A conditional Pax6 depletion study with no morphological effect on the adult mouse corneal epithelium

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Abstract

Objective: The corneas of heterozygous Pax6+/− mice develop abnormally and deteriorate further after birth but it is not known whether the postnatal deterioration is predetermined by abnormal development. Our objective was to identify whether depletion of Pax6 in adult mice caused any corneal abnormalities, similar to those in Pax6+/− mice, where Pax6 levels are low throughout development and adulthood. We used two tamoxifen-inducible, Cre-loxP experimental strategies to deplete Pax6 either ubiquitously or in a restricted range of cell types.

Results: In a preliminary study, ubiquitous depletion of Pax6 by tamoxifen treatment of E9.5 CAG-CreERT2/−;Pax6fl/fl embryos affected eye development. Tamoxifen treatment of 12-week old, adult CAG-CreERT2/−;Pax6fl/+ and CAG-CreERT2/−;Pax6fl/fl mice resulted in weak and/or patchy Pax6 immunostaining in the corneal epithelium but caused no corneal abnormalities. GFP staining in tamoxifen-treated CAG-CreERT2/−;RCE:loxP reporter mice was also patchy. We attribute patchy Pax6 staining to mosaic deletion of the Pax6fl allele, probably caused by mosaic CAG-CreERT2 expression. In a parallel study, we treated adult Krt19-CreERT2/−;Pax6fl/+ mice with tamoxifen to try to deplete Pax6 in limbal epithelial stem cells (LESCs) which replenish the corneal epithelium. However, Pax6 staining remained strong after a 12-week chase period so the Krt19-CreERT2 transgene may have failed to target LESC.

Keywords: Cornea, Corneal epithelium, Cre-loxP, CAG-CreERT2, Krt19-CreERT2, Pax6, Mouse, Mosaic transgene expression

Introduction

The mouse cornea comprises an outer epithelium of 5–6 cell layers, a thick stroma and an inner endothelium. The corneal epithelium is maintained by limbal epithelial stem cells (LESCs), in the ring-shaped limbus, which is a transition zone between the corneal epithelium and the conjunctiva. The LESCs replace themselves and produce transient (or transit) amplifying cells (TACs) that move centripetally across the basal corneal epithelium and produce more differentiated daughter cells, which move apically, through the epithelial layers, and are shed from the surface [1].

The Pax6 gene, encoding the Pax6 transcription factor, is expressed in the brain, pancreas, olfactory system and several eye tissues, including the corneal and limbal epithelia, and is critical for eye development [2]. Low levels of Pax6 throughout development of heterozygous Pax6+/− mice cause small eyes, aniridia plus lens and corneal defects [3–8]. The newborn Pax6+/− cornea has a thin epithelium and the adult cornea deteriorates further, because the epithelium is poorly maintained and limbal blood vessels invade the stroma [6–8]. The adult Pax6+/− corneal epithelium is thin and fragile, cell turnover is elevated, centripetal movement is disrupted and goblet cells accumulate. The expression of keratin 12 (K12), which is regulated by Pax6 [9], is delayed and immunostaining is weak and patchy [6–8, 10, 11]. Indirect evidence suggests that reduced Pax6 causes LESC deficiency in both...
were bred by crossing CreER expression of GFP were bred by crossing CAG-CreER Esr1*)5Amc) [13] to heterozygous Pax6fl/ mice. We used one experimental strategy to deplete Pax6 ubiquitously and another to deplete Pax6 in LESC.

Main text
Materials and methods
Mice
To deplete Pax6 ubiquitously, CAG-CreERTg/−;Pax6fl/+ mice were produced by crossing hemizygous CAG-CreERTg/− mice (formal transgene name: Tg(CAG-cre/Erz1)5Amc) [13] to heterozygous Pax6fl/+ mice (formal name: Pax6tm1Led/+ ) [14]. Superscript symbols ‘Tg’/−’ and ‘−/−’ are used to distinguish hemizygous CAG-CreERTg/− mice and non-transgenic CAG-CreERTg/−/− siblings.

 Keratin 19 (Krt19 gene; K19 protein) is expressed in the basal epithelium of the mouse limbus and conjunctiva but not the cornea [15]. To try to target LESC in the limbal epithelium, Krt19-CreERTg/−;Pax6fl/+ mice were produced by crossing hemizygous Krt19-CreERTg/− mice (formal transgene name: Krt19tm1(cre/ERT)Ggu ) [16] to heterozygous Pax6fl/+ mice.

RCE:loxP mice have the R26R CAG-boosted EGFP (RCE) reporter allele with an upstream loxP-flanked STOP cassette (formal transgene name: Gt(Rosa)26Sor1tm1.1(CAG-EGFP/FlStop) [17]). CAG-CreERTg/−;RCE:loxP mice with tamoxifen-inducible expression of GFP were bred by crossing CAG-CreERTg/− and RCE:loxP mice. Krt19-CreERTg/−;RCE:loxP mice were bred by crossing Krt19-CreERTg/− and RCE:loxP mice.

Mice were maintained on a predominantly CBA/Ca genetic background and genotyped by PCR [13, 14, 16]. Some additional samples from mice on a CD-1 or (C57BL/6 × CBA/Ca)F1 genetic background from other studies [18, 19], were also analysed.

To activate CreER in adult mice, tamoxifen (Sigma-Aldrich) was freshly prepared in corn oil (25–40 mg/ml) by sonication in a 40 °C water bath and adjusted to 100 μg/g body weight in 0.1 ml. Mice of both sexes were injected intraperitoneally with tamoxifen at 12 weeks on 5 consecutive days and analysed 3 days later (no chase group) or after chase periods of 6 or 12 weeks. Control mice were injected with 0.1 ml corn oil. Mice were cull by cervical dislocation, following overdose of gaseous halothane, and eyes were enucleated. Procedures for the induction of Cre expression in embryos at embryonic day (E) 9.5 and the subsequent collection of E13.5 fetal samples are described elsewhere [19]. Tamoxifen treatment causes CreER to move to the nucleus and recombine loxP sites to convert the functional Pax6floxed allele to a Pax6null allele or express the GFP lineage marker in the target cells and their progeny. This should occur in all cell types in CAG-CreERTg/− mice, because CreER is expressed ubiquitously from the CAG promoter, but only in specific cell types in Krt19-CreERTg/− mice.

Analysis
Tissue samples were fixed in 4% paraformaldehyde overnight at 4 °C. Fetal heads were processed to OCT compound and stored frozen before cryosections were cut and stained with haematoxylin and eosin (H&E) [19, 20]. Adult eyes were processed to paraffin wax, then 7 μm sections were cut, mounted on glass slides and stained with H&E or periodic acid-Schiff (PAS) stain [20]. Stained sections were photographed and measured using a Zeiss Axioplan-2 microscope and calibrated Zeiss Axiovision 4.8 digital camera system. Numerical data are included in Additional file 1 and measurements were compared by Student’s t-test.

Immunohistochemistry methods are described elsewhere [20]. Briefly, wax sections, mounted on glass slides were heat-treated to unmask antigens, then incubated with blocking serum, followed by primary antibody, biotinylated secondary antibody, avidin–biotin reagent and 3,3′-diaminobenzidine (DAB) stain. Sections were then lightly counterstained with haematoxylin, dehydrated and slides were mounted with DPX mounting medium under coverslips. Negative control slides were treated with blocking serum instead of primary antibody. The antibodies used for Pax6 and K12 immunostaining were as described elsewhere [20], except that the secondary antibody was biotinylated rabbit anti-mouse, diluted 1:200 (E0433 from Dako, Ely, UK). For GFP immunostaining, the primary antibody was rabbit anti-GFP diluted 1:500 (ab290 from Abcam, Cambridge, UK) and the secondary antibody was biotinylated goat anti-rabbit, diluted 1:200 (Sc-2012 from SantaCruz Biotechnology, Heidelberg, Germany).

Results
Ubiquitous depletion of Pax6 in embryos
For another study, CAG-CreERTg/−;Pax6fl/+ and CAG-CreERTg/−;Pax6fl/+ embryos were exposed to tamoxifen at E9.5 and culled at E13.5 [19]. By E13.5 tamoxifen-treated CAG-CreERTg/−;Pax6fl/+ fetuses (with two floxed Pax6 alleles) had smaller eyes and lenses than CAG-CreERTg/−;Pax6floxed/+ fetuses (Additional file 1 and Additional file 2: Fig. S1). This showed that tamoxifen-mediated depletion of Pax6 could affect eye development.
Ubiquitous depletion of Pax6 in adults

The preliminary result with fetal eyes encouraged us to investigate whether tamoxifen-mediated, depletion of Pax6 in adults caused any corneal abnormalities, similar to those in Pax6<sup>+/−</sup> mice, where Pax6 is low throughout development. Pax6-depletion in adults is unlikely to reproduce Pax6<sup>+/−</sup> developmental defects but adult corneal deterioration could be mediated via Pax6-deficiency in adult LESCs, the LESC niche, the corneal epithelium or other ocular tissues [5, 10]. We compared the effects of tamoxifen treatment of CAG-CreER<sup>Tg/−;Pax6fl/+</sup> mice to several genotype and treatment controls, which were included to control for any unexpected effects of the CAG-CreER transgene or the floxed Pax6<sup>fl</sup> allele alone [20–24].

Following tamoxifen treatment at 12 weeks and a 6-week chase, Pax6 immunostaining was positive in the corneal epithelia of all the control combinations (Fig. 1a–g). Although immunohistochemistry was not quantified, Pax6 appeared to be weak and/or patchy in the corneal epithelia of CAG-CreER<sup>Tg/−;Pax6fl/+</sup> mice, treated with tamoxifen as adults (Fig. 1h–l). However, eye and corneal morphology appeared grossly normal (apart from some processing artefacts), with no blood vessels visible in the cornea (Fig. 2). PAS-positive goblet cells were not detected in the corneal epithelium (data not shown) and there was little or no effect on K12 staining (Fig. 1m–t). Similar results were obtained after a 12-week chase and, again, corneal morphology appeared grossly normal (Fig. 3). For comparison, previously published corneal histology and immunostaining are shown for wild-type Pax6<sup>+/+</sup> and heterozygous Pax6<sup>+/−</sup> eyes in Additional file 2: Fig. S2. GFP immunostaining of eyes from tamoxifen-treated CAG-CreER<sup>Tg/−;RCE:loxP</sup> reporter mice showed mosaic expression in the corneal epithelium (Additional file 2: Fig. S3).

We did not include CAG-CreER<sup>Tg/−;Pax6fl/fl</sup> mice, with two floxed Pax6<sup>fl</sup> alleles, in the main study because...
severe, global depletion of Pax6 in these mice results in diabetes [18]. However, Pax6 immunostaining of corneas from CAG-CreER$^{Tg/-}$;Pax6$^{fl/+}$ mice, produced for another study [18], showed that Pax6 protein was not eliminated following tamoxifen treatment and a 6-week chase period (Additional file 2: Fig. S4).

**Targeting Pax6-depletion to LESCs**

To try to deplete Pax6 in LESCs we treated Krt19-CreER$^{Tg/-}$;Pax6$^{fl/+}$ mice with tamoxifen at 12 weeks and analysed the results after a 12-week chase period, to allow sufficient time for treated LESCs to replenish the corneal epithelium. Pax6 and K12 staining appeared normal and no corneal morphological abnormalities were seen (Additional file 2: Fig. S5). Tamoxifen-treated Krt19-CreER$^{Tg/-}$;RCE:loxP reporter mice produced patchy GFP reporter immunostaining in the conjunctiva, some sparse staining in the limbus but no staining in the corneal epithelium (Additional file 2: Fig. S6).

**Discussion**

Tamoxifen treatment to deplete Pax6 in E9.5 CAG-CreER$^{Tg/-}$;Pax6$^{fl/+}$ embryos affected eye morphology by E13.5 but did not prevent lens development. The lens...
placode forms around E9.5 and is absent in Pax6<sup>−/−</sup> homozygotes [25], so E9.5 tamoxifen-treatment was probably too late to prevent lens development in the CAG-CreER<sup>Tg−/−</sup>;Pax6<sup>fl/−</sup> embryos but we did not investigate whether any Pax6 remained at E13.5.

As tamoxifen treatment of Krt19-CreER<sup>Tg−/−</sup>;RCE:loxP mice did not produce any GFP-positive corneal epithelial cells after a 12-week chase, it is likely that this strategy failed to target LESCs. With hindsight, the Krt14-Cre-ER<sup>Tg−/−</sup> mouse may have been more suitable for targeting expression to LESCs, as this has been successful in lineage tracing experiments [26, 27].

The normal corneal morphology of the tamoxifen-treated adult CAG-CreER<sup>Tg−/−</sup>;Pax6<sup>fl/−</sup> mice is consistent with the possibility that normal Pax6 levels are not required in the adult to maintain the corneal epithelium. This would suggest that deterioration of the adult Pax6<sup>+/−</sup> corneal epithelium was predetermined during development. However, we also need to consider technical explanations for our results, particularly as corneal

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**Fig. 3** Histology and immunohistochemistry of CAG-CreER<sup>Tg−/−</sup>;Pax6<sup>fl/−</sup> tissues after a 12 week chase period. a–d H&E stained sections of whole eyes (a, b) and central cornea (c, d) of corn oil treated controls and tamoxifen treated CAG-CreER<sup>Tg−/−</sup>;Pax6<sup>fl/−</sup> mice, 12 weeks after treatment. e–h Immunostained sections (brown DAB endpoint) for Pax6 (e, f) and keratin 12 (g, h) 12 weeks after treatment. Scale bars: a (for a, b) = 500 μm; c (for c, d); e (for e, f) and g (for g, h) = 50 μm. CO corn oil treatment, Tx tamoxifen treatment.
effects of Pax6-deficiency have now been corrected successfully in adult mice [28, 29].

One possibility is that our investigation was under mined by mosaic deletion of the Pax6 allele. The presence of some Pax6-positive cells in the corneal epithelia of tamoxifen-treated CAG-CreER⁺⁻/−;Pax6Δ/Δ mice suggests that mosaicism occurred in these mice as well as CAG-CreER⁺⁻/−;RCE:loxP reporter mice. We, therefore, suggest that mosaic Pax6 deletion was caused by mosaic CAG-CreER⁺⁻ transgene expression but recombination of Pax6Δ/Δ;loxP sites could also be inefficient for other reasons. Mosaic Pax6Δ/Δ deletion would lead to a mixture of Pax6Δ/Δ; Pax6Δ/Δ and Pax6Δ/Δ cells in CAG-CreERTg⁺⁻;Pax6Δ/Δ mice or a mixture of Pax6Δ/Δ and Pax6Δ/Δ cells in CAG-CreER⁺⁻;Pax6Δ/Δ mice.

The effects of mixtures of wild-type and Pax6Δ/Δ or Pax6Δ/Δ cells in ocular tissues, have been investigated using mouse chimaeras. Eye development was abnormal in Pax6Δ/Δ ↔ Pax6Δ/Δ chimaeras [30–33] but Pax6Δ/Δ ↔ Pax6Δ/Δ chimaeras had normal eyes [5, 10, 33] with normal corneal morphology [10]. It was suggested that unknown signals from wild-type cells in the cornea and/or other ocular tissues might rescue the Pax6Δ/Δ cells [10]. This might also occur in tamoxifen-treated CAG-CreERTg⁺⁻;Pax6Δ/Δ corneas if the conditional Pax6Δ allele is not deleted in all cells.

Although the corneal epithelium of tamoxifen-treated CAG-CreERTg⁺⁻;Pax6Δ/Δ mice contained many Pax6-positive cells (Additional file 2: Fig. S4), no Pax6 protein was detected by immunofluorescence in most pancreatic islet cells in equivalent mice from the same study [18]. Apparent differences in frequencies of Pax6-positive cells between these two tissues may reflect genuine biological differences rather than technical differences in detecting Pax6-positive cells. Mosaic CAG-CreER⁺⁻ expression might be more common and/or recombination of loxP sites less efficient in the corneal epithelium than pancreatic islets, resulting in mosaic Pax6Δ deletion in the cornea.

Mosaic reporter expression also occurred in the corneal epithelium of CAG-CreER;R26R-LacZ and CAG-CreER;R26R-mT/mG reporter mice in a lineage-tracing experiment [34]. This lineage tracing experiment was undertaken after the present study and, with hindsight, it would be worth investigating whether mosaic transgene expression is more common for specific tissues and/or specific CAG-CreER;loxP combinations.

**Limitations**

We evaluated corneal histology, the absence of goblet cells and K12 immunohistochemistry. Future investigations could include additional endpoints and markers.

In lineage-tracing experiments, labelled cells produced by tamoxifen-treated LESCs took at least 14 weeks to replace the whole corneal epithelium [26, 27, 34]. Thus, although our chase time of 12 weeks should have identified corneal defects attributable to Pax6-depletion in LESCs or the niche, it might not have been sufficient to produce the maximum effects.

We did not investigate how effectively Pax6 protein was depleted in other CAG-CreERTg⁺⁻;Pax6Δ/Δ ocular tissues or whether mosaic transgene expression occurred in those tissues. Also, DNA or RNA methods were not used to confirm that at least some floxed Pax6Δ alleles were converted to Pax6Δ in the corneal epithelium.

**Additional files**

Additional file 1. Fetal eye measurements.

Additional file 2: Fig S1. E13.5 fetal eye morphology following tamoxifen treatment at E9.5. Fig. S2. Previously published histology and immunohistochemistry of adult wild-type and heterozygous Pax6Δ/Δ mouse eyes. Fig. S3. Expression of GFP reporter in corneal epithelium of CAG-CreERTg/+; RCE:loxP mice after tamoxifen treatment and different chase periods. Fig. S4. Pax6 immunohistochemistry of CAG-CreERTg/+; Pax6Δ/Δ and CAG-CreERTg⁺⁻;Pax6Δ/Δ corneal epithelia after a 6-week chase period. Fig. S5. Histology and immunohistochemistry of Krt19-CreERTg⁺⁻; Pax6Δ/Δ corneas after a 12 week chase period. Fig. S6. Expression of GFP reporter in corneal epithelium of Pax6Δ/Δ; RCE:loxP mice after tamoxifen treatment and different chase periods.

**Abbreviations**

DAB: 3,3’-diaminobenzidine; DPX: diptyrene plasticizer xylene; E: embryonic day; EGFP: enhanced green fluorescent protein; GFP: green fluorescent protein; H&E: haematoxylin and eosin; K12: keratin 12, K19: keratin 19; LESC: limbal epithelial stem cell; OCT compound: optimal cutting temperature compound; PAS: periodic acid-Schiff; TAC: transient (or transit) amplifying cell.

**Authors’ contributions**

JMD produced and analysed the results, produced the figures and wrote most of the first draft. MM and DJK provided samples, produced in other studies, for evaluation. DJK designed and supervised the study that provided tamoxifen-treated embryos. JMC and REH supervised and designed supervision of the main study. JDW designed and supervised part of the main study. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
The numerical data generated and analysed during this study are included in the additional information files of this published article.

Consent for publication
Not applicable.

Ethics approval and consent to participate
Mice were bred and maintained at the University of Edinburgh, UK. Animal work was approved by the University of Edinburgh Ethical Review Committee and performed in accordance with UK Home Office Regulations under UK Home Office licences PPL 60/3635 and PPL 60/4302. Samples produced in other studies were from mice regulated by UK Home Office licences PPL 60/3785 and PPL 60/3913.

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