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# FoxC1 activates limbal epithelial stem cells following corneal epithelial debridement

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## Abstract

Limbal epithelial stem cells are not only critical for corneal epithelial homeostasis but also have the capacity to change from a relatively quiescent mitotic phenotype to a rapidly proliferating cell in response to population depletion following corneal epithelial wounding. Pax6+/− mice display many abnormalities including corneal vascularization and these aberrations are consistent with a limbal stem cell deficiency (LSCD)phenotype . FoxC1 has an inhibitory effect on corneal avascularity and a positive role in stem cell maintenance in many tissues. However, the role of FoxC1 in limbal epithelial stem cells remains unknown. To unravel FoxC1's role(s) in limbal epithelial stem cell homeostasis, we utilized an adeno-associated virus (AAV) vector to topically deliver human FOXC1 proteins into Pax6+/− mouse limbal epithelium. Under unperturbed conditions, overexpression of FOXC1 in the limbal epithelium had little significant change in differentiation (PAI2, Krt12) and proliferation (BrdU, Ki67). Conversely, such overexpression resulted in a marked increase in the expression of putative limbal epithelial stem cell markers, N-cadherin and Lrig1. After corneal injuries in Pax6+/− mice, FOXC1 overexpression enhanced the behavior of limbal epithelial stem cells from quiescence to a highly proliferative status.

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Declaration of competing interest

All the authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Overall, the treatment of AAV8-FOXC1 may be beneficial to the function of limbal epithelial stem cells in the context of a deficiency of Pax6 function.

**Keywords**

limbal stem cell deficiency; Pax6; corneal wounding; proliferation; FoxC1

The corneal epithelium is a self-renewing tissue, thus a balance between cell loss and cell renewal is required for homeostasis. Self-renewing tissues are, by definition, governed by stem cells. Corneal epithelial homeostasis is maintained by a population of epithelial stem cells preferentially located in the basal layer of the limbal epithelium. These cells: (i) rarely cycle (detected as label-retaining cells); (ii) have vast proliferative capacity; (iii) have a large nuclear:cytoplasmic ratio; (iv) are relatively biochemically primitive; (v) contain specific genes (e.g., ABCB5, TSPAN7, ATF3) that regulate stem cell proliferative status; and, (vi) express putative limbal epithelial stem cell markers (e.g., N-cadherin, Lrig1) (Hayashi et al., 2007; Kaplan et al., 2019). Alterations in the limbal epithelial stem cell population result in profound negative effects on the corneal epithelium. For example, when limbal stem cells are compromised, delayed or incomplete corneal epithelial regeneration following corneal wounding occurs. A persistent corneal epithelial defect is another consequence of altered limbal stem cell physiology. When the limbal stem cell niche becomes compromised a myriad of abnormalities occur, including the appearance of goblet cell clusters in the peripheral and central corneal epithelium (conjunctivalization), corneal opacification, inflammation, corneal angiogenesis, and eventual scarring (Dong et al., 2018; Stepp and Zieske, 2005). Collectively this condition is known as limbal stem cell deficiency (LSCD).

Corneal angiogenesis is a major contributing factor to LSCD (Le et al., 2018; Sejpal et al., 2013; Tseng, 1989). FoxC1 is a member of the large Forkhead box transcription factor family, which regulate an array of fundamental processes, including cell fate determination, proliferation, and differentiation. Interestingly, some family members, including FoxC1, have recently been implicated in controlling corneal vascularity. Specifically, FoxC1 expression in the corneal stroma is essential for the establishment and maintenance of corneal avascularity during development and in postnatal life (Seo et al., 2012). Interestingly, overexpression of FOXC1 increases the proportion of cells expressing putative cancer stem cell markers (such as breast cancer, non-small cell lung cancer) (Gilding and Somervaille, 2019). Such an increase in the cancer stem cell-like population is associated with enhanced cancer cell proliferation and survival (Gilding and Somervaille, 2019). More importantly, it has been predicted that N-cadherin and Lrig1, two putative limbal epithelial stem cell markers, are target genes of FoxC1 (Rouillard et al., 2016). Downregulation of FoxC1 in limbal epithelium reduces Pax6(Li et al., 2021), a key regulator for corneal epithelial homeostasis(Ramaesh et al., 2003). However, the role of FoxC1 in limbal epithelial stem cells remains unknown.

To further our knowledge on the etiology and treatment of LSCD, an experimental model would be of great utility. To this end, it has been reported that corneal epithelial homeostasis

is abnormal in Pax6$^{+/-}$ mice (Ramaesh et al., 2003). There is an imbalance in cell gain vs cell loss, the corneal epithelium is thinner due to reduced layers, goblet cells are present in the corneal epithelium, and these mice have corneal angiogenesis(Ramaesh et al., 2003). This constellation of abnormalities suggests a phenotype consistent with LSCD. Since FoxC1 contributes to corneal avascularity, and Pax6$^{+/-}$ mice display aberrant corneal angiogenesis, we reasoned that FoxC1 might be a unique treatment in controlling corneal angiogenesis as well as LSCD in these mice.

FoxC1 expression was elevated in Pax6+/− mouse limbal epithelium compared to wild type (WT) (Fig. 1A). This may suggest a compensation effect. Since FoxC1 positively regulates Pax6 expression in limbal epithelial cells(Li et al., 2021), there is a negative feedback regulatory loop between FoxC1 and Pax6. This negative feedback loop can limit the expression level of such compensatory induction of FoxC1 and thus such limited induction of FoxC1 expression doesn't restore the LSCD in Pax6+/− mice. Thus, we determined whether AAV8-FOXC1 treatment could suppress the abnormal formation of the ocular epithelium in Pax6$^{+/-}$ mice since FOXC1 expression in AAV8 vector is not regulated by Pax6. To facilitate delivery of the adeno-associated virus 8 (AAV8) to the corneal stroma, the corneal epithelium was removed by debridement from 21-day-old Pax6$^{+/-}$ and wild-type mice. The corneal stroma was treated by either AAV8-GFP (control eye) or AAV8-FOXC1 (U.S. Patent Number: 11,001,618) (infected eye) at a dosage of [1 X $10^9$μg/ul]. Previous studies demonstrated that this dosage resulted in an effective infection into the corneal stroma by an AAV8 drop delivery method (Sharma et al., 2010). At 14 days post-infection, eyes were harvested and analyzed by immunohistochemistry for FOXC1 expression. AAV-FOXC1 treatment markedly increased FOXC1 in Pax6+/− limbal epithelium compared to AAV-GFP treated Pax6+/− mice (Fig. 1A). Initially, we investigated whether FOXC1 overexpression would affect proliferation and/or differentiation in Pax6$^{+/-}$ mice(Hill et al., 1991) compared to wild-type mice. We evaluated expression patterns of differentiation (PAI2, Krt12), proliferation (BrdU, Ki67) and stem cell markers (N-cadherin, Lrig1) in Pax6$^{+/-}$ mice. Interestingly, there was not a significant difference in the expression levels of the differentiation markers in corneal and limbal epithelia between AAV-GFP (control) and AAV-FOXC1 infected Pax6$^{+/-}$ mice (Figs. 1 D, E). We also found no significant change in Ki67+ and BrdU+ cells (Figs. 1 F, G) in the limbal epithelium of Pax6$^{+/-}$ mice between AAV-GFP and AAV-FOXC1 infected eyes. However, we did observe a marked increase in the expression of putative limbal epithelial stem cell markers, N-cadherin and Lrig1 in AAV-FOXC1 infected Pax6$^{+/-}$ limbus (Figs. 1 B, C). Such an increase in the expression of putative stem cell markers in Pax6+/− mouse limbal epithelium is consistent with the in vitro observations showing that knockdown of FoxC1 in both submerged and 3D cultures of limbal epithelial cells decreased the expression of putative stem cell markers (such as KRT15, GPHA2 and CDH2) (GSE155773) (Li et al., 2021). Collectively, these observations indicate that while FOXC1 overexpression in the Pax6$^{+/-}$ mice may not markedly affect the proliferative or differentiative status of the corneal epithelium, but such overexpression may be beneficial in the preservation of limbal epithelial stem cell properties.

One of the salient features of limbal epithelial stem cells is their capacity to change from a relatively quiescent mitotic phenotype to a rapidly proliferating cell in response to population depletion due to corneal epithelial wounding. In this scenario, limbal epithelial

stem cells give rise to numerous transient amplifying (TA) cells, which migrate centripetally to re-epithelialize the corneal surface (Cotsarelis et al., 1989; Lehrer et al., 1998). Thus, to test whether FoxC1 plays a positive role in activating limbal epithelial stem cells, we examined whether FOXC1 overexpression could enhance the activation of limbal epithelial stem cells following central corneal epithelial debridement in $Pax6^{+/-}$ mice. Two weeks after being transduced with either AAV8-GFP or AAV8-FOXC1, the central corneal epithelium of the $Pax6^{+/-}$ mice was removed using a diamond burr. Twenty-four hours post-wounding, mice received an I.P. injection of BrdU (50 mg/kg). One hour later, mouse eyes were harvested, fixed in formalin, embedded in paraffin blocks, sectioned, and processed for the detection of BrdU+ cells. Immunostaining for BrdU revealed that FOXC1 overexpression slightly increased the number of BrdU+ cells in the limbal epithelium of FOXC1-treated wild type (WT) mice (Figs. 2A, B, E) compared to GFP-treated WT but such an increase was not statistically significant. The limbal epithelium of the $Pax6^{+/-}$-GFP-treated mice contained few BrdU positive cells (Figs. 2C, E), whereas the $Pax6^{+/-}$-FOXC1-treated mice had a marked increase in BrdU+ cells (Figs. 2D, E). These findings clearly indicate that overexpression of FOXC1 in Pax6+/− mouse eyes, enhances the response of limbal epithelial stem cells to wounding and corroborates the immunohistochemical findings suggesting that FOXC1 overexpression may restore the function of limbal epithelial stem cells in the LSCD mouse limbus. FOXC1 has been shown to play a role in stem cell maintenance in other tissues. For example, FOXC1 plays a significantly positive role in the self-renewal of multipotent arachnoid-pia stem cells (APSCs) and contributes to embryonic and adult neurogenesis(Lee et al., 2019). In endothelial cells, overexpression of FoxC1 promoted survival and neovascularization of mesenchymal stem cells (MSCs) under hypoxic coculture conditions(Ji et al., 2021). In hair follicle stem cells (HFSC), loss of FoxC1 resulted in the disruption of stem cell quiescence and aberrant proliferation during hair cycles (Wang et al., 2016).

Our findings indicate that after corneal injuries in Pax6+/− mice, FOXC1 overexpression via AAV8 enhances the response of limbal epithelial stem cells (i.e., proliferation) to wounding. Overall, the treatment of AAV8-FOXC1 may be beneficial to patients with aniridia, a disease involving Pax6.

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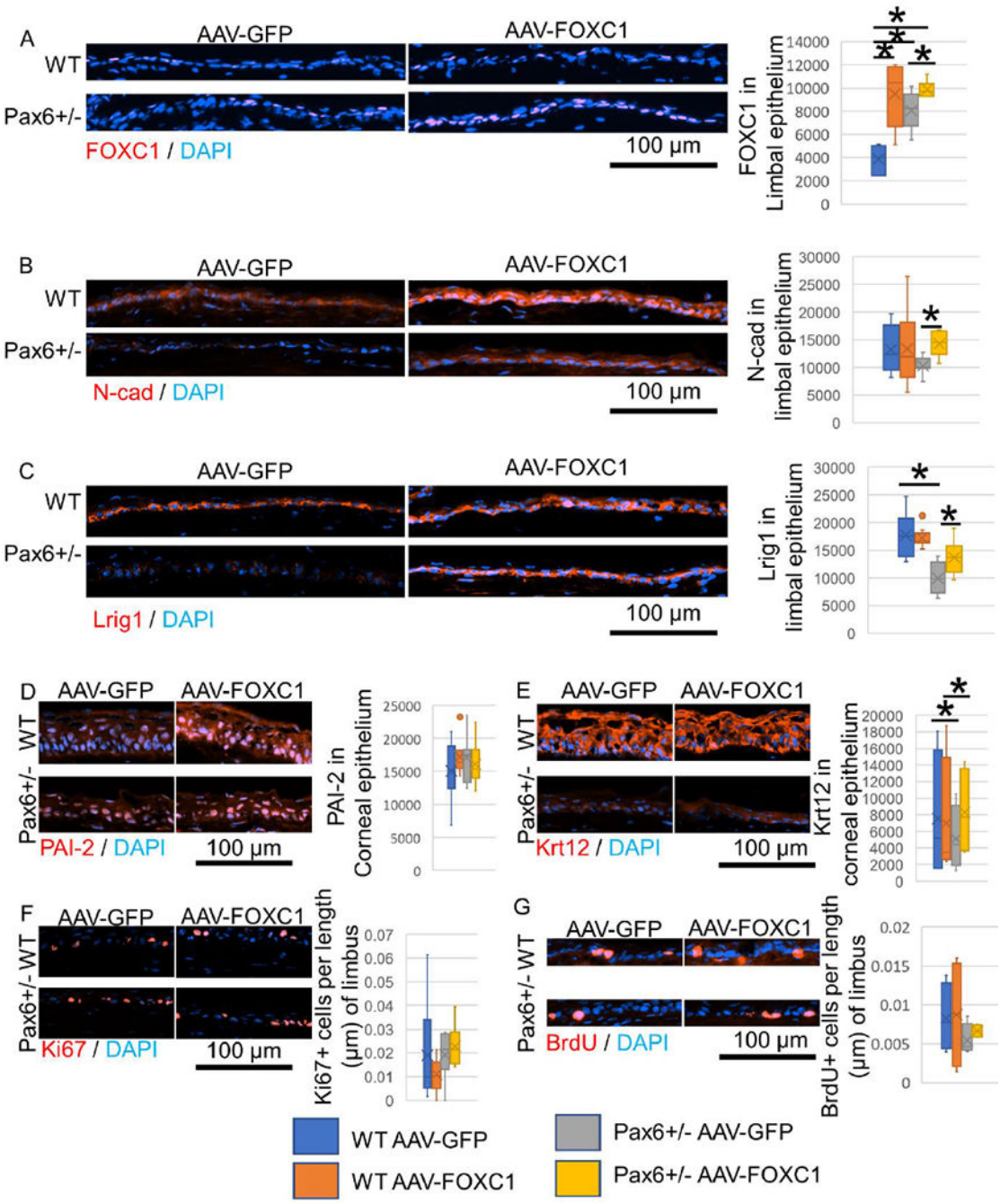


**Figure 1.**

Delivery of Foxc1 in limbal epithelium of Pax6+/− mice using AAV increases the expression of stem cell markers. (A-G) 21-day-old mice were infected with AAV-Foxc1 or AAV empty vector (control). Fourteen days after infection, mice received an I.P. injection of BrdU (50 mg/kg). One hour later, mouse eyes were harvested and subjected to immunostaining for Foxc1 (A), N-cadherin (N-cad, B), Lrig1 (C), PAI-2 (D), Krt12 (E), Ki67 (F), BrdU (G). Quantification of the relative fluorescent intensity was conducted using ImageJ. n = 12; *, $p < 0.05$.

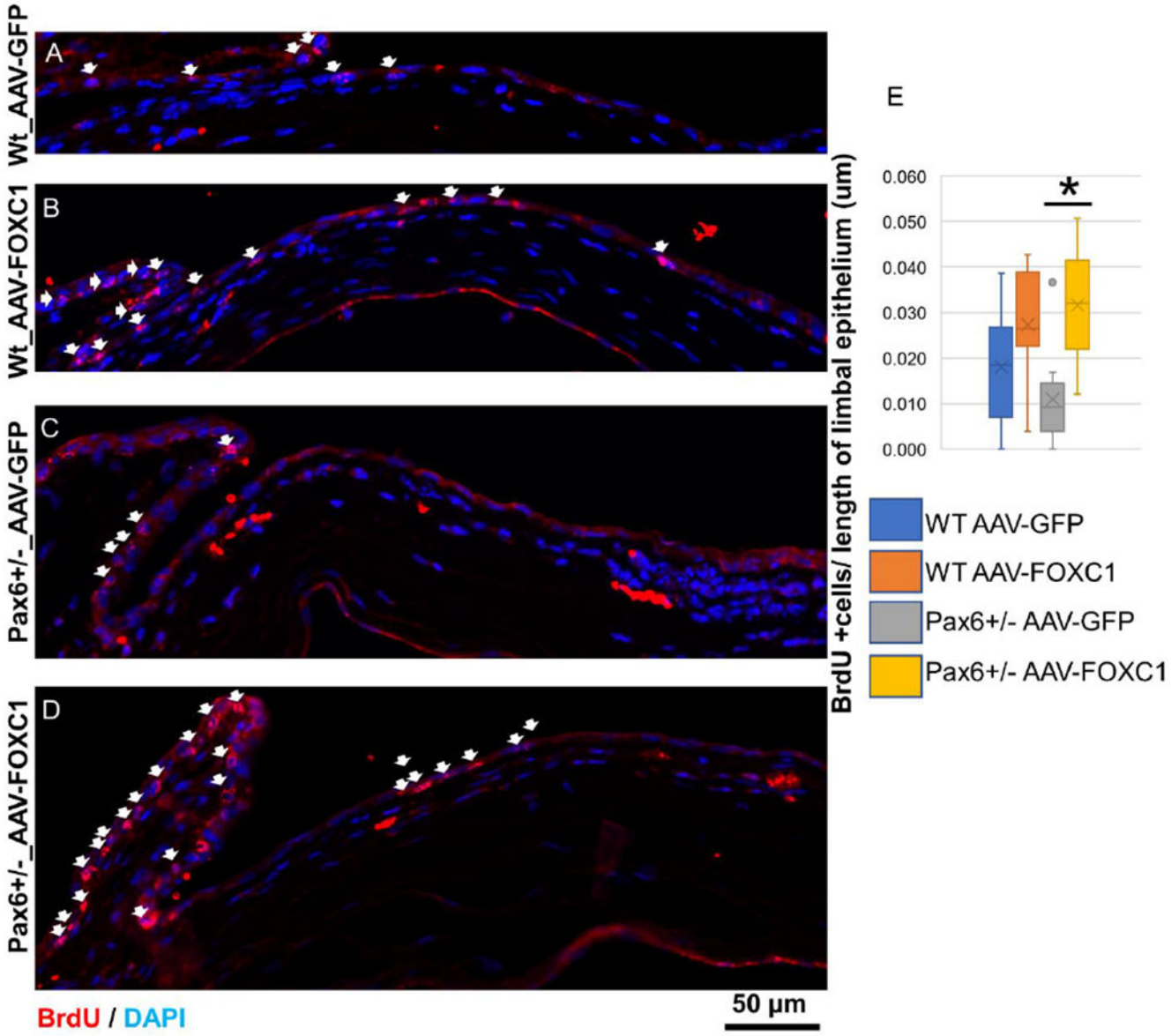


**Figure 2.**

Delivery of Foxc1 in limbal epithelium of Pax6+/− mice using AAV promotes the response of limbal epithelial stem cells to wounding. Twenty-one-day old mice were infected with AAV-Foxc1 or AAV-GFP (control). Fourteen days after infection, mice were subjected to debridement wounding in the central corneal epithelium using a diamond burr. Twenty-four hours later, mice received an I.P. injection of BrdU (50 mg/kg). One hour later, mouse eyes were harvested and subjected to immunostaining for BrdU. Quantification of the BrdU+ cells was conducted using ImageJ (L). n = 8; *, $p < 0.05$.