Patterning the optic neuroepithelium by FGF signaling and Ras activation

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SUMMARY

During vertebrate embryogenesis, the neuroectoderm differentiates into neural tissues and also into non-neural tissues such as the choroid plexus in the brain and the retinal pigment epithelium in the eye. The molecular mechanisms that pattern neural and non-neural tissues within the neuroectoderm remain unknown. We report that FGF9 is normally expressed in the distal region of the optic vesicle that is destined to become the neural retina, suggesting a role in neural patterning in the optic neuroepithelium. Ectopic expression of FGF9 in the proximal region of the optic vesicle extends neural differentiation into the presumptive retinal pigment epithelium, resulting in a duplicate neural retina in transgenic mice. Ectopic expression of constitutively active Ras is also sufficient to convert the retinal pigment epithelium to neural retina, suggesting that Ras-mediated signaling may be involved in neural differentiation in the immature optic vesicle. The original and the duplicate neural retinae differentiate and laminate with mirror-image polarity in the absence of an RPE, suggesting that the program of neuronal differentiation in the retina is autonomously regulated. In mouse embryos lacking FGF9, the retinal pigment epithelium extends into the presumptive neural retina, indicating a role of FGF9 in defining the boundary of the neural retina.

Key words: Optic vesicle, Neural retina, Retinal pigment epithelium, Neural patterning, Retinal development, Lens differentiation, FGF9, Ras, Mouse

INTRODUCTION

During vertebrate embryogenesis, the neuroectoderm gives rise to a variety of neural and non-neural tissues in the central nervous system (CNS). Examples of non-neural tissues derived from the neuroectoderm include the choroid plexus in the brain and the retinal pigment epithelium (RPE) in the eye. Very little is known about how the neuroectoderm is specified to become either neural or non-neural tissue and how boundaries between these tissues are determined. The vertebrate retina provides a model system to study these processes. During vertebrate development, the optic vesicle grows out from the diencephalon. As the optic vesicle contacts the surface ectoderm, it starts to invaginate to form the optic cup (Barnstable, 1991). The inner layer of the optic cup becomes the neural retina and the outer layer becomes the non-neural RPE. The margin between these two layers gives rise to non-neural iris and ciliary epithelia. Early explant and transplant studies have demonstrated that the optic vesicle neuroepithelium is capable of becoming either neural retina or RPE, depending on its environment (Stone and Steinitz, 1957; Stroeva, 1960; Coulombre and Coulombre, 1965; Stroeva and Mitashov, 1983). It has been postulated that the neural retina and RPE cell fates are influenced by signals from the

surface ectoderm and periocular mesenchyme, respectively (Coulombre and Coulombre, 1965; Pittack et al., 1997; Nguyen and Arnheiter, 2000). However, the relevant signaling molecules remain unknown.

Members of the fibroblast growth factor (FGF) family can contribute to neural induction during vertebrate development (Tanabe and Jessell, 1996). FGF signaling induces posterior neural tissue from gastrula stage ectoderm in *Xenopus* (Tannahill et al., 1992; Slack and Isaacs, 1994; Lamb and Harland, 1995; Cox and Hemmati-Brivanlou, 1995; Launay et al., 1996; Holowacz and Sokol, 1999) and in chick (Storey et al., 1998; Alvarez et al., 1998). Several studies suggest that FGF signaling may also be involved in neural differentiation in the optic vesicle. It has been shown that FGF1 and FGF2 can induce early RPE from chick embryos to become neural retina-like tissue in vivo (Park and Hollenberg, 1989; Park and Hollenberg, 1991; Hyer et al., 1998) and in vitro (Pittack et al., 1991; Guillemot and Cepko, 1992). Early RPE from rat (Zhao et al., 1995; Zhao et al., 1997) or *Xenopus* (Sakaguchi et al., 1997) embryos can also transdifferentiate to neural retina when stimulated by FGF2.

Another member of the FGF family, FGF9, has been shown to be expressed during retinoic acid-induced neuronal differentiation of murine embryonal carcinoma P19 cells (Seo

and Noguchi, 1995). FGF9, originally named glia-activating factor (GAF), was first identified from a human glioma cell line (Miyamoto et al., 1993). Although FGF9 does not contain a consensus signal peptide sequence for secretion, it has been shown to be efficiently secreted (Miyamoto et al., 1993; Lovicu and Overbeek, 1998) via an alternative secretory pathway (Naruo et al., 1993; Song and Slack, 1996; Revest et al., 2000). FGF9 can bind to and activate FGF receptor 2 (FGFR2) and receptor 3 (FGFR3) (Hecht et al., 1995; Santos-Ocampo et al., 1996; Ornitz et al., 1996). Mouse *Fgf9* gene maps to chromosome 14 near the *Ctla6* locus (Colvin et al., 1999). Its mRNA transcripts are expressed in multiple tissues during embryonic development, including intermediate mesoderm, ventricular myocardium, lung pleura, skeletal myoblasts in the limb bud, spinal cord motoneurons, olfactory bulb and gut lumenal epithelium (Colvin et al., 1999). Mice homozygous for a targeted disruption of *Fgf9* gene exhibit lung hypoplasia and early postnatal death (Colvin et al., 2001a) and male to female sex reversal (Colvin et al., 2001b).

To investigate the effects of members of the FGF family on ocular development, we generated transgenic mice expressing different FGFs in the eye using a lens-specific αA-crystallin promoter (Lovicu and Overbeek, 1998). Surprisingly, in one of the FGF9 transgenic families (OVE1070), the dorsal half of the RPE was converted to a second layer of neural retina. This phenotype was found to correlate with inappropriate transient expression of the transgene in the dorsal presumptive RPE. To test the implications of this discovery, expression of FGF9 was targeted intentionally to the presumptive RPE using a tyrosinase-related protein 2 (TRP2) promoter (Zhao and Overbeek, 1999). We now report detailed characterization of the ocular phenotypes in these transgenic families. In addition, we have generated transgenic mice that express a constitutively active *Ras* gene using the TRP2 promoter. Transient activation of Ras is sufficient to convert the presumptive RPE into neural retina, suggesting that the neuronal differentiation pathway in the early optic neuroepithelium may be specified through activation of Ras by a receptor tyrosine kinase. Cells in both the original and RPE-derived neural retinae differentiated and laminated to form three distinct cell layers. The cellular polarity of the duplicate neural retina is a mirror image of that of the endogenous neural retina. The ability of the original and ectopic neural retinae to proliferate and differentiate in the absence of RPE indicates that retinal growth and differentiation are autonomously programmed. Embryos lacking FGF9 show abnormal differentiation of the anterior retina, indicating that FGF9 plays a role in defining the boundary between the neural retina and the RPE in embryonic mouse eyes.

MATERIALS AND METHODS

Generation and genotyping of transgenic mice

To express FGF9 in the developing lens, a mouse FGF9 cDNA (Santos-Ocampo et al., 1996) was inserted downstream of a 0.3 kb αA-crystallin promoter (CPV2) (Lovicu and Overbeek, 1998) and upstream of a 0.8 kb fragment containing the SV40 small t intron and early region polyadenylation sequences (SV40 pA). To express Ras in the presumptive RPE, genomic sequences encoding an activated human RAS (*ras*T24) (Taparowsky et al., 1982; Capon et al., 1983) were inserted downstream of a 1.7 kb TRP2 promoter (Zhao and Overbeek, 1999) and upstream of the SV40 pA. DNA constructs were injected into one-cell stage embryos from FVB/N female mice and the embryos were transferred to pseudopregnant ICR females (Hogan et al., 1994; Taketo et al., 1991). Transgenic embryos and mice were identified by polymerase chain reaction (PCR) with genomic DNA extracted from mouse tails. A pair of primers specific to the SV40 pA sequences were used for the PCR (Lovicu and Overbeek, 1998).

Generation of chimeric embryos

Aggregation chimeras were generated as previously described (Robinson et al., 1995). Briefly, FVB/N females were mated with either OVE1070 males or ROSA26 males. ROSA26 mice express the reporter gene *lacZ* ubiquitously (Friedrich and Soriano, 1991). Early morula stage embryos were collected from the females at 55-60 hours postcopulation. Embryos from the two different matings were aggregated in pairs and cultured for 24 hours in microwells covered with paraffin oil at 37°C in a humidified 5% CO$_2$ incubator. Aggregated embryos that reached the blastocyst stage were transferred to the uteri of pseudopregnant ICR females. The embryos were collected at E12.5, assayed for *lacZ* expression in X-gal solution, and sectioned for histological examination (see below).

Histology, immunohistochemistry and in situ hybridization

Mouse embryos or postnatal eyes were fixed in 10% formalin overnight at room temperature and then rinsed in 70% ethanol for 24 hours. The tissues were next dehydrated in a series of increasing concentrations of ethanol, cleared in xylene and embedded in paraffin wax. Sections (5 µm) were cut on a microtome for Hematoxylin/Eosin staining, immunohistochemistry or in situ hybridization. Before these procedures, tissue sections were dewaxed with xylene and rehydrated using decreasing concentrations of ethanol.

For immunohistochemistry, tissue sections were treated first with 3% H$_2$O$_2$ in 10% methanol for 15 minutes to quench endogenous peroxidase activity and then with 5% normal goat serum (NGS) in phosphate-buffered saline (PBS) for 15 minutes to block nonspecific antibody binding. The tissue sections were then incubated at room temperature sequentially with the primary antibody for 1 hour, biotinylated secondary antibody to the primary antibody for 1 hour, and finally biotinylated horseradish peroxidase-avidin complex from the VECTASTAIN ABC kit (Vector Laboratories). Peroxidase activity was visualized by incubation with diaminobenzidine tetrahydrochloride (DAB). For fluorescent antibody labeling, tissue sections were incubated with fluorecein-conjugated secondary antibodies. Slides were coverslipped with 50% glycerol and examined under blue light illumination.

In situ hybridization was performed as previously described (Robinson et al., 1995; Robinson et al., 1998). Briefly, plasmids containing SV40 pA or mouse cDNAs for *Fgf9*, *Cdh3* (*P-cadherin*), *Rax* (*Rx*), *Chx10*, *Pax6*, *Atoh7* (*Math5*) and *Mitf* were linearized by restriction endonuclease digestions. Antisense ^{35}S-UTP-labeled RNA probes were synthesized from T7, T3 or SP6 RNA polymerase promoters. After hybridization to tissue sections, unhybridized probes were removed by rinsing in 2×SSC buffer before and after RNase treatment. Tissue sections were then dehydrated and coated with Kodak NTB-2 emulsion for autoradiography. The slides were developed, stained with Hematoxylin and mounted with a coverslip for examination of silver grains under dark-field illumination. The images were collected using a CCD camera (Optronics Engineering, Goleta, CA). Dark-field images were typically pseudocolored as red (to highlight the silver grains) and were superimposed onto the corresponding bright-field images using Photoshop software (Adobe, San Jose, CA).

Cell proliferation assay

Pregnant female mice were injected intraperitoneally with 100 µg 5-bromo-2′-deoxyuridine (BrdU) (Sigma, St Louis) per gram body weight supplemented with 10 µg/g 5-fluoro-2′-deoxyuridine in PBS (Sigma, St Louis). The female mice were sacrificed and embryos were

collected one hour after BrdU injection. The embryonic tissues were fixed, processed, embedded and sectioned as described above. Antibody to BrdU (Dako, Carpenteria, CA) was used to assay for patterns of BrdU incorporation as previously described (Lovicu and Overbeek, 1998). The procedure for immunohistochemistry was the same as described above except that tissue sections were pretreated with 0.02% pepsin (Sigma, St. Louis) in 2 N HCl for 20 minutes at room temperature after the H_2O_2 treatment.

X-gal staining

Mouse embryos or eyes at various developmental stages were collected and fixed in 2% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) for 1 hour at 4°C as described by Yokoyama et al. (Yokoyama et al., 1992). After three 30 minute rinses (in 0.01% sodium deoxycholate, 0.02% NP-40, 2 mM $MgCl_2$, and 0.1 M phosphate buffer pH7.3), β-galactosidase activity was visualized in X-gal (5-bromo-4-chloro-3-indolyl β-D-galactoside) staining solution (0.1M phosphate buffer pH 7.3, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 1 mg/ml X-gal) overnight at 4°C. The stained tissues were rinsed in phosphate-buffered saline (pH 7.4) and stored in 70% ethanol. The tissues were processed with HistoClear (National Diagnostics, Atlanta, GA) and embedded in paraffin for sectioning. Tissue sections were counter-stained with Nuclear Fast Red (Poly Scientific, Bay Shore, NY).

RESULTS

Transgenic mice

Eight transgenic families were generated from construct CPV2-FGF9 which contains a lens-specific αA-crystallin promoter (CPV2) linked to the coding sequences for mouse FGF9 (Lovicu and Overbeek, 1998). Premature differentiation of lens epithelial cells was observed in six out of eight transgenic families (Lovicu and Overbeek, 1998). Unexpectedly, in the dorsal region of embryonic eyes from one of the families (OVE1070), a layer of neural tissue, morphologically similar to neural retina, formed in place of the RPE (Fig. 1A). This ectopic neural retina will be referred to as RPE-derived neural retina (rNR). The same RPE phenotype was observed in mice heterozygous or homozygous for the transgene. Unlike the modified dorsal RPE, the ventral RPE of these transgenic mice remained as a pigmented epithelium (Fig. 1A). In the wild-type mice, the entire RPE is a pigmented epithelium (Fig. 1B). No duplicated neural retina was found in any of the other transgenic families generated from the CPV2-FGF9 construct (data not shown).

Loss of RPE phenotype

To characterize the altered RPE differentiation in family OVE1070, we assayed for changes of RPE-specific markers in the developing eyes. The OVE1070 mice were cross-bred to mice from a transgenic line that expresses the reporter gene *lacZ* in the RPE (and other pigmented cells) under the control of the TRP2 promoter (Zhao and Overbeek, 1999). Expression of the TRP2-lacZ transgene was first detected in the presumptive RPE at 9.5 days of gestation (E9.5) and persisted in the RPE postnatally (Zhao and Overbeek, 1999). At E10.5, in embryos carrying both the CPV2-FGF9 and the TRP2-*lacZ* transgenes, *lacZ* expression was absent in the presumptive dorsal RPE (Fig. 1C). For embryos carrying only TRP2-*lacZ*, *lacZ* expression was detected throughout the entire prospective RPE (Fig. 1D).
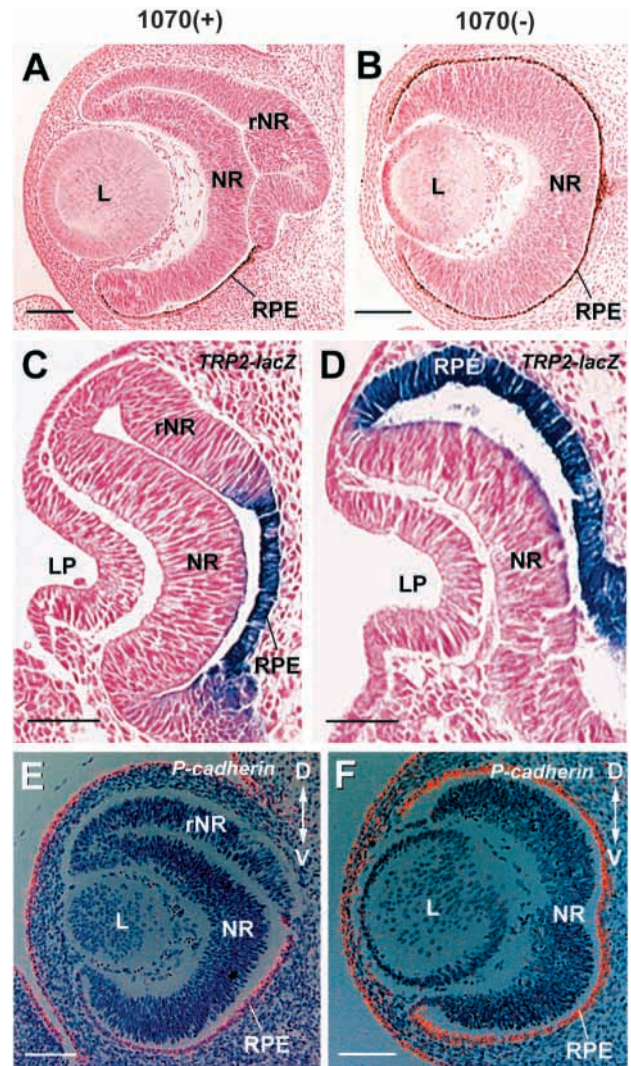

Fig. 1. Altered RPE differentiation in family OVE1070. Embryos, transgenic (A,C,E) and non-transgenic (B,D,F) for CPV2-FGF9 (OVE1070), were assayed for ocular pigmentation (A,B), for expression of the TRP2-*lacZ* transgene (C,D), and by in situ hybridization for expression of *Cdh3* (*P-cadherin*) (E, F). OVE1070 mice were mated to a C57BL/6 partner to produce pigmented offspring. In E13.5 transgenic embryos, the dorsal RPE (rNR) lost its pigmentation and became a neural retina-like tissue (A). By contrast, the entire RPE in non-transgenic embryos was pigmented (B). When OVE1070 mice were mated with TRP2-*lacZ* transgenic mice, the offspring carrying both CPV2-FGF9 and TRP2-*lacZ* lost β-gal activity in the dorsal presumptive RPE (rNR) at E10.5 (C). In offspring carrying TRP2-*lacZ* only, the entire prospective RPE expressed *lacZ* (D). In E12.5 OVE1070 embryos, *Cdh3* (*P-cadherin*) expression (red) was attenuated in the dorsal presumptive RPE (rNR) (E), while in wild-type embryos, *Cdh3* was expressed in the entire RPE (F). *Cdh3* (*P-cadherin*) expression was also detected in the corneal and conjunctival epithelia in both types of embryos. D-V, dorsal-ventral orientation; L, lens; LP, lens pit; NR, neural retina; rNR, RPE-derived neural retina. Scale bars: 50 μm in C,D; 100 μm in A,B,E,F.

We also assayed by in situ hybridization for changes in the expression pattern of Cdh3 (P-cadherin), a Ca^{2+}-dependent cell adhesion molecule normally expressed in the RPE and other

Fig. 2. Expression of transgenic and endogenous FGF9. In family OVE1070, transgene expression was first detected in the dorsal presumptive RPE (rNR) at E10.5 by in situ hybridization using an SV40 riboprobe (A). At E11.5, the transgene remained expressed in the dorsal rNR and was also expressed in the posterior of the lens vesicle (LV) (B). By E12.5, transgene expression was turned off in the rNR and became restricted to the lens (C). In chimeric embryos (OVE1070: ROSA26) (D), clusters of β-gal-positive cells (arrows) were present in the rNR, indicating that cells from ROSA26 mice (blue cells) were induced to become neural retinal cells by the transgenic FGF9 secreted from neighboring cells. During normal eye development (E,F), expression of endogenous *Fgf9* was detected in the prospective neural retina but not in the prospective RPE at E10.0 (E) and E11.5 (F). *Fgf9* is also expressed in the ventral (yellow arrow in E) but not dorsal (yellow arrowhead) region of the optic stalk. Abbreviations: D-V, dorsal-ventral orientation; L, lens; LP, lens pit; LV, lens vesicle; NR, neural retina; rNR, RPE-derived neural retina. Scale bars: 50 μm in A,B,D-F; 100 μm in C.
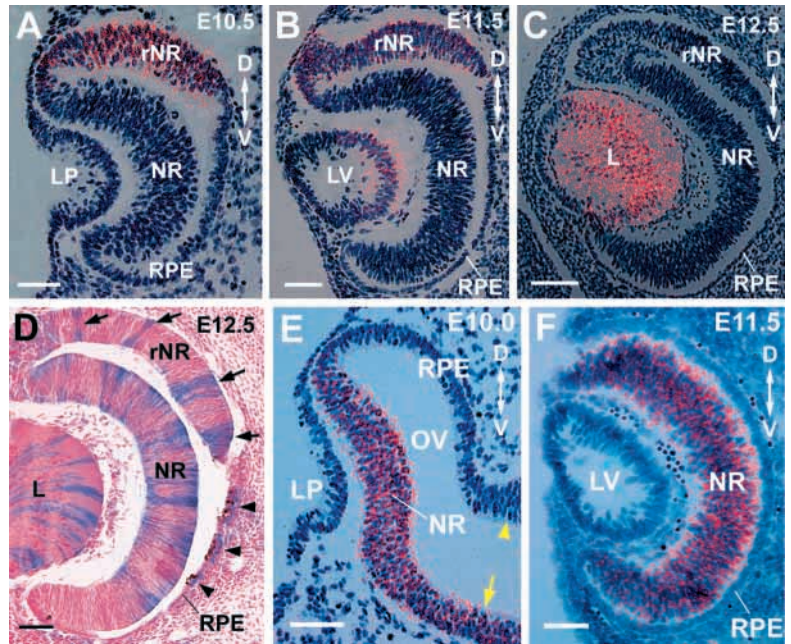
epithelial tissues (Nose and Takeichi, 1986; Nose et al., 1987; Radice et al., 1997). In E12.5 transgenic embryos, *Cdh3* (*P-cadherin*) was expressed in the unaffected ventral RPE but not in the dorsal rNR (Fig. 1E). In wild-type E12.5 eyes, the mRNA was detected throughout the RPE (Fig. 1F). The expression pattern of *Cdh3* (*P-cadherin*) in the corneal and conjunctival cells was not altered in the transgenic embryos.

Expression and secretion of transgenic FGF9

The localized and early alteration in RPE differentiation was difficult to explain since the αA-crystallin promoter normally targets transgene expression specifically to the lens beginning at E11.5 (Robinson et al., 1995; Lovicu and Overbeek, 1998). To determine whether the unique phenotype in family OVE1070 might be the result of inappropriate transgene expression (i.e. expression outside of the lens), we carried out in situ hybridization on ocular sections from transgenic embryos using an SV40 pA riboprobe (Fig. 2A-C). The results revealed that the CPV2-FGF9 transgene was expressed in the dorsal region of the presumptive RPE at E10.5 (Fig. 2A). At E11.5, transgene expression persisted in the dorsal RPE and was also detected in the posterior cells of the lens vesicle as expected (Fig. 2B). At E12.5, transgene expression was no longer detectable in the presumptive RPE and became restricted to the lens (Fig. 2C). In situ hybridization on eye sections at later stages of development (E13.5 and E15.5) confirmed that transgene expression was subsequently restricted to the lens (data not shown). In other transgenic families carrying the CPV2-FGF9 construct, transgene expression was detected only in the lens (data not shown). The unique pattern of transgene expression in family OVE1070 strongly suggests that the unexpected phenotype in this family is caused by ectopic FGF9 expression in the presumptive RPE. The transient expression of the *Fgf9* transgene in the dorsal presumptive RPE of OVE1070 mice is presumably due to the influence of endogenous regulatory elements near the transgene integration site.

To test whether the transgenic FGF9 functions as a secreted protein, we generated chimeric embryos by aggregating eight-cell stage embryos from OVE1070 (albino) and ROSA26 (pigmented) mice. ROSA26 mice express *lacZ* ubiquitously (Friedrich and Soriano, 1991). X-gal staining of chimeric embryos revealed blue cells from ROSA26 mice and unstained cells from OVE1070 (Fig. 2D). Clusters of blue and non-blue cells exhibited the same morphology in the rNR (arrows in Fig. 2D), indicating that the ROSA26 cells had responded to the transgenic FGF9 secreted from neighboring OVE1070 cells. In the ventral RPE, blue ROSA26 cells were pigmented as expected (Fig. 2D).

Conversion to a neural retinal cell fate

One of the distinctive differences between the neural retina and the RPE in the developing eye is the rate of cell proliferation. Retinal neuroblasts proliferate rapidly while the RPE cells differentiate as a monolayer and display a low rate of cell proliferation. The ectopic neural retina in OVE1070 mice was found to have high levels of BrdU incorporation, analogous to that of the endogenous neural retina (Fig. 3A). By contrast, very few cells showed BrdU incorporation in the unaltered ventral RPE (Fig. 3A).

To determine whether the rNR differentiated in a similar fashion to the normal neural retina, we used immunohistochemistry to examine the expression of known neuronal markers. β-tubulin III is a molecular marker for retinal ganglion cells in the developing eye (Zhao and Barnstable, 1996). At E15.5, this marker was expressed not only in the inner neural retina but also in the rNR, indicating retinal ganglion cells had been generated in both tissues (Fig. 3B). The localization of β-tubulin III-positive cells shows that the cellular polarity of the ectopic neural retina is a mirror image of the original neural retina.

In postnatal OVE1070 mice, cells in the rNR show evidence of lamination, forming cellular layers analogous to those of the normal neural retina, including a ganglion cell layer, an inner
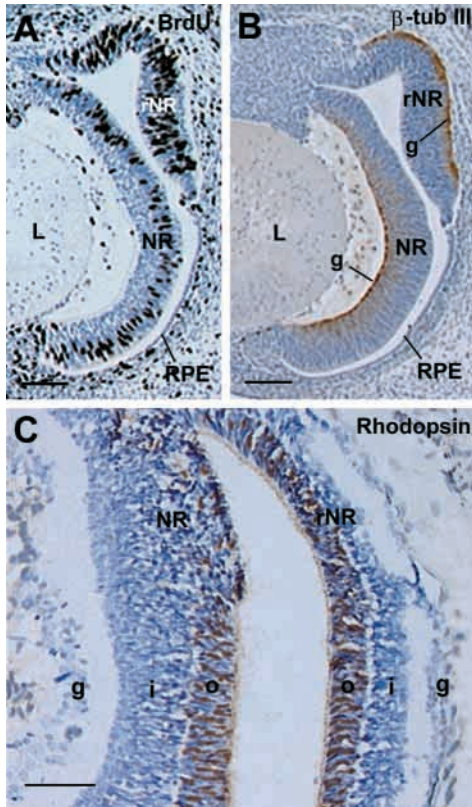
Fig. 3. Neural patterning in OVE1070 transgenic eyes. In E15.5 OVE1070 embryos, BrdU incorporation was detected in a high percentage of the cells in the RPE-derived neural retina (rNR), analogous to the original neural retina (NR) (A). Very few cells in the unaffected ventral RPE were actively proliferating (A). Immunohistochemistry with antibody to neuron-specific β-tubulin III showed that retinal ganglion cells (g) had been generated in both the NR and rNR at E15.5 (B). The tissue morphology and the absence of ganglion cells in the anterior margin between NR and rNR indicate that this tissue was not converted to neural retina. At postnatal day 6 (C), both the NR and rNR became laminated and formed three distinct cellular layers referred to as the ganglion cell layer (g), the inner nuclear layer (i) and the outer nuclear layer (o). Immunohistochemistry with antibody to rhodopsin showed that photoreceptors were born and located basally in both retinae (C). Abbreviations: L, lens; Scale bars: 50 μm in C; 100 μm in A,B.

nuclear layer and an outer nuclear layer (Fig. 3C). By postnatal day 6, immunohistochemistry using an antibody to rhodopsin confirmed the presence of differentiating rod photoreceptors in both the endogenous and RPE-derived retinae (Fig. 3C). Together, these results indicate that the rNR develops in a manner similar to that of the normal neural retina, with comparable cell proliferation, differentiation and lamination, but a mirror-image polarity.

In order to confirm that the altered RPE differentiation in family OVE1070 is truly due to ectopic FGF9 expression and to test whether the ventral part of the presumptive RPE can also respond to FGF9 stimulation, we intentionally targeted FGF9 expression to the presumptive RPE using the TRP2 promoter (Zhao and Overbeek, 1999). In these mice, the entire presumptive RPE is affected (Zhao and Overbeek, 1999).

Altered expression of transcription factors

To further characterize the molecular changes that accompanied the phenotypic switch of the RPE in OVE1070 mice, we examined, by in situ hybridization, expression of a panel of transcription factors implicated in eye development. Homeobox transcription factors Rax (Rx) and Chx10, and basic helix-loop-helix (bHLH) transcription factor Atoh7 (Math5) are required for normal eye development and/or retinal neurogenesis (Furukawa et al., 1997; Mathers et al., 1997; Liu et al., 1994; Burmeister et al., 1996; Kanekar et al., 1997; Brown et al., 1998). *Rax* (*Rx*), *Chx10* and *Atoh7* (*Math5*) are normally expressed in the neural retina but not in the RPE in developing eyes (Fig. 4A,C,G). In OVE1070 transgenic embryos, *Rax* (*Rx*), *Chx10* and *Atoh7* (*Math5*) are ectopically expressed in the rNR (Fig. 4B,D,H). The level of *Rax* (*Rx*) mRNA in the ectopic neural retina is similar to that in the endogenous neural retina while the level of *Chx10* expression is lower in the rNR. *Atoh7* (*Math5*) expression in the endogenous neural retina of the transgenic mice appears to be reduced (Fig. 4H).

The paired-box transcription factor Pax6 is also required for normal eye development (Hill et al., 1991; Walther and Gruss, 1991; Grindley et al., 1995). In wild-type embryos, *Pax6* is expressed in the lens epithelium, in the neural retina, and in the anterior region of the RPE (white arrowheads in Fig. 4E) but not in the posterior RPE (yellow arrowheads in Fig. 4E). In OVE1070 embryos, *Pax6* expression is activated throughout the presumptive RPE (Fig. 4F). *Pax6* expression in the lens epithelial cells is turned off (Fig. 4F) when these cells prematurely differentiate into fiber cells (Lovicu and Overbeek, 1998).

The bHLH zipper protein MITF is expressed in pigmented cells and is important for their differentiation and function (Hodgkinson et al., 1993; Nakayama et al., 1998). In normal embryonic eyes, *Mitf* transcripts are detected in the RPE (Fig. 4I). In OVE1070 embryos, *Mitf* mRNA levels per cell in the dorsal RPE were reduced compared with the unaltered ventral region (Fig. 4J), indicating that decreased MITF expression accompanies the switch in the differentiation program.

Ras-mediated induction of ectopic neural retina

FGF receptors are receptor tyrosine kinases (RTKs) that can activate different signal transduction pathways upon binding by FGF ligands. One of the RTK signaling pathways is the Ras-mediated Raf-Mek-mitogen-activated protein kinase (MAPK) pathway (Szebebyi and Fallon, 1999). To determine whether the conversion of the presumptive RPE to neural retina might be mediated by Ras activation, we generated transgenic embryos expressing an activated human *RAS* oncogene (*ras*T24) (Taparowsky et al., 1982; Capon et al., 1983; Schaffner et al., 1993) under the direction of the TRP2 promoter. In E16.5 transgenic embryos, the presumptive RPE was converted to a second layer of neural retina (Fig. 5A), analogous to the TRP2-FGF9 results (Zhao and Overbeek, 1999). To rule out the possibility that the action of *ras*T24 was simply to stimulate cell proliferation, we carried out immunohistochemistry using the anti-β-tubulin III antibody. Positive fluorescent labeling indicated that ganglion cells were generated in the ectopic neural retina (rNR) as well as in the endogenous neural retina (Fig. 5B). The rNR again developed
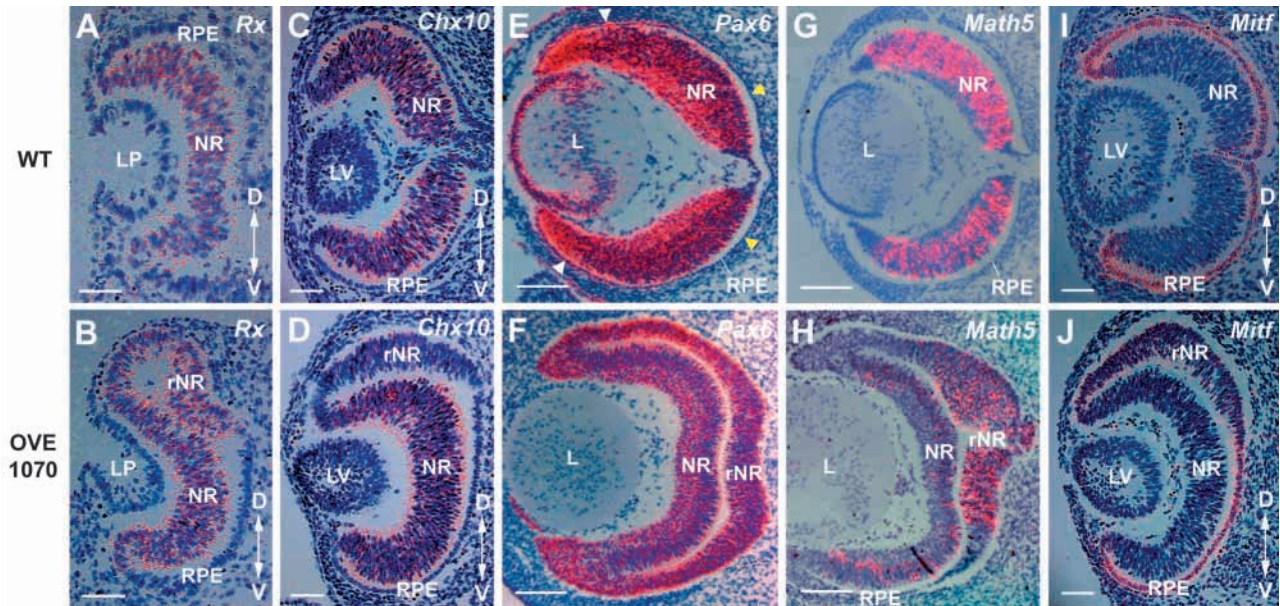
Fig. 4. Expression of retinal transcription factors in wild-type (top panels) and in OVE1070 transgenic (bottom panels) embryos. In situ hybridization was performed to assay for expression of *Rax* (*Rx*) at E10.5 (A,B), *Chx10* at E11.5 (C,D), *Pax6* (E, F) and *Atoh7* (*Math5*) (G,H) at E13.5, and *Mitf* at E11.5 (I,J). Expression of neuronal genes (*Rx*, *Chx10*, *Pax6* and *Math5*) was activated in the RPE-derived neural retina (rNR), while *Mitf* expression was downregulated. In wild-type eyes (E), *Pax6* was expressed in the lens epithelium and neural retina, and in the anterior (white arrowheads) but not in the posterior region (yellow arrowheads) of the RPE. D-V, dorsal-ventral orientation; L, lens; LP, lens pit; L, lens vesicle. Scale bars: 50 µm in A-D,I,J; 100 µm in E-H.

with a mirror-image polarity relative to the endogenous neural retina. BrdU incorporation showed that a large number of cells were actively proliferating in both neural retinae (Fig. 5C). These results suggest that the effect of FGF9 on the presumptive RPE is to activate a Ras-dependent signal transduction pathway, resulting in commitment to neuronal differentiation.

Expression of endogenous *Fgf9*

To assess whether endogenous FGF9 might play a role during normal mouse eye development, we carried out in situ hybridization to examine the pattern of endogenous *Fgf9*

expression at early stages of ocular development (Fig. 2E, F). *Fgf9* expression is not detectable in the developing eye at E9.5 (data not shown). By E10, *Fgf9* is expressed in the distal region of the optic vesicle as it invaginates to form the neural retina (Fig. 2E). *Fgf9* is also expressed in the ventral (yellow arrow) but not dorsal (yellow arrowhead) region of the optic stalk (Fig. 2E). No expression was detected in the prospective RPE. *Fgf9* expression persisted in neural retina of E11.5 embryos (Fig. 2F). *Fgf9* expression in the embryonic mouse retina has also been reported in an independent study (Colvin et al., 1999). The pattern of *Fgf9* expression suggests that it may be involved in neuronal differentiation.

Fig. 5. Induction of neural retina from the presumptive RPE by TRP2-rasT24. Hematoxylin/Eosin staining of an E16.5 transgenic embryo showed that nearly the entire presumptive RPE was converted to a second neural retina (rNR) (A). Antibody labeling (green fluorescence) using anti-β-tubulin III indicated that ganglion cells had differentiated in both the endogenous neural retina (NR) and rNR (B, boxed region in A). BrdU incorporation showed that numerous cells in the rNR were actively proliferating, analogous to the cells in the original neural retina (NR) (C). Postmitotic ganglion cells (g) were readily visible in the NR, but not as prevalent in the rNR (red arrowhead). Blood cells (arrows in B,C) showed autofluorescence. L, lens. Scale bars: 50 µm in B,C; 100 µm in A.
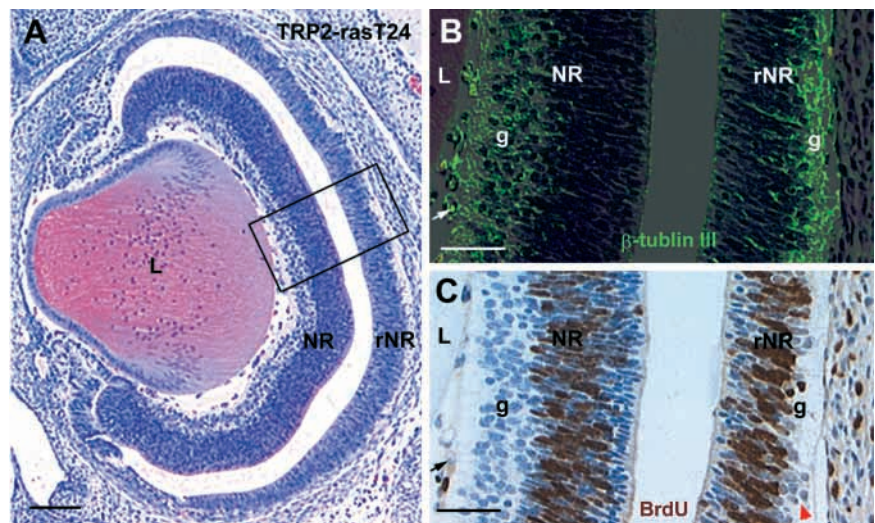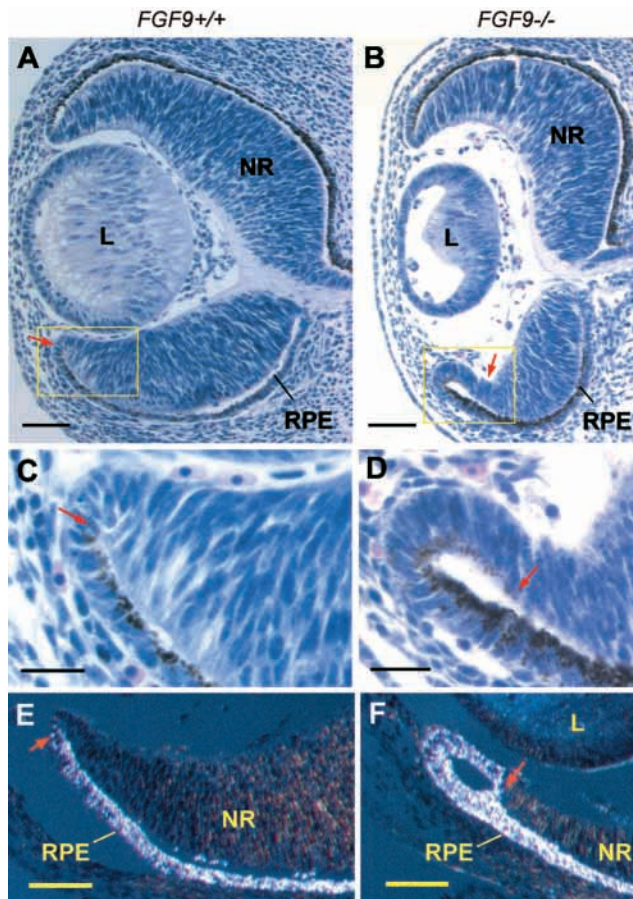
Fig. 6. Expansion of RPE into the presumptive neural retina in
Fgf9⁻/⁻ embryos. In wild-type E13.5 eyes (A,C), the neural retina
(NR) is morphologically distinguished from the RPE. The anterior
boundary of the RPE (arrow) is clearly defined by the shape of the
cells and the presence of pigment granules. In E13.5 *Fgf9⁻/⁻*
embryos, the anterior border of the ventral neural retina is
consistently affected (B,D). The RPE extends around the anterior
margin and occupies a small zone on the inner layer of the optic cup
(arrows in B,D). High-magnification images of the boxed regions in
A,B are shown in C,D, respectively. Dark-field images of the wild-
type (E) and mutant (F) eyes clearly show extension of pigmentation
around the anterior margin in *Fgf9⁻/⁻* embryos. Scale bars: 60 µm in
A,B; 20 µm in C,D; 40 µm in E,F.

FGF9-null mutant embryos

To determine whether FGF9 is required for normal neural
patterning in the optic vesicle, we analyzed the eyes of *Fgf9*
knockout embryos. In all the E13.5 *Fgf9⁻/⁻* embryos analyzed
(*n*=6), the anterior margins of the ventral and nasal neural
retina were abnormal (Fig. 6). The anterior RPE boundary
appeared to have shifted into the presumptive neural retina
(compare arrows in Fig. 6A and 6B). Pigmentation extends
into the presumptive neural retina in the *Fgf9⁻/⁻* embryos but
not in the control eyes (Fig. 6C-F). These results indicate that
endogenous FGF9 is not required for initial induction of
neural retina but its expression helps to define the boundary
of the neural retina. In some of the *Fgf9⁻/⁻* embryos, lens
fiber cells appear underdeveloped (Fig. 6B), suggesting that
FGF9 may also stimulate differentiation of primary lens fiber
cells.

DISCUSSION

Autonomous regulation of retinal differentiation

The present study demonstrates that transient expression of an
FGF (FGF9) and transient activation of Ras (less than 48
hours) are sufficient not only to alter the differentiation
pathway of the presumptive RPE but to initiate nearly all
aspects of retinal differentiation. Not only does this process
involve downregulation of RPE-specific markers (Fig. 1, Fig.
4J), but the newly differentiated neural tissue exhibits
molecular characteristics of the normal neural retina (Fig. 3,
Fig. 4). In situ hybridization shows that expression of the *Fgf9*
transgene in the presumptive RPE is turned off by E12.5 in
family OVE1070 (Fig. 2A-C). Nevertheless, the dorsal RPE
continues to develop as neural retina, indicating that sustained
FGF9 stimulation is not necessary for retinal development.
Further evidence that a transient signal is sufficient to switch
the RPE differentiation pathway comes from the transgenic
mice that express FGF9 or activated Ras under the control
of the TRP2 promoter. These findings are consistent with
an earlier in vitro study showing that FGF2-induced
transdifferentiation of rat RPE to neural retina continued even
after FGF2 was removed from the culture medium (Zhao et al.,
1995). These observations suggest that after an initial induction
signal is received, the neural retina develops and differentiates
autonomously.

Previous in vitro studies have shown that cultured retinal
explants or RPE-derived neural retina can give rise to a variety
of differentiated neural retinal cells (Sparrow et al., 1990; Zhao
et al., 1995). The present study demonstrates that neural retinal
cells can differentiate, establish polarity and laminate in vivo
in the absence of RPE. At postnatal stages, both the original
and RPE-derived neural retinae in our transgenic mice were
thinner than the wild-type neural retina (data not shown).
Therefore, although RPE is not required for initial retinal cell
differentiation and lamination in the mouse, it is essential for
photoreceptor maturation and/or survival.

MITF in RPE differentiation

The bHLH transcription factor MITF is encoded by a gene
located at the microphthalmia (*mi*) locus (Hodgkinson et al.,
1993). MITF is important for differentiation of the neural
crest-derived melanocytes and the neuroepithelium-derived
RPE (Steingrimsson et al., 1994; Nakayama et al., 1998).
Developmental defects in mice with mutations at the *mi* locus
include loss of pigmentation and reduced eye size. In Japanese
quail with the *silver* mutation, defects reminiscent of *mi* mutant
mice are observed in neural crest-derived and ocular tissues
(Araki et al., 1998; Mochii et al., 1998). One of the *silver*
abnormalities is the formation of a second neural retina from
the presumptive RPE. It has also been reported recently that a
second neural retina is formed from the dorsal presumptive
RPE in a strain of *mi* mutant mice (Bumsted and Barnstable,
2000). These observations have demonstrated that MITF is
essential for normal RPE differentiation and loss of its activity
can result in adoption of a neural retinal cell fate by a subset
of the presumptive RPE cells. In transgenic embryos from
family OVE1070, expression of *Mitf* was downregulated but
not completely turned off in the RPE region exposed to FGF9
(Fig. 4J). This may help explain an earlier observation that FGF
stimulation is required for a certain period of time before the

conversion of RPE to neural retina becomes irreversible in culture (Zhao et al., 1995). The remaining MITF in OVE1070 transgenic embryos might also be responsible for the lower level of *Chx10* expression in the FGF9-stimulated RPE (Fig. 4D). It is possible that FGF stimulation may not directly block *Mitf* expression, but may instead interfere with the transcriptional function of the protein.

FGF9 in eye development

The gain-of-function and loss-of-function studies with FGF9 in mouse eyes, taken together with the timing and localization of endogenous FGF9 expression in the optic vesicle, indicate an autocrine/paracrine function for this molecule in neural patterning during eye development. FGF9 is unlikely to be the initial inducer of neural retina because the onset of its expression at E10 is too late for initial induction. Neural retinal markers such as Chx10 are already expressed in the prospective neural retina by E9.5 (Liu et al., 1994). Therefore, upregulation of FGF9 appears to be a downstream response to the initial inductive signal. FGF9 appears to participate in fine-tuning the boundary between the neural retina and the RPE.

We show that endogenous FGF9 is expressed in the prospective neural retina in the optic vesicle and optic cup during normal eye development (Fig. 2E,F). As FGF9 has been shown to be a secreted protein in previous studies (Miyamoto et al., 1993; Lovicu and Overbeek, 1998) and in this study (Fig. 2D), this raises the question of why the endogenously expressed FGF9 in the neural retina has no effect on RPE differentiation during normal eye development. It is known that the high affinity of FGFs for extracellular matrix, particularly heparan sulfate proteoglycans, can severely limit their diffusion in interstitial spaces. As a result, FGFs often exert their effects very close to their site of production (Ornitz, 2000). Expression of the *Fgf9* transgene in OVE1070 transgenic embryos co-localizes with the phenotypic change in the presumptive RPE, suggesting that no significant diffusion of transgenic protein occurs (Fig. 2A,B). We also found that mRNA levels of endogenous *Fgf9* in the prospective neural retina were quite low. This may further help to limit diffusion of FGF9 protein in vivo. Even if some FGF9 protein did manage to diffuse into the open space in the optic vesicle, its concentration might be too dilute to affect RPE differentiation (Fig. 2E). By the time when the neural retina comes into close contact with the RPE (Fig. 2F), the RPE may have further differentiated as pigmented cells and lost its responsiveness to FGF9. A previous in vitro study (Zhao et al., 1995) demonstrated that there was a narrow window of time during which early rat RPE could respond to FGF signal and become neural retina and that FGF stimulation had no apparent effect on the RPE at later stages. It is also possible that FGF9 is a cell surface protein, rather than secreted one. Alternatively, endogenous FGF9 may be secreted only basally by retinal cells, i.e. into the vitreous, so the RPE is not exposed to the FGF signal.

Role for Ras in retinal induction

Signaling pathways downstream of FGF receptors can be Ras dependent or Ras independent (Szebenyi and Fallon, 1999). For example, FGF-stimulated neurite growth is Ras independent and is mediated by factors such as phospholipase Cγ and diacylglycerol lipase (Doherty and Walsh, 1996; Hall

et al., 1996). Our data suggest that the switch of RPE differentiation to a neuronal fate by FGF9 stimulation is mediated by the Ras-Raf-MAPK pathway. Transient activation of Ras is sufficient to initiate a full neuronal differentiation program in the presumptive RPE.

During gastrulation in vertebrates, the embryonic ectoderm gives rise to epidermal progenitors on the ventral side and neural progenitors on the dorsal side. Studies with *Xenopus* embryos have shown that bone morphogenetic proteins (BMPs) induce epidermis and inhibit neural differentiation (Hemmati-Brivanlou and Melton, 1997). We hypothesize that a similar mechanism may also apply to cell fate determination within the optic vesicle neuroepithelium. During ocular development, several members of the BMP family including BMP4 and BMP7 are expressed in the RPE and prospective iris and ciliary epithelia (Dudley and Robertson, 1997), suggesting that BMPs could provide the signals to specify optic vesicle to become pigmented epithelium. Unfortunately, targeted mutagenesis of *Bmp4* or *Bmp7* in mice has not shed light on this because of early embryonic lethality (Dunn et al., 1997) or degeneration of early ocular structures (Dudley et al., 1995; Luo et al., 1995). FGF9 may antagonize the BMP signaling and maintain the neuronal differentiation pathway in the prospective neural retina. FGFs have been shown to oppose the effects of BMP2 and BMP4 during limb development (Niswander and Martin, 1993; Ganan et al., 1996). It is possible that FGF-initiated RTK/MAPK signaling blocks BMP function by preventing nuclear translocation of Smad protein(s) (Kretzschmar et al., 1997).

In summary, we show that FGF9 signaling is sufficient but not essential for the induction of neural retina. Transient activation of Ras is also sufficient to induce neural retina, suggesting that the endogenous inductive signal activates a Ras-dependent signaling pathway and that Ras activation by factors other than FGFs can also induce the switch in developmental fate of the RPE. Once initiated, the program for retinal differentiation and lamination appears to proceed autonomously. Based on the mirror-image polarity of the duplicate neural retina, polarizing signals must be provided by the apical-basal asymmetry of the neuroectoderm, rather than by external signals from surrounding tissues. Endogenous FGF9 may work in conjunction with BMPs to define the precise boundary between neural and non-neural tissues in the developing eye.

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