Signaling and transcriptional regulation in early mammalian eye development: a link between FGF and MITF

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Accepted 27 May; published on WWW 20 July 2000

SUMMARY
During vertebrate eye development, the optic vesicle is partitioned into a domain at its distal tip that will give rise to the neuroretina, and another at its proximal base that will give rise to the pigmented epithelium. Both domains are initially bipotential, each capable of giving rise to either neuroretina or pigmented epithelium. The partitioning depends on extrinsic signals, notably fibroblast growth factors, which emanate from the overlying surface ectoderm and induce the adjacent neuroepithelium to assume the neuroretinal fate. Using explant cultures of mouse optic vesicles, we demonstrate that bipotentiality of the optic neuroepithelium is associated with the initial coexpression of the basic-helix-loop-helix-zipper transcription factor MITF, which is later needed solely in the pigmented epithelium, and a set of distinct transcription factors that become restricted to the neuroretina. Implantation of fibroblast growth factor-coated beads close to the base of the optic vesicle leads to a rapid downregulation of MITF and the development of an epithelium that, by morphology, gene expression, and lack of pigmentation, resembles the future neuroretina. Conversely, the removal of the surface ectoderm results in the maintenance of MITF in the distal optic epithelium, lack of expression of the neuroretinal-specific CHX10 transcription factor, and conversion of this epithelium into a pigmented monolayer. This phenomenon can be prevented by the application of fibroblast growth factor alone. In Mitf mutant embryos, parts of the future pigment epithelium become thickened, lose expression of a number of pigment epithelium transcription factors, gain expression of neuroretinal transcription factors, and eventually transdifferentiate into a laminated second retina. The results support the view that the bipotent optic neuroepithelium is characterized by overlapping gene expression patterns and that selective gene repression, brought about by local extrinsic signals, leads to the separation into discrete expression domains and, hence, to domain specification.

Key words: Retinal pigment epithelium, Neuroretina, Neuroepithelium, Optic vesicle culture, Transcription factor, Transcriptional repression, Mouse, Cell-fate specification

INTRODUCTION
The development of the vertebrate eye, much as that of many other organs, is the result of complex, reciprocal interactions between tissues of distinct embryological origin. The lens, for instance, is derived from the surface ectoderm that overlies the optic vesicle, but without the juxtaposition of a normal optic vesicle lens development is perturbed (Spemann, 1901; Lewis, 1904; Li et al., 1994; Porter et al., 1997; Furuta and Hogan, 1998; Kamachi et al., 1998). Conversely, without a proper lens placode the optic vesicle remains rudimentary, and an optic cup with a well-defined neuroretinal layer and a pigmented layer does not form (Holtfreter, 1939; Hyer et al., 1998). Even though these tissue interactions have long been noted and characterized extensively, it remains a challenge to sort out the molecular mechanisms responsible for them. In particular, much needs to be learned about the nature of the relevant signaling molecules and how the cells interpret them to develop into the eye’s many distinct structures.

Here we focus on the factors that specify the neuroretina (NR) and the retinal pigment epithelium (RPE). These two tissues, though distinct in form and function, have a common precursor, the cells of the anterior neuroepithelium that become specified in an eye field. Normally, the epithelium forms an NR in the distal (inner) layer of the eye cup and an RPE in the proximal (outer) layer. However, there is a species-specific period during which the fates of these two tissues remain reversible. For instance, when in amphibians the eye is experimentally inverted so that the outer layer comes to lie adjacent to the lens primordium, this outer layer may develop into a complete retina rather than an RPE (Mikami, 1939). These and many other observations (Pitack et al., 1997; Hyer et al., 1998) suggest that the neuroepithelium of the optic vesicle is initially bipotential and that its separation into NR and RPE domains is mediated by extrinsic factors that emanate from the surface ectoderm.

Among the factors potentially responsible for this effect, fibroblast growth factors (FGFs) are prime candidates. Both
FGF1 and FGF2 are expressed in the surface ectoderm (de Longh and McAvoy, 1993; Pittack et al., 1997) and their receptors, FGFR1 and FGFR2, are expressed in the developing optic vesicle (Wanaka et al., 1991; Tcheng et al., 1994). RPE, when exposed to FGF2, develops into NR (Park and Hollenberg, 1989; Pittack et al., 1991, 1997; Guillemot and Cepko, 1992; Zhao et al., 1995), and FGF1 is involved in early optic vesicle patterning in the chick (Hyer et al., 1998). Furthermore, FGFs have well-established roles in patterning of other tissues in both invertebrates and vertebrates. Whether FGFs play similar roles in the patterning of the mammalian optic vesicle, however, remains unknown. Neither FGF1 or FGF2 knockout mice nor FGF1/FGF2 double knockout mice have overt eye defects (Dono et al., 1998; Ortega et al., 1998; Miller et al., 2000). Thus, in the mouse, FGFs may either have redundant functions or may collectively be unimportant for cell-fate decisions in the optic vesicle.

Much as determination of the nature of the relevant signaling molecules is crucial to our understanding of eye development, so is identification of the cell-intrinsic factors, including particular transcription factors, through which these signaling molecules may act. A multitude of transcription factors specifically involved in the various developmental steps of eye formation have recently been identified from spontaneous and induced mutations, predominantly in mice. For instance, the homeodomain protein RX is needed for optic vesicle formation (Mathers et al., 1997), the lim-homeodomain protein LHX2 (Porter et al., 1997) and the paired-domain protein PAX6 (Hill et al., 1991) are needed for optic cup formation, and the homeodomain protein CHX10 controls the growth of the NR and the differentiation of retinal interneurons (Burmeister et al., 1996). Based on analyses in dissociated cultures, several transcription factors, including MITF on which the present study focuses, have been implicated in cell-fate decisions in cultures of dissociated optic cups (Mochii et al., 1998a; Holme et al., 2000), but none so far has been shown to regulate the separation into NR and RPE in the intact optic vesicle.

We have recently demonstrated that the basic-helix-loop-helix-zipper (bHLH-Zip) transcription factor MITF (Hodgkinson et al., 1993; Hughes et al., 1993), in addition to regulating the development of neural crest-derived melanocytes, also controls the development of the RPE. Such a role is not surprising given Mitf’s prominent expression in the RPE (Hodgkinson et al., 1993; Nakayama et al., 1998). In fact, when rendered non-functional in embryos with microphthalmia mutations, the RPE hyperproliferates, remains unpigmented and displays areas developing into a second retina (Packer, 1967; Scholtz and Chan, 1987; Mochii et al., 1998b; Nakayama et al., 1998; Bumsted and Barnstable, 2000). Intriguingly, in the mouse, Mitf mRNA expression starts in the entire optic vesicle and thus precedes the separation into presumptive NR and RPE; only later is its expression restricted to the presumptive RPE (Nguyen et al., 1997; Bora et al., 1998; Nakayama et al., 1998). It is likely, therefore, that the progressive restriction of Mitf expression to the future RPE is instrumental for the separation of the mammalian optic vesicle into NR and RPE.

In the present paper we use wild-type and Mitf mutant mouse embryos and explant cultures of optic vesicles to demonstrate that FGFs from the surface ectoderm partition the neuroepithelium into NR and RPE, and that they do so by regulating the expression of Mitf. The results suggest a model in which the initial coexpression of transcription factors marks undifferentiated, bipotential neuroepithelial precursor cells, and the region-specific repression of distinct transcription factors leads to lineage commitment.

**MATERIALS AND METHODS**

**Mice and harvest of embryos**

Embryos were harvested at E9.5 in Hepes-buffered DMEM supplemented with 10% fetal bovine serum (FBS). The head bands containing the optic vesicles were dissected and cultured in 10% FBS-supplemented UltraCulture medium (BioWhittaker) on floating 0.45 µm Nucleopore membranes (Corning #110607) coated with 10 ng/ml poly-D-Lysine (Sigma). Heparin-coated acrylic beads (125-200 µm diameter, Sigma H5263) were soaked in 5 µl of 1 mg/ml of human recombinant FGF1, FGF2, murine recombinant FGF (all from Gibco) or bovine serum albumin (BSA; fraction V, Sigma) for 1 hour (Niswander et al., 1993) and implanted into specific locations around the optic vesicle, as indicated in the text. Removal of the surface ectoderm was done as described (Furuta et al., 1998). For immunolabeling, the cultured explants were fixed in 4% paraformaldehyde overnight, cryoprotected in sucrose, embedded in OCT compound and sectioned at 14°C.

**Tissue preparations for histological analysis and in situ hybridization**

Embryo preparations, immunolabeling and in situ hybridizations were done as described (Hodgkinson et al., 1993; Chan et al., 1995; Nakayama et al., 1998). The following mouse probes, all described previously, were used: Mitf (Hodgkinson et al., 1993), Dct (Opdecamp et al., 1997), Chx10 (Liu et al., 1994), Crx (Furukawa et al., 1997), Six3 (Oliver et al., 1995), Pax6 (Walther and Gruss, 1991). The templates for Otx2 and FGF15 are EST cDNA clones (accession numbers AA199520 and AA051675, respectively) obtained from Genome Systems Inc. and confirmed by sequencing.

**Antibodies and immunohistochemistry**

The following antibodies were used: for horizontal cells, NF160 (Sigma); for amacrine cells, syntaxin, VC1.1 (Sigma); for bipolar cells, anti-Chx10 (R. McClunes); for rod outer segments K42-41c; for rod outer and inner segments, R2-15 and R2-12 (Röhllich et al., 1989) (P. Hargrave). Rabbit antiserum to chicken PAX6 (cross-reacting with mouse PAX6) was a gift from S. Saule. Monospecific Anti-FGF1 and anti-FGF2 rabbit sera were from R&D, and rabbit anti-calretinin serum was from Chemicon. Rabbit anti-DCT was a gift of V. Hearing. All primary antibodies were used at 1:100 dilution in 10% normal goat serum. Secondary antibodies were appropriate FITC-conjugated affinity-purified goat immunoglobulins (Sigma). Sections were viewed using a Reichert-Jung Polyvar microscope equipped for epiphluorescence and differential interference contrast and photographic images were processed digitally.

**Dil labeling**

For cell labeling in optic vesicle cultures, DiI crystals (D3911, 3582 M.-T. T. Nguyen and H. Arnheiter
RESULTS

Expression of MITF and FGF during early eye development

We first determined the expression patterns of MITF RNA and protein during early eye development. Fig. 1A shows that initially, at the 22-somite stage, wild-type MITF RNA was expressed throughout the optic vesicle (OV). Shortly thereafter (Fig. 1B, 26-somite stage), its levels were reduced in the distal optic vesicle (dOV) adjacent to the surface ectoderm (SE) but maintained in the proximal optic vesicle (pOV). This differential expression pattern was further accentuated at the cup stage (Fig. 1C) where the future NR did not express MITF while the future RPE did. Protein expression patterns faithfully tracked RNA expression (Fig. 1D-F). In optic vesicles of Mitf null embryos (Mitf<sup>vga</sup>-<sup>9/vga</sup>-<sup>9</sup>), no immunofluorescent label was detected, indicating that the antiserum was specific (Fig. 1G). In Mitf<sup>mi-ew/mi-ew</sup> embryos, which synthesize an MITF protein deficient in DNA binding, the shift from expression in the entire optic vesicle to the presumptive RPE was as in wild type, indicating that the mechanisms regulating MITF expression remained intact (Fig. 1H-I). Even though a cup was formed, however, it was missshapen, and the mutant protein, rather than being concentrated in the cell nuclei, filled both nuclei and cytoplasm as observed previously in the RPE at E14.5 (Nakayama et al., 1998). In addition, some areas in the dorsal part appeared thickened and displayed reduced levels of MITF (Fig. 1I, arrow, see below).

We then labeled similar sections for FGF1 and FGF2 protein (Fig. 2A,B). The antibodies used in these studies recognize these proteins selectively and gave low level staining in the entire vesicle area. However, the surface ectoderm overlying the optic vesicle, though not the more dorsal and ventral surface ectoderm, was particularly intensely stained. Similar expression patterns have been seen for FGF2 in chick (Pittack et al., 1997) and FGF1 in rat (de Longh and McAvoy, 1993). In addition, in situ hybridizations (not shown) confirmed previous observations (McWhirter et al., 1997) that another member of the FGF family, FGF15, is expressed in the distal optic vesicle beginning at E9.5. Thus, several FGFs are expressed in the surface ectoderm or the distal optic vesicle and thus at the right time and place to potentially play a role in MITF regulation and optic vesicle patterning.

**FGFs downregulate MITF in the presumptive RPE**

To test the potential role of FGFs in downregulating MITF, we dissected the head portions of E9.5 embryos and established explant cultures that retained the morphology of the budding optic vesicles. Over a period of 3 days, each culture formed two optic cups, which became pigmented in their proximal parts (Fig. 3A,C,E). Immunolabeling of cryosections of these cultures showed that the eye cups were composed of an MITF-negative lens vesicle (lv, Fig. 3B) that later detached from the surface ectoderm to form a lens (l, Fig. 3D,F), an MITF-positive layer reminiscent of RPE, and an MITF-negative layer reminiscent of NR (Fig. 3B,D,F). Pigmentation of the RPE started at 2 days and became prominent at 3 days. At 2 days, the NR layer became weakly positive for calretinin, a neuronal marker (not shown). Staining for PAX6 (NR and RPE) and CHX10 (NR) (see Figs 4 and 5) confirmed previous in vivo observations (Li et al., 1994; Liu et al., 1994).
With this system in hand, we then implanted growth factor-coated acrylic beads immediately after establishing the cultures. The beads were placed close to the area where the outer MITF-positive layer would normally develop and were left in place until the cultures were harvested 1-3 days later. In all of 21 vesicles given beads coated with BSA and 21 of 22 vesicles given beads coated with EGF, development of a pigmented layer was not perturbed (a typical example is shown in Fig. 4A, arrow). With FGF1-coated beads, however, 16 of 18 bead-exposed vesicles showed no pigmentation, and with FGF2-coated beads, 25 of 29 remained unpigmented (a typical example is shown in Fig. 4B, arrow).

To test whether the lack of pigmentation was correlated with a disturbance in gene expression, the cultures were harvested at various time points, cryosectioned, and the sections stained for MITF, PAX6 and CHX10 protein. As shown in Fig. 4C for a culture implanted with a control bead and harvested at 3 days, MITF staining was, as expected, restricted to the RPE layer, PAX6 staining (Fig. 4E) was seen in the surface ectoderm, lens vesicle, NR and RPE, and CHX10 staining was found only in the NR (Fig. 4G). In contrast, when the bead was coated with FGF1, there was no MITF staining and the RPE layer became thickened, resembling the NR layer in appearance and thickness (Fig. 4D). Both NR and RPE layer were positive for PAX6 (Fig. 4F) and CHX10 (Fig. 4H). These results suggest that in the presumptive RPE, FGFs downregulate MITF, upregulate CHX10, and lead to increased cell numbers, thus allowing the RPE to initiate differentiation along the neuroretinal pathway.

**Removal of the surface ectoderm leads to inversion of the optic vesicle**

If exposure to FGFs downregulates MITF expression, upregulates CHX10 expression, and eliminates pigmentation in the presumptive RPE, then the removal of the surface ectoderm, i.e. the removal of a physiological source of FGF along with any other signals emanating from this part of the ectoderm, should have the opposite effects. We thus removed the surface ectoderm by a combination of enzymatic and physical means from E9 (20-22 somite stage) wild-type embryos. This time point marks a stage at which there is no direct contact between optic vesicle and surface ectoderm. The procedure left the optic vesicle intact (compare Fig. 5A with B) and allowed for pigmentation to develop over a 3-day period (Fig. 5C-E, left side) but had the consequence that no lens vesicle and no optic cup were formed. Immunolabeling showed that the PAX6 positive surface ectoderm had been removed entirely without damage to the distal layer (Fig. 5F,K,P). The distal layer soon became covered with unlabeled mesenchymal cells from the surrounding tissue (see for instance Fig. 5G, arrow). More importantly, the distal layer, which over 2-3 days should have developed into the MITF-negative presumptive NR and should have stayed unpigmented, remained a single cell layer, retained expression of MITF (Fig. 5G-I), and starting at day 2 became pigmented (Fig. 5H,I). This layer remained positive for PAX6 (Fig. 5K-N) but soon lost the initial staining for CHX10 (compare Fig. 5P with Q-S). Thus, rather than developing into NR, the distal layer switched its fate to that of RPE.

**Fig. 2.** FGF protein expression in the mouse embryonic optic vesicle area (wild type, E9.5). (A) FGF1, (B) FGF-2. Note predominant expression in the surface ectoderm overlying the optic vesicle. Bar, 50 μm.

**Fig. 3.** Development of pigmented eye structures in embryonic explant cultures. The head bands of wild-type embryos were harvested at E9.5 and cultured for 1 day (A,B), 2 days (C,D) or 3 days (E,F). (A,C,E) View in dissection scope; (B,D,F) cryostat sections immunostained for MITF protein (green). Arrows in A mark lens placodes, and arrowheads in A,C and E the progressively pigmented optic cup. Note similarities to the in vivo situation (Fig. 1) in morphology, MITF expression, and RPE pigmentation. SE, surface ectoderm; lv, lens vesicle; l, lens; NR neuroretina; RPE, retinal pigment epithelium. Bar, 50 μm (B,D,F).
Interestingly, during this time period, the normally cell-free space in the vesicle’s interior became progressively filled with MITF-negative, unpigmented cells (Fig. 5G-I). These cells were positive for PAX6 (Fig. 5L-N), suggesting they were contributed from the PAX6-positive proximal layer, and they also acquired CHX10 expression (Fig. 5Q-S). Serial sectioning of vesicles harvested over a 3-day time period confirmed that these cells resulted from progressive proliferations and infoldings of the proximal layer, i.e. the presumptive RPE. This interpretation was consistent with numerous DiI tracing experiments (examples of which are depicted in Fig. 6A,B), which suggested that the inner cells (x in Fig. 6A,B) are neither derived from the distal layer nor the surrounding mesenchyme nor from the more proximal neuroepithelium of the optic stalk and brain. Thus, by morphology and gene expression, these cells resembled an NR that was derived from the presumptive RPE and was localized on the wrong (proximal) side of the pigmented layer.

If the interpretation is correct that FGF signals from the surface ectoderm are thus responsible for optic vesicle patterning, then replacing the removed surface ectoderm with FGF alone should at least partially restore the development of the distal optic vesicle into presumptive NR. This was indeed the case. While BSA-coated control beads placed in front of an optic vesicle lacking a surface ectoderm did not inhibit the distal pigment formation (Fig. 5C, arrow), placing an FGF1- or FGF2-coated bead in this location resulted in a thickened distal layer that lacked MITF expression, retained PAX6 and CHX10 expression, and remained unpigmented (Fig. 5D,J,O,T; arrow indicates FGF2-coated bead). Not unexpectedly, however, simply replacing the removed surface ectoderm with a source of FGF did not restore the normal formation of a cup and did not allow the proximal layer to become pigmented. Interestingly, EGF-coated beads reduced distal pigmentation almost to the same extent as FGF (Fig. 5E, arrowheads) and led to changes in gene expression (not shown) similar to those observed with FGF. This result is different from those obtained with EGF-coated beads placed in the back of vesicles with intact surface ectoderm where pigmentation was not disturbed (see above). The difference in EGF response between the proximal layer that would go on to form the pigmented RPE in the presence of the surface ectoderm, and the distal layer that would become pigmented after removal of the surface ectoderm, suggests that the two layers, though each bipotential, are not entirely equivalent. This non-equivalence is reflected in the fact, for instance, that expression of CHX10 or FGF15 is restricted to the distal layer even before MITF downregulation effects the observed cell-fate switches.

Nevertheless, the two layers were similar with respect to the non-uniform intracellular distribution of the pigment granules. As is well known for the in vivo situation at the cup stage, RPE pigment granules are concentrated at the epithelium’s apical side, adjacent to the NR (see for instance, Fig. 4C). In the pigmented cells that

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**Fig. 4.** FGF downregulates MITF in RPE and interferes with pigmentation. Wild-type cultures were established as for Fig. 3, but single BSA- or FGF-coated beads were implanted at the beginning of the culture period proximal to the area of the optic vesicle. The cultures were harvested for cryosectioning at 3 days. (A,C,E,G) Implantation of BSA-coated control bead (arrows). (B,D,F,H) Implantation of FGF2-coated bead (arrows). (A,B) View in dissection scope. Note the presence of pigmentation on both implanted and unimplanted sides in A and on the unimplanted side but not on the side implanted with the FGF2-coated bead in B. (C,D) Staining for MITF. Note absence of MITF and relative thickening of both neuroretina (NR) and retinal pigment epithelium (RPE) in D compared to C. (E,F) Immunostaining for PAX6. Note PAX6 expression in surface ectoderm (SE), lens (l), NR and RPE in vesicles implanted with control or FGF2-coated beads. (G,H) Immunostaining for CHX10. Note staining in NR but not RPE in control and staining in both NR and RPE in FGF2 bead-implanted side. Bar, 50 μm (C-H).
were generated from the distal optic vesicle after surface ectoderm removal, the granules were still concentrated at the apical side (see for instance, Fig. 5I). This observation suggested that the basal-to-apical polarity of these pigmented cells was maintained irrespective of whether they were derived from the proximal optic vesicle, as is normally the case, or from its distal part, as was experimentally achieved.

Taken together, these results suggest that removing the

Fig. 5. After removal of the surface ectoderm, the distal optic vesicle maintains MITF expression, loses CHX10 expression, and becomes a pigmented monolayer. Wild-type cultures were established as for Fig. 3. The surface ectoderm was removed on both sides of the explants and in some cultures, growth factor-coated beads were implanted on one side (arrows in C-E,J,O,T) at the beginning of the culture period. (A-E) View in dissection scope. (A) Fresh explant, OV not manipulated. (B) Appearance immediately after removal of the surface ectoderm. (C) At 3 days, note pigment on both sides, including where a BSA-coated control bead had been implanted after surface ectoderm removal. (D) Note the presence of pigment on unimplanted side but near absence of pigment where an FGF2-coated bead had been implanted. (E) Note the presence of pigment on the unimplanted side but near absence of pigment where an EGF-coated bead had been implanted. (F-T) Immunolabeled cryosections. (F-I) Development of optic vesicle after surface ectoderm removal, labeled for MITF. Note the maintenance of MITF expression and development of pigmentation in distal optic vesicle, which becomes covered with unlabeled mesenchymal cells (arrow in G). (K-N) Similar sections, labeled for PAX6. Note PAX6-positive cells filling the originally cell-free space proximal to the distal layer. (P-S) Similar sections, labeled for CHX10. At 0 days, CHX10 is expressed in the distal layer but later is downregulated in this layer and upregulated in the PAX6-positive proximal cells. (J,O,T) Effect of FGF2-coated bead after surface ectoderm removal. FGF2 interferes with MITF expression (J), but PAX6 (O) and CHX10 (T) staining are seen in the entire optic vesicle. dOV, distal optic vesicle; pOV, proximal optic vesicle; me, mesenchyme. Bar, 50 μm (F-T).
Mitf mi

in embryos homozygous for the semidominant RPE proliferation is an early sign of RPE pathology, not only RPE. Furthermore, an RPE-to-retina transition was also achieved at physiological levels and may not readily result in the previously described semidominant Mitf alleles (see below).

To characterize the events in cell fate changes in Mitf mutant RPE in more detail, we analyzed in vivo RNA or protein expression of several marker genes at various stages of embryonic and postnatal development. Besides the markers used above in explant cultures (Mitf, Pax6 and Chx10), we included Otx2, which encodes a homeodomain transcription factor expressed in RPE (Bovolenta et al., 1997), Six3, the mammalian homolog of the Drosophila eye developmental gene sine oculis, which at E9.5 is expressed in the surface ectoderm and the entire optic vesicle but at E14.5 is restricted to the NR and lens (Oliver et al., 1995) and Crx, which is required for the differentiation of rod and cone photoreceptor cells and normally begins to be expressed at E12.5 in the neuroblast layer of the NR but is negative in RPE (Furukawa et al., 1997). For postnatal stages, we used markers for specific retinal interneurons such as amacrine, horizontal and bipolar cells, and antibodies labeling rods and, selectively, rod outer segments.

The results of the analysis in embryos are shown in Fig. 7. In the leftmost column, sections through wild-type eyes are shown for comparative NR labeling, not RPE labeling, since RPE labeling is obscured by pigmentation. However, the mutant eyes shown in the middle and rightmost column are unpigmented, and hence RPE labeling in these eyes is specific for the genes in question. The results, shown for either Mitf vga−9 or Mitf Mi-wh as indicated in Fig. 7, were confirmed for Mitf Mi-wh and can be summarized as follows. While the principle separation into a retinal and RPE layer was maintained in mutant, the RPE progressively thickened, particularly in the dorsal part. The first sign of abnormal gene expression was the downregulation of MITF in the dorsal part, which had started already at E10.5 (see Fig. 1I, arrow), became prominent at E12.5 (Fig. 7B), and was complete at E14.5 (Fig. 7C). Concomitantly, the thickened dorsal and proximal parts of the mutant RPE also lost expression of Otx2 (Fig. 7E,F), similar to observations recently made in E13 embryos homozygous for Mitf mi (Bumsted and Barnstable, 2000). Expression of DCT, an enzyme involved in pigment synthesis, was likewise lost (not shown). Of note, however, the areas of the RPE that escaped the dorsal thickening retained expression of all of these RPE markers, suggesting that functional MITF is not required for their expression. Expression of Pax6, which is normally progressively lost in wild-type RPE, stayed high throughout the mutant RPE, and the neuroretinal markers Six3, Chx10 and Crx, not found in wild-type RPE, were all expressed throughout the mutant RPE. These results suggested that the mutant RPE, particularly its thickened dorsal parts, initiates the differentiation along the NR fate.

To determine the further fate of the mutant RPE, we examined subsequent embryonic stages and labeled postnatal day (PN) 0-14 eyes for retinal interneurons including amacrine, horizontal and bipolar cells. The expanded dorsal region of the RPE remained easily identifiable and at PN 0 showed labeling for the three retinal cell types, as did the retina in wild type (Fig. 8A-C) and in mutant (Fig. 8D-F). In fact, the thickened RPE (labeled ‘second retina’ in Fig. 8D-F) appeared layered but the layers were topologically inverted with respect to those of the primary retina. This topological inversion was consistent with earlier observations in Mitf Mi-mi mutant mice (Scholtz and

surface ectoderm, and with it a source of FGFs, leads to the formation of an inverted optic vesicle whose distal part resembles RPE and whose proximal part resembles NR. The formation of a distal pigment layer can be prevented with just FGF alone.

**Mitf mutant RPE proliferates, expresses neuroretinal markers and transdifferentiates into a second retina**

While the above experiments showed that FGF signals from the surface ectoderm regulate MITF expression and affect cell fate decisions, they did not formally establish a causal link between the two events. The view that MITF is the crucial cell-intrinsic factor through which extrinsic signals work would be supported, though, if deliberate MITF expression, on its own, turned NR into RPE, or if elimination of MITF turned RPE into NR. Since ectopic MITF expression may not easily be achieved at physiological levels and may not readily result in a NR-to-RPE transition against the physiological action of FGF, we restricted our analysis to the fate of the Mitf mutant RPE.

Using cyclinD1 staining, we first confirmed that excessive RPE proliferation is an early sign of RPE pathology, not only in embryos homozygous for the semidominant Mitf alleles Mitf Mi and Mitf Mi-wh (Packer, 1967) but also for the null allele Mitf vga−9. CyclinD1 marks cells in S phase and is downregulated in mitosis. It is prominently expressed in many cells in the NR for whose development it is required (Fantl et al., 1995; Sicinski et al., 1995), but is seen in relatively few cells in the wild-type RPE. In mutant RPE, however, the frequency of cyclinD1 positive cells was similar to that in NR (not shown). Furthermore, an RPE-to-retina transition was also seen in Mitf vga−9/vga−9 mice and thus was not a peculiarity of

**Fig. 6.** Cells filling the inner vesicle space after surface ectoderm removal are not derived from the distal neuroepithelium or from the proximal mesenchyme or optic stalk. Dil crystals were placed over (A) the distal optic neuroepithelium (for 1-2 minutes) or (B) behind the optic vesicle (left in place) and the cultures were kept for 2 days before cryosectioning. Note that the PAX6/CHX10-positive cells identified in Fig. 5 (marked x) are not labeled, suggesting they are neither derived from the distal layer nor from the proximal mesenchyme or adjacent areas such as the optic stalk. Note that, at the stage shown, the distal optic vesicle has not yet accumulated pigment.
Chan, 1987) and a recent study (Bumsted and Barnstable, 2000), where in addition rods lacking outer segments were identified at PN 17. Beyond PN 14, however, the second retinæ showed complex foldings, making their clear identification difficult, and they soon degenerated. In fact, in the absence of a normal RPE, the primary retina became disorganized as well and lacked expression of rod outer segments (not shown).

Taken together, the above results suggest that irrespective of whether Mitf is downregulated experimentally by FGF, or rendered non-functional by mutation, presumptive RPE cells change their gene expression profile and may switch their fate towards that of neuroretina. In vivo, the loss of wild-type Mitf may eventually lead to the development of a second retina, albeit one that is restricted to the dorsal area of the RPE, suggesting that the loss of functional Mitf, though crucial, is not sufficient for this in vivo transdifferentation.

DISCUSSION

It is well established that the division of the optic neuroepithelium into two domains, the presumptive NR and the RPE, is critical for the development of the vertebrate eye. Here we describe experiments whose design was prompted by the notion that the early optic neuroepithelium is bipotential, capable of giving rise to either NR or RPE, and that this bipotentiality may be reflected in the fact that distinct transcription factors, which are later needed separately in NR and RPE, are initially coexpressed. The results of these experiments support the view that in the mouse, the division into NR and RPE is effectively controlled by extrinsic factors, notably FGFs, which emanate from the surface ectoderm and regulate an intrinsic factor, the transcription factor MITF, which ultimately specifies the RPE.

A first set of experiments addressed the

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**Fig. 7.** Molecular markers of in vivo RPE transdifferentiation in Mitf mutant embryos. Pictures in the left column represent wild-type embryos and those in the right two columns embryos homozygous for the Mitf alleles indicated. The rightmost column shows higher magnifications of areas of transition between RPE and thickened parts that transdifferentiate into neuroretina. All pictures represent embryonic eyes from E14.5 except (A,B,G,H), which are from eyes at E12.5. Labeling was for the markers indicated on the left. MITF and PAX6 were labeled by the immunoperoxidase reaction, the remainder of markers by in situ hybridization. Note that in wild-type RPE, pigmentation obscures specific labeling, and so the insets in A and G show a small area labeled by immunofluorescence. Based on earlier studies of the genes labeled by in situ hybridization, only Otx2 is expressed in the RPE at the developmental stage shown (E14.5) while Six3, Chx10 and Crx are not expressed in the RPE. Mitf mutant RPE is not pigmented and so does not obscure the label. Arrows in B and C show downregulation of MITF expression (see text for details). Bar, 100 μm (left and middle column), 40 μm (right column).
Role of FGF and MITF in eye development

importance of the surface ectoderm in principle. Normally, the optic vesicle forms an NR domain at the distal tip where it comes in contact with the surface ectoderm, and an RPE domain at its proximal base where it connects to the optic stalk. When the surface ectoderm was physically ablated in explant cultures, the two domains still formed, only they were now inverted, with a pigmented monolayer resembling RPE located at the vesicle’s tip and a multicellular layer resembling NR located at its base. This inversion occurred rapidly and cannot be explained by an involution of the vesicle followed by de novo generation of the two layers from pre-existing, committed precursors. Rather, it appears that the optic neuroepithelium of mammals, much as that of lower vertebrates, is capable of giving rise to either NR or RPE and, consequently, needs to be instructed by local signals to develop into the types of cells appropriate for their anatomical location. If the surface ectoderm were the only source of local signals for NR specification, however, its removal should leave the optic vesicle incapable of forming NR. Since NR still formed, it follows that NR inducing signals must originate at other sites as well and gain in prominence in the absence of the surface ectoderm. Such signals might emanate from the surrounding mesenchyme, the neuroepithelium of the brain, or the novel, distal RPE. In fact, it is thought that the apposition of RPE and NR may help to mutually reinforce the specification of these two layers (Orts-Llorca and Genis-Galvez, 1960; Sheedlo and Turner, 1996) and thus, it is conceivable that after removal of the surface ectoderm, the newly formed distal RPE may cooperate with proximal signals to induce the proximal cells to express NR markers. Intriguingly, in the chick, in contrast to our observations in the mouse, removal of the surface ectoderm led to the generation of an epithelium in which neuronal cells were interspersed with pigmented cells, which were not arranged as cuboidal epithelium (Hyer et al., 1998). This difference may reflect the fact that chick embryos do not show the initial pan-vesicular expression of Mitf (Mochii et al., 1998a), and since the removal of the surface ectoderm may not induce Mitf, just not suppress it, presumptive chicken NR cells may not differentiate into pigmented cells without the action of additional RPE inducing signals.

A second set of experiments suggested that the relevant extracellular signals from the surface ectoderm are members of the FGF family of ligands. Both FGF1 and FGF2 were expressed at higher concentrations in the surface ectoderm than in the neuroepithelium itself or the surrounding mesenchyme. In addition, while the neuroepithelium was thickened in response to FGFs in the distal as well as the proximal retina, the thickened part of the transdifferentiated RPE (second retina) and are contiguous with parts of the RPE that retain a more RPE-like, though unpigmented, appearance (not shown). Arrows indicate interneurons in the lower nuclear layer, arrowheads (A and D) indicate displaced amacrine cells in the ganglion cell layer, rpe, retinal pigment epithelium; inl, inner nuclear layer; gcl, ganglion cell layer. Bar, 40 μm (A-C); 50 μm (D-F).

Fig. 8. Neuroretinal markers in wild-type (WT, A-C) and Mitf mutant eye (Mitf<sup>vga<sup>-9</sup></sub>) (D-F), labeled at postnatal day 0 for the markers indicated. (D-F) Note that in the dorsal part of the Mitf mutant eyes, the layers of the specific retinal cells are duplicated. The duplicated layers are derived from the thickened part of the transdifferentiated RPE (second retina) and are contiguous with parts of the RPE that retain a more RPE-like, though unpigmented, appearance (not shown). Arrows indicate interneurons in the lower nuclear layer, arrowheads (A and D) indicate displaced amacrine cells in the ganglion cell layer. rpe, retinal pigment epithelium; inl, inner nuclear layer; gcl, ganglion cell layer. Bar, 40 μm (A-C); 50 μm (D-F).

Fig. 9. Schematic representation of how selective gene repression leads to domain specification in the optic vesicle. In both (A) and (B), the optic vesicle initially expresses the RPE transcription factor MITF along with NR transcription factors. As the vesicle comes into contact with the surface ectoderm (SE), signals from the ectoderm downregulate MITF in the future NR. Where MITF expression is not downregulated, an RPE develops. In model A, FGF1 and FGF2 directly downregulate MITF. The more general model in B suggests that other surface ectodermal factors (marked X) induce the adjacent neuroepithelium one or several factors (marked Y, potentially including FGF15), which in turn lead to downregulation of MITF.
proximal domain, suggesting that the appropriate receptors and signal transduction machineries were available at either location, only the domain closest to the surface ectoderm normally gives rise to the NR. Furthermore, the development of a pigmented monolayer, either starting out from the presumptive RPE or, after ablation of the surface ectoderm, from the presumptive NR, could be prevented by the local application of FGF alone. Consistent with this observation, activation of FGF receptors in the presumptive RPE by ectopic expression of FGF9 in transgenic mice has recently been shown to result in a second retina (Zhao and Overbeek, 1999), suggesting that FGFs may be sufficient to trigger the presumptive RPE to form a retina. As discussed below, however, other signals from the surface ectoderm may conceivably have similar effects as FGFs.

A third set of experiments led to the conclusion that FGFs (or ligands with similar activities) affect these developmental cell fate choices by regulating the expression of Mitf. This conclusion was based on the following lines of reasoning. Where FGF, whether derived from the surface ectoderm or from experimentally implanted beads, were able to prevent the formation of an RPE, they also induced the disappearance of Mitf, and where Mitf stayed on, an RPE developed. Beyond this correlation, we also found that when Mitf was missing, as in Mitf<sup>−/−</sup>/vga<sup>−/−</sup> mice, or was crippled in its DNA binding and transcriptional activity, as in Mitf<sup>fluor</sup>/Mitf<sup>fluor</sup> or Mitf<sup>mi-ew</sup>/Mitf<sup>mi-ew</sup> mice, the RPE, at least its dorsal part, lost the expression of RPE-specific genes, including that of Mitf itself, and gained the expression of NR-specific genes, including that of Six3, Chx10 and Crx. In fact, this dorsal part thickened and then became a laminated second retina not unlike that induced by the above mentioned ectopic FGF9 expression (Zhao and Overbeek, 1999). The results suggest that the formation of a second retina from mutant RPE depends on additional local signals, such as BMP7 or other factors expressed on the dorsal side of the cup (Wawersik et al., 1999; Koshiba-Takeuchi et al., 2000). Nevertheless, the fact that mutations in a single gene, Mitf, are capable of inducing the transdifferentiation of the presumptive RPE into an NR suggests that Mitf is pivotal in RPE specification. It remains an open question, though, whether ectopic expression of wild-type Mitf, experimentally uncoupled from its negative control by FGFs, would be sufficient to induce the presumptive NR to form an RPE against the action of FGFs.

The results can be interpreted as schematically depicted in Fig. 9. The model builds on previous models in the chick, which highlighted the importance of the surface ectodermal FGFs in the induction of the NR (Pittack et al., 1997; Hyer et al., 1998), and extends these models to the mouse and to the regulation of distinct transcription factors involved in cell-fate determination. The simplest explanation (Fig. 9A) is that the distal neuroepithelium, which initially coexpresses transcription factors that later become NR- and RPE-specific, becomes activated by surface ectodermal FGFs, which leads to transcriptional repression of a presumably eye-specific Mitf promoter and the concomitant disappearance of MITF protein. Consequently, two domains are formed, one MITF-negative but retaining the expression of transcription factors regulating the development of the NR, and one MITF-positive and specifying the RPE. This model makes no assumptions as to the functional interrelations of the factors that are initially coexpressed, but it suggests, of course, that FGFs may induce transcription factors that serve as transcriptional repressors of Mitf; and conversely, that MITF may directly or indirectly lead to transcriptional repression of NR-inducing factors.

The fact that undifferentiated, bi- or multipotential precursor cells initially show overlapping gene expression patterns, which for subsequent lineage commitment are sorted out by selective gene repression, is reminiscent of other systems in both invertebrates and vertebrates. During vertebrate neurulation, for instance, transcription factor genes such as Pax3 and Pax7 initially have broad expression patterns that encompass both the future dorsal and future ventral domains; only later do they become restricted to the dorsal neural tube through repression by sonic hedgehog signaling in the ventral neural tube and maintenance by BMPs in the dorsal neural tube (for a review, see Lee and Jessell, 1999). Singling-out of distinct cell fates by repression of transcription factors also seems to operate, for instance, in the vertebrate hematopoietic system (Nutt et al., 1999).

Equally consistent with our results, and representing a more general explanation, is the model depicted in Fig. 9B. Here, the relevant factors from the surface ectoderm need not be FGFs; they only need to be capable of activating signaling pathways with similar consequences in the adjacent neuroepithelium. This view is supported by the demonstration that EGF was apparently as potent as FGF in inhibiting pigment formation in the distal optic vesicle after surface ectoderm removal (although EGF had no such effect on the normal future RPE). According to this scenario, then, the experimental application of FGFs would simply mimic the signaling effects of other factors. Evidently, such factors could induce distinct FGFs or functionally related ligands in the adjacent neuroepithelium. An excellent candidate for an FGF interposed between the surface ectoderm and MITF is FGF15, which is expressed specifically in the NR domain (McWhirter et al., 1997). Although the model in Fig. 9B may not seem conceptually different from that in Fig. 9A, it would accommodate the fact that mice with null mutations in FGF1 or FGF2, alone or even in combination, apparently do not show overt alterations in eye development.

In conclusion, in the mouse, the division of the optic neuroepithelium into a future NR domain and an RPE domain, and hence the formation of a proper eye, critically depends on the negative regulation of Mitf. The results suggest that when left unchecked, MITF might usurp the future NR and at best disturb its proper growth, but at worst turn it into a pigmented layer unsuitable to function as a retina.

We thank Drs Constance Cepko, Peter Gruss, Paul Hargrave, Vincent Hearing, Roderick McNees and Simon Saule for probes and antibodies, Story Landis and Ronald McKay for discussions, and Monique Dubois-Dalcq, Lynn Hudson and Ward Odenwald for critically reading the manuscript.

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