Basic fibroblast growth factor induces retinal pigment epithelium to generate neural retina in vitro

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Summary
During embryogenesis, the cells of the eye primordium are initially capable of giving rise to either neural retina or pigmented epithelium (PE), but become restricted to one of these potential cell fates. However, following surgical removal of the retina in embryonic chicks and larval amphibians, new neural retina is generated by the transdifferentiation, or phenotypic switching, of PE cells into neuronal progenitors. A recent study has shown that basic fibroblast growth factor (bFGF) stimulates this process in chicks in vivo. To characterize further the mechanisms by which this factor regulates the phenotype of retinal tissues, we added bFGF to enzymatically dissociated chick embryo PE. We found that bFGF stimulated proliferation and caused several morphological changes in the PE, including the loss of pigmentation; however, no transdifferentiation to neuronal phenotypes was observed. By contrast, when small sheets of PE were cultured as aggregates on a shaker device, preventing flattening and spreading on the substratum, we found that a large number of retinal progenitor cells were generated from the PE treated with bFGF. These results indicate that bFGF promotes retinal regeneration in vitro, as well as in ovo, and suggest that the ability of chick PE to undergo transdifferentiation to neuronal progenitors appears to be dependent on the physical configuration of the cells.

Key words: fibroblast growth factor, neural retina, pigment epithelium, regeneration, retina.

Introduction
The neural retina and the pigmented epithelium (PE) develop from a region of the anterior neural plate that has been determined at gastrulation to give rise to ocular tissue (see Lopashoz and Stroeva, 1964, for review). Experiments in both frog and chick embryos have indicated that the state of determination of the eye primordia is not immediately fixed; instead, there is a certain amount of lability in whether the cells will adopt a PE phenotype or a neural retinal phenotype (Coulombre and Coulombre, 1965). Even after the cells of the pigmented epithelial layer have become clearly different morphologically and biochemically from the cells of the neural retina, they retain the capacity to replace the sensory retinal cells after retinal damage by a process of phenotypic switching known as transdifferentiation (for reviews, see Lopashov and Stroeva, 1964; Reyer, 1977; Okada, 1980; Reh et al. 1987; Coulombre and Coulombre, 1965).

Although little is known concerning the molecular factors controlling this process, previous experiments in our laboratory supported the hypothesis that the extracellular matrix (ECM) is important in the transdifferentiation of pigment epithelial cells into neuronal progenitors during retinal regeneration. In vitro experiments, in which larval Rana pipiens pigment epithelial cells were cultured on a variety of different substrata, showed that laminin and the EHS–sarcoma basement membrane preparations (a complex mixture of extracellular matrix molecules composed primarily of laminin) were effective promoters of neuronal transdifferentiation, while the other substrata that we tried were not. More recently, another component of the ECM has also been implicated in the switch in phenotype from PE cell to neuronal progenitor. Basic fibroblast growth factor (bFGF), a heparan-binding peptide mitogen concentrated in many basement membranes (Baird and Ling, 1987; Folkman et al. 1988; Gonzalez et al. 1990; Saksela and Rifkin, 1990), has been shown to promote retinal regeneration in embryonic chicks in vivo, presumably from the pigment epithelial cell layer (Park and Hollenberg, 1989).

To test directly whether bFGF promotes new neural retina formation from the pigment epithelium, or alternatively from another source within the embryonic eye, we cultured the pigment epithelial cells from chicks of a variety of different stages in the presence of bFGF. We found that the pigment cells from early embryonic stages can give rise to new neural retina when cultured
with bFGF, but, surprisingly only when cultured as aggregates in suspension cultures, and not when dissociated and allowed to spread onto a substratum.

**Materials and methods**

**Dissociated pigment epithelial cell culture**

Embryonic chick eyes, from Hamburger and Hamilton stages 24 to 34, were removed and dissected to separate the PE from the neural retina using fine watchmaker’s forceps. The PE was dissociated as follows: (1) the sheets of PE were transferred to a solution of Ca$^{2+}$/Mg$^{2+}$-free Hank’s buffered salt solution (HBSS) and incubated for 5 min at 37°C; (2) trypsin was added to a final concentration of 0.25% and the incubation was continued for another 5 min; (3) the trypsin was then inhibited by the addition of 1% fetal bovine serum (FBS); and (4) the cells were pelleted and resuspended in fresh F12/DMEM medium (GIBCO), containing 1–10% FBS, 1 mM Hepes, antibodies (penicillin-streptomycin, 100 units ml$^{-1}$; 100 μg ml$^{-1}$), and 0.6% glucose. In our initial experiments, we plated cells at high density, i.e. the pigment epithelia from four eyes of chick embryos at stages 24–34 were dissociated and plated into 24 wells in a single culture plate. In a second series of experiments, a much lower density of cells was obtained in the following way: (1) the cells were transferred to a plastic Petri dish for 30 min to allow aggregation of the single cells into small clusters of 10–20 cells each; (2) then, using a micromanipulator-controlled micropipette and an inverted microscope, the clusters of pigmented cells were individually transferred to wells of a 24-well culture plate containing media. The substrata onto which the cells were plated were prepared as follows: Matrigel (Collaborative Research, Boston, MA) was diluted in HBSS 1:100 and incubated for 5 min at 37°C; (2) trypsin was used to coat either the plastic surface of the wells directly or, glass coverslips that had been placed into the wells; in both cases, the Matrigel solution was allowed to incubate in the wells for >30 min at 37°C, and then most of the solution was removed, and the remainder left to dry in the well. Cultures were maintained at 5% CO$_2$. bFGF (R&D Research, Minneapolis, MN) was added to the wells when the cells were fed, three times each week (10–100 ng ml$^{-1}$). After culture periods of up to 30 days, the cells were fixed with 4% paraformaldehyde for 15 min, washed with PBS and processed for immunocytochemistry.

**Pigment epithelial explant cell culture**

For the explant experiments, strips of PE were obtained from embryonic chicks in the same manner as described above. The tiny strips of PE were placed directly in 24-well culture plates containing 1 ml of medium and mounted on a shaking device (Nutator) at 37°C for up to 2 weeks. In each experiment, approximately half of the wells were treated with bFGF (10–100 ng ml$^{-1}$) while the other wells were used as controls. All wells were examined daily on an inverted microscope for evidence of neuronal transdifferentiation. For immunocytochemical analysis, cultures were fixed in 4% paraformaldehyde for 20 min, frozen at −70°C, and sectioned on a cryostat. Sections from control and bFGF-treated wells were then processed for immunohistochemistry as described below. To label the proliferating cells within the explants, [3H]thymidine (1 μCi ml$^{-1}$ of a 6.7 Ci mmol$^{-1}$ solution, New England Nuclear) was added to some of the cultures for 12 h. At the end of the incubation period, the cultures were fixed, sectioned on a cryostat, labelled with NF-M antiserum, as described below and processed for autoradiography by standard methods.

**Neural retinal cell culture**

As a control experiment, we also examined the effects of bFGF on the neuroepithelial cells of chick retina. Neural retinas from the same eyes from which the PE was obtained (i.e., stage 24) were placed into Ca$^{2+}$/Mg$^{2+}$-free HBSS for 15 min, followed by treatment with 0.25% trypsin for an additional 15 min. The trypsin was then inactivated by the addition of 1% FBS. The cells were subsequently resuspended in fresh medium and gently triturated to yield a single-cell suspension. An equal number of cells was plated into wells of 24-well plates and the cells were cultured for one to five days on a shaking device, as has been described for the PE cultures. Half of the wells received 20 ng of bFGF every other day. The number of cells/well was determined each day, in triplicate, by counting with a hemacytometer. Since the cells typically reaggregated within a short period of time in the cultures, they were redissociated prior to counting, as described above.

**Immunohistochemistry**

The cultures were then incubated with various neuron-specific antibodies to reveal the composition of the cultured cells. Rabbit antisera to neural cell adhesion molecule (N-CAM, a gift from Dr A. Acheson, University of Alberta, Edmonton), neuron-specific enolase (NSE, purchased from Dakopatts, Denmark) and the 160X10$^6$/M, subunit of neurofilament protein (NF-M, generously supplied by Dr G. Bennett, University of Florida) were diluted to approximately 10 μg ml$^{-1}$ prior to use. A monoclonal antibody raised against the cellular retinaldehyde-binding protein was the gift of Dr J. Saari (University of Washington), and diluted to 1 μg ml$^{-1}$ prior to use. All antibodies were visualized via a biotin–streptavidin–fluorochrome (Molecular Probes, Eugene OR) complex. Omission of the primary antibody eliminated the observed immunoreactivity.

**Results**

bFGF causes dissociated chick PE cells to undergo several morphological changes, but they do not shift their phenotype to neuronal progenitors

Retinal pigment epithelial cells were plated on Matrigel, polylysine or untreated tissue culture plastic and observed daily over the course of 2 to 4 weeks. Fig. 1A shows a typical small cluster of PE cells from a stage 24 chick embryo one day after plating onto Matrigel, while Fig. 1B shows the same culture two weeks later. Without the addition of bFGF, the cultures retained their pigmentation and cuboidal morphology even though they proliferated rapidly and eventually covered the well (Fig. 1B). This morphology was similar regardless of the substratum onto which the cells were plated, and so we conclude that Matrigel does not promote neuronal transdifferentiation in dissociated chick PE, as it does in the larval frog. By contrast, PE cells that were treated with bFGF underwent several obvious morphological changes (Fig. 1C–F). The bFGF-treated PE cells lost their pigmentation and frequently formed large, loosely adherent balls of cells. Although these balls were reminiscent of similar aggregates found in transdifferentiating frog PE (Reh et al. 1987), we never observed neurites extending from them.
Fig. 1. Effects of bFGF on dissociated cell cultures of embryonic chick pigment epithelial cells. Small clusters of PE cells (A, C, E) were individually placed into wells of a 24-well tissue culture plate and cultured for up to two weeks in the presence or absence of bFGF (10 ng ml⁻¹). PE clusters that were not bFGF-treated gave rise to confluent monolayers of cells that retained their cuboidal morphology and dark pigmentation (B). C and E show two different bFGF-treated PE clusters one day after plating onto Matrigel, while D and F, respectively, show the same clusters 14 days later. In the cultures treated with bFGF, most of the cells lost their pigment, and formed large aggregates or balls (D and F); however, these balls showed no immunohistochemical or morphological evidence of neuronal or neuroepithelial phenotypes. Scale bar=30 μm for A, B, C and E and 35 μm for D and F.

To characterize further the cells within these balls, we carried out a series of immunohistochemical studies. We found that neither the control nor bFGF-treated cultures containing the non-pigmented balls were immunoreactive for any of the following neuronal antigens: N-CAM, NSE, A2B5, neurofilament or opsins (rho 4D2). In some cases, the cultures containing the balls were sectioned (see Methods) and examined for
the formation of a neuroepithelium characteristic of transdifferentiation in ovo, but we found no evidence of such a morphology.

In an attempt to reconcile the inability of dissociated chick PE to transdifferentiate in response to bFGF, we considered two alternative possibilities. First, it is possible that the cells that give rise to the regenerated retina in vivo were derived from a particularly immature region of the pigmented epithelium, such as that found along the ventral fissure or the anterior margin of the eye. We tested this possibility by selecting these regions of PE during the dissection and culturing these cells in the presence of bFGF, following enzymatic dissociation. We found that the cells derived from the ventral fissure or the anterior margin behaved in exactly the same way as that described for the dissociated PE from the other regions of the retina, described in the previous section (data not shown).

**PE cells transdifferentiate to neuronal progenitors in response to bFGF when cultured as an explant**

An alternative explanation for the failure of dissociated PE cells to transdifferentiate into retinal neuronal progenitors can be found in an observation made by several experimental embryologists following transplantation of eye primordia (see Lopashov and Stroeva, 1964). When eyes develop in ectopic locations, the PE layer will form neural retina only when it retains its epithelial structure. If this layer flattens out onto an adjacent surface the cells will remain pigmented. We therefore examined the ability of PE explants, obtained from stage 24-28 chick embryos, to undergo transdifferentiation to neural retina when exposed to bFGF in a shaker culture. Under these conditions, strips of PE consisting of approximately 5000 cells and some of their associated mesenchymal/scleral cells, remain suspended in the media and do not attach to the bottom of the culture plate. Fig. 2A shows one of the explants after four days in culture, showing that under control conditions (no bFGF added) the cells remain aggregated and retain their pigmentation in culture. By contrast, when 20 ng of bFGF is added to the cultures daily a dramatic morphological change occurs in the cells. Within the first day in culture, some of the bFGF-treated explants began to lose their pigmentation and the cells within the explants became more columnar in their appearance. After four days of exposure to bFGF, approximately 40% of the explants have large regions within them that have lost all evidence of pigmentation and begin to resemble a neuroepithelium which is very reminiscent of the sensory retina (Fig. 2D,E). Typically, the explants that underwent this morphological change retained some pigmented cells after 4 days; however, after 7 days in culture some of the explants no longer showed any signs of pigmented cells. This apparent morphological transdifferentiation was never observed in cultures that were not treated with bFGF.

In addition, neither NGF (Fig. 2D), EGF (Fig. 2C) nor TGF-β (not shown) elicited the same response as bFGF. The TGF-β and NGF treated explants were identical to untreated explants, while those explants treated with EGF appeared to grow in size, but did not lose their pigmentation.

Although the retina can be easily separated from the PE during dissection, it is possible that a few neuroepithelial cells remained associated with the PE explants and bFGF induced these remaining cells to proliferate rapidly. In order to determine whether the PE strips contained small pieces of neural retina that were not removed in the dissection, we randomly selected a number of PE strips and sectioned them at two microns instead of culturing them. In addition, PE strips that were cultured without bFGF were also sectioned and stained with neuron specific antibodies. We examined the sections of the control explants for the presence of neuronal progenitors. We found no evidence of retinal neurons or neuroepithelial cells in any of these control explants. In addition, we added bFGF to cultures of chick retina from the same stages to determine whether this factor is mitogenic for retinal neuroepithelial cells or promotes the survival of cryptic neuroepithelial progenitor cells. When bFGF or EGF was added to stage 27 retinal cells, cultured either as aggregates from initially dissociated cells, or as small explants, we found no significant differences in the cell number between the bFGF-treated wells and the control wells, even after 5 days in culture (1.3 ± .38 x 10⁶ for control and 1.3 ± .19 x 10⁶ for bFGF-treated wells). Therefore, it is unlikely that the bFGF acts in our cultures to stimulate the proliferation of a small number of cryptic progenitors to generate the extensive neuroepithelium that we have observed.

**Immunocytochemical analysis of PE explant cultures**

In order to characterize further the morphological changes that we observed with bFGF treatment, some of the explants were fixed and sectioned for immunocytochemical analysis. We found that most of the cells within the bFGF-treated explant were now apparently organized in a pseudostratified columnar epithelium similar to that normally found in the embryonic retina. In addition, most of the explants still contained some pigmented cells and some transitional cells which have a cuboidal morphology but no pigmentation (arrowhead, Figs 3 and 4A-D). Fig. 3 shows an example of a bFGF-treated explant, labelled with a monoclonal antibody directed against the cellular retinaldehyde-binding protein (CRALBP), found in PE cells of all species that have been examined (Deleceuw et al. 1990). As the PE cells lose their pigmented, cuboidal phenotype, and begin to resemble the cells of the neuroepithelium, they lose their immunoreactivity for this antigen. Fig. 4 shows a section through another bFGF-treated explant, labelled with an antiserum directed against N-CAM (Fig. 4B,D). While the pigmented cells (PE) within the explants are not immunoreactive for N-CAM, as the cells begin to resemble a neuroepithelium in response to the bFGF, they also show a marked increase in the level of N-CAM immunoreactivity.

We also labelled sections from bFGF-treated and control explants with an antiserum directed against neuron-specific enolase (NSE). Fig. 5A shows a typical
Fig. 2. Effects of bFGF on embryonic chick PE explants cultured as nonadherent explants. After four days in culture, untreated PE explants remain as round aggregates that retain their pigmentation (A). The cultures treated with bFGF showed a distinct morphological response after 4 to 7 days in vitro with the addition of bFGF to the medium; the cells began to lose some of their pigmentation (D,E). In (E), the degree of retinal transdifferentiation is quite extensive after seven days in culture with bFGF. Neither NGF (B) nor EGF (C) (both at 10 ng/ml) appears to elicit the same response in the PE explants as bFGF after seven days in culture. Scale bar=90 μm for A–C, 215 μm for D and 275 μm for E.
Fig. 3. bFGF-treated PE explants were sectioned and processed for immunohistochemistry with antibodies to CRALBP (a gift from Dr J. C. Saari). A and B show corresponding phase and fluorescent micrographs, respectively, of a bFGF-treated PE explant. C and D are a higher magnification of the same explant. CRALBP immunoreactivity is present in the pigmented, cuboidal cells (pe) but is absent from the newly generated non-pigmented regions (nr). The arrowheads point to the transitional zone between the pigmented, CRALBP-immunoreactive cells and the nonpigmented columnar epithelium that is devoid of CRALBP immunoreactivity. Scale bar=75 μm for A and B, 30 μm for C and D.

PE explant immediately after dissection. Fig. 5B shows the same explant treated with bFGF after 6 days in vitro. This same explant was fixed and sectioned as previously described and labelled with antibodies directed against NSE. Fig. 5C shows the typical morphology observed in bFGF-treated explants. The arrowheads in Fig. 5C and D point out small areas within the explant of PE cells that have retained their pigmentation. Note that these cells do not label with anti-NSE. The cells within the explant that do label with anti-NSE are the cells that have the characteristic pseudostratified columnar epithelial appearance (Fig. 5C) that also label with anti-N-CAM. None of the control explants showed any evidence of neuroepithelium formation after 14 days in culture (Fig. 5E), and never exhibit any NSE or N-CAM immunoreactivity (data not shown). These control explants instead form a pigmented epithelium (pe) surrounding a core of mesenchymal/scleral cells (Fig. 5F).

An additional neuron-specific antiserum that we used to analyze the explants was directed against the 160kD subunit of neurofilament protein (NF-M), which has been previously localized to newly postmitotic, migrating retinal ganglion cells, in addition to the mature cells in the ganglion cell layer (Bennett and DiLullo, 1985; McLoon and Barnes, 1989). We labelled some of the sections of bFGF-treated explants after 6 days in culture and sections from untreated control explants cultured for a similar period, with an antiserum directed against NF-M. Fig. 6A, B and C show the typical staining pattern that we observed in a section through part of the unpigmented, columnar, neuroepithelium of a bFGF-treated explant. Most of the NF-M immunoreactive cells are arranged in a layer along the inner surface (gel), similar to the retinal ganglion cell layer of the normally developing retina (Fig. 6A, B); however, NF-M immunoreactive cells with a bipolar morphology are also present within the neuroepithelium and presumably represent migrating ganglion cells (Bennett and DiLullo, 1985; Barnes and McLoon, 1989). In tangential sections through the presumptive ganglion cell layer in these explants, there are NF-M immunoreactive cells that resemble ganglion cells that have long processes extending from the cell body (Fig. 6C). The sections of the control explants did not have any cells immunoreactive for NF-M (data not shown).

To understand better the pattern of new cell addition
in the cultures, explants that had been in vitro for 5 days were treated with [3H]thymidine for 12 h, and then fixed, sectioned, labelled with the antiserum directed against NF-M and processed for autoradiography (Fig. 7). Fig. 7A shows that there are a large number of [3H]thymidine-labelled cells (primarily S-phase progenitor cells) in the newly generated retinal neuroepithelium. It appears that the majority of the [3H]thymidine-labelled cells are in a midradial position within the neuroepithelium, the same position that they would occupy in normal embryonic retina (Kahn, 1974). In addition, there appears to be a central to peripheral gradient of labelled cells, reminiscent of that found in vivo. After 12 h of labelling, there are already some postmitotic, [3H]thymidine-labelled retinal ganglion cells that have yet to migrate from the presumptive ventricular surface. This can be seen in Fig. 7B, where the same section is labelled with anti-NF-M antiserum. The arrowhead points to a cell that is both positive for NF-M and labeled with [3H]thymidine. Explants that were cultured without bFGF were also labelled with [3H]thymidine for 12 h; in these control cultures there were many [3H]thymidine-labelled pigmented epithelial cells, as well as cells within the presumptive sclera. However, the percentage of [3H]thymidine labelled cells was much lower than in the bFGF-treated explants (data not shown).

Age dependence of bFGF effects
The ability of PE to transdifferentiate into neuroepithelial cells and regenerate the sensory retina has been previously shown to be an age-dependent process. Coulombre and Coulombre (1965) showed that regeneration of retina from PE occurred only in chick
embryos of stages 21–27. We therefore tested whether the explant cultures mimicked the in ovo situation, by culturing PE strips from chick embryos at progressively later stages (stages 24–35). Fig. 8A,B shows the data from two experiments in which PE explants from two different embryonic stages were cultured in the presence of bFGF. The explants were carefully examined each day and their morphological changes were assigned to one of the following categories; '1' (solid bars) represents explants that remain pigmented and unchanged in morphology, similar to the control (for example, see Fig. 2A); '2' (cross-hatched bars) was used to describe explants that showed areas of nonpigmented, proliferating cells, but had no evidence of neuroepithelial formation or immunoreactivity for various neuronal antigens; '3' (open bars) denotes cases where the explant had large areas of nonpigmented neuroepithelial tissue, typical of those that demon-
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Stratified immunoreactivity for the neuronal antigens in sections (see Fig. 2D,E). The graph in Fig. 8A shows the morphological characterization of explants obtained from a stage 24 chick embryo. After two days in culture less than 25% of the explants remain in category '1', 50% are in category '2', and a few already show extensive transdifferentiation (category '3'). After four days in vitro, all of the explants are in either category '2' or '3'. On day six, the majority showed extensive areas of nonpigmented neuroepithelial tissue characterized by category '3'. By contrast, Fig. 8B shows the results from explants derived from chick embryos at stage 27. On day two all of the explants remain unchanged and are in category '1', on day four, more than half of the explants are still in category '1'; however, a few display the morphology characteristic of category '2'. On day six, the results are the same as day four. These results demonstrate that the explants behave similarly to PE in ovo, with respect to their ability to transdifferentiate into neuroepithelial precursors and that this ability is progressively restricted as the embryonic development progresses.

We also quantified the response of the PE to other growth factors using the same scale. Fig. 8C shows PE explants from stage 24 embryos that remain in category '1' for the duration of their culture regardless of addition of EGF, NGF or TGF-β (all at 20ng ml⁻¹ every 48h), while 50% of the explants treated with bFGF showed extensive transdifferentiation in this same experiment after six days in vitro. In another experiment, we asked whether TGF-β might exhibit a synergistic inductive response when combined with bFGF, as has been found for mesodermal induction. We found no evidence for synergism between these factors and, in fact, the TGF-β appeared to antagonize the ability of bFGF to induce neuroepithelial cells from PE (data not shown).

**Discussion**

The present study evaluated the ability of chick PE to transdifferentiate into neuronal tissue in vitro after administering bFGF to PE cell cultures from embryonic stages 24 to 34. The PE was dissected from the neural retina and underlying mesenchymal tissue and cultured for periods of up to 30 days on various substrata. Proliferation, loss of pigmentation and some morphological changes occurred, but there was no evidence of transdifferentiation into neuronal phenotypes. Since previous transplantation experiments indicated that the PE will not generate neural retina under conditions
Fig. 7. Cell proliferation continues at a high rate in the bFGF-treated cultures. PE explants, cultured in the presence of bFGF for five days, were labelled with [3H]thymidine for 12h and then fixed, sectioned, processed for autoradiography and immunohistochemistry with anti-NF-M antiserum. In the bFGF-treated explants exhibiting large areas of newly generated neuroepithelium, large numbers of [3H]thymidine labeled cells can be seen localized to middle of the neuroepithelium (A), although a few labelled cells can be observed at the presumptive ventricular surface. These are presumably cells that have recently completed, or are still in, the mitotic phase of the cell cycle. Some of the [3H]thymidine-labelled cells were also immunoreactive for NF-M (arrow) indicating that new neurons are still being generated at this time (B). Scale bar=40µm.

where it spreads or flattens on adjacent tissues (see Lopashov and Stroeva, 1964 for review), we also cultured explants of PE from the posterior eye in 24-

Fig. 8. The ability of PE explants to form a neuroepithelium in culture is dependent on the stage of the embryo from which the cells are derived, as well as the presence of bFGF in the culture medium. (A) Bar graph showing the percentage of stage 24 explants with a morphology of either type '1' (PE phenotype, similar to controls; solid bars), type '2' (transitional morphology, early phases of transdifferentiation to retinal neuroepithelium, cross hatched bars), or type '3' (fully transdifferentiated neuroepithelium, open bars) as a function of days in culture with exposure to bFGF at a concentration of 20 ng ml⁻¹. In the stage 24 cultures, a progressively greater percentage of the explants (up to 50%) show a fully transdifferentiated phenotype with increasing time in culture; however, culture periods longer than 6 days did not result in a further increase in this percentage. (B) A similar graph for stage 27 embryonic PE, under identical culture conditions; no examples of transdifferentiation to neuroepithelium are observed in explants from this stage after 6 days of in vitro exposure to bFGF. Higher concentrations (up to 100 ng ml⁻¹) or longer incubation periods (up to 14 days) did not result in the formation of neural retina in any of the explants from this stage. (C) A similar rating scale was applied to cultures, from a stage 24 embryo, in which other growth factors were added to the medium, and the morphology of the explants was scored after 6 days in culture. Once again, bFGF promoted the transdifferentiation of 50% of the explants to neural retina, while EGF- and NGF-treated cultures retained their control PE appearance.
well plates mounted on a shaking device to prevent attachment. Under these conditions, we found that up to 50% of the explants in a given experiment showed extensive transdifferentiation of the PE into a neuroepithelium, with all of the characteristics of the normal neural retina that we assayed.

These results confirm and extend those obtained previously by other investigators, most notably Coulombre and Coulombre (1965), Tsunematsu and Coulombre (1981), and Park and Hollenberg (1989), and support the idea that bFGF is an important regulator of cell phenotype during retinal development. Coulombre and Coulombre (1965) were the first to demonstrate that embryonic chick retina regenerates from the PE layer in a manner similar to that of the amphibian. Surgical removal of the retina in chick embryos from Hamburger and Hamilton stages 21–28 caused the remaining PE layer to differentiate into neural retina, but only when a small piece of the original neural retina or otocyst was reintroduced into the damaged eye. Recently, Parks and Hollenberg (1989) tested whether the active substance released by the transplanted retina in the Coulombres' experiment was bFGF by implanting slow release gels that contained this growth factor into the optic cup after retinal removal. They found that only those eyes from embryonic chick stages 21–28 that were treated with bFGF showed histological evidence of regeneration.

There are several possibilities as to why only 50% of the PE strips within a given experiment showed extensive transdifferentiation. One possibility is that the amount of bFGF available to the cells was limiting; however, higher concentrations of bFGF (up to 100 ng ml⁻¹) did not result in the generation of neural retina in a greater percentage of the explants. In addition, combining the bFGF with TGFβ, which has been shown to potentiate the effects of bFGF in mesodermal induction (Rosa et al. 1988), also failed to cause neural retinal formation in the remaining 50% of the explants. Another possibility is that, at stage 24, many of the PE cells are already irreversibly committed to the PE phenotype. Previous in vivo studies of retinal regeneration found a gradual decline in the ability of the retina to regenerate from the PE with increasing embryonic stage. Perhaps if we were able to dissect the PE from embryos at stages earlier than Hamburger & Hamilton stage 24, as is possible for in ovo regeneration experiments (Coulombre and Coulombre, 1965), we would be able to see a greater percentage of the explants fully transdifferentiate. Lastly, it is possible that explants randomly distribute with the pigment cells either external or internal to the associated mesenchymal cells. In light of this possibility, we have observed that, in all of the transdifferentiated explants that we have sectioned, the mesenchymal cells are at the core. Conversely, the bFGF-treated explants that do not transdifferentiate, have the PE cells at the core and are surrounded by mesenchymal cells. When the PE cells are at the core, they also lose their typical epithelial structure. We are currently investigating the last possibility, to determine what aspects of the epithelial structure change under these two alternative conditions of folding.

The fact that the neuroepithelium generated by bFGF-treated PE is organized like a normal embryonic retina is also of interest. Based on the presence of a normal pattern of lamination in sections of the regenerated retina, Coulombre and Coulombre (1965) and Parks and Hollenberg (1989) came to a similar conclusion. In both of these studies, the authors also found that the orientation of the regenerated retina was inverted from the normal, with the photoreceptor outer segments in the vitreous and the ganglion cell layer adjacent to the mesenchymal cells of the sclera. We have found, with immunohistochemical identification of the different retinal cell types, that the organization of the retina derived from the PE in these cultures is also oriented with the ganglion cell layer adjacent to the mesenchymal cells of the sclera, similar to that observed for retinal regeneration in ovo.

Although the molecular basis of this switch in phenotype is currently unknown, evidence from the frog suggested that some components in the extracellular matrix, including laminin, are important regulators of this process. During the initial stages of retinal regeneration in Rana pipiens tadpoles, we observed that the first new neuronal progenitors always arose in association with the vitreal vascular membrane (Reh and Nagy, 1987), suggesting to us that the composition of this vitreal vascular ECM might somehow differ from that which the cells were normally in contact with on Bruch's membrane. When the PE cells were exposed to these factors during regeneration, the pigmented epithelial phenotype shifted to the neuronal progenitor phenotype. Initial biochemical characterization of this membrane revealed that it contained a considerable amount of laminin (Reh et al. 1987) and heparan sulfate proteoglycan (Reh and Nagy, unpublished observations), and so we proposed that these molecules might be important regulators of the process of transdifferentiation. This idea was tested by in vitro experiments, in which larval Rana pigment epithelial cells were cultured on a variety of different substrata, including solubilized basement membrane from EHS-sarcoma tumours and laminin that had been purified from the same source. While laminin and the EHS basement membrane were effective promoters of neuronal transdifferentiation, the other substrata that we tried were not. This finding further supports the hypothesis that retinal regeneration is initiated by changes in the composition of the extracellular matrix that the PE cells contact early in this process. In this context, it is interesting to note that HSPG forms complexes with both laminin (Mathews and Patterson, 1986) and bFGF (Baird and Ling, 1987; also see above).

In the chick embryo, as well, there is heterogeneity in the composition of the basement membrane which PE and neural retinal cells contact. We have found that fibronectin and laminin are not homogeneously distributed in the basal lamina surrounding the developing eye; for example, fibronectin immunoreactivity is only associated with Bruch's membrane, and ends abruptly
at the vitreal retinal surface (Pittack and Reh, unpublished observations). During eye development, differences in position within the optic primordia may translate into differences in local inductive molecules, i.e., cells at the interface with ectoderm could be exposed to higher concentrations of laminin and/or bFGF than cells surrounded by mesenchyme. The pigmented phenotype might require a matrix low in bFGF/HSPG/laminin (and relatively higher in fibronectin) to develop, while the neuroepithelial phenotype would require contact with an environment rich in laminin, HSPG and bFGF. Alternatively, a matrix rich in HSPG could selectively convert the low-affinity bFGF receptors to high affinity receptors in those cells directly contacting this matrix (Yayon et al. 1991). Matrix-dependent receptor activation could also explain the age dependency of transdifferentiation, since in situ techniques have shown that pigment epithelial cells throughout the relevant stages of chick embryogenesis express FGF receptors (Heuer et al. 1990a,b). As these two cell types become differentiated, they reinforce the initial matrix differences leading to further heterogeneity in the basement membranes. Stabilization of phenotype leads to the independence of the pigment epithelial cell's state of commitment from the microenvironmental factors in both chicks and mammals; urodeles and larval anurans retain their dependence on these local environmental factors for the stability of their phenotype. During regeneration of the retina in the frog, the PE cells migrate to the vitreal vascular membrane and respond to the laminin/HSPG (and possibly also its associated bFGF) by proliferating and forming neuroepithelial cells. In the early embryonic chick, after retinal removal, no vitreal vasculature remains and so either bFGF or a little piece of retina are required to stimulate neuroepithelial cell formation; however, after the PE phenotype is stabilized, manipulations in the concentrations of microenvironmental factors, such as bFGF, will no longer elicit their transdifferentiation into neural progenitor cells.

One of the most interesting findings of the present study is the fact that the PE cells will only transdifferentiate into neural retinal progenitors when they are exposed to bFGF while in their epithelial structure. Flattening and spreading of either dissociated cells or of the explants precludes this particular response to bFGF, though the cells still respond to the growth factor by increasing their rate of proliferation. Why do PE only transdifferentiate when in an epithelial form rather than when flattened out? We can think of three possibilities. First, it could be that some other cell type within the explant is essential for the transdifferentiation of PE to neuronal epithelial cells, and these are removed in dissociated cell cultures. This is unlikely, since the explants that have settled down on the surface also fail to generate neural retina and show no evidence of neuronal transdifferentiation, even though their cellular composition is identical to those explants that do produce neural retina when they remain suspended in the medium. Second, there may be a critical number of cells required for the transdifferentiation to occur; Edelman (1988) has proposed that only aggregates can respond to inductive signals and that the role of CAMs or morphoregulatory molecules is to force cells into these aggregates prior to the presence of an inductive signal. However, it is unlikely that a critical number of PE cells is all that is required for bFGF to exert its effects, since in high-density dissociated cultures the cells do not transdifferentiate. Third, cytoskeletal changes associated with flattening may influence expression of particular genes necessary for transdifferentiation to occur; cell morphology has been shown to affect gene expression in many systems (see Hay, 1981 for a review).

Whichever of these possibilities turns out to be the case, it is clear from the experiments we have described above that the PE of the chick can be induced to undergo a dramatic transdifferentiation in vitro when bFGF is added to the culture medium. This phenotypic switching should enable a further understanding of the molecular basis for neuronal determination.

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