrane) (Fig. 3A, B). The inner epithelial layer consisted of pigmented cells that were positively stained for RPE65, a specific marker of RPE cells (Fig. 3C). The layer was always found to have developed on laminin-immunostained structures (Fig. 3C, D, E).

In most cases, the retinal vascular membrane remained in the cavity during the surgical operation for retinectomy, possibly due to its firm attachment to the iris tissue (Figs. 1 and 2A). The RPE layer remained as a single pigmented epithelial layer and did not undergo de-pigmentation nor transdifferentiate to the retina inside the cell layer (Fig. 2B, D, E).

By PD 10, the inner layer (a newly formed epithelial layer) was composed of a single-cell or two-cell layer and, in most cases, was still separated from the RPE layer (Fig. 2). Numerous pigmented cells were found within the epithelium. In some cases, this epithelial layer, presumably regenerating NR, was closely apposed to the RPE (Fig. 2E). The inner epithelial layer was always thicker at the marginal zone than at the central zone. At around PD 20, the inner layer showed a multi-cellular layer and many single cells with melanin granules were still found in the vitreous space between the inner layer (presumably regenerating NR) and RPE layer (Fig. 4A, B, C). By PD 40, the inner epithelial layer, now apparently a regenerating NR,

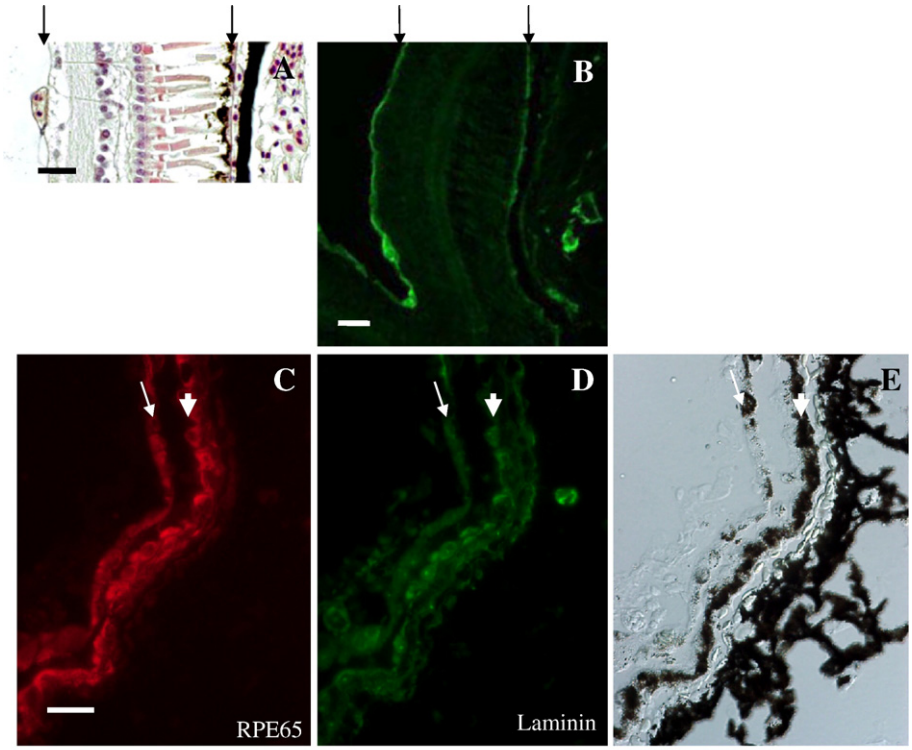


Fig. 3. Laminin and RPE65 distribution in regenerating retinas at Day 10 after retinectomy. (A) Normal *Xenopus* retina stained with hematoxylin and eosin. Two arrows indicate RVM (retinal vascular membrane) and Bruch's membrane. Both membrane structures are clearly immunoreactive for laminin as indicated by arrows in (B). (C, D, E) Retinectomized eye at Day 10. RPE65, laminin and Nomarsky differential image of the same area at the central (posterior) part of the eye. Arrows and arrowheads indicate the newly formed pigmented epithelium and the original RPE layer, respectively. Both layers are positively stained for RPE65 and laminin. Scale bars in A and B are 30 μm, and bar in C is 20 μm.

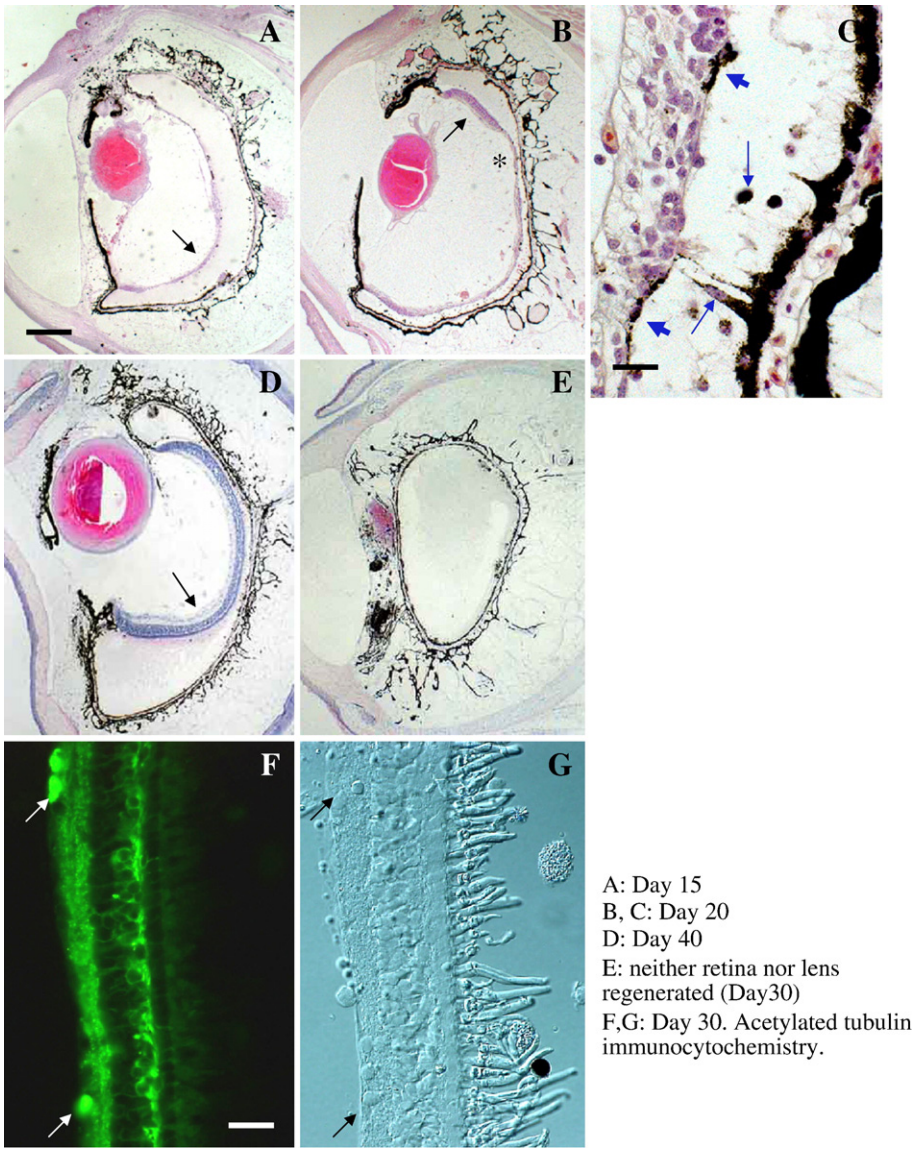


Fig. 4. Later stage of retinal regeneration in *X. laevis* eyes (4 to 5 months after metamorphosis). (A) Day 15, (B, C) Day 20, (D) Day 40 and (E) Day 30 after retinectomy. (A) By Day 15, lens structures are well recognized with intense eosin staining. Regenerating retinas (arrows) are still thin and are separated from RPE layer. (B) By Day 20, the laminar structure of the regenerating retina is partially developed at the periphery (arrow). The area indicated by asterisk is shown in (C) at a higher magnification. (C) A few RPE cells are found in the space between RPE layer and the retinal epithelium as shown by blue arrows. Some cells extend from RPE layer to the retinal layer (indicated by lower blue arrow). Blue-colored arrowheads indicate pigmented cells attached to the retinal layer. (D) By Day 40 a well-stratified retinal structure develops as shown by an arrow. (E) In some cases neither the retina nor lens regenerates, the eye cavity being occluded by connective tissue derived from the choroid tissue. (F, G) Laminar structure of the regenerating retina at Day 30 is well identified with acetylated tubulin staining. Arrows indicate immunoreactive ganglion cell bodies. G is a Nomarsky differential image of the same area. Scale bar in A is 200 μm and is applied to B, D, E. Scale bars in C and F are 20 μm.

consisted of complete laminar structures, in some cases, similar to those observed in the intact eye, although the newly formed NR was still separated from the RPE layer at the marginal zones (Fig. 4D, F, G). The RPE layer did not show significant morphological changes (such as depigmentation), always remained pigmented, and were intensely stained for RPE65, indicating that RPE cells do not transdifferentiate into retinal cells inside of their original site.

RPE65 is a specific marker for retinal pigmented cells, since none of the iris and ciliary pigmented epithelial cells and melanocytes in the choroids were stained for RPE65. Immunocytochemical staining for RPE65 revealed that the inner pigmented layers were also positively stained, similarly to the RPE layer, suggesting that these pigmented epithelial cells were derived from RPE cells (Figs. 2 and 5). The inner epithelial layers on the retinal vascular membrane, however, were not continuously stained for RPE65, suggesting that cells originating from other sources like the ciliary marginal cells were intermingled with RPE cells (Figs. 2C and 4B).

Expression of Pax6, a crucial gene for retinal regeneration, was examined immunocytochemically (Hitchcock et al., 1996). In normal retina, ganglion cells as well as amacrine cells were

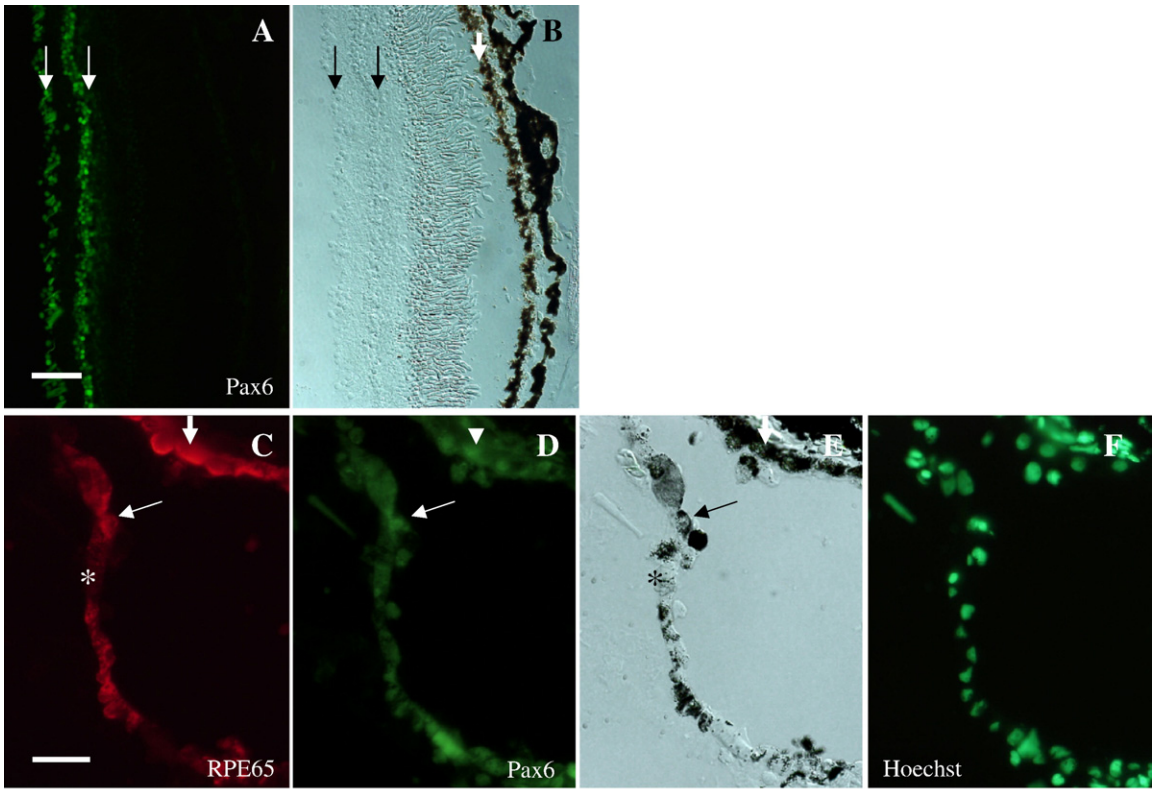


Fig. 5. RPE65 and Pax6 immunocytochemistry in the regenerating retina at Day 10 after retinectomy. (A, B) Pax6 staining and Nomarsky differential image of normal *Xenopus* retina. Arrows indicate two layers of positively stained ganglion and amacrine cells. (C, D, E, F) A newly formed pigmented layer (asterisk) and the original RPE layer (arrowhead) at Day 10 after retinectomy. Pigmented cells in both layers are mostly doubly stained for RPE65 and Pax6, as shown by arrows. Scale bars in A and C are 50 μm and 20 μm, respectively.

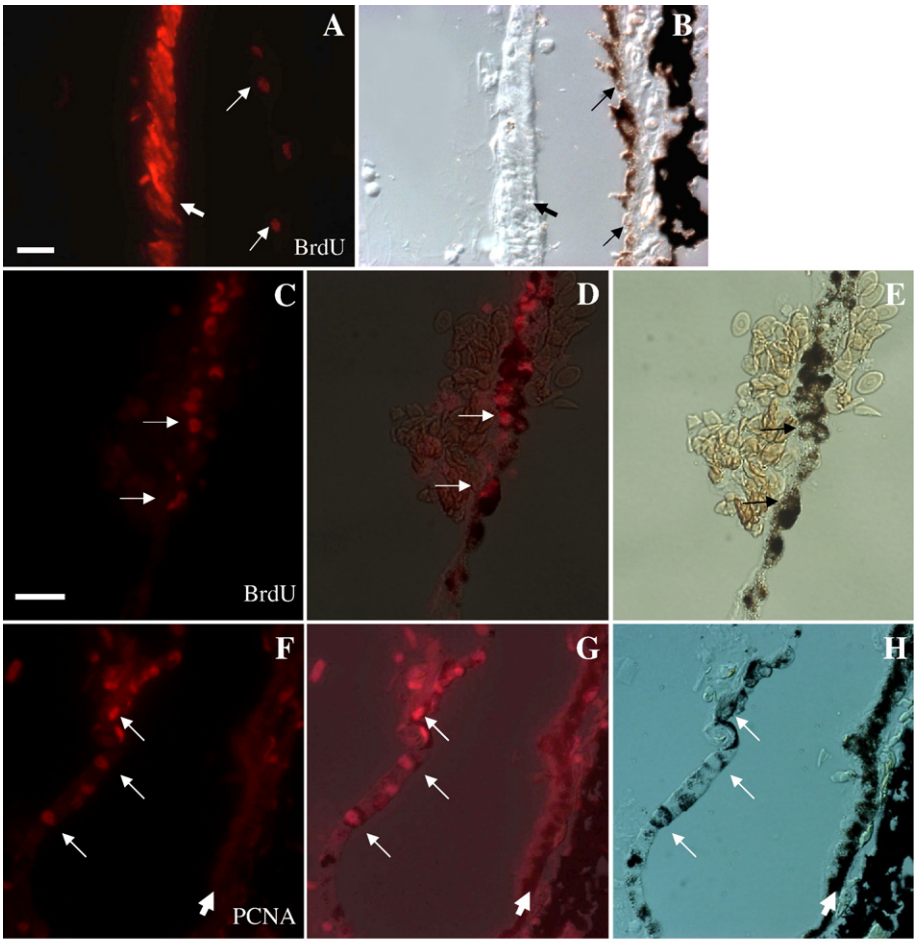
positively stained (Fig. 5A, B) and other retinal cells and RPE cells were negative (Kaneko et al., 1999). At PD 10, it was found that newly formed epithelial layers as well as the RPE layer were positively stained for Pax6 (Fig. 5). In both layers, pigmented cells were doubly stained for RPE65 and Pax6, suggesting that RPE cells undergo transdifferentiation.

To summarize these observations, the RPE layer was always unchanged and the newly formed epithelium (the regenerating NR) emerged on the remaining retinal vascular membrane. Epithelial cells constituting this layer were considered to be derived from both RPE cells and cells in the ciliary marginal zone. Numerous isolated pigmented cells were found in the vitreous space between the inner epithelial layer and the RPE, seemingly detached from the RPE layer and migrating to the inner layer (Figs. 2B, E and 4C). At the same time, stem cells in the marginal zone and peripherally located RPE cells might have migrated on the retinal vascular membrane toward the central zone to regenerate peripheral retinal tissues.

BrdU labeling experiments showed that at the early stage of retinal regeneration (PD 10), a few RPE cells in the peripheral RPE layer were labeled for BrdU (Fig. 6A, B), indicating that RPE cells were proliferating to produce surplus cells, and numerous cells in the regenerating retina at the peripheral region were intensely labeled for BrdU (Fig. 6A). The pigmented cells in the newly formed inner epithelial layer were also labeled for BrdU, suggesting that these pigmented cells were now undergoing the early phase of transdifferentiation (Ikegami et al., 2002) (Fig. 6C, D, E). These pigmented cells were also positively stained for PCNA, a proliferating cell marker (Fig. 6F, G, H).

Transdifferentiation of cultured RPE cells into neural cells

RPE cells from mature *X. laevis* were examined as to whether they retain the potency to transdifferentiate into neural cells under tissue culture conditions (Figs. 7 and 8). This culture system has been established in newt ocular tissues and



A, B: Regeneration at the peripheral region at Day 10.
C, D, E: BrdU labeled cells in a newly formed RPE layer at the central area
F, G, H: PCNA-positive cell nuclei in a newly formed RPE layer at the central area.

Fig. 6. Detection of proliferating cells by BrdU labeling and PCNA staining at Day 10 after retinectomy. (A, B) At the peripheral area close to the iris, numerous cells in a thick epithelial layer (arrowhead), probably derived from the ciliary marginal cells, are intensely stained for BrdU. A few cell nuclei in the RPE layer are also stained (arrows). B shows Nomarsky differential image of A. (C, D, E) At the central area, pigmented cells in a newly formed epithelium are stained for BrdU (arrows in C, D and E). D is an overlaid image of C and E. (F, G, H) Cells in the pigmented layer of the central area are also stained for PCNA. Nuclei of pigmented cells are positively reacted for PCNA (arrows). Arrowheads indicate RPE layer. G is an overlaid image of F and H. Scale bar in A is 20 μm. Bar in C is 20 μm and applied to F.

newt RPE cells were shown to proliferate and transdifferentiate into neural cells (Mitusda et al., 2005). *X. laevis* eyes, 3 to 4 months after metamorphosis, were enucleated and the anterior part of the eyeball including the lens was removed. The NR and sclera were then removed carefully and the remaining RPE-choroid tissues were laid on a filter membrane and cultured for 30 days (Fig. 7). At around Day 6 *in vitro*, RPE cells migrated out from the periphery of the explants (Fig. 7A) and by Day 30, many of these cells had extended long branching processes that were positively stained for neural markers such as acetylated tubulin and neurofilament (Fig. 7C, D, E).

RPE sheets alone were then isolated by treating RPE-choroid tissues with dispase. Single RPE sheets were cultured according to the procedure previously described (Mitusda et al., 2005). By Day 3, RPE cells had extended out from the periphery of the sheet but still remained as a simple epithelial layer (Fig. 8A). At around Day 30, RPE cells remained pigmented and no neuronal differentiation could be seen, although cells had proliferated, as shown by BrdU labeling (Fig. 8B, C). When cultured in the presence of FGF-2 and IGF-1, some of the RPE cells became de-pigmented, extended long processes, and were positively stained for acetylated tubulin (Fig. 8D, E, F). These observations suggest that RPE cells from mature *X. laevis* proliferate and differentiate into neural cells under culture condition in a similar fashion to the newt.

Lens regeneration in lentectomized animals

Lens regeneration was observed in half of the cases (33 out of 65 cases) in operated animals. They were subjected to surgical operation between 3 and 9 months after metamorphosis, and both the lens and NR were removed (Fig. 9). When only the lens was removed without removing the retina, lens regeneration was observed in most cases (9 out of 11). Lens regeneration was much faster and the lens was much

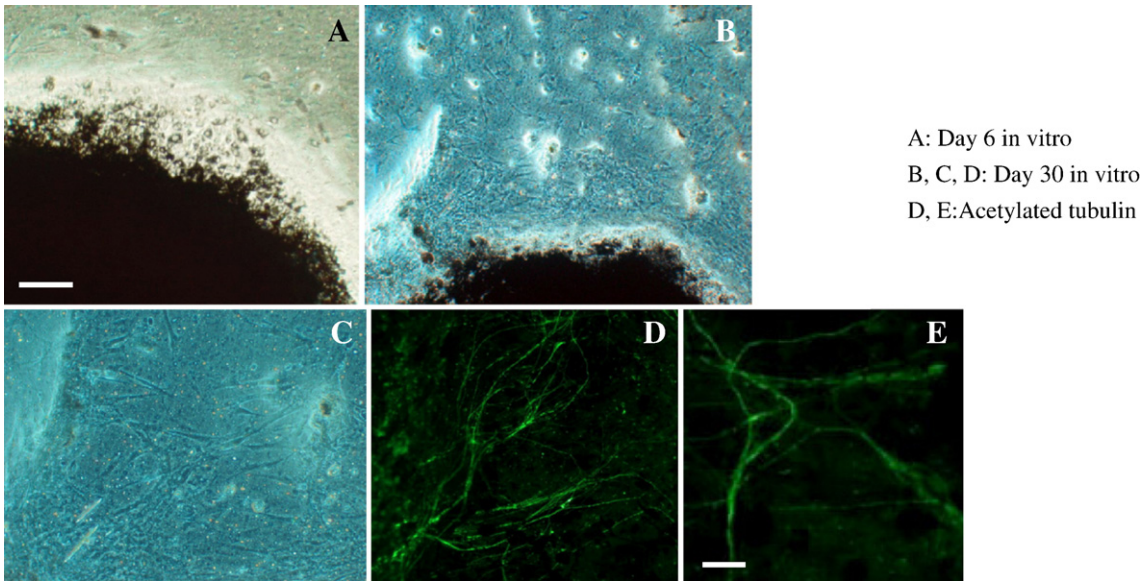


Fig. 7. Organotypic cultures of the RPE with attached choroid. RPE sheet with choroid was cultured on a filter membrane. (A) Day 6 in vitro (B, C, D, E) Day 30 in vitro. RPE cells begin to migrate out onto the filter membrane by Day 6, where they start to depigment and transform into fiber-like cells with long branching processes. (D, E) shows a culture stained with anti-acetylated tubulin. Scale bar in A is 50 μm and is applied to B, C, D. Bar in E is 10 μm.

bigger than was observed in animals which had been subjected to removal of both the lens and NR, suggesting a possibility that a retinal factor(s) is necessary for lens regeneration. As early as post-operative Day 5, a lens capsule-like structure with attached cells was often observed, and some cells within the epithelium-like cell arrangement were positively stained with anti-whole newt lens crystalline antibody (Fig. 9A, B, C). These stained cells were often located posteriorly. By PD 10, an elliptical irregular lens structure was observed and crystalline expression was found throughout the whole regenerating lens (Fig. 9D, E, F). On PD 30, the regenerating lens was almost similar to the intact one. Throughout lens regeneration, both the cornea and iris appeared morphologically intact. These observations suggest that a small number of lens cells within the remaining lens capsule might have proliferated and differentiated to lens fiber cells to re-construct the whole lens structure.

Discussion

In anuran amphibians including *X. laevis*, it is generally accepted that neither the retina nor lens regenerates after metamorphosis. Our data demonstrate, for the first time, that mature *X. laevis* (3 to 9 months after metamorphosis) can regenerate both the retina and lens after the surgical removal of these tissues, regardless of the animal stages. Transdifferentiation of RPE into neural retina in the urodele has been reviewed many times (Reyer, 1977; Okada, 1991; Mitashov, 1997; DelRio-Tsonis and Tsonis, 2003; Hitchcock et al., 2004); in newt retinal regeneration, RPE is the major source of regenerating NR. RPE cells in the epithelium become depigmented at the initial stage of regeneration and start proliferating to form the retinoblastic layer, which subsequently develops into a new NR (Hasegawa, 1958; Reyer, 1971; Keefe, 1973). In addition to this RPE transdifferentiation, the neuroblastic stem cells in the ciliary marginal zone also contribute to retinal regeneration (Levine, 1975). The present observations show that the process involved in the regeneration of the retina differs between *X. laevis* and the newt; in *X. laevis*, RPE cells in the epithelium always remained pigmented as a single layer, and the regenerating NR layer was found in the vitreous space on the retinal vascular membrane. This membranous structure consists of a basement membrane and numerous blood capillaries, and forms the inner limiting membrane of the retina in the intact eye. The retinal vascular membrane was left in place when the retina was surgically removed using the present procedure, because the membrane is attached firmly to the iris tissue. We supposed that in cases where no retinal regeneration was found, the retinal vascular membrane had been removed together with the retinal tissue. This may be the most likely reason that previous studies were unsuccessful in retinal regeneration of adult *X. laevis*, since retinal tissues were normally removed by air suction through a fine tipped pipette. When the retinal vascular membrane was removed intentionally, retinal regeneration was not observed. Thus, the vascular membrane is considered to play a crucial role in retinal regeneration of *X. laevis*.

Retinal regeneration in a mature anuran amphibian (*Rana esculenta*) was reported when only the retinal quadrant was removed (Lombardo, 1969). Although it is not clearly described as to whether RPE cell transdifferentiation plays a part in this case, we speculate that this might be partly due to the remaining retinal vascular membrane. Under certain artificial conditions, RPE cells from adult frogs (*Rana temporaria*) were shown to retain a retinal regenerative potential, in which RPE explants were transplanted into the embryonic ocular chamber (Lopashov and Sologub, 1972).

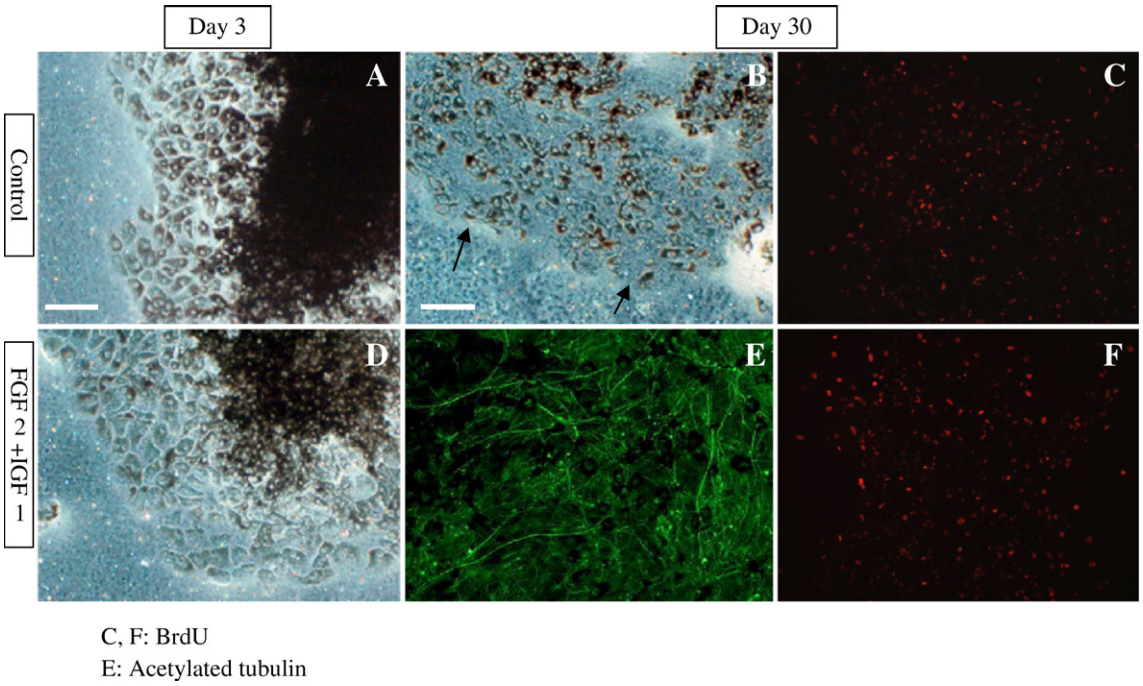


Fig. 8. Organotypic cultures of isolated RPE on Day 3 (A, D) and on Day 30 (B, C, E, F). RPE sheet was separated from the choroid and cultured on a filter membrane. (A, B, C) show cultures without FGF-2 and IGF-1, and (D, E, F) show cultures with both factors. (C, F) show BrdU labeling and (E) shows acetylated tubulin staining. In control cultures (A, B), RPE cells proliferated but remained mostly pigmented, while addition of FGF-2 and IGF-1 induces RPE cells to proliferate, depigment and differentiate into neural cells, as shown by acetylated tubulin immunocytochemistry (E). Arrows in B indicate the peripheral edge of the epithelial sheet. Scale bar in A is 20 μm and is applied to D. Scale bar in B is 30 μm and is applied to C, E, F.

In vivo transdifferentiation of RPE cells regenerate the retina on the retinal vascular membrane

In the early phase of *X. laevis* retinal regeneration, we found that pigmented cells formed a simple, flat epithelial sheet on the retinal vascular membrane and that this epithelial structure was positively stained for RPE65, an RPE-specific substance. This is a good indication that favors RPE cells as the source of regenerating NR. These cells were positively stained for Pax6, a crucial gene for retinal regeneration (Hitchcock et al., 1996; Kaneko et al., 1999; Arresta et al., 2005), and they were also proliferative as shown by BrdU labeling and PCNA immunostaining. At the same time, it is also possible that retinal stem cells, if any, residing within the retinal tissue of adult *X. laevis*, might be mobilized for regeneration. This, however, did not occur in the present case, because such stem-like cells residing in the inner nuclear layer appeared not to have been left in the vitreous chamber when the whole retinal tissue was surgically removed. Such stem-like cells have not been well characterized in the retina of adult *X. laevis*, although in the newt, proliferating cells in the inner nuclear layer have been reported to replace partially damaged retina (Grigorian et al., 1996). From our present observations, we suggest that some of the RPE cells detach from Bruch's membrane of the RPE layer and move inwardly toward the remaining retinal vascular membrane to adhere to the membrane, where they commence transdifferentiation (Fig. 10). The detachment of RPE cells from Bruch's membrane appears to also be a necessary prerequisite for transdifferentiation in the newt (Ikegami et al., 2002); newt retinal stem cells produced by RPE transdifferentiation do not leave the epithelium but stay within the epithelial layer, forming a pseudostratified epithelium. The most basally located cells that adhere to Bruch's membrane become re-pigmented and other cells that have no contact with Bruch's membrane are subjected to the retinal fate. In *Xenopus* retinal regeneration, besides centrally located RPE cells, a few RPE cells at the peripheral region appear to migrate onto the vascular membrane together with the ciliary marginal cells. The present model for *X. laevis* retinal regeneration is basically similar to that suggested for retinal regeneration in *R. catesbieanna* or *Rana pipiens* tadpoles (Reh and Nagy, 1987; Nagy and Reh, 1994), where retinal tissues are subjected to degeneration by a transient interruption of the blood supply.

There arises a question, then, as to what role the retinal vascular membrane plays in RPE transdifferentiation in *X. laevis*. Does it provide a permissive condition as a cell substratum or afford any substantial factors for transdifferentiation? The vascular membrane is intensely stained for laminin, which appears to have a promoting effect on RPE transdifferentiation and is also an important co-factor for the activation of FGFs (Werb and Chin, 1998). This, however, does not explain the role of the vascular membrane in the present case, since Bruch's membrane was also intensely stained for laminin. An *in vitro* culture study using RPE tissues of larval *X. laevis* does not support the positive role of laminin (Sakaguchi et al., 1997). It can be speculated that once RPE cells detach from Bruch's membrane, they can initiate transdifferentiation under the effect of FGF-2, presumably derived from the choroid, and

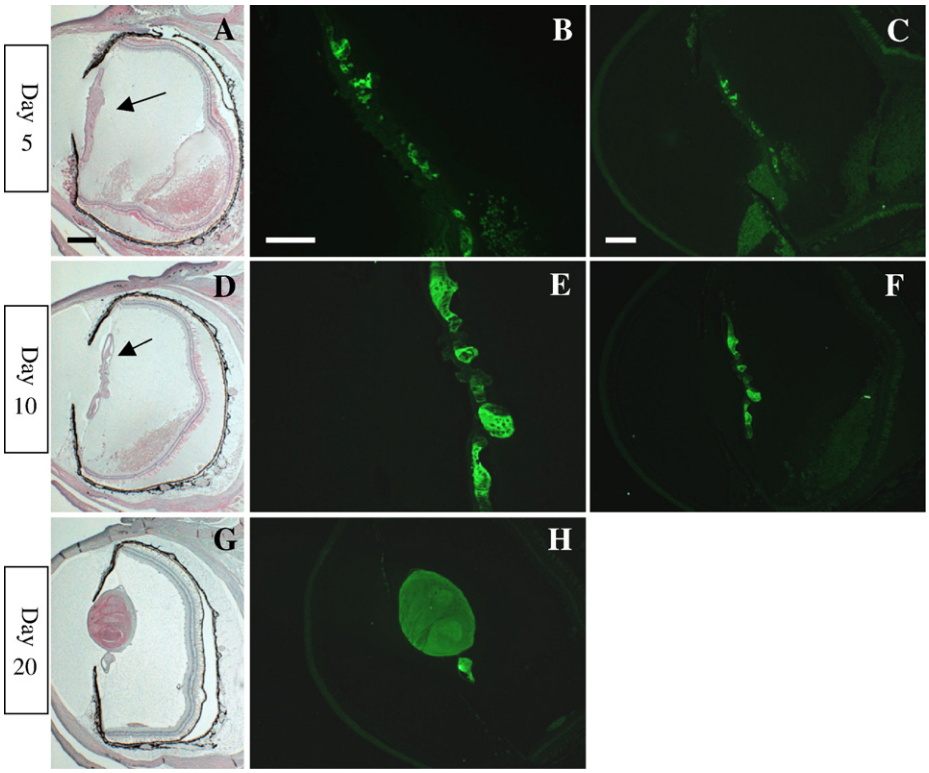


Fig. 9. Immunocytochemical detection of crystalline in regenerating lens in *X. laevis* eyes (4 months after metamorphosis). After lentectomy lens capsule-like structures are observed to adhere to the iris portion (arrows in A, D). (B, C) By Day 5 after lentectomy, some cells in the capsule are already positively stained with an antibody specific for newt lens. The stained cells are always located at the posterior side (B). (E, F) By Day 10 more cells are stained for crystalline. (G, H) By Day 20, the lens structure grows and shows a cuboidal shape. Lens fiber cells are stained intensely, while lens epithelium is devoid of staining. Scale bar in A is 200 μm and is applied to D, G. Scale bar in B is 50 μm and is applied to E. Scale bar in C is 100 μm and is applied to F, H.

the retinal vascular membrane affords a substratum for RPE cells to form a new epithelial layer. A more precise study is definitely necessary to characterize the molecular nature of the retinal vascular membrane.

In vitro transdifferentiation of RPE cells to neural cells requires factors from the choroid

The present *in vitro* study provides evidence that RPE cells from adult *X. laevis* can transdifferentiate into retinal neurons, since the cultured materials are free of both retinal cells and ciliary marginal cells. We have performed RPE cultures from *X. laevis* under the same conditions as we previously reported in the newt RPE (Mitusda et al., 2005). This enabled us to compare the whole processes of RPE transdifferentiation between the two species, whereby we found no substantial differences between them; RPE cells do not transdifferentiate into neural cells when cultured alone (without the choroid), although RPE cells from *X. laevis* do proliferate, as shown by BrdU labeling, while newt RPE cells do not proliferate. The role of the choroid was suggested to promote RPE transdifferentiation by sending signals like FGF-2 (Mitusda et al., 2005). This will also be the case in *X. laevis*, as shown by the present culture study, and was also previously suggested in a culture study of larval frog RPE (Sakaguchi et al., 1997). Further culture studies need to examine the expression patterns of early transcription factors that are required for the proliferation and differentiation of retinal progenitor cells and compare these patterns between the two animal models.

Besides RPE cells, the retinal stem cells in the CMZ (ciliary marginal zone) appear to contribute to the regenerating NR. Neurogenesis in the amphibian retina occurs partly during embryonic stages but mostly takes place post-embryonically by means of the addition of new cells from the CMZ (Straznicky and Gaze, 1971; Reh and Fisher, 2001; DelRio-Tsonis and Tsonis, 2003). In tadpoles whose retinas have been partly damaged either mechanically or chemically, cells in the CMZ migrate and act as retinal stem cells to replace damaged retina (Reh and Nagy, 1987). These cells in the CMZ are mitotically active in mature *X. laevis* (Straznicky and Hiscock, 1984). In the present study, epithelial cells on the retinal vascular membrane were often unpigmented at the periphery (close to the iris) and continuous with the iris epithelium. Such unpigmented epithelium in this area was usually thicker (two-cell or multi-cellular layer) than that at the central zone, probably due to a higher mitotic activity of the ciliary marginal cells than that of RPE cells.

Lens repair is promoted by retinal factors

In the present study, it was also found that *X. laevis* retains its ability to regenerate a whole lens after metamorphosis.

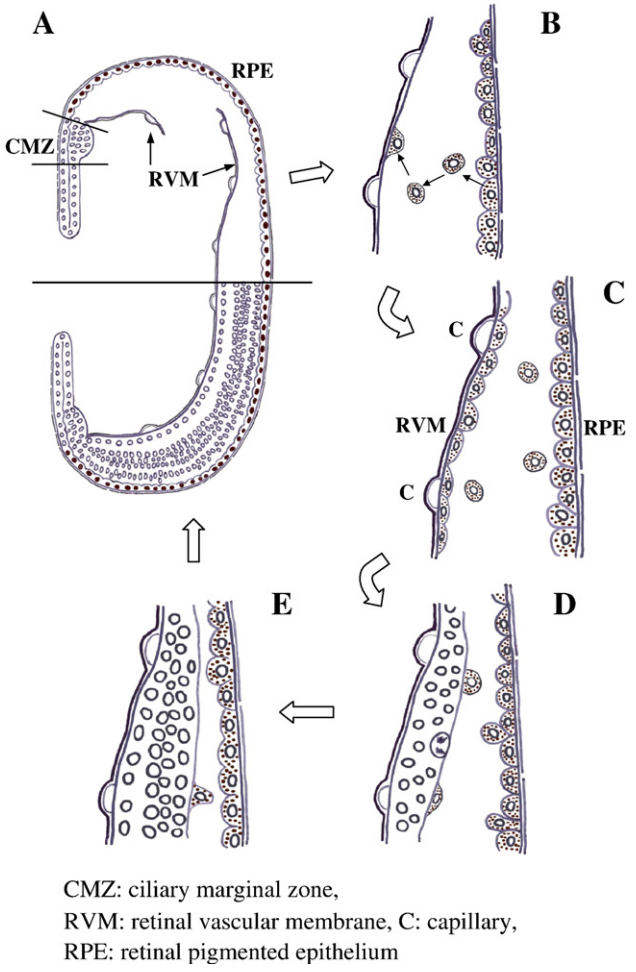


Fig. 10. Schematic diagram of retinal regeneration in *X. laevis*. The upper part in (A) shows retinectomized eye cavity, and the lower shows intact one. Cells from two origins regenerate the retina; ciliary marginal cells and the retinal pigmented epithelial cells. The CMZ (ciliary marginal zone) partially remains after retinectomy with the present surgical procedure, and CMZ stem cells initiate migration on the RVM (retinal vascular membrane) to the posterior direction. (B) At the same time, some of RPE cells leave RPE layer, migrate and attach to the RVM, where they form a new RPE layer as indicated in (C). Numerous capillaries (indicated as C) are seen in RVM. RPE cells on the RVM proliferate and transdifferentiate to neural retinal precursor cells (D, E). RPE cells that were positively stained for RPE65 are shown by brown-colored nuclei or pigmented granules in the cytoplasm.

Presently, we have no direct evidence for the cellular source of the regenerating lens structure. The iris epithelium, the dorsal region of which is involved in lens regeneration in the newt, does not appear to fulfill the same role in *X. laevis*, because it did not show any morphological change during the experimental period. The cornea also appeared to be morphologically intact during the whole period. A candidate tissue is the remaining lens epithelium included in the lens capsule. As shown in the present study, some epithelial cells were positive for the lens specific antibody as early as on Day 5 after lentectomy. These positively stained epithelial cells were arranged within a small capsule-like vesicle. Each lens capsule, with a small number of lens cells, was left in the ocular chamber with the present procedure, since the capsule is firmly attached to the iris tissue. Such remaining cells may still retain the capacity to proliferate and differentiate into lens fibers and finally to regenerate the whole lens structure. If so, this is not a transdifferentiation process but is rather considered as repair by a process of lens cell proliferation and differentiation. There are some reports in the rabbit and cat that describe lens regeneration when the anterior and posterior capsular bags are left intact in the vitreous fluid (Gwon et al., 1993). In anuran larva, lens regeneration occurs from the outer cornea epithelium, and retinal factors appear to be necessary prerequisites (Filoni et al., 1982; Schaefer et al., 1999). Our study also suggests that retinal factors are important for the present lens regeneration (or lens repair) from lens epithelial cells, because reconstruction of the lens structure was much faster and the lens was much bigger when the lens alone was removed than that found in animals which had undergone removal of both the lens and NR.

Recent progress in retinal regeneration studies have revealed a wide spectrum of regenerative potentials sequestered in vertebrate eyes including mammals (Raymond and Hitchcock, 1997; Tropepe et al., 2000; DelRio-Tsonis and Tsonis, 2003), though we are still far from a complete understanding of the molecular mechanisms involved in regeneration. Since there is a large volume of literature concerning the molecular genetics of *X. laevis*, this animal will be a useful model for the molecular analysis of the amphibian retina and lens regeneration. This, in turn, will contribute enormously to understanding the whole story of vertebrate lens and retinal regeneration.

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