CMLS, Cell. mol. life sci. 53 (1997) 921–928
1420-682X/97/120921-08 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Lens fibre transdifferentiation in cultured larval *Xenopus laevis* outer cornea under the influence of neural retina-conditioned medium

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Received 25 June 1997; received after revision 2 September 1997; accepted 25 September 1997

Abstract. The outer cornea of larval *Xenopus laevis* can reprogram cell differentiation when cultured in medium conditioned by *X. laevis* neural retina (XRCM) or by *Rana esculenta* neural retina (RRCM). Under these experimental conditions corneal cells showed the same series of cytological changes of fibre cell differentiation observed during ontogenesis and in vivo lens regeneration: enlargement of nuclei and nucleoli, increase of ribosomal population (cytoplasm basophilia), cell elongation, gradual loss of basophilic properties and acquisition of acidophilic properties for crystallin synthesis and accumulation. These events were completely dependent on XRCM or RRCM, suggesting that the neural retina secretes a factor(s) which initiates and sustains lens fibre transdifferentiation of the corneal epithelial cells. This culture system appears to be a suitable one for investigating the control of lens fibre transdifferentiation in vitro.

Key words. Lens; transdifferentiation; *Xenopus*.

At present the available data concerning lens regeneration in Anura show that the phenomenon is restricted to larval *X. laevis* (for a review see [1, 2]). After lentectomy *X. laevis* larvae are able to regenerate a lens from outer cornea (squamous stratified epithelium two layers thick, bounded by a basement membrane) through a typical sequence of phases [3]. There is evidence that lens regeneration in this species occurs through a transdifferentiation process of corneal cells under the influence of a factor produced by the neural retina (for a review see [4]). Though the exact nature of the retinal factor has not yet been determined, some experimental results indicate that it has a protein nature; pellets of whole protein complement of the eye cup induce lens-forming transformation of the outer cornea [5] when implanted between the outer and inner cornea.

The regenerative capacity, which is also present in the pericorneal epidermis, decreases during the larval period, and it disappears at metamorphosis when the outer cornea undergoes substantial structural changes [3]. Some experimental data suggest that the lens transdifferentiation process of the outer cornea requires a sequence of interactions extending over a long period of time during which the retinal factor must be present until complete lens transdifferentiation of cornea is achieved [6]. Although the lens transdifferentiative capacity of the outer cornea appears to be limited to larval *X. laevis*, the lens-inducing capacity of neural retina is widely present both in larval and adult Anura [2, 7, 8].

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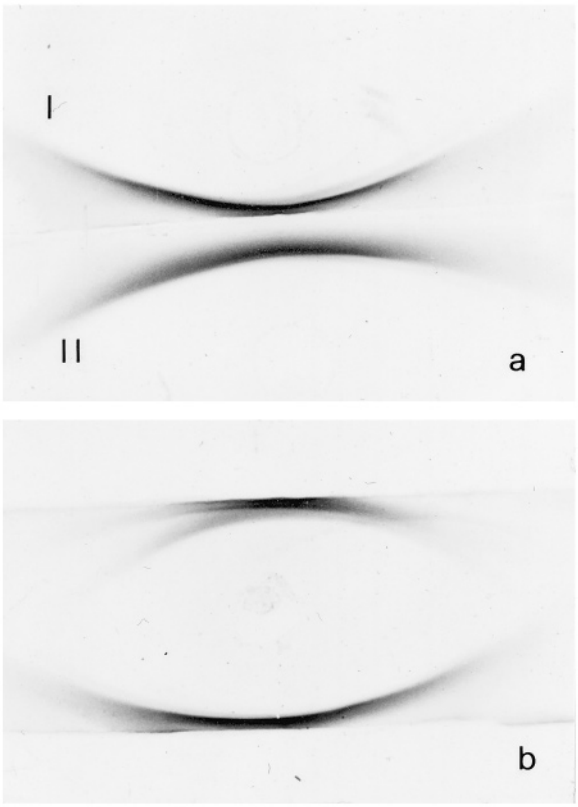

Figure 1. Immunoelectrophoresis in 1% agarose gel with 0.05 mM Veronal buffer, pH 8.4. (*a*) I. *X. laevis* total lens proteins against *X. laevis* anti-total lens protein antibody. II. *X. laevis* total lens proteins against *R. esculenta* anti-γ-crystallin antibody. (*b*) *X. laevis* total lens proteins against *R. esculenta* anti-γ-crystallin antibody.

The crucial role played by neural retina in the lens transdifferentiation of corneal cells has been confirmed by in vitro studies [9]. Lens-forming transformation of the outer cornea was completed in vitro when this tissue was cultured within the lentectomized eye cup. Fibre cells showing positive immunofluorescence with anti-total lens protein antibody were also observed when the outer cornea was cultured with isolated neural retina. In contrast, no lens fibre was formed when the outer cornea was cultured alone. Moreover, lens-forming structures originating from cornea at different times after lentectomy, regressed completely when isolated in vitro.

Lens transdifferentiation of the outer cornea in vivo and in vitro appears to occur without physical contact between this tissue and neural retina, suggesting that neural retina produces a diffusible factor needed to trigger reprogramming differentiation and maintenance of the lens-forming structure.

Recent experimental data [10] reveal that under in vitro conditions lens transdifferentiation of the outer cornea can be induced by bovine brain-derived acidic fibroblast growth factor (aFGF). These data together with the fact that the presence of FGFs and their receptors in ocular tissues of vertebrates has been evidenced in various studies (for a review see [11]), and that FGFs appear to be involved in the lens differentiation of mouse lens epithelial cells [12], suggest that one member of FGF family (for a review see [13–15]) could be the factor on which lens regeneration in larval *Xenopus* depends.

Because we are interested in determining the nature and mechanism of action of the putative inducing factor, we decided to establish a culture system with a powerful inducing capacity. In the present study we show that adult *X. laevis* and *R. esculenta* neural retina-conditioned media (XRCM and RRCM) initiate fibre transdifferentiation in explant cultures of larval *Xenopus* outer cornea as determined by cell phenotype changes and initiation of crystallin synthesis. We used *X. laevis* anti-total lens protein antibodies to detect total lens crystallins and *R. esculenta* anti-γ-crystallin antibodies to detect γ-crystallin. The validity of using heterologous anti-γ-crystallin antibody against *X. laevis* lens was confirmed.

Materials and methods

Animals. Three experiments were carried out. In all experiments larvae of *X. laevis* at stage 51–52 (according to Nieuwkoop and Faber [16]) were used. All larvae were obtained from a single pair after amplexus and ovulation induced by gonadotropic hormones (Profasi,

Table 1. Experiment 1 (control experiment): simple lentectomy.

Days after operation	No. of cases examined	No. of regenerates	Lens regeneration stage 2 3 4 5
3	6	3	1 2
5	6	4	1 3
7	6	6	5 1
10	6	6	2 4
15	6	6	6

Table 2. Experiment 2 (control experiment): explant culture of outer cornea and pericorneal epidermis.

Days of culture	No. of cases	Lens fibres formation	%
4	40	0	0
10	60	0	0

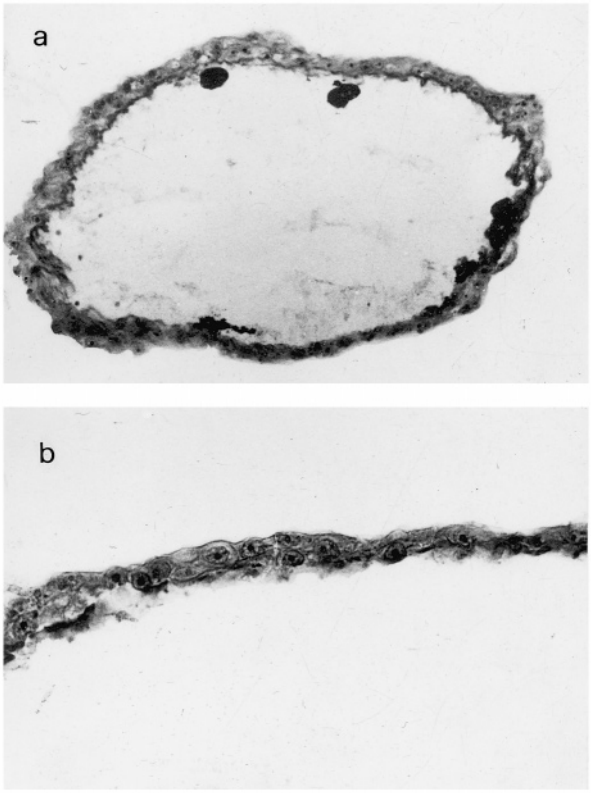

Figure 2. Experiment 2: explant culture of outer cornea and pericorneal epidermis. (*a*) Four-day culture: the cultured tissues originated hollow vesicles maintaining the original histological epithelial structure, ×600. (*b*) Ten-day culture: in some cases the volume of the cells, nuclei and nucleoli clearly increased, ×1100.

Serono). Neural retina used for the preparation of conditioned medium was isolated from adult *X. laevis* and *R. esculenta*. In all experiments larvae and adult were anaesthetized with MS 222 1:2000 and 1:1000 respectively before operation and fixation.

Histological methods. Larvae and explant cultures were fixed overnight in 95% ethanol at 4 °C, embedded in paraffin, cut into 5-µm serial sections and stained by the Mallory-Azan method according to Heidenhain [17].

Preparation of anti-total lens protein antibody. *Xenopus laevis* adults were sacrificed, and lenses were removed and freed from other tissues. They were washed three times in ice-cold 5 mM phosphate buffer, pH 7, and stored at −20 °C. Soluble lens proteins were obtained by homogenization in the same buffer; insoluble materials were removed by centrifugation following the procedure of McDevitt and Brahma [18]. A mixture of complete Freund's adjuvant and 500 mg of lens proteins was injected three times into rabbits over a period of 3 weeks. The animals were bled 1 week after a booster injection without adjuvant.

The serum obtained was tested against the homologous antigen by immunoelectrophoresis in 1% agarose gel with 0.05 mM Veronal buffer, pH 8.4, on microscope slides at 5 V/cm. After the electrophoretic run the immune serum was added and allowed to diffuse overnight at 18 °C. Staining was performed with Coomassie Brilliant Blue R-250.

Preparation of anti-γ crystallin antibody. An antibody directed against purified *R. esculenta* γ-crystallins was used to detect *X. laevis* γ-crystallins. The validity of this approach was confirmed by immunoelectrophoretic analyses of various combinations of *R. esculenta* and *X. laevis* antigens and antibodies (fig. 1), all of which confirmed cross-reactivity (fig. 1).

Similar results were obtained by McDevitt and Brahma [18], who demonstrated the cross-reactivity and resultant identical immunofluorescence profile of an antibody directed against purified *R. pipiens* γ-crystallins and *X. laevis* γ-crystallins. Nöthiger and McDevitt [19] also showed cross-reactivity of purified *R. pipiens* γ-crystallins and *Notophthalmus viridescens* γ-crystallins.

This *R. esculenta* γ-crystallin antigen was obtained by successive DEAE-cellulose and DE-32 chromatography of adult *R. esculenta* total lens proteins as described in a previous paper [20], and the specific homologous antibody was produced as described above for anti-total lens antibody. The specificity of the anti-γ-crystallin antibody was tested staining all sections of the eye.

Retrospective immunofluorescence staining. Retrospective immunofluorescence was effected by the method of Mikailov and Gorgolyuk [21]. For the immunochemical analysis, the preparations were immersed in chilled xylene to remove Canada balsam (10 °C, 2–7 h) and then washed with 95% ethanol (two to three changes, 1 h each) and three changes of buffered saline (pH 7.1, 30 min each).

Anti-total lens protein or anti-γ-crystallin antibody was used as the first antibody, according to the 'sandwich' method of Weller and Coons [22]. Before use, anti-total lens protein antibody was cross-absorbed with adult and larval lentectomized *X. laevis* tissue powder. The preparations were then treated with commercial goat anti-rabbit gammaglobulin antibody conjugated with fluorescein isothiocyanate (Pierce no. 31572).

As a preliminary control before the retrospective immunofluorescence test, normal *X. laevis* eyes were incubated with rabbit non-immune serum and then with

Table 3. Experiment 3: explant culture of outer cornea and pericorneal epidermis with XRCM.

Days of culture	No. of cases	Lens fiber formation	%	Primary nucleus-like structures	%
3	20	0	0	0	0
4	20	18	90	0	0
5	20	16	80	0	0
7	20	19	95	11	55
10	20	18	90	14	70

goat anti-rabbit gammaglobulin antibody conjugated with fluorescein isothiocyanate. Negative results were obtained in this immunofluorescence test.

Experiment 1 (control experiment): Simple lentectomy of larval *X. laevis* at stage 51–52 (30 operations). This experiment was carried out to determine the percentage of *X. laevis* larvae showing lens regeneration, as this percentage varies considerably [23]. The lenses were removed by the operative technique described in a previous paper [1]. Operations were carried out in Holtfreter's solution, and after operation the larvae were maintained in Holtfreter's solution for 3 days before transfer to springwater. Entire larvae were fixed in Bouin's solution 3, 5, 7, 10 and 15 days after operation, in groups of six individuals.

Experiment 2 (control experiment): Explant culture of outer cornea and pericorneal epidermis (100 cases). The larvae were immersed for 30 s in 1% Euclorin solution (Zambeletti), then rinsed three times in sterile Holtfreter's solution. 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). A small incision was made in the dorsal pericorneal epidermis, and the outer cornea was gently separated from the inner cornea with a thin hook-shaped tungsten needle. The cornea and a small portion of pericorneal epidermis were removed through a circular incision made using iridectomy scissors. The tissues removed were rinsed four times in Leibovitz L 15 (Flow) diluted with water (2:1) and containing 100 U/ml penicillin, 100 μg/ml streptomycin and 1.5% glutamine (200 mM). The explanted tissues were placed in a plastic culture dish (3.5×10 mm, Falcon Plastics) containing 2 ml of medium Leibovitz L 15 diluted with water (2:1), 100 U/ml penicillin, 100 μg/ml streptomycin, 1.5 glutamine (200 mM) and 10% inactivated fetal bovine serum. Approximately 2 ml of medium was used per dish containing ten cultures. Culture medium was renewed daily. Cultures were terminated after 4 (50 explants) and 10 days (50 explants).

Experiment 3: Explant culture of outer cornea and pericorneal epidermis (200 cases) in neural retina-conditioned medium. The retina-conditioned medium was obtained from neural retina explant cultures of adult *X. laevis* (XRCM) (48 eyes), and from retina explant cultures of adult *R. esculenta* (RRCM) (48 eyes). After lentectomy the retina was removed with a sharp-edged tungsten loop. Immediately after isolation of the retina, the pars nervosa retinae was surgically separated from the pars iridic retinae. Forty-eight retinas cut into several pieces were cultured in 20 ml of Leibovitz L 15 added with 10% of inactivated fetal bovine serum. The medium was replaced completely every 24 h for 6 days. The medium harvested during this period was decanted, centrifuged at 200*g* for 5 min followed by 40,000*g* for 60 min. The final supernatant was stored at −20 °C. Immediately before use the conditioned medium was diluted in an equal volume of Leibovitz L 15 and filter-sterilized. Explants of outer cornea and pericorneal epidermis, obtained as in the experiment 2, were placed in a plastic organ culture dish (35×10 mm, Falcon Plastics). Approximately 2 ml of conditioned medium was used per dish containing ten cultures. The culture medium was renewed daily. One hundred explants were cultivated in XRCM and in RRCM. Cultures were terminated after 3, 4, 5, 7 and 10 days, in groups of 20 explants.

Results

Experiment 2 (control experiment): Simple lentectomy. In 25 out of the 30 cases examined (83%) the outer cornea underwent lens-forming transformation (table 1). Results on the regenerative process were consistent with those reported by Freeman [3]. The staging of new-forming lenses was carried out according to Freeman [3].

Experiment 2 (control experiment): Explant culture of outer cornea and pericorneal epidermis. None of the 100 cases examined after 4 (40 cases) and 10 (60 cases) days showed outer corneal lens-forming transformations (table 2). In many cases the cultivated tissues originated hollow vesicles (fig. 2a), but the outer cornea and pericorneal epidermis maintained the same epithelial tissue organization as that of a normal eye. The only noticeable cellular structural change sometimes observed was a notable volumetric increase of the double epithelial sheet (fig. 2b). The indirect immunofluorescence test gave negative results in all 50 cultures examined (not shown). These results are consistent with those we obtained previously [9].

Table 4. Experiment 3: explant culture of outer cornea with RRCM.

Days of culture	No. of cases	Lens fibre formation	%	Primary nucleus-like structures	%
3	20	0	0	0	0
4	20	16	80	0	0
5	20	19	95	0	0
7	20	17	85	9	53
10	20	19	95	16	84

Experiment 3: Explant culture of outer cornea and pericorneal epidermis with neural retina-conditioned medium. Explant cultures of the outer cornea and pericorneal epidermis in the presence of XRCM or RRCM showed the same clear changes both in morphological appearance and in crystallin contents (tables 3 and 4). Histological examination showed that after 3 days the cultivated tissues gave rise to solid aggregates that progressively increased in volume due to cell proliferation (fig. 3, a and b).

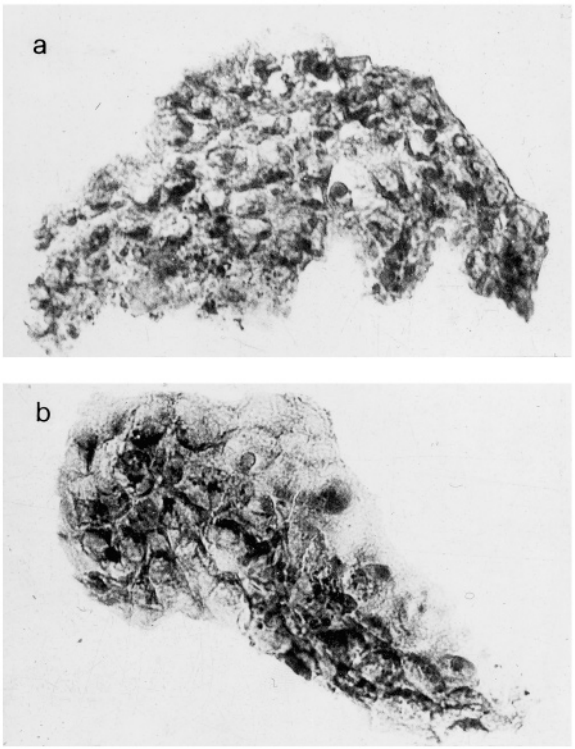

Figure 3. Experiment 3: explant culture of outer cornea and pericorneal epidermis with neural retina-conditioned medium. Three-day culture, the cultured tissues originated a solid aggregate which progressively increased in volume. (*a*) XRCM, (*b*) RRCM; ×1100.

Typical cytological signs of lens differentiation [3, 24–27] such as enlargement of nuclei and nucleoli, increase of ribosomal population (cytoplasmic basophilia), cell elongation, gradual loss of basophilic properties and acquisition of acidophilic properties for crystallin synthesis and accumulation [25, 28] were observed beginning from 4 days culture (fig. 4a). As a rule the entire cultivated tissue underwent lens-forming transformation, giving rise to very thick and acidophilic cell aggregates (fig. 5, c to d). However, these lens-forming structures never organized into normal lens, the lens fibres were arranged irregularly and a lens epithelium was not observed (fig. 4, a to d). The abnormal histological organization of the lens-forming structures prevented us from applying Freeman's method for staging lens regenerating structures in vivo [3]. The progressively higher degree of lens differentiation of the lens-forming structures during the culture period was revealed by gradually increasing cytoplasmic acidophilia, disappearance of lens fibre cell nuclei and lens fibres aggregating into so-called primary lens fibre nucleus, similar to lens development and lens regeneration in vivo.

All specimens of the different fixation groups were analyzed by the indirect immunofluorescence method, half of the cases with anti-total lens protein antibody, the other half with anti-γ-crystallin antibody. The immunofluorescence method showed the specificity of the lens-forming structures and confirmed the histological observations. All the explants forming lens fibres (tables 3 and 4) showed positive reactions. With anti-total lens protein antibody the first positive immunofluorescence reaction observed under our experimental conditions was in the 4-day explant cultures (fig. 5a). Subsequently, the explant cultures showed positive cells which progressively increased in number and intensity (fig. 5c).

The first positive immunofluorescence reaction with anti-γ-crystallin antibody appeared in the 4-day explant cultures (fig. 5b). The extent of the positive area was approximately the same as that showing the positive reaction with anti-total lens protein antibody (fig. 5d).

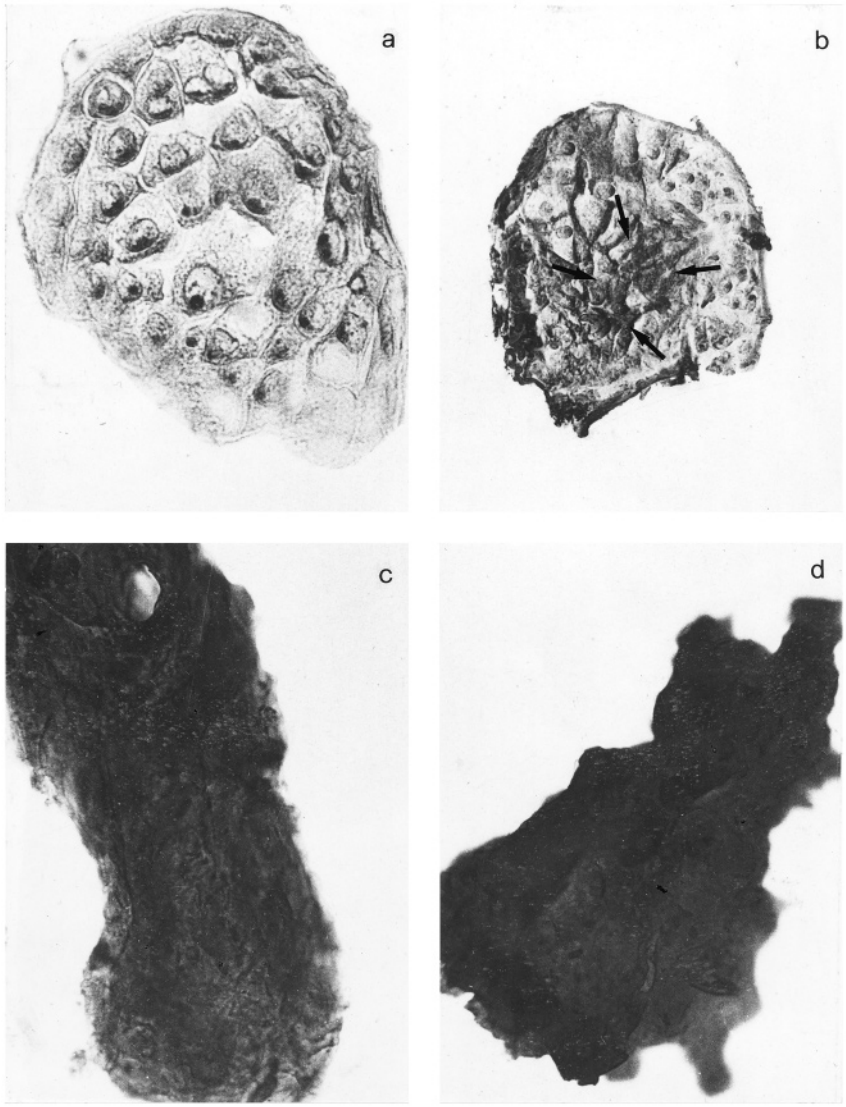

Figure 4. Experiment 3: explant culture of outer cornea and pericorneal epidermis with neural retina-conditioned medium. (*a*) XRCM: typical cytological signs of lens differentiation could be observed after 4 days in culture, ×1100. (*b*) RRCM: seven-day culture. The progressively higher degree of lens differentiation was revealed by gradual disappearance of lens fibre cellular nuclei and by increasing acidophilia (arrows), ×600. (*c*, *d*) Ten-day culture. Progressive lens fibres differentiation gave rise to lens fibers aggregates similar to large primary lens fibre nuclei, ×1100.

Discussion

The results obtained in the present study show that the epithelial corneal cells of larval *X. laevis* can transdifferentiate into lens fibres when cultivated in the presence of neural retina-conditioned medium obtained from retina explants of adult *R. esculenta* (RRCM) and *X. laevis* (XRCM). Under the present experimental conditions, corneal cells undergo the same series of cytological changes of fibre differentiation as those observed during ontogenesis [25] and in vivo regeneration [3], in particular enlargement of nuclei and nucleoli, increase in ribosomal population (cytoplasm basophilia), cell elongation, gradual loss of basophilic properties and acquisition of acidophilic properties for crystallin synthesis and accumulation [18, 28].

During lens regeneration from the cornea of larval *X. laevis*, Brahma and McDevitt [28] observed the first positive immunofluorescence with anti-total lens-protein antibody and with *R. pipiens* anti-γ-crystallin antibody at early stage 4 of regeneration [3]. At this stage of regeneration, the lens-forming structure consists of cells with a large round nucleus near the centre. This is the first sign of lens fibre differentiation. In the

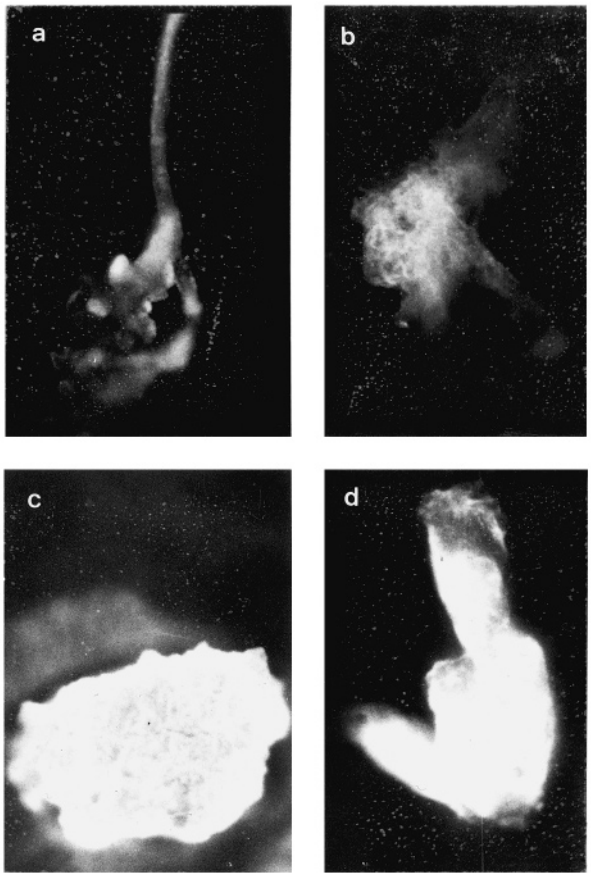

Figure 5. Experiment 3: explant cultures of outer cornea and pericorneal epidermis with neural retina-conditioned medium. The lens-forming structures showed the first positive immunofluorescence reaction with absorbed anti-total lens protein antibody (*a*) and with anti-γ-crystallin antibody (*b*) after 4 days in culture. The explant cultures were completely positive for anti-total lens protein antibody (*c*) and for anti-γ-crystallin antibody (*d*) after 10 days in culture. (*a*, *c*) XRCM; (*b*, *d*) RRCM.

present research the first positive reaction with anti-total lens protein antibody, and with *R. esculenta* anti-γ-crystallin antibody, appeared in the 4-day explant cultures. Although the abnormal histological organization of the lens-forming structure prevented staging according to Freeman's method, the cells did show nuclear features typical of lens-regenerating structures at stage 4. These results indicate that the ontogeny of lens crystallins during in vitro lens transdifferentiation of corneal epithelial cells is the same as that observed during lens regeneration, indicating that the γ-crystallins are among the first – if not the first – crystallins to appear (for a review see [29]).

Whatever molecular mechanisms underlie fibre transdifferentiation, our culture system confirms that they are initiated by the neural retina and shows that the neural retina releases a factor(s) into the culture medium that is able to trigger and sustain the lens transdifferentiation of the corneal cells independent of any contact between this tissue and the neural retina.

Previous experimental results obtained in vivo indicate that in the normal eye (non-lentectomized) of *X. laevis* larvae such a factor(s) would be secreted into the vitreous chamber, but its interaction with corneal cells is prevented by the intact inner cornea (mesenchymal) and lens, both of which prevent the factor(s) needed to trigger lens transdifferentiation from spreading from the vitreous chamber toward the anterior chamber. The outer cornea undergoes lens transdifferentiation only when it communicates directly with the vitreous chamber (for a review see [4]). The capacity of adult *R. esculenta* and *X. laevis* retina-conditioned medium to promote lens transdifferentiation of larval *Xenopus* cornea shown in this study, together with previous results obtained in grafting experiments of cornea and lentectomized eyes in various species of larval Anura and *Xenopus*, indicates that the retinal factor(s) promoting lens transdifferentiation of outer cornea is generally present in larval and adult anuran eye (for a review see [2]). At present it is quite difficult to explain why the retinal factor(s) inducing lens transdifferentiation of the outer cornea is present in the eyes of many larval and adult Anura, whereas the capacity to regenerate a lens in anuran larvae so far tested is present only in larval *X. laevis*. One possible hypothesis might be that the retinal factor(s) is a ubiquitous factor having one or more different actions in the normal vertebrate eye. Actually, there is some evidence to support this hypothesis.

Barritault and co-workers [30] showed that the extract of the bovine adult retina (RE) can stimulate the growth and morphology of bovine epithelial lens cells in vitro. Since RE-like activity is also found in other eye tissues from bovine and other species, the authors postulated that there is a ubiquitous growth factor in the eye – called eye-derived growth factor (EDGF) – which may play an important role in the physiology and pathology of the eye. McAvoy and co-workers [31–34] showed that epithelial cells isolated from neonatal rat lens undergo changes characteristic of fibre differentiation when cultured with neural retina, neural retina-conditioned medium or bovine acidic and basic FGF. Cuny and co-workers [35] cultured the dorsal iris of *N. viridescens* in a medium added with various concentrations of EDGFs. They observed that both EDGF II, the retinal form of aFGF, and EDGF III, the non-retained retinal factor after heparin affinity chromatography, stimulate lens regeneration.

In a previous paper [10] we reported that aFGF (bovine brain-derived) can induce lens transdifferentiation of larval *X. laevis* outer cornea in vitro. This result, together with the fact that the presence of FGFs and their receptors in ocular tissues of vertebrates has been evidenced in various studies [11], suggests that one member

of the FGF family could be the factor on which in vivo lens regeneration depends. However, this is still a working hypothesis, as no FGF-like factor has been isolated yet in the larval *X. laevis* eye.

The results presented in this paper show that cytological changes and synthesis of crystallins of the outer cornea occur in the same order in vitro as in vivo, that the 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.

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