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Identification of Candidate KLF4 Target Genes Reveals the Molecular Basis of the Diverse Regulatory Roles of KLF4 in the Mouse Cornea

Shivalingappa K. Swamynathan^{1,*}, Janine Davis², and Joram Piatigorsky²

¹ University of Pittsburgh School of Medicine, Department of Ophthalmology, 203 Lothrop Street, Room 1025, Pittsburgh PA-15213

² Laboratory of Molecular and Developmental Biology, National Eye Institute, National Institutes of Health, Bethesda MD-20892

Abstract

Purpose—Krüppel-like factor KLF4 plays a crucial role in the development and maintenance of the mouse cornea. Here, we have compared the wild type (WT) and *Klf4*-conditional null (*Klf4*CN) corneal gene expression patterns to understand the molecular basis of the *Klf4*CN corneal phenotype.

Methods—Expression of more than 22,000 genes in 10 WT and *Klf4*CN corneas was compared by microarrays, analyzed using BRB ArrayTools and validated by Q-RT-PCR. Transient cotransfections were employed to test if KLF4 activates the aquaporin-3, Aldh3a1 and TKT promoters.

Results—Scatter plot analysis identified 740 and 529 genes up- and down-regulated by more than 2-fold, respectively, in the *Klf4*CN corneas. Cell cycle activators were upregulated while the inhibitors were downregulated, consistent with the increased *Klf4*CN corneal epithelial cell proliferation. Desmosomal components were downregulated, consistent with the *Klf4*CN corneal epithelial fragility. Downregulation of aquaporin-3, detected by microarray, was confirmed by immunoblot and immunohistochemistry. Aquaporin-3 promoter activity was stimulated 7–10 fold by cotransfection with pCI-KLF4. Corneal crystallins Aldh3A1 and TKT were downregulated in the *Klf4*CN cornea and their respective promoter activities were upregulated 16- and 9-fold by pCI-KLF4 in co-transfections. Expression of epidermal keratinocyte differentiation markers was affected in the *Klf4*CN cornea. While the cornea specific keratin-12 was downregulated, most other keratins were upregulated, suggesting hyperkeratosis.

Conclusions—We have identified functionally diverse candidate KLF4 target genes, revealing the molecular basis of the diverse aspects of the *Klf4*CN corneal phenotype. These results establish KLF4 as an important node in the genetic network of transcription factors regulating the corneal homeostasis.

Keywords

Cornea; Development; KLF4; Microarray

*Corresponding Author: Shivalingappa K. Swamynathan, University of Pittsburgh School of Medicine, Department of Ophthalmology, Eye and Ear Institute, 203 Lothrop Street, Room 1025, Pittsburgh PA-15213, Phone: 412-802-6437, Fax: 412-647-5880, Swamynathansk@upmc.edu.

Introduction

Clear vision requires proper development and maintenance of the cornea, a multilayered tissue comprising an outer stratified squamous epithelium, an inner monolayered endothelium and a central stroma with regularly arranged collagen lamellae sparsely populated by keratocytes. The molecular and cellular mechanisms involved in the development and maintenance of the transparency, refractive and barrier functions of the cornea are exquisitely regulated^{1–8}. Defective development and/or maintenance of the cornea result in severe defects in vision^{9, 10}. Different transcription factors influencing corneal morphogenesis and their target genes are known^{11–25}. In spite of these advances, our knowledge of the genetic network of transcription factors regulating embryonic morphogenesis, postnatal maturation and maintenance of cornea remains incomplete.

KLF4, a member of the Krüppel-like transcription factors (KLF) subfamily of Cys2-His2 zinc finger proteins capable of binding the “GT box” or “CACCC” element, is one of the most highly expressed transcription factors in both 9 day- and 6 week-old mouse cornea^{20, 26–29}. During development, the expression of KLF4 begins in a stripe of mesenchymal cells extending from the forelimb bud to the developing eye around embryonic day (E) 10³⁰. In the adult mouse, KLF4 is widely expressed in post-mitotic epithelia of diverse tissues, including skin, gastrointestinal tract and cornea^{27, 31, 32}. *Klf4* null mice die within 15 hours after birth due to late stage defects in skin barrier formation³³. *Klf4* conditional null (*Klf4*CN) corneas derived by mating *Klf4-LoxP* mice³⁴ with *Le-Cre* mice^{17, 35} develop multiple ocular defects including corneal epithelial fragility, stromal edema, smaller, vacuolated lens, and loss of conjunctival goblet cells³⁶. In order to investigate the changes in gene expression underlying the *Klf4*CN corneal phenotype, we have compared the gene expression patterns of the wild type and *Klf4*CN corneas by microarray hybridization in the present report. Our results show that KLF4 plays a significant role in maintenance of corneal homeostasis by regulating a wide array of genes encompassing a diverse spectrum of functional subgroups such as regulators of cell proliferation, cell adhesion molecules, corneal crystallins, components of epithelial barrier function and regulators of stromal hydration.

Materials and Methods

Conditional deletion of *Klf4*

*Klf4*CN mice were generated on a mixed background by mating *Klf4^{loxP/loxP}*, *Le-Cre*^{-/-} mice with *Klf4^{loxP/loxP}* mice as described before^{34, 35} a mixed, ³⁶. This mating scheme generated equal numbers of *Klf4^{loxP/loxP}*, *Le-Cre*^{-/-} (*Klf4*CN) and *Klf4^{loxP/loxP}* (control) offspring. Genomic DNA isolated from tail clippings was assayed for the presence of the *Klf4-LoxP* and *Le-Cre* transgenes by PCR using specific primers. Mice studied here were maintained in accordance with the guidelines set forth by the Animal Care and Use Committee of the National Eye Institute, NIH, and the ARVO statement for the use of animals in ophthalmic and vision research.

Isolation of total RNA, quality control, labeling and microarray analysis

In the present analysis, we used the whole cornea, comprising epithelial cells, stromal keratocytes and endothelial cells as well as a small number of infiltrating leukocytes. Similar microarray analyses of whole corneas have proven useful in identifying the corneal responses to *Aspergillus fumigatus*³⁷ or *Pseudomonas aeruginosa*³⁸ infections, diabetic conditions³⁹ and in characterizing the healing process following laser ablation⁴⁰ or keratectomy⁴¹. Five age-matched 8 week-old wild type and *Klf4*CN mice each were used for comparison of corneal gene expression by microarray analysis. Two dissected corneas from each mouse were combined for isolation of total RNA using the RNeasy Mini kit (Qiagen, Valencia, CA). The

RNA was sent to Expression Analysis (Durham, NC) who confirmed the integrity of the isolated total RNA using Agilent Bioanalyzer (Supplementary Figure 1) and subsequently performed the microarray hybridizations. Labeled samples were hybridized with Affymetrix Mouse 430 2.0 arrays containing 45,101 panels, each targeting a specific nucleic acid sequence. In these arrays, approximately 22,000 transcripts identified by Entrez Gene numbers are redundantly targeted by 41,400 panels: the remaining panels target relatively less characterized sequences. The raw data obtained from Expression Analysis was analyzed using BRB ArrayTools software available from NCI, NIH. Microarray data was normalized using median over entire array, and filtered using the following criteria. Genes were excluded from analysis if (1) the P value was greater than 0.005, (2) the % data missing or filtered out were greater than 50%, (3) greater than 20% of expression data values had more than 1.5-fold change in either direction from the median value, or (4) the detection call was “absent” in more than 50 % in both WT and *Klf4*CN. The microarray results provided in this report are all log transformed.

Validation of microarray results using RT-PCR and real time quantitative RT-PCR

Total RNA isolated from the wild type or *Klf4*CN corneas was quantified, the concentration adjusted with RNase-free water to 100 ng/μl and one step RT-PCR performed using 100 ng total RNA and Ready-To-Go RT-PCR beads (Amersham Pharmacia Biotech, Piscataway, NJ). In order to distinguish the products amplified from the contaminating genomic DNA if any, from those originating from the mRNA, the forward and reverse primers used in RT-PCR were picked from adjacent exons. The sequence of primers used for RT-PCR is provided in Supplementary Table 1. The RT-PCR products were separated on a 1.5% agarose gel using TBE buffer.

Applied Biosystems (Foster City, CA) was the source of the reagents, equipment and software for TaqMan gene expression quantitative real time RT-PCR assays (Q-RT-PCR). cDNA was generated using High Capacity cDNA Archive Kit and total RNA isolated from pooled corneas of 10 wild type or *Klf4*CN mice. Q-RT-PCR assays with pre-standardized gene-specific probes for different transcripts were performed in 7900HT thermocycler using 18S rRNA as endogenous control; the results were analyzed using SDS software version 2.1.

Immunoblots and immunohistochemistry

Total protein was extracted by homogenizing dissected corneas in 8.0 M urea, 0.08% Triton X-100, 0.2% sodium dodecylsulfate, 3% β-mercaptoethanol, and proteinase inhibitors and quantified by the bicinchoninic acid method (Pierce, Rockford, IL). Equal amounts of protein were electrophoresed in sodium dodecyl sulfate-polyacrylamide gels, transferred to polyvinylidene difluoride membranes and subjected to immunoblot analysis. Rabbit anti-aquaporin-3 (Calbiochem, La Jolla, CA), and anti-actin antibody (Sigma Chemical Company, St. Louis, MO) were used as primary antibodies at a 1:1,000 dilution in PBST. Horseradish peroxidase-coupled anti-rabbit immunoglobulin G (Amersham Biosciences, Piscataway, NJ) was used as a secondary antibody at a 1:5,000 dilution. Immunoreactive bands were visualized by chemiluminescence using Super Signal West Pico solutions (Pierce, Rockford, IL).

For immunohistochemistry, 10 μm-thick cryosections from OCT-embedded eyeballs were fixed in freshly prepared buffered 4% paraformaldehyde for 30 min, blocked with 10% sheep serum in PBST for 1 h at room temperature in a humidified chamber, washed twice with PBST for 5 min each, incubated with a 1:100 dilution of the primary antibody for 1 h at room temperature, washed thrice with PBST for 10 min each, incubated with secondary antibody (Alexafluor 555-coupled goat anti-rabbit IgG antibody; Molecular Probes, Carlsbad, CA) at a 1:300 dilution for 1 h at room temperature, washed thrice with PBST for 10 min each, mounted

with Prolong Gold antifade reagent with DAPI (Molecular Probes, Carlsbad, CA), and observed with a Zeiss Axioplan 2fluorescence microscope.

Reporter vectors, cell culture, and promoter activities

Mouse genomic DNA was used to amplify different promoter fragments used in cotransfection assays reported here. Aquaporin3 (Aqp3) –502/+42 bp and –262/+42 bp promoter fragments were amplified by using the downstream Aqp3 +42/+22C HindIII (+42 ATGCAAGCTTGTCCGGCGCGTACGAGTGC +22C) and upstream Aqp3 –502/–482 XhoI (–502 ATGCCTCGAGCACGAAGCGCTGGTGAATTC –482) or Aqp3 –262/–245 XhoI (–262 ATGCCTCGAGGGAGACCGCTTGCTCTTC –245) primers. Transketolase (TKT) –518/+104 bp promoter fragment was amplified by using upstream TKT –518/–491 KpnI (–518 GGCCGGTACCGGCAAACCCAGTAATCTC –491) and downstream TKT +104/+87 HindIII (+104 GGCCAAGCTTCCTTCCATGGCGTGGTAGG +87) primers. Aldh3a1 –1050/+3486 bp promoter fragment was isolated as described previously (Davis et al. 2007, Invest. Ophthalmol. Vis. Sci., in press). These promoter fragments were cloned upstream of the luciferase reporter gene in pGL3Basic vector (Promega, Madison, WI) to generate the reporter vectors. Full length KLF4 was transiently expressed using the CMV promoter in pCI-Klf4. Monkey kidney Cos7 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, penicillin and streptomycin at 37°C in a humidified chamber containing 5% CO₂ in air. Simian virus SV40-transformed human corneal epithelial (HCE) cells⁴² were grown at 37°C in Ham's F-12 supplemented with 10% fetal bovine serum, 0.5% (vol/vol) dimethyl sulfoxide, cholera toxin (0.1 µg/ml), epidermal growth factor (10 ng/ml), insulin (5 µg/ml), gentamicin (40 µg/ml), and glutamine (20 mM) in a humidified chamber containing 5% CO₂ in air. Mid-log phase cells in six-well plates were transfected with 0.5 µg of pAldh3A1-Luc or pTKT-Luc or pAqp3-Luc, along with 10 ng pRL-SV40 (Promega, Madison WI, for normalization of transfection efficiency) and 0.5 µg of pCI or pCI-Klf4, using 3 µl of Eugene 6 reagent (Roche Molecular Biochemicals). After 2 days, cells were washed with cold PBS and lysed with 500 µl of passive lysis buffer (Promega, Madison, WI). The lysate was clarified by centrifugation and 50 µg protein in the lysate was analyzed using a dual-luciferase assay kit (Promega, Madison WI) and a Victor microplate luminometer (Perkin-Elmer). The measurement was integrated over 10 seconds with a delay of 2 seconds. Results from at least three independent experiments, normalized for transfection efficiency using the SV40 promoter-driven *Renilla* luciferase activity, were used to obtain mean promoter activities and standard deviation. Fold-activation was determined by dividing mean promoter activity by the promoter activity without added pCI/pCI-KLF4.

Results

Microarray analysis and validation of results

In order to obtain mechanistic insight into the diverse ocular surface phenotype observed in the *Klf4*CN mice³⁶, we compared the gene expression patterns between the wild type and *Klf4*CN corneas by microarray hybridization. Scatter plot analysis of the 6333 genes that passed the filtering criteria described in Materials and Methods showed that 529 genes were downregulated and 740 genes were upregulated by more than 2-fold in the *Klf4*CN compared to the wild type corneas (Fig. 1, Supplementary Tables 2A and 2B). Microarray results were validated by quantitative real time RT-PCR comparison of expression levels of 19 different genes (Table 1). There was a general conformity between the microarray and Q-RT-PCR results, indicating that the microarray results reflect the extent of changes in gene expression in the *Klf4*CN corneas (Table 1). The candidate KLF4 target genes, whose expression was significantly affected in the *Klf4*CN corneas, fall into different functional subgroups regulating diverse functions such as cell proliferation, apoptosis, development, immune response and barrier function. Below, we have analyzed the microarray results further, to correlate different

aspects of the *Klf4*CN corneal phenotype with specific changes in gene expression, thus revealing the diverse contributions of KLF4 to corneal physiology.

Role of KLF4 in regulating the progression of corneal epithelial cell cycle

In view of the increased rate of *Klf4*CN corneal epithelial cell proliferation³⁶, we examined the expression pattern of a subset of genes known to participate in or regulate cell cycle. Several cell proliferation activators were upregulated (Table 2A) while cell cycle suppressors were downregulated (Table 2B) in the *Klf4*CN cornea, compared to the wild type. Important activators of cell cycle that were upregulated include cyclin D2, cyclin-dependent kinase 6, receptor type protein tyrosine phosphatase-b and -c, and FMS like tyrosine kinase-1 (Table 2A). Significantly downregulated inhibitors of cell cycle include retinoblastoma-1 (Rb) and cyclin-dependent kinase inhibitor 1a (p21) (Table 2B). Upregulation of activators of cell cycle and downregulation of inhibitors of cell cycle are consistent with the increased cell proliferation in the *Klf4*CN corneal epithelium³⁶.

Role of KLF4 in regulation of epithelial cell-cell adhesion

In spite of the increased rate of cell proliferation, the *Klf4*CN cornea possessed fewer epithelial cell layers than the wild type, suggesting reduced cell-cell adhesion at the *Klf4*CN ocular surface³⁶. To explore this possibility further, we examined the expression of different components of desmosomes, the intercellular adhesion complexes prevalent in the stratified epithelia⁴³. Expression of most of the desmosomal components was significantly downregulated in the *Klf4*CN cornea, strongly supporting the notion that compromised cell-cell adhesion is responsible for the reduced numbers of *Klf4*CN corneal epithelial cell layers (Fig. 2A). Comparison of expression of these desmosomal components in the wild type and *Klf4*CN corneas by semi-quantitative RT-PCR validated the microarray results (Fig. 2B).

Role of KLF4 in regulation of corneal hydration

Aquaporin-1, -3 and -5 are responsible for keeping the hydrophilic corneal stroma from abnormal swelling^{44, 45}. Previously, we have reported that the downregulation of *Aqp5* may be responsible for the *Klf4*CN corneal stromal edema³⁶. In addition to confirming the downregulation of *Aqp5*, the present microarray analysis revealed that the expression of *Aqp3* also is reduced in the *Klf4*CN cornea to about half of that in the wild type, while the expression of *Aqp1* remained relatively unaffected. Downregulation of *Aqp3* transcripts and the 38 kDa *Aqp3* protein was validated by Q-RT-PCR (Table 1· Fig. 3A) and immunoblot analysis (Fig. 3B), respectively. Equal loading of proteins for immunoblot analysis was confirmed by stripping the membrane of antibodies and reprobing with an anti-actin antibody, which did not show any difference between wild type and *Klf4*CN corneas (Fig. 3B). Immunohistochemistry confirmed the reduction in the amount of *Aqp3* in the *Klf4*CN corneal epithelium (Fig. 3C) consistent with the reduced amount of *Aqp3* in the *Klf4*CN corneal extracts compared with the wild type extracts (Fig. 3B). In view of the reduced stratification of the *Klf4*CN corneal epithelium (Fig. 3C), it was uncertain to what extent the reduced *Aqp3* protein band in the immunoblot (Fig. 3B) was due to reduced expression of *Aqp3* in the epithelium or to the relatively large stromal cell representation in the *Klf4*CN corneal extracts inasmuch as stromal cells do not express *Aqp3*^{46, 47; present data}, yet contain actin which was used for normalization. In order to more directly test if KLF4 stimulates *Aqp3* promoter activity, we utilized co-transfection assays in cultured cells. Both -502/+42 bp and -262/+42 bp *Aqp3* promoter activities were upregulated 8 to 9-fold by co-transfection with pCI-KLF4, indicating that the KLF4 binding sites reside within the -262/+42 bp region of the *Aqp3* proximal promoter (Fig. 3D). Taken together, these results show that *Aqp3* promoter is regulated by KLF4 and suggest that the cumulative effect of reduced expression of *Aqp3* and *Aqp5* is responsible for the observed *Klf4*CN stromal edema³⁶.

Role of KLF4 in regulation of expression of corneal crystallins

Corneas, like lenses, accumulate unusually high proportions of a few water-soluble proteins termed corneal crystallins, in a taxon-specific manner⁴⁸. In the mouse corneal epithelium, aldehyde dehydrogenase-3a1 (Aldh3a1) and transketolase (TKT) comprise 20–50% and 10–15% respectively, of the water-soluble protein^{49, 50}. Microarray comparison indicated that the expression of Aldh3a1 and TKT genes was downregulated in the *Klf4*CN cornea to 90% and 30% of the wild type, respectively (Fig. 4A). This downregulation was estimated more quantitatively by Q-RT-PCR, which showed that the expression of Aldh3a1 and TKT genes in the *Klf4*CN cornea was downregulated to 41 % and 56 % of the wild type, respectively (Fig. 4B). In order to directly test the influence of KLF4 on Aldh3A1 and TKT promoter activities, we utilized cotransfection assays in cultured Cos7 cells, in which the –1050/+3486 bp Aldh3A1 promoter and –518/+104 bp TKT promoter activities were upregulated by 16- and 9- fold, respectively, in cells co-transfected with pCI-*Klf4*, compared to those co-transfected with pCI (Fig. 4C). Taken together, these results suggest that KLF4 contributes to the corneal transparency and refractive properties, by activating the expression of corneal crystallins Aldh3A1 and TKT.

Role of KLF4 in regulating the barrier function of corneal epithelium

Several genes encoding proteins related directly or indirectly to epithelial barrier function were downregulated in the *Klf4*CN cornea. Uroplakin 3B, an integral membrane protein that contributes to the apical membrane permeability barrier function⁵¹, as well as gastrophilin-1 (also known as antrum mucosal protein-18), a mitogenic protein abundantly expressed in the superficial gastric epithelium and down-regulated in gastric carcinoma⁵², were significantly downregulated in the *Klf4*CN cornea (Supplementary Table 2B). The expression of a large number of immune response related genes was affected in the *Klf4*CN cornea (Supplementary Tables 2A and 2B). The upregulated immune response related genes belonged to diverse groups such as complement components, chemokines and chemokine receptors. Infiltration of the immune cells into the *Klf4*CN cornea in response to the inflammatory signals generated by the fragile corneal epithelium and edematous stroma may account for the observed upregulation of the immune response related genes in the *Klf4*CN cornea.

Keratins, the fibrous intermediate-filament protein polymers, play critical roles in maintenance of the epithelial cell structure, protecting them from mechanical trauma. Previously, we have shown that *Krt12* promoter is bound and upregulated by KLF4, and that the downregulation of *Krt12* may be responsible for the *Klf4*CN corneal epithelial fragility³⁶. Microarray analysis showed that while *Krt12* was indeed downregulated, most of the other keratins were upregulated, indicating hyperkeratosis in the *Klf4*CN cornea (Fig. 5A). Semi-quantitative RT-PCR assays validated these microarray results (Fig. 5B).

Role of KLF4 in regulation of genes involved in mouse epidermal keratinocyte differentiation

In view of the fact that KLF4 plays a crucial role in the mouse epidermal development³³, we examined the expression pattern of different genes involved in the mouse epidermal keratinocyte differentiation. Oncogenes *Ets1* and *Jun*, as well as tumor necrosis factor receptor *Tnfrsf1b*, were upregulated, while the expression of epidermal growth factor (*egf*), protein kinase-C β 1 and mitogen activated protein kinase kinase-6 (*Mapkk6*) were downregulated by more than 2-fold in the *Klf4*CN cornea, compared to the wild type (Supplementary Table 2A; Table 3). Expression of epidermal keratinocyte differentiation markers, such as involucrin and transglutaminase was affected in the *Klf4*CN cornea. Expression of several members of the *Sprr* family of proteins, constituents of the cornified envelope in the skin, was upregulated in the *Klf4*CN cornea (Supplementary Table 2A; Table 3).

Role of KLF4 in the regulation of gene regulatory networks in the cornea

In order to understand the impact of conditional deletion of *Klf4* on gene regulatory networks in the cornea, we examined the expression levels of different transcription factors in the *Klf4*CN cornea. Downregulation of the paired box-homeobox transcription factor Pax6, central to eye development⁵³, by more than 2-fold as detected by microarray analysis (Table 4), was validated by Q-RT-PCR (Table 1). In addition, special AT-rich sequence binding protein-1 (SATB1), which organizes nuclear architecture and recruits chromatin remodeling factors to specific promoters^{54–56}, was downregulated by approximately 3-fold (Table 4). Another significantly downregulated transcription factor, inhibitor of DNA binding-2 (Id2), is known to contribute to epithelial cell differentiation and suppress tumor formation⁵⁷. In contrast, several stress response related transcription factors such as Ahr⁵⁸, E2F5 and EGR1⁵⁹, NFκB2⁶⁰ and ATF3⁶¹ were significantly upregulated in the *Klf4*CN cornea, compared to the wild type cornea (Table 4).

Discussion

Previously, we demonstrated that the structural integrity of the mouse corneal epithelium, stroma and endothelium was affected when the *Klf4* gene was deleted, indicating that KLF4 plays a crucial role in the development and maintenance of the mouse cornea³⁶. We also demonstrated that the expression levels of Krt12 and Aqp5 are reduced in the *Klf4*CN cornea, consistent with the *Klf4*CN corneal epithelial fragility and stromal edema, respectively. Here, we have employed microarray analysis to obtain a comprehensive view of the changes in corneal gene expression upon deletion of *Klf4*. Our findings reveal the molecular basis of the wide ranging influence of KLF4 on corneal homeostasis and identify candidate target genes of KLF4 in the adult mouse cornea.

Previous attempts at KLF4 target gene profiling used a human cell line containing inducible KLF4^{62, 63}, or transgenic mice over-expressing KLF4 in the epidermis under the control of keratin-5 promoter (Krt5-KLF4) and the *Klf4*^{−/−} mice⁶⁴. In addition to being largely consistent with the earlier reports, our results identify novel KLF4 target genes with specialized functions in the mouse cornea, such as aquaporins-3 and -5, corneal crystallins Aldh3a1 and transketolase, and desmosomal components.

Consistent with the established mechanism of suppression of cell proliferation by KLF4^{65, 66}, cyclin-D2 was upregulated and cdkn1a (p21) was downregulated in the *Klf4*CN cornea. Furthermore, our results are consistent with the previous report that several other inhibitors of cell division are upregulated, and activators of cell cycle are downregulated by KLF4⁶³, suggesting additional mechanisms by which KLF4 can suppress the progression of cell cycle (Tables 2A and 2B).

Many members of the large family of keratins, the major components of the intermediate filaments protecting the epithelial cells from mechanical and non-mechanical stresses⁶⁷, are upregulated in the *Klf4*CN corneas. In contrast to our results, most keratins were upregulated by KLF4 in a KLF4 inducible cell culture system⁶². This difference could be due to the different experimental systems used and/or to an indirect hyperkeratinizing response in the *Klf4*CN cornea as a consequence of the loss of corneal barrier function.

A striking feature of our results is the large number of genes whose expression is affected by the absence of KLF4 in the mouse cornea. While some of these genes are likely to be direct targets of KLF4, the remaining genes may be indirect targets through other transcription factors regulated by KLF4. The expression of Pax6 is reduced to about half in the *Klf4*CN cornea compared to the wild type cornea. Thus, Pax6 target genes are likely to be included in the list of genes affected by the absence of KLF4. In this regard, it is noteworthy that the Pax6(+/-)

corneal epithelium appears remarkably similar to the *Klf4*CN corneal epithelium with fewer cell layers, roughened ocular surface, and epithelial vacuolation^{15, 68}. Other transcription factors whose expression in the *Klf4*CN cornea is reduced (Id2, SATB1, and Elk4) or increased (NFκB, Sox4, Atf3, C/EBPδ, GATA2, Ahr and Egr1) may indirectly contribute to the list of genes with changed expression in the *Klf4*CN cornea.

In addition to the changes discussed above, the expression of many genes with no established functions in the cornea and/or no apparent relevance to the *Klf4*CN corneal phenotype was significantly affected in the *Klf4*CN cornea. Three members of the Ly6/Plaur domain containing proteins- Slurp1, one of the most abundant transcripts in the mouse cornea²⁷, a ligand for nicotinic acetylcholine receptors and a late marker of epidermal differentiation associated with the inflammatory palmoplantar keratoderma disease *Mal de Meleda*^{69–71}, Lynx1 (also a ligand for nicotinic acetylcholine receptors⁷²), and Lypd2 were significantly downregulated in the *Klf4*CN cornea (Supplementary Table 2B). Similarly, the expression of 15 and 9 different members of the solute carrier family of proteins was up- and down- regulated respectively, in the *Klf4*CN compared to the wild type cornea (Supplementary Tables 2A and 2B). Whether these changes contribute to any aspect of the *Klf4*CN corneal phenotype remains to be established.

The results presented in this report show that KLF4 coordinately regulates functionally related subsets of genes such as those contributing to the control of corneal epithelial cell cycle progression, intercellular adhesion, corneal crystallins, Ly6/Plaur domain containing proteins Slurp1, Lypd2 and Lynx1^{69–72} and the small proline-rich proteins (SPRR), the primary constituents of the cornified cell envelope and integral components of the surface barrier^{73, 74}. We have also shown that KLF4 stimulates the promoter activities of aquaporin-3 and -5³⁶, and corneal crystallins Aldh3A1 and TKT in cultured cells. It remains to be established if KLF4 plays a direct role in the coordinate regulation of the remaining groups of genes whose expression is affected in the *Klf4*CN cornea. A fraction of the observed changes in gene expression could be indirect, such as a response to the inflammatory conditions caused by the fragile *Klf4*CN corneal epithelium. The loss of epithelial barrier function may be responsible for the overexpression of several stress related genes in the *Klf4*CN cornea, such as the antioxidant enzyme ceruloplasmin that is upregulated in different neurodegenerative disorders including glaucoma^{75, 76}, arachidonate lipoxygenase-12 and -15, which promote epithelial wound healing and host defense⁷⁷, and carbonic anhydrase-2, -12, and -13, regulators of corneal ion transport, that are overexpressed in human glaucoma^{78, 79} (Supplementary Tables 2A and 2B).

In summary, the changes in gene expression patterns detected by the present microarray analysis are consistent with the phenotypic changes in the *Klf4*CN cornea. Our results show that KLF4 contributes to corneal homeostasis by coordinately regulating the expression of subsets of genes involved in specific functions such as progression of cell cycle, cell-cell adhesion, epithelial barrier formation, corneal crystallins and maintenance of corneal hydration. Taken together with our earlier report³⁶, the present studies establish KLF4 as an important node in the genetic network of transcription factors required for proper development and maintenance of the ocular surface.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Scatter plot analysis of 6333 genes passing the filter described in Materials and Methods. Expression of 529 genes was downregulated and 740 genes upregulated by more than two folds in *Klf4CN* compared to the wild type corneas. The expression of about 80% of the genes (5064 genes) passing the filter remained relatively stable with less than 2-fold change.

A

Gene Name	Gene Symbol	WT	<i>Klf4</i> CN	Fold Difference
Desmoglein 1a	Dsg1a	13.22	10.91	0.20
Desmoglein 1b	Dsg1b	6.46	5.98	0.61
Desmocollin 3	Dsc3	11.07	9.84	0.43
Desmoplakin	Dsp	7.83	7.21	0.53
Plakoglobin	Jup	8.20	7.78	0.65

B

Figure 2.

A. Expression levels of different desmosomal components in the wild type and *Klf4*CN corneas. The values shown are log transformed.

B. Validation of downregulation of genes encoding desmosomal components in the *Klf4*CN compared to the wild type corneas, by semi-quantitative RT-PCR. M, Molecular weight markers; WT, wild type; CN, *Klf4* conditional null.

Figure 3.

Aquaporin-3 expression is reduced in the *Klf4CN* relative to the wild type cornea.

A. Q-RT-PCR comparison of expression levels of Aqp3 in the wild type and *Klf4CN* corneas.

B. Immunoblot analysis of Aqp3 levels in total proteins extracted from wild type and *Klf4CN* corneas. The blot probed with anti-Aqp3 antibody (on the left) was stripped of the antibody and re-probed with anti-actin antibody (on the right) to ensure equal loading of proteins.

C. Immunohistochemistry with anti-Aqp3 antibody. Images in the top row show nuclei stained with DAPI, while those in the bottom row show fluorescence coming from the secondary antibody bound to the primary anti-Aqp3 antibody. The expression of Aqp3 localized to the epithelial and endothelial cell membranes in the wild type (bottom, left), is reduced in the

*Klf4*CN cornea (bottom, center). The sections processed in a similar manner without the primary antibody served as controls (bottom, right).

D. Aquaporin-3 promoter is stimulated by KLF4 in cultured human corneal epithelial cells cotransfected with Aqp3-Luc and pCI-KLF4.

Figure 4.

Expression of corneal crystallins *Aldh3a1* and *TKT* is regulated by KLF4

A. Comparison of expression of *Aldh3a1* and *TKT* in the wild type (WT) and *Klf4* conditional null (*Klf4CN*) cornea, as detected by microarray.

B. Q-RT-PCR comparison of expression of *Aldh3a1* and *TKT* in WT and *Klf4CN* corneas.

C. *Aldh3a1* and *TKT* promoter activities are stimulated by KLF4 in Cos-7 cells upon cotransfection with pCI-KLF4.

A

Gene	WT	<i>Klf4</i> CN	Fold upregulation
keratin 4	10.64	13.22	5.98
keratin 6a	10.27	10.44	1.19
keratin 6b	10.52	10.76	1.26
Keratin 7	10.27	13.10	7.14
keratin 8	7.47	11.82	20.38
keratin 14	12.40	14.37	3.91
keratin 15	14.04	15.27	2.34
keratin 16	7.34	10.06	6.60
keratin 17	6.74	12.60	57.79
keratin 18	8.14	10.12	3.96
keratin 19	11.75	14.67	7.59
keratin 23	9.34	11.78	5.45

B

Figure 5.

A. Expression levels of different keratins in the wild type and *Klf4*CN corneas. The values shown are log transformed.

B. Validation of upregulation of genes encoding different keratin genes in the *Klf4*CN compared to the wild type corneas, by semi-quantitative RT-PCR. M, Molecular weight markers; WT, wild type; CN, *Klf4* conditional null.

Table 1

Validation of microarray analysis results by real time quantitative RT-PCR of selected genes.

Gene Symbol	Fold Difference as Measured by	
	Microarray	Q-RT-PCR
Alox12e	19.64	61.34
Alox15	86.21	137.26
Aqp3	0.44	0.45
Aqp5	0.09	0.24
Krt1-12	0.91	0.33
Krt1-17	57.79	80.59
Krt2-4	5.98	10.7
Lumican	1.08	1.36
CcnD2	5.86	5.71
Lamb1-1	0.29	0.34
Muc1	3.94	7.04
Spr2A	77.43	20.01
ALDH3A1	0.92	0.41
ELF3	0.76	0.65
IRF1	0.6	0.56
Pax6	0.38	0.55
SLURP1	0.02	0.1
GSTO1	1.33	0.84
SPARC	1.41	1.7

Table 2

Table 2A. Expression levels of genes involved in cell cycle regulation, upregulated in the *Klf4*CN compared to the wild type corneas. The values shown for the wild type and *Klf4*CN cornea are log transformed.

Description	Gene symbol	WT	<i>Klf4</i> CN	Fold Difference
cyclin D2 (Validated by Real time Q-RT-PCR)	<u>Ccnd2</u>	6.86	9.41	5.86
cyclin-dependent kinase 6	<u>Cdk6</u>	9.34	10.71	2.58
protein kinase, cGMP-dependent, type II	<u>Prkg2</u>	5.80	8.29	5.64
protein kinase, cAMP dependent regulatory, type I, alpha	<u>Prkar1a</u>	5.68	6.86	2.27
FMS-like tyrosine kinase 1	<u>Flt1</u>	5.64	6.95	2.48
protein kinase C, mu	<u>Prkcm</u>	5.71	7.20	2.80
Protein tyrosine phosphatase, receptor type, B	<u>Ptprb</u>	5.84	7.25	2.66
protein tyrosine phosphatase, receptor type, C	<u>Ptprc</u>	6.04	8.41	5.19
early growth response 1	<u>Egr1</u>	9.70	11.38	3.20
Jun oncogene	<u>Jun</u>	9.69	10.86	2.26
expressed in non-metastatic cells 1, protein	<u>Nme1</u>	11.70	12.77	2.11
platelet derived growth factor receptor, beta polypeptide	<u>Pdgfrb</u>	8.08	9.25	2.26
Eph receptor A4	<u>Epha4</u>	9.38	10.72	2.52

Table 2B. Expression levels of genes involved in cell cycle regulation, downregulated in the *Klf4*CN compared to the wild type corneas. The values shown for the wild type and *Klf4*CN cornea are log transformed.

Description	Gene symbol	WT	<i>Klf4</i> CN	Fold Difference
Eph receptor B6	<u>Ephb6</u>	8.07	6.95	0.46
ephrin A1	<u>Efna1</u>	10.04	8.9	0.45
ephrin B1	<u>Efnb1</u>	9.12	7.96	0.45
Cyclin-dependent kinase inhibitor 1a (p21)	<u>cdkn1a</u>	11.76	10.92	0.56
adenylate kinase 1	<u>Ak1</u>	9.41	8.11	0.41
NIMA (never in mitosis gene a)-related expressed kinase 3	<u>Nek3</u>	9.11	7.4	0.31
macrophage stimulating 1 receptor (c-met-related tyrosine kinase)	<u>Mst1r</u>	8.9	7.89	0.5
protein kinase C, beta 1	<u>Prkcb1</u>	7.56	6.53	0.49
protein kinase C, iota	<u>Prki</u>	10.67	9.28	0.38
non-catalytic region of tyrosine kinase adaptor protein 1	<u>Nck1</u>	11.46	10.44	0.49
Rous sarcoma oncogene	<u>Src</u>	9.01	7.99	0.49
fibroblast growth factor receptor 2	<u>Fgfr2</u>	10.28	9.08	0.43
RAN binding protein 2	<u>Ranbp2</u>	5.5	4.19	0.4
retinoblastoma 1	<u>Rb1</u>	6.48	4.24	0.21
growth arrest and DNA-damage-inducible 45 alpha	<u>Gadd45a</u>	10.95	9.82	0.46
growth arrest specific 2	<u>Gas2</u>	8.55	7.32	0.43

Table 3

Expression levels of mouse epidermal keratinocyte differentiation markers in the wild type and *Klf4*CN corneas.

The values shown for the wild type and *Klf4*CN cornea are log transformed.

Description	Gene symbol	WT	<i>Klf4</i> CN	Fold Difference
Tumor necrosis factor receptor superfamily, member 1b	Tnfrsf1b	5.91	7.30	2.62
E26 avian leukemia oncogene 1, 5' domain	Ets1	6.84	8.32	2.77
Jun oncogene	Jun	9.69	10.86	2.26
Small proline-rich protein 2a	Spr2a	5.96	12.24	77.43
Small proline-rich protein 2f	Spr2f	5.64	10.41	27.25
Small proline-rich protein 2d	Spr2d	2.26	5.49	25.06
Cystatin B	Cstb	9.91	10.30	1.47
Transglutaminase 1	Tgm1	5.56	6.02	1.58
Transglutaminase 2	Tgm2	9.08	9.57	1.63
epidermal growth factor	Egf	8.31	7.02	0.41
protein kinase C, beta 1	Prkcb1	2.02	1.88	0.49
Mitogen activated protein kinase kinase	Map2k6	8.39	7.37	0.49
Involucrin	Ivl	5.78	5.18	0.55
Transglutaminase 4	Tgm4	5.43	5.15	0.75
Cystatin A	Csta	9.59	9.10	0.61
Cystatin E/M	Cst6	9.98	8.03	0.26

Table 4

Comparison of expression levels of selected transcription factors in the wild type and *Klf4*CN corneas. The values shown for the wild type and *Klf4*CN cornea are log transformed.

Description	Gene symbol	WT	<i>Klf4</i> CN	Fold Difference
Upregulated Genes				
SRY-box containing gene 4	Sox4	4.49	7.52	8.19
Nuclear Factor Kappa b2	Nfkb2	5.31	7.49	4.52
Activating transcription factor 3	Atf3	5.96	7.73	3.41
E2F transcription factor 5	E2f5	6.08	7.92	3.57
Aryl-hydrocarbon receptor	Ahr	9.87	11.36	2.82
Ribosomal protein S9	Rps9	10.01	12.97	7.81
Early growth response 1	Egr1	9.70	11.38	3.20
CCAAT/enhancer binding protein (C/EBP), delta	Cebpd	8.86	11.41	5.86
Eukaryotic translation initiation factor 4E member 3	Eif4e3	6.13	8.48	5.10
GATA binding protein 2	Gata2	6.11	7.41	2.47
Downregulated Genes				
Paired box gene 6	Pax6	11.02	9.62	0.38
Special AT-rich sequence binding protein 1	Satb1	9.17	7.14	0.24
Inhibitor of DNA binding 2	Id2	12.35	10.56	0.29
Hepatic leukemia factor	Hlf	13.11	11.88	0.43
ELK4, member of ETS oncogene family	Elk4	5.2	3.5	0.31
kinase 4	Eif2ak4	9.30	8.12	0.44