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Effects of aberrant \textit{Pax6} gene dosage on mouse corneal pathophysiology and corneal epithelial homeostasis

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\textbf{Abstract}

\textbf{Background:} Altered dosage of the transcription factor PAX6 causes multiple human eye pathophysiologies. \textit{PAX6} heterozygotes suffer from aniridia and aniridia-related keratopathy (ARK), a corneal deterioration that probably involves a limbal epithelial stem cell (LESC) deficiency. Heterozygous \textit{Pax6$^{+/\text{Sey-Neu}}$} (\textit{Pax6$^{+/}$}) mice recapitulate the human disease and are a good model of ARK. Corneal pathologies also occur in other mouse \textit{Pax6} mutants and in \textit{PAX77$^{9\gamma}$} transgenics, which over-express \textit{Pax6} and model human \textit{PAX6} duplication.

\textbf{Methodology/Principal Findings:} We used electron microscopy to investigate ocular defects in \textit{Pax6$^{+/}$} heterozygotes (low \textit{Pax6} levels) and \textit{PAX77$^{9\gamma}$} transgenics (high \textit{Pax6} levels). As well as the well-documented epithelial defects, aberrant \textit{Pax6} dosage had profound effects on the corneal stroma and endothelium in both genotypes, including cellular vacuolation, similar to that reported for human macular corneal dystrophy. We used mosaic expression of an \textit{X}-linked \textit{LacZ} transgene in \textit{X}-inactivation mosaic female (\textit{XLacZ$^{9\gamma}$} / $^{9\gamma}$) mice to investigate corneal epithelial maintenance by LESC clones in \textit{Pax6$^{+/}$} and \textit{PAX77$^{9\gamma}$} mosaic mice. \textit{PAX77$^{9\gamma}$} mosaic, over-expressing \textit{Pax6}, produced normal corneal epithelial radial striped patterns (despite other corneal defects), suggesting that centripetal cell movement was unaffected. Moderately disrupted patterns in \textit{Pax6$^{+/}$} mosaics were corrected by introducing the \textit{PAX77} transgene (in \textit{Pax6$^{+/}$}, \textit{PAX77$^{9\gamma}$} mosaic). \textit{Pax6$^{+/}$}, \textit{XLacZ$^{9\gamma}$} mosaic mice (heterozygous for the \textit{Pax6$^{ec9\delta}$} missense mutation) showed more severely disrupted mosaic patterns. Corrected corneal epithelial stripe numbers (an indirect estimate of active LESC clone numbers) declined with age (between 15 and 30 weeks) in wild-type \textit{XLacZ$^{9\gamma}$} mosaics. In contrast, corrected stripe numbers were already low at 15 weeks in \textit{Pax6$^{+/}$} and \textit{PAX77$^{9\gamma}$} mosaic corneas, suggesting \textit{Pax6} under- and over-expression both affect LESC clones.

\textbf{Conclusions/Significance:} \textit{Pax6$^{+/}$} and \textit{PAX77$^{9\gamma}$} genotypes have only relatively minor effects on LESC clone numbers but cause more severe corneal endothelial and stromal defects. This should prompt further investigations of the pathophysiology underlying human aniridia and ARK.

\textbf{Introduction}

The \textit{Pax6} gene encodes the \textit{Pax6} transcription factor with key regulatory roles in eye development [1–5]. Abnormal expression results in a spectrum of ocular pathophysiologies, some of which are directly linked to the protein level [6–9]. Some corneal abnormalities associated with \textit{Pax6} mutations occur during development and others result from inadequate tissue maintenance.

It is widely accepted that, during adult corneal epithelial homeostasis, cell production is initiated by limbal epithelial stem cells (LESCs) in the limbus at the corneoscleral junction [10–14]. LESC clones produce transient (or transit) amplifying cells (TACs) that migrate centripetally, dividing a few times before terminally differentiating. As TACs differentiate they lose contact with the basal epithelium, move apically, and are desquamated from the surface layer [15,16]. Epithelial abnormalities could be caused by defects in LESC or epithelial cell proliferation, movement or loss.

Centripetal movement in the mouse corneal epithelium has been demonstrated directly in several experimental systems [17,18] and indirectly by the postnatal switch from a randomly orientated mosaic pattern to radial stripes in \textit{X}-inactivation mosaics [19–21] and lentivirus-labelled lineages [22]. Radial stripes emerging from the periphery and extending towards the central cornea from ~5 weeks are thought to represent clones of centripetally migrating epithelial cells produced after LESC activation. Numerical analysis of these striping patterns provides...
an indirect estimate of the number of coherent clones of LESC
cells that maintain the corneal epithelium [19–21].

Pax6 is widely expressed during eye development [23] and
continues in several adult tissues, including the corneal, limbal and
corneoconjunctival epithelium [24]. Absence of Pax6 causes anophthalmia in
both mice [1] and humans [23]. Eye development is highly sensitive to Pax6 dose and haploinsufficiency in human PAX6 heterozygotes is characterised by aniridia and other ocular abnormalities [26–29]. Heterozygosity for mouse Pax6 null mutations, such as Pax6(Eye) [1,29,30], Pax6(Eye)−/+ [1,31] and Pax6(Eye)−/− [32], causes similar abnormalities and small-eyes. Ocular phenotypes produced by hypomorphic Pax6 alleles showed that surface ectoderm derivatives are more sensitive to Pax6 levels than optic
vesicle derivatives [6]. Pax6 mouse mutations often cause different eye phenotypes from null mutations [33–35]. For example, Pax6(Eye)−/− heterozygous mice have small, abnormal eyes with cornealvascularisation from fetal stages and pigmenta-
tion (but no goblet cells) within the cornea [36].

Pax6(Eye)−/− mice have corneal and other anterior segment abnormalities [37–46] and some have been characterised by electron microscopy (EM) [38,47,48]. The postnatal corneal deterioration in Pax6(Eye)−/− mice is equivalent to that seen in human aniridia-related keratopathy (ARK), which has been attributed to LESC deficiency. This is based entirely on indirect evidence such as the presence of goblet cells [49] and clinical results for limbal transplants [50] because there are currently no suitable LESC markers. Quantitative analysis of mosaic corneal epithelial patterns in mouse X-inactivation mosaics and chimeras also suggests that Pax6(Eye)−/− mice have fewer active clones of LESC than normal species. However, the stripe pattern is disrupted, implying that cell movement is abnormal so Pax6(Eye)−/− corneal deterioration probably involves additional factors.

Over-expression of Pax6 in hemizygous PAX7(Tg−/−) mice with 5–7 copies of human Pax6 [8] also causes eye abnormalities on a wild-type (WT) background and provides a model for human PAX6 gene duplication [7]. The abnormalities overlap with those produced by heterozygous Pax6(Eye)−/− mice (low Pax6 levels) but there are significant differences and genetic background modulates the phenotype [8,52–55]. Pax6 levels are increased less than gene copy numbers predict [52,54,55] but the PAX7 transgene can rescue various Pax6(Eye)−/− and Pax6(Eye)−/−/−
ocular phenotypes [8].

The present study had three aims. (1) To investigate the effects of altered Pax6 dose on the corneal stroma and endothelium in Pax6(Eye)−/− and PAX7(Tg−/−) mice using EM, to complement previous studies of effects on the corneal epithelium. (2) Given that our previous clonal analysis of X-inactivation mosaics identified abnormalities of corneal epithelial maintenance in Pax6(Eye)−/− mice [51], to investigate whether Pax6 over-expression causes similar abnormalities in PAX7(Tg−/−) mice. (3) To investigate whether this effect can be rescued by combining the PAX7 transgene with a Pax6(Eye)−/− genotype. We identified previously unreported corneal endothelial and stromal abnormalities in both genotypes by EM. Furthermore, our qualitative analysis of X-inactivation mosaics implied that cell movement was normal during corneal epithelial maintenance in PAX7(Tg−/−) mice (unlike Pax6(Eye)−/− heterozygotes) and our quantification suggested that in younger mice LESC clone numbers were reduced in both Pax6(Eye)−/− and PAX7(Tg−/−) genotypes. The PAX7 transgene normalised both the qualitative and quantitative defects in Pax6(Eye)−/− corneas.

Results

We used scanning electron microscopy (SEM) and transmission electron microscopy (TEM) to compare the structure of corneas from WT, Pax6(Eye)−/− and PAX7(Tg−/−) mice. We also analysed mosaic patterns in the corneal epithelia of WT, Pax6(Eye)−/− and PAX7(Tg−/−) X-inactivation mosaics to compare effects of Pax6 doses on corneal epithelial cell movement (from mosaic patterns) and LESC clone numbers (from quantitative analysis of corrected stripe numbers).

PAX7(Tg−/−) corneal epithelial cells have abnormally large microvilli and less pronounced cell junctions

On the CBA/Ca background Pax6(Eye)−/− and PAX7(Tg−/−) eyes were smaller than WT (Fig. 1A–C) and PAX7(Tg−/−) mice had microcorneas and a pronounced ring around the corneoscleral junction (Fig. 1C). SEM of the superficial corneal epithelial cells showed that, the WT corneal epithelium consisted of large polygonal cells with tightly opposed cell junctions (Fig. 1D) and numerous microvilli (Fig. 1G). Despite reported increased sloughing of Pax6(Eye)−/− corneal epithelial cells [38], the epithelia of the Pax6(Eye)−/− specimens analysed by SEM appeared similar to WT (Fig. 1E,H). However, the PAX7(Tg−/−) epithelial cells had a more irregular surface (Fig. 1E,I), indistinct cell junctions (Fig. 1F) and larger microvilli (Fig. 1I). Other corneal epithelial abnormalities have been well described, so further EM work focused on the corneal stroma and endothelium. Previously unreported abnormalities are discussed below and summarised in Table 1.

Pax6(Eye)−/− and PAX7(Tg−/−) corneal endothelial cells are severely abnormal

SEM examination revealed serious abnormalities in both Pax6(Eye)−/− and PAX7(Tg−/−) corneal endothelia. The WT corneal endothelial cells were hexagonal (mean diameter, 18.7±2.35 μm; Fig. 2A,D) whereas Pax6(Eye)−/− cells were larger (23.75±2.3 μm; Fig. 2B,E) and slightly irregularly shaped. The PAX7(Tg−/−) endothelium was...
highly irregular with indistinct cell borders that were only visible at higher magnification (Fig. 2C,F). The WT endothelial cells had either no vacuoles or very small vacuoles (Fig. 2D,G), whereas the Pax6+/− and PAX77Tg+/− endothelial surfaces appeared highly irregular by SEM (Fig. 2E,F) and TEM revealed large intracellular vacuoles across the entire endothelium in each case (Fig. 2H,I). In PAX77Tg+/− corneal endothelia the vacuoles seemed smaller towards the central cornea (data not shown). These results imply that Pax6 over- and under-expression both produced significant endothelial defects but over-expression produced a more severe phenotype.

**Pax6+/− and PAX77Tg+/− corneal stromas are abnormal**

Marked abnormalities occurred in the corneal stromas of both the Pax6+/− and PAX77Tg+/− mice. TEM showed that keratocytes in the WT stroma were normal with numerous cell organelles (Fig. 3A) but Pax6+/− keratocytes had large intracellular vacuoles (Fig. 3B,C) and PAX77Tg+/− keratocytes had smaller vacuoles (Fig. 3D). Subjectively both Pax6+/− and PAX77Tg+/− stromas also appeared to be more highly innervated with nerve cells (Fig. 3E,F) than in the WT stroma but this was not quantified.

**Over-expression of Pax6 in PAX77Tg+/− mice does not disrupt corneal epithelial maintenance**

To compare the effects of different Pax6 doses on corneal epithelial maintenance by analysis of striped patterns in X-inactivation mosaics, we crossed PX77Tg+/− and Pax6+/− females to XLacZ+/− males carrying the X-linked LacZ transgene and analysed patterns in the corneal epithelia of XLacZ+/− X-inactivation mosaic females, as described elsewhere [19–21]. WT, XLacZ+/− mosaics corneas showed radial striping patterns (Fig. 4A,B) whereas Pax6+/−, XLacZ+/− mosaics produced more irregular patterns (Fig. 4C,D) as demonstrated previously.

Table 1. Main new abnormalities in Pax6+/− and PAX77Tg+/− corneas identified by electron microscopy.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Wild-type</th>
<th>Pax6+/−</th>
<th>PAX77Tg+/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size of microvilli on surface of epithelial cells</td>
<td>normal</td>
<td>normal</td>
<td>large</td>
</tr>
<tr>
<td>Intracellular vacuoles in stromal keratocytes</td>
<td>none</td>
<td>yes (large)</td>
<td>yes (small)</td>
</tr>
<tr>
<td>Presence of nerve cells in stroma</td>
<td>present</td>
<td>more frequent*</td>
<td>more frequent*</td>
</tr>
<tr>
<td>Intracellular vacuoles in corneal endothelium</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Corneal endothelial cell size and shape</td>
<td>regular/hexagonal</td>
<td>large/irregular</td>
<td>indistinct boundaries</td>
</tr>
</tbody>
</table>

*Nerve cell numbers appeared more frequent in Pax6+/− and PAX77Tg+/− corneal stromas but they were not quantified.

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Figure 2. Electron microscopy of corneal endothelium. A–C. SEM micrographs of (A) WT, (B) Pax6+/− and (C) PAX77Tg+/− corneal endothelial cells. WT endothelial cells have a regular hexagonal shape, Pax6+/− cells have an irregular vacuolated appearance and are larger than normal and PAX77Tg+/− endothelial cells have an irregular vacuolated appearance, are irregular in shape and the cell borders are difficult to resolve. Scale bars = 10 µm. D–F. Higher power SEM micrographs of (D) WT, (E) Pax6+/− and (F) PAX77Tg+/− corneal endothelial cells showing that although both Pax6+/− and PAX77Tg+/− corneal endothelial cells are vacuolated and irregular in shape the cell borders are more distinct in Pax6+/− cells. Scale bars = 10 µm. G–I. TEM micrographs of (G) WT, (H) Pax6+/− and (I) PAX77Tg+/− corneal endothelial cells (shown above Descemet’s membrane and stroma) at the periphery of the cornea. Pax6+/− and PAX77Tg+/− endothelial cells contain large vacuoles. Scale bars = 1 µm. doi:10.1371/journal.pone.0028895.g002

Figure 3. Electron microscopy of corneal stroma. TEM micrographs of (A) WT corneal stroma showing normal keratocyte morphology with no vacuoles; (B,C) Pax6+/− corneal stroma showing keratocytes with very large vacuoles; (D) PAX77Tg+/− corneal stroma showing keratocyte with small vacuoles; (E) Pax6+/− corneal stroma showing nerve cell; (F) PAX77Tg+/− corneal stroma showing nerve cell. Abbreviations: *v, vacule; *n, nerve cell. Scale bars = 500 nm in A–D and 1 µm in E and F. doi:10.1371/journal.pone.0028895.g003
cells (Fig. 5). We, therefore, treated the stripes as 2-dimensional patterns and reduced them to a 1-dimensional count for quantification (see Materials and Methods).

**Over-expression of Pax6 in PAX77<sup>9/w</sup> mosaic corneas results in fewer corneal stripes**

Although stripe patterns in PAX77<sup>Tg/-</sup>, XLacZ<sup>Tg/-</sup> mosaics appeared normal, a quantitative analysis was undertaken to identify any subtle differences that might suggest that stem cell clones were affected. The observed number of radial stripes in the corneal epithelium was converted to a corrected stripe number to compare LESC clone numbers between different groups (see Materials and Methods). A preliminary experiment, using PAX77<sup>Tg/-</sup> mice on an outbred CD-1 genetic background showed that the corrected stripe number per eye was significantly lower in PAX77<sup>Tg/-</sup>, XLacZ<sup>Tg/-</sup> mosaics than WT, XLacZ<sup>Tg/-</sup> controls at 15 weeks (Table 3). This difference remained significant after correcting for the smaller circumferences of PAX77<sup>Tg/-</sup> corneas, suggesting that PAX77<sup>Tg/-</sup>, XLacZ<sup>Tg/-</sup> corneas were maintained by fewer active LESC clones than normal.

Although the corrected stripe number does not provide a direct estimate of LESC numbers, it can be used to compare LESC clones between different groups of mice. The corrected stripe number is an estimate of the number of corneal epithelial clones, each of which will be produced by an active coherent clone of LESCs in the limbus. A difference in corrected stripe number, therefore, predicts a difference in the number of active LESC clones and this could reflect a difference in active LESC numbers and/or a change in the LESC distribution (number of LESC per clone).

The preliminary results were confirmed and extended in a second experiment, using PAX77<sup>Tg/-</sup> mice on an inbred CBA/Ca genetic background and analysing corneas at both 15 and 30 weeks. The mean corrected stripe number per eye (or per mm circumference) was significantly lower in PAX77<sup>Tg/-</sup>, XLacZ<sup>Tg/-</sup> mosaics than WT, XLacZ<sup>Tg/-</sup> controls at 15 weeks (Fig. 6). It declined between 15 and 30 weeks in WT, XLacZ<sup>Tg/-</sup> controls, as reported previously [19,21], but no reduction occurred in the PAX77<sup>Tg/-</sup>, XLacZ<sup>Tg/-</sup> mosaics, so by 30 days the PAX77<sup>Tg/-</sup> corrected stripe number was not significantly different from controls (Fig. 6C,D). This suggests that PAX77<sup>Tg/-</sup>, XLacZ<sup>Tg/-</sup> corneas were maintained by fewer active LESC clones than normal at 15 weeks (as in the preliminary experiment, Table 3) but, unlike WT, this did not decline further between 15 and 30 weeks.

The PAX77 transgene normalises corneal circumference and corneal epithelial maintenance in Pax<sup>6<sup>-/-</sup></sup> heterozygotes

The additional Pax6 expression produced by the PAX77<sup>Tg/-</sup> transgene can rescue abnormal ocular phenotypes in Pax6<sup>-/-</sup> mice [8]. To determine whether the PAX77<sup>Tg/-</sup> transgene could also rescue the abnormal Pax6<sup>-/-</sup> striped pattern in mosaics (implying abnormal corneal epithelial maintenance; Fig. 4E,F), we undertook a third experiment which compared striping patterns in the four XLacZ<sup>Tg/-</sup> mosaic genotypes produced by crosses of Pax6<sup>-/-</sup> females and PAX77<sup>Tg/-</sup>, XLacZ<sup>Tg/-</sup> males. The stripe patterns for the three genotypes already examined in earlier experiments (Fig. 4A–F) were reproduced at both 15 and 30 weeks in the third experiment (Fig. 7A-C) although on the genetic background produced by this three-way cross, mice over-expressing Pax6 (PAX77<sup>Tg/-</sup>, Pax6<sup>+/</sup>, XLacZ<sup>Tg/-</sup>) had smaller corneas (Fig. 7C; compare Figs. 6B and 8A). In PAX77<sup>Tg/-</sup>, Pax6<sup>+/</sup>, XLacZ<sup>Tg/-</sup> mosaics, where the PAX77 transgene is expressed in a Pax<sup>6<sup>-/-</sup></sup> background (Fig. 4G,H), mosaic eyes showing variation in mosaic patterns among the four different genotypes: (A,B) WT, XLacZ<sup>Tg/-</sup>; (C,D) Pax6<sup>+/</sup>, XLacZ<sup>Tg/-</sup>; (E,F) PAX77<sup>Tg/-</sup>, XLacZ<sup>Tg/-</sup> and (G,H) Pax6<sup>+/</sup>, XLacZ<sup>Tg/-</sup>. Scale bar = 1 mm.

doi:10.1371/journal.pone.0028895.g004 [19,21,51]. Strikingly, despite corneal defects (Tables 1 and 2), the PAX77<sup>Tg/-</sup>, XLacZ<sup>Tg/-</sup> mosaic eyes exhibited a qualitatively normal striped phenotype with radial stripes extending from the limbal region (Fig. 4E,F), consistent with normal centripetal movement from the presumptive LESCs. In contrast, the Pax6<sup>+/</sup>, XLacZ<sup>Tg/-</sup> mosaic corneal patterns appeared as an abnormal mosaic patchwork rather than radial stripes (Fig. 4G,H), so were not included in the quantitative analyses of stripe numbers described below.

In sections of stained XLacZ<sup>Tg/-</sup> corneas from WT, Pax6<sup>+/</sup>- and PAX77<sup>Tg/-</sup> animals, clones of β-gal positive cells were aligned vertically with little overlap of β-gal positive and β-gal negative

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**Figure 4. Mosaic patterns in the corneal epithelium.** Representative images of β-gal staining in the corneal epithelia of X-inactivation mosaic eyes showing variation in mosaic patterns among the four different genotypes: (A,B) WT, XLacZ<sup>Tg/-</sup>; (C,D) Pax6<sup>+/</sup>, XLacZ<sup>Tg/-</sup>; (E,F) PAX77<sup>Tg/-</sup>, XLacZ<sup>Tg/-</sup> and (G,H) Pax6<sup>+/</sup>, XLacZ<sup>Tg/-</sup>. Scale bar = 1 mm. doi:10.1371/journal.pone.0028895.g004
The PAX77 transgene restores corneal epithelial stripe numbers in Pax6+/−, PAX77g+/- mice

Although the mosaic corneal stripe patterns appeared normal in PAX77+/−, Pax6+/−, XLacZ−/− mosaics (Fig. 7), the second experiment shows that this may mask quantitative differences (Fig. 6C,D). Thus, to investigate whether the striped pattern was normalised quantitatively as well as qualitatively by the presence of the PAX77 transgene in PAX77+/−, Pax6+/−, XLacZ−/− mosaics, we analysed the corrected stripe number per cornea for the four genotypes at 15 and 30 weeks of age (Fig. 8). At 15 weeks, corrected stripe numbers in PAX77+/−, Pax6+/−, XLacZ−/− mosaics were not significantly different from WT, XLacZ−/− mosaics, but were significantly higher than in either PAX77+/−, Pax6+/−, XLacZ−/− or PAX77+/−, Pax6+/−, XLacZ−/− mosaics (Fig. 8B). The corrected stripe number later declined in both PAX77+/−, Pax6+/−, XLacZ−/− and WT, XLacZ−/− mosaics and by 30 weeks there were no significant differences among the four genotypes. When results were expressed as corrected stripes per mm of corneal circumference (Fig. 8C), the PAX77+/−, Pax6+/−, XLacZ−/− and WT, XLacZ−/− mosaics again had more stripes than the other genotypes at 15 weeks but these differences failed to reach significance. Nevertheless, corrected stripe numbers in PAX77+/−, Pax6+/−, XLacZ−/− mosaics were quantitatively similar to those in WT, XLacZ−/− mosaics, suggesting that the PAX77 transgene had rescued the low 15-week Pax6+/− stripe number (estimated LESC clone number) as well as the qualitative mosaic pattern.

Table 2. Comparison of maintenance of corneal epithelium in adult Pax6+/− and PAX77g+/- mice with low and high doses of Pax6 respectively.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Pax6+/−</th>
<th>PAX77g+/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of corneal epithelial cell layers</td>
<td>Reduced [37,38]</td>
<td>slightly reduced [54]</td>
</tr>
<tr>
<td>Fragility of corneal epithelium</td>
<td>fragile [38]</td>
<td>moderately fragile [56]</td>
</tr>
<tr>
<td>Cell proliferation in basal corneal epithelium</td>
<td>increased [38,39]</td>
<td>increased [54]</td>
</tr>
<tr>
<td>Corneal opacity and vascularisation</td>
<td>yes [37,43]</td>
<td>no [54,55]</td>
</tr>
<tr>
<td>Abnormal corneal epithelial wound healing</td>
<td>yes [40,42,54]</td>
<td>yes [54]</td>
</tr>
<tr>
<td>Goblet cells in basal corneal epithelium</td>
<td>present [37]</td>
<td>absent [54,55]</td>
</tr>
<tr>
<td>Mosaic pattern in corneal epithelium (15 &amp; 30 weeks)</td>
<td>disrupted [20]*</td>
<td>normal*</td>
</tr>
<tr>
<td>Stripe number in mosaic corneal epithelium (15 weeks)</td>
<td>reduced [20]*</td>
<td>reduced*</td>
</tr>
<tr>
<td>Stripe number in mosaic corneal epithelium (~30 weeks)</td>
<td>reduced (28 wks) [20] or normal (30 wks)*</td>
<td>normal*</td>
</tr>
</tbody>
</table>

*Reported in the present study.

doi:10.1371/journal.pone.0028895.t002

Figure 5. Sections of β-gal stained mosaic corneal epithelia.
Representative images of β-gal stained corneal sections showing vertical alignment of β-gal-positive epithelial cells across the full thickness of the epithelium in eyes expressing various levels of Pax6. (A,B) WT, XLacZ−/−; (C,D) Pax6+/−, XLacZ−/− and (E,F) PAX77g+/−, Pax6+/− corneal stromas (C,D) probably reflect the greater permeability of the thin Pax6+/− corneal epithelium to X-gal stain during whole-mount staining. Scale bar = 100 μm.

doi:10.1371/journal.pone.0028895.g005

Discussion

This study identified new corneal abnormalities, particularly in the stroma and endothelium, in both Pax6+/− and PAX77g+/− mice, which respectively under- and over-express Pax6. Analysis of mosaic corneal patterns showed cell movement during corneal epithelial maintenance was affected in Pax6+/− but not PAX77g+/− mice and implied that LESC clones were affected in both Pax6+/− and PAX77g+/− mice at 15 weeks.

Morphological abnormalities of Pax6+/− and PAX77g+/− corneas

The abnormally large microvilli and indistinct cell junctions of PAX77+/− corneal epithelial cells are consistent with fragility tests suggesting cell adhesion may be affected [36]. Significant new morphological abnormalities were identified in the corneal stroma and endothelium of both Pax6+/− and PAX77+/− mice including intracellular vacuoles. There is some evidence that Pax6 is expressed weakly and transiently in the mouse fetal corneal stroma [47,57] and chimera experiments imply that Pax6
functions cell-autonomously in developing stromal keratocytes or their progenitors [57]. However, it is not known whether the keratocyte and endothelial abnormalities described here are primary defects of altered Pax6 dose in these lineages or an indirect effect mediated via another tissue. The corneal endothelium controls corneal hydration and nutrition via fluid transport. Excessive hydration may cause corneal stromal haze or corneal oedema, as in some human corneal stromal dystrophies. Similar corneal endothelial and stromal keratocyte vacuolation also occurs in human macular corneal dystrophy [58,59]. If the corneal endothelium and/or stroma of Pax6+/− human aniridia patients are affected like the Pax6−/− mice described here, this might have important clinical implications and could underlie some of the abnormal phenotypes associated with aniridia-related keratopathy.

Effects of Pax6 on corneal epithelial cell movement and maintenance

The normal radial striped corneal epithelial pattern of WT X-inactivation mosaics was disrupted in Pax6+/− mice, implying that corneal epithelial cell movement is abnormal [51] but it is unclear whether this is caused by some intrinsic abnormality or a response to chronic wounding of a thin and fragile cornea. Although maintenance of the Pax6+/− corneal epithelium is not entirely normal (Table 2), Pax6+/− mosaics had completely normal radial striped patterns, implying that centripetal cell movement is unaffected by the higher Pax6 dose. In contrast, the pattern in corneas of Pax6Leca+/+ mosaics resembled the randomly orientated pattern of patches seen in young WT corneas before stripes emerge. This suggests that movement of cells from the limbus is severely reduced and that the Pax6Leca+/+ corneal epithelium may be maintained by proliferation from within the epithelium, perhaps because of an extreme LESC deficiency. This possibility has previously been suggested to explain a similar phenotype in Drosophila homoeozygotes [60].

Evidence for effects of Pax6 dose on limbal stem cell clones

Our quantitative analysis of striped patterns in Pax6+/− and Pax77+/− X-inactivation mosaics showed that, at 15 weeks, the corrected stripe numbers were lower than in WT mosaics, even after correcting for differences in corneal circumference, implying that at this age there were fewer clones of active LESCs maintaining the corneal epithelium. However, no such difference was seen at 30 weeks because, by this age, the stripe number had declined in WT mosaics but the stripe numbers did not decline between 15 and 30 weeks in Pax6+/− and Pax77+/− mosaics.

The decline in corrected stripe number in WT XLacZ− mice implies that there is an age-related decline in active LESC clones. This may reflect a decline in LESC numbers or activity but it could also be explained by a drift in LESC clone distributions. If LESCs can divide either symmetrically to produce one LESC and one TAC or asymmetrically to produce either two LESC or two TACs then the pattern of active stem cell clones may follow a pattern of neutral drift as suggested for some other stem cell systems, including spermatogonial stem cells [61] and intestinal crypts [62,63]. Over time, stem cells may be lost and replaced by their neighbours. On this basis, clones of stem cells will expand and contract stochastically and some clones will be lost (e.g. if a β-gal negative LESC clone, that is flanked by two β-gal positive clones, is lost the β-gal negative stripe will be lost and the two flanking β-gal positive stripes will merge into one larger one).

The lower corrected stripe number in Pax6+/− and Pax77+/− XLacZ− mosaics compared to WT mosaics at 15 weeks can be explained in several ways. It is possible that there are initially fewer LESC clones in Pax6+/− and Pax77+/− eyes, either because fewer LESCs are specified, or activated or because the LESCs are grouped into fewer larger clones. Alternatively, LESC clone numbers may initially be similar in all groups (before 15 weeks) but the decline may begin earlier or be more rapid in Pax6+/− and Pax77+/− mice, so by 15 weeks the LESC clone number is similar to that in a 30 week WT mouse. It is not clear why the Pax6+/− and Pax77+/− LESC clone numbers do not continue to decline after 15 weeks, but it has been suggested that this might be related to a minimum required for corneal epithelial maintenance [21]. Regardless of the explanation it is clear that, at 15 weeks, LESC clones numbers and/or distributions are different in Pax6+/− and Pax77+/− mice. However, as this difference is no longer detectable at 30 weeks the difference is short-lived and may have little biological significance.

The presence of goblet cells in the human corneal epithelium is often cited as evidence of LESC deficiency [49,64]. However, the quantitative analysis of Pax6+/− and Pax77+/− mosaics indicates LESC clones are similarly affected in both genotype at 15 weeks (Fig. 8B,C) but only Pax6+/− mice have goblet cells in the corneal epithelium (Table 2). This difference highlights the need for reliable LESC markers.

The Pax77 transgene compensates for defects of corneal maintenance in Pax6+/− X-inactivation mosaics

On a Pax6+/− background, the Pax77 transgene rescued the abnormal stripe patterns that normally occur in Pax6+/− heterozygotes and are attributed to low Pax6 levels. Quantitative analysis showed that the Pax77 transgene also normalised the putative deficiency in active stem cell clones (reduced stripe number) that occurs in Pax6+/− heterozygotes at 15 weeks. These results imply that restoring the Pax6 dose to a more normal level corrects abnormalities of corneal cell maintenance as well as the developmental ocular defects demonstrated previously [8].

It is widely believed that stem cell deficiency causes most corneal abnormalities in ARK. However, our quantitative analyses of mosaic patterns suggest that Pax6+/− and Pax77+/− mice have only relatively modest reductions in LESC clone numbers. In contrast, both Pax6+/− and Pax77+/− mice have severe corneal

Table 3. Preliminary comparison of corrected stripe number in corneal epithelia of wild-type, XLacZ−/− and Pax77+/−, XLacZ−/− X-inactivation mosaics at 15 weeks.

<table>
<thead>
<tr>
<th></th>
<th>Wild-type</th>
<th>Pax77+/−</th>
<th>Significance (t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of eyes</td>
<td>27</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Corrected stripe number per eye (mean ±SEM)</td>
<td>79.97 ± 3.65</td>
<td>54.07 ± 3.43</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>Corrected stripe number per mm corneal circumference</td>
<td>7.65 ± 0.35</td>
<td>5.93 ± 0.45</td>
<td>P = 0.005</td>
</tr>
</tbody>
</table>

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Figure 6. Quantitative comparisons of PAX77^+/+ X LacZ^+/+ and PAX77^−/− X LacZ^−/− eyes at 15 and 30-weeks. (A) WT Eye mass increased significantly between 15 and 30 weeks (2-way ANOVA P<0.0001, results of relevant post-hoc tests are shown in the figure). (B) Corneal circumference differed significantly between WT and PAX77^+/+ XLacZ^+/+ at 15 and 30 weeks but the increase in circumference between 15 and 30 weeks was only significant for WT (2-way ANOVA P<0.0001, results of relevant post-hoc tests are shown in the figure). (C) The mean corrected stripe number was significantly higher in the 15-week WT corneas than the 30-week WT or PAX77Tg^−/− eyes at 15 and 30 weeks but the increase in stripe number between 15 and 30 weeks was only significant for WT (2-way ANOVA P<0.0001, results of relevant post-hoc tests are shown in the figure). (D) The mean corrected stripe number was significantly higher in the 15-week WT corneas than the 30-week WT or PAX77Tg^−/− group (2-way ANOVA P<0.0001, results of relevant post-hoc tests are shown in the figure). For each comparison there were 14–36 eyes per group: 22 15-week WT, 36 30-week WT, 14 15-week PAX77Tg^−/− and 20 30-week PAX77Tg^−/−. Significant P-values for Tukey’s HSD post-hoc tests are shown: ns = not significant; *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. For all post-hoc tests see Tables S1, S2, S3, S4.

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Figure 7. Representative images of β-gal staining in the corneal epithelia of X-inactivation mosaic eyes expressing different levels of Pax6. (A) WT (PAX77^+/+, Pax6^+/+, XLacZ^+/+) eyes exhibit ordered radial stripes of clonally related epithelial cells. (B) PAX77^−/−, Pax6^−/−, XLacZ^−/− eyes are smaller and stripping patterns are disrupted, normal radial stripes are only rarely observed. (C) In eyes over-expressing Pax6 (PAX77^+/+, Pax6^+/+, XLacZ^+/+) the corneal epithelial diameter is smaller in comparison to the overall eye size (microcornea) but normal radial stripe patterns are visible. (D) PAX77^+/+, Pax6^+/+, XLacZ^+/+ corneas appear normal both in size and stripe phenotype. Scale bar = 1 mm.

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Materials and Methods

Consumables were purchased from Sigma (Poole, UK) and procedures carried out at room temperature, unless stated otherwise.

Ethics statement

All animal work was approved by a University of Edinburgh internal ethics committee and was performed in accordance with institutional guidelines under license by the UK Home Office (project license number PPL 60/3635).

Animals and genetic crosses

Mice were maintained in animal facilities of the College of Medicine and Veterinary Medicine, University of Edinburgh. Heterozygous Pax6^+/+ X LacZ mice (abbreviated to Pax6+/+) and wild-type (WT, Pax6^+/+) littermates were produced from Pax6^+/+ female × Pax6^+/− male crosses on a CBA/Ca genetic background and genotyped by PCR as described previously [3]. Heterozygous Pax6^+/+ XLacZ mice, on a mixed genetic background, were provided by Prof. Ian Jackson and Dr Sally Cross (MRC, Human Genetics Unit, Edinburgh) and maintained as a closed, random-bred colony by crossing Pax6^+/+ XLacZ and WT mice within the colony. Outbred CD-1 mice carrying the Pax77 transgene which expresses 5–7 copies of the human Pax6 gene [8] were provided by Professor Veronica van Heyningen and Dr Dirk A. Kleinjan (MRC Human Genetics Unit, Edinburgh) and the transgene was transferred to the inbred CBA/Ca strain by genetic crosses as reported previously [55]. In the present study we designated mice hemizygous for the Pax77 transgene as Pax77^+/− (because the use of ‘Tg’ to designate presence of the transgene is less ambiguous than ‘+’ used in our previous ‘Pax77^+/−’ notation [55]) and we designate non-transgenic littermates as Pax77^−/−. The founder colony is designated CD1-Pax77^+/− and was maintained by CD1 × CD1-Pax77^+/− crosses. The derived congenic stock is...
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designated CBA-PAX77Tg and was maintained by CBA/Ca × CBA-PAX77Tg crosses. Hemizygous PAX77Tg mice and WT, PAX77Tg littersmates were genotyped by PCR as described previously [55]. No homozygous PAX77Tg transgenic mice were used in this study.

H253 strain mice [65], ubiquitously expressing the Tg(Hmgcr-lacZ)$^{H253Sest}$, X-linked nLacZ transgene (abbreviated to XLacZ), were obtained from the MRC Mammalian Genetics Unit, Harwell, UK, as strain FTH, and maintained on a genetic background that was predominantly a mixture of C57BL/6 and CBA/Ca inbred strains. Males and females hemizygous for this X-linked transgene are designated respectively XLacZ$^{TLac}$ and XLacZ$^{SLac}$; female homozygotes are designated XLacZ$^{TLac/SLac}$. X-inactivation mosaics hemizygous for the Pax6$^{Sey-Neu}$ null mutation and the Pax6$^{Sey-Neu}$ missense mutation, plus WT littermate controls, were produced from Pax6$^{Sey-Neu}$ female × XLacZ$^{TLac}$ male and Pax6$^{Sey-Neu}$ female × XLacZ$^{SLac}$ male crosses respectively.

In a preliminary experiment, PAX77Tg, XLacZ$^{TLac}$ and WT control PAX77Tg, XLacZ$^{TLac}$ X-inactivation mosaic females were produced using the original CD1-PAX77Tg stock in XLacZ$^{TLac}$ female × CD1-PAX77Tg male and CD1-PAX77Tg female × XLacZ$^{SLac}$ male crosses. Once the PAX77Tg transgene had been bred onto the CBA/Ca genetic background, female PAX77Tg, XLacZ$^{TLac}$ and PAX77Tg, XLacZ$^{SLac}$ littersmates were produced from crosses between CBA-PAX77Tg females and hemizygous XLacZ$^{TLac}$ and XLacZ$^{SLac}$ males for a second experiment. In a third experiment, crosses between Pax6$^{Sey-Neu}$ females and hemizygous CBA-PAX77Tg, XLacZ$^{TLac}$ males were used to produce 4 types of XLacZ$^{TLac}$ X-inactivation mosaic females: (1) PAX77Tg, Pax6$^{Sey-Neu}$, XLacZ$^{TLac}$, (2) PAX77Tg, Pax6$^{Sey-Neu}$, XLacZ$^{STac}$, (3) PAX77Tg, Pax6$^{Sey}$, XLacZ$^{TLac}$, and (4) PAX77Tg, Pax6$^{Sey}$, XLacZ$^{SLac}$.

Electron microscopy

Eyes from adult (8–22 weeks old) Pax6$^{Sey-Neu}$ (Pax6$^{Sey}$) and WT (Pax6$^{Sey}$) littersmates plus PAX77Tg and WT (PAX77Tg) littersmates, all on a CBA/Ca genetic background, were enucleated and fixed in 2.5% (w/v) glutaraldehyde in 0.1 M sodium cacodylate buffer prior to processing for scanning electron microscopy as described elsewhere [59]. Whole eyes were washed three times in cacodylate buffer for 15 minutes. Samples were
post-fixed in 2% (w/v) osmium tetroxide for 3 hours and washed again in cacodylate buffer before being passed through a graded ethanol series.

For scanning electron microscopy (SEM), samples were transferred to propylene oxide twice for 20 minutes each time. They were placed in a solution containing 50% propylene oxide and 50% Araldite resin (Agar Scientific, UK) overnight, after which they were transferred to 100% resin and infiltrated overnight under agitation. The samples were embedded in moulds containing fresh resin and polymerised at 60°C for 24–36 hours. Ultra-thin sections (50–70 nanometres thick) were cut on a Leica Ultracut E microtome, collected on naked copper grids and counterstained for 1 hour each with 1% vanadyl sulphate and phosphotungstic acid and then 15 minutes with Reynolds’ lead citrate prior to examination on a JEOL JSM 5600 scanning electron microscope.

Clonal analysis of X-inactivation striping patterns

X-gal staining of XLacZTg/+ eyes and the acquisition of images have been described previously [19,21]. Striping patterns were analysed automatically as described in Mort [66]. Photographs of eyes were taken so that the entire cornea was visible and were then cropped to the edge of the corneal epithelium and analysed using ImageJ, a freeware software package designed by Wayne Rasband for the National Institute of Health (NIH), USA (http://rsb.info.nih.gov/ij/). The observed number of radial stripes in the corneal epithelium was corrected for the probability that stripes would contain multiple adjacent β-gal-positive corneal epithelial clones. This involved dividing the observed mean width by the function 1/(1−p), where p is the proportion of β-gal-positive cells around the circumference as described previously [19–21]. The corrected stripe number provides an estimate of the total number of active corneal epithelial coherent clones (both β-gal positive and β-gal negative) per circumference. This is useful for comparing numbers of active clones of stem cells between different groups but because the number of stem cells per coherent clone may vary it is not a direct estimate of the number of active stem cells. For the preliminary experiment mosaic corneal patterns were analysed manually at 15 weeks using Adobe Photoshop software as described previously [19]. For later mosaic analyses performed at both 15 and 30 weeks, the ImageJ plug-in ‘Clonal Tools’ [66] was used in batch mode to analyse all the images automatically. Where correction for the actual circumference was required this was calculated by dividing the number of corrected stripes by the circumference of each eye measured using ImageJ.

Histology

Whole eyes dissected at 15 and 30 weeks after birth were fixed and stained for β-gal reporter activity using X-gal as described previously [19,21]. X-gal stained eyes were embedded in paraffin wax and 7 μm sections were cut on a microtome, mounted on standard microscope slides and counterstained with eosin and neutral red as described previously [21].

Statistics

2-way ANOVAs and Tukey’s HSD post-hoc tests were calculated using R statistical software (http://www.r-project.org/). Student’s t-tests were calculated using Microsoft Excel.

Supporting Information

Table S1 Multiple comparisons of PAX77Tg/+ and PAX77−/− eyes mass. (See Fig. 6A.) (PDF)

Table S2 Multiple comparisons of PAX77Tg/+ and PAX77−/− corneal circumference. (See Fig. 6B.) (PDF)

Table S3 Multiple comparisons of PAX77Tg/+ and PAX77−/− corrected stripe number. (See Fig. 6C.) (PDF)

Table S4 Multiple comparisons of PAX77Tg/+ and PAX77−/− corrected stripe number per mm circumference. (See Fig. 6C.) (PDF)

Table S5 Multiple comparisons of WT, Pax6+/+, PAX77Tg/+ and Pax6−/− PAX77Tg−/− corneal circumference. (See Fig. 8A.) (PDF)

Table S6 Multiple comparisons of WT, Pax6+/+, PAX77Tg/+ and Pax6−/− PAX77Tg−/− corrected stripe number. (See Fig. 8B.) (PDF)

Table S7 Multiple comparisons of WT, Pax6+/+, PAX77Tg/+ and Pax6−/− PAX77Tg−/− corrected stripe number per mm circumference. (See Fig. 8C.) (PDF)

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Author Contributions

Conceived and designed the experiments: JW RM JC NF SM RH. Performed the experiments: RM AB FM JC PD. Analyzed the data: RM JC. Contributed reagents/materials/analysis tools: JW RM NF. Wrote the paper: RM JW.

References


