PAX6 is a master regulatory gene involved in neuronal cell fate specification. It also plays a critical role in early eye field and subsequent limbal stem cell (LSC) determination during eye development. Defects in PAX6 cause aniridia and LSC deficiency in humans and the eye development. Defects in Pax6 cause aniridia and LSC and subsequent limbal stem cell (LSC) determination during fate specification. It also plays a critical role in early eye field of Cellular and Molecular Medicine, and Ming Zhang

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Background: PAX6 is a master regulatory gene involved in eye development.

Results: PAX6 is expressed in the primitive eye cup and later in corneal tissue progenitors in early embryonic development.

Conclusion: PAX6 plays a critical role in limbal stem cell and corneal epithelial fate determination.

Significance: These findings provide important insight into corneal homeostasis and disease.

The corneal epithelium is crucial to the maintenance of corneal transparency, which is in turn essential for vision. It is maintained by a population of stem cells found in the corneal limbus, or margin, known as limbal stem cells (LSCs).5 LSCs continuously differentiate into corneal epithelial cells (CECs), a unique non-keratinized epithelial cell type that is arranged in an orderly fashion on the corneal surface and allows light transmission. A deficiency in LSCs causes the corneal surface to turn into an opaque keratinized skin-like epithelium. This type of corneal surface is seen in severe ocular surface diseases such as corneal dermoids, Stevens-Johnson syndrome, and ocular surface squamous cell carcinoma, which can all lead to incurable blindness (2).

Originating from the surface ectoderm during development, both corneal and skin epithelia are stratified. Their stem cells are maintained by the transcription factor p63 in the keratin 5 (K5)-positive basal cell layer of both the corneal limbus and epidermis (3–7). However, there are also marked differences between these two cell populations. During differentiation, skin epidermal stem cells (SESCs) move vertically upward from the basal to the suprabasal layers (8, 9), during which time K5 is replaced by K1 and K10, which are expressed exclusively in the mature superficial skin epithelium (10). In contrast, LSCs undergo centripetal migration for several millimeters to the central cornea, during which time it undergoes differentiation, and K5 and K14 are replaced by K3 and K12, which are expressed exclusively in the superficial corneal epithelium (11, 12).

PAX6 is a key gene required for fate determination and maintenance of the corneal epithelium. It is expressed in the ectoderm, which gives rise to epithelial cells of the cornea. Heterozygous null mutations of PAX6 in Sey (Small eye) mice

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5 The abbreviations used are: LSC, limbal stem cell; CEC, corneal epithelial cell; K, keratin; SES, skin epidermal stem cell; P, postnatal day; E, embryonic day.
result in corneas with decreased expression of K12 and conjunctival invasion (1, 13). Dkk2 is another gene that plays an important role in the development of the ocular surface epithelium. Earlier studies showed that when PAX6 expression is absent, Dkk2-null eyes demonstrate a cornea-to-skin fate change (14). Although PAX6 plays an important role in corneal fate, little is known about how PAX6 maintains LSCs and ensures their further differentiation to CECs. In this study, we explored the function of PAX6 and potential signaling pathways in corneal LSC lineage determination, and we use this information to elucidate the pathology of corneal surface disease.

### Experimental Procedures

**Animals**—ROSA<sup>mT/mG</sup> mice were described previously (28) and maintained as homozygotes. The P0–3.9-GFPCre mice (generated by R. L. M.), expressing an EGFP-Cre recombinase fusion protein under the control of the Pax6 P0 enhancer, were maintained on an FVB background and PCR-genotyped as described (29).

**Lineage Tracing**—Lineage tracing experiments were performed by crossing homozygous GFP reporter mice (ROSA<sup>mT/mG</sup>) with lens-specific Cre transgenic mice (P0–3.9-GFPCre), in which Cre expression is under the control of mouse Pax6 ectoderm enhancer. Eyes were dissected at postnatal day (P) 1 and P60 and fixed overnight in 4% formaldehyde. Tissues were then incubated in 10% sucrose and embedded in optimal cutting temperature medium for cryosectioning. Frozen sections were washed in PBS and imaged on a Zeiss Axio Imager fluorescence microscope.

**Isolation and Culture of Human LSCs and SESCs**—Post-mortem human eyeballs were obtained from eye banks; human epidermis was obtained from donor skin biopsy of eyelids. Limbal regions were excised and washed in cold PBS with 100 IU of penicillin and 100 μg/ml streptomycin. After limbal regions were cut into small pieces, cell clusters were obtained by 0.2% collagenase IV digestion at 37 °C for 2 h. This was followed by further digestion with 0.25% trypsin/EDTA at 37 °C for 15 min to obtain single cells. Primary cells were seeded on plastic plates coated with 2% growth factor-reduced Matrigel (354230, BD Biosciences). Primary human SESCs were isolated from the interfollicular epidermis using the same procedure.

Culture medium for both LSCs and SESCs contained DMEM/nutrient mixture F-12 and DMEM (1:1) with 1:100 g/ml insulin, 0.4 μg/ml hydrocortisone, 10<sup>−10</sup> M cholera toxin, and 2 × 10<sup>−10</sup> M 3,3′,5-triiodo-t-thyronine. To differentiate LSCs, cells were cultured in CnT-30 (CELLnTEC) for 8–12 days.

**Real-Time Quantitative PCR**—An RNase kit (Qiagen) was used to isolate RNA. On-column DNase digestion was performed. A SuperScript III reverse transcriptase kit (Invitrogen) was used for cDNA synthesis following the manufacturer's instructions. A real-time PCR system (Applied Biosystems) was used to perform quantitative PCR. Forty cycles of amplification were carried out using gene-specific primers (Table 1) and Power SYBR Green PCR Master Mix. Measurements were performed in triplicates and normalized to endogenous GAPDH levels. Relative -fold change in expression was calculated using the ΔΔC<sub>T</sub> method (C<sub>T</sub> < 30). Data are shown as means ± S.D. based on three replicates.

**Lentiviral RNAi**—The PAX6 gene was targeted using lentiviral shRNAs that were cloned into a pLKO.1 plasmid between AgeI and EcoRI. shRNA targeting sequences for gene-specific knockdowns were 5′-CGTCCATCTTTGCTTGGGAAA-3′ and 5′-AGTCTGAGAGACCCATTATC-3′. In all gene knockdown experiments, we used a lentiviral pLKO.1-puro non-target shRNA control plasmid (Sigma) encoding a shRNA that does not target any known genes from any species as a negative control. For preparation of lentiviral shRNA particles, replication-incompetent lentiviral particles were packaged in 293T cells by co-transfection of shRNA constructs with a packaging mixture (9:1 pCMV-D8.2 and pCMV-ΔSVSVG). Virus was harvested twice at 48 and 72 h post-transfection. Cells were infected with the lentivirus for 16–20 h with fresh medium containing individual virus and Polybrene at a final concentration of 8 μg/ml. The infected cells were further selected with 2 μg/ml puromycin for 48 h.

**Immunofluorescence and Confocal Microscopy**—Cells were fixed in 4% paraformaldehyde for 15 min at room temperature, permeabilized with PBS containing 0.3% Triton X-100 for 10 min, and blocked in PBS containing 5% bovine serum albumin and 0.3% Triton X-100. The cells were incubated with primary antibodies for 18 h at 4 °C, washed three times in PBS, and incubated with secondary antibody for 1 h. Cell nuclei were counterstained with DAPI. Immunofluorescence of paraffin-embedded tissue sections was accomplished by standard deparaffinization, followed by the same immunofluorescence protocol as described above.

The following antibodies were used: mouse anti-p63 monoclonal antibody, rabbit anti-K5 monoclonal antibody, and mouse anti-K10 monoclonal antibody (MA1-21871, RM210650, and MS611P0, Thermo Fisher Scientific); rabbit anti-PAX6 polyclonal antibody (PRB-278P, Covance); mouse anti-K1 monoclonal antibody (sc-376224, Santa Cruz Biotechnology); and mouse anti-K3/K12 monoclonal antibody and rabbit anti-K12 monoclonal antibody (ab68260 and ab124975.

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**TABLE 1**

| Gene     | Forward primer   | Reverse primer   |
|----------|------------------|------------------|
| GAPDH    | GAGTCAACGGATTTGCTGGCT | CTACACGACGCTTTCCTCTCA |
| k1       | CAGCGTACATTGCTGAGATCAGG   | CTCTGCTGCCACAGCTGCTGA |
| k3       | AGCTGACCTACGAGCTGAGTGA   | CTCTGCTGCCACAGCTGCTGA |
| K10      | CCCTGCTTCGAGATCGACAATGCC | ATCTCCAGGTCAGCCTTGGTCA |
| K12      | AGCCAGAACGGAAGGACGCTGAGA | ACCCGTCGGTCTGGAGACTGAA |
| PAX6     | TGGTCCACGCAGATGTTGAGT    | TTTCCCAAGCAAGATGAGAC |

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8–12 days.
Abcam). Secondary antibodies (Alexa Fluor 488 or 568-conjugated anti-mouse or anti-rabbit IgG, Invitrogen) were used at a dilution of 1:500. Images were obtained using an Olympus FV1000 confocal microscope.

**Microarray Data Analysis**—Total RNA was isolated from LSCs and SESCs as in our previous study (15). Raw data were deposited in the Gene Expression Omnibus database under accession number GSE32145. Microarray-based gene expression data produced in this study were normalized across samples and subjected to average linkage hierarchical clustering using the Cluster 3.0/TreeView software package (16). Selection of genes belonging to the Wnt and Notch pathways was performed based on previous studies. Expression values were overlaid on a network generated using the GeneSet analysis tool for the Reactome network (17) using the Reactome Functional Interaction plugin for Cytoscape 3 (18).

**Results**

**PAX6 and p63 Expression during Ocular Development in Mice**—To elucidate the function of PAX6 and p63 during cornea development, we first studied their expression profiles from embryonic day (E) 12.5 to E18.5 in mice. At E12.5, strong PAX6 expression was detected in the eye field and particularly in the early corneal epithelium, whereas p63 expression was negative (Fig. 1A). In contrast, p63-positive cells appeared at the ocular surface and expanded to the limbus and cornea later, at E14.5, following the development and fusion of the eyelid (Fig. 1B). PAX6 expression was restricted to the eye field during ocular development (Fig. 1, A–D). In addition, we performed lineage tracing experiments using a Pax6 promoter driving a GFP reporter. We observed intensive GFP expression in the corneal epithelium of ROSA<sup>tm1TagG</sup>;<sup>PAX6-GFPcre</sup> mice at P1 and P60 (Fig. 1E). These results suggest a central role of PAX6 in LSCs and the corneal epithelium, from early developmental stages to adulthood.

**PAX6 and p63 Expression in Human Corneal and Skin Epithelia**—We observed that P63, a master regulator of squamous epithelial cell development, was expressed mainly in the basal layer of both the limbus and skin epidermis, suggesting the similarity of these two epithelial cell types. However, although PAX6 was highly expressed in the epithelial layers of the cornea, it was undetectable in the skin epidermis in human adults (Fig. 2). For further characterization of the skin epithelial and corneal epithelia, we performed immunostaining for tissue-specific keratins. LSCs migrated to the central cornea.
upon differentiation, with K3 and K12 as cornea-specific markers (Fig. 2A), whereas skin epidermis-specific keratins (K1 and K10) were expressed in the suprabasal epidermal layers (Fig. 2B), and PAX6 and p63 co-localized in the corneal limbus (Fig. 3A). We further isolated and cultured LSCs and SESCs in vitro. LSCs could be expanded and identified by PAX6 and p63 expression (Fig. 3B), and SESCs could be identified by p63 and K5 expression (Fig. 3C).

**PAX6 Is Essential in Corneal Cell Fate Determination**—To investigate the role of PAX6 in determining corneal cell fate, we used a lentivirus-mediated PAX6 knockdown in human LSCs. We purified LSCs with knockdown of PAX6 by puromycin selection. RNA was extracted from both stem cells and differentiated cells; the expression levels of related genes were compared by quantitative PCR. Two different short hairpin RNAs (shRNAs) for PAX6 were used and showed similar results. Although 4.5-fold knockdown of PAX6 in LSCs did not produce a proliferation defect with active Ki67 expression (Fig. 4A), the cornea-specific markers K3 and K12 were significantly down-regulated upon differentiation by 17.7- and 14.5-fold ($p < 0.05$), respectively, compared with controls. In contrast, skin-specific K1 and K10 expression was up-regulated by 4.1- and 4.4-fold ($p < 0.05$), respectively (Fig. 4B). These results indicate that loss of PAX6 in LSCs leads to skin-like differentiation.

**Loss of PAX6 in Human Congenital Limbal Dermoid Tissue**—To determine the clinical relevance of PAX6 expression in LSCs and CECs, we studied human corneal limbal dermoids, which exhibited skin epidermis pathology with vascularization and disorganized cells in the stroma (Fig. 5A). We found that PAX6 expression was completely absent in an area of corneal dermoids (Fig. 5B). Moreover, we observed localized expression of p63 and K5 in the basal layer (Fig. 5B) and skin-specific keratins K1 and K10 in the suprabasal layer (Fig. 5B). These results suggest a conversion of corneal epithelial cells to skin-like epithelial cells in patient tissues during development and strongly support the essential role of PAX6 in CEC fate determination.

**Signaling Pathways in LSC Fate Determination**—To further determine the functional characteristics that control corneal cell fate commitment, we sought to identify signaling pathways that might be differentially activated in LSCs and SESCs. We performed RNA expression analyses using microarrays on LSCs and SESCs, followed by gene ontology and pathway analyses using DAVID (19, 20). We identified subsets of genes that showed at least 2-fold expression differences between LSCs and SESCs, resulting in a total of 1185 genes. This analysis identified...
numerous GO terms and signaling pathways affecting many cellular and metabolic processes (data not shown). In particular, however, the Notch, Wnt, and TGF-β pathways emerged as important from this analysis, consistent with their critical roles in the self-renewal and lineage commitment of stem cells from a variety of tissues (21–23), including the epithelium. Graphical representation of expression changes for distinct members of these pathways is provided in Fig. 6.

**Discussion**

A clear transparent cornea is maintained by self-renewal of LSCs and their differentiation into CECs (24, 25). These two processes must be highly organized to maintain the integrity and homeostasis of the corneal epithelium. Pathological changes in LSCs can lead to a loss of transparency in the cornea and cause partial or complete blindness (2, 15). In this study, we found that PAX6 is essential for the maintenance of LSC characteristics and their further commitment to the CEC lineage. Of note, although p63 is well documented as a master gene of self-renewal and differentiation for common squamous epithelia such as in the cornea, dermis, and prostate (4–6), we observed that p63 was expressed after PAX6, which implicates PAX6 expression as a central event in corneal cell fate control.

PAX6-deficient LSCs in culture exhibit a skin-like epithelial cell fate as indicated by a switch in keratin expression upon differentiation, specifically replacement of cornea-specific K3/K12 by skin-specific K1/K10. Furthermore, PAX6 is absent in corneal dermoid tissue, a congenital teratoma that switches cornea into the skin lineage. This is consistent with the recent finding of PAX6 down-regulation in abnormal epidermal differentiation, such as that seen in Stevens-Johnson syndrome, chemical burns, aniridia, and recurrent pterygium (2).

Wnt and Notch signaling has been shown to be critical for the self-renewal and lineage commitment of epithelial cells in embryogenesis and stem cells from a variety of tissues (21–23). Both signaling pathways are complex, with different receptors, ligands, co-activators, and inhibitory proteins. For example, Notch1 helps maintain CEC fate during repair in injured mouse corneas (27). The influence of Wnt signaling on ocular surface development has also been extensively reported. Wnt4 is expressed in human fetal corneas and adult basal LSCs (26). DKK2, an antagonist of canonical Wnt signaling, is required for accurate development of the ocular surface epithelium in mice by regulation of Wnt/β-catenin activity (14). In addition, our previous work suggested a central role of the Wnt7A-PAX6 axis in CEC fate determination, in which SESC could be converted to LSC-like cells by overexpression of PAX6 (15). In the present study, we have provided further evidence for the importance of the Wnt and Notch signaling pathways by comparison of gene expression profiles between LSCs and SESC. Further investi-
gation to define the function of these genes in corneal epithelium specification may allow us to better understand and manipulate the corneal diseases.

Taken together, our data indicate a critical role of PAX6 in LSCs and corneal epithelial fate determination. Furthermore, we have identified a key role for PAX6 in the determination of the corneal epithelial phenotype. Understanding how PAX6 controls corneal cell fate will provide important insight into corneal homeostasis and disease and aid in developing new therapeutic strategies for the treatment of common corneal diseases.

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