Trans-differentiation of iris pigmented epithelial cells of *Euphlyctis cyanophlyctis* tadpoles into lens *in vitro*


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Received 15 May 2009; revised 16 September 2009

Meshed pigmented iris epithelium along with neural retina of tadpoles of the frog *Euphlyctis cyanophlyctis* were found to undergo dedifferentiation and subsequently transdifferentiate into lens in culture medium. During lag period, depigmentation (dedifferentiation) occurred in many cells. When culture became confluent 3-4 weeks after seeding tiny lens like structures differentiated from foci of cultured pigmented iris epithelium cells. The percentage of lens formation was higher in vitamin A treated cases. The culture system appears to be a suitable for investigating the changes occurred during trans-differentiation of pigmented epithelial cells into lens.

Keywords: Frog, Lens, Pigmented epithelial cells, Trans-differentiation

One of the most intriguing cellular events is the transdifferentiation of one terminally differentiated cell type to another. Lens regeneration in adult Urodele amphibians occurs through such an event. After lentectomy the pigmented epithelial cells (PECs) at the dorsal margin of iris undergo a reprogramming that directs them to proliferate, dedifferentiate and finally transdifferentiate into lens cells, producing a lens vesicle. This vesicle continues to grow and the cells of the vesicle begin to differentiate into the lens epithelium and lens fibers and thus give rise to the new lens. Among anurans, lens regeneration appears to be an extremely limited phenomenon. *Xenopus laevis* larvae are capable of regenerating a lens after lentectomy. This remarkable process results in trans-differentiation of the epithelial cells of the outer cornea which are able to reprogram and differentiate into the lens under the influence of the neural retina.

In *vitro* experiments have also revealed that the outer cornea of larval *Xenopus laevis* undergoes lens transdifferentiation when isolated and cultured in the presence of neural retina. As far as urodele are concerned, newts and other salamandridae can regenerate a new lens from the dorsal iris margin. The lens regeneration is stimulated by neural retina both in the eye and iris organ culture. Cultured dorsal iris of *Notophthalmus viridescens* in a medium supplemented with various concentration of eye derived growth factors, significantly stimulated lens regeneration. The present study has been undertaken to report lens regeneration from pigmented epithelial cells of iris under the influence of neural retinal factors even in anuran tadpoles of *E. cyanophlyctis* in culture medium. Effect of vitamin A on percentage of lens formation from iris PECs in culture medium has been also studied.

Materials and Methods

Three toe stage young tadpoles of *E. cyanophlyctis* were used. In all experiments tadpoles were anaesthetised with MS 222 (Sandoz) 1:2000 before operation and fixation. Following three experiments were performed:

Experiment-I

This experiment was carried out on 60 young tadpoles (three toe stage). The lentectomy was done under stereoscopic binocular microscope. A longitudinal slit was made in the cornea of right eye

Abbreviations: IR=Iris ring, EB=Eye ball, CO=Cornea, L=Lens, ME=Meshed; extract of the tissue, CD=Culture dish, NR=Neural retina, DPC=Depigmented cells, MPECs=Meshed pigmented epithelial cells, MP=Melanin pigments, LFCs=Lens forming cells, ALFCA=Acidophilic lens forming cell aggregate, RL=Regenerated lens, LLS=Lens like structure, PLF=Primary lens fiber, SLF=Secondary lens fiber, and LE=Lens epithelium
extending across the middle of the pupillary space. The lens was extracted through incision. Of the operated animals, 30 were treated with vitamin A solution (30 IU/ml) and remaining 30 were kept untreated and served as control. The operated animals of both treated and untreated groups were preserved at different time intervals in Bouin’s solution for histological evaluation. Experiment was terminated on day 40 after operation.

Experiment-II (Fig. 1)

In this experiment transdifferentiative ability of iris PECs into lens in vitro was studied. For this purpose iris explants of three toe stage young tadpoles were employed.

The tadpoles were immersed in 1% Euclorine solution for 30 sec, rinsed thrice in sterile Holtfreter’s solution and anaesthetized with MS 222 (Sandoz) 1:2000. Operations were carried out in sterile Holtfreter’s solution containing 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml Fungizone (GIBCO). After removing cornea, a complete iris ring was operated through a circular incision made with iridectomy scissors. The removed tissues were rinsed four times in Leibovitz L 15 diluted with sterile water (2:1) and containing 100 U/ml penicillin and 100 μg/ml streptomycin.

Culture medium—Leibovitz L 15 diluted with sterile water (2:1) and containing 100 U/ml penicillin 100 μg/ml streptomycin and 10 % inactivated foetal calf serum was used.

Culture methods—Explanted tissues (meshed iris PECs) were placed in a plastic organ culture dish (35 × 10 mm Falcon plastics) with culture medium. Vitamin A (30 IU/ml) was supplemented to the culture medium for treated group. The culture medium was renewed on every 2nd day. Cultures were terminated after 5, 10, 15 and 40 days.

Histological methods— Cultures were fixed in 95% ethanol at 4°C, embedded in paraffin, cut into 7 μm serial sections and stained with Haematoxyline and counter stained with Eosin.

Experiment III (Fig. 2)

In vitro culture of meshed iris PECs associated with neural retina (NR) were employed in this experiment.

The neural retina was removed with a sharp edged tungsten loop. For neural retina operation a small incision was made into the limbus and cornea, the equatorial part of the eye surrounding ora serrata dissected and discarded and the lens and vitreous were removed to expose the retina. The NR was removed with micro forceps and special care was taken to ensure removal of the NR without RPE cells, which are already heavily pigmented at this developmental stage. Immediately after its isolation,
the *pars* neurale retinae was surgically separated from the *pars* iridea retinae. The iris was removed as described under experiment II. Both the removed tissues, viz neural retina and iris were meshed with culture medium. The meshed cellular extract of iris and neural retinae was cultured. The remaining techniques followed for experiment III are same as described for experiment II.

Results

Experiment-I—Lens regeneration *in vivo* under the influence of vitamin A. (Simple lentectomy of 3 toe stage tadpoles of *E. cyanophlyctis*).

The lens regenerative power was found in 3 toe stage young tadpoles of *E. cyanophlyctis* (Group A) (Table 1). Vitamin A induced lens regeneration from dorsal iris PECs (Group B) and increased the percentage of lens regeneration: it was 86.6% in treated tadpoles and 53.3% in untreated control tadpoles (Table 1). The morphological features like shape, size and transparency of regenerated lens were found similar to that of normal intact lens. The regenerated lens was found normal in its function which was tested experimentally. Normal response was obtained when intact eye was closed by putting black tape on it. Histological changes occurred during lens regeneration were found almost similar in both treated as well as untreated control group animals.

In both, control as well as vitamin-A treated group animals lens regeneration occurred from pigmented epithelial cells of dorsal iris. Histological observations revealed that once the lens is removed the process of regeneration initiated by dedifferentiation of the dorsal iris pigment epithelium.

After lentectomy the two layers of pigmented epithelia of dorsal iris began to thicken and the nuclei of iris cells changed their shape. Cleft appears between two laminae of dorsal iris (Fig. 3). The pupillary margin of the iris became knob like. The formation of this knob like structure continued until the free margin became a swollen loop like structure (Fig. 4). Scattered mitotic figures were also observed. Formation of the lens vesicle by the depigmented progenies of the iris cells was evident between 5 and 10 days (Fig. 5). Between 10 and 15 days after lentectomy the internal layer of the lens vesicle thicken. Following that period lens fibers were produced in the vesicle. Cells began to elongate and entered the lumen of the vesicle (Fig. 6). The lumen was filled by primary lens fiber nuclei before the secondary lens fiber began to form (Fig. 7). Later the secondary lens fibers began to differentiate and grow around the central nucleus and the regenerated lens became a better defined structure. In the next stage the regenerated lens get detached from the dorsal iris and returned to its normal position (Fig 8). At last the nuclei of the secondary lens fibers progressively disappeared.

Experiments II and III (Transdifferentiation of PECs into lens *in vitro*)

The results obtained from these two experiments are presented in the Table 2. The results show that lens forming transformation ability is found in iris PECs of the young tadpoles (3 toe stage) of *E. cyanophlyctis*. Vitamin A accelerated the percentage of lens forming transformation. It was in 82% cases of vitamin A rich culture medium and 52% cases in untreated control group explants (Table 2).

Results of the experiment III showed that lens forming transformation ability of iris PECs increased in presence of neural retinal cells. In experiment III meshed iris PECs were cultured along with the meshed neural retinal cells. Here also the percentage of lens formation was higher in vitamin A supplemented culture media. (Table 2). It was 94% (experiment III B) in vitamin A rich culture medium and 75% (experiment III A) in untreated control group culture media. The regenerated lenses developed in the culture medium indicated essentially the same histological pattern as in the normal lens regeneration. However, there are some differences between the lens developed *in vitro* and the lens regenerate developed normally in the ocular environment after lentectomy. That is to say, lens developed in culture medium was always smaller in

<table>
<thead>
<tr>
<th>Group*</th>
<th>Day of preservation</th>
<th>No. of operated animals preserved</th>
<th>Number of Normal Lens regenerates</th>
<th>Non-Regenerates</th>
<th>Lens Regeneration (% )</th>
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<tr>
<td>A</td>
<td>5</td>
<td>10</td>
<td>4</td>
<td>53.3</td>
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<tr>
<td>(untreated)</td>
<td>7</td>
<td>5</td>
<td>3</td>
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<td>15</td>
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<td>40</td>
<td>15</td>
<td>10</td>
<td>05</td>
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<tr>
<td>B (Vitamin A treated)</td>
<td>5</td>
<td>03</td>
<td>2</td>
<td>86.6</td>
<td></td>
</tr>
<tr>
<td>A treated</td>
<td>7</td>
<td>04</td>
<td>01</td>
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*Total 30 animals were used in each group*
size and generally ellipsoidal in shape and its epithelium occupied less area than in the normal lens regenerates. Results clearly revealed that neural retinal cells are beneficial to iris PECs for lens forming transdifferentiation ability.

Histological examination showed that the changes occurred during lens regeneration (transdifferentiation of pigmented epithelial cells into lens) were almost similar in both in vivo (experiment I) and in vitro (experiments II and III). The iris (or iris PECs + neural retina) was isolated from the eye ball as mentioned earlier (Figs 1 and 2). The tissues was meshed and inoculated in the culture media. The iris PECs grow slowly and stably maintain the phenotype in a standard culture medium. After one week the PECs grow rapidly and dedifferentiate (Figs 9 and 10). The cells of meshed PECs extract shed off their melanin pigments (Fig. 10). Many foci of typical epithelial cells appeared. After 10 days of lentectomy most of the explants showed lens forming transformation particularly in vitamin A supplemented cultures. The process can be considered as differentiation of pigmented epithelial cells into lens forming cells (Figs 11 and 12). After 10-15 days the cells started to form aggregates (Fig. 12). These aggregates were easily distinguishable from adjacent mass of cells and continued to grow and progressively increased in volume due to cell proliferation.

Typical cytological signs of lens differentiation such as enlargement of nuclei, nucleoli, cell elongation, gradual loss of basophilic properties and acquisition of acidophilic properties for crystalline synthesis and accumulation were observed beginning from 5 days culture (Figs 11, 12 and 13).

The cultivated tissues of experiments II & III (both control and vitamin A treated) underwent lens

<table>
<thead>
<tr>
<th>Group</th>
<th>Experiment</th>
<th>Days of culture</th>
<th>No. of culture examined</th>
<th>No. of regenerates</th>
<th>Lens regeneration (%)</th>
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<tr>
<td>A</td>
<td>Culture of iris PECs in control standard culture medium</td>
<td>5</td>
<td>20</td>
<td>10</td>
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<td>A2: Culture of iris PECs in the medium supplemented with vitamin A</td>
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<td>20</td>
<td>15</td>
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<td>22</td>
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<td>B</td>
<td>Culture of iris PECs associated with meshed neural retina extract in control standard culture medium</td>
<td>5</td>
<td>20</td>
<td>15</td>
<td>92.5</td>
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<td>15</td>
<td>20</td>
<td>20</td>
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</tr>
<tr>
<td>B2: Culture of iris PECs associated with meshed neural retina Medium supplemented with vitamin A</td>
<td>5</td>
<td>20</td>
<td>18</td>
<td>97.5</td>
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Fig. 3—Microphotograph of a section passing through lentectomized eye of 3 days vitamin A treated tadpole of *E. cyanophlyctis* showing cleft formation in between two laminae of dorsal iris (400 ×).

Figs 4 and 5—Microphotographs of sections passing through lentectomized eye of 5 days (Fig. 4)/7 days (Fig. 5) vitamin A treated tadpoles of *E. cyanophlyctis* showing formation of lens vesicle at the tip of dorsal iris.

Note that in Fig. 4—the tip of the iris has dedifferentiated cells (lens forming cells) and early lens vesicle enclosing lens forming cells (LFC) (400 ×). Fig 5- shows 7 days post lentectomized eye with lens vesicle. Lens fiber differentiation begins at posterior part of the vesicle (400 ×).

Fig. 6—Microphotograph of a section passing through the lentectomized eye of 15 days vitamin A treated tadpole showing newly formed regenerated lens. Figure shows the early differentiation of lens fibers in regenerated lens. (200 ×).

Fig. 7—Microphotograph of a section passing through the lentectomized eye of 15 days vitamin A treated tadpoles showing lens fiber differentiation in regenerated lens (400 ×). Note the definite formation of the lens epithelium and differentiation of primary lens fibers. (400 ×).

Fig. 8—Microphotograph of a section passing through the regenerated lens in 40 days vitamin A treated froglet. Note the regenerated lens depicting the advanced stage of growth, Secondary lens fibers are compactly arranged in normal concentric fashion (200 ×). Inset-photograph shows a hand section of the lentectomized eye with regenerated lens.
forming transformation, giving rise to very thick and acidophilic cell aggregates (Figs 12 and 13). However, these lens forming structures never organized into normal lens in untreated control group explants (the lens fibers being oriented in the anterior-posterior plane rather than concentrically) (Figs 14, 15 and 16). But in the culture which was supplemented with vitamin A (of both experiments II and III) and neural retinal cells cultivated with iris PECs transdifferentiated into a complete normal lens in 5 cases out of 20 (Fig. 17).

Discussion

The results obtained in the present study show that vitamin A induced and accelerated the percentage of lens regeneration in the young tadpoles of _E. cyanophlyctis_ in both _in vivo_ as well as _in vitro_ condition. Results also revealed that iris PECs of young tadpoles of _E. cyanophlyctis_ have the ability to transdifferentiate into lens. Presence of neural retinal cells increased the percentage of lens forming transformation ability of iris PECs in the both untreated control and vitamin A treated explants.

Lens regeneration from non-ocular tissue (dorsal iris) has been well documented in urodele amphibians. Although lens regeneration from the pupillary margin of the dorsal iris has not yet been found in anuran, there is evidence in the tadpole stage of the anuran _Diemyctylus pyrrhogaster_ that the capacity for lens formation is apparently hidden in the pigment epithelium of the iris and retina.

The pigment cells have a dual capacity to form either retinal tissue or lenses when transplanted into lentectomized eye. Recently, Goswami also reported that the iris PECs of the tadpoles of _E. cyanophlyctis_ have the ability to transdifferentiate into lens in _in vivo_ and in transplantation set up. In the present study lens regeneration occurred from PECs of dorsal iris rather cornea. It is evident from the results obtained from _in situ_ and _in vitro_ study done on young tadpoles. Thus, the present result provides substantial evidence for the cell type conversion of iris pigmented epithelial cells into lens. Similar to present results, Yamada, also reported that following depigmentation PECs redifferentiate into lens fiber cells and synthesize crystalline proteins. This process is dependent upon retina _in vivo_ and _in vitro_.

In the present study it was found that the percentage of lens formation was much high in organ culture dish which was containing both iris PECs and neural retina tissue. The retina acts indirectly by promoting passage of retinal factors to the iris epithelial cells. It is also possible that the retina acts directly by instructing the iris cells to redifferentiate into lens (tested the effects of eye derived mitogen substances as well as other mitogens on regeneration of lens from iris in organ culture). Connely and Green have shown that crude extract of retina can enhance lens registration in culture. In the present study mitogenic substance vitamin A was found to enhance the percentage of lens regeneration _in vivo_ as well as _in vitro_. It was 86.6, 86.2 and 97.5% in vitamin A treated cases of experiment I, II and III respectively in comparison to untreated control groups (53.3, 55 and 92.5% in respective experiments). How vitamin A affects lens regeneration is still not well known. However, Chytill and Ong suggested that retinoids enter the cells via some surface receptor or by lipophilic intercalation through the membranes and then bind to cytoplasm binding proteins (RABP). Petkovitch et al. also reported two such binding proteins one for retinoic acid, cellular retinoic acid binding protein (CRABP) and one for retinal cellular retinal binding protein (CRBP). The complex then transported to the nuclei where it ultimately alters the pattern of gene activity. Thus it can be suggested that retinoids play an important role in transmission of their legends from the cell membrane to the nucleus where the pattern of gene activity may be altered. Recently Jangir et al. and Amit et al. studied the beneficial effect of vitamin A on lens regeneration in amphibians and mammals as well. The accelerating influence of retinoic acid on lens regeneration has
Fig. 15—Microphotograph of a section passing through the newly developed lens from the explant of meshed iris + neural retina in the culture on day 15 after operation (400 ×). Note the lens forming structure containing primary lens fibers.

Fig. 16—Microphotograph of a section passing through the lens regenerate from the explant (iris + neural retina) on day 40 after operation (400 ×). Note well differentiated secondary lens fibers arranged in the antero-posterior plane rather than concentrically.

Fig. 17—Photograph of a lens regenerate of normal status from the explant (iris + neural retina in vitamin A containing culture medium) (Experiment III). Note that the regenerated lens encapsulated with normal lens epithelium and containing well differentiated lens fibers oriented in concentric manner.

been also reported. Tsonis et al. 28 suggested that when the function of retinoid receptors was impaired by using RAR antagonist, the process of lens regeneration was dramatically affected.

The present results show that some of the anuran species like E. cyanophlyctis have the capacity for lens regeneration from iris PECs similar to urodele amphibians. The results also show that cytological changes and lens fiber differentiation of pigmented epithelial cells occur in the same order in vitro as in vivo, that the neural retinal factor on which lens transdifferentiation depends is a diffusible factor and that this culture system represents a suitable in vitro model for investigating the inducing retinal factor in lens regeneration. It can be concluded that the culture system provides a useful opportunity for analyzing cellular and molecular mechanism involved in each step of differentiation. This will be solving the problem of cataracts, artificial lens and open a new vast field of transdifferentiation.

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