KLF4 Plays an Essential Role in Corneal Epithelial Homeostasis by Promoting Epithelial Cell Fate and Suppressing Epithelial–Mesenchymal Transition

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Purpose. The purpose of this study was to test the hypothesis that KLF4 promotes corneal epithelial (CE) cell fate by suppressing the epithelial–mesenchymal transition (EMT), using spatiotemporally regulated CE-specific ablation of *Klf4* in $Klf4^{\Delta/\Delta CE}$ ($Klf4^{LoxP/LoxP}$/$Krt12^{rtTA/rtTA}$/*Tet-O-Cre*) mice.

Methods. CE-specific ablation of *Klf4* was achieved by feeding $Klf4^{\Delta/\Delta CE}$ mice with doxycycline chow. The wild-type (WT; normal chow-fed littermates) and the $Klf4^{\Delta/\Delta CE}$ histology was compared by hematoxylin and eosin–stained sections; EMT marker expression was quantified by quantitative PCR, immunoblots, and immunofluorescent staining; and wound healing rate was measured by CE debridement using Algerbrush. KLF4 and EMT markers were quantified in human corneal limbal epithelial (HCLE) cells undergoing TGF-β1–induced EMT by quantitative PCR, immunoblots, and immunofluorescent staining.

Results. The epithelial markers E-cadherin, Krt12, claudin-3, and claudin-4 were down-regulated, whereas the mesenchymal markers vimentin, β-catenin, survivin, and cyclin-D1 and the EMT transcription factors Snail, Slug, Twist1, Twist2, Zeb1, and Zeb2 were up-regulated in the $Klf4^{\Delta/\Delta CE}$ corneas. The $Klf4^{\Delta/\Delta CE}$ cells migrated faster, filling 93% of the debrided area within 16 hours compared with 61% in the WT. After 7 days of wounding, the $Klf4^{\Delta/\Delta CE}$ cells that filled the gap failed to regain epithelial characteristics, as they displayed abnormal stratification; down-regulation of E-cadherin and Krt12; up-regulation of β-catenin, survivin, and cyclin-D1; and a 2.5-fold increase in the number of proliferative $Ki67^{+}$ cells. WT CE cells at the migrating edge and the HCLE cells undergoing TGF-β1–induced EMT displayed significant down-regulation of KLF4.

Conclusions. Collectively, these results reveal that KLF4 plays an essential role in CE homeostasis by promoting epithelial cell fate and suppressing EMT.

Keywords: KLF4, epithelial–mesenchymal transition, E-cadherin, β-catenin, survivin, cyclin-D1

Epithelial cells derived from the ectoderm during embryonic development line the outer layer of the body and internal organs, serving an important barrier function. Unique properties of epithelial cells depend on their gene expression profile that is regulated by a set of epithelium-enriched transcription factor families including E26 transformation-specific (ETS), p63, activating protein-2 (AP2), and krüppel-like factors (KLFs).[1–4] Disruption of epithelial gene expression pattern results in severe abnormalities including epithelial–mesenchymal transition (EMT), a process by which sedentary epithelial cells transition to more motile, invasive mesenchymal cells.[5,6] Epithelial cells undergoing EMT are transcriptionally reprogrammed to undergo morphologic and molecular changes toward a mesenchymal phenotype by SNAIL, TWIST, ZEB, and FOX transcription factor family members.[7] At the cellular level, EMT is associated with increased proliferation and migration, elevated resistance to senescence, and apoptosis.[8–10] At the molecular level, it is characterized by down-regulation of the epithelial markers E-cadherin, claudin-3, and claudin-4, coupled with up-regulation of mesenchymal markers β-catenin, survivin, vimentin, and cyclin-D.[11–13] EMT plays an important role during embryogenesis and in epithelial wound healing in adults.[6,14] However, EMT in adults can have undesirable consequences as seen during pathologic fibrosis, metastasis, and epithelial tumor invasion.[14–16] Although EMT has been studied extensively, a complete understanding of the molecular mechanisms that govern EMT remains elusive.[17]

The cornea, the outermost part of the eye, is composed of an anterior stratified squamous epithelium, central stroma, and posterior endothelium. The corneal epithelium (CE) is a highly stratified tissue with columnar basal cells glued to the underlying basement membrane through hemidesmosomes, three to four layers of wing cells that tightly adhere and communicate with each other and the basal cells through desmosomes and gap junctions, respectively, and one to two layers of the most superficial, terminally differentiated cells that

express tight junctions and form a watertight outermost barrier.[18] Adult CE surface needs to be continually renewed as the superficial cells are lost by sloughing.[19] CE stem cells residing in the limbal epithelial niches give rise to transient amplifying cells that further divide and migrate centripetally and upward as they differentiate, ultimately replacing the superficial cells. Thus, CE homeostasis depends on an exquisitely regulated balance between cell proliferation, migration, and differentiation.[20–22] Several proteins including corneal crystallin Aldh3a1,[23,24] extracellular matrix component lumican,[25] and secreted signaling molecule Wnt7a[26] contribute toward CE homeostasis. Although recent studies have unraveled the network of transcription factors involved in corneal development,[27] large gaps remain in our understanding of regulation of adult CE homeostasis.

Krüppel-like factor 4 (KLF4), a zinc finger transcription factor, is essential for epithelial cell differentiation and homeostasis in diverse epithelial tissues.[28–31] KLF4 promotes epithelial cell fate by up-regulating epithelial genes while down-regulating mesenchymal genes.[32,33] KLF4 is suppressed in metastasis and EMT and is mutated, down-regulated, or lost in many cancers including ovarian, urothelial, hepatocellular, and breast cancer cells.[34–37] KLF4 is abundantly expressed in the ocular surface, conditional ablation of which resulted in fragile CE with disrupted epithelial barrier function, stromal edema, and depletion of conjunctival goblet cells.[38–41] Spatiotemporally regulated CE-specific ablation of *Klf4* altered CE barrier function and disrupted homeostasis in a manner reminiscent of EMT, leading to squamous metaplasia.[42] Although KLF4 is abundantly expressed in the CE and is important for CE homeostasis, the mechanism(s) by which it promotes CE epithelial fate remains unknown. Here, we report that the epithelial markers E-cadherin, claudin-3, claudin-4, and keratin-12 are down-regulated, whereas mesenchymal markers vimentin, β-catenin, survivin, and cyclin-D1 are up-regulated on spatiotemporally regulated ablation of *Klf4*, suggesting that KLF4 plays an essential role in CE homeostasis by promoting epithelial cell fate and suppressing EMT.

Materials and Methods

Mice

All studies were conducted with 8- to 10-week-old mice of mixed background. *Klf4* was ablated in a spatiotemporally regulated manner in the CE by feeding ternary transgenic $Klf4^{\Delta/\Delta CE}$ ($Klf4^{LoxP/LoxP}/Krt12^{rtTA/rtTA}/Tet\text{-}O\text{-}Cre$) mice with doxycycline chow for at least a month as previously described.[42–44] Littermates fed with normal chow served as wild-type (WT) controls. All procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Human Corneal Limbal Epithelial Cells

Human corneal limbal epithelial (HCLE) cells obtained from Ilene Gipson (Harvard University, Boston, MA, USA) were maintained as previously described.[45] Briefly, HCLE cells were cultured in keratinocytes-serum free medium (KSFM) supplemented with calcium chloride (0.3 M), epidermal growth factor (0.2 ng/mL), and brain pituitary extract. To induce EMT, 70% confluent HCLE cells were treated with 10 ng/mL of TGF-β1 for 48 hours, following which they were imaged for morphologic changes and processed to evaluate KLF4 levels and EMT markers by quantitative PCR (qPCR), immunoblots, and immunofluorescent staining.

Antibodies

Antibodies used in this study were commercially obtained. Their source, catalog numbers, and the host in which they were raised are listed in Supplementary Table S1.

Corneal Epithelial Debridement

Corneal epithelial debridement was carried out as previously described[46] in mice anesthetized by intraperitoneal injection of ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight), followed by topical application of proparacaine eye drops. A circular area of 1.5 mm diameter of cornea was demarcated using trephine blade, and the epithelium was removed using Algerbrush, taking care to not damage the basement membrane. The extent of CE migration was quantified by staining the debrided area at 0 and 16 hours after wounding with 2 μL 1% sodium fluorescein for 1 minute, rinsing with PBS, and photographing under blue light using a slit-lamp biomicroscope. Eyes were collected 16 hours or 7 days after wounding for further analysis.

Histology

Enucleated mouse eyes were fixed in freshly prepared 4 % para-formaldehyde (Sigma-Aldrich Corp., St. Louis, MO, USA) in PBS (pH 7.4) and embedded in paraffin. Central corneal 5-μm-thick sections were stained with hematoxylin and eosin (H&E) or periodic acid-Schiff's reagent (PAS) using standard protocols. An Olympus BX60 microscope (Olympus America, Inc., Allentown, PA, USA) with Spot digital camera (Spot Diagnostics Instruments, Inc., Sterling Heights, CA, USA) was used to capture images, which were further processed using Adobe Photoshop and Illustrator (Adobe Systems, San Jose, CA, USA).

Isolation of Total RNA and qPCR

RNA isolated from dissected mouse corneas or HCLE cells treated with and without TGF-β1 using the EZ-10 spin column total RNA mini-prep kit (Bio Basic, Inc., Amherst, NY, USA) was used for cDNA synthesis with mouse Maloney leukemia virus reverse transcriptase (Promega, Madison, WI, USA). SYBR Green gene expression assays were performed in triplicate in an ABI StepOne Plus thermocycler using appropriate endogenous controls (Applied Biosystems, Foster City, CA, USA). The sequence of different primers used for qPCR is shown in Supplementary Table S2.

Immunoblots

WT or $Klf4^{\Delta/\Delta CE}$ corneas were homogenized in urea buffer (8.0 M urea, 0.8 % Triton X-100, 3 % β-mercaptoethanol, 0.2 % SDS, and protease inhibitors). HCLE cells treated with and without TGF-β1 were lysed using urea buffer. After removing the insoluble material by centrifugation, total protein in the supernatant was quantified, and 10 μg separated on 4% to 12% gradient polyacrylamide gels using 3-(*N*-morpholino)propanesulfonic acid (MOPS)/2-(N-morpholino) ethanesulfonic acid (MES) buffer, and electrophoretically blotted onto polyvinylidine fluoride (PVDF) membranes. The membranes were blocked with Odyssey blocking solution for 1h at room temperature, followed by overnight incubation with primary antibody at 4°C, washed three times with phosphate buffered saline with 0.1% Tween-20 (PBST) for 5 minutes each, incubated with fluorescently labeled secondary antibody (goat anti-rabbit IgG, or donkey anti-goat IgG) at a 1:20,000 dilution for 1 hour at room temperature, washed three times with PBST for 5 minutes each followed by a wash with PBS to remove

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traces of Tween-20. Blots were scanned on Odyssey scanner (Li-Cor Biosciences, Lincoln, NE, USA), and densitometric measurements of the immunoreactive band intensities were performed using Image J software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). β-Actin was used as a loading control for normalizing the data.

Immunofluorescent Staining

Eight-micrometer-thick sections from OCT-embedded eyeballs were fixed in freshly prepared buffered 4% paraformaldehyde for 10 minutes, washed three times for 5 minutes each with PBS (pH 7.4), and permeabilized (0.1% Triton X-100 in PBS) when necessary, followed by three washes of 5 minutes each with PBS, treated with glycine for 20 minutes, followed by three washes with PBS, blocked (10% goat or donkey serum in PBS) for 1 hour at room temperature in a humidified chamber, washed twice with PBS for 5 minutes each, incubated with the appropriate dilution of the primary antibody for 2 hours at room temperature, washed three times with PBS for 5 minutes each, incubated with appropriate secondary antibody (Alexafluor 546-coupled goat anti-rabbit IgG, and Alexafluor 488-coupled donkey anti-goat IgG; Molecular Probes, Carlsbad, CA, USA) at a 1:400 dilution for 1 hour at room temperature, washed three times with PBST, counterstained with 4,6-diamidino-2-phenylindole (DAPI), mounted with Aqua-Poly/Mount (Polysciences, Warrington, PA, USA), and imaged using Olympus IX81 microscope (Olympus America, Inc.). HCLE cells grown on coverslips were processed as above. The mean proliferative index in the WT and *Klf4*$^{\Delta/\Delta CE}$ corneas was determined by dividing the number of Ki67-positive cells by the total number of epithelial cells in multiple fields from different sections stained independently. The relative fluorescence intensity was quantified using Metamorph software.

Statistical Analysis.

The results presented here are representative of at least three independent experiments and shown as mean ± SEM. Statistical significance was tested by Student's *t*-test, with $P \leq 0.05$ considered statistically significant.

Results

CE-Specific Ablation of *Klf4* Results in Altered CE Gene Expression Favoring EMT

Considering that the ablation of *Klf4* resulted in decreased expression of tight junction proteins ZO-1 and Dsg and up-regulation of MMP-9, and compromised barrier function reminiscent of EMT,[42] we examined the expression of EMT-associated transcription factors Snail, Slug, Twist1, Twist2, Zeb1, and Zeb2 in WT and *Klf4*$^{\Delta/\Delta CE}$ corneas. qPCR revealed a strong up-regulation of each of these transcription factors, suggesting that the *Klf4*$^{\Delta/\Delta CE}$ cells are undergoing EMT (Fig. 1A). Immunoblots probing the lysates from the WT and *Klf4*$^{\Delta/\Delta CE}$ corneas revealed that the expression of Slug and Twist1 is up-regulated by 3.9- and 3.6-fold, respectively (Figs. 1B, 1C). Consistent with these results, the expression of epithelial markers claudin-3 and -4 and E-cadherin was significantly down-regulated and that of the mesenchymal marker vimentin was up-regulated in the *Klf4*$^{\Delta/\Delta CE}$ corneas (Fig. 2A). As the intermediate filament protein vimentin, a major cytoskeletal component of mesenchymal cells, is considered a bona fide marker for EMT,[6,14,17,47,48] we further evaluated its expression in the *Klf4*$^{\Delta/\Delta CE}$ corneas. Immunoblots revealed a robust increase

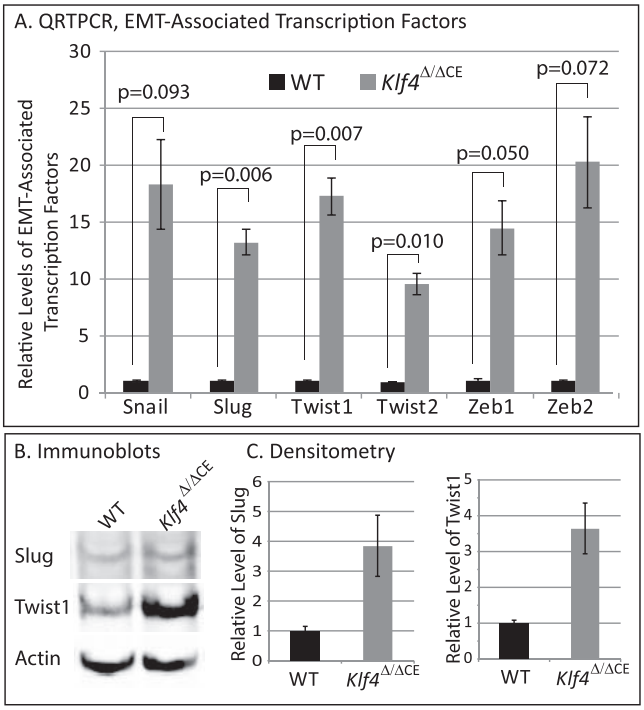

Figure 1. Up-regulation of EMT-associated transcription factors in the *Klf4*$^{\Delta/\Delta CE}$ CE. (**A**) qRT-PCR for EMT transcription factors. qPCR was performed in duplicate using three different pools of WT and *Klf4*$^{\Delta/\Delta CE}$ corneal cDNA, each generated using total RNA from two corneas of different mice. Results shown are mean ± SEM. $P \leq 0.05$ was considered statistically significant. The sequence of oligonucleotide primers used is shown in Supplementary Table S1. (**B**) Immunoblots for representative EMT-transcription factors Slug and Twist1. The blot was stripped of the antibody and reprobed with anti-actin antibody for normalization. (**C**) Densitometric scan from three independent replicates using actin as a loading control. Results shown are mean ± SEM.

in the expression of vimentin in the *Klf4*$^{\Delta/\Delta CE}$ corneas (Figs. 2B, 2C), which was further confirmed by immunofluorescent stain (Fig. 2D). Taken together, these results suggest that CE-specific ablation of *Klf4* results in altered gene expression favoring EMT.

As down-regulation of E-cadherin is an invariable feature of EMT,[7,17,47,49] and KLF4 is known to regulate E-cadherin expression in mammary epithelial cells,[13] we further examined the expression of E-cadherin in the adult WT and *Klf4*$^{\Delta/\Delta CE}$ corneas. Immunoblots revealed that E-cadherin protein levels in the *Klf4*$^{\Delta/\Delta CE}$ corneas are decreased to 19% of that in the WT corneas (Figs. 3A, 3B). Immunofluorescent stain with anti–E-cadherin antibody revealed abundant expression of E-cadherin in the WT CE, where it was predominantly localized on the cytoplasmic membrane (Fig. 3C). In contrast, it was expressed at low to undetectable levels in the *Klf4*$^{\Delta/\Delta CE}$ CE, where it was sparsely dispersed in the cytoplasm, consistent with the loss of epithelial phenotype and EMT in *Klf4*$^{\Delta/\Delta CE}$ corneas (Fig. 3C).

Considering that E-cadherin and β-catenin are normally tethered together at the epithelial cell membrane; loss of E-cadherin releases β-catenin into the cytoplasm and nucleus, which in turn promotes EMT; and the aberrant nuclear localization of β-catenin is often associated with CE neoplasia,[50] we next examined whether β-catenin expression is altered in the *Klf4*$^{\Delta/\Delta CE}$ CE. Immunoblots revealed that the expression of β-catenin is up-regulated by 2.5-fold in the *Klf4*$^{\Delta/\Delta CE}$ compared with the WT (Figs. 4A, 4B). Immunofluorescent stain confirmed elevated expression and the nuclear translocation

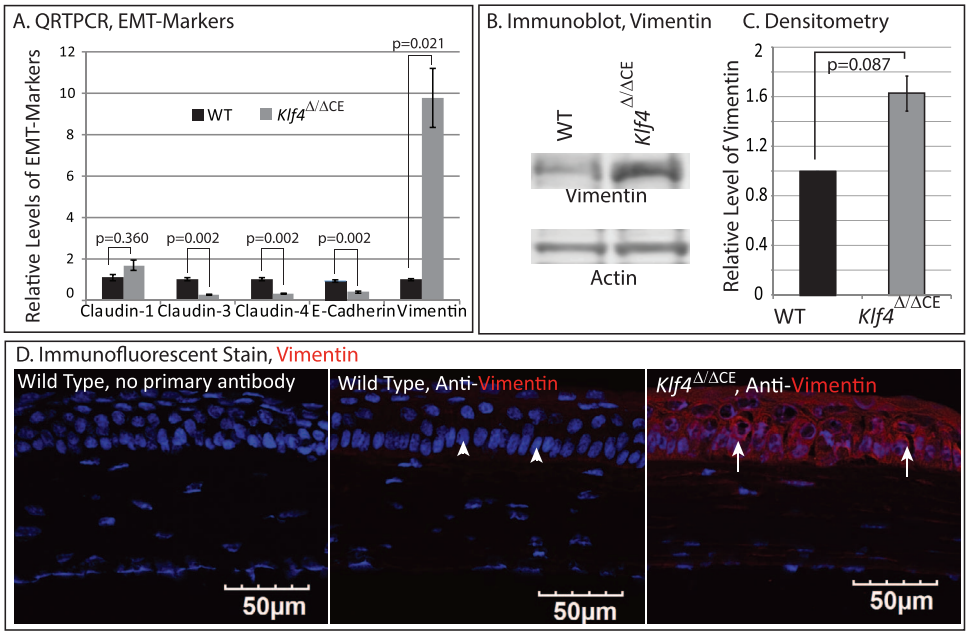

Figure 2. Down-regulation of epithelial markers and up-regulation of mesenchymal marker vimentin in the *Klf4*$^{\Delta/\Delta CE}$ CE. (**A**) qRT-PCR for EMT markers. qPCR was performed in duplicate using three different pools of WT and *Klf4*$^{\Delta/\Delta CE}$ corneal cDNA, each generated using total RNA from two corneas of different mice. (**B**) Immunoblot shows increased expression of vimentin in the *Klf4*$^{\Delta/\Delta CE}$ CE compared with the WT. (**C**) Histogram showing densitometric quantitation from three independent replicates, using actin as a loading control. Results shown are mean ± SEM; $P \leq 0.05$ was considered statistically significant. (**D**) Immunofluorescent stain shows robust expression of vimentin in the *Klf4*$^{\Delta/\Delta CE}$ (*arrows*) but not the WT CE (*arrowheads*). No primary antibody control is shown.

of β-catenin in the *Klf4*$^{\Delta/\Delta CE}$ cells (Fig. 4C). Together with the up-regulation of vimentin and down-regulation of E-cadherin in the *Klf4*$^{\Delta/\Delta CE}$ corneas, these results demonstrate that Klf4 is essential for maintenance of CE phenotype and that the absence of Klf4 leads to EMT.

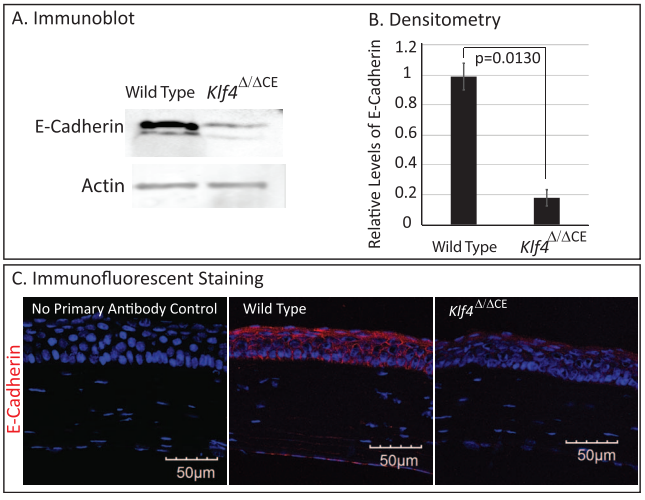

Figure 3. E-cadherin is down-regulated in the *Klf4*$^{\Delta/\Delta CE}$ CE. (**A**) Immunoblot shows decreased expression of E-cadherin in the *Klf4*$^{\Delta/\Delta CE}$ CE compared with the WT. (**B**) Histogram showing densitometric quantitation from three independent replicates, using actin as a loading control. Results shown are mean ± SEM; $P \leq 0.05$ was considered statistically significant. (**C**) Immunofluorescent stain shows decreased expression of E-cadherin in the *Klf4*$^{\Delta/\Delta CE}$ compared with the WT CE. Note that E-cadherin is localized predominantly on the cell membranes in the WT but not the *Klf4*$^{\Delta/\Delta CE}$ CE.

As elevated proliferation is a key feature of EMT[6,47] and KLF4 regulates epithelial cell proliferation and differentiation,[28] both of which are influenced by survivin and cyclin-D1,[51,52] we next evaluated their expression in *Klf4*$^{\Delta/\Delta CE}$ corneas. Immunofluorescent stain revealed significantly increased expression of survivin and cyclin-D1 in the *Klf4*$^{\Delta/\Delta CE}$ cells compared with the WT (Fig. 5). Taken together with our previous results that revealed increased number of Ki67^{+} proliferative cells in the *Klf4*$^{\Delta/\Delta CE}$ CE,[42] these results demonstrate that Klf4 suppresses proliferation and promotes differentiation in the WT CE by suppressing the expression of cycin-D1 and survivin.

KLF4 Is Down-Regulated in HCLE Cells Undergoing TGF-β1–Induced EMT

To test whether the corollary is true with respect to the role of KLF4 in EMT, we evaluated the levels of KLF4 in HCLE cells undergoing TGF-β1–induced EMT. EMT in TGF-β1–treated HCLE cells was confirmed by their elongated morphology (Fig. 6A), decreased expression of E-cadherin, and increased nuclear localization of β-catenin (Fig. 6B). Both qPCR and immunoblot revealed significantly decreased expression of KLF4 transcript (25% of the control) and protein (35% of the control) in these cells (Fig. 6C), which was further confirmed by immunofluorescent stain (Fig. 6C.iv). On the basis of these results, we conclude that KLF4 is significantly down-regulated in HCLE cells undergoing TGF-β1–induced EMT, consistent with its role in promoting CE phenotype by suppressing EMT.

Increased Rate of Migration and Failure of Re-Epithelialization in the Debrided *Klf4*$^{\Delta/\Delta CE}$ CE

Epithelial wound healing and re-epithelialization depend on well-coordinated cell migration.[53,54] Considering that the cells

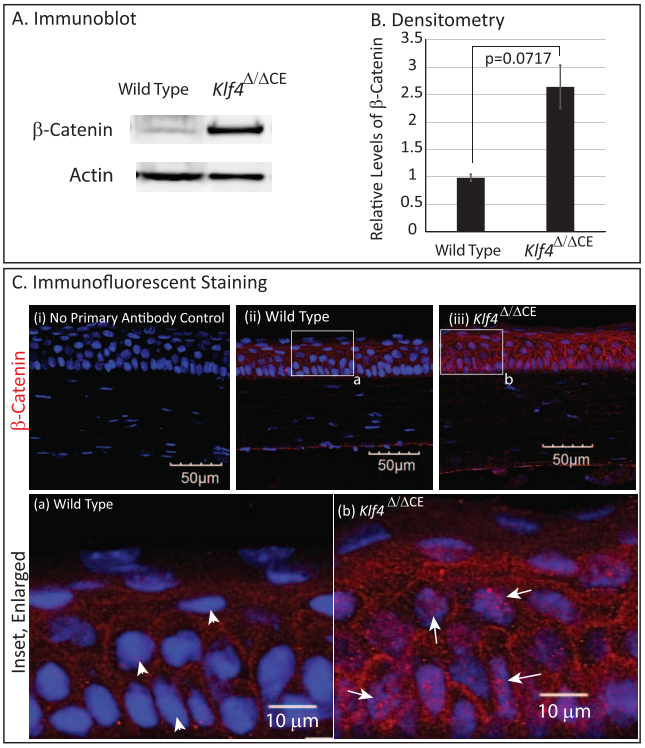

Figure 4. Increased expression and nuclear translocation of β-catenin in *Klf4*$^{\Delta/\Delta CE}$ CE. (**A**) Immunoblot shows increased expression of β-catenin in the *Klf4*$^{\Delta/\Delta CE}$ CE compared with the WT. (**B**) Histogram showing densitometric quantitation from three independent replicates, using actin as a loading control. Results are mean ± SEM; $P \leq 0.05$ was considered statistically significant. (**C**) Immunofluorescent stain shows increased expression and nuclear translocation of β-catenin in *Klf4*$^{\Delta/\Delta CE}$ (*arrows*) compared with the WT CE, where the base level expression of β-catenin is mostly localized to the cell membrane and not the nucleus (*arrowheads*). The two panels in the bottom show enlarged images of the corresponding inset regions from **Cii** and **Ciii**.

flanking the wound area undergo transient EMT facilitating rapid proliferation and migration,[14,16,55] and that the results above revealed that Klf4 suppresses CE EMT (Figs. 1–6), we next determined whether postdebridement re-epithelialization is abnormal in the absence of Klf4. We generated a 1.5-mm-diameter circular wound in the WT and *Klf4*$^{\Delta/\Delta CE}$ CE using Algerbrush and evaluated the extent of their closure after 16 hours by fluorescein staining. The *Klf4*$^{\Delta/\Delta CE}$ cells migrated faster, filling 93% of the debrided area within 16 hours of wounding compared with 61% in the WT, suggesting that the absence of Klf4 resulted in faster migration of CE (Figs. 7A, 7B). Histology revealed the persistence of open wound in the WT central cornea unlike that in the *Klf4*$^{\Delta/\Delta CE}$ CE, which was completely covered (Fig. 7C). Histologic examination after 7 days of wound healing revealed that the repopulated *Klf4*$^{\Delta/\Delta CE}$ CE was thicker, with many more cell layers of altered morphology than the WT (Fig. 7C). Immunofluorescent staining with anti-laminin antibody confirmed that the epithelial debridement did not affect the basement membrane (Fig 7D). Next, we tested whether the expression of Klf4 is modulated in the WT CE cells undergoing transient EMT at the migrating edge during wound healing. Immunofluorescent stain with anti-Klf4 antibody revealed markedly decreased expression of Klf4 at the migrating edge in the WT CE cells at 16 hours after debridement compared with those away from the wound, where it was abundantly expressed (Fig. 7E). Thus, migrating cells undergoing EMT at the wound edge transiently lose the expression of Klf4. Taken together, the faster migration and abnormal re-epithelialization of the *Klf4*$^{\Delta/\Delta CE}$ cells and the transient loss of Klf4 expression in the migrating edge of the WT CE are consistent with a role for Klf4 in suppressing EMT.

KLF4 Regulates Proliferation in Newly Re-Epithelialized CE Through Survivin and Cyclin D1

Previously, we reported elevated cell proliferation in unwounded *Klf4*$^{\Delta/\Delta CE}$ CE.[42] To determine whether the newly migrated *Klf4*$^{\Delta/\Delta CE}$ CE cells in the wounded area also proliferate

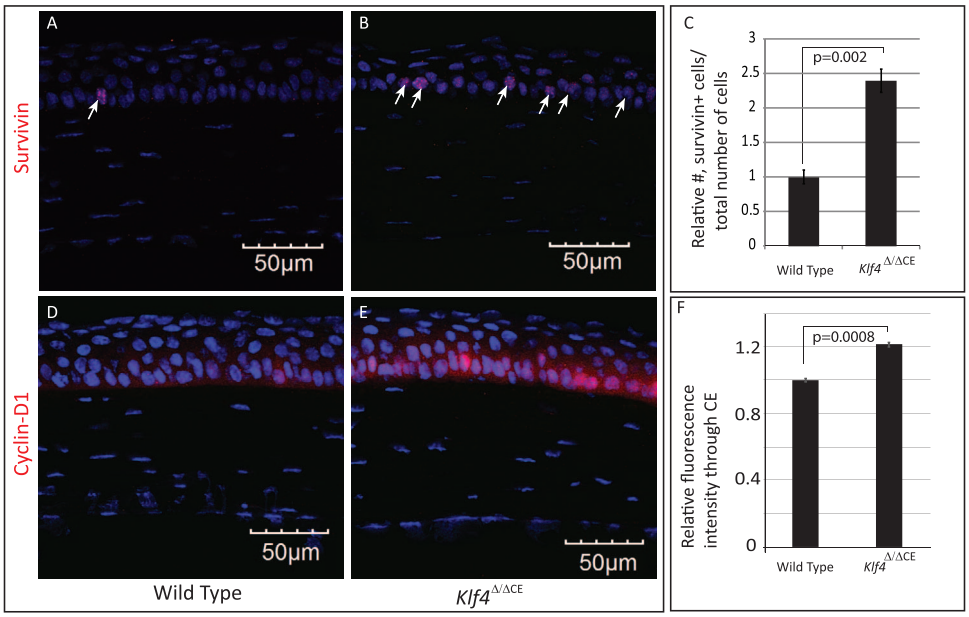

Figure 5. Altered expression of survivin and cyclin-D in the *Klf4*$^{\Delta/\Delta CE}$ CE. (**A–C**) Increased number of survivin-positive cells in the *Klf4*$^{\Delta/\Delta CE}$ compared with the WT CE, revealed by immunofluorescent staining. Corresponding histogram shows the mean number of survivin-positive cells per unit area, using data from three independent replicates. (**D–F**) Increased expression of cyclin-D1 in the *Klf4*$^{\Delta/\Delta CE}$ cells compared with the WT. Corresponding histograms show relative fluorescence intensities measured throughout the CE. Results are mean ± SEM; $P \leq 0.05$ was considered statistically significant.

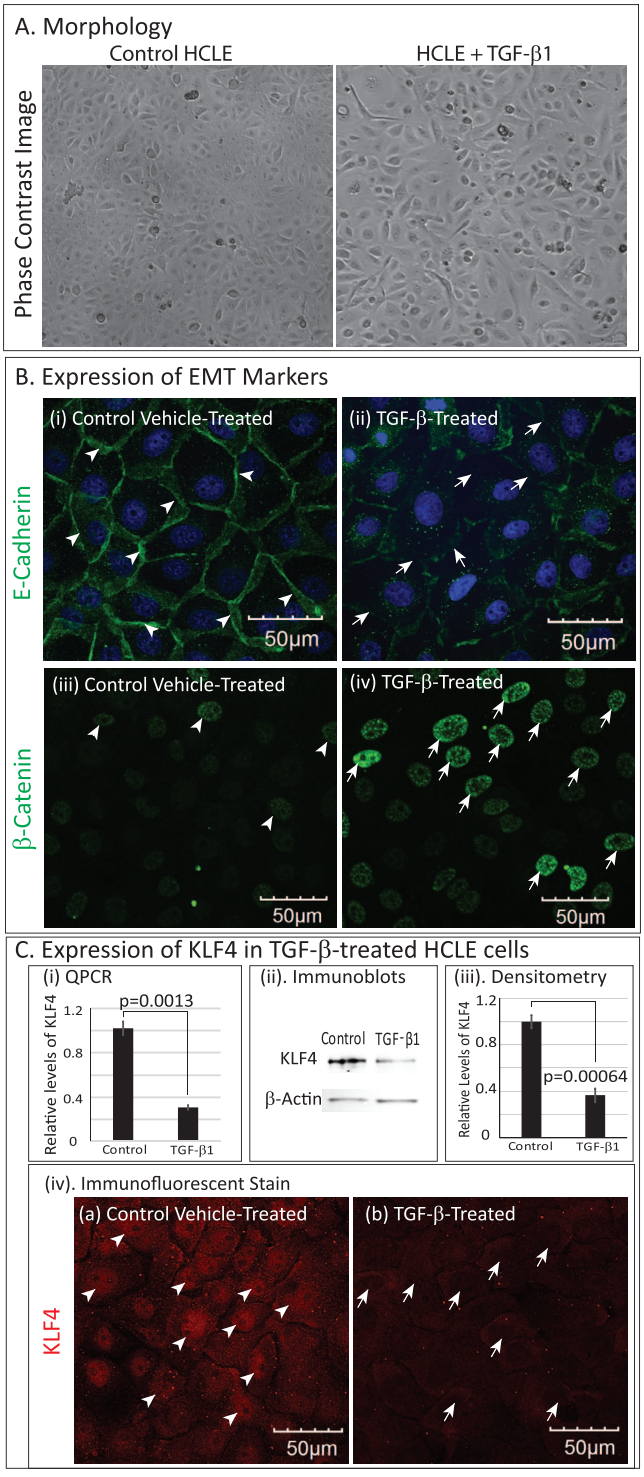

Figure 6. KLF4 is down-regulated in HCLE cells undergoing TGF-β1–induced EMT. (**A**) Phase contrast images of control HCLE cells and those treated for 48 hours with TGF-β1. Note that the TGF-β1–treated HCLE cells are elongated and more spindle shaped compared with the untreated control. (**B**) Immunofluorescent stain reveals decreased expression of epithelial marker E-cadherin and increased expression, as well as nuclear localization of mesenchymal marker β-catenin in TGF-β1–treated HCLE cells (*arrows*) compared with the vehicle-treated control cells (*arrowheads*). (**C.i**) qPCR showing the relative *KLF4* mRNA levels in the control and TGF-β1–treated HCLE cells. (**C.ii**) Immunoblot probed with anti-KLF4 antibody, showing the decreased expression of KLF4 in TGF-β1–treated HCLE cells compared with the control. (**C.iii**) Histogram showing densitometric quantitation from three independent immunoblots. Results are mean ± SEM; $P \leq 0.05$ was considered statistically significant. (**C.iv**) Immunofluorescent stain for KLF4 in HCLE cells treated with and without (control) TGF-β1. *Arrows* in **C.iv.a** point to nuclear expression of KLF4 in control vehicle-treated HCLE cells. *Arrowheads* in **C.iv.b** point to the nuclei of TGF-β1–treated HCLE cells that lack KLF4 expression.

abnormally, we performed immunofluorescent staining with anti-Ki67 antibody. After 7 days of wounding, the newly repopulated *Klf4*$^{\Delta/\Delta CE}$ CE harbored 2.5-fold more Ki67-positive cells than their WT counterparts (Fig. 8). Additionally, the number of survivin-positive cells was increased by 2.8-fold and the relative fluorescence intensity for cyclin-D1 immunostain was significantly increased to 1.4-fold in the *Klf4*$^{\Delta/\Delta CE}$ CE (Fig. 8). Although Ki67, survivin, and cyclin-D1 expression was restricted to the basal epithelial layer in the WT, it was also detected in the suprabasal cells in the *Klf4*$^{\Delta/\Delta CE}$ CE (Fig. 8), consistent with dysregulated proliferation in newly repopulated cells. Together, these results suggest that Klf4 regulates proliferation in the newly repopulated CE through survivin and cyclin-D1.

Migrated *Klf4*$^{\Delta/\Delta CE}$ Cells Fail to Regain CE Properties Following Gap Filling

During WT CE wound healing, migrating cells undergo transient EMT, which is reversed once the gap is filled.[14,55] To determine whether the *Klf4*$^{\Delta/\Delta CE}$ cells can regain CE properties, we next evaluated the identity of re-epithelialized CE after 7 days of wounding. Unlike their WT counterparts that quickly regained the expression of Klf4 and CE-specific marker Krt12 in the newly repopulated cells, the *Klf4*$^{\Delta/\Delta CE}$ cells lacking Klf4 displayed negligible Krt12 expression (Fig. 9). Consistent with these results, intact membranous expression of E-cadherin, a hallmark epithelial characteristic evident in the WT CE, was not observed in the *Klf4*$^{\Delta/\Delta CE}$ cells (Figs. 10A–C). Finally, increased expression of β-catenin was observed in *Klf4*$^{\Delta/\Delta CE}$ CE, compared with the WT (Figs. 10D–F). Thus, the *Klf4*$^{\Delta/\Delta CE}$ cells unlike the WT fail to regain epithelial characteristics after the gap is filled. Together, these results provide evidence that Klf4 plays an essential role not only in normal homeostasis but also in ensuring CE re-epithelialization following wound healing.

Discussion

The corneal epithelium plays an essential role in vision by providing a smooth curved surface that refracts light appropriately with minimal scatter, serving as a barrier to environmental toxins and microbial pathogens, secreting mucins, defensins, SLURP1, lacritin, and other important proteins to the tear film,[56–58] and serving as surrogate Schwann cells for their sensory nerves.[59] Being the most anterior cellular layer of the eye, the CE is subject to frequent abrasions and exposure to physical, chemical, and biological insults and is continuously renewed as the most superficial cells are sloughed off. As such, well-orchestrated CE homeostasis is essential for preserving sight. Recent studies have begun to shed light on the genetic network of transcription factors that regulate CE development and homeostasis.[27,49] Our previous studies demonstrated that Klf4 is essential for both maturation and maintenance of the mature CE.[42] Data presented in this report reveal that the *Klf4*$^{\Delta/\Delta CE}$ CE display decreased expression of epithelial markers E-cadherin and claudins, up-regulation of mesenchymal markers β-catenin, vimentin, survivin, and cyclin-D1, overexpression of EMT-associated transcription factors, and

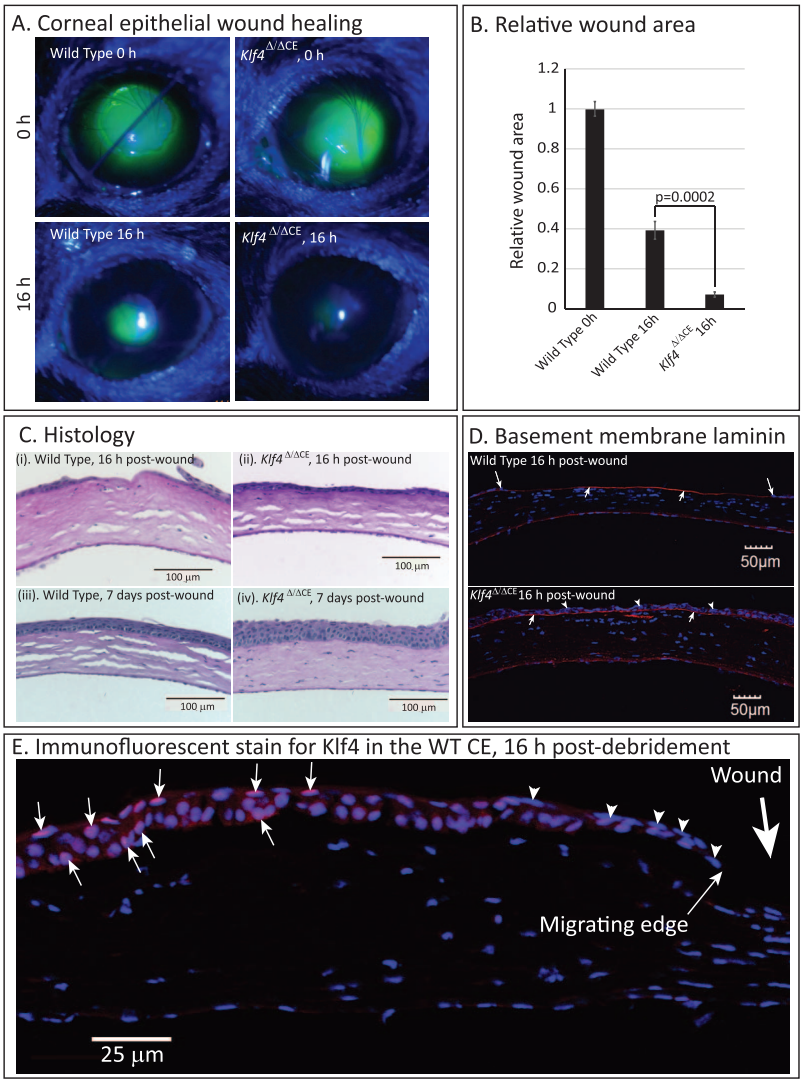

Figure 7. After debridement, *Klf4*$^{\Delta/\Delta CE}$ cells migrate faster and display abnormal morphology. (**A**) Representative images showing the wounded area detected by fluorescein staining at 0 and 16 hours after debridement in the WT and *Klf4*$^{\Delta/\Delta CE}$ CE. (**B**) Histogram showing the relative wound area (fluorescein-stained area) at 0 and 16 hours after debridement in the WT and *Klf4*$^{\Delta/\Delta CE}$ CE. (**C**) Histology of debrided WT and *Klf4*$^{\Delta/\Delta CE}$ corneas at 16 hours and 7 days after debridement. (**D**) Immunofluorescent stain with anti-laminin antibody at 16 hours after debridement reveals intact basement membrane in debrided WT and *Klf4*$^{\Delta/\Delta CE}$. Note that the debrided gap remains in the WT, but not the *Klf4*$^{\Delta/\Delta CE}$ CE at 16 hours after debridement. (**E**) Immunofluorescent stain with anti-Klf4 antibody at 16 hours after debridement reveals markedly decreased expression of Klf4 in the WT CE cells at the migrating edge (*arrowheads*), unlike the cells away from the wound (*arrows*) where it is abundantly expressed.

faster wound closure, consistent with these cells undergoing EMT in the absence of Klf4. These signs of EMT were exaggerated in wounded *Klf4*$^{\Delta/\Delta CE}$ CE, and the migrated *Klf4*$^{\Delta/\Delta CE}$ cells failed to revert to epithelial phenotype after 7 days of wound healing, providing evidence for the essential role of Klf4 in CE homeostasis. Moreover, HCLE cells undergoing TGF-β–induced EMT expressed significantly lower levels of KLF4. Taken together, these results demonstrate that KLF4 plays an essential role in CE homeostasis by promoting epithelial cell fate and suppressing EMT (Fig. 11).

The most common features of EMT displayed by the *Klf4*$^{\Delta/\Delta CE}$ cells include loss of cell–cell adhesion accompanied by down-regulation of adherens junction molecule E-cadherin, tight junction molecules claudins, ZO1, and occludin, and desmosomal components Dsg and Dsp[42,60–63]; reorganization of the intermediate filaments of the cytoskeleton caused by down-regulation of keratins and overexpression of vimentin; and increased proliferation and motility caused by up-regulation of cyclin-D1 and survivin.[7,47,49,64] These changes are also consistent with a less differentiated status for *Klf4*$^{\Delta/\Delta CE}$ cells with increased life span resulting in abnormal stacking of CE layers, and squamous metaplasia reported earlier.[42]

EMT is associated with elevated expression of a group of transcription factors including members of the Snail, Twist, and Zeb families.[17,65] Considering that the overexpression of each of these transcription factors can induce EMT on its own,[60] their combined up-regulation provides strong evidence in support of EMT in the *Klf4*$^{\Delta/\Delta CE}$ CE. Snail and Slug are both known to repress the expression of E-cadherin, whereas KLF4 activates it. Whether the down-regulation of E-cadherin observed in the *Klf4*$^{\Delta/\Delta CE}$ cells is a direct outcome of the absence of Klf4 or caused indirectly by up-regulation of Snail and Slug remains to be tested. Determination of the hierarchical position of KLF4 in this network of EMT-associated

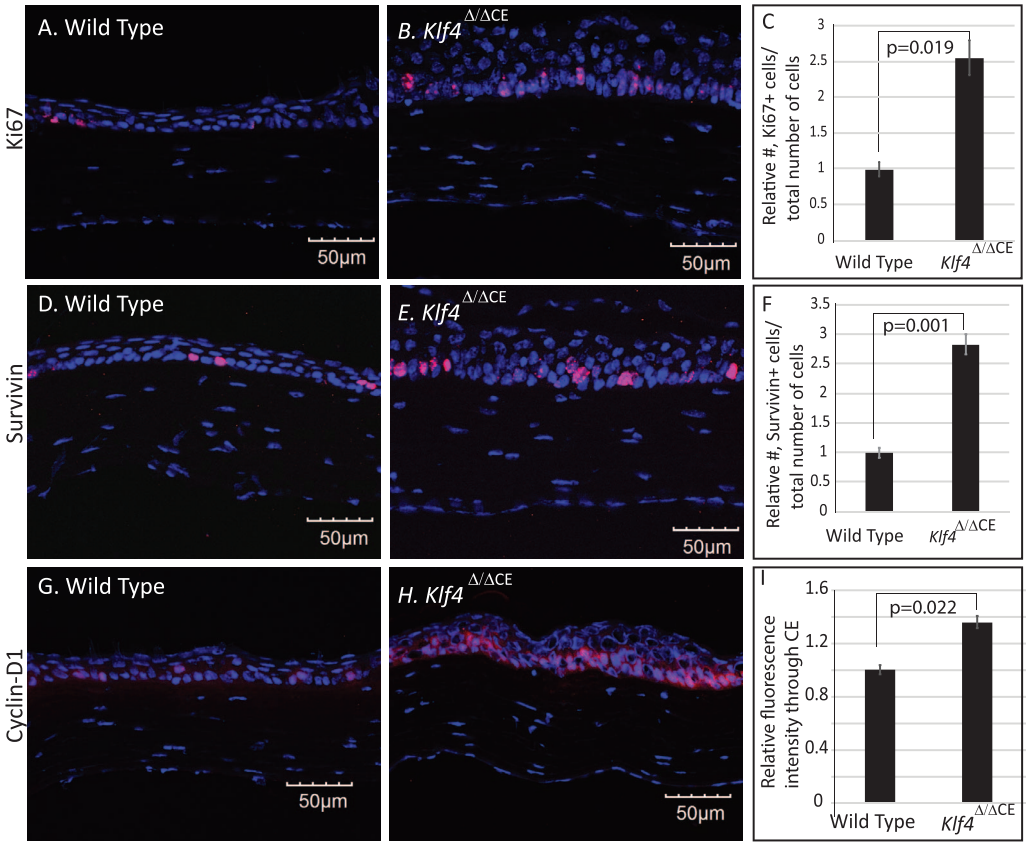

Figure 8. Enhanced signs of EMT in *Klf4*$^{\Delta/\Delta CE}$ CE after 7 days of wounding. Immunofluorescent stain and corresponding histogram showing the relative expression of EMT-associated proteins (**A–C**) Ki67, (**D–F**) survivin, and (**G–I**) cyclin-D1 in the WT and *Klf4*$^{\Delta/\Delta CE}$ cornea. Results presented in histograms are mean ± SEM. $P \leq 0.05$ was considered statistically significant.

transcription factors, as well as any crosstalk, and feedback regulation among them is likely to help clarify this relationship.

Dysregulation of the CE cell proliferation results in a wide range of potentially blinding disorders collectively termed ocular surface squamous neoplasia (OSSN).[66,67] OSSN represents the third most common oculoorbital tumors in elderly patients, after melanoma and lymphoma.[68] When the dysplastic CE cells undergo EMT and gain metastatic potential, they can penetrate the basement membrane, leading to the formation of squamous cell carcinomas (SCCs). Despite their clinical relevance, molecular mechanisms underlying OSSN and SCCs are not well understood. Further studies are required to examine whether the expression and/or activity of KLF4 is altered in OSSN and SCCs.

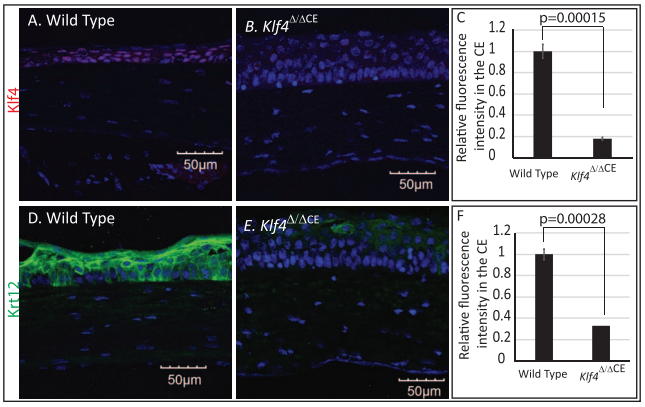

Figure 9. Migrated *Klf4*$^{\Delta/\Delta CE}$ CE cells fail to revert to epithelial phenotype. Immunofluorescent staining and histograms of corresponding fluorescence intensities of re-epithelialized WT and *Klf4*$^{\Delta/\Delta CE}$ cells 7 days after corneal debridement with (**A–C**) anti-KLF4 antibody and (**D–F**) CE-specific marker anti–keratin-12. Results presented in histograms are mean ± SEM. $P \leq 0.05$ was considered statistically significant.

Although the involvement of KLF4 in EMT has been studied previously in other tissues including the colon, breast, gastric, and lung epithelia,[8–14,34–36,48,69–76] this is the first report of this critical function in the CE that plays an essential role in vision.[19] EMT is involved in the formation of myofibroblasts that promote fibrotic lesions with excessive accumulation of fibrogenic extracellular matrix, tissue contraction, and impaired functions.[77–79] Previous studies on CE EMT have focused on its involvement in pathogenesis of pterygium[12] and OSSN.[50] Whether persistent CE EMT if left untreated for extended duration induces the formation of myofibroblast-like cells leading to stromal fibrosis, a major cause of corneal blindness in the world, remains to be tested.[80]

The CE cells normally undergo transient EMT during early stages of wound healing, reversing it in the final stages to undergo mesenchymal–epithelial transition (MET).[55] The *Klf4*$^{\Delta/\Delta CE}$ cells displayed exaggerated EMT on CE debridement by displaying faster migration, stacking of additional cell layers, altered morphology, and high proliferation coupled with increased survivin and cyclin-D1 expression. Moreover, 7 days

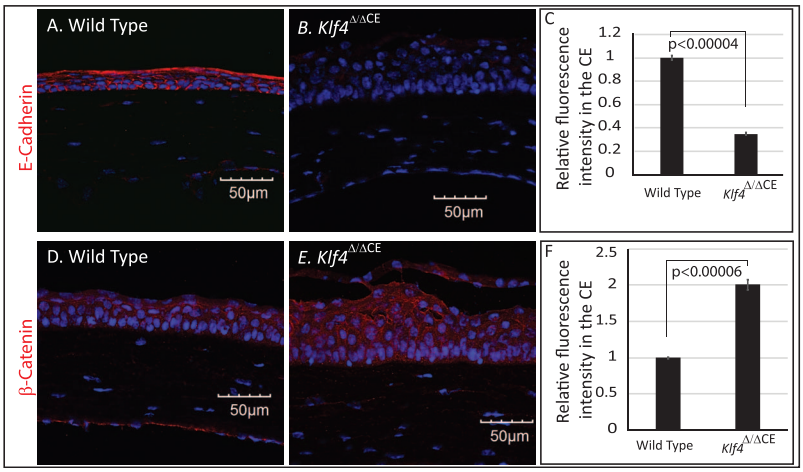

Figure 10. Migrated *Klf4*$^{\Delta/\Delta CE}$ CE cells maintain mesenchymal characteristics. Images showing immunofluorescent stain and histogram of corresponding fluorescence intensities for epithelial marker E-cadherin (**A–C**) and mesenchymal marker β-catenin (**D–F**) in the WT and *Klf4*$^{\Delta/\Delta CE}$ corneas 7 days after wounding. Results presented in histograms are mean ± SEM. $P \leq 0.05$ was considered statistically significant.

after epithelial debridement, the migrated *Klf4*$^{\Delta/\Delta CE}$ cells failed to express E-cadherin and keratin-12, indicating that KLF4 is required for the reversal of transient EMT (MET) to regain epithelial features in the final stages of epithelial wound healing.

In summary, our findings demonstrate that spatiotemporally restricted ablation of *Klf4* pushes the CE cells toward EMT, resulting in less differentiated mesenchymal-like cells with increased capacity for proliferation and migration, reminiscent of squamous metaplasia. Together with previous reports, our data presented here provide evidence that KLF4 promotes CE cell fate by suppressing EMT, possibly by Wnt/β-catenin signaling and regulating survivin and cyclin-D1 in CE cells. The significance of our findings is further enhanced by the likelihood that KLF4 plays a similar role in other tissues where it is expressed including the skin, lung, intestines, and the colon by suppressing EMT and promoting the epithelial phenotype.[34,76,81,82]

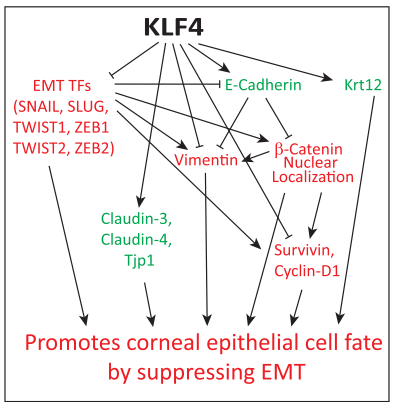

Figure 11. Schematic representation of a model for the role of Klf4 in promoting CE cell fate by suppressing EMT. The results presented in this manuscript demonstrate that Klf4 suppresses the expression of EMT transcription factors, vimentin, cyclin-D1, and survivin (indicated in *red* and connected by *blunt-ended lines*) and activates the expression of tight junction proteins Tjp1, claudin-3, and claudin-4 and CE markers Krt12 and E-cadherin (indicated in *green* and connected by *pointed arrows*). Collectively, these results reveal that KLF4 promotes corneal epithelial cell fate by suppressing EMT.

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