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Disruption of eyelid and cornea morphogenesis by epithelial β-catenin gain-of-function

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Purpose: To examine the developmental pathobiology of the eyelid and the cornea caused by epithelial β-catenin gain-of-function (gof) during mouse embryogenesis.
Methods: Compound mutant mice (*Ctnnb1*^{GOFOSE}, gof of β-catenin in the epidermis and the ocular surface epithelium) were generated by time-mating keratin 5-promoter-Cre recombinase (*Krt5-Cre*) and *Ctnnb1*$^{fE3/WT}$ (floxed exon 3 of *Ctnnb1*) mice. Eyes obtained from wild-type (WT) and mutant embryos at various gestation stages until E18.5 were examined with histology and immunohistochemistry. The ultrastructure of the ocular tissues of the E18.5 embryos was also examined.
Results: Expression of the gof-β-catenin mutant protein in the epidermis severely impaired eyelid morphogenesis at E15.5, E17.5, and E18.5. The mutant stroma exhibited impaired keratocyte differentiation with accelerated cell proliferation and reduction in the accumulation of collagen type I. The mutant embryos also showed hyperproliferative nodules in the ocular surface epithelia with anomaly of cornea-type epithelial differentiation and the absence of the epithelial basement membrane.
Conclusions: Expression of the gof-β-catenin mutant protein in basal epithelial cells disrupts eyelid and cornea morphogenesis during mouse embryonic development due to the perturbation of cell proliferation and differentiation of the epithelium and the neural crest-derived mesenchyme.

Embryonic eyelid morphogenesis is regulated by a complex interaction of epidermis and neural crest-derived mesenchyme, which is precisely orchestrated by growth factors [1,2]. A report showing that fibroblast growth factor 10 (FGF10) is essential to eyelid morphogenesis was followed by a publication that reported the bone morphogenetic protein (BMP) signal is located downstream of the FGF10 signal for eyelid morphogenesis [1,3,4]. We previously reported that transforming growth factor α (TGF-α) is involved in the migration of the neural crest–derived mesenchyme during eyelid morphogenesis, while TGF-β is also reportedly involved in eyelid morphogenesis [5,6]. At the signaling level, the loss of mitogen-activated protein kinase kinase kinase 1 (MAP3K1) reportedly impairs eyelid closure during embryonic development in mice [7,8]. Gene ablation of sphingosine 1-phosphate receptors also leads to the failure of eyelid closure during mouse embryonic development, via inhibition of MAP3K1 signaling [9].

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Signaling-activated by β-catenin plays pivotal roles in regulating cell lineage differentiation or cell fate in tissue morphogenesis during embryonic development [10,11]. Canonical Wnt/β-catenin pathways activated by Wnt binding to Frizzled family receptors, leading to dissociation of the Dvl, GSK-3β, and Axin complex, result in the inhibition of serine/threonine phosphorylation encoded within exon 3 of the *Ctnnb1* allele, which prevents β-catenin degradation via ubiquitination and leads to nuclear translocation of β-catenin [12]. Specific target proteins of the Wnt/β-catenin signal regulate self-renewal and differentiation of various tissue-specific stem cells in adult somatic tissues, suggesting involvement in maintenance of tissue homeostasis [10-12]. Abnormal and/or excessive activation of β-catenin signaling is, in turn, reportedly involved in the development and metastasis of certain malignant tumors [13]. We previously reported that keratin 12 promoter-driven conditional gain-of-function (gof) of β-catenin in the cornea epithelium (Krt12$^{rtTA/Wt}$/tetO-Cre/*Ctnnb1*$^{floxE3/Wt}$ triple transgenic mouse) exhibited tumor-like hyperproliferation of the corneal epithelium and increased the proliferation of stromal cells in mouse embryos [14]. In this mouse line, abnormalities were not observed in the conjunctiva and the eyelid.

Because MAP3K1 positively modulates Wnt-dependent gene expression via interaction with axin1, it is likely that Wnt/β-catenin signaling is involved in the impairment of eyelid morphogenesis in MAP3K1 null mice [7,8,15]. Inhibition of Tcf3-β-catenin by the homozygous Tcf3 knock-in mutant ($Tcf3^{\Delta N/\Delta N}$) also results in a defect in embryonic eyelid closure in mice, which supports the previous notion [16]. A mouse line introduced with a null mutation of Dkk-2 also shows impaired eyelid morphogenesis during embryonic development [17]. These reports suggest that the involvement of epithelial β-catenin signaling in mouse eyelid tissue morphogenesis should be investigated further. To understand the effects of gof-β-catenin signaling in the epithelium on mouse eyelid morphogenesis, we created a conditional mutant mouse line with the gof allele of β-catenin by deleting exon 3 (floxed E3 β-catenin) with keratin 5 promoter-driven Cre recombinase in basal epithelial cells and examined morphogenesis of the eyelid and the ocular surface, that is, the cornea and the conjunctiva during embryonic development. Our data indicate that the keratin 5 promoter mutants exhibit a corneal phenotype similar to that of keratin 12-deriven gof-β-catenin, but they manifest marked impairment in eyelid morphogenesis.

METHODS

The experiments were approved by the DNA Recombination Experiment Committee and the Animal Care and Use Committee of Wakayama Medical University. The experiments performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Conditional mutant mice: Genetically modified mouse lines of *Krt5-Cre* and $Ctnnb1^{fE3/WT}$ (*Ctnnb1*-floxed Exon3) were previously described [18,19]. Cre excises exon 3 of the $Ctnnb1^{fE3}$ allele, leading to the accumulation of active β-catenin in the cells. The genotypes were identified with PCR using oligonucleotide primers specific for each transgene as previously reported [20]. Primers were designed based on the mouse β-catenin sequence (DDBJ/EMBL/GenBank accession No. M90364): BCAT-S1 (5'-ATG GCT ACT CAA GCT GAC CTG ATG G-3') and BCAT-AS1 (5'-TTA CAG GTC AGT ATC AAA CCA GGC C-3'). PCR was for 30 cycles of 94 °C for 30 s, 60 °C for 90 s and 72 °C for 2 min. In all experiments, the detection time of the vaginal plug was defined as E0.5 of embryonic development. The E13.5, E15.5, E17.5, and E18.5 embryos are described below. Embryos at E13.5 and E15.5 were labeled with maternal i.p., administration of bromo-deoxyuridine (BrdU) as previously reported [21].

Histology and immunohistochemistry: The mutant (*Krt5-Cre/*$Ctnnb1^{(ex3)fl/WT}$) mouse dies at birth due to epidermis defects since Krt5 is ubiquitously expressed by all stratified epithelia. The eyes and eyelids of the mutant embryos and wild-type (WT) littermates at E13.5, 15.5, and 17.5 were processed for paraffin embedding, and E18.5 embryos were used for the electron microscopic examination. The number of embryos used were E13.5 (n=2), E15.5 (n=2), E17.5 (n=5), and E18.5 (n=4) in the WT and E13.5 (n=2), E15.5 (n=2), E17.5 (n=5), and E18.5 (n=4) in mutants. Deparaffinized sections (5 µm) were processed for hematoxylin and eosin (HE) staining. Immunohistochemistry was also performed with antibodies against β-catenin (1:1,000 in PBS [1X; 137 mM NaCl, 2.70 mM KCl, 7.75 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.5], BD Biosciences, San Jose, CA), keratin 12 [22], keratin 14 (1:200 in PBS, Sigma-Aldrich, St. Louis, MO), collagen type I (1:200 in PBS, Southern Biotech, Birmingham, AL), laminin (1:25 in PBS, Sigma-Aldrich), and keratocan [23]. Secondary antibodies were conjugated with either peroxidase or Alexa-Fluor 555. Nuclei were counterstained with either methylgreen or 4,6-diamidino-2-phenylindole (DAPI), respectively. Immunodetection of BrdU in cell nuclei was performed according to the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany) as previously reported [24]. The eyes and eyelids of E18.5 embryos of both genotypes (n=2) were observed under routine transmission electron microscopy as previously reported [21]. Briefly, tissues were fixed in 2% glutaraldehyde in 0.1 M phosphate-buffer (pH 7.4) for 48 h and then post-fixed in 1% osmium tetroxide. Then the samples were embedded in Epon mixture. Ultrathin sections were cut, electron-stained, and observed under transmission electron microscopy.

RESULTS

Eyelid morphogenesis: Expression of gof-β-catenin driven by the *Krt5* promoter in basal epidermal cells perturbed eyelid morphogenesis under the examination of the gross appearance of the eyelids and the external ocular structure (Figure 1). At E13.5, no obvious abnormalities were observed in eyelid elongation (not shown). As early as E15.5, elongation of eyelid tissues was somewhat impaired (Figure 1B) compared with a WT littermate (Figure 1A). At E17.5 (Figure 1C) and E18.5 (Figure 1D), the eyelid fused in the WT littermate, while the mutant of gof-β-catenin in basal epidermal cells exhibited an eye-open phenotype at these embryonic day points (Figure 1E, F).

HE histology of the eyelid anlage seemed to be similar between the WT and mutant embryos at E13.5 (Figure 2A,B). However, the mesenchymal cells in the periocular tissue of

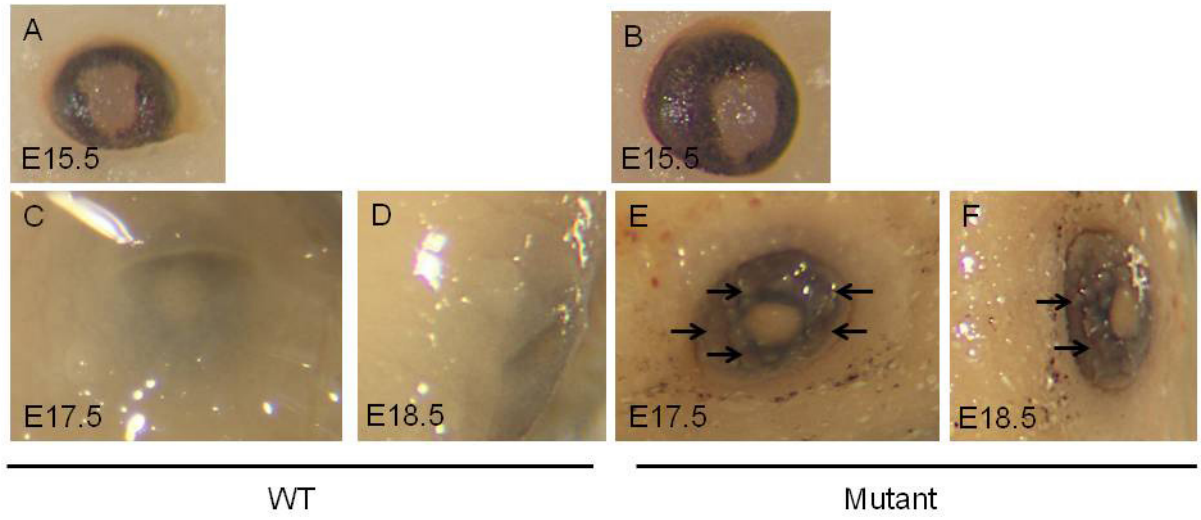

Figure 1. Gross appearance of eyes of a WT embryo and a mutant embryo with gof-β-catenin in epithelial tissues. At embryonic day (E) 15.5, the cornea is not covered by eyelids in the wild-type (WT) (**A**) and mutant embryos (**B**). At E17.5 and E18.5, the upper and lower eyelids are fused to each other, covering the cornea, in the WT eye (**C**, **D**), while no eyelid structure is observed in the mutant embryo (**E**, **F**).

the mutant embryo (Figure 2B) seemed to be more densely packed compared with the WT littermate at this embryonic stage (Figure 2A). Morphogenesis of the upper and lower eyelids was severely impaired in the mutant embryo of gof-β-catenin at E15.5 (Figure 2D) compared with the WT embryo (Figure 2C). At E17.5 and E18.5, eyelid fusion can be readily seen in the wild-type embryo (Figure 2E,G), while in the mutant the cornea is not covered by fused eyelids (Figure 2F,H) with an uneven corneal epithelium (described in detail below). Higher magnification observation showed that the mesenchymal cells in the periocular tissue of a mutant embryo (Figure 2H) still seemed to be more densely packed compared with the WT littermate (Figure 2G) at E18.5 similar to embryos at earlier embryonic day points.

Characterization of tissue phenotype was then performed with ultrastructural observation and immunohistochemistry. Transmission electron microscopy showed abnormal epidermal cell differentiation (loss of stratification or cornification in the superficial epithelial layer) and reduction in the collagenous matrix in the dermis of an E18.5 mutant embryo (Figure 3D) compared with that of a WT littermate (Figure 3C).

Immunohistochemistry detected nuclear accumulation of β-catenin in the focal nodular structures of the proliferating epidermal keratinocytes of the eyelid skin of the mutant embryo at E18.5 (Figure 3H), while faint staining for β-catenin was seen in the epidermis of the WT embryos (Figure 3G). The eyelid epidermis in the E18.5 WT and knockout (KO) embryos (Figure 3I, J) was not labeled for vimentin, and vimentin-labeled cells (presumably fibroblasts) were distributed in the dermis. At E15.5, the cell proliferation rate of the eyelid epidermis determined with BrdU labeling was comparable between the WT (Figure 3 K) and mutant embryos (Figure 3L).

Ocular surface epithelium: We then examined abnormalities of the ocular surface epithelium in detail. HE staining histology showed that the thickness of the corneal epithelium was similar between the WT (Figure 4A) and mutant (Figure 4B) embryos at E13.5. The cell density of the stromal cells was higher in the mutant embryo compared with the stroma of the WT littermate throughout the embryonic stages (Figure 4A-H, discussed later). At E15.5, the WT corneal epithelium remained thin (Figure 4C). At this time point, the epithelium of a mutant mouse embryo seemed thicker with multilayerization than that in the WT control, and localized multilayerization was observed (Figure 4D). Gross examination of the cornea of the E17.5 embryos showed opaque tumor-like nodules on the open corneal surface without overlaying eyelids (Figure 1E); in contrast, the eyelids were well developed in the WT littermate (Figure 1C). Focal nodular epithelial hyperproliferation was observed in the corneal epithelium in the mutant embryo (Figure 4F,H) but not in the WT embryo (Figure 4E,G).

Immunohistochemistry detected obvious β-catenin staining in the corneal epithelium of the mutant embryo (Figure 4J), but not in the WT embryo, at E13.5 (Figure 4I). At E15.5, β-catenin was detected in the WT corneal epithelium, which showed a monolayer appearance (Figure 4K). β-catenin

staining was markedly observed in the corneal epithelium, which was thicker than the normal one. Immunoreactivity for β-catenin was observed in cell–cell borders among each epithelial cell and was prominently accumulated in the cytoplasm and/or nuclei of the focal proliferative nodules in the corneal epithelium of the mutant embryos at E15.5 (Figure 4L). At E17.5, β-catenin was detected in the cell–cell border of the basal cells of the WT corneal epithelium (Figure 4M). At this embryonic day point, accumulation of β-catenin was still observed in the cytoplasm and/or nuclei of the epithelial cell nodule but not observed in the thick epithelium among each nodules (Figure 4N).

Ultrastructural observation showed a regular arrangement of stratified epithelial cells on the surface of the WT cornea at E18.5 (Figure 5C). In the E18.5 mutant embryos, the superficial epithelial cells of the corneal epithelium failed to assume the flattened characteristic shape of the stratified superficial epithelium seen in the WT cornea (compare Figure 5D to Figure 5C). The E18.5 mutant epithelium lacked a subepithelial basement membrane at the interface with the underlying stroma (Figure 5F), while the WT epithelium had an obvious basement membrane structure (arrows, Figure 5E).

Because the β-catenin signal is closely related to cell differentiation and cell phenotype control, the epithelial–mesenchymal transition (EMT), we examined whether gof-β-catenin affected differentiation of the corneal epithelium by using immunohistochemical analysis of intermediate filament components. We first examined the characteristics of epithelial differentiation by examining the expression pattern of keratin 12, the marker of cornea-type epithelium differentiation, and keratin 14. In WT embryos, the keratin 12 protein was negative in the cornea at E13.5 (Appendix 1, panel A), and expression in the corneal epithelium was detected at E15.5 (Appendix 1, panel B); marked expression was detected at E17.5 (Figure 6A). However, the keratin 12 protein was not detected in the corneal epithelium throughout E13.5–17.5 (Figure 6B). Keratin 14 was observed in the basal cells of the WT corneal epithelium (Appendix 1, panel C). Keratin 14 was markedly expressed in epithelial nodules (Appendix 1, panel

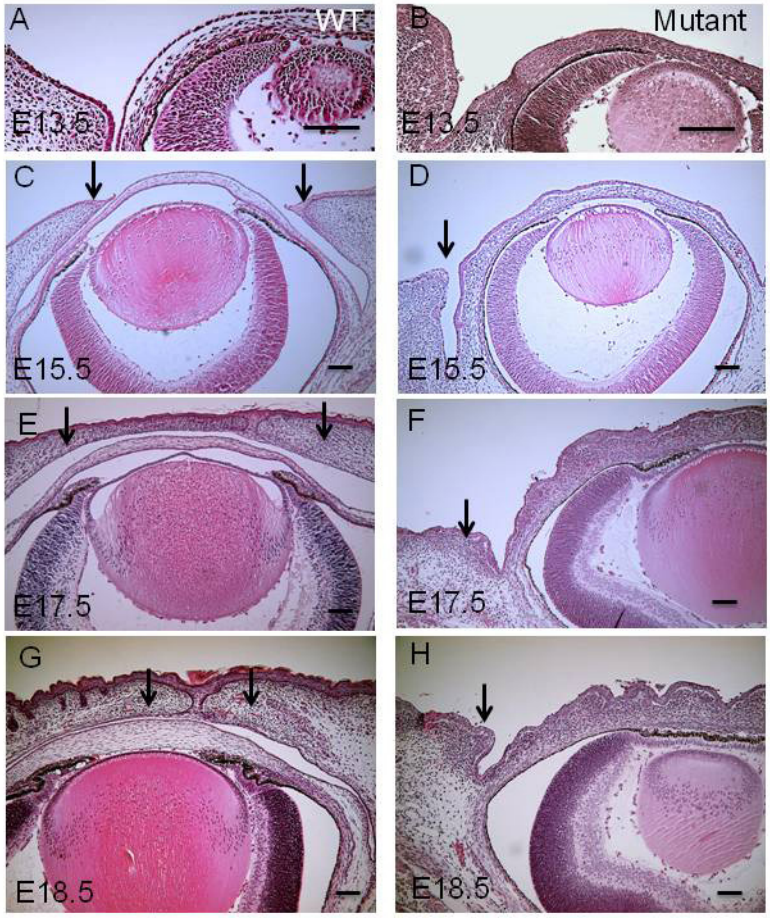

Figure 2. HE histology of eyes of a WT embryo and a mutant embryo with gof-β-catenin in epithelial tissues. Hematoxylin and eosin (HE) histology shows a clear difference in the cellular architecture of the eyelid anlage and the cornea between the wild-type (WT) embryo and the mutant embryo with gain-of-function (gof)-β-catenin. No obvious difference in the structure of the eye and eyelids between the WT (**A**) and mutant embryos (**B**) was observed at E13.5. At E15.5, the eyelid (arrows) development is impaired (**D**) compared with the WT tissue (**C**). At E17.5 and E18.5, the cornea is completely covered with fused eyelids (arrows) in the WT embryo (**E**, **G**), while no eyelids, but just anlage (arrow) of an eyelid, are observed in the mutant (**F**, **H**). The surface of the cornea is thicker with an irregular surface in the mutant eye while smooth curvature is seen in the WT embryo (compare **H** to **G**). Bar, 100 µm.

D). Focal hyperproliferation of the conjunctival epithelium was also observed in the mutant embryos (Appendix 1, panel E).

Impairment of intraepithelial differentiation from the basal layer toward the superficial layer might be associated with changes in cell proliferation, which can be examined with BrdU labeling. At E13.5, the incidence of BrdU-labeling in the embryonic corneal epithelium was similar between the WT and mutant embryos (Appendix 1, panel F,G). At E15.5, many epithelial cells were labeled with BrdU immunohistochemistry in the mutant corneal epithelium (Figure 7B,D), while such BrdU-labeled cells were less frequently observed in the WT corneal epithelium (Figure 7A,C).

Corneal stroma: In our previous study, we did not fully examine corneal stroma abnormalities in the keratin 12 promoter-driven conditional expression of gof-β-catenin (*Krt12*$^{rtTA/Wt}$/tet-O-Cre/*Ctnnb1*$^{floxE3/Wt}$ triple transgenic) mouse embryos [14]. In the present study, the mutant embryo with *Krt5*-Cre-driven gof-β-catenin overexpression induced hypercellularity with increased thickness in the stroma as early as E13.5 (Figure 4A,B) along with epithelial β-catenin immunostaining (Figure 4I-P). An ultrastructural examination was performed with the E18.5 embryos to examine the potential anomaly of keratocytes and the extracellular matrix. The WT corneal stroma exhibited a well-organized lamellar structure of keratocytes and collagen fibers at E18.5 (Figure

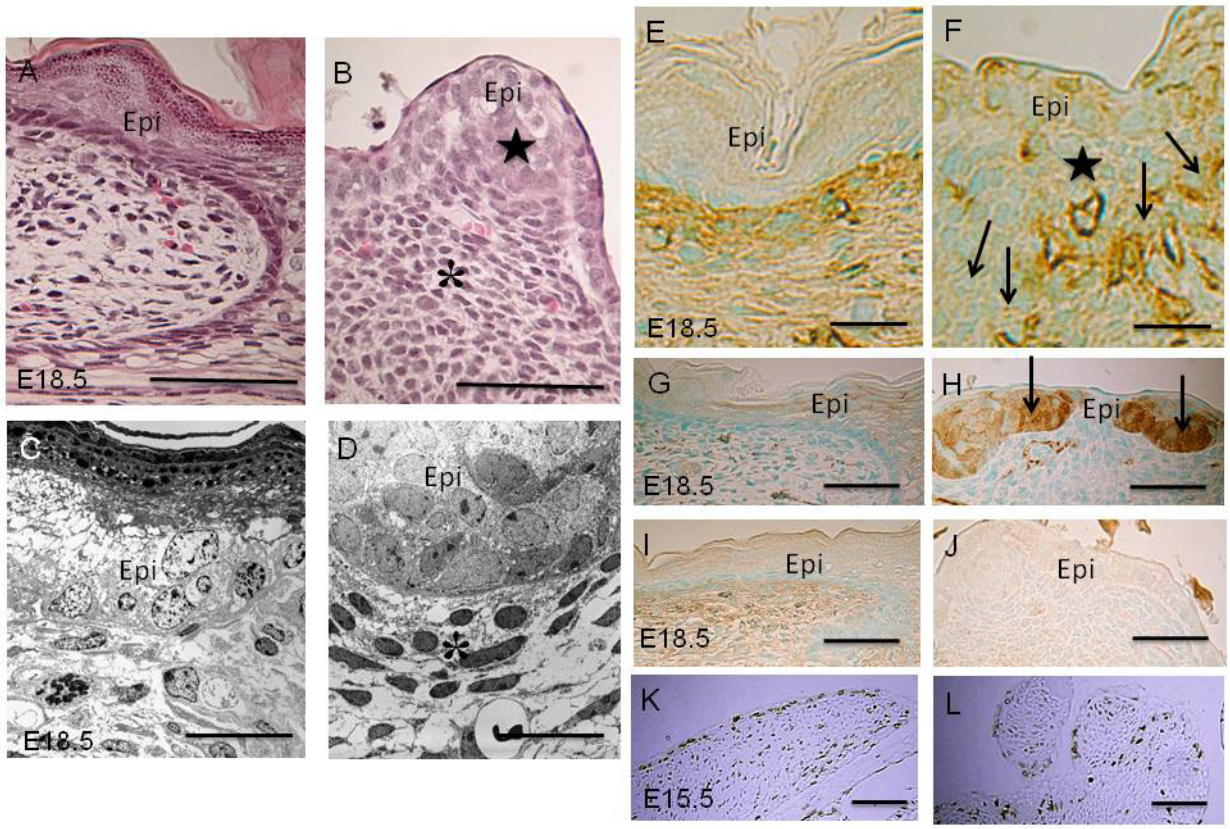

Figure 3. Ultrastructural and immunohistochemical examinations of impaired eyelids in mutant mice. Hematoxylin and eosin histology shows a clear difference in the cellular architecture of the epidermis and the mesenchyme of the eyelid (anlage) between the wild-type (WT) embryo (**A**) and the mutant embryo (**B**) with gain-of-function (gof)-β-catenin at E18.5. Nodular structures (srar) are observed in the mutant epidermis, but not in the WT epidermis. Mesenchymal cells are more densely packed (asterisk) in the mutant eyelid anlage than in the WT eyelid. Ultrastructural observation showed loss of stratification or intraepidermal differentiation in the upper layers with relatively round basal-like cells (Epi) and reduction of the accumulation of the collagenous matrix in the dermal tissue (asterisks) in an E18.5 mutant embryo (**D**) compared with the WT tissue (**C**). Immunohistochemistry shows laminin is well condensed in the subepithelial basement membrane zone in the WT embryo (**E**), while immunoreactivity for laminin is disrupted (arrows) beneath the epithelial nodular structure in the mutant eyelid anlage (**F**). The accumulation of β-catenin is detected in the nodular structures of the epidermis of the mutant embryo at E18.5 (arrows, **H**), while faint staining for β-catenin was seen in the epidermis of the WT embryo (**G**). The eyelid epidermis in the E18.5 WT and mutant embryos is not labeled for vimentin (**I**, **J**). The population of bromo-deoxyuridine (BrdU)-labeled epidermal keratinocytes was similar in the eyelid epidermis of the WT (**K**) and mutant embryos (**L**) at E15.5. Bar, 5 µm (**C**, **D**); 20 µm (**E**, **F**); 50 µm (**A**, **B**, **G**–**J**); 100 µm (**K**, **L**).

8A), while the keratocytes of the mutant stroma showed a disorganized distribution with less extracellular matrix in between the cells (Figure 8B). Such abnormality was more prominent in the anterior stroma than in the posterior stroma (Figure 5D). Neovascularization was also observed in the mutant stroma (Figure 8B).

To characterize the level of differentiation or maturation of keratocytes and the nature of the extracellular matrix of the corneal stroma, we then performed immunohistochemistry for the extracellular matrix components. Expression of keratocan is a hallmark of keratocyte-type differentiation. At E13.5, the corneal stroma of the WT and mutant littermates was not labeled with anti-keratocan antibodies (Appendix 1, panel H,I). At E15.5 (Appendix , panel J) and E17.5, immunoreactivity for keratocan was detected in the stroma of the wild-type mouse (Figure 9A) but completely absent in the mutant stroma (Figure 9B). We then evaluated the maturation of the stromal extracellular matrix by examining the protein expression of collagen type I (the major collagen in the corneal stroma).

Collagen type I was not detected at E13.5 but was readily observed at E15.5 (Appendix 1, panel E) and E17.5 in the WT embryos (Figure 9C). Epithelial gof-β-catenin completely abolished accumulation of collagen I in the corneal stroma at E17.5 (Figure 9D). These findings indicate that gof-β-catenin disrupted the keratocyte-type differentiation of corneal stromal cells.

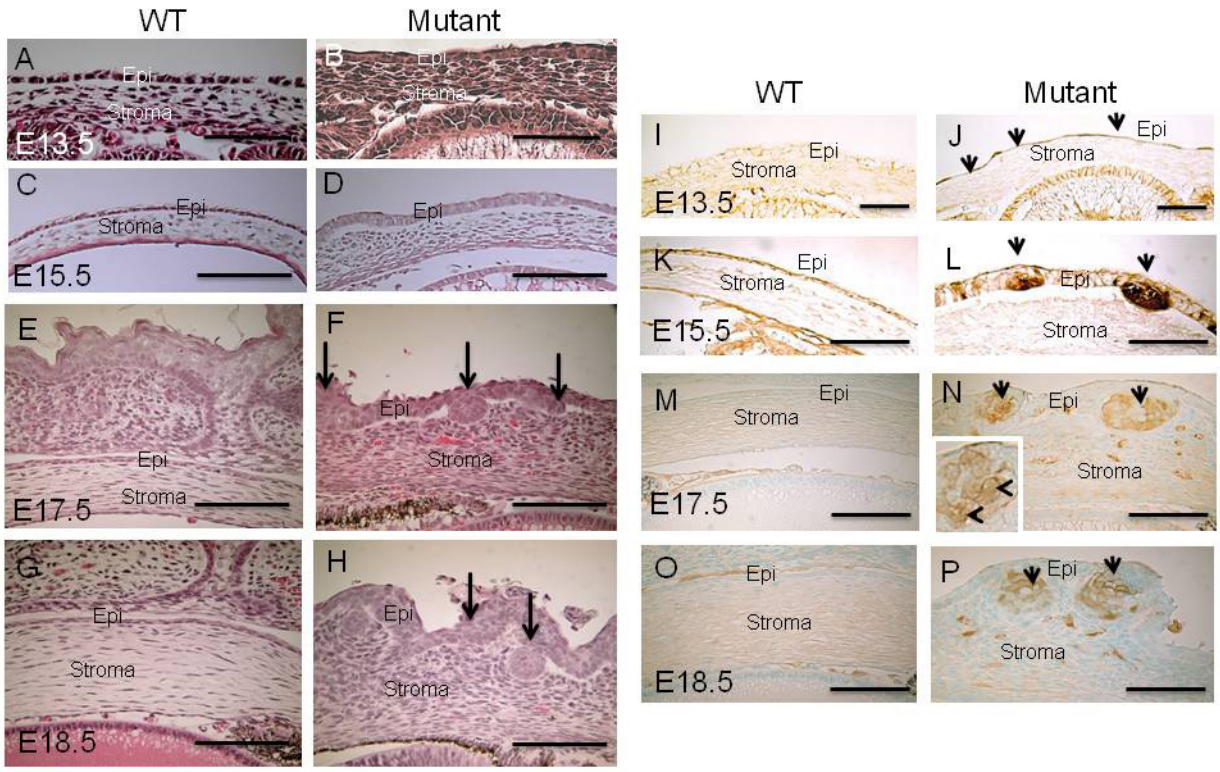

Figure 4. Effect augmented β-catenin on histology of the cornea epithelium. a. Hematoxylin and eosin (HE) staining histology suggested that at E13.5 there is no obvious difference of the corneal epithelial morphology between a wild-type (WT) littermate and the mutant of gof-β-catenin in epithelial tissue (**A**, **B**). At E15.5, the WT corneal epithelium is thin (**C**), while the epithelium of the mutant mouse embryo seemed thicker than that in the WT control, and localized multilayerization was observed (**D**). At E17.5 and E18.5, focal epithelial cell nodules (arrows) are observed in the corneal epithelium in the mutant embryo (**F, H**), and such structures are not seen in the WT tissue (**E**, **G**). Throughout the embryonic day points, cell density in the corneal stroma seems higher than that of the WT stroma. Bar, 50 µm (**A**, **B**); 100 µm (**C**–**H**). Expression of β-catenin is not seen in the corneal epithelium of the WT embryo (**I**) at E13.5. Obvious β-catenin immunoreactivity is observed in the mutant corneal epithelium (arrows) at this day point (**J**). At E15.5, β-catenin is detected in the WT corneal epithelium (**K**). Marked β-catenin staining is detected in the thickened epithelium and more markedly in the focal cellular nodules (arrows) in the epithelium of the mutant embryo (**L**). At E17.5 and E18.5, the expression of β-catenin is faint in the WT corneal epithelium (**M, O**), while the mutant epithelium exhibits β-catenin immunoreactivity in the proliferative nodules (arrows), but not the thickened epithelium among each nodule (**N, P**). The insert in Frame F indicates that β-catenin immunoreactivity is detected in the cytoplasm and nuclei (arrows) of the epithelial cells in the nodular structure. Epi, corneal epithelium; Bar, 50 µm (**A**, **B**, **I**, **J**); 100 µm (**C**–**H, K-P**).

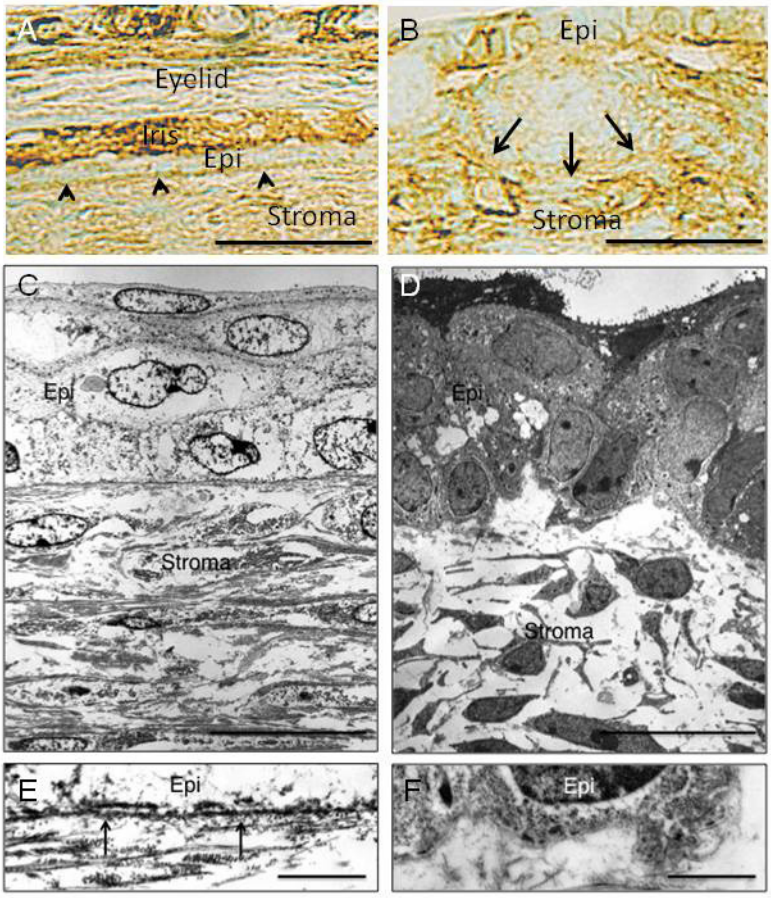

Figure 5. Immunohistochemistry for laminin in the E18.5 corneal epithelium and its ultrastructural histology. Immunohistochemistry detects laminin in the epithelial basement membrane of the corneal epithelium (arrowheads) in the E18.5 wild-type (WT) embryo (**A**), while the epithelial nodules that grows downward to the stroma lacks a laminin-basement membrane (arrows; **B**). Ultrastructural observation shows that the corneal epithelium (**C**, Epi) forms stratification of the intraepithelial-differentiated cell in the WT tissue, while it consists of disarranged spheroidal epithelial epidermal cells (**D**, Epi) in the mutant tissue. Mutant epithelial cells in the mutant mouse lack upward differentiation. The epithelial–stromal interface is irregular in the mutant tissue with disorganized stromal connective tissue (Stroma) in the mutant cornea. Higher magnification observation shows that the WT corneal epithelium (Epi) has a basement membrane (arrows) between stroma (**E**), while the basal epithelial cells (Epi) lacks this structure at the interface with the underlying stroma in the mutant (**F**). Bar, 50 µm (**A**, **B**); 5 µm (**C**, **D**); 500 nm (**E**, **F**).

DISCUSSION

Our primary interest was to understand how augmented β-catenin signaling in the basal epithelial cells of the stratified epithelium, for example, the epidermis and the ocular surface epithelia, affects the embryonic development of whole ocular tissues. We used *Krt5-Cre* mice to drive *Ctnnb1*$^{fE3/WT}$ for this purpose because our previous study with *Krt12-Cre* mice showed an increase in the β-catenin signal in the corneal epithelium, but not in the conjunctiva and the eyelid epidermis. Our major purpose included understanding the mechanisms of pathogenic consequences induced by stabilized epidermal β-catenin-mediated signals in mouse eyelid morphogenesis. Our results showed the constitutive excess

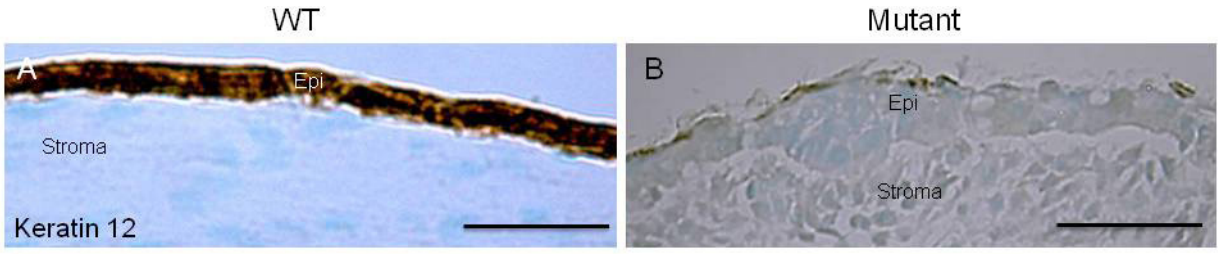

Figure 6. Expression pattern of intermediate filament components in the corneal epithelium at E17.5. The wild-type (WT) corneal epithelium (**A**, Epi) is labeled for keratin 12, while keratin 12 is not detected in the mutant corneal epithelium (**B**, Epi). Stroma, corneal stroma; Bar, 50 µm.

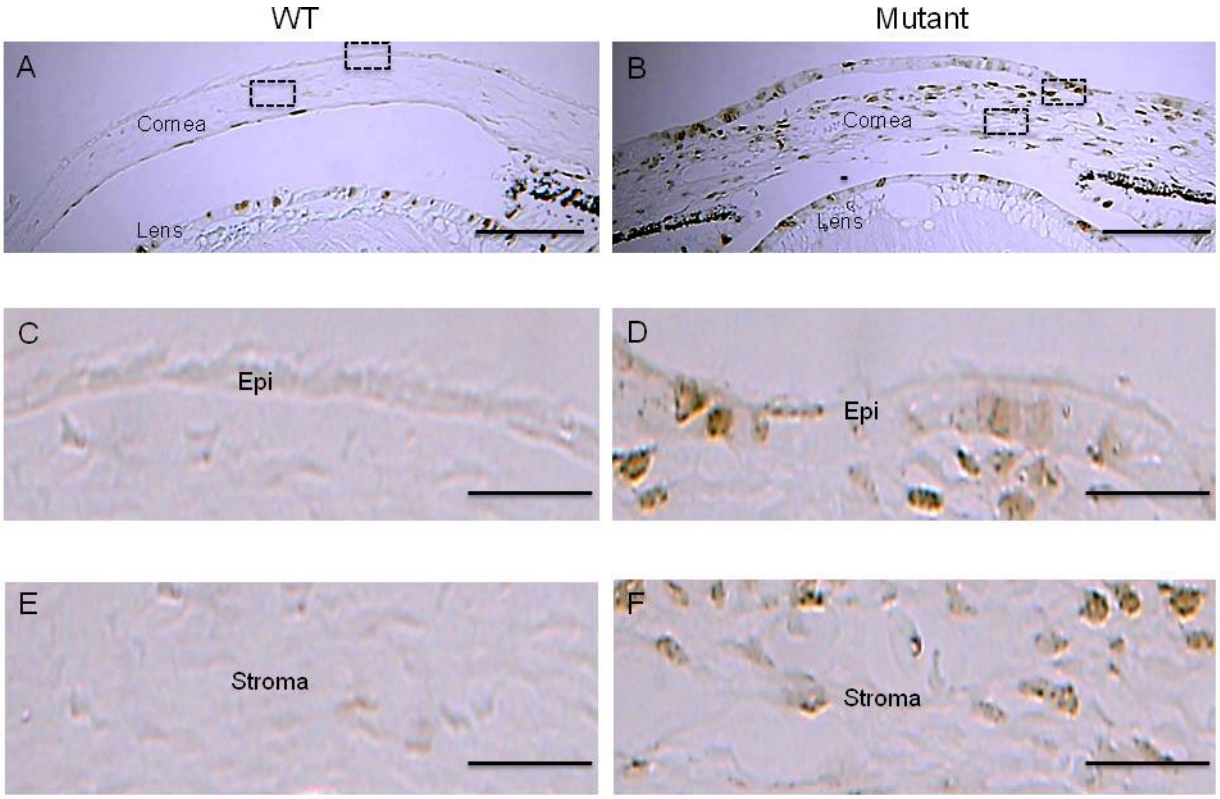

Figure 7. Cell proliferation by the detection of incorporation of BrdU. At E15.5, quite a few cells in the epithelium (Epi) and stroma are labeled for bromo-deoxyuridine (BrdU) in the wild-type (WT) cornea (**A**), while incidence of BrdU-labeled cells is frequently observed in a mutant embryo (**B**). Frames **C**, **D**, **E**, and **F** indicate the boxed areas in Frames A and B, respectively. Bar, 100 µm (**A**, **B**), 10 µm (**C**–**F**).

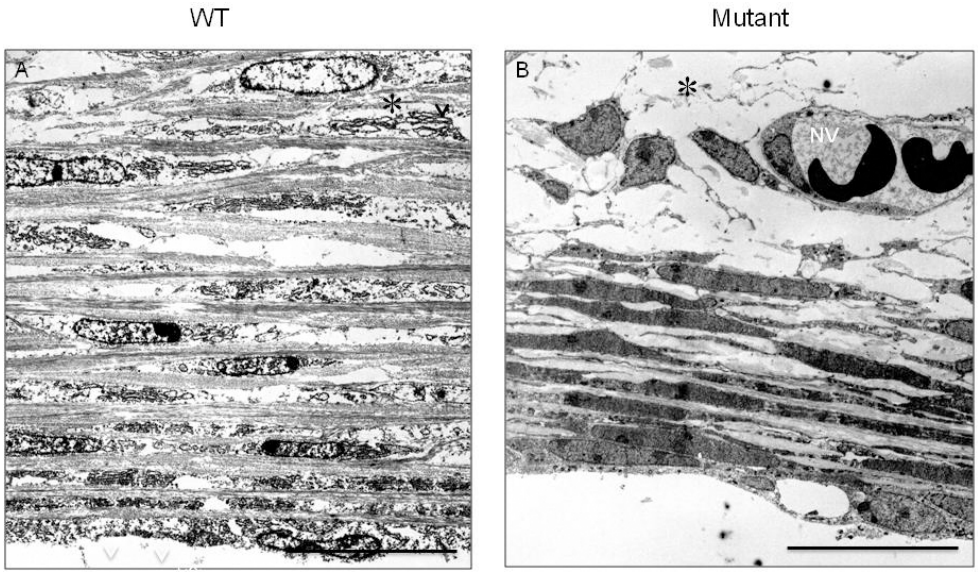

Figure 8. Ultrastructure of the posterior corneal stroma of WT and mutant embryos at E18.5. **A**: Lamellar structure of piled elongated keratocytes and collagenous connective tissue are observed in the posterior stroma of the wild-type (WT) embryo cornea. **B**: This lamellar structure is not observed in the anterior stroma of the mutant cornea (asterisk). Neovascularization (NV) is also seen in the mutant stroma. Bar, 5 µm.

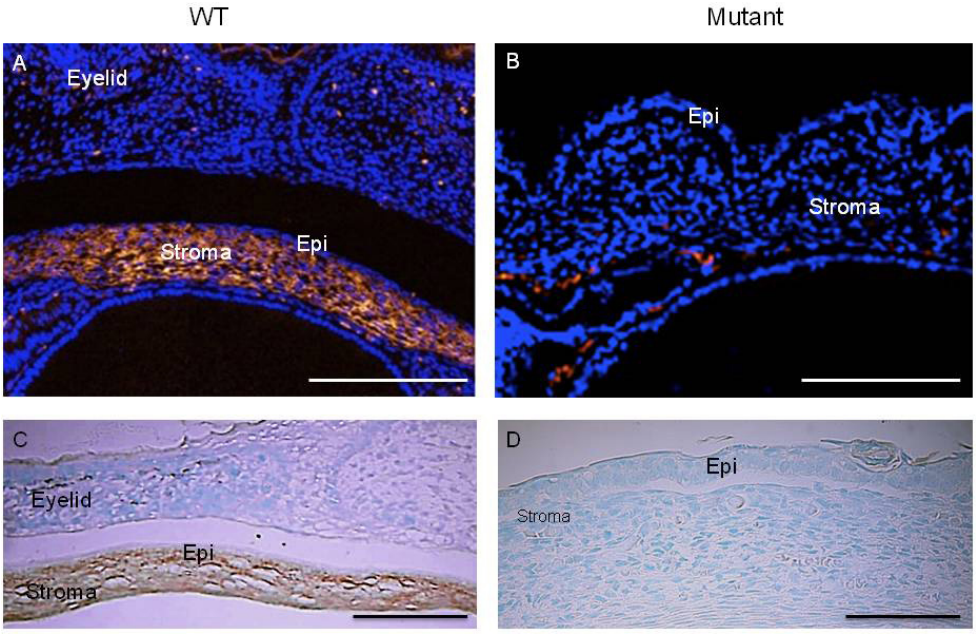

Figure 9. Expression pattern of keratocan and collagen type I in embryonic corneal stroma at E17.5. Stroma of the wild-type (WT) cornea is labeled for keratocan (**A**), while keratocan is not detected in the mutant corneal stroma (**B**). The entire corneal stroma of the WT embryo is positive for collagen type I (**C**). The mutant stroma lacks collagen I (**D**). Epi, corneal epithelium; Bar, 100 µm.

β-catenin-mediated signal in the epithelial tissues disrupted morphogenesis of the eyelid in addition to ocular surface epithelia and the underlying mesenchyme.

Eyelid morphogenesis: Embryonic morphogenesis of the eyelid is a complex, yet orchestrated, process governed by the interaction between epithelium derived from the surface ectoderm and mesenchymal cells of neural crest origin. Several investigations including literature mentioned in the Introduction suggest β-catenin-mediated signals in the epidermis are involved in eyelid morphogenesis. Null mutation of Dkk-2 impairs eyelid morphogenesis, which also prompted us to hypothesize that expression of β-catenin in periocular epidermal keratinocytes might have adverse consequences on eyelid formation [17]. Eyelid formation is essential to the morphogenesis of ocular adnexal tissues, for example, the meibomian gland or the extraocular muscle.

The present study showed that constitutive activation of β-catenin signaling in the basal epidermal epithelial cells interrupted eyelid morphogenesis. In the WT mouse embryo, the eyelid folds begin to grow at E15.5 and fuse by E17.5 as observed with the gross appearance. Eyelid morphogenesis was morphologically impaired in the mutant with epidermal gof-β-catenin at E15.5, although gof-β-catenin was activated at E13.5. The exact mechanism(s) by which the excess β-catenin signal in the epidermis impairs the eyelid formation must be investigated. Ultrastructural observation showed abnormal epidermal cell differentiation (loss of organized stratification in the upper layers and ectopic vimentin expression). The finding suggests epidermal keratinocytes with excess β-catenin signal might induce the epithelial–mesenchymal transition and might affect organized keratinocyte movement for eyelid morphogenesis. Accumulation of dermal collagen bundles also seemed to be impaired in the eyelid mesenchyme of the mutant embryo compared with the WT embryo, suggesting epidermal–mesenchymal (neural crest–derived cell) crosstalk; growth factors/cytokines expressed by mutant epithelial cells might affect the behavior of mesenchymal cells. Further studies are needed to investigate whether this abnormal structure of the epidermis is the direct consequence of impaired keratinocyte migration during eyelid formation.

Conjunctiva: We previously reported the expression of gof-β-catenin in the limited area of the corneal epithelium in mice by using tet-On (*Krt12-rtTA/tet-O-Cre/Ctnnb1*$^{fE3/WT}$) mice that exhibited intraepithelial neoplasm-like lesions in the corneal epithelium similar to that seen in the present study using *Krt5-Cre/ Ctnnb1*$^{fE3/WT}$ mice [14]. In addition, we observed lesions in the epidermis of the eyelid and the conjunctival epithelium in the present study. The mutant gof-β-catenin (*Krt5-Cre/Ctnnb1*$^{fE3/WT}$) embryos also manifested abnormalities in conjunctival differentiation; focal hyperproliferative nodules formed in the mutant conjunctival epithelium such as the corneal epithelium (Appendix 1, panel E).

Ocular surface epithelium: An in vitro study conducted by other investigators showed that Wnt/β-catenin signaling has an inhibitory effect on the differentiation of corneal epithelium–stem cells, and promotes epithelial cell proliferation and default epithelium differentiation [25]. Results of the current

in vivo experiments do not contradict these cell culture findings. Namely, gof-β-catenin impaired differentiation of the corneal epithelium in association with the presence of hyperproliferative spots and increased epithelial intracellular layerization in the corneal epithelium as evaluated with immunohistochemistry and ultrastructural observation [26]. The phenotype seen in the corneal epithelium of the current mutant mouse embryos was similar to that observed in gof-β-catenin in the corneal epithelium. *Krt12*$^{rtTA/WT}$*/TC/Ctnnb1*$^{fE3/WT}$ triple transgenic gof-β-catenin mice did not completely abolish the expression of keratin 12, the cornea-type epithelium differentiation marker [27]. However, expression of keratin 12 was not detected in the current mutant embryos at E17.5 while it was readily seen in WT embryos at E15.5, suggesting that gof-β-catenin in limbal basal cells shuts down cornea-type epithelial differentiation. Ultrastructural observation showed the subepithelial basement membrane disappeared, which coincides with a previous finding that β-catenin signaling leads to upregulated expression of matrix metalloproteinase-7, which may account for the EMT of the corneal epithelium [14]. Targeting β-catenin as a strategy for treating ocular surface neoplasms should be examined.

Corneal stroma: The current finding of the increased proliferation of corneal stromal cells with epithelial gof-β-catenin is consistent with our previous result of hyperproliferation in stromal cells in *Krt12*$^{rtTA/Wt}$/tet-O-Cre/*Ctnnb1*$^{floxE3/Wt}$ triple transgenic mouse embryos [14]. However, we failed to fully examine the nature of the corneal stromal matrix in the previous mutants. The present study showed an abnormally increased cell population in the mutant corneal stroma at E13.5. This abnormality is somewhat different from those observed in *Krt12*-rtTA-driven mutants, which can in part be explained by the fact that keratin 5 is expressed at E12.5 several days ahead of keratin 12 around E13.5 to 14.5. Thereafter, perturbation of the peridermal epithelium by gof-β-catenin may potentially affect the migration and differentiation of periocular mesenchymal cells of neural crest origin, which contribute to the formation of ocular surface stroma, as well as the eyelid, during embryonic development. This possibility is substantiated by observations with transmission electron microscopy and immunohistochemistry: an irregular arrangement of embryonic keratocytes without expression of keratocan and collagen type I in the mutant embryo stroma [23]. The corneal endothelium seemed ultrastructurally well developed even in the mutant embryos at E17.5.

In Summary, further study is needed to uncover the roles of β-catenin signaling in ocular Development. But we conclude that excess β-catenin signaling in the epidermis and corneal epithelium impairs eyelid morphogenesis in addition to affecting morphogenesis of the cornea and the conjunctiva in mice.

APPENDIX 1.

To access the data, click or select the words "Appendix 1." **A**: In WT embryos, the keratin 12 protein was negative in the cornea at E13.5. **B**: In WT embryos, the keratin 12 immunoreactivity is observed in the corneal epithelium at E15.5. **C**: Keratin 14 was observed in the basal cells of the WT corneal epithelium at E17.5. **D**: Keratin 14 was markedly expressed in mutant epithelial nodules at E17.5. **E**: Focal hyperproliferation of the conjunctival epithelium was also observed in the mutant embryos at E17.5. **F** and **G**: At E13.5, the incidence of BrdU-labeling in the embryonic corneal epithelium was similar between the WT and mutant embryos. **H** and **I**: At E13.5, the corneal stroma of the WT and mutant littermates was not labeled with anti-keratocan antibodies.**J**: At E15.5, the corneal stroma of the WT was labeled with collagen type I. Scale bar represents 10 µm (**A-J**).

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