Development 114, 743-754 (1992)
Printed in Great Britain © The Company of Biologists Limited 1992

Retinal fate and ganglion cell differentiation are potentiated by acidic FGF in an *in vitro* assay of early retinal development

FRANÇOIS GUILLEMOT* and CONSTANCE L. CEPKO

Department of Genetics, Harvard Medical School, 25 Shattuck Street, Boston MA 02115, USA

*Present address: Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Avenue, Toronto, M5K1X5, Canada

Summary

One of the earliest events in vertebrate eye development is the establishment of the pigmented epithelium and neural retina. These fundamentally different tissues derive from the invaginated optic vesicle, or optic cup. Even after achieving a fairly advanced state of differentiation, the pigmented epithelium exhibits the same potential as the optic cup in that it can "transdifferentiate" into neural retina. C. M. Park and M. J. Hollenberg (*Dev. Biol.* 134, 201-205, 1989) discovered that administration of basic fibroblast growth factor, coupled with retinal removal, could trigger this transformation *in vivo*. We have developed a quantitative *in vitro* assay to study the role(s) of the fibroblast growth factor (FGF) family in this phenomenon and more generally in early retinal development. We found that several aspects of the process, including inhibition of pigmented epithelium differentiation, proliferation, and conversion to a retinal fate, were not strictly correlated. Both acidic and basic FGFs were found to potentiate all aspects of the process, with acidic FGF being 4 to 20 times more potent than basic FGF for inhibition of pigmentation and induction of retinal antigens. Depending upon its concentration, acidic FGF induced from 40% to 80% of the cells in the explants to produce antigens normally expressed by retinal ganglion cells, the first cell type to be generated in retinal development. Expression of such a ganglion cell marker could be directly stimulated in non-dividing cells as well as in dividing cells, indicating that conversion from the pigmented epithelial to retinal fate did not require cell division. These data suggest that acidic FGF, or a related molecule, may function in establishment of retinal fate from the optic cup. This effect may be directly or indirectly mediated by induction of retinal ganglion cell fate among multipotent progenitor cells.

Key words: fibroblast growth factors, pigmented epithelium, neural retina development, *in vitro* assay.

Introduction

The neural retina has been frequently used as a model of development of the vertebrate central nervous system (CNS). Both the neural retina (NR) and the apposed pigmented epithelium (PE) derive from a lateral evagination of the neural tube, the optic vesicle. The NR originates from the distal portion of the optic vesicle, after it invaginates to form the inner layer of the optic cup, while the PE derives from the proximal portion, or outer layer of the cup. The NR and PE thus have a nearly common origin and show an early divergence of fate. The PE however exhibits a remarkable plasticity in the initial stages of development: it can be induced to "transdifferentiate" into NR, even after it has started to differentiate into PE (Coulombre and Coulombre, 1965; Detwiler and Van Dycke, 1954). This plasticity is lost upon further maturation in birds and mammals, but is kept throughout life in some urodeles. In the chick embryo, the PE is able to generate neural retina tissue until about embryonic day 4 (E4) (Coulombre and Coulombre, 1965; Stone, 1950; Stroeva, 1962).

One of the first indications of a different fate for the two layers of the optic cup is an increase in proliferation of the neuroepithelial cells of the presumptive NR relative to proliferation in the neuroepithelium of the presumptive PE. This is rapidly followed by the birth of the first ganglion cells which immediately initiate differentiation, as marked by expression of neurofilament and the marker RA4, and by axonal outgrowth (Hinds and Hinds, 1974; Kahn, 1973; McLoon and Barnes, 1989). Development of the NR is then characterized by generation of a variety of neuronal types that form the well defined laminae of the mature retina. The sequence of generation of retinal neurons has been studied in several vertebrate species. Some aspects of the sequence are well conserved throughout vertebrate evolution; ganglion cells are always the first to be born, and bipolar cells are always among the last

(reviewed by Altshuler et al., 1991). Lineage studies using retroviral markers in rodents and chick (Turner and Cepko, 1987; Turner et al., 1990; Fekete and Cepko, unpublished data), and intracellular injections in the frog (Wetts and Fraser, 1988; Holt et al., 1988) have shown that single retinal progenitor cells can give rise to radial clones containing many different types of neurons. These studies have clearly demonstrated that the last division of a progenitor can give rise to two different cell types. Thus, retinal cell fate can be determined at or after the final cell cycle, possibly through interactions among neighbouring cells.

The anlage of NR and PE in the optic vesicle are in contact with head ectoderm and neural-crest-derived mesenchyme, respectively. Transplantation studies carried out in amphibians and in the mouse embryo have suggested an influence of these surrounding tissues on induction of either NR or PE from a common primordium (Detwiler and Van Dyke, 1954; Lopashov, 1963). In addition, the anterior neural plate, and to some extent the presumptive NR, play a role in the formation of the lens from head ectoderm, a classical example of embryonic induction (Spemann, 1905; Grainger et al., 1988). Thus, transplantation experiments as well as lineage analysis have underlined the importance of the interaction of cells with their environment in the control of cell proliferation, determination, and differentiation in eye development. However, very little is known of the molecules involved in these processes.

The presence of several members of the fibroblast growth factor (FGF) family has been demonstrated in NR during embryonic development. Basic FGF (bFGF), the most ubiquitous molecule of the family, is present as early as E2 in the CNS of avian embryos (Kalcheim and Neufeld, 1990) and has been reported to be within NR at least as early as E11 (Mascarelli et al., 1987). Acidic FGF (aFGF) transcripts are found in chick CNS at low levels between E3 and E9, and at increased levels at later stages of development; in general, aFGF gene expression seems to be restricted to the central and peripheral nervous system, throughout chick development (Schnurch and Risau, 1991). At E11, aFGF is, like bFGF, present in high amounts in the eye (Mascarelli et al., 1987). Another FGF-family member, int-2, shows a very specific pattern of expression in several locations of the CNS in the mouse embryo, including in the neuroblastic layer of the retina between E14.5 and P1 (Wilkinson et al., 1989). Finally, transcripts for an FGF receptor are also present in the embryonic retina in chicken (Heuer et al., 1990) and rat (Wanaka et al., 1991). The functions of the FGF family in the development of the retina are largely unknown, but a few clues from disparate systems have recently been reported. A role for aFGF and/or basic FGF (bFGF) in survival and axonal regeneration of mature ganglion cells has been demonstrated both *in vivo* and *in vitro* (Lipton et al., 1988; Sievers et al., 1987). bFGF has been shown to be mitogenic for retinal germinal cells in the rat embryo (Lillien and Cepko, unpublished data). Finally bFGF has been shown to induce the transdifferentiation of PE into NR in E4 chick embryos *in vivo* as well as *in vitro*, after removal of the original retina (Park and Hollenberg, 1989; Pittack et al., 1991).

The lack of data on the role of growth and differentiation factors in retinal development is partially due to the fact that explants of early retina undergo neuronal differentiation *in vitro* without requirement for added factors (Sparrow et al., 1990; our unpublished observations). Presumably, factors required for neuronal development are present and active within such explants. In contrast, immature PE explants do not autonomously undergo neuronal development when cultured in the absence of exogenous factors, but, as shown here, will do so if cultured in the presence of FGFs. We have exploited this observation by developing an *in vitro* assay which should allow a dissection of the effects of FGF molecules on early retinal development. In the present report, we used this system to analyse the effect of aFGF and bFGF on the acquisition of retinal fate and on the differentiation of ganglion cells in PE explants. The neuroepithelium of the presumptive PE of E2.5 chicken embryos was found to respond to low levels of FGFs by differentiating into neural retina, and aFGF was found to be more potent than bFGF in this process. A striking effect of these molecules was on the generation of ganglion cells. aFGF appeared to initiate the differentiation of retinal ganglion cells independently from its effects on the pigmentation and proliferation of explanted PE cells. Thus aFGF, or a related molecule, may be a factor important in commitment to the retinal fate and commitment and/or differentiation of ganglion cells.

Materials and methods

Animals and materials

Fertilized eggs were incubated at 38°C in a humidified atmosphere. Embryos were staged according to Hamburger and Hamilton (1951). Components of the serum-free medium were purchased from Sigma; bovine brain acidic fibroblast growth factor from Research and Development and human recombinant basic fibroblast growth factor from Amgen. The horseradish peroxidase-coupled avidin-biotin staining system was the ABC kit of Vector laboratories. Biotinylated anti-mouse immunoglobulin antibody and texas red-conjugated avidin were also from Vector. Fluorescein-conjugated anti-BrdU antibody was from Becton Dickinson. The 8A1 (anti-NF68) and HPC1 monoclonal antibodies were gifts from Dr. C. J. Barnstable (Barnstable et al., 1985), the RA4 antibody from Dr. S. C. McLoon (McLoon and Barnes, 1989), the SB1 20.11 (anti-chicken Thy1) antibody from Dr. P. L. Jeffrey (French et al., 1987) and the anti-BrdU antibody from Dr. D. Mason. The anti NF200 antibody was from Sigma and the anti-HNK1 (Leu 7) antibody was from Becton Dickinson.

Dissection and explant culture

The explant cultures were established from chicken embryos incubated for 2.5 days (stages 17-18) or 3.5 to 4.5 days (stages 21-24). At stage 17-18, the only morphological difference between presumptive PE and NR is the greater thickness of NR as a result of a higher rate of proliferation; at stage 21 and older, in addition to a greater difference in thickness between

the two tissues, PE starts to show pigmentation. The embryos were collected in phosphate-buffered saline (PBS). The eyes were dissected with forceps, and transferred into Hank's medium (Gibco). Most of the mesenchyme and ectoderm surrounding the eye were then dissected away and the eye was transferred to 4% pancreatin (Sigma) in Hank's medium for 5 minutes at room temperature. The enzymatic digestion of the explant was stopped by a short passage in Dulbecco modified Eagle's medium (DME) plus 10% calf serum, and then a rinse in Hank's medium. The mesenchyme surrounding the optic cup (including the choroid), the ectoderm and the lens were then removed using stainless steel pins. The parts of the optic cup where the inner wall (neural retina) and outer wall (pigmented epithelium) are connected (i.e. the most peripheral part of the cup and the choroid fissure) were separated with pins. The pigmented epithelium could then be detached easily as a sheet from the neural retina without any attached retinal cells, as determined by microscopic observation.

The PE explant was then transferred into a 35 mm culture dish (Costar) coated with bovine type 1 collagen (Collaborative Research), flattened using pins with the outer surface against the surface of the dish, and allowed to adhere by incubation in a tissue culture incubator for 3 hours in a minimum volume of serum-free culture medium containing 0.2% methyl cellulose (Fluka A.G.). Prewarmed serum-free culture medium (1 ml) containing the desired concentration of factor was added. This medium was as described in Lillien et al. (1988). When acidic FGF was used, 10 μg/ml heparin (Sigma) was also added, and the same concentration of heparin was then used in control experiments without added growth factor. Half of the medium containing the appropriate concentration of factor was replaced every other day. At the end of the culture period, explants were washed and dissociated by incubation in 0.5% trypsin + EDTA (Gibco) for 15-30 minutes at 37°C. Enzymatic digestion was stopped by addition of 1 volume of 10% CS in DME, cells were suspended by gentle pipetting, centrifuged and resuspended in serum-free medium containing 0.2% methyl cellulose. 20 μl of the cell suspension were plated on collagen-coated coverslips in 24-well plates, and the cells allowed to adhere for 3 hours. Up to 6 coverslips were prepared from one dissociated explant (stage 17 PE explants typically contained about 10,000 cells after 3-4 days in culture). Cells were then fixed by addition of 200 μl of 4% paraformaldehyde to the wells for 10 minutes at room temperature, followed by one rinse in 50% methanol in PBS, and storage in 100% methanol at −20°C before staining. When cells were prepared for Thy1 staining, staining directly followed fixation with 4% paraformaldehyde and a rinse in PBS. Neural retina, used for antibody staining experiments and [^{3}H]thymidine incorporation experiments, were obtained from eye primordia and were dissociated in the same way as for PE explants as described above. To identify mitotic cells expressing neurofilament, PE explants or neural retinas were pulsed with 5 μCi/ml [^{3}H]thymidine for 1 hour, then dissociated as described above, and cell suspensions were plated for 30 minutes on poly-L-lysine (Sigma) coated coverslips before fixation.

Antibody staining

The following antibodies: 8A1 (ascites fluid used at 1:100 dilution), anti-NF200 (ascites fluid, 1:200), HNK1 (1:20), RA4 (hybridoma supernatant, 1:300), anti-Thy-1 (0.1 μg/ml), anti-BrdU (hybridoma supernatant, 1:2) were diluted in DME plus 10% CS, and applied for 30-45 minutes at room temperature. Most antibodies were visualized using a biotinylated anti-mouse Ig antibody and horseradish peroxidase-coupled avidin-biotin complex ABC kit, Vector), followed by revelation of HRP activity with DAB. Anti-Thy-1 antibody was visualized by anti-mouse Ig antibody and Texas Red-coupled streptavidin, and anti-HNK1 was visualized with an HRP-coupled anti-mouse IgM antibody.

Anti-BrdU staining was preceded by a 30 minute incubation in DNAse I at 25 μg/ml in PBS with 10 mM $MgCl_2$ and 40 μg/ml BSA, and a 5 minute rinse in PBS. Double staining for BrdU and NF68 was obtained using biotinylated anti-mouse Ig antibody and Texas Red-avidin to reveal NF staining and fluorescein-conjugated anti-BrdU antibody for BrdU staining.

Coverslips were mounted in gelvatol and examined with a Zeiss Axiophot microscope.

Results

FGFs induce neural development in explants of pigmented epithelium

PE explants from either stage 17-18 of Hamburger and Hamilton (HH) (E2.5-3) or stage HH 24-25 (E4-E4.5) chicken embryos (Hamburger and Hamilton, 1951) were separated from both the NR and the underlying mesenchyme, and cultured as monolayers in serum-free medium on collagen-coated dishes. Without added growth factor, the younger explants (stage 17-18), which were initially unpigmented (pigmentation starts at stage 20 *in vivo*), acquired a distinct pigmentation within 2 days. Similarly, the older explants (stage 24-25), which were initially pigmented, remained largely pigmented in these conditions (Fig. 1A). When acidic or basic FGF (FGFs) were added to stage 17-18 explants, pigmentation never appeared and the culture progressively lost its epithelial appearance; after 3 to 4 days, some cells showed a neuronal morphology (Fig. 1B,E). In older (stage 24-25) PE cultures treated with aFGF or bFGF, some regions of the cultures lost their flat aspects and within 12 hours developed folds and bulbs generally devoid of pigmentation. If the stage 24-25 cultures contained serum in addition to an FGF, they developed complex folds within one week (Fig. 1D), whereas in serum-free medium containing FGFs, these folds usually stopped development after 3-4 days.

To determine whether the morphological changes in FGF-treated PE cultures reflected neuronal development, immunocytochemistry was carried out with antibodies specific for neural markers. Stage 24 PE explants were grown for 10 days in 100 ng/ml aFGF and 10% calf serum, and sections from these cultures were stained and analyzed. Positive staining was observed for widely expressed neural markers such as HNK1 (Fig. 2A) or N-CAM (not shown), and for cell type-specific neuronal markers such as HPC1, recognizing amacrine cells and possibly horizontal cells in retina (Fig. 2B). These sections also showed that the folded structures observed after several days in culture were multilayered (Fig. 2A,B), and displayed some organization reminiscent of retinal structure, with HPC1-positive cells mostly present in the basal part of the explant (Fig. 2B). Since the initial explant was a monolayer, this observation suggested that neuronal differentiation in the PE explant was accompanied by extensive prolifer-

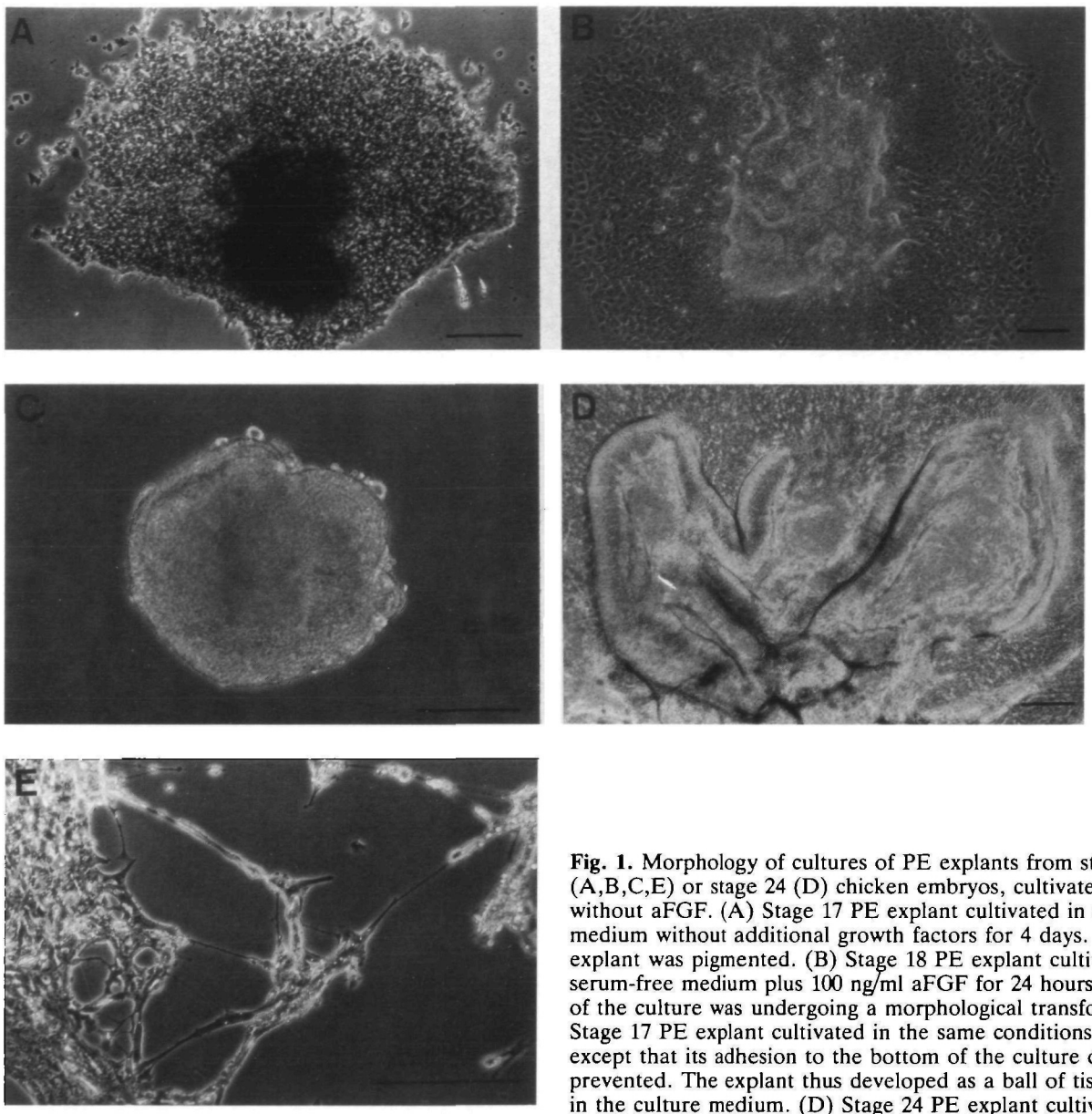

Fig. 1. Morphology of cultures of PE explants from stage 17-18 (A,B,C,E) or stage 24 (D) chicken embryos, cultivated with or without aFGF. (A) Stage 17 PE explant cultivated in serum-free medium without additional growth factors for 4 days. The entire explant was pigmented. (B) Stage 18 PE explant cultivated in serum-free medium plus 100 ng/ml aFGF for 24 hours. The center of the culture was undergoing a morphological transformation. (C) Stage 17 PE explant cultivated in the same conditions as in (B) except that its adhesion to the bottom of the culture dish was prevented. The explant thus developed as a ball of tissue floating in the culture medium. (D) Stage 24 PE explant cultivated in DME containing 10% fetal calf serum and 100 ng/ml aFGF for 4 days. Part of the culture was undergoing extensive morphological transformation. (E) Stage 17 PE explant cultivated in the same conditions as in (B) for 4 days. The explant lost its epithelial structure, and some cells at the periphery of the culture adopted a neuronal morphology. Scale bar=200 μm.

ation. This was confirmed by 2 hr of BrdU incorporation followed by anti-BrdU immunocytochemistry, which showed that numerous nuclei had incorporated BrdU in those parts of the cultures that had undergone a morphological transformation in response to FGF (Fig. 2C). The sections also showed a continuity between the pigmented monolayer that persisted in part of the FGF-treated cultures and the unpigmented multilayered structures (Fig. 2C,D). This observation indicates that the latter structures developed from the PE and not from neural retinal cells that may have remained attached to the PE explant after dissection. The observation of a significant number of cells (4 to 5%) showing both pigmentation and the neuronal marker neurofilament 68 K (NF68) after dissociation of cultures treated with low concentration of FGFs (see below and Fig. 3C,D) further demonstrated that the cells undergoing differentiation in these cultures derived from the PE and not from retinal germinal cells present in the PE sheet which had been selectively stimulated to proliferate and differentiate by FGFs.

Dose-response of PE explants to FGFs: inhibition of pigmentation and neurofilament expression are not strictly correlated

In order to further analyze the effect of FGFs on PE explants, and to compare the development of these cultures and retinal development *in vivo*, we attempted

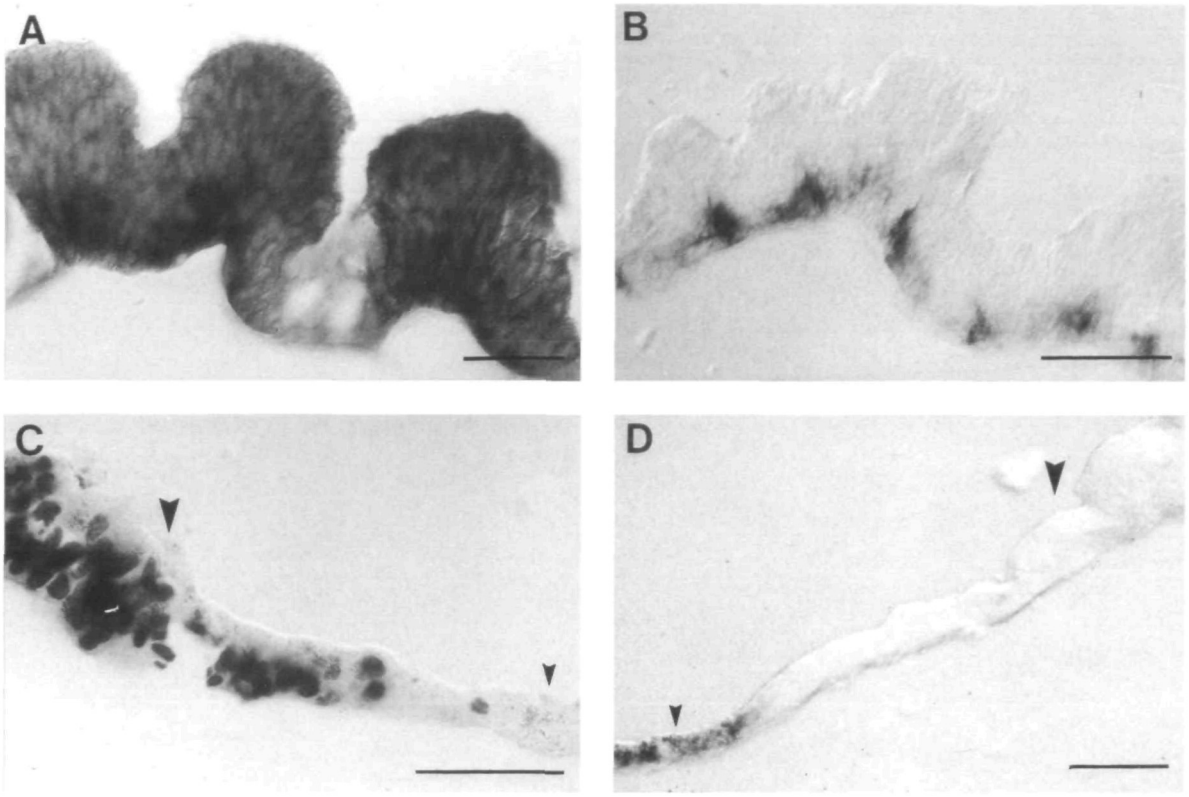

Fig. 2. Immunostaining of sections of stage 24 PE explants cultured for 10 days in DME plus 10% FCS and 100 ng/ml bFGF, and undergoing neural development. (A) Anti-HNK1 antibody, a widespread neural marker, stained the plasma membrane of most of the cells of this portion of explant, revealing a multilayered structure. (B) Anti-HPC1 antibody, an amacrine cell marker in NR, labeled only scattered cells on the basal part of sections, revealing some organization in the retina-like structures of the explants. (C) Anti-BrdU staining on a culture pulsed with 10 μM BrdU for 2 hours before fixation. The area undergoing transformation contained numerous dividing cells (large arrowhead) whereas the portion that remained flat and pigmented did not contain any (small arrowhead). (D) Control staining without first antibody shows the transition between a part of the culture which was flat and pigmented (small arrowhead) and an area which had undergone morphological transformation and lost pigmentation (large arrowhead). Scale bar=50 μm.

to quantify this assay. At the end of the culture period, explants were dissociated with trypsin and single cell suspensions were allowed to adhere for 3 hours to coverslips that were then fixed and processed for immunocytochemistry. Two markers were studied initially: (1) pigmentation as a marker of PE differentiation, and (2) NF68 expression as a marker of neuronal differentiation. This last marker was chosen because it is the earliest neuronal marker expressed in NR, and because it is rapidly expressed at a detectable level in recently differentiated ganglion cells.

The cells containing pigment granules (Fig. 3A) or expressing NF68 (Fig. 3B) were counted in explant cultures treated with increasing doses of either aFGF of bFGF. When treated with bFGF for 3-4 days, stage 17-18 PE explants showed a dose-dependent inhibition of pigmentation and a concomitant increase in number of NF68-positive cells (Fig. 4A). At the highest concentrations tested (20 and 100 ng/ml), none or few pigmented cells remained in the cultures, but the expression of NF68 did not reach a plateau. Such concentrations are already much higher than the optimal concentration of bFGF in most bioassays (typically 0.1-0.5 ng/ml, Gospodarowicz et al., 1987). This suggested that, although bFGF inhibited pigmentation and induced NF68 expression, it might mimic related factors, such as aFGF, which could be more potent in this assay.

Stage 17-18 PE cultures treated with aFGF also showed a dose-dependent decrease of pigmentation and a correlated increase of NF68 expression (Fig. 4B). However, total inhibition of pigmentation and maximal NF68 expression were achieved at lower concentrations than with bFGF. For example, at 20 ng/ml aFGF, pigmentation was totally inhibited and the percentage of NF68+ cells was close to its maximum with 70±10, whereas at the same concentration of bFGF, 11±7% cells were pigmented and only 38±9% were NF68+. Thus aFGF was more potent than bFGF in this assay. Furthermore, in contrast to the response to bFGF, the response to increasing concentrations of aFGF was clearly not linear. The percentage of NF68+ cells reached a plateau (38 to 44% labelled cells) between 0.4 and 5 ng/ml aFGF, whereas at the higher concentrations of 20 to 100 ng/ml aFGF, 70-80% of cells in 3-4 day-old cultures were NF68+.

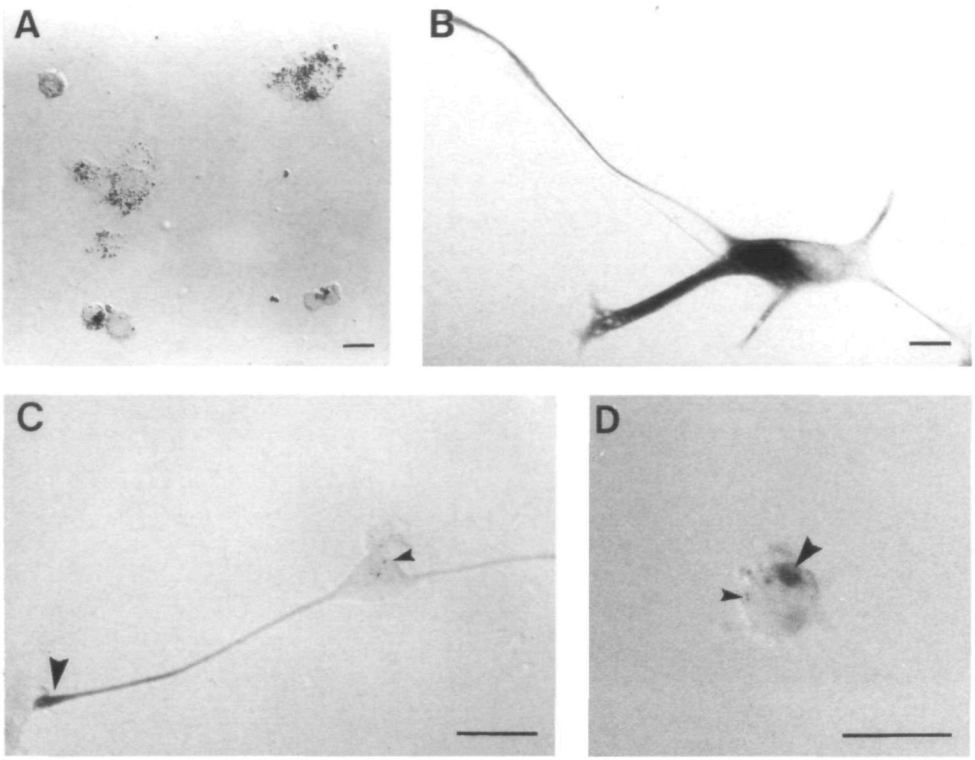

Fig. 3. Pigmentation and NF68 immunostaining of cells from PE explants dispersed after culture in various conditions. (A) Stage 17 explant cultivated in serum-free medium for 4 days. All the cells were pigmented and did not stain for NF68. (B) A cell from a stage 17 explant cultured in serum-free medium plus 20 ng/ml aFGF for 4 days. This cell showed NF68 staining in the cell body and the processes and no pigmentation. (C) A cell from a stage 24 PE explant (initially pigmented) cultivated in serum-free medium plus 5 ng/ml aFGF for 4 days. This cell was positive for NF68 staining (large arrowhead) and also contained pigment granules (small arrowhead). (D) A cell from a stage 17 PE explant (initially unpigmented) cultured in serum-free medium plus 0.4 ng/ml aFGF. This cell also showed both NF68 staining (large arrowhead) and pigment granules (small arrowhead). Scale bar=10 μm.

In addition to the different percentages of NF68+ cells, PE explant cultures in high and low concentrations of aFGF tended to differ in morphology after several days in culture. In low concentrations of aFGF, some of the explants formed balls of cells of variable size in the center of the culture. In high concentrations of aFGF, the cultures were flat and tended to dissociate more extensively. One possibility was that the apparent differences in the extent of cell-cell contacts could affect neuronal differentiation; if this was the case, the variations of NF68 expression may only be an indirect effect of the addition of different concentrations of aFGF to the culture. We thus modified the conditions of some explant cultures in order to test this hypothesis. Adherence of some explants was prevented by immediate addition of culture medium and occasional pipetting; they then rounded up and developed as a ball of tissue floating in the culture medium (Fig. 1C). Under these conditions, the explants cultured in the presence of 1.5 or 100 ng/ml aFGF had a similar morphology and presumably the same extent of cell-cell contacts (not shown). Dissociation of these cultures at different times showed that they were healthy and that markers for different neuronal populations were expressed in synchrony with adherent cultures (not shown). The percentage of NF68+ cells after 4 days was essentially the same in floating and adherent cultures at both 1.5 ng/ml (39±3 and 41±7, respectively) as well as 100 ng/ml aFGF (66±7 and 76±6, respectively). Thus extensive flattening of 100 ng/ml aFGF-treated cultures compared with 1.5 ng/ml aFGF-treated cultures could not account for the increased proportion of NF68+ cells.

While the number of NF68+ cells remained constant between 0.4 and 5 ng/ml aFGF, the number of pigmented cells decreased progressively, showing that these two parameters were not strictly inversely correlated. In keeping with this notion, some cells in cultures of stage 17 PE, which were initially not pigmented, were found both pigmented and NF68+ after treatment with low concentrations of FGFs, as already mentioned (Fig. 3D). This latter finding further indicated that PE and NR differentiation pathways were not mutually exclusive at the initial stages of differentiation.

Time course of expression of ganglion cell markers in response to aFGF

As different proportions of cells expressing NF68 were observed in 3-4 day- old cultures treated with low (1.5

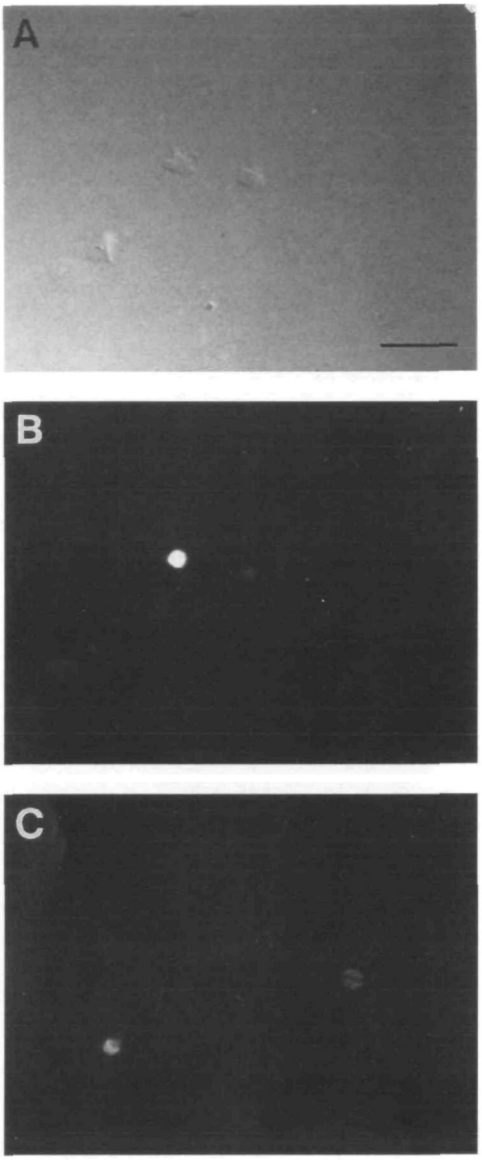

Fig. 7. BrdU incorporation and NF68 expression in cells from a culture treated with aFGF for 12 hours. PE cultures were treated with 100 ng/ml aFGF for 12 hours in the continuous presence of 10 μM BrdU. These cultures were then dispersed and stained for NF68 expression (B) and BrdU incorporation (C). Nomarski of the same field (A). Scale bar=25 μm.

Expression of NF68 by dividing cells in vitro *and* in vivo

The comparison of the profiles of BrdU incorporation and NF68 expression over time in PE cultures in 100 ng/ml aFGF (Fig. 5B) suggested that a significant proportion of the NF68+ cells must have been dividing after 2 to 4 days in culture. To directly verify this observation and compare the onset of NF expression with respect to cell proliferation in FGF-treated cultures and in NR *in vivo*, the following experiments were performed: three day-old cultures continuously treated with either 1.5 or 100 ng/ml aFGF, as well as normal NR explants from stage 17 to stage 25 embryos,

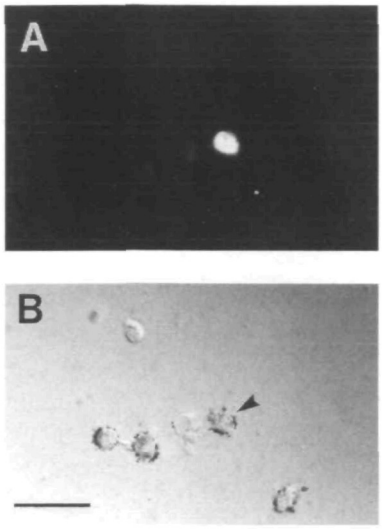

Fig. 8. Expression of NF68 and [^{3}H]thymidine incorporation in E4 chick NR cells. The NR was explanted for 1 hour in culture medium in presence of 5 μCi [^{3}H]thymidine before dissociation, and stained for NF68 expression (A). The cells were then processed for autoradiography and exposed for 3 days (B). A cell which incorporated the labeled nucleotide and which was NF68+ is shown by an arrowhead. Scale bar=25 μm.

were exposed to [^{3}H]thymidine for 1 hour and then dissociated. The cell suspensions were plated for 30 minutes, fixed and stained for NF68 expression, and then processed for autoradiography. In all cases, a significant number of NF68+ cells had incorporated [^{3}H]thymidine (Fig. 8). The proportion of NF68+, 3H thy+ cells in the NR remained approximately constant, from 14% to 18% of all NF68+ cells at all stages examined between stages HH16 (E2) and HH25 (E5). The proportion of double positive cells was similar (18±1%) in 100 ng/ml aFGF-treated cultures and lower (9%) in 1.5 ng/ml aFGF-treated cultures. Thus, in normal NR and in FGF-treated cultures, a significant number of cells expressing the differentiation marker NF68 were in the S phase of the cell cycle.

In summary, the two previous experiments show that aFGF induced cells of the explants to express NF68 without these entering the cell cycle (Fig. 7), but it also induced NF68 expression in cells that were dividing, and/or induced mitosis in NF68+ cells (Fig. 8). Thus induction of NF68 expression and proliferation were not correlated in this system.

Discussion

When cultivated in the absence of FGFs, explants of differentiated PE maintain the characteristics of this tissue, i.e. pigmentation and an epithelial structure. Upon addition of FGFs, however, PE explants can transdifferentiate and develop into retinal tissue (this article and Pittack et al., 1991), as previously discovered for *in vivo* transdifferentiation of PE by bFGF (Park and Hollenbeck, 1989). In addition, we observed

that when the neuroepithelium of undifferentiated, or presumptive PE at optic cup stage (HH stage 17-18), was cultivated in the presence of FGFs, it showed no sign of PE differentiation, but underwent the typical steps of early retinal development. We thus exploited the FGF-dependence of neural development in this assay to analyse some of the effects of these molecules in early retinal development.

FGFs and the choice between PE and NR fates

The effects of FGFs in this culture system suggest that these molecules are able to direct the choice of fate of the bipotential neuroepithelium of the immature PE, by promoting the neural pathway and inhibiting the PE pathway. Since members of the FGF family are present in the CNS (Kalcheim and Neufeld, 1990; Risau et al., 1988; Schnurch and Risau, 1991), including the NR (Mascarelli et al., 1987; Wilkinson et al., 1989), at early stages of development, these molecules are likely candidates for controlling some of the initial steps in eye development. In order to allow normal PE differentiation *in vivo*, one can speculate that FGF molecules may be absent, or present in an inactive form, in the part of the optic vesicle that generates the PE. Alternatively, a factor present during normal development, but missing in the experimental conditions (e.g. because of its retinal origin) may suppress the action of FGFs on presumptive PE, allowing PE differentiation to proceed normally *in vivo*. Thus, differential expression, activation or suppression of FGF molecules in the neuroepithelium of the optic vesicle or in the surrounding tissues (head ectoderm versus head mesenchyme) may be a key control mechanism in the induction of different fates followed by different regions of the optic vesicle.

FGFs induce NF expression independently from proliferation and inhibition of pigmentation

The simultaneous effect of FGFs on a complex series of events including cell proliferation, neuronal differentiation and inhibition of pigmentation, can be interpreted in different ways. One possibility is that these different processes belong to a common pathway and FGFs, by exerting direct control at one key point, could trigger the variety of responses observed *in vitro*. Alternatively, FGFs could independently control several of these processes, which may be part of one, or more than one, pathway. In the first hypothesis, a simple model of FGF action in this system would be control of retinal development through stimulation of proliferation in PE explants. In this model, neuronal differentiation would be correlated with a high proliferation rate of the neuroepithelium of the optic vesicle, and PE differentiation with a slowing down or arrest of proliferation. This hypothesis is especially appealing as NR proliferates more than PE at the earliest stage of optic cup formation, and FGFs are known mitogens for many cell types, including neuronal progenitors. However this simple model cannot pertain. *In vitro*, there were different dose-response curves for inhibition of pigmentation, proliferation, and neuronal differentiation (Figs 4, 5A,B). In addition, these parameters were not strictly correlated when followed in individual cells. For example, in cultures treated for 12 hours with aFGF, most NF68+ cells had not entered the cell cycle; reciprocally, most cells having incorporated BrdU did not express NF68 (Fig. 7). Moreover, in cultures treated with low concentrations of FGFs, some cells were both pigmented and NF68+ (Fig. 3D). These data demonstrate that division of presumptive PE cells was not required for differentiation along the neuronal pathway, and that neuronal differentiation is controlled at least partially independently from proliferation by FGFs in this system.

Increasing concentrations of aFGF induce different proportions of neurofilament+ cells

The dose-response curve of stage 17 PE explant cultures to aFGF (but not bFGF) showed a clear 2 step-increase in the proportion of NF-expressing cells (Fig. 4B), and at high concentrations of FGFs, 60 to 80% of the cells in explants expressed 3 ganglion cell markers: NF68, RA4 and Thy 1 (Fig. 6). These observations raise several points. Firstly, the highest concentrations of factor used in this experiment (up to 100 ng/ml) were much higher than the concentrations of acidic or basic FGF optimal in most *in vitro* biological assays (Gospodarowicz et al., 1987) and were presumably higher than those found *in vivo* at these early stages of development. It is worth noting, however, that increasing doses of bFGF in the same range of concentrations as in the experiment mentioned above have been shown to trigger qualitatively and quantitatively different responses in at least two other systems, lens development (Mc Avoy and Chamberlain, 1989) and mesoderm induction in *Xenopus* (Slack et al., 1987; Godsave and Slack, 1991). For example, bFGF induces mostly ventral type mesoderm at concentrations from 0.15 to 5 ng/ml, muscle differentiation between 2 and 60 ng/ml, and notochord differentiation between 10 and 60 ng/ml (Godsave and Slack, 1991).

Secondly, the dose response of PE explants to aFGF shows that different percentages of cells respond to aFGF at different concentrations. This observation suggests a cellular as well as molecular heterogeneity of the neuroepithelium of the PE. The fact that aFGF was 4 to 20 times more potent than bFGF in inhibiting PE differentiation, as well as in inducing neuronal differentiation in presumptive PE cultures, suggests that cells of the PE explants express a receptor and/or a transduction machinery that is more sensitive to aFGF than to bFGF. Furthermore, the observation of distinct effects obtained with different concentrations of aFGF may indicate the presence of more than one receptor, or different forms of the same receptor, one with high affinity and another with lower affinity for aFGF. A low affinity form of a receptor for bFGF has been observed in rat fetal hippocampal cells, and the same or a very similar molecule is apparently present in mesenchymal cells where it shows a 100 times higher affinity for bFGF (Walicke et al., 1988). Alternatively, at high concentration, aFGF could bind to a receptor for a distinct but

related molecule. In addition to aFGF and bFGF, the only other member of the FGF family known to be present in the developing retina is the product of the proto-oncogene int-2. Interestingly, int-2 transcripts are present in the neuroblastic layer of the mouse retina between E14.5 and a few days after birth, i.e. at the time and place where ganglion cells begin to differentiate (Wilkinson et al., 1988). int-2 is thus a good candidate to mediate *in vivo* some of the effects observed with aFGF in this *in vitro* assay, particularly in ganglion cell differentiation.

Thirdly, the astonishing proportion of ganglion cells induced in response to high concentrations of FGFs can be interpreted in two ways. It could either be an artefact of this *in vitro* culture system, or may reveal a direct effect of an FGF-like molecule on the determination of ganglion cells. FGF-like molecules may also influence proliferation and/or determination of progenitors for other retinal cell types, indirectly affecting the proportion of ganglion cells in the explants. Further studies will be necessary to clarify these issues.

Expression of NF68 by dividing progenitors

In the PE cultures and in NR explanted for short periods, we observed a consistent proportion (9-19%) of cells expressing NF68 while still in the S phase of the cell cycle. The presence of NF+ dividing cells has already been reported for the chick NR (Sechrist, 1968), as well as for other regions of the CNS (Tapscott et al., 1981). One interpretation of this observation is that NF68 expression may be a marker of commitment to the ganglion cell fate by mitotic progenitors. Alternatively, ganglion cell commitment and differentiation may start only after the last division of a progenitor, and NF68 expression by mitotic neuroepithelial cells would not be restricted to ganglion cell progenitors. Descriptions of early stages of retinal development in chick and mouse using electron microscopy (Hinds and Hinds, 1974) and immunochemistry (McLoon and Barnes, 1989) support the notion that some mitotic progenitors are commited to the ganglion cell lineage before their last division. On the other hand, lineage analysis conducted in rodent retina with retroviral vectors or in amphibians with injections of tracers (Holt et al., 1988; Turner and Cepko, 1987; Turner et al., 1990; Wetts and Fraser, 1988) suggest that commitment to a cell fate, which includes horizontal cells, ganglion cells, bipolar cells, muller glia, cones, rods and amacrine cells, could occur during or after the last cell division. Unfortunately, the lineage data concerning ganglion cells are difficult to interpret because they are very limited in the number of observations of ganglion cells. This is presumably due to the fact that up to 50% of ganglion cells normally die during development, and because ganglion cells constitute a small proportion of retinal cells. It is thus possible that ganglion cells, which are the first cells to be generated in the retina of all vertebrates and which show similarities with other early born "macroneurons", with long, projection axons (e.g. motoneurons of the spinal chord) (Jacobson, 1989), are determined at an earlier stage than the other retinal cell types. Further lineage analysis of chick retina, in particular at early developmental stages, will be necessary to examine this possibility.

In conclusion, we have developed a culture system in which development of the NR from the bipotential neuroepithelium of the early PE is FGF-dependent. This situation is favorable for dissecting the multiple functions that FGF molecules seem to have early in retinal development. In this article, we show that aFGF is able to induce differentiation of ganglion cells independently from proliferation. Interestingly, addition of aFGF to PE explant cultures also leads to the appearance of differentiation markers for subsequent retinal cell populations (Fig. 2B and not shown), with the same timing as observed during *in vivo* NR development. Thus this assay system may also be suited for the analysis of the signals or conditions required for generation of retinal cell populations other than ganglion cells.

We thank Drs. C. Barnstable, P. L. Jeffrey, D. Mason and S. C. McLoon for the gift of 8A1, anti-Thy-1, anti-BrdU and RA4 antibodies, respectively; members of the lab, in particular L. Lillien, for discussions and helpful suggestions during the course of this work, and D. Altschuler, D. Fekete, L. Lillien, C. Tabin, D. Wu and X. Yang for critical reading of the manuscript. F.G. was supported by the Centre National de la Recherche Scientifique, France, and a fellowship of the European Molecular Biology Organization. This research was supported by grant ROI EY 08064 to C. L. C.

References

Altshuler, D. M., Turner, D. L. and Cepko, C. L. (1991). Specification of cell type in the vertebrate retina. In *Development of the Visual System*, (eds D. M. Lam and C. J. Shatz) pp 37-58. MIT Press.

Barnstable, C., Hofstein, R. and Akagawa, G. (1985). A marker of early amacrine cell development in rat retina. *Dev. Brain Res.* **20**, 286-291.

Cohen, J., Burne, J. F., Winter, J. and Bartlett, P. (1986). Retinal ganglion cells lose response to laminin with maturation. *Nature* **322**, 465-467.

Coulombre, J. L. and Coulombre, A. J. (1965). Regeneration of neuronal retina from the pigmented epithelium in the chick embryo. *Dev. Biol.* **12**, 79-92.

Detwiler, S. R. and Van Dyke, R. H. (1954). The induction of neural retina from the pigment epithelial layer of the eye. *J. Exp. Zool.* **126**, 135-150.

Dutting, D., Gierer, A. and Hansman, G. (1983). Self-renewal of stem cells and differentiation of nerve cells in the developing chicken retina. *Dev. Brain Res.* **10**, 21-32.

French, P. W., Sheppard, A. M. and Jeffrey, P. L. (1987). Neural membrane glycoproteins associated with chicken Thy-1: an anti-idiotypic study. *Brain Res.* **420**, 324-334.

Godsave, S. F. and Slack, J. M. W. (1991). Single cell analysis of mesoderm formation in the *Xenopus* embryo. *Development* **111**, 523-530.

Gospodarowicz, D., Ferrara, N., Schweigerer, L. and Neufeld, G. (1987). Structural characterization and biological functions of Fibrobast Growth Factor. *Endocrine Reviews* **8**, 95-114.

Grainger, R. M., Henry, J. J. and Henderson, R. A. (1988). Reinvestigation of the role of the optic vesicle in embryonic lens induction. *Development* **102**, 517-526.

Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49-92.

Heuer, C. G., von Bartheld, C. S., Kinoshita, Y., Evers, P. C. and Bothwell, M. (1990). Alternating phases of FGF receptor and NGF receptor expression in the developing chicken nervous system. *Neuron* **5**, 283-296.

Hinds, J. W. and Hinds, P. L. (1974). Early ganglion cell differentiation in the mouse retina: an electron microscopic analysis utilizing serial sections. *Dev. Biol.* **37**, 381-416.

Holt, C. E., Bertsch, T. W., Ellis, H. M. and Harris, W. A. (1988). Cellular determination in the Xenopus retina is independent of lineage and birth date. *Neuron* **1**, 15-26.

Jacobson, M. (1989). *Developmental Neurobiology*, New York, Plenum Press.

Kahn, A. J. (1973). Ganglion cell formation in the chick neural retina. *Brain Res.* **63**, 285-290.

Kalcheim, R. and Neufeld, G. (1990). Expression of basic fibroblast growth factor in the nervous system of early avian embryos. *Development* **109**, 203-215.

Lillien, L. E., Sendtner, M., Rohrer, H., Hughes, S. M. and Raff, M. C. (1988). Type-2 astrocyte development in rat brain cultures is initiated by a CNTF-like protein produced by type-1 astrocytes. *Neuron* **1**, 485-494.

Lipton, S. A., Wagner, J. A., Madison, R. D. and D'Amore, P. A. (1988). Acidic fibroblast growth factor enhances regeneration of processes by postnatal retinal ganglion cells in culture. *Proc. Natl. Acad. Sci. USA* **85**, 2388-2392.

Lopashov, G. V. (1963). *Developmental Mechanisms of Vertebrate Eye Rudiments*. McMillan (Pergamon), New York.

Mascarelli, F., Raulais, D., Counis, M. F. and Courtois, Y. (1987). Characterization of acidic and basic fibroblast growth factors in brain, retina and vitrous of chick embryo. *Bioch. Bioph. Res. Commun.* **146**, 478-486.

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

McLoon, S. C. and Barnes, R. B. (1989). Early differentiation of retinal ganglion cells: an axonal protein expressed by premigratory and migrating retinal ganglion cells. *J. Neurosc.* **9**, 1424-1432.

Park, C. M. and Hollenberg, M. J. (1989). Basic Fibroblast Growth Factor induces retinal regeneration *in vivo*. *Dev. Biol.* **134**, 201-205.

Pittack, C., Jones, M. and Reh, T. A. (1991). Basic fibroblast growth factor induces retinal pigment epithelium to generate neural retina *in vitro*. *Development* **113**, 577-588.

Risau, W., Gautschi-Sova, P. and Bohlen, P. (1988). Endothelial cell growth factors in embryonic and adult chick brain are related to human acidic fibroblast growth factor. *EMBO J.* **7**, 959-962.

Schnurch, H. and Risau, W. (1991). Differentiating and mature neurons express the acidic fibroblast growth factor gene during chick neural development. *Development* **111**, 1143-1154.

Sechrist, J. W. (1968). Neurocytogenesis I. Neurofibrils, neurofilaments, and the terminal mitotic cycle. *Am. J. Anat.* **124**, 117-134.

Sievers, J., Hausmann, B., Unsicker, K. and Berry, M. (1987). Fibroblast Growth Factors promote the survival of adult rat retinal ganglion cells after transection of the optic nerve. *Neurosc. Let.* **76**, 157-162.

Slack, J. M. W., Darlington, B. F., Heath, H. K. and Godsave, S. F. (1987). Heparin binding growth factors as agents of mesoderm induction in early *Xenopus* embryo. *Nature* **326**, 297-300.

Sparrow, J. R., Hicks, D. and Barnstable, C. J. (1990). Cell commitment and differentiation in explants of embryonic rat neural retina. Comparison with the developmental potential of dissociated retina. *Dev. Br. Res.* **51**, 69-84.

Spemann, H. (1905). Uber Linsenbildung nach experimenteller Entfernung der primaren Linsbildungszellen. *Zool. Anz.* **28**, 419-432.

Stone, L. S. (1950). The role of retinal pigmented cells in regenerating neural retinae of adult salamander eyes. *J. Exp. Zool.* **113**, 9-31.

Stroeva, O. G. (1962). Experimental analysis of the eye morphogenesis in mammals. *J. Embryol. Exp. Morphol.* **8**, 349-368.

Tapscott, S. J., Benett, G. S. and Holtzer, H. (1981). Neuronal precursor cells in the chick neural tube express neurofilament proteins. *Nature* **292**, 836-838.

Turner, D. L. and Cepko, C. L. (1987). A common progenitor for neurons and glia persists in rat retina late in development. *Nature* **328**, 131-136.

Turner, D. L., Snyder, E. V. and Cepko, L. C. (1990). Lineage-independent determination of cell type in the embryonic mouse retina. *Neuron* **4**, 833-845.

Walicke, P. A., Feige, J.-J. and Baird, A. (1988). Characterization of the neuronal receptor for basic Fibroblast Growth Factor and comparison to receptors on mesenchymal cells. *J. Biol. Chem.* **264**, 4120-4126.

Wanaka, A., Milbrandt, J. and Johnson Jr, E. M. (1991). Expression of FGF receptor gene in rat development. *Development* **111**, 455-468.

Wetts, R. and Fraser, S. E. (1988). Multipotent precursors can give rise to all major cell types of the frog retina. *Science* **239**, 1142-1145.

Wilkinson, D. G., Bhatt, S. and McMahon, A. P. (1988). Expression of the FGF-related proto-oncogene int-2 suggests multiple roles in fetal development. *Development* **105**, 131-136.

(Accepted 5 December 1991)