A comparative study of amphibian retinal regeneration by tissue culture technology

Masasuke Araki
Developmental Neurobiology Laboratory, Department of Biological Sciences
Nara Women’s University, Nara 630-8506, Japan

Summary
Amphibian retinal regeneration has been intensely studied, using the urodele as a model organism. Transdifferentiation of retinal pigment epithelium (RPE) into retinal stem cells play a crucial role in regeneration. Recently, it was found that anurans (Xenopus laevis) in the adult stage also can regenerate the retina similarly to the newt. This provides a new tool of a model for the molecular mechanisms involved in retinal regeneration. A precise analysis of the initial cell dynamics in vivo and under culture conditions indicates that the interaction of RPE cells with extracellular cues, such

Correspondence/Reprint request: Dr. Masasuke Araki, Developmental Neurobiology Laboratory, Department of Biological Sciences, Nara Women’s University, Nara 630-8506, Japan. E-mail: masaaraki@cc.nara-wu.ac.jp
as the choroid tissue and basement membranes, has an important role in the transdifferentiation of RPE. Further development of culture methods now gives a system in which the RPE monolayer can regenerate into a well-organized structure consisting of all layers of the retina, providing an excellent culture model for retinal regeneration.

**Introduction**

Urodelian amphibians possess a prominent capacity to regenerate organs when they are partially damaged or are lost. Anurans also have this ability but only in their embryonic stages. The ocular tissues such as lens, cornea, and retina have been experimental materials most intensively examined in regeneration studies since the initial observations by biologists in the 19th century (Philipeaux, 1880; Colucci, 1891; Wolff, 1895). In recent years many different animal species from fish to mammals have been studied to search for their regeneration potential. Numerous review articles have repeatedly described details of the phenomena (Eguchi, 1979; Stroeva and Mitashov, 1983; Okada, 1991; Hitchcock and Raymond, 1992; Park and Hollenberg, 1993; Mitashov, 1997; Reh and Levine, 1998; Raymond and Hitchcock, 1997) and in recent years more information has accumulated on the molecular bases of retinal regeneration (Del Rio-Tsonis and Tsonis, 2003; Hitchcock et al., 2004; Araki, 2007a).

The vertebrate eyes are planned on a common developmental progression and built also in a common structural arrangement. Similar molecular mechanisms in part also underlie the development and regeneration of the vertebrate retina (Araki, 2007b). Vertebrate retinas basically have the same physiological functions, and researchers have been utilizing a variety of animal species for investigation. Retinal regeneration, however, seems to have diverse aspects depending on species; in some animals like urodeles a complete retina regenerates after surgical removal of the whole retina, but most other animals are unable to undergo such a complete retinal regeneration except during embryonic stages in certain species. In some species like fish or avians, the retina can be repaired by intraretinal stem cells after it is partially injured. More recently, evidence has been found for the existence of retinal stem cells in mammals, including humans. Various culture technologies are excellent approaches for the regeneration studies, because we are able to compare results from different animal models under the artificially organized conditions. Such comparative studies will bring us a basic understanding on the cellular and molecular mechanisms involved in vertebrate retina regeneration. The purpose of this review is to provide a comparative analysis of retina regeneration in urodele and anuran under the culture condition as well as in vivo circumstances so as to obtain a common view on vertebrate retina regeneration. We particularly want to focus on transdifferentiation of retinal pigment epithelium (RPE).
Regeneration of vertebrate retina

Retinal regeneration can take place to a certain extent in some vertebrates, depending on animal species and in a perfect manner only in the urodele amphibian. These include fish, amphibians, birds and mammals, although regeneration is restricted to certain stages of development in most animals (Del Rio-Tsonis and Tsonis, 2003; Reh and Fischer, 2001; Okada, 2004). Partial retina regeneration has been described in many adult animal species, such as fish, amphibians and birds; cells sequestered in the retinal tissue including rod precursors or Müller glial cells are the sources of regenerating retina in addition to retinal stem cells or precursor cells in the ciliary marginal zone (CMZ) (Reh and Levine, 1998; Fischer and Reh, 2002). In teleost fish such as goldfish, for instance, retinal regeneration occurs either after surgical removal of a portion of the central part of the retina or pharmacological lesioning by ouabain that destroys the neural retina but leaves rod cells intact (Hitchcock and Raymond, 1992). The rod precursors, in addition to the stem-like cells located within the retina (in the inner nuclear layer), appear to be the origin of retinal regeneration.

Urodelian amphibians like the newt are unique animals that possess the ability even in the adulthood to regenerate the whole retina after its complete removal. No other animals had been shown to have this ability until recently when Yoshii et al. (2007) reported that anuran amphibian, *Xenopus laevis*, have a similar ability at the post-metamorphosis stage. Previously, it was reported that retinal regeneration occurs only in embryonic stages of anuran amphibians and in embryonic avian embryos after whole retinal removal. In the latter case, however, exogenous administration of cell growth factor (FGF2) is necessary (Park and Hollenberg, 1989; 1993). In amphibian retina regeneration at the mature stages, the cellular sources are the RPE cells that subsequently undergo differentiate into retinal progenitor cells. Stem cells in the CMZ that contribute to the ongoing growth of the retina also participate in retinal regeneration of these animals, particularly in anurans (Hitchcock et al., 2004).

Mammals also possess cells that can proliferate and differentiate into various types of retinal cells under certain conditions of cell cultures; these are iris epithelial cells or ciliary pigmented epithelial cells. Such findings open the possibility of clinically oriented autologous grafts for retinal regeneration (Ahmad et al., 1999; Haruta et al., 2001), although it appears impossible, at the moment, to restore the large part of damaged retinal tissue in mature mammals. Details on this topic of mammalian retina regeneration will be mentioned in another chapter of this book.

It is apparent that retinal regeneration takes place by means of several different mechanisms and from cell sources depending on animal species. Retina can be replaced or repaired by RPE cells via a transdifferentiation process or by stem-like cells in the CMZ and/or within the retina. Different
animals use different strategies, and regeneration also depends on the age of the animal, although potent cells are retained in a wide variety of animals. In the following part of this article, we will focus the transdifferentiation process of RPE cells that are the main source of retinal regeneration in amphibians such as the newt, *Cynops pyrrhogaster* and African toad frog, *Xenopus laevis*. Retina regeneration in these animals can take place through transdifferentiation of RPE cells but seemingly involve different cellular steps in various species. We will first discuss the regeneration processes of amphibian retinas briefly and then describe some experimental approaches including tissue cultures, used by the author’s group.

**Amphibian retinal regeneration**

Amphibians, both urodels and anurans, have long been the animals most extensively studied for retinal regeneration with over a century (Fig. 1; Fig. 2) (see reviews by Okada, 1991; Mitashov, 1997; Hitchcock et al., 2004). Numerous studies on the newt retina regeneration have revealed that RPE cells proliferate and differentiate to regenerate the retina following its surgical removal (Hasegawa, 1958; Keefe, 1973a; 1973b; Levine, 1974, 1975; Reyer 1971; 1977; Stone, 1950a, 1950b). It is apparent that intracellular interaction and distribution of cytoskeletons in RPE cells are largely affected during retina regeneration (Fig. 1B, C) (Chiba and Saito, 2000; Umino and Saito, 2002), but these are not yet well investigated, particularly at the initial stage of regeneration. In spite of many morphological studies, including electron microscopic observations and immunocytochemical staining, there have been virtually no reports on the morphologic changes of RPE at the initial step. Since upregulation of certain genes in RPE cells is considered to take place right after retinectomy, more intensive studies are needed to examine the initial changes of RPE cells at the molecular level. It is of interest to mention that RPE cells become labeled almost simultaneously with BrdU at an interval of 4-5 days after retinectomy (Ikegami et al., 2002). Once they initiate cell division, RPE cells produce retinal stem cells. Therefore, the initial period of 4-5 days is a sort of ‘latent period’, in which several genes must be activated for the sequential events of regeneration. After that period expression patterns of several genes have been described such as Pax6 and Notch (Kaneko et al., 1999; 2001). An attempt to find early-activated genes has been done and several candidate genes have been described (Goto et al., 2006), although their functions in retinal regeneration have not been determined.

Complete retinal regeneration in adult animals following surgical removal of whole retinal tissue has been considered to take place only in certain urodele amphibians. In contrast, it was considered that anuran amphibian species retain the ability only in the larval stages and that during metamorphic stage, they
Newt retina regeneration

Figure 1. Newt retinal regeneration. Newt retinal regeneration at different stages after the surgical removal of the retina is shown in the light micrographs from A to F. The retinal pigment epithelial (RPE) cells appear to be irregularly arranged at day 5 (yellow arrow in B) which then become more epithelial at day 10 (arrow in C). At the same time, RPE cells become de-pigmented. Then, a multi-stratified epithelium is formed, separating a pigmented single epithelium layer at the basal side (arrow in E). In the neuroepithelial layer cells are randomly arranged. About one month later, a well stratified retinal tissue and a RPE have developed (arrow in F). Hematoxylin and eosin staining. (A, B) Day 5, (C) Day 10, (D, E) Day 14, (F) Day 28. (from Araki, 2007).

quickly lose the ability to regenerate the retina, only retaining the ability to repair partially-damaged retinal tissue (Lombardo, 1969). We recently showed that *X. laevis* retain the ability to regenerate the retina even in mature animals following the surgical removal (Fig. 2) (Yoshii et al, 2007). This finding leads us to examine further the possibility that regeneration potency can be potentiated more widely in other higher vertebrates.

In spite of a volume of descriptive studies on urodele RPE transdifferentiation, we still have very little information on the cellular and molecular mechanisms involved in amphibian retina regeneration (Eguchi, 2004; Okada, 2004). The finding of complete retinal regeneration in two different amphibian species provides a new tool for the molecular analysis of regulatory mechanisms. At the same time, *X. laevis* is a suitable animal for transgenic analysis and this may enable us to search for genes and their regulative expression responsible for retinal regeneration.
Xenopus retinal regeneration

Figure 2. Xenopus retinal regeneration. Retinal regeneration of adult *X. laevis* is shown at different stages after the surgical removal of the retina. (A, B) An early stage of retinal regeneration at day 7 after retinectomy. Arrows indicate retinal vasular membranes (RVM). The area indicated by asterisk in (A) is shown in (B) at a higher magnification. In (B), a new flat epithelial layer consisting of pigmented epithelial cells is juxtaposed with the retinal pigmented epithelium (RPE) (yellow arrow). Blue arrow indicates single pigmented cell located between the RPE and RVM. (C, D) Retinal regeneration at day 20. By this time, the laminar structure of the regenerating retina is partially developed at the periphery (arrow in C). The area indicated by asterisk is shown in (D) at a higher magnification. (D) 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-color arrowheads indicate pigmented cells attached to the retinal layer. (from Yoshii et al., 2007).

RPE cell dynamics at the initial phase of transdifferentiation

To begin with this part, the author wants to tell the reason that we started to study *X. laevis* retina regeneration and finally came to find that *X. laevis* can regenerate the retina after complete retina removal.
Numerous review articles have indicated that anurans were considered not to be able to regenerate the retina. Only at the larval stage was it believed to be possible in certain species (Stroeva and Mitashov, 1983; Reh and Nagy, 1987). As mentioned below, we had been working on the newt RPE using tissue culture techniques and found that RPE cells do proliferate and differentiate into neuronal cells under certain culture conditions (Ikegami et al., 2002; Mitsuda et al., 2005). While we were working on the newt RPE, we considered that the tissue culture technique could be used with *X. laevis* RPE to test whether they can transdifferentiate into neural cells or not. The results showed, as shown below, that *X. laevis* RPE cells (3 to 4 months after metamorphosis) behave quite similarly to the newt RPE *in vitro*, suggesting that mature *X. laevis* retain an ability to regenerate the retina. If *X. laevis* can not regenerate retina in vivo, this might indicate that *X. laevis* RPE cells manifest their ability of transdifferentiation only under culture conditions.

In our initial attempts to study retinal regeneration in *X. laevis*, we found that immature retina was occasionally observed in retinectomized eyes at 30 days after operation and we finally found that retina can regenerate if the retinal vascular membrane is left in the posterior eye chamber (Fig. 2). In our experiments, 120 animals in total, between 3 and 9 months after metamorphosis, were surgically operated on to remove both the lens and retina (Yoshii et al., 2007). At around post-operative day 30 (PD 30), both the 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 also regenerated. No profound difference was observed in the regeneration process, regardless of the animal stages. In the rest of the animals (20 out of 65), neither the lens nor NR could be seen, and the vitreous cavity was usually occluded by incision closure in these unsuccessful cases. Shortly after retinectomy (PD7-10), a pigmented cell layer became apparent in the vitreous chamber, facing the original RPE layer (Fig. 2B). As a result, two pigmented epithelial layers were observed, separated from each other, and facing each other. During the subsequent period, this newly formed epithelial layer (inner layer) appeared to undergo transdifferentiation into the neural retina. Cells in the two pigmented epithelial layers are positively stained for both RPE65, a specific marker of RPE, and Pax6, indicating that the pigmented cells in the inner layer do not originate from the iris pigmented epithelial cells but from RPE cells (Yoshii et al., 2007). RPE cells in the newly formed layer actively proliferate as shown by BrdU uptake and intense staining for PCNA, a cell proliferation marker. How could RPE cells form the new epithelial layer?

When the retina of *Rana* tadpoles is devascularized by complete removal of the eyeball and restoring it into the orbit, new retina regenerates following
Figure 3a. 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-color nuclei or pigmented granules in the cytoplasm. (from Yoshii et al., 2006)

Figure 3b. RPE transdifferentiation in the newt. In the newt retina regeneration, RPE cells become more loosely adhered each other soon after the retinectomy. At about day 5 cells initiate proliferation and become de-pigmented and show more well packed epithelial structure. RPE cells do not leave the epithelium and within the epithelium, cells at the most basal side (attaching to the Bruch’s membrane) become pigmented. More apically located cells now form a stratified epithelial structure. IML: inner limiting membrane. (from Araki, 2007).
retina degeneration (Reh and Nagy, 1987). In that regeneration, it was observed that RPE cells detach from the epithelium, phagocyte degenerating retinal cells during migration and finally re-attach to the retinal vascular membrane. A partial retinal regeneration was reported in adult *Rana* when a radial incision or partial retinal removal was made (Lombardo, 1969). These studies indicate that some anurans retain the ability to regenerate the retina in oculo under certain conditions.

Fig. 3 summarizes the results of *X. laevis* retinal regeneration. During regeneration processes, the RPE layer always remains pigmented (that is, RPE cells do not undergo depigmentation) and the newly formed epithelium (the regenerating NR) emerges on the remaining retinal vascular membrane. Epithelial cells constituting this layer are considered to originate from RPE cells and partly from cells in the CMZ. Numerous isolated pigmented cells are found in the vitreous space between the inner epithelial layer and the RPE, seemingly having detached from the RPE layer and migrated to the inner layer. RPE cells anchored by the retinal vascular membrane initiate cell proliferation and transdifferentiate into retinal cells. They finally regenerate the whole retina structures. It is particularly interesting to draw attention to the fact that no retinal regeneration occurs at the original RPE layer. It appears that for retinal regeneration RPE cells must detach from the RPE layer, be deprived of cell-cell interaction by migrating alone and must attach to the retinal vascular membrane.

A retinal vascular membrane (RVM) consisting of the inner limiting membrane (basement membrane) and capillaries is considered to supply an anchoring base for migrating RPE cells, because when the RVM was intentionally removed, no pigmented layer was formed, resulting in no regeneration. The RVM and Bruch’s membrane are both positively stained for laminin. In contrast to these observations in anurans, the newt RPE cells always transdifferentiate within the epithelium, as if they remained firmly fixed to each other or to the basement membrane. As the cells in the epithelium undergo transdifferentiation, they become depigmented and proliferate. It is possible that asymmetrical cell division gives rise to two daughter cells, one adhering to the basement membrane and the other free from the membrane. The latter cell type then develops into retinal stem cells to regenerate the retinal structure. An important question remains to be solved in the anuran case why RPE cells at the original RPE layer do not transdifferentiate in situ; instead they only do so after migrating to the RVM. Some ECM components, characteristic to RVM, appear to be needed for RPE cell transdifferentiation and formation of retinal stratified structure. For the moment, we do not know about the molecular nature characteristic of the RVM. Alternatively, any molecular component in the anuran RPE basement membrane may maintain the properties characteristic of RPE cells.
In vitro approach to RPE transdifferentiation

Numerous culture techniques have been utilized in amphibian RPE to elucidate the cellular and molecular mechanism involved in transdifferentiation. Intraocular implantation (vitreous chamber culture of tissues) was applied to ocular tissues such as the iris pigmented epithelium and RPE and it has been shown that pigmented epithelium from tadpoles of *X. laevis* and *Rana temporaria* transdifferentiate to the retina (Lopashov and Sologub, 1972; Bosco, 1988; Arresta et al., 2005). It is also reported that these tissues from adult anurans can transdifferentiate into the retina under a certain condition (Sologub, 1977; Bosco, 1988). Although vitreous chamber culture is suitable to ensure retinal histogenesis (formation of sublayers), artificial culture methods are more widely used for the cell and molecular analysis of RPE transdifferentiation (Mitsuda et al., 2005). Reh and his colleagues developed cell and tissue culture methods for anuran RPE and obtained evidence that extracellular matrix molecules like laminin and heparan sulfate proteoglycans play a crucial role in larval *Rana* transdifferentiation (Reh et al., 1987; Nagy and Reh, 1994). RPE cells from the newt were also cultured after tissues were dissociated, and voltage-gated inward currents were observed with time in culture (Sakai and Saito, 1997). Under dissociate culture condition, however, newt RPE cells do not manifest neuronal morphology. This was also the case in RPE cells from chick embryos; bFGF fails to induce neuronal transdifferentiation when RPE is cultured as dissociated cells (Pittack et al., 1991). These results indicate that cell-cell communication in RPE cells are a pre-requisite for transdifferentiation.

While we were searching for the key substances involved in retinal regeneration in the newt, we utilized a tissue culture method originally developed for tissue culture of embryonic chick RPE (Araki et al., 1998; 2002); RPE sheets were isolated and cultured on culture filter membrane without dissociation. This method enabled us to analyze the cellular mechanisms involved in transdifferentiation in the Silver homozygote mutant of the quail under culture conditions. Intraocular administration of FGF2 into retinectomized chicken eye chambers induced the remaining RPE to transdifferentiate into retina at an early stage of development (Coulombre and Coulombre, 1970; Park and Hollenberg, 1989). In the Silver homozygote, RPE cells autonomously transdifferentiate to neural retina during embryonic development, resulting in a double NR at the posterior region of the eye (one original and the other newly formed retinal layer from RPE). The RPE transdifferentiation begins at about 4 days of incubation and occurs only at the posterior central region of the eye (Araki et al., 1998 and 2002; Mochii et al., 1998a and 1998b). We were interested to learn whether tissue interaction between RPE and the neighboring choroid tissue has any role in transdifferentiation.
With our culture method, tissues consisting of RPE and choroid cells were cultured and the results were compared with that obtained by single cultures of isolated RPE. It was shown that RPE with the choroid tissue transdifferentiate to retina, while isolated RPE did not (Araki et al., 2002), suggesting that the mesenchymal tissues play a crucial role in RPE transdifferentiation. Similar tissue interaction also appears to play an important role in the amphibian transdifferentiation.

**Tissue culture study of RPE from different amphibian species enables us to compare their transdifferentiation ability under the same condition**

The newt RPE was cultured under the artificial culture condition described above, and it was found that RPE cells transdifferentiate into various retinal cells including photoreceptors when RPE and the choroid were cultured together (Ikegami et al., 2002). When RPE was removed from the choroid by a treatment with dispase and was cultured alone, RPE cells neither proliferated nor differentiated into neural cells and remained epithelial (Fig. 4). The RPE sheets were then co-cultured with individual choroids by separating these two tissues by a membrane filter. The results showed that RPE cells became depigmented and proliferated, suggesting that some diffusible substances from the choroid are involved in RPE-choroid interaction (Mitsuda et al., 2005). Among various growth-promoting factors examined, FGF2 had a drastic effect on RPE cell proliferation and neuronal differentiation. Addition of FGF signaling pathway inhibitors like SU5402 or U0126 suppressed RPE proliferation and neuronal differentiation. These results indicated that the choroid plays an essential role in newt retinal regeneration. FGF2 appears to induce some neurogenic genes including Pax6 upregulated in RPE cells. It is still unknown whether FGF2 gene is upregulated in the choroid or not when the retina is removed. Our preliminary search for FGF2 protein by specific antibody against newt FGF2 showed that numerous cells in the choroid become immunoreactive (data not shown).

As mentioned above, while we were working on the newt, we were also interested in the anurans. Our culture method provides a good experimental system to compare the RPE properties of the two animal species (Fig. 4; Fig. 5). The RPE-choroid tissues from mature *X. laevis* were laid on a filter membrane and cultured for 30 days. At around Day 6 in vitro, RPE cells migrated out from the periphery of the explants and began to proliferate as indicated by BrdU labeling. By Day 30, numerous migrated cells extended long branching processes, positively stained for neural markers such as acetylated tubulin and neurofilament.
Figure 4. Organotypic culture of isolated newt RPE sheet. (A ~ E) Isolation of newt RPE sheet from the choroid and culturing of isolated RPE. (A) A tissue consisting of RPE and choroid (sclera had been removed) stained for Azan. (B) Dispase treatment removed RPE from the choroid. The Bruch’s membrane stained in blue color is found at the choroid (arrow). (C) Isolated RPE sheet. No other cells from the choroid were found. (D) Isolated RPE sheet cultured on the membrane for 2 days showed an epithelial feature which remained on Day 30 as seen in (E). Scale bar in A is 100 µm and is applied to B, and C. Scale bar in D is 100 µm and is applied to E. (F ~ K) Isolated RPE sheets were cultured with FGF-2 and examined for cell proliferation and neural differentiation. Numerous cells extended long neurite-like processes (F) and some of them were stained either for Syntaxin1A (I), acetylated tubulin (J) or neurofilament 200kD (K). While RPE cells cultured without FGF-2 did not labeled for BrdU (G), numerous cells were labeled for BrdU when cultured with FGF-2, indicating they were proliferating in the culture (H). Scale bar in F is 100 µm. Bar in G is 250 µm for B and 50 µm for C. Bar in I is 50 µm and is applied to E and F. (from Mitsuda et al., 2005).

RPE sheets were then isolated by treating RPE-choroid tissues with dispase and cultured (Fig. 5). By Day 3, RPE cells had extended out from the periphery of the sheet but still remained as a simple epithelial layer. At around Day 30, RPE cells remained pigmented and no neuronal differentiation could be seen, although cells had proliferated, as shown by BrdU labeling (Figs. 8B, C).
In vitro analysis of amphibian retinal regeneration

Figure 5. Organotypic culture of isolated Xenopus RPE sheet. (A, D) Day 3 and (B, C, E, F) Day 30. RPE sheet was separated from the choroid and cultured on a filter membrane. (A, B, C) show cultures without FGF2 and IGF1, 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 FGF2 and IGF1 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 20µm and is applied to D. Scale bar in B is 30µm and is applied to C, E, F. (from Yoshii et al., 2007).

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. These observations suggest that RPE cells from mature X. laevis proliferate and differentiate into neural cells under culture condition in a similar manner to the newt.

Lens regeneration of the newt has been a paradigm of experimental tissue regeneration, and recent studies have shown that FGF2 triggers lens regeneration in the iris epithelium of the newt eye (Hayashi et al., 2004). After the lens removal, lens regeneration appears to proceed in two steps, the FGF2-dependent proliferation of iris pigmented epithelium followed by expression of Wnt2b and Frizzled4 in the dorsal half of the iris (Hayashi et al., 2006). Whether activation of Wnt or other signaling pathway secondarily occurs during amphibian retina regeneration remains to be studied, particularly in X. laevis regeneration, where RPE cells in a newly formed epithelial sheet on RVM must have a different set of gene expression from RPE on Bruch’s membrane.
Further application of the method: 3D-reconstruction of retinal tissues

The culture method described above has developed an experimental system to analyze the cellular dynamics and to compare them among different animal species. Although RPE cells do proliferate and differentiate into neuronal cells under the culture conditions, retinal histogenesis could not be realized in that condition. Instead, cells extend over the filter membrane. Another type of culture is needed for the understanding of in vivo retina regeneration. Reaggregation culture technique has been utilized to develop in \textit{vitro} histogenesis of retinal structure using embryonic retinal cells (Layer et al., 2002), although this method has not yet applied to amphibian regeneration study. By culturing \textit{Xenopus} embryonic RPE with rotation culture method, neurons and glial cells differentiate in cell aggregates that show partially organized structure (Sakaguchi et al., 1997).

We have developed a new method to ensure the 3D reconstruction of retinal structure regenerating from the RPE monolayer sheet under artificial conditions. In our preliminary experiments, retinal vascular membranes were isolated from \textit{X. laevis} eyes and were then put over the RPE sheet cultured on the filter. In this method RPE cells differentiate to a multi-cellular layered structure. On substituting an artificially reconstructed gel form for the retinal vascular membrane, a complete retinal structure developed from RPE sheets (data not shown). Morphological examination of the cultured RPE indicates that RPE cells actually transdifferentiate into retinal stem cells to reconstruct the whole retinal structure including ganglion cells, amacrine cells, Müller cells and rods. In the development of the retina, the inner limiting membrane including the basement membrane functions as an anchorage for retinal cells and is essential for the survival of the ganglion cells (Halter et al., 2005). Disruption of basement membrane leads to dramatic aberrations in retinal histogenesis (Halter et al., 2001). The present 3D reconstruction method for RPE culture provides the RPE cell-derived retinal stem cells with physical cues for retinal histogenesis.

By this method it may be possible to understand the whole processes of amphibian retinal regeneration under culture conditions. The extrinsic factors that surround RPE and the cellular and molecular mechanisms involved in transdifferentiation and histogenesis of regenerating retina are of particular interest. At the same time the method enables us to make an artificially reconstructed retina, although we have not yet determined whether the retinal ganglion cells extend axons and make a proper connection with the target region of the brain.
Conclusion and perspectives: A hypothetical mechanism underlying RPE transdifferentiation

Obviously tissue interaction plays an important role in RPE transdifferentiation as shown by the culture studies and also in vivo observations. The choroid makes a direct contact with RPE and appears to play a crucial role in the initial step of transdifferentiation. The choroid will be the source of FGF2, a trigger signal for transdifferentiation. It needs to be determined in the further studies which cell type is responsible for the synthesis of FGF2 and by what mechanism the signal is transduced to the RPE cells. Apparently, RPE cells never initiate any morphological and cell dynamic changes unless the retina is surgically removed. The retina-RPE interaction might be necessary for RPE cells to maintain the RPE-specific phenotypes by continuous expression of certain transcription factors like Mitf. It is speculated that retinectomy in the eye or isolation of RPE tissues for culture will downregulate some RPE specific genes. At the same time, FGF2 in the choroid is transmitted to RPE cells. This, in turn, upregulates several transcription factors, including Pax6 that are necessary for retinal regeneration. FGF2 has been shown previously to upregulate genes for cell growth and neurogenesis (Azuma et al., 2005; Spence et al., 2007). The most important question is what is the signal by which the retina exerts its effect on RPE. This question will be addressed using the culture system.

Removal of the retina may induce alterations in cell-cell communication of RPE cells. Only few studies have been done on the morphological changes in RPE cells right after retinectomy, such as junctional complex and cell adhesion molecules. Following retinectomy in X. laevis, RPE cells detach from the epithelium and initiate migration. This apparently deprives RPE cells of direct cell communication. In vitro studies show that RPE cells do not proliferate when newt RPE is isolated and cultured as an epithelial sheet, while they begin proliferation when cultured on the choroid. RPE cells transdifferentiate to neuronal cells only after they migrate out from the periphery of the graft as single cells. All these observations clearly indicate that cell communication form of RPE cells regulate their dynamic state.

Although RPE cells behave seemingly differently at the initial stage of regeneration between urodele and anuran amphibians, many common features are noticed in retinal regeneration of the two animal species and culture studies indicate that there are no fundamental differences in the cell dynamics particularly at the initial step of transdifferentiation. The seemingly different cell behavior between the newt and Xenopus RPE cells observed in vivo regeneration is probably not due to intrinsic cell properties but reflects some differences in the extrinsic cues such as ECM components. It is becoming increasingly important to compare retinal regeneration among a variety of animal models. The culture technology will provide an excellent approach to
address the important question of what mechanisms are involved in different vertebrates; in certain animal species complete retina regeneration occurs, while in other species like mammals it never happens in vivo. In future studies we will be able to answer this question, why does it happen?

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