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Role of SH2-Containing Tyrosine Phosphatase Shp2 in Mouse Corneal Epithelial Stratification

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Purpose. Shp2 protein tyrosine phosphatase mediates a wide variety of receptor tyrosine kinases (RTK) cell signaling. Herein, we investigate the role of Shp2 in corneal morphogenesis and homeostasis.

Methods. *Shp2* was conditionally knocked out ($Shp2^{cko}$) in *Krt14-rtTA;tet-O-Cre;*$Shp2^{f/f}$ triple transgenic mice administrated with doxycycline (Dox) from postnatal day 1 (P1) to P10, P15, and P25, respectively. In addition, corneal epithelial debridement was performed in adult (P42) mice treated with or without Dox for 8 days (from P42–P50). Mouse eyes were then subjected to histology and immunohistochemistry.

Results. $Shp2^{cko}$ revealed impaired stratification of conjunctival and corneal epithelia during morphogenesis. Likewise, $Shp2^{cko}$ failed to restore epithelial stratification after a corneal epithelial wound in adult $Shp2^{cko}$. At the cellular level, the ratio of proliferating cell nuclear antigen (PCNA-positive)/total basal cells remained unchanged, but cells in G2/M (survivin-positive) phase was significantly increased in $Shp2^{cko}$ as compared with those in the control littermate. Interestingly, deltaN-p63 (ΔNp63) expression and the asymmetric division of the basal cells were coincidentally dampened in $Shp2^{cko}$. Transmission electron microscopic study showed that desmosome and hemidesmosome densities were reduced in the corneal epithelium of $Shp2^{cko}$. Immunohistochemistry also demonstrated that expression of E-cadherin/β-catenin junction and laminin-β1 was extensively downregulated in $Shp2^{cko}$. On the other hand, corneal epithelium lacking *Shp2* remained positive for K14, Pax-6, and keratin 12 (K12), suggesting that *Shp2* was dispensable for the corneal epithelial-type differentiation.

Conclusions. These data argued that *Shp2* deficiency predominantly impacted p63-dependent cell division and cell adhesive ability, which resulted in the impairment of stratification during corneal epithelial development and wound healing.

Keywords: Shp2, corneal epithelium, stratification, knockout animals, p63

The stratification of epithelial cells forms a multilayer that serves as a primary barrier to prevent the invasion of pathogens and antigens from external environment, such as skin and ocular surface, where the stratification of epithelium can avoid epithelial cell defect duo to continuous exfoliation of superficial cells.[1] However, the cellular and molecular mechanisms by which epithelial stratification is initiated during development and maintained have not been clearly identified. Previous studies have found that Shp2, a member of the protein tyrosine phosphatase family is ubiquitously expressed and plays a fundamental role in many cell signal pathways.[2–5] One of these is the Ras/MAPK pathway, which impacts several facets of cellular behaviors, such as proliferation, apoptosis, and migration.[6–8] All of these would influence cell stratification.

Shp2 naturally remains in a folded conformation as its two N-terminal SH2 domains bind to the central protein tyrosine phosphatase domain, keeping the phosphatase in an auto-inhibited state. Shp2 is activated in the presence of phosphotyrosine residues of the epidermal growth factor (EGF) receptors when extracellular ligands bind. When Shp2 binds to the phospho-EGF receptor (active form), other scaffolding proteins, such as Grb2 and Gab1, are able to aggregate and continue signal transduction down the pathway.[9–11] Because Shp2 interacts directly with activated EGFR, these findings suggest that the presence of the tyrosine phosphatase is essential to the Ras/MAPK pathway and maybe, therefore, necessary for the development and maintenance of proper epithelial stratification.

Using the corneal epithelium as a model, the present study aims to identify the role of Shp2 in corneal epithelial stratification in the corneal morphogenesis and homeostasis during wound healing. Two strains of transgenic mice, *K14-rtTA;tetO-Cre;*$Shp2^{flox/flox}$ and *K14-rtTA;tetO-Cre;*$Shp2^{flox/+}$, with the latter as the control, were employed in this study. Our

results revealed that *Shp2* was required for the initiation of corneal epithelial stratification, which occurred just before eyelid opening in the second week of postnatal age (P) in rodents.[1] *Shp2* was also indispensable for the restratification of epithelium during epithelial debridement wound healing. A possible mode of action of *Shp2* in these processes is also discussed.

Materials and Methods

Mice

Compound transgenic mouse strain *K14-rtTA;tetO-Cre; Shp2$^{flox/flox}$* was generated through the natural mating of single transgenic mouse lines *K14-rtTA*,[12] *tetO*-Cre,[13] and *Shp2^{floxed}*,[14] as seen in Figure 1A. *K14-rtTA;tetO-Cre;Shp2$^{flox/flox}$* transgenic males were mated with *K14-rtTA;tetO-Cre;Shp2$^{flox/+}$* females. The resulting mouse littermates were induced at P0 since previous studies have shown that ablation of Shp2 during the early embryonic stage results in an open eyelid defect at birth,[14] which could introduce other factors that might impact the proper formation of the corneal epithelium. Therefore, to ensure that the data was solely attributed to the gene ablation, the mice were induced at P1, and their corneas were collected at P10, P15, and P25 for immunostaining. All mice were maintained in mixed genetic background. Experimental animals were housed under pathogen-free conditions in accordance with institutional guidelines. Animal care and use conformed to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Cincinnati.

Administration of Doxycycline (Dox)

To induce *Shp2* gene ablation in adult animals, *K14-rtTA; tetO-Cre;Shp2$^{flox/flox}$* mice (P42) were injected once intraperitoneally (IP) with Dox (80 μg/g body weight; Clontech Laboratories, Mountain View, CA) dissolved in 1× PBS (pH 7.4) at a concentration of 10 mg/mL and fed Dox-chow (1 g/kg chow; Bioserv, Frenchtown, NJ)[15] ad libitum. Control animals were injected with PBS and fed regular chow. Neonates (P1) were administrated with Dox by feeding the nursing mother with Dox chow ad libitum.

Histology Analysis

Mouse samples were fixed overnight in 4% paraformaldehyde (PFA) in PBS, followed by dehydration in a series of ascending alcohol and paraffin embedding. Deparaffinized sections (5 μm) were stained with hematoxylin and eosin (H&E) or periodic acid Schiff reagent (PAS).

Immunofluorescence and Immunohistochemistry

Tissue sections (5 μm) were deparaffinized, rehydrated, and subjected to antigen retrieval in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween, pH 6.0) at boiling temperature for 30 minutes. Eyelid sections were then blocked with 3% BSA in PBS containing 0.025% Nonidet P40 for 1 hour at room temperature, then incubated overnight at 4°C with the primary antibodies diluted in the same buffer. After three washes with PBST (PBS, 0.1% Tween 20), slides were incubated at room temperature for 1 hour with AlexaFluor-488- or AlexaFluor-555-conjugated secondary antibodies (Invitrogen, Inc., Carlsbad, CA) and 1 μg/mL 4′,6-diamidino-2-phenylindole (DAPI) as a nuclear counterstain, washed with PBST again, and mounted with Mowiol (Sanofi-Aventis, Paris, France). Sections were examined and photographed using a Zeiss microscope equipped with a camera (Axiocam Mrm; Carl Zeiss, Thornwood, NY). For data acquisition, we used the Axiovision 4.6 software (Carl Zeiss). The following antibodies were used: rabbit anti–β-Catenin antibody (#C2206, Sigma-Aldrich, St. Louis, MO); rabbit anti–keratin 12 (K12) antibody[16]; rabbit anti-K14 (PRB-155P; Covance, Princeton, NJ), rabbit anti-K18 (clone E431-1, cat# 04-586, EMD; Millipore Corporation, Billerica, MA); rabbit anti-Shp2 (#3752; Cell Signaling Technology, Danvers, MA), rabbit anti-Survivin (clone 71G4B7; Cell Signaling Technology); mouse anti-p63 monoclonal antibody (4A4; Neomarkers, Fremont, CA); mouse anti–proliferating cell nuclear antigen (PCNA, PC10:sc-56; Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti–E-cadherin (clone 67A4; Chemicon, Inc., Temecula, CA), goat anti–laminin-β1 (sc-6018; Santa Cruz Biotechnology, Inc.), Secondary Alexa488-, Alexa555-labeled antibodies were purchased from Molecular Probes, Inc. (Eugene, OR).

Detection of Apoptosis In Situ

Cell apoptosis status in Dox-treated and nontreated groups were subjected to TUNEL assay using Click-iT TUNEL Assay kit (Invitrogen, Inc.) according to the manufacturer's protocol.

Transmission Electron Microscopy

Cornea samples were obtained and fixed in 0.1M cacodylate buffer (pH7.4) containing 3% glutaraldehyde and 2% paraformaldehyde for 2 hours at 4°C and then were preserved in 0.1 M cacodylate buffer (pH 7.4) containing 0.5% glutaraldehyde at 4°C overnight. After refixation in 1% osmium tetraoxide (OsO4) for 1 hour at 4°C, cornea samples were washed in 0.1 M cacodylate buffer (pH 7.4) three times for 10 minutes, and then dehydrated in a graded ethanol series and embedded in Epon 812 epoxy resin (Polysciences, Inc., Warrington, PA). Ultrathin 50-nm sections were stained with uranyl acetate and lead citrate and images were photographed with a Hitachi 7500 Transmission Electron Microscope (Hitachi, Tokyo, Japan) equipped with AMT Digital camera.

Wound Healing Experiment

A 2-mm diameter central corneal epithelial debridement was generated in the right eye using Algerbrush II Rust Ring Remover (Alger Equipment Co., Inc., Lago Vista, TX) and mice were then separated into two groups. One group of mice was fed with Dox (+Dox) chow and another group of mice was fed with regular chow (–Dox) for 8 days.

Statistical Analysis

A two-tailed Student's *t*-test (Excel; Microsoft, Redmond, WA) was used in the analysis of the percentage of PCNA-, deltaN-p63 (ΔNp63)-, and survivin-positive cells. All quantification data are presented as mean ± SEM. Student's *t*-test was used to analyze the significance of difference; *P* less than 0.05 was considered statistically significant.

Results

K14+ Cell Type–Specific and Dox-Inducible Ablation of Shp2 Gene in the Transgenic Mouse

Conventional *Shp2* knockout mice died early in the embryonic development. To examine the function of Shp2 in ocular surface epithelial morphogenesis, we generated a *K14-rtTA;*

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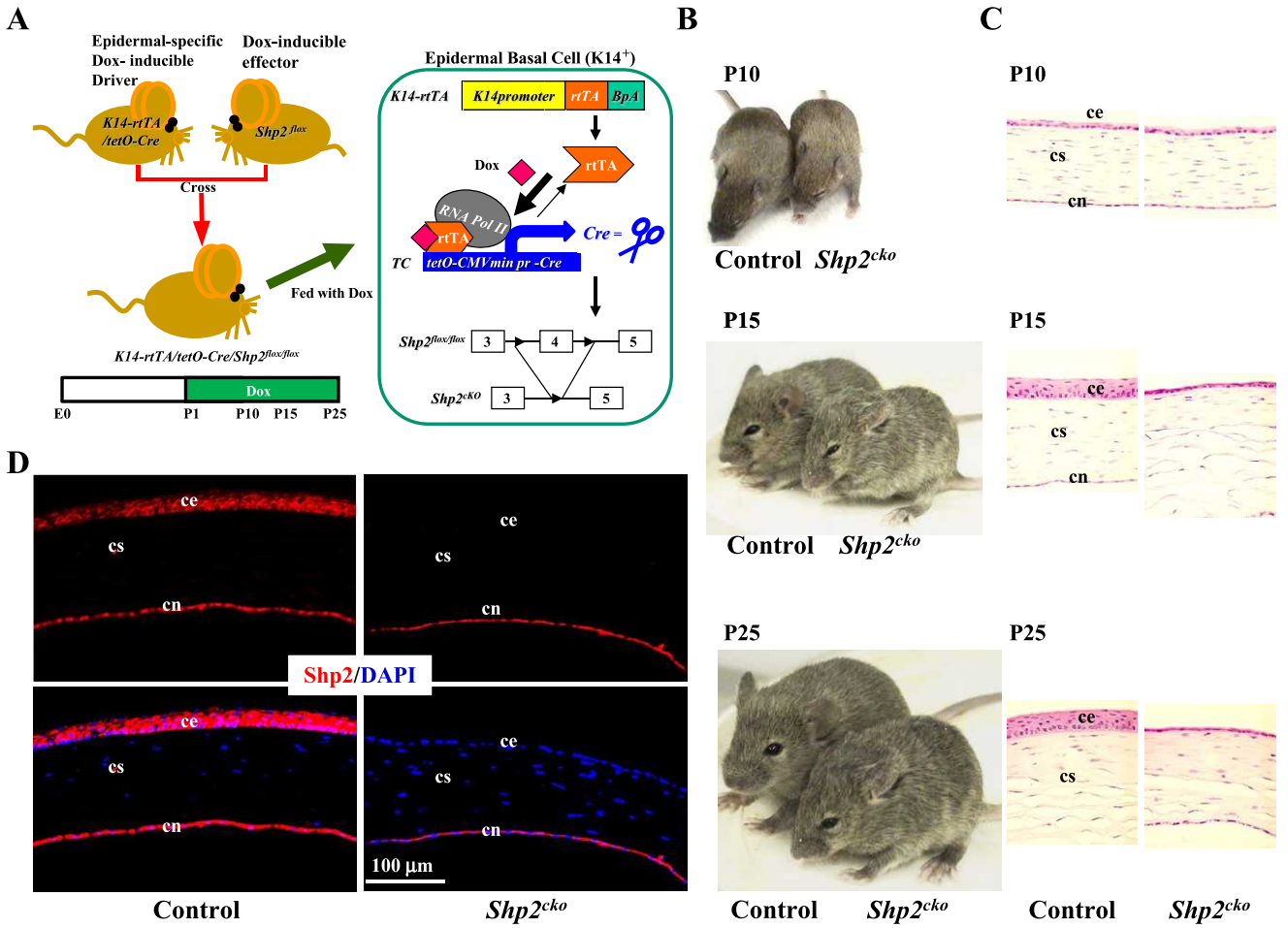

Figure 1. K14+ cell type–specific and Dox-inducible ablation of *Shp2* gene in the transgenic mouse. (**A**) Schematic presentation showing Dox-dependent ablation of *Shp2* gene alleles. Double transgenic mouse, *K14-rtTA;TC*, serves as epidermal-specific Dox-inducible driver. *Shp2 floxed* allele (*Shp2$^{f/f}$*) mouse line serves as Dox-inducible effector. Intercross between *K14-rtTA;TC* and *Shp2$^{f/f}$* generated triple transgenic mice *K14-rtTA;TC;Shp2$^{f/f}$*. Administration of Dox resulted in the deletion of exone4 of the *Shp2* gene only in K14-positive basal cells in all stratified epithelial tissues including skin and ocular surface epithelia. (**B**) *K14-rtTA;TC;Shp2$^{f/+}$* (control) and *K14-rtTA;TC;Shp2$^{f/f}$* (*Shp2^{cko}*) mouse littermates administered with Dox in the dam via mother's milk from P0 to P10, P15, and P25, respectively. It should be noticed that mice lacking *Shp2* alleles exhibited wavy fur at P15 and P25 as compared with the control littermates. This partially recapitulated phenotype in *Waved-1* (*TGF-α* deficient) and *Waved-2* (*EGFR* deficient) mouse strains. (**C**) Hematoxylin and eosin histologic analysis of corneal stratification corresponding to (**B**). (**D**) Immunohistochemistry showed that immunoreactivity of Shp2 protein was completely undetectable only in the corneal epithelium, but remained strong and positive in the corneal endothelium of the Dox-treated *K14-rtTA;TC;Shp2$^{f/f}$* mouse (compare *right panels* to *left panels*). Abbreviations: ce, corneal epithelium; co, cornea; cs, corneal stroma; cn, corneal endothelium. *Scale bar*: 100 µm in (**D**).

tetO-Cre;Shp2$^{flox/flox}$ triple–transgenic mouse strain, in which conditional *Shp2* ablation was induced in basal cells of the stratified epithelia upon Dox treatment (Fig. 1A). We first administered Dox from embryonic day 0.5 (E0.5) to P0 (at birth) and noticed that, unlike *K14-rtTA;tetO-Cre;Shp2$^{flox/+}$* (control) littermate with closed eyelid, *K14-rtTA;tetO-Cre;Shp2$^{flox/flox}$* (*Shp2^{cko}*) displayed an open eyelid phenotype (Supplementary Fig. S1) and died within 24 hours due to defects in esophagus and upper airway (unpublished data). To avoid neonatal death and to allow the investigation of corneal epithelium stratification occurring just prior to eyelid opening at P12 to P14 in the mouse,[1,17,18] we administered mouse neonates with Dox from P1 via nursing mother fed Dox–chow as described in Materials and Methods. With this protocol of Dox treatment, *Shp2^{cko}* mice could survive and remained healthy during the weaning stage (up to P25), but gradually became moribund after a couple of days of weaning. This was also due to obstruction of the esophagus, which inhibited food uptake (data not shown). Therefore, we examined *Shp2^{cko}* mice and their control littermates at P10, P15, and P25, respectively. We did not find a significant difference in general appearance between control and *Shp2^{cko}* at P10. However, wavy fur was noticed in *Shp2^{cko}* at P15 and P25, which partially resembled the phenotypes of *TGF-α* (*Wave-1*) and *EGFR* (*Wave-2*) deficient mice[19–21] (Fig. 1B). This observation suggested that Shp2 was likely to mediate TGF-α/EGFR signaling pathway in epidermal and ocular surface tissues.

Shp2 Deficiency Caused Failure of Ocular Surface Epithelial Stratification

Histologic analysis revealed that corneal epithelium consisting of two cell layers at P9 stratified to four to five cell layers at P15 and to six to seven cell layers at P25 in the control mice. In contrast, corneal epithelium failed to do so and remained at one to two cell layer(s) in *Shp2^{cko}* at P15 and P25 (Fig. 1C; Supplementary Fig. S2). Immunohistochemical staining with anti-Shp2 antibody confirmed that the Shp2 protein was totally undetectable in the corneal epithelium, but remained strong and positive in the corneal endothelium of the *Shp2^{cko}* mouse

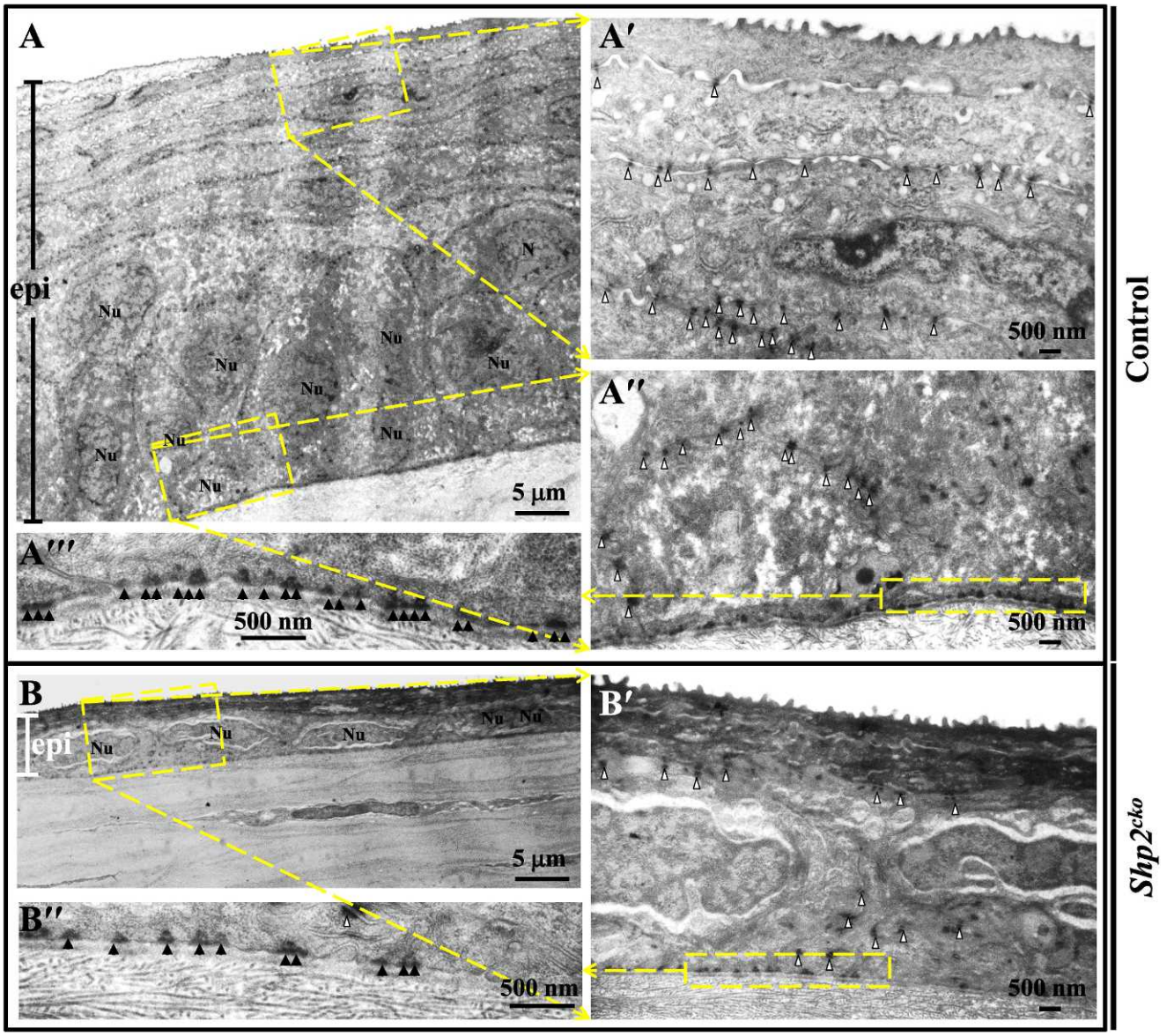

FIGURE 2. *Shp2* deficiency reduced the density of desmosome between cells and hemidesomesone between cell-matrix. Transmission electronic micrographs of control group (**A**, **A′**, **A″**, **A‴**) and *Shp2*^{cko} (**B**, **B′**, **B″**). Control corneal epithelium exhibited seven to eight cell layers at P25 (**A**), but displayed only one to two cell layers in *Shp2*^{cko} (**B**). Unlike control epithelium, which showed distinct cell morphology of different layers (i.e., basal columnar cells), suprabasal wing cells, and superficial flattened cells, *Shp2* deficient epithelium exhibited only flattened cells. It should be noticed that the density of desmosome (*open triangles*) between cells and hemidesmosome (*arrowheads*) between cell and basement membrane were dramatically reduced in *Shp2* deficient corneal epithelium. No early phase of apoptosis with condensed or peripheralized chromatin was found. Likewise, no apparent autophagosome membranes showed in the *Shp2* deficient corneal epithelium. Abbreviations: Nu, nucleus; epi, epithelium.

at P25. (Fig. 1D). In addition to the corneal epithelium, other ocular surface epithelia, including eyelid mucocutaneous and conjunctival epithelium, also failed to stratify into multilayers. Moreover, the ectoderm-derived meibomian gland and goblet cells were malformed in the *Shp2*^{cko} ocular surface epithelia (Supplementary Figs. S3, S4). In this paper, we focused on the stratification of the corneal epithelium.

Shp2 Deficiency Lost Polarized Basal Cells and Reduced the Density of Desmosomes Between Cells and Hemidesmosomes Between Cell-Matrix

Next, we took a closer look regarding the ultrastructure of corneal epithelium under a transmission electron microscope as shown in Figure 2. The corneal epithelium exhibited seven to eight cell layers in the control mice. In contrast, the corneal epithelium of *Shp2*^{cko} mice only displayed two cell layers at P25 (Fig. 2A versus Fig. 2B). The corneal epithelium of *Shp2*^{cko} mice lost the distinctive polymorphic epithelium from basal to superficial layers and only flattened cells were exhibited in the cornea of the *Shp2*^{cko} mice (Fig. 2B). Moreover, the density of desmosomes between epithelial cells and hemidesmosomes between epithelial cells and the basement membrane (BM) were obviously reduced in *Shp2*^{cko} mutants as compared with the control littermates (Fig. 2B″ versus Fig. 2A‴). However, no early phase of apoptosis with condensed or peripheralized chromatin was found. Likewise, no apparent autophagosome membranes showed in either control or *Shp2*^{cko} corneal epithelia. These data suggested that *Shp2* deficiency might

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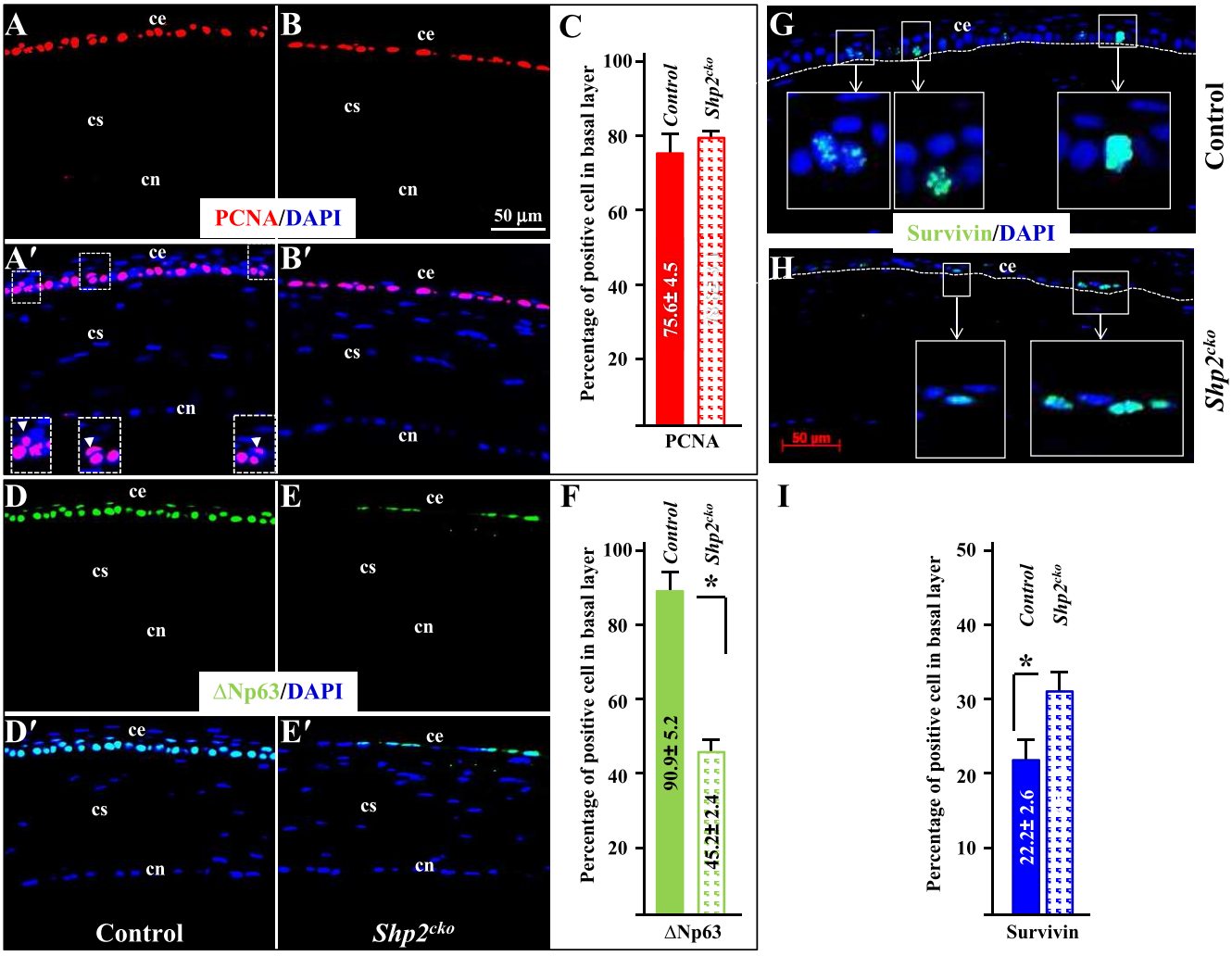

FIGURE 3. Role of *Shp2* in cell proliferation and division of the corneal epithelial basal cells. Immunofluorescent stained micrographs of PCNA (**A**, **A′**, **B**, **B′**), ΔNp63 (**D**, **D′**, **E**, **E′**), and survivin (**G**, **H**) in control (**A**, **A′**, **D**, **D′**, **G**) and *Shp2*^{cko} (**B**, **B′**, **E**, **E′**, **H**) mice at P25. Noted that Shp2 deficiency did not impact cell proliferative activity (compare **B**, **B′** with **A**, **A′**), but downregulated ΔNp63 (compare **D**, **D′** with **C**, **C′**). Histogram of quantitative analyses represents the percentage of cells positive for PCNA (**C**) and ΔNp63 (**F**) in the corneal epithelial basal layer. The percentage of survivin-positive (G2/M of the cell cycle) basal cells in *Shp2*^{cko} was significantly increased as compared with that of control cornea (**G**, **H**, **I**). More interestingly, basal cells of the control cornea underwent division perpendicular to basement membrane (*dotted lines*) to move up, while cell division orientation in *Shp2*^{cko} is parallel to basement membrane (**I**). *Scale bars*: 50 μm. *$P < 0.05$.

impact cell polarity, cell–cell and cell-matrix adhesion, but little cell death (please see below).

Role of Shp2 in Proliferation and Division Orientation of Corneal Epithelial Basal Cells

Given that epithelial stratification has been at least in part conferred by the balanced symmetric cell division (SCD) and asymmetric cell division (ACD) of the basal cells,[22,23] we performed immunofluorescence staining to detect PCNA and ΔNp63 to examine whether impaired stratification in *Shp2*^{cko} mice was attributed to defects in epithelial cell proliferation and/or division orientation. We found that 75.6 ± 4.5% and 78.8 ± 3.1% of the total basal cells in the control and *Shp2*^{cko} corneal epithelia, respectively, were PCNA-positive (Figs. 3A–C). Notably, some cells right above their basal layer were also PCNA-positive in the control group, but little of such were found in the *Shp2*^{cko} cornea (compare insets of Figs. 3A′ and 3B′). Given that ΔNp63 is required for the cell cycle progression, ACD, and epidermal stratification,[24–26] as we expected, ΔNp63-positive cells were significantly reduced from 90.9 ± 5.2% in the control cornea down to 45.2 ± 2.4% in those of the *Shp2*^{cko} (Figs. 3D–F). We then performed immunohistochemistry to examine the expression of survivin, which is only expressed in the G2/M phase and localizes to the mitotic spindle by interaction with tubulin during mitosis.[27] We found that the percentage of survivin-positive cells/total basal cells increased from 22.2 ± 2.6% in the control cornea up to 31.3 ± 2.6% in those of the *Shp2*^{cko} (Figs. 3G–I). More interestingly, most of the basal cells in corneal epithelium of control underwent perpendicular cell division, while those in the *Shp2*^{cko} turned into a parallel orientation relative to the BM (compare Figs. 3G and 3H). These data strongly suggested that *Shp2* deficiency not only affected cell cycle progression through G2/M phase, but also dramatically reduced ACD, leading to the impaired stratification.

Role of Shp2 in Cornea-Type Differentiation

We next investigated whether corneal epithelial differentiation was altered by *Shp2* ablation. As shown by the immunofluo-

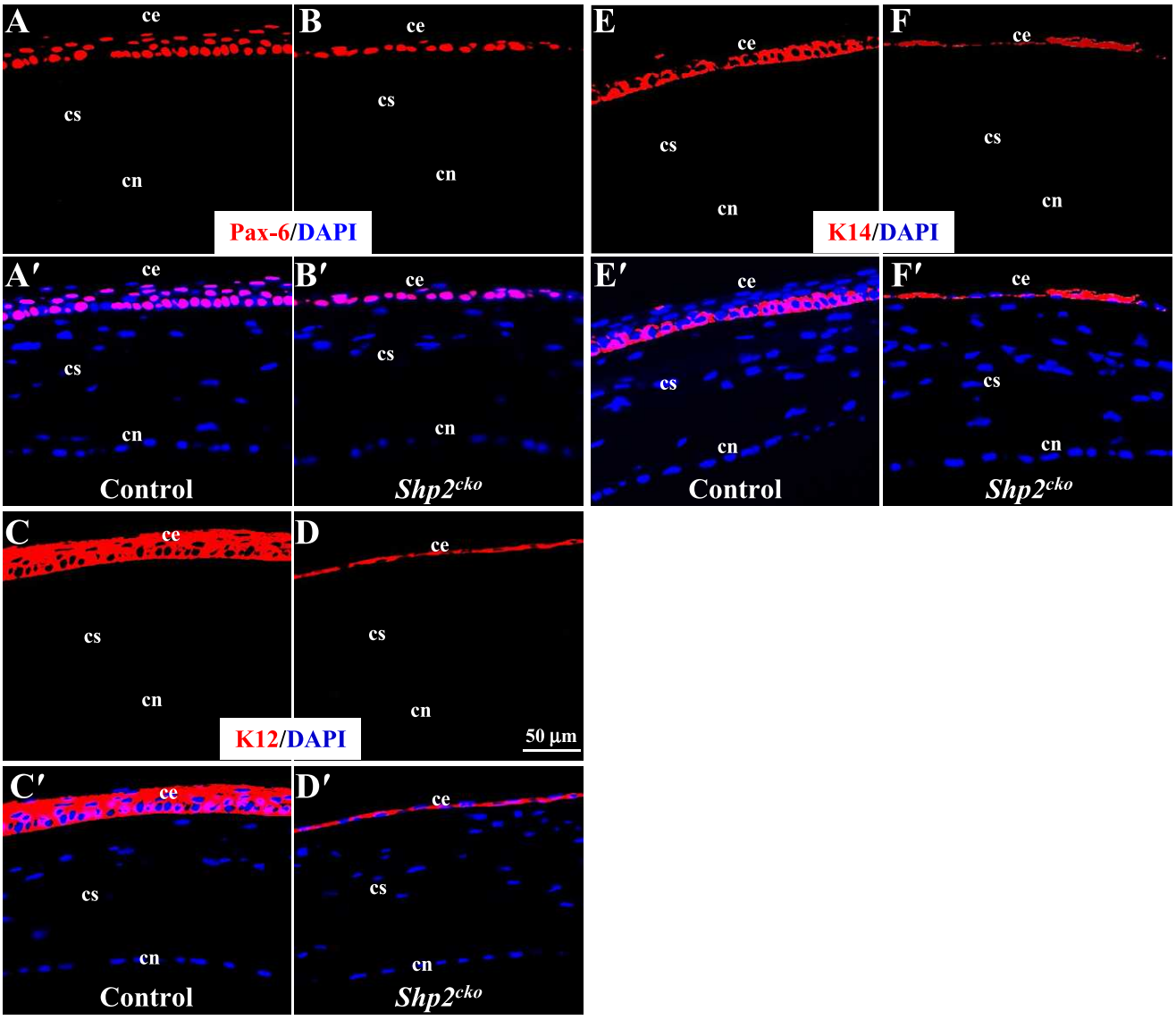

FIGURE 4. *Shp2* deficiency did not impact cornea-type differentiation. Immunofluorescent-stained micrographs of Pax-6 (**A**, **A′**, **B**, **B′**), K12 (**C**, **C′**, **D**, **D′**), and K14 (**E**, **E′**, **F**, **F′**) in control (**A**, **A′**, **C**, **C′**, **E**, **E′**) and *Shp2*^{cko} (**B**, **B′**, **D**, **D′**, **F**, **F′**) mice at P25. Noted that Shp2 deficiency did not impact Pax-6 or K12 (markers for corneal epithelium), but downregulated the expression of K14, an epithelial progenitor cell marker. *Scale bars*: 50 < μm.

rescence staining, Pax-6[28–29] (a transcription factor essential for ocular surface epithelial differentiation) and K12, the marker of cornea-type epithelium differentiation, remained strongly positive in the nonstratified epithelium of the *Shp2*^{cko} cornea (Figs. 4B, 4B′, 4D, 4D′). Interestingly, keratin 14 (Krt14) expressions, which was positive for all basal cells in the control cornea (Figs. 4E, 4E′) was interrupted along the corneal epithelium (Figs. 4F, 4F′) in the cornea of *Shp2*^{cko} mice. These data illustrated that *Shp2* deficiency did not impact cornea-type differentiation but downregulated the expression of K14, an epithelial progenitor cell marker.

Shp2 Deficiency Altered Cell–Cell and Cell-Matrix Adhesion

Because of the downregulation of desmosomes and hemidesmosomes observed in corneal epithelium of the *Shp2*^{cko} mice, we examined cell adhesive functions. Immunofluorescence staining unambiguously demonstrated that the expression of E-cadherin, a calcium-dependent cell–cell adhesion molecule[30] (Figs. 5B, 5B′) and β-catenin (Figs. 5D, 5D′) are dramatically downregulated in *Shp2*^{cko}. Furthermore, laminin-β1,[31] an extracellular matrix (ECM) component of the epithelium BM was completely diminished in corneal epithelium of *Shp2*^{cko} (Figs. 5F, 5F′). These data argued that *Shp2* deficiency might compromise cell–cell and cell-BM adhesive function. Taken together, Shp2 function played a pivotal role in the epithelium stratification during normal corneal morphogenesis.

Shp2 Is Required for the Restoration of Stratification After Corneal Epithelial Debridement

To address the necessity of *Shp2* for maintaining corneal epithelial homeostasis, *K14-rtTA;tetO-Cre;Shp2*$^{flox/flox}$ triple-transgenic mice were subject to central corneal epithelial abrasion and then were treated with Dox (*Shp2*^{cko}) or with regular chow (control) for 8 days as shown in Figure 6A. In a naïve cornea, Cre recombinase effectively knocked out *Shp2*

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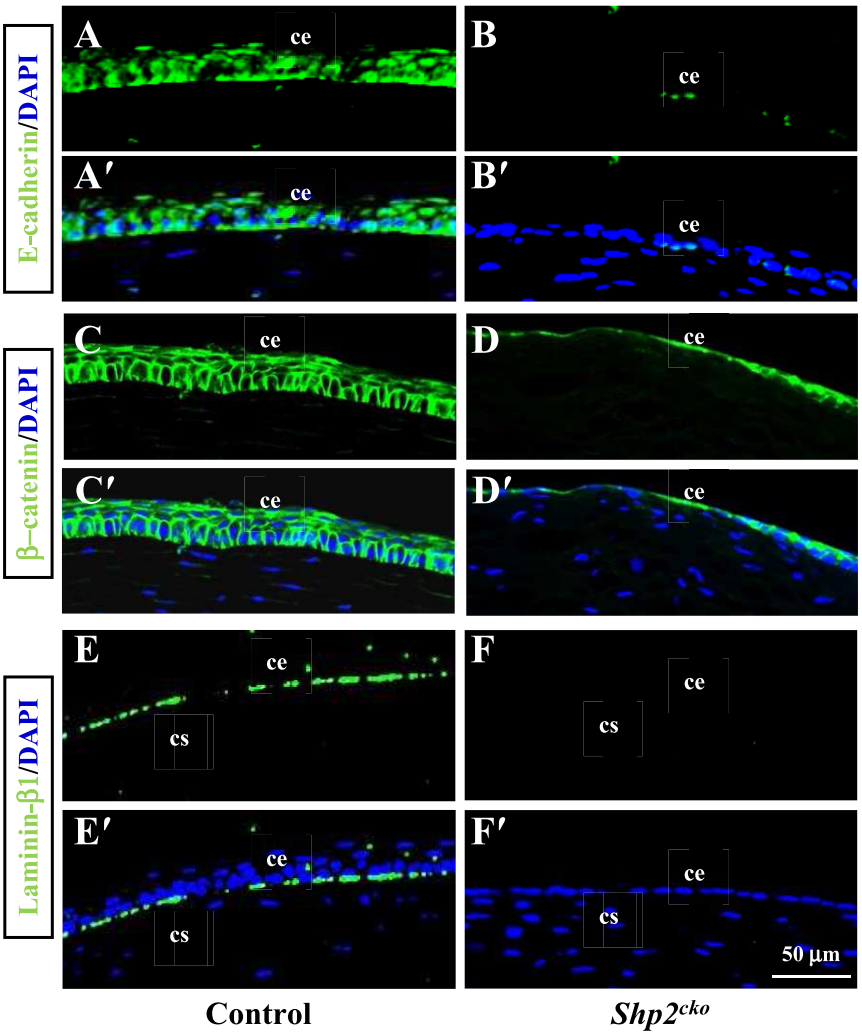

Figure 5. Shp2 deficiency altered cell–cell and cell-matrix adhesion. Immunofluorescent-stained micrographs of E-cadherin (**A**, **A′**, **B**, **B′**), b-catenin (**C**, **C′**, **D**, **D′**), and laminin-β1 (**E**, **E′**, **F**, **F′**) in control (**A**, **A′**, **C**, **C′**, **E**, **E′**) and *Shp2*^{cko} (**B**, **B′**, **D**, **D′**, **F**, **F′**) mice administered with Dox from P1 to P25. It should be noticed that *Shp2* deficiency drastically downregulated the expression of cell adhesive molecules E-cadherin/β-catenin and basement membrane component laminin-β1 in corneal epithelium. *Scale bars*: 50 < μm.

alleles from the basal epithelial cells upon Dox treatment (Supplementary Fig. S5C, arrows). This resulted in the loss of basal lamellar structure (compare Figs. 6D and 6B, arrowheads), but did not crushed down stratified epithelium. In wounded eye, the initial re-epithelialization to cover the 2-mm diameter wound area behaved no differently between +Dox and –Dox as judged by the corneal epithelial fluorescein staining at 24 hours after wound healing (data not shown). This result suggested that Shp2 was not required for cell migration during early phase wound closure. Therefore, we did not attempt to compare the migrating leading edge within 6 to 24 hours after the wound. However, when examining if the epithelium could restore seven to eight layers and display typical basal lamellar structure 8 days after epithelial abrasion during wound healing, we noticed that these only happened when *Shp2* remained intact in the absence of Dox (Fig. 6C, arrowheads). In contrast, epithelial restratification was compromised (Fig. 6E) when *Shp2* was ablated in the presence of Dox (Supplementary Fig. S5C). Concomitantly, laminin-β1 expression was drastically reduced in the unwounded eye (compare Supplementary Figs. S5G and S5E) and completely diminished during wound healing (Supplementary Fig. S5H) when *Shp2* was ablated, while laminin-β1 expression was upregulated during epithelial restratification (Supplementary Fig. S5F) when Shp2 was expressed (Supplementary Fig. S5B). Moreover, as shown in Supplementary Figure 6E, 80.0 ± 5.3% (+Dox) versus 82.7 ± 3.5% (–Dox) of basal cells in the unwounded eye and 76.3 ± 3.4% (+Dox) versus 78.5 ± 2.1% (–Dox) of basal cells in the wounded eye were PCNA-positive, suggesting that proliferative activity was not altered when *Shp2* was ablated in the unwounded cornea (compare Supplementary Figs. S6C and S6A) and in corneal restratification during wound healing (compare Supplementary Figs. S6D and S6B). On the other hand, as revealed in Figure 8E, the percentage of ΔNp63-positive cells was reduced approximately 3.3-fold (from 63.9 ± 4.4% to 19.4 ± 4.1%) when *Shp2* was ablated in the unwounded cornea (compare Supplementary Figs. S6C′ and S6A′). More interestingly, we found that nearly half (48.7 ± 4.8%) of the re-epithelialized epithelial cells expressed ΔNp63 (Supplementary Fig. S6B′), but only 3.6 ± 2.5% of those were ΔNp63-positive when *Shp2* was ablated during wound healing (Supplementary Fig. S6D′). Taken together, these data argued that *Shp2* deficiency caused

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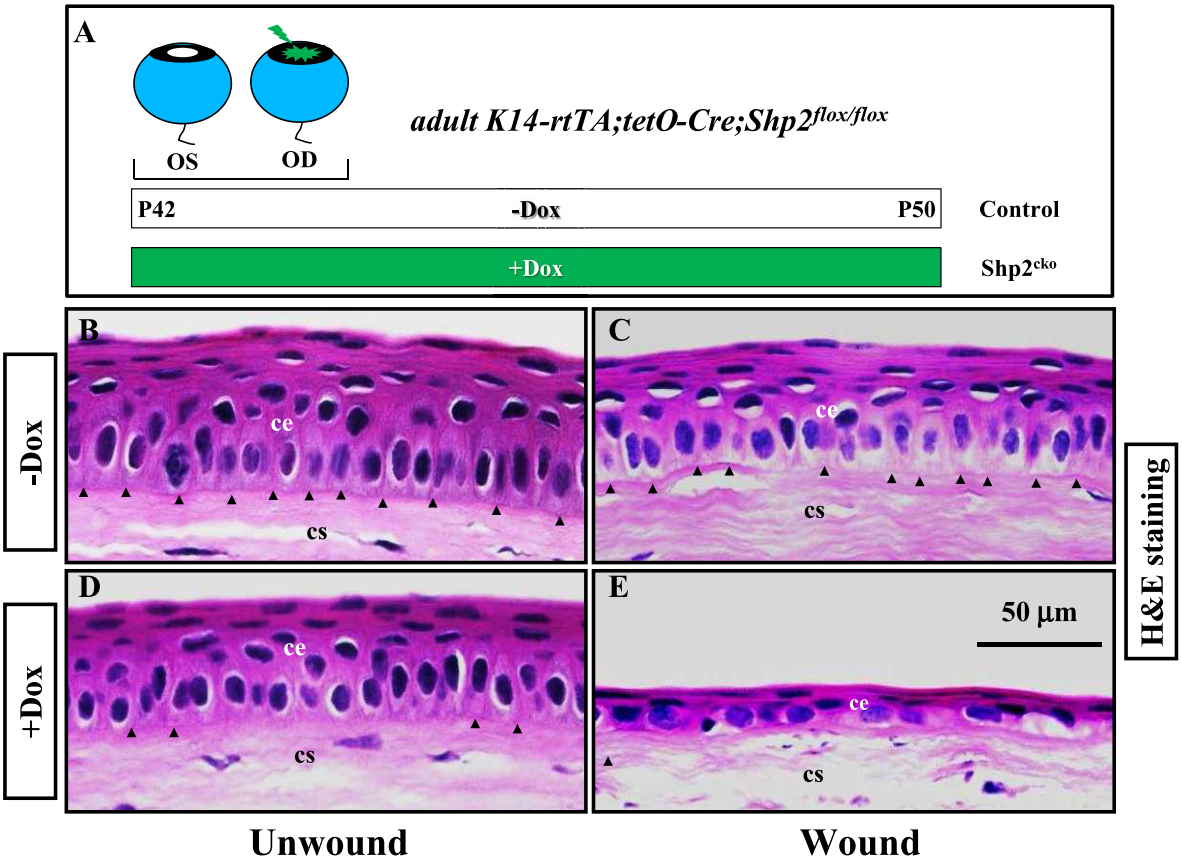

Figure 6. Shp2 is required for the restoration of stratification after corneal epithelial debridement. Adult (P42) *K14-rtTA;tetO-Cre;Shp2$^{f/f}$* mice were administered with or without Dox (80 μg/g body weight) via intraperitoneal injection. A 2-mm diameter central corneal epithelial debridement was generated in right eye (OD) with Algerbrush II Rust Ring Remover. Left eyes (OS) were wounded. Mice were then separated into two groups; group I mice were fed with regular chow (–Dox) and group II mice were fed with Dox chow (+Dox) for 8 days (**A**). Histologic analysis was performed by H&E staining (**B**–**E**). Noted that +Dox predominantly affected basal lamellar formation, albeit no significant changed of epithelium thickness in unwounded cornea (comparing *arrowheads* shown in [**D**] with [**B**]). Epithelium stratification was restored in wounded cornea in which basal lamellar formation was also seen in –Dox (**C**). In contrast, only two to three cell layers were formed without basal lamellar formation in +Dox cornea (**E**).

downregulation of ΔNp63 and the impairment of stratification during corneal epithelial wound healing.

Discussion

The stratified, nonkeratinized corneal epithelium serves as a barrier that contributes to maintain corneal transparency and rigidity, which warrant normal visual function. In mice, the anatomic cornea demarcation from conjunctiva and the expression of cornea-type specific keratin 12 coincide with eyelid closure at E14.5.[32] At this stage, the primitive corneal epithelium consists of two cell layers and remains constant until the time of eyelid opening in P11 to P12. Interestingly, just prior to eyelid opening, corneal epithelium quickly stratify from two cell layers to up to six to seven cell layers at P25 and maintains as such throughout their lifetime[1] (Fig. 1). Similarly, experimental corneal epithelial wound healing recapitulates, at least in part, the developmental process of corneal epithelial cell stratification by re-epithelializing the denuded surface with two cell layers and then stratifying to restore the full thickness of a healthy corneal epithelium[33] (Fig. 6). Therefore, epithelial stratification in corneal morphogenesis and homeostasis may be achieved by the similar mechanism.

Growth factors including the EGF family, keratinocyte growth factor, hepatocyte growth factor, insulin-like growth factor (IGF) insulin, TGF-β, and so on, have been shown to involve in corneal epithelial development and wound healing,[34] but the key intracellular molecule that transmits signaling to control epithelial stratification has not been documented. In this study, our data argued that Shp2 might serve as a hub for various growth factors to relay the cellular signaling needed for cornea morphogenesis and homeostasis. The mutant strain in which *Shp2* was conditionally knocked out in K14-positive basal cells exhibited wavy fur that resembled a *TGF-α* KO phenotype. As for ocular surface tissues, *TGF-α* knockout animals displayed uniformly thinner corneal epithelia than normal mice.[19] No stratification took place throughout the ocular surface epithelium of *Shp2^{cko}* mice and defects in their derived glands and goblet cells were observed. This suggested that in addition to TGF-α, the aforementioned growth factors might combine efforts to achieve ocular surface epithelial morphogenesis. Perhaps Shp2 directly integrates and relays signaling from these growth factors down to the nucleus for gene regulation and tissue morphogenesis. It should be noted that unlike *TGF-α* KO corneas,[20] which revealed profound inflammation, little inflammation was found in those of *Shp2^{cko}* mice. Therefore, corneal inflammation seen in *TGF-α* KO corneas[20] might not be triggered by the epithelium but stroma or other cell types. The phenotypes presented in this study were not likely attributed to the inflammation, but to the autonomous effect of *Shp2* deficiency.

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The processes of establishing a stratified corneal epithelium have been termed "XYZ hypothesis"—X, the proliferation of basal epithelial cells; Y, the contribution to the cell mass by centripetal movement of peripheral cells; and Z, the epithelial cell loss from the surface.[35] The underlying molecular genetics of each component of this hypothesis has never been examined; however, we found that *Shp2*^{cko} failed to develop stratified, nonkeratinized ocular surface epithelia during development and to restore stratification after a corneal epithelium debridement wound. In line with the hypothesis, the X component in *Shp2*^{cko} cornea remained as active as a control cornea (Fig. 3C). Likewise, the Z component was probably unaffected, since we did not detect any cell death from *Shp2*^{cko} cornea (data not shown). However, the Y component was significantly influenced as the total epithelial basal cell count was dramatically reduced in *Shp2*^{cko} cornea, which might be due to the decrease of ΔNp63-positive cells from *Shp2* deficiency (Fig. 3F). It has been shown that ΔNp63 isoforms promoted cell cycle progression through a dominant negative effect on p53[36] as well as transcriptional repression of p21 (CIP1/WAF1).[37] Therefore, downregulation of ΔNp63 resulting from *Shp2* deficiency perhaps slowed down or arrested cell cycle progression, leading to the overall cell mass reduction of the corneal epithelium. This statement is in agreement with the most recent published paper by Günschmann et al.[38] in which conditional deletion of insulin/IGF-1 signaling (IIS) resulted in a mitotic checkpoint arrest and impaired epidermal stratification through FoxO-mediated p63 inhibition. Moreover, since p63 is required for proper cell division orientation and indeed plays an essential role for ACD of the epithelial basal cells during epithelial stratification and morphogenesis,[25,39] reduction of p63 in *Shp2*^{cko} might result in a biased loss of ACD, leading to impaired stratification. Given that Shp2 can relay receptor tyrosine kinases (RTK) signaling, including IIS from membrane to nucleus,[40] therefore, it makes sense that *Shp2*^{cko} manifests a similar phenotype to that of the loss of *IIS* and *p63* gene, respectively, in corneal stratification.

In epidermal tissues, p63 is required for proliferation, stem/progenitor cells regeneration, differentiation, and apoptosis during development. Embryonic stratification failed to initiate at E12.5 in mice lacking p63 and led to defects in limb, craniofacial, and all stratified epithelia and their derivatives such as mammary, lacrimal, and salivary glands.[41] These data demonstrated that p63 was an ectodermal-specific gatekeeper of epidermal fate during development.[42,43] Ocular surface epithelia differentiation occurred at E14.5, but stratification began at approximately P12,[44] therefore, the interpretation to studies concerning the role of p63 in the developing corneal epithelium at E12.5 to P0 should attribute to the cell fate determination and differentiation, but not to the cell dividing event. In our study, *Shp2* deficiency was induced postnatally before stratification in differentiated ocular surface epithelia. We concluded that corneal epithelial stratification largely relied on p63-dependent ACD but not on cell differentiation as the mono/bilayer epithelium remained positive for Pax-6, K12, and K14 in *Shp2*^{cko} mice (Fig. 4). On the other hand, it has been documented that ΔNp63 isoforms upregulate cell adhesion molecules, increasing cellular adhesion in various epithelial cells.[45–47] We observed a downregulation of desmosome and hemidesmosome as well as downregulation of E-cadherin, β-catenin, and laminin-β1 expression in *Shp2* deficient corneal epithelium. This suggested that in addition to affecting cell division orientation, *Shp2*^{cko} compromised cell–cell and cell-BM adhesion and led to the impairment of corneal epithelium stratification.

Previous studies have documented the three typical phases of corneal epithelial wound healing that occur after epithelial debridement: (1) cell migration, (2) cell proliferation, and (3) cell differentiation to restore corneal homeostasis and barrier function.[33] Immediately following epithelial abrasion, re-epithelialization is initiated by the adjacent K12^{+} superficial cell sheet, which migrates downwards to cover the wound area. Since the superficial cells had functioning Shp2, migration and re-epithelialization were fine. However, the subsequent transient amplifying (TA) cell sheet (K14^{+}) migration underneath the K12^{+} sheet was impaired due to the downregulation of p63 and the deficiency of hemidesmosomes, desmosomes, and the BM ECMs such as laminin-β1. We concluded that Shp2 function was to lay down a good foundation and relatively tight anchors for the basal cell to form a stratified epithelium. Nevertheless, it remains unknown (1) how Shp2 regulates desmosome components and ECMs of BM? Is it a transcriptional event and/or cytoskeletal event? (2) What is the downstream signaling event of Shp2? Is this mediated through Ras-Mek-Erk?[10] We are currently crossing *K14-rtTA;TC;Shp2*$^{f/f}$ into *Rosa*$^{Kras(G12D)}$ (constitutively active)[48] to see if the phenotype can be rescued and to what extent. If so, Shp2-Ras-Mek-Erk axis plays an essential role in corneal epithelial stratification in vivo; otherwise, other signaling molecules such as focal adhesion kinase, nuclear factor of activated T cell, signal transducer and activator of transcriptions may be involved in the epithelial stratification. The feasibility of *K14-rtTA;tetO-Cre;Shp2*$^{flox/flox}$ mouse strain in conjunction with other Dox-inducible transgenic and/or gene-targeted floxed alleles will help elucidate the molecular mechanism(s) underlying corneal epithelium stratification and ocular surface morphogenesis.

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References

1. Zieske JD. Corneal development associated with eyelid opening. *Int J Dev Biol*. 2004;48:903–911.
2. Feng GS, Hui CC, Pawson T. SH2-containing phosphotyrosine phosphatase as a target of protein-tyrosine kinases. *Science*. 1993;259:1607–1611.
3. Feng GS, Pawson T. Phosphotyrosine phosphatases with SH2 domains: regulators of signal transduction (review article). *Trends Genet*. 1994;10:54–58.
4. Freeman RM Jr, Plutzky J, Neel BG. Identification of a human src homology 2-containing protein-tyrosine-phosphatase: a putative homolog of Drosophila corkscrew. *Proc Natl Acad Sci U S A*. 1992;89:11239–11243.
5. Vogel W, Lammers R, Huang J, et al. Activation of a phosphotyrosine phosphatase by tyrosine phosphorylation. *Science*. 1993;259:1611–1614.
6. Shi ZQ, Yu DH, Park M, et al. Molecular mechanism for the Shp-2 tyrosine phosphatase function in promoting growth factor stimulation of Erk activity. *Mol Cell Biol*. 2000;20:1526–1536.

7. Neel, BG, Gu, H, Pao, L. The 'Shp'ing news: SH2 domain-containing tyrosine phosphatases in cell signaling. *Trends Biochem Sci*. 2003;28:284–293.
8. Yang W, Klaman L, Chen B, et al. An Shp2/SFK/Ras/Erk signaling pathway controls trophoblast stem cell survival. *Dev Cell*. 2006;10:317–327.
9. Dance M, Montagner A, Salles JP, et al. The molecular functions of Shp2 in the Ras/Mitogen-activated protein kinase (ERK1/2) pathway. *Cell Signal*. 2008;20:453–459.
10. Matozaki T, Murata Y, Saito Y, et al. Protein tyrosine phosphatase SHP-2: a proto-oncogene product that promotes Ras activation. *Cancer Sci*. 2009;100:1786–1793.
11. Grossmann KS, Rosário M, Birchmeier C, et al. The tyrosine phosphatase Shp2 in development and cancer. *Adv Cancer Res*. 2010;106:53–89.
12. Nguyen H, Rendl M, Fuchs E. Tcf3 governs stem cell features and represses cell fate determination in skin. *Cell*. 2006;127: 171–183.
13. Perl AK, Wert SE, Nagy A, et al. Early restriction of peripheral and proximal cell lineages during formation of the lung. *Proc Natl Acad Sci U S A*. 2002;99:10482–10487.
14. Zhang EE, Chapeau E, Hagihara K, et al. Neuronal Shp2 tyrosine phosphatase controls energy balance and metabolism. *Proc Natl Acad Sci U S A*. 2004;101:16064–16069.
15. Chikama T, Liu CY, Meij JTA, et al. Excess FGF-7 in corneal epithelium causes corneal intraepithelial neoplasia in young mice and epithelium hyperplasia in adult mice. *Am J Pathol*. 2008;172:638–649.
16. Liu CY, Zhu G, Converse R, et al. Characterization and chromosomal localization of the cornea-specific murine keratin gene Krt1.12. *J Biol Chem*. 1994;269:24627–24636.
17. Chung EH, Bukusoglu G, Zieske JD. Localization of corneal epithelial stem cells in the developing rat. *Invest Ophthalmol Vis Sci*. 1992;33:2199–2206.
18. Song J, Lee YG, Houston J, et al. Neonatal corneal stromal development in the normal and lumican-deficient mouse. *Invest Ophthalmol Vis Sci*. 2003;44:548–557.
19. Mann GB, Fowler KJ, Gabriel A, et al. Mice with a null mutation of the TGF alpha gene have abnormal skin architecture, wavy hair, and curly whiskers and often develop corneal inflammation. *Cell*. 1993;73:249–261.
20. Luetteke NC, Qiu TH, Peiffer RL, et al. TGF alpha deficiency results in hair follicle and eye abnormalities in targeted and waved-1 mice. *Cell*. 1993;73:263–278.
21. Luetteke NC, Phillips HK, Qiu TH, et al. The mouse waved-2 phenotype results from a point mutation in the EGF receptor tyrosine kinase. *Genes Dev*. 1994;8:399–413.
22. Poulson ND, Lechler T. Robust control of mitotic spindle orientation in the developing epidermis. *J Cell Biol*. 2010;191: 915–922.
23. Williams SE, Beronja S, Pasolli HA, et al. Asymmetric cell divisions promote Notch-dependent epidermal differentiation. *Nature*. 2011;470:353–358.
24. Senoo M, Pinto F, Crum C, et al. p63 is essential for the proliferative potential of stem cells in stratified epithelia. *Cell*. 2007;129:523–536.
25. Lechler T, Fuchs E. Asymmetric cell divisions promote stratification and differentiation of mammalian skin. *Nature*. 2005;437:275–280.
26. Koster MI, Roop DR. Mechanisms regulating epithelial stratification. *Annu Rev Cell Dev Biol*. 2007;23:93–113.
27. Caldas H, Jiang Y, Holloway MP, et al. Survivin splice variants regulate the balance between proliferation and cell death. *Oncogene*. 2005;24:1994–2007.
28. Davis J, Duncan MK, Robison WG Jr, et al. Requirement for Pax6 in corneal morphogenesis: a role in adhesion. *J Cell Sci*. 2003;116:2157–2167.
29. Collinson JM, Quinn JC, Hill RE, et al. The roles of Pax6 in the cornea, retina, and olfactory epithelium of the developing mouse embryo. *Dev Biol*. 2003;255:303–312.
30. Hulpiau P, van Roy F. Molecular evolution of the cadherin superfamily. *Int J Biochem Cell Biol*. 2009;41:349–369.
31. Aumailley M, Bruckner-Tuderman L, Carter WG, et al. A simplified laminin nomenclature. *Matrix Biol*. 2005;24:326–332.
32. Kurpakus MA, Maniaci MT, Esco M. Expression of keratins K12, K4 and K14 during development of ocular surface epithelium. *Curr Eye Res*. 1994;13:805–814.
33. Suzuki K, Saito J, Yanai R, et al. Cell-matrix and cell-cell interactions during corneal epithelial wound healing. *Prog Retin Eye Res*. 2003;22:113–133.
34. Yu FS, Yin J, Xu K, et al. Growth factors and corneal epithelial wound healing. *Brain Res Bull*. 2010;81:229–235.
35. Thoft RA, Friend J. The X, Y, Z hypothesis of corneal epithelial maintenance. *Invest Ophthalmol Vis Sci*. 1983;24:1442–1443.
36. Schavolt KL, Pietenpol JA. p53 and Delta Np63 alpha differentially bind and regulate target genes involved in cell cycle arrest, DNA repair and apoptosis. *Oncogene*. 2007;26: 6125–6132.
37. Westfall MD, Mays DJ, Sniezek JC, et al. The ΔNp63α phosphoprotein binds the p21 and 14-3-3σ promoters in vivo and has transcriptional repressor activity that is reduced by Hay-Wells syndrome-derived mutations. *Mol Cell Biol*. 2003; 23:2264–2276.
38. Günschmann C, Stachelscheid H, Akyüz MD, et al. Insulin/IGF-1 controls epidermal morphogenesis via regulation of FoxO-mediated p63 inhibition. *Dev Cell*. 2013;26:176–187.
39. Poulson ND, Lechler T. Robust control of mitotic spindle orientation in the developing epidermis. *J Cell Biol*. 2010;191: 915–922.
40. Myers MG Jr, Mendez R, Shi P, et al. The COOH-terminal tyrosine phosphorylation sites on IRS-1 bind SHP-2 and negatively regulate insulin signaling. *J Biol Chem*. 1998;273: 26908–26914.
41. Yang A, Schweitzer R, Sun D, et al. p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. *Nature*. 1999;398:714–718.
42. Shalom-Feuerstein R, Lena AM, Zhou H, et al. ΔNp63 is an ectodermal gatekeeper of epidermal morphogenesis. *Cell Death Differ*. 2011;18:887–896.
43. Romano RA, Smalley K, Magraw C, et al. ΔNp63 knockout mice reveal its indispensable role as a master regulator of epithelial development and differentiation. *Development*. 2012;139:772–782.
44. Findlater GS, McDougall RD, Kaufman MH. Eyelid development, fusion and subsequent reopening in the mouse. *J Anat*. 1993;183:121–129.
45. Carroll DK, Carroll JS, Leong CO, et al. p63 regulates an adhesion program and cell survival in epithelial cells. *Nat Cell Biol*. 2006;8:551–561.
46. Lopardo T, Lo Iacono N, Marinari B, et al. Claudin-1 is a p63 target gene with a crucial role in epithelial development. *PLoS One*. 2008;3:e2715.
47. Ferone G, Mollo MR, Thomason HA, et al. p63 control of desmosome gene expression and adhesion is compromised in AEC syndrome. *Hum Mol Genet*. 2013;22:531–543.
48. Jackson EL, Willis N, Mercer K, et al. Analysis of lung tumor initiation and progression using conditional expression of oncogenic K-ras. *Genes Dev*. 2001;15:3243–3248.

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