Effects of Homeobox Genes on the Differentiation of Photoreceptor and Nonphotoreceptor Neurons

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PURPOSE. The homeobox genes Pax6 and Chx10 are diffusely expressed in proliferating, undifferentiated retina neuroepithelial cells. Distinct, topographically specific expression patterns emerge, however, as postmitotic cells become organized into layers. The hypothesis that the product of each gene may be necessary for the differentiation of particular nonphotoreceptor neuron subsets and that their absence may be required for progenitor cells to differentiate as photoreceptors was tested in this study.

METHODS. Neural retinas from 5-day-old chick embryos were dissociated, cultured at low density, and cotransfected with a plasmid expressing the green fluorescent protein (GFP)-reporter gene, and a plasmid expressing Pax6, Chx10, Optx2, or the control gene lacZ. After further culture, the cells were fixed and processed for the detection of cell-specific markers.

RESULTS. Nonphotoreceptor neurons increased threefold with Chx10 and almost sixfold with Pax6, compared with cells transfected with lacZ. The frequency of GFP(+) cells immunoreactive with the ganglion cell-specific antibody RA4 was unchanged by Chx10, but was increased twofold by Pax6. Conversely, Chx10 and Pax6 expression diminished the photoreceptor population to approximately 35% and 15% of control values, as determined by morphologic analysis, visinin immunocytochemistry, and peanut lectin binding. Optx2 had some inhibitory effects on photoreceptor differentiation, which were accompanied by marked increases in the frequency of morphologically undifferentiated cells.

CONCLUSIONS. These results are consistent with the hypothesis that Chx10 and Pax6 promote the differentiation of nonphotoreceptor neurons while inhibiting the differentiation of photoreceptor cells. (Invest Ophthalmol Vis Sci. 2002;43:3522-3529)

The mature neural retina is composed of six major classes of postmitotic, differentiated cells, that occupy precise positions within its distinctively laminated structure and are linked by a complex network of connections. In the early embryo, however, the future retina consists of a morphologically homogeneous, proliferating, pseudostratified epithelium. The cellular and molecular mechanisms that specify the differentiation of neural retina cell types from this simple neuroepithelium have not been fully elucidated, although it is clear that they involve both microenvironmental signaling molecules and intracellular genetic mechanisms. Several DNA-binding transcription factors have been shown to control cell differentiation through the coordinated activation of networks of cell-specific genes in the Drosophila compound eye (reviewed in Refs. 5.6). Members of several transcriptional regulator families have also been detected in the vertebrate retina, and gain-or loss-of-function experiments have shown the involvement of several of them in retinal cell differentiation (reviewed in Refs. 3,7–9). Candidate genes identified in the chick retina include Eya2,10 SOH1,11 GH6,12 Casb1,13 Six4,14 Six3 and Six9,15 NeuroD,16 cNSCI1,17 Optx2/Six6,18 NeuroM,19 and otx2,20 in addition to the genes that are the subject of this article.

This laboratory is investigating the role of the homeobox genes Pax6 and Chx10 in chick embryo retinal development. Pax6 is the vertebrate homologue of the Drosophila eyeless gene. Its ectopic expression into embryonic cells leads to the development of histologically normal eyes in Drosophila and Xenopus.21,22 Pax6 mutations cause the small-eye phenotype in mice and a related complex syndrome in humans.23,24 Chx10 belongs to the paired family of homeobox transcription factors.25,26 Abnormalities in mouse Chx10 are responsible for the ocular retardation phenotype.27,28

In situ hybridization signals for Pax6 and Chx10 are diffusely distributed in the proliferating, undifferentiated neuroepithelium of the chick embryo at embryonic days (ED) 3 and 4.26 Distinct, topographically specific expression patterns of these genes, however, are detectable as postmitotic cells become organized into layers between ED 6 and 8. Pax6 concentrates at these stages in prospective amacrine and ganglion cells and at low levels in horizontal cells, whereas Chx10 is observed in putative bipolar and Müller cells. Presumptive photoreceptors, located in the outer nuclear layer, are completely devoid of hybridization signals for these genes.26 We report here the results of gain-of-function experiments showing that Pax6- or Chx10-transfected chick embryonic precursor cultures have a marked increase in the frequency of retinal progenitors that differentiate as nonphotoreceptor neurons, with concomitant decrease in the frequency of cells differentiating as photoreceptors. These results are consistent with the hypothesis that the products of these homeobox genes may be necessary for the differentiation of progenitor cells into particular subsets of nonphotoreceptor neurons and that their absence may be required for progenitor cells to differentiate as photoreceptors.2,26

MATERIALS AND METHODS

Materials

Reagents and materials were purchased from the following suppliers: B&G Eggs (Stevens, PA), regular grade White Leghorn chick embryos; Clontech (Palo Alto, CA), pEGFP-N1; Gene Therapy Systems (San Diego, CA), GenePORTER, a gene transfection reagent; Vector (Burlingame, CA), biotinylated goat anti-rabbit IgG, rhodamine-avidin d, and
rhodamine-labeled peanut lectin; Amersharm (Piscataway, NJ), PKC-1 antibody; Jackson Immunoresearch (West Grove, PA), goat anti-mouse IgG Cy5. Rabbit (polyclonal) anti-aminomethyluracil (GABA) and all other materials were purchased from Sigma (St. Louis, MO).

Cell Culture
White Leghorn chick embryos were used for all experiments. Retinal cell cultures were prepared as described.29 Embryonic days (ED) 5 or 6 neural retinas, free of pigment epithelial cells and other eye tissues, were dissociated enzymatically, and the resultant cells were resuspended in medium M199 supplemented with 100 μg/mL penicillin, 2 mM glutamine, 100 μg/mL linoleic acid-bovine serum albumin, 1 μg/mL fetal calf serum and seeded at a density of 8.0 × 10^5 cells per 35-mm polyornithine-treated dish. Cultures were incubated at 57°C in an atmosphere of 5% CO2 in air.

In Vitro Transfection
Pilot transfection studies were performed with several commercially available reagents, according to their respective manufacturer’s instructions. The protocol recommended for the transfection reagent (GenePORTER; Gene Therapy Systems) was eventually adopted with some modifications.30 Twenty-four hours after culture onset, the medium from the cultures was collected and saved (conditioned medium, described later) and was replaced with 2 mL of serum-free medium M199 supplemented with 100 μg/mL linoleic acid-bovine serum albumin, 100 μg/mL penicillin, 2 mM glutamine, 16.6 × 10^-7 M insulin, 4 × 10^-8 M progesterone, 4 × 10^-8 M putrescine, 6 × 10^-8 M selenium, and 12.5 × 10^-6 M transferrin.31 After a 5- to 10-minute incubation, this medium was replaced with 1 mL of transfection cocktail, consisting of similarly supplemented 109 medium, containing 7 μL GP reagent and 5 μL DNA preparation. The latter included 340 ng pEGFP-N1 plasmid (Clontech), which expresses the green enhanced green fluorescent protein (GFP) reporter, and 670 ng of a plasmid that expresses either one of the chicken homeobox genes (Pax6, Chx10) or the control gene lacZ, which encodes the enzyme β-galactosidase (β-gal). An additional group of experiments was performed under similar conditions to compare Pax6, lacZ, and Optx2.18 In all cases, the genes were expressed from the same cytomegalovirus (CMV) promoter. The components of the transfection cocktail were preincubated together for 25 minutes before their addition to the culture cells, to facilitate the formation of DNA-lipid complexes. The treated cultures were returned to the incubator for 3.5 to 4.0 hours, after which the transfection medium was replaced with 2 mL of conditioned medium, and the dishes were returned to the incubator for an additional 72 hours. Pilot experiments showed that cells survived much better after being refed with conditioned medium than with fresh medium. At the end of the culture period, the transfected cultures were fixed in 4% paraformaldehyde for 30 minutes and processed for immunocytochemistry as described in the following section. Some cultures were processed for peanut lectin binding before fixation.

Immunocytochemistry
Paraformaldehyde-fixed dishes were blocked for 1 hour at room temperature with either 10% goat serum and 0.25% Triton X-100 in PBS, or a 5% solution of nonfat dried milk, clarified by centrifugation, in PBS-Tween (0.1%). They were then incubated overnight at 4°C in primary antibody in 2% goat serum and 0.05% Triton X-100 in PBS. The antibodies were rabbit anti-chicken visinin antibody at 1:100 (a gift from Art Polans, University of Wisconsin),32 RA4 mouse anti-intermediate filament antibody at 1:200 (a gift from Steve McLoon, University of Minnesota),33 rabbit anti-GABA antibody (1:500–1:8000; Sigma), and mouse anti cellular retinoic acid–binding protein (CRABP, a gift from Jack Saari, University of Washington; 1:200).34 Primary antibody binding was detected either with sequential 1-hour incubations in a 1:100 dilution of biotinylated goat anti-rabbit, followed by 1:100 dilution of rhodamine-avidin D, or with IgG-cyanine3–labeled secondary antibodies. Fluorescence was investigated with an epifluorescence microscope (Nikon, Melville, NY) and recorded photographically (Ektachrome 400 ASA; Eastman Kodak, Rochester, NY), developed at (800 ASA), or with a digital camera (Spot RT; Diagnostic Instruments, Sterling Heights, MI).

Peanut Lectin Binding
Cultures were washed twice in Hanks’ balanced salt solution (HBSS) and incubated for 15 minutes at room temperature with 50 μg/mL rhodamine-labeled peanut lectin in HBSS.33 At the end of the lectin incubation, the dishes were rinsed twice in HBSS and fixed in paraformaldehyde.

Analysis of Cultures
Cells were identified and quantitated by phase contrast microscopy as either photoreceptors, nonphotoreceptor multipolar neurons, or morphologically undifferentiated cells, according to published criteria.35,36 Total number and relative frequency of each cell type was determined by scanning the dish systematically from left to right and top to bottom, and counting 10 to 20 randomly selected fields under 400× magnification, corresponding to 1:1300 to 1:650 of the dish area. Transfected cells were identified by bright GFP fluorescence in the fluorescein channel, and immunoreactivity was detected in the rhodamine channel. The frequency of immunoreactive cells in the transfected population within each culture was determined by analyzing 200 GFP(+) cells. In selected cases, 200 GFP(−) cells were also counted to evaluate the behavior of untransfected cells. In all cases, observers were unaware of the identity of the samples. Experiments were repeated at least twice, with triplicate dishes for each condition. Results are expressed as the mean ± SD. Treatment groups were compared using analysis of variance (ANOVA). Pair-wise comparisons were made according to the least-significant-difference method, and significance was determined at α = 0.05.

RESULTS
Characterization of the Culture Preparations Used for Transfection Experiments
Dissociated cells isolated from ED 5 to 6 chick embryo retinas were used for most experiments. This immature donor tissue contains a mixture of proliferating neuroepithelial cells and postmitotic progenitors which, with the exception of some ganglion cells, remain morphologically undifferentiated.37 The cells appear process-free and have a circular outline at the beginning of culture.2,36 Some changes in cell morphology were observed after 24 hours in vitro (the stage when the transfection treatment was applied), but it was not possible at this stage to distinguish conclusively the photoreceptors from nonphotoreceptor neurons (Fig. 1A). These two cell categories, however, become readily identifiable with further culturing (reviewed in Ref. 2). As shown in Figures 1B and 1C, after 4 days in culture, photoreceptors appeared as elongated, polarized cells, with a highly compartmentalized phenotype and a single, short neurite; nonphotoreceptor neurons were identified by a large cell body and the presence of several neurites. The cells were also be readily recognized by fluorescence microscopy when they expressed GFP (Figs. 1D, 1E). Both cell types were further characterized using cytochemical methods (reviewed in Ref. 2 and described later). Approximately 50% of the cultured cells remained morphologically undifferentiated. In ED 5 cultures, photoreceptors were much more abundant than nonphotoreceptor neurons, but this ratio was reversed in ED 8 cultures.38,39

Pilot and Control Experiments
Pilot cotransfection experiments on ED 5, 6, and 8 retinal cultures all suggested that nonphotoreceptor neurons were
Quantitative Morphologic Analysis

GFP(+) cells expressing nonphotoreceptor neuronal phenotypes appeared more abundant in cultures transfected with either Pax6 or Chx10 than in the lacZ control. Experiments with ED 5 cells showed the greatest effects, and therefore ED 5 cells were used for the remainder of the study. Control experiments showed that the transfection reagent (GenePORTER; Gene Therapy Systems) treatment necessary for transfection did not have deleterious effects on the cultures, as evaluated by counting the total number of cells and the relative frequency of photoreceptors, nonphotoreceptor neurons, and undifferentiated cells in untreated and mock-transfected cultures (Fig. 2A). All cultures transfected with the various plasmids under investigation had similar numbers of GFP(-) cells (Fig. 2B). The relative frequency of GFP(-), untransfected photoreceptors, nonphotoreceptor neurons, and morphologically undifferentiated cells was also similar in Chx10-, Pax6-, and lacZ-treated cultures (Fig. 3A).

Cytochemical Analysis of Retinal Cells Transfected with Pax6, Chx10, or LacZ

The effects of Pax6 and Chx10 on retinal cell differentiation were also investigated by determining the frequency of cells expressing cell-specific markers. Visinin is a calcium-binding protein expressed in embryonic photoreceptor cells shortly after terminal mitosis, both in vivo and in culture.
Visinin-immunoreactive cells are shown in Figure 4. Quantitative analysis showed that more than 60% of the GFP(+) cells appeared visinin(+) in lacZ-transfected cultures (Fig. 5). Only 45% and 33% of the GFP(+) cells were visinin(+) in Chx10- and Pax6-transfected cultures, respectively. These values were statistically different from lacZ, as well as between Chx10 and Pax6. The same was true for results obtained with cultures processed cytochemically with rhodamine-labeled peanut lectin, which binds selectively to differentiating photoreceptors. RA4 immunoreactivity appeared localized to neurite processes of some retinal ganglion cells.33 RA4 immunoreactivity appeared localized to neurite processes of some retinal neurons in culture (Fig. 4C). Approximately 10% of the GFP(+) cells were RA4(+) in either lacZ- or Chx10-transfected cultures, but there was a statistically significant twofold increase (to 20% ± 4%) in double-positive—

![Figure 2](image-url)

**Figure 2.** (A) Transfection reagent’s effects on ED 5 chick retinal cultures (GenePORTER, Gene Therapy Systems). Twenty-four hours after culture onset, replicate dishes were either undisturbed, or mock transfected with the reagent. Seventy-two hours later, the cultures were fixed and analyzed quantitatively to determine the numbers of photoreceptors, nonphotoreceptor neurons, and morphologically undifferentiated cells. No significant changes were caused by treatment of cultures with the reagent. (B) Comparison of the frequency of GFP(+) cells in cultures cotransfected with lacZ, Chx10, or Pax6. The frequency of transfected cells was similar in all three experimental groups.

![Figure 3](image-url)

**Figure 3.** Effects of Chx10 or Pax6 transfection on the differentiation of chick embryo retina progenitor cells in culture. Twenty-four hours after culturing began, replicate ED 5 cultures were cotransfected with plasmids expressing GFP plus a second plasmid expressing either lacZ, Chx10, or Pax6. The cultures were terminated 72 hours after transfection. (A) In all three experimental groups, GFP(−) cells (those that failed to be transfected) showed similar frequencies of photoreceptors, nonphotoreceptor neurons, and morphologically undifferentiated cells. (B) In lacZ-transfected cultures, the GFP(+) population had a similar relative frequency of photoreceptors, nonphotoreceptor neurons, and morphologically undifferentiated cells as the GFP(−) population within the same cultures. In contrast, cells cotransfected with GFP and Chx10 had significantly fewer photoreceptors and significantly more nonphotoreceptor neurons than both lacZ-transfected cells and GFP(−) cells within Chx10-treated cultures. The decrease in photoreceptors and increase in nonphotoreceptor neurons was even more marked in cells cotransfected with GFP and Pax6. Pax6 also triggered a decrease in the frequency of morphologically undifferentiated cells. The Pax6-induced population changes were also significantly different from the GFP(−) population within the same cultures and from the GFP(+) populations in lacZ-transfected cultures. *Significant difference compared with lacZ; #significant difference between Chx10 and Pax6.
Control Experiments with Optx2

Optx2 is a homeobox gene belonging to the sine oculis-six family; it is expressed in the neural retina but not in the pigment epithelium and has the capacity to induce the expression of retinal properties in pigment epithelial cells in culture. Its function in the retina, however, has not been established. We therefore decided to perform transfection experiments with an Optx2 expression plasmid, to determine whether its effects would be similar to those of Pax6, a homeobox gene from a different family. Some similarities and several important differences were observed between the two homeobox genes in a comparison of cultures transfected with GFP plus either lacZ, Pax6, or Optx2. Pax6 and Optx2, for example, caused similar decreases in the frequency of cells differentiating morphologically as photoreceptors in the cultures (Table 1). When photoreceptor differentiation was evaluated immunocytochemically, however, the frequency of GFP(+) cells expressing visinin or peanut lectin in Pax6-transfected cultures (19% ± 7% and 6% ± 3%, respectively) was several fold lower than in lacZ-transfected cultures (70% ± 2% and 47% ± 6%, respectively). Optx2-transfected cultures, however, had frequencies of 52% ± 12% and 26% ± 3% for visinin and peanut lectin, respectively. These values were significantly lower than in lacZ cultures, but were also significantly higher than in Pax6-treated cultures. Other significant differences between Pax6 and Optx2 were also observed. Thus, GFP(+) cells expressing a neuronal phenotype increased from 27% ± 7% in lacZ cultures to 71% ± 5% in Pax6 cultures, but only to 35% ± 3% in Optx2 cultures, with all the differences being statistically significant. In contrast, Optx2 caused a significant increase in the frequency of GFP(+) cells that retained a morphologically undifferentiated phenotype (to 58% ± 3%, compared with 56% ± 6% in the lacZ control), whereas Pax6 actually caused a decrease in such cells (to 23% ± 5% of the GFP(+) population).

DISCUSSION

In this study we used lipid-mediated transfection of cultured chick retinal cells to investigate the effects of homeobox genes on retinal cell differentiation. Morphologic analysis, together with investigation of cell-specific markers by immunocytochemistry and lectin cytochemistry, showed that Pax6 and Chx10 inhibited the differentiation of retinal progenitor cells as photoreceptors, while stimulating their differentiation as nonphotoreceptor neurons. Optx2, which belongs to a different family of homeobox genes, had some inhibitory effects on photoreceptor development, but they were accompanied by marked increases in morphologically undifferentiated, rather than neuronal cells. Some methodological considerations appear relevant for the interpretation of these results. The experimental paradigm used involves cotransfection of the retinal progenitor cells with two plasmids, one encoding a reporter gene GFP, and one encoding the gene of interest (Pax6, Chx10, and Optx2 or lacZ, which served as a negative control). Previous studies using the same transfection technique showed that coexpression of two genes encoded in separate plasmids occurred in more than 80% of the transfected cells. Given that plasmids for GFP and the test gene of interest were mixed in a 1:2 ratio in the present experiments, it appears reasonable...
to assume both genes were expressed in GFP(+) cells. In agreement with the same study,36 we observed that the transfection reagent (GenePORTER, Gene Therapy Systems)-mediated transfection was devoid of detectable toxicity. The frequency of GFP(+) cells, moreover, was similar in cultures cotransfected with GFP plus any of the test genes. It must be noted that changes in cell differentiation occurred in fewer than 100% of the transfected cells. This may have been caused by differences in the degree of plasticity and commitment of each progenitor cell at the time of transfection, in the amount of gene product synthesized in each transfected cell, in possible interactions between the exogenous gene and endogenous transcription factors present in each cell or by a combination of these variables.

As indicated in the introduction, the distribution of Pax6 and Chx10 mRNAs in the developing retina suggests two complementary hypotheses.2,26 One hypothesis predicts that overexpression of these genes in retinal progenitor cells would inhibit photoreceptor differentiation. Our Pax6 and Chx10 results are consistent with this prediction. The inhibitory effects of Pax6 and Chx10 on the retinal progenitor cells’ ability to develop as photoreceptors could be demonstrated using morphologic criteria, as well as by peanut lectin binding and visinin immunoreactivity, two markers expressed at early stages of photoreceptor differentiation both in vivo and in vitro.35,40,42 The decrease in photoreceptor frequency ranged between 30% and 60% of the control cultures and was consistently and significantly more pronounced in Pax6- than in Chx10-transfected cultures. Pax6- and Chx10-induced decreases in photoreceptor frequencies were significant, both in GFP(+) cells in cultures cotransfected with the lacZ control gene and in GFP(-) cells within cultures treated with Pax6 or Chx10. From these observations, we propose that both Pax6 and Chx10 have the capacity to inhibit not only the expression of some photoreceptor-specific marker genes, but also the complex morphogenetic mechanisms involved in the structural differentiation of progenitor cells as photoreceptors.35,46 Given that both homeobox genes are diffusely expressed in immature progenitor cells,26 it appears reasonable to postulate that their downregulation may be an important step that allows progenitor cells to follow a photoreceptor developmental pathway. Pax6 and Chx10 overexpression into cultured retinal progenitor cells also led to increases in the frequency of progenitor cells differentiating as nonphotoreceptor neurons. Morphologic analysis, a well-established and reliable method for identifying cells with nonphotoreceptor neuronal phenotypic properties,31 showed that such cells were significantly more frequent in Pax6- or Chx10-transfected cells than in untransfected cells in the same cultures or than in lacZ-transfected cells in control cultures. Immunohistochemical analysis did not disclose significant changes in the frequency of amacrine-like neurons. However, the monoclonal antibody RA4, which recognizes an intermediate filament epitope expressed by retinal ganglion cells in the early stages of their differentiation,35 showed that RA4(+) neurons were at least twice as frequent among cells transfected with Pax6 as in cells transfected with either Chx10 or lacZ. The differential effect of Pax6 and Chx10 on this marker is consistent with the previous finding

![Figure 5](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932911/) 

**Table 1. Effects of Optx2 and Pax6 Transfection on the Differentiation of Retinal Progenitor Cells in Culture**

<table>
<thead>
<tr>
<th>Transfection Treatment</th>
<th>Morphologically Undifferentiated</th>
<th>Neurons</th>
<th>Photoreceptors</th>
<th>Visinin(+)</th>
<th>Peanut Lectin(+)</th>
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<tbody>
<tr>
<td>lacZ</td>
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<td>27 ± 7</td>
<td>38 ± 4</td>
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<td>35 ± 3†</td>
<td>8 ± 1†</td>
<td>52 ± 12†</td>
<td>26 ± 3†</td>
</tr>
<tr>
<td>Pax6</td>
<td>23 ± 5†</td>
<td>71 ± 5†</td>
<td>7 ± 1†</td>
<td>19 ± 7†</td>
<td>6 ± 3†</td>
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Twenty-four hours after beginning cell culture, replicate ED5 cultures were cotransfected with plasmids expressing GFP plus a second plasmid expressing either lacZ, Optx2, or Pax6. The cultures were terminated 72 hours after transfection. The data represent the percentage of GFP(+) cells that express a particular phenotype.

† Significant difference from lacZ.

* Significant difference between Optx2 and Pax6.
that Pax6 is expressed in developing ganglion cells in vivo, but Chx10 is not.\textsuperscript{26} We were unable to investigate the potential role of Chx10 in bipolar cell differentiation because of our failure to obtain detectable staining with antibodies against PKC-1.

The role of Pax6 in the determination of retinal cell fates is much less clear than in other aspects of eye development, because functional studies have not disclosed a consistent picture. Conditional gene disruption experiments in the mouse, for example, have shown that retinal progenitor cells become restricted to the exclusive generation of amacrine cells in the absence of Pax6.\textsuperscript{47} Targeted overexpression of Pax6 during retinal development in Xenopus failed to produce a significant shift of retinal precursors toward neuronal or away from photoreceptor fates,\textsuperscript{48} in contrast to our own results. Pax6 inhibitory effects on photoreceptor development were also observed in transgenic mice carrying the human Pax6 locus on yeast artificial chromosomes.\textsuperscript{49} Additional work is needed to further evaluate these discrepancies, which could be due to species and/or methodological differences, as well as to interactions between Pax6 and other transcriptional regulators.\textsuperscript{50} Some ambiguity exists also in the case of Chx10. During retinal development in both the chicken and mouse, Chx10 expression is concentrated in the region of the inner nuclear layer where bipolar and Müller cells develop.\textsuperscript{25,26,50} Despite the absence of bipolar cells in Chx10 mutants,\textsuperscript{27} it has been suggested that this gene promotes Müller cell development (rather than bipolar neurons) in mouse retinas that do not possess the transcriptional regulators Mash1 and Mash2.\textsuperscript{45} Our own findings indicate that Chx10 stimulates the development of neuronal cells, without the appearance of cells with the typical phenotype of cultured chick Müller cells.\textsuperscript{51–55} Although we were unable to determine whether the neuronal cells that developed after transfection with Chx10 expressed bipolar markers.

The possibility must be considered that the effects of Pax6 and Chx10 are nonspecific. It could be argued, for example, that overexpression of any transcription factor of the homeobox family would result in inhibition of photoreceptor differentiation and that the cultures perhaps cannot do anything other than turn into more neuronal cells when photoreceptor development is inhibited. Although this possibility cannot be formally dismissed, several aspects of our results appear to argue against it. First, the inhibitory effects of Pax6 on photoreceptor differentiation and its stimulatory effects on neuronal differentiation were significantly stronger than those of Chx10. Pax6, moreover, had a stimulatory effect on the expression of the ganglion cell marker RA4 that was not observed with Chx10. Finally, Optx2 resembled Pax6 in its capacity to inhibit photoreceptor differentiation as evaluated using morphologic criteria, but its effects on the cytotoxic differentiation of these cells were significantly weaker than those of Pax6 and were accompanied by increases in the frequency of morphologically undifferentiated cells. Taken together, these observations suggest that the effects of these transcriptional regulators on cell differentiation are, at least to some degree, specific.

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**References**


