The Requirement of Pax6 for Postnatal Eye Development: Evidence from Experimental Mouse Chimeras

Shengxiu Li, Dan Goldowitz, and Douglas J. Swanson

PURPOSE. The small eye mouse mutant (Sey) is caused by a mutation of the Pax6 gene. Previous studies, in which aggregation chimeras were used, have demonstrated that Sey/Sey cells contribute poorly to the neural retina forming small clumps of cells restricted to the inner retina at embryonic day 16.5. In addition, Sey/+ cells are absent from the lens epithelium during this embryonic period and postnatally. This study was conducted to determine the fates of these Sey/Sey and Sey/+ cells with continued development in chimeric mouse eyes.

METHODS. Observations were made on heterozygous and homozygous Sey cells in chimeric eyes from postnatal day (P)0 to P10.

RESULTS. In Sey/Sey→wild-type (wt) chimeras, all Sey/Sey cells originating from retinal progenitor cells died at perinatal times. The only remaining Sey/Sey cells in the neural retina were associated with blood vessels, including vascular endothelial cells, pericytes, astrocytes, and microglia, which have extraretinal origins. In contrast, Sey/+ cells formed all retinal cell classes. As previously reported, Sey/Sey cells were absent from the lens and corneal epithelium. However, in contrast to previous reports, Sey/+ cells contributed to the lens epithelium as well as corneal tissues, and Sey/Sey cells were absent from the anterior retinal pigment epithelium.

CONCLUSIONS. All evidence showed that, when Pax6 is absent at the initial stages of the development, Sey/Sey cells that contribute to the neural retina die, even when wild-type cells are available to provide normal environmental cues. (Invest Ophthalmol Vis Sci. 2007;48:3292–3300) DOI:10.1167/iovs.06-1482

Pax6, a gene containing paired-box and homeobox motifs, is crucial for normal development of the brain, pancreas, and eye.1 Pax6 is expressed very early in the evaginating optic vesicle and subsequently in the entire retinal progenitor population before neuronal differentiation.2–4 With development, Pax6 expression is eventually limited to retinal ganglion cells and amacrine cells.5–9 The phenotype of the small eye (Sey) mutant mouse, results from an autosomal dominant mutation of the Pax6 gene.9–12 The Sey homozygote has virtually no eyes, whereas the heterozygote has microphthalmia with distinctive colobomas.7 In the human, the defect in the Pax6 gene is responsible for aniridia and other ocular phenotypes.10–16 Therefore, Sey mice serve as an important model for exploring human aniridia and other developmental eye defects.

Because Sey homozygous mutants die perinatally, before the completion of retinal development, the details of the role of Pax6 in retinal cell fate determination remain unclear.23 In one study, Marquardt et al.17 found that Pax6 activity is necessary for the generation of multipotential cell types in the retina. In contrast, Philips et al.18 found that Pax6-null cells can colonize the ganglion cell layer and give rise to a precocious but nonspecified population of neurons that do not survive past embryonic day (E)13.

Studies in which experimental mouse chimeras are used are an excellent way to adjudicate this question. Collinson et al.19 examined embryonic day 16.5 Pax6-null chimeric retinas and found scattered clusters of Pax6-null cells in both the inner nuclear and ganglion cell layers. Similarly, Collinson et al.20 identified ectopic, placodelike structures associated with the developing lens in the embryonic chimera. However, the fate and phenotype of these cells and structures were not determined at later time points. In contrast, cells in Pax6 heterozygotes contribute normally to all eye tissues, but were absent from the lens epithelium at E16.5 and onward.21 In the present study, we made and examined Pax6 mutant chimeras from postnatal day 0 (P0) to P10. We found that Sey/Sey retinal neurons did not survive past birth. A small population of Pax6-null cells was found in the retina that contributed to the blood vessel–associated cells that have their origins outside the retina. Furthermore, in contrast to previous reports, Sey/+ cells did contribute to the lens epithelium and Sey/Sey cells did not contribute to the anterior retinal pigment epithelium (RPE).

METHODS

Animals

Experimental animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Two strains of mice possessing different Pax6-null alleles were used in generating experimental chimeras, the Pax6WT strain (obtained from Robert Grainger and Marilyn Fisher, University of Virginia, maintained on a mixed genetic background) and the Pax6SeyNeu strain (obtained from Brigid Hogan, Duke University, Durham, NC, maintained on an ICR background22). The Pax6SeyNeu strain was crossed to a GFP transgenic reporter line (chick β-actin:GFP maintained on a 129/ICR background23) to generate the Pax6WTNeu line that provides a constitutively expressed GFP marker for cells derived from the Pax6 strain embryos. For the wild-type component of experimental chimeras, we used the Rosa26 mouse24 or FVB transgenic (chick β-actin:GFP) mouse (Liu et al., manuscript in preparation). Efforts were made to minimize animal suffering and to reduce the number of animals used, and all studies were conducted in accordance with University of Tennessee and NIH policies on the ethical use of animals in research.
Aggregation Chimeras

Experimental mouse chimeras were generated as described previously. In brief, four to eight-cell embryos carrying the mutant component (Sey/Sey, Sey+/+, +/+ +/) were cultured, together with wild-type embryos overnight. After successful fusion, blastocysts were transplanted into the uterine horn of pseudopregnant host ICR females. To generate Pax6-null mutant embryos we mated Pax6<sup>Sey</sup> × Pax6<sup>E<sub>N<sub>eu</sub></sub></sup> heterozygotes (in most cases these also carried the GFP transgene). The Sey and Sey-Neu alleles produced a similar phenotypic outcome in the eye and allowed us to obtain unequivocal genotyping of the chimeric mice (described later). In addition, chimeras were generated that had the +/+ , Sey/+ , or Sey-Neu/+ alleles contributed by the Sey matings, and these mice served as control animals for comparison to Pax6-null chimeras. Most chimeras were made using Rosa26 embryos as the wild-type component providing a β-galactosidase marker in all wild-type cells which complemented the actin-GFP marker carried by the Sey-derived embryos.

Immunostaining of the Pax6 Chimeric Eye Sections

Chimeras were perfusion fixed at postnatal day (P)0 or P10 with 4% paraformaldehyde, and then eyeballs were cryoprotected in 30% sucrose solution and cryosectioned. The reagents used for the immunostaining are as follows: a polyclonal rabbit anti Pax6 (1:8000, Covance, Princeton, NJ) which recognizes the C-terminal portion of the protein which is absent in Pax6-null mutants; a monoclonal rat anti-CD34 (1:100, Abcam, Cambridge, MA) was used to identify vascular endothelial cells; a monoclonal mouse anti α-actin (1:1000, Chemicon, Temecula, CA) to identify the vascular pericytes; a monoclonal mouse anti β-tubulin III (1:5000, Covance) to identify retinal ganglion cells (RGCs); a monoclonal mouse anti synaptin 1A isoform to identify rod bipolar cells (1:5000, Sigma-Aldrich, St. Louis, MO) to identify amacrine cells and horizontal cells; a monoclonal mouse anti-synaptophysin (1A) isoform to identify rod bipolar cells (1:5000, Sigma-Aldrich, St. Louis, MO) to identify amacrine cells and horizontal cells; a monoclonal mouse anti-ED1 (1:100, Serotec, Raleigh, NC) to identify microglia; and a monoclonal mouse anti-GFAP (1:4 of Ready for Use; Thermo, Waltham, MA) and a monoclonal mouse anti β-tubulin III (1:5000, Sigma-Aldrich, St. Louis, MO) to identify astrocytes. Biotinylated-horse anti mouse IgG, biotinylated-goat anti rabbit IgG, biotinylated-rabbit anti rat IgG, and horseradish peroxidase (HRP)-conjugate avidin-biotin complex (Vector Laboratories, Burlingame, CA) were used, followed by 3,3′-diaminobenzidine (DAB) staining and observation by light microscopy. For immunofluorescent staining, Alexa Fluor 488 conjugate rabbit anti-GFP (1:1000), Alexa Fluor 568 conjugate streptavidin (1:1000), and TOTO-3 (1:500; Invitrogen-Molecular Probes, Eugene, OR), were used for double or triple labeling. The results were then observed by confocal microscope (Bio-Rad Laboratories, Hercules, CA).

Phenotyping of the Sey Chimeras and Their Retinas

A general indication of the percentage of chimerism was determined by assessing the level of GFP expression in lenses and tails of P0 and P10 chimeras, as well as by coat color, when contributing embryos had pigment differences. We also made a general assessment of external and cellular features of regions known to be affected by the Sey mutation. These included the nares and olfactory bulb; eyelids, eye, retina, and lens; and various regions of the brain (see the Results section).

Genotyping of the Pax6 Chimeras

We took advantage of the availability of the two mutant alleles of Pax6 to determine the precise genotype of the mutant component of the chimeras. Thus, Pax6-null chimeras were created only when the embryos were double mutants for each Pax6 allele. The Pax6<sup>Sey</sup> and Pax6<sup>E<sub>N<sub>eu</sub></sub></sup> alleles were detected by using a mutagenically separated PCR technique (MS-PCR), as previously described. In brief, PCR genotyping of chimeras was performed on DNA from tails verified to contain cells of the mutant lineage. PCR reactions were run with multiplexing primer sets specific for each Pax6 allele under conditions for each allele. PCR products were resolved using a 3% SRF agarose gel run in 1× TBE (Tris-boric acid-EDTA). Under ideal conditions, this PCR genotyping can detect a contribution of mutant cells down to 10% chimerism. Below that threshold, we relied on additional phenotypic information to suggest Sey homozygosity (described earlier).

RESULTS

General Overview of Pax6 Chimeras

In Sey/Sey embryos, eyes are absent, whereas in the Sey+/ embryo they are smaller than normal. Although Sey/Sey mutant mice are born dead (or die shortly after birth), the addition of

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**Table 1. Summary of the Pax6-Null Chimeras Used for Detailed Analysis**

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Age</th>
<th>Genotype*</th>
<th>Chimerism Percentage†</th>
<th>Neural Retina</th>
<th>Lens</th>
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<tbody>
<tr>
<td>699</td>
<td>P0</td>
<td>Sey/Sey</td>
<td>++ +</td>
<td>Folding</td>
<td>No lens</td>
</tr>
<tr>
<td>700</td>
<td>P0</td>
<td>Sey/Sey</td>
<td>++ +</td>
<td>Folding</td>
<td>No lens</td>
</tr>
<tr>
<td>1865</td>
<td>P0</td>
<td>Sey/Sey</td>
<td>UND</td>
<td>Folding</td>
<td>No lens</td>
</tr>
<tr>
<td>698</td>
<td>P0</td>
<td>Sey/Sey</td>
<td>++ +</td>
<td>Folding</td>
<td>1 Lens, small</td>
</tr>
<tr>
<td>1867</td>
<td>P0</td>
<td>Sey/Sey</td>
<td>+</td>
<td>Folding</td>
<td>1 Lens, small</td>
</tr>
<tr>
<td>1701</td>
<td>P0</td>
<td>Sey/Sey</td>
<td>++</td>
<td>Folding</td>
<td>2 Lenses in 1 eye, 1 lens in other side, small</td>
</tr>
<tr>
<td>1868</td>
<td>P0</td>
<td>Sey/Sey</td>
<td>+</td>
<td>Folding</td>
<td>2 Lenses in 1 eye, 1 lens in other side, small</td>
</tr>
<tr>
<td>1702</td>
<td>P0</td>
<td>Sey/+</td>
<td>++</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>1703</td>
<td>P0</td>
<td>+/+</td>
<td>++ +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>1704</td>
<td>P0</td>
<td>+/+</td>
<td>++ +</td>
<td>+</td>
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</tr>
<tr>
<td>1741</td>
<td>P10</td>
<td>Sey/Sey/Neu</td>
<td>80</td>
<td>Folding</td>
<td>1 Lens, small</td>
</tr>
<tr>
<td>1350</td>
<td>P10</td>
<td>Sey/Sey</td>
<td>40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1352</td>
<td>P10</td>
<td>Sey/Sey</td>
<td>10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Estimates of the percentage of mutant cells contributing to each chimera at P0 were determined based on whole-body GFP signal and are denoted as + (low), ++ (medium), or +++ (high). At P10, chimerism was based on percentages that were defined based on coat color and/or GFP signal in tail biopsy specimens.

† Genotype determined via ms-PCR.
Table 2. Summary of Control P10 Chimeras

<table>
<thead>
<tr>
<th>n</th>
<th>Genotype</th>
<th>Chimerism Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>Sey/+</td>
<td>10–90</td>
</tr>
<tr>
<td>6</td>
<td>SeyNu/+</td>
<td>15–90</td>
</tr>
<tr>
<td>8</td>
<td>+/+</td>
<td>2–70</td>
</tr>
</tbody>
</table>

Overall summary of the P10 Sey/+ and +/+ chimeras that were used in the current analysis. n, the total number of chimera; chimerism percentage is the upper and lower boundaries of chimeras in the analysis (detailed in Supplementary Table S1, http://www.iovs.org/cgi/content/full/48/7/3292/DC1).

wild-type cells in experimental chimeras results in extending survival beyond parturition. This survival occurs despite the embryos’ displaying a range of severe to mild craniofacial and obvious eye defects, especially in a high percentage of homozygous mutant chimeras. At P0, some chimeras showed signs of respiratory and digestive complications, and most often these mice were taken for histologic assessment, to avoid their loss due to death. Typically, these neonates were shown to be high-percentage Sey/Sey^{Neu} chimeras, and thus our P0 samples were biased toward chimeras with high percentages of mutant cells. Several chimeras, however, survived well into the postnatal period and were taken at P10, three of which were observed in the present study. The chimeras in this group showed overt but subtle eye and/or brain defects (forebrain and cerebellum). Some of those seen unilaterally were also shown to be composed of Sey/Sey^{Neu} cells. For convenience, all compound heterozygous (Sey/Sey^{Neu}) chimeras are designated as Sey/Sey in the Results and Discussion sections. In Table 1, we summarize the 13 chimeras that were analyzed in this study. In Table 2, we outline the large number of P10 heterozygous and wild-type chimeras that were also assessed in this analysis.

Eyes of Sey/Sey^{+/+} wild-type (wt) chimeras show a wide range of variable phenotypes: from severe defects such as absence of eye tissues, malformed iris, and retinal folding, to a normal appearance. At P0, Sey/Sey^{+wt} chimeras are skewed toward containing a high percentages of mutant cells (as noted earlier) and the eyes in these mice are either absent or much smaller, with severe defects. At P10, Sey/Sey^{+wt} chimeras have normal-appearing eyes. We found that the eyes of our Sey/+^{+wt} chimeras had a normal appearance (Fig. 1), as was reported by Collinson et al.

Analysis of Extraretinal Eye Tissues in Sey Chimeric Mice

Cornea. All seven of the Sey/Sey^{+wt} chimeras at P0 had severe abnormalities of the eye that were probably due to the high percentage of mutant cells in these chimeras. Sey/Sey cells were absent in the corneal epithelium, but the contribution of mutant cells in corneal endothelium and stroma was variable at P0. In some samples, Sey/Sey cells were seen in the corneal endothelium and stroma (data not shown), but in other samples, Sey/Sey cells were not detected in these structures, even though the presence of the cells was evident in other surrounding tissues of the eye (Fig. 2). In P10 Sey/Sey^{+wt} chimeras with normal-looking eye structures, Sey/Sey cells still did not contribute to the corneal epithelium, but they did contribute to the corneal endothelium and corneal stroma (Figs. 3B, Bf). The presence of Sey/Sey cells in the corneal endothelium and stroma, but absence in the corneal epithelium is in accordance with the expression pattern of Pax6, where positive immunostaining is found in the corneal epithelium but not in the corneal endothelium and stroma (Fig. 4).

Lens. There was a range in the severity of lens abnormalities in the P0 Sey/Sey^{+wt} chimeras. In three of seven P0 homozygous mutant chimeras, the lens was absent from both eyes (Fig. 2A). In the other Sey/Sey^{+wt} chimeras, there were one to two smaller lenses. However, no Sey/Sey cells contributed to the lens epithelium or lens fibers compared with control chimeras (Fig. 2). In contrast, Sey/+ cells were competent to colonize the lens epithelium and lens fiber at both E15 and P0 (data not shown).

All three of the P10 Sey/Sey^{+wt} chimeras had relatively normal-looking eyes, even though they spanned a wide range of percentage of mutant cell chimerism (from 10% to 80%). In none of these chimeras could we find Sey/Sey cells contributing to the lens epithelium or lens fibers (Figs. 3B, Bd, Be). In contrast to the mutant chimeras and to the results of Collinson et al., Sey/+ cells were present in the lens epithelium and fibers in all the Sey/+^{+wt} chimeras examined (Figs. 3A, Aa, Ab).

Retinal Pigment Epithelium. Pax6 protein was strongly expressed in the iris and nearby anterior RPE at E15 through P10 (Figs. 5A–C), but was very weakly expressed in the posterior RPE (image not shown) as has been reported for Pax6 mRNA expression. There was a colocalization of GFP and Pax6 in the pigmented RPE in the iris and anterior RPE adjacent to the iris in Sey/+ chimeras (Figs. 5D–G) and wild-type chimeras (data not shown). However, in all P0 and P10 Sey/Sey chimeras, there were no GFP^{+} cells in the RPE of the iris and ciliary body and anterior RPE near the iris (Figs. 5H–K). Furthermore, in the two severely abnormal P0 Sey/Sey chimeras (Fig. 2A), no pigmented RPE was present. Thus, Pax6-null cells do not appear capable of contributing to the anterior RPE and pigmented iris.

Analysis of the Retina in Sey Chimeric Mice

As we found in the lens in P0 mutant chimeras, the neural retinas from all individuals showed severe abnormalities. In each of these eyes, portions of the neural retina were irregularly folded creating rosettes around the lens or lenses and these rosettelike folds were also seen in mutant chimeric eyes lacking a lens. In all chimeras that survived past P0, the neural retinas looked normal, even when our indicators of chimerism suggested that a large component of the animal was derived from a mutant embryo (e.g., up to 80% based on coat color).
Inasmuch as chimera lethality may be due to an abundance of Sey/Sey cells in the brain, the fact that these animals survived to P10 suggests that the contribution of Sey/Sey cells to the brain is substantially less than to coat color (data not shown).

In P0 and P10 chimeric retinas of the Sey/+ lineage, the GFP+ Sey/+ cells formed radial cell columns that spanned the inner to outer extent of the retinas, similar to those seen in wild-type chimeras. Within these Sey/+–derived retinal columns, the cells coexpressed GFP along with various markers, including those for retinal neurons: β-tubulin III for RGCs, syntaxin 1A for the amacrine cells, and PKC for the rod bipolar cells (Fig. 6) and glutamine synthetase for the Müller cells, GAD67 for the GABAergic amacrine cells, and Ki67 for the proliferating cells (data not shown). Therefore, Sey/+ cells contribute to most cell classes, if not all, that make up the neural retina.

In contrast to heterozygous chimeras, in the Sey/Sey chimeric retina there were no radial columns of Sey/Sey GFP+ cells at either P0 or P10. Only a few scattered GFP+ cells were observed in the Sey/Sey chimeric retinas, none of which stained for the classic retinal neuronal markers described earlier (Fig. 6). In fact, only one to two neuronlike GFP-labeled Sey/Sey cells was detected in one P0 mutant chimeric retina (Fig. 7). These cells appeared to have a neuronal morphology but were not double labeled with β-tubulin III (data not shown). In addition, the cells appeared to be undergoing cell death by virtue of the condensed and fragmented appearance of their nuclei in TOTO-3-stained tissue (Figs. 7B–D). Most of the scattered GFP+ cells found in the Sey/Sey chimeric retinas did not have a neuronal appearance, but rather appeared to be associated with tubelike or vascular cell-like structures scattered in the inner retina. In the P0 chimera, most of these GFP+ cells...
profiles were found at the vitreous surface of retinas and seemed to be extensions from the vessels on the vitreous surface (Fig. 8B). In P10 mutant chimeric retinas, the GFP+ vessel related-profiles were scattered on the vitreous surface, in the inner and outer borders of the inner nuclear layer (INL), and sometimes radially extended across the retina (Fig. 6). Thus, these Pax6-null derived cells most likely represent derivatives that contribute to the penetrating retinal blood vessels.

To characterize the cellular phenotype of the GFP+ Pax6-null cells in chimeric retinas we used markers for blood vessel–related components including CD34, a vascular endothelial cell marker; α-actin, a vascular pericyte marker; GFAP, a marker of astrocytes; and ED1, a marker of microglia. The vascular endothelial cell marker, CD34, labeled these GFP+ profiles, suggesting that some of the cells are of endothelial origin (Fig. 8). Furthermore, we found that the GFP-labeled cells in mutant chimeric retinas also colocalized with α-actin, GFAP, and ED1 (Fig. 9), which suggests that the Sey/Sey cells in the chimeric retina consist of vascular endothelial cells, vessel pericytes, astrocytes, and microglia.

**DISCUSSION**

The analysis of chimeras provides a unique experimental opportunity to examine many issues in development, in the in vivo context, that would be otherwise impossible to investigate. For example one can assess the importance of cell–cell interactions, cell autonomy, and developmental competition in tissue morphogenesis. The variable colonization of tissue by each of the donor populations provides multiple windows to obtain a more comprehensive view of development. Another advantage of chimeric mice, which we used in the present study, is that they afford postnatal analyses of mutations that cause perinatal lethality. In this study, we used experimental mouse chimeras to enable the analysis of postnatal eyes to provide insights into the role of Pax6 in eye development and the diseases found in Pax6-null/+ chimeric eyes. In a chimera approach, we can ask the intriguing question of how many and what type of cells are necessary for proper development of an organ structure (in this case the developing eye). Extremes in the contribution of cells in a chimera push the question of what proportion of wild-type cells can protect against the mutant disease or what proportion of mutants cells are necessary to recruit the mutant phenotype.

As found previously, there is impressive plasticity in retinal development, such that even with as much as 80% to 90% Sey/+ cells contributing to the chimeric organism, the wild-type component results in a normal-appearing eye. The plasticity of wild-type cells in the developing retina extends to the presence of Sey/Sey cells in the chimera. In this study, we found that in a P10 chimera with up to 80% Sey/Sey cells, the eyes are quite normal in appearance, by virtue of the nearly normal anterior retinal pigment epithelium (RPE) (Fig. 5). In P10 Sey/Sey chimeras, Pax6-null cells do not contribute to anterior RPE, whereas Pax6-null cells do not. The transmission images (A, D, H) show the pigmented anterior RPE (black arrows). (A–C) Pax6 immunostaining in and E15 eye section with background of transmission image shows that Pax6 is positive in the anterior RPE (white arrows). (D–G) In P0 Sey/+ chimeras, double labeling of GFP and Pax6 immunostaining with background of the transmission image showed that Sey/+ cells contribute to anterior RPE (arrowbeads). (H–K) In P0 Sey/Sey chimeras, Pax6-null cells do not contribute to anterior RPE, but do contribute to choriocapillaris adjacent to the RPE and to blood vessels in the ciliary body (open arrowbead). Scale bars, 50 μm.
complete exclusion of Sey/Sey cells from the eye. In contrast, in our P0 chimeras we found that even a relatively low percentage of Sey/Sey cells contribute to several cell types of the neural retina while Sey/Sey cells do not. Double labeling of GFP and β-tubulin III (Tub), a marker for retinal ganglion cells (A, B); Syntaxin (Syn), a marker for amacrine cells (C, D); or PKC, a marker for rod bipolar cells (E, F) in chimeric retinas at P0 and P10. (A) Radial columns of Sey/+ cells (GFP+) are clearly evident in chimeras indicating the normal participation of Sey/+ cells in generating all cell types including retinal ganglion cells (red). (B) Only a limited number of cells from the Sey/Sey lineage were found in the neural retina but they were present in larger numbers in the vitreous. (C-F) Syntaxin is expressed in amacrine cells and PKC in rod bipolar cells. Sey/+ cells contribute to both of these cell classes as seen in columns of GFP+ cells. However, we found no contribution of Sey/Sey cells to either of the amacrine or rod bipolar cell class. Genotypically, Sey/Sey cells are limited to the vitreal surface of the retina and the innermost and outermost parts of the INL. The location of these cells is coincident with retinal blood vessels and they appear to have a vessellike appearance (arrows) and see endothelial immunocytochemistry that better documents this point in Figure 8, 9. Ch, choroids; GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; NBL, neuroblast layer; ONL, outer nuclear layer. Scale bar, 100 μm.

Chimeras in the Analysis of Pax6 Gene Action in Retinal Development

No neuronal cells of Sey/Sey origin survived in the chimeric neural retina and any neuronlike cells that colonized this tissue appeared to die perinatally. The Sey/Sey cells that were main-
tained in the chimeric neural retina were blood vessel-related, including vascular endothelial cells, pericytes, astrocytes, and microglia. It is generally accepted that none of these cells are intrinsic to the retina, but originate and migrate into the retina from the optic nerve during development.58,59 These results expand the findings of Collinson et al.,19 who found that Sey/Sey cells contribute only poorly to the neural retina, forming small clumps of cells that were restricted to the ganglion cell layer at E16.5. It is possible that the small clusters of cells identified by Collinson et al. are represented by the dying neuronlike cells we observed or alternatively, that the clumps of cells they found in the inner retina represent the cells that are recruited to participate in the formation of the retinal vasculature in the mutant chimera.

In a previous study of Pax6 knockout mice, a large cohort of Pax6-null retinal progenitor cells were found to differentiate precociously (a day earlier than normal at E10) and subsequently disappeared and presumably died at E13.16 This result, in conjunction with ours and those of Collinson et al.19 suggest that the lack of Pax6 eventually leads to the death of these retinal progenitor cells during development. A difference in the timing of the demise of these retinal progenitor cells between the Pax6 knockout embryos and Sey/Sey chimeric retinas may suggest, however, that the relatively normal optic vesicles provided by wild-type cells in the chimera may delay the death of Sey/Sey cells, but do not rescue them from their intrinsically guided death.

One hypothesis that we entertained at the outset of these studies was provided by the work of Marquardt et al.,17 who showed that on Pax6 inactivation by conditional gene targeting after an early point of progenitor differentiation (at approximately E10.5), Pax6-null cells were channeled into the amacrone cell lineage but could form no other retinal cell type. Thus, the undifferentiated mutant retinal cells described by Collinson et al.19 could eventually have become the amacrine cells described by Marquardt et al.,17 but this is not what we found in the postnatal chimera. The fundamental difference in the experimental paradigms that yield different results is informative as to the role of Pax6 in retinal neuron cell fate determination. In one case, Pax6 expression is present in all retinal precursors until inactivation at a specific time,17 whereas in the chimeric retina, Pax6 expression is either normal or absent in different subsets of cells from the beginning of retinal development (present study).19 In the former case, Pax6-null cells can differentiate into amacrine cells, whereas in the latter case Sey retinal cells cannot survive. Therefore, wild-type cells cannot ameliorate the inability of Sey cells to differentiate, and this fact points to the basic cell autonomy of Pax6 action in retinal development. Thus, Pax6 is critical for retinal progenitor cells to proceed through initial development18 and an additional role of Pax6 may be in later stages of neuronal subtype determination.17

The importance of the interaction between developing optic cup and lens placode has long been thought of as a key factor in promoting the development of the early retina. However, it has been challenged by recent studies after specific inactivation of Pax6 activity in the eye surface ectoderm to arrest the development of the lens.17,40 These studies found that the developing lens is not necessary to instruct the differentiation of the neural retina but rather is essential for the correct placement of a single retina in the eye. In the present study, we confirmed that Sey/Sey cells were eliminated from the lens epithelium. The severity of the lens defects varied in Sey/Sey chimeras, from individual eyes with no lens to those with two smaller lenses. Of importance, in some mutant chimeras that lacked a lens, there was still a well-developed retina. This result confirms the findings of Ashery-Padan and Gruss.3
that retinogenesis is intrinsic and independent from interaction with the developing lens.

**Requirement of Pax6 Activity for Contribution of Cells in the Lens and Corneal Epithelium**

We found that no Sey/Sey cells contributed to the chimeric lens, in agreement with Collinson et al.\textsuperscript{19}. In the highest percentage chimeras, no lens tissue developed. Because we examined only late-stage (postnatal) animals, we have no direct evidence as to whether lens formation is initiated and then regresses or whether no lens tissue forms at all. Collinson et al.\textsuperscript{20} suggested that Pax6-null cells can contribute to early lens formation but then are excluded from the lens at later times. Collinson et al.\textsuperscript{19} also suggested that ectopic placodes composed of exclusively mutant cells are seen early but must undergo regression or apoptosis, as they are no longer detected in older chimeras. We also found a unique developmental response to the presence of Pax6-null cells in the formation of multiple lenses in high-percentage mutant chimeras. In high-percentage mutant chimeras, Collinson et al.\textsuperscript{19,20} found that Sey/Sey cells could not contribute to placodal structures and that multiple, ectopic placodes were formed from wild-type cells. The distinct possibility exists that the multiple lenses that we observe could have arisen from the continued development of these ectopic placodes.

Our finding that Sey/+ cells are present in the lens epithelium and lens fibers in E15, P0, and P10 chimeras does not agree with previous findings that Sey/+ cells are virtually absent from the lenses of E16.5 and older chimeras.\textsuperscript{21} An explanation of the differing results could be the cell marker used for identifying the cells of the Sey lineage. In the studies of Collinson et al.\textsuperscript{19,20} the cells were labeled using in situ detection of a globin transgene repeated element that results in a single puncta of label in the nucleus. In contrast, the GFP-transgene used in the present study results in labeling that was visible throughout the cell. The possibility of falsely identifying Sey/+ cells by GFP immunolabeling is therefore likely to be rare. It may also be more likely that a false negative could be obtained using the globin transgene repeat as the marker. Another possibility for the differing results could be the percentages of chimerism of the mice that are analyzed. In our study, we observed 21 samples of P10 Sey/+ chimeras, with chimerism percentages ranging from 10% to 90%. All samples were found to have Sey/+ cells contributing to the lens epithelium and fibers. Considering the existence of lens in the heterozygotes in most previous studies, although the lens is smaller, the Sey/+ have the ability to form the lens. Therefore,
we are confident in concluding that \( \text{Sey}^+ \) contribute to the lens in chimeras. However, a recently published paper describes the lack of lenses in a heterozygous \( \text{Sey}^{Dey} \) mutant small eye mouse. The \( \text{Sey}^{Dey} \) mutant is a large deletion within chromosome 2 that encompasses several genes (including \( \text{Wt1} \) and \( \text{reticulocalbin} \)) as well as the \( \text{Pax6} \) locus. Thus, the different lens phenotype of the \( \text{Sey}^{Dey} \) and the \( \text{Pax6Sey} \) mice could relate to an interaction of \( \text{Pax6} \) with these other potentially lost genes.

In our \( \text{Sey} / \text{Sey} \) chimeras, we found mutant cells present in the corneal stroma and endothelium, but they were absent from the corneal epithelium. These findings agree, in large part, with Collinson et al. who found that, in the chimera setting, \( \text{Sey}/\text{Sey} \) cells were markedly underrepresented from the corneal epithelium but might be present in the corneal stroma and endothelium. These results make sense in the context that \( \text{Pax6} \) is strongly expressed in corneal epithelial cells but is not expressed in cells from corneal stroma and endothelium.

**Pax6 in Iris and Anterior RPE Development**

Few studies have focused on \( \text{Pax6} \) in the RPE. It has been demonstrated that in the early optic cup, \( \text{Pax6} \) mRNA is expressed in both prospective RPE and neural retina layers. By E15.5, \( \text{Pax6} \) expression in the RPE is seen only in anterior regions near the rim of the optic cup. The role of \( \text{Pax6} \) in development of the pigmented iris and RPE has been discussed by Collinson et al. who suggested that within \( \text{Sey}/\text{Sey} \) ut chimeras RPE development is delayed and only a proportion of the \( \text{Pax6} \)-null cells produce pigment in the RPE. Their evidence indicates that \( \text{Pax6} \) is not completely essential for pigment expression in these animals. This finding is in contrast to our results demonstrating that no \( \text{Pax6} \)-null cells contribute to the pigmented cells of the RPE in or near the iris. Although in our P0 and P10 chimeras some GFP-immunostaining (as a marker indicating \( \text{Sey}/\text{Sey} \)-derived cells) is apparent in the layers of the RPE, under close examination using confocal microscopy we found that these cells are not in the RPE but are localized to the adjacent choriocapillaris. Thus, our data suggest that \( \text{Pax6} \) is necessary in the development of the pigmented iris and anterior RPE. The apparent discrepancy between our current results and those of Collinson et al. may be due to the degeneration of \( \text{Pax6} \)-null cells between E16.5 when some mutant cells are reported to reside in the RPE and the postnatal times, when we saw no such cells in the chimeric retina. Alternatively, the difference in findings may be due to our enhanced capability of observing cell genotype and phenotype in the same section. Our conclusion of the requirement of \( \text{Pax6} \) for anterior RPE (including iris) development is consistent with the facts that RPE and neural retina are all derived from the same bipotent retinal progenitors and \( \text{Sey}/\text{Sey} \) mutant animals do not have any eye tissues, including RPE, remaining.

The examination of postnatal \( \text{Sey}/\text{Sey} \) chimeras presents another chapter in the fascinating story of the role of \( \text{Pax6} \) in eye development. The question of the possible contribution of \( \text{Sey}/\text{Sey} \) cells to the neural retina is resolved. \( \text{Pax6} \) is a key intrinsic factor in early retinogenesis. When \( \text{Pax6} \) is absent at the outset of development, all retinal cells, except the vasculature-related components extrinsic to the retina, die even when a wild-type developmental environment is provided in chimeras. In addition, \( \text{Pax6} \) is essential for normal lens architecture and absence of \( \text{Pax6} \)-expressing cells in the chimera can result in aberrant lens and cornea development. \( \text{Pax6} \) is also necessary for the development of the anterior RPE.
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References