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 Klf4FACS (Klf4Cre/LoxP/ Krt12DTA/DTA/ 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, βR1, and βR2; (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, βR1, and βR2 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
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 transcripts. (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 40x; scale bar: 40 μm.
reminiscent of EMT,9,10 (2) the loss of KLF4 exacerbates oncogenic TGF-β signaling in hepatocellular carcinomas,37,38 (3) TGF-β–induced EMT is accompanied by KLF4 downregulation in both HCLE cells10 and prostate tumors,10,45 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 D/D CE (Klf4 LoxP/LoxP/Krt12rtTA/rtTA/Tet-O-Cre/C0) mice of a mixed genetic background with doxycycline chow as detailed previously.9 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 described46 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).47 This resulted in the addition of a single N-terminal amino acid (proline) on KLF4. 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-4), or a scrambled negative control shRNA (shRNA-5; Qiagen, Germantown, MD, USA). The transfected cells were selected using media containing 1 mg/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), immunoblots, 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 Klf4<sup>D<sub>D</sub>/D<sub>D</sub></sup> 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 were lysed in RIPA buffer with protease inhibitors and phosphatase inhibitors (Thermo Fisher Scientific, Waltham, MA, USA). A total of 20 to 30 μg total protein in the supernatant was separated on 4 % to 12 % gradient polyacrylamide gels using 2-(N-morpholino)ethanesulfonic acid (MES) or 3-(N-morpholino)propanesulfonic acid (MOPS) buffer and transferred onto polyvinylidine fluoride (PVDF) membranes. The membranes were blocked, incubated overnight with primary antibody at 4 °C, washed thrice with PBST, blocked with 10 % goat or donkey serum in PBS for 1 hour at 23 °C, washed thrice with PBST, and incubated with the appropriate Alexafluor-conjugated secondary antibody (Molecular Probes, Carlsbad, CA, USA) for 1 hour at 23 °C, washed twice with PBS, and incubated with 4,6-diamidino-2-phenylindole (DAPI) and mounted with Aqua-Poly/Mount (Polysciences, Warrington, PA, USA). Images were acquired using an Olympus IX81 microscope (Olympus America, Inc., Center Valley, PA, USA). Image processing was performed using FV10-ASW4.2.
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 \( P \leq 0.05 \) 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-/- CE corneas. qPCR revealed statistically significant upregulation of TGF-β1, -β2, -βR1, and -βR2 in the Klf4-/- CE corneas compared with the control (Fig. 1A). Consistently, immunofluorescent stain revealed significant upregulation of TGF-β1-β2, -βR1, and -βR2 expression in Klf4-/- CE corneas (Figs. 1B, 1C), indicating that...
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 TGF-β1 and -β2 expression in the HCLE-KLF4 compared with the HCLE-WT cells (Fig. 2E). Together with Fig. 1, these results suggest that KLF4 negatively regulates TGF-β signaling within the CE.

In a reciprocal approach, we knocked down the endogenous 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, -βR1, and -βR2 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. 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 Klf4Δ/ΔCE 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 Klf4Δ/ΔACE corneas (Figs. 4Ai, Aii). In contrast, phospho-Smad2/3 (pSmad2/3) levels were significantly increased, with elevated nuclear translocation in the Klf4Δ/ΔACE compared with the control corneas (Figs. 4Aiii, 4Aiv, 4B). Although there was no significant change in the overall levels of Smad4, it was predominantly localized in the nucleus in the Klf4Δ/ΔACE corneas (Figs. 4Aiv, 4Avi). 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 Klf4Δ/ΔACE and HCLE cells. Immunofluorescent stain revealed significant decrease in SMAD7 expression in Klf4Δ/ΔACE 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. 2B), showing significant decrease in SMAD7 expression in HCLE-KD compared with the control cells. Images were acquired at 20×; scale bar as shown. (D) qPCR confirming the downregulation of SMAD7 transcripts in HCLE-KD compared with the control cells. Data show results from two independent experiments performed in triplicate and reported as means ± SEM.

**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. 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−/− corneas.8,9,11 To clarify this, we evaluated p16 and p27 expression in Klf4−/− corneas compared with the control or in HCLE-KLF4 compared with the HCLE-WT. Change in p27 levels was not significant. Data show results from three independent experiments reported as mean ± SEM. Images were acquired at 40×; scale bar: 40 μm for control and Klf4−/− cells. For HCLE-WT and HCLE-KLF4 cells, images were acquired at 20×; scale bar: 60 μm.

FIGURE 7. KLF4 regulates p16 and p27. (A) Immunofluorescent stain for p16 (i, ii) and p27 (iii, iv) in control and Klf4−/− corneas. (B) Immunofluorescent stain in HCLE-KLF4 and HCLE-WT cells for p16 (i, ii) and p27 (iii, iv). Bar graphs on the right indicate significant decrease in p16 levels in Klf4−/− compared with the control or in HCLE-KLF4 compared with the HCLE-WT. Change in p27 levels was not significant. Data show results from three independent experiments reported as mean ± SEM. Images were acquired at 40×; scale bar: 40 μm for control and Klf4−/− cells. For HCLE-WT and HCLE-KLF4 cells, images were acquired at 20×; scale bar: 60 μm.

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

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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.9,10 Here, we provide evidence that (1) activation of Smad2/3-mediated TGF-β signaling underlies the initiation of EMT upon downregulation of Klf4, and (2) decreased expression of CDK inhibitors p16 and p27 underlies the higher rate of proliferation in Klf4−/− 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).

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KLF4 and TGF-β Crosstalk in Corneal Epithelium

Figure 8. Crosstalk between KLF4 and TGF-β signaling plays an important role in CE homeostasis. In homeostatic condition, KLF4 controls cell proliferation by regulating proper cellular levels of SMAD7, p16, and p27. SMAD7 inhibits TGF-β signaling cascade suppressing EMT, whereas p16 and p27 regulate cell cycle progression. Decreased levels of KLF4 result in downregulation of SMAD7, which in turn leads to activation of TGF-β signaling and EMT, as well as downregulation of p16 and p27 that leads to dysregulated cell cycle progression through the G1/S phase.

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 creates a pool of differentiated myofibroblast-like cells that secrete extracellular matrix proteins, leading to fibrosis. 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 hyper-proliferation of CE cells likely due to the release of p16 and p27-mediated cell cycle arrest. KLF4 suppresses cyclinD1 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. 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 EGF 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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