**Chx10 repression of Mitf is required for the maintenance of mammalian neuroretinal identity**

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Summary

During vertebrate eye development, the cells of the optic vesicle (OV) become either neuroretinal progenitors expressing the transcription factor Chx10, or retinal pigment epithelium (RPE) progenitors expressing the transcription factor Mitf. Chx10 mutations lead to microphthalmia and impaired neuroretinal proliferation. Mitf mutants have a dorsal RPE-to-neuroretinal phenotypic transformation, indicating that Mitf is a determinant of RPE identity. We report here that Mitf is expressed ectopically in the Chx10null, Chx10null;or−/− neuroretina (NR), demonstrating that Chx10 normally represses the neuroretinal expression of Mitf. The ectopic expression of Mitf in the Chx10null, Chx10null;or−/− NR deflects it towards an RPE-like identity; this phenotype results not from a failure of neuroretinal specification, but from a partial loss of neuroretinal maintenance. Using Chx10 null and Mitf transgenic and mutant mice, we have identified an antagonistic interaction between Chx10 and Mitf in regulating retinal cell identity. FGF (fibroblast growth factor) exposure in a developing OV has also been shown to repress Mitf expression. We demonstrate that the repression of Mitf by FGF is Chx10 dependent, indicating that FGF, Chx10 and Mitf are components of a pathway that determines and maintains the identity of the NR.

Key words: Mouse, Mitf, Chx10, FGF, RPE, Neuroretina

Introduction

In vertebrate eye development, anterior ectodermal tissue undergoes specification to form the three major structures of the eye: the NR and the RPE, both of which originate from the neuroectodermal tissue of the OV; and the lens, which develops from the surface ectoderm (Chow and Lang, 2001). Recent evidence suggests that eye cell fate decisions begin early in embryogenesis and occur through a series of narrowing cell commitments (Baker and Bronner-Fraser, 2001; Zuber et al., 2003). However, the binary cell fate decision through which an OV neuroectodermal cell becomes either a neuroretinal or an RPE cell is poorly characterized, especially at the molecular level.

The majority of studies examining RPE versus neuroretinal cell identity decisions have focused on the ability of differentiated or developing RPE cells to ‘transdifferentiate’ into neuroretinal cells in response to external signals (Reh and Pittack, 1995). The neuroretinal-inducing signal appears to come from the surface ectoderm (Mikami, 1939), suggesting a model in which surface ectodermal signals induce the underlying OV neuroectoderm to become NR rather than RPE. Although FGF has long been considered the surface ectodermal signal that induces neuroretinal cell fate, recent evidence suggests that surface ectodermal FGFs (or factors mimicking FGF signaling) pattern and organize the RPE and NR in the OV, rather than inducing neuroretinal cells (Hyer et al., 1998; Nguyen and Arnheiter, 2000). This model is consistent with the step-wise specification of neuroretinal and RPE cells that begins early in embryogenesis, before the OV stage.

Of the regulatory molecules implicated in RPE cell specification, the basic helix-loop-helix leucine-zipper transcription factor Mitf is the best characterized. Mitf is considered to be crucial for the acquisition and maintenance of RPE cell identity. In the mouse, Mitf is expressed throughout the neuroectoderm of the E9.0 OV, and is subsequently downregulated in the presumptive NR at E9.5 (Nguyen and
Arnheiter, 2000). However, Mitf expression continues in the RPE and ciliary margin throughout early eye development. Mutations in mouse Mitf result in microphthalmia, a lack of RPE cell differentiation and an RPE-to-neuroretinal change in cell identity in the dorsal region of the eye (Bumstead and Barnstable, 2000; Hodgkinson et al., 1993; Hughes et al., 1993; Nguyen and Arnheiter, 2000). Other studies suggest that Mitf is involved in OV organization and pattern formation in response to external signals. Mitf expression is repressed by surface ectodermal FGF (Nguyen and Arnheiter, 2000), and upregulated by activin signals from the mesenchyme surrounding the RPE (Fuhrmann et al., 2000). In addition, Pax2, Pax6 (Baumer et al., 2003), Otx1, Otx2 (Martinez-Morales et al., 2003; Martinez-Morales et al., 2001), Gas1 (Lee et al., 2001) and Ap2a (West-Mays et al., 1999) are necessary for RPE cell identity; Pax2, Pax6 and the Otx genes are upstream of Mitf.

Apart from the role of FGF in regulating neuroretinal cell identity, little is known about the mechanisms of neuroretinal transformation in chick (Toy et al., 1998). In the mouse, transcription factor Six6/Optx2 leads to an RPE-to-NR transformation (Burmeister et al., 1996). One potential target was the early eye-specifying transcription factor Six6/Optx2 in the presumptive NR, and expressed specifically in the presumptive NR, beginning at E9.5 (Liu et al., 1994). A possible regulatory relationship between Six6/Optx2 and Mitf, which is upregulated by activin signals from the mesenchyme surface ectoderm (Nguyen and Arnheiter, 2000), at the time extinction of the presumptive NR (in the distal OV) is normally extinguished at E9.5 (Nguyen and Arnheiter, 2000), at the time that Chx10 expression in the distal OV is first observed (Liu et al., 1994). Mitf expression is repressed by surface ectodermal FGF (Nguyen and Arnheiter, 2000), and upregulated by activin signals from the mesenchyme surrounding the RPE (Fuhrmann et al., 2000). In addition, Pax2, Pax6 (Baumer et al., 2003), Otx1, Otx2 (Martinez-Morales et al., 2003; Martinez-Morales et al., 2001), Gas1 (Lee et al., 2001) and Ap2a (West-Mays et al., 1999) are necessary for RPE cell identity; Pax2, Pax6 and the Otx genes are upstream of Mitf.

We considered the Chx10 homeodomain transcription factor to be a strong candidate regulator of neuroretinal specification. In the mouse, ectopic expression of Ras (Zhao et al., 2001) or Mek1 (Galy et al., 2002) results in RPE-to-NR changes in cell identity, presumably by mimicking the reception of an FGF signal from the surface ectoderm. In addition, misexpression of the early eye-specifying transcription factor Six6/Optx2 leads to an RPE-to-NR transformation in chick (Toy et al., 1998). In the mouse, however, no loss-of-function studies have identified a NR-specifying molecule.

We examined the expression of candidate Chx10 target genes in the developing eye of the homozygous recessive Chx10<sup>−/−</sup/> mouse (Burmeister et al., 1996). One potential target was the key eye development transcription factor gene Mitf, because mutations in this gene, like mutations in Chx10, are also associated with a small eye phenotype (Hodgkinson et al., 1993; Hughes et al., 1993). A possible regulatory relationship between Chx10 and Mitf in early stages of mouse eye development is also suggested by the fact that Mitf expression in the presumptive NR (in the distal OV) is normally extinguished at E9.5 (Nguyen and Arnheiter, 2000), at the time that Chx10 expression in the distal OV is first observed (Liu et al., 1994).

Materials and methods

Animals

Chx10<sup>−/−</sup>/+, Chx10<sup>−/−</sup>/A+ (129/Sv), Mitf<sup>−/−</sup> (B6C3He) and C57BL/6 mice were obtained from the Jackson Laboratories (Bar Harbor, ME). Mice were placed together in the evening and females were considered to be at 0.5 days of gestation (E0.5) when a vaginal plug was detected the following morning. Mice were euthanised by cervical dislocation.

Chx10<sup>−/−</sup> mice were genotyped as previously described (Burmeister et al., 1996). Mitf<sup>−/−</sup> mice were genotyped by PCR amplification with mouse Mitf intron 6 forward (5′-GTTGTTG-CTCTAGCTACAAATG-3′) and exon 7 reverse (5′-CGGATCA-TTTGACTTGGG-3′) primers. Genotypes were determined by heteroduplex analysis on a 9% acrylamide gel. Heterozygous individuals yielded multiple bands instead of a single 200 bp band that arises from homozygous normal or mutant mice.

In situ hybridization and immunofluorescence

Embryos were processed and sectioned, and in situ hybridization and immunofluorescence were performed as previously described (Chow et al., 2001).

Mitf, Tyr, Tyrp1, Otx, Math5 (Atoh7 – Mouse Genome Informatics) and human MITF (see below) expression was identified using mRNA from full-length cDNAs; Chx10 expression was examined using a 3′ UTR probe (Liu et al., 1994). The localization of Mitf protein was determined using rabbit anti-Mitf antibodies (Nguyen and Arnheiter, 2000) pre-adsorbed to mouse embryo powder (see below), at a dilution of 1:25. Chx10, Pax6 and NCAM proteins were identified with affinity-purified anti-Chx10 (Liu et al., 1994), anti-Pax6 (a gift from G. Mastick) and anti-NCAM (Santa Cruz Biotechnology, Santa Cruz CA, H-300) rabbit polyclonal antibodies. The final dilutions of these primary antibodies were 1:500, 1:500 and 1:100, respectively. Islet1 protein was identified with an anti-Islet1 (Developmental Studies Hybridoma Bank, University of Iowa; 39.4D5) mouse monoclonal antibody at a dilution of 1:100. Notch1 was identified using a goat anti-Notch1 (C-20; 1:25; Santa Cruz Biotechnology, Santa Cruz, CA; sc-6014) polyclonal antibody. Secondary AlexaFluor488-conjugated F(ab′)<sub>2</sub> fragment goat anti-mouse IgG (Molecular Probes, Eugene, OR) and secondary Cy3-conjugated goat anti-rabbit and anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) and donkey anti-goat antibodies (Jackson ImmunoResearch, West Grove, PA) were used at a dilution of 1:100. Stained sections were examined on a LSM 510 Zeiss confocal microscope and on a Nikon Eclipse E-1000 conventional upright fluorescence microscope.

Anti-Mitf antibody was adsorbed on acetone-extracted E15.5 Mitf<sup>−/−</sup>/+ embryo powder in phosphate-buffered saline (PBS) and stored in 10% goat serum, 0.05% sodium azide/PBS.

Hematoxylin and Eosin staining

To perform Hematoxylin and Eosin staining, paraffin wax-embedded sections were deparaffinized in xylene and rehydrated in a graded ethanol series, immersed in xylene and mounted in Permount (Fisher Scientific).

NR-MITF transgenic mice

The NR-MITF construct consisted of the human PAX6 neuroretnal enhancer and minimal β-globin promoter driving the expression of the human M-MITF cDNA (without exon 6) followed by an SV40 polyadenylation signal (see Fig. S1 in the supplementary material).

A human M isoform MITF (M-MITF) cDNA was obtained by generating a hybrid EST clone (due to an EST mutation: 5′ EST Accession Number N36632, 3′ EST Accession Number N34462). The 5′ and 3′ sequences were linked using a ClaI site at nucleotide 478 of the M-MITF cDNA. The sequence starting at nucleotide 68 of the cDNA (without exon 6) followed by an SV40 polyadenylation signal (see Fig. S1 in the supplementary material).
spliced exon 6. This plasmid was digested with AccI, blunted and digested with HindIII.

As the putative neural retinal enhancer (NRE) of the human PAX6 gene is located in intron 4 (Kammend et al., 1999), a 6 kb EcoRI fragment encompassing exons 3, 4 and part of exon 5 of the PAX6 gene was identified and subcloned into the pBlueScript-SK (pBS-SK) cloning vector (Stratagene). This fragment was then excised as a NotI fragment for subsequent ligation into the NotI site of the p1229 reporter plasmid that contains the minimal β-globin promoter, the lacZ gene and the polyadenylation sequence from SV40. Transgenic studies demonstrated that this PAX6 enhancer is sufficient to direct expression in the developing mouse neuroretinal epithelium as detected by staining for lacZ expression (G.C.S., unpublished). The β-globin promoter from PAX6-NRE-β-globin-lacZ-SV40 was amplified from this plasmid with the primers T7 (5′-GAAGAAGCGGCGACACAGG-3′) and lacZ5Rev (5′-AAGTTGGGTAACGCGAGGG-3′). This PCR product was then digested with AccI and HindIII and subcloned into pBS-SK digested with SacI and HindIII to generate the plasmid BMpBS-SK. The SV40 polyadenylation sequence was amplified from PAX6-NRE-β-globin-lacZ-SV40 with the primers SV40Fwd (5′-CCATCGATCCGGGCAGGCCATGTCTGC-3′) and T3 (5′-AATTAACCTCTACTAAAGGG-3′). This PCR product was then digested with Clal and HindIII and subcloned into pBS-SK to generate the plasmid SV40pBS-SK. SV40pBS-SK was digested with HindIII and the liberated fragment was ligated into the HindIII site of BMpBS-SK to generate BMpSpBS-SK. The PAX6-NRE-β-globin-lacZ-SV40 construct was digested with NotI and SalI to remove all inserted sequences, which were replaced with the NotI-SalI fragment from BMpSpBS-SK, thus generating the plasmid BMSpBS-SK. The 6 kb PAX6-NRE was ligated into the NotI site of BMS to generate the NR-MITF construct (see Fig. S1 in the supplementary material). The NR-MITF insert was released with SalI prior to microinjection.

Fig. 1. Mitf is ectopically expressed in the Chx10or-J/or-J NR. (A) In the eye of an E10.5 wild-type (+/+ ) embryo, Mitf mRNA is present only in the presumptive RPE (arrow). (B) In an E10.5 Chx10or-J/or-J (J/J) mutant eye, Mitf is expressed normally in the presumptive RPE (arrow), and ectopically in the NR (arrowhead). (C) By E13.5, Mitf is localized to the RPE and presumptive ciliary margin (arrow) in a wild-type embryo. (D) E13.5 Chx10or-J/or-J animals continue to misexpress Mitf in the NR (arrowhead). In addition, the presumptive ciliary margin domain (arrow) is expanded, as evidenced by a larger area of increased Mitf expression. (E) Mitf protein levels mirror the RPE and presumptive ciliary margin (arrow) Mitf mRNA expression domain in a wild-type E13.5 embryo. (F) Mitf protein is synthesized in the E13.5 Chx10or-J/or-J NR (arrowhead) and the enlarged presumptive ciliary margin region can also be clearly identified (arrow). Scale bars: 25 μm in A,B,D; 32 μm in C; 50 μm in E,F.

The purified NR-MITF insert was injected into both C57BL/6;SIL (Hospital for Sick Children Transgenic Facility) and C57BL/6;C3H (Ottawa Health Research Institute) donors; germine-transmitting NR-MITF mice were identified using Southern blot analysis. EcoRI-digested genomic DNA was probed with radiolabeled full-length M-MITF cDNA; NR-MITF-positive individuals yielded 1 kb and 5 kb bands. Transgenic mice were also identified by PCR using primers in exon 3 (5′-CCAGCGGATTAGACACACTTAC-3′) and exon 9 (5′-GTGCTCCGTCTTTCATGTC-3′), resulting in a 1 kb product.

RPE-CHX10 transgenic mice

The RPE-CHX10 construct consisted of the mouse Dct enhancer/promoter driving the expression of the human CHX10 cDNA (see Fig. S1 in the supplementary material).

The Dct enhancer/promoter construct (pPdct) was a generous gift from T. Hornyk. This construct contains ~3.3 kb of sequence upstream of the open reading frame of the mouse Dct gene in a pcDNA vector backbone (Hornyk et al., 2001). To generate the human CHX10 cDNA construct, a second XhoI site was inserted 3′ to a 3.1 kb human CHX10 cDNA in pBS-ks to generate CHX10-2Xho. CHX10-2Xho was digested with XhoI to release the CHX10 cDNA, which was then ligated to XhoI-digested pDct to generate RPE-CHX10 (see Fig. S1 in the supplementary material). The RPE-CHX10 insert was released with SalI prior to microinjection.

The purified RPE-CHX10 insert was injected into C57BL/6;C3H donors (Ottawa Health Research Institute) and germline-transmitting mice were identified. RPE-CHX10 mice were then genotyped using EcoRI-digested genomic Southern blots probed with a radiolabeled full-length human CHX10 insert. RPE-CHX10-positive individuals yielded a major 3 kb band, and minor undigested bands that varied with the individual transgenic mouse lines. RPE-CHX10-positive mice were also identified by PCR using primers located in the homeodomain (5′-GAAGAAGCGGCGACACAGG-3′) and CVC domain (5′-CCATCGATCCGGGCAGGCCATGTCTGC-3′), resulting in a 350 bp product.

Results

Chx10 represses Mitf in eye development

We examined the expression of Mitf in wild-type and Chx10or-J/or-J mutant eyes, and confirmed that Mitf is normally expressed in the RPE and the ciliary margin (Nguyen and Arnheiter, 2000) (Fig. 1A,C,E). In the Chx10or-J/or-J embryo, however, we unexpectedly found that both the Mitf mRNA and protein are ectopically expressed in the NR, from at least E10.5 until P0 (Fig. 1B,D,F; data not shown), whereas the expression of Mitf in the RPE appears unchanged. The ectopic expression of Mitf in the developing Chx10or-J/or-J NR establishes that one function of Chx10 in eye formation is to repress, directly or indirectly, the expression of Mitf in neuroretinal progenitor cells. This finding is consistent with a report that the CHX10
protein acts as a transcriptional repressor in vitro (Bremner et al., 1997). We investigated the ability of CHX10 to repress human MITF directly using MITF promoter-driven (Fuse et al., 1996; Takeda et al., 2002; Udono et al., 2000) luciferase reporter gene expression in cultured RPE (D407, ARPE19), melanocyte (melan-a), neural (NG108) or fibroblast (COS7) cells. We examined the M-, D- and H-MITF promoters because each possesses a CHX10 consensus DNA-binding site (TAATTAGG/C/T) (Percin et al., 2000). However, baseline promoter activity was detected only with the H-MITF construct. The co-transfection of CHX10 and the H-MITF construct did not result in significant repression of the H-MITF promoter (data not shown).

The Chx10-mediated repression of Mitf expression may normally prevent Mitf from implementing an RPE differentiation program in the developing NR. The failure to repress Mitf expression in Chx10or-J/or-J neuroretinal progenitors might therefore be associated with additional evidence of differentiation of the mutant NR towards an RPE progenitors during optic cup development (Packer, 1996). Conversely, some of the phenotypes observed in the Mitfmi/mi mouse eye may require the ectopic Chx10 expression observed in the RPE. These considerations predicted that the eyes of double Mitfmi/mi;Chx10or-J/or-J mutants may have at least partial correction of the phenotypes seen in both Chx10or-J/or-J and Mitfmi/mi mutant eyes. In fact, double Mitfmi/mi;Chx10or-J/or-J mutants were reported previously to have increased retinal progenitor cell proliferation and normalization of the size of the NR and RPE (Konyukhov and Sazhina, 1966). To corroborate these findings using mice with a well-defined Chx10 allele (or′), we generated Mitfmi/mi;Chx10or-J/or-J mice and confirmed that the eyes of these double mutant animals are larger than those of Chx10or-J/or-J mice (Fig. 3A-D), that the NR displays near-normal lamination (Fig. 3C,D,D′), and that the dorsal RPE is a normal-appearing monolayer (Fig. 3E-H). The requirement for Mitf function in the Chx10or-J/or-J NR phenotype was also evident from the significant improvement of neuroretinal lamination in Chx10or-J/or-J mice heterozygous for the Mitfmi allele (Fig. 3C). In addition, as expected (Bone-Larson et al., 2000; Green et al., 2003) (e.g. see Fig. 3E-H), inspection of the Mitfmi/mi;Chx10or-J/or-J phenotype in two different backgrounds (50% 129/SvJ; 50% B6C3He and >90% 129/SvJ) confirmed that Mitf and Chx10 interact genetically, that Mitf is at least partially necessary for the Chx10or-J/or-J phenotype, and that Chx10 is necessary for the Mitfmi phenotype.

**Chx10 and Mitf are necessary for their reciprocal mutant eye phenotypes**

The ectopic expression of Mitf in the Chx10or-J/or-J retina suggested that some of the phenotypic features of the Chx10or-J/or-J eye phenotype might be the result of Mitf neuroretinal expression. For example, the decreased proliferation of Chx10or-J/or-J neuroretinal progenitors may reflect a Mitf-dependent constraint on proliferation, as Mitf-expressing RPE progenitors normally proliferate less than neuroretinal progenitors during optic cup development (Packer, 1967). Conversely, some of the phenotypes observed in the Mitfmi/mi mouse eye may require the ectopic Chx10 expression observed in the RPE. These considerations predicted that the eyes of double Mitfmi/mi;Chx10or-J/or-J mutants may have at least partial correction of the phenotypes seen in both Chx10or-J/or-J and Mitfmi/mi mutant eyes. In fact, double Mitfmi/mi;Chx10or-J/or-J mutants were reported previously to have increased retinal progenitor cell proliferation and normalization of the size of the NR and RPE (Konyukhov and Sazhina, 1966). To corroborate these findings using mice with a well-defined Chx10 allele (or′), we generated Mitfmi/mi;Chx10or-J/or-J mice and confirmed that the eyes of these double mutant animals are larger than those of Chx10or-J/or-J mice (Fig. 3A-D), that the NR displays near-normal lamination (Fig. 3C,D,D′), and that the dorsal RPE is a normal-appearing monolayer (Fig. 3E-H). The requirement for Mitf function in the Chx10or-J/or-J NR phenotype was also evident from the significant improvement of neuroretinal lamination in Chx10or-J/or-J mice heterozygous for the Mitfmi allele (Fig. 3C). In addition, as expected (Bone-Larson et al., 2000; Green et al., 2003) (e.g. see Fig. 3E-H), inspection of the Mitfmi/mi;Chx10or-J/or-J phenotype in two different backgrounds (50% 129/SvJ; 50% B6C3He and >90% 129/SvJ) confirmed that Mitf and Chx10 interact genetically, that Mitf is at least partially necessary for the Chx10or-J/or-J phenotype, and that Chx10 is necessary for the Mitfmi phenotype.

**Chx10 and Mitf function antagonistically to regulate retinal cell identity**

The repression both of the size of the presumptive ciliary margin and of neuroretinal Mitf expression by Chx10 suggested that Chx10 and Mitf may act in opposition during early eye development, a model supported by the rescue in double Mitf;Chx10 mutant animals of both the RPE and neuroretinal phenotypes. We therefore hypothesized that the role of Chx10 may be to define or maintain neuroretinal identity either by repressing Mitf, or by other mechanisms, and that the role of Mitf is to define or maintain an RPE identity.
This hypothesis also raises the possibility that the ratio of Mitf:Chx10 gene products in the developing eye, rather than the absolute level of expression of the Mitf or Chx10 genes, is the crucial determinant of retinal cell identity. To examine these concepts, we asked whether the ectopic expression of transgenic human MITF in the developing NR, and of human CHX10 in the developing RPE, were sufficient to determine RPE and neuroretinal cell identity, respectively (see Fig. S1 in the supplementary material).

The eyes of mice either hemizygous (see Fig. S1 in the supplementary material) or homozygous (data not shown) for the NR-MITF transgene were normal. Thus, in a wild-type background, the transgenic expression of MITF in the developing NR failed to alter retinal cell identity. We then considered that this failure resulted from the activity of the wild-type Chx10 gene. We therefore increased the gene dosage of neuroretinal MITF:Chx10 by crossing mice carrying the NR-MITF/+ transgene to animals heterozygous or homozygous for mutant Chx10, and examined the resulting retinal phenotypes at P0. In animals hemi- or homozygous for the human MITF transgene and carrying one Chx10 wild-type allele (NR-MITF/++; Chx10<sup>pro-lor</sup>/+ and NR-MITF/NR-MITF; Chx10<sup>pro-lor</sup>/+), no changes were observed in the NR (data not shown). By contrast, the NR in NR-MITF/++; Chx10<sup>pro-lor</sup>/+ individuals in two independent transgenic mouse lines changed dramatically, to a pigmented monolayer or PML (Fig. 4A-D). The PML resembled a mature RPE, not only in being a highly pigmented monolayer but also in not expressing the neuroretinal marker protein, Notch1 (see Fig. S2 in the supplementary material). By contrast, the cells of the PML were still partially neuroretinal in identity, because they expressed both the neural marker NCAM (Fig. 4E,F) and the neuroretinal protein Pax6 (Fig. 4G,H). In addition, the PML has not begun to differentiate into the earliest retinal cell type, the ganglion cell, as it did not express Isl1 (see Fig. S2 in the supplementary material).

To determine whether the formation of the PML in NR-MITF/++; Chx10<sup>pro-lor</sup>/+ mice represented a change in neuroretinal specification, or a later change in the identity of a properly specified NR because of a failure of maintenance of neuroretinal identity, we examined the developing retinas of NR-MITF/++; Chx10<sup>pro-lor</sup>/+ mice at E11.5. These retinas were histologically normal, and expressed the neuroretinal markers Rax (E11.5; Fig. 4I,J) and Math5 (E16.5; see Fig. S2 in the supplementary material), but not the RPE marker Dct (E11.5; Fig. 4K,L). Thus, the change in neuroretinal phenotype resulted from the ‘transdifferentiation’ of a normally specified NR to a PML after E11.5. The transdifferentiation occurs incrementally, first noticeable as a decrease in the size of the NR and a subsequent increase in pigmentation that is apparent by about E16.5 (see Fig. S3 in the supplementary material) and
appears to progress from a peripheral-to-central fashion, expanding from the ciliary margin region. Taken together, these results demonstrate, first, that an increase in the level of Mitf:Chx10 gene products in the developing optic cup can dramatically alter the identity of NR progenitors towards an RPE-like phenotype, and second, that the increased expression of Mitf/MITF gene products in the developing NR is insufficient to confer an RPE identity on cells that have been specified to become NR.

We similarly found that the eyes and retinas of animals either hemizygous (see Fig. S1 in the supplementary material) or homozygous (data not shown) for the RPE-CHX10 transgene in a wild-type background were also normal. Thus, either the absolute level of CHX10 expression achieved in the developing RPE of wild-type mice, or the ratio of CHX10:Mitf gene products, was insufficient to alter cell identity. To evaluate the latter possibility, we increased the ratio of RPE-CHX10 transgene:wild-type Mitf by crossing mice carrying the RPE-CHX10 transgene onto mice heterozygous for a semi-dominant mutant allele of Mitf, the mi allele. At P0, the retinas of RPE-CHX10/+;Mitf^{mi/+} mice had a significant decrease in the overall level of RPE pigmentation compared with Mitf^{mi/+} mice (Fig. 4M,N). Most notable, however, was the transformation of the dorsal RPE in RPE-CHX10/+;Mitf^{mi/+} animals to a thickened multicellular layer (Fig. 4O-R). This thickened structure was a neuroretinal-like layer (NRLL), as it expressed the progenitor markers Pax6, Rax, mouse Chx10 (Fig. 4S-V) and Notch1 (see Fig. S2 in the supplementary material) and had begun to differentiate, as shown by the presence of Islet1-expressing cells (see Fig. S2 in the supplementary material). The similarity of the NRLL to the transdifferentiated neuroretinal phenotype found in the dorsal RPE of mice with a severe loss of Mitf function is striking (Nguyen and Arnheiter, 2000; Packer, 1967). All of the above findings suggest that Mitf and Chx10 act antagonistically in the developing eye, and that the
levels of Mitf:Chx10 gene products in the optic cup are major determinants of retinal development.
**Fig. 6.** Chx10 lies downstream of FGF in mouse neuroretinal cell identity decisions. (A) An OV culture from a wild-type animal (+/+), dissected at E9-9.5 and grown for 3 days in the presence of a bovine serum albumin-coated bead (BSA) results in normal development of the NR (arrowhead) and RPE (arrow), including pigmentation and expression of Mitf protein (green). (B) By contrast, a culture of wild-type OV in the presence of an FGF2-coated bead results in a change in cell identity from an RPE to a NR (arrow), as evidenced by a loss of pigmentation and greatly reduced Mitf expression, although the NR appears normal (arrowhead). (C) A Chx10or-J/or-J OV cultured with an FGF2-coated bead has no effect on the identity of the RPE, as shown by the pigmentation of the RPE and the expression of Mitf (arrow). The ectopic Mitf is clearly apparent in the Chx10or-J/or-J NR (arrowhead). (D) Graphical representation of the percent frequency of normal RPE pigmentation in optic vesicle cultures incubated with beads coated in BSA, FGF1, or FGF2. Genotype is indicated by bar color (black, wild-type; grey, Chx10or-J/or-J). The asterisks represent experiments that were not performed, rather than a value of zero. The numbers above the bars indicate the number of individuals tested. RPE pigmentation: +/- BSA, 10/10; +/- FGF2, 4/28. Scale bars: 20 μm in A-C.

**Chx10 lies downstream of FGF in neuroretinal cell identity maintenance**

FGF has been shown to induce and organize neuroretinal cells (Hyer et al., 1998; Nguyen and Arnheiter, 2000; Park and Hollenberg, 1989), and has recently been shown to repress the expression of Mitf in the OV (Nguyen and Arnheiter, 2000). Our demonstration that Chx10 is also required to repress Mitf expression in the developing NR suggested that FGF lies upstream of Chx10 and Mitf in regulating retinal identity decisions, and that FGF represses Mitf by first activating the expression of Chx10 in the OV. This model is supported by the fact that the removal of surface ectodermal FGF leads to the formation of ‘salt and pepper’ neuroretinas (Hyer et al., 1998), by first activating the expression domains of Mitf, Otx2, and Chx10 in OVs derived from wild-type and Chx10 or-J/or-J mice. In wild-type mouse OV cultures, the exposure to FGF leads the developing RPE to become an ectopic NR that does not express the Mitf protein (Fig. 6A, B). By contrast, FGF fails to induce an ectopic NR in the OV of Chx10or-J/or-J mice, which develops instead into a pigmented RPE that expresses Mitf (Fig. 6C). The dependency of the FGF-induced repression of Mitf on Chx10 suggests that Chx10 lies downstream of FGF1 or FGF2 in a regulatory pathway that defines neuroretinal identity.

**Discussion**

**Chx10, Mitf and the Chx10or-J/or-J mutant phenotype**

The ectopic expression of Mitf in the Chx10or-J/or-J NR establishes that Chx10 represses the neuroretinal transcription of Mitf, either directly or indirectly. Chx10-mediated repression of Mitf would account for the specific downregulation of Mitf expression in the presumptive NR in normal eye development, at the time that Chx10 expression is initiated (Liu et al., 1994; Nguyen and Arnheiter, 2000). Although we could not demonstrate it, the Chx10-mediated repression of Mitf may indeed be direct, but might require eye-specific co-factors. Alternatively, Chx10 may act in the NR through another Mitf promoter (Oboki et al., 2002; Takeda et al., 2002; Takemoto et al., 2002).

The absence of ectopic neuroretinal expression of the Mitf-regulated pigmentation enzyme transcripts in the Chx10or-J/or-J mouse may reflect low levels of Mitf expression, or to the absence of essential co-factors. The lack of ectopic Otx1 and Otx2 expression in the Chx10 mutant NR argues that Chx10 may specifically repress Mitf, rather than downregulating an entire RPE developmental program. However, the expansion of the expression domains of Mitf, Tyr, and Dct in the ciliary margin of the Chx10or-J/or-J eye indicates that Chx10 is required not only to repress the neuroretinal expression of Mitf, but also to constrain the size of the presumptive ciliary margin. Thus, Chx10 and Mitf may regulate ciliary margin size in an antagonistic manner.

We confirmed and extended earlier findings (Konyukhov and Sazhina, 1966) that the expression of Mitf modifies the Chx10 mutant phenotype and contributes to the failure of lamination and the hypocellularity of the Chx10 mutant. We propose that the proliferation defects in the Chx10or-J/or-J NR may result from the shift towards an RPE cell identity, an identity normally associated with decreased proliferation (at least compared to the NR) (Green et al., 2003; Packer, 1967). The Chx10-mediated regulation of proliferation is mediated, at least partially, through p27kip1 (Green et al., 2003), and may also be Mitf dependent. Consistent with a possible Mitf-dependent regulation of p27kip1, Chx10 or-J/or-J mice express the p27kip1 family member (Goding et al., 2004).

We also demonstrated that Chx10 is required for the RPE-hyperproliferation phenotype of the Mitfmutant, suggesting that Mitf may normally repress Chx10 activity in the developing RPE. However, Chx10 expression is not ectopically expressed throughout the entire RPE in Chx10 mutants early in eye development (data not shown), arguing that the repression of Chx10 activity in the Mitfmutant RPE is not mediated by the repression of Chx10 expression. Mitf may therefore repress neuroretinal cell identity in the developing RPE by some other mechanisms that repress both Chx10 activity indirectly and the
function and/or expression of other neuroretinal specification genes also expressed in the Mitf/mi RPE (Nguyen and Arnheiter, 2000). Finally, the relatively normal eye that results from the Chx10:Mitf double mutant emphasizes that although Chx10 and Mitf are essential for the maintenance of retinal identity, other genes must also be involved in these processes and can at least partially compensate for loss of Chx10 and Mitf.

**Chx10 and Mitf function antagonistically in a pathway with FGF to regulate retinal cell maintenance**

Through the use of transgene expression in Chx10 and Mitf mutant mice, we established that Chx10 and Mitf act antagonistically during development to regulate the acquisition and/or maintenance of retinal cell identity. However, neither transgenic RPE CHX10 nor neuroretinal MITF were sufficient to alter retinal cell identity in a wild-type context. The levels of the NR-MITF and RPE-CHX10 transgenes, their spatiotemporal expression patterns, or the cellular environment may have limited the effects of the transgenes. Although high levels of Mitf expression are sufficient to establish pigment cell identity in some contexts (Lister et al., 1999; Planque et al., 2004; Planque et al., 1999; Tachibana et al., 1996), the levels of ectopic Mitf expression achieved in those studies (using strong promoters) are likely to have been higher than those obtained in the NR from the human PAX6 enhancer we used (Kammandel et al., 1999). Similarly, the Dct promoter-enhancer RPE-CHX10 construct we used was unable to change RPE identity in wild-type mice. This result contrasts with the finding that a Dct promoter-enhancer construct driving RPE Fgf9 or Ras conferred an RPE-to-NR change in a wild-type background (Zhao and Overbeek, 1999). The differences between these results may be due to variations in transgene expression levels or to differences in the roles of Chx10 versus Fgf9 and Ras in neuroretinal development. For example, ectopic RPE Fgf9/Ras may mimic the combined role of various FGFs or other ligands activating receptor tyrosine kinases [e.g. epidermal growth factor (EGF), see below] that confer neuroretinal cell identity.

We established that Chx10 is required for neuroretinal maintenance, at least in some genetic backgrounds, as a PML instead of a differentiated NR formed in Chx10<sup>−/−</sup>;Mitf<sup>−/−</sup> mutants. A similar PML was observed in the great majority (74-100%) of NR-MITF/+;Chx10<sup>−/−</sup> animals in which the dose of MITF:Chx10 genes was high. In these transgenic mice the NR was properly specified and initially normal in appearance, but development of the ciliary margin, which forms the boundary of the NR and RPE. In any case, these results provide strong evidence that Chx10 and Mitf act antagonistically in the regulation of retinal cell identity.

The inability of FGF to change the RPE to a NR in Chx10<sup>−/−</sup> optic vesicles indicates that Chx10 transduces the surface ectodermal FGF signal and represses Mitf in the OV. Our proposal that FGF and Chx10 act within a single pathway is supported by the similar neuroretinal phenotypes – a 'salt and pepper' mixture of pigmented (presumed RPE) cells and un-pigmented (presumed NR) cells – observed in both the majority of Chx10<sup>−/−</sup> mutants in a mixed genetic background and in developing chick eyes from which the proposed FGF source (the surface ectoderm) was removed (Hyer et al., 1998). That cells of the OV lacking either Chx10 or an FGF signal appear to become either NR or RPE cells further argues that the FGF→Chx10→Mitf pathway we identified is necessary for neuroretinal maintenance rather than specification. The disorganization of the neuroretinal and RPE...
cells in optic vesicles without FGF or Chx10 also highlights the need for this pathway in organizing and patterning the already-specified NR and RPE in the developing OV.

The role of FGF in patterning and organizing the OV was defined by in vitro loss-of-function studies (Hyer et al., 1998; Nguyen and Arnheiter, 2000). By contrast, FGF overexpression induces the transdifferentiation of RPE into NR (Fig. 6B) (Hyer et al., 1998; Nguyen and Arnheiter, 2000; Park and Hollenberg, 1989). This contradiction may be resolved by a model in which FGFs function redundantly in eye development, consistent with the lack of phenotypes in Fgf1 and/or Fgf2 mutants (Dono et al., 1998; Miller et al., 2000; Ortega et al., 1998). In this model, some FGFs, including at least FGF1 and FGF2 from the surface ectoderm (Nguyen and Arnheiter, 2000) organize and pattern the OV by activating Chx10. Subsequently, Chx10 or other FGF-induced genes in the NR may then activate Fgf8 (Vogel-Hopker et al., 2000; Fgf9 (Zhao et al., 2001) and/or Fgf15 (McWhirter et al., 1997; Thut et al., 2001) to further develop and maintain the NR. It is entirely conceivable, however, that Fgf8, Fgf9 or Fgf15 also act upstream of Chx10, as they will activate the same signaling cascades as FGF1 or FGF2. Indeed, FGFs themselves may not be the surface ectodermal signals that organize the OV. Rather, the FGFs may mimic another surface ectodermal ligand by activating the FGF receptor tyrosine kinase signaling pathway. However, no other known ligand is capable of causing RPE-to-NR transdifferentiation (including EGF, insulin, NGF, Fgf8, Fgf9 and TGFß1) (Nguyen and Arnheiter, 2000). We suggest, therefore, that FGFs or other unidentified molecule(s) organize the OV by activating Chx10, which then represses Mitf.

The mechanisms regulating retinal cell identity

On the basis of our work, we propose that major function of Chx10 is to prevent Mitf from imposing an RPE cell identity on the developing NR (Fig. 7). Alternatively, Chx10 may also have pro-neuroretinal functions apart from the repression of Mitf, and the balance of the Chx10-dependent neuroretinal and Mitf-dependent RPE regulatory activities is a key determinant of retinal cell identity. Although Chx10 and Mitf are essential for the maintenance of retinal cell identity, it has been established that other genes participate in regulating cell identity, including the early eye transcription factor SIX6/Optix (Toy et al., 1998), FGF family members, Gas1 (Lee et al., 2001) and Ap2a (West-Mays et al., 1999).

A recent study, complimentary to the work reported here, has also demonstrated that the Chx10(+/−;lor-J) NR undergoes transdifferentiation to become an RPE-like structure (Rowan et al., 2004).

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/131/1/177/DC1

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