Original Article

KLF4 prevents epithelial to mesenchymal transition in human corneal epithelial cells *via* endogenous TGF-β2 suppression

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ABSTRACT

Introduction: Krüppel-like factor 4 (KLF4) is considered one of the Yamanaka factors, and recently, we and others have shown that KLF4 is one of the transcription factors essential for reprogramming non-human corneal epithelial cells (HCECs) into HCECs. Since epithelial to mesenchymal transition (EMT) suppression is vital for homeostasis of HCECs *via* regulation of transcription factors, in this study, we aimed to investigate whether KLF4 prevents EMT in HCECs and to elucidate the underlying mechanism within the canonical TGF-β signalling pathway, which is involved in corneal epithelial wound healing.
Methods: HCECs were collected from cadaver donors and cultivated. We generated *KLF4*-knockdown (KD) HCECs using siRNA transfection and analysed morphology, gene or protein expression, and endogenous TGF-β secretion. *KLF4* was overexpressed using lentiviral *KLF4* expression vectors and underwent protein expression analyses after TGF-β2 treatment.
Results: *KLF4*-KD HCECs showed a fibroblastic morphology, downregulation of the epithelial markers, keratin 12 and keratin 14, and upregulation of the mesenchymal markers, fibronectin 1, vimentin, N-cadherin, and *SLUG*. Although *E-cadherin* expression remained unchanged in *KLF4*-KD HCECs, immunocytochemical analysis showed that E-cadherin—positive adherens junctions decreased in *KLF4*-KD HCECs as well as the decreased total protein levels of E-cadherin analysed by immunoblotting. Moreover, within the TGF-β canonical signalling pathway, TGF-β2 secretion by HCECs increased up to 5 folds, and several TGF-β—associated markers (*TGFB1*, *TGFB2*, *TGFBR1*, and *TGFBR2*) were significantly upregulated up to 6 folds in the *KLF4*-KD HCECs. SMAD2/3, the main signal transduction molecules of the TGF-β signalling pathway, were found to be localised in the nucleus of *KLF4*-KD HCECs. When *KLF4* was overexpressed, cultivated HCECs showed upregulation of epithelial markers, keratin 14 and E-cadherin, indicating the contributory role of KLF4 in the homeostasis of human corneal epithelium *in vivo*. In addition, *KLF4* overexpression in HCECs resulted in decreased SMAD2 phosphorylation and altered nuclear localisation of SMAD2/3, even after TGF-β2 treatment.
Conclusions: These results show that KLF4 prevents EMT in HCECs and suggest a novel role of KLF4 as an endogenous TGF-β2 suppressor in the human corneal epithelium, thus highlighting the potential of KLF4 to prevent EMT and subsequent corneal fibrotic scar formation by attenuating TGF-β signalling.

1. Introduction

The corneal epithelium, consisting of several squamous epithelial layers, forms the outermost surface of the cornea. Epithelial to mesenchymal transition (EMT) is the process by which cells lose their epithelial characteristics and gain mesenchymal properties. In human corneal epithelial cells (HCECs), EMT suppression supports a healthy and transparent corneal epithelium, which is vital for homeostasis of HCECs *via* regulation of transcription factors [1,2]. Recently, we and others demonstrated that corneal epithelial sheets can be obtained from human induced pluripotent stem cells or non-corneal epithelial cells [1,3,4], and EMT suppression is also a vital condition for maintaining such corneal sheets.

Krüppel-like factor 4 (KLF4) is a zinc finger-containing transcription factor that regulates cell growth, proliferation, and differentiation [5–7], and is considered one of the Yamanaka reprogramming factors [8]. KLF4 has also been shown as one of the transcription factors essential in reprogramming non-HCECs into HCECs and maintaining human corneal epithelium homeostasis [1,4]. In addition, our previous findings using cap analysis of gene expression (CAGE) revealed that KLF4 co-regulates HCEC-specific genes in conjunction with PAX6, another key transcription factor in HCECs [9].

KLF4 has been shown to regulate EMT and function as an oncogene or a tumour-suppressor gene depending on the cell type [10–13]. In the murine corneal epithelium, Klf4 is reported to play an important role in cell differentiation and maintenance of the barrier function or epithelial characteristics [2,14,15], and one report shows that KLF4 suppresses mesenchymal properties [2]. Whilst EMT is also involved in corneal epithelial wound healing [16,17], the detailed mechanism underlying the role of KLF4 between this EMT suppression and wound healing has not yet been fully elucidated. Since TGF-β signalling pathway is well known to drive EMT [18,19], in this study, we investigated the involvement of *KLF4* within EMT in HCECs, and the underlying mechanism of the TGF-β signalling pathway. By elucidating the underlying mechanism, KLF4 can be explored as a therapeutic target for corneal wound healing.

2. Materials and methods

2.1. Cell culture

Research-grade corneoscleral rims of human cadaver donors were procured from the eye bank CorneaGen Inc. (Seattle, WA, USA) and were handled in accordance with the tenets of the Declaration of Helsinki. Corneal limbal cells were carefully collected as previously described [9]. Cells were seeded at 1×10^4 cells/cm^2 onto plastic tissue culture plates coated with 0.5 μg/cm^2 laminin 511 (iMatrix-511, Nippi, Tokyo, Japan). Cells were cultured in corneal epithelium maintenance medium (CEM), as it is reported to be suitable for HCEC maintenance [20], and incubated with 5% CO$_2$ at 37 °C. CEM consists of DMEM:F-12 medium (1:1) containing L-glutamine and 2.438 g/L sodium bicarbonate (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 2% B-27 (Thermo Fisher Scientific), 10 μM of Y-27632 (Wako Pure Chemical Industries, Osaka, Japan), 10–20 ng/mL of human recombinant KGF/FGF-7 (R&D Systems, Minneapolis, MN, USA), and 1% Penicillin-Streptomycin (Thermo Fisher Scientific).

2.2. RNA interference

Small interfering RNAs (siRNAs) against *KLF4* (5′-GCAGCUU-CACCUAUCCGAUTT-3′) and a negative control siRNA (Silencer®

Select Pre-designed (Inventoried) siRNA) were purchased from Thermo Fisher Scientific. When HCECs were confluent, they were treated with TrypLE™ Express (Thermo Fisher Scientific) for 20 min at 37 °C, to create a single-cell suspension and seeded at 2.5×10^4 cells/cm^2 onto 0.5 μg/cm^2 laminin 511-coated plastic tissue culture plates. Seeded cells were transfected with 30 nM KLF4 siRNA using Lipofectamine™ RNAiMAX Transfection Reagent (Thermo Fisher Scientific) as per the manufacturer's instructions. Immediately after seeding, HCECs were transfected at day 0, the CEM medium was changed, and the HCECs were transfected again on days 2 and 4, to sustain the knockdown. On day 7, the supernatant was collected, and the HCECs were harvested for further analysis.

2.3. Viral transduction

KLF4 was subcloned into the CSIV-CMV-MCS-IRES2-Venus vector, which was kindly provided by RIKEN BioResource Center, as previously described [4]. EmGFP was subcloned into the CSIV-CMV-IRES2-Venus vector as control. Both lentiviral vectors were co-transfected into 293T cells with pCMV-VZV-G-RSV-Rev and pCAG-HIV-gp using X-tremeGENE™ 9 DNA Transfection Reagent (Roche Applied Science, Manheim, Germany). Virus-containing supernatants were ultra-centrifuged in an Optima L-90K Preparative Ultracentrifuge for 1.5 h at $50,000 \times g$ at 4 °C. When the HCECs were sub-confluent, they were treated with TrypLE™ Express, seeded at 1×10^4 cells/cm^2 onto 0.5 μg/cm^2 laminin 511-coated plastic tissue culture plates, and subjected to viral treatment with 6 μg/mL of polybrene (Nacalai Tesque, Kyoto, Japan). HCECs were incubated for 24 h, the medium was changed, and the HCECs were further incubated for 48 h before collection.

2.4. TGF-β treatment

After 72 h of cultivation, *KLF4*-overexpressing cells and control HCECs were pre-starved for 2 h in DMEM:F12 medium (1:1) supplemented with 0.1% bovine serum albumin (Merck & Co., Kenilworth, NJ, USA) and then treated with TGF-β2 (10 ng/mL). TGF-β2–treated cells were harvested at 30 min and 4 h and analysed for immunocytochemistry or immunoblotting. SB431542, a potent and specific inhibitor of TGF-β type I receptor kinases (Wako Pure Chemical Industries; 10 μM), was used for inhibition of TGF-β signalling.

2.5. Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated from the corneal epithelial cells using RNeasy Plus Micro Kit (QIAGEN, Hilden, Germany). cDNA synthesis and quantitative PCR were performed as described previously [4]. TaqMan™ Gene Expression Assay probes (Thermo Fisher Scientific) are provided in Supplementary Fig. 1. *GAPDH* was used as the reference gene, and the relative percentages of the desired markers were determined.

2.6. Immunoblotting

Total protein was extracted from cultivated HCECs using RIPA Buffer (Nacalai Tesque) or Laemmli sample buffer for detection of phosphorylated SMAD2, and immunoblotting was performed as previously described [21]. Briefly, 10 μg of protein was subjected to SDS-PAGE and then electro-transferred. Immunoblot membranes containing protein samples were treated with antibodies at dilutions detailed in Supplementary Figs. 2 and 3. ACTB was used as reference control and protein expression levels relative to those of

ACTB were appropriately calculated using densitometric analyses using Image Lab software (BIO-RAD, Hercules, CA) as a ratio of desired protein to control protein.

2.7. Immunocytochemistry and high-content imaging assay

Immunocytochemistry was performed as previously described [3], using primary antibodies (Supplementary Fig. 2), with Alexa Fluor 488, 568, or 647-conjugated secondary antibodies (Thermo Fisher Scientific) at 1:200 dilution. Images were captured using an inverted microscope (Axio Observer. Z1, D1, Carl Zeiss, Jena, Germany). Cells were also viewed using the Operetta high-content imaging system (PerkinElmer, Waltham, MA, USA) to determine cell numbers, cell size, cell roundness, and cell border fluorescence intensity using the Harmony Software (PerkinElmer). Details of the imaging assay are listed in Table 1.

2.8. Immunoassays

Cell culture supernatants were analysed to measure total TGF-β1 and TGF-β2 using the Human TGF-β1 and TGF-β2 Quantikine ELISA kits (R&D systems) respectively, according to the manufacturer's instructions. For latent TGF-β release, samples were acidified with HCl and neutralised with NaOH. The minimum detectable dose was 31.3 pg/mL for both TGF-β1 and TGF-β2. Optical densities were measured at 540 nm with correction at 570 nm using a multiplate reader (2030 Multilabel Reader ARVO™ X4, PerkinElmer).

2.9. Statistical analysis

Data were analysed using JMP version 13.0 (SAS Institute, Cary, NC, USA). One-way analysis of variance or Mann Whitney tests were performed as appropriate. For multiple non-paired comparisons, Steel's tests were performed for each gene as appropriate. $P < 0.05$ was considered significant.

3. Results

3.1. KLF4 knockdown caused loss of phenotype in corneal epithelial cells

To determine the role of KLF4 in the process of corneal epithelial homeostasis, we generated KLF4-knockdown (KLF4-KD) HCECs using siRNA. KLF4-KD was successful in HCECs up to day 7 in vitro, as confirmed by qPCR, immunoblotting, and immunocytochemistry (Fig. 1a). In addition, cell morphology and gene expression of non-transfected HCECs compared to those of control siRNA transfected HCECs, displayed no major differences (Supplementary Figs. 4 and 5). Therefore, we used control siRNA as a comparative of KLF4 siRNA. On day 7, KLF4-KD HCECs displayed notable morphological changes to elongated and asymmetrical cells, compared to the classical polygonal and rounded control HCECs (Fig. 1b); roundness and cell size were quantified using high-content imaging (Fig. 1c). The percentage of round cells among KLF4-KD HCECs was significantly lower than that among control HCECs. Further quantification analyses of morphology revealed that on day 7, KLF4-KD mean cell size was twice that of HCEC controls, and the number of KLF4-KD HCECs was half that of control HCECs (Fig. 1c), over an equal surface area.

3.2. KLF4 KD promoted EMT in corneal epithelia

As KLF4 KD altered the morphology of HCECs, indicating EMT, we examined the gene expression of EMT-associated markers. In KLF4-KD HCECs, the epithelial genes keratin 14 (KRT14) and keratin 12 (KRT12) were significantly downregulated, whereas E-cadherin (CDH1) showed no significant changes (Fig. 2a). In addition, all the mesenchymal genes analysed, namely fibronectin 1 (FN1), vimentin (VIM), N-cadherin (CDH2), and SLUG, were significantly upregulated (Fig. 2a). Immunoblotting revealed that KRT14, KRT12, and CDH1 protein levels were reduced in KLF4-KD HCECs, compared to those in the control cells, and that FN1 and VIM proteins were expressed only in KLF4-KD HCECs (Fig. 2b). Immunocytochemical analysis showed that CDH1 adherens junction decreased in KLF4-KD HCECs (Fig. 2c). Immunofluorescence quantification revealed that membrane CDH1 in KLF4-KD HCECs was significantly lower than that in the controls (Fig. 2c bar graph).

3.3. KLF4 inhibits EMT via activation of TGF-β signalling pathway in corneal epithelia

The TGF-β signalling pathway plays a major role in EMT [18,19], and we observed EMT in KLF4-KD HCECs; therefore, we further investigated the involvement of the TGF-β canonical pathway. TGF-β1 was not detectable in control or KLF4-KD HCECs but TGF-β2 expression in KLF4-KD HCECs was significantly higher than that in the control cells (Fig. 3a). In addition, the expression of TGFB1, TGFB2, TGFBR1, and TGFBR2 increased significantly in KLF4-KD HCECs, compared to that in control cells (Fig. 3b). Interestingly, the upregulation of TGFB1, TGFB2, TGFBR1, and TGFBR2 had a trend to be repressed by SB431542, a potent and specific inhibitor of TGF-β type I receptor kinases (Supplementary Fig. 6). The expression of SMAD2/3, the main signal transduction molecules in the TGF-β signalling pathway, was higher in KLF4-KD HCECs than in the control cells; however, the difference was not statistically significant (Fig. 3c). Immunocytochemistry analyses revealed the nuclear localisation of SMAD2/3 proteins in KLF4-KD HCECs, compared to the diffuse nuclear and cytoplasmic localisation in control HCECs (Fig. 3d).

3.4. KLF4 overexpression enhanced corneal epithelial characteristics

As previously demonstrated, KLF4 is highly expressed in HCECs in vivo [4], whereas expression in vitro is comparatively low. As such, cultivated HCECs were transfected with KLF4 or control lentivirus to elucidate the specific contribution toward corneal epithelium homeostasis. We generated KLF4-overexpressing (KLF4+) HCECs and confirmed KLF4 overexpression using qRT-PCR, immunoblotting, and immunocytochemistry (Fig. 4a and Supplementary Fig. 7). GFP-positive KLF4+ HCECs appeared slightly bulging compared with GFP-positive control HCECs (Fig. 4b). Epithelial genes KRT14 and CDH1 were upregulated in KLF4+ HCECs (Fig. 4c upper panels), and the same tendency was detected at the protein level (Fig. 4d). Mesenchymal genes revealed no significant changes in KLF4+ HCECs (Fig. 4c lower panels).

Table 1
Details of high-content imaging system assays.

| Purpose	Methods
Single cell size	Cell nuclei stained with Hoechst Cytoplasm stained with keratin 14 (KRT14)
Ratio of round cells	Step 1: Analyse relative roundness index: '1' represents a circle and '0' represents a line Step 2: Count the number of round cells with a roundness index >0.9 and width:length ratio >0.5
Cell border fluorescence intensity	Cell nuclei stained with Hoechst Cytoplasm stained with KRT14 Trace region of cell border for membrane fluorescence quantification
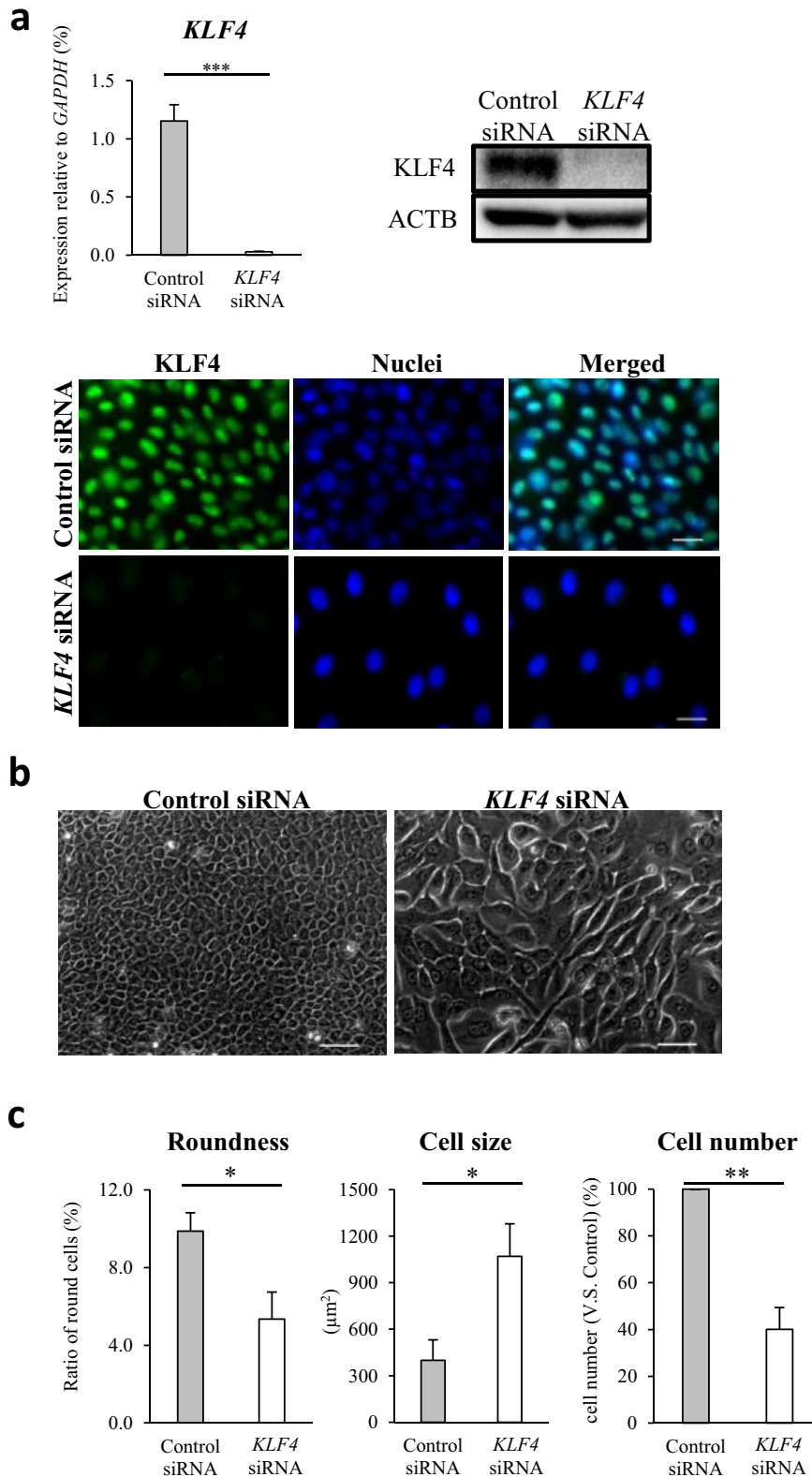
Fig. 1. *KLF4* knockdown (KD) altered the morphology and increased cell size of human corneal epithelial cells (HCECs). (a) Compared to HCECs transfected with control siRNA, complete *KLF4* KD was confirmed *via* qRT-PCR (biological $n = 8$, Mann Whitney test), and at the protein level *via* immunoblot and immunofluorescence (representative of three independent experiments; scale bars, 20 μm). (b) Morphology of HCECs transfected with control siRNA (left panel) and siRNA against *KLF4* (*KLF4* siRNA; right panel). *KLF4* KD led to the loss of the classical polygonal HCEC morphology, forming asymmetrical and elongated cells (representative of three independent experiments; scale bars, 50 μm). (c) The percentage of round cells in *KLF4*-KD HCECs was significantly lower than that in control siRNA transfected HCECs (left panel). In addition, the mean size was significantly higher (middle panel) and cell number was significantly lower (right panel) in *KLF4*-KD HCECs, than in control siRNA transfected HCECs (biological $n = 6$, Mann Whitney test). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.
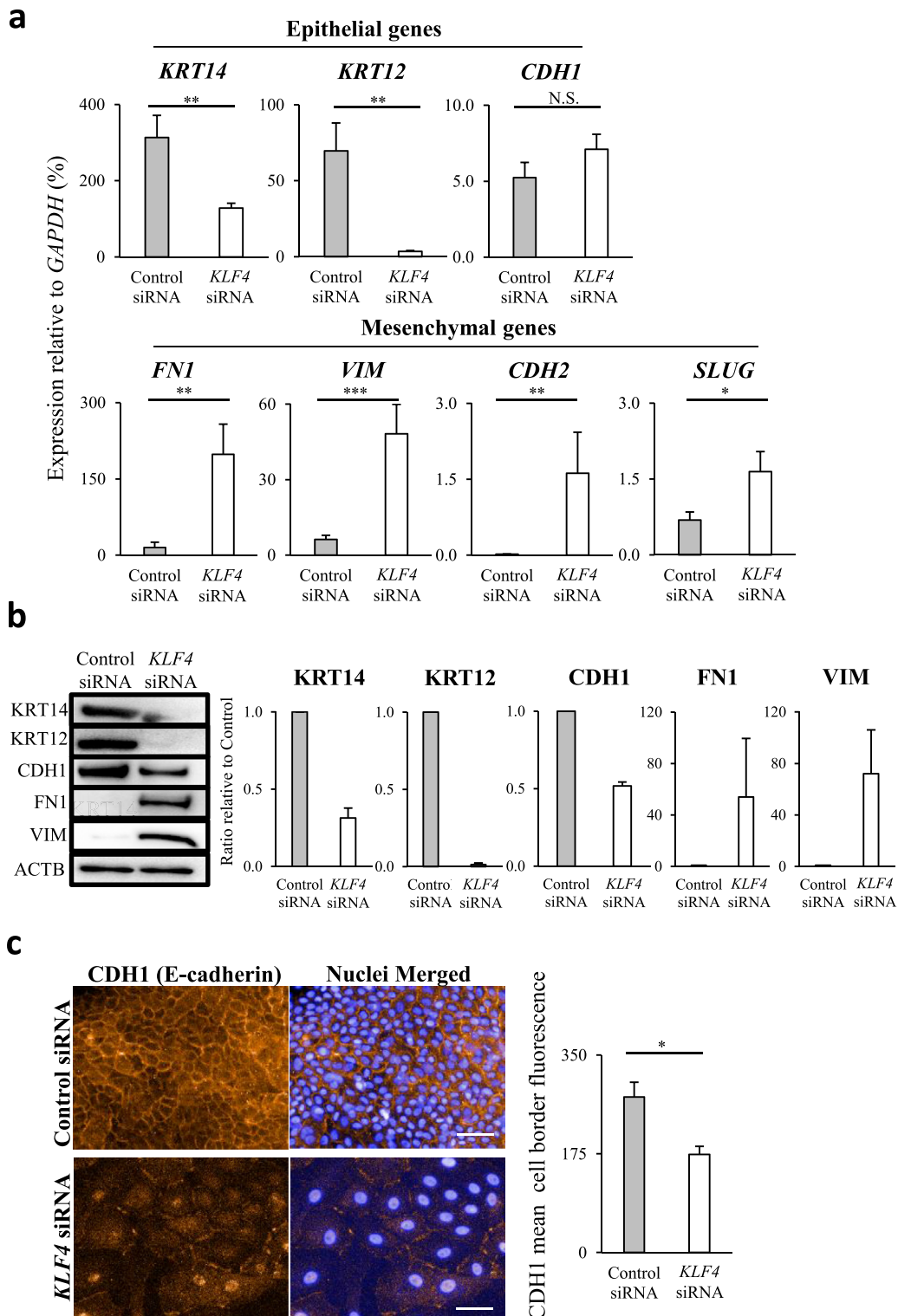
Fig. 2. *KLF4* knockdown (KD) promoted epithelial to mesenchymal transition in corneal epithelia. (a) *KLF4* KD in human corneal epithelial cells (HCECs) significantly downregulated the epithelial genes *KRT14* and *KRT12* but had no significant effect on *CDH1*. Conversely, the mesenchymal genes *FN1*, *VIM*, *CDH2*, and *SLUG* were significantly upregulated (biological $n = 8$, Mann Whitney test, N.S., not significant). (b) In *KLF4*-KD HCECs, KRT14, KRT12, and CDH1 protein levels decreased and FN1 and VIM protein was detected. Immunoblots are representative of each sample that was used for densitometric analyses (bar graph), relative to control siRNA (biological $n = 3$). (c) Immunofluorescence staining for CDH1 (E-cadherin) adherens junction did not show a clearly defined border in *KLF4*-KD HCECs (left panels; scale bars, 50 μm). Immunofluorescence quantification revealed that membrane CDH1 intensity in *KLF4*-KD HCECs was significantly lower than that in control siRNA transfected HCECs (right bar graph; biological $n = 5$, Mann Whitney test). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.
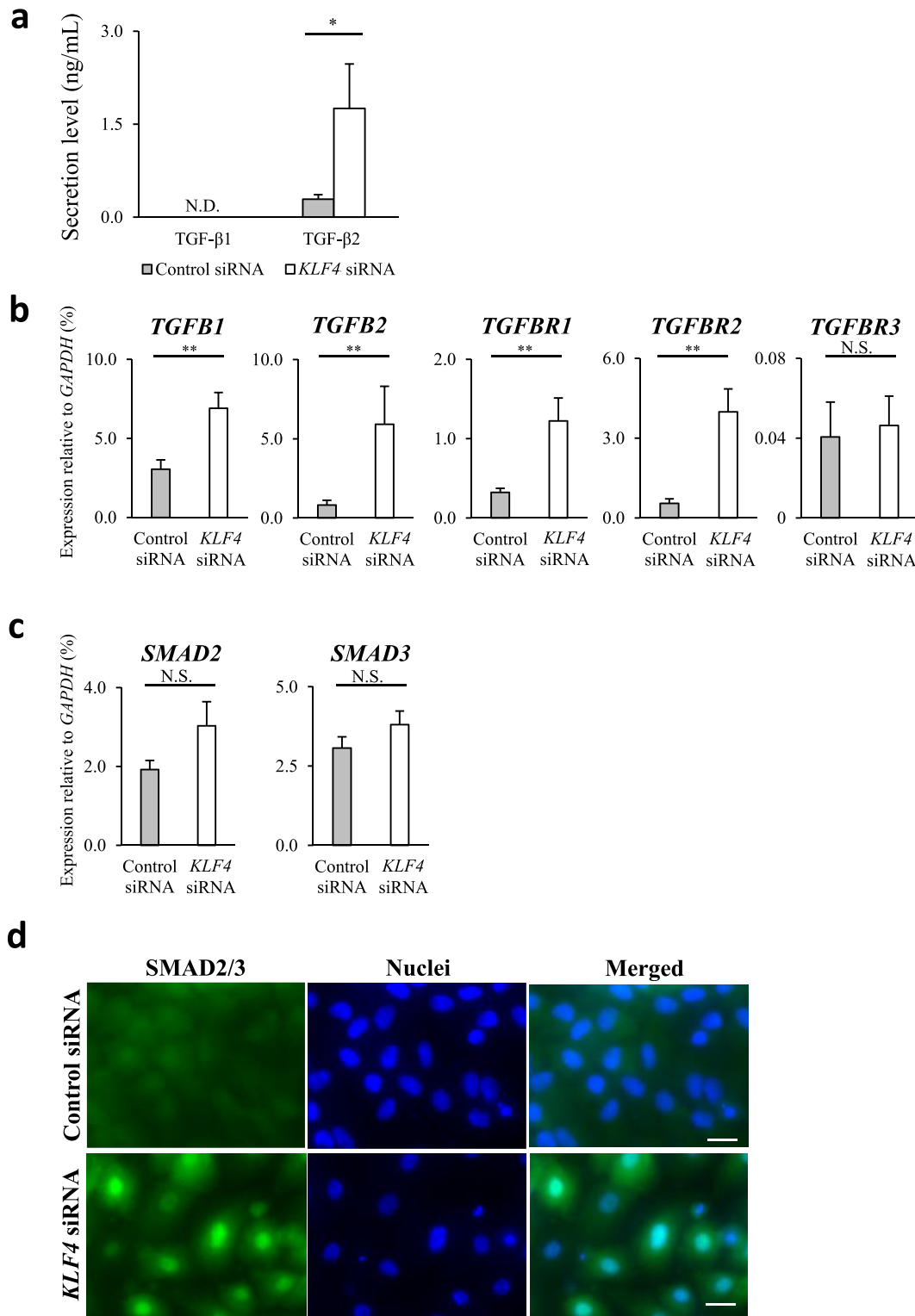
Fig. 3. KLF4 inhibited epithelial to mesenchymal transition *via* TGF-β2 activation in HCECs. (a) TGF-β2 secretion by human corneal epithelial cells (HCECs) increased significantly in *KLF4*-knockdown (*KLF4*-KD) HCECs (biological $n = 5$; Mann Whitney test, N.D., not detected). (b) Expression of *TGFB1*, *TGFB2*, *TGFBR1*, and *TGFBR2* was significantly upregulated in *KLF4*-KD HCECs (biological $n = 8$, Mann Whitney test, N.S., not significant). (c) Expression of *SMAD2* and *SMAD3* was not significantly different (biological $n = 8$, Mann Whitney test, N.S., not significant). (d) Immunocytochemistry for SMAD2/3 displayed intense nuclear staining in *KLF4*-KD HCECs but diffused staining in control siRNA transfected HCECs. (representative of three independent experiments; scale bars, 20 μm). *$P < 0.05$ and **$P < 0.01$.
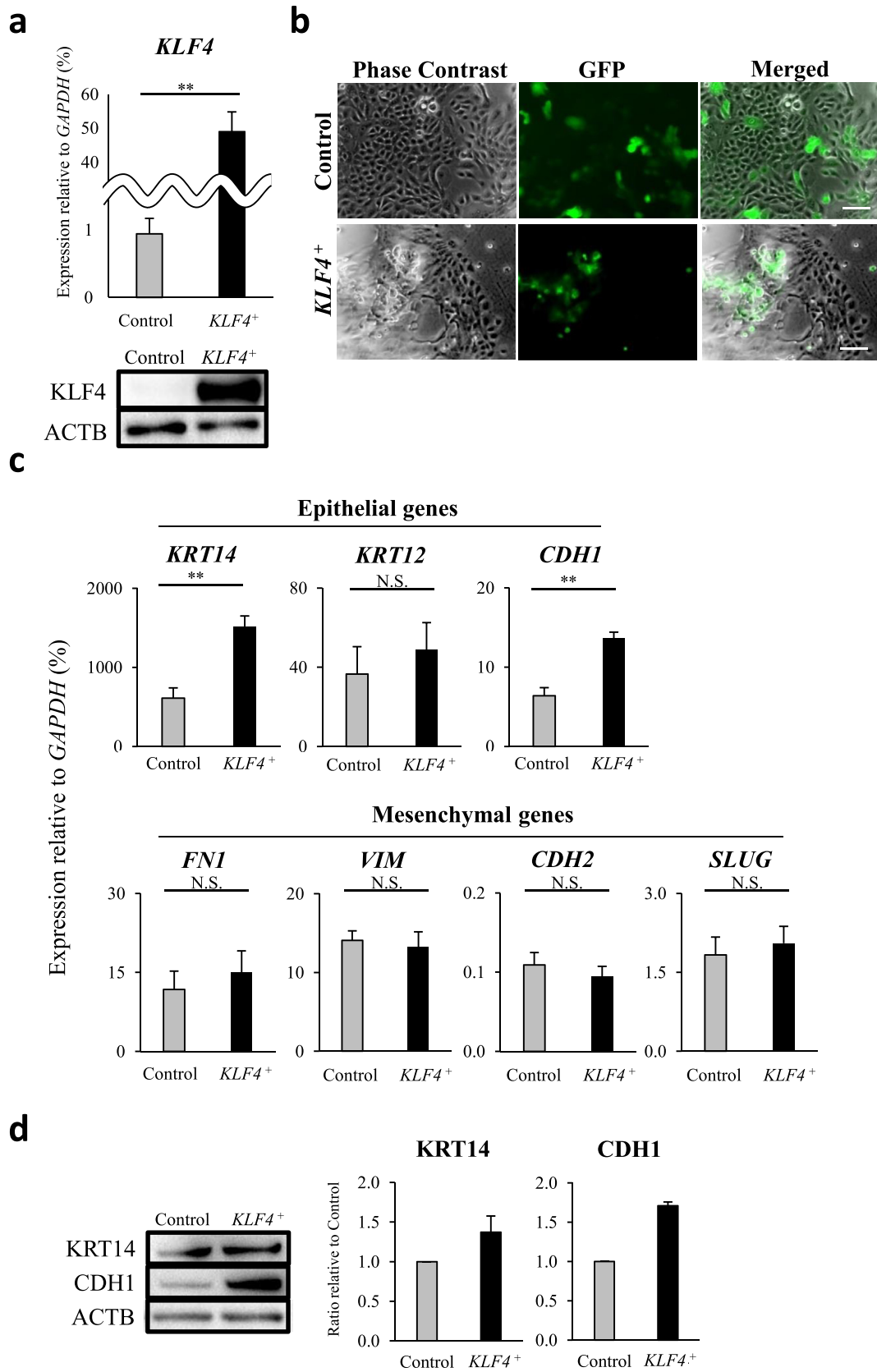
Fig. 4. KLF4 enhanced human corneal epithelium characteristics. (a) HCECs were transfected with control vector (Control) or *KLF4* vector (*KLF4*+). *KLF4* overexpression was confirmed by qRT-PCR (biological $n = 5$, Mann Whitney test) and immunoblotting (biological $n = 3$). (b) Lentiviral-positive GFP-*KLF4*+ cells appeared slightly bulging compared to GFP-control HCECs (representative of three independent experiments; scale bars, 100 μm). (c) Epithelial markers *KRT14* and *CDH1* were significantly upregulated in *KLF4*+ HCECs, whilst mesenchymal markers displayed no relevant differences (biological $n = 6$, Mann Whitney test, N.S., not significant). (d) KRT14 and CDH1 protein levels were increased by *KLF4* overexpression (biological $n = 3$). **$P < 0.01$.

3.5. Nuclear localisation of SMAD2/3 induced by TGF-β2 treatment was inhibited in KLF4-overexpressing HCECs

As TGF-β2 is known to be released from corneal epithelial cells after basement membrane injury [22,23], we hypothesised that KLF4 inhibits the TGF-β2−induced EMT in the corneal epithelium by inhibiting the nuclear localisation of SMAD2/3. Therefore, we treated KLF4+ HCECs with TGF-β2. Immunocytochemistry revealed that the nuclear localisation of SMAD2/3 proteins post−TGF-β2 treatment was inhibited in the GFP-KLF4+ HCECs, compared to that in the GFP-control HCECs (Fig. 5a). As it is well known that TGF-β activates SMAD2/3 phosphorylation and its nuclear localization [18,19], we further investigated the phosphorylation level of SMAD2 in KLF4+ HCECs to identify the precise TGF-β signalling event inhibited by KLF4. SMAD2 phosphorylation was detected as early as 30 min post−TGF-β2 treatment in control and KLF4+ HCECs; however, the phosphorylation level in KLF4+ HCECs was less than 20% of that in the control HCECs. At 4 h post−TGF-β2 treatment, phosphorylation levels returned to pre-treatment levels in KLF4+ HCECs, but were reduced to approximately half in the control cells (Fig. 5b). These results indicate that KLF4+ HCECs were resistant to TGF-β2-induced EMT.

4. Discussion

In the current study, we investigated the involvement of KLF4 in EMT in HCECs and elucidated the mechanisms underlying the role of KLF4 in homeostasis maintenance, suggesting that the use of KLF4 may serve as a novel approach for EMT suppression in the field of regenerative medicine in maintaining cultivated corneal epithelial cell sheets. We also elucidated that KLF4 prevents EMT by suppressing TGF-β2 secretion, which has a vital role in corneal wound healing, via the canonical TGF-β signalling pathway. We demonstrated EMT in HCECs by KLF4 KD via morphological and gene expression analysis. EMT is generally detected as changes in gene expression or protein level; however, we additionally demonstrated that cell morphology analysis using high-content imaging assay can be a useful tool for quantification of EMT.

Here, we showed that KLF4 KD in HCECs decreased the gene expression and protein levels of the epithelial markers KRT12 and KRT14. These results support the previous findings that KLF4 directly binds and transactivates the promoter of KRT12 [24], and that KLF4 overexpression upregulates KRT14 expression [4]. Although CDH1 expression remained unchanged in KLF4-KD HCECs, membrane CDH1 protein levels were significantly decreased as well as total protein levels of CDH1, suggesting that KLF4 may regulate the expression of CDH1 or the post-transcriptional recruitment of CDH1 to the cell membrane via other factors.

On the other hand, upregulation of mesenchymal markers in KLF4-KD HCECs was observed to occur via promotion of the TGF-β signalling pathway. We demonstrated increased secretion of TGF-β2 by KLF4-KD HCECs due to activation of the TGF-β signalling pathway, implying that KLF4 KD promoted EMT, which is known to be maintained by an autocrine TGF-β loop [25−27]. Contrary to previous reports, we did not detect TGF-β1 secretion [28,29]; however, we and others previously reported that TGF-β2 plays a major role in normal corneal epithelial maintenance and enhances corneal epithelial wound healing [30−32]. During corneal epithelial wound healing after epithelial debridement, KLF4 expression is lost in the cells migrating for repair, compared with that in the normal corneal epithelial cells [2]; therefore, our result, together with this aforementioned finding, indicates that higher secretion of TGF-β2 by migrating cells could enhance corneal epithelial wound
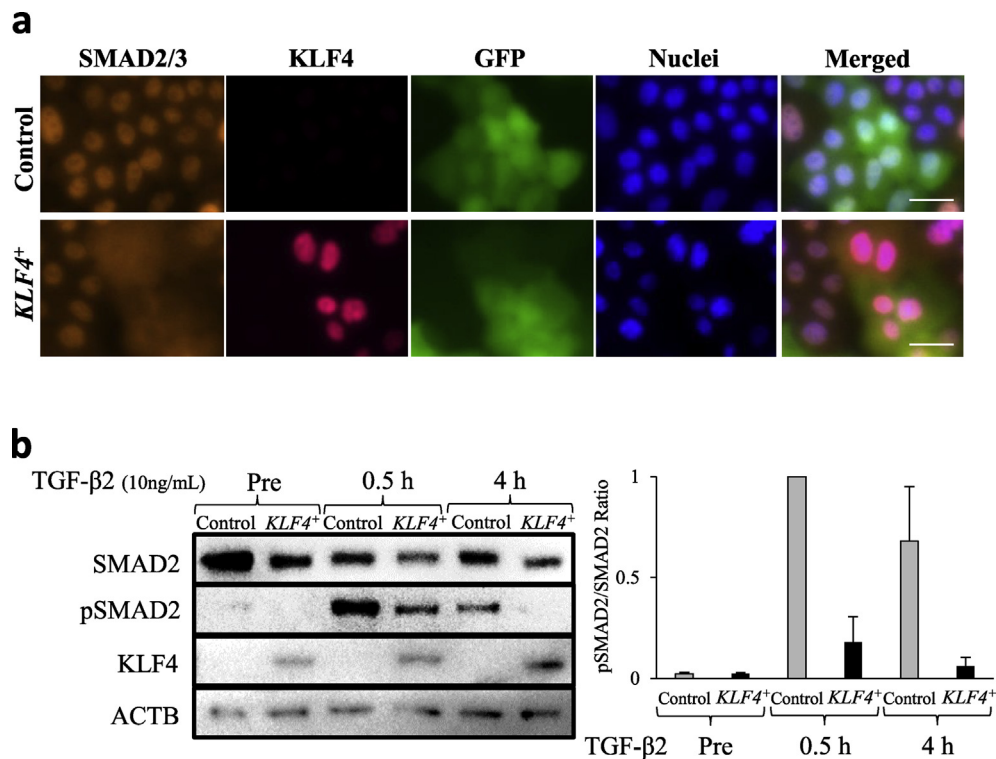

Fig. 5. TGF-β2 treatment-induced nuclear localisation of SMAD2/3 was inhibited in KLF4-overexpressing (KLF4+) HCECs. (a) Immunocytochemistry revealed the nuclear localisation of SMAD2/3 proteins after TGF-β2 treatment was inhibited in GFP-KLF4+ HCECs, compared with that in the GFP-control HCECs (representative of three independent experiments; scale bars, 10 μm). (b) Following TGF-β2 treatment (10 ng/mL), levels of phosphorylated SMAD2 (pSMAD2) decreased in KLF4+ HCECs, compared with that in the control. After 4 h, the levels returned to the pre-treatment levels in KLF4+ HCECs (ACTB reference control; biological $n = 3$).

healing. Following corneal injury with disruption of the basement membrane, TGF-β2 is released by the corneal epithelium into the corneal stroma, inducing a subpopulation of keratocytes to undergo transformation into myofibroblasts and form a fibrotic scar [22,23] that may directly cause vision impairment. Therefore, attenuation of TGF-β expression and signalling may provide means to counteract fibrotic changes, and *KLF4* may play an important role in penetrating wound healing essential for preventing corneal scar formation by repressing TGF-β2 secretion.

In this study, we did not investigate the detailed mechanism underlying the function of KLF4 as a transcription factor in suppressing phosphorylation and nuclear localisation of SMAD2/3, the main signal transduction molecule in the TGF-β signalling pathway. However, since recent reports demonstrated that KLF4 suppresses TGF-β signalling by transcriptional activation of SMAD7, an inhibitory SMAD [33,34], we can speculate that *KLF4* KD led to SMAD7 downregulation, causing nuclear localisation of SMAD2/3 in *KLF4*-KD HCECs, whilst *KLF4* overexpression led to SMAD7 upregulation, causing inhibition of nuclear localisation of SMAD2/3 or decrease of SMAD2 phosphorylation in $KLF4^+$ HCECs treated with TGF-β2.

5. Conclusions

Our findings demonstrate that *KLF4* prevented EMT *via* inhibition of the TGF-β signalling pathway in human corneal epithelia, resulting in the suppression of TGF-β2 secretion, thereby providing a foundation for developing *KLF4*-based approaches to prevent corneal scar formation.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2019.08.003.

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