Italian Journal of Zoology

ISSN: 1125-0003 (Print) 1748-5851 (Online) Journal homepage: http://www.tandfonline.com/loi/tizo20

In vivo and *in vitro* experimental analysis of lens epithelium differentiative capacity in *Xenopus laevis*

Daniela Willems , Fulvio Chiacchera & Luigi Bosco

To cite this article: Daniela Willems , Fulvio Chiacchera & Luigi Bosco (2004) *In vivo* and *in vitro* experimental analysis of lens epithelium differentiative capacity in *Xenopus laevis* , Italian Journal of Zoology, 71:3, 181-189, DOI: 10.1080/11250000409356571

To link to this article: https://doi.org/10.1080/11250000409356571

Ital. J. Zool., 71: 181-189 (2004)

In vivo and *in vitro* experimental analysis of lens epithelium differentiative capacity in *Xenopus laevis*

DANIELA WILLEMS
FULVIO CHIACCHERA
LUIGI BOSCO
Department of Ecology and Sustainable Economic Development,
Università della Tuscia,
Via S. Giovanni Decollato 1, I-01100 Viterbo (Italy)

ABSTRACT

In the present study, the differentiative capacities of *Xenopus laevis* lens epithelium were tested by isolating it at different stages of development and implanting it autoplastically and homoplastically into the enucleated orbit. The autonomous differentiative capacity of this tissue was also tested in explants cultured *in vitro* in Leibovitz L 15 with fetal bovine serum added, or serum-free. The results obtained from *in vivo* experiments showed that whatever were the developmental stages, the eye cup was not necessary for differentiation of lens epithelium into lens fiber cells; this suggested that this tissue has an intrinsic capacity to differentiate into lens. Furthermore, lens epithelium showed an autonomous lens differentiation capacity also when isolated and cultured *in vitro*, with or without addition of fetal bovine serum.

KEY WORDS: Differentiation - Lens - Epithelium.

(Received 28 July 2003 - Accepted 4 December 2003)

INTRODUCTION

During development of vertebrate embryos, the first sign of lens morphogenesis is the appearance of a thickening (lens placode) of cephalic ectoderm in the area of apparent contact between ectoderm and eye vesicle (diencephalic outpouching). Immediately after its formation, the lens placode rolls up to form the lens vesicle which detaches from the surrounding ectoderm and occupies its definitive location in the eye cavity. These morphogenetic events are coupled with the differentiation of the lens vesicle posterior wall cells, which typically elongate to form the primary lens fiber nucleus (Woerdman, 1953; Papaconstantinou, 1967; Piatigorsky, 1981). The equatorial epithelial cells are added to the posterior compartment through division and elongation into secondary fiber cells located in the peripheral or cortex region.

Lens development is the result of inductive interactions by which the presumptive lens ectoderm becomes able to form a lens (Liedke, 1951, 1955; Jacobson, 1955, 1958, 1963a, b, c; Reyer, 1958a, b). In the past, a critical role in the formation of lens was attributed to the inductive action of the optic vesicle (Herbst, 1901; Spemann, 1901, 1938). However, the formation of free lenses in absence of the optic vesicle has been reported (for a review, see Jacobson & Sater, 1988). This finding was ascribed to an inductive action of dorsolateral-endomesodermal tissues on the presumptive lens ectoderm occurring before the formation of the optic vesicle. More recently obtained experimental data (Henry & Grainger, 1987, 1990; Grainger *et al.*, 1988) in *Xenopus laevis* (Daudin, 1802) show that in lens morphogenesis the presumptive anterior neural plate is an essential early lens inductor and that the dorsolateral-endomesodermal tissues and the optic vesicle are not individually sufficient inductors of the lens. The presumptive lens ectoderm isolated at early stages of development and cultured with the adjacent neural plate tissues is able to form a lens vesicle which shows a lens-cell specificity by immunofluorescence testing with anti-lens antibody.

There is evidence that the optic cup environment induces cell elongation and primary fiber formation (for a review, see McAvoy, 1980). The prolonged dependence of lens fiber cell elongation and differentiation upon retinal influence was demonstrated experimentally in chick (Coulombre & Coulombre, 1963) and mouse (Muthukkaruppan, 1965) embryos. A factor called lentropin, that promotes cell elongation and crystallin synthesis in primary explants of embryonic chick lens epithelia, was partially purified from the vitreous body (Beebe *et al.*, 1980). Subsequently, lentropin was confirmed to be IGF1 (Beebe *et al.*, 1987).

After the completion of morphogenesis, the lens continues to grow throughout life thanks to the proliferating activity of lens epithelium. Analysis of the DNA synthesis pattern (Harding *et al.*, 1959) and mitosis (Von Sallman, 1952) of lens epithelium indicated that lens

cell proliferation in adults is confined to a narrow band of cells in the pre-equatorial region of the lens, called "germinative zone", and the cells originating from these divisions move below the equator into the transitional zone, where they differentiate into lens fibers (for a review, see McAvoy, 1980; Reddan, 1982).

In order to determine whether lens epithelial cells of vertebrates were able or not to grow and differentiate autonomously, lens epithelium was isolated *in vivo* and *in vitro*. Takeichi (1970) observed differentiation of fiber cells from lens epithelium of six-day-old chick embryo when it was transplanted onto the chorio-allantoic membrane. When explanted, it was able to differentiate into lens fiber cells if cultured *in vitro* (Piatigorsky *et al.*, 1973). Although *in vitro* primary explants of lens epithelium cells lose the ability to differentiate into lens fiber cells near the time of hatching (Piatigorsky & Rothschild, 1972), long-term cultures of dissociated cells from hatched chicken will develop fiber cells in the form of lentoid bodies (Okada *et al.*, 1971).

As regards Amphibia, McDevitt & Yamada (1969) in a short communication reported that the lens epithelium of larval *Rana pipiens* will differentiate, or "modulate", under specific tissue culture conditions, to form cells showing the morphological and biochemical/immunological characteristics of lens fiber cells. By contrast, mature lens epithelial cells of amphibians proliferate rather than elongate when maintained in organ culture (Rothstein *et al.*, 1965; Reddan & Rothstein, 1966). However, a systematic research on the differentiative capacity of isolated lens epithelium of a single anuran species at different stages of development has not been effected. In the present study, we tested this capacity in lens epithelium isolated from larval *X. laevis*.

MATERIAL AND METHODS

Animals

Post-metamorphic (one year after metamorphosis) and larvae of *X. laevis* at stages 50, 53, 56, and 59 (according to Nieuwkoop & Faber, 1956) were used. All larvae were obtained from a single pair after gonadotropic hormones-induced amplexus and ovulation (Profasi, Serono). In all experiments post-metamorphic animals and larvae were anaesthetized with MS 222 1:1500 and 1:2000, respectively, before operation and fixation.

Obtaining lens epithelium

After a small incision in the dorsal pericorneal epidermis, the outer and inner cornea were removed through a circular incision made with iridectomy scissors. In this way the lens was exposed and it was possible to apply a micro-pad imbued with 1% Nile blue sulfate (a vital stain widely used to draw the maps of presumptive regions during ontogenesis) in Holtfreter's solution onto the central region of the lens. The dye diffused in the lens epithelium but a clear, more intense spot remained in the center. The lens was removed using the same operative technique described in a previous paper (Bosco, 1988a), placed into a plastic tissue culture dish containing full-strength Holtfreter's solution and the fiber mass was surgically separated from the epithelium. The epithelium was placed capsule-down on the surface of the culture dish and the peripheral region, surrounding the central intensively labeled area (anterior region of the lens epithelium), was trimmed away with a micro-scalpel.

Histological methods

Larvae and cultures were fixed in 95% ethanol overnight at 4 °C, embedded in paraffin, cut into 5-µm serial sections and stained using the Mallory-Azan method according to Heidenhain (1915).

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 (Bosco *et al.*, 1997), and Western blotting (Fig. 1), all of which confirmed cross-reactivity.

Similar results were obtained by McDevitt & Brahma (1973), who demonstrated the cross-reactivity and resultant identical immunofluorescence profile of an antibody directed against purified *R. pipiens* and *X. laevis* γ crystallins. Nöthiger *et al.* (1971) evidenced also cross-reactivity of purified *R. pipiens* and *Notophthalmus viridescens* γ crystallins.

Rana esculenta adults were sacrificed, and lenses were removed and freed from other tissues. Then 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 & Brahma (1973). The γ crystallin antigen was obtained by successive DEAE-cellulose and DE 32 chromatography of the total lens proteins, as described in a previous paper (Bosco & Venturini, 1988). A mixture of complete Freund's adjuvant and 500 µg of γ crystallin lens proteins was injected three times into rabbits over a period of three weeks. The animals were bled one week after a booster injection without adjuvant. The serum obtained was tested against the homologous antigen by immunoelectrophoresis in 1% agarose gel

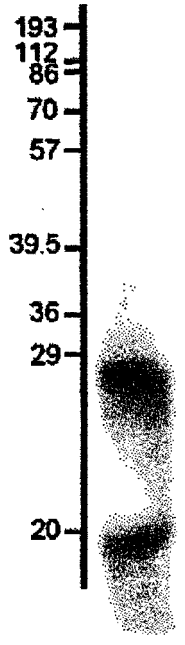

Fig. 1 - Western blot of *X. laevis* whole crystallins stained with *R. esculenta* anti-γ-cristallin antibody raised in rabbit. Two main bands were evident between 20 and 27 kDa, corresponding to the molecular mass range of amphibian crystallins.

with 0.05 mm Veronal buffer pH 8.4 on microscope slides at 5 V/cm. Staining was performed with Coomassie brillant blue R-250. The serum was also tested by Western blotting.

Western blotting

Larval *X. laevis* lenses were homogenized in 5 mM phosphate buffer pH 7 and centrifuged at 50,000 *g* for 60 min. After electrophoretic separation with SDS PAGE (15% acrylamide), proteins were transferred onto nitrocellulose membrane and treated with rabbit anti-γ-crystallin antibody (1:500) after blocking with 3% non fat dried milk. As secondary antibody, anti-rabbit Ig labelled with horseradish peroxidase was used. Reacted antibody was revealed by ECL method (Amersham, UK).

Two main bands were evident between 20 and 27 kDa (Fig. 1), corresponding to the molecular mass range of amphibian γ crystallins (McDevitt & Brahma, 1982; De Jong *et al.*, 1994).

The specificity of the anti-γ-crystallin antibody was tested by staining sections of the normal eye, where only lens fibers were positively labeled.

Retrospective immunofluorescence staining

Retrospective immunofluorescence was effected by the method of Mikailov & Gorgolyuk (1979). For immunochemical analysis, preparations were immersed in chilled xylene to remove Canada balsam (10 °C, 2-7 h) and then washed with ethanol (two, three changes 1 h each) and three changes of buffered saline (pH 7.1, 30 min each).

Anti-γ-crystallin antibody was used as the first antibody in the "sandwich" method of Weller & Coons (1954). After incubation with the first antibody, sections were incubated with a Fluorescein Isothiocyanate (FITC) conjugated secondary antibody (FITC conjugated goat anti-rabbit gamma globulin antibody Pierce No. 31572). Before immunofluorescence, in order to test the specificity of the antiserum used, sections of normal eyes of larval *X. laevis* were incubated either with anti-γ-cristallin antibody, or with pre-immune serum, and then all sections were incubated with FITC conjugated secondary antibody. Sections incubated with pre-immune serum gave negative results. Immunofluorescence was also effected on the lens epithelium immediately after its isolation (Fig. 2) (twenty cases). This also gave a negative immunofluorescence reaction (Fig. 3).

Implant of lens epithelium into the enucleated orbit

Left eye cup, lens and inner cornea were removed through a dorsal incision using the operative technique described elsewhere (Bosco *et al.*, 1993), and the previously isolated lens epithelium was implanted into the enucleated orbit (Fig. 2).

The operated animals were fixed at two, three, four, five, and seven days after implantation in groups of twelve individuals.

Four experimental conditions were considered:

- experiment I: autoplastic implant of lens epithelium in larval *X. laevis* at stage 50 (60 cases);
- experiment II: homoplastic implant of lens epithelium from larval *X. laevis* at stage 54 into the enucleated orbit of larvae at stage 50 (60 cases);
- experiment III: homoplastic implant of lens epithelium from larval *X. laevis* at stage 56 into the enucleated orbit of larvae at stage 50 (60 cases);
- experiment IV: homoplastic implant of lens epithelium from one year metamorphosed *X. laevis* into the enucleated orbit of larvae at stage 50 (60 cases).

Fig. 2 - Lens epithelium implanted into the enucleated orbit of larvae at stage 50, fixed immediately after operation (×1200).

Fig. 3 - Lens epithelium immediately after its isolation showed a negative staining by indirect immunofluorescence with anti-γ-crystallin antibody (×1200).

Explant culture of lens epithelium

Before isolation of lens epithelium, donor larvae of *X. laevis* at stage 56 were immersed for 30 sec 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). Isolated lens epithelium was rinsed four times in Leibovitz 15 (L 15; Flow) diluted with water (2:1) containing 100 U/ml penicillin, 100 µg/ml streptomycin, and 1.5% glutamine (200 mM); then it was placed in a plastic culture dish (3.5 × 10 mm Falcon Plastics) containing 2 ml of L 15 medium diluted with water (2:1), 100 U/ml penicillin, 100 µg/ml streptomycin, and 1.5% glutamine (200 mM).

Lens epithelium was cultured in L 15 supplemented with 10% inactivated fetal bovine serum (experiment V, batch 1, sixty cases) or in serum-free L 15 (experiment V, batch 2, sixty cases). Culture medium was renewed daily. Cultures were terminated after three, five, and seven days.

To ascertain the histological nature of the lens epithelium fragments explanted and used in the experiments, we carried out a thorough histological analysis of the serial sections of lens epithelium fragments implanted into the enucleated orbit of fifteen larvae fixed immediately after the operation. We chose the method reported above to effect controls since lens epithelial fragments, when implanted into the enucleated orbit, can be processed more fruitfully for histological examination.

Histological analysis showed that the lens epithelial fragments were made up exclusively by lens cubical epithelial cells (Fig. 2).

RESULTS

Implant of lens epithelium into the enucleated orbit

In all implantation experiments (experiments I, II, III, IV) lens epithelium formed aggregates of lens fibers (lentoids), regardless of the stage of development of donors (Table I). Histological examination of lens epithelia fixed two, three, four, five, and seven days after implantation into the orbit, showed that the differentiative process, which gave rise to the lens fibers, essentially followed the same pathway in all experiments.

Two days after operation, the implanted lens epithelium had originated a solid vesicle (Fig. 4A) in which the proliferative activity was evident. In the cases fixed three days after the operation, the newly formed aggregates showed a considerable volumetric increase, and the typical cytological signs of lens differentiation (Freeman, 1963; Papaconstantinou, 1967; Eguchi & Okada, 1973; Yamada *et al.*, 1973; Piatigorsky, 1981) such as cell elongation, granular cytoplasm, and increasing cytoplasmic acidophilia, were clearly observed (Fig. 4B). In most cases, almost the entire newly formed cell aggregate underwent lens fiber differentiation, and in the inner part of the newly formed lens structures, formation of "primary lens fiber nucleus" could be observed (Fig. 4B). Histological examination of implanted tissues fixed four days after operation revealed that the lens differentiation process affected the entire newly forming structures, but the histological organization was not normal: primary and secondary lens fibers were normally arranged but coexisted with lens fibers oriented in the anterior-posterior plane rather than concentrically, and the typical lens epithelium (peripheral continuous monolayer of cubical epithelial cells) was not present (Fig. 4C, D). In the following days (five and seven days after operation) the newly forming lens structures did not undergo substantial structural changes, but the lens fibers progressively showed an increasing cytoplasmic acidophilia, revealing an active crystallin synthesis, and nuclei of primary fibers began to disappear (Fig. 5A-D). The first positive immunofluorescence reaction with anti-γ-crystallin antibody was observed in the newly formed structures three days after operation (Fig. 6) confirming the lens specificity of newly formed structures (Fig. 7).

TABLE I - *Implant of lens epithelium into the enucleated orbit. Lens fiber differentiation with reference to days after the operation. The percentage of cases of primary nucleus-like structure formation was calculated from the number of cases which formed lens fibers.*

Experiment	Days after operation	*n*	Lens fiber formation	%	Primary nucleus-like structures	%
I	2	12	0	0	0	0
	3	12	9	75	7	78
	4	12	9	75	8	89
	5	12	11	92	10	91
	7	12	10	83	10	100
II	2	12	0	0	0	0
	3	12	10	83	8	80
	4	12	9	75	8	89
	5	12	11	92	10	91
	7	12	12	100	11	92
III	2	12	0	0	0	0
	3	12	9	75	8	89
	4	12	11	92	8	73
	5	12	10	83	10	100
	7	12	11	92	9	82
IV	2	12	0	0	0	0
	3	12	10	83	9	90
	4	12	11	92	9	82
	5	12	9	75	8	89
	7	12	11	92	10	91

The outer cornea covering the enucleated orbit containing the implanted lens epithelium did not show any sign of lens differentiation throughout experimental period; the only histological modification observed in some cases was a variable scar thickening (Figs 4B-D, 5A-D). The outer cornea gave a negative reaction in the indirect immunofluorescence test with anti-g-crystallin antibody (Figs 6, 7).

Explant culture of lens epithelium

Batch 1 - Culture of lens epithelium in L 15 supplemented with 10% inactivated fetal bovine serum

Under these experimental conditions, the cultured

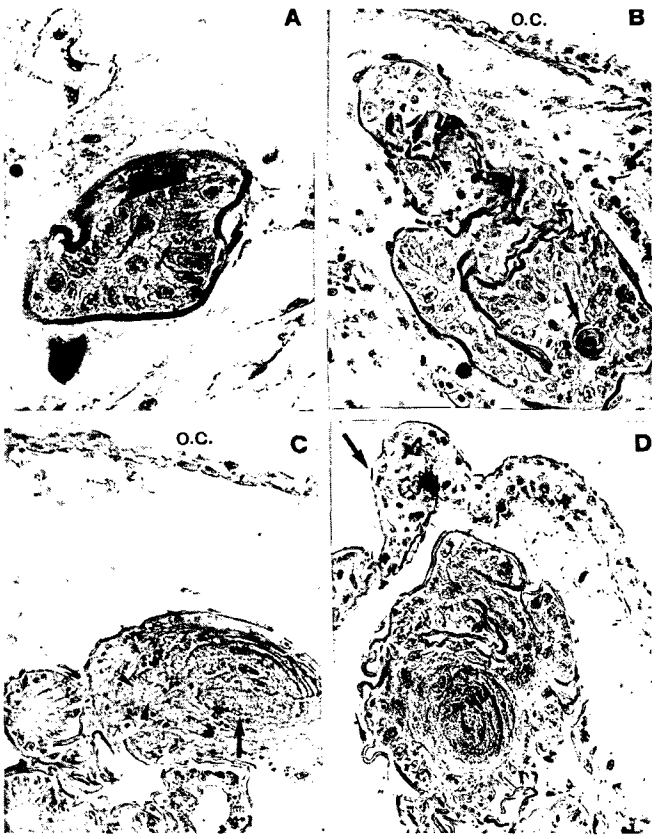

Fig. 4 - Implant of lens epithelium into the enucleated orbit. **A**, experiment I, autoplastic implant of lens epithelium in larval *X. laevis* at stage 50. Two days after the operation: the implanted lens epithelium had originated a solid vesicle in which the proliferative activity was evident (arrows) (×600). **B-D**, experiment III, homoplastic implant of lens epithelium from larval *X. laevis* at stage 56 into the enucleated orbit of larvae at stage 50. **B**, three days after the operation: typical cytological signs of lens differentiation can be observed; in the inner part of newly formed structures the formation of the "primary lens fiber nucleus" is under way (arrow). The outer cornea (o.c.) overlying the enucleated orbit does not show any sign of lens differentiation (×600). **C**, four days after operation: the lens differentiation process affects the whole newly formed structure; lens fibers normally arranged (arrow) coexist with not normally oriented lens fibers (arrowheads). The outer cornea (o.c.) does not show any sign of lens differentiation (×750). **D**, four days after the operation: in some cases the pericorneal epidermis shows a non specific scar thickening (×750).

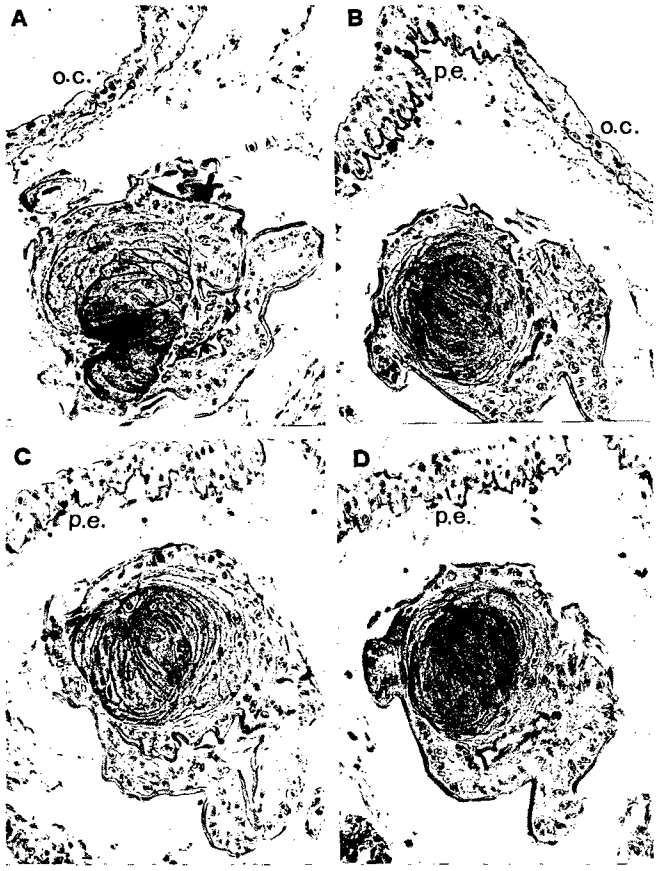

Fig. 5 - Implant of lens epithelium into the enucleated orbit. **A-D**, The lens fibers show an increasing cytoplasmic acidophilia and the nuclei of the primary fibers begin to disappear (×750). **A**, experiment I, autoplastic implant of lens epithelium in larval *X. laevis* at stage 50. Five days after operation. **B**, experiment II, homoplastic implant of lens epithelium from larval *X. laevis* at stage 54 into the enucleated orbit of larvae at stage 50. Five days after operation. **C**, experiment III, homoplastic implant of lens epithelium from larval *X. laevis* at stage 56 into the enucleated orbit of larvae at stage 50. Five days after operation. **D**, experiment IV, homoplastic implant of lens epithelium from one year metamorphosed *X. laevis* into the enucleated orbit of larvae at stage 50. Seven days after operation. Abbreviations: o.c., outer cornea; p.e., pericorneal epidermis.

lens epithelium expressed lens differentiation in thirty-four out sixty explants (Table II). Lens epithelia fixed after three days in culture had formed solid actively proliferating aggregates; the histological organization of these newly formed structures was closely similar to that observed in the implant experiments into the enucleated orbit fixed two days after the operation. Histological examination of cultured lens epithelium fixed after a five-day culture showed that the cell aggregates gave rise to well-differentiated lens fiber cells. In most cases, the whole tissue underwent lens differentiation. The degree of differentiation of the newly formed structures was high. This was revealed as a gradual disappearance of lens fiber cellular nuclei and lens fibers aggregating into the so-called "primary lens fiber nucleus", but in all cases the lens differentiating structures showed an abnormal arrangement of fibers; in many cases the primary nucleus-like structures were surrounded by less thickened lens fibers (Fig. 8). The presence of lens epithelium, as previously defined, was never observed.

Immunofluorescence with anti-γ-crystallin antibody showed the specificity of the lens-differentiating structures and confirmed the histological observations (Fig. 9). The newly formed structures, examined after seven days in culture, did not show any substantial structural difference with those observed after five days in culture.

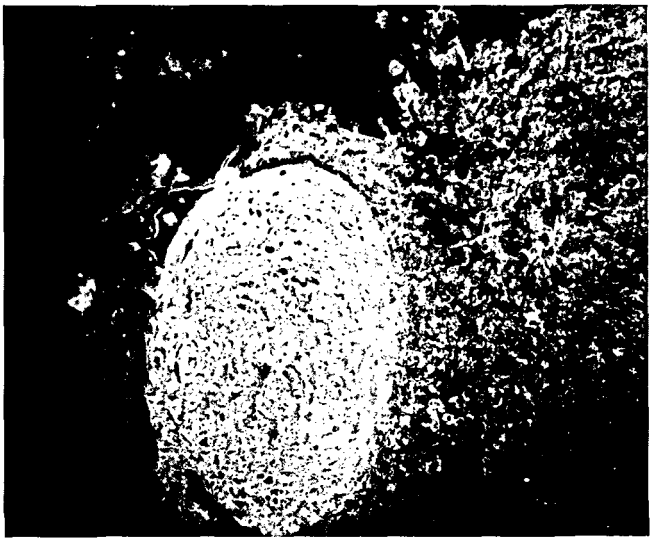

Fig. 6 - Experiment III: implant of lens epithelium from larval *X. laevis* into the enucleated orbit of larvae at stage 50. Three days after operation. The newly formed structure is clearly positive to the immunofluorescence test for anti-γ-crystallins (×1200).

Batch 2 - Culture of lens epithelium in serum-free L 15

Under these experimental conditions, the cultured lens epithelium expressed lens differentiation in thirty out sixty explants (Table II). The differentiative process of the newly formed aggregates occurred with the same series of changes observed in batch 1, but in all cases the extent of the newly formed aggregates was constantly very reduced. Although we did not effect a cell count, this feature could be clearly evaluated by comparison with the extent of the newly formed cell aggregates obtained in batch 1 (Figs 9-10). Immunofluorescence performed with anti-γ-crystallin antibody showed the specificity of the newly formed structures (Fig. 10).

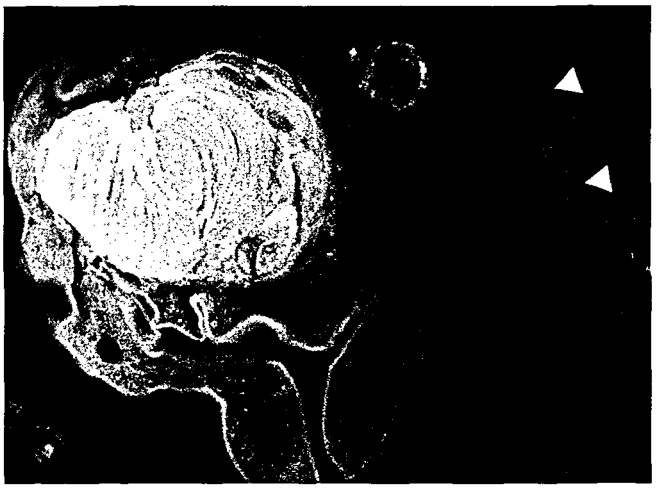

Fig. 7 - Experiment III: implant of lens epithelium from larval *X. laevis* at stage 56 into the enucleated orbit of larvae at stage 50. Five days after the operation: the lens specificity is confirmed by the positive reaction with the indirect immunofluorescence test with anti-γ-crystallin antibody. The outer cornea (arrowheads) gives a negative reaction (×1200).

Fig. 8 - Experiment V, batch 1: explant culture of lens epithelium from larval *X. laevis* at stage 56. Five-day culture: lens-forming structure containing lens fibers irregularly arranged and primary nucleus-like structure (arrows; ×750).

DISCUSSION

The results obtained in this study show that the lens epithelium of *X. laevis* at larval stages 50-53-56-59 and post-metamorphic (one year after metamorphosis) is able to grow and differentiate into lens fibers, when isolated from the eye and implanted into the enucleated orbit. Lens fiber differentiation was also observed when lens epithelium was isolated and cultured *in vitro* in a medium either supplemented with 10% fetal bovine serum or serum-free. Both under *in vivo* and *in vitro* experimental conditions, lens epithelium undergoes a series of cytological changes that are characteristic of fiber differentiation (Freeman, 1963; Papaconstantinou, 1967; Eguchi & Okada, 1973; Yamada *et al.*, 1973; Piatigorsky, 1981): 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 (McDevitt & Brahma, 1973; Brahma & McDevitt, 1974). In several experiments indi-

TABLE II - *Experiment V: explant culture of lens epithelium from larval* X. laevis *at stage 56.*

Experimental batch	Days in culture	*n*	Lens fiber formation	%	Primary nucleus-like	%
1	3	20	0	0	0	0
	5	20	16	80	14	87
	7	20	18	90	15	83
2	3	20	0	0	0	0
	5	20	15	75	14	93
	7	20	16	80	13	81

Fig. 9 - Experiment V, batch 1: explant culture of lens epithelium from larval *X. laevis* at stage 56. Five-day culture: immunofluorescence with anti-γ-crystallin antibody shows the specificity of the lens-differentiating structure (×750).

Fig. 10 - Experiment V, batch 2: explant culture of lens epithelium from larval *X. laevis* at stage 56. Five-day culture: lens epithelium cultured in serum-free L 15, originated a structure very reduced in size but showing intense labeling by immunofluorescence with anti-γ-crystallin antibody (×1200).

rect immunofluorescence staining was used to study lens crystallins ontogeny in different amphibian species, both during development and lens regeneration (for a review, see McDevitt & Brahma, 1982). In these studies, antibodies directed against total soluble lens proteins as well as γ crystallins were used in homologous and heterologous combinations. The results obtained showed that γ crystallins are amongst the first crystallins to appear, and they are confined only to fiber cells (McDevitt *et al.*, 1969; McDevitt & Brahma, 1973, 1979, 1982). All the above reported data evidenced that γ crystallins may be considered useful markers of lens fiber differentiation. In the present study, immunofluorescence with an antibody specific for γ crystallins was employed to detect the presence of these proteins in the newly formed structures. Immunofluorescence performed on lens epithelium immediately after its isolation gave a negative reaction, while the newly formed structures, under the present experimental conditions, showed a clear positive reaction.

In vivo prolonged dependence of lens growth and lens fiber differentiation upon retinal influence was demonstrated by the classic lens inversion experiments of Coulombre & Coulombre (1963), where they turned the lens of a five-day-old chick embryo through 180°, so that the epithelium which normally faced away from the eye cup was then facing it. Under the influence of the new environment the epithelial cells of the central zone elongated and differentiated into lens fiber cells. Subsequently, data obtained in mouse and chick under different experimental conditions confirmed these results (Muthukkaruppan, 1965; Philpott & Coulombre, 1965; McAvoy, 1980; Beebe *et al.*, 1980). The results obtained in experiments I-IV of the present study show that the lens epithelium of *X. laevis* can grow and differentiate into lens fibers even when it is not under direct retinal influence. Different explanations are possible: the eye of *X. laevis* could be a "system" substantially different from that of chick and mouse; the influence exerted by the retina on differentiation of lens epithelium pointed out in chick and mouse might not be the same as in *X. laevis*. Another possibility, which has not been rigorously excluded by the present study is that the retinal factor(s) influencing lens fiber differentiation or its equivalent was present in the enucleated orbit. In this case the ability of the retina to induce lens fiber differentiation should not be considered exclusive to this tissue.

Although it has been pointed out that the differentiation of lens epithelial cells into lens fibers *in vivo* appears to be regulated in some way by retinal influence, there is evidence that when chick lens epithelial cells are isolated in tissue and cell culture in a medium supplemented with different amounts of serum, these cells can differentiate into lens fibers or express the specific markers of lens differentiation (Okada *et al.*, 1971, 1973; Piatigorsky & Rothschild, 1972; Piatigorsky *et al.*, 1972, 1973; Creighton *et al.*, 1976). These findings indicate that the transformation of lens epithelial cells into fibers results from a programmed differentiation which can take place autonomously in tissue and cell culture (Pi-

atigorsky *et al.*, 1973). However, it was pointed out that lens fiber differentiation of lens epithelial cells may be stimulated *in vitro* by different agents present in the serum added to culture medium (Piatigorsky, 1981).

The results obtained in experiment V show that lens epithelial cells of larval *X. laevis* can differentiate into lens fibers in tissue culture in a medium supplemented with serum (batch 1) or a serum-free one (batch 2). When lens epithelium was cultured in a serum-free medium, it expressed high levels of γ crystallins, as revealed by the immunofluorescence. However, under these culture conditions the cell aggregates were manifestly reduced in size, so the presence of serum appears to influence the growth of the newly formed cell aggregates rather than the expression of lens differentiative markers. With regard to this, the lens epithelium of *X. laevis* seems to have an autonomous differentiative capacity not revealed in other vertebrates. Mouse lens epithelial explants maintained in serum-free medium remained as a monolayer of cubical epithelial cells (Lovicu *et al.*, 1995).

In the last ten years the differentiation of rat lens epithelium into lens fibers has been the object of a specific series of investigations. It has been shown that in culture, both basic Fibroblast Growth Factor (bFGF) and acidic Fibroblast Growth Factor (aFGF) induce lens epithelial cells to proliferate, migrate, and differentiate into lens fibers in a progressive dependent manner (Chamberlain & McAvoy, 1987, 1989; McAvoy & Chamberlain, 1989). Fibroblast growth factors have been found in ocular media, in lens cells and throughout the lens capsule (Schulz *et al.*, 1993). On the basis of these results, it was postulated (Schulz *et al.*, 1993) that the normal lens polarity is determined by differences in FGF bio-availability in the two ocular media. More recently (Lovicu *et al.*, 1995), it was evidenced that the vitreous, but not the aqueous humour, induces morphologic changes characteristic of fiber differentiation and accumulation of β-crystallin, and using a variety of techniques it was shown (Schulz *et al.*, 1993) that FGF activity levels are higher in vitreous than in aqueous humour.

These results suggest that in rodents, FGFs and their distribution in the eye play a necessary and sufficient role in the lens fiber differentiative process of lens epithelium.

In chicken embryo, lens fiber differentiation of lens epithelium seems to be regulated quite differently. The growth factors necessary for lens fiber cell differentiation, proliferation, and survival *in vivo* appear to be other than FGFs, i.e., insulin-like growth factor, IGF1, and IGF2, even though it cannot be ruled out that FGFs are involved in the same aspects of lens fiber cell differentiation (Beebe *et al.*, 1987; Huang *et al.*, 2003).

The results obtained in the present study indicate that in *X. laevis* differentiation of lens epithelial cell into lens fiber can occur independently of exogenous stimuli. As a consequence, the failure of central lens epithelial cells to differentiate into lens fiber in the normal eye could be attributed to the action of an inhibitor, or generically the aqueous humour could be an environment that does not allow the expression of the lens differentiative capacity (a non-permissive environment).

On the other hand, the autonomous capacities shown by lens epithelium to differentiate *in vitro* do not rule out that *in vivo* proliferation of equatorial lens epithelial cells and expression of differentiative capacities could be regulated temporally and spatially by the action of growth factors such as FGFs.

REFERENCES

Beebe D. C., Feagans D. E., Jebens H. A. H., 1980 - Lentropin: a factor in vitreous humor which promotes lens fibers cell differentiation. Proc. Natl. Acad. Sci. U.S.A., 77: 490-493.

Beebe D. C., Silver M. H., Belcker K. S., 1987 - Lentropin, a protein that controls lens fiber formation, is related functionally and immunologically to the insulin-like factors. Proc. Natl. Acad. Sci. U.S.A., 84: 2327-2330.

Bosco L., 1988a - The problem of lens regeneration in anuran amphibian tadpoles. Acta Embryol. Morphol. Exp., 1: 25-38.

Bosco L., 1988b - Transdifferentiation of ocular tissues in larval *Xenopus laevis*. Differentiation, 39: 4-15.

Bosco L., Testa O., Venturini G., Willems D., 1997 - Lens fiber transdifferentiation in cultured larval *Xenopus laevis* outer cornea under the influence of neural retina-conditioned medium. Cell. Mol. Life Sci., 53: 921-928.

Bosco L., Valle C., Willems D., 1993 - *In vivo* and *in vitro* experimental analysis of lens regeneration in larval *Xenopus laevis*. Dev. Growth Differ., 35: 257-270.

Bosco L., Venturini G., 1988 - Separation and characterization of the lens proteins of the amphibian *Rana esculenta*. Atti Accad. Naz. Lincei Rend. Cl. Sci. Fis. Mat. Nat., 82: 353-359.

Brahma S., McDevitt D. S., 1974 - Ontogeny and localization of the lens crystallins in *Xenopus laevis* lens regeneration. J. Embryol. Exp. Morphol., 3: 783-794.

Chamberlain C. G., McAvoy J. W., 1987 - Evidence that fibroblast growth factor promotes lens fiber differentiation. Curr. Eye Res., 6: 1165-1168.

Chamberlain C. G., McAvoy J. W., 1989 - Induction of lens fiber differentiation by acidic and basic fibroblast growth factor (FGF). Growth Factors, 1: 125-134.

Coulombre J., Coulombre A. J., 1963 - Lens development: fiber elongation and lens orientation. Science, 142: 1489-1490.

Creighton M. O., Mousa G. Y., Trevithick J. R., 1976 - Differentiation of rat lens epithelial cells in tissue culture. (I) Effects of cell density, medium and embryonic age of initial culture. Differentiation, 6: 155-167.

De Jong W. W., Lubsen N. H., Kraft H. J., 1994 - Molecular evolution of the eye lens. Progr. Retin. Eye Res., 13: 391-442,

Eguchi G., Okada T. S., 1973 - Differentiation of lens tissue from the progeny of chick retinal pigment cells culture *in vitro*: a demonstration of a switch of cell types in clonal cell culture. Proc. Natl. Acad. Sci. U.S.A., 70: 1495-1499.

Freeman G., 1963 - Lens regeneration from cornea in *Xenopus laevis*. J. Exp. Zool., 154: 39-65.

Grainger R. M., Henry J. J., Henderson R., 1988 - Reinvestigation of the role of the optic vesicle in embryonic lens induction. Development, 192: 517-526.

Harding C. V., Donn A., Srinivasan D. R., 1959 - Incorporation of thymidine by injured lens epithelium. Exp. Cell Res., 18: 582-585.

Heidenhain, M. 1915 - Über die mallorysche Bindegewebsfärbung mit Karmin und Azokarmin als Vorfarben. Z. Wiss. Mikrosk., 32: 361-372.

Henry J. J., Grainger R. M., 1987 - Inductive interactions in the spatial and temporal restriction of lens-forming potential in embryonic ectoderm of *Xenopus laevis*. Dev. Biol., 124: 200-214.

Henry J. J., Grainger R. M., 1990 - Early tissue interactions leading to embryonic lens formation in *Xenopus laevis*. Dev. Biol., 141: 149-163.

Herbst C., 1901 - Formative Teize in der tierischen Ontogenese. Arth. Georgi, Leipzig.

Hoperskaya O. A., Zviadadze K. G., 1981 - Transdifferentiation of adult frog iris in retina or lens by exogeneous influences. Dev. Growth & Differ., 23: 201-213.

Huang J. X., Feldmeier M., Shui Y. B., Beebe D. C., 2003 - Evaluation of Fibroblast Growth Factor signaling during lens fiber cell differentiation. Invest. Ophthalmol. Vis. Sci., 44: 680-690.

Hyatt G. A., Beebe D. C., 1993 - Regulation of lens cell growth and polarity by embryo-specific growth factor and by inhibitors of lens cell proliferation and differentiation. Development, 117: 701-709.

Jacobson A. G., 1955 - The roles of the optic vesicle and other head tissues in lens induction. Proc. Natl. Acad. Sci. U.S.A., 41: 522-525.

Jacobson A. G., 1958 - The roles of neural and non-neural tissues in lens induction. J. Exp. Zool., 139: 525-557.

Jacobson A. G., 1963a - The determination and positioning of the nose lens and ear. I. Interaction within the ectoderm and between the ectoderm and underlying tissues. J. Exp. Zool., 154: 273-284.

Jacobson A. G., 1963b - The determination and positioning of the nose lens and ear. II. The role of the endoderm. J. Exp. Zool., 154: 285-292.

Jacobson A. G., 1963c - The determination and positioning of the nose lens and ear. III. Effects of reversing the antero-posterior axis of epidermis, neural plate, and neural folds. J. Exp. Zool., 154: 293-304.

Jacobson A. G., Sater A. K., 1988 - Features of embryonic induction. Development, 104: 341-359.

Liedke K. B., 1951 - Lens competence in *Amblystoma punctatum*. J. Exp. Zool., 117: 573-591.

Liedke K. B., 1955 - Studies on lens induction in *Amblystoma punctatum*. J. Exp. Zool., 130: 353-379.

Lovicu F. J., Chamberlain C. G., McAvoy J. W., 1995 - Differential effects of aqueous and vitreous on fiber differentiation and extracellular matrix accumulation in lens epithelial explants. Invest. Ophthalmol. Vis. Sci., 36: 1459-1469.

McAvoy J. W., 1980 - Induction of the eye lens. Differentiation, 17: 137-149.

McAvoy J. W., Chamberlain C. G., 1989 - Fibroblast growth factor (FGF) induces different responses in lens epithelial cells depending on its concentration. Development, 107: 221-228.

McDevitt D. S., Brahma S. K., 1973 - Ontogeny and localization of the crystallins during embryonic lens development in *Xenopus laevis*. J. Exp. Zool., 186: 127-140.

McDevitt D. S., Brahma S. K., 1979 - Embryonic appearance of α, β and γ crystallins in the periodic albinism (a^p) mutant of *Xenopus laevis*. Differentiation, 14: 107-112.

McDevitt D. S., Brahma S. K., 1982 - Ontogeny and localization of the crystallins in eye lens development and regeneration. *In:* D. S. McDevitt (ed.), Cell biology of the eye. Academic Press, London, pp. 143-191.

McDevitt D. S., Meza I., Yamada T., 1969 - Immunofluorescence localization of the crystallins in amphibian lens development, with special reference to the gamma crystallins. Dev. Biol., 19: 581-607.

McDevitt D. S., Yamada T., 1969 - Acquisition of antigenic specificity by amphibian lens epithelial cells in culture. Am. Zool., 9: 1130-1131.

Mikailov M., Gorgolyuk N. A., 1979 - Retrospective immunofluorescence of specific antigens in stained and balsam embedded sections of developing amphibians lens. Experientia, 36: 113-116.

Muthukkaruppan V., 1965 - Inductive tissue interaction in the development of the mouse lens *in vitro*. J. Exp. Zool., 159: 269-288.

Nieuwkoop P. D., Faber J., 1956 - Normal table of *Xenopus laevis* (Daudin). North Holland Pub. Co., Amsterdam.

Nöthiger R., McDevitt D. S., Yamada T., 1971 - Comparison of γ crystallins from frog and newt. Exp. Eye Res., 12: 94-98.

Okada T. S., Eguchi G., Takeichi M., 1971 - The expression of differentiation by chicken lens epithelium in *in vitro* cell culture. Dev. Growth & Differ., 13: 323-336.

Okada T. S., Eguchi G., Takeichi M., 1973 - The retention of differentiated properties by lens epithelial cells in clonal cell culture. Dev. Biol., 34: 321-333.

Papaconstantinou J., 1967 - Molecular aspects of lens cell differentiation. Science, 156: 338-345.

Philpott J. W., Coulombre A. J., 1965 - Lens development. II. Differentiation of embryonic chick lens epithelial cells *in vivo* and *in vitro*. Exp. Cell Res., 38: 635-644.

Piatigorsky J., 1981 - Lens differentiation in vertebrates. Differentiation, 19: 134-153.

Piatigorsky J., Rothschild S. S., 1972 - Loss during development of the ability of chick embryonic lens cells to elongate in culture: inverse relationships between cell division and elongation. Dev. Biol., 28: 382-389.

Piatigorsky J., Rothschild S. S., Milstone L. M., 1973 - Differentiation of lens fibers in explanted embryonic chick lens epithelia. Dev. Biol., 34: 334-335.

Piatigorsky J., Webster H. D., Craig S. P., 1972 - Protein synthesis and ultrastructure during the formation of embryonic chick lens fibers *in vivo* and *in vitro*. Dev. Biol., 27: 176-189.

Reddan J. R., 1982 - Control of cell division in the ocular lens, retina and vitreous humor. *In:* D. S. McDevitt (ed.), Cell biology of the eye. Academic Press, London, pp. 193-242.

Reddan J. R., Rothstein H., 1966 - Growth dynamics of an amphibian tissue. J. Cell Physiol., 67: 307-318.

Reyer R. W., 1958a - Studies on lens induction in *Amblystoma punctatum* and *Triturus viridescens viridescens*. I. Transplants of prospective belly ectoderm. J. Exp. Zool., 138: 505-555.

Reyer R. W., 1958b - Studies on lens induction in *Amblystoma punctatum* and *Triturus viridescens viridescens*. II. Transplants of prospective lateral head ectoderm. J. Exp. Zool., 139: 137-180.

Rothstein H., Lauder J. N., Weinsieder A., 1965 - *In vitro* culture of amphibian lenses. Nature, 19: 1267-1269.

Schulz M. W., Chamberlain C. G., De Jong R. U., McAvoy J. W., 1993 - Acidic and basic FGF in ocular media and lens: implications for lens polarity and growth patterns. Development, 118: 117-126.

Spemann H., 1901 - Über Korrelationen in der Entwicklung des Auges. Verh. Anat. Ges., 15: 61-69.

Spemann H., 1938 - Embryonic development and induction. University Press, New Haven.

Takeichi M., 1970 - Growth of the chicken embryonic lens transplanted onto the chorio-allantoic membrane. Dev. Growth & Differ., 12: 21-30.

Von Sallman L., 1952 - Experimental studies on early lens changes after roentgen irradiation. III. Effects of X-irradiation on mitotic activity and nuclear fragmentation of lens epithelium in normal and cystein-treated rabbit. Arch. Ophtalmol., 5: 560-567.

Weller T. H., Coons A. H., 1954 - Fluorescent antibody studies with agents of *varicella* and *herpes zoster* propagated *in vitro*. Proc. Soc. Exp. Biol. Med., 86: 789-794.

Woerdman M. W., 1953 - The differentiation of the crystalline lens. J. Embryol. Exp. Morphol., 1: 301-305.

Yamada T., Reese D. H., McDevitt D. S., 1973 - Transformation of iris into lens *in vitro* and its dependency on neural retina. Differentiation, 1: 65-82.