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Differentiation of Lens-Like Structures from Newt Iris Epithelial Cells *In Vitro*

(cytodifferentiation/determination/lens regeneration/eye)

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ABSTRACT **Dissociated cells of pigmented iris epithelium from adult newts grew intensively in monolayer cultures after a lag of two to three weeks. During the lag period, depigmentation occurred in many cells. When cultures became confluent five to six weeks after seeding, many tiny lens-like structures (30–70 per plate) differentiated from dense foci of amelanotic epithelial cells. These lens-like structures appeared in all cultures originated from cells of ventral as well as dorsal iris. The identification of these structures as lens was established by both immunological and ultrastructural techniques.**

The phenomenon of Wolffian lens regeneration in newts has attracted the interest of developmental biologists since Wolff's description of it in 1895 (1), for it provides an opportunity to study a switch in differentiation from pigmented iris cells to lens (2–6). However, the analysis has not yet been done thoroughly at the cellular level, since most studies were conducted *in vivo* where the phenomenon involves interactions among different cell types (2–9). What is most needed is a simple cell culture system that extends studies that have already been conducted with organ culture techniques with varying degrees of success (10, 11, 27, 28).

We have shown that the switch from pigmented retinal cells of 9-day-old check embryos into lens cells occurs in clonal cell culture without interactions with other cell types (14). The question then arises whether this approach can be extended to iris epithelial cells of newts which possess the capacity for Wolffian regeneration *in vivo*. This communication describes the differentiation of lens-like structures in cultures of dissociated iris epithelial cells of adult newts.

MATERIALS AND METHODS

Twenty-five to 50 adult newts, *Cynops* (*Triturus*) *pyrrhogaster* were used in each experiment. Isolated whole eyes were sterilized by three 1-min immersions in 70% ethanol. The iris-rings (*iris pars iridica*) were then removed (11) and treated in an enzyme mixture that contained 5 parts of 0.4% (v/v) trypsin (Difco, 1:250) and one part of 0.08% (v/v) collagenase (Worthington) in solution A for 60 to 90 min at 25°. Solution A was composed of Ca^{++}- and Mg^{++}-free modified Hanks' (diluted with triple glass-distilled water to 80% strength) balanced salt solution. The adherent iris stroma was then cleanly removed from the iris-ring. For dissociation, isolated, clean iris epithelia were incubated again in the same enzyme mixture for 20 min. More than 99.5% of cells in the resulting suspension were pigmented. In some experiments the isolated iris-rings were divided into dorsal and ventral halves before dissociation and cells from each half were separately cultured. Culture dishes were 3.5 cm Falcon plastic ware. Diluted Leibovitz medium L-15 (12) was used as the culture medium and was prepared by dissolving 0.894 g of commercial Leibovitz L-15 powder (GIBCO) in 90 ml of triple-distilled water; this was supplemented with 10 ml of fetal calf serum (GIBCO, Control No. A322102), 3200 IU of penicillin, and 4 mg of streptomycin before filtration by Millipore filter.

For the immunological identification of lens products in cultured cells, antisera were prepared in rabbits against the 15,000 × *g* supernatant of adult newt lenses in 20 mM Tris·HCl buffer (pH 7.5) and against the α-, β-, and γ-crystallin fractions, respectively. The separation of crystallins from the whole lens extract was made by column chromatography through Sephadex G-50 and G-200, and DEAE-Sephadex A-50 according to established methods (13). Sixteen rabbits were injected, one each with 40, 5, 3, and 1 mg of each protein fraction, respectively. Each rabbit received injections of protein with an incomplete Freund's adjuvant at intervals of two weeks. Antisera were usually obtained 10 days after the second injection. In immunodiffusion tests (15) anti-whole lens extract and anti-α-crystallin cross-reacted faintly with extracts of heterologous organs of newts. This cross-reactivity was removed by absorbing the antisera with saline extracts of liver homogenate of adult newts. Cross-reactions of anti-α-crystallin with the heterologous crystallins were also removed by absorption with β-crystallin solution, whereas anti-β-crystallin and anti-γ-crystallin did not cross-react with the heterologous test-antigens (Fig. 1).

For immunodiffusion tests of lens antigens from cultured cells, all four types of antisera were used. Anti-whole lens extract, conjugated with fluorescein isothiocyanate, was used to stain cultures fixed with absolute ethanol at −20°C and postfixed with the same reagent for 20 min at room temperature. Often, smear preparations of freshly dissociated cells and of cells harvested from cultures were made on microscope slide glasses, and were stained by the fluorescent antibody

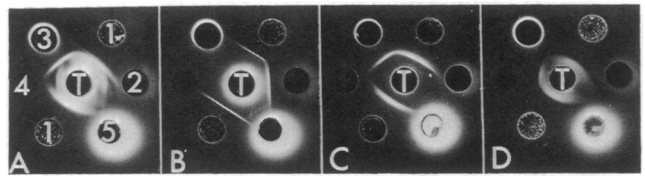

FIG. 1. Relative specificity of antisera revealed by immunodiffusion: the central well (T) contains anti-whole lens-extract (A), anti-α-crystallin (B), anti-β-crystallin (C), and anti-γ-crystallin (D). Test antigens: 1: whole lens extract, 2: α-crystallin, 3: β-crystallin, 4: γ-crystallin, 5: liver extract.

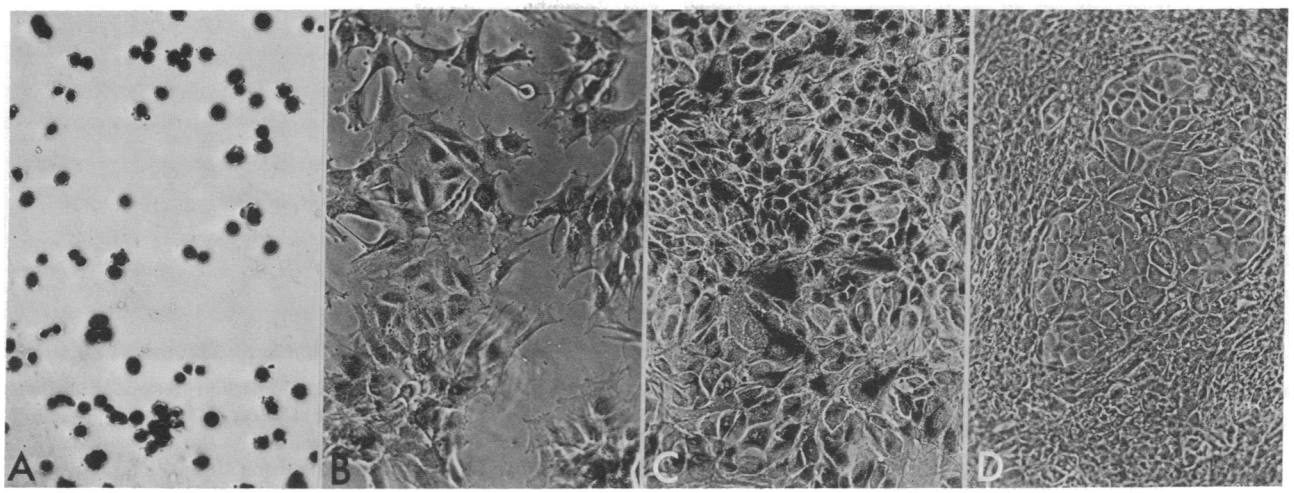

FIG. 2. Iris epithelial cells cultured *in vitro* (phase microscopy, × 100). (A) Heavily pigmented cells attached to the dish on the 2nd day of culture. Some cells are spreading. (B) Actively growing cells in the log phase of growth, 20 days after inoculation. (C) Well-developed monolayer consisting of typical epithelial cells which are repigmenting. (D) Monolayer of nonpigmented cells with foci of packed cells.

technique after fixation as above. The usual controls for specificity of the fluorescent antibody were prepared according to Nairn (16).

For observations by electron microscopy, cultures with lens-like structures were fixed *in situ* (17) with 3.0% glutaraldehyde (pH 7.2) for 90 min and postfixed with 1.0% OsO_4, embedded in Epon 812, and sectioned.

RESULTS

Cell culture

Cells (0.2 to 1.0 × 10^5) were inoculated into 2.0 ml of medium in 3.5-cm plastic dishes. About one-half of the cells attached to the plastic dish during the first three days (Fig. 2A). Thereafter they began to spread gradually and discharge their pigment granules (14, 18–20). Mitosis was first observed in the cultures about 10 days after seeding; therefore the number of cells per dish always decreased in this early phase of culture (Fig. 3). From two weeks onwards, the number of depigmented and lightly pigmented cells increased (Fig. 2B). Concomitant with cell proliferation, foci consisting of coherent nonpigmented epithelial cells developed (Fig. 2D). Both the size and number of these foci increased rapidly; they were usually surrounded by more or less elongated cells which did not seem to proliferate actively. Some of these epithelial foci became pigmented again (Fig. 2C) (14, 18); others remained amelanotic, consisting of cells adhering to each other firmly. When the rate of increase in cell number declined, as the confluent state was reached (Fig. 3), usually 5 to 6 weeks after inoculation, the cells in many of the nonpigmented epithelial foci began to pile up and to become elongated (Fig. 4). These structures resemble the lentoid bodies that develop in cultures of chick lens epithelial cells (21, 22) and of chick pigmented retinal cells (14).

The processes leading to the formation of lens-like structures from pigmented cells of iris were similar regardless of whether dorsal or ventral cells were used in starting the cultures (Fig. 3), although ventral cells always showed a slightly higher growth rate in the log phase than the dorsal cells. In

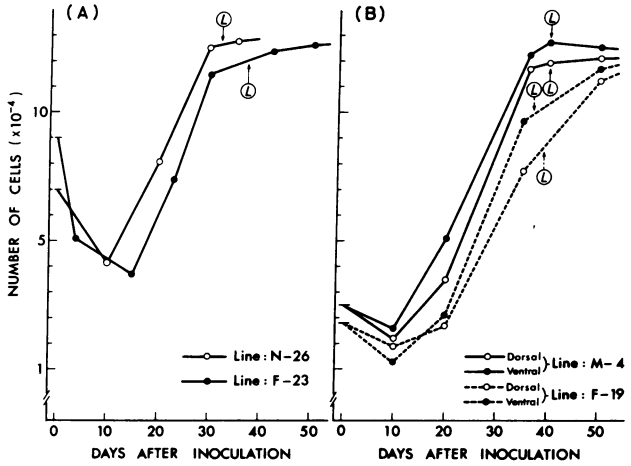

FIG. 3. Growth curves of primary cultures. (A) Two cultures of iris epithelial cells dissociated from the whole iris-ring. (B) Parallel cultures of dorsal and ventral iris epithelial cells. The lens-like structures first appeared at the point indicated by L.

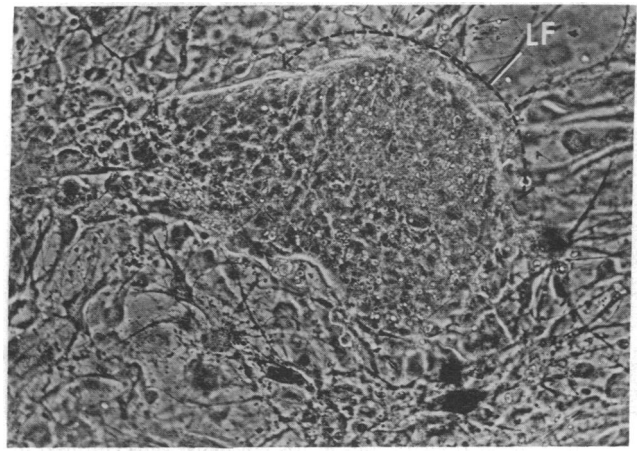

FIG. 4. Well-formed lens-like structure developing in culture. The part consisting of well differentiated lens fiber cells is indicated by LF (phase microscopy, × 100).

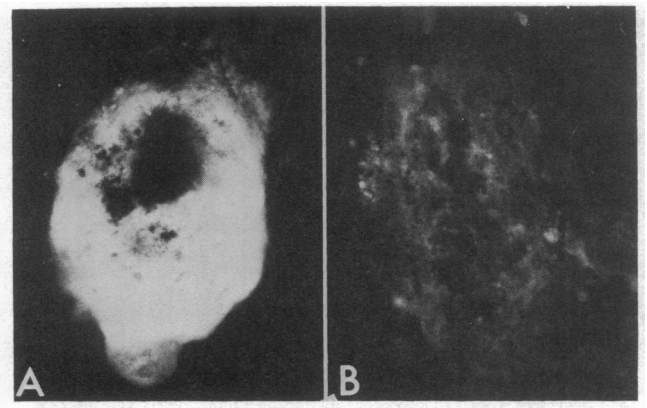

FIG. 5. Lens-like structures stained with fluorescent antiserum against whole lens extract (A) and with nonimmunized rabbit serum globulin conjugated with fluorescein isothiocyanate (B). (× 120).

each of six experiments, in all cultures plated longer than 50 days, lens-like structures were invariably formed; their numbers were about 30 to 70 per plate.

In order to confirm the pigment cell origin of lens-like structures formed *in vitro*, the following test was made in three experiments. Soon after seeding, well separated clusters consisting of only heavily pigmented epithelial cells were marked and further outgrowth from these marked clusters was followed daily. In 37 of 85 marked clusters, lens-like structures appeared.

Identification of lens structures

Fluorescent Antibody Staining. Well-developed lens-like structures showed specific fluorescence when stained with fluorescent antibody against the whole extract of newt lens (Figs. 5A and B). Fluorescent antibody staining was also applied to smear preparations. None of the freshly isolated iris epithelial cells reacted with the fluorescent antibody. Cells harvested from the cultures after 30 and 50 days, respectively, reacted positively (Table 1). A small number of positive cells appeared in cultures after 30 days, although visible

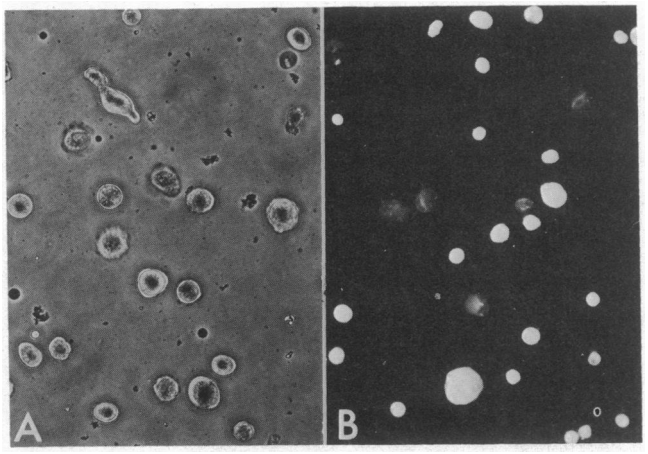

FIG. 6. Smear preparations of iris epithelial cells cultured for 50 days. (A) Freshly dissociated cells. (B) Cells showing specific fluorescence; stained after fixation of another smear preparation from the same cell suspension used for (A). (× 120).

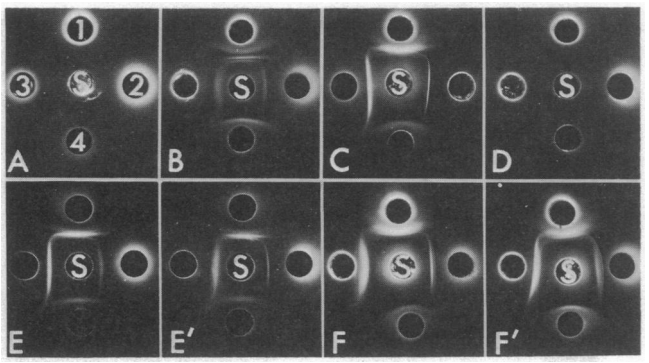

FIG. 7. Results of immunodiffusion tests: Iris epithelium (A), lens regenerant *in vivo* 25 days after lens removal (B), normal full-grown lens (C), cells from dorsal iris cultured for 30 days (dorsal) (D), cells cultured for 40 days (E: dorsal and E′: ventral), cells cultured for 50 days (F: dorsal and F′: ventral). S, test antigens. 1, antiserum against whole lens extract; 2, anti-α-crystallin; 3, anti-β-crystallin; and 4, anti-γ-crystallins. The arrangement of antisera is the same in all tests. No positive reactions were detected in A and D against any antisera.

lens structures had not yet developed at this stage *in vitro*. In 50-day cultures derived from both the ventral and dorsal iris epithelium, more than 50% of cells contained lens-specific antigens (Fig. 6).

Immunodiffusion Tests. About 1×10^4 cells were harvested at 30, 40, and 50 days, homogenized with 0.3 ml of a 50 mM Tris·HCl buffer (pH 7.5) and put in each well of an Ouchterlony immunodiffusion plate on microscope slides. A typical example of an immunodiffusion test is shown in Fig. 7. Positive reactions to all four antisera were recorded with homogenates of cells from cultures kept for more than 40 days and containing lens-like structures. The pattern of reactions was essentially the same as that seen with homogenates of 25-day-old lens regenerants developed in eyes *in situ* (23, 24) and with normal lens (Fig. 7). The reaction of antisera against homogenate of iris epithelial cells was completely negative.

Electron Microscopy. Observations on thin sections of well-differentiated lens-like structures revealed that the cells constituting such structures had the highly specialized ultrastructural characteristics of cortical lens fibers of lenses *in situ* (25, 26). The cytoplasm consisted mainly of polysomes of 8 to 12 ribosomal units and of homogeneous fine structures of fibrous nature (Fig. 8). A small number of disintegrating mitochondrial profiles were observed. Membrane systems such

TABLE 1. *The presence of lens antigen in cultured iris epithelial cells as revealed by staining with fluorescent antibody to whole lens extract*

	Cultured for 30 days		Cultured for 50 days	
Line: M4D/V	Dorsal	Ventral	Dorsal	Ventral
Total number of cells counted	596	601	1287	731
Number of positive cells (%)	33 (5.5)	17 (2.6)	881 (68.4)	383 (52.4)
Number of negative cells (%)	563 (94.5)	584 (97.4)	406 (31.6)	348 (47.6)

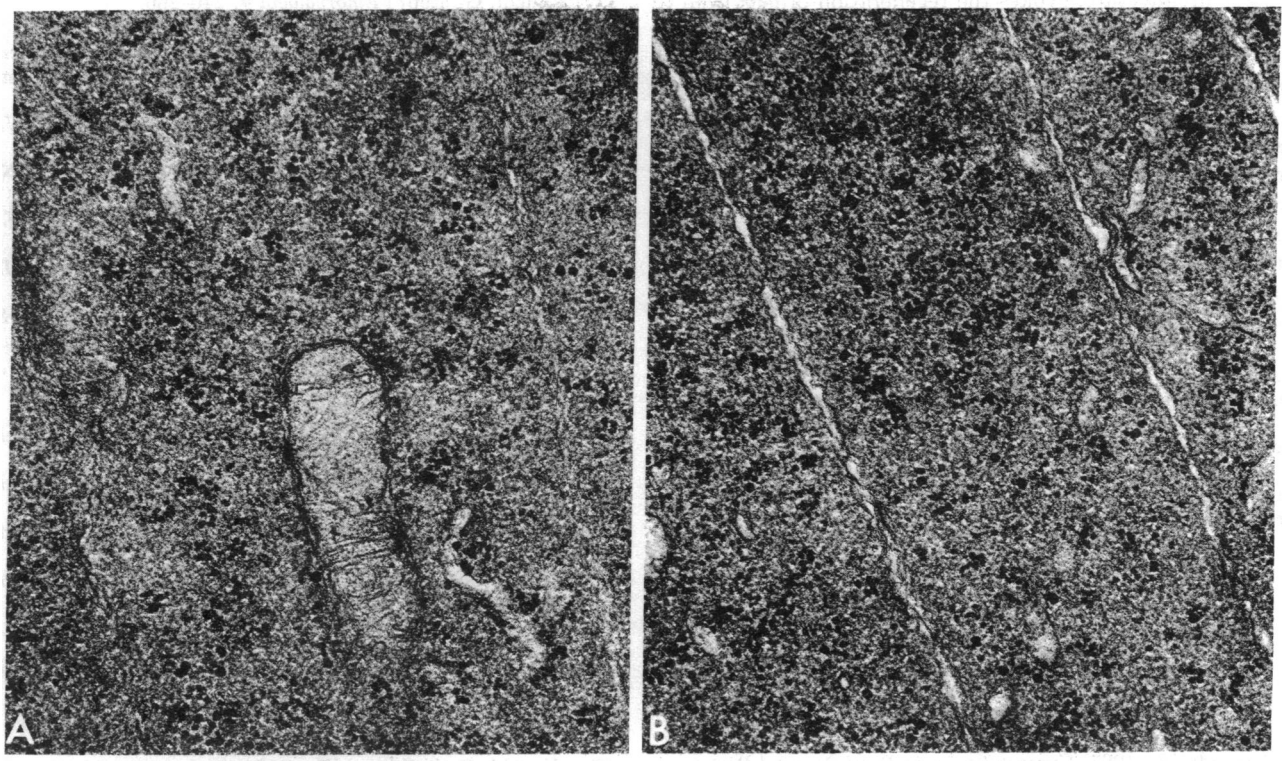

FIG. 8. Ultrastructural profiles of lens-like structures differentiated from iris epithelial cells cultured *in vitro* (A) and of cortical lens fibers developed in the newt eye *in situ* (B). In both figures the cytoplasm has a few disintegrating mitochondria and is nearly free of endoplasmic reticula, but contains a large number of polysomes.

as endoplasmic reticulum and Golgi complex were scarce. It is noteworthy that the surfaces of these lens-like structures were sometimes covered by an acellular, thick, fibrous structure resembling the lens capsule.

DISCUSSION

The present experiments have demonstrated the appearance of lens-like structures after prolonged cultivation of pigmented epithelial cells from the iris of adult newts. The specific lens properties of these structures were demonstrated both by electron microscopy and immunological techniques. The question arises whether such structures were derived from pigmented epithelial cells and not from inadvertently contaminating lens cells. All cultures were started from carefully cleaned iris epithelial cells, of which almost all were pigmented. The possible contamination of lens cells was checked by staining smear preparations of the original inocula with fluorescent antibody and no positive cells were observed. Iris homogenate did not cross-react with antisera against whole lens extracts and crystallin fractions in immunodiffusion tests (Fig. 7A). Immunologically positive cells appeared only after a prolonged period in culture. Lens differentiation from carefully marked clusters consisting of only pigmented cells occurred in many cases. Thus, it can be safely concluded that the lens-structures differentiated from the progeny of pigmented epithelial cells of the iris cultured *in vitro*. Successful cultivation of dissociated newt iris-epithelial cells has been reported recently by Horstman and Zalik (19); however, these authors did not observe the appearance of lens-like structures in their cultures.

The role of an inductive influence of the neural retina has been emphasized in studies of lens differentiation from the regenerant *in situ* (2–6), and has been confirmed in *in vitro* studies in which tissue fragments were used (10, 11, 27, 28). Therefore, the present observations of autonomous lens differentiation stand in sharp contrast to earlier works. Differences in culture methods could explain these differences. We have used monolayer cultures of dissociated cells rather than integrated iris explants. Also, variation in batches of fetal calf serum cannot be excluded here.

In the regeneration of lens *in situ*, DNA replication always precedes the differentiation of lens cells (6, 8, 29–31). In cultures of dissociated iris, cells underwent an actively growing phase before lens specificities were expressed. The switch to lens from newt iris cells occurred in about one-half the time required for the similar switch from chick pigmented retina (14). However, transformation *in vitro* in newts was slower than lens regeneration *in situ;* possibly the absence of other cells to interact with iris cells may result in a delay in "switching."

In our cultures, lens structures were invariably formed from cells of both the dorsal and the ventral iris epithelium, whereas, lens regeneration *in situ* usually occurs only from the dorsal part of the iris. In reflecting on this point, the following observations may be relevant. After lentectomy, a "loosening" of cell association was observed in the dorsal marginal iris, while the characteristic tissue architecture of the ventral iris epithelium was well maintained (7, 8, 32). The present cell cultures started from enzymatically dissociated cells of dorsal and ventral iris, and we may speculate that changes in the properties of cell surfaces (33) provide the signal for expressing lens potentials in cells of the ventral iris. The fact that the administration of a potent carcinogen, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, which must drastically disturb the orig-

inal tissue architecture, evokes the regeneration of lens from the ventral iris *in situ* (34) supports the above speculation. Success in eliciting lens from the ventral iris by inserting a celluloid septum to divide the pupil into two subdivisions (35) can be interpreted similarly.

Recently, a low frequency of lens differentiation was obtained in culture of intact pieces but not of dissociated cells of the ventral iris (28). This occurred, however, under the influence of neural retinae.

The present cell culture system should provide a useful opportunity for studying the conditions under which the switching occurs.

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