KLF4 Regulates Corneal Epithelial Cell Cycle Progression by Suppressing Canonical TGF-β Signaling and Upregulating CDK Inhibitors P16 and P27

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PURPOSE. Krüppel-like factor 4 (KLF4) promotes corneal epithelial (CE) cell fate while suppressing mesenchymal properties. TGF-β plays a crucial role in cell differentiation and development, and if dysregulated, it induces epithelial-mesenchymal transition (EMT). As KLF4 and TGF-β regulate each other in a context-dependent manner, we evaluated the role of the crosstalk between KLF4 and TGF-β-signaling in CE homeostasis.

METHODS. We used spatiotemporally regulated ablation of Klf4 within the adult mouse CE in ternary transgenic Klf4Krt12rtTA/rtTA/ Tet-O-Cre (Klf4LoxP/LoxP/ Krt12rtTA/rtTA/ Tet-O-Cre) mice and short hairpin RNA (shRNA)-mediated knockdown or lentiviral vector-mediated overexpression of KLF4 in human corneal limbal epithelial (HCLE) cells to evaluate the crosstalk between KLF4 and TGF-β-signaling components. Expression of TGF-β signaling components and cyclin-dependent kinase (CDK) inhibitors was quantified by quantitative PCR, immunoblots, and/or immunofluorescent staining.

RESULTS. CE-specific ablation of Klf4 resulted in (1) upregulation of TGF-β1, -β2, -β1, and -β2; (2) downregulation of inhibitory Smad7; (3) hyperphosphorylation of Smad2/3; (4) elevated nuclear localization of phospho-Smad2/3 and Smad4; and (5) downregulation of CDK inhibitors p16 and p27. Consistently, shRNA-mediated knockdown of KLF4 in HCLE cells resulted in upregulation of TGF-β1 and -β2, hyperphosphorylation and nuclear localization of SMAD2/3, downregulation of SMAD7, and elevated SMAD4 nuclear localization. Furthermore, overexpression of KLF4 in HCLE cells resulted in downregulation of TGF-β1, -β1, and -β2 and upregulation of SMAD7, p16, and p27.

CONCLUSIONS. Collectively, these results demonstrate that KLF4 regulates CE cell cycle progression by suppressing canonical TGF-β signaling and overcomes the undesirable concomitant decrease in TGF-β-dependent CDK inhibitors p16 and p27 expression by directly upregulating them.

Keywords: KLF4, TGF-β, SMAD, EMT, corneal epithelium, squamous neoplasia

The stratified squamous corneal epithelium (CE) plays an important role in protecting the rest of the eye from external threats and ensuring a clear and refractile optical path for the incident light to reach the photoreceptors in the retina. A proper balance between proliferation in the basal cell layers and differentiation of the suprabasal cells is necessary for CE homeostasis. Disruption of this balance results in a spectrum of tumors collectively called ocular surface squamous neoplasia (OSSN). Solar ultraviolet (UV) radiation, human immunodeficiency virus (HIV), and human papilloma virus (HPV) infections are considered the main risk factors for OSSN. Despite their devastating effects on vision, the pathophysiology of OSSN remains understudied, and their molecular underpinnings are unexplored. Krüppel like-factor-4 (KLF4) and KLF5, two of the most highly expressed transcription factors in the cornea, play important roles in CE homeostasis. KLF4 regulates its target gene expression by binding GC-rich sequences such as CACCC elements and promotes epithelial cell fate by suppressing epithelial-mesenchymal transition (EMT). The expression and function of KLF4 is regulated in a context-dependent manner at the level of transcription, mRNA- and protein-stability, and by post-translational modification. KLF4 is induced by serum starvation, oxidative stress, sodium butyrate, selenium, IFNγ, and cAMP but is suppressed by TGF-β. Within the CE, KLF4 is expressed at a low level in a mosaic pattern in the basal epithelial cells and in a uniformly robust pattern in the differentiated superficial cell layers consistent with its prodifferentiation role. Although dele-
tion, mutation, or altered expression of KLF4 is associated with different tumors, its involvement in OSSN has not been investigated.

TGF-β signaling plays a crucial role in epithelial cell growth, proliferation, differentiation, and development, and if dysregulated, it induces epithelial-mesenchymal transition (EMT). TGF-β pathway is disrupted in different cancers including hepatocellular, colorectal, gastrointestinal, and head and neck squamous cell carcinomas. Different steps of tumor progression, including tumor initiation, stemness, invasion, metastasis, and resistance to therapy are associated with specific transitional states of EMT defined by unique transcriptional landscapes regulated by EMT transcription factors such as Zeb1, Zeb2, Snail, Slug, Twist1, and Twist2. Previously, we reported that CE-specific ablation of Klf4 results in dysregulated cell proliferation, loss of epithelial features, and gain of mesenchymal characteristics.

KLF4 and TGF-β Crosstalk in Corneal Epithelium

**Figure 1.** Klf4ΔACE cells display increased expression of TGF-β1, -β2, -βR1, and -βR2. (A) qPCR reveals that the Klf4ΔACE corneas display significant upregulation of TGF-β1, -β2, -βR1, and -βR2 transscripts. (B) Immunofluorescent stain confirms the elevated CE expression and localization of TGF-β1, -β2, -βR1, and -βR2 in Klf4ΔACE (v–viii) compared with the control (i–iv) cornea. (C) Bar graph showing quantified relative fluorescence intensity. Significant increase in TGF-β1, -β2, -βR1, and -βR2 was observed in Klf4ΔACE corneas compared with the control. Data show results from three independent experiments performed in duplicate and reported as means ± SEM. Images were acquired at 40×; scale bar: 40 μm.
reminiscent of EMT, the loss of KLF4 exacerbates oncogenic TGF-β signaling in hepatocellular carcinomas, and TGF-β-induced EMT is accompanied by KLF4 downregulation in both HCLE cells and prostate tumors. Here, we tested the hypothesis that KLF4 promotes the antitumorigenic environment and contributes to CE homeostasis by suppressing TGF-β signaling and upregulating cell cycle inhibitors. Our results indicate that KLF4 promotes the CE phenotype by suppressing SMAD2/3-mediated TGF-β signaling and overcomes the undesirable concomitant decrease in TGF-β-dependent expression of p16 and p27 by directly upregulating them.

**METHODS**

**Mice**

CE-specific ablation of Klf4 was achieved by feeding 8- to 10-week-old ternary transgenic Klf4 LoxP/LoxP/Krt12rtTA/rtTA/Tet-O-Cre/ mice of a mixed genetic background with doxycycline chow as detailed previously. Age-matched littermates fed with regular 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.

**HCLE Cells**

HCLE cells (from Dr. Ilene Gipson, Harvard University, Cambridge, MA, USA) were cultured as previously described in keratinocyte serum-free medium (Gibco-Invitrogen Corp., Rockville, MD, USA) supplemented with 25 µg/mL bovine pituitary extract, 0.2 ng/mL epidermal growth factor, and 0.3 M calcium chloride in a humidified incubator with 5% CO2 at 37°C.

**Lentiviral Vector Construction and Transduction in HCLE Cells**

A lentiviral vector containing cytomegalovirus (CMV) promoter-directed green fluorescent protein (GFP) and KLF4 (pLenti-CMV-GFP-T2A-KLF4) was constructed by cloning PCR-amplified KLF4 and GFP cDNAs using primers that inserted the ribosome skipping motif peptide T2A (see Supplementary Table S1 for primer sequences) in the plasmid pLenti-CMV-GFP-Puro (Addgene, Cambridge, MA, USA). Lentiviral packaging was performed in Lenti-X 293T cells with Lenti-X Packaging Single Shots (TaKaRa, Kusatsu, Japan). HCLE cells at 70% confluence were infected with pLenti-CMV-GFP or pLenti-CMV-GFP-T2A-KLF4 lentivirus at a multiplicity of infection of 3 in the presence of 6 µg/mL polybrene. After 5 days of transduction, GFP-positive cells were sorted via flow cytometry and propagated to generate HCLE-WT or HCLE-KLF4 cells, respectively.

**Short hairpin RNA-Mediated KLF4 Knockdown**

HCLE cells at 70% to 80% confluence were transfected with plasmids encoding short hairpin RNA targeting KLF4 (shRNA-1-4), or a scrambled negative control shRNA (shRNA-5; Qiagen, Germantown, MD, USA). The transfected cells were selected using media containing 1 µg/mL G418 for 2 weeks. Mixed pools of the shRNA-transfected KLF4 knocked down HCLE cells (HCLE-KD) were tested for KLF4 expression by quantitative PCR (qPCR), immunobots, and immunofluorescent staining and used in further experiments.
Antibodies

All antibodies used are presented in Supplementary Table S2 and were previously tested and confirmed to cross-react with corresponding mouse antigens.

Total RNA Isolation and qPCR

Total RNA from mouse corneas or HCLE cells was isolated using EZ-10 spin column total RNA mini-prep kit (Bio Basic, Inc., Amherst, NY, USA) and used for cDNA synthesis with mouse Maloney leukemia virus reverse transcriptase (Promega, Madison, WI, USA). qPCR was performed using SYBR Green gene expression assays in triplicate using an ABI StepOne Plus thermocycler with appropriate endogenous controls (Applied Biosystems, Foster City, CA, USA). The sequence of primers used for qPCR is shown in Supplemental Table S1.

ImmunobLOTS

Immunoblots were carried out as detailed previously. WT or Klf4Δ/Δ corneas were homogenized in urea buffer (8.0 M urea, 0.1% Triton X-100, 3% β-mercaptoethanol, 0.2% SDS, and protease inhibitors). HCLE cells were lysed in RIPA buffer with protease inhibitors and phosphatase inhibitors (Thermo Fisher Scientific, Waltham, MA, USA). qPCR was performed using SYBR Green gene expression assays in triplicate using an ABI StepOne Plus thermocycler with appropriate endogenous controls (Applied Biosystems, Foster City, CA, USA). The sequence of primers used for qPCR is shown in Supplemental Table S1.

Immunofluorescent Staining

HCLE cells grown on coverslips or 8-μm-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), permeabilized (0.1% Triton X-100 in PBS), and washed thrice with PBS for 5 minutes each. Samples were then treated with glycine for 20 minutes, washed with PBS, blocked with 10% goat or donkey serum in PBS for 1 hour at 23°C, washed twice with PBS, and incubated with the appropriate dilution of the primary antibody for 2 hours at 23°C or overnight at 4°C. After further washing in PBS, the slides were incubated with appropriate Alexafluor-conjugated secondary antibody (Molecular Probes, Carlsbad, CA, USA) and quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA). β-Actin was used as a loading control for normalizing the data.

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Viewer software. After converting the images to grayscale, their relative fluorescence intensity was quantified using Image J software (National Institutes of Health).

**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* ≤ 0.05 considered statistically significant.

**RESULTS**

**KLF4 Negatively Regulates the Expression of TGF-β1, -β2, and Their Receptors in the CE**

Three lines of evidence warranted a further examination of the relationship between KLF4 and TGF-β signaling within the CE: (1) KLF4 inhibits EMT by upregulating epithelial genes and suppressing mesenchymal genes; (2) TGF-β induces EMT by suppressing KLF4; and (3) KLF4 and TGF-β regulate each other in a context-dependent manner. Toward this, we quantified TGF-β signaling components in Klf4+/ACE corneas. qPCR revealed statistically significant upregulation of TGF-β1, -β2, -βR1, and -βR2 in the Klf4+/ACE corneas compared with the control (Fig. 1A). Consistently, immunofluorescent stain revealed significant upregulation of TGF-β1-β2, -βR1, and -βR2 expression in Klf4+/ACE corneas (Figs. 1B, 1C), indicating that...

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**Figure 4.** Canonical Smad2/3 signaling pathway is activated in Klf4+/ACE cells. (A) Expression of Smad2/3, pSmad2/3, and Smad4 in Klf4+/ACE and control CE visualized by immunofluorescent stain. (B) Bar graph shows fluorescence intensity, pSmad2/3:Smad2/3 ratio (left), and Smad4 (right). Data show results from three independent experiments reported as means ± SEM. Image acquired at 40×; scale bar: 40 μm.

**Figure 5.** SMAD2/3 signaling pathway is activated in HCLE-KD cells. (A) Immunoblots showing pSMAD2/3 and total smad2/3 in control and HCLE-KD cells. β-Actin serves as loading control. (B) Bar diagram shows the quantified densitometric ratio of pSmad2/3:Smad2/3. Data show results from three independent experiments reported as mean ± SEM. (C) Immunofluorescent stain showing increased expression and localization of pSMAD2/3 in shRNA-2 and -4. No nuclear pSMAD2/3 was observed in controls. (D) Immunofluorescent stain showing increased nuclear translocation of SMAD4 in HCLE-KD cells compared with the control. Images were acquired at 20×; scale bar: 60 μm.
KLF4 negatively regulates TGF-β1, -β2, and their receptors within the CE.

To further confirm the role of KLF4 in regulating TGF-β signaling pathway in CE, we overexpressed KLF4 in HCLE cells using lentiviral transduction. qPCR revealed a 67-fold increase in KLF4 transcripts in HCLE-KLF4 cells compared with the HCLE-WT control (Fig. 2A). Robust overexpression and predominantly nuclear accumulation of KLF4 in HCLE-KLF4 cells were confirmed by immunoblots and immunofluorescent stain, respectively (Figs. 2B, 2C). qPCR also revealed that KLF4 overexpression resulted in a significant decrease in TGF-β1 (0.26-fold), TGF-β2 (0.89-fold), TGF-βR1 (0.44-fold), and TGF-βR2 (0.29-fold) in HCLE-KLF4 compared with the HCLE-WT cells, concomitant with a significant 15-fold increase in SMAD7, a cellular inhibitor of TGF-β signaling (Fig. 2D). Immunofluorescent stain displayed a consistent decrease in KLF4 expression levels in HCLE cells using plasmids expressing different anti-KLF4 shRNAs. qPCR revealed efficient knockdown of KLF4 in HCLE cells transfected with anti-KLF4 shRNA-1 (by 80%), -shRNA-2 (by 85%), and -shRNA-4 (by 82%), but not in those transfected with the control scrambled shRNA-5 (by 15%) compared with the untransfected cells (Fig. 3A). Immunoblots confirmed efficient knockdown of KLF4 protein in HCLE cells transfected with shRNA-2 (by 97%) and -4 (by 85%), but only a moderate decrease in those transfected with shRNA-1 (by 60%) and a marginal decrease in those transfected with control shRNA-5 (by 20%; Fig. 3B), which was further corroborated by immunofluorescent stain (Fig. 3C). qPCR also revealed a significant increase in TGF-β1, -β2, -β1R, and -β2R transcripts in shRNA-2- and -4-transfected cells compared with shRNA-5 or control HCLE cells (Fig. 3D), which was further confirmed by immunofluorescent stain (Fig. 3E). Taken together, these results are consistent with a strong inverse relationship between of KLF4 and TGF-β signaling within the CE cells.

Klf4 Ablation Activates SMAD2/3-Mediated Canonical TGF-β Signaling

TGF-β mediates its activity either by canonical SMAD signaling or via the noncanonical mitogen-activated protein kinase pathway.50 To determine which pathway is activated on Klf4 ablation within the CE, we next examined Smad2/3 expression and phosphorylation status in the control and Klf4ΔACE corneas and in shRNA-2-, shRNA-4-, or shRNA-5-transfected HCLE cells. Immunofluorescent stain revealed no significant change in Smad2/3 expression in the Klf4ΔACE corneas (Figs. 4Ai, Aii). In contrast, phosphoSmad2/3 (pSmad2/3) levels were significantly increased, with elevated nuclear translocation in the Klf4ΔACE compared with the control corneas (Figs. 4Aiii, 4Av, 4B). Although there was no significant change in the overall levels of Smad4, it was predominantly localized in the nucleus in the Klf4ΔACE corneas (Figs. 4Av, 4Aiii). Consistent with these results, immunoblots and immunofluorescent stain revealed elevated Smad2/3 phosphorylation and nuclear localization in shRNA-2- and shRNA-4-transfected HCLE cells compared with those transfected with scrambled shRNA-5 and the untransfected controls (Figs. 5A–5C). Furthermore, Smad4 was predominantly localized in the nucleus in shRNA-2- and -4-transfected HCLE cells compared with the shRNA-5 control transfected cells (Fig. 5D). Together, these results indicate that KLF4 knockdown activates SMAD2/3-mediated canonical TGF-β signaling.

KLF4 Inhibits TGF-β Signaling by Upregulating SMAD7 Expression

As qPCR revealed a robust 15-fold increase in expression of TGF-β signaling inhibitor SMAD7 in HCLE-KLF4 cells (Fig. 2B), we further examined Smad7 in Klf4ΔACE and HCLE cells. Immunofluorescent stain revealed significant decrease in SMAD7 expression in Klf4ΔACE corneas and in shRNA-2 and -4 HCLE cells compared with the controls (Figs. 6A, 6B). Reciprocally, SMAD7 expression was significantly upregulated in HCLE-KLF4 cells (Fig. 6C). Collectively, these results indicate that KLF4 controls SMAD2/3-mediated TGF-β signaling in CE cells by upregulating SMAD7.

KLF4 Upregulates Cell Cycle Arrest Proteins p16 and p27

TGF-β signaling upregulates cyclin-dependent kinase (CDK) inhibitors p16 and p27 in different cell types.51–54 Considering that the results above suggest that TGF-β signaling is activated...
upon suppression of KLF4, it was not clear how Klf4 ablation results in increased proliferation in Klf4<sup>−/−</sup>CE cells. To clarify this, we evaluated p16 and p27 expression in Klf4<sup>−/−</sup>CE and HCLE-KLF4 cells. Klf4<sup>−/−</sup>CE corneas displayed a decrease in p16 and p27 expression compared with the control (Fig. 7A). Consistent with these results, HCLE-KLF4 cells expressed increased levels of p16 (2.5-fold) and p27 (1.5-fold) compared with the control HCLE-WT cells (Fig. 7B). Taken together, these results suggest that KLF4 overcomes the undesirable effect of unabated cell proliferation upon suppression of TGF-β signaling by promoting the expression of CDK inhibitors p16 and p27, which in turn inhibit cell cycle progression.

**DISCUSSION**

Previously we reported that Klf4 ablation results in CE hyperplasia with compromised barrier function, increased proliferation, and migration, consistent with EMT commonly associated with squamous cell carcinomas. Here, we provide evidence that (1) activation of Smad2/3-mediated TGF-β signaling underlies the initiation of EMT upon down-regulation of Klf4, and (2) decreased expression of CDK inhibitors p16 and p27 underlies the higher rate of proliferation in Klf4<sup>−/−</sup>CE corneas (Fig. 8). Collectively, these results suggest that KLF4, when present in normal levels, suppresses TGF-β signaling by promoting the expression of inhibitory SMAD7 and overcomes the undesirable concomitant decrease in TGF-β-dependent expression of p16 and p27 by directly upregulating them (Fig. 8).

The unique positioning of proliferating and differentiated cells in the complex architecture of stratified squamous corneal epithelium makes it a useful model to understand the important biological events essential for epithelial homeostasis and pathology. KLF4 and TGF-β both influence cell proliferation and serve as anti-or protumorigenic agents in a context-dependent manner. For example, TGF-β regulates KLF4
positively in vascular smooth muscle cells and negatively in mink lung epithelial cells. Although the individual roles of KLF4 and TGF-β are well known in the cornea, the crosstalk between them was not studied previously. Our observation of altered expression of canonical TGF-β signaling pathway components in Klf4−/− corneas and HCLE-KLF4 cells provides evidence that KLF4 helps maintain appropriate levels TGF-β signaling in CE cells. In corneas subjected to injuries, and/or pathologic conditions, elevated TGF-β coupled with lower KLF4 levels initiates EMT and tumorigenic signaling.

TGF-β functions through the SMAD-dependent or SMAD-independent signaling cascade, depending on the cellular context. We observed increased SMAD2/3 phosphorylation in Klf4−/− corneas and upon shRNA-mediated knockdown of KLF4 in HCLE (HCLE-KD) cells, suggesting that canonical SMAD signaling is activated. The increased shuttling of Smad4 to the nucleus in Klf4−/− cornea, an essential step for nuclear entry of pSmad2/3, provides a favorable platform for transcription of TGF-β target genes. SMAD7 inhibits TGF-β signaling by interacting with TGF-β receptors, impeding with TGF-β receptor-SMAD2/3/SMAD4 complex formation. Upregulation of SMAD7 in HCLE-KLF4, and its downregulation in Klf4−/− corneas as well as HCLE-KD cells, suggests that KLF4 upregulates SMAD7, which in turn suppresses TGF-β signaling in CE cells (Fig. 8). Taken together, the lower levels of SMAD7 and increased pSMAD2/3 and nuclear SMAD4 in HCLE-KD cells and Klf4−/− corneas provide evidence that KLF4 regulates SMAD-mediated TGF-β signaling in CE cells.

The data presented here demonstrate that Klf4−/− corneas display elevated TGF-β signaling that induces EMT. EMT also results in apoptosis in the presence of TGF-β in many cell types. The sustained presence of TGF-β is also known to carve out a small population of proliferative cells that can evade EMT-induced apoptosis. Our previous data and current observations indicate no signs of growth arrest or apoptosis in Klf4−/− corneas. Instead, we observed hyperproliferation of CE cells likely due to the release of p16 and p27-mediated cell cycle arrest. KLF4 suppresses cyclin-D1 and promotes p16 and p27, resulting in cell cycle arrest at G1/S, which further results in cellular senescence and arrested growth. The loss of KLF4 creates a proliferative milieu by downregulating p16 and p27 and favors mesenchymal phenotype by elevating TGF-β signaling, creating a pathologic environment conducive for squamous neoplasia.

Corneal tumors are relatively rare despite frequent exposure of the cornea to adverse conditions such as UV radiation, chemical and physical insults, pathogens, and mutagens. Ocular surface squamous neoplasia (OSSN), a rare form of ocular tumor in elderly patients, occurs mainly due to UV exposure, P53 mutation, and hereditary deficiency of DNA damage. Although OSSN is visually debilitating and clinically relevant, the lack of appropriate study systems and low abundance of clinical samples makes it difficult to understand the molecular mechanisms that underlie OSSN. Considering that KLF4 is highly expressed in the CE cells, and its ablation results in EMT, it is likely that KLF4 serves as a strong tumor suppressor in CE cells, protecting them from frequent oncogenic challenges.

In summary, this report provides evidence that KLF4 regulates corneal epithelial cell cycle progression by suppressing canonical TGF-β signaling and upregulating CDK inhibitors P16 and P27. Interestingly, the related transcription factor KLF5 also serves as a tumor suppressor by downregulating EGFR signaling and upregulating p15, indicating that multiple redundant mechanisms operate in concert to regulate cell proliferation. Although KLF4, KLF5, and TGF-β are highly expressed in the cornea, and are frequently altered in many cancers, their involvement in OSSN is not well established. It would be worthwhile examining human OSSN samples to further verify our findings, as better understanding of the crosstalk between KLF4, KLF5, and TGF-β in the CE would help us further unravel their involvement in epithelial tumor development and progression.

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