Primary defects in the lens underlie complex anterior segment abnormalities of the *Pax6* heterozygous eye

J. Martin Collinson*†, Jane C. Quinn*, Malcolm A. Buchanan‡, Matthew H. Kaufman‡, Sarah E. Wedden‡, John D. West*§, and Robert E. Hill†

*Department of Reproductive and Developmental Sciences and ‡Department of Biomedical Sciences, Genes and Development Group, University of Edinburgh, Hugh Robson Building, George Square, Edinburgh EH8 9XD, Scotland, United Kingdom; and †Comparative and Developmental Genetics Section, Medical Research Council, Human Genetics Unit, Crewe Road, Edinburgh EH4 2XU, Scotland, United Kingdom

Edited by Jeremy Nathans, Johns Hopkins University School of Medicine, Baltimore, MD, and approved June 5, 2001 (received for review March 26, 2001)

We describe lens defects in heterozygous small eye mice, and autonomous deficiencies of *Pax6*$^{+/-}$ cells in the developing lens of *Pax6*$^{+/+}$ ↔ *Pax6*$^{+/-}$ chimeras. Two separate defects of the lens were identified by analyzing the distribution of heterozygous cells in chimeras: *Pax6*$^{+/-}$ cells are less readily incorporated into the lens placode than wild type, and those that are incorporated into the lens are not maintained efficiently in the proliferating lens epithelium. The lens of chimeric eyes is, therefore, predominantly wild type from embryonic day 16.5 onwards, whereas heterozygous cells contribute normally to all other eye tissues. Eye size and defects of the iris and cornea are corrected in fetal and adult chimeras with up to 80% mutant cells. Therefore, these aspects of the phenotype may be secondary consequences of primary defects in the lens, which has clinical relevance for the human aniridia (*PAX6*$^{+/-}$) phenotype.

PAX6 is expressed in the developing lens, inner and pigmented layers of the iris [I(i) and I(p), respectively] and ciliary body, the corneal epithelium, and the developing retina (1–4). Heterozygous null mutations in *PAX6* cause a range of sight-threatening abnormalities in the anterior segment of human eyes, including iris hypoplasia (aniridia), cataract, incomplete separation of the lens from the cornea (Peter's anomaly), iridolenticular and iridocorneal adhesions, corneal opacities, and vascularization of the peripheral cornea (autosomal-dominant keritits; refs. 5–9). There are also degenerative aspects of the phenotype (early onset glaucoma and optic nerve hypoplasia) that may be secondary to the primary developmental defects.

These defects are shared with the small eye (*Pax6*$^{+/-}$) mouse, a model of the human syndromes (10–12). The small eye mouse model will aid in understanding the etiology of *PAX6*-associated disease and, in addition, provide insights into other anterior-segment abnormalities.

Previously, we have investigated the roles of *Pax6* in the eye by studying the developmental potential of homozygous *Pax6*$^{-/-}$ cells in chimeras (13, 14). By comparing the behavior of wild-type (wt) and mutant cells in chimeras, it is possible to assess the ability of mutant cells to contribute to eye tissues and to participate in inductive interactions when in direct competition with wt cells. The production of mosaic mice with tissue-specific knockouts of *Pax6* in the lens or retina has complemented our chimeric approach (15, 16). Together, these studies have revealed requirements for *Pax6* for the maintenance of lens competence, the interaction between the lens placode and the optic vesicle, and the subsequent differentiation of the lens and retina. It is possible, therefore, that abnormalities in the *heterozygous* eye could result from defects in several eye tissues at several stages of development.

The developmental mechanisms underlying the heterozygote phenotype are poorly understood, although lens placode formation is delayed in *Pax6*$^{+/-}$ mice, leading to a 50% reduction in the number of cells in the lens during early embryogenesis (17). Defects in the expression of genetic or biochemical markers have also been reported in small eye ocular tissues (17, 18).

For this study, we tested the hypothesis that the phenotypic abnormalities in the *Pax6*$^{+/-}$ eye are attributable to cell-autonomous effects that lead to a failure of mutant cells to contribute properly to, or be fully maintained in, affected tissues. This hypothesis predicts that *Pax6*$^{+/+}$ and *Pax6*$^{+/-}$ cells would behave differently in anterior segment structures of *Pax6*$^{+/+}$ ↔ *Pax6*$^{+/-}$ chimeras. We found lens defects in small eye mice and autonomous deficiencies of *Pax6*$^{+/-}$ cells in the lenses of chimeras, whereas, in contrast, heterozygous cells contributed normally to all other ocular tissues. The small eye phenotype was corrected in chimeras with up to 80% *Pax6*$^{+/-}$ cells in which the lens epithelium was wt. We conclude that abnormalities in other eye tissues (in small eye mice and, by extrapolation, human patients) arise as secondary consequences of primary defects in the lens.

Methods

Analysis of the small eye Mice. *Pax6*$^{Sey/+}$ embryos allocated for semithin histological analysis were fixed in 2.5% (vol/vol) glutaraldehyde in sodium cacodylate buffer (pH 7.3). Heads were bisected and washed three times in 10% (vol/vol) ethanol, then three times in 100% ethanol. After transfer to propylene oxide, heads were embedded in Araldite (York Survey Supply Centre, York, U.K.). Transverse sections of 1 μm were cut and stained with toluidine blue.

Production and Analysis of Chimeras. Mouse stocks, their maintenance, and the production of chimeras by aggregation of eight-cell embryos from pigmented small eye and albino *Pax6*$^{+/+}$ matings ([*Pax6*$^{Sey\text{-}Neu1/+}$, *C*/*C*, *Gpi1*$^{b/b}$ × *Pax6*$^{Sey/+}$, *C*/*C*, *Tg*/*Tg*, *Gpi1*$^{b/b}$] ↔ [*Pax6*$^{+/+}$, *c*/*c*, *Gpi1*$^{a/a}$ × *Pax6*$^{+/+}$, *c*/*c*, *Gpi1*$^{a/a}$]) have been described diagrammatically (13). The reiterated β-globin transgene *TgN(Hbb-b1)83Clo* (*Tg*) is carried homozygously by the *Pax6*$^{Sey/+}$ male studs and used as a nuclear marker for cells derived from the *Pax6*$^{Sey\text{-}Neu1/+}$, *C*/*C*, *Gpi1*$^{b/b}$ × *Pax6*$^{Sey/+}$, *C*/*C*, *Tg*/*Tg*, *Gpi1*$^{b/b}$ eight-cell embryo, whatever their *Pax6* genotype.

Chimeric embryos were dissected into cold PBS and staged according to criteria of forelimb development (19). The head was removed and fixed in 4% (vol/vol) paraformaldehyde overnight at 4°C, before processing to wax. Tail tissue was removed for PCR genotyping, as described (14). The use of two mutant alleles of *Pax6* allowed homozygous mutant chimeras (*Pax6*$^{Sey/Sey\text{-}Neu1}$

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: wt, wild type; E*n*, embryonic day *n*; *Tg*, β-globin transgene *TgN(Hbb-b1)83Clo;* TUNEL, terminal deoxynucleotidyltransferase-mediated UTP end labeling; Brd-Urd, 5-bromodeoxyuridine; RPE, retinal pigmented epithelium; I(p), pigmented layer of iris; I(i), inner layer of iris.

§To whom reprint requests should be addressed. E-mail: John.West@ed.ac.uk.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

www.pnas.org/cgi/doi/10.1073/pnas.161144098

↔ *Pax6*$^{+/+}$) to be distinguished from heterozygous chimeras (*Pax6*$^{+/+}$ ↔ *Pax6*$^{Sey/+}$ and *Pax6*$^{+/+}$ ↔ *Pax6*$^{Sey\text{-}Neu1/+}$) by PCR. Only the heterozygous chimeras were used in the present study.

Tissues from the limbs and trunk were taken for colorimetric quantitative GPI1 analysis (20), giving a global value of the percentage of cells derived from each aggregated eight-cell embryo in the chimera. For all aggregations, the eight-cell embryo derived from the wt mating was *Gpi1*$^{a/a}$, and the eight-cell embryo derived from the *Pax6*$^{+/Sey\text{-}Neu1}$ × *Pax6*$^{Sey/+}$ mating was *Gpi1*$^{b/b}$. The percentage contribution of GPI1-B was measured by electrophoresis for all resulting chimeras—those with a higher percentage of GPI1-B are composed primarily of cells derived from the small eye mating and vice versa.

Histological analysis of the distribution of cells derived from the *Pax6*$^{Sey\text{-}Neu1/+}$ × *Pax6*$^{Sey/+}$ embryo was facilitated by performing DNA *in situ* hybridization on 7-μm sections with digoxygenin-labeled β-globin probe to detect the reiterated *Tg* transgene (21).

After *in situ* hybridization, the percentage of *Tg*$^{+}$ cells in various tissues of the eye was calculated. Primary estimates of the percentage of hybridization-positive cells were corrected as follows on a tissue-by-tissue basis to allow for failure to detect the signal in some *Tg*$^{+}$ cells caused by sectioning through the nucleus. The apparent percentage of hybridization-positive nuclei in each tissue of (*Pax6*$^{Sey\text{-}Neu1/+}$, Tg$^{-/-}$) × (*Pax6*$^{Sey/+}$, *Tg*$^{+/+}$) embryos (nonchimeric, 100% *Tg*$^{+}$) was counted, and these percentages were used as correction factors when counting the hybridization-positive cells in the same tissues in chimeras. Statistical analysis of count data was performed with STATVIEW statistical software (Abacus Concepts, Berkeley, CA).

Measurement of Adult Eye Size. Eyes were excised and placed in PBS. With the cornea uppermost, the diameter was measured three times by using a dissecting microscope with a graticule, rotating the eye on the vertical axis 120° between each measurement. The mean diameter was taken.

Terminal Deoxynucleotidyltransferase-Mediated UTP End Labeling (TUNEL). The *In Situ* Cell Death Detection Kit, Fluorescein (Roche Molecular Biochemicals) was used to label apoptotic cells in tissue sections. Dewaxed sections were exposed to proteinase K (80 μg/ml) for 20 min at room temperature. Slides were washed twice in PBS. One slide then was treated with DNase1 (Roche Molecular Biochemicals) as a positive control (50 units/ml DNase1 in 50 mM Tris·HCl, pH 7.5/1 mM $MgCl_2$/1 mg/ml BSA for 30 min at 37°C, followed by two 5-min washes in PBS). TUNEL labeling was performed according to the manufacturer's instructions. Slides then were washed two times for 5 min in PBS and mounted in Vectashield (Vector Laboratories). It was possible to perform the *in situ* protocol before TUNEL labeling to identify heterozygote cells in sections of chimeric eyes.

5-Bromodeoxyuridine (BrdUrd) Labeling. Pregnant mice were injected with 0.2 ml of 10 mg/ml BrdUrd in saline and killed after 45 min, and the fetuses were excised. Heads were fixed for 3 hr in 4% (vol/vol) paraformaldehyde and processed to wax; other tissues were used for PCR genotyping. BrdUrd-staining with diaminobenzidine substrate has been described (22).

Results

Developmental Defects in the Eyes of *Pax6*$^{+/-}$ Embryos. Comparison of microphthalmic heterozygous *Pax6*$^{+/-}$ embryos between embryonic day (E)12.5 and E17.5 with wt littermates revealed consistent defects of the lens and anterior segment (Fig. 1). We found a phenotype that partly mirrors human *PAX6*$^{+/-}$ phenotypes: a persistent plug of ectoderm linking the corneal and anterior lens epithelia, extensive vacuolation of the primary lens fibers; reduced anterior chamber, with multiple sites of adhesion between the anterior surface of the lens and the corneal endothelium; persistence of the lens vesicular cavity; and delayed fusion of the eyelids. The *Pax6*$^{+/-}$ lens is developmentally delayed and shows evidence of degeneration. The lens-corneal bridge is a center of high apoptosis but normal proliferation (Fig. 1 *g* and *h*). In the nonchimeric small eye, this structure may act as a sink for lens epithelial cells. It is not possible to say to what extent this cell death may affect the eventual lens size.

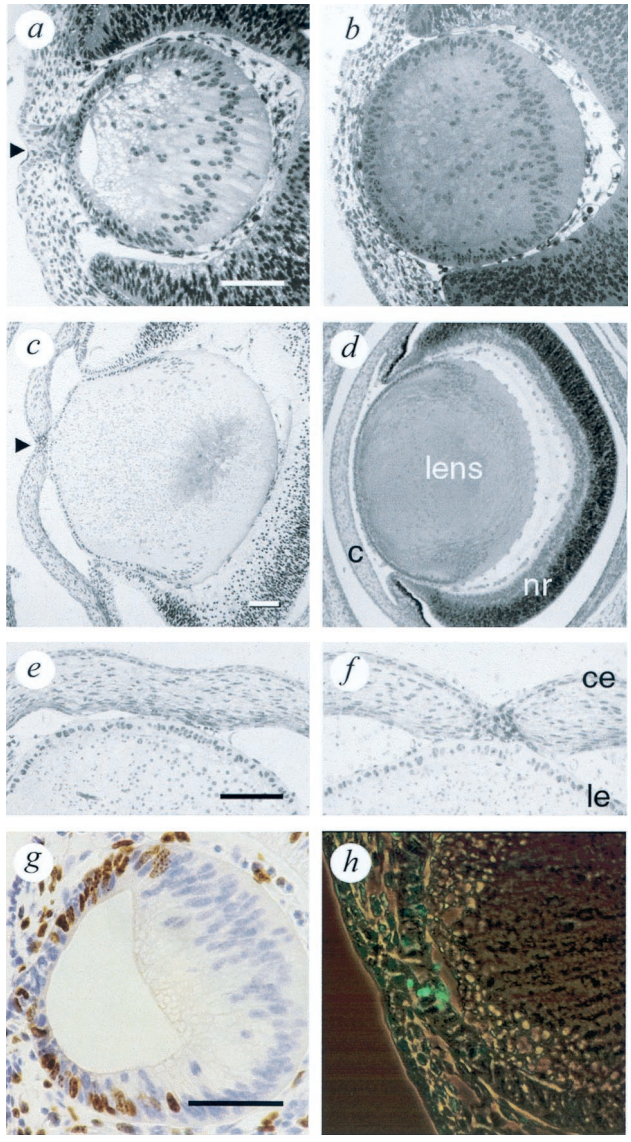

Fig. 1. Development of the *Pax6*$^{+/-}$ lens. Plastic sections (1 μm) of eyes from small eye and wt mice, at E13.5 (*a* and *b*) and E16.5 (*c–f*). (*a*) Extensive vacuolation of the small eye lens is seen, with persistence of a lens-corneal plug (arrowhead); (*b*) a wt littermate. (*c–f*) Lens-corneal plug, an adhesion between the corneal epithelium and the lens epithelium (arrowhead) persists at E16.5. (*d*) A wt E16.5 eye. (*e*) Multiple adhesions between the lens and corneal endothelium in the E16.5 *Pax6*$^{+/-}$ eye with reduction of the anterior chamber. (*g*) BrdUrd staining (brown) in E12.5 lens shows no defects in the pattern of proliferation of *Pax6*$^{+/-}$ eyes. The proliferation index showed no difference between wts and heterozygotes (see text), in agreement with ref. 17. (*h*) Fluorescence TUNEL in the E12.5 *Pax6*$^{+/-}$ lens shows apoptotic cells only in the lens-corneal bridge. Apoptotic cells were not found in the lens epithelium of E12.5 wt *Pax6*$^{+/+}$ eyes. c, cornea; nr, neural retina; ce, corneal epithelium; le, lens epithelium. (Bars = 50 μm.) Figs. 1 *a–f* are reproduced from M.A.B.'s BSc. thesis (1995, Univ. of Edinburgh, Scotland, U.K., unpublished).

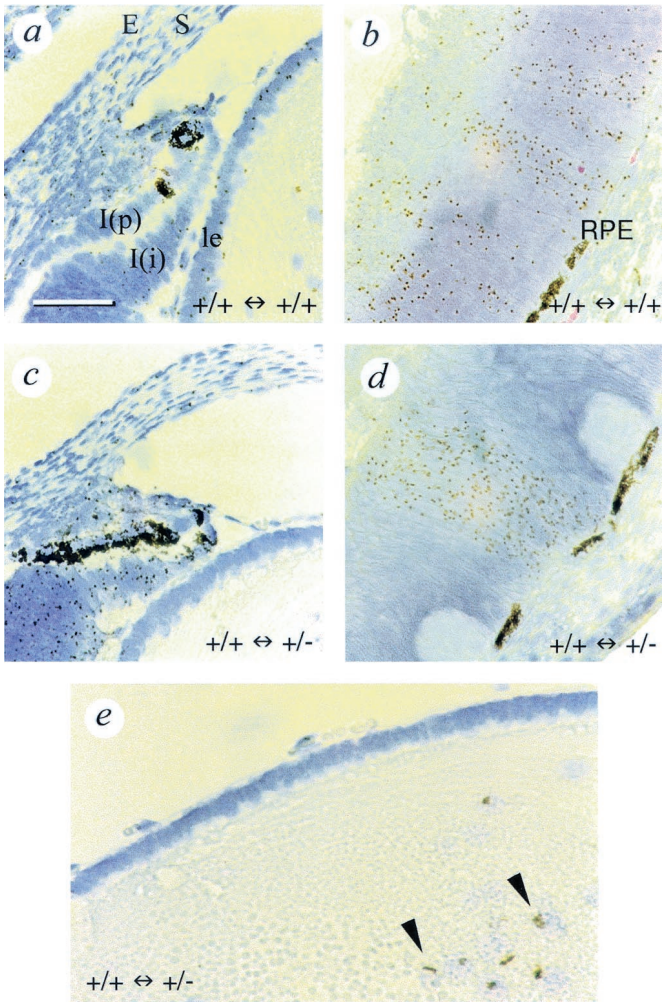

Fig. 2. Pax6$^{+/-}$ cells contribute to all chimeric eye tissues except the lens epithelium. Histological sections of the lens epithelium, iris, and cornea (*a*, *c*, and *e*) and retina (*b* and *d*) of *Pax6*$^{+/+}$ ↔ *Pax6*$^{+/+}$ (*a* and *b*) and *Pax6*$^{+/+}$ ↔ *Pax6*$^{+/-}$ (*c*–*e*) E16.5 aggregation chimeras. *Tg*$^{+}$ cells derived from the aggregated *Pax6*$^{+/Sey\text{-}Neu1}$ × *Pax6*$^{Sey/+}$ embryo (whether *Pax6*$^{+/-}$ or *Pax6*$^{+/+}$) are visualized by *in situ* hybridization and have a brown spot in the nucleus (see *Methods*). Pigmented cells in the iris and retinal pigmented epithelium (RPE) are also derived from the *Pax6*$^{+/Sey\text{-}Neu1}$ × *Pax6*$^{Sey/+}$ embryo. (*e*) Arrowheads indicate small numbers of enucleating *Tg*$^{+}$ fibers in the lens of a *Pax6*$^{+/+}$ ↔ *Pax6*$^{+/-}$ chimera that was 77% heterozygous cells. The anterior epithelium is entirely wt. E, corneal epithelium; S, corneal stroma; le, lens epithelium. (Bar = 40 μm.)

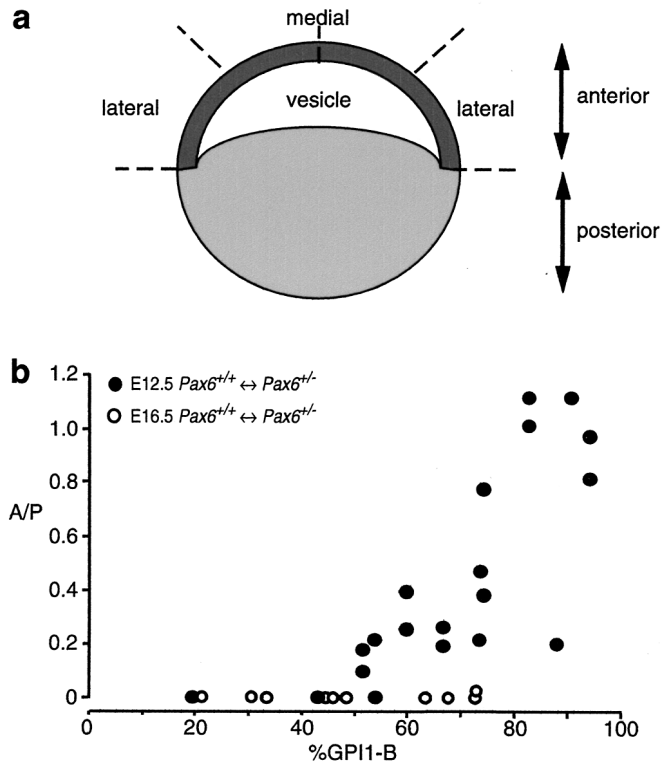

Fig. 3. Loss of heterozygous cells from the anterior lens epithelium after lens vesicle closure. The percentages of *Tg*$^{+}$ cells were compared between the anterior epithelium and posterior lens fibers of *Pax6*$^{+/+}$ ↔ *Pax6*$^{+/-}$ chimeras at E12.5 and E16.5. (*a*) Representation of the lens at E12.5. The area defined as anterior (lens epithelium) is shaded dark gray. Posterior area (lens fibers) is shaded pale gray. Near-central sections of the lens epithelium were also divided into defined lateral and medial quartiles. (*b*) The percentage of *Tg*$^{+}$ cells anteriorly (*A*) was divided by the percentage of *Tg*$^{+}$ cells posteriorly (*P*) for each *Pax6*$^{+/+}$ ↔ *Pax6*$^{+/-}$ lens, and the ratio *A*/*P* was plotted against fetal percentage of GPI1-B (*Methods*). Analysis at E12.5 showed that, in chimeras that were <50% *Pax6*$^{+/-}$ cells, these cells are lost from the lens epithelium (although normally detectable in the posterior lens fibers). In contrast, increasing numbers of *Pax6*$^{+/-}$ cells remained in the anterior lens of chimeras that had a >50% GPI1-B contribution. By E16.5 (○), the elimination of *Pax6*$^{+/-}$ cells from the epithelium was almost complete, showing that elimination was ongoing at E12.5. *A*/*P* ratio for *Pax6*$^{+/+}$ ↔ *Pax6*$^{+/+}$ control lenses was mean ± SEM = 0.994 ± 0.029 and was not affected by the percentage of GPI1-B.

To investigate the cell autonomous roles of *Pax6* in defects of various eye tissues further, 29 *Pax6*$^{+/+}$ ↔ *Pax6*$^{+/+}$ "control chimeras" and 70 *Pax6*$^{+/+}$ ↔ *Pax6*$^{+/-}$ "heterozygous chimeras" were made and analyzed at E10.5, E12.5, and E16.5.

An Autonomous Failure of *Pax6*$^{+/-}$ Cells to Contribute Normally to the Lens Placode. In other studies (14), we detected no difference between the behavior of wt and heterozygous cells in chimeric embryos at E9.5. To investigate whether heterozygous cells are as competent as wt cells to populate the invaginating lens placode, E10.5 heterozygous chimeras were compared with control chimeras. After *in situ* hybridization for the β-globin transgene *Tg* to detect the cells in the chimera that were derived from the *Pax6*$^{Sey/+}$ × *Pax6*$^{+/Sey\text{-}Neu1}$ eight-cell embryo (see *Methods*), the percentage of *Tg*$^{+}$ cells in the lens placode (*L*) was divided by the percentage of *Tg*$^{+}$ cells in the dorsal head surface epithelium (*H*), which does not express *Pax6*. In control chimeras, the mean (± SEM) of the *L*/*H* ratio was 1.047 ± 0.069 (n = 13). For heterozygous chimeras, *L*/*H* = 0.690 ± 0.093 (n = 18), indicating significant underrepresentation of *Pax6*$^{+/-}$ cells in the lens placode (Mann–Whitney *U* test; P = 0.014). This result demonstrates an autonomous deficiency of *Pax6*$^{+/-}$ cells at or immediately after lens placode formation—they are not incorporated into the lens placode as readily as wt cells. A recent study (17) reported a delay in lens placode induction in *Pax6*$^{+/-}$ mice but could not demonstrate whether this delay was a failure of induction by the optic vesicle or an autonomous failure of the lens placode. Our data show that the developmental defect resides in the placode.

An Autonomous Defect of the Heterozygous Lens Epithelium. A striking feature of E16.5 eyes was a near-complete absence of *Pax6*$^{+/-}$ cells from the lenses of heterozygous chimeras (Fig. 2). Those *Pax6*$^{+/-}$ cells that populate the lens placode at E10.5 were rarely seen in the E16.5 lens. In *Pax6*$^{+/+}$ ↔ *Pax6*$^{+/+}$ chimeras, wt *Tg*$^{+}$ cells were distributed throughout all tissues, including the anterior lens epithelium, in proportions that would be expected from the global composition of the embryo (percentage of

Table 1. Heterozygous cells are not underrepresented in the iris (I) or corneal epithelium (E) of chimeras

Chimeric eye	%I(p)*	%I(i)†	%RPE‡	I(p)/RPE	I(i)/RPE	E§	S¶	E/S
Control (wild-type) chimeras								
MC268 (L)	6.57	6.21	14.60	0.45	0.43	14.40	12.68	1.13
MC268 (R)	8.62	5.67	8.97	0.96	0.63	19.61	24.66	0.80
MC232 (L)	33.05	33.01	30.52	1.09	1.08	41.28	37.74	1.09
MC232 (R)	45.48	55.07	51.90	0.89	1.06	37.85	35.43	1.07
MC228 (L)	40.05	34.84	30.33	1.33	1.08	15.31	14.70	0.99
MC228 (R)	36.47	25.45	39.31	0.93	0.65	14.21	12.88	1.10
MC328 (L)	7.30		5.42	1.35		5.43	5.83	0.93
MC328 (R)	2.67	3.93	3.62	0.74	1.08	5.19	5.39	0.95
MC310 (L)	58.37	34.09	49.46	1.18	0.69	45.82	53.80	0.84
MC310 (R)	22.85	26.55	31.77	0.72	0.84	34.77	35.33	1.01
Mean ± SEM	—	—	—	0.96 ± 0.090	0.84 ± 0.082	—	—	0.99 ± 0.036
Heterozygous chimeras								
MC262 (L)	15.31	23.19	13.61	1.12	1.70	14.89	24.07	0.61
A/M9 (L)	14.92	24.34	15.33	0.97	1.59	4.49	13.06	0.34
A/M3 (L)	25.61		37.76	0.68		13.22	12.76	1.04
A/M3 (R)	37.10	25.68	25.44	1.46	1.01			
A/M4 (L)	32.41	56.51	38.75	0.84	1.46	16.95	6.75	2.51
MC231 (L)	25.75	23.11	23.97	1.07	0.97	25.50	23.01	1.11
MC231 (R)	23.14	40.09	23.44	0.99	1.71	22.94	25.47	0.91
MC269 (L)	30.08	25.09	32.18	0.93	0.78			
MC264 (L)	16.11	14.79	15.89	1.01	0.93	20.87	17.09	1.22
MC264 (R)	15.76	15.75	16.27	0.97	0.97	9.68	7.63	1.27
MC274 (L)	61.32	48.82	51.37	1.19	0.95	40.54	57.64	0.70
MC311 (L)	48.20	43.68	46.46	1.04	0.94	48.32	45.35	1.07
MC311 (R)	50.00	8.87	52.26	0.96	0.17	29.91	29.01	1.03
MC322 (L)	73.65	62.67	53.05	1.39	1.18	33.24	35.05	0.95
MC322 (R)	82.72	71.13	71.97	1.15	0.99	46.89	18.43	2.55
Mean ± SEM	—	—	—	1.05 ± 0.051	1.10 ± 0.11	—	—	1.18 ± 0.18

Mann Whitney *U* tests comparing the ratios for wt and heterozygous chimeras showed no significant difference between the medians of $I(p)/RPE^{+/+}$ and $I(p)/RPE^{+/-}$ ($P = 0.35$), $I(i)/RPE^{+/+}$ and $I(i)/RPE^{+/-}$ ($P = 0.19$), or $E/S^{+/+}$ and $E/S^{+/-}$ ($P = 0.71$). *t* tests on log-transformed data also showed no significant differences in the means (not shown). Note that these are specific tests for underrepresentation of $Pax6^{+/-}$ cells in tissues that are overtly affected in heterozygotes, and would not necessarily identify more general loss of cells from the whole retina or eye.

*% I(p), % pigmented cells in pigmented layer of iris.

†%I(i), %Tg^+ cells in inner layer of iris.

‡%RPE, % pigmented cells in RPE.

§E, %Tg^+ cells in corneal epithelium.

¶S, %Tg^+ cells in corneal stroma.

GPI1-B; 13 eyes examined; Fig. 2*a*). Clonally derived stripes of $Pax6^{+/+}$, Tg^+ cells were seen across all layers of the neural retina (Fig. 2*b*). In heterozygous chimeras, the distribution of $Pax6^{+/-}$, Tg^+ cells was identical to that of the wt controls (Fig. 2 *c* and *d*), except that $Pax6^{+/-}$ Tg^+ cells were largely absent from the chimeric lenses. $Pax6^{+/-}$ cells were not detected in the anterior epithelium (18 eyes; Fig. 2*c*) and were only occasionally detected in the lens fibers (3 of 18 eyes; Fig. 2*e*).

Analysis of lenses of heterozygous chimeras at E12.5 showed that, although $Pax6^{+/-}$ cells were present in the posterior lens fibers, they were underrepresented and undergoing clearance in the anterior epithelium (Fig. 3). Hence, loss of heterozygous cells from the lens epithelium, which is the sole source of proliferating cells (Fig. 1*g*) to replace terminally differentiating lens fibers, results in the production of a largely wt lens.

The mechanism of elimination of $Pax6^{+/-}$ cells from the lens epithelium was investigated. Apoptosis and proliferation in lenses of wt, heterozygous, and chimeric embryos were studied at E10.5 to E16.5. In agreement with previous work (17), the rates and pattern of proliferation of cells in the heterozygous lens were not significantly different from those of wt. BrdUrd labeling in the E12.5 lens epithelia was 44.0 ± 2.46% ($n = 6$) for $Pax6^{+/+}$ and 42.6 ± 1.60% ($n = 8$) for $Pax6^{+/-}$ ($t = 1.22$, $P = 0.26$).

The persistent lens-corneal bridge of $Pax6^{+/-}$ embryos was a region of high apoptosis (Fig. 1). No chimera retained a lens-corneal bridge after E12.5, even when the corneal epithelium (but not the lens epithelium) was predominantly mutant, suggesting that the bridge is a result of autonomous deficiency in the lens. No apoptotic elimination of heterozygous cells from chimeric lens epithelia was detected (data not shown).

The distribution of heterozygous cells in the lens epithelium of high-percentage chimeras at E12.5 was nonrandom; the remaining $Pax6^{+/-}$ cells were distributed near the lateral edge of the epithelium. The epithelia in tissue sections through the middle of $Pax6^{+/+} \leftrightarrow Pax6^{+/-}$ and control chimeric lenses were analyzed. The percentage of Tg^+ cells was calculated in defined medial (*m*) and lateral (*l*) regions of the epithelium (see Fig. 3*a*). For control chimeras, the mean m/l ratio was 1.061 ± 0.107 ($n = 7$), indicating a random distribution of Tg^+ wt cells. The mean m/l ratio for $Pax6^{+/+} \leftrightarrow Pax6^{+/-}$ chimeras was 0.181 ± 0.119 ($n = 8$). The difference between the two groups was highly significant (Mann–Whitney *U* test, $P = 0.0005$).

During normal lens development, cells at the periphery of the epithelium stop dividing, differentiate as lens fibers, and extrude their nuclei. Taken together, our results demonstrate a persistent autonomous defect of heterozygous cells in the anterior lens epithelium. $Pax6^{+/-}$ cells are not lost but segregate peripherally and preferentially move out of the epithelium between E12.5 and E16.5, where they differentiate, enucleate, and become unde-

DEVELOPMENTAL BIOLOGY

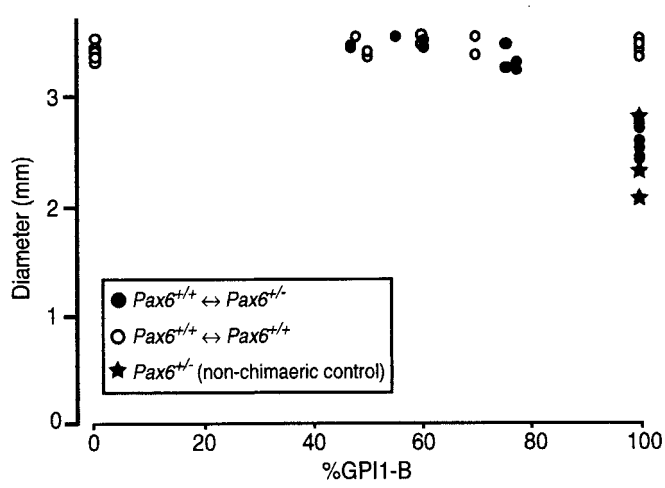

Fig. 4. Eye size is corrected in adult chimeras. Mean equatorial eye diameter (D) was measured before fixation for adult (7 months) $Pax6^{+/+} \leftrightarrow Pax6^{+/+}$ (○) and $Pax6^{+/+} \leftrightarrow Pax6^{+/-}$(●) chimeras (0 to 100% GPI1-B), as well as control nonchimeric $Pax6^{+/-}$ eyes (⋆). D was plotted against the percentage of GPI1-B for each eye.

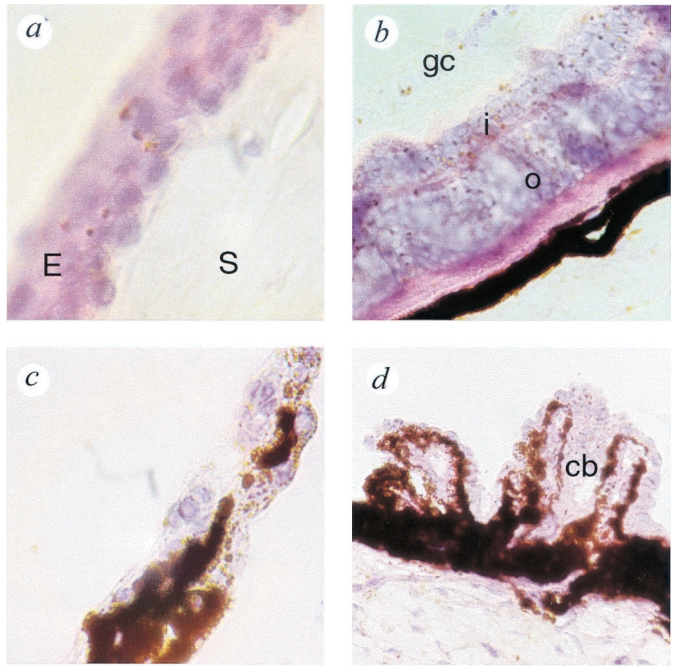

Fig. 5. Sections of adult chimeric eye tissues. The contribution of heterozygous cells (pigmented or Tg^{+}) is maintained in adulthood in corneal epithelium (*a*), all layers of the retina (*b*), all layers of the iris (*c*), and in ciliary body in adult $Pax6^{+/+} \leftrightarrow Pax6^{+/-}$ chimeras (*d*). Pigmentation in the iris is derived both from the RPE-derived layer and the neural crest-derived stromal outer layer. $Pax6^{+/-}$ cells contribute to both layers. The chimera was 74% GPI1-B. E, corneal epithelium; S, corneal stroma; gc, ganglion cell layer; i, inner nuclear layer; o, outer nuclear layer; cb, ciliary body.

tectable by *in situ* hybridization. Hence, all lens fibers laid down subsequently by the epithelium are wt.

***Pax6*$^{+/-}$ Cells Contribute Normally to All Other Chimeric Eye Tissues.** For the series of E16.5 $Pax6^{+/+} \leftrightarrow Pax6^{+/-}$ and control chimeras, the contribution of Tg^{+} cells to the corneal epithelium (which expresses *Pax6*; E) was compared with the contribution to the corneal stroma (that does not express *Pax6*; S). The presence of pigmentation in appropriate tissues can be used as a marker of cells derived from the $Pax6^{Sey/+} \times Pax6^{+/Sey\text{-}Neu1}$ eight-cell embryo (homozygous for the pigmentation gene, *C*/*C*). The contributions to the developing I(p) and I(i) were compared with the contribution to the RPE (which expresses *Pax6* at low levels and is not overtly affected in the heterozygous small eye mouse). There was no evidence of underrepresentation of heterozygous cells in the corneal epithelium or either of the *Pax6*-expressing layers of the iris, compared with the controls (Table 1). Thus, $Pax6^{+/-}$ cells are able to participate normally in the early stages of cornea and iris development.

Correction of the Adult small eye Phenotype. To investigate further the development of the $Pax6^{+/-}$ phenotype, 28 chimeras were analyzed 7 months postnatally (Fig. 4). Control chimeric eyes were relatively invariant in size. $Pax6^{+/-}$ eyes (100% GPI1-B) were significantly smaller as expected ($t = 12.37$, $P < 0.0001$). $Pax6^{+/+} \leftrightarrow Pax6^{+/-}$ eyes ($n = 12$) from 40–80% GPI1-B were of wt diameter, not of intermediate sizes. Eye diameters of $Pax6^{+/+} \leftrightarrow Pax6^{+/+}$ and $Pax6^{+/+} \leftrightarrow Pax6^{+/-}$ chimeras did not differ significantly ($t = 0.63$, $P = 0.53$). $Pax6^{+/+} \leftrightarrow Pax6^{+/-}$ eyes were, therefore, of normal wt size. The requirement of only 20–25% $Pax6^{+/+}$ cells in the chimeric eye, which has been shown (Figs. 2 and 3) to be sufficient to correct the phenotype of the lens epithelium, is thus sufficient also to correct microphthalmia.

Heterozygous cells contributed well to the ciliary body, iris, corneal epithelium, and all layers of the retina in adult chimeras (Fig. 5). These chimeric eyes showed no obvious examples of iris hypoplasia, cataracts, or retention of a lens-corneal bridge (defects that are found with very high penetrance in our small-eye stock). Thus, in addition to eye size, other pathological features can be rescued functionally in $Pax6^{+/+} \leftrightarrow Pax6^{+/-}$ chimeras in which the lens epithelium is wt.

Discussion

Histological study of $Pax6^{+/-}$ embryos from E12.5 demonstrated that the $Pax6^{+/-}$ lens is developmentally delayed and degenerate, with vacuolation that may underlie the later occurrence of cataracts. Subsequent chimeric data demonstrated two defects of the $Pax6^{+/-}$ lens: (*i*) a cell autonomous deficiency at lens placode formation, such that heterozygous cells contribute less readily to the invaginating lens placode at E10.5; and (*ii*) after closure of the lens pit, a cell autonomous defect of heterozygous cells in the lens epithelium, such that $Pax6^{+/-}$ cells segregate and move prematurely into the posterior of the lens.

The failure of $Pax6^{+/-}$ cells to contribute normally to the lens placode provides a mechanism to explain the delayed appearance of this placode reported in $Pax6^{+/-}$ mice (17). It suggests this failure is not one of induction by the optic vesicle but a defect of the lens-competent cells.

There are no abnormalities in cell proliferation in the small-eye lens, nor is there any general increase in apoptosis that might have explained the preferential loss of heterozygous cells from the chimeric lens epithelium. The defect in the heterozygous lens epithelium is consistent with an altered profile of cell-surface molecules, leading to the segregation of heterozygous cells in chimeras and their exclusion from the lens epithelium. This defect is consistent with other evidence that *Pax6* controls the expression of cell-adhesion molecules and affects the interaction of cells with each other or the extracellular matrix (23–26). Our study extends these observations about the importance of correctly regulated *Pax6* expression for cell behavior, and we postulate that changes in cell adhesion caused by a haploinsufficiency of *Pax6* have functional implications for the development of the heterozygous lens.

Analysis of $Pax6^{+/+} \leftrightarrow Pax6^{-/-}$ chimeras demonstrated roles for PAX6 in all eye tissues where the gene is expressed (previous

studies and data not shown). The hypothesis that drove the current experiments was that the abnormal phenotype of *Pax6*$^{+/-}$ eyes was caused by a haploinsufficiency that would be reflected in chimeras by an inability of heterozygous cells to contribute fully or be properly maintained in all affected tissues. Contrary to our hypothesis, at E16.5 we were unable to detect any underrepresentation of heterozygous cells in anterior segment tissues other than the lens. We showed that heterozygous cells compete well with wt in the ciliary body, iris, and cornea of chimeric eyes up to 7 months postnatally.

There may be subtle defects in the kinetics of differentiation of *Pax6*$^{+/-}$ cells in the iris, cornea, or ciliary body that we have not detected. It is clear, however, that any defects within these tissues are qualitatively different from the primary functional consequences of *Pax6* heterozygosity in the lens.

The astonishing correlation between the correction of the embryonic lens phenotype and subsequent normalization of adult eye morphology in chimeras that are otherwise up to 80% *Pax6*$^{+/-}$ suggests that the lens abnormalities are primary defects in the heterozygous small eye phenotype. It is uncertain why affected small eye tissues are primarily those of the anterior segment, but the lens produces growth factors (27) and influences the development of the ciliary body, iris, and cornea (28–31). Although a layered optic cup can form in the absence of a lens (13, 15), there is experimental evidence that the lens plays a major role in directing the development of other eye tissues (32, 33). *Pax6* has fundamental roles in lens development (refs. 13–15 and this study). Therefore, we postulate that reduction of *Pax6* gene dosage compromises the ability of the lens to instigate inductive interactions that organize the anterior segment. The link that we have demonstrated between lens phenotype and eye size is consistent with previous experimental data and may be mediated by means of the induction of ciliary body function (34–36). Therefore, our data, although surprising, fall within the overall picture of previous data that have demonstrated the importance of the *Pax6* for lens development and the role of the lens in organizing eye development.

We have shown that wt cells are at a selective advantage over heterozygotes in the developing chimeric lens. By extrapolating our results to shared aspects of the human *PAX6*$^{+/-}$ phenotype, we suggest that partial therapeutic correction of the lens in affected individuals identified by prenatal diagnosis (37) could significantly ameliorate several of the clinical defects responsible for poor eyesight, ocular degeneration, and blindness.

We thank Jean Flockhart and Margaret Keighren for their expert help. We are grateful to Denis Doogan, Jim Macdonald, Maureen Ross, and Ted Pinner for specialist assistance. Preliminary studies of cell proliferation in *Pax6*$^{+/-}$ mice were performed by Richard Redfern, Richard Newman, and John Collin. The work was supported by Medical Research Council Award G9630132 (to J.D.W.).

1. Walther, C. & Gruss, P. (1991) *Development (Cambridge, U.K.)* **113,** 1435–1449.
2. Grindley, J. C., Davidson, D. R. & Hill, R. E. (1995) *Development (Cambridge, U.K.)* **121,** 1433–1442.
3. Hitchcock, P. F., Macdonald, R. E., van de Ryt, J. T. & Wilson, S. W. (1996) *J. Neurobiol*. **29,** 399–413.
4. Koroma, B. M., Yang, J.-M. & Sundin, O. H. (1997) *Invest. Ophthalmol. Visual Sci.* **38,** 108–120.
5. Ton, C. C. T., Hirovenen, H., Miwa, H., Weil, M., Monaghan, A. P., Jordan, T., van Heyningen, V., Hastie, N. D., Meijers-Heijboer, H., Dreschler, M., *et al*. (1991) *Cell* **76,** 1059–1074.
6. Hanson, I., Fletcher, J., Jordan, T., Brown, A., Taylor, D., Adams, R., Punnett, H. & van Heyningen, V. (1994) *Nat. Genet.* **6,** 168–173.
7. Mirzayans, F., Pearce, W. G., MacDonald, I. M. & Walter, M. A. (1995) *Am. J. Hum. Genet.* **57,** 539–548.
8. Churchill, A. J. & Booth, A. (1996) *Br. J. Ophthalmol.* **80,** 669–673.
9. Hamburg, A. (1967) *Am. J. Ophthalmol.* **64,** 729–733.
10. Roberts, R. C. (1967) *Genet. Res.* **9,** 121–122.
11. Hill, R. E., Favor, J., Hogan, B. L. M., Ton, C. C. T., Saunders, G. F., Hanson, I. M., Prosser, J., Jordan, T., Hastie, N. D. & van Heyningen, V. (1991) *Nature (London)* **354,** 522–525.
12. Jordan, T. (1993) Ph.D. thesis (Univ. of Edinburgh, Scotland, U.K.).
13. Quinn, J. C., West, J. D. & Hill, R. E. (1996) *Genes Dev.* **10,** 435–446.
14. Collinson, J. M., Hill, R. E. & West, J. D. (2000) *Development (Cambridge, U.K.)* **127,** 945–956.
15. Ashery-Padan, R., Marquardt, T., Zhou, X. & Gruss, P. (2000) *Genes Dev.* **14,** 2701–2711.
16. Marquardt, T., Ashery-Padan, R., Andrejewski, N., Scardigli, R., Guillemot, F. & Gruss, P. (2001) *Cell* **105,** 43–55.
17. van Raamsdonk, C. D. & Tilghman, S. M. (2000) *Development (Cambridge, U.K.)* **127,** 5439–5448.
18. Pritchard, D. J., Clayton, R. M. & Cunningham, W. L. (1974) *Exp. Eye Res.* **19,** 335–340.
19. Wanek, N., Muneoka, K., Holler-Dinsmore, G., Burton, R. & Bryant, S. V. (1989) *J. Exp. Zool.* **249,** 41–49.
20. West, J. D. & Flockhart, J. H. (1994) *Genet. Res.* **63,** 87–99.
21. Keighren, M. & West, J. D. (1993) *Histochem. J.* **25,** 30–44.
22. Sharpe, R. M., Turner, K. J., McKinnell, C., Groome, N. P., Atanassova, N., Millar, M. R., Buchanan, D. L. & Cooke, P. S. (1999) *J. Androl.* **20,** 94–101.
23. Duncan, M. K., Kozmik, Z., Cveklova, K., Piatigorsky, J. & Cvekl, A. (2000) *J. Cell Sci.* **113,** 3173–3185.
24. Sivak, J. M., Mohan, R., Rinehart, W. B., Xu, P. X., Maas, R. L. & Fini, M. E. (2000) *Dev. Biol.* **222,** 41–54.
25. Meech, R., Kallunki, P., Edelman, G. M. & Jones, F. S. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 2420–2425.
26. Stoykova, A., Gotz, M., Gruss, P. & Price, J. (1997) *Development (Cambridge, U.K.)* **124,** 3765–3777.
27. de Iongh, R. & McAvoy, J. W. (1993) *Dev. Dyn.* **198,** 190–202.
28. Stroeva, O. G. (1963) *Dokl. Acad. Nauk SSSR* **151,** 464–467.
29. Genis-Galvez, J. M. (1966) *Nature (London)* **210,** 209–210.
30. Beebe, D. C. (1986) *Trans. Ophthalmol. Soc. U.K.* **105,** 123–130.
31. Beebe, D. C. & Coats, J. M. (2000) *Dev. Biol.* **220,** 424–431.
32. Coulombre, A. J. & Coulombre, J. L. (1964) *J. Exp. Zool.* **156,** 39–47.
33. Jeffrey, W. R. (2001) *Dev. Biol.* **231,** 1–12.
34. Stroeva, O. G. & Panova, I. G. (1983) *J. Embryol. Exp. Morphol.* **75,** 271–291.
35. Bard, J. B. L. & Ross, A. S. A. (1982) *Dev. Biol.* **92,** 73–86.
36. Porte, A., Stoeckl, M. E., Brini, A. & Metais, P. (1968) *Arch. Ophthalmol.* **28,** 259–282.
37. Churchill, A. J., Hanson, I. M. & Markham, A. F. (2000) *Ophthalmology* **107,** 1153–1156.