

Direct transdifferentiation in the vertebrate retina

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ABSTRACT Transdifferentiation is the process by which differentiated cells alter their identity to become other, distinct cell types. The conversion of neural retina into lens epithelium is one of the most spectacular examples of transdifferentiation. We show that the redirection of cell fate from neural retina to lens and subsequent transdifferentiation is independent of cell replication as it occurs in growth-arrested cell populations. Using DNA ratiometry of individual cells in these cultures we show that, indeed, individual amitotic cells do transdifferentiate. Hence, choice of fate in transdifferentiating cells does not rely on a "community effect" but instead can be categorized as a «leadership effect». For lack of overt lens progenitors, and most importantly, for its mitotic independence, we conclude that lens colony formation *in vitro* does occur by direct transdifferentiation and not by clonal proliferation of progenitor cells.

KEY WORDS: *differentiation, transdifferentiation, cell proliferation*

Introduction

The differentiated state of cells *in vivo* is very stable as if fixed by a series of commitments that result, hypothetically, from the cell's progression through cycles. Although, both traditionally and theoretically, cell differentiation has been linked to cell division (Holtzer *et al.*, 1975) the two, however, can be separated experimentally (Cooke, 1973; Gurdon and Fairman, 1986; Grainger and Gurdon, 1989; Kato and Gurdon, 1993; Neuhaus and Fedoroff, 1994). Blau, using cell fusion to produce heterokaryons, showed that DNA replication is not necessary for silencing and activation of differentiation-specific genes *in vitro* (Blau, 1989). Like differentiation, transdifferentiation - a process requiring sequential silencing and activation of differentiation-specific genes (Okada, 1991; Okada and Yasuda, 1993) - has been, by intuition, associated with cell replication. Transdifferentiation usually occurs as a proliferation-dependent change involving intermediate phenotypes which form as cells undergo rounds of DNA synthesis and proliferation (Nathanson, 1986; Okada, 1986; Eguchi and Kodama, 1993). In fact, Eguchi's group (Mochii *et al.*, 1988a,b; Agata *et al.*, 1993) has elegantly shown that transdifferentiation of retinal pigment epithelium into lens proceeds *via* an intermediate, dedifferentiated cell type in which the *c-myc* gene is activated but neither the pigment epithelial- nor lens-specific genes are.

It has been postulated, however, that transdifferentiation may also occur directly, without DNA replication (Okada, 1986; Beresford, 1990). Direct transdifferentiation has, so far, been shown unequivocally only in invertebrates (Schmid, 1992). In vertebrates, the embryonic retina has been a favored model of transdifferentiation. The neural retina derives from neuroepithe-

lium, hence it diverges from ectodermally derived lens early in development. Nevertheless, it has been known for some time now that neural retina can transdifferentiate into lens (Moscona, 1957; Okada, 1976; Pritchard *et al.*, 1978; Thomson *et al.*, 1978; Yasuda *et al.*, 1978), reviewed in (Okada, 1991; Eguchi and Kodama, 1993; Kodama and Eguchi, 1994). Transdifferentiation of neural retina may possibly occur not by activation of silent genes, but by enhancing the expression of genes that are already active, albeit at low levels (de Pomerai and Clayton, 1978; Errington *et al.*, 1985; Kondoh and Okada, 1986; Kondoh *et al.*, 1987). This implies that transdifferentiation of the neural retina into lens epithelium might occur without DNA replication. While this implication appeared in the literature (Moscona, 1957; Pritchard *et al.*, 1978; Pritchard, 1981; Moscona *et al.*, 1983; Okada, 1986), it has never been rigorously examined. It has, however, been reported that transdifferentiation of cornea into lens in *Xenopus laevis* can occur in the absence of cell proliferation (Filoni *et al.*, 1995; Bosco *et al.*, 1997). The key questions regarding transdifferentiation of neural retina into lens are the existence of cryptic lens progenitor cells in the neural retina proper and the requirement of proliferation for the transdifferentiation. In the present paper, while we do not directly address the notion of progenitor existence, we nevertheless do not find support for it and we conclusively show lack of the requirement of cell division for transdifferentiation.

Abbreviations used in this paper: ED, Embryonic Day; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; MEM, minimum essential medium; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TRITC, tetramethyl rhodamine isothiocyanate.

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Fig. 1. Differential interference contrast of lentoid bodies. After ca. 3 weeks in culture, cells of explanted neural retina form prominent lentoid bodies. As the lentoid bodies shown are quite large the underlying layer of neural retina is out of focus. Bar, 100 μm .

Results

In the present work, we have used the neural retina cell system that transdifferentiates *in vitro* into lens epithelium to establish whether or not a change in cell fate is dependent on cell divisions. The end product of transdifferentiation in culture, that is formation of lens, could be detected by the appearance of *lentoid bodies*, i.e., unmistakable refractile cell aggregates (Fig. 1) that contain high levels of lens-specific proteins, crystallins. With antibodies against β_1 crystallin we were able to identify small cell clusters and, most importantly, single cells which had committed to the lens fate as early as after 9 days after plating (Fig. 2). Except for this high expression of β_1 crystallin, we could not detect any other morphological indication of transdifferentiation.

Next, to verify if cell proliferation is a requirement for transdifferentiation of neural retina into lens epithelium we investigated transdifferentiation in growth-arrested cultures. To inhibit cell growth we tested several mitotic blockers and nocodazole (10^{-3} - 10^{-6} mg/ml) and arabinoside cytosine (5×10^{-5} - 5×10^{-6}) gave the best combination of effectiveness and toxicity (Table 2). Nocodazole was selected for further use because of superior cell viability which was ~97% in 10^{-6} mg/ml and ~95% in 10^{-5} mg/ml. Nocodazole at 10^{-4} mg/ml or more as well as arabinoside cytosine were toxic in the long term (Liu *et al.*, 1986). Nocodazole at 10^{-5} mg/ml was selected for its immediate and complete anti-mitotic effect without affecting cell viability. Table 3 shows that the growth-arrested cultures of neural retina transdifferentiated at the same rate as the non-treated control cultures. To estimate crystallin abundance in these cultures we used Western blotting. To obtain a measure of crystallin content normalized for gel loading we performed double labeling of nitrocellulose filters with anti- β_1 crystallin and anti-actin antibodies. Crystallin abundance dramatically increased in both proliferating and arrested cultures (Fig. 3).

Next we related the phenotype of an individual cell to its proliferative history. This was done using a single cell DNA ratiometry

technique. Both control and growth-arrested cultures were vitally labeled with a blue-fluorescent DNA probe, Hoechst 33258. The cultures, after growing for different time periods, were fixed, permeabilized and double labeled with the red-fluorescent nucleic acid probe, propidium iodide, and anti- β_1 crystallin followed by a secondary antibody conjugated to a green-fluorescent tag, FITC and analyzed with a fluorescence microscope. The phenotype of *individual* cells was then determined: β_1 crystallin abundance (green channel) indicated if a cell had transdifferentiated. The number of DNA replication rounds a cell underwent was calculated as the «Red:Blue DNA ratio». In crystallin-negative cells the Red:Blue DNA ratio increased steadily in control cultures while in growth-arrested cells the ratio remained at levels indicating lack of division (Fig. 4). The DNA ratio in crystallin-positive cells from control cultures increased moderately, indicating that these cells had undergone one or two mitoses before or during transdifferentiation. In growth-arrested cultures we detected single crystallin-positive cells as well as their colonies. In crystallin-positive cells from growth-arrested cultures the DNA ratio remained relatively stable at levels indicating no divisions.

Discussion

Neural retina in an early culture comprises: (I) sheets of flat cells derived from the same progenitor cells as the Müller glia (Li and Sheffield, 1984; Moyer *et al.*, 1990) and (II) neuroblastic cells sitting on top of the flat cells. The neuroblasts are lost within a few weeks *in vitro*. Both the presumptive (Araki *et al.*, 1979) and definitive (Moscona, 1986) Müller cells can convert into lens epithelium but this potential diminishes with age *in vivo* and number of subcultures *in vitro* (de Pomerai and Clayton, 1978; Okada, 1980; Clayton *et al.*, 1986, 1991; Patek *et al.*, 1993). By a classical definition, transdifferentiation is the process by which differentiated cells change their identity and convert into other, also differentiated but distinct, cell types. As ED4.5 neural retina cells used in our experiments are far from being fully differentiated, it is questionable whether or not the term of "transdifferentiation" applies. Nonetheless, irrespective of their eventual fate, the cells of ED4-5 neural retina are not destined to form lens epithelium. It is this switch in cell fate that we refer to as "transdifferentiation" throughout the manuscript.

Transdifferentiation is an environmentally driven process and its efficacy depends on a wide number of factors and culture conditions (de Pomerai *et al.*, 1977, 1982; Pritchard *et al.*, 1978; de Pomerai and Clayton, 1980; Moscona and Degenstein, 1981; Moscona *et al.*, 1983; Moscona, 1986; Karim *et al.*, 1987; Karim and de Pomerai, 1990). Formation of lens epithelium in culture manifests itself by the appearance of *lentoid bodies*, i.e., unmistakable refractile cell aggregates (Fig. 1) that contain high levels of lens-specific proteins, crystallins. Thanks to efforts of Clayton, Okada and their collaborators, the identity of lentoid bodies in terms of their retinal origin, lens authenticity and crystallin profile is well established (de Pomerai *et al.*, 1977; Okada, 1977; de Pomerai and Clayton, 1978; Pritchard *et al.*, 1978; Thomson *et al.*, 1978;

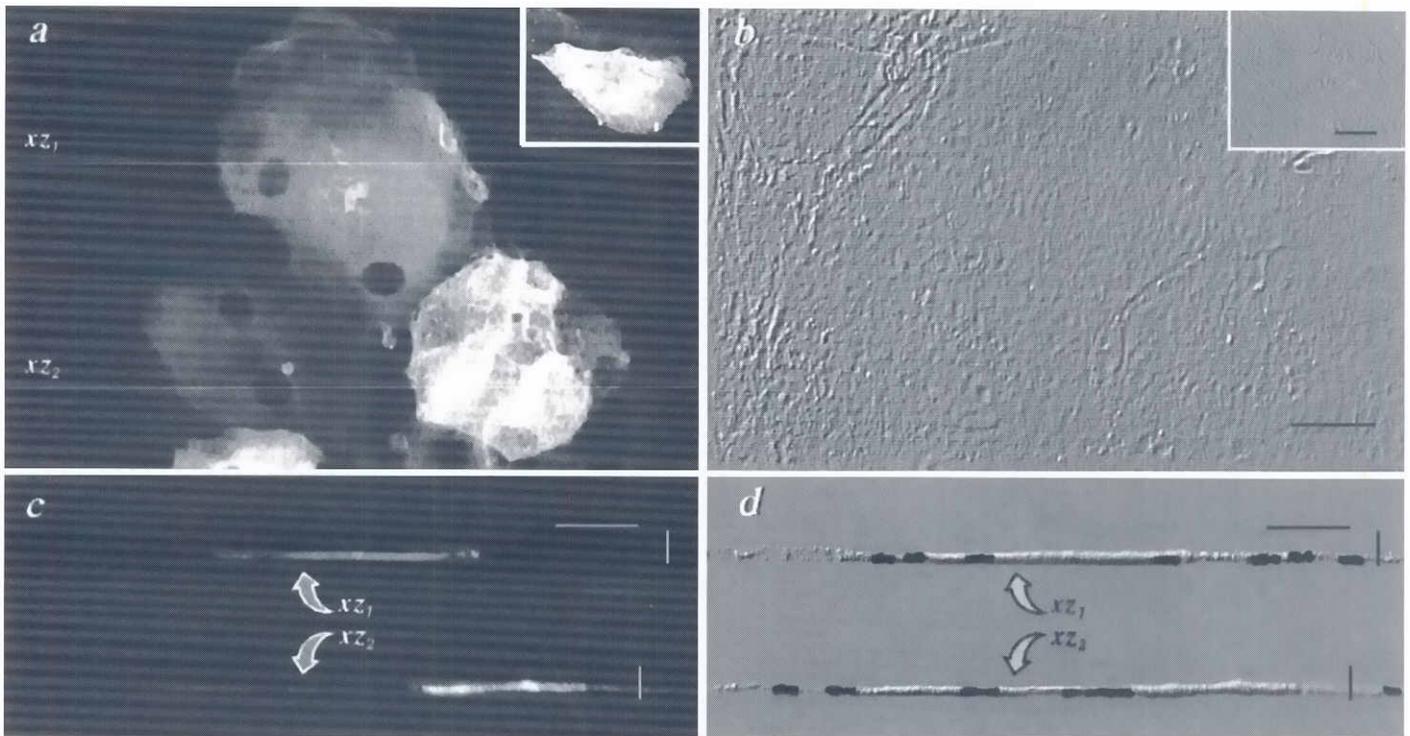


Fig. 2. Immunodetection of crystallin in transdifferentiating cultures of neural retina. Immunofluorescence with anti- β_1 crystallin antibodies (**a,c**) shows large differences in crystallin expression between individual cells in transdifferentiating cultures (**a**). No other morphological differences can be detected between these cells (**b**). Insert in (**a**) shows a single cell expressing high levels of crystallin, while an insert in (**b**) shows that this cell is indistinguishable from its neighbors. Confocal cross-sections were reconstructed from stacks of scan lines taken at the positions marked by lines in (**a**) to show the transdifferentiating cells in side view (**c**). Each of the cross-sections was merged with the corresponding image of propidium iodide-labeled nuclei in the same location and a shear-subtracted image was created to show details of the cell sheet (**d**). Other than the intensity of the anti-crystallin labeling, there is no substantial difference between the cells across these fields of view. Hence, the increase in β_1 crystallin labeling seen in (**a**) is due to high crystallin content in these cells and not due to increase in their thickness. Horizontal bars, 25 μm ; vertical bars, 10 μm .

Araki *et al.*, 1979).

Since the first description by Moscona (Moscona, 1957), it has been occasionally implied that transdifferentiation of neural retina into lens may be direct, as it occurs in "dissociated cells from differentiated, postmitotic neural retina" (Moscona *et al.*, 1983) or in very crowded cultures (Pritchard *et al.*, 1978; Pritchard, 1981). However, the number of neuroblastic cells in persisting culture has no influence on the subsequent lens formation (Moscona *et al.*, 1983). The implication of the directness of lens transdifferentiation has not been verified directly, however. Here, we used the cultured neural retina cell system to establish whether or not a change in their fate is dependent on cell divisions.

Decision to change fate occurs at a single cell level

To localize precursors of conspicuous lentoid bodies in transdifferentiating cultures we used β_1 crystallin as a lens marker because (I) both β_1 crystallin RNA and protein that might be expressed in non-lenticular cells are totally lost during the first days of culture (de Pomerai *et al.*, 1977; Patek *et al.*, 1993) and (II) β_1 crystallin appears well after α and δ crystallins during transdifferentiation (Araki *et al.*, 1979; Clayton *et al.*, 1986) thus being an indicator of terminal lens differentiation. Accordingly, shortly after establishing in culture, neither neural retina cells express crystallin and all are glial fibrillary acidic protein (GFAP)-positive (not shown). In cultured neural retina basal expression of

crystallins appear between the second and third week *in vitro* (Clayton *et al.*, 1986; Patek *et al.*, 1993). Because we detected β_1 crystallin-positive single cells and cell clusters in early (= 9-day old) cultures that did not display any other indication of transdifferentiation, we have concluded that change in commitment from Müller glia fate to lens fate and transdifferentiation of glial neuroepithelium into lens epithelium occur well before lentoid bodies are formed.

It has been hypothesized that the potential of non-lenticular cells to transdifferentiate into lens is related to (I) the level of crystallin expression in these cells and (II) their number (Clayton *et al.*, 1979, 1986; Clayton, 1988; Head *et al.*, 1991). We, however, often detected single crystallin-rich cells that transdifferentiated into lens but were surrounded by crystallin-negative neighbors (Fig. 2) as initially all cells in the cultures were crystallin-negative. The absence of crystallin in the majority of cells in our cultures was to be expected as the basal expression of the protein and its message is initially downregulated in culture (de Pomerai *et al.*, 1977; Patek *et al.*, 1993). Does this mean that these single crystallin-positive cells are cryptic lens «progenitor» cells hidden in the neural retina, and which can be induced to grow into lentoids? This is unlikely as extensive work of Okada and his collaborators who specifically addressed this issue found no evidence for existence of such lens progenitors in the neural retina (Okada, 1980, 1983; Okada *et al.*, 1982). Alternatively, did the crystallin-positive cells acquire the

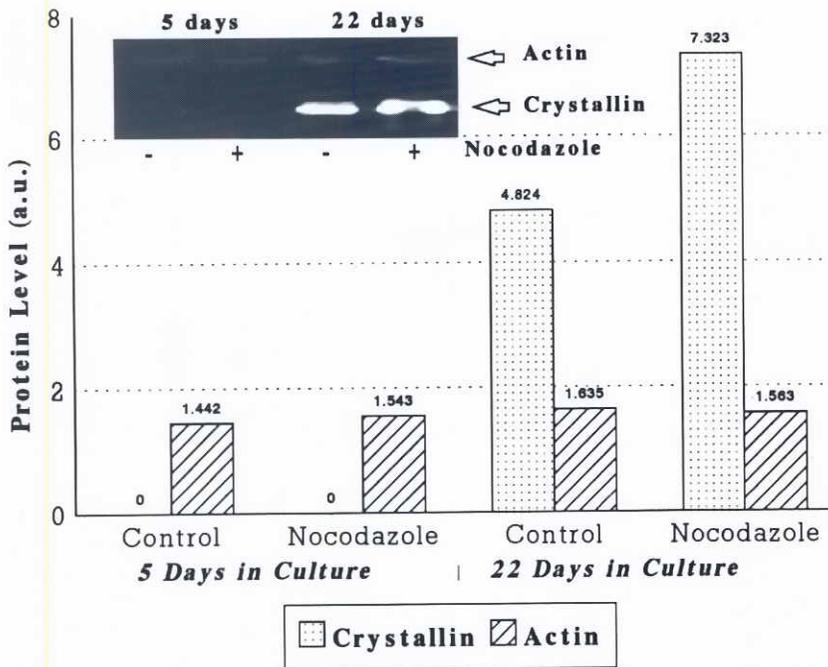


Fig. 3. Densitometric analysis of double label immunoblotting of crystallin and actin expression in total cell extracts of neural retina cultures. Insert shows a typical immunoblot. β_1 crystallin is not detectable in the non-transdifferentiating (5 days) cultures. The protein is expressed in transdifferentiating (22 days) cultures and it is much more abundant in growth-arrested than in control cultures. Expression of actin does not change appreciably. a.u. - arbitrary units.

potential to transdifferentiate into lens by proliferation? To directly address the requirement of proliferation for the latter alternative we investigated transdifferentiation in growth-arrested cultures of neural retina.

Decision to change fate occurs independently of cell replication

To inhibit cell growth we tested several mitotic blockers and nocodazole (10^{-3} - 10^{-6} mg/ml) and arabinoside cytosine (5×10^{-5} - 5×10^{-6} mg/ml) gave the best combination of effectiveness and toxicity (Table 2). Nocodazole was selected for further use because of superior cell viability which was ~97% in 10^{-6} mg/ml and ~95% in 10^{-5} mg/ml. Nocodazole at 10^{-4} mg/ml or more as well as arabinoside cytosine were toxic in the long term (Liu *et al.*, 1986). Nocodazole at 10^{-5} mg/ml was selected for its immediate and complete anti-mitotic effect without affecting cell viability. Table 3 shows that the growth-arrested cultures of neural retina transdifferentiated at the same rate as the non-treated control cultures as determined by the lentoid body count. Furthermore, crystallin abundance, as determined by Western blotting, dramatically increased in both proliferating and growth-arrested cultures. Hence, we have proved that lentoid body formation occurs in growth-arrested cell populations.

The lentoid body count is an excellent morphological measure of overt transdifferentiation in the neural retina cell population and immunoblotting with anti- β_1 crystallin is its good biochemical measure. Neither technique can resolve, however, events occurring during transdifferentiation at the single cell

level. Therefore, we next related the phenotype of an individual cell to its proliferative history. Of several methods used to assess cell proliferative behavior, use of antibodies against proliferating cell nuclear antigen (Ogata *et al.*, 1987) would not give us any insight into cell's proliferative history, [3 H]thymidine could not be used in our long-term experiments and, finally, even a transient exposure to bromodeoxyuridine inhibits retinal differentiation (Mayerson and Moscona, 1979; Moscona *et al.*, 1981). Therefore, we designed a single cell DNA ratiometry technique. Using the Red:Blue DNA ratio, we found that, in crystallin positive cells from growth-arrested cultures the ratio did not change appreciably, thus indicating no cell divisions. Hence, each of these cells, whether single or in a colony, individually underwent direct transdifferentiation.

Crystallin is 1.5-2 times more abundant in growth-arrested than in proliferating cultures that transdifferentiated (Fig. 3), but the numbers of lentoid bodies formed in a given time are the same for both culture types (Table 3). Furthermore, crystallin content of lentoid bodies determined after 22 days in culture by fluorescence ratiometry is the same in growth-arrested cultures (average grey level= 121 ± 3) and in proliferating cultures (average grey level= 121 ± 2). The difference in crystallin content between control and growth-arrested cultures implies either the difference in rate of cell proliferation between transdifferentiating and non-transdifferentiating cells in control cultures or induction of crystallin synthesis by growth arrest in growth-arrested cultures.

As the lentoid body counts are the same for proliferating and growth-arrested cultures the latter possibility is unlikely. More importantly, the fact that the lentoid body counts are the same for control and growth-arrested cultures implies the existence of a predetermined number of cells with the potential to transdifferentiate. Clayton (Clayton *et al.*, 1986) found that 15% of cells in the neural retina express δ crystallin in 3.5-day old embryo that could comprise a pool of transdifferentiation-competent cells. It has been reported that, in the retina, only Müller glia and their progenitors are capable of transdifferentiation into lens (Moscona and Linser, 1983; Okada,

TABLE 1

CORRELATION BETWEEN THE NUMBER OF CELL DIVISION AND THE RED: BLUE DNA RATIO IN CULTURES OF NEURAL RETINA CELLS

Normalized values of Red: Blue DNA ratio	Number of cell divisions			
	<1	2	3	>4
Minimal Red: Blue DNA ratio	1.22	1.66	2.76	5.01
Maximal Red: Blue DNA ratio	1.46	3.02	3.76	6.07

1991). If this is correct, our data suggest that the degree of competence for lens transdifferentiation varies greatly among progenitors of Müller glia. Neural retina cells normally never transdifferentiate in the eye; it is thus the transfer to culture («culture shock») that activates a transdifferentiation programme. Here we conclusively show that amitotic cells derived from ED4.5 neural retina cells transdifferentiate. Thus, neither transdifferentiation nor subsequent lentoid body formation require proliferation of «progenitor» cells.

“Sociology” of cell fate choice

In growth-arrested cultures, we see colonies of transdifferentiating cells. How can colonies of transdifferentiated cells be formed in the absence of cell proliferation? The colony nature of lentoid body formation argues for the existence of localized microenvironments that would induce transdifferentiation of competent cells. By microenvironment we understand factors that may be either environmental, or intracellular or combinatorial in nature. In fact Lillien has elegantly demonstrated that not only concentration of a morphogen but level of expression of its receptor will affect cell fate choice in the retina (Lillien, 1995). The nature of intracellular factor(s) that may be involved in cell fate choice during retinal transdifferentiation is obscure, however.

TABLE 2

EFFICACY OF INHIBITION OF NEURAL RETINA CELL PROLIFERATION¹ BY VARIOUS TREATMENTS

Day	Control	Nocodazole 5x10 ⁻⁶	Nocodazole 5x10 ⁻⁵	Nocodazole 5x10 ⁻⁴	Nocodazole 5x10 ⁻³	Ara-C ² 5x10 ⁻⁴
1	100	100	100	100	100	100
3	122	111	81	54	41	68
6	383	331	31	11	9	31
11	577	371	35	12	10	27

¹ The number of cell plated onto the dishes on day 1 of culture was taken as 100% in each case and the effects of experimental treatments are expressed as its percentage.

² Ara-C: arabinoside cytosine

Responsiveness of competent cells to an inductive signal can be also greatly affected by their number, which is known as a «community effect» (Gurdon, 1988; Gurdon *et al.*, 1993). The community effect requires a competent cell to be in a «community» of similar cells to be able to respond to an inductive signal. A community, in the case of mesodermal induction, is ~100 cells in amphibians (Gurdon, 1988) and ~40 in mammals (Cossu *et al.*, 1995). However, as we have demonstrated existence of single transdifferentiated cells, we consider «community-based» regulation of cell fate choice in the transdifferentiating retinal cell system unlikely.

In painstaking experiments Pritchard (1981) showed that during transdifferentiation of neural retina into retinal pigment epithelium «leader cells» cells begin to synthesize pigment and next a «wave of recruitment» spreads centrifugally from a transdifferentiation

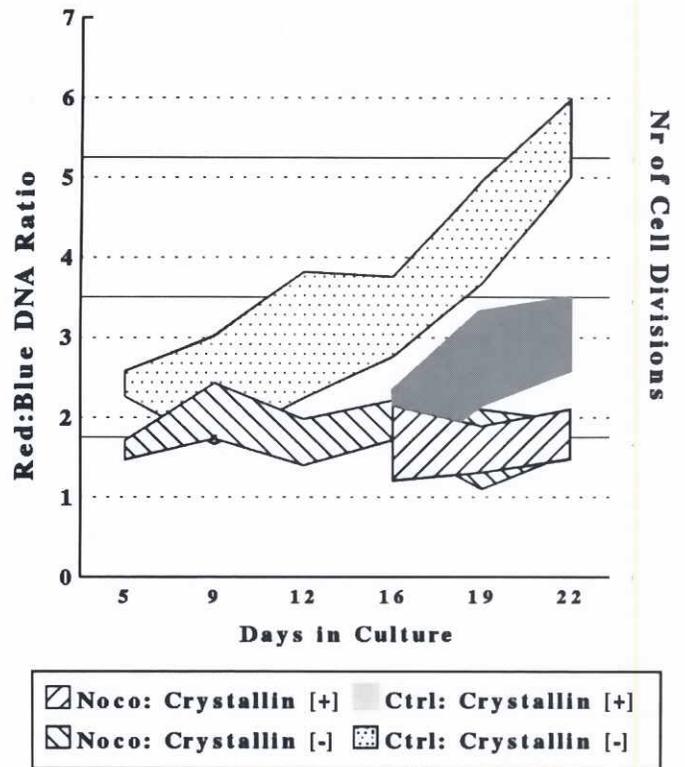


Fig. 4. DNA ratiometry to correlate the crystallin content and proliferative history in individual cells. The «Red:Blue DNA ratio» is a measure of the predicted number of divisions that a cell have undergone in culture. This graph shows that cells that transdifferentiated into the crystallin-positive lens cells did so without divisions in nocodazole-treated cultures. The transdifferentiated cells from the control cultures withdrew from the cell cycle either before or during transdifferentiation. The graph shows a range (standard deviation) of ratio values. The respective sample sizes for the day 5,9,12,16 and 22 were: 36,156,47,60,37, and 32 for control crystallin-negative cells; 55,37,32,112,16 and 87 for nocodazole-treated crystallin-negative cells; 0,0,0,16,14 and 12 for control crystallin-positive cells; 0,0,0,37,11 and 55 for nocodazole-treated crystallin-positive cells.

centre «persuading» cells in contact with the most recent «converts» to follow their example. We have shown that even a cell shape change occurring stochastically in a cell sheet may be sufficient to induce transdifferentiation competence (Zhou and

TABLE 3

FREQUENCY OF LENTOID BODY FORMATION IN GROWTH-ARRESTED AND CONTROL NEURAL RETINA CULTURES

Expt # ¹	Control	Nocodazole
1	12.8±1.6 ²	15.9±1.8
2	11.4±2.6	13.5±1.4
3	15.5±2.6	12.0±2.3

¹ 100 counts were done per experiment.

² Number of lentoid bodies (mean ± SD) per 4 mm² of dish surface.

Opas, 1994). Thus, we infer that a similar «leadership effect» may be taking place in cultures of neural retina transdifferentiating into lens epithelium.

Collectively, commencement of transdifferentiation appears to be a stochastic process that relies on a pool of pluripotent cells and which is driven by discrete stimuli acting at a single cell level.

Materials and Methods

Materials

SDS-PAGE¹ reagents were purchased from Bio Rad (Mississauga, Ont.) or Sigma (Sigma, St. Louis, MO). Enhanced Chemiluminescence Western blotting detection system was purchased from Amersham (Oakville, Ont.). Propidium iodide, Texas Red and Hoechst 33258 were purchased from Molecular Probes, Inc. (Eugene, OR). Nocodazole, arabinoside cytosine and anti-actin antibody were from Sigma Chemical Co. (St. Louis, MO). a minimal essential medium (α MEM) and heat-inactivated fetal bovine serum (FBS) were from Gibco BRL (Canadian Life Technologies Inc., Burlington, Ont. Canada). Fluorescein isothiocyanate (FITC)- or tetramethyl rhodamine isothiocyanate (TRITC)-conjugated donkey-anti-rabbit or -mouse antisera were purchased from Bio/Can (Mississauga, Ont.). A rabbit polyclonal anti- β_1 crystallin antibody specific for chick crystallin was a gift of Dr. Sam Zigler (NIH).

Preparation of cells

Primary neural retina cultures from 4.5-day old chick embryos were established in ϕ 10 cm Petri dishes as described (Opas and Dziak, 1994). Briefly, fertilized eggs were incubated for 4.5 days in a humidified atmosphere at 38°C. Eyes from chick embryos were enucleated, than an incision was made through the sclera and the lens and vitreous were removed thus forming an eyecup. The neural retina was collected with a hand-held micropipette by applying mouth suction. The tissue fragments were pooled in medium (MEM supplemented with 10% FBS), dispersed by gentle mechanical agitation and plated into ϕ 10 cm tissue culture dishes at a «density» of 6 eyecups per dish. Neither fungizone nor antibiotics were used in the primary culture. The primary cultures were grown for two weeks until confluent. Next, the cultures were trypsinized and replated into ϕ 3.5 cm tissue culture dishes at a density of 250,000 cells per dish. We used secondary cultures of chick embryo neural retina to select against neuroblastic cells that linger in primary neural retina cultures. The cultures were maintained α MEM containing 10% FBS without antibiotics at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

To inhibit cell growth, the cell cultures were maintained in α MEM containing 10% FBS and antibiotic mixture supplemented with nocodazole at a concentration of 10⁻⁵ mg/ml. The medium was changed every three days.

For cell counting, the cultures were prepared in ϕ 35 mm tissue culture dish as described above. At selected time intervals, non-adherent cells were washed off the dishes with a brief rinse of cold medium and the remaining adherent cells were trypsinized and counted (in triplicate) on a Coulter Counter. Average population doubling times were calculated from growth curves (Patterson, 1979) to be circa 102 h.

Fluorescent labeling of cells

The cells were grown for 21 days and then fixed, permeabilized and labeled as described (Opas and Dziak, 1994). The anti- β_1 crystallin antibody (1:50 in PBS) was followed by FITC-conjugated donkey anti-rabbit antiserum (1:30 in PBS). Total cellular protein in fixed and permeabilized cells was labeled with Texas Red (30 ng/ml in PBS for 30 min at 37°C). We considered it important to demonstrate that the cells that heavily labeled with the anti-crystallin antibody did so because of high crystallin content and not due to optical artifacts or a random cell shape change (e.g., substantial thickening) of cells expressing basal levels. To obtain a measure of crystallin content in the cells we performed fluores-

cence ratio measurements in which the emission intensity of the crystallin immunolabel in a particular cell was divided by the emission intensity of a total protein marker, Texas Red, in the same cell. In this manner, crystallin content was normalized to the protein content in the same cells. Confocal microscopy was performed using a Bio-Rad MRC-600 microscope equipped with a Krypton-Argon laser. To obtain the crystallin: total protein ratios the crystallin fluorescence was divided by that of Texas Red and multiplied by a constant factor of 64. Nonspecific fluorescence in the FITC channel was measured by omitting primary antibodies from the labeling procedure and its maximal ratio value (40) was then subtracted from each data point. For each data point the following culture areas were measured: 5 and 9 days: 124,848 μ m²; 12 and 16 days: 93,636 μ m²; 19 days: 249,696 μ m²; 22 days: 218,484 μ m². This is equivalent to a count of >80 cells at the beginning of the experiment and of >700 at its end. The average grey levels of emission ratios for crystallin-negative cells on day 5, 9, 12, 16, 19 and 22 of culture were 15 \pm 4, 15 \pm 5, 26 \pm 9, 26 \pm 6, 27 \pm 9 and 32 \pm 6 respectively. The average grey levels of emission ratios for crystallin-positive cells on day 12, 16, 19 and 22 of culture were 120 \pm 4, 120 \pm 6, 124 \pm 2, 122 \pm 3, respectively. Thus it became clear that the cells that heavily labeled with the antibody did so because of high crystallin content.

To visualize DNA in living cells they were treated with Hoechst 33258 (25 μ g/ml in PBS for 30 min at 37°C) or fixed, permeabilized, digested with 0.2 mM RNase for 2 h and stained with propidium iodide (1 μ g/ml in PBS for 15 min at 37°C).

SDS-PAGE and immunoblotting

Total cell lysates from cultures grown with or without 10⁻⁵ mg/ml nocodazole were prepared as described (Tharin *et al.*, 1995) and analyzed by SDS-PAGE (10% acrylamide) as described by Laemmli (1970). Resolved proteins were transferred to nitrocellulose membranes according to the method of Towbin *et al.* (Towbin *et al.*, 1979) and double labeled with antibodies against β_1 crystallin (1:400) and non-muscle actin (1:100). Lysate volumes were adjusted to give 40 μ g of total cellular protein loaded per lane. Proteins were detected using Enhanced Chemiluminescence system according to the manufacturer's instructions. The protein content of each band was densitometrically quantified (using Image-1 program, West Chester, PA) in arbitrary units, each of which equals 103 pixels with a grey level \geq 35 within the digitized image of a band.

Single cell DNA ratiometry

The cultures were set up at different times to be processed simultaneously. Both nocodazole-treated and control cultures were *vitaly* pre-labeled on day 3 *in vitro* with Hoechst 33258. At the end of the experiment the cells were fixed, permeabilized and double labeled for crystallin and propidium iodide as described above. For each individual cell, the number of DNA replication rounds it underwent was determined by calculating «Red:Blue DNA ratio». This was obtained by dividing the current DNA content of the cell nucleus (propidium-labeled DNA: red channel emission) by the remaining amount of pre-labeled DNA (Hoechst-labeled DNA: blue channel emission). To relate the number of cell divisions in culture to the Red:Blue DNA ratio the same was calculated using cells that underwent a known number of divisions (Table 1). Images were collected with a SIT (Venus Scientific) camera and fed into the Image-1 frame grabber/digitizer. a 2FL epifluorescence condenser on a Zeiss (Don Mills, Ont.) Standard WL microscope was used to host a triple wavelength barrier filter and dichroic mirror (both from set # XF56, Omega Optical, Brattleboro, VT) and a slider containing three separate narrow band interference excitation filters 350FS10-21 (Omega Optical), BP 485/20 (Zeiss) and BP446/12 (Zeiss). This arrangement assured pixel registration of red and blue DNA images as well as the spectral separation of DNA images from that of crystallin (green).

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