Increased Fragility, Impaired Differentiation, and Acceleration of Migration of Corneal Epithelium of Epiplakin-Null Mice

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Purpose. To investigate the effects of gene ablation of epiplakin on the homeostasis of corneal epithelium in mice.

Methods. Light and transmission electron microscopic histology, immunohistochemistry, and real-time RT-PCR were carried out to evaluate the effects of the loss of epiplakin on structure and gene expression of cell–cell adhesion-related components in mice. Integrity against mechanical intervention and wound-healing response of corneal epithelium were also tested.

Results. Epiplakin protein was detected in the cells of the basal layer of corneal epithelium. Morphologically basal-like cells were observed in the suprabasal layer of adult epiplakin-null corneal epithelium, suggesting an impaired intraepithelial cell differentiation. Such abnormality was not detected in mice before the age of postnatal day 14. Epiplakin-deficient epithelium exhibits fragility against mechanical intervention as compared with wild-type epithelium. Although cell proliferation is suppressed, migration-dependent wound healing is promoted in epiplakin-null epithelium. E-cadherin expression was suppressed by the loss of epiplakin in the epithelium.

Conclusions. Lacking epiplakin affects cell differentiation of the corneal epithelium, as well as its proliferation activity and its structural integrity. The mechanism of acceleration of cell migration in the epiplakin-null corneal epithelium is to be further investigated, although suppression of expression of E-cadherin might be included.

Keywords: epiplakin, knockout, corneal epithelium, differentiation, fragility, wound healing, mouse

Corneal epithelium is a kind of a nonkeratinizing stratified squamous epithelium that consists of multiple layers from basal cells to superficial cells. Epithelial cells are connected to each other via intercellular connecting systems to maintain epithelial integrity.[1,2] Such systems include tight junction, adherens junction, desmosome, and gap junction. Each apparatus is also involved in the maintenance of barrier function of the corneal epithelium.

Desmosome is composed of transmembrane proteins that are connected to intermediate filaments of keratin members.[3,4] Epiplakin (EPPK) is one such intermediate filament-related component and was originally identified as an autoantigen that reacted with serum from an individual with subepidermal blistering disease.[5,6] Human EPPK is a 552-kDa protein that is expressed in various epithelial tissues (i.e., epidermis, esophagus, outer root sheath of hair follicles, and mucous epithelial cells). EPPK is homologous to plectin and other members of the plakin family.[5,6]

We previously generated a mouse line that lacks EPPK.[7] Studies by using this mouse line showed that lacking EPPK accelerates migration of epidermal keratinocytes in mice in vivo and also showed that this is also the case in outgrowth of keratinocytes from explanted skin tissue in vitro, although the exact mechanism of the phenomenon is to be revealed.[7]

Corneal epithelium differs from epidermis in many aspects and lacks keratinization and epithelial cell papillae. Localization of stem cells also differs between epidermis and corneal epithelium. Expression pattern of EPPK and its roles in the corneal epithelium remain to be investigated. In the present study, we therefore first examined if EPPK is expressed in mouse corneal epithelium. We then examined the roles of EPPK in intraepithelial differentiation and integrity of corneal epithelium, as well as its wound-healing response by using an EPPK-deficient mouse.

Materials and Methods

Experimental protocols and the use of experimental mice were approved by the DNA Recombination Experiment Committee and the Animal Care and Use Committee of Wakayama Medical University and conducted in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Table 1. Targets of Immunohistochemistry and the Sources of the Antibodies

Bullous pemphygoid antigen 230 (BP230 donated by Yoshiaki Hirako, Nagoya University School of Medicine, Aich, Japan)
Keratin 12[11] (donated by Winston Kao, University of Cincinnati)
Keratin14 (diluted 1:20 in PBS; Sigma-Aldrich, St. Louis, MO)
Desmocollin-1 (diluted 1:1; Progen Biotechnik, Heidelberg, Germany),
Desmocollin-2 (diluted 1:20; Progen Biotechnik),
Desmoplakin-1 (diluted 1:20; Progen Biotechnik),
Desmoplakin-2 (diluted 1:20; Progen Biotechnik),
Plakoglobin (diluted 1:10; Progen Biotechnik),
Occludin (diluted 1:100; Zymed, South San Francisco, CA)
E-cadherin (diluted 1:100; Santa Cruz Biotechnology, Santa Cruz, CA)

Immunohistochemistry for EPPK in Corneal Epithelium

A round epithelial defect 2.0 mm in diameter was produced in the cornea of an adult male C57BL/6 mouse and the animals were killed at 12 or 24 hours of healing (EPPK-null mice were obtained from the Department of Dermatology, Faculty of Medicine, Oita University; Yuhu City, Japan; $n = 3$).[8] Four eyes of four animals were used at each time point. The healing eyes, as well as uninjured control eyes, were fixed in 4% paraformaldehyde and then embedded in paraffin as previously reported.[9] Immunohistochemistry was carried out in paraffin sections with an anti-EPPK antibody as previously reported.[9] The antibody complex with a peroxidase-conjugated secondary antibody was visualized with 3,3′-diaminobenzidine (DAB) reaction as previously reported.[9]

Histology and Immunohistochemical Analysis of EPPK-Deficient Corneal Epithelium

Histological observation by light and transmission electron microscopy (Olympus, Tokyo, Japan) was carried out to evaluate structure (or differentiation) of corneal epithelium of an EPPK-deficient mouse.

The eye of an adult EPPK-null (knockout [KO]) mouse of the background of C57BL/6 (wild-type [WT], $n = 10$) and an age-matched control WT littermate mouse ($n = 10$) were obtained. Samples were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer and embedded in paraffin.[8,9] Paraffin sections (5.0-μm thick) were stained in hematoxylin and eosin (HE) and processed for 4′,6-diamidino-2-phenylindole (DAPI) nuclear staining in order to observe the morphology of the nuclei in the epithelium. Differentiation of a basal cell to a suprabasal cell is known to be associated with flattening of the nucleus. Therefore, the ratio of horizontal/vertical length of the basal or suprabasal cells was obtained to examine if lacking EPPK affects differentiation of a basal epithelial cell to a suprabasal cell. Data were analyzed by using unpaired Student's *t*-test.

Similarly, the eyes of WT and KO mice at postnatal day 1 (P1), P7, and P14 were also processed for HE as well as processed for DAPI nuclear staining in order to observe the shape of the nuclei in the epithelium. The ratio of horizontal/vertical length of the cell nuclei in the corneal monolayer epithelia of P1 and P7 mice and in the suprabasal epithelial cells of the central cornea at P14 was obtained. Six eyes of three mice of each genotype were used. Twenty nuclei in the central cornea were enrolled in the examination in each cornea.

As for the ultrastructural observation, the corneas of KO and WT mice ($n = 4$ in each genotype) were fixed in 2.0% glutaraldehyde and then postfixed in osmium tetroxide. The specimens were embedded in Epon mixture. Ultrathin sections were cut, electron-stained, and observed under transmission electron microscopy.[10] Ultrastructure of corneal epithelial cells, desmosomes, and hemidesmosomes were examined.

Table 2. Primers for Real-Time RT-PCR

E-cadherin	Mm01247357_ml
Desmoglein-1α	Mm00809994_sl
Desmoglein-1β	Mm00839130_mH
Desmoplakin	Mm01351876_ml
GAPDH	Mm03302249 gl

Immunohistochemistry

Specimens were also immunostained by using peroxidase-DAB reaction as previously reported.[8,9] Protein expression of cell–cell connection-related components and cytoskeletal components were examined. Antibodies used are listed in Table 1.

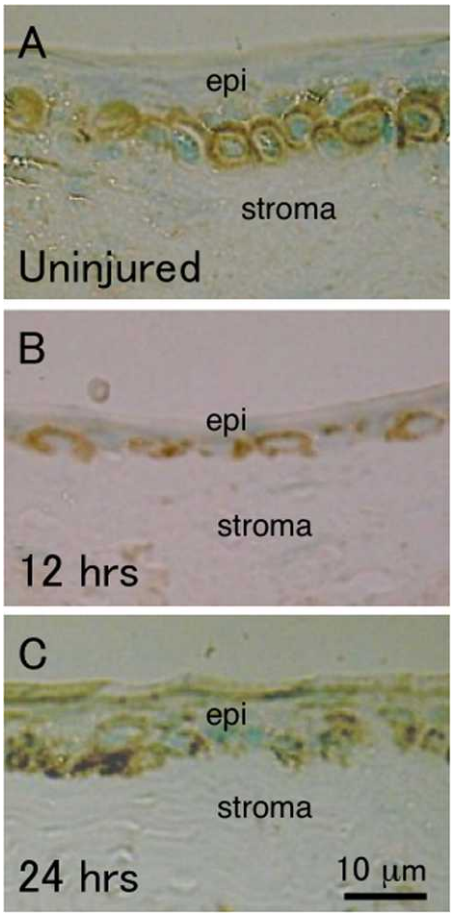

Figure 1. Immunohistochemical detection of EPPK in the corneal epithelium of a WT mouse. (**A**) Immunohistochemistry detected EPPK protein in mainly the cell–cell junction zone of the basal cells and some of the suprabasal cells of uninjured epithelium of a WT mouse. (**B**) The cytoplasm of migrating monolayer epithelium on the central epithelial defect was also labeled with anti-EPPK antibody at 12 hours post debridement. (**C**) After the epithelium recovered the stratification, the basal cells in the regenerated epithelium were found to be positive for EPPK at 24 hours. epi, epithelium; *Scale bar*: 10 μm.

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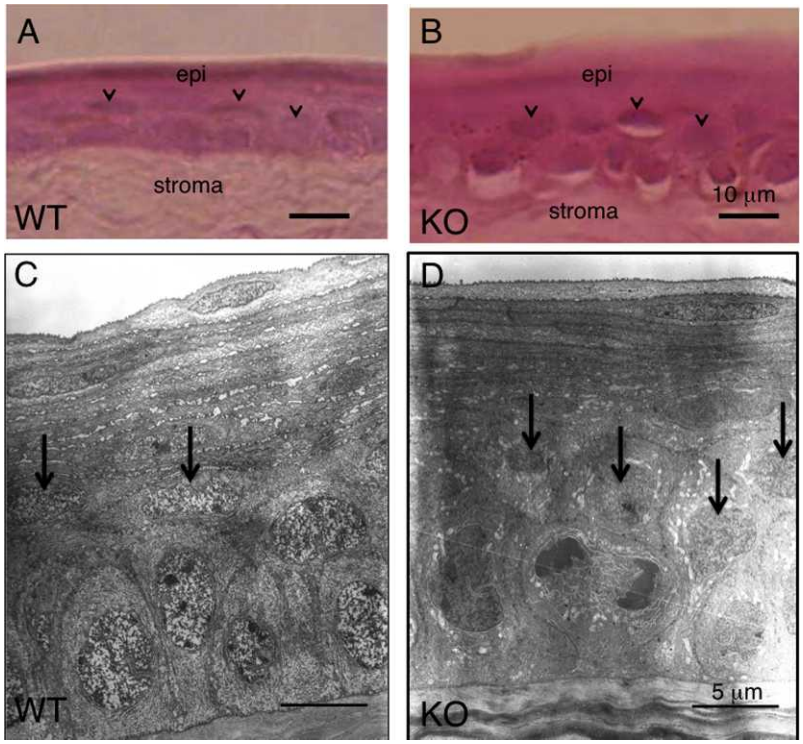

FIGURE 2. Histological alternation of corneal epithelium of an EPPK-null (KO) mouse. (**A**) Light microscopy by HE staining showed that the normal corneal epithelium of a WT mouse consisted of basal cells, wing cells, and superficial cells. The relatively flattened wing (suprabasal) cells (*arrowheads*) were observed above the single layer of the basal cells with a relatively round nucleus in each in a WT corneal epithelium. (**B**) In a KO epithelium, the cells with an oval or less flattened nucleus were observed above the basal cells (*arrowheads*). (**C**) Under ultrastructural observation, the cells just above the basal cells were relatively flattened (*arrows*) in the epithelium of a WT mouse. (**D**) On the other hand, cells with oval nuclei were observed above the basal layer. epi, epithelium; *Scale bars*: 10 μm (**A**, **B**); 5 μm (**C**, **D**).

Real-Time RT-PCR

To examine if mRNA expression of cell-cell connection-related components (i.e., E-cadherin, desmoglein-1 [1α and 1β], or desmoplakin) is affected by the loss of EPPK, we ran Taqman real-time RT-PCR for these components in RNA samples obtained from WT and KO corneas as previously reported.[9] Delta-delta threshold cycle program was used with the internal control of the expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data were analyzed by using unpaired Student's *t*-test. Primers used were obtained from Applied Biosystems, Inc. (Foster City, CA) and are listed in Table 2.

Examination of Fragility of the Corneal Epithelium

To test if lacking EPPK affects the integrity of the corneal epithelium in mice, we provided a mechanical challenge to the corneal epithelium as previously reported.[12] The corneal surface of adult WT and KO mice, under general anesthesia, was gently brushed (10 times) with a surgical micro-sponge as previously reported. After mice were killed, the eye was enucleated and embedded in Epon mixture as described above. Semithin sections were first cut and stained with toluidine blue for light microscopy. Then ultrathin sections were cut and observed under transmission electron microscopy.

Epithelial Wound Healing in a Cornea of a KO Mouse

A round epithelial defect 2.0 mm in diameter was produced in the central cornea of an adult KO or an adult WT mouse (n = 14 in each genotype at each time point) by using a trephine (2.0 mm in diameter) and a scalpel, while mice were under general anesthesia and using topical anesthesia, as previously reported.[8] The remaining defect was evaluated at 6, 12, 18, 24, 30, 36, and 48 hours after wounding. The data were analyzed by ANOVA and taken as significant with P less than 0.05.

Cell Proliferation in the Healing Corneal Epithelium

A round epithelial defect was produced in the central cornea of a KO or a WT mouse (n = 12 in each genotype) by using a trephine (2.0 mm in diameter) and a scalpel, while mice were under general anesthesia and using topical anesthesia, as described above. The mice were killed at 12, 24, and 36 hours after wounding, following labeling with intraperitoneal bromodeoxyuridine (BrdU) 2 hours before being killed.[13] Four corneas were used at each time point in WT or KO mice. Both genotypes of mice (n=4 in each genotype) without epithelial debridement were also labeled with BrdU 2 hours prior being killed. Paraffin sections were stained for incorporated BrdU by using an antibody against BrdU (1:10; Roche Diagnostics, Mannheim, Germany) as previously reported.[13] The data were analyzed by ANOVA and taken as significant with P less than 0.05.

RESULTS

Protein Expression of EPPK in the Corneal Epithelium of a WT Mouse

Immunohistochemistry detected EPPK protein in mainly the cell–cell junction zone of the basal cells and some of the

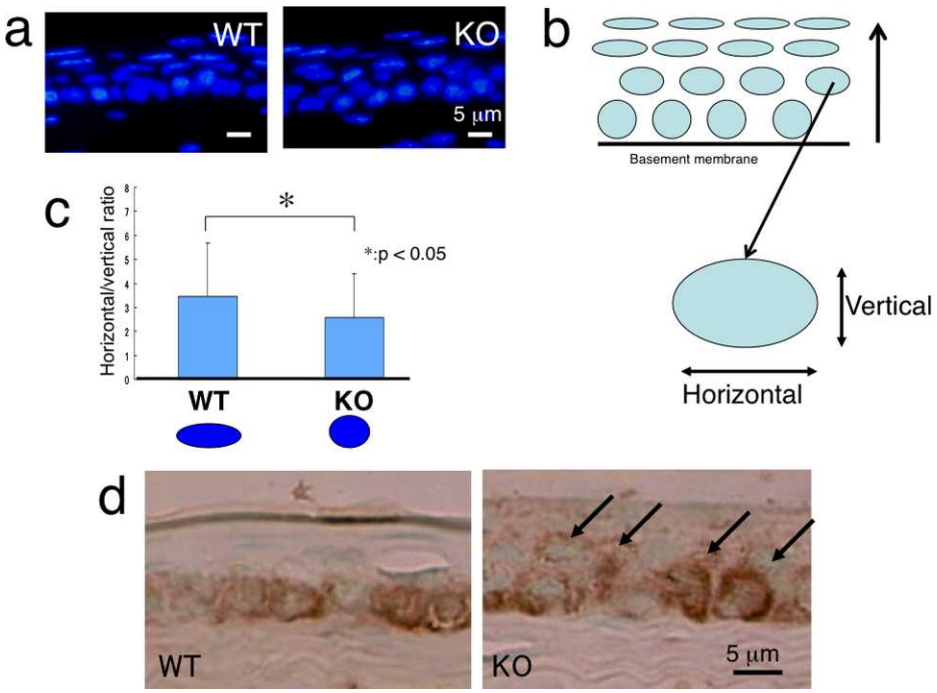

Figure 3. Analysis of differentiation of suprabasal cells of a KO mouse corneal epithelium. (**a**) In order to evaluate the morphology of the nuclei of the basal and suprabasal cells of the corneal epithelium, DAPI nuclear staining was employed. *Scale bar*: 5 μm. (**b**) The ratio of horizontal/vertical length of the nucleus of a suprabasal or basal cell was obtained to evaluate the intraepithelial differentiation of an epithelial cell. The *arrow* indicates the direction of cell movement from basal to suprabasal layer. (**c**) The ratio of horizontal/vertical length of the nuclei of suprabasal cells was significantly higher in corneal epithelium of adult WT mice than in that of adult KO mice. $^*P < 0.05$ by unpaired Student's *t*-test. *Bar*, SE. (**d**) Keratin 14, a marker of basal epithelial cell, was detected in the basal cells of the corneal epithelium of a WT mouse, while both basal and suprabasal cells (*arrows*) were labeled for keratin 14 in the corneal epithelium of a KO mouse. *Scale bar*: 5 μm.

suprabasal cells of uninjured epithelium of an adult WT mouse (Fig. 1A). The cytoplasm of migrating monolayer epithelium on the central epithelial defect was also labeled with anti-EPPK antibody at 12 hours post debridement (Fig. 1B). After the epithelium recovered the stratification, the basal cells in the regenerated epithelium were found to be positive for EPPK at 24 hours (Fig. 1C).

Histological Alternation of Corneal Epithelium by Lacking EPPK

Light microscopy by HE staining showed that the normal corneal epithelium of a WT mouse consisted of basal cells, suprabasal (wing) cells, and superficial cells. The relatively flattened suprabasal (wing) cells were observed above the single layer of the basal cells with a relatively round nucleus in each in a WT corneal epithelium (Fig. 2A). In a KO epithelium, the cells with an oval or less flattened nucleus were observed in the suprabasal layer above the basal cells (Fig. 2B).

To further observe the detailed structure of the corneal epithelium, transmission electron microscopy was carried out. As observed under HE light microscopy the cells just above the basal cells (i.e., suprabasal cells) were relatively flattened in the epithelium of a WT mouse (Fig. 2C). On the other hand, cells with oval nuclei were observed in the suprabasal layer above the basal layer (Fig. 2D).

To evaluate the morphology of the nuclei of the basal and suprabasal cells of the corneal epithelium, DAPI nuclear staining was used (Fig. 3a). In the normal corneal epithelium, the nucleus becomes more flattened along with maturation or differentiation toward the upper layer until reaching the superficial layer (Fig. 3b). The ratio of horizontal/vertical length of the nuclei of suprabasal cells was significantly higher in corneal epithelium of adult WT mice than in that of adult KO mice (Fig. 3c).

These light and electron microscopic findings suggest an impairment of differentiation from a basal cell to a suprabasal cell in corneal stratified squamous epithelium; the presence of a multilayer (mainly double layer) of basal-type cells in a KO corneal epithelium while such cells form a single layer of basal cells in WT corneal epithelium. To examine if this hypothesis is real, we conducted an immunohistochemistry for keratin 14, a marker for basal cells of the squamous epithelium in a KO mouse. The results indicated that keratin 14 was detected in the basal cells of the corneal epithelium of a WT mouse, whereas both basal and suprabasal cells were labeled for keratin 14 in the corneal epithelium of a KO mouse (Fig. 3d).

Age Dependency of the Abnormality of Corneal Epithelium of a KO Mouse

To examine if the abnormal cell differentiation from basal cells to suprabasal cells depends on the age of the mouse, we observed HE histology, as well as nuclear morphology analysis by DAPI dye stain at the ages of P1, P7, and P14 (Fig. 4a). The results indicated that there was no statistical difference of the horizontal/vertical ratio of the nuclei of the monolayer epithelial cells at P1 and P7, as well as the suprabasal cells of the corneal epithelium at P14 between WT and KO mice (Fig. 4b).

Cornea-Type Differentiation and Cell–Cell Junction Proteins in the Epithelium

Expression of keratin 12 indicates the cornea-type epithelial differentiation. Corneal epithelium of both WT and KO mice were labeled for keratin 12 (data not shown).

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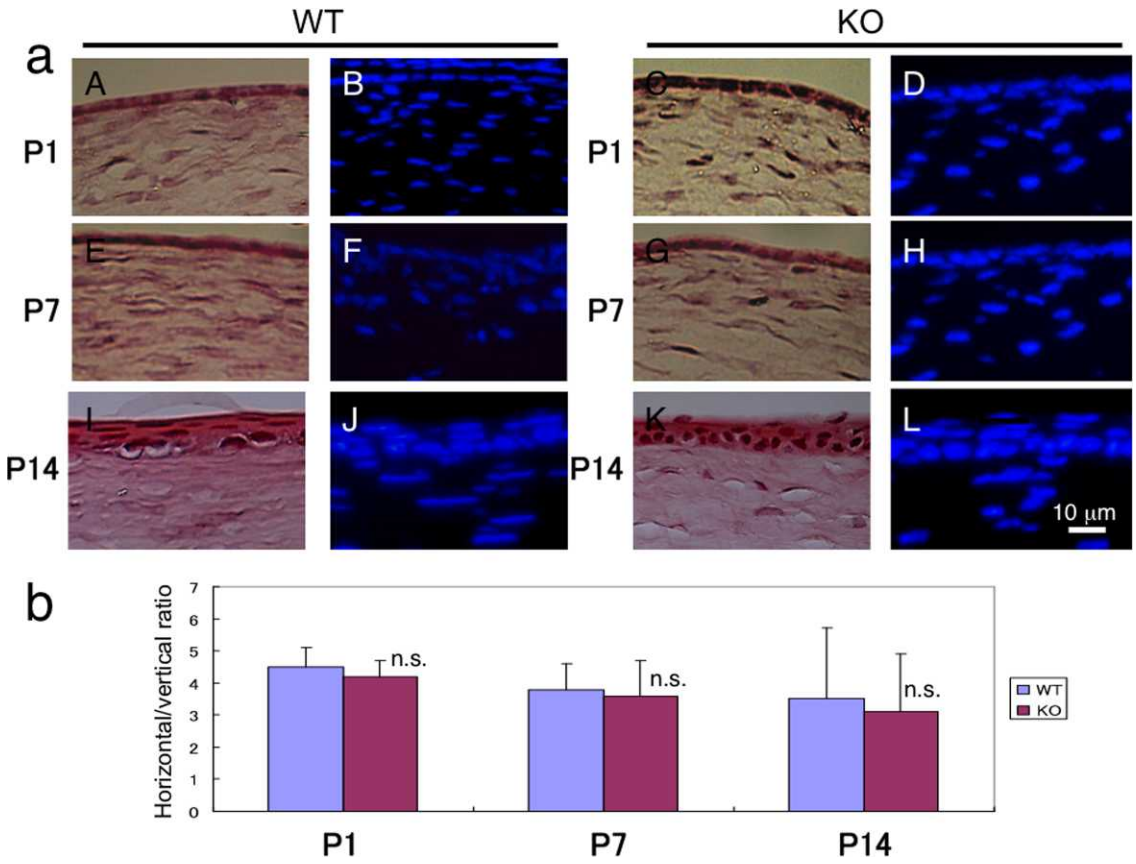

FIGURE 4. Age dependency of the abnormality of corneal epithelium of a KO mouse. (**a**) We observed HE histology as well as nuclear morphology by DAPI dye stain at the ages of P1, P7, and P14 in order to examine if the abnormal cell differentiation depends on the age of the mouse. Frames (**A–D**, **E–H**), or (**I–L**), indicate the histology of the corneal epithelium and anterior stroma of P1, P7, or P14 mouse, respectively. Frames (**A**, **B**, **E**, **F**, **I**, **J**) or Frames (**B**, **D**, **G**, **H**, **K**, **L**) show WT or KO cornea, respectively. *Scale bar*: 10 μm. HE staining (**A**, **C**, **E**, **G**, **I**, **K**); DAPI staining (**B**, **D**, **F**, **H**, **J**, **L**). (**b**) There was no statistical difference at these age points, the horizontal/vertical ratio, the nuclei of the monolayer epithelial cells at P1 and P7, as well as the suprabasal cells of the corneal epithelium at P14 between WT and KO mice. n.s., not significant. *Bar*, SE.

Because EPPK is a member of intermediate filament-related components, we then examined if the loss of EPPK affects the structure of desmosome and hemidesmosome in terms of cytoskeletal anchoring. Ultrastructural observation of a desmosome showed that the keratin bundle adhering to desmosome between the neighboring basal cells appeared reduced in a KO corneal epithelium (Fig. 5B) as compared with in a WT epithelium (Fig. 5A). Such difference was not noted in upper layers (data not shown). Then, we examined if structure of a hemidesmosome is affected by the loss of EPPK. Immunolocalization of BP230 antigen was similarly detected in the basement membrane zone between basal epithelial cells and stroma both in WT (Fig. 5C) and KO corneas (Fig. 5D). However, ultrastructural observation of the basement membrane showed that the keratin bundle adhering to hemidesmosome looks less condensed in a KO corneal epithelium (Fig. 5F) as compared with in a WT epithelium (Fig. 5E).

Because expression of a desmosomal component might reportedly affect the expression level of other desmosomal or cell–cell junctional components,[14] we then examined if lacking EPPK might affect the expression pattern of such components by using real-time RT-PCR and immunohistochemistry.

Immunohistochemistry first showed a decreased immunoreactivity for E-cadherin in corneal epithelium of a KO mouse (Fig. 6aB) as compared with a WT mouse epithelium (Fig. 6aA). Protein expression of desmocollin-1 (not shown), desmocollin-3 (not shown), desmoplakin-1 (Figs. 6aC, 6aD), desmoplakin-2 (not shown), desmoglein-1 (Figs. 6aE, 6aF), desmoglein-2 (not shown), plakoglobin (not shown), and occludin (not shown) in corneal epithelium seemed similar to each other between WT and KO mice.

Real-time RT-PCR also showed that mRNA expression of E-cadherin was suppressed by the loss of EPPK, whereas that of desmoglein-1 (1α and 1β) or desmoplakin-1 was not affected by EPPK gene knockout in a mouse cornea (Fig. 6b).

Fragility of the Corneal Epithelium in an EPPK-Deficient Mouse

EPPK is a desmosome- and intermediate filament-related component, suggesting that EPPK-null epithelium might be fragile against a mechanical stress. Gentle brushing with a surgical micro-sponge did not severely damage the structure of the corneal epithelium in a WT mouse (Fig. 7A). On the other hand, the cells in the superficial layer and wing cell (suprabasal) layer were removed by gentle brushing in a KO epithelium (Fig. 7B). Transmission electron microscopy showed that each layer of the epithelial cells was well maintained following brushing in a WT epithelium, although partial separation occurred between cells of the superficial layer (Fig. 7C). In a KO epithelium one or two layer(s) of basal or suprabasal epithelial cells were found to be in the original position following the treatment and upper layer cells were

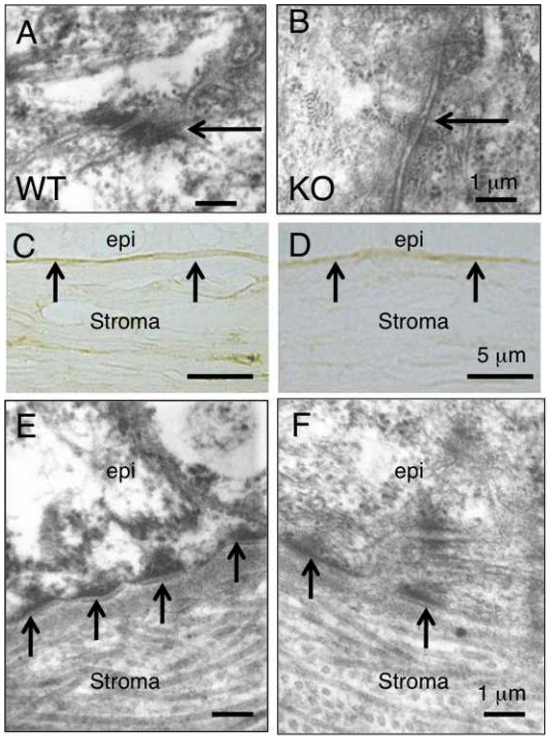

FIGURE 5. Ultrastructure of desmosomes and hemidesmosomes. Ultrastructural observation of a desmosome (*arrows*) between the basal cells showed that the keratin bundle adhering to desmosome between the neighboring basal cells looks reduced in a KO (**B**) corneal epithelium as compared with a WT (**A**) epithelium. *Scale bar*: 1 μm. Immunolocalization of BP230 antigen was similarly detected (*arrows*) in the basement membrane zone between basal epithelial cells and stroma both in WT (**C**) and KO (**D**) corneas. *Scale bar*: 5 μm. The keratin bundle adhering to hemidesmosomes (*arrows*) looks less in a KO (**F**) corneal epithelium as compared with in a WT (**E**) epithelium. *Scale bar*: 1 μm.

found to be removed (Fig. 7D). Higher magnification examination revealed that cell surface microplicae of a superficial layer cell were well maintained following brushing in a WT epithelium (Fig. 7E). The cell membrane of the ablated cells including relatively intact desmosomes remained to the neighboring cells in a KO epithelium following the treatment (Fig. 7F and inset). This finding suggests that integrity of the framework of the cytoplasm or connection between cytoskeletal components and cell membrane might be one of the main points that was impaired by the loss of EPPK.

Wound Healing of an Epithelial Defect in a Cornea of a KO Mouse

The round epithelial defect 2.0 mm in diameter produced in the center of the cornea was gradually recovered with the migrating remaining epithelium from outside the defect (Fig. 8a). At 36 hours post wounding the defect was closed in both WT and KO mice (Fig. 8a). At this time point, the recovered epithelium faintly stained with green fluorescein in a KO mouse. At 48 hours post wounding, the epithelia of both WT and KO mice never stained for the dye. The healing rate of the corneal epithelial defect in a KO mouse seemed overall higher as compared with a WT mouse. The size of the remaining defect was significantly smaller in a KO mouse cornea as compared with a WT cornea at 18 hours post wounding ($P < 0.05$) (Fig. 8b).

Cell Proliferation in Healing Corneal Epithelium Following Wounding

The incidence of proliferating cells in the uninjured KO corneal epithelium as detected by BrdU labeling was similar in an uninjured corneal epithelium of a WT mouse (Figs. 8c, 8d). The numbers of BrdU-labeled epithelial cells increased at 12 and 24 hours following wounding in both WT and KO corneas. The incidence of BrdU-labeled cells in the healing cornea epithelium was significantly lower in KO mice as compared with WT mice at 24 hours post wounding ($P < 0.05$) (Figs. 8c, 8d).

DISCUSSION

The present study was undertaken to uncover the roles of EPPK in homeostasis of corneal epithelium by using a mouse line with EPPK deficiency. Here EPPK was detected mainly in the basal cells of WT corneal epithelium. In epidermis, EPPK is reportedly expressed in the upper layer more markedly as compared with the basal layer.[5,6] The exact reason for this difference is to be studied, although the presence/absence of keratinization might be related to this difference.

First, we examined if lacking EPPK affects the morphogenesis of corneal epithelium by employing histology. Light microscopic histology with HE staining and ultrastructural observation both suggested the presence of morphologically basal-like cells above the layer of real basal cells (suprabasal layer). Morphological evaluation of the shape of the DAPI-labeled nuclei of basal and suprabasal cells of the corneal epithelium was performed; the nuclei of both basal and suprabasal cells of the KO corneal epithelium were more markedly round, or less flattened, as compared with those of the WT cornea. These findings suggest the presence of basal-like cells in the suprabasal layer of the KO epithelium. To confirm this notion we then performed immunohistochemistry for keratin 14, a marker for basal cells of the stratified epithelium, and observed the expression of keratin 14 in the cells above the basal cell layer. This indicates the presence of basal-like cells in the suprabasal layer of the corneal epithelium and that the loss of EPPK impairs the normal differentiation of stratified epithelium of the cornea. Explanation includes that loss of EPPK expression affects regulatory volume ion transport mechanism activity leading to a change in nuclear shape. Such a change may be associated with an increase in keratin 14 expression. Such multilayerization of keratin 14–positive cells is not observed in KO epidermis.[15] The phenotype of impaired intraepithelial differentiation of corneal epithelium was observed in adult mice, but not mice until P14, indicating that the abnormality is age-dependent.

The present study also revealed the fragility of the corneal epithelium in the absence of EPPK. A gentle brushing readily damaged the corneal epithelium of a KO mouse, but not in a WT mouse. Ultrastructural observation of the basal cells of the corneal epithelium showed less keratin fiber adhering to the desmosomes in a KO mouse. A high-power magnification observation showed the cell membrane of a damaged and removed cell remains adhered to a neighboring cell. This might suggest the presence of abnormal or attenuated cytoskeletal framework in the cytoplasm. The resistance of the epidermal tissue against a mechanical intervention has not been tested. In epidermis, but not in cornea, fragility was induced by the loss of other components of a desmosome, desmocolollin-1 or plakophilin-1, suggesting desmosomal abnormality impairs the integrity of the epithelial architecture.[16,17] A similar finding was reported in an in vitro experiment; the loss of plakoglobin attenuates desmosome-intermediate filament connection, lead-

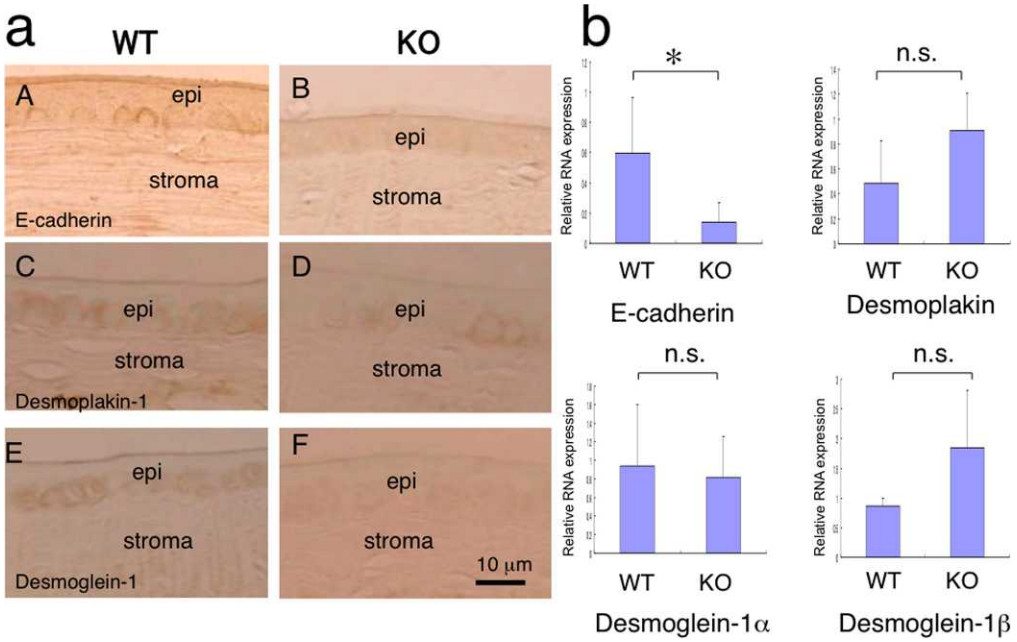

Figure 6. Expression of cell–cell junction-related components in corneal epithelium. (**a**) Immunohistochemistry showed a decreased protein expression of E-cadherin in corneal epithelium of a KO (**B**) mouse as compared with a WT (**A**) epithelium. Protein expression of desmoplakin-1 and desmoglein-1 in corneal epithelium was similar to each other between WT (**C**, **E**) and KO (**D**, **F**) mice. epi, epithelium; *Scale bar*: 10 μm. (**b**) Real-time RT-PCR also showed that mRNA expression of E-cadherin was suppressed by the loss of EPPK, whereas that of desmoglein-1α, desmoglein-1β, or desmoplakin-1 was not affected by EPPK gene knockout in a mouse cornea. $^*P < 0.05$; *Bar*, SE.

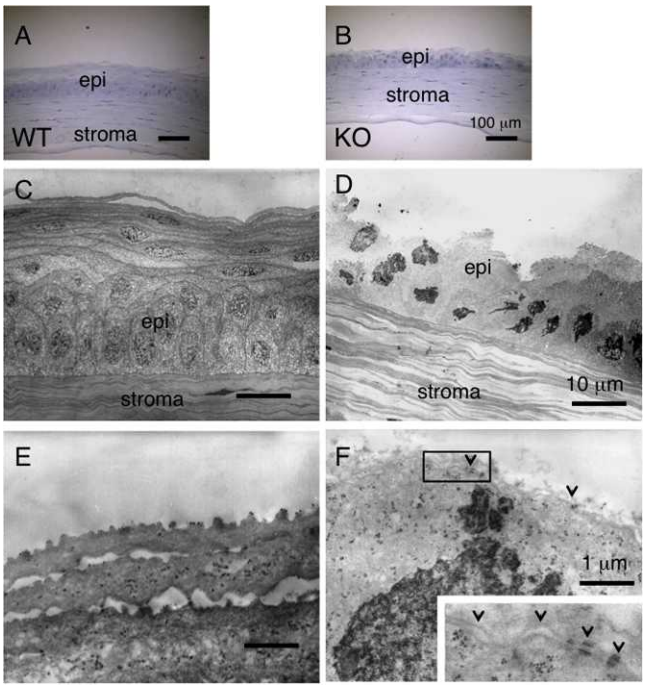

Figure 7. Fragility of the corneal epithelium in a KO mouse. Light microscopy with Toluidine blue staining shows that gentle brushing with a surgical micro-sponge did not severely damage the structure of the corneal epithelium in a WT mouse (**A**). On the other hand, the cells in the superficial layer and wing cell (suprabasal)-layer were removed by gentle brushing in a KO epithelium (**B**). Ultrastructural observation indicates that the WT epithelium (**C**) maintains the original layered structure even after the mechanical intervention of brushing, whereas the epithelial structure is severely damaged by the treatment in a KO mouse (**D**). The nuclei of the KO epithelium are pyknotic. Higher-magnification transmission electron microscopy showed that cell surface microplicae of a superficial layer cell were maintained following brushing in a WT epithelium (**E**). In a KO epithelium, the cell membrane of the ablated cells, including relatively intact desmosomes, remained to the neighboring cells ([**F**], *arrowheads* in *inset*). The *inset* in (**F**) shows the higher magnification view of the quadrangle. epi, epithelium; *Scale bars*: 100 μm (**A**, **B**), 10 μm (**C**, **D**), 1 μm (**E**, **F**).

ing to fragility in cultured keratinocytes.[14] It was previously reported that the loss of keratin 12, a corneal epithelium-specific keratin, results in the attenuated integrity (thus fragile) in mice, although the epithelium of an EPPK-null mouse cornea was labeled with antikeratin 12 antibody. Because EPPK is involved in the architecture of intermediate filament cytoskeleton, the epithelial fragility in an EPPK-null mouse is considered to be consistent with the finding in a keratin 12 null mouse.[12] EPPK was originally identified as an autoantigen in a patient with a subepidermal blistering disease. The present ultrastructural examination suggests less keratin bundle adhesion to a hemidesmosome. This finding is consistent with the reduced diameter of keratin filaments in KO keratinocytes.[18] However, the KO mouse does not develop epithelial defects in cornea, as suggested by the finding that basal cells seem to adhere well to the basement membrane, as shown by the brushing experiment.

Expression of keratin 12 was similarly detected in a KO epithelium (data not shown) as discussed above, indicating that the loss of EPPK does not impair cornea-type epithelial differentiation. To further evaluate the differentiation pattern of the corneal epithelium of a KO mouse, we then examined the expression pattern of cell–cell junction-related components by using immunohistochemistry and real-time RT-PCR. Our unpublished data showed that knockdown of EPPK by using a small interfering RNA procedure in an immortalized

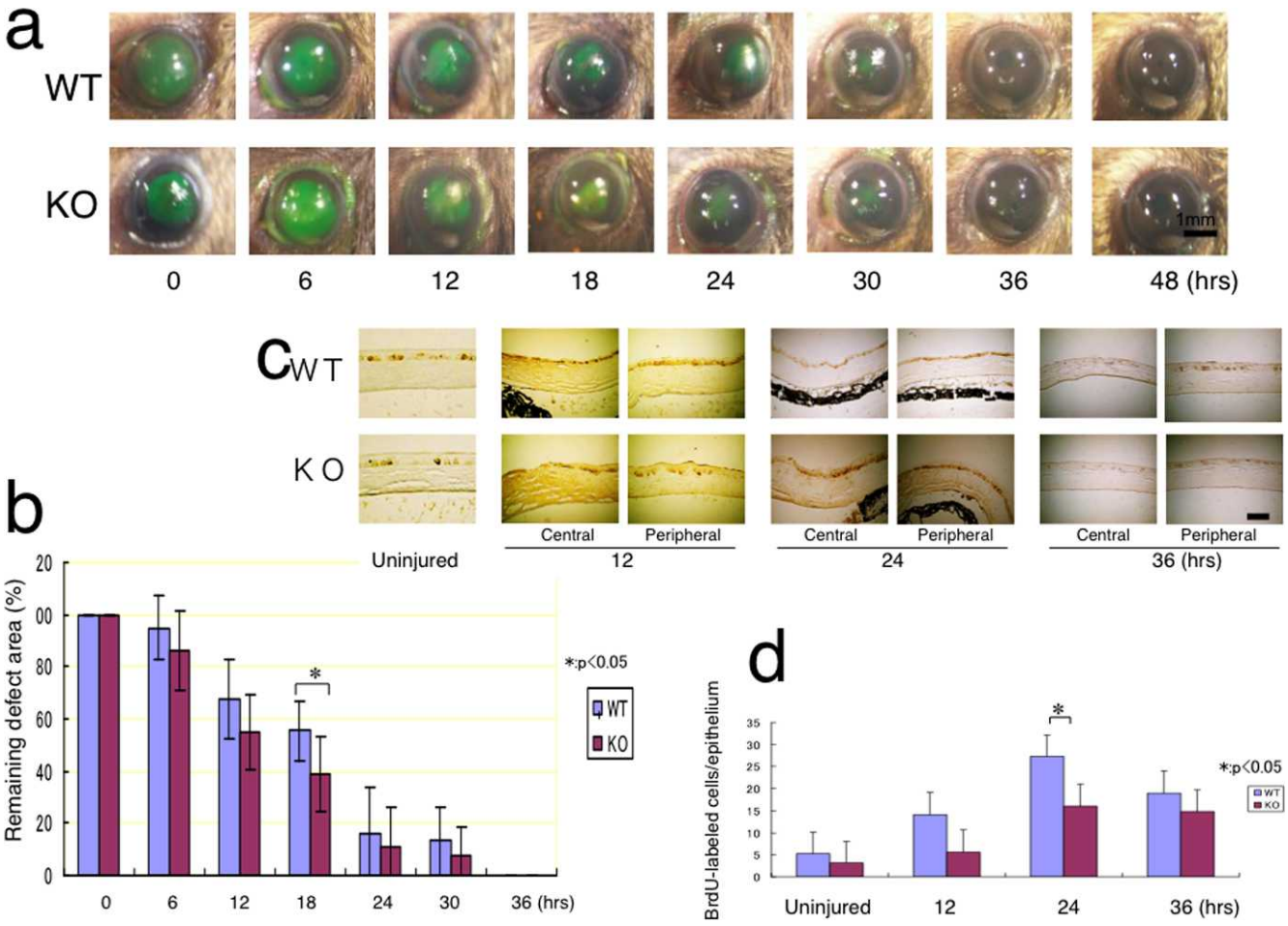

Figure 8. Wound healing of an epithelial defect in a cornea of a KO mouse. (**a**) The round epithelial defect (stained with green fluorescein) produced in the center of the cornea was gradually recovered with the migrating remaining epithelium from outside the defect. Thirty-six hours post wounding, the defect was closed in both WT and KO mice. The healing rate of the corneal epithelial defect in a KO mouse seemed overall higher as compared with a WT mouse. *Scale bar*: 1 mm. (**b**) The size of the remaining defect was significantly smaller in a KO mouse cornea as compared with a WT cornea at 18 hours post wounding (*$P < 0.05$). *Bar*, SE. (**c**) Immunodetection of BrdU incorporated in uninjured corneal epithelium as well as those at 12, 24, and 36 hours post wounding in WT and KO mice. Central, healing epithelium and peripheral healing epithelium were shown at each time point. *Scale bar*: 20 μm. (**D**) The numbers of BrdU-labeled epithelial cells increased at 12 and 24 hours following wounding in both WT and KO corneas. The number of proliferating cells in the whole corneal epithelium at 24 hours after injury was significantly lower in a KO mouse as compared with that of a WT mouse (*$P < 0.05$ by ANOVA). The BrdU-labeled cells in an uninjured epithelium and those at 12 and 36 hours post injury were similar between WT and KO mice. *Bar*, SE.

corneal epithelial cell line reduced the mRNA and protein expression level of E-cadherin. The results obtained by immunohistochemistry of in vivo specimens showed no difference of the staining intensity for desmocollin-1, desmocollin-3, desmoglein-1 (α and β), desmoplakin-1, plakoglobin, and occluding between WT and KO corneal epithelia. However, immunoreactivity for E-cadherin was less marked in a KO epithelium as compared with that of a WT corneal epithelium. To examine if the protein expression as detected by immunohistochemistry depends on mRNA expression, we also ran real-time RT-PCR for mRNA of E-cadhesin, desmoplakin, and desmoglein-1 (1α and 1β), and showed that E-cadherin mRNA expression was suppressed by the loss of EPPK. An in vitro experiment previously showed that expression of a desmosome-related component affects E-cadherin expression; overexpression of desmoglein-3 decreases E-cadherin expression.[19] The mechanism of suppression of E-cadherin expression by the loss of EPPK is to be explored.

The incidence of BrdU-labeled cells in an uninjured KO epithelium was similar to that in a WT cornea. However, uptake of BrdU was significantly less in the EPPK-null corneal epithelium than in a WT epithelium under the healing condition (i.e., at 24 hours). However, the exact mechanism of how the loss of EPPK suppresses cell proliferation activity in healing corneal epithelium is yet to be uncovered. It is known that a component of the desmosome affects cell cycle in epithelial tissue positively or negatively.[20] For example, epidermal hyperplasia is observed in a desmocolin-1–null mouse.[16] Desmoplakin also negatively regulates keratinocyte cell proliferation by, in part, regulating cell cycle progression.[21] On the other hand, overexpression of desmoglein-2 or -3 induces keratinocyte hyperproliferation in the epidermis, suggesting that the desmocolins-2/3 positively regulate cell cycle progression.[22,23] However, the present study showed that the expression of these cell–cell junction components, except for desmoglein-1 that was not examined, were not altered by the loss of EPPK. Thus, lacking EPPK might directly

affect cell cycle regulation. Except for possible relationship between desmosomal components and cell cycle regulation, expression of keratin 14 reportedly affects cell proliferation; keratin 14 expression positively correlates with cell proliferation.[24] However, the present study showed proliferation activity of EPPK-null corneal epithelium was less as compared with that in a WT epithelium, even though KO epithelium contains keratin 14–positive suprabasal cells above the basal layer.

It was reported that deficiency of E-cadherin promotes the conversion of an epithelial cell to that of a more migratory phenotype or accelerates epithelial-mesenchymal transition, a process through which an epithelial cell type transforms its phenotype to be more mesenchymal-like.[25,26] Suppression of E-cadherin therefore might accelerate movement of corneal epithelium in a KO mouse. To explore this hypothesis, we then examined the effect of lacking EPPK on epithelial wound healing in the cornea in vivo. The healing of a round epithelial defect in the cornea was more rapid in a KO mouse as compared with a WT mouse. The BrdU-labeled experiment showed that lacking EPPK attenuates the cell proliferation activity in the injured/healing epithelium of the cornea, as in an uninjured one. This indicates that acceleration of repair of the epithelial defect was caused by stimulation of epithelial cell migration, but not cell proliferation, in the absence of EPPK. However, there is a possibility that the mechanism of promotion of migration of corneal epithelial cells is attributable to multiple mechanisms. It is known that alteration of expression of cell–cell connection-related components affect cell migration. For example, protein expression of desmosomal components is reduced in corneal epithelium during wound healing in rats.[27] The in vitro study presented further evidence that desmosomal components negatively modulate epithelial cell migration; lacking a component of desmosome, plakophilin-1 or plakoglobin, accelerates cell migration of cultured epidermal keratinocytes.[28,29] It has also been shown that desmosomal molecules are downregulated in certain cancers, such as squamous cell carcinomas; suppression of expression of plakophilin-3, another desmosomal component, reduces cell adhesion of a neoplastic cell and promotes metastasis.[30] In turn, overexpression of desmosomal cadherins has been inversely correlated with invasive potential and reduced motility in neoplastic cells.[31,32] As for the multilayerization of keratin 14–labeled cells in a KO epithelium that was discussed above, a reduction of E-cadherin expression and an impairment of desmosome architecture might have allowed the basal cells to simply move upward without undergoing differentiation.

It was suggested that fewer keratin 6 bundles might explain the acceleration of cell migration in KO epidermis, because lacking keratin 6 reportedly promotes epidermal keratinocyte migration.[7] However, our unpublished data from an in vitro cell culture experiment showed EPPK knockdown did not affect the protein expression level of keratin 6. Because cell adhesion might affect cytoplasmic signaling activation, further study is needed to examine the relationship between EPPK deficiency and cell migration–related signals.

In conclusion, the loss of EPPK affects morphogenesis of the corneal epithelium as well as its integrity. The mechanism of acceleration of cell migration in the KO corneal epithelium is to be further investigated, although suppression of expression of E-cadherin might be included.

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