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Original Article

Formation of retinal pigment epithelium *in vitro* by transdifferentiation of neural retina cells

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ABSTRACT Chick embryonic neural retina (NR) dedifferentiates in culture and can transdifferentiate spontaneously into retinal pigment epithelium (RPE). Both, primary RPE and transdifferentiated RPE (RPE^t), are characterized by pigmentation, expression of RPE-specific protein, eRPE$_{AG}$ and lack of expression of the neural cell adhesion molecule, NCAM. In contrast, NR cells are unpigmented and express NCAM but not eRPE$_{AG}$. Functionally, both primary RPE and the RPE^t cells display a pH_i response to bFGF, which is different from that of the NR. We used these characteristics to distinguish cell types in primary cultures of chick NR and follow the changes in phenotype that occur during transdifferentiation. We show that the RPE^t forms as small "islands" in the packed regions of the primary, "mother" NR cell sheets, in a stochastic process. Because of a small number of cells involved in the initiation of the transdifferentiation we refer to it as a "leader effect" to contrast it with the "community effect" which requires many competent cells to be present in a group to be able to respond to an inductive signal. The RPE^t then expands centrifugally and underneath the surrounding NR sheet. To determine if the RPE^t maintains its identity in isolation while displaying the RPE-typical phenotypic plasticity, we explanted the islands of RPE^t and treated half of them with bFGF. The untreated RPE^t maintained its closely packed, polygonal pigmented phenotype but the bFGF-treated RPE^t transdifferentiated into a non-pigmented, NR-like phenotype, indicating that RPE^t encompasses the full differentiation repertoire of native RPE.

KEY WORDS: *transdifferentiation, chick, retina, RPE, intracellular pH*

Introduction

During the eye development a series of inductive events cause differentiation of several distinct ocular phenotypes from an initially nondescript neuroepithelium. While general agreement exists as to the importance of soluble factors and contact phenomena in normal eye development, their identities, individual roles and combined effects have just started to be unravelled. To add to the complexity of the system, the eye phenotypes are not firmly predetermined at early stages of development and they can convert or "transdifferentiate" into each other (Okada *et al.*, 1979; Okada, 1980; Bosco, 1988). The early embryonic retina has displays especially high level of plasticity as any of the retinal cell subpopulations may transdifferentiate (Okada *et al.*, 1979; Okada, 1980; Moscona, 1986; Okada and Yasuda, 1993; Eguchi and Kodama, 1993; Reh and Pittack, 1995; Zhao *et al.*, 1997). One group of soluble factors of an indisputable importance in the retinal differentiation and transdifferentiation is fibroblast growth factor (FGF) family (Gao and Hollyfield, 1995; Zhao and Barnstable, 1996; Pittack *et al.*, 1997), for pre-1995 literature see an excellent review by Reh and Pittack (1995). While the importance of contact phenomena has been implied in the retinal differentiation and transdifferentiation (Tsunematsu and Coulombre, 1981; Opas, 1989; Buse and De Groot, 1991; Reh, 1992; Buse *et al.*, 1993; Zhou and Opas, 1994; Opas and Dziak, 1994; Opas, 1994a; Opas, 1994b; Pittack *et al.*, 1997) neither detailed experimental evidence nor general hypotheses for its mechanism(s) abound. Importance of the extracellular matrix in transdifferentiation has been discussed by Eguchi (1998). Thus, *in vitro* model cell systems, in spite of their obvious limitations, may be found useful in providing basic concepts regarding the role of cell-cell and cell-substratum adhesion in the retinal development.

Within a few days, primary cultures of embryonic neural retina (NR) form sheets of dedifferentiated flat glial cells with overlying

Abbreviations used in this paper: AM, acetoxymethyl; DMSO, dimethyl sulfoxide; FBS, foetal bovine serum; bFGF, basic fibroblast growth factor; FITC, fluorescein isothiocyanate; Ig, immunoglobulin; MEM, minimum essential medium; NCAM, neural cell adhesion molecule; NR, neural retina; pH_i intracellular pH; RPE, retinal pigment epithelium; RPE^t, transdifferentiated retinal pigment epithelium; eRPE$_{AG}$, embryonic RPE antigen; SNAFL, seminaphthofluorescein; TRITC, tetramethyl rhodamine isothiocyanate.

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Fig. 1. Differential interference contrast images of formation of islands of tightly packed, polygonal and pigmented RPE^t cells by primary cultures of NR. *Initially, after about two weeks of culture, circular areas containing tightly packed and polygonal cells are easily discerned and betray prospective RPE islands* **(A)**. *Such islands become pigmented after ca. three weeks in vitro* **(B)**. *After and additional two weeks (about five-six week in culture), distinct RPE cell areas are easily detected by their heavy pigmentation.* **(C)** *shows such an area of the RPE^t surrounded by a multilayer of unpigmented NR cells. Scale bar, 100 μm.*

neuroblastic cells that are lost within a few weeks *in vitro* (Combes *et al.*, 1977; Li and Sheffield, 1986a; Li and Sheffield, 1986b). The glial cells are derived from the same progenitor cells as the Müller cells (Li and Sheffield, 1984; Moyer *et al.*, 1990). Although our cultures are not strictly differentiated NR (NR becomes post-mitotic and fully differentiated only after embryonic day 13; we used 6 day-old embryos), we refer to the Müller cell-derived cell populations collectively as the "NR" throughout. Such NR, in culture, is pluripotential in that it may transdifferentiate into melanin-producing retinal pigment epithelium (RPE) or lens epithelium and form lentoid bodies. Transdifferentiation of the NR into lens occurs spontaneously *in vitro* (Pritchard *et al.*, 1978; Okada *et al.*, 1979; de Pomerai and Clayton, 1980; Okada, 1980; Moscona and Degenstein, 1981; de Pomerai *et al.*, 1982; Moscona, 1986; Okada and Yasuda, 1993), and soluble factors, in particular FGFs, enhance lentoid body formation in transdifferentiating cultures (Karim and de Pomerai, 1990; Tcheng *et al.*, 1994). The flat glial cells of NR may, however, convert into RPE cells (Pritchard *et al.*, 1978; Okada *et al.*, 1979; Okada, 1980; Pritchard, 1981). While lens formation by transdifferentiation of the NR has been extensively studied, transdifferentiation of the NR into RPE received only limited attention.

Adhesion is important in the regulation of retinal development (Getch and Steinberg, 1986; Lagunowich and Grunwald, 1989; Adler, 1993) where the neural cell adhesion molecule, NCAM, plays a role (Schlosshauer *et al.*, 1984; Brackenbury *et al.*, 1984; Hoffman *et al.*, 1986; Neugebauer *et al.*, 1988; Bartsch *et al.*, 1989). The expression of NCAM is developmentally regulated (Neill and Barnstable, 1990) and in the chick embryo, NCAM is expressed abundantly by the NR (Cole and Glaser, 1984; Schlosshauer *et al.*, 1984) but not at all by the RPE (Rizzolo *et al.*, 1994; Opas and Dziak, 1994). Thus, in the chick embryonic eye, NCAM expression is a useful marker for the identification of cells with a neural phenotype (Zhou and Opas, 1994; Opas and Dziak, 1994). Also, primary RPE and transdifferentiated RPE (denoted by RPE^t) cells show a similar pH_i responsiveness to bFGF which is distinctly different from that of the primary NR: RPE cells respond to bFGF with only a brief and transient increase in pH_i, while in the primary NR, in response to bFGF, the pH_i oscillates, then rises steadily and stabilizes at an elevated level (Zhou and Opas, 1994).

Formation of lens epithelium from the NR has been studied extensively and it is certain by now that it involves a *bona fide* transdifferentiation. This is not the case for transdifferentiation of NR into the RPE. The objective of this work was to examine the origin of transdifferentiated cells and interrelations between the original NR cells and the resulting RPE^t cell sheets. We have used (i) melanogenesis, (ii) characteristics of pH_i responses to bFGF and (iii) expression of either the RPE-specific protein, eRPE$_{AG}$ (Chu and Grunwald, 1990), or the NR-specific protein, NCAM, to distinguish cell phenotypes in cultures of NR that contain transdifferentiated cells. We show that the transdifferentiating cells down regulate NCAM and up regulate the RPE-specific eRPE$_{AG}$ and, using confocal optical sectioning, we show that the RPE^t cell sheet and the primary NR develop into a complex multilayered neuroepithelium. Using these observation we discuss possible role of contact phenomena in the recent model of retinal development (Pittack *et al.*, 1997).

Results

Primary cultures of NR cells attach and spread during the first week in culture. As we frequently change the growth medium during the first two weeks of culture, loss of neuroblastic cells growing on top of the glia is accelerated. After ca. 2 weeks in culture, areas containing tightly packed and polygonal cells are easily found (Fig. 1A). About one week later such areas commence to be pigmented (Fig. 1B) and after another couple of weeks distinct RPE cell areas are well formed (Fig. 1C). Usually these areas appear as isolated colonies, or islands, of RPE cells within the NR

TABLE 1

PH_i OF RPE AND NR CELLS IN RELATION TO THE PROJECTED CELL AREA

Cell Morphology	Projected Cell Area (μm^2)	pH_i of RPE	pH_i of NR
Cuboidal	400-1000	7.13±0.03	7.12±0.02
Crowded	1500-3000	7.22±0.05	7.21±0.05
Well Spread	> 4000	7.31±0.03	7.30±0.03

All differences are statistically significant at $P < 0.05$.

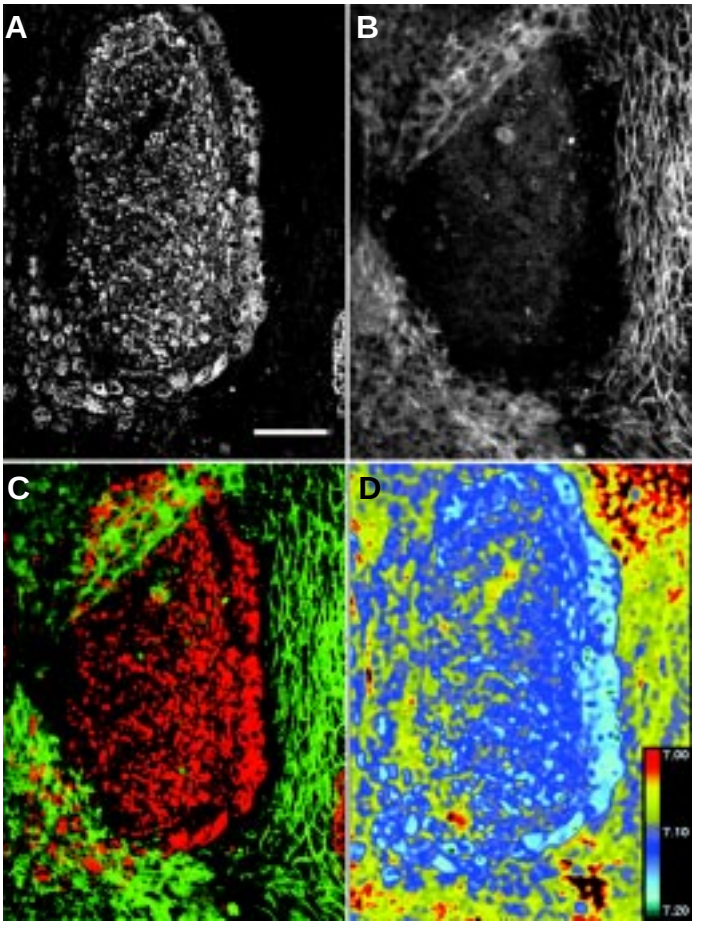

Fig. 2. Distribution of cell type-specific markers and the resting pH_i in the island of RPE^t formed within the mother sheet of primary NR cells after 3 weeks in culture. (A,B) *Immunofluorescence images of the same colony after double labelling with antibodies to eRPE*$_{AG}$ **(A)** *and NCAM* **(B).** *Note that even the flatter RPE^t cells located at the edge of the RPE island abundantly express eRPE*$_{AG}$*, leaving no doubt as to their identity.* **(C)** *is the result of merging (A) with (B) to show spatial separation of the two markers: yellow denotes overlap of eRPE*$_{AG}$ *and NCAM labels. As the label pattern is not exactly the same in the single label pictures (A,B), the yellow colour in (C) shows areas of tight overlap of layers of cells bearing two antigens and not a single layer of cells bearing two antigens. A ratio image of pH_i in the same RPE^t cell colony is shown in* **(D)**. *The RPE^t cells in the central region of the colony have an average pH_i of 7.13 ± 0.03. The flat RPE^t located at the lower edge of the colony have a pH_i of 7.22 ± 0.05. The NR cells surrounding the RPE^t cells have an average pH_i of 7.12 ± 0.02. Scale bar, 100 μm.*

monolayer. The RPE islands are readily detectable by their pigmentation as our dissection procedure ensures the absence of any pigmented cells in the initial cultures. After about three weeks in culture, Müller cells of the NR start to transdifferentiate into lens cells (or bottle cells) and form numerous lentoid bodies. Often the RPE islands are surrounded by lentoid bodies at different stages of formation (not shown).

NR transdifferentiates into the RPE^t and upregulates the RPE marker

Colonies were double labelled with anti-NCAM and 3c10 antibodies recognizing the eRPE$_{AG}$. Fig. 2 is the same field of view showing the RPE^t colony and the adjacent primary NR. The RPE^t cells express the RPE-specific eRPE$_{AG}$ (Fig. 2A) but no longer express NCAM (Fig. 2B). It must be stressed that, in early (less than a week old) cultures, there are no cells that express eRPE$_{AG}$ but all cells express NCAM. A merged image of NCAM (green) versus eRPE$_{AG}$ (red) distribution (Fig. 2C) shows that the two markers are expressed in well-demarcated areas. In only a few regions at the periphery of the RPE island both markers overlap (yellow). The overlap may indicate either co-expression of the two markers in the same cells or an overlap of sheets of cells expressing single markers or both. Fig. 2D shows the resting pH_i throughout the same field of view. The cuboidal RPE^t cells have similar pH_i (average = 7.13 ± 0.03) to the adjacent packed NR cells (average = 7.12±0.02). Interestingly, cells at the lower edge of the RPE^t cell area are much flatter than the cells from its centre and also have higher pH_i (7.22 ± 0.05) than the pH_i of the packed NR and that of the RPE^t. Nevertheless, these flat RPE^t cells abundantly express the eRPE$_{AG}$ (Fig. 2A). Note that we refer to tightly packed cells, as are found in the centre of a colony, as “cuboidal” throughout.

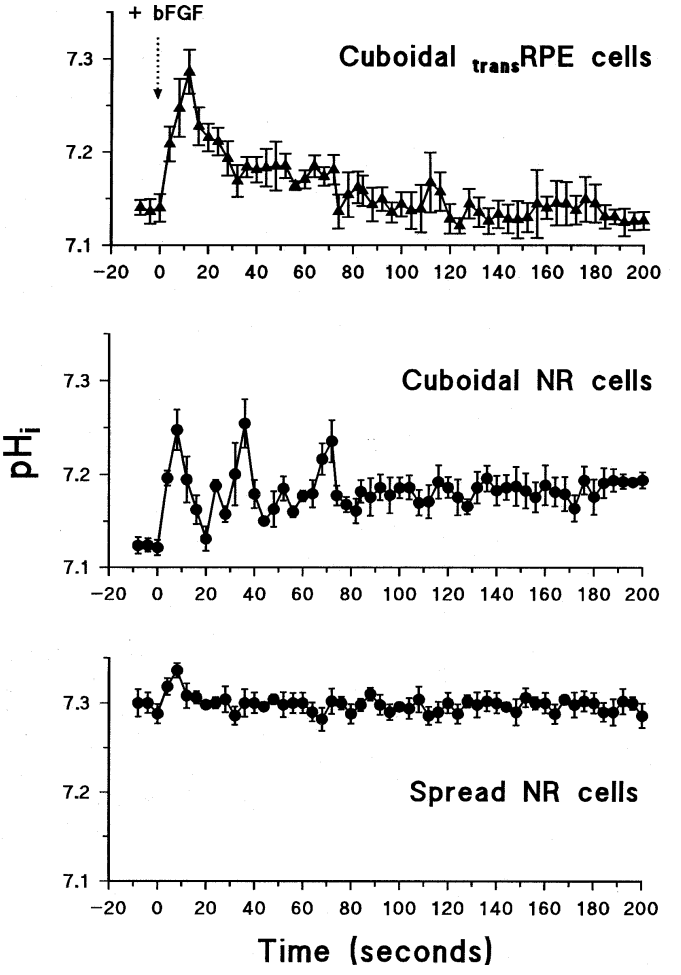

Fig. 3. Changes in pH_i of the NR and RPE^t cells in response to bFGF. *Data represent pH_i (mean ± s.e.m.) as a function of time after the addition of bFGF at time 0. Both cuboidal NR and RPE^t cells respond with a pH_i increase (by ≈0.11-0.15 pH unit) within the initial 1.1/2 minutes after the addition of bFGF. In contrast to the RPE^t, the pH_i of the cuboidal NR cells oscillates in response to bFGF and then stabilizes at a more alkaline level.*

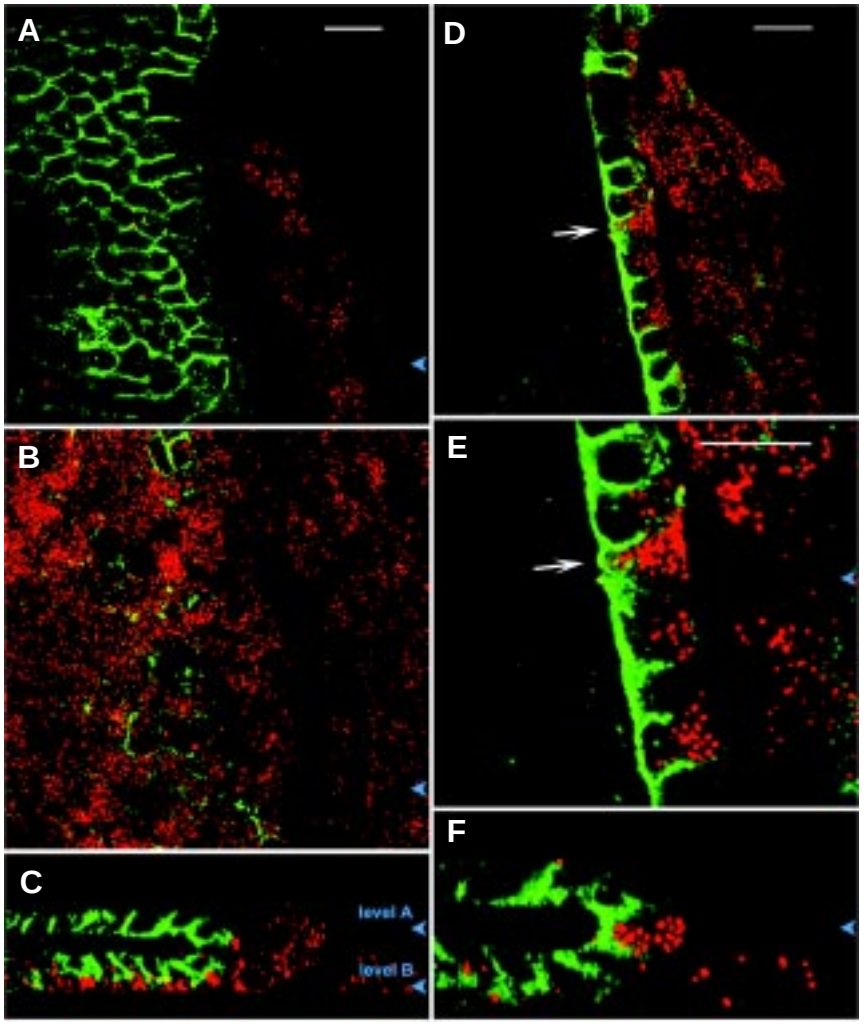

Fig. 4. Double labelling immunofluorescence images showing the spatial arrangement of the NR and RPE^t cells in the transitional NR➡RPE zone of the colony. **(A,B,D,E)** *"X-Y" confocal optical sections taken in a plane parallel to the substratum.* **(C,F)** *"X-Z" confocal optical cross-section taken in a plane perpendicular to the substratum. In each of the panels, anti-NCAM labelling is shown in green while eRPE$_{AG}$ distribution is shown in red. Yellow indicates coexpression of both antigens.* **(A,B)** *A pair of X-Y sections taken through the uppermost* **(A)** *and bottom* **(B)** *layers of the same NR➡RPE transition zone. Most of the cells within the uppermost plane of focus (A) are NCAM-positive NR cells; eRPE$_{AG}$-positive RPE^t cells are absent from this plane. In contrast, all of the cells in the basal plane (B) are NCAM-negative RPE^t cells that strongly express eRPE$_{AG}$.* **(C)** *An X-Z optical cross-section through the cell layers in the same NR➡RPE transition zone scanned along the position indicated by arrowheads in A and B. It is evident that two layers of NCAM-positive NR cells abut a single layer of eRPE$_{AG}$-positive RPE^t cells. The arrowheads with "level A" and "level B" labels indicate height at which pictures shown in (A) and (B) were recorded.* **(D)** *Low and* **(E)** *high magnification X-Y sections taken through the cells at the very edge of a NR cell sheet fold in the NR➡RPE transition zone. It is evident that many of these cells express both NCAM and eRPE$_{AG}$ in a polarized manner: one of them is indicated by an arrow. The RPE-specific antigen, eRPE$_{AG}$, predominates at the side of the cell facing the RPE^t while NCAM is strongly concentrated at the opposite side adjacent to the primary NR (Fig. 4E).* **(F)** *An X-Z optical cross-section through the cell layers shown in (E) at the position indicated by the arrowhead and it shows the arrow-marked cell in a profile view. Scale bars: A-D, 50 μm, E-F, 25 μm.*

pH_i in transdifferentiating cultures correlates with the degree of cell spreading and not the phenotype

A common and immediate response to most growth factors is an activation of the Na^+/H^+ antiporter (Moolenaar, 1986; Grinstein *et al.*, 1989; Li *et al.*, 1991) and cell responsiveness to soluble factors can be modulated by adhesion and the degree of spreading (Ingber and Folkman, 1989; Sutton *et al.*, 1991; Schubert and Kimura, 1991). Usually, spread cells have a higher intracellular pH (pH_i) than round ones (Margolis *et al.*, 1988; Schwartz *et al.*, 1989; Schwartz *et al.*, 1991; Yoshida *et al.*, 1993; Wu *et al.*, 1994). We have found this to be the case for both NR and RPE as well. Besides being affected by the degree of cell spreading, the resting pH_i is can also be cell-type specific (Ingber *et al.*, 1990; Mariot *et al.*, 1991; Yoshida *et al.*, 1993; Isfort *et al.*, 1993; Zhou *et al.*, 1995). We therefore examined the resting pH_i of RPE and NR cells in relation to the degree of spreading in cuboidal cells in the centre of a colony, crowded 2-3 rows away from the edge of a colony and fully spread cells at the edge of a colony. Primary NR and primary RPE cells that have spread to the same degree have similar pH_i. The relationship between pH_i and projected cell area of both NR and RPE is summarized in Table 1. While all the data shown are statistically significant form each other we, however, we do not believe that the differences in pH_i between cells in the same size category are biologically meaningful. From it is also clear that the resting pH_i of both NR and RPE cells drops as the cells pack.

The responsiveness of RPE^t and NR cells to bFGF differs

The time course of pH_i changes in response to bFGF was examined in both RPE^t cells and primary NR cells. To allow rigorous comparison, ratio images were recorded simultaneously in both cell types from the same field of view in the same culture. Furthermore, the same cells were next double labelled with anti-NCAM and 3c10 antibodies to ensure their identity. During the initial 30 seconds after the addition of bFGF, alkalinization waves occur in both cuboidal RPE^t and cuboidal NR cells (Fig. 3). In RPE^t cells the pH_i peaks (pH 7.32 ± 0.04) in the first 20 seconds then recovers gradually, regaining the resting level after about 2 minutes. In contrast, the differentiated NR cells respond with up to three alkalinization waves within the first minute. After 1-2 minutes, the pH_i in the differentiated cuboidal NR rises and stabilizes at a slightly more alkaline level (by pH≤0.10).

Spatial relationship between primary NR and RPE^t cells

The RPE^t cells are always found as islands in the packed regions of the NR mother cell sheets, sometimes after only two weeks in culture. The RPE^t cells form small colonies, which

expand centrifugally. After ca. 3 weeks in culture, the RPE^t sheets appear to extend underneath the NR cell sheets. To reconstruct the spatial arrangement of the cell layers in our cultures we used confocal optical sectioning after double labelling for NCAM and eRPE$_{AG}$. Fig. 4 shows the spatial relationship between the RPE^t cells and the neighbouring NR cells in the transitional (NR➡RPE) zone of the colony. Figs. 4A and 4B show two optical sections of the same area, taken at two different focal levels in a plane parallel to the substratum, through either the uppermost layers (A) or the basal layers (B) of the NR➡RPE zone. From these pictures it is clear that the primary NR cells that are NCAM-positive are located above and abutting the eRPE$_{AG}$-positive RPE^t cell layer. The multilayered arrangement of the border between the primary NR cells and the RPE^t cells is obvious in Fig. 4C. Fig. 4C shows an X-Z optical cross-section (i.e. taken in a plane perpendicular to the substratum at the position indicated by arrowheads in A and B) of the same area in the NR➡RPE zone. Folding of the NR cell sheet into a bilayer resting on the RPE cell monolayer is evident. Many cells located at the very border between the primary NR cells and the RPE^t cells express both NCAM and eRPE$_{AG}$ (Fig. 4D). Importantly, the distributions of NCAM and eRPE$_{AG}$ in the same transdifferentiating cells are polarized. The RPE-specific antigen, eRPE$_{AG}$, predominates at the side of the cell facing the RPE^t while NCAM is strongly concentrated at the opposite side adjacent to the primary NR (Fig. 4 E,F).

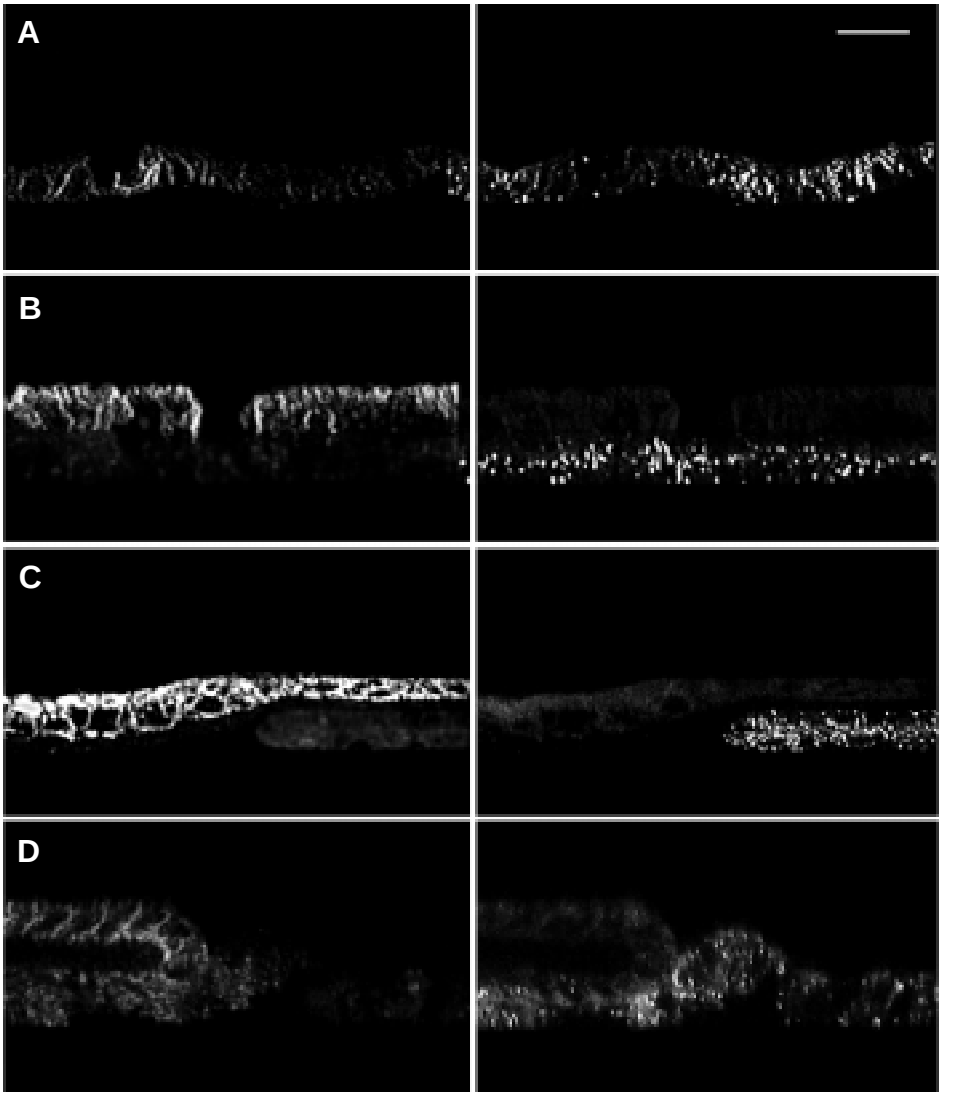

Fig. 5. Double labelling immunofluorescence images showing progression through the stages of development of multilayers. *All panels show X-Z optical cross-sections taken in a plane perpendicular to the substratum. Anti-NCAM labelling is shown on the left and the corresponding eRPE$_{AG}$ distribution in the same areas on the right.* **(A)** *shows that in the early stages of transdifferentiation (2-3 weeks in vitro), some of the cells comprising the monolayer have lost NCAM and become eRPE$_{AG}$-positive.* **(B)** *The very centre of a small RPE^t colony at an early stage of transdifferentiation. A bilayered arrangement, with the RPE^t cells underlying the NR layer, is evident.* **(C)** *The edge of the RPE^t colony at a later stage of transdifferentiation (4-5 weeks in vitro): a sheet of eRPE$_{AG}$-positive and NCAM-negative RPE^t lies underneath the NCAM-positive primary NR layer.* **(D)** *At a yet later stage (7 or more weeks in vitro), the upper NR layer folds and forms a bilayered sheet of NCAM-positive cells overlying a single-cell thick sheet of RPE^t cells. At this time many cells in the NR➡RPE transition zone express both eRPE$_{AG}$ and NCAM. Scale bar, 40 μm.*

The RPE^t islands, which expand outwards and underneath the primary, mother NR cells, form a single-cell thick sheet. We followed the spatial arrangement of the RPE^t cells for up to 8 weeks in culture. At the early stages of transdifferentiation, two to three weeks *in vitro*, the cells down regulate NCAM and some of them start to express eRPE$_{AG}$ (Fig. 5A) while still remaining unpigmented. These cells increase in number, pack and form discernible RPE^t colonies. Next, layers are formed in large areas of the NR➡RPE transition zone by continued proliferation of the NCAM-positive NR cell layer above the underlying RPE^t monolayer (Fig. 5 B,C). The mechanism of the layering process is not clear but it is obvious from Fig. 5D that the edge of the overlying NR has folded to form a bilayered sheet of NCAM-positive cells resting on the underlying NCAM-negative RPE^t. However, as such simple and well demarcated folds were rarely seen, we surmise that the layering process can be, and in fact often is, more complex than this figure shows. Nevertheless, late into the transdifferentiation (six to seven weeks *in vitro*), at the edges of the RPE^t areas, two tissue layers are always formed: a multilayer of NCAM-positive primary NR cells resting on a monolayer of RPE^t cells.

Explantation of RPE^t islands from the mother NR sheet: the phenotype of RPE^t cells is stable and bFGF induces their secondary transdifferentiation into the NR

Of those explanted RPE^t cells grown without bFGF, most retained the pigmented, polygonal RPE phenotype, forming striking, pig-

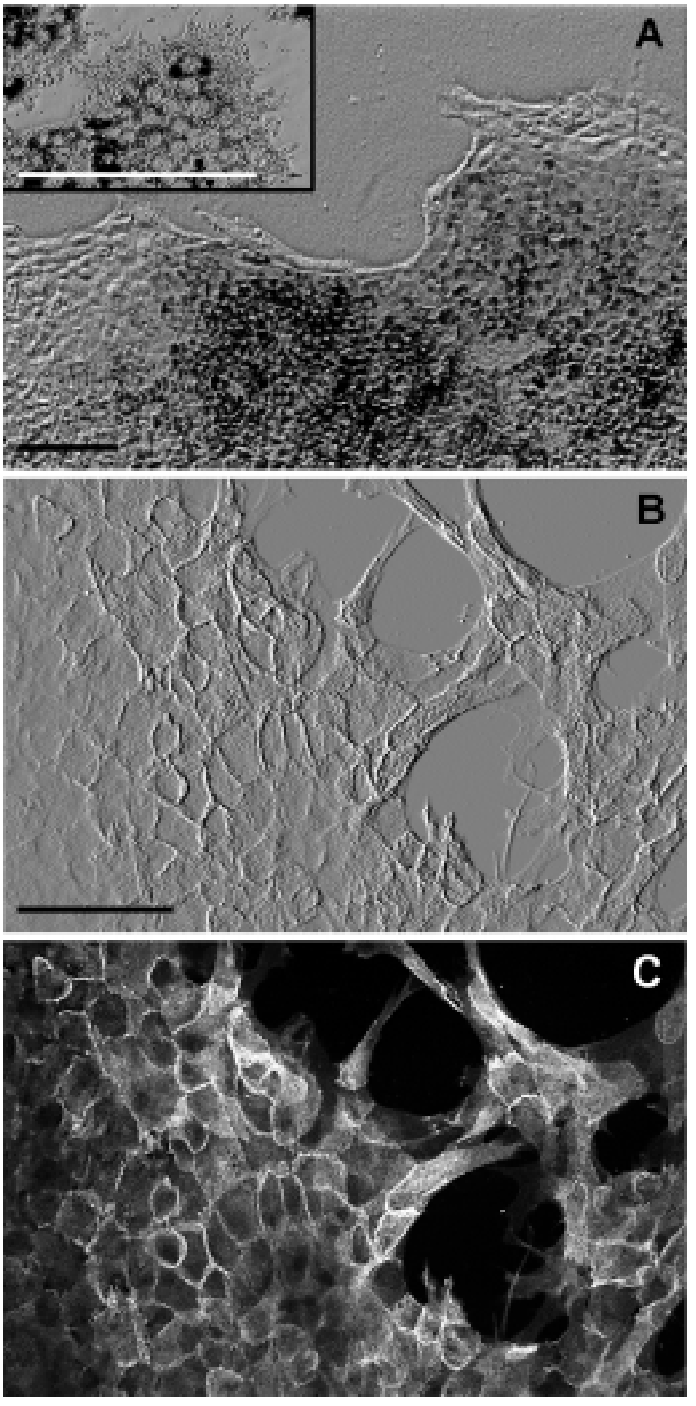

Fig. 6. Effect of bFGF on explanted RPE^t colonies. *Differential interference contrast images of explanted RPE^t colonies cultured (A) without and (B,C) with bFGF. Insert in (A) shows freshly explanted pigmented RPE^t cells.* **(A)** *Four weeks after explantation, many of these cells still maintain packed, pigmented and differentiated RPE phenotype. Explanted RPE^t cells grown in bFGF for four weeks after explantation become* **(B)** *depigmented and more fusiform and* **(C)** *acquired NCAM. Scale bars, 100 μm.*

mented, evenly round colonies, a part of which is shown in Fig. 6A (insert). All of the explanted RPE^t cells grown in bFGF lost their pigment, acquired NCAM (Fig. 6C) and proliferated and spread in a manner resembling that of the primary NR (Fig. 6B). Eventually lentoid bodies in various stages of development were seen in most of these colonies (not shown).

In both groups (i.e. with and without bFGF) there was a minority of colonies that were not easily identifiable as either RPE or NR/Müller cells and it is likely that the excised RPE^t may have undergone a partial dedifferentiation in the absence of bFGF as they normally do *in vitro* (Turksen *et al.*, 1983; Opas *et al.*, 1985). Nevertheless, it is clear that the transdifferentiated RPE maintains its phenotypic plasticity in that after explantation, it can revert to the original NR-like phenotype under the influence of bFGF.

Discussion

We have used a culture system in which NR spontaneously transdifferentiates into RPE. The RPE cells are concluded to be derived from the NR as our dissection ensures the lack of contamination by the original RPE cells. The cells of the RPE layer have all become heavily pigmented by the 6th day of development (Stroeva and Mitashov, 1983). This is a *bona fide* transdifferentiation as it can also be achieved in clonal culture (Okada *et al.*, 1979).

Identity of the RPE^t

Formation of the RPE^t is accompanied by the loss of NCAM from the cell surface, the induction of melanogenesis and the upregulation of eRPE$_{AG}$. The downregulation of NCAM points only to the loss of neural characteristics, whereas the induction of melanogenesis and the upregulation of eRPE$_{AG}$ positively identify the transdifferentiating cells as RPE. The appearance of pigment granules on E3.5 distinguishes the RPE from any other cell type in the retina. The nature of the antigen seen by the 3c10 antibody and referred to by us as eRPE$_{AG}$ is currently not known as this antibody does not immunoblot nor immunoprecipitate (Grunwald, personal communication). Nevertheless, eRPE$_{AG}$ is expressed **exclusively** by the RPE and is already abundant by E3 (Chu and Grunwald, 1990). Because we use E6 NR to establish cultures and we do not detect any pigmented or eRPE$_{AG}$-bearing cells there, we are confident that the RPE^t originates from the NR.

The resultant RPE^t cells are phenotypically stable in that they maintain the RPE phenotype after isolation and explantation. These cells also display phenotypic plasticity as they can undergo a secondary transdifferentiation into the NCAM-positive NR phenotype under the influence of bFGF or they may differentiate into lens epithelium (Okada, 1980).

It is well established that a dynamic profile of changes in pH_i in response to soluble factors is often specific to a cell type (Moolenaar, 1986; Ingber *et al.*, 1990; Schwartz *et al.*, 1991; Schwartz, 1993). The lack of a stable pH_i elevation in response to bFGF is common to both RPE^t and primary RPE cells (Zhou and Opas, 1994). In contrast to the primary RPE, pH_i in the RPE^t peaks transiently in response to bFGF. It is not surprising to fine minor differences between primary and RPE^t since transdifferentiation may be considered a new, redirected differentiation (Okada, 1986; Nathanson, 1986). Hence, although the primary and RPE^t are not physiologically identical, we infer that the RPE^t are *bona fide* RPE cells because their morphological and antigenic profiles are identical.

Phenomenology of the NR➡RPE transdifferentiation

The RPE cells are first seen as isolated islands, therefore the decision to switch from the NR fate to the RPE fate occurs locally. Does this decision to dedifferentiate occur first, followed by the

choice of fate or is the cell fate determined first and followed by transdifferentiation? It appears from work on RPE transdifferentiation by Eguchi and his collaborators that a decision to dedifferentiate has to be undertaken before a choice of cell fate can be made (Eguchi and Kodama, 1993; Agata *et al.*, 1993). Whether or not this is also the case for the NR➡RPE transdifferentiation still remains to be determined.

The RPE^t cell colonies are initiated in the packed regions of the primary, mother NR cell sheets. The cessation of mitosis is probably essential for melanin synthesis to occur (Pritchard, 1981), thus pigmentation begins in non-dividing cells in the centres of these packed regions. Pritchard (1981) suggests that melanin synthesis begins in a minority of "pigment leader cells", which act as centres of influence on neighbouring "potential" pigment cells and that controlling the tricarboxylic acid cycle is also an important step in the transdifferentiation of NR to RPE.

The sheets of RPE^t extend centrifugally and their edges are often found underneath the primary NR cells. The overlying NR in the NR/RPE transition zone then forms a multilayered structure, the origin of which can sometimes be traced to a distinct bilayer. This is highly reminiscent of the folding of the cell layers, which occurs during optic vesicle invagination and the formation of the optic cup by proliferation of the germinal neuroepithelium. The mechanism of the layering process *in vitro* is not clear, but appears to be due to physical deformation that occurs when a sheet (the NR) is pulled at either end, thus stretching and elongating it to the extent that it is "rucked up" into longitudinal folds. This implies rearrangement and/or loss of adhesions between the overlying and underlying cells. When one considers that *in vivo* the Müller cells elongate and eventually lie with their longitudinal axis perpendicular to the RPE sheet (i.e. radially, between the scleral and vitreal layers) it is perhaps not surprising that they should lose contact, for the most part, with the RPE layer within the two-dimensional confines of a culture dish.

Pritchard (1981) observed that multilayers are produced by the upward extrusion of cells due to their continuing expansion in packed areas to the extent that a cell or group of cells is "pushed" upwards. We have seen single raised cells in X-Z optical cross-sections of these cultures (Zhou and Opas, 1994), but we do not know whether these raised cells (which have a much lower pH_i than their neighbours) correspond to the upwardly extruded cells referred to by Pritchard. Perhaps these are the precursors of bottle cells? Folding of the NR, by physical manipulation of the cell sheet in culture, has been shown to enhance lentoid body formation (Clayton *et al.*, 1977). Pritchard *et al.* (1978) noted that lentoids develop from the upper regions of multilayers whereas pigment epithelium forms only among cells which are in contact with the vessel surface.

Attachment and soluble factors in regional specification during transdifferentiation and histodifferentiation of retinal tissues

Extracellular matrix (Eguchi, 1998) as well as attachment factors such as NCAM play an important role in the retina (Brackenbury *et al.*, 1984; Hoffman *et al.*, 1986; Neugebauer *et al.*, 1988; Bartsch *et al.*, 1989). NCAM expression in the retina is developmentally regulated (Neill and Barnstable, 1990) and its role has been hypothesized to be an instructive one (Rutishauser, 1992). NCAM expression is upregulated during transdifferentiation of RPE into NR (Opas and Dziak, 1994). Thus, our present data showing NCAM downregulation during transdifferentiation of the NR into RPE are consistent with the aforementioned findings. Cells at the early stages of transdifferentiation often express markers characteristic of both the starting and the destination phenotype (Guillemot and Cepko, 1992; Opas and Dziak, 1994). Accordingly, in the present cell system we also find cells located at the border between the primary NR cells and RPE^t cells that express both NCAM and the $eRPE^{AG}$. Importantly, NCAM and $eRPE^{AG}$ are polarized in these cells, with $eRPE^{AG}$ predominating at the sides of the cells facing the RPE^t, whereas NCAM is strongly expressed at the cell apices facing the primary NR cells. This is reminiscent of cultured rat RPE, which expresses NCAM (Neill and Barnstable, 1990) unlike chick RPE (Rizzolo *et al.*, 1994; Opas and Dziak, 1994), and in which NCAM is present basolaterally but it becomes polarized apically when the RPE is brought into contact with NR (Gundersen *et al.*, 1993). Thus by analogy, in our system, contact with NCAM-positive primary NR may induce polarized NCAM expression in cells of a destabilized, transitional phenotype. It appears that the presence of a basal layer of RPE cells stimulates histodifferentiation of the overlying NR cells with which they are in contact. Indeed, the importance of such contact phenomena for early retinal histodifferentiation is well established (Johnston *et al.*, 1979; Buse and De Groot, 1991; Reh, 1992; Buse *et al.*, 1993; Layer and Willbold, 1993; Pittack *et al.*, 1997). In our experiments, morphology and immunohistochemistry of transdifferentiating cultures of NR indicate that at least some aspects of retinal histogenesis are being recapitulated *in vitro*.

Why do the retinal tissues transdifferentiate in the first place? After a "culture shock" of retinectomy, the cells *in vitro* destabilize and appear to regain some pluripotential characteristics of the immature tissue. At this stage, different combinations of environmental factors can promote differentiation of cells in several directions. Spontaneous transdifferentiation into lens epithelium appears to be a default pathway as NR grown under several different culture conditions (different ECM substrata ± FGFs, ± insulin, ± phorbol ester) will always produce lentoid bodies (E. Dziak, unpublished observations). In the case of FGF-stimulated transdifferentiation of RPE into the NR the true inductive stimuli are obscure. The presence of anti-FGF antibodies does not affect chick RPE differentiation (Pittack *et al.*, 1997) and differentiated chick RPE cells are unresponsive to bFGF (Zhou and Opas, 1994; Opas and Dziak, 1994). Therefore, FGF acts not on the fully differentiated RPE but only on those cells that have been stimulated to change their identity (Zhou and Opas, 1994; Opas, 1994b). In the RPE cells, it is the cell shape itself that determines resting pH_i and responsiveness to FGF (Zhou and Opas, 1994), therefore cell shape may be a sufficient inductive stimulus. Also, the RPE cells extend as if they had to be "primed" for the subsequent events by stretching. Whether this involves strain-sensitive signal transduction only or also strain-sensitive gene regulation remains unknown. Extended cells acidify and start to express the neural marker, NCAM. Formation of the NR by transdifferentiation of RPE requires extensive cell proliferation of the newly formed neuroepithelium. Hence, in the absence of growth-stimulatory effect of FGF, full histodifferentiation does not occur. In contrast, stimulation of proliferation of neuroepithelial cells by FGF results in formation of the stratified NR (Park and Hollenberg, 1989; Park and Hollenberg, 1991; Pittack *et al.*, 1991; Guillemot and Cepko, 1992; Hyuga *et al.*, 1993; Opas and Dziak, 1994; Zhao *et al.*, 1995).

In the present experiments, as small (several cells in diameter) RPE^t islands can form at any location across the NR cultures, it appears that NR➡RPE transdifferentiation is a stochastic process,

most likely initiated at a single cell level by a change in the local micro environment. We shall refer to this mode of response as a "leader effect". This is by contrast to the "community effect" which requires that many (100 or more) competent cells be present at a given site to be able to respond to an inductive signal (Gurdon, 1988). According to Pritchard's hypothesis (Pritchard, 1981), "pigment leader cells" appear and influence neighbouring cells to convert to the RPE fate and the RPE^t cell sheet is formed. It is tempting to hypothesize that contact of NR cells with the RPE^t layer initiates its further histodifferentiation into the stratified "retinal" structure. This hypothesis derives support form the observation that the presence of the RPE cell layer is necessary for the proper retinal stratification both *in vitro* (Vollmer *et al.*, 1984) and *in vivo* (Raymond and Jackson, 1995). In an elegant study of FGF effects on the retinal histodifferentiation and transdifferentiation, Pittack *et al.* (1997) present a model of retinal development in which contact of the neuroepithelium of the optic vesicle with FGF-rich ectoderm induces histodifferentiation of the NR, while extraocular mesenchyme provides neuroepithelium of the more medial regions of the optic vesicle with hypothetical signal(s) to histodifferentiate into the RPE. While bFGF seems to be the signalling molecule responsible for the NR development (Pittack *et al.*, 1997), any mesenchyme appears to be able to provide a signal necessary for RPE development (Buse *et al.*, 1993). Is it not the absence of a "neural" signal then which is responsible for RPE fate? Based on the present data, we think that it is possible to supplement the model of Pittack *et al.* (1997) with the role of contact phenomena in the development and maintenance of retinal phenotypes. After induction of the NR by FGF and invagination of the optic vesicle into the optic cup, contact of the developing NR with the RPE is necessary for further retinal histodifferentiation. On the other hand, the absence of neuralizing signals (such as NCAM, for example) from the neighbouring mesenchyme instructs the RPE of its position and, possibly, its identity.

Materials and Methods

Materials

Seminaphthofluorescein (SNAFL)-calcein acetoxymethyl (AM) ester was purchased from Molecular Probes, Inc. (Eugene, OR). Dimethyl sulfoxide (DMSO), Triton X-100 and nigericin were from Sigma Chemical Co. (St. Louis, MO). Human recombinant bFGF, alpha-minimal essential medium (αMEM), heat-inactivated fetal bovine serum (FBS) and antibiotic mixtures for cell culture were from Gibco BRL (Canadian Life Technologies Inc., Burlington, Ont.). A rabbit antiserum specific for chick NCAM (serum R04-6-GH) was a gift of Dr U. Rutishauser (Case Western Reserve University, Cleveland, OH) and a monoclonal antibody, 3c10, against chick RPE determinants expressed exclusively between embryonic days 3 and 17 (E3-E17) (Chu and Grunwald, 1990) was a gift of Dr G. B. Grunwald (Thomas Jefferson University, Philadelphia, PA). This antibody does neither immunoblots nor immunoprecipitates. The antigen recognized by the 3c10 monoclonal antibody is referred to as $eRPE_{AG}$ throughout the paper. FITC- or TRITC-conjugated donkey-anti-rabbit or -mouse antisera were purchased from Bio/Can (Mississauga, Ont.). All MEM-HEPES media used for pH_i measurement were $NaHCO_3$ free. Amino-acid free MEM-HEPES was used for dye loading. For pH_i calibration, the MEM-HEPES was modified by replacing all NaCl with KCl (K^+-MEM-HEPES).

Preparation of cells

Fertilized chick eggs were obtained from a local hatchery and maintained in an egg incubator for 6 days in a humidified atmosphere at 38°C. All dissections were carried under a dissecting microscope under aseptic conditions in α-MEM supplemented with 100 IU penicillin, 0.25 μg fungizone and 100 μg streptomycin per ml.

For preparation of the NR cell cultures the embryos were decapitated and retinectomy was performed as described previously (Opas and Dziak, 1988; Opas and Dziak, 1994). Briefly, a small incision was made into the limbus and cornea, the equatorial part of the eye surrounding ora serrata dissected and discarded, and the lens and vitreous were removed to expose the retina. The NR was removed with micro forceps and special care was taken to ensure removal of the NR without any RPE cells, which are already heavily pigmented at this developmental stage. It is quite possible to obtain RPE cell-free preparations of NR as intercellular adhesion between RPE cells is very strong, while RPE-NR adhesion is extremely weak at this developmental stage. Following visual inspection for cleanness of the retinectomy the NR cell sheets were then pooled in serum-free α-MEM and dissociated into a suspension by a short (2-3 min) treatment with trypsin-EDTA accompanied by gentle mechanical agitation. Cells and cell clumps were then pooled and incubated in 100% FBS for 10-20 min, after which the FBS was replaced with growth medium consisting of α-MEM containing 10% FBS and antibiotic mixture (100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 0.25 μg ml^{-1} fungizone). The cells were plated onto coverslips in 60 mm culture dishes and maintained in culture at 37°C in a humidified atmosphere of 95% air and 5% CO_2 with the medium changed every two or three days for up to two months.

Immunofluorescent labelling of cells for confocal microscopy

Cells grown on coverslips were fixed in 3.7% formaldehyde in PBS for 10 minutes. After permeabilization with 0.1% Triton X-100 in PBS (2 minutes) and three rinses in PBS, the cells were incubated with a mixture of primary antibodies for 1 hour at room temperature. Cells were washed three times (5 minutes each) with PBS containing 1% FBS. They were then incubated in PBS containing FITC-conjugated donkey-anti-rabbit IgG (1:40 dilution) and TRITC-conjugated donkey-anti-mouse IgG (1:30 dilution) for 1 hour at room temperature. After washing (as above), the cover slips were then mounted on glass slides and fluorescence images collected with the FITC and TRITC filter sets of the Bio-Rad MRC-600 laser scanning confocal microscope.

Fluorescent dye loading and pH_i measurement and calibration

Cells were loaded with the fluorescent pH-indicator SNAFL-calcein AM, based on a method described in detail elsewhere (Zhou *et al.*, 1995). Cover slips with packed cell colonies were rinsed twice (5 minutes each) with $NaHCO_3$-free αMEM with 20 mM HEPES (MEM-HEPES, pH 7.36), then incubated (room temperature, 30-40 minutes) in amino-acid and serum-free MEM-HEPES containing 5 μM SNAFL-calcein AM prepared immediately before use from a 1 mM stock solution in DMSO. Loaded cells were rinsed twice with MEM-HEPES and further incubated in the same medium containing 2% FBS for about 10 minutes at room temperature to allow deestrification and equilibration. The cells were sufficiently labelled after a 30 minutes incubation with 5 μM SNAFL-calcein AM - higher concentrations or longer incubation times resulted in overloading of the dye into the nucleus and dye compartmentalization into membrane-bound organelles, which affects the accuracy of pH_i calibration (Zhou *et al.*, 1995). Using ratio imaging confocal microscopy, pH_i measurements and calibration were performed exactly as described previously(Zhou and Opas, 1994). pH of the culture medium during these measurements was 7.36. All pH_i values throughout this paper are expressed as mean ± s.e.m. with $n \geq 15$, unless otherwise indicated. Statistical analysis was performed by means of Student's *t*-test for paired observations. To eliminate errors caused by photobleaching, photobleaching assays were performed to determine the average rate of photobleaching as described previously (Zhou and Opas, 1994).

Projected cell area measurements

To correlate pH_i to the degree of cell spreading we measured areas of optical cross-sections of at least 15-20 cells per experiment in each of the 3 regions (tightly packed, cuboidal cells in the centre, crowded cells 2-3

rows away from the edge and fully spread cells at the edge) of a cell colony. This was done with a BIO-RAD COMOS imaging program. Since we measured areas of optical cross-sections of cells we refer to them as the Projected Cell Areas throughout. In parallel, average grey levels in cells with different Projected Cell Areas were measured and converted into pH_i as described above. The measurements were performed on the following three morphologically different groups of cells:

(i) tightly packed cells in the centre of a colony with Projected Cell Areas of < 1000 μm^2 ("cuboidal");
(ii) cells 2-3 rows away from the edge of a colony with Projected Cell Areas of 1500-3500 μm^2 ("crowded"); and
(iii) fully spread cells at the edge of a colony with Projected Cell Areas of > 4000 μm^2 ("spread").

Explantation of RPE^t colonies

Using the x10 objective of a phase-contrast microscope and under sterile conditions, a glass micropipette with blunted tip was used to excise RPE^t colonies from the mother cell sheet. Care was taken to remove only the pigmented, polygonal cells from the centre of a colony. The resulting clumps of RPE^t cells (generally 20-50 cells in each clump) were then transferred to individual wells of a 24-well plate, each containing a glass coverslip and fresh medium (αMEM + 10% FBS). After 24 - 48 hours, 1 $\mu g\ ml^{-1}$ bFGF was added to half of the clumps of cells that had adhered and begun to proliferate and spread. The "new" colonies, each on a separate coverslip, were then cultured in the presence or absence of bFGF for a further 2-4 weeks.

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