Int. J. Dev. Biol. 40: 833-844 (1996)



# Mechanisms of retina regeneration in urodeles

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**ABSTRACT This review is a comparative analysis of retina regeneration in amphibians, fish, birds, and mammals. Special attention is given to the newt, which, unlike other vertebrates, retains the capacity for the regneration of eye structures during all life. The review focuses on the sources of the cells which contribute to retina regeneration, the factors which control the process, and the genes expressed during the course of regeneration.**

KEY WORDS: *retina regeneration, cell sources, signal molecules, gene expression, amphibians, chick embryos, adult fish*

## Introduction

Adult newts possess remarkable potential for eye regeneration. For example, the ability to perfectly regenerate the eye lens and the retina was discovered a long time ago. Dinsmore (1992), reviewing the history of studies on regeneration, noted that the first investigation on regeneration of eye tissues in amphibians (newts) was performed and published in 1781 by Charles Bonnet, a famous Swiss naturalist of the eighteenth century. His results were confirmed a hundred years later, when methods of experimental morphology replaced plain macroscopic observations. Studies of Philipeaux (1880), Griffini and Marcchio (1889), Colucci (1891), and Wolff (1895) demonstrated perfect regeneration of the lens and retina in newts. Specialists regard the end of the nineteenth century as the initial period of experimental morphological research on lens and retina regeneration in amphibians. A vast experimental material accumulated since that time has been repeatedly reviewed in detail (Mangold, 1931; Reyer, 1954, 1962, 1977; Stone, 1959; Lopashov and Stroeva, 1964; Yamada, 1966, 1967, 1977, 1982, 1986, 1989; Eguchi, 1979, 1986, 1988, 1993; Stroeva and Mitashov, 1983; McDevitt, 1989; Okada, 1991; Hitchcock and Raymond, 1992; Park and Hollenberg, 1993). Each of these reviews reflects contemporary trends and achievements in these studies. More recently new experimental data have been obtained. Studies on regeneration of the retina in amphibians, fish, and birds have been particularly revealing.

Eyes of all vertebrates are built from a common plan (Fig. 1). The retina of adult newts, which will receive detailed attention in this review, consists of the following layers (from the basal surface, bounded by the internal limiting membrane, to the apical surface, bounded by the external limiting membrane): the ganglion cell layer, the inner plexiform layer, the inner nuclear layer, the outer plexiform layer, the outer nuclear layer, and the layer of rods and cones (Fig. 2). Outer segments of rods and cones are in direct contact with a single layer of heavily pigmented cuboidal epithelial cells called the retinal pigment epithelium (RPE). RPE cells perform several physiological functions: they are involved in the process of vision, supply nutrients to the retina, and also phagocytize shed discs of rod and cone outer segments.

The purpose of this review is to provide a comparative analysis of retina regeneration in amphibians (Urodele and Anura), fish, birds, and mammals. Attention will be focused on the sources of cells which contribute to retina regeneration, factors controlling this process, and genes expressed during the process. Data on lens regeneration have been comprehensively discussed in a recent review by Eguchi (1993) and will be used here only as applied to the problem of mechanisms responsible for retina regeneration.

## Transdifferentiation of retinal pigment epithelium cells in vertebrate embryos

Analysis of the regeneration potential of eye tissues traditionally begins with examining peculiarities of their development, as well as changes in the regeneration capacity that accompany differentiation of eye tissues during the embryonic period. Such an approach is quite appropriate, because it provides a deep insight into the mechanisms of regeneration.

The vertebrate eye rudiment forms as an evagination (outgrowth) of the diencephalon, the posterior portion of the forebrain. At the optic vesicle stage, the rudiment consists of a single layer of nondifferentiated neuroepithelial cells. The distal portion of the eye vesicle invaginates, and the rudiment develops into the optic cup. Its inner layer (the distal portion of the optic vesicle) subsequently becomes the retina, whereas the outer layer forms the RPE. Due

*Abbreviations used in this paper:* RPE, retinal pigment epithelium; DOPA, dihydroxyphenylalanine; N-CAM, neural cell adhesion molecules; aFGF, bFGF, acidic (basic) fibroblast growth factor; GFAP, glial fibrillar acidic protein; NFP, neurofilament proteins; EGF, epithelial growth factor; RT-PCR, reverse transcriptase polymerase chain reaction.

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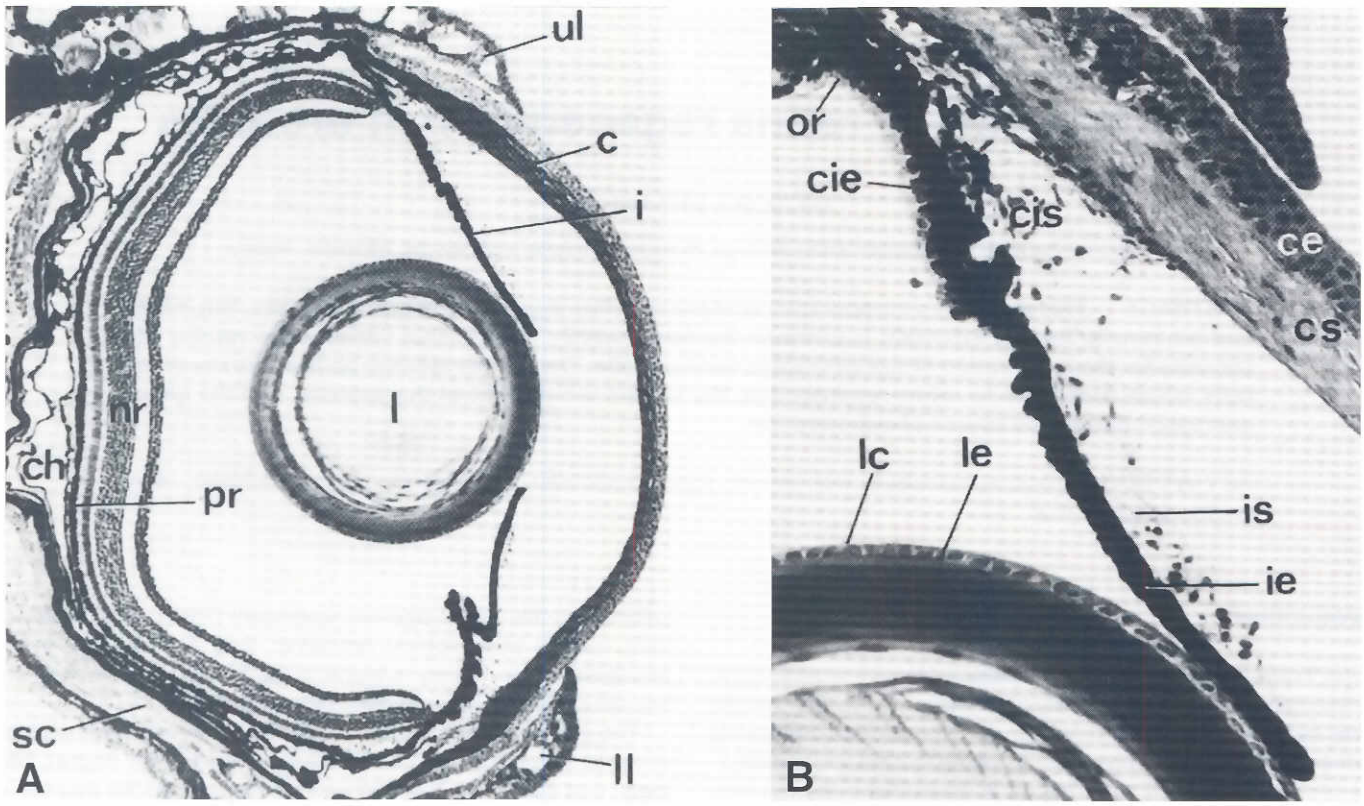


**Fig. 1. Histological preparations of the eye of the adult newt, *Notophthalmus viridescens*.** **(A)** *Vertical meridional section through the eye. c, cornea; l, lens; i, iris; nr, neural retina; pr, pigment epithelium; ch, choroid; ll, lower lid; sc, scleral; ul, upper lid.* **(B)** *Detailed representation of the dorsal iris region. cie, ciliary epithelium; le, lens epithelium; lc, lens capsule; ie, iris epithelium; cis, stroma of ciliary body; is, iris stroma; or, ora serrata; ce, corneal epithelium; cs, corneal stroma (substantia propria) (from Reyer, 1977).*

to invagination, the retina acquires inverted polarity (with respect to RPE). Intense proliferation and cell differentiation in the inner layer result in its histological stratification, whereas the outer layer persists as a simple epithelium. Thus, the retina and RPE are derived from the same rudiment. Since the retina has inverted polarity, its apical surface is represented by the external limiting membrane (which passes through the layer of rods and cones), whereas the inner limiting membrane (adjacent to the ganglion cell layer) corresponds to the basal surface of the retina. Cell divisions in the developing retina rudiment proceed at the apical surface, as the result of interkinetic migration of nuclei. This is also characteristic of the developing rudiment of the central nervous system.

At early stages of embryonic development, RPE demonstrates a remarkable plasticity: its cells respond to the inducing stimuli by transdifferentiating into retinal cells. This phenomenon was observed for vertebrates of all studied groups, including fish (Dabagyan, 1959, 1960; Sologub, 1975), newts and axolotls (Dragomirow, 1936; Detwiler and Van Dyke, 1953, 1954), frogs (Ikeda, 1937; Lopashov, 1949, 1955; Lopashov and Sologub, 1972; Reh and Nagy, 1987; Reh *et al.*, 1987), chicken (Orts-Llorca and Genis-Galvez, 1960; Coulombre and Coulombre, 1965, 1970; Tsunematsu and Coulombre, 1981; Park and Hollenberg, 1989, 1991; Pittack *et al.*, 1991; Reh *et al.*, 1991; Guillemot and Cepko, 1992), and rats (Stroeva, 1960). In frogs, axolotls, fish, birds, and mammals, this capacity is lost in the course of development, but in certain urodeles RPE retains it for life. In adult newts *(T. cristatus, T. vulgaris, T. pyrrhogaster, Pleurodeles waltl, Notophthalmus viridescens)* RPE is the primary cell source for regenerating the retina after its damage or removal. RPE cells transdifferentiate during regeneration into cells of the neuroepithelial retina rudiment. To gain an insight into mechanisms of retina regeneration in adult urodeles, it is necessary to reveal factors responsible for formation of neuroepithelial rudiment because this event is crucial for the process of regeneration. A comparative analysis of peculiarities of RPE cell transformations in frog larvae, chick embryos, mammalian fetuses, as well as in adult newts and fish will also be presented.

## What are the sources of the cells contributing to regeneration of the retina in adult newts?

Several moments in the history of studies on retina regeneration in the newt deserve special attention. For more than a hundred years of studies, views on the sources of the cells contribution to retina regeneration have been mutually contradictory. (Philipeaux, 1880; Griffini and Marcchio, 1889; Fujita, 1913; Wachs, 1920; Stone and Zaur, 1940; Stone, 1950a,b; Hasegawa, 1958, 1965; Gaze and Watson, 1968; Mitashov, 1968; Reyer, 1971, 1977; Keefe, 1973a-d; Levine, 1975, 1977; Stroeva and Mitashov, 1983; Klein *et al.*, 1990). In the absence of reliable cell markers, researchers attributed the source, more or less convincingly, to weakly differentiated precursor cells located at the peripheral growth zone of the retina or, the opposite, to differentiated RPE cells only (Fig. 3). In the mid-1970s, it appeared that this discussion was over: specialists accepted that both views are correct: the retina of adult

newts regenerates due to cell proliferation in the growth zone of the retina as well as in RPE (see Reyer, 1977; Stroeva and Mitashov, 1983). However, recent data, obtained in experiments with amphibians, fish, birds, and mammals, have provided evidence that eye growth and retina regeneration (provided the latter is possible) depend on different cell sources. Analysis of mechanisms controlling these processes is impossible without reevaluating the data on the sources of cells which contribute to retina regeneration.

Numerous experiments with [$^3$H]thymidine, retroviruses, and fluorescent dyes used as nuclear and cytoplasmic cell markers have shown that, in all vertebrates studied, the peripheral zone of the retina contains weakly differentiated precursor cells, i.e., nondifferentiated neuroblasts or germinal neuroepithelial cells (Hollyfield, 1968, 1971; Straznicky and Gaze, 1971; Johns, 1977; Beach and Jacobson, 1979; Morris and Cowan, 1984; Hunt *et al.*, 1987; Reh, 1987, 1992; Turner and Cepko, 1987; Holt *et al.*, 1988; Reh and Radtke, 1988; Wetts *et al.*, 1989; Fekete *et al.*, 1990; Turner *et al.*, 1990; Wetts and Fraser, 1988, 1991). This cell population is heterogeneous and includes uni- and multipotent cells which can differentiate into rods and cones, interneurons, and glial cells. Such precursor cells provide material for growth of the retina during embryonic and postembryonic development of the eye. The growth zone determines the potential of the vertebrate eye for growth. This potential can differ sharply in animals of different ages and , depends on the number of cells produced by this zone. In adult newts, precursor cells located at the periphery of the retina also provide for normal growth of the eye (Gaze and Watson, 1968; Svistunov and Mitashov, 1985). After the removal of the retina or its damage under the conditions of ischemia, they supply cells for regenerating only the peripheral part of the retina (Fig. 3). This fact reflects the growth potential of the peripheral zone: complete regeneration of the retina only on the basis of precursor cells of the growth zone is never, however, achieved. Conclusive data to support this proposition were obtained in experiments with *Xenopus* and axolotl (Reyer, 1977; Levine, 1981; Mitashov and Maliovanova, 1982; Svistunov and Mitashov, 1985). In these amphibians, RPE cells are not involved in retina regeneration, and cell proliferation in the growth zone leads to the formation of only a small regenerate. As a result, restoration of the retina is incomplete. Apparently, the retina of these amphibians regenerates only up to the capacity of the cell population of the growth zone. This is evidence that the number of precursor cells in this zone is sufficient for growth of the retina during individual development, but not for its complete regeneration later on. Regeneration of the central part of the retina depends on cells source in a single layer of RPE. Even if cells of the growth zone are removed, the complete restoration of the retina is possible due only to RPE cells.

The role of RPE as the principal cell source for retina regeneration was demonstrated using various experimental models, including the complete or partial surgical removal of the retina, cutting of the optic nerve and the ophthalmic artery, transplantation of RPE cells in the eye cavity of the newt, and transplantation of the whole eye. In experimental morphological studies on retina regeneration, researchers used melanin granules as a natural cytoplasmic marker for tracing the gradual transformation of RPE cells into neuroepithelial cells. During this process, RPE cells gradually lose pigment and begin to proliferate. The initial melanin content in these cells is so high that depigmentation takes a long time. When the developing neuroepithelial rudiment is two to three cell layers thick, its cells contain enough pigment for tracing their gradual

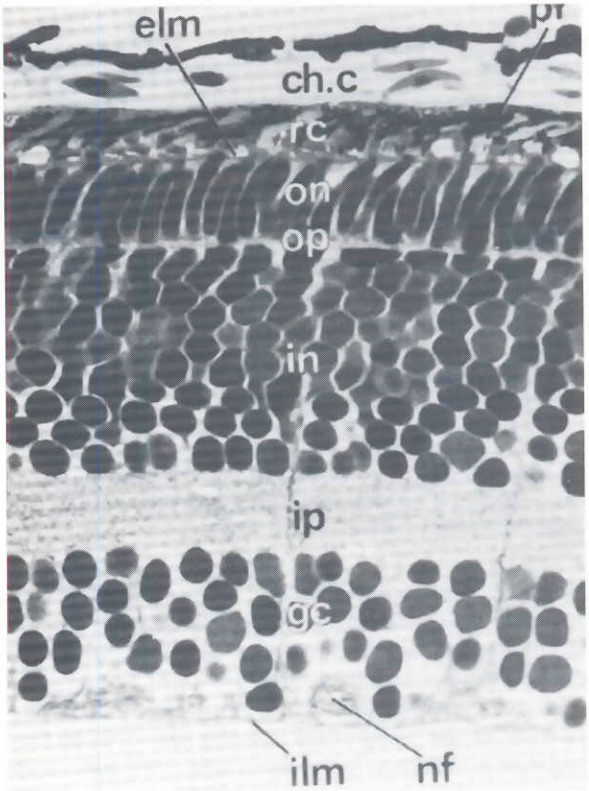


**Fig. 2. Histological preparation of neural and pigmented retina.** *ilm, internal limiting membrane; nf, nerve fiber layer; gc, ganglion cell layer; ip, inner plexiform layer; in, inner nuclear layer; op, outer plexiform layer; on, outer nuclear layer; elm, external limiting membrane; rc, layer of rods and cones; pr, pigmented retinal epithelium; ch.c, choriocapillaris (from Reyer, 1977).*

displacement from the RPE layer to the eye cavity. At this stage, histological preparations clearly show the boundary between the pigmented neuroepithelium derived from RPE (in the central part of the eyeground) and neuroepithelium formed by the growth zone, whose cells contain no pigment.

Subsequent studies provided conclusive evidence for the role of RPE in retina regeneration. Methods used in these experiments included [$^3$H]thymidine labeling of cell nuclei (Mitashov, 1968; Reyer, 1971, 1977; Keefe, 1973a,d; Stroeva and Mitashov, 1983) and labeling of the cytoplasm with monoclonal antibodies which bind specifically to RPE cells (Klein *et al.*, 1990).

The retina was removed from adult newts, proliferating RPE cells were labeled with [$^3$H] thymidine, and animals were fixed several days after surgery, during formation of the neuroepithelial rudiment. This rudiment contained labeled cells, but the label was diluted due to preceding cell divisions. Comparing the intensity of labeling in initial RPE cells and in newly formed neuroepithelial cells clearly showed that neuroepithelium was derived from RPE cells (Stroeva and Mitashov, 1970, 1983; Reyer, 1971; Keefe, 1973a,d). Experiments with the monoclonal antibody REP-1 against specific cytoplasmic antigens of RPE cells confirmed this conclusion (Klein *et al.*, 1990).

Melanin is a specific product of RPE cells, and [$^3$H]-labeled dihydroxyphenylalanine (DOPA), a specific melanin precursor, fortunately proved to be a convenient cytoplasmic marker, which allowed us to estimate the number of RPE cells forming the

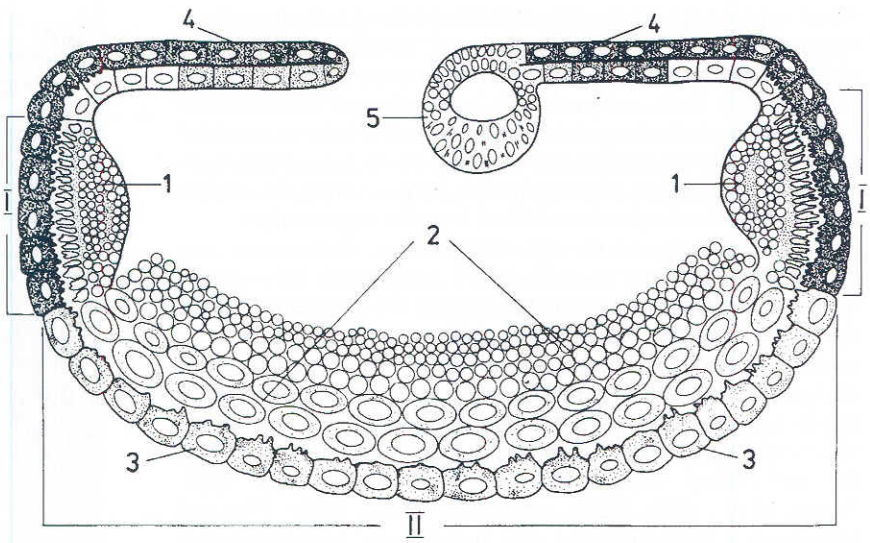


**Fig. 3. Schematic drawing of regenerating adult newt eye.** *I, peripheral zone; II, central part of the eyeground;* **(1)** *neuroepithelial rudiment formed from ora serrata;* **(2)** *neuroepithelial rudiment formed from RPE cells;* **(3)** *RPE cells;* **(4)** *iris;* **(5)** *regenerating lens. At early stages of retina regeneration, all RPE cells at the eye periphery incorporate [$^3$H]DOPA, [$^3$H]thymidine labeling index being 4-6%. During the formation of neuroepithelial rudiment, [$^3$H]DOPA is not incorporated in cells of the central part of the eyeground; [$^3$H]thymidine labeling index in RPE is 40 to 50%, whereas in the neuroepithelial rudiment it is 70 to 80%. Cells of the two-layered neuroepithelial rudiment contain melanin granules, because the rudiment is formed from RPE, but these granules are absent from the multilayered rudiment. The total duration of the cell cycle in the neuroepithelium is 1.5-2.2 times shorter than that in RPE. Expression of NFP, N-CAM, and GFAP proteins is observed in the neuroepithelial rudiment but not in RPE (from Stroeva and Mitashov, 1983).*

neuroepithelial rudiment (Mitashov, 1976, 1980). Analysis of changes in the rate of melanin synthesis during retina regeneration revealed an interesting peculiarity (Fig. 3). RPE cells involved in the formation of neuroepithelial rudiment, which are located in the central zone of the eyeground, do not incorporate [$^3$H]DOPA. That is, synthesis of the component specific for RPE in this cell subpopulation is inhibited. At the same time, RPE cells located at the periphery of the eye do not participate in retina regeneration: the small peripheral portion of the neuroepithelial rudiment develops due to proliferation of weakly differentiated cells of the growth zone (see Fig. 3). Peripheral RPE cells instead undergo partial depigmentation and immediately respond by resuming melanin synthesis. The latter is registered on autoradiographs by [$^3$H]DOPA incorporation into the cytoplasm of these cells. Data on [$^3$H]DOPA incorporation shows that 60 to 70% RPE cells do not incorporate the label. These cells, therefore, are involved in forming the neuroepithelial rudiment (Fig. 3). This proportion can vary, depending on the experimental model (e.g. removal of the retina vs. cutting of the optic nerve and the ophthalmic artery). Experiments in which RPE cells were dissected from the central zone of the eye without any admixture of retina cells and transplanted into the eye cavity of the newt also proved that all these cells participate in retina regeneration (Stone and Steinitz, 1957; Mitashov and Grigoryan, 1984).

How does the neuroepithelial retina rudiment form from RPE cells? Data obtained using different experimental approaches suggest two principal mechanisms. The first mechanism operates after surgical removal of the retina. RPE cells begin to lose pigment and proliferate. Mitotic spindles in certain proliferating RPE cells are perpendicular to the choroid. As a result, one of daughter cells is displaced into the eye cavity and becomes neuroepithelial, whereas another partially loses pigment but remains in the RPE layer. All such experiments demonstrated that the neuroepithelial rudiment develops asynchronously. In crested newts *(Triturus cristatus)*, for example, RPE cells proliferated mainly in two zones: around the optical nerve and around the annular blood vessel. In contrast, in experiments which prevent blood supply to the eye, the first step is retina degeneration accompanied by an intense and distant migration of pigmented cells from the RPE layer into the eye cavity. These cells lose pigment, proliferate, and form neuroepithelium, which gives rise to the new retina. Cells remaining in the RPE layer phagocytize components of the degenerating retina, thus functioning similarly to macrophages that migrate into the eye cavity. These cells also partially lose pigment granules and then become pigmented again, restoring the initial RPE layer (Keefe, 1973c).

The problem concerning the probable existence in the adult newt's eye of nondifferentiated cells, which provide the source for retina regeneration, has long been discussed in literature. Keefe (1973d) in his electron microscope studies discovered that, in adult crested newts *(Triturus cristatus)*, the outer nuclear layer of the retina contains small oval cells, which he classified as weakly differentiated specialized cells. Attempts to reveal the role of these cells in retina regeneration were unsuccessful, and their origin remains obscure. The structure of these cells suggests that they probably belong to cells actively migrating from the circulatory system rather than to neural cells.

The RPE cell population includes no particular subpopulation of weakly differentiated cells that could serve as a specific cell source for regeneration. An important characteristic of RPE is the macrophage-like functioning of its cells, which becomes particularly obvious in experiments which sever the ophthalmic artery. The proportions of RPE cells involved in phagocytizing components of the destroyed retina and in forming the neuroepithelial rudiment are probably determined at random.

## Retina regeneration in frog larvae

Data described above raise the question as to whether the ability of differentiated RPE cells to produce neuroepithelial cells, which restore the retina, is unique.

For a long time, retina regeneration in adult newts had been regarded as a unique phenomenon, and the ability of RPE cells to transdifferentiate remained open to question. However, several experimental models which have been recently developed using specific markers *in vivo* and *in vitro*, provided evidence that differentiated RPE cells do participate in retina regeneration in animals other than newts.

One of these models is retina regeneration in frog (*Rana catesbeiana*) larvae. As in the adult newt eye, preventing blood

supply to the larval frog retina leads to its degeneration. Subsequently, the retina regenerates due to transdifferentiation of RPE cells (Reh and Nagy, 1987). The neuroepithelial rudiment also develops similarly to that in the adult newt eye. Reh and Nagy (1987), analyzing in detail the detachment of RPE cells from Bruch's membrane and their subsequent migration into the eye cavity, found that migrating cells come into contact with the vascular membrane, which retains normal structure. The authors regarded this event as principal for the formation of neuroepithelium, because cells contacting the vascular membrane lose pigment, proliferate, and become germinal neuroepithelial cells. Cultivating RPE of *R. catesbeiana* larvae on a laminin-containing substrate, Reh *et al.* (1987) obtained its more advanced transdifferentiation into neuroepithelial cells. Since the vascular membrane has an increased laminin content, this fact was regarded as evidence that laminin induces transdifferentiation of RPE cells. The effect of laminin is realized indirectly, via stimulation of RPE cell proliferation.

Normal RPE cells do not react with antibodies 2D3 to neuroblast-specific antigens (N-CAMs). Positive staining with these antibodies was observed only in cells forming neuroepithelial rudiment. The similarity of retina regeneration in frog larvae and in adult newts is also manifested in the formation of two retina rudiments. The first rudiment develops due to cell proliferation in the ciliary growth zone, thus demonstrating the growth potential of the peripheral zone of the retina in this amphibian species. The second rudiment develops from RPE cells. Subsequently, both rudiments join into a single strand of proliferating neuroepithelial cells. These transformations of RPE cells in the larval frog eye are similar to those observed in the adult newt eye during the formation of neuroepithelial rudiment in experiments which prevent blood supply to the eye. In other frog species *(Rana esculenta, R. terrestris,* and *R. temporaria)*, RPE cells do not transdifferentiate into neuroepithelium. The retina regenerates due only to growth potential of the peripheral zone. The size of the regenerate depends on the stage of development of larvae, but complete regeneration of the retina is never observed (Dabagyan and Sheresheva, 1966; Dabagyan and Tret'yakova, 1968; Stroeva and Mitashov, 1981).

## Retina regeneration in chick embryos

Another remarkable model of retina regeneration is transformation of differentiating RPE cells into the retina in chick embryos (Orts-Llorca and Genis-Galvez, 1960; Coulombre and Coulombre, 1965, 1970; Tsunematsu and Coulombre, 1981; Park and Hollenberg, 1989, 1991; Pittack *et al.,* 1991; Reh *et al.,* 1991; Guillemot and Cepko, 1992). The main cytological processes in this model are similar to those characteristic of retina regeneration in newts and frog larvae and include cell depigmentation and proliferation and development of the neuroepithelial rudiment and its differentiation into rods and cones, interneurons, and glial cells. In chick embryos, however, RPE cells do not dissociate from the RPE cell layer to form the neuroepithelial rudiment, as they do in adult newts and frog larvae. Instead, the entire RPE begins to proliferate and produces in the eye cavity the second cell layer, which becomes the neuroepithelial rudiment.

Two peculiarities of this model attract attention. The first is that retina regeneration in chick embryos does not occur spontaneously but depends on external stimuli. In particular, the role of such stimuli is played by fibroblast growth factors (aFGF and bFGF). In experimental protocols these factors were introduced into the eye cavity after their adsorption onto a carrier, from which they gradually diffused (Park and Hollenberg, 1989, 1991), or they were simply added to the culture medium during cultivation of RPE fragments (Pittack *et al.,* 1991; Reh *et al.,* 1991; Guillemot and Cepko, 1992). In the cultivated RPE fragments, cells proliferate only at the periphery (rather than throughout the explant, as they do *in vivo*), and it is possible to trace a gradual transformation of simple RPE into a strand of stratified neuroepithelium.

Another peculiarity, which becomes most evident *in vivo*, is that the regenerating retina is inverted: rod and cone outer segments face the vitreous body rather than RPE and choroid as they do in the normal eye. Moreover, the entire RPE layer becomes transformed into stratified neuroepithelium, so that the regenerated organ contains no RPE. A logical explanation for this phenomenon, proposed by Coulombre and Coulombre (1965, 1970), is that polarity of the regenerating retina exactly corresponds to polarity of RPE cells, from which it is formed. As noted above, the retina develops from the invaginated portion of the eye rudiment and, therefore, has inverted polarity (with respect to RPE). Consequently, polarity of the RPE-derived retina, because it remains the same as in the initial tissue, becomes inverted with respect to polarity of the normal retina.

In experiments performed *in vitro*, polarity of the regenerated retina was also opposite that of the normal retina (Tsunematsu and Coulombre, 1981; Pittack *et al.,* 1991). However, high doses of aFGF administered to the eye cavity led to the formation of two oppositely oriented retinas: one inverted, devoid of RPE (in the central part of the eyeground) and one "normal," underlain by RPE fragments (at the periphery of the eye) (Park and Hollenberg, 1991). Subsequently, the role of RPE in determining polarity of regenerating retina in chick embryos received experimental confirmation (Wolburg *et al.,* 1991).

Inversion of polarity during retina regeneration via transdifferentiation of the whole RPE layer into neuroepithelium is a general trend. It was also demonstrated for mammals with colobomas, in which RPE cells around the choroid fissure proliferate and transdifferentiate into the retina (Szilly, 1924; Mann, 1957). Another example is transdifferentiation of RPE into neuroepithelium in experiments with rat eye rudiments cultivated in the anterior eye chamber of adult rats (Stroeva, 1960).

## Retina regeneration in adult fish

The adult fish retina is also capable of regeneration. However, cell sources of regeneration are different from those in newts, frog larvae, or chick embryos. The regeneration potential of eye tissues of such fish as *Carassius auratus* or *Salmo gairdneri* has been known for more than 25 years (Lombardo, 1968, 1972; Maier and Wolburg, 1979; Kurz-Isler and Wolburg, 1982). Early experiments on retina regeneration in fish after its removal or damage by cytotoxic agents revealed the role in this process of precursor cells located in the peripheral growth zone of the retina, as in other vertebrates. The same cells provide for normal growth of the retina throughout life (Johns, 1977; Johns and Easter, 1977; Meyer, 1978). Early studies also demonstrated the presence of proliferating cells within the outer nuclear layer of the differentiated retina. Their authors, in the absence of reliable cell markers, classified these cells as differentiated and concluded that differentiated cells of fish retina undergo a certain dedifferentiation and form an

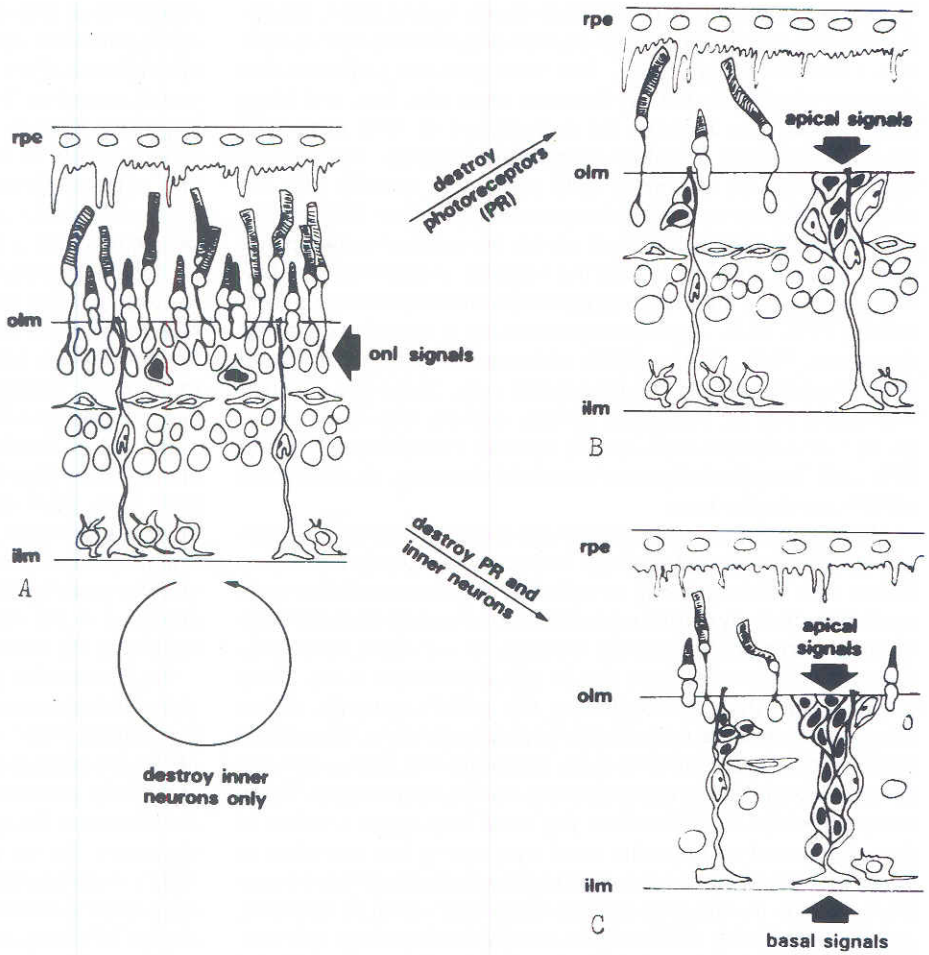


**Fig. 4. Scheme of retina regeneration in adult fish and of probable inducing signals involved in this process. (A)** *When rods are destroyed, they regenerate from rod precursor cells. The same cells produce rods during the normal growth of the retina. Signals inducing differentiation of rod precursors come from the local microenvironment (onl signals).* **(B)** *When both types of photoreceptor cells (PR) are destroyed, proliferating rod precursors form clusters at the outer limiting membrane and subsequently differentiate into both rods and cones. Inducing signals come from the outer limiting membrane and extracellular matrix of the photoreceptor cell layer.* **(C)** *When photoreceptor cells are destroyed together with neurons of the inner nuclear layer, rod precursors form clusters across the whole retina. Inducing stimuli are provided by apical and basal signals. Rod precursors and regenerating neuroepithelial cells are shown with black nuclei; nuclei of Muller cells are lobular and have prominent nucleoli. ilm, inner limiting membrane; onl, outer nuclear layer; olm, outer limiting membrane; PR, photoreceptor cells; rpe, retinal pigment epithelium (from Braisted et al., 1994). For more detailed explanations, see the text.*

additional cell source for regeneration (Kurz-Isler and Wolburg, 1982).

However, subsequent detailed studies, involving electron microscopy and autoradiography with nuclear and cytoplasmic markers, allowed identification of these cells as specialized precursors that give rise to rods during the normal development of fish retina (Sandy and Blaxter, 1980; Johns and Fernald, 1981; Johns, 1982; Raymond and Rivlin, 1987; Raymond, 1985, 1990, 1991). The fish eye grows throughout life, and these precursors replenish rods during the eye growth. It is these specialized precursor cells that provide the only source for regeneration of the outer and inner nuclear layers after the removal or damage of the retina (Fig. 4) (Raymond *et al.*, 1988; Braisted and Raymond, 1990; Raymond, 1991; Hitchcock and Raymond, 1992; Braisted *et al.*, 1994).

The use of bromodeoxyuridine and monoclonal antibodies to opsin allowed Knight and Raymond (1990) to characterize these precursors as multipotent stem cells that give rise to cones as well as to rods. Cellular events in the regenerating retina of adult fish depend on the extent of its damage. The greater the damage to the retina, the more profound its regeneration. If rods are destroyed (Fig. 4A), their precursors proliferate and differentiate into rods only. If both rods and cones are destroyed (Fig. 4B), rod precursors come into contact with the outer limiting membrane. In the normal retina, nuclei of cones are located at this membrane. Contact of intensely proliferating rod precursors with the apical surface of the retina changes their fate: as the result, these cells differentiate into cones as well as into rods. After destruction of rods, cones, and the inner nuclear layer (Fig. 4C), proliferating cells spread throughout the surface of the retina and, in this case, give rise to regenerating rods, cones, and neurons of the inner nuclear layer.

## Signal molecules controlling retina regeneration in vertebrates

Comparative analysis of retina regeneration in vertebrates shows that this process can be completed only in the presence of cells providing for regeneration itself as well as for growth of the organ. These are either RPE cells (in embryos of fish, chicken, axolotls, and mammals, and in amphibian larvae and adult newts; see Fig. 3) or specialized rod precursors (in adult fish; see Fig. 4). In both cases, the initial cells undergo gradual transformation, change their fate, and acquire characteristics of the presumptive neuroepithelium (Figs. 3 and 4). Let us consider the main regulatory events in the RPE and rod precursor populations separately and then compare them.

First, it is evident that the initial RPE cells are differentiated. However, the degree of their differentiation at embryonic stages (retina regeneration in axolotls, newts, fish, chicken, and mammals), larval stages (frogs), and in adult animals (newts) is different. This depends on factors directly controlling gene expression,

factors stabilizing the differentiated state of cells, the intensity of cell interactions and cell-substrate contacts, etc. Eguchi and his coworkers identified a glycoprotein (80-200 kDa), produced by pigment epithelium cells of the retina and iris, which is capable of maintaining the differentiated state of these tissues (Imokawa *et al.,* 1992; Eguchi, 1993). A similar glycoprotein was previously found in limb tissues of the adult newt, both in the intercellular space and on cell membranes (Imokawa and Eguchi, 1992; Imokawa *et al.,* 1992). At early stages of lens regeneration, this glycoprotein disappears from the dorsal iris. The authors suggested that the removal of the factor abolishes the differentiated state of cells, making them capable of forming the lens rudiment. Similar events, associated with the removal of stabilizing factor, were also observed at early stages of limb regeneration in adult newts (Eguchi, 1993).

The factor maintaining the differentiated cell state is produced in pigment epithelium of the developing eye during the period when the retina is already stratified (Eguchi, 1993). This fact suggests that the factor is probably absent from pigment epithelium of amphibians, fish, birds, and mammals at earlier stages of development, at which RPE cells demonstrate a high regeneration potential. It is quite likely that the absence of this factor is exactly responsible for the regeneration capacity of pigment epithelium in early embryos.

In adult newts and frog larvae, RPE cells probably lose this glycoprotein during their detachment from the cell layer and displacement into the eye cavity, where they form the neuroepithelial rudiment. Early neuroepithelial cells already produce antigens specific for the neural lineage. We used antibodies to glial fibrillar acid protein (GFAP), neurofilament proteins (NFP), and neural cell adhesion molecules (N-CAM) to determine, whether the initial RPE cells produce any antigens found in the normal differentiated retina (Mitashov *et al.,* 1993, 1995a; Markitantova *et al.,* 1995). The results obtained with normal RPE cells and cells at early stages of transdifferentiation were completely negative. Antigens specific for neural tissues appeared in the neuroepithelial rudiment only when it formed one- to two-layered depigmented retinal rudiment (Mitashov *et al.,* 1995a). The expression of antigens specific for differentiated retina cells (e.g., of opsin) was detected in the first rods appearing after proliferation of neuroepithelial cells and the formation of a multilayered retinal regenerate (Bugra *et al.,* 1992).

Similar data have also been obtained for regenerating retina of frog larvae and fish. Rod precursors in fish do not produce opsin, the protein specific for differentiated rods (Knight and Raymond, 1990). In frog larvae, the expression of antigens specific for cells of the neural lineage was also detected in depigmented cells of the developing neuroepithelial rudiment but not in the initial RPE cells (Reh and Nagy, 1987). Thus, the early depigmented retinal rudiment consists of cells with traits specific for neuroepithelium. Regeneration is the result of a long period of cell depigmentation, detachment from the RPE layer, and migration into the eye cavity. All these events are accompanied by changes in the type of cell interactions and contacts with the substrate, which is different in the choroid adjacent to RPE and in the eye cavity (Ortiz *et al.,* 1992; Mitashov *et al.,* 1993). Changes also concern the rate of cell proliferation in the RPE layer and in the neuroepithelial rudiment formed in the eye cavity (Stroeva and Mitashov, 1983; see Fig. 3). Genes encoding RPE-specific antigens are switched off, whereas other genes, responsible for synthesis of antigens specific for neuroepithelium and differentiating retina, become activated (Markitantova *et al.,* 1995; Mitashov *et al.,* 1995a).

***What factors are responsible for changes in the cell fate that lead to the formation of different neuron types, rods and cones, and glial cells during retina regeneration?***

First we consider the model of cell transdifferentiation in the chick embryo. Retina regeneration *in vivo* is achieved via the formation of neuroepithelial rudiment, like in adult newts and frog larvae. The rudiment appears due to the effect of growth factors (aFGF and bFGF) and active proliferation of the initial RPE cells. Experiments *in vitro* showed, however, that differentiated ganglion cells appear both among the progeny of proliferating RPE cells and among non-proliferating RPE cells, after their partial depigmentation, although the proportion of such cells is rather small (4-5%) (Guillemot and Cepko, 1992). Such a result suggested that growth factors stimulate differentiation into ganglion cells (which are the first to differentiate during both normal development and regeneration of the retina) directly in RPE cells or in their progeny after proliferation. Moreover, RPE cells of chick and mammalian embryos were shown to contain transcripts of genes encoding FGF receptors (Noji *et al.,* 1990; Bost *et al.,* 1992; Jacquemin *et al.,* 1993; Tcheng *et al.,* 1994; Torriglia *et al.,* 1994).

These data suggest two alternative processes leading to transdifferentiation of RPE cells during retina regeneration: either activation of growth factors preexistent in RPE cells or their diffusion into these cells from outside (Guillemot and Cepko, 1992). Unfortunately, mechanisms responsible for the effect of growth factors as morphogens determining cell differentiation along a certain lineage remain virtually unknown. The only data available concern specific binding of FGFs with the inner limiting membrane of the retina, Bruch's membrane adjacent to RPE, RPE cells, and inner and outer plexiform layers (Jeanny *et al.,* 1987; Schweigerer *et al.,* 1987; Cirillo *et al.,* 1990).

***What is the situation with RPE of adult newts, in which retina regenerates perfectly?***

We analyzed early stages of retina regeneration (before neuroepithelial rudiment formation) in immunochemical experiments with antibodies to bFGF and observed an intense positive reaction in the RPE, the ciliary zone of the iris, macrophages entering the eye cavity from circulation, the intercellular matrix, and the choroid (Mitashov *et al.,* 1993; Markitantova *et al.,* 1995). Thus, we demonstrated the presence of bFGF in RPE and also in the cells migrating into the eye cavity. Circulating macrophages also showed a strong positive reaction for tenascin, the glycoprotein with multiple EGF-specific repeats that have an effect on cell proliferation. An intense expression of bFGF molecules in RPE cells and, subsequently, also in the retina rudiment and macrophages suggests that in newts, as in chick embryos, growth factors act as morphogenetic substances determining changes in the fate of RPE cells. Since macrophages also contain growth factors, their interaction with cells of RPE and the retina rudiment can probably result in the additional secretion of growth factors and other substances promoting RPE conversion into neuroepithelium. Secretory cells of the ciliary body may provide another source of factors with biological activity. RPE cells also produce factors stimulating neural cell differentiation (Tombran-Tink *et al.,* 1991, 1992; Steele *et al.,* 1992). These data are evidence that RPE can

contain intracellular factors responsible for cell differentiation along the neural lineage, which become activated in the neuroepithelial rudiment.

Experimental data obtained thus far suggest the following scheme of events leading to RPE transdifferentiation into cells of the neuroepithelial rudiment followed by the formation of differentiated retina (summarized in Fig. 5).

The key stage in retina regeneration is the formation of neuroepithelial rudiment (rNE), an intermediary structure. The initial RPE cells change their differentiation pathway after receiving a morphogenetic signal conveyed by FGFs and as yet unidentified intra- and extracellular biologically active factors (bAFs). Intense proliferation of rNE cells leads to the formation of multilayered neuroepithelium (mNE). Substances stimulating their proliferation probably also belong to growth factors (GFs). Cells of the ganglion, inner and outer nuclear layers and glial cells differentiate under the effect of factors produced as the result of cell interactions (cell-to-cell signals, CCS). This leads to the formation of differentiated neural retina (dNR).

Experiments confirmed that this scheme reflects the actual sequence of events during retina regeneration in adult newts and also in fish, amphibians, birds, and mammals at early stages of development.

In adult fish, the putative inducing signals responsible for retina regeneration depend on the severity of tissue damage. If the latter is limited to rods only, signals like mitogens and differentiation factors come from the local microenvironment (Fig. 4A). If both rods and cones are damaged, these signals come from the intercellular space and the outer limiting membrane (Fig. 4B). If rods and cones are destroyed together with neurons of the inner nuclear layer, the inducing signals are produced on both apical and basal surfaces of the retina (Fig. 4C). The nature of these signals is as yet unknown, but recent experimental data suggest that they may be growth factors (Negishi and Shinagawa, 1993).

Although retina regeneration in these two models is based on different cell sources (RPE cells and rod precursors), cell transformations associated with the shift to a new differentiation pathway are quite similar in both cases. One more fact, relevant for comparative characterization of the role of growth factors in determining cell fate, is that transdifferentiation of chick embryonic RPE cells into lens cells during cultivation *in vitro* also proceeds under the influence of growth factors (Hyuga *et al.,* 1993).

The final question concerns the role of the growth zone in retina regeneration. In adult newts and fish, as already noted, it depends on the growth potential of precursor cells located in this zone. Proliferation of these cells is controlled by diffusible growth factors found in the extracellular matrix and RPE (Lillien and Cepko, 1992).

## Gene expression during retina regeneration

One more way to identify morphogenetic factors involved in regeneration is to analyze gene expression during this process. Studies in this field are at the initial stage. First, it is necessary to develop approaches to recognizing genes activated in the course of retina regeneration.

Considerable progress in the analysis of gene expression during embryogenesis was achieved in experiments with *Drosophila melanogaster.* Numerous studies provided for identification of several gene families responsible for different stages of embryonic development. Success in this research depended largely

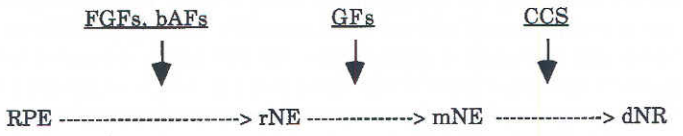


**Fig. 5. A scheme of main cellular events during transdifferentiation of RPE**. *For more detailed explanation, see the text.*

on the existence of many mutant *Drosophila* lines characterized in detail, including lines carrying mutations with the maternal effect and mutations specifically disturbing early embryogenesis (Lewis, 1978; Kaufman, 1983; Gehring, 1987).

During recent years, an increasing number of researchers have addressed the problem of gene expression during the normal eye development. Success in this field, as in studies on the genetic control of individual development, is associated with mutations that allow identification of homeobox genes with known regulatory functions (Matsuo, 1993; Beebe, 1994; Dorn *et al.,* 1994; Quiring *et al.,* 1994; Tremblay and Gruss, 1994; Zuker, 1994; Heberlein *et al.,* 1995). The research team headed by W. Gehring obtained spectacular results concerning ectopic eye development in *Drosophila* wings, limbs, and antennae. Their data provided conclusive evidence for the existence of master genes controlling a cascade of genes responsible for eye morphogenesis (Barinaga, 1995; Halder *et al.,* 1995). Such a function is attributed to *Drosophila* gene *ey (eyeless)* and its homologs *Sey (Small eye* or *Pax-6)* in mice and *Aniridia* in humans.

Thus, genes controlling morphogenesis of such a complex organ as the eye were identified; but does this mean the existence of corresponding genes for regeneration of eye structures?

Because mutations affecting regenerative processes have not been revealed, it is apparent that research on genetic mechanisms of regeneration should involve the development of alternative methods (not based on mutation analysis) for identifying corresponding control genes. At present, we can distinguish two main approaches to identification of genes expressed during regeneration. One of them involves producing a library of gene sequences expressed in regenerating structures and its subsequent screening with the use of known *Drosophila* or vertebrate probes. In experiments on amphibian limb regeneration, this approach allowed the researchers to trace the expression of homeobox genes (Savard *et al.,* 1988; Brockes, 1989, 1992; Tabin, 1989; Beauchemin and Savard, 1992; Simon and Tabin, 1993; Beauchemin *et al.,* 1994) and genes encoding keratin (Ferretti *et al.,* 1991), retinoic acid receptors (Ragsdale *et al.,* 1989), tenascin (Onda *et al.,* 1991), and FGF receptors (Poulin *et al.,* 1993).

Screening of genomic libraries of regenerating organs with certain probes sometimes led to identification of genes belonging to previously known families. Analysis of their expression at different stages of regeneration has not yet provided enough data for discussing one of the principal problems of regeneration: *expression of which genes is responsible for triggering regeneration of a certain organ (tissue) or, in other words, do particular "regeneration-specific" genes really exist?*

Key events in the regeneration of an organ can be under the control of a specific set of genes that have no immediate homologues in representatives of other taxonomic groups. Identification of such genes is impossible without using specific methods for revealing differential gene expression. A promising method is

subtractive hybridization of cDNA sequences specific for cells of different populations (Duguid and Dinauer, 1990; Timblin *et al.*, 1990) or tissue types (Belyavsky *et al.*, 1989). Its various modifications involve "subtraction" of expressed sequences that are common for both samples and subsequent cloning (or the use as a probe) of the cDNA fraction that is rich in sequences specific for only one sample and, hence, unaffected by subtraction (Luk'yanov *et al.*, 1994).

The regenerating newt eye is one of those rare models perfectly suitable for such an analysis (Mitashov *et al.*, 1992, 1995b,c,d; Kazanskaya *et al.*, 1995). Comparing gene expression in RPE, retina, and retina regenerates is an obvious way to identify genes specifically expressed in each structure. However, our first experiments with this method were performed using a simpler model of lens regeneration (Mitashov *et al.*, 1994, 1995b; Kazanskaya *et al.*, 1995). An important point is that one of analyzed cDNA fragments was expressed in the early neuroepithelial rudiment. Remember also that, in adult newts, the lens regenerates from the dorsal but not from the ventral part of the iris.

Total RNAs, isolated from the dorsal and ventral parts of the adult newt iris on day 14 after lens removal, were used for synthesizing "dorsal" and "ventral" cDNAs. Their subtractive hybridization allowed us to obtain two amplified cDNA samples enriched with sequences specific for respective zones of the iris. These cDNAs were cloned in pTZ19R vector. Each of two resulting libraries contained approximately 400 clones, 96% of which were recombinant. Screening the libraries with the use of "dorsal" and "ventral" cDNA samples as probes revealed ten clones predominantly hybridizing with the dorsal (four clones) or ventral probe (six clones). For further analysis, we selected only two clones, one from the dorsal and one from the ventral group. Structural characterization of clones showed that they carry insertions (459 and 306 bp, respectively) with sequences from the 3' regions of cDNA of corresponding genes. Sequence analysis of insertions was performed according to Sanger (see Maniatis *et al.*, 1982). Comparison of their sequences with those included in the Gene Bank database did not reveal any cases of significant homology. We designated the studied sequences as LeR1 (from Lens Regeneration) and VeR1 (from Ventral iris during Regeneration).

Expression of these genes during regeneration was studied by analyzing samples of different eye tissues in RT-PCR with *LeR1*- and *Ver1*-specific primers. *LeR1* expression was also revealed in the regenerating retina, at the stage when the neuroepithelial rudiment consists of two to three cell layers. Blotting of newt and Rana temporaria genomic DNA samples with *LeR1* DNA probe, performed using the standard procedure (Maniatis *et al.*, 1982), revealed the presence in both samples of identical 600 and 800bp bands. Such a result is evidence for evolutionary conservatism of the *LeR1* sequence. Thus, analysis of clones resulting from subtractive hybridization showed that at least two clones contain sequences of genes specifically activated in the course of regeneration. Note also that identified genes become activated at early stages of retina regeneration in cells of the neuroepithelial rudiment.

*What is the function of these genes?* Is their activation associated with the expression of neural and glial proteins (NFP, N-CAM, and GFAP) in neuroepithelial cells, which we identified in our experiments? Unfortunately, these questions still remain unanswered. Continuing our studies in this field, we have analyzed LeR1 and VeR1 expression during the early development of the newt. In the near future, we plan to obtain full-size cDNA copies of these genes and to further analyze their functioning.

*Acknowledgments*

*Our original research reviewed in this paper has been supported by the Russian Foundation for Fundamental Research (grant 93-04-07376 and 96-04-48446). I am grateful to Ms. Susan Duhon and Professor G.M. Malacinski for their help in preparing this manuscript for publication.*

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