Fibroblast growth factors are necessary for neural retina but not pigmented epithelium differentiation in chick embryos

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SUMMARY

During eye development, optic vesicles evaginate laterally from the neural tube and develop into two bilayered eye cups that are composed of an outer pigment epithelium layer and an inner neural retina layer. Despite their similar embryonic origin, the pigment epithelium and neural retina differentiate into two very distinct tissues. Previous studies have demonstrated that the developmental potential of the pigmented epithelial cells is not completely restricted; until embryonic day 4.5 in chick embryos, the cells are able to switch their phenotype and differentiate into neural retina when treated with fibroblast growth factors (FGF) (Park, C. M., and Hollenberg, M. J. (1989).Dev. Biol. 134, 201-205; Pittack, C., Jones, M., and Reh, T. A. (1991).Development 113, 577-588; Guillemot, F. and Cepko, C. L. (1992). Development 114, 743-754). These studies motivated us to test whether FGF is necessary for neural retina differentiation during the initial stages of eye cup development. Optic vesicles from embryonic day 1.5 chick were cultured for 24 hours as explants in the presence of FGF or neutralizing antibodies to FGF2. The cultured optic vesicles formed eye cups that contained a lens vesicle, neural retina and pigmented epithelium, based on morphology and expression of neural and pigmented epithelium-specific antigens. Addition of FGF to the optic vesicles caused the presumptive pigmented epithelium to undergo neuronal differentiation and, as a consequence, a double retina was formed. By contrast, neutralizing antibodies to FGF2 blocked neural differentiation in the presumptive neural retina, without affecting pigmented epithelial cell differentiation. These data, along with evidence for expression of several FGF family members and their receptors in the developing eye, indicate that members of the FGF family may be required for establishing the distinction between the neural retina and pigmented epithelium in the optic vesicle.

Key words: FGF, chick, eye development, differentiation, optic vesicle, fibroblast growth factor

INTRODUCTION

Experimental embryology has shown that many sequential inductions between the neural tube and the surrounding tissues of the embryo are necessary for the more detailed pattern of the central nervous system (Holtfreter and Hamburger, 1955). For example, the experiments of Holtfreter demonstrated that the notochord, somites and ectoderm are all important for the development of the dorsal ventral axis of the spinal cord (see Holtfreter and Hamburger, 1955 for review). Some of the molecules expressed in the tissues surrounding the neural tube that control its development have recently been identified. Sonic hedgehog, expressed in the notochord, and later the floorplate, has a ventralizing activity (Johnson and Tabin, 1995; Roelink et al., 1995), while both BMP4 and BMP7 expressed in the ectoderm, activate dorsal genes in the neural tube (Liem et al., 1995).

The development of the eye also appears to require several different inductive interactions for the various tissues to form properly. Although the cells of the optic vesicle are committed to give rise to ocular phenotypes (see Reh, 1992 for review of earlier literature), if this region of the neurula is stripped of its epidermis and mesenchyme, differentiation is arrested at the optic vesicle stage; "the naked eye primordium does not differentiate into tapetum (pigment epithelium), multilayered retina, rods and cones, and it fails to fold inwards into an eye cup" (Holtfreter, 1939). One of the first events in ocular development that requires interactions with the surrounding tissues, is the differentiation of the simple neuroepithelium of the optic vesicle, into the neural retina and pigmented epithelium. At the time that the optic vesicles form, the anterior part of the optic vesicle normally forms the neural retina, while the posterior part of the optic vesicle gives rise to the pigment epithelium. Although derived from the same region of the neural tube, these two tissues are quite distinct; the neural retina is a multilayered structure containing the photoreceptors and neurons necessary for vision, while the pigmented epithelium is a single layer of non-neuronal, pigmented, cuboidal cells, necessary for support of the retinal photoreceptors.

The commitment to form either neural retina or pigmented epithelium is not immediately fixed, however, and there is a period of development during which the fates of these two tissues can be reversed. If the optic cup in an amphibian embryo is transplanted in such a manner that the posterior cell
layer (presumptive pigment epithelium) is adjacent to the ectoderm, a fully differentiated retina will form from this layer (Detwiler and van Dyke, 1953; 1954). Conversely, under some conditions of transplantation, the retina will fail to form and both layers of the optic cup give rise to pigmented epithelial cells (Holtfreter, 1939; Loposhov and Stroeva, 1964). For chicks and mammals, the fate of these cells becomes restricted to either the pigmented epithelial or neural retinal cell fate shortly after the formation of the optic cup (Orts-Llora and Genis-Galvez, 1960; Lopashov and Stroeva, 1964; Coulombre and Coulombre, 1965). However, in amphibians, even after the cells of the pigment epithelial layer have their fully differentiated function, they retain the capacity to replace the neural retinal cells after retinal damage (Stone, 1950a,b; 1960; Reyer, 1977; Okada, 1980; Reh and Nagy, 1987; Reh et al., 1987). Thus, in addition to demonstrating that both the anterior and posterior walls of the optic vesicle have the ability to generate either neural retina or pigmented epithelium, these transplant studies demonstrated that this developmental decision is controlled by factors in the adjacent tissues.

One clue about a possible factor involved in the development of the neural retina comes from experiments done several years ago by Carol Park and Martin Hollenberg (1989). As noted above, if the neural retina is surgically removed from chick embryos shortly after optic cup formation, the cells of pigmented epithelium have the capacity to regenerate neural retina (see for e.g. Orts-Llora and Genis-Galvez, 1960; Coulombre and Coulombre, 1965; Tsunematsu and Coulombre, 1981). Park and Hollenberg (1989) found that this regeneration of neural retina from the pigmented epithelium was stimulated by fibroblast growth factor 2 (FGF2, also known as basic FGF). After removing the retina from chick embryos, Park and Hollenberg found that retinal regeneration was stimulated in those eyes in which they implanted FGF2 containing slow release gels. Since this initial study, a number of laboratories have confirmed and extended these results (Pittack et al., 1991; Reh et al., 1991; Guillemot and Cepko, 1992; Opas and Dziliak, 1994). The effect appears to be specific to FGF, since other growth factors, such as EGF, NGF, and TGFβ do not induce the pigmented epithelium to form neural retina (Park and Hollenberg, 1989; Pittack et al., 1991; Guillemot and Cepko, 1992).

The distribution of FGFs and their receptors in the developing ocular tissues are also consistent with a role for these molecules in neural retinal induction. In the rat, De Longh and McAvoy (1993) demonstrated strong immunoreactivity of FGF1 (acidic FGF) in the lens ectoderm at the point of contact with the optic vesicle. Furthermore, receptors for FGF, which are members of the receptor tyrosine kinase family, have also been localized to the developing nervous system in particular. FLG (CEK1), which binds both FGF1 and FGF2 (see Johnson and Williams, 1993 for review), is expressed in the neural retina and pigmented epithelium in the eye cups of rat and chick embryos (Heuer et al., 1990; Wanaka et al., 1991; Tcheng et al., 1994). Also consistent with the possibility that FGF may be important in neural retina induction during the initial stages of eye development, are a number of studies that have found FGF family members are important during several inductive interactions in the embryo, such as mesoderm induction in Xenopus (Kimelman and Kirschner, 1987; Slack, 1987), limb bud development (Cohn et al., 1995; Fallon et al., 1994) and inner ear induction (Represa et al., 1991). In addition, there is also recent evidence suggesting that FGF may be involved in neural induction in Xenopus (Launay et al., 1996; Lamb and Harland, 1995).

In light of this evidence, we hypothesized that FGF may be involved in setting up the initial phenotypic distinction between the pigmented epithelium and the neural retina in the optic vesicle. To directly test this hypothesis, we determined the effects of exogenous FGF, as well as neutralizing antibodies, raised against FGF, on the differentiation of the neural retina and pigmented epithelium during eye cup formation in cultured optic vesicles.

**MATERIALS AND METHODS**

**Optic vesicle, pigment epithelium and neural retina cultures**

Optic vesicles from E1.5 (Hamburger and Hamilton stages 9-10) chicks were removed with fine tungsten needles. Care was taken to dissect away the attached diencephalon as much as the surrounding mesenchyme as possible, however, the overlying ectoderm (future lens) was left attached. The optic vesicles were then cultured for 24 hours in DMEM/F12 medium (GIBCO) containing 1% fetal bovine serum, 1 mM Hepes, 0.6% glucose and antibiotics (penicillin/streptomycin, 100 units/ml: 100 mcg/ml). For some experiments, the optic vesicles were cultured for 36 hours and the media was supplemented with B-27 (GIBCO). The cultures were maintained in 1 ml of media in single wells of a 24-well plate at 5% CO2 in a 37°C humidified incubator. To prevent the explants from adhering to the bottom of the well, the plates were placed on a shaking device (Nutator). From each embryo, one optic vesicle was treated with 30 ng/ml FGF1 or FGF2 (R&D Systems) or with one of three different neutralizing antibodies to FGF2: (1) A mouse monoclonal antibody to human recombinant FGF2 (mAb 148.2.1.1, a gift from Dr C. Hart, Zymogenetics) that has been previously shown to specifically cross-react with chicken FGF2 (Savage et al., 1993); (2) a mouse monoclonal antibody to recombinant Xenopus FGF2 (a gift from Dr M. Kirschen, Harvard University) or (3) a mouse monoclonal antibody to human FGF2 (F8; Sigma Immunochemicals). The FGF2 antibodies were used at a concentration of 10 µg/ml in the optic vesicle cultures. In these experiments, one optic vesicle from the embryo was treated with either FGF or the antibodies to FGF2 and the other vesicle was left untreated and served as the control. In order to study cell proliferation, BrdU (Becton-Dickson) was added to some of the optic vesicles at the time of culture at a final concentration of 40 nM. Immediately following the culture period, the optic cups and pigmented epithelial cell strips were fixed with Bouin’s fixative for 10 minutes, rinsed in 100% ethanol and rehydrated through an ethanol gradient. The samples were washed with phosphate-buffered saline solution (PBS), cryoprotected with 20% sucrose, frozen at ~70°C in OCT compound (Tissue Tech) and sectioned at 12 µm to prepare them for immunocytochemical analysis. In some cases, the samples were fixed with 4% paraformaldehyde/4% sucrose for 30 minutes, washed in PBS and cryoprotected as described above.

In order to more carefully examine the morphological changes that the optic vesicles undergo, the explants were cultured as described above, fixed in Bouin’s fixative, rinsed in 100% ethanol and prepared for plastic embedding. The tissue was first infiltrated and then embedded in Historesin (Reichert-Jung) following the manufacturers protocol. 1 µm sections were cut using a microtome and mounted onto slides treated with 3-aminopropyltriethoxysilane (SIGMA).

For the experiments designed to demonstrate the blocking effects of the FGF antibodies, we used a retinal regeneration assay previously
In order to determine the change in the number of differentiated neurons within the neural retina or pigmented epithelium of the optic cups, the labelling was carried out according to the directions as described above. FGF2 (20 mg/ml) and/or the FGF2 antibodies (20 ng/ml, final concentration) mentioned above, were added to the culture medium.

For the experiments designed to examine the effects of the FGF2 antibodies on neurotrophin expression in cultured neural retina, whole neural retinas from an E3.5 chick were dissected free from the lens, pigment epithelium and surrounding extraocular tissue and cultured as explants as described above for the optic vesicles. The neural retina explants were maintained as explants for 36 hours on a Nutator in 1 ml of the same media with the addition of 30 mg/ml of one of the two recombinant human FGF2 antibodies. After the culture period, the retinas were dissociated as follows. (1) The retinas were transferred to a solution of Ca2+-Mg2+-free Hank's buffered salt solution (HBSS) with the addition of trypsin to a final concentration of 0.25% and incubated at 37°C for 10 minutes. (2) Following a gentle titration, the trypsin was inhibited by the addition of 1% FBS and (3) the cells were pelleted and resuspended in fresh F12/DMEM medium containing 1% FBS. The cells were then plated onto poly-L-lysine-coated coverslips in 24-well plates and allowed to adhere for 2 hours, after which the cultures were fixed in 4% paraformaldehyde for 15 minutes, washed in PBS and processed for immunohistochemistry.

Immunohistochemistry

To determine the differentiation of neuronal and pigmented epithelium phenotypes, cryostat sections of the optic cups and pigmented epithelium explants or coverslips with the dissociated retinal cells, were incubated with various neuron-specific and pigmented epithelial cell-specific antibodies. The following antibodies were used: (1) a mouse monoclonal to the RMO-160-Mr subunits of neurofilament protein, RMO-270 (from Dr. V. Lee, University of Pennsylvania), (2) TuJ1, a mouse monoclonal to neuron-specific class III β-tubulin (Lee et al., 1990) (a gift from Dr. A. Frankfurter, University of Virginia), (3) 3C10, a mouse monoclonal to an antigen expressed on pigmented epithelial cells (Chu and Grunwald, 1990) and (4) a rabbit antiserum to type II collagen (a gift from Dr. L. Sandell, University of Washington). A rat-specific anti-BrDU IgG (from Accurate Chemicals) was used to identify mitotically active cells in the optic vesicles. All antibodies were used at a concentration of approximately 10 mg/ml and visualized via a biotin-streptavidin-fluorochrome complex (Cappell). Omission of the primary antibody eliminated the observed immunoreactivity. In order to count cell numbers in the sections of optic vesicles or the coverslips containing the dissociated neural retinas, the cells were labeled with 4,6-diamidino-2-phenylindole (DAPI, Sigma) by incubation for 15 minutes (diluted 1:1000 in PBS) in order to examine cell viability.

To determine the expression of FGF2 in optic vesicles, similar fixation, cryoprotection and sectioning methods as described above were employed on optic vesicles from E1.5 chick embryos. Both FGF2 antibodies were used at a concentration of approximately 10 ng/mL and visualized via a biotin-streptavidin-fluorochrome complex as described above.

Analysis of apoptosis in vitro

We determined the number of apoptotic cells in the culture with the TUNEL method using terminal transferase to incorporate digoxigenin-labelled nucleotides into the DNA of dying cells (Apoptag kit from Oncor). The labelling was carried out according to the directions of the manufacturer. The number of labelled cells for each experimental condition was determined in six randomly selected fields within the neural retina or pigmented epithelium of the optic cups.

Optic vesicle and pigmented epithelium analysis

In order to determine the change in the number of differentiated neurons, RMO-270-immunoreactive neurons were counted in the neural retina and pigmented epithelium of control and treated optic cup sections. The optic cups were serially sectioned and the total number of neurons was derived by counting the number of RMO-270-immunoreactive cells in the neural retina or pigmented epithelium of every section from the eye cup. The area of pigmented epithelium in the optic cups was determined by measuring the area of 3C10-immunoreactive staining in each section using an Image Analyzer attached to an integrating CCD camera (MTI). The total area of 3C10 immunoreactivity from all the sections was calculated using the NIH Image program. If more than 20% of the sections from either the control or treated optic vesicle had been lost during the immunolabelling procedure, the sample was omitted from the data set.

Immunoblotting

FGF1 and FGF2, diluted in media containing 1% fetal bovine serum, were diluted 1:1 with SDS sample buffer and run on a 15% SDS-PAGE (500 ng per lane). Tissues from E4.0 chick embryos were homogenized in lysis buffer and run on the same gel. The proteins were transferred to nitrocellulose using a basic transfer buffer consisting of 25 mM ethanolamine, 25 mM glycine, 20% HPLC grade methanol, pH 9.5. The filters were incubated with the FGF2 antibodies (described above) and the immunoreactive proteins were visualized with an alkaline phosphatase-conjugated goat anti-mouse secondary antibody (Sigma).

RESULTS

Expression of FGF2 in optic vesicles

Based on the effects of FGF on retinal regeneration from the pigmented epithelium (see above), we hypothesized that FGF is necessary for neural retinal differentiation.
may be important in setting up the original distinction between the neural retina and pigmented epithelium in the optic vesicle. Although FGF has been reported to be expressed in the lens ectoderm adjacent to the developing rat optic vesicle (De Iongh and McAvoy, 1993), it is not known if the chick embryo has a similar distribution. Therefore, we examined the distribution of FGF during early chick eye development with immunohistochemistry.

We incubated sections of embryonic chicks during optic vesicle and optic cup stages with either of two different monoclonal antibodies directed specifically against FGF2 (Savage et al., 1993). Fig. 1A shows that the FGF2 immunoreactivity (with mAb 148.2.1.1) is present at a low level throughout the neuroepithelium of the optic vesicle, including the presumptive pigmented epithelium; however, there is much stronger immunoreactivity in the head ectoderm at the point where the most anterior region of the optic vesicle, the presumptive neural retina, is in close contact with during its outgrowth. As a control, the antibodies were incubated with an excess of FGF2 protein and immunoprecipitated prior to incubation with the section and this abolished the immunoreactivity (data not shown). The FGF2 immunoreactivity in the ectoderm of the chick at this stage of development is consistent with previous reports using anti-FGF2 antibodies in chick embryos (Savage et al., 1993) and FGF2 mRNA in situ hybridization and northern analysis (Savage and Fallon, 1995; Zuniga et al., 1993).

The specificity of the antibodies used for immunostaining was further established by incubating western blots of purified FGF and chick embryo tissues with the anti-FGF2 antibodies (Fig. 1B; see also Savage et al., 1993 for mAb 148.2.1.1). Although some tissues also showed some higher molecular weight bands (see for example, Brigstock et al., 1990), an 18x10^3 Mr band was present in the tissue extracts (Fig. 1B). The FGF2 antibodies that we used throughout our experiments, appear to be specific to FGF2 and do not recognize FGF1 (Fig. 1B, and see also Savage et al., 1993), FGF4 or FGF5 (see Savage et al., 1993).

**Optic vesicles cultured for 24 hours develop into optic cups similar to those in vivo**

To examine the effects of FGF on neural retina and pigmented epithelium differentiation prior to the formation of an optic cup, we developed an in vitro culture method that recapitulates the early stages of eye development. Optic vesicles from E1.5 chick embryos, along with their attached ectoderm, were dissected from the diencephalon and cultured as explants. After a period of one to two days in culture, the optic vesicles formed three-layered eye cups with a neural retina, a pigmented epithelium and a lens vesicle. Fig. 2 shows comparable micrographs of normal in vivo eye development (Fig. 2A-D) and an optic cup that
FGF is necessary for neural retinal differentiation

Developed in vitro from an optic vesicle that was cultured for 24 hours (Fig. 2E-H). Although considerably smaller, eye cups that develop in vitro are morphologically similar to normal eyes at a similar stage of development (Fig. 2A); they form (1) a pseudostratified neuroepithelium, characteristic of the neural retina (nr), (2) a more flattened epithelium, resembling a pigmented epithelium (pe), and (3) a lens placode (1).

In order to determine the extent of neural retina differentiation in the cultured eye cups, we used RMO-270, a monoclonal antibody directed against the middle subunit (160×10^3 M_r) of neurofilament (NF-M) (Lee et al., 1987). Fig. 2B shows an E2.5 eye cup labeled with NF-M. The arrows point to several NF-M-immunoreactive cells in the central neural retina, the majority of which are presumably migrating through the neuroepithelium from the ventricular zone to the future ganglion cell layer. These NF-M-immunoreactive cells are likely to be differentiating ganglion cells based on their morphology, position in the developing retina and the timing of their differentiation (Kahn, 1973; Bennet and DiLulio, 1985; Spence and Robson, 1989). In the chick retina, ganglion cells are the only cells that express neurofilament proteins and, during development, the cells begin to express ganglion cell-specific antigens immediately following their terminal division, prior to migration from the ventricular zone (Waid and McLoon, 1995). For comparison, Fig. 2F shows NF-M immunoreactivity in an eye cup that developed in vitro from a cultured optic vesicle. Many NF-M-immunoreactive cells are also present in the neuroepithelium of the cultured eye cup in the central neural retina (arrows).

In addition to the formation of a neural retina, a pigmented epithelium developed in the cultured eye cups. Fig. 2C shows an E2.5 eye cup immunolabeled with 3C10, a monoclonal antibody directed against an uncharacterized protein in pigmented epithelial cells (Chu and Grunwald, 1990). The epithelium adjacent to the neural retina is immunoreactive for 3C10; the cells in this layer have thus begun to differentiate as pigmented epithelial cells, even though at this stage in development, pigmented epithelial cells have not yet begun to express melanin granules (Stroeva and Mitashov, 1983). The 3C10 immunoreactivity stops at the anterior margin of the eye cup, where the pigmented epithelium and the neural retina join (arrows, Fig. 2C). The presumptive pigmented epithelium of the cultured optic vesicle cells also begin to express 3C10 immunoreactivity within the culture period. Fig. 2G shows a cultured eye cup immunolabeled with 3C10. Like the in vivo eye cup at this stage, the cells in the epithelium surrounding the neural retina were 3C10 immunoreactive, indicating that they have begun to differentiate as pigmented epithelial cells.

We also labeled sections of the control and cultured eye cups with a polyclonal antibody directed against type II collagen (Wood et al., 1991). Fig. 2D shows that in an E2.5 eye cup, type II collagen is localized to the basement membrane surrounding the eye cup and, in particular, it is concentrated between the neural retina and lens (arrows). Fig. 2H shows that, in a cultured optic vesicle, the type II collagen staining is also concentrated between the lens and neural retina after 24 hours in vitro (arrows). This antibody was useful for verifying the position of the neural retina in the cultured optic vesicles.

![Fig. 3. Effects of FGF on neural retina and pigmented epithelium formation in optic vesicles cultured as explants for 24 hours.](image)
The optic vesicle cultures can be maintained for at least two days, and development proceeds, albeit at a slower rate than that observed in vivo. We have not observed obvious lamina- tion of the retina in these cultures, and the photoreceptor-specific antigens that we have tested (visinin, PNA and rod-specific opsins) do not appear to be expressed after 2 days. Nevertheless, these cultures are useful for examining the effects of factors on the early development of the eye and the lens.

**Fibroblast growth factor promotes neural differentiation in the presumptive pigmented epithelium of the optic cup**

To determine whether FGF promotes neural retinal formation, we cultured optic vesicles in the presence of exogenous FGF (either FGF1 or FGF2). In all of our experiments, one optic vesicle from each embryo was treated with FGF while the other vesicle from the same embryo was not treated with FGF and thereby served as a negative control. When the optic vesicles were cultured in FGF, there were several significant changes in both the pigmented epithelium and neural retina.

Addition of FGF to the optic vesicles causes NF-M-positive cells to appear in the presumptive pigment epithelial layer. An example of this is shown by comparing a control eye cup (Fig. 3A) with an FGF2-treated eye cup (Fig. 3B,C). In the untreated eye cups, there are no NF-M-immunoreactive cells in the presumptive pigment epithelial layer, although the neural retina has several NF-M-immunoreactive cells in the neuroepithelium (arrows) in the central neural retina. By comparison, examples of FGF2-treated optic vesicles are shown in Fig. 3B,C. In the FGF-treated vesicles, the presumptive pigmented epithelium did not form its characteristic flattened epithelium, but instead formed a columnar epithelium (Fig. 3F) with extensive NF-M and TuJ1 (neuron-specific β-tubulin) immunolabeling (Fig. 3B and C, respectively). The NF-M and neuron-specific β-tubulin-immunoreactive cells in the pigmented epithelium had characteristics similar to those found in the neural retina; the cells have extended processes and appear to be migrating across the neuroepithelium. The pigmented epithelium looks so similar to the neural retina that the eye cups appeared as a double retina. Concurrent with the expression of NF-M and neuron-specific tubulin in the pigmented epithelium, these cells have also lost their 3C10 labeling (Fig. 3E, arrows) as compared to untreated cultured vesicles (Fig. 3D). In some cases, addition of FGF2 to the optic vesicles caused a complete loss of 3C10 staining. However, in most cases, there were still a few 3C10-immunoreactive cells in the central regions of the presumptive pigmented epithelium. We obtained similar results with either FGF1 or FGF2, although we did not carry out extensive dose-response experiments necessary to rule out a quantitative difference between the factors.

To quantify the effects of FGF (FGF1 and FGF2) on ocular development, we serially sectioned the optic cups, labelled every section with the NF-M (RMO-270) or 3C10 antibodies and either (1) counted the number of NF-M-immunoreactive cells in the neural retina and presumptive pigmented epithelium, or (2) measured the area of the 3C10 staining as described in the Methods. Fig. 4A is a graph of the number of NF-M-immunoreactive cells in the control and FGF-treated optic cups. There is a significant increase in the number of NF-M-immunoreactive cells in both layers of the optic cups of FGF-treated vesicles (n=16, P=0.012 using the Student’s t-test) and a significant decrease in the amount of 3C10 immunoreactivity (shown in Fig. 4B) (n=32, P=0.014, student’s t-test). In nearly half of the optic vesicles pairs (7/16), there was a complete loss of 3C10 staining. Thus, FGF prevents the normal differentiation of the presumptive pigmented epithelium, and causes this layer to develop a neural retinal phenotype.

**Antibodies to FGF2 block FGF-induced regeneration of neural retina from pigmented epithelium explants**

The above results indicate that exogenous FGF is capable of promoting neuronal differentiation in both layers of the developing optic cup. In order to determine whether FGF is necessary for the formation of neural retina and plays a role in the induction of neural retina, we attempted to block the formation of neural retina by culturing the optic vesicles in the presence of antibodies that neutralize FGF2.

Previous experiments from several laboratories have shown that FGF can induce newly differentiated pigmented epithelial cells to regenerate a neural retina (Park and Hollenberg, 1989; Pittack et al., 1991; Guillemot and Cepko, 1992). We used this...
assay to determine whether the antibodies to FGF2 were capable of blocking a biological response to this factor. Pigmented epithelium explants from E3.5 chick embryos cultured in the presence of FGF2, regenerated neural retina after 4 days (Fig. 5A,B). However, this response was blocked by the antibodies to FGF2. Fig. 5C shows a pigmented epithelium explant after 4 days in vitro incubated with both FGF2 and an antibody against recombinant *Xenopus* FGF2. The other two antibodies to FGF2 also blocked neural retina regeneration from pigmented epithelium (data not shown). These explants remained pigmented and did not show evidence of neural retina formation (Fig. 5D). Thus, these antibodies could be used to test for a function of FGF in neural retinal development in optic vesicles.

**FGF antibodies inhibit neural retinal development in optic vesicles**

To determine whether FGF is required for neuronal development, the optic vesicles from E1.5 embryos were cultured in separate wells and the FGF2 neutralizing antibodies described above were added to one optic vesicle, while the other vesicle served as a paired control. After 24 hours in culture, both the control vesicles (Fig. 6A,C,E) and those treated with FGF2 antibodies (Fig. 6B,D,F) appeared morphologically similar, and we were still able to identify a lens vesicle and the two layers of the optic cup in both explants (Fig. 6A,B). However, when the vesicles were labeled with antibodies to ganglion cells or pigmented epithelium (NF-M and 3C10, respectively), we found that the presence of antibodies to FGF2 effectively blocked the initial differentiation of neural retina. Fig. 6C,D shows an example of an optic vesicle treated with an FGF2 antibody after 24 hours in vitro. When labelled with neurofilament antibody, the anti-FGF2-treated vesicle (Fig. 6D) had no NF-M-immunoreactive cells (see Fig. 2F for comparison). In 5/14 cases, treatment with FGF2 antibodies resulted in the complete absence of NF-M-immunoreactive cells in the neural retina while, in the remaining cases, the number of NF-M-immunoreactive cells was substantially reduced as compared with the control vesicle from the same embryo. We quantified this effect by counting the number of NF-M-immunoreactive cells in the treated and control vesicles in serial sections of pigmented epithelium through the cultures. The results of these cell counts are summarized for all cases in Fig. 7 (n=24, P=0.007, Student’s t-test), and show that neutralizing antibodies to FGF2 significantly inhibit neural retina differentiation in the developing optic vesicle.

By contrast to the effect on neural retinal formation, the differentiation of the pigmented epithelium proceeds normally in the presence of the FGF2 antibodies. Fig. 6E and F show an FGF2 antibody-treated vesicle after 24 hours, sectioned and labeled with 3C10 antibody to detect pigmented epithelium. The 3C10 labeling is similar in the treated (Fig. 6F) as in the control vesicles (see Fig. 2G for comparison). Thus, the antibodies to FGF2 do not affect the initial differentiation of the pigmented epithelium in the optic vesicle cultures.

We have shown that antibodies to FGF2 causes a decrease in the number of NF-M-immunoreactive cells in the neural retina without affecting the differentiation of the pigmented epithelium. It is possible that this result was due to a general effect of culturing the optic vesicles in the antibodies rather than a specific effect of the FGF2 antibodies. In order to address this question, we performed the same experiment as described above, but using a non-specific antibody, mouse IgG. Following the 24 hour culture period, the control, untreated and IgG-treated vesicles were sectioned and processed for immunoreactivity with antibodies to NF-M. The number of NF-M-immunoreactive cells in the neural retina of the resultant eye cups were counted as previously described. We found there to be no significant difference in the number of NF-M-immunoreactive cells in the IgG-treated (8±1 cell/section) and control (5±0.5 cells/section) vesicle (see Table 1).
Antibodies to FGF2 do not affect cell proliferation or cell death

The normal development of the pigmented epithelium in the treated vesicles indicates that the FGF2 neutralizing antibodies do not have any non-specific toxic effects on the cultures; however, it is possible that the absence of NF-M-immunoreactive cells in the eyes was due to the death of cells in the prospective neural retina, rather than blocking neural differentiation. To control for this possibility, we labeled the optic vesicle sections with a nuclear marker, DAPI in order to show the overall morphology of the optic cups. Optic vesicles treated with FGF2 antibodies develop into an optic cup that forms a neural retina, pigmented epithelium and lens vesicle comparable to untreated control optic vesicles.

Fig. 6. FGF2 antibodies block neural retina formation.
(A,C,E) Control untreated optic vesicle and (B,D,F) FGF antibody-treated optic vesicles after 24 hours in vitro. (A,B) Stained with the nuclear marker DAPI in order to show the overall morphology of the optic cups. Optic vesicles treated with FGF2 antibodies develop into an optic cup that forms a neural retina, pigmented epithelium and lens vesicle comparable to untreated control optic vesicles. (C,D) Corresponding phase and fluorescent micrographs, respectively, of an FGF2 antibody-treated optic vesicle processed for immunohistochemistry with NF-M. In this case, NF-M-immunoreactive cells were completely absent. The immunoreactivity in the basement membrane surrounding the optic cup is due to the secondary antibody binding to the FGF2 antibody, also a mouse monoclonal. (E,F) Corresponding phase and fluorescent micrographs of an optic vesicle cultured with antibodies to FGF2 and processed for immunohistochemistry with the pigmented epithelial cell marker, 3C10. The arrows point to the 3C10-immunoreactive pigmented epithelium.

Addition of FGF2 antibodies to optic vesicles cultured for 24 hours in vitro inhibits the formation of the neural retina, as shown by the decrease in the number of NF-M-immunoreactive cells in the eye cups. The FGF2 antibody does not increase apoptosis or affect cell proliferation or cell number in the optic vesicles. In addition, the FGF2 antibodies do not alter the expression of NF-M in cultured E3.5 neural retina explants. Culturing the optic vesicles in mouse IgG, does not affect the formation of the neural retina in the cultured optic vesicles. Optic vesicles were dissected from an E1.5 embryo and cultured in FGF2 neutralizing antibodies or IgG antibodies for 24 hours prior to immunohistological analysis. The results of the optic vesicle experiments are expressed as cells/section except in the case of DAPI where the results are expressed as cells/360 \( \mu \text{m}^2 \). The results of the neural retina experiments (E3.5) are expressed as cells/360 \( \mu \text{m}^2 \). Control optic vesicles and neural retina were untreated.

Table 1. Effects of FGF2 neutralizing antibodies on NF-M expression, apoptosis, cell proliferation and cell number in cultured optic vesicles, and NF-M expression in E3.5 neural retina

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<th>Control</th>
<th>FGF2 antibody</th>
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<tr>
<td>Optic vesicle</td>
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<tr>
<td>NF-M+</td>
<td>4±0.7</td>
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<td>Apogat</td>
<td>18±2</td>
<td>15±2</td>
<td>0.248</td>
<td>18</td>
</tr>
<tr>
<td>BrdU</td>
<td>87±14</td>
<td>84±15</td>
<td>0.911</td>
<td>5</td>
</tr>
<tr>
<td>DAPI</td>
<td>27±2</td>
<td>31±2</td>
<td>0.564</td>
<td>16</td>
</tr>
<tr>
<td>E3.5 NR</td>
<td>25±3</td>
<td>29±2</td>
<td>0.386</td>
<td>48</td>
</tr>
</tbody>
</table>

| Optic vesicle          |         |               |        |       |
| NF-M+                  | 5±0.5   | 8±1           | 0.097  | 27    |

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Fig. 7. Addition of FGF2 antibodies inhibits the development of the neural retina in cultured optic vesicles. Addition of antibodies raised against FGF2 to optic vesicles cultured for 24 hours as explants results in a significant decrease in the number of NF-M-positive cells in the neural retina of the eye cups. Error bars: \( \pm \) s.e.m. of 14 separate experiments.
DISCUSSION

The present study investigated whether members of the FGF family are necessary for neural retina and/or pigmented epithelium development during eye cup formation using cultures of isolated optic vesicles. Three lines of evidence indicate that FGF is important in the induction of neural retina. (1) We have found FGF2 immunoreactivity in the head ectoderm of the chick embryo at the stages of optic cup formation. (2) Addition of FGF (FGF1 or FGF2) to the optic vesicles during the culture period causes the presumptive pigmented epithelium to differentiate as neural retina. (3) Addition of FGF2 neutralizing antibodies to the optic vesicles inhibited neural retina development, but did not interfere with normal development of the pigmented epithelium.

Extraocular factors control cell fates in the developing eye

Many years ago, the results of embryological experiments demonstrated that the specific microenvironments surrounding the pigmented epithelium and neural retina are important for the development of these two tissues (see Lopashov and Stroeva, 1964 for review). If the optic vesicle is flattened and surrounded entirely with mesenchyme, only pigmented epithelium will develop (Lopashov and Stroeva, 1964). Alternatively, when the optic vesicle is rotated 180°C such that the presumptive pigmented epithelium now lies next to the head ectoderm, a secondary retina is induced from the pigmented epithelium (Dragomirov, 1957). In addition, grafting the optic vesicle to other regions in the embryo, in particular the otic vesicle, will also induce the presumptive pigmented epithelium to differentiate into a neural retina (Detwiler and van Dyke, 1953; Lopashov and Stroeva, 1964). These studies suggest that the differentiation of neural retina and pigmented epithelium is not due strictly to intrinsic differences between these two tissues, but rather that specific inducing substances are produced by the tissues surrounding the optic vesicle to pattern the differentiation of the eye. The extraocular mesenchyme appears to be important for pigmented epithelium differen-
that show that FGF is important in regulating cell phenotype presumptive pigment epithelium in the FGF-treated eye cups retina. Concurrent with the expression of NF-M, the pre-epithelium that the eye cups looked like a double-layered reactivity was so extensive in the presumptive pigment epithelial morphology, and, in some cases, the NF-M immunoreactivity was so extensive in the presumptive pigmented epithelium to form a NF-M-epithelium, and a lens vesicle. Addition of FGF (FGF1 or FGF2) is necessary for normal retinal formation

FGF and its receptors are expressed in the developing ocular tissues

The results that we have obtained are consistent with the hypothesis that FGF may be an important inducer of neural retina during development. Several members of the FGF receptor family of tyrosine kinases, along with their ligands, have been shown to be expressed in the developing rat, mouse and chicken eye cups (Heuer et al., 1991; Wanaka et al., 1991; Tcheng et al., 1994; Kalcheim and Neufeld, 1990; De Iongh and McAvoy, 1993; Consiglio et al., 1993; this study). Both the present study and De Iongh and McAvoy (1993) have shown FGF immunoreactivity in the lens ectoderm overlying the optic vesicle, consistent with its role in lens development and as an inducer of neural retina. However, while De Iongh and McAvoy (1993) found FGF1 expression in the ectoderm of the rat embryo, Savage and Fallon (1995), as well as our own studies, have shown FGF2 expression in the ectoderm in the chick embryo. It is possible that FGF1 plays an inductive role for the retina in the rat, similar to what we have found for FGF2 in the chick embryo. However, it is also possible that variations in the epitopes recognized by the antibodies, differences in sensitivity in the detection methods, different immunocytochemical techniques used and species differences may all contribute to the observed differences.

Our results showing that FGF2 is expressed in the ectoderm are consistent with the hypothesis that FGF may act as an inducer of neural retina since transplant studies have shown that the signal for neural retinal formation in widely distributed in the embryonic ectoderm. It is also interesting in this regard that int-2, another member of the FGF family of factors, is expressed in the developing rhombencephalon (Wilkinson et al., 1989; Represa et al., 1991), another region of the embryo with neural retinal inducing activity (Detwiler and van Dyke, 1953).

FGF is necessary for normal retinal formation

Exogenous FGF2 acts on the undifferentiated cells of the optic vesicle to induce the presumptive pigmented epithelium to form neural retina. In our control experiments, we showed that optic vesicles with the attached head ectoderm and surrounding mesenchyme, cultured for 24 hours, develop into eye cups that are morphologically similar to eye cups that developed in vivo, containing a neural retina, pigmented epithelium, and a lens vesicle. Addition of FGF (FGF1 or FGF2) to these optic vesicles during the culture period caused the presumptive pigmented epithelium to form a NF-M-expressing cells and class III β-tubulin (TuJ1)-immunoreactive columns of neuroepithelium. The pigment epithelium in the FGF-treated eye cups lost the characteristic flattened epithelial morphology, and, in some cases, the NF-M immuno-activity was so extensive in the presumptive pigment epithelium that the eye cups looked like a double-layered retina. Concurrent with the expression of NF-M, the presumptive pigment epithelium in the FGF-treated eye cups showed a significant decrease in 3C10 expression and in some cases, there was a complete loss of 3C10.

These results extend those obtained by other investigators that show that FGF is important in regulating cell phenotype later in development during retinal regeneration. Studies by Park and Hollenberg (1989), Pittack et al. (1991), Guillemot and Cepko (1992) and Opas and Dziak (1994) have shown that members of the FGF family, in particular FGF1 and FGF2, cause pigmented epithelium to regenerate neural retina up to E4.5. Several other growth factors that are present in the developing eye were tested for their ability to induce pigmented epithelial cells to form neural retina in vivo and in vitro, however, only FGF has this effect (Park and Hollenberg, 1991; Pittack et al., 1991).

We have also provided evidence that FGF is necessary for normal neural retina formation. Addition of FGF2 neutralizing antibodies to the optic vesicles inhibited neural retina development. By comparison with controls, optic vesicles cultured with FGF2 antibodies had significantly fewer NF-M-immunoreactive cells in the retina; in some cases, no NF-M-immunoreactive cells were present in the vesicles treated with FGF2 antibodies. The loss of differentiated neurons in the presumptive neural retinal layer was likely due to the inhibition of neural retinal differentiation and not due to suppression of NF-M expression in ganglion cells, since we found no effect of these antibodies on the number of NF-M-immunoreactive cells in later staged retinas (see Results). By contrast, FGF2 antibody treatment did not appear to affect the development of the pigment epithelium in the eye cups and 3C10 immunoreactivity was expressed by the cells in this layer.

Although difficult to quantify in the optic vesicle cultures, it appeared that FGF treatment also caused an increase in the number of neurofilament-immunoreactive ganglion cells in the presumptive neural retina by specifically promoting their differentiation. This is consistent with the earlier report of Guillemot and Cepko (1992), in which addition of FGF to pigmented epithelium not only causes the pigmented epithelial cells to regenerate neural retina, but a higher percentage of the cells in the neural retina differentiate into retinal ganglion cells (Guillemot and Cepko, 1992). However, it is also possible that the increase in neurofilament-immunoreactive cells is secondary to a mitogenic effect of FGF on the cultures. Nevertheless, these results suggest that the induction of the neural retina and specifically retinal ganglion cells may occur through the same factor, an hypothesis consistent with previous reports in mammals that ganglion cells are the “default” phenotype of the early retina (Reh and Kjavin, 1989; Reh, 1992).

Specification of the different regions of the optic vesicle by extrinsic factors

During the development of the eye, the optic vesicle gives rise to the neural retina, the pigmented epithelium and the optic stalk. Previous reports have demonstrated that Sonic hedgehog, expressed first in the prechordal plate, and then later in the ventral diencephalon, in the development of the optic stalk (Ekker et al., 1995; MacDonald et al., 1995; Chiang et al., 1996). In this report, we have demonstrated that FGF2, expressed in the lens ectoderm, is necessary for the development of the neural retina.

Our results can be interpreted in the following model, shown in Fig. 9. As the optic vesicles grow out laterally from the neural tube, the leading edge (future neural retina) is in close contact with the overlying ectoderm. A localized source of
FGF from the ectoderm (stippled) promotes neural retinal development in this region. The medial surface of the optic vesicle does not receive this signal, but instead is surrounded by extracellular mesenchyme, which might release signals that induce the pigmented epithelium.

When the optic vesicle is isolated from surrounding extracellular tissue, the pigmented epithelium also fails to develop. At present, the factors in the extracellular tissue necessary for the development of the pigmented epithelium are still largely unknown. One possibility is activin, a member of the transforming growth factor family, which acts along with FGF in the process of mesoderm induction (Thomsen et al., 1990). In this model, activin induces the mesenchyme to secrete FGF, which in turn promotes the development of the pigmented epithelium. Activin has been shown to be expressed in the pigmented epithelium and, more importantly, in the surrounding mesenchyme in mouse embryos (Feijen et al., 1994). Concurrent with the expression of activin at this stage in development, is the expression of the activin receptor type II in the pigment epithelium in mouse embryos (Feijen et al., 1994). Consistent with a role for activin in the process of pigment epithelial cell differentiation, we found that activin can block FGF-induced retinal regeneration from E3.5 pigmented epithelium (Pitack, 1995). Thus, while our results provide support that FGF is an inducer of the neural retina, it is likely that additional factors in the local microenvironment of the optic vesicle are necessary for appropriate cell fate decisions during ocular development.

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