Hemizygous Le-Cre Transgenic Mice Have Severe Eye Abnormalities on Some Genetic Backgrounds in the Absence of LoxP Sites

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Abstract

Eye phenotypes were investigated in Le-Cre\textsuperscript{gr−}; Pax6\textsuperscript{fl/+} mice, which were expected to show tissue-specific reduction of Pax6 in surface ectoderm derivatives. To provide a better comparison with our previous studies of Pax6\textsuperscript{fl−−} eye phenotypes, hemizygous Le-Cre\textsuperscript{gr−} and heterozygous Pax6\textsuperscript{fl/+} mice were crossed onto the CBA/Ca genetic background. After the Le-Cre transgene had been backcrossed to CBA/Ca for seven generations, significant eye abnormalities occurred in some hemizygous Le-Cre\textsuperscript{gr−}; Pax6\textsuperscript{fl/+} controls (without a floxed Pax6\textsuperscript{fl} allele) as well as experimental Le-Cre\textsuperscript{gr−}; Pax6\textsuperscript{fl/+} mice. However, no abnormalities were seen in Le-Cre\textsuperscript{gr−}/+; Pax6\textsuperscript{fl/+} or Le-Cre\textsuperscript{gr−}/+; Pax6\textsuperscript{fl/+} controls (without the Le-Cre transgene). The severity and frequency of the eye abnormalities in Le-Cre\textsuperscript{gr−}; Pax6\textsuperscript{fl/+} control mice diminished after backcrossing Le-Cre\textsuperscript{gr−} mice to the original FVB/N strain for two generations, showing that the effect was reversible. This genetic background effect suggests that the eye abnormalities are a consequence of an interaction between the Le-Cre transgene and alleles of unknown modifier genes present in certain genetic backgrounds. The abnormalities were also ameliorated by introducing additional Pax6 gene copies on a CBA/Ca background, suggesting involvement of Pax6 depletion in Le-Cre\textsuperscript{gr−}; Pax6\textsuperscript{fl/+} mice rather than direct action of Cre recombinase on cryptic pseudo-loxP sites. One possibility is that expression of Cre recombinase from the Pax6-Le regulatory sequences in the Le-Cre transgene depletes cofactors required for endogenous Pax6 gene expression. Our observation that eye abnormalities can occur in hemizygous Le-Cre\textsuperscript{gr−}; Pax6\textsuperscript{fl/+} mice, in the absence of a floxed allele, demonstrates the importance of including all the relevant genetic controls in Cre-loxP experiments.

Introduction

\textit{Tg(Pax6-cre,GFP)1Pgr} transgenic mice (hereafter abbreviated to Le-Cre transgenic mice) express Cre recombinase from Pax6-Le tissue-specific regulatory elements (the Pax6 surface ectoderm enhancer and P0 promoter) in the pancreas and developing head surface ectoderm from embryonic day (E) 8.75 [1]. In their original study, Ashery-Padan et al. produced Le-Cre\textsuperscript{gr−} mice; Pax6\textsuperscript{fl−−/−} mice, which were hemizygous for the Le-Cre transgene and carried both the Pax6\textsuperscript{fl−−/−} null and floxed Pax6\textsuperscript{fl} alleles [1]. Pax6 was deleted in the head surface ectoderm lineage early in development, demonstrating that, in the absence of Pax6, the lens fails to develop and the optic cup develops abnormally. Subsequently Le-Cre transgenic mice have been widely used to delete floxed alleles of other genes in the developing surface ectoderm including \textit{Tgpa2a} [2], \textit{Fgfr2} [3], \textit{Ctnmb} (previously \textit{Catnb}; β-catenin) [4], \textit{Six3} [5], \textit{Klf4} [6,7], \textit{Klf5} [8,9], \textit{Ptn} [10], \textit{Spry1} and \textit{Spry2} [11], \textit{Rac1} [12], \textit{Ndst1} (in a \textit{Ndst2}−−/− background) [13], \textit{Ilk} [14], \textit{Vegfa} [15] and \textit{Cited2} (either alone or in combination with floxed \textit{Vgf2} or floxed \textit{Hsf1}a) [16,17,18].

We conditionally excised a single Pax6\textsuperscript{null} allele in the head surface ectoderm and derivatives (lens, conjunctiva and corneal epithelium) of Le-Cre\textsuperscript{gr−}; Pax6\textsuperscript{fl−−/−} mice to reduce Pax6 levels in these tissues rather than completely delete it. Pax6\textsuperscript{null} levels are reduced globally in Pax6\textsuperscript{fl−−/−} mice, which are heterozygous for any Pax6\textsuperscript{fl−} null allele (e.g. Pax6\textsuperscript{fl−−/−y} or Pax6\textsuperscript{fl−/−y,Nos}). This global reduction results in a complex combination of abnormal phenotypes in the fetal and adult cornea, which disrupts corneal homeostasis [19,20,21,22] and affects wound healing [23,24]. We aimed to analyse the consequences of tissue-specific depletion of Pax6 on the corneal phenotype and compare this to the previously reported consequences of globally reducing Pax6 levels in Pax6\textsuperscript{fl−−/−} heterozygotes, thereby distinguishing abnormalities caused by low levels of Pax6 in the surface ectoderm lineage from those caused by low levels of Pax6 in other tissues, such as the optic cup. As the lens produces growth factors and probably influences the development of other anterior segment tissues [25,26], we...
anticipated that depletion of Pax6 in the surface ectoderm tissues might also affect neighbouring tissues not derived from this lineage. This could occur if reduced Pax6 affected the production of signalling molecules or if Pax6 was itself secreted extracellularly, as reported for the developing chick nervous system [27].

Two previous studies used a similar approach and showed that reducing Pax6 levels in the surface ectoderm of Le-CreTg/+; Pax6fl/+ eyes was sufficient to produce a number of developmental abnormalities that are characteristic of Pax6−/− heterozygotes. Some of these directly affected the lens and corneal epithelium (e.g. the lens was small and often remained attached to the cornea), suggesting an autonomous requirement for normal Pax6 gene dosage during development of these tissues [28]. However, other abnormalities suggested that normal Pax6 gene dosage in the surface ectoderm tissues is also required for normal development of some neighbouring tissues, implying a non-cell-autonomous mode of action. The trabecular meshwork and Schlemm’s canal failed to develop and adhesions occurred between the peripheral iris and cornea causing the iridocorneal angle to be closed in the adult Le-CreTg/+; Pax6fl/+ eye [29]. These abnormalities suggest that reduced Pax6 gene dosage in the surface ectoderm tissues can cause abnormalities in the neighbouring ocular mesenchyme.

We planned to extend these experiments and use Le-CreTg/+; Pax6fl/+ eyes with a panel of morphological, immunohistochemical and wound-healing endpoints to consider a wider range of abnormal corneal phenotypes, as previously reported for adult Pax6−/− heterozygotes. To allow direct comparisons with phenotypes in our previous studies of Pax6−/− mice on a predominantly CBA/Ca genetic background, we crossed the Le-Cre transgene onto this genetic background. After several generations, some hemizygous Le-CreTg/+; Pax6fl/+ control mice had eye abnormalities even though they did not carry a floxed Pax6 allele. Although eye abnormalities have been observed in homozygous Le-CreTg/+ mice on some genetic backgrounds, this has only been reported as a brief abstract [30] and we are not aware of any previous reports of eye abnormalities in hemizygous Le-CreTg/+ mice. The unexpected control phenotype in our study was only identified because all three genetic controls (Le-CreTg+; Pax6fl/+; Le-Cre−/−; Pax6fl/+ and Le-Cre−/−; Pax6fl/+ ) were examined alongside experimental Le-CreTg+; Pax6fl/+ eyes throughout the experiment, as the genetic background changed.

We have characterised the eye defects in Le-CreTg+; Pax6fl/+ mice so that others are aware of the genetic background issues with the Le-Cre transgene. It is possible that some previous reports of Le-Cre-mediated conditional gene deletions may have conflated the abnormal eye phenotypes, resulting from a deleted floxed gene, with unexpected abnormal phenotypes, arising from effects of the Le-Cre transgene itself.

Materials and Methods

Ethics Statement

All the animal work in this study was approved by the University of Edinburgh Ethical Review Committee (applications PL21–06 and PL26–11) and performed in accordance with UK Home Office regulations under project license numbers PPL 60/3635 and PPL 60/4302. Mice were killed by cervical dislocation following inhalation of gaseous isoflurane anaesthetic. The highest standards of animal care were maintained throughout the study.

Experimental animals

FVB/N mice were purchased from Charles River UK and other mice were bred and maintained in the Biomedical Research Facilities of the University of Edinburgh. Heterozygous Pax6mut/+ (abbreviated to Pax6mut/+ ) mice [31] were obtained from Prof David Price (University of Edinburgh, UK) on an outbred CD1 genetic background. Hemizygous Le-CreTg−/− mice (full name Tg(Pax6-cre,GFP)1Pgr; MGI number 3045749) [1] were obtained from Dr Ruth Ashery-Padan and Prof Peter Gruss (Max Plank Institute for Biophysical Chemistry, Gottingen, Germany) on an inbred FVB/N genetic background. (We use the genotype notation Le-CreTg−/+ to represent mice homozygous for the Le-Cre transgene, Le-CreTg−/− for hemizygous mice and Le-CreTg+/− for mice without the transgene.) We crossed both stocks to CBA/Ca to make the genetic background more consistent with our previous studies with Pax6mut−/− (Pax6mut−/−) mice. (Unlike CBA/J, FVB/N and some CD1 mice, the CBA/Ca inbred strain does not carry the Pde6brd1 (retinal degeneration mutation.) Le-CreTg−/−; Pax6mut−/+ and Le-CreTg−/−; Pax6mut−/− mice were intercrossed to produce offspring of four genotypes, with or without the Le-Cre transgene and with or without the Pax6mut floxed allele of Pax6: (i) Le-CreTg−/−; Pax6mut−/+; (ii) Le-CreTg−/−; Pax6mut−/−; (iii) Le-Cre−/−; Pax6mut−/+ and (iv) Le-Cre−/−; Pax6mut−/− (which is wild-type, WT). For the initial crosses (stage 1), the Le-CreTg−/− mice used had been crossed to CBA/Ca for 3 or 4 generations (denoted as N3–N4) and the Pax6mut−/− mice had been crossed to CBA/Ca for 1 or 2 generations. The average genetic background of the stage-1 progeny was estimated as approximately 78% CBA/Ca, 5% FVB and 17% CD1 (Table S1 and Fig. 1A). Wound healing studies were undertaken with stocks that had been backcrossed to CBA/Ca for a few more generations (Le-CreTg−/− at N6; Pax6mut−/− at N3). This is designated stage 2 and the average genetic background was estimated as approximately 93% CBA/Ca, 0.8% FVB and 6% CD1 (Table S1 and Fig. 1B). Further investigations of embryonic day (E)12.5 fetal stages to postnatal day (P) 10 and adults were undertaken in stage 3 after further backcrossing (Le-CreTg−/− at N7–N8; Pax6mut−/− at N5–N6). The average genetic background of the stage-3 progeny was estimated as approximately 98% CBA/Ca; 0.3% FVB and 1.5% CD1 (Table S1 and Figs. 1C,D). A final comparison (stage 4) was made using Le-Cre−/− mice that had been backcrossed to CBA/Ca for 8 generations and then crossed to FVB/N for 2 generations to change the genetic background and Pax6mut−/− mice that had been crossed to CBA/Ca for 5 or 6 generations. The average genetic background of the stage-4 progeny was estimated as approximately 61% CBA/Ca, 38% FVB and 1.2% CD1 (Table S1 and Fig. 1F). Mice were genotyped by polymerase chain reaction (PCR) [1,31]. The study was conducted continuously, Le-Cre mice used were all derived from a single backcross line and mice were bred from June 2010 to October 2012 as shown in Table S1. Experiments were all performed by the same person (NJD), using the same methods throughout the study.

Heterozygous Pax6mut−/− mice (abbreviated to Pax6mut−/+ ) were maintained by crossing them to inbred CBA/Ca mice and were considered congenic on that strain (>20 backcross generations). Heterozygotes were distinguished from wild-type littermates by eye size and genotypes were confirmed by PCR [32]. Mice carrying the Pax7 transgene, comprising 5–7 copies of the human Pax7 gene [33] were obtained from Prof Veronica van Heyningen and Dr Dirk A. Kleinjan (MRC Human Genetics Unit, Edinburgh) on an outbred CD1 genetic background. (We use the genotype notation Pax7Tg−/+ to represent mice homozygous for the Pax7 transgene, Pax7Tg−/− for hemizygous mice and Pax7−/− for mice without the transgene.) Hemizygous Pax7Tg−/− mice used in this study had been backcrossed to the CBA/Ca genetic background for at least 20 generations (CBA/Ca-Pax7Tg−/− congenic strain) and were identified by their small eye size with genotypes confirmed by PCR as described previously [34,35]. The genetic background of
Le-Cre^{Tg-}; Pax6^{fl/+} mice used for crosses with CBA/Ca-PAX77^{Tg/} (Le-Cre^{Tg-} at N6; Pax6^{fl/+} at N5) was approximately 98% CBA, 0.4% FVB and 1.6% CD1. The genetic background of mice produced by Le-Cre^{Tg-}; Pax6^{fl/+} × Pax77^{Tg/} crosses was estimated as approximately 99% CBA/Ca, 0.2% FVB/N and 0.8% CD1 (Table S1 and Fig. 1E) and is considered equivalent to the stage 3 genetic background.

Adult mice were killed by cervical dislocation, following inhalation of gaseous isoflurane anaesthetic, at 12 weeks and their eyes were removed immediately, weighed and fixed in 4% PFA. Only one eye per mouse (the right eye) was examined histologically or by immunohistochemistry so the numbers of eyes examined is also equal to the numbers of mice examined.

Histology

Samples were fixed in 4% PFA overnight at 4°C, processed to wax and 7 μm sections cut. Adult eyes were cut in an anterior-posterior plane to include cornea, lens and retina. To avoid adult lenses shattering the wax block was kept wet during sectioning. Embryonic heads were cut anterior-posterior in a horizontal plane so sections through the developing eye included cornea, lens, and retina. Histological features were compared using standard haematoxylin and eosin (H & E) staining methods. For Periodic acid-Schiff’s (PAS) staining, slides were washed in periodic acid for 15 minutes, rinsed in water and transferred to Schiff’s reagent for 5 minutes.

Morphometric measurements

Corneal diameters were measured using a stereomicroscope fitted with an eyepiece graticule. Tissue sections of adult eyes were viewed under a Zeiss Axiosplan 2 compound microscope (x 40 objective) and captured images of the cornea measured using a calibrated Zeiss Axiovision 4.8 digital camera system. Sections from the central cornea were measured in six regions (2 peripheral, 2 intermediate and 2 central), mean thicknesses were calculated for peripheral, intermediate and central corneal epithelium. Cell layers were also counted for peripheral, intermediate and central regions in the same sections.

Immunohistochemical staining

Sections were de-waxed in histoclear and re-hydrated through a graded alcohol series to water and incubated in 3% hydrogen peroxide in methanol for 20 minutes rehydrated in 70% ethanol and washed in phosphate buffered saline (PBS). Antigen unmasking was performed by incubating slides in 0.01 M citrate buffer.
(pH 6.0) in a water bath heated to 95°C for 35 minutes; slides were then allowed to cool for 20 min. and washed in PBS. Sections were treated with 10% blocking serum (species according to secondary antibody), 0.1% bovine serum albumin (BSA) in PBS for 1 hour at room temperature. Sections were incubated overnight at 4°C in the appropriate primary antibody, diluted in blocking serum, as follows. (The following primary antibodies were used. Pax6 staining: Developmental Studies Hyridoma Bank, University of Iowa diluted 1:500, Cytokeratin 12 (K12); Santa Cruz Biotechnology sc-17101 diluted 1:500, Cytokeratin 5 (K5); Abcam ab53121 diluted 1:100, Cytokeratin 19 (K19); LifeSpan Biosciences LS-C3372 diluted 1:200). Slides were washed in PBS and incubated in blocking serum for 10 min. then incubated with secondary antibody, diluted in blocking serum, for 45 min. at room temperature. (The following secondary antibodies were used. Pax6 staining: Vector Labs BA-9200 biotinylated goat anti-mouse diluted 1:200, K12: Vector Labs BA-5000, biotinylated rabbit anti-goat IgG diluted 1:200, K5 and K19: Vector Labs BA-1000, biotinylated goat anti-rabbit IgG diluted 1:200). Slides were washed in PBS and incubated with avidin-biotin reagent (ABC RTU Vectastain, Vector Labs PK-7100). Antibody was then visualised by 3,3-diaminobenzidine (DAB) stain (5.9 ml 20 mM Tris pH 7.6, 100 µl 50 mg/ml DAB, 1 µl H2O2) and slides were lightly counterstained with haematoxylin, dehydrated and coverslips were mounted with DPX mounting medium. Control slides were treated with blocking serum in place of primary antibody but otherwise treated identically.

β-galactosidase and alkaline phosphatase histochemical staining on frozen sections

To prepare frozen tissue sections, samples were fixed in 4% PFA overnight, washed three times for 15 minutes in PBS and cryoprotected by treating with 15% sucrose in PBS for 1 hour at 4°C and then 30% sucrose overnight at 4°C. Tissues were incubated in optimal cutting temperature compound (OCT) at 4°C for 1 hour before they were embedded in OCT over dry ice. Blocks were stored at −80°C and warmed to −20°C before cryosections were cut at 10 µm, air dried for at least 1 hour and stored at −20°C.

Prior to staining, sections were fixed again with 0.2% gluteraldehyde in ice cold PBS for 10 min. For β-galactosidase (β-gal) staining, slides were washed three times for 5 min. in β-gal wash buffer (2 mM, 0.01% sodium deoxycholate, 0.02% Nonidet-P40 (NP-40) in 100 mM sodium phosphate, pH 7.3) and then incubated in β-gal stain (0.5 mg/ml X-gal, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide in β-gal wash buffer) for 4–6 h. at 37°C, while protected from light. Slides were rinsed in PBS, dehydrated through graded ethanol and coverslips were mounted with DPX mounting medium. For alkaline phosphatase (AP) staining, following fixation in 0.2% gluteraldehyde, slides were washed three times for 5 min. in PBS. Endogenous phosphatase activity was inactivated by incubating slides in PBS at 70–75°C for 30 min., slides were rinsed in PBS and washed in AP buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 10 mM MgCl2) 10 min. Slides were incubated with nitro-blue tetrazolium and 3-bromo-4-chloro-3’-indolyolphosphate (NBT/BCIP) stain for 10–30 min. at room temperature until the colour developed, washed in PBS, dehydrated and coverslips were mounted with DPX mounting medium.

Conical epithelial wound healing

Mice were killed and a wound was made in the central region of the cornea using a 1 mm diameter trephine blade, under a dissecting microscope while the eye was in situ. The area within the wound was debrided with an ophthalmological scalpel and the wound was visualized with fluorescein dye and photographed using a digital camera mounted on a dissecting microscope. Eyes were enucleated and placed in culture wells (cornea facing up) in corneal culture medium (CCM) [37] and kept in standard culture conditions for 24 h. The wound area was visualised and photographed at specific time points during culture and the wound diameter was measured using Adobe Photoshop CS v8.

Statistical analysis

Left and right eyes were analysed separately because their genotypes are identical so they are not completely independent samples. Non-parametric Kruskal-Wallis tests and Dunn’s multiple comparison post-hoc tests were performed using the statistics package GraphPad Prism 5.0c (GraphPad Software Inc., San Diego, USA). Fisher’s Exact tests were calculated using an on-line statistics calculator (http://vassarstats.net/odd2x2.html). The raw data are included in Supplementary Data S1.

Results

Morphology of Le-CreTg+; Pax6fl/+ eyes from stage-1 crosses

Eyes from the experimental and three control genotypes produced by Le-CreTg+; Pax6fl/+Le-Cre−/−; Pax6fl/+ crosses were compared in four discrete stages as the genetic background changed, as explained in the Materials and Methods section. The average genetic background of the stage-1 progeny was approximately 78% CBA/Ca, 5% FVB/N and 17% CD1 (Fig. 1A) and results for this stage are summarised in Figs. 2–6 and Table 1. (The four genotypes are colour-coded in each figure to help distinguish them).

Haematoxylin and eosin staining of histological sections (one eye per mouse) showed that control eyes (Le-Cre−/−; Pax6fl/+; Le-Cre−/−; Pax6fl/+ and Le-CreTg+; Pax6fl/+; Le-CreTg+; Pax6fl/+; Pax6fl/+; Le-CreTg+; Pax6fl/+; Pax6fl/+ were morphologically normal (Figs. 2A–C), apart from two of the six Le-CreTg+; Pax6fl/+ eyes examined, in which the irido-corneal angle appeared at least partly closed due to adhesion between the iris and peripheral cornea (Fig. 2D). All eight experimental Le-CreTg+; Pax6fl/+ eyes examined were morphologically abnormal but they varied in severity (Figs. 2E,F). Lenses were small, malformed, vacuolated and not always entirely contained within a capsule. Lens-corneal plugs (persistent lens stalks) were present in some cases (Fig. 5M), as described previously for both Pax6fl/+ [26,38] and Le-CreTg+; Pax6fl/+ mice. Retinal dysgenesis occurred in all Le-CreTg+; Pax6fl/+ samples examined and varied from mild folding to swirling to more severe abnormalities. In most cases, there was no pupil and pigmented tissue adhered to the corneal endothelium, which may have been a persistent, pigmented pupillary membrane, as reported for Pax6fl/+ heterozygotes [39]. There were also irido-lenticular and irido-corneal adhesions and in some cases the irido-corneal angle appeared closed and the ciliary body appeared abnormal or hypoplastic (Fig. 2F). For frequency comparisons, eye morphology was classified as normal, mildly abnormal or severely abnormal (Table 1). For these stage-1 crosses, the frequency of eyes with severely abnormal eyes was significantly greater than in the pooled group of Le-Cre−/−; Pax6fl/+ and Le-Cre−/−; Pax6fl/+ controls without the Le-Cre transgene (0/6) by Fisher’s Exact test for Le-CreTg+; Pax6fl/+ eyes (6/8; P = 0.0007) but not for Le-CreTg+; Pax6fl/+ eyes (0/6; P = 1.0000).

In stage 1, Le-CreTg+; Pax6fl/+ eye size varied and some were significantly smaller than the three control genotypes, which were all similar, as shown for eye mass in Figs. 3A,D,G. For some Le-CreTg+; Pax6fl/+ mice, the mass of left and right eyes differed
markedly, implying that both genetic and stochastic differences can affect eye size. Comparisons of eye diameter and corneal diameter measurements (Figs. S1 and S2) showed the same trends as eye mass.

Abnormalities and immunohistochemistry of Le-Cre<sup>Tg<sup>−</sup>−</sup>; Pax<sup>6<sup>fl/fl</sup></sup> corneas (stage-1 crosses)

The original focus of this study was the corneal epithelium, which was thinner in the experimental Le-Cre<sup>Tg<sup>−</sup>−</sup>; Pax<sup>6<sup>fl/fl</sup></sup> group than the three control groups at stage-1 (Figs. 4A–D) and this is shown quantitatively in Fig. 6. As reported for Pax<sup>6<sup>Sey-Neu</sup></sup> heterozygotes [19], ectopic goblet cells were identified by PAS staining in the corneal epithelium of two of the six Le-Cre<sup>Tg<sup>−</sup>−</sup>; Pax<sup>6<sup>fl/fl</sup></sup> eyes examined (Fig. 4L) but none were seen in 18 control eyes (six for each genotype; Figs. 4I–K). The corneal epithelium of all four genotypes stained positively for Pax6 (Figs. 4E–H) and keratin 5 (K5; Figs. 5A–H). Staining of keratin 19 (K19), which is normally expressed in the mouse conjunctiva and limbus but not in the central cornea [40], was detected in conjunctival-limbal region of all four groups (Figs. 5N–Q). As expected, it was absent from the central cornea of the three control groups (Figs. 5I–K) but patchy staining occurred in the central corneal epithelium of the experimental Le-Cre<sup>Tg<sup>−</sup>−</sup>; Pax<sup>6<sup>fl/fl</sup></sup> mice (Figs. 5L,M), as reported previously for Pax<sup>6<sup>Sey-Neu</sup></sup> heterozygotes [41], suggesting that the corneo-limbal boundary may be indistinct. The presence of K19 staining and goblet cells in the central corneal epithelium is consistent with either conjunctivalisation or abnormal differentiation of the Le-Cre<sup>Tg<sup>−</sup>−</sup>; Pax<sup>6<sup>fl/fl</sup></sup> corneal epithelium. As expected, keratin 12 (K12) immunostaining was detected throughout the corneal epithelium of all three controls but, unexpectedly, was completely absent from the corneal epithelium of all six Le-Cre<sup>Tg<sup>−</sup>−</sup>; Pax<sup>6<sup>fl/fl</sup></sup> eyes examined (Figs. 5R–Y). This is a significantly more severe phenotype than reported for Pax<sup>6<sup>Sey-Neu</sup></sup> heterozygotes [19] [22]. Moreover, the presence of abnormalities (closure of irido-corneal angles) in some Le-Cre<sup>Tg<sup>−</sup>−</sup>; Pax<sup>6<sup>fl/fl</sup></sup> control eyes suggested that depletion of Pax6 in the surface...
Figure 3. Variation in eye mass for different genotypes at different stages of the study. (A–F) Mass of left (A–C) and right (D–F) eyes of 12-week old mice from Le-Cre<sup>+/+</sup>; Pax6<sup>−/−</sup> × Le-Cre<sup>−/−</sup>; Pax6<sup>−/−</sup> crosses on different genetic backgrounds: (A,D) stage 1 crosses (B,E) stage 3 crosses (C,F) stage 4 crosses. (G–I) The percentage eye mass difference, calculated for each mouse as (larger eye mass - smaller eye mass) / 100(larger eye mass).
Corneal wound healing in Le-Cre<sup>Tg<sup>-/-</sup></sup>; Pax6<sup>fl/+</sup> eyes (stage-2 crosses)

Ex-vivo corneal epithelial wound healing, in the four groups, produced by Le-Cre<sup>Tg<sup>+/+</sup></sup>; Pax6<sup>fl/+</sup> × Le-Cre<sup>-/-</sup>; Pax6<sup>fl/+</sup> crosses, was compared to wound healing in CBA/Ca- Pax6<sup>fl/+;Sey-Neu<sup>+</sup></sup> heterozygotes (Fig. S3). The Le-Cre<sup>Tg<sup>/-</sup></sup>; Pax6<sup>fl/+</sup> and Le-Cre<sup>-/-</sup>; Pax6<sup>fl/+</sup> mice had been crossed to CBA/Ca for more generations and the genetic background had increased from approximately 78% CBA/Ca to approximately 93% CBA/Ca (Figs. 1A,B) so this was considered to be a new stage of the study (stage 2). The trajectory of wound healing was quite variable within groups and some CBA/Ca- Pax6<sup>fl/+;Sey-Neu<sup>-</sup></sup> wounds increased in size during the first 6 hours, suggesting greater corneal epithelial fragility. Comparisons of the frequencies of wounds that healed within 24 hours are shown both separately for left and right eyes and for pooled samples of left and right eyes in Table S2. In each case there is a trend for fewer wounds to close in CBA/Ca- Pax6<sup>fl/+;Sey-Neu<sup>-</sup></sup> positive controls and Le-Cre<sup>Tg<sup>+/+</sup></sup>; Pax6<sup>fl/+</sup> experimental mice than in Le-Cre<sup>-/-</sup>; Pax6<sup>fl/+</sup>, Le-Cre<sup>-/-</sup>; Pax6<sup>fl/+</sup> or Le-Cre<sup>Tg<sup>+/+</sup></sup>; Pax6<sup>fl/+</sup> control groups but most differences failed to reach significance by Fisher’s Exact tests unless results for left and right eyes were pooled. Wound healing abnormalities in Pax6<sup>fl/+;Sey-Neu<sup>-</sup></sup> mice have been attributed to reduced Pax6 levels [23,24]. As wound-healing was similar in Pax6<sup>fl/+;Sey-Neu<sup>-</sup></sup> and Le-Cre<sup>Tg<sup>+/+</sup></sup>; Pax6<sup>fl/+</sup> mice, the wound-healing abnormalities in Pax6<sup>fl/+;Sey-Neu<sup>-</sup></sup> mice are likely to be at least partly mediated intrinsically via the surface ectoderm lineage.

Morphology of experimental Le-Cre<sup>Tg<sup>-/-</sup></sup>; Pax6<sup>fl/+</sup> eyes is abnormal by E16.5 (stage 3 crosses)

We next compared eye morphology (one eye per mouse) in a developmental series of H & E stained sections of fetal and juvenile control Le-Cre<sup>-/-</sup>; Pax6<sup>fl/+</sup> and experimental Le-Cre<sup>Tg<sup>+/+</sup></sup>; Pax6<sup>fl/+</sup> eyes (Fig. 7) to identify when the abnormalities seen in adult Le-Cre<sup>Tg<sup>+/+</sup></sup>; Pax6<sup>fl/+</sup> experimental mice (Fig. 2) arose. By this stage, Le-Cre<sup>Tg<sup>+/+</sup></sup>; Pax6<sup>fl/+</sup> and Le-Cre<sup>-/-</sup>; Pax6<sup>fl/+</sup> mice had been crossed to CBA/Ca for more generations and the genetic background had increased from approximately 93% to approximately 98% CBA/Ca (Figs. 1B,C) so this was considered to be...

**Figure 4. Histology and immunohistochemistry of adult corneas of four genotypes from Le-Cre<sup>Tg<sup>+/+</sup></sup>; Pax6<sup>fl/+</sup> × Le-Cre<sup>-/-</sup>; Pax6<sup>fl/+</sup> crosses in stage 1** (A–D) H & E stained corneas showing normal morphology in controls (A–C) but reduced numbers of corneal epithelial layers in the Le-Cre<sup>Tg<sup>+/+</sup></sup>; Pax6<sup>fl/+</sup> cornea shown in (D). (E–H) Pax6 immunostaining (brown endpoint) in the corneal epithelium of all four genotypes. (I–L) Periodic acid-Schiff (PAS) staining (purple-magenta endpoint) in cornea showed goblet cells were absent from the corneal epithelium of the three control genotypes (I–K) and some experimental Le-Cre<sup>Tg<sup>+/+</sup></sup>; Pax6<sup>fl/+</sup> eyes (not shown) but present in other Le-Cre<sup>-/-</sup>; Pax6<sup>fl/+</sup> eyes (arrows in L). Scale bars: A–H = 20 μm, I–L = 50 μm. **Abbreviations:** en, corneal endothelium; ep, corneal epithelium; st, corneal stroma.

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Figure 5. Immunohistochemistry for keratins 5, 19 and 12 in epithelia of the adult ocular surface of four genotypes from Le-CreTg/2; Pax6+/+ crosses in stage 1. (A–H) Keratin 5 (K5) immunostaining (brown endpoint) shows K5 is present in the central (A–D) and peripheral (E–H) corneal epithelium of all four groups. (I–Q) K19 immunostaining (brown endpoint) in the central corneal epithelium (I–M) shows K19 is absent in the three control groups (I–K) but patchy staining (arrows) is present in the Le-CreTg/2; Pax6fl/+ central corneal epithelium (L,M). The cornea shown in M has a lens-corneal plug. In the peripheral corneal epithelium (N–Q), K19 is present in some cells of the limbus (and conjunctiva) in all four groups. (R–X) K12 immunostaining (brown endpoint) in the central corneal epithelium (R–U) and peripheral corneal and limbal epithelium (V–Y) shows K12 is absent from the Le-CreTg/2; Pax6+/+ ocular surface epithelium (UY) but present in the corneal epithelium of all three control groups (R–T and V–X). Scale bars = 50 μm. Abbreviations: co, cornea; cj, conjunctiva; lcp, lens-corneal plug; li, limbus.

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Eye Abnormalities in Le-Cre Mice

Figure 6. Variation in corneal epithelial thickness in different genotypes from stage-1 crosses. Variation in corneal thickness in 12-week-old mice of different genotypes from Le-Cre\textsuperscript{Tg}\textsuperscript{+/–}; Pax6\textsuperscript{fl/+} \times Le-Cre\textsuperscript{−/−}; Pax6\textsuperscript{fl/+} stage 1 crosses. Results are means of two measurements at the periphery, intermediate and central regions of 4–6 eyes (1 eye per mouse) per genotype. Abbreviations: WT \textsuperscript{+/+} is Le-Cre\textsuperscript{−/−}; Pax6\textsuperscript{fl/+}, WT fl/+ is Le-Cre\textsuperscript{−/−}; Pax6\textsuperscript{fl/+}, Cre \textsuperscript{+/+} is Le-Cre\textsuperscript{Tg}\textsuperscript{+/−}; Pax6\textsuperscript{fl/+} and Cre fl/+ is Le-Cre\textsuperscript{Tg}\textsuperscript{−/+}; Pax6\textsuperscript{fl/+}. Results for all four genotypes were compared by non-parametric Kruskal-Wallis (KW) tests separately for each corneal region (P-values are shown in the figure) and results for WT fl/+; Cre \textsuperscript{+/+} and Cre fl/+ were compared to WT \textsuperscript{+/+} by Dunn’s multiple comparison post-hoc test. * P<0.05; ** P<0.01.

stage 3. Abnormalities were first detected as disorganised lenses in E16.5 Le-Cre\textsuperscript{Tg}\textsuperscript{−/−}; Pax6\textsuperscript{fl/+} eyes (Fig. 7F) whereas Le-Cre\textsuperscript{−/−}; Pax6\textsuperscript{fl/+} control eyes were normal (Fig. 7E). By P2 abnormalities in Le-Cre\textsuperscript{Tg}\textsuperscript{−/−}; Pax6\textsuperscript{fl/+} eyes were very obvious. Eyes and lenses were smaller than in controls, the retina was dysplastic (retinal swirls) and pigmented iris like-tissue adhered to the corneal endothelium (Fig. 7H).

Eye morphology is abnormal in Le-Cre\textsuperscript{Tg}\textsuperscript{−/−}; Pax6\textsuperscript{fl/+} controls as well as Le-Cre\textsuperscript{Tg}\textsuperscript{−/−}; Pax6\textsuperscript{fl/+} experimental mice from stage-3 crosses

At P10 we also examined the other two control groups (Le-Cre\textsuperscript{−/−}; Pax6\textsuperscript{fl/+} and Le-Cre\textsuperscript{Tg}\textsuperscript{−/−}; Pax6\textsuperscript{fl/+}). As expected, all five of the experimental Le-Cre\textsuperscript{Tg}\textsuperscript{−/−}; Pax6\textsuperscript{fl/+} eyes examined were abnormal (Figs. 7M,N). More surprisingly, some P10 control Le-Cre\textsuperscript{16.5}; Pax6\textsuperscript{16.5} eyes from stage-3 crosses also displayed a much more obvious abnormal phenotype than the partly closed irido-corneal angles seen in stage 1 adults. Three of the five Le-Cre\textsuperscript{16.5}; Pax6\textsuperscript{16.5} control eyes examined appeared normal (Fig. 7K) but two had abnormal lenses (Fig. 7L). In contrast, all the Le-Cre\textsuperscript{−/−}; Pax6\textsuperscript{fl/+} and Le-Cre\textsuperscript{Tg}\textsuperscript{−/−}; Pax6\textsuperscript{fl/+} controls examined (4 eyes per genotype) appeared normal (Figs. 7L).
Table 1. Summary of adult eye morphology frequency results at different stages of the study.

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<th>% severe</th>
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For morphology classification, “Mild” indicates mildly abnormal morphology and “Severe” indicates severely abnormal morphology.

<sup>P</sup>-values for stage-1, stage-3 and stage-4 crosses are for Fisher’s Exact tests of the proportion of severely abnormal eyes tested against the proportion in the Le-Cre<sup>+</sup>; Pax6<sup>+</sup> plus the Le-Cre<sup>-/-</sup>; Pax6<sup>-/-</sup> control groups. For the PAX77 crosses, <sup>P</sup>-values are for Fisher’s Exact tests of the proportion of severely abnormal eyes for Le-Cre<sup>-/-</sup>; Pax6<sup>-/-</sup>; PAX77<sup>-/-</sup> versus Le-Cre<sup>+</sup>; Pax6<sup>+</sup>; PAX77<sup>-/-</sup> and for Le-Cre<sup>-/-</sup>; Pax6<sup>-/-</sup>; PAX77<sup>-/-</sup> versus Le-Cre<sup>+</sup>; Pax6<sup>-/-</sup>; PAX77<sup>-/-</sup>.

<sup>*</sup>P<0.05;

<sup>***</sup>P<0.001; N/A = not applicable.

<sup>†</sup>Two Le-Cre<sup>+</sup>; Pax6<sup>-/-</sup> mice from stage-1 crosses had a very mild phenotype (irido-corneal angles appeared partly closed) – see Fig. 2D.

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Figure 7. Morphology of fetal and juvenile eyes and Z/AP reporter expression in adult eyes from mice produced in stage 3. (A–H) Comparison of morphology in H & E stained histological sections of control Le-Cre\textsuperscript{-/}; Pax6\textsuperscript{+/+} and experimental Le-Cre\textsuperscript{+/-}; Pax6\textsuperscript{+/-} fetal and neonatal eyes from Le-Cre\textsuperscript{+/+}; Pax6\textsuperscript{+/+} x Le-Cre\textsuperscript{-/}; Pax6\textsuperscript{+-} stage-3 crosses at E12.5 to P2 showing lens abnormalities in Le-Cre\textsuperscript{+/-}; Pax6\textsuperscript{+/-} eyes at E16.5 (F) and more extensive ocular abnormalities in Le-Cre\textsuperscript{+/-} ; Pax6\textsuperscript{+/-} eyes at P2 (H). (I–N) Comparison of morphology in H & E stained histological sections of all three control genotypes from Le-Cre\textsuperscript{+/+}; Pax6\textsuperscript{+/+} x Le-Cre\textsuperscript{-/}; Pax6\textsuperscript{+-} stage-3 crosses at P10 showing normal morphology of (I) Le-Cre\textsuperscript{+/-}; Pax6\textsuperscript{+/-}, (J) Le-Cre\textsuperscript{-/}; Pax6\textsuperscript{+/-} and (K) one Le-Cre\textsuperscript{+/-}; Pax6\textsuperscript{+/-} control and (M,N) eyes from Le-Cre\textsuperscript{+/-}; Pax6\textsuperscript{+/-} experimental mice in stage 3 of this study. The lenses in I-K are normal but the lens shown in (J) was damaged during sectioning. (O,P) Histochemical staining for (O) placental alkaline phosphatase (blue endpoint) and (P) \(\beta\)-galactosidase staining. Colour code of panel lettering: black = Le-Cre\textsuperscript{+/+}; Pax6\textsuperscript{+/-}, red = Le-Cre\textsuperscript{+/-}; Pax6\textsuperscript{+/-}, green = Le-Cre\textsuperscript{+/-}; Pax6\textsuperscript{+/+}, purple = Le-Cre\textsuperscript{+/-}; Z/AP.

Abbreviations: AP, alkaline phosphatase staining; \(\beta\)-gal = \(\beta\)-galactosidase staining. Colour code of panel lettering: black = Le-Cre\textsuperscript{+/+}; Pax6\textsuperscript{+/-}, red = Le-Cre\textsuperscript{+/-}; Pax6\textsuperscript{+/-}, green = Le-Cre\textsuperscript{+/-}; Pax6\textsuperscript{+/+}, purple = Le-Cre\textsuperscript{+/-}; Z/AP.

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two appeared normal (Fig. 8C). The types of abnormalities seen in the stage-3 Le-Cre\textsuperscript{+/-}; Pax6\textsuperscript{+/-} control mouse was similar to those reported for some heterozygous Pax6\textsuperscript{+/-} mice (e.g. small disorganised lens, thinner corneal epithelium, irido-corneal adhesions and irido-lenticular adhesions). The experimental Le-Cre\textsuperscript{+/-}; Pax6\textsuperscript{+/-} eyes displayed a range of severe abnormalities (Figs. 8D,H) that were similar to those seen in stage-1 experimental Le-Cre\textsuperscript{+/-}; Pax6\textsuperscript{+/-} eyes (Figs. 2E,F).

For stage-3 crosses, the frequency of eyes with severe abnormalities was significantly greater than in the two control groups without the Le-Cre transgene (0/12) by Fisher’s Exact test for both Le-Cre\textsuperscript{+/-}; Pax6\textsuperscript{+/-} and Le-Cre\textsuperscript{-/}; Pax6\textsuperscript{+/-} eyes (3/6; \(P = 0.0245\) in both cases) as shown in Table 1. Although the frequency of Le-Cre\textsuperscript{+/-}; Pax6\textsuperscript{+/-} eye abnormalities was higher at stage 3 than stage 1 this difference did not reach statistical significance either for all abnormalities (4/6 vs. 2/6; \(P = 0.5671\)) or severe abnormalities (3/6 vs. 0/6; \(P = 0.1818\)). However, as noted above, eye abnormalities were significantly more frequent in Le-Cre\textsuperscript{+/-}; Pax6\textsuperscript{+/-} than the two control groups without the Le-Cre transgene at stage 3 (4/6 vs. 0/12; \(P = 0.0049\) for all abnormalities and 3/6 vs. 0/12; \(P = 0.0245\) for just the severe abnormalities) but not at stage 1 (2/6 vs. 0/12; \(P = 0.0980\) for all abnormalities and 0/6 vs. 0/12; \(P = 1.0000\) for severe abnormalities). This comparison indicates that the trend for more Le-Cre\textsuperscript{+/-}; Pax6\textsuperscript{+/-} eye abnormalities at stage 3 than stage 1 is significant.
As in the stage-1 cross, Pax6 immunostaining occurred in the corneal epithelium of all four genotypes from the stage-3 crosses (Figs. 8I–L). K12 immunostaining was examined in six eyes from each of the four genotypes. K12 was detected throughout the corneal epithelium of all three controls (Figs. 8M–O), including the four Le-CreTg/+; Pax6+/+ eyes that were morphologically normal.

Figure 8. Morphology of eyes and corneal immunostaining for Pax6 and K12 from adults in stage 3. (A–H) H & E stained sections of adult eyes from stage-3 crosses (with a predominantly CBA/Ca genetic background) showing normal morphology of (A,E) Le-Cre<sup>−/−</sup>; Pax6<sup>+/+</sup>, (B,F) Le-Cre<sup>−/−</sup>; Pax6<sup>−/−</sup> and (C) an Le-Cre<sup>−/−</sup>; Pax6<sup>−/−</sup> control eye but abnormal morphology of (G) another Le-CreTg<sup>Tg−/−</sup>; Pax6<sup>−/−</sup> control eye and a range of morphological abnormalities in (D,H) experimental Le-CreTg<sup>Tg−/−</sup>; Pax6<sup>−/−</sup> eyes. (I–L) Pax6 immunostaining (brown endpoint) in the corneal epithelium of all four genotypes. (M–P) K12 immunostaining (brown endpoint) in the central corneal epithelium shows K12 is present in the corneal epithelium of all three control eyes but absent from the Le-CreTg<sup>Tg−/−</sup>; Pax6<sup>−/−</sup> corneal epithelium. (Q–S) Low power views of K12 immunostaining (brown endpoint) across the whole corneal epithelium showing (Q) strong staining in an Le-Cre<sup>−/−</sup>; Pax6<sup>−/−</sup> control eye, (R) no staining in an Le-CreTg<sup>Tg−/−</sup>; Pax6<sup>−/−</sup> eye and (S) weak and patchy staining in a heterozygous Pax6<sup>−/−</sup>x<sup>xNeu</sup> eye (arrow shows a K12-positive region). (T) Higher power view of a K12-positive region in the corneal epithelium of an adult Pax6<sup>−/−</sup>x<sup>xNeu</sup> heterozygote. Scale bars: A–P (shown in the first panel of each row) and T = 200 μm, Q–S (shown in Q) = 500 μm.

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abnormal, but it was again undetectable in the corneal epithelium of all the experimental Le-Cre<sup>Tg</sup> −/−; Pax6<sup>fl/fl</sup> eyes examined (Fig. 6P). Figs. 8S,T show weak and patchy K12 immunostaining in the corneal epithelium of an adult Pax6<sup>fl/fl;Gnas</sup> mouse [17-21 weeks old] that was congenic on a CBA/Ca genetic background (>20 generations of backcrosses). This differs from both the strong staining in Le-Cre<sup>Tg</sup> −/−; Pax6<sup>+/+</sup> controls (Figs. 8M,Q) and the undetectable staining of Le-Cre<sup>Tg</sup> +/+; Pax6<sup>fl/fl</sup> eyes (Figs. 8P,R) from stage-3 crosses, with a similar genetic background.

To check whether the Le-Cre transgene was being ectopically expressed we stained separate sections of eyes from Le-Cre<sup>Tg</sup> −/−/Z/ AP reporter mice for alkaline phosphatase and β-galactosidase expression. These mice had a similar genetic background to other stage 3 mice (estimated as approximately 97% CBA/Ca, 0.3% FVB/N and 2.3% C57BL/6J; Fig. 1E). The Z/AP reporter mouse uses a double reporter system to provide an assay for Cre-mediated recombination of lacZ sites. Before recombination, cells express lacZ but upon recombination the lacZ reporter is excised and lacZ expression is replaced with alkaline phosphatase (AP) expression [36]. Alkaline phosphatase was expressed in the corneal epithelium and lens of Le-Cre<sup>Tg</sup> −/−; Z/ AP eyes (Fig. 7O), confirming that Cre-recombinase was expressed appropriately. These stage-3 Le-Cre<sup>Tg</sup> −/−; Z/ AP eyes had abnormal lenses and AP-positive cells were not all contained within a lens capsule. β-galactosidase staining on sections of the same eyes revealed lacZ expression in the retina where, as expected, no Cre-mediated excision had occurred (Fig. 7P). Thus, despite the morphological abnormalities there was no evidence for ectopic Le-Cre expression in these stage-3, Le-Cre<sup>Tg</sup> −/−; Z/AP reporter mice.

**Eye morphology in Le-Cre<sup>Tg</sup> −/−; Pax6<sup>+/+</sup> control eyes is improved by changing the genetic background (stage-4 crosses)**

Comparisons of eye morphology in mice from crosses in stages 1 and 3 suggested that continued backcrossing to CBA/Ca was associated with a marked increase in abnormalities in control Le-Cre<sup>Tg</sup> −/−; Pax6<sup>+/+</sup> eyes. In stage 4 of this study we, therefore, investigated whether increasing the contribution of the FVB/N genetic background, which was originally used to maintain the Le-Cre transgene, would reduce the level of abnormalities. Le-Cre<sup>Tg</sup> −/−; Pax6<sup>+/+</sup> mice that had been backcrossed to CBA/Ca for 8 generations (99.6% CBA and 0.4% FVB) were backcrossed to FVB for a further two generations and then crossed to Le-Cre<sup>Tg</sup> −/−; Pax6<sup>+/+</sup> mice that had been backcrossed to CBA/Ca for 5 or 6 generations to produce four genotypes with genetic backgrounds estimated as 61% CBA, 38% FVB and 1% CD1 (Fig. 1F). This resulted in a significant improvement in morphology of control Le-Cre<sup>Tg</sup> −/−; Pax6<sup>+/+</sup> eyes, although experimental Le-Cre<sup>Tg</sup> −/−; Pax6<sup>fl/fl</sup> eyes remained severely abnormal (Figs. 9A-F). A mildly abnormal phenotype (mild lens abnormalities and slight retinal swelling) was detected in 2 of the 6 Le-Cre<sup>Tg</sup> −/−; Pax6<sup>−/+</sup> control eyes examined (Fig. 9D) but the other 4 eyes were normal (Fig. 9C).

For stage-4 crosses, the frequency of eyes with severely abnormal eyes was significantly greater than in the two control groups without the Le-Cre transgene (0/3) by Fisher’s Exact test for Le-Cre<sup>Tg</sup> −/−; Pax6<sup>fl/fl</sup> eyes (4/6; P = 0.0150) but not for Le-Cre<sup>Tg</sup> −/−; Pax6<sup>−/+</sup> eyes (0/6; P = 1.0000) as shown in Table 1. Although the frequency of Le-Cre<sup>Tg</sup> −/−; Pax6<sup>−/+</sup> eye abnormalities was lower at stage 4 than stage 3 this difference was not significant either for all abnormalities (2/6 vs. 4/6; P = 0.5671) or severe abnormalities (0/6 vs. 3/6; P = 0.1818). However, the trend for fewer Le-Cre<sup>Tg</sup> −/−; Pax6<sup>−/+</sup> eye abnormalities at stage 4 is supported by comparisons between Le-Cre<sup>Tg</sup> −/−; Pax6<sup>−/+</sup> eyes and the two control groups without the Le-Cre transgene at stages 3 and 4. The frequency was significantly higher among Le-Cre<sup>Tg</sup> −/−; Pax6<sup>−/+</sup> eyes at stage 3 (4/6 vs. 0/12; P = 0.0049 for all abnormalities and 3/6 vs. 0/12; P = 0.0245 for severe abnormalities) but not at stage 4 (2/6 vs. 0/8; P = 0.1645 for all abnormalities and 0/6 vs. 0/8; P = 1.0000 for severe abnormalities).

The improved morphology of the control Le-Cre<sup>Tg</sup> −/−; Pax6<sup>−/+</sup> eyes, in stage-4 crosses, was accompanied by a more normal and less variable eye size, as demonstrated by measurements of eye mass (Figs. 3C,F,I), eye diameter (Fig. S1) and corneal diameter (Fig. S2). Experimental Le-Cre<sup>Tg</sup> −/−; Pax6<sup>−/+</sup> eyes from this stage-4, FVB cross remained small but were less variable in size than those from stage 1 and stage 3 crosses (Fig. 3, Figs. S1 and S2). Pax6 and K12 immunostaining results (data not shown) were unchanged from results shown Figs. 4, 5 and 8 for stages 1 and 3.

**Increased Pax6 expression provides a partial rescue of Le-Cre<sup>Tg</sup> −/−; Pax6<sup>−/+</sup> and Le-Cre<sup>Tg</sup> −/−; Pax6<sup>−/+</sup> abnormal eye phenotypes on a predominantly CBA/Ca genetic background**

To determine whether increased Pax6 levels could rescue the eye abnormalities seen in Le-Cre<sup>Tg</sup> −/−; Pax6<sup>−/+</sup> controls from stage-3 crosses, we crossed Le-Cre<sup>Tg</sup> −/−; Pax6<sup>−/+</sup> mice with approximately 98% CBA/Ca genetic background to CBA/Ca-PAX77<sup>Tg</sup> −/− mice (on an almost 100% CBA/Ca genetic background). This transgene contains the human Pax6 gene so over-expresses Pax6 [33] and affects the eye and corneal phenotypes [42] [34]. The genetic background of the progeny was approximately 99% CBA/Ca, 0.2% FVB and 0.8% CD1 (Fig. 1G). Comparisons of eye phenotypes of control Le-Cre<sup>Tg</sup> −/−; Pax6<sup>−/+</sup>; PAX77<sup>Tg</sup> −/− (without the PAX77 transgene) and Le-Cre<sup>Tg</sup> −/−; Pax6<sup>−/+</sup>; PAX77<sup>Tg</sup> +/+ eyes (with the PAX77 transgene) and comparisons of experimental Le-Cre<sup>Tg</sup> −/−; Pax6<sup>−/+</sup>; PAX77<sup>Tg</sup> −/− and Le-Cre<sup>Tg</sup> −/−; Pax6<sup>−/+</sup>; PAX77<sup>Tg</sup> −/+ eyes are shown in Figs. 9G-J. As expected, the abnormal phenotypes of the experimental Le-Cre<sup>Tg</sup> −/−; Pax6<sup>−/+</sup> eyes (with reduced Pax6 levels in the surface ectoderm) was partially rescued by the presence of the PAX77 transgene (which provides additional Pax6) as shown in Figs. 9G-H.

Although the frequency of severely abnormal eyes was lower for Le-Cre<sup>Tg</sup> −/−; Pax6<sup>−/+</sup>; PAX77<sup>Tg</sup> −/− mice with the PAX77 transgene (0/3) than for Le-Cre<sup>Tg</sup> −/−; Pax6<sup>−/+</sup>; PAX77<sup>Tg</sup> −/+ mice without PAX77 (3/3) these differences did not reach significance by a 2-tailed Fisher’s Exact test (P = 0.1000; Table 1) but were bordering on significance by a 1-tailed test (P = 0.05). Interestingly, the abnormal phenotypes of the control Le-Cre<sup>Tg</sup> −/−; Pax6<sup>−/+</sup> eyes were also partially rescued by the presence of the PAX77 transgene even though the genetic background was quite similar to that of the stage 3 crosses (Figs. 9I-J). Thus, the frequency of severely abnormal eyes was lower for Le-Cre<sup>Tg</sup> −/−; Pax6<sup>−/+</sup>; PAX77<sup>Tg</sup> −/+ mice with PAX77 transgene (0/3) than for Le-Cre<sup>Tg</sup> −/−; Pax6<sup>−/+</sup>; PAX77<sup>Tg</sup> −/+ mice without PAX77 (2/3) but again the small sample size lacked statistical power and these differences did not reach significance by Fisher’s Exact test (P = 0.4000; Table 1).

In both Le-Cre<sup>Tg</sup> −/−; Pax6<sup>−/+</sup>; PAX77<sup>Tg</sup> −/− and Le-Cre<sup>Tg</sup> −/−; Pax6<sup>−/+</sup>; PAX77<sup>Tg</sup> −/+ genotypes, the most noticeable improvement in the phenotype attributable to the presence of the PAX77 transgene was normalisation of lens size and morphology and absence of retinal dysgenesis (swirling and folding), although iridocorneal adhesions remained and the retina appeared thinner than normal. The retinal hypoplasia might reflect elevated Pax6 levels in the neuroectoderm lineage because the PAX77 transgene will produce a global increase in Pax6 but this will only be balanced by Pax6 depletion, caused by the Le-Cre<sup>Tg</sup> −/−; Pax6<sup>fl/fl</sup> genotype, in
the surface ectoderm derivatives. Even if Pax6 levels are more normal in the surface ectoderm lineage they are likely to be abnormally high in the tissues derived from the neuroectoderm of the optic cup. The striking partial rescue of control Le-Cre\textsuperscript{Tg\textsuperscript{-}}; Pax6\textsuperscript{+/+} eye phenotypes by the PAX77 transgene on a predominantly CBA/Ca genetic background suggests that low Pax6 levels may mediate the abnormal phenotype of control Le-Cre\textsuperscript{Tg\textsuperscript{-}}; Pax6\textsuperscript{+/+} eyes as well as the experimental Le-Cre\textsuperscript{Tg\textsuperscript{-}}; Pax6\textsuperscript{+/+} eyes.

**Discussion**

**Eye abnormalities in Le-Cre\textsuperscript{Tg\textsuperscript{-}}; Pax6\textsuperscript{+/+} experimental mice from stage-1 crosses**

Pax6 was expected to be reduced to heterozygous levels in only the surface ectoderm derivatives of Le-Cre\textsuperscript{Tg\textsuperscript{-}}; Pax6\textsuperscript{+/+} mice, so it was predicted that the range of abnormalities would be either similar to those reported for Pax6\textsuperscript{+/+} heterozygotes (if all their eye abnormalities were mediated via the surface ectoderm derivatives) or less extensive than those reported for Pax6\textsuperscript{+/−} (if some Pax6\textsuperscript{−} eye abnormalities were mediated via depletion of Pax6 in other lineages). Unexpectedly, the experimental Le-Cre\textsuperscript{Tg\textsuperscript{-}}; Pax6\textsuperscript{+/+} mice appeared more abnormal, in some respects, than the eye phenotypes reported for global depletion of Pax6 in heterozygous Pax6\textsuperscript{+/−} (Pax6\textsuperscript{+/−}) mice [19,22], as shown in Table 2. For example, K12 staining was undetectable in the Le-Cre\textsuperscript{Tg\textsuperscript{-}}; Pax6\textsuperscript{+/−} corneal epithelium.

This striking and unexpected result was seen both at stage 1 (~78.1% CBA/Ca) and stage 3 (~98.1% CBA/Ca). As far as we are aware, this has not been reported previously for any heterozygous Pax6\textsuperscript{+/−} mice, regardless of the Pax6 null allele involved. It is a significantly more severe phenotype than reported for Pax6\textsuperscript{+/−} heterozygotes, which showed patchy, weak to strong K12 staining at a similar age on a genetic background comprising 75% CBA/Ca and 25% C57BL [22]. K12 staining was also detectable in corneal epithelia of adult Pax6\textsuperscript{+/−} heterozygotes that were congenic on a CBA/Ca genetic background (Figs. 8S,T). K12 is a marker for corneal-type epithelial differentiation [43] and is regulated by Pax6 [44,45,46]. The absence of K12 indicates that corneal epithelial differentiation is abnormal and implies that experimental
**Table 2.** Comparison of eye phenotypes in control Le-Cre<sup>Tg<sup>1</sup></sup>; Pax6<sup>fl/fl</sup>; Le-Cre<sup>Tg<sup>1</sup></sup>; Pax6<sup>fl/fl</sup> and different Pax6 heterozygous mice.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Le-Cre&lt;sup&gt;Tg&lt;sup&gt;1&lt;/sup&gt;&lt;/sup&gt;; Pax6&lt;sup&gt;fl/fl&lt;/sup&gt; (from stage 1 crosses)</th>
<th>Le-Cre&lt;sup&gt;Tg&lt;sup&gt;1&lt;/sup&gt;&lt;/sup&gt;; Pax6&lt;sup&gt;fl/fl&lt;/sup&gt; (from stage 1 crosses)</th>
<th>Pax6&lt;sup&gt;Sey-Neu&lt;/sup&gt;</th>
<th>Pax6&lt;sup&gt;Sey+&lt;/sup&gt;</th>
<th>Pax6&lt;sup&gt;fl/+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genetic background</td>
<td>~78% CBA, ~5% FVB &amp; ~17% CD1</td>
<td>~78% CBA, ~5% FVB &amp; ~17% CD1</td>
<td>75% CBA &amp; 25% C57BL&lt;sup&gt;*&lt;/sup&gt; or 100% CBA&lt;sup&gt;**&lt;/sup&gt;</td>
<td>CD1 (outbred)</td>
<td>mixed (unspecified)</td>
</tr>
<tr>
<td>Reference</td>
<td>present study</td>
<td>present study</td>
<td>[19, 22, 41]</td>
<td>[47]</td>
<td>[39]</td>
</tr>
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</table>

**Phenotypes**

1. Eye size (mass or diameter) | normal (Fig. 3) | some are small (Fig. 3) | small | small | very small |
2. Corneal epithelial layers | normal (Figs. 4-6) | reduced (Figs. 4-6) | reduced | reduced | reduced |
3. Keratin 12 immunostaining in cornea | positive staining (Figs. 5T, X) | absent (Figs. 5U, Y) | reduced staining | ND | ND |
4. Keratin 19 immunostaining in cornea | limbus not cornea | limbus & patchy in cornea | limb & cornea<sup>**</sup> | ND | ND |
5. Goblet cells in corneal epithelium | present (Fig. 4K) | present (Fig. 4L) | present | ND | absent |
6. Blood vessels in cornea | none seen | none seen | present in some | present | present very early |
7. Lens structure | normal (Figs. 2C, D) | cataracts and abnormal (Figs. 2E, F) | cataracts | cataracts | cataracts and vacuolated. |
8. Lens-corneal plug in corneal epithelium (persistent lens stalk) | absent (Figs. 2A, 5) | lens-corneal plug in some corneas (Fig. 5M) | lens-corneal plug present | lens-corneal plug in some corneas | absent |
9. Kerato-lenticular adhesions or strands | absent | some adhesions | strands | adhesions | |
10. Irido-corneal adhesions (anterior synchia) or strands | mostly absent | some adhesions | adhesions | adhesions | adhesions and strands |
11. Irido-corneal angles | mostly open angles (Fig. 2C) but some appear partly closed (Figs. 2D, 5G, X) | Most eyes severely abnormal and angles closed (Figs. 2E, S) | closed angles | closed angles | closed angles |
12. Irido-lenticular adhesions (posterior synchia) | absent (Figs. 2C, D) | some adhesions present (Fig. 2F) – also see point 13 | absent | adhesions present | adhesions present |
13. Iris | normal (Figs. 2C, D) | malformed iris & pigmented pupillary membrane (Figs. 2F, S) | hypoplastic | hypoplastic | malformed iris & pigmented pupillary membrane |
14. Ciliary body | normal (Figs. 2C, D) | sometimes abnormal or hypoplastic (Fig. 2E) | normal | hypoplastic | malformed |
15. Retina | normal (Figs. 2C, D) | dysplastic (Figs. 2E, F) | normal | dysplastic | dysplastic |

*C57BL<sup>*</sup> is derived from the UK C57BL/Grfa strain via C57BL/Ola and is closely related to the C57BL/6 strain [69].

**Genetic background for Pax6<sup>fl/+Neu</sup>** K19 study was 100% CBA/Ca [41]. ND, not done.

Le-Cre<sup>Tg<sup>1</sup></sup>; Pax6<sup>fl/+</sup> corneas are more severely affected than Pax6<sup>fl/+Neu</sup> heterozygotes.

Some stage-1 Le-Cre<sup>Tg<sup>1</sup></sup>; Pax6<sup>fl/+</sup> abnormalities appear more similar to those described for Pax6<sup>fl/flNeu</sup> heterozygotes [39] or Pax6<sup>fl/fl</sup> heterozygotes on an outbred CD1 background [47] (Table 2), although K12 immunohistochemistry was not evaluated in either of these studies. Even during the first stage of our study, some Le-Cre<sup>Tg<sup>1</sup></sup>; Pax6<sup>fl/+</sup> controls appeared slightly abnormal, as they had partially closed irido-corneal angles. Thus, the greater abnormalities in Le-Cre<sup>Tg<sup>1</sup></sup>; Pax6<sup>fl/+</sup> eyes compared to Pax6<sup>fl/flNeu</sup> mice could be mediated by additional, unknown effects specific to the Le-Cre transgene. Conversely, corneal neovascularisation occurs in Pax6<sup>fl/+</sup> mice but was not seen in the Le-Cre<sup>Tg<sup>1</sup></sup>; Pax6<sup>fl/+</sup> experimental mice analysed, suggesting either that it is mediated by lineages other than the surface ectoderm or the Le-Cre<sup>Tg<sup>1</sup></sup>; Pax6<sup>fl/+</sup> genetic background is less-permissive for this phenotype than the genetic background used for the Pax6<sup>fl/+</sup> studies.

**Eye abnormalities in Le-Cre<sup>Tg<sup>1</sup></sup>; Pax6<sup>fl/+</sup> control mice from stage-3 crosses**

After further backcrosses to CBA/Ca mice, eyes of Le-Cre<sup>Tg<sup>1</sup></sup>; Pax6<sup>fl/+</sup> experimental mice from stage 3 crosses were again severely affected but now some Le-Cre<sup>Tg<sup>1</sup></sup>; Pax6<sup>fl/+</sup> controls also had small and abnormal eyes. The types of abnormalities seen in these Le-Cre<sup>Tg<sup>1</sup></sup>; Pax6<sup>fl/+</sup> control mice were similar to those reported for some heterozygous Pax6<sup>fl/+</sup> mice. We attribute these abnormalities to the presence of the Le-Cre transgene because no
abnormalities were seen in the Le-Cre<sup>+/−</sup>; Pax6<sup>+/+</sup> or Le-Cre<sup>−/−</sup>; Pax6<sup>+/+</sup> controls without the Le-Cre transgene. We also observed some variability in eye mass between left and right eyes from the same Le-Cre<sup>+/−</sup>; Pax6<sup>+/−</sup> mice, indicating that stochastic variability occurs as well as variability attributed to differences in genetic background. Liu et al. [5] reported variation for lens abnormalities in Le-Cre; Six3<sup>Lox/Lox</sup> mice, which they attributed to variability in the timing and extent of Le-Cre activity on the NMRI genetic background that was used. However, stochastic variation in eye size has also been reported for mice with reduced or elevated Pax6 levels, which is not controlled by Cre-recombinase [34,39].

Reducing Le-Cre eye abnormalities by manipulating the genetic background or Pax6 levels

When Le-Cre<sup>+/−</sup>; Pax6<sup>+/−</sup> mice from generation 8 were crossed to FVB/N for two generations before crossing them to Le-Cre<sup>−/−</sup>; Pax6<sup>+/+</sup> mice (stage 4 experiments), the eye abnormalities in Le-Cre<sup>+/−</sup>; Pax6<sup>+/−</sup> control progeny were less frequent and much less severe than in stage 3. This partial rescue of the eye phenotype implies that a genetic background effect is involved and the abnormal stage-3 Le-Cre<sup>−/−</sup>; Pax6<sup>+/+</sup> phenotype is not caused by an inherited mutation. Together these results suggest that the eye abnormalities are mediated by an interaction between the Le-Cre<sup>+/−</sup> transgene and alleles of unknown modifier genes present in certain genetic backgrounds, including CBA/Ca. Finally, introduction of the Pax7 transgene to the Le-Cre<sup>+/−</sup>; Pax6<sup>+/−</sup> genotype also reduced the severity of the eye abnormalities suggesting that increasing the Pax6 level could counteract the effects of the Le-Cre<sup>+/−</sup> transgene on a largely CBA/Ca genetic background.

Abnormalities in other Cre-loxP experiments

A number of possible pitfalls with Cre-loxP experiments have been identified and reviewed elsewhere [48,49,50]. These include (i) unexpected Cre expression patterns in somatic tissues; (ii) unexpected Cre expression in the germ line leading to deletion of floxed genes in all cells of individuals in the next generation [51,52]; (iii) variation among floxed alleles in their sensitivity to Cre-mediated recombination and (iv) toxic effects of Cre. The genetic background may affect the pattern of loxP recombination [53] but it is not known whether this acts by altering Cre-recombinase expression (i) or another mechanism. Collectively, these problems indicate that Cre-loxP experiments are not always as robust as is often assumed. In our experiments, the abnormalities occurred in Le-Cre<sup>+/−</sup>; Pax6<sup>+/−</sup> but not Le-Cre<sup>−/−</sup>; Pax6<sup>+/−</sup> controls, implicating the Le-Cre transgene rather than loxP.

One type of Cre toxicity involves the action of Cre recombinase on cryptic pseudo-loxP sites, which are present in the endogenous mouse genome [34,55] but, to have any detectable effect, this may require prolonged expression of high levels of Cre recombinase [56,57,58]. Such action may result in mutations and chromosomal abnormalities, by introducing single-stranded and double-stranded DNA breaks even if Cre-mediated recombination does not occur [55,59]. This has been implicated as the cause of sterility in mice expressing Cre recombinase in sperm mitochonds [60]. In somatic cells a common phenotypic effect is likely to be reduced cell proliferation and cell cycle arrest [57,61] leading to increased cell death. This may be relatively well tolerated and it has been suggested that many Cre-expressing transgenic strains that appear superficially normal are not actually completely normal, as often the effects of Cre cytotoxicity would be ameliorated by a combination of developmental selection and adaptation [48]. Nevertheless, abnormal phenotypes have been reported as a result of Cre expression in somatic cells, including (i) brain damage (e.g. reduced neuronal proliferation, increased aneuploidy and apoptosis, microencephaly and hydrocephaly) [61,62,63], (ii) damage to the retinal pigment epithelium [64], (iii) glucose intolerance [65], (iv) cardiomyopathy leading to heart failure [66] and (v) tetraploidy among epidermal keratinocytes [59]. Activation of CreER<sup>132</sup> transgenes with tamoxifen treatment has also been associated with abnormalities in transgenic mice, including reduced proliferation and increased apoptosis in haematopoietic tissues [63,67]. There was no clear evidence of a genetic background effect in these studies although this possibility was considered for glucose intolerance, which was observed on two different genetic backgrounds [65].

Possible causes of eye abnormalities in Le-Cre<sup>+/−</sup>; Pax6<sup>+/−</sup> control mice

The examples discussed above are thought to arise by action of Cre recombinase on endogenous pseudo-loxP sites. However, the eye abnormalities reported here for Le-Cre<sup>+/−</sup>; Pax6<sup>+/−</sup> mice are phenotypically very similar to those seen in Pax6<sup>+/−</sup>-heterozygotes [19,22], which suggests that a different mechanism may be involved. The observed reduction in Le-Cre<sup>+/−</sup>; Pax6<sup>+/−</sup> abnormalities seen after crossing Le-Cre<sup>+/−</sup> to FVB for two generations makes it unlikely that the abnormal phenotype is caused by permanent DNA damage of the sort expected for action of Cre recombinase on off-target, pseudo-loxP sites. Furthermore, the amelioration of the eye abnormalities in Le-Cre<sup>+/−</sup>; Pax6<sup>+/−</sup> mice by crossing to CBA/Ca-PAX7<sup>−/−</sup> suggests that the mechanism may act by affecting the dose of Pax6.

We have not investigated the mechanism underlying the Le-Cre<sup>+/−</sup>; Pax6<sup>+/−</sup> eye abnormalities directly but the observation that the eyes of Le-Cre<sup>+/−</sup>; Pax6<sup>+/−</sup>; Pax7<sup>−/−</sup> mice (produced by crosses of Le-Cre<sup>+/−</sup>; Pax6<sup>+/−</sup> with CBA/Ca-PAX7<sup>+/−</sup>-transgenic mice) were less severe than in Le-Cre<sup>−/−</sup>; Pax6<sup>+/−</sup>; Pax7<sup>−/−</sup> mice (without the PAX7 transgene) are instructive. Firstly, crosses with Le-Cre<sup>−/−</sup>; Pax6<sup>+/−</sup> mice, containing both Le-Cre and a floxed target gene, risk causing additional abnormalities if the Le-Cre transgene is ectopically expressed in the germline (as discussed above) but none was seen. Secondly, as the PAX7<sup>−/−</sup> mice were on a CBA/Ca genetic background (=20 backcross generations), the Le-Cre<sup>−/−</sup>; Pax6<sup>+/−</sup>; Pax7<sup>−/−</sup> mice had no less CBA/Ca genome in their genetic background but should have higher Pax6 levels than stage-3 Le-Cre<sup>+/−</sup>; Pax6<sup>+/−</sup> control mice without the PAX7 transgene. The results, therefore, argue that the eye abnormalities in our stage-3 Le-Cre<sup>−/−</sup>; Pax6<sup>+/−</sup> mice could be caused by a mechanism that involves Pax6 depletion. The Le-Cre integration site remains unknown and although it is possible that insertion of the Le-Cre transgene into the mouse genome disrupts expression of Pax6 or a gene that affects Pax6 expression, this seems unlikely because the genetic background effect is reversible. Also, homozygous Le-Cre<sup>+/−</sup> mice are viable, so if the transgene insertion disrupted expression of an endogenous gene it would have to specifically affect Pax6 expression and cause eye defects without being homozygous lethal.

In our view, a more likely possibility is that expression of Cre recombinase, from the Pax6-Le regulatory sequences in the Le-Cre transgene, competes with the endogenous Pax6 gene for co-factors required for Pax6 gene expression, so reducing Pax6 production from the endogenous gene. This possibility also predicts that abnormalities observed in Le-Cre<sup>+/−</sup>; Pax6<sup>+/+</sup> and Le-Cre<sup>+/−</sup>; Pax6<sup>+/−</sup> eyes are a consequence of altered Pax6 expression in the surface ectoderm lineage. Consistent with this, Le-Cre<sup>+/−</sup>; Pax6<sup>+/−</sup> eye phenotypes were similar to some of the abnormalities reported for Pax6<sup>−/−</sup> heterozygotes [19,22] and
Le-Cre<sup>+/−</sup>; Pax6<sup>fl/+</sup> phenotypes were more severe than those in Pax6<sup>+/−</sup> mice. The types of eye abnormalities (microphthalmia plus cataracts) reported in abstract form for Le-Cre<sup>Tg</sup>/Le homozygotes on a mixed genetic background [30] are also consistent with an effect that is mediated via altered Pax6 levels but less penetrant on FVB than some other genetic backgrounds. This mechanism would predict that use of Pax6-Le regulatory sequences to drive other transgenes (apart from Pax6 itself) would have similar consequences on certain genetic backgrounds.

**Genetic background effects on eye phenotype**

Differences in genetic background also affects the phenotype of Pax6<sup>+/−</sup> homozygotes [68] and PAX7<sup>+/−</sup> transgenics [34] and probably explains why some reported phenotypes of heterozygous Pax6<sup>Sey/+</sup> mice are more severe than others [47]. The existence of variants of unknown modifier genes in different genetic backgrounds which influence the expressivity and penetrance of abnormal ocular phenotypes has been proposed previously to explain genetic background effects in Le-Cre<sup>Tg</sup> homozygotes (reported in abstract form [30]) and also Pax6<sup>Sey/+</sup> or PAX7<sup>+/−</sup> mice [34]. If the genetic background modulates the level or effectiveness of Pax6, a genetic background that decreased the effective Pax6 level is likely to increase the abnormalities in Le-Cre<sup>Tg/Tg</sup>; Pax6<sup>−/+</sup>; Le-Cre<sup>Tg</sup>; Pax6<sup>−/+</sup> and Pax6<sup>−/+</sup> mice but would probably reduce the severity of the abnormalities in PAX7<sup>+/−</sup> transgenic mice, which are caused by elevated levels of Pax6.

Whatever mechanism is involved, our observation that eye abnormalities can occur in hemizygous Le-Cre<sup>Tg</sup> mice on some genetic backgrounds, in the absence of a floxed allele, serves as a cautionary tale for future studies with Le-Cre mice, almost all of which use Le-Cre<sup>Tg</sup> hemizygotes. Offspring with deleted floxed alleles are analysed in most Le-Cre experiments but these are also Le-Cre<sup>Tg</sup> hemizygotes and are often mixed on genetic backgrounds. Furthermore, many published Cre-loxP studies give incomplete details about which controls are analysed or do not include a Cre-positive control without a floxed allele. For example, a review of studies with a different Cre transgene showed that only 6/22 (27%) included a Cre-positive control without a floxed allele [65]. Some of the Le-Cre experiments cited in the Introduction also failed to include this type of control and/or used Le-Cre mice maintained on a mixed genetic background. Intriguingly, some of these studies reported ocular phenotypes for Le-Cre-induced conditional knockouts that appear to overlap with those seen in our Le-Cre<sup>Tg/Tg</sup>; Pax6<sup>−/+</sup> controls. This implies that the authors should consider whether any of these phenotypes could have been produced by the type of unexpected Le-Cre effects that we have described rather than by deletion of the floxed allele. In conclusion, our results highlight the importance of including all the relevant controls in Cre-loxP experiments.

**Supporting Information**

**Figure S1** Variation in eye diameter for different genotypes on different genetic backgrounds. (A–F) Diameter of left (A–C) and right (D–F) eyes of 12 week old mice from Le-Cre<sup>Tg/Tg</sup>; Pax6<sup>−/+</sup> and × Le-Cre<sup>Tg/Tg</sup>; Pax6<sup>−/+</sup> crosses on different genetic backgrounds: (A,D) stage 1 crosses (B,E) stage 3 crosses (C,F) stage 4 crosses. (G–I) The percentage corneal diameter difference, calculated for each mouse as (larger cornea diameter – smaller cornea diameter) ×100/(larger cornea diameter). Abbreviations: WT +/+ is Le-Cre<sup>Tg/Tg</sup>; Pax6<sup>−/+</sup>; WT fl/+ is Le-Cre<sup>Tg/Tg</sup>; Pax6<sup>−/+</sup>; Cre +/+ is Le-Cre<sup>Tg/Tg</sup>; Pax6<sup>−/+</sup> and Cre fl/+ is Le-Cre<sup>Tg/Tg</sup>; Pax6<sup>−/+</sup>. Results for all four genotypes were compared by non-parametric Kruskal-Wallis (KW) tests separately for each stage of the study (P-values are shown in the figure) and results for WT fl/+; Cre +/+ and Cre fl/+ were compared to WT +/+ by Dunn’s multiple comparison post-hoc test: *P<0.05; **P<0.01; ***P<0.001.

**Figure S2** Variation in corneal diameter for different genotypes on different genetic backgrounds. (A–F) Diameter of left (A–C) and right (D–F) corneas of 12 week old mice from Le-Cre<sup>Tg/Tg</sup>; Pax6<sup>−/+</sup> and × Le-Cre<sup>Tg/Tg</sup>; Pax6<sup>−/+</sup> crosses on different genetic backgrounds: (A,D) stage 1 crosses (B,E) stage 3 crosses (C,F) stage 4 crosses. (G,I) The percentage corneal diameter difference, calculated for each mouse as (larger cornea diameter – smaller cornea diameter). Abbreviations: WT +/+ is Le-Cre<sup>Tg/Tg</sup>; Pax6<sup>−/+</sup>; WT fl/+ is Le-Cre<sup>Tg/Tg</sup>; Pax6<sup>−/+</sup>; Cre +/+ is Le-Cre<sup>Tg/Tg</sup>; Pax6<sup>−/+</sup> and Cre fl/+ is Le-Cre<sup>Tg/Tg</sup>; Pax6<sup>−/+</sup>. Results for all four genotypes were compared by non-parametric Kruskal-Wallis (KW) tests separately for each stage of the study (P-values are shown in the figure) and results for WT fl/+; Cre +/+ and Cre fl/+ were compared to WT +/+ by Dunn’s multiple comparison post-hoc test: *P<0.05; **P<0.01; ***P<0.001.

**Figure S3** Comparison of corneal epithelial wound healing rates among different groups (from stage 2 crosses). (A–C) Mean corneal epithelial wound diameters at different times for (A) left eyes, (B) right eyes and (C) both eyes for each of the five groups compared. (D–H) Corneal epithelial wound for individual corneas for (D) Le-Cre<sup>Tg/Tg</sup>; Pax6<sup>−/+</sup> (E) Le-Cre<sup>Tg/Tg</sup>; Pax6<sup>−/+</sup> (F) Le-Cre<sup>Tg/Tg</sup>; Pax6<sup>−/+</sup> (G) Le-Cre<sup>Tg/Tg</sup>; Pax6<sup>−/+</sup> and (H) Pax6<sup>Sey/+</sup>-New. Abbreviations: WT +/+ is Le-Cre<sup>Tg/Tg</sup>; Pax6<sup>−/+</sup>; WT fl/+ is Le-Cre<sup>Tg/Tg</sup>; Pax6<sup>−/+</sup>; Cre +/+ is Le-Cre<sup>Tg/Tg</sup>; Pax6<sup>−/+</sup>; Cre fl/+ is Le-Cre<sup>Tg/Tg</sup>; Pax6<sup>−/+</sup> and Pax6<sup>−/+</sup> is Pax6<sup>Sey/+</sup>-New.

**Table S1** Genetic backgrounds of mice used in different stages of the study.

**Table S2** Frequency of healed wounds after 24 hours.

**Supplementary Data S1** Supplementary Data for Figs. 3 & 6 and Figs. S1, S2 & S3.

**Author Contributions**

Conceived and designed the experiments: JDW REH JMC NJD. Performed the experiments: NJD. Analyzed the data: NJD JMC REH. Contributed reagents/materials/analysis tools: REH JDW. Wrote the paper: JDW NJD. Revised first draft of manuscript: JDW NJD JMC REH.

**Acknowledgments**

Dedication

This paper is dedicated to the memory of our friend and colleague, Dr. Thaya Ramashe, who taught us so much and died far too young. She is sorely missed by us all.

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References


68. Quinn JC, West JD, Kaufman MH (1997) Genetic background effects on dental and other craniofacial abnormalities in homozygous small eye (Pax6<sup>Sey</sup>/Pax6<sup>Sey</sup>) mice. Anat Embryol 196: 311–323.