Transdifferentiation of the retina into pigmented cells in ocular retardation mice defines a new function of the homeodomain gene Chx10

Sheldon Rowan1, C.-M. Amy Chen1,*, Tracy L. Young1, David E. Fisher2 and Constance L. Cepko1,†

1Department of Genetics and Howard Hughes Medical Institute, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, MA 02115, USA
2Department of Pediatric Oncology, Dana Farber Cancer Institute, 44 Binney Street, Boston, MA 02115, USA
*Present address: Hydra Biosciences, 790 Memorial Drive, Suite 203, Cambridge, MA, 02139, USA
†Author for correspondence (e-mail: cepko@genetics.med.harvard.edu)

Accepted 10 June 2004
Development 131, 5139-5152
Published by The Company of Biologists 2004
doi:10.1242/dev.01300

Summary
The homeodomain transcription factor Chx10 is one of the earliest markers of the developing retina. It is required for retinal progenitor cell proliferation as well as formation of bipolar cells, a type of retinal interneuron. or1 (ocular retardation) mice, which are Chx10 null mutants, are microphthalmic and show expanded and abnormal peripheral structures, including the ciliary body. We show here, in a mixed genetic background, the progressive appearance of pigmented cells in the neural retina, concomitant with loss of expression of retinal markers. Fate mapping analysis using a multifunctional Chx10 BAC reporter mouse revealed this process to be direct transdifferentiation of retinal cells into pigmented cells. Microarray and in situ hybridization analyses revealed a complex program underlying the transdifferentiation. This program involved the expansion of expression of genes normally found only in the periphery into central regions of the eye. These genes included a transcription factor controlling pigmentation, Mitf, and the related factor Tfec (Tfec – Mouse Genome Informatics), which can activate a melanogenic gene expression program. Misexpression of Chx10 in the developing retinal pigmented epithelium (RPE) caused downregulation of Mitf, Tfec, and associated pigment markers, leading to a nonpigmented RPE. These data link Chx10 and Mitf to maintenance of the neural retina and RPE fates respectively. Further, they suggest a new role for Chx10 in maintenance of compartment boundaries in the peripheral retina.

Key words: BAC transgenic, Mitf, or1, Fate mapping, Microarray, Compartments

Introduction
The vertebrate retina and retinal pigmented epithelium (RPE) derive from the optic vesicle, an evagination of the developing forebrain. As the optic vesicle grows and encounters signals from the prospective lens ectoderm, the domains that form the neural retina and RPE become established (reviewed by Chow and Lang, 2001). The distal optic vesicle thickens and becomes the neural retina, while the immediate proximal optic vesicle gives rise to the RPE. The interface between these two domains forms the peripheral retina, a region that gives rise to several pigmented and nonpigmented structures, including the iris and ciliary body. Several transcription factors, many of them homeodomain-containing factors, have been shown to play important roles in the specification and development of the optic vesicle (Chow and Lang, 2001). Mutations in these genes in mice or humans usually lead to anophthalmia or microphthalmia (Graw, 2003). Other transcription factors contribute to the definition of optic vesicle compartments. Two notable transcription factors, Chx10 and Mitf, are expressed in the developing optic vesicle in patterns that are either temporally or spatially mutually exclusive (Bora et al., 1998; Liu et al., 1994; Nguyen and Arnheiter, 2000). Mutations of both Chx10 and Mitf are responsible for classically defined mouse ocular phenotypes affecting development of the retina or RPE, respectively.

Chx10, a paired-like homeodomain transcription factor, is expressed in the presumptive neural retina, probably in response to inductive signals from the prospective lens ectoderm (Liu et al., 1994; Nguyen and Arnheiter, 2000). In this respect, Chx10 is the earliest characterized specific marker of retinal progenitor cells. Fibroblast growth factor (Fgf) signals from the surface ectoderm may play a role in patterning the domains of the optic vesicle and may be required for Chx10 expression in the presumptive neural retina (Nguyen and Arnheiter, 2000). In mice, as well as the chick, Chx10 is expressed in what appears to be nearly all retinal progenitor cells, but is absent from all of the postmitotic cell types except for bipolar interneurons (Belecky-Adams et al., 1997; Burmeister et al., 1996; Chen and Cepko, 2000) and a subset of Müller glia (Rowan and Cepko, 2004). Mutations in Chx10 cause the ocular retardation phenotype in mice, including the or1 mutant, which has a spontaneous mutation that leads to a premature stop codon and failure to produce Chx10 protein.
(Burmeister et al., 1996). This phenotype correlates with a reduction in proliferation of retinal progenitor cells, especially those in the periphery of the retina (Bone-Larson et al., 2000; Burmeister et al., 1996). The central retina does appear to undergo differentiation, but the retina differentiates into a poorly laminated structure (Bone-Larson et al., 2000; Burmeister et al., 1996). Molecular analysis has revealed the absence of bipolar cells in these retinas, although all other cell types could be found (Burmeister et al., 1996). Several peripheral structures of the eye are abnormal in or^1 mice, including the ciliary body and iris, and the lens is cataractous (Bone-Larson et al., 2000; Tropepe et al., 2000). The ciliary body appears to be expanded at the periphery of the retina, but does not undergo proper morphogenesis. Chx10 expression has not been analyzed in these peripheral structures in the mouse. In the chick, however, Chx10 has proven to be a useful marker of cells in the peripheral retina and nonpigmented cells of the ciliary body (Fischer and Reh, 2003; Kubo et al., 2003).

It has been known for several decades that ocular retardation mutant mice can show a range of variability in severity of the phenotype, predicting the presence of genetic interactors (Osipov and Vakhрушева, 1983). In fact, genetic interactions between ocular retardation mutants and a number of microphthalmia mutants, including the fidget mouse and two different naturally occurring Mitf mutant mice, microphthalmia (Mitf^M6) and white (Mitf^Wb), have been observed (Koniukhov and Sazhina, 1985; Koniukhov and Ugol’kova, 1978; Koniukhov and Sazhina, 1966). In each case, the ocular retardation small eye and microphthalmia small eye phenotypes were ameliorated in the double mutant animals. The genetic interactions between Mitf and Chx10 mutants indicate a functional antagonism between Mitf and Chx10, a supposition supported by their mutually exclusive expression patterns. Genetic modifiers for or^1 mutants have been identified that affect the proliferation of progenitor cells, as well as the lamination of the differentiated retina. One set of modifiers was observed by crossing 129/Sv mice, the original background of or^1 mice, to Mus musculus castaneus. These mice had intermediate-sized, well-laminated retinas, but the peripheral retina remained unpatterned and the ciliary body did not form (Bone-Larson et al., 2000). A partially rescued or^1 phenotype was also observed in crosses with p27 nullizygous mice. These retinas also were intermediate in size and had lamination, although they did not generate bipolar cells (Green et al., 2003).

Mitf is a basic helix-loop-helix (bHLH)-Zip transcription factor that acts as a master regulator of pigment cell development, and is expressed throughout neural crest-derived melanocytes and the RPE (Hodgkinson et al., 1993). Mitf is initially expressed throughout the optic vesicle, but its expression is rapidly restricted to the proximal part of the optic vesicle, the presumptive RPE (Bora et al., 1998; Nguyen and Arnheiter, 2000). The restriction of Mitf expression both spatially and temporally correlates with induction of Chx10 expression in the presumptive neural retina. While Mitf is not required for specification of the RPE, it is essential for its differentiation and maintenance (Bumsted and Barnstable, 2000; Nakayama et al., 1998; Nguyen and Arnheiter, 2000). A regulatory pathway upstream of Mitf expression has been characterized involving repressor activities of Pax6 and Pax2 as well as Otx1 and Otx2 (Baumer et al., 2003; Martinez-Morales et al., 2003; Martinez-Morales et al., 2001), but the transcriptional pathway leading to repression of Mitf within the distal optic vesicle has not been identified. It is possible that either signaling upstream of Chx10 or Chx10 itself is responsible for the downregulation of Mitf in this domain. A fascinating feature of the developing RPE is its ability to transdifferentiate into retinal cells under the appropriate circumstances. This has been observed following the addition of Fgf to RPE in vivo or in culture (Guillemot and Cepko, 1992; Park and Hollenberg, 1989; Pittack et al., 1991). Most studies have focused on chick RPE, which maintains the ability to transdifferentiate at later embryonic stages. Rodent RPE has the ability to transdifferentiate into retina in culture, but this capacity is lost relatively early in development (Nguyen and Arnheiter, 2000; Zhao et al., 1995). Transgenic mice have been generated that overexpress Fgf9 in developing RPE, and these mice show dramatic RPE to retina transdifferentiation (Zhao et al., 2001). Mitf mutants show spontaneous transdifferentiation of dorsal RPE (Bumsted and Barnstable, 2000; Nguyen and Arnheiter, 2000), while mice lacking a cell cycle inhibitor, Gas1, have ventral RPE transdifferentiation (Lee et al., 2001). Mitf function appears to be a key target in transdifferentiation. In the chick, forced expression of Mitf prevents RPE transdifferentiation in the presence of Fgf, although on its own, Mitf does not cause retinal effects (Mochii et al., 1998). Mutation or overexpression of factors thought to be upstream of Mitf, such as Pax2 and Pax6, have phenotypes consistent with their action being mediated by Mitf. Thus, Mitf appears to function in part as a safeguard against transdifferentiation of the RPE into the retina.

A full understanding of the role of Chx10 in retinal development would require understanding its biochemical functions and the genes it regulates. To date this has been lacking. Two candidate Chx10 target genes have been identified. One gene, Foxn4, is co-expressed in progenitor cells with Chx10, and is highly downregulated in the or^1 mutant (Gouge et al., 2001). Another candidate gene is cyclin D1, whose RNA levels are significantly decreased in the or^1 mice, possibly leading to derepression of p27 (Green et al., 2003). In neither case is it known if these effects reflect direct regulation by Chx10. To further our understanding of the function of Chx10 and how it might act through downstream targets, we characterized the fate of cells within the Chx10-expressing domain in or^1 mutant mice. In addition, we present a microarray analysis of or^1 mutant versus wild-type retinas. We show that several genes are upregulated in the or^1 mutant, including those associated with peripheral retina development and those controlling pigmentation. Examination of the developing periphery of or^1 retinas revealed the progressive expansion of pigmented cells towards the center of the retina at the expense of retinal progenitor cells. Using a multifunctional Chx10 BAC transgenic mouse reporter, we demonstrate that these pigmented cells transdifferentiated from retinal cells. Peripheral markers also expand centrally in the or^1 retinas, suggesting a new function for Chx10 in maintaining boundaries in the peripheral retina. Finally, we show that ectopic Chx10 in the developing chick RPE causes lack of pigmentation of the RPE, perhaps through direct repression of Mitf. These studies link Chx10 and Mitf together as critical determinants in maintenance of the retina and RPE respectively.
Materials and methods

Transgenic and mutant mice

or<sup>l</sup> mice were obtained from Jackson Laboratories and were maintained on a 129/Sv background before being crossed to Chx10 BAC transgenic mice. R26R mice (a gift from R. Awatramani and S. Dynecki, Harvard Medical School, Boston, MA, USA), created by P. Soriano (Soriano, 1999), were maintained on a C57BL/6 background before being crossed to Chx10 BAC mice. Mitf<sup>gfp</sup> mice (a gift from H. Arnheiter and L. Lamoureux, NIH, Bethesda, MD, USA) have been described previously (Hodgkinson et al., 1993), and were maintained on a C57BL/6 background. Chx10 BAC transgenic mice are described elsewhere (Rowan and Cepko, 2004), and were maintained on a mixed background containing FVB, 129/Sv, SJL and C57BL/6.

Immunohistochemistry and X-gal histochemistry

For antibody staining, cryosections were prepared and stained as described previously (Chen and Cepko, 2002). Primary antibodies used were 1:1000 rabbit anti-GFP (Molecular Probes) and 1:500 rabbit anti-β-gal (5′3′) Secondary antibodies were used 1:250 Cy2- or Cy3-conjugated goat anti-rabbit (Jackson Immunologicals). Following antibody staining, 4′,6-diamidino-2-phenylindole (DAPI) was applied to stain nuclei (Sigma), and the sections were coverslipped and mounted in Gel/Mount (Biomeda). Dissociated cells were prepared by removing the lens and cornea from E17.5 or<sup>l</sup> eyes and dissociating the remaining tissue using papain (Worthington) as described previously (Chen and Cepko, 2002). Tissue sections or dissociated cells were stained for β-galactosidase using standard methods (Kwan et al., 2001). Tissue was stained overnight at 37°C, washed in PBS, coverslipped, and mounted in Gelvatol (Air Products).

Microarray analysis

Retinal RNA samples were isolated from E12.5 or E13.5 or<sup>l</sup> mutant embryos using the Trizol reagent (Gibco). Retinal RNA samples of E13.5 wild-type 129/Sv or E12.5 Swiss Webster were used as comparisons. Each RNA sample was pooled from an entire litter. Care was taken to avoid contamination from the RPE and lens. 0.3-0.7 µg of total RNA was RT-PCR amplified for 14 to 18 cycles using the SMART amplification kit according to the manufacturer’s instructions (Clontech). PCR-amplified cDNA from each experiment and control pair was Klenow labeled with Cy5 and Cy3, respectively, and color swapped with Cy3 and Cy5, respectively as described (Livesey et al., 2000). Labeled probe was then hybridized to microarray slides spotted with 11,500 cDNA clones from the brain molecular anatomy project (BMAP) library (kind gift of B. Soares, University of Iowa, see http://trans.nih.gov/bmap/index.htm for details) and 500 cDNA clones of known identity from our lab collection (list available by request). Slides were printed and hybridized as described (Dyer et al., 2003; Livesey et al., 2004). Slides were then scanned on an Axon Instruments GenePix 4000 scanner and images were analyzed using the GenePix Pro software package. Gene expression data were uploaded to the AMAD database for data management and filtering. Gene expression ratios were normalized after filtering the data to remove low-intensity and poor-quality spots.

In situ hybridization

Section in situ hybridization was performed as previously described (Murtaugh et al., 1999) using 20 µm cryosections from OCT-embedded tissue. Whole-mount in situ hybridization was performed as previously described (Chen and Cepko, 2000). Riboprobes labeled with DIG were detected with NBT/BCIP (Sigma). Riboprobes, gene nomenclature, and relevant references are described in Table S1 (supplementary material).

Cloning of a partial chick Tfec cDNA

Total RNA from E4 chicks was isolated using the Trizol reagent according to the manufacturer’s instructions (Life Technologies) and cDNA was generated using superscript II reverse transcriptase (Life Technologies). cDNA was amplified using degenerate primers to the Mitf family bHLH domain as described elsewhere (Rehli et al., 1999). PCR products were subcloned and sequenced to determine their identity: 3/16 colonies coded for a bHLH domain that shared higher homology to Tfec orthologs than Mitf. The remaining clones encoded Mitf. 5′ RACE was performed using the Marathon cDNA Amplification Kit (Clontech) with the following gene-specific primer: 5′-TCACACGAGATACGCGGAGCAATGG-3′ to obtain a partial chick Tfec cDNA, which was subcloned into pCR2.1 (Invitrogen) and sequenced. This sequence has been submitted to Genbank as accession number AY502941. Using available EST data, a full-length chick Tfec cDNA was compiled and the nucleotide sequence data are available in the Third Party Annotation Section of the DDBJ/EMBL/GenBank databases under the accession number TPA: BK004078

In ovo electroproportion

Electroportations were performed by injecting 0.5 µg of pMiw-Chx10 (1.3 Kb EcoRI/KpnI coding sequence fragment) along with 0.25 µg pMiw-GFP expression vector (Schulte et al., 1999) into the right optic vesicle of HH stage 9-10 chick embryos as described previously (Chen and Cepko, 2002). Embryos were harvested at stage 15/16. Electroporated embryos with normal eye sizes and strong GFP fluorescence (visualized on a Leica MZFLIII dissecting microscope) were further analyzed by whole-mount in situ hybridization analysis.

Results

Progressive hyperpigmentation in the or<sup>l</sup> eye

Changes in rates of proliferation and differentiation in the periphery of the or<sup>l</sup> retina have been described in the 129/Sv genetic background (Burmeister et al., 1996). In the studies presented here, or<sup>l</sup> animals on the 129/Sv background were crossed into a mixed genetic background that contained contributions from SJL, C57BL/6, FVB and 129/Sv strains. In contrast to crosses of or<sup>l</sup> mutants with Mus musculus castaneus or p27 nullizygous mice, which have ameliorated or<sup>l</sup> phenotypes, a more severe phenotype was observed. Differences in eye size were apparent at E11.5 (data not shown), and by E14.5, the or<sup>l</sup> eyes were significantly smaller than or<sup>l/+</sup> littermates (Fig. 1A,B). The peripheral of the retina was particularly thin, consistent with a decrease in the proliferation rate in the periphery. The peripheral RPE appeared to expand into the neural retina as pigment granules were detected (Fig. 1B, arrow). This expansion of pigmented cells continued more centrally as the eye continued to develop (Fig. 1D,F, arrows). In the periphery of the retina, extensive pigmentation was observed, resulting in what appeared to be a trilayered structure at P1 (Fig. 1F). More centrally, the pigmentation in the retina was lighter. The sections shown in Fig. 1 and those that were analyzed by in situ hybridization were taken from central parts of the or<sup>l</sup> eye and showed a less dramatic extent of pigmentation than is typically observed in the rest of the eye. Attempts to dissect retinas and RPE in older or<sup>l</sup> mice frequently proved unsuccessful. Some eyes had very thin retinas surrounded by masses of pigment cells. Sections through a P15 or<sup>l</sup> mouse revealed that virtually the entire retina had become pigmented in a multilayered structure (Fig. 1G,H). In some central sections, a thin layer of nonpigmented cells could be observed (Fig. 1H, arrowheads).
Transdifferentiation of the retina into pigmented cells in or^l^ mutants

While expansion of the ciliary body has been characterized before in or^l^ mice, the source of the expanded population was not clear. We sought to determine whether the pigmented cells in the or^l^ retina arose via transdifferentiation of the retina, using a bacterial artificial chromosome (BAC) transgenic reporter mouse expressing both enhanced green fluorescent protein (GFP) fused to Cre recombinase, and alkaline phosphatase, under Chx10 control (Fig. 2A). These mice were crossed with the R26R reporter mouse that expresses β-galactosidase (β-gal) following Cre-mediated excision of a transcriptional stop cassette (Fig. 2A), and therefore permanently marks progeny of Cre-expressing cells. Thus, cells that expressed Chx10 at the time of analysis could be detected using GFP (localized to the nucleus), while all cells that derived from Chx10-expressing cells could be analyzed for expression of β-gal using either histochemical X-gal staining or by anti-β-gal staining.

In or^l^/P1 mice, GFP was readily detectable throughout the outer neuroblastic layer (ONBL) in the retina, where progenitor cells are located, and in the non-pigmented ciliary body epithelium (npcbe) (Fig. 2C). Consistent with early expression of Chx10 throughout progenitor cells, all of the different cell layers in the retina were β-gal-positive as determined by X-gal staining (Fig. 2B) or antibody staining for β-gal protein (Fig. 2G). The npcbe also stained positive for β-gal, with high levels transcribed from the Rosa26 promoter in the more central part of the npcbe (Fig. 2B,G). Chx10 protein was detected in the same pattern as GFP in or^l^/+ mice (data not shown). GFP and β-gal were not detected in pigmented cells in the developing ciliary body or in stromal cells outside the ciliary body. Analysis in or^l^/or^l^ P1 eyes revealed the presence of GFP in retinal cells in the central part of the retina (Fig. 2E). The levels of GFP were markedly lower in or^l^/or^l^ retinas than or^l^/+ retinas, and within or^l^/or^l^ retinas, the levels were higher in the central retina than the periphery, where pigmentation was observed. However, GFP was detected in the lightly pigmented cells of or^l^/or^l^ retinas near the periphery, showing that these cells were derived from Chx10-expressing cells (Fig. 2D,E arrows). Lack of GFP reporter activity in the heavily pigmented cells could indicate that GFP reporter activity was repressed in these tissues, that their origin was not a Chx10-expressing cell type, or that GFP fluorescence was obscured by the heavy pigmentation. Therefore, the R26R fate mapping cross was performed to determine what cells derived from Chx10-expressing cells. X-gal staining was found to be punctate and was detected in subsets of pigmented cells (Fig. 2D). To better visualize the extent of β-gal expression, immunofluorescence was performed using an anti-β-gal antibody. Analysis of the heavily pigmented peripheral region of the or^l^ eye, from the same section as Fig. 2D and at higher magnification, revealed that many pigmented cells did express β-gal protein, demonstrating their Chx10-expressing origin (Fig. 2I, also see below). Notably, the RPE and parts of the pigmented ciliary body did not express β-gal in these fate-mapping experiments. Overall, the presence of a largely β-gal-positive peripheral showed the extent of retina to pigmented cell transdifferentiation in the or^l^ eye.

To better illustrate the pattern of neopigmentation, or^l^ mutant Chx10 BAC transgenic animals were immunofluorescently stained for GFP at P0 (Fig. 2K) or P17 (Fig. 2M). In both cases, regions of mostly non-pigmented neuroepithelium included some lightly pigmented cells (Fig. 2J,L arrows). These regions expressed high amounts of GFP (Fig. 2K,M), demonstrating that they expressed Chx10. Furthermore, GFP-positive nuclei could be observed surrounded by pigment granules. At P1, the newly transdifferentiating region was still peripherally located within the eye, whereas by P17 it was more central, illustrating the progressive feature of transdifferentiation in the or^l^ eye.

In order to unambiguously determine whether β-gal- or GFP-positive cells were pigmented, as well as quantify the extent of transdifferentiation, E17.5 Chx10 BAC/R26R double

Fig. 1. Progressive pigmentation in or^l^ retinas. (A-H) Eyes were sectioned to analyze for pigmentation in or^l^/+ heterozygous (A,C,E) or or^l^/or^l^ homozygous (B,D,F,G,H) littermates. Ages are indicated on the figure. (A,B) Insets show a full section of the eye (note scale bar differences). (B,D,F) Arrows indicate the central limit of ectopic pigmentation in the retinas of or^l^ mutants. (D) The lens is marked by a thin black line in the or^l^ eye at E17.5. (G,H) The peripheral retina in or^l^ mutants shows ectopic pigmentation, increasing over time until most of the retina appears pigmented at P15. (H) The presumptive retina is marked with arrowheads. Scale bar is 100 μm.
Transdifferentiation in \( or^J \) mice

Examination of \( \beta \)-gal-positive cells under Nomarski illumination revealed that many of these cells (59/100) were pigmented, with some of them heavily pigmented (Fig. 2N, arrowheads). Similar analysis of GFP-positive cells also indicated that a significant, but smaller number of cells (18/100), contained pigment granules. These GFP-positive pigmented cells were more lightly pigmented than typical pigmented cells (Fig. 2Q arrowhead and insert) and were probably cells in the process of transdifferentiation. Histochemical staining of dissociated cells for \( \beta \)-gal also revealed cells that were clearly pigmented and expressing \( \beta \)-gal (Fig. 2R,S).

Identification of genes affected in the \( or^J \) mutant
To identify genes affected in the \( or^J \) mutant, a microarray analysis was performed comparing wild type to \( or^J \) retinas.
RNA samples were compared at E12.5 or E13.5 in development, very early time points following the onset of Chx10 expression. At this time, the difference in eye size is small and transdifferentiation is not observed (data not shown). Table 1 shows the genes that were upregulated greater than 1.7-fold in the orJ versus wild-type retinas and Table 2 shows the genes that were downregulated 0.5-fold compared to wild-type retinas. These cutoffs were arbitrarily determined, and a number of genes with ratios below the cutoffs showed expression differences as analyzed by in situ hybridization (see below). A more complete list of putatively affected genes is shown in Tables S2 and S3 (supplementary material).

Table 1. Genes upregulated in orJ mutants by microarray analysis

<table>
<thead>
<tr>
<th>UNIQID</th>
<th>Name</th>
<th>E12.5 Average</th>
<th>E13.5 Average</th>
<th>Average</th>
<th>s.d.</th>
</tr>
</thead>
<tbody>
<tr>
<td>AI852420</td>
<td>Leucine-rich repeat protein 3, neuronal</td>
<td>2.69</td>
<td>3.55</td>
<td>3.27</td>
<td>0.62</td>
</tr>
<tr>
<td>AI851397</td>
<td>Gap-junction membrane channel protein α1</td>
<td>3.06</td>
<td>2.563</td>
<td>2.73</td>
<td>0.54</td>
</tr>
<tr>
<td>AI843797</td>
<td>Hemoglobin α, adult chain 1</td>
<td>2.04</td>
<td>2.678</td>
<td>2.47</td>
<td>0.88</td>
</tr>
<tr>
<td>AI845279</td>
<td>RIKEN cDNA 2700084L06 gene</td>
<td>2.005</td>
<td>2.585</td>
<td>2.39</td>
<td>0.93</td>
</tr>
<tr>
<td>AI854349</td>
<td>Serum/glucocorticoid regulated kinase</td>
<td>2.41</td>
<td>2.345</td>
<td>2.37</td>
<td>0.37</td>
</tr>
<tr>
<td>AI854777</td>
<td>I-xB α chain mRNA</td>
<td>1.915</td>
<td>2.558</td>
<td>2.34</td>
<td>0.95</td>
</tr>
<tr>
<td>AI847573</td>
<td>Protease, serine, 25</td>
<td>1.455</td>
<td>2.688</td>
<td>2.28</td>
<td>1.29</td>
</tr>
<tr>
<td>AI838302</td>
<td>CD63 antigen</td>
<td>1.575</td>
<td>2.58</td>
<td>2.25</td>
<td>0.58</td>
</tr>
<tr>
<td>AI846734</td>
<td>Growth arrest specific 1 (Gas1)</td>
<td>2.28</td>
<td>1.598</td>
<td>1.83</td>
<td>0.41</td>
</tr>
<tr>
<td>AI838156</td>
<td>Carbonic anhydrase 8</td>
<td>1.195</td>
<td>2.133</td>
<td>1.82</td>
<td>0.99</td>
</tr>
<tr>
<td>AI848419</td>
<td>H3 histone, family 3A</td>
<td>1.58</td>
<td>1.903</td>
<td>1.79</td>
<td>0.34</td>
</tr>
<tr>
<td>AI852943</td>
<td>Chondroitin sulfate proteoglycan 5</td>
<td>1.585</td>
<td>1.82</td>
<td>1.74</td>
<td>0.32</td>
</tr>
<tr>
<td>AI854206</td>
<td>Hexosaminidase B</td>
<td>1.655</td>
<td>1.785</td>
<td>1.74</td>
<td>0.23</td>
</tr>
<tr>
<td>AI834768</td>
<td>RIKEN cDNA 1810027120 gene</td>
<td>1.835</td>
<td>1.66</td>
<td>1.72</td>
<td>0.29</td>
</tr>
<tr>
<td>AI845761</td>
<td>Rho, GDP dissociation inhibitor (GDI)</td>
<td>1.72</td>
<td>1.705</td>
<td>1.71</td>
<td>0.25</td>
</tr>
<tr>
<td>AI853186</td>
<td>Fatty acid binding protein 3, muscle and heart</td>
<td>1.775</td>
<td>1.658</td>
<td>1.7</td>
<td>0.48</td>
</tr>
<tr>
<td>AI850915</td>
<td>UDP-glucose ceramide glucosyltransferase</td>
<td>2.2</td>
<td>1.448</td>
<td>1.7</td>
<td>0.41</td>
</tr>
<tr>
<td>AI851397</td>
<td>Gap-junction membrane channel protein α1</td>
<td>2.005</td>
<td>2.585</td>
<td>2.39</td>
<td>0.93</td>
</tr>
<tr>
<td>AI852420</td>
<td>Leucine-rich repeat protein 3, neuronal</td>
<td>2.69</td>
<td>3.55</td>
<td>3.27</td>
<td>0.62</td>
</tr>
</tbody>
</table>

Genes listed are those upregulated at least 1.7-fold. The last three genes in the table were confirmed to be upregulated by in situ hybridization analysis, although they were below the 1.7-fold cutoff. Genes shown in bold have been analyzed by in situ hybridization. Genes listed without unique ID are full-length clones from within our laboratory. The average of E12.5 was determined from one hybridization and its color swap. The average from E13.5 was determined from two hybridizations and their color swaps. The total average was calculated from all six data points and is shown with its standard deviation (s.d.). Individual data points and Unigene numbers are included in Table S2.

Downregulated genes (Table 2) with known functions fitted into a more definite pattern than upregulated genes. The bHLH transcription factors, Neurod1 and Math3, are required for neuronal differentiation and were highly downregulated. Several other genes found to be downregulated corresponded to structural genes and known markers (e.g. Gap43 and Snap25) of neurons. In situ hybridization analysis of a number of genes identified in Table 2 showed significant downregulation of these genes (see below). Two markers of cell cycle progression, cyclin D1 and E2f1, were downregulated according to the microarray analysis, although less so than the cutoff assigned to Table 2. Cyclin D1 has previously been shown to be downregulated in orJ retinas (Green et al., 2003).

In situ hybridization analysis of affected genes

In order to verify that some genes identified as differentially regulated by microarray analysis were changed in expression (in bold). The genes that were tested by in situ hybridization from the array list were verified to be differentially expressed in the orJ mutant (see below). One particular gene, Crhbp, evaluated because of a known expression pattern in the peripheral retina (Blackshaw et al., 2004), was verified to be upregulated in the orJ mutant, even though the fold upregulation by microarray analysis was 1.29. This suggested that a high percentage of the genes identified by microarray analysis might be significantly differentially expressed in the orJ mutant.
level, and to better identify genes that may play a role in transdifferentiation, in situ hybridization analysis was performed (Fig. 3; see Fig. S1 in the supplementary material). Several additional genes not present on the microarray were analyzed because of their previously characterized expression patterns. In situ hybridizations were performed at E14.5, when early phenotypes were apparent, and at E17.5, when transdifferentiation was at an intermediate stage (see Fig. 1).

To better describe the patterns that were observed, the peripheral eye was subdivided into four separate zones (see Fig. 4A). Zone 1 is in the peripheral retina and normally does not express pigment markers. Zone 2 is peripheral to zone 1 and contains non-pigmented cells that directly abut pigmented cells. Zone 3 consists of pigmented cells at the periphery of the RPE, where the tissue is thickened. Zone 4 contains loosely packed pigment cells, presumably neural crest-derived melanocytes. Within the neural retina (i.e. the tissue that abuts zone 1 and extends more centrally), specific expression patterns were observed in the outer neuroblastic layer (ONBL), containing a mixture of progenitor cells, newly postmitotic cells, and differentiating photoreceptors, and the inner neuroblastic layer (INBL), where postmitotic amacrine and ganglion cells reside. Summaries of the expression patterns in Fig. 3 are listed in Fig. 4B. References of known expression patterns or functions are included in Table S1 (supplementary material).

Class I: ONBL-expressed genes
In or/+/ retinas, Chx10 was expressed at E14.5 and E17.5 throughout the ONBL in progenitor cells, and extended peripherally into zone 1, but was not expressed in zone 2 (Fig. 3A). In or/− or J/− eyes, its expression was restricted centrally at E14.5, but its expression levels appeared normal. At E17.5, Chx10 was only detected in a few cells in the center of the or/− mutant retina. A large number of genes expressed in the ONBL exhibited similar patterns to that of Chx10 in the or/− mutant retina. These included genes controlling retinal progenitor cell function or proliferation (Notch1, E2f1, cyclin E1, cyclin D1), bHLH transcription factors (Neurod1 and Math5), and genes associated with cell cycle exit (Btg2 and Dll1) (see Fig. S1A-H in the
supplementary material). A number of these genes (Neurod1, Btg2, cyclin D1 and E2f1) were identified as dependent on Chx10, by the microarray analysis as described above. Based on in situ hybridization analysis, RNA levels did not appear significantly altered within expressing cells, suggesting that the decrease of RNA levels was due to a decreased number of cells expressing these genes. Together, these data indicate a progressive loss of retinal progenitor cell identity and neurogenesis in the orl mutant.

Most genes analyzed with ONBL expression patterns were not expressed in the zones of the peripheral eye in orl/+ mice, or were only expressed transiently in the peripheral retina (E2f1, cyclin D1 and cyclin E1) with the exception of Chx10. To determine whether Chx10 was differentially regulated in orl mutants from other ONBL-expressed genes because of its peripheral expression, Hes1, a gene expressed similarly to Chx10 was analyzed (see Fig. S11 in the supplementary material). Hes1 expression in the orl mutant behaved like that of Chx10 rather than other ONBL-expressed genes (see Fig. S11 in the supplementary material), suggesting that peripheral gene expression was differentially affected in the orl mutant.

Class II: INBL- and INBL+ONBL-expressed genes

The loss of expression of genes enriched in retinal progenitor cells, as well as genes that play a role in neurogenesis, suggested that the genesis of retinal neurons might be affected in orl mutant retinas. Since a number of genes found by microarray analysis to be downregulated in orl mutants were markers of early-born neurons, we used in situ hybridization to examine genes expressed in the INBL. Gap43, Brn3b (Pou4f2 – Mouse Genome Informatics) and Snap25 were expressed in the INBL at E14.5 and E17.5 in orl/+ heterozygotes (Fig. 3C and Fig. S1J,K in the supplementary material). Gap43 was restricted to only a few cells in the INBL in the central retina in orl mutant retinas (Fig. 3C). Similar results were obtained for Brn3b and Snap25 (see Fig. S1J,K in the supplementary material). This decrease in the number of cells expressing markers of retinal ganglion cells and amacrine cells correlated with the restricted location and reduced number of cells expressing ONBL markers, and was further indicated by the finding of a hypocellular INBL in orl mutant retinas.

Statl, one of three stathmin family member genes observed by microarray analysis to be downregulated, was evaluated by in situ hybridization. In orl/+ eyes, stathmin 1 was expressed throughout the INBL and in many ONBL cells at E14.5 and E17.5 (see Fig. S1L in the supplementary material). In orl mutant retinas, stathmin 1 was expressed in a similar fashion to the combination of Gap43/Brn3b and Neurod1, and in a much-reduced number of cells, which was especially apparent at E17.5 (see Fig. S1L in the supplementary material). The paired box transcription factor Pax6 was expressed throughout the ONBL and INBL, and at high levels in zones 1, 2 and 3 (see Fig. S1M in the supplementary material). Unlike statl 1, Pax6 appeared slightly upregulated in orl mutant retinas and persisted even in pigmented regions. These data, like the comparison of Chx10 and Hes1 with other ONBL-expressed genes, suggest that the peripheral component of Pax6 may have contributed to the high expression level of Pax6 in the orl mutant retina.

Class III: Peripherally-expressed genes

Gas1 and p57 were both found to be significantly upregulated in the orl mutant microarray analysis. In situ detection of Gas1 at E14.5 in orl/+ eyes showed expression exclusively in the peripheral retina in zones 1 and 2, but not in pigmented cells (Fig. 3D). At E17.5, it remained highly expressed in these zones and was also expressed at low levels in the ONBL in orl/+ eyes. In orl eyes, Gas1 was highly upregulated in the peripheral retina and expressed at lower levels in more central parts (Fig. 3D). At E17.5, Gas1 was highly upregulated throughout the entire orl retina. Another gene also functioning as a cell cycle inhibitor, p57, was highly expressed in the peripheral retina and showed a similar expression pattern to Gas1 in orl/+ and orl eyes (see Fig. S1N in the supplementary material). A gene not represented on the microarrays, but with known important roles in ciliary body development, Otx1, was analyzed by in situ hybridization, and it too showed an expression pattern in orl/+ and orl eyes highly similar to Gas1 and p57 (see Fig. S1O in the supplementary material).

To address the role of gene expression specific to zone 1, Wdf6, a potential tumor suppressor gene and a peripheral retinal marker (J. Trimarchi and C.L.C., unpublished), was analyzed by in situ hybridization (Fig. 3E). The limits of Wdf6 expression defined zone 1 (Fig. 3E, arrows). In the orl retina...
Table 2. Genes downregulated in or/ mice by microarray analysis

<table>
<thead>
<tr>
<th>UniQID</th>
<th>Name</th>
<th>E12.5 Average</th>
<th>E13.5 Average</th>
<th>Average</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>AI852799</td>
<td>Synuclein γ</td>
<td>0.29</td>
<td>0.23</td>
<td>0.25</td>
<td>0.06</td>
</tr>
<tr>
<td>AI849972</td>
<td>Neurogenic differentiation 1 (Neurod1)</td>
<td>0.23</td>
<td>0.26</td>
<td>0.25</td>
<td>0.04</td>
</tr>
<tr>
<td>AI841966</td>
<td>Protein kinase, AMP-activated, γ1 chain</td>
<td>0.33</td>
<td>0.23</td>
<td>0.26</td>
<td>0.06</td>
</tr>
<tr>
<td>AI836519</td>
<td>Stathmin-like 2</td>
<td>0.29</td>
<td>0.27</td>
<td>0.28</td>
<td>0.03</td>
</tr>
<tr>
<td>AI840806</td>
<td>ALL1-fused gene from chromosome 1q</td>
<td>0.31</td>
<td>0.29</td>
<td>0.3</td>
<td>0.05</td>
</tr>
<tr>
<td>AI841303</td>
<td>Growth associated protein 43 (Gap43)</td>
<td>0.33</td>
<td>0.39</td>
<td>0.37</td>
<td>0.06</td>
</tr>
<tr>
<td>AI835930</td>
<td>EST</td>
<td>0.28</td>
<td>0.46</td>
<td>0.4</td>
<td>0.12</td>
</tr>
<tr>
<td>AI848983</td>
<td>Microtubule-associated protein 1 B</td>
<td>0.44</td>
<td>0.38</td>
<td>0.4</td>
<td>0.06</td>
</tr>
<tr>
<td>AI851775</td>
<td>B-cell translocation gene 2, anti-proliferative (Btg2)</td>
<td>0.17</td>
<td>0.53</td>
<td>0.41</td>
<td>0.19</td>
</tr>
<tr>
<td>AI841267</td>
<td>Stathmin-like 3</td>
<td>0.57</td>
<td>0.34</td>
<td>0.42</td>
<td>0.14</td>
</tr>
<tr>
<td>AI843116</td>
<td>Neurogenic differentiation 4 (Math4)</td>
<td>0.44</td>
<td>0.44</td>
<td>0.43</td>
<td>0.07</td>
</tr>
<tr>
<td>AI849592</td>
<td>Melanoma antigen, family D, 1</td>
<td>0.44</td>
<td>0.43</td>
<td>0.44</td>
<td>0.08</td>
</tr>
<tr>
<td>AI844983</td>
<td>ELAV (Drosophila)-like 4 (Hu antigen D)</td>
<td>0.43</td>
<td>0.5</td>
<td>0.47</td>
<td>0.06</td>
</tr>
<tr>
<td>AI848368</td>
<td>Tubulin, α8</td>
<td>0.44</td>
<td>0.5</td>
<td>0.49</td>
<td>0.08</td>
</tr>
<tr>
<td>AI850105</td>
<td>Synaptosomal-associated protein 25 (Snap25)</td>
<td>0.44</td>
<td>0.52</td>
<td>0.49</td>
<td>0.12</td>
</tr>
<tr>
<td>AI841233</td>
<td>Potassium voltage-gated channel, Shaw-related, 4</td>
<td>0.51</td>
<td>0.49</td>
<td>0.5</td>
<td>0.06</td>
</tr>
<tr>
<td>AI846286</td>
<td>β-1,3-glucuronyltransferase 1</td>
<td>0.57</td>
<td>0.48</td>
<td>0.5</td>
<td>0.12</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td></td>
<td>0.35</td>
<td>0.59</td>
<td>0.51</td>
<td>0.17</td>
</tr>
<tr>
<td>E2F1 (human)</td>
<td></td>
<td>0.78</td>
<td>0.88</td>
<td>0.84</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Genes listed are those downregulated at least 0.5-fold, an arbitrary cut-off point. The last two genes are significantly downregulated, as determined by in situ hybridization analysis. Genes shown in bold were analyzed by in situ hybridization. Genes listed without unique IDs are full-length clones from within our laboratory clones. Note that the E2F-1 clone on the array was a full-length human cDNA. The average from E12.5 was determined from one hybridization and its color swap. The average from E13.5 was determined from two hybridizations and their color swaps. The total average was calculated from all six data points and is shown with its standard deviation (s.d.). Individual data points and Unigene numbers are included in Table S3.

Class IV: RPE transcription factors

To examine changes in genes expressed in zones 2 and 3 and evaluate factors involved in controlling pigmentation, Mitf, Tfec and Otx2 were examined by in situ hybridization. In or/+ eyes, Mitf was expressed throughout the RPE at E14.5 and at higher levels in peripheral RPE in zone 3 and zone 2 as well (Fig. 3F). Mitf was also expressed in migrating melanoblasts and in zone 4 at E17.5. Mitf expression abutted Chx10 in the peripheral retina at E14.5 and E17.5, and was never detected in wild-type retinas. In the or/ retina, Mitf was ectopically expressed at high levels in the retina, in a manner similar to Gas1, while there was a lessened expression in the center. However, by E17.5, Mitf was highly upregulated throughout the entire or/ retina (Fig. 3F). Tfec was the most upregulated gene as evaluated by microarray analysis. At E14.5, in or/+ eyes, Tfec was barely detectable in zone 2 and was not detected at E17.5 (apparent staining is likely to be background) (Fig. 3G). In the or/ retina, Tfec was ectopically expressed in the retina, but more peripherally than Mitf and at lower levels. At E17.5, Tfec was moderately expressed throughout the entire retina in or/ eyes. In or/+ eyes (Fig. 3G), Otx2 was expressed in both the ONBL and RPE, including zone 3, at E14.5, but not the peripheral retina (see Fig. S1R in the supplementary material). The Otx2 expression pattern mimicked that of ONBL-specific genes in the or/ retina, except that Otx2 was detected in the periphery in lightly pigmented cells in the peripheral retina only at E14.5.

Activation of Mitf target genes in transdifferentiation

To address whether ectopic Mitf and Tfec in the or/ retina was sufficient to activate their cognate target genes, Mitf target genes were analyzed by in situ hybridization. Mitf target genes were analyzed by in situ hybridization at E17.5, during an intermediate stage of transdifferentiation (Fig. 5). Trpm1 is a direct target of Mitf and depends on Mitf for expression in the developing RPE. Like Mitf, Trpm1 was expressed in zone 2 in or/+ heterozygotes and was ectopically expressed throughout the retina in or/ mutants, including unpigmented cells (Fig. 5A). In contrast, Dct was highly expressed in pigmented cells in or/ retinas as well as in regions of non-pigmented cells in the central retina. Its expression correlated with regions that had lost neural retinal identity, but had not yet undergone pigmentation, and complemented the expression of ONBL and INBL markers (Fig. 5B). Tyrosinase, the rate-limiting enzyme in melanin synthesis, was only expressed in pigmented cells in both or/+ heterozygotes and or/ mutant retinas (Fig. 5C). Therefore, different classes of Mitf target genes define a progressive program of transdifferentiation beginning with direct Mitf target genes like Trpm1, then proceeding to express Dct, and finally tyrosinase, coinciding with the appearance of pigment. This ordered gene expression progression was also observed at E14.5 in or/ mutant retinas (see Fig. S2A-C in the supplementary material).

While RPE genes, including Mitf and melanin synthesis
enzymes, were generally not represented on the microarray, one that was on the array and showed orl upregulation was Cdb3 (a melanoma antigen in humans). By in situ hybridization, Cdb3 was upregulated in the orl retina, similarly to Trpm1 (see Fig. S2D,E in the supplementary material), and was dramatically downregulated in Mitf-deficient RPE, especially at E14.5 when Tfec was only weakly expressed (see Fig. S3A,C in the supplementary material), suggesting it is a direct target of Mitf and possibly Tec. In contrast, Tfec expression was not dependent on Mitf (see Fig. S3B in the supplementary material), and Otx2 was neither upregulated in the orl retina, nor was it altered in Mitf-deficient RPE (see Fig. S3E in the supplementary material). Other genes expressed in zone 1 and zone 2, including p57, Pax6 and Crhbp were also unaffected in the peripheral retina in Mitf nullizygous mice (see Fig. S3F-H in the supplementary material), suggesting their upregulation in the orl retina was a consequence of their peripheral expression and not indirectly through Mitf.

**Chx10 is sufficient to repress the expression of RPE genes**

Microarray and in situ hybridization analysis pointed to Mitf family members as possible key target genes for Chx10 regulation. To determine if the expression of Chx10 could lead to repression of these genes, Chx10 was misexpressed in the developing RPE in chick by in ovo electroporation. A plasmid encoding chick Chx10, along with another plasmid encoding GFP as a cotransfection reporter, were transduced into early chick optic vesicles by electroporation, and embryos were allowed to develop for 1 day in ovo (Fig. 6A). Non-transfected optic vesicles from the same embryo (Fig. 6B) or electroporations with GFP alone (Fig. 6C) were used as controls and showed identical marker expression (compare Fig. 6F,I,L,O,R,U to Fig. 6E,H,K,N,Q,T).

Electroporation of Chx10 resulted in a strong downregulation of Mitf (Fig. 6G) and Tfec (Fig. 6J). Additionally, a direct target gene of Mitf in chick RPE, Mmp115, and an additional RPE marker Dct, were also strongly downregulated upon Chx10 misexpression (Fig. 6D,M). Mmp115 was analyzed in sections and showed strong downregulation only in the transfected eye (Fig. 6W,X) and within regions of the RPE that were electroporated, as judged by GFP antibody staining. The retinal progenitor cell markers Six3 (Fig. 5P) and Chx10 (Fig. 5S) were unaffected by Chx10 misexpression. The restriction of Six3 and Chx10 to the neural retina also confirmed that transdifferentiation was not occurring upon Chx10 misexpression, in contrast to misexpression of other retinal homeobox transcription factors in the RPE (C.-M.A.C, and C.L.C., unpublished) (Toy et al., 1998). These data place Mitf and Tfec as potentially direct target genes for Chx10 repression in the absence of a direct cell fate change.

**Discussion**

**Transdifferentiation of retina to pigment cells in the orl mutant**

The data presented here demonstrate that the retina in the orl mutant mouse directly transdifferentiates into pigmented cells. This is the first description of retinal tissue becoming pigmented in vivo, whereas a number of studies have shown the opposite transition. It is difficult to know whether transdifferentiation in either direction is a physiologically relevant process, or simply the result of aberrant development. There is some suggestion that transdifferentiation of RPE could serve as a source of retinal cells in regeneration (Fischer and Reh, 2001), but there is no evidence to suggest that retinal cells ever change their identity to pigmented cells. Rather, this transdifferentiation seems to be specific to loss of Chx10. Nonetheless, individuals with diseases of the RPE might benefit from this observation when engraftment of RPE cells is being considered.

Several lines of evidence support the notion that the neural retina directly transdifferentiates into pigmented cells. However, the additional expression domain of Chx10 in the nonpigmented ciliary body epithelium raises the possibility that the transdifferentiation observed is one of nonpigmented ciliary body cells into pigmented cells. In this model, the neural retina would die and be replaced with an expanding population of pigment-fated cells. The fate mapping analysis and extensive expression studies presented here do not support this alternative model. Firstly, in situ hybridizations and microarray data do not indicate that pigment-fated cells undergo the rapid proliferation required to populate the orl retina, nor do they indicate abnormally high rates of cell death in the neural retina. Specifically, markers of proliferation, including E2f1, cyclin E1, and cyclin D1, were downregulated in regions of the orl retina undergoing pigmentation, while cell cycle inhibitors,
including p57 and Gas1, were concomitantly upregulated in these regions. Secondly, analysis of markers from the peripheral retina, particularly in zone 2, showed their expansion into parts of the retina that also expressed bona fide neural retina markers well before transdifferentiation was observed. The overall temporal pattern of gene expression and high number of fate-mapped pigmented cells even at E17.5 support the direct transdifferentiation of neural retinal cell into pigmented cells.

**A molecular program of transdifferentiation**

Genes expressed in either the RPE or different peripheral regions of the retina were upregulated in the orJ mutant retina and were identifiable by microarray analysis. By analyzing candidate genes and other genes with known expression patterns by in situ hybridization, we have formed a model for transdifferentiation that also possibly explains why it occurs progressively (Fig. 4A). The model designates zones of gene expression for purposes of description, although as discussed below, these zones may represent functional compartments (except zone 4, which is not part of the neuroepithelium). Fig. 4B is a tabular summary of some of the genes presented in this study (Fig. 3, Fig. 5 and Fig. S1,S2 in the supplementary material) and their inclusion in the different zones.

The expansion and ectopic expression of genes normally restricted to zone 2 of the peripheral retina appears to be an early event in the orJ retina. The upregulation, however, was not uniform and was stronger in the periphery than the center. While Mitf, and some of its target genes, showed broad upregulation, other melanogenic genes were restricted to peripheral regions, and thus pigmentation did not occur in all Mitf-expressing cells. Where zone 1 genes and zone 2 genes then began to overlap (shown as yellow in Fig. 4A), Dct expression became pronounced. Shortly thereafter, tyrosinase was expressed and the tissue became pigmented and adopted a zone 3-like appearance. Concurrent with pigmentation was a downregulation of genes expressed in zone 1, as well as Gas1 and p57. The next progression occurred within zone 1 which began to expand centrally. As new parts of the retina began coexpressing zone 1 and zone 2 genes, Dct expression expanded centrally and marked non-pigmented cells programmed to initiate pigmentation. The cells did not initiate pigmentation until tyrosinase, the rate-limiting enzyme in melanin production, became expressed.
It is striking how the progression of transdifferentiation occurred so slowly. Even as late as P17, there were still regions of the retina just beginning to undergo pigmentation (see Fig. 2L). The rate-limiting step appears related to the expansion of zone 1, as zone 2 completely expanded throughout the retina well before pigmentation reached this area. The central boundary of zone 1 was defined by the expression of progenitor cell genes in the ONBL. The downregulation of ONBL-expressed genes was observed in the peripheral retina prior to those regions acquiring pigmentation, and by E17.5, the entire retina appeared to lose neural identity. These events may precipitate loss of the central boundary of zone 1 and lead to the expansion of zone 1 into central parts of the retina. Determination of the ONBL-expressed genes that are responsible for defining the boundary with zone 1 will yield interesting insights into the mechanism of compartmentalization of the retina.

**Genetic modifiers of the or6 phenotype**

Modifiers of the or6 phenotype have been described that partially rescue eye size and lamination. In neither case was the peripheral or6 phenotype rescued (Bone-Larson et al., 2000; Green et al., 2003). This raised the possibility that another factor could compensate for some Chx10 functions in the central but not peripheral retina. One would then predict that in the genetic background employed here, the compensating factor was detrimentally affected, leading to a more severe phenotype. A candidate gene for this function is the Chx10 homolog, Vsx-1. Vsx-1 in other organisms including chick and zebrafish is co-expressed with Chx10 in retinal progenitor cells, but has not been detected in mammalian progenitor cells (Chen and Cepko, 2000; Chow et al., 2001; Passini et al., 1997). We performed in situ analysis for Vsx-1 and did not see it expressed in either or6/+ mice or or6 mice on any background, probably ruling out Vsx-1 as a modifying allele (data not shown).

An alternative possibility for a genetic modifier relates to the extent of upregulation of Mitf and other zone 2 genes. Mitf is normally not expressed at any level in the retina, thus Mitf upregulation in the or6 mutant may be influenced by genetic modifiers in terms of the spatial limits of Mitf expression or the absolute quantity of RNA. One might then predict that Mitf in rescued or6 mice might only be ectopically expressed in the periphery and/or may be expressed at lower levels. A further prediction is that broad and strong overexpression of Mitf in a mildly affected or6 strain would lead to a more dramatic phenotype. Interestingly, Mitf was one of the candidate genes preliminarily identified by Bone-Larson and colleagues as a modifier of the or6 phenotype (Bone-Larson et al., 2000).

Another candidate gene that might control the degrees of severity of the or6 transdifferentiation phenotype is the Mitf-related transcription factor Tfec. Normally, Tfec is only transiently expressed in the RPE and zone 2, and its ectopic expression in the retina along with Mitf might have distinct consequences. It is also notable that Tfec appeared to be upregulated less centrally than Mitf at E14.5, possibly marking the region of the retina actively undergoing transdifferentiation. Gas1 is also an interesting candidate, not only because of its characterized role as a cell cycle inhibitor, but also its functions in the RPE. Mice lacking Gas1 show transdifferentiation of the ventral RPE to neural retina, suggesting a role for Gas1 in ventral RPE identity (Lee et al., 2001). Mitf mutant mice also show transdifferentiation of RPE to neural retina, but this occurs only dorsally (Bumsted and Barnstable, 2000). Thus, the concurrent expression of Gas1 and Mitf in the retina of or6 mice may have a distinct effect on cell fate, functioning in an opposite direction to their loss-of-function phenotypes. Finally, there remains the possibility that mesenchymal cells play a role in controlling the spatial and temporal features of transdifferentiation, and these functions could be altered by genetic background.

**Transcriptional targets of Chx10**

One of the goals of or6 microarray analysis was to identify genes that may be directly controlled by Chx10. If Chx10 functioned as a transcriptional activator, one would predict that direct target genes would be downregulated in the or6 retina. A large number of downregulated transcripts were observed by microarray analysis, but the ones studied in greater detail by in situ hybridization were not necessarily downregulated on a per cell transcript level, but rather through a reduction in the number of cells expressing them. Many of these downregulated genes were markers of neurogenesis and early differentiation. Further characterization of genes downregulated in the or6 retina may give new insights into this molecular program.

Genes upregulated in the or6 retina were the focus of this study and are candidate targets for repression by Chx10, e.g. Mitf, Tfec, Gas1, p57 and Otx1. However, some upregulated genes are likely targets of Mitf-dependent activation rather than repression by Chx10. Genes like Trpm1 and Cd63 are probably upregulated through Mitf transcription. Trpm1, in particular, is transcriptionally activated in a concentration-dependent fashion by Mitf and is not expressed in Mitf mutant RPE (Miller et al., 2004). In this respect, analysis of or6 mice by microarray analysis would be an ideal way to identify new target genes of Mitf and Tfec, provided the array had a large set of RPE genes. Neither Gas1, p57 nor Tfec require Mitf for their expression and therefore their misexpression in or6 mice is probably secondary to, or independent of, Mitf expression (see Fig. S3 in the supplementary material). However, p57 is expressed elsewhere in the retina in subsets of progenitor cells, and Gas1 is expressed in the ONBL at E17.5 in cells that potentially express Chx10, so Gas1 and p57 may not be ideal candidate target genes for repression by Chx10.

The finding that misexpression of Chx10 in developing chick RPE led to a depigmentation and downregulation of Mitf, Tfec and other pigment markers further supports the notion that Mitf and Tfec are direct targets of Chx10. Overexpression of a number of transcription factors in the RPE (e.g. Six3, Six6 and Rav/Rxs) can lead to depigmentation, but this usually occurs via transdifferentiation of the RPE to retina (C.M.A.C. and C.L.C., unpublished) (Toy et al., 1998). RPE made to express Chx10 seemed to maintain proper RPE identity, at least as judged by the lack of conversion to a retinal identity as viewed through expression of Six3 and Chx10. These data further suggest that the aberrant expression of Mitf, Tfec and their target genes was not secondary to alterations in the center-periphery pattern also observed in the or6 mutant. Mitf has been implicated as a Chx10 target gene previously, based on its temporal and spatial expression in early eye development. The boundary between Chx10 and Mitf persists throughout eye development (discussed further below). An attractive model then is that
Chx10 functions in the optic vesicle to repress Mitf and permanently keep it repressed in the retina. This repression does not require continuous Chx10 expression, as mature retinal cell types that lose Chx10 expression do not ever express Mitf. Possible derepression of Mitf in the optic vesicle in orf mice may help explain the broad ectopic expression of Mitf in the retina of these mutants. Identification of a physiologically meaningful Chx10 binding site in Mitf regulatory regions is a necessary next step in testing this hypothesis of direct regulation by Chx10.

Insights into other Chx10 mutant phenotypes

The results of this study provide new insights into a number of phenotypes observed in mice and humans lacking Chx10 function. Human patients with mutations in Chx10 present with microphthalmia, cataracts and iris abnormalities (Percin et al., 2000). While microphthalmia is expected, based upon the earlier characterization of the orf phenotype, the anterior eye phenotypes were unexplained. Conversion of nonpigmented ciliary body epithelium into pigmented tissue might disrupt formation and function of the ciliary body and iris. Even in the orf animals rescued by a Mus musculus castaneus genetic background, the ciliary body did not form properly, although the iris partially developed (Bone-Larson et al., 2000). Overt hyperpigmentation was not observed in these eyes, suggesting that Chx10 may play a direct role in differentiation of the ciliary body where it is expressed in the presumptive nonpigmented ciliary body epithelium.

An intriguing observation made in orf mice was the presence of a five-fold increase in the number of retinal stem cells compared to wild-type counterparts (Tropepe et al., 2000). Retinal stem cells derive from cells in the pigmented ciliary body in mammals, which may be a homolog of the ciliary marginal zone of fish and amphibians (Perron and Harris, 2000). It was proposed that retinal progenitor cells might negatively regulate the number of stem cells in the retina, and as a consequence of fewer progenitor cells in the orf retina, more stem cells would be produced. Another possibility might be that the retinal cells that transdifferentiated into pigment cells form a larger niche for retinal stem cells, or themselves become retinal stem cells. The possibility that retinal progenitor cells could become retinal stem cells via transdifferentiation opens up new experimental possibilities in studying retinal stem cells.

Compartment boundaries in the peripheral retina

The study of compartment boundaries has been elegantly conducted in Drosophila using classical genetics and molecular markers (see Dahmann and Basler, 1999; Wolpert, 2003). Much less is known in vertebrate systems about where compartments are found and how they are regulated. Several aspects of the data presented here provide evidence for compartment boundaries in the peripheral retina. First, early gene expression patterns in the peripheral retina were largely maintained. For example, Notch1, Fgf15, and several bHLH factors examined were never expressed in the peripheral zones in the retina. Wdfy1 also was never expressed outside of zone 1, although Crrh and Igf2 showed some zone 2 expression. Not only was the spatial organization maintained, but most markers of the periphery remained expressed in the mature ciliary body. Second, a combination of expression and fate-mapping analysis of Chx10 at the periphery revealed it to be expressed in zone 1 throughout development. Fate mapping analysis using R26R in wild type or orf mutant heterozygotes showed that labeled cells stayed within their boundaries and labeled cells were never observed to be pigment cells. This finding strongly implicates a boundary between future pigmented and nonpigmented cells within an initially nonpigmented population, and suggests that even if some cell mixing occurs, the eventual fate of a cell is still lineage-fixed relative to its initial specification. Third, loss of function of Chx10 in the orf mutant caused normally peripherally restricted genes to become expanded to the center of the retina. Therefore, Chx10 function appears linked to maintaining at least one important boundary in the peripheral retina. Strikingly, the most peripherally expressed genes, in zone 2, expanded more dramatically and temporally before zone 1 cells in the orf mutant. This finding implies that formation and/or maintenance of the boundary between zone 1 and zone 2 requires Chx10.

We are grateful to R. Awatramani, S. Dynecki, and P. Soriano for R26R mice; H. Arnheiter and L. Lamoreux for Mitfpros mouse; and G. Eguchi, H. Arnheiter and A. Miller for riboprobe templates. We thank R. Livesey for assistance with microarray analyses and J. Trimarchi and A. Jadhav for generously sharing many riboprobes. C. Hershey performed crosses of Mitfpros mice and A. Shaw assisted with animal husbandry. Finally, we are grateful to A. Jadhav and J. Trimarchi for critical review of this manuscript and the members of the Cepko and Tabin labs for encouragement and helpful discussions. S. Rowan was a predoctoral fellow of the Howard Hughes Medical Institute during a portion of these studies. D. E. Fisher is the Jan and Charles Nierenberg Fellow at the Dana Farber Cancer Institute. C. L. Cepko is an investigator of the Howard Hughes Medical Institute.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/131/20/5139/DC1

References


