

RESEARCH ARTICLE

Wnt/ β -catenin signaling modulates corneal epithelium stratification via inhibition of Bmp4 during mouse development

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ABSTRACT

The development of organs with an epithelial parenchyma relies on reciprocal mesenchymal-epithelial communication. Mouse corneal epithelium stratification is the consequence of a coordinated developmental process based on mesenchymal-epithelial interactions. The molecular mechanism underlying these interactions remains unclear. The Wnt/ β -catenin signaling pathway is involved in fundamental aspects of development through the regulation of various growth factors. Here, we show that conditional ablation of either β -catenin (*Ctnnb1^{CKO}*) or co-receptors *Lrp5/6* (*Lrp5/6^{CKO}*) in corneal stromal cells results in precocious stratification of the corneal epithelium. By contrast, ectopic expression of a murine *Ctnnb1* gain-of-function mutant (*Ctnnb1^{GOF}*) retards corneal epithelium stratification. We also discovered that Bmp4 is upregulated in the absence of β -catenin in keratocytes, which further triggers ERK1/2 (Mapk3/1) and Smad1/5 phosphorylation and enhances transcription factor p63 (Trp63) expression in mouse corneal basal epithelial cells and in a human corneal epithelial cell line (HTCE). Interestingly, mouse neonates given a subconjunctival BMP4 injection displayed a phenotype resembling that of *Ctnnb1^{CKO}*. Conditional ablation of *Bmp4* eradicates the phenotype produced in *Ctnnb1^{CKO}* mice. Furthermore, ChIP and promoter-luciferase assays show that β -catenin binds to and suppresses *Bmp4* promoter activity. These data support the concept that cross-talk between the Wnt/ β -catenin/Bmp4 axis (in the stromal mesenchyme) and Bmp4/p63 signaling (in the epithelium) plays a pivotal role in epithelial stratification during corneal morphogenesis.

KEY WORDS: Precocious epithelium stratification, Mesenchymal-epithelial interactions, Ocular surface, Wnt/ β -catenin signaling, Bmp4, Mouse

INTRODUCTION

The development of organs composed of an epithelial parenchyma is dependent on reciprocal mesenchymal-epithelial interactions. Tissue morphogenesis, cell differentiation and migration of both epithelium and mesenchyme are disrupted once the interactions are affected (Cunha et al., 2004). The vertebrate cornea is ideal for studying mesenchymal-epithelial interactions (Wilson et al., 1999).

It is a transparent tissue located on the anterior ocular surface and consists of a stratified non-keratinized epithelium, a thick stroma scattered with keratocytes and a single-cell-layer endothelium (Zieske, 2004). The outermost cell layers of the corneal epithelium serve as a protective barrier against fluid loss and pathogen penetration. This biological function is established through a complex and precisely coordinated program of epithelial stratification (Koster and Roop, 2007), which is mainly dependent on communication between the epithelium and the stromal mesenchyme during development (Liu et al., 1999). It has been suggested that bidirectional mesenchymal-epithelial interactions depend on soluble growth factors/cytokines, which are tightly regulated by as yet unknown signaling transduction networks (Kao, 2010). Identification of these signaling transduction networks would facilitate our understanding of the intricate mechanism of corneal epithelium stratification at both the cellular and molecular level.

Wnt/ β -catenin signaling plays a crucial role in controlling cell fate, differentiation, proliferation and apoptosis during development (Nüsslein-Volhard and Wieschaus, 1980), stem cell maintenance (Zhang et al., 2014) and adult tissue homeostasis (Logan and Nusse, 2004). This signaling activity is initiated by Wnt ligands binding to a dual receptor complex comprising Frizzled and either low-density lipoprotein receptor-related protein 5 (Lrp5) or Lrp6, resulting in the stabilization and nuclear translocation of β -catenin, which further binds with T cell factor/lymphoid enhancer factor (TCF/LEF) to regulate the transcription of downstream target genes (Clevers and Nusse, 2012). It has been reported that several ocular tissues express multiple components of the Wnt/ β -catenin signaling pathway during eye development (Ang et al., 2004; Borello et al., 1999). Gain- and loss-of-function studies reveal that Wnt/ β -catenin signaling is involved in coordinating vital processes during the development of ocular tissue, such as eye field formation and neural retina specification (Fuhrmann, 2008; Mukhopadhyay et al., 2006). However, the function of Wnt/ β -catenin signaling in corneal development is poorly understood.

Bone morphogenetic proteins (BMPs) have been extensively studied in the regulation of cell differentiation and morphogenesis of several tissues, including the eye during development (Furuta and Hogan, 1998; Huang and Klein, 2004; Wang et al., 2014). Signaling interactions between the BMP and Wnt/ β -catenin signaling pathways have been observed in fruit flies, amphibians, zebrafish and mammals (Huang and Klein, 2004; Zhu et al., 2014). For example, in *Xenopus* Wnt/ β -catenin signaling contributes to the repression of Bmp4 on the dorsal side of the embryo and sensitizes the ectoderm to respond to neural-inducing signals (Baker et al., 1999). In the mouse dental mesenchyme, Wnt/ β -catenin signaling regulates Bmp4 expression during incisor development (Fujimori et al., 2010). However, the possible cross-talk between these two signaling pathways during mouse corneal epithelium stratification has not been elucidated.

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It has been shown that loss of *Dkk2*, an antagonist of canonical Wnt signaling pathways, suppresses corneal differentiation during mouse development (Gage et al., 2008; Mukhopadhyay et al., 2006). This strongly suggests that canonical Wnt signaling might play a crucial role in ocular surface epithelia development. In this study, we discovered that an intimate interrelationship between canonical Wnt and *Bmp4* in stromal keratocytes is key to controlling the adjacent epithelial stratification during corneal development. We showed that conditional ablation of Wnt/ β -catenin signaling in stromal keratocytes results in the activation of *Bmp4* signaling, which led to precocious corneal epithelium stratification via upregulation of the transcription factor p63 in corneal basal epithelial cells.

RESULTS

Wnt/ β -catenin signaling activity in stromal keratocytes is inversely correlated with corneal epithelium stratification

To establish baseline information regarding the activity of Wnt/ β -catenin signaling in the mouse ocular surface, we took advantage of the knock-in *Axin2^{lacZ}* mouse line as a reporter of canonical Wnt signaling by X-gal staining. Interestingly, we found that as the corneal epithelium stratified and more cell layers formed the activity of Wnt/ β -catenin signaling gradually reduced from P1 to P21 (Fig. 1). Paraffin sections clearly revealed positive X-gal staining in the corneal epithelium, stroma and endothelium at P1. It was dramatically reduced in stromal keratocytes at P7 and disappeared at P21 (supplementary material Fig. S1). This finding was confirmed by immunofluorescence staining using a rabbit antibody (mAb D13A1) that only recognizes β -catenin that has not been phosphorylated by glycogen synthase kinase 3 β (Gsk3 β) and thus is functionally active in cell-cell adhesion and/or the canonical Wnt signaling pathway (Fig. 1). The β -catenin expression pattern did not change in the corneal epithelium from P1 to P21. However, nuclear localization of β -catenin in the stroma was easily detected at P1 and P7 and then steadily decreased by P21 (Fig. 1F-J).

Deletion of β -catenin in stromal keratocytes promotes corneal epithelium stratification

This dynamic change of Wnt/ β -catenin signaling activity in the cornea, especially in the stromal cells, prompted us to investigate its function during corneal development because there is a striking inverse correlation between Wnt/ β -catenin signaling activity and the process of corneal epithelium stratification during mouse ocular surface morphogenesis. To study the potential role of Wnt/ β -catenin signaling in corneal stromal keratocytes during corneal development, we employed a loss-of-function approach by crossing *KR; tetO-Cre (TC)* with the *Ctnnb1^{fl/fl}* mouse line to generate an inducible triple-transgenic mouse line, *KR; TC; Ctnnb1^{fl/fl}*, in which the *Ctnnb1* gene is deleted in corneal stromal keratocytes upon Dox induction (Fig. 2A).

Immunofluorescence staining confirmed that β -catenin was indeed deleted in corneal stromal keratocytes (compare Fig. 2C,E with 2B,D) upon Dox induction from P0 to P10. Surprisingly, conditional knockout of β -catenin (*Ctnnb1^{CKO}*) in corneal stromal keratocytes resulted in a thicker epithelium, with 4-5 epithelial layers compared with 2-3 cell layers in littermate controls (compare Fig. 2C,E with 2B,D and supplementary material Fig. S2B with S2A). Knocking out *Ctnnb1* from E12.5, prior to the expression of endogenous keratocan at E13.5 (Shiraishi et al., 1998), resulted in a more dramatic phenotype. Unlike the controls, which had 1-2 cell layers (Fig. 3A,A'), the mutant corneal epithelium had 4-5 cell layers at P0 (Fig. 3B,B'). Transmission electron microscopy (TEM) further confirmed these observations. At birth, controls had 1-2 epithelial cell layers without a clear basement membrane (Fig. 3C,C'), whereas multiple epithelial cell layers were present in the *Ctnnb1^{CKO}* mutant and these showed distinct morphological differences in the various layers, i.e. basal columnar cells, suprabasal (wing) cells and flattened superficial cells (Fig. 3D). Additionally, the mutant cornea had a distinct basement membrane with the appearance of hemidesmosomes (arrowheads in Fig. 3D'), which were not clearly defined in the control.

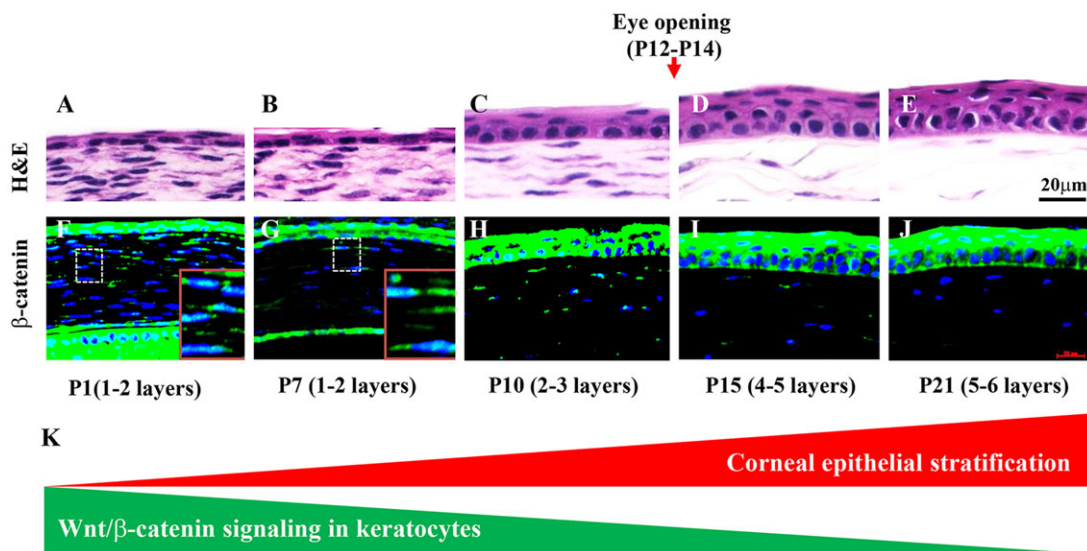


Fig. 1. Wnt/ β -catenin activity in stromal keratocytes is inversely correlated to corneal epithelium stratification. (A-E) Corneal epithelium stratification is a postnatal event. From P1 to P7 the mouse epithelium consists of 1-2 cell layers. Just before eyelid opening at P10, the epithelium begins to stratify. At P15 it increases to 4-5 cell layers and, by P21, there are 5-6 layers. (F-J) Expression of the active form of β -catenin (immunofluorescence, green) is found in the corneal epithelium and stroma from P1 to P21. Note that the β -catenin expression pattern did not change in the corneal epithelium from P1 to P21, but exhibited a dynamic change in the stroma. Nuclear localization of β -catenin was detected in the stroma at P1 to P7 and gradually decreased through P21. Boxed regions (F,G) are shown at higher magnification in insets. Blue, cell nuclei (DAPI). (K) Schematic representation of the reduction of Wnt/ β -catenin activity coinciding with epithelial stratification. Scale bars: 20 μ m.

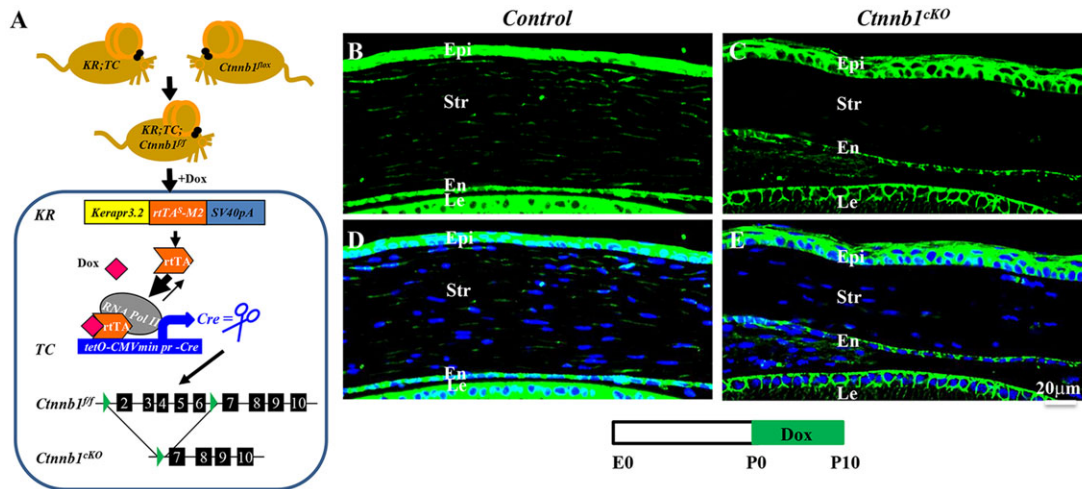


Fig. 2. Conditional knockout of β -catenin ($Ctnnb1^{cKO}$) in corneal stromal keratocytes. (A) Ablation of $Ctnnb1$ in keratocytes of the triple-transgenic mouse line $KR; TC; Ctnnb1^{fl/fl}$, which was generated by crossing the Dox-inducible driver mouse $KR; TC$ with the $Ctnnb1^{fl/fl}$ mouse. Administration of Dox resulted in ablation of $Ctnnb1$ only in keratocan-positive keratocytes. (B-E) $Ctnnb1^{cKO}$ and control littermates were treated with Dox from P0 to P10. β -catenin expression (green) in stromal keratocytes was analyzed by immunofluorescence staining of corneal sections at P10. β -catenin was absent in the stroma of $Ctnnb1^{cKO}$ (compare C,E with B,D). DAPI, blue. epi, corneal epithelium; str, stroma; en, endothelium; le, lens.

The expression of the corneal epithelial differentiation marker keratin 12 (Krt12) was unchanged in the $Ctnnb1^{cKO}$ cornea (supplementary material Fig. S2C,D). Interestingly, a triple-transgenic β -catenin gain-of-function mouse strain, $KR; TC; Ctnnb1^{\Delta E3}$ ($Ctnnb1^{cGOF}$), that expressed a stabilized β -catenin in corneal stroma when Dox was administered from E12.5 to P21, showed an impairment of corneal epithelial stratification in that the corneal epithelium remained at 1-2 cell layers (supplementary material Fig. S3). Taken together, these data indicate that deletion of $Ctnnb1$ in corneal stromal keratocytes triggers precocious corneal epithelial cell stratification.

Deletion of $Ctnnb1$ in keratocytes promotes corneal epithelial cell proliferation

An increase in the number of epithelial cell layers in the $Ctnnb1^{cKO}$ mouse suggested that there was an increase in cell proliferation. To test this, immunofluorescence staining against endogenous proliferating cell nuclear antigen (Pcna) and G1/S-specific cyclin D1 (*Ccnd1*) was performed. Our data showed a 2-fold increase in the number of Pcna-positive cells in the basal epithelium of the $Ctnnb1^{cKO}$ mouse as compared with littermate controls (Fig. 4). Additionally, *Ccnd1* expression was significantly increased in the $Ctnnb1^{cKO}$ mutant compared with littermate controls (supplementary material Fig. S4). These results indicated that loss of β -catenin in the corneal stroma results in the promotion of cell proliferation in basal epithelial cells, which is associated with precocious corneal epithelium stratification in the $Ctnnb1^{cKO}$ mutant.

Bmp4 expression is upregulated in the $Ctnnb1^{cKO}$ mutant, which triggers corneal epithelial stratification

To understand the molecular mechanism(s) by which β -catenin inactivation in stromal keratocytes results in precocious corneal epithelial stratification, we screened a mouse cytokine primer library to evaluate the expression of growth factors/cytokines in primary cultured $Ctnnb1^{cKO}$ cells versus controls. The $Ctnnb1^{cKO}$ mutant cells showed changes in the expression of various cytokines and growth factors, including a 2.5-fold increase in Bmp4 expression (Fig. 5A). It has been documented that Bmp4 is a

downstream target of Wnt/ β -catenin signaling (Baker et al., 1999; Kim et al., 2002). The elevation of Bmp4 expression after $Ctnnb1$ ablation was further confirmed at the mRNA level by qRT-PCR (supplementary material Fig. S5A) and at the protein level by western blotting (Fig. 5C,D). Immunofluorescence staining showed that Bmp4 expression was upregulated in the stroma and epithelium of the $Ctnnb1^{cKO}$ mutants (supplementary material Fig. S5B). These data imply that Bmp4 might be the key factor that induces corneal epithelial stratification upon β -catenin inactivation in stromal keratocytes.

To determine if exogenous Bmp4 could prompt corneal epithelial stratification *in vivo*, human recombinant BMP4 was injected into the subconjunctival space in C57BL/6J neonates every other day from P0 to P8 and corneal histology was examined at P10. As shown in Fig. 5, H&E staining as well as TEM images showed that addition of BMP4 resulted in an increase in the number of corneal epithelial cell layers compared with controls injected with PBS (Fig. 5E-H).

Next, we generated compound quadruple-transgenic mice, $KR; TC; Ctnnb1^{fl/fl}; Bmp4^{fl/fl}$ ($Bmp4^{cKO}; Ctnnb1^{cKO}$), to examine whether simultaneous deletion of both *Bmp4* and *Ctnnb1* in corneal stromal keratocytes could counteract the precocious epithelial stratification seen in $Ctnnb1^{cKO}$. As expected, the $Bmp4^{cKO}; Ctnnb1^{cKO}$ mouse showed normal corneal epithelium stratification (supplementary material Fig. S6). These *in vivo* and *in vitro* data indicate that Bmp4 is an important mediator of the Wnt/ β -catenin signaling pathway in the corneal stroma that is required for corneal epithelial cell stratification during corneal development. This also suggests that the signals that initiate corneal epithelial stratification might come from the corneal stroma.

Bmp4, as the direct effector of Wnt/ β -catenin signaling in the corneal stroma, triggers corneal epithelial stratification

To elucidate the molecular mechanism by which β -catenin signaling regulates Bmp4 expression in corneal stromal keratocytes, we performed a luciferase assay following transient co-transfection of the mouse *Bmp4* promoter (7.0 kb) and luciferase (*pGL3.-Bmp4pr-Luc*) plasmids into the HTK cell line. Our data showed inhibition of *Bmp4* promoter activity in the presence of β -catenin. *Bmp4* promoter activity was reduced to less than 10% when co-transfected

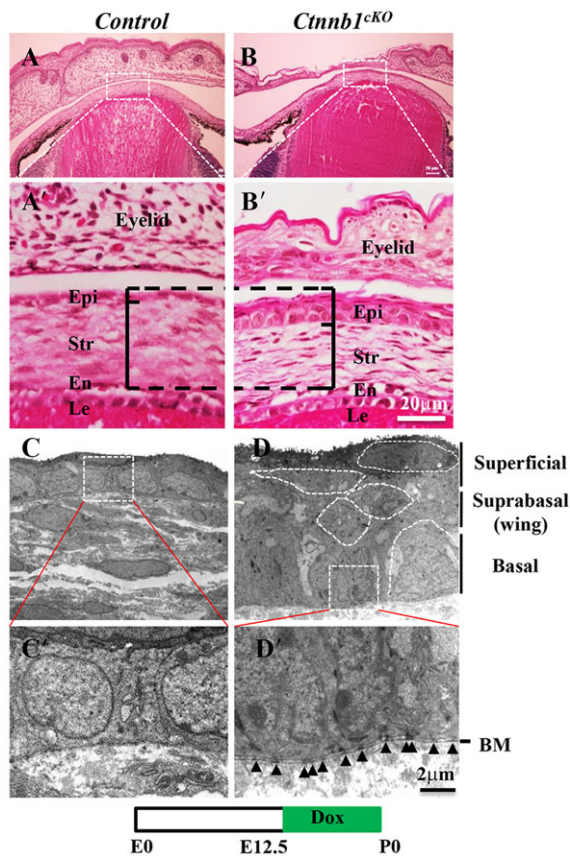


Fig. 3. *Ctnnb1* ablation in keratocytes results in precocious epithelial stratification at birth. (A-B') H&E staining revealed that, unlike the littermate controls which had 1-2 cell layers (A,A'), the mutant corneal epithelium had 4-5 cell layers (B,B') at birth following induction at E12.5. (C-D') TEM images showing a 1- to 2-cell-layer corneal epithelium without a clearly defined basement membrane in controls (C,C'). By contrast, the *Ctnnb1*^{CKO} mutant had multiple cell layers displaying distinct cell morphologies, i.e. basal columnar cells, suprabasal wing cells and flattened superficial cells (D). In addition, the *Ctnnb1*^{CKO} mutant had a clearly defined basement membrane containing hemi-desmosomes (arrowheads in D'), which were not apparent in the control.

with β -catenin and *pGL3.-Bmp4pr-Luc* (Fig. 6A). Next, NIH3T3 cells transfected with β -catenin and human TCF4 expression plasmids were used for ChIP analysis. Our data showed that the anti-TCF4 antibody was able to pull down the DNA sequence

corresponding to the TCF/LEF binding site of the mouse *Bmp4* promoter (Fig. 6B,C), suggesting that the complex formed by β -catenin/TCF/LEF binds to the mouse *Bmp4* promoter *in vivo*. Together, these data indicate that *Bmp4* expression is directly regulated by Wnt/ β -catenin signaling.

Bmp4, as a member of the TGF β superfamily, binds to *Bmpr1* and *Bmpr2*, transmitting signals via Smad and/or the MAP kinase pathways to effect target genes (Miyazono et al., 2010). Next, we attempted to determine which signaling pathway *Bmp4* signals through to regulate corneal epithelial stratification. HTCE cells were treated with human recombinant BMP4 followed by analysis by western blot and immunofluorescence staining. Western blot data clearly showed that the phosphorylation levels of both ERK1/2 (Mapk3/1 – Mouse Genome Informatics) and Smad1/5 were transiently increased at different time points after BMP4 protein treatment (Fig. 7A,B). ERK1/2 responded to BMP4 more rapidly than Smad1/5 (Fig. 7A-D), which is consistent with the results from immunofluorescence staining (Fig. 7E,F). Collectively, the *in vivo* and *in vitro* data indicated that, in the absence of β -catenin in the corneal stromal keratocytes, *Bmp4* suppression is lifted allowing it to serve as a paracrine factor triggering corneal epithelial stratification.

The activation of *Bmp4* signaling in the corneal stroma caused by deletion of β -catenin enhances p63 expression in corneal epithelial basal cells

It has been established that the transcription factor p63 (Trp63 – Mouse Genome Informatics) is essential for initiating epithelial stratification during cornea and skin development and for maintaining the proliferative potential of the basal layer keratinocytes (Koster et al., 2004; Ng et al., 2013; Zhu et al., 2014). Two main isoforms are encoded by the *p63* gene: TAp63 and Δ Np63 (Yang et al., 1998). To explore the potential involvement of p63 in the precocious corneal epithelial stratification observed in *Ctnnb1*^{CKO} mutants, immunofluorescence staining with antibody against the two isoforms of p63 was performed to detect *p63* expression, induced from E0 to E16.5. The data showed a concomitant increase in Smad1/5 and ERK1/2 phosphorylation, as well as an increase in p63-positive cells, in the mutant corneal epithelium when compared with littermate controls (Fig. 8G, also compare Fig. 8A,C,E with 8B,D,F and supplementary material Fig. S7A,C,E with S7B,D,F). Likewise, p63 expression was upregulated in mouse corneal basal epithelial cells subcutaneously injected with BMP4 protein (supplementary material Fig. S8A,B). Furthermore, Δ Np63 expression was

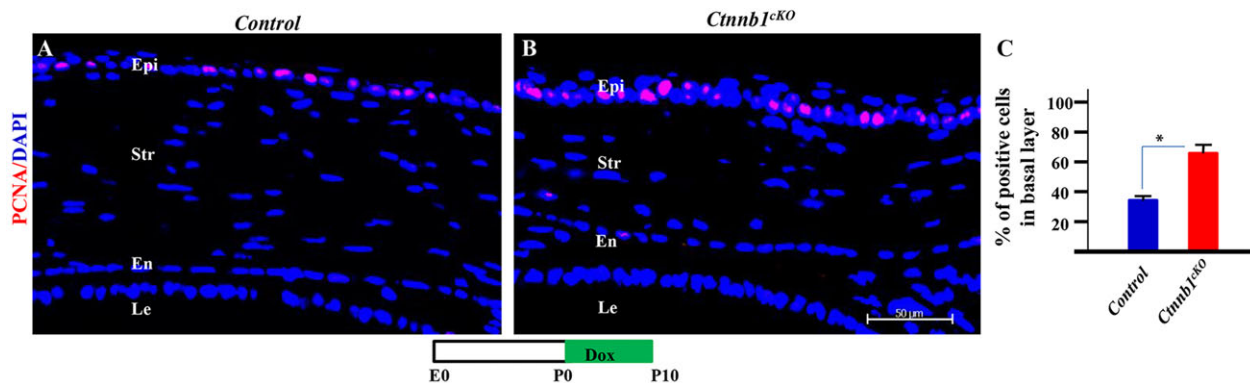


Fig. 4. *Ctnnb1* ablation in keratocytes enhances corneal epithelial cell proliferation. (A,B) Immunofluorescence micrographs of a P10 cornea probed with anti-Pcna showing that loss of *Ctnnb1* in stromal keratocytes increased epithelial cell proliferation. (C) Quantification of the number of Pcna-positive cells showed a significant increase in the *Ctnnb1*^{CKO} mutant. **P*<0.05. Mean \pm s.e.m.

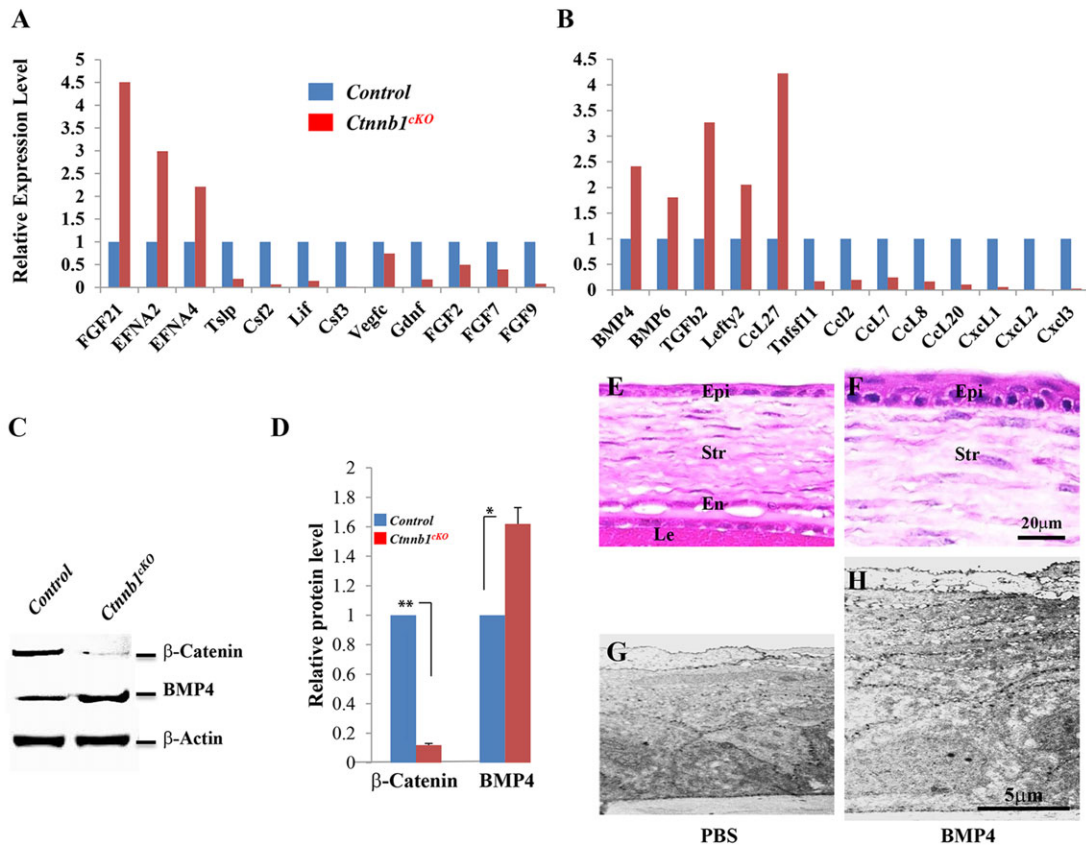


Fig. 5. Differential expression of growth factors/cytokines upon *Ctnnb1* ablation. (A,B) *Ad-Cre-GFP*-treated or *Ad-GFP*-treated primary cell cultures of stromal keratocytes isolated from *Ctnnb1^{fl/fl}* mice at P0 were screened using a mouse cytokine primer library. The *Ctnnb1^{cKO}* mutant showed changes in the expression of various cytokines and growth factors, including a 2.5-fold increase in *Bmp4*. (C) Western blotting analyses confirmed the increase in *Bmp4* after *Ctnnb1* ablation. β -actin provided a loading control. (D) Statistical analysis of western blot results showed that *Bmp4* expression was increased at the protein level. (E-H) Histological analysis of corneas from P10 mice treated with recombinant *Bmp4* (10 ng/ml) or PBS (control) every other day from P0 to P8. H&E staining showed that *Bmp4* administration results in an increase in the number of epithelial cells (3-4 cell layers; compare E with F). Likewise, TEM showed that *Bmp4* administration resulted in a much thicker epithelium (H) than that of the control (G). * $P < 0.05$, ** $P < 0.01$. Mean \pm s.e.m.

significantly elevated in HTCE cells treated with different dosages of *Bmp4* protein (supplementary material Fig. S8C,D). These data suggested that lifting the suppression of *Bmp4* signaling in *Ctnnb1^{cKO}* mice resulted in upregulation of p63 expression, which promoted corneal epithelial stratification.

Wnt/ β -catenin signaling in keratocytes negatively regulates *Bmp4* signaling in the epithelium during corneal development

It is well documented that β -catenin is the core mediator of the canonical Wnt signaling pathway, while *Lrp5* and *Lrp6* are the

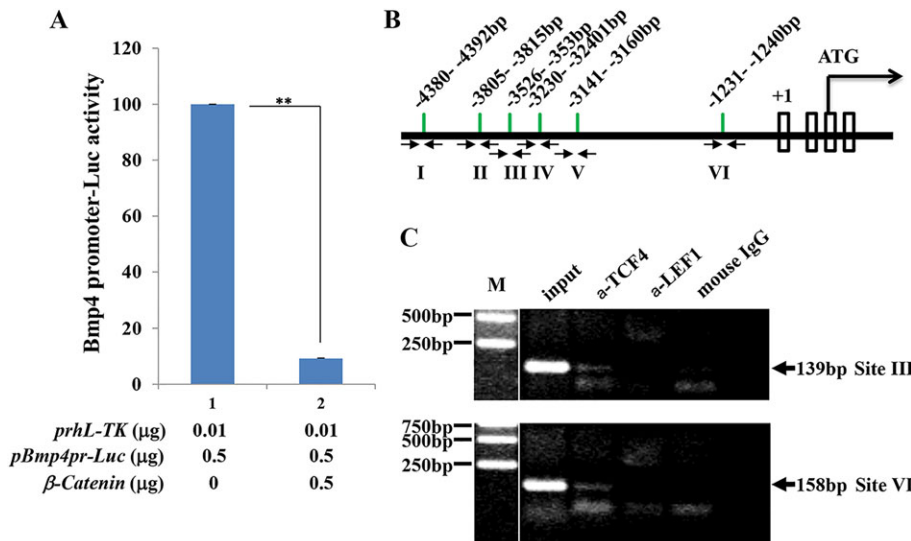


Fig. 6. β -catenin binds and suppresses *Bmp4* promoter activity *in vitro*. (A) β -catenin inhibits *Bmp4* promoter activity. A luciferase assay was performed by co-transfecting HTK cells with the plasmids indicated. The presence of β -catenin resulted in a 90% decrease in *Bmp4* promoter activity. (B) Potential TCF/LEF binding sites (I to VI) in relation to the transcription start site (+1) are indicated within the 5 kb 5' flanking regulatory region of mouse *Bmp4*. Arrows show the location of PCR primers used in the ChIP assay. (C) ChIP analysis of TCF/LEF binding to the mouse *Bmp4* 5' flanking region. Note that TCF4 binds to two of the six potential sites (III and VI), while neither LEF1 nor mouse IgG bound to any of the sites in the *Bmp4* 5' flanking regulatory region. M, DNA size marker. ** $P < 0.01$. Mean \pm s.e.m.

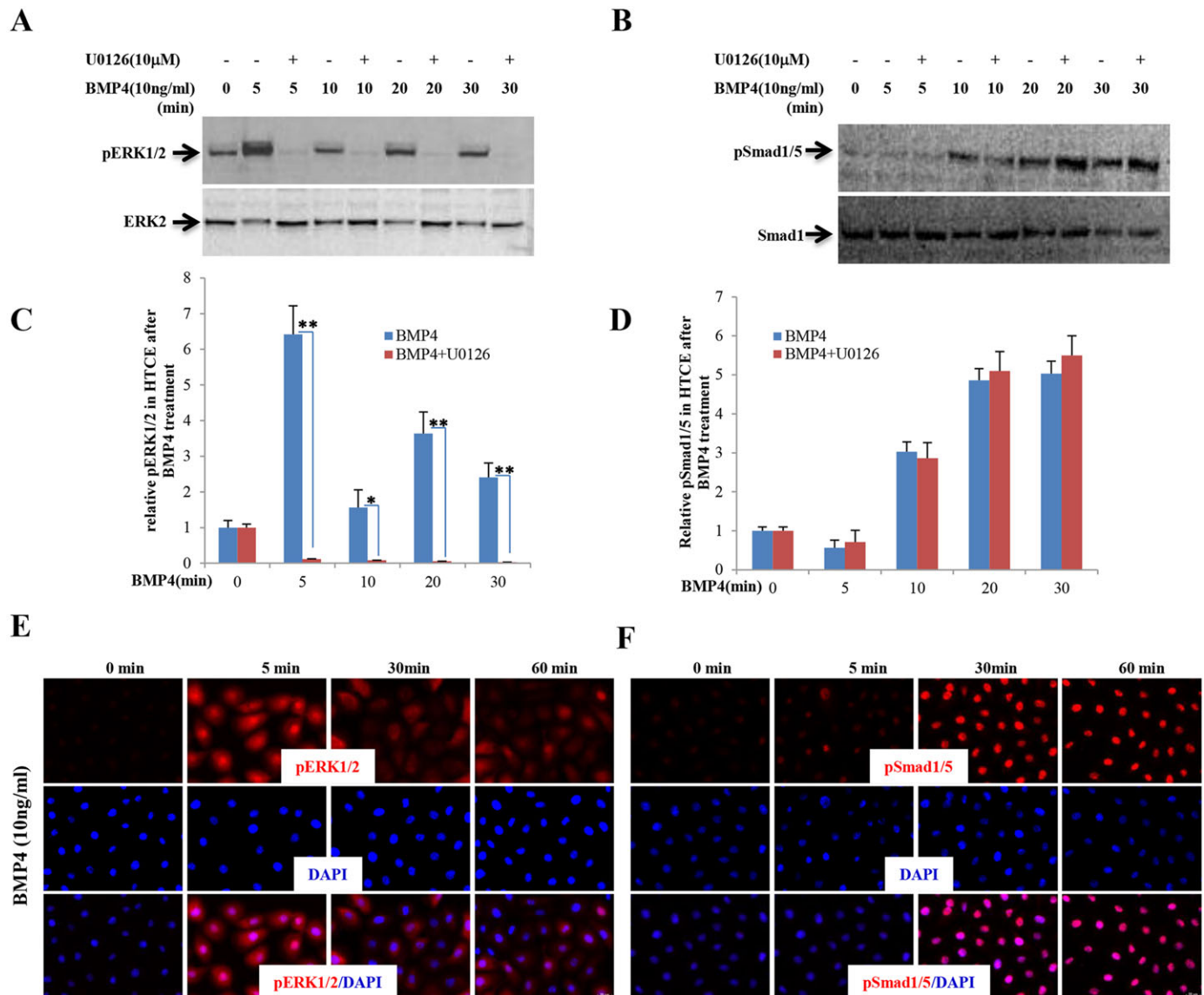


Fig. 7. BMP4 triggers ERK1/2 and Smad1/5 phosphorylation in HTCE cells. (A,B) Dynamic changes in phosphorylation of ERK1/2 and Smad1/5 in HTCE cells after BMP4 treatment were confirmed by western blot. ERK1/2 responded to BMP4 treatment within 5 min and then became dephosphorylated, whereas Smad1/5 phosphorylation began after 10 min treatment. The specific MEK inhibitor U0126 impeded ERK1/2 phosphorylation but had no effect on Smad1/5 phosphorylation. (C,D) Statistical analysis showing the ratios of phosphorylated versus non-phosphorylated ERK1/2 and Smad1/5. (E,F) Immunocytofluorescence staining confirmed the dynamic change in phosphorylation of ERK1/2 and Smad1/5 in HTCE cells triggered by BMP4. * $P < 0.05$, ** $P < 0.01$. Mean \pm s.e.m.

co-receptors of Wnt ligands. Therefore, knockout of either β -catenin or *Lrp5/Lrp6* will block the Wnt signaling pathway. We asked whether the phenotype observed in the *Ctnnb1^{CKO}* line could be recapitulated in mice lacking *Lrp5/Lrp6* in the stromal keratocytes. *KR; TC; Lrp5^{f/f}; Lrp6^{f/f}* transgenic mice were generated by natural mating between *KR, TC, Lrp5^{f/f}* and *Lrp6^{f/f}* mice (Fig. 9A). No overt phenotype was observed in the *Lrp5* or *Lrp6* single-knockout mice (data not shown); however, loss of both *Lrp5* and *Lrp6* resulted in a phenotype similar to that observed in the *Ctnnb1^{CKO}* mutant (compare Fig. 9C with 9B). These data suggest that the canonical Wnt/ β -catenin signaling pathway in cornea stromal keratocytes plays a pivotal role in triggering corneal epithelial stratification.

DISCUSSION

Our results provide, for the first time, direct genetic evidence that Wnt/ β -catenin signaling in stromal keratocytes regulates corneal

epithelial stratification via the *Bmp4*→*p63* axis. Using compound transgenic mouse models we demonstrated that inactivation of the canonical Wnt signaling pathway in cornea stromal keratocytes via conditional disruption of the key mediator β -catenin or of co-receptors *Lrp5/6* of the Wnt signaling pathway can lift the repression of *Bmp4* in a naive situation. Following derepression, *Bmp4* then acts as a paracrine growth factor triggering corneal epithelial stratification through upregulation of transcription factor *p63* expression. These data present an excellent example of the coordinated interaction of the stroma and epithelium by means of cross-talk between the Wnt/ β -catenin and *Bmp4* signaling pathways, which is essential for corneal epithelial maturation.

Multiple components of the canonical Wnt signaling pathway are expressed in the developing ocular surface (Ang et al., 2004; Borello et al., 1999; Liu et al., 2003); however, in the cornea the exact components of this pathway are not well characterized. In this

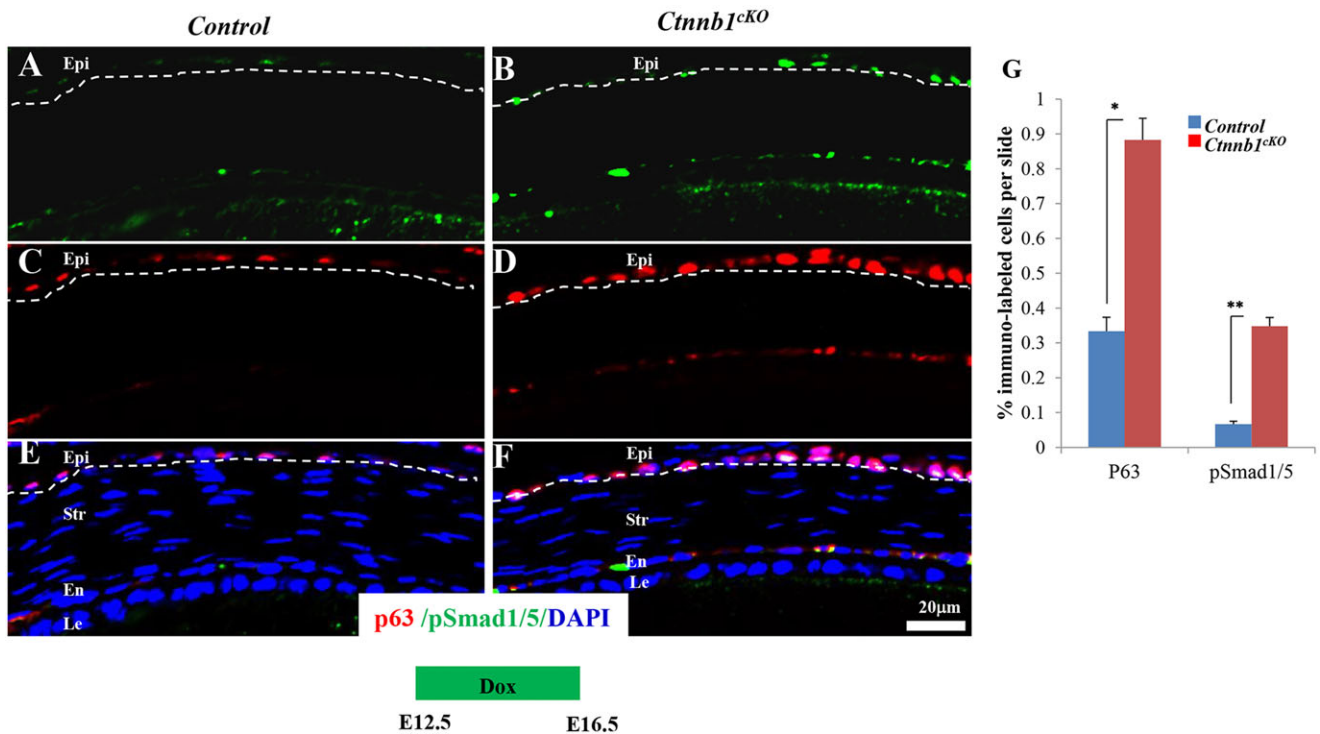


Fig. 8. *Ctnnb1* ablation in keratocytes results in Smad1/5 phosphorylation and enhances p63 expression. (A-F) Immunofluorescence staining showed that at E16.5 more p63-labeled basal cells and more pSmad1/5-labeled basal cells were detected in *Ctnnb1^{cKO}* mutant corneal epithelium than in the littermate controls. Note that p63 expression was increased in the mutant and that this colocalized with Smad1/5 phosphorylation. The dashed line delineates the corneal epithelium layer. (G) Statistical analysis of the immunofluorescence staining showed that Smad1/5 phosphorylation and p63 expression were upregulated in the mutant. * $P < 0.05$, ** $P < 0.01$. Mean \pm s.e.m.

study, we found that canonical Wnt signaling is dynamically decreased in the cornea stroma from P1 to P21, as revealed by whole-mount X-gal staining of the *Axin2^{lacZ}* mouse cornea and immunofluorescence staining for the active form of β -catenin. This dynamic change in activity is in agreement with the notion that canonical Wnt signaling must be repressed in the cornea for proper development, as reported for the *Dkk2* knockout mouse (Mukhopadhyay et al., 2006) and in *Pitx2*-deficient mice (Gage

et al., 2008). In addition, it has been shown that ectopic expression of the stable form of β -catenin in the differentiated cornea epithelial cells using *Krt12 rtTA* driver mice leads to corneal intraepithelial neoplasia (Zhang et al., 2010). In the current study, loss of β -catenin in the cornea stroma resulted in precocious epithelial stratification. Furthermore, canonical Wnt/ β -catenin signaling activity forms a gradient from the peripheral limbal region to the central cornea (supplementary material Fig. S1). This suggests that canonical Wnt/

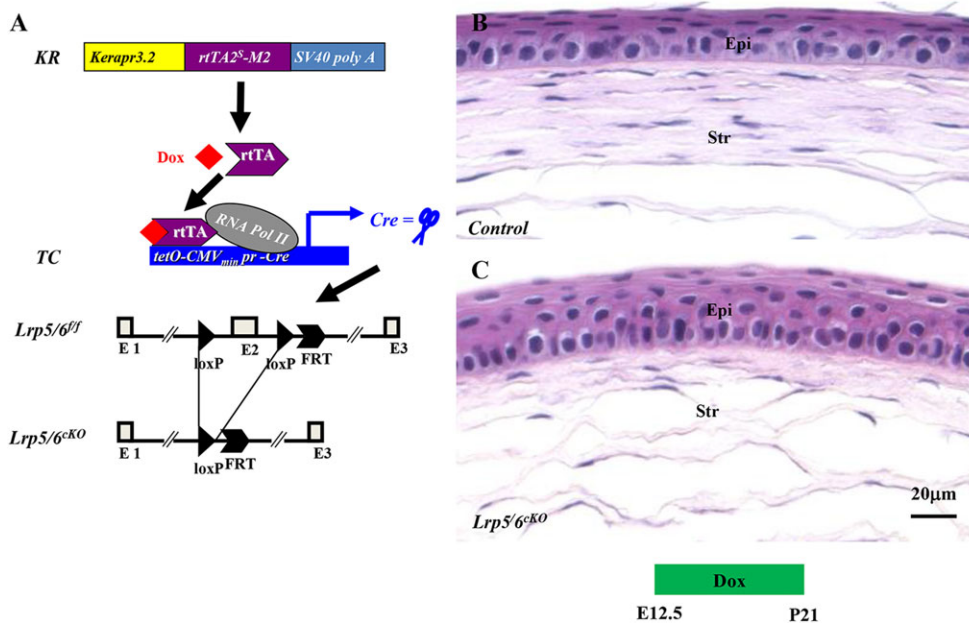


Fig. 9. Deletion of *Lrp5* and *Lrp6* in stromal keratocytes recapitulates the *Ctnnb1^{cKO}* phenotype. (A) Schematic representation of Dox-dependent deletion of *Lrp5* and *Lrp6* in stromal keratocytes via the Cre-loxP system. (B, C) A thicker epithelium is observed in *Lrp5^{cKO}* and *Lrp6^{cKO}* (*Lrp5/6^{cKO}*) double mutants (C) than in littermate controls (B).

β -catenin signaling has to be precisely and stringently controlled in the stroma for corneal epithelial stratification.

Our current focus considers the canonical Wnt signaling pathway and its regulation in the corneal stroma during development. A variety of secreted Wnt inhibitors, including Dkk2, have been identified that regulate Wnt signaling either by binding to Wnt ligands or to their receptors (Hsieh et al., 1999; Mao and Niehrs, 2003; Rattner et al., 1997). It has previously been reported that Dkk2 expression in the corneal and limbal epithelium is required to control Wnt activity in the stroma, which contributes to the formation of the corneal epithelium (Mukhopadhyay et al., 2006). Based on all these observations, we propose that Dkk2 in the corneal epithelium may modulate the activity of Wnt/ β -catenin signaling in the stroma, which is essential for releasing the inhibition of Bmp4 expression. Bmp4, in turn, prompts corneal epithelial stratification through p63. Therefore, the signaling communication involved in corneal epithelial stratification via the Wnt signaling pathway may follow 'epithelium-stroma-epithelium' interaction. This will be explored in the future via *in vitro* and *in vivo* experiments that include utilizing compound transgenic mouse models to modify the expression of Wnt signaling inhibitors, such as Dkk2, in the corneal epithelium.

The Wnt signaling pathway is essential for the regulation of multiple processes during eye development, such as tissue specification, morphogenetic movement, cellular proliferation and differentiation (Fuhrmann, 2008). Most of the previous genetic studies examining the role of Wnt/ β -catenin signaling in the cornea have been carried out in the mouse corneal epithelium (Chikama et al., 2008; Mukhopadhyay et al., 2006; Zacharias and Gage, 2010; Zhang et al., 2010). We took advantage of a unique transgenic mouse driver line, *KR* [in which a keratocan gene regulatory cassette drives a mutant reverse tetracycline transactivator minigene (*rtTA*)], to delete β -catenin and/or Lrp5/6 specifically in corneal stromal keratocytes and, surprisingly, observed a phenotype: mutant neonates exhibited a thicker corneal epithelium. We also noticed an anomaly in the mutant eyelid (Fig. 3B) that caused eyelid opening at P1 to P5 (data not shown). Earlier eyelid opening in the neonatal mutant is unlikely to play a role in the formation of the thicker corneal epithelium because we observed a similar corneal epithelium phenotype in mutant mice when induced after eyelid fusion (Fig. 2; supplementary material Fig. S2).

In our cytokine cDNA library screening experiment, in addition to the upregulation of Bmp4, we observed simultaneous trends in the upregulation of cytokines and growth factors, such as fibroblast growth factor 21 (Fgf21), chemokine (c-c motif) ligand 27 (Ccl27), ephrin A2 (Efna2) and ephrin A4 (Efna4). Fgf21 acts as a metabolic regulator and plays a key role as an anti-diabetic and anti-obesity agent (Kharitonov et al., 2005; Woo et al., 2013). Ccl27 plays a role in T cell-mediated inflammation in the skin (Homey et al., 2002). Based on their function, it is unlikely that Fgf21 and Ccl27 are directly involved in the precocious corneal epithelial stratification seen in the absence of β -catenin. Nonetheless, Wnt signaling is essential for neural development and corneal nerve fibers exert important trophic influences on the corneal epithelium (Ille and Sommer, 2005). Efna2 and Efna4, which are membrane-bound proteins, play a crucial role during the development of the nervous system (Flanagan and Vanderhaeghen, 1998). The upregulation of Efna2 and Efna4 in the β -catenin mutant cells might not be related to corneal epithelial stratification. Among all of the upregulated cytokines in the β -catenin mutant, only Bmp4 has previously been reported to be a downstream target of Wnt/ β -catenin signaling (Baker et al., 1999; Fujimori et al., 2010). Therefore, we addressed the possible involvement of Bmp4 in the observed phenotype and found that Bmp4 indeed plays an important role in corneal epithelium development. Nevertheless, further in-depth investigation will be required to establish whether any of the additional factors mentioned above, which are impacted in transcriptional regulation by β -catenin, has any precise function in corneal development.

Our data from *in vitro* and *in vivo* experiments suggest that the change in Bmp4 expression in β -catenin mutant mice may be the consequence of loss of Wnt/ β -catenin signaling. This implies that during corneal development communication between the stroma and the epithelium is essential for the initiation of corneal epithelial stratification. We propose a model whereby cross-talk between the Wnt/ β -catenin and Bmp4 signaling pathways in the stroma and epithelium is essential for mouse corneal development (Fig. 10). Under normal developmental conditions, Wnt/ β -catenin signaling is active in the cornea stromal keratocytes at early developmental stages and this is responsible for suppressing Bmp4 expression in the keratocytes. Before eyelid opening, Wnt/ β -catenin signaling is

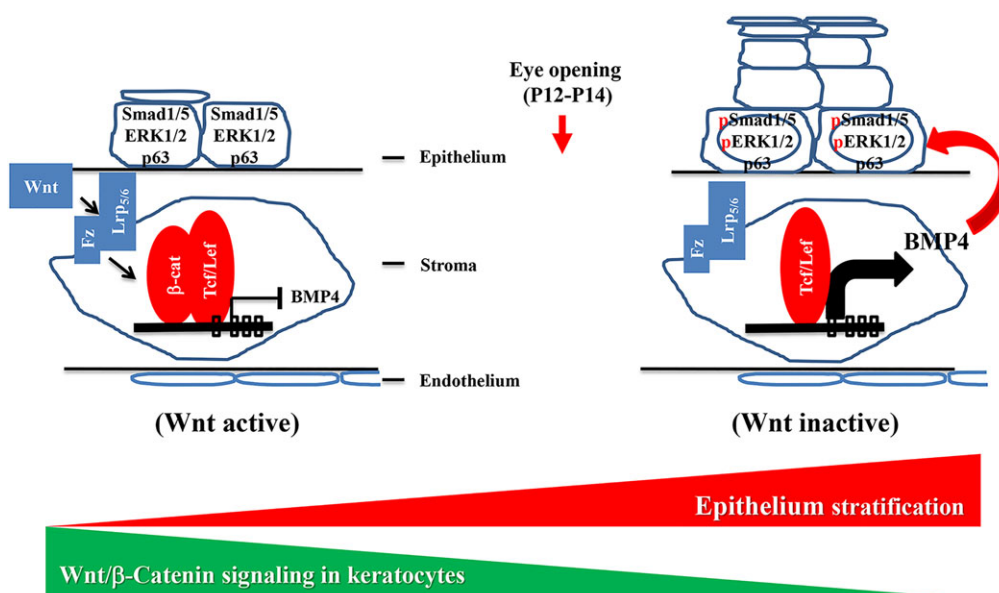


Fig. 10. Model of the interaction between corneal stroma (canonical Wnt signaling) and epithelium (Bmp4 signaling) during mouse corneal development. Before stratification (left), Wnt/ β -catenin signaling is active, which suppresses Bmp4 expression. Before eyelid opening (right), the Wnt/ β -catenin signaling pathway is downregulated leading to the relief of Bmp4 suppression. Bmp4 then triggers epithelial cell stratification via p63. Fz, Frizzled.

gradually inactivated by some unknown mechanism, which results in lifting of the inhibition and an increase in *Bmp4* expression. *Bmp4*, as a paracrine growth factor, then stimulates p63 expression in the corneal epithelium, which initiates corneal epithelial stratification.

MATERIALS AND METHODS

Mouse strains and genotyping

The genetically modified mouse lines *KR* (keratocan promoter-driven rTA; Zhang et al., 2011), *tetO-Cre* (Perl et al., 2002), *Ctnnb1^{fllox}* (Brault et al., 2001), *Ctnnb1^{flloxE3}* (Harada et al., 1999), *Bmp4^{fllox}* (Liu et al., 2004), *Axin2^{lacZ}* (Lustig et al., 2002), *Lrp5^{fllox}* (Joeng et al., 2011) and *Lrp6^{fllox}* (Joeng et al., 2011) have been described previously. Compound transgenic mice were generated via natural mating. All mice were bred at the Animal Facility of the University of Cincinnati Medical Center. Experimental procedures for handling mice were approved by the Institutional Animal Care and Use Committee, University of Cincinnati/College of Medicine. Transgene alleles were identified by PCR genotyping with tail DNA using specific primer pairs (supplementary material Table S1).

Administration of doxycycline and recombinant BMP4 protein

To induce the conditional knockout of *Ctnnb1*, *Lrp5/6*, *Bmp4* or ectopic expression of *Ctnnb1^{cGOF}* in mice from embryonic day (E) 12.5 to various developmental stages, including E16.5, postnatal day (P) 0, P10 and P21, pregnant dams were given an intraperitoneal (IP) injection of doxycycline [Dox; 80 µg/g body weight in 1× phosphate buffered saline (PBS), pH 7.4; Clontech Laboratories] followed by administration of Dox chow (Dox diet #AD3008S, Custom Animal Diets, Bangor, PA, USA) *ad libitum*. Neonatal mice at birth were induced through milk-nursing following a Dox IP injection of the mother and feeding with Dox chow *ad libitum*. Control animals were littermates with either single or double transgene(s).

To administer BMP4 protein, C57BL/6J neonates were given a subconjunctival injection with human recombinant BMP4 protein (10 ng/ml in PBS, 20 µl per eye; R&D Systems, 314-BP/CF) every 48 h from P0 to P8. Mice were sacrificed at P10 and the enucleated eyeballs were subjected to immunohistochemical examination.

X-gal staining for detection of β-galactosidase activity

Enucleated eyes were fixed in 4% paraformaldehyde in 1× PBS pH 7.4 (PFA/PBS) for 30 min at 4°C followed by incubation in X-gal staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl₂, 0.02% NP-40, 0.01% sodium deoxycholate, 0.4 mg/ml X-gal in PBS) overnight at room temperature. The eyes were postfixed in 4% PFA/PBS overnight at 4°C and examined by microscopy as whole-mount tissue or paraffin sections.

Histological analysis

Mouse samples were fixed overnight in 4% PFA/PBS, followed by paraffin embedding. Deparaffinized sections (5 µm) were stained with Hematoxylin and Eosin (H&E).

Transmission electron microscopy (TEM)

Mouse cornea samples were fixed in 0.1 M cacodylate buffer (pH 7.4) containing 3% glutaraldehyde and 2% paraformaldehyde for 2 h at 4°C and then preserved in 0.1 M cacodylate buffer (pH 7.4) containing 0.5% glutaraldehyde at 4°C overnight. After refixation in 1% osmium tetroxide for 1 h at 4°C, cornea samples were washed in 0.1 M cacodylate buffer (pH 7.4) for 10 min three times, and then dehydrated in a graded ethanol series and embedded in Epon 812 epoxy resin (Electron Microscopy Sciences, #14120). Ultrathin 50 nm sections were stained with uranyl acetate and lead citrate and images were photographed with a Hitachi 7500 transmission electron microscope equipped with an AMT digital camera.

Mouse corneal stroma fibroblast primary cell culture

Corneal epithelium and Descemet's membrane were removed from the cornea of newborn mice *Ctnnb1^{fllox/fllox}* (*Ctnnb1^{fl/fl}*) with a scalpel blade. The

remaining stromal tissue was digested overnight with 2.0 mg/ml collagenase and 0.5 mg/ml hyaluronidase in DMEM (Gibco-BRL) at 37°C. Isolated cells were washed in DMEM and cultured in DMEM supplemented with 10% fetal calf serum (Gibco-BRL) and 1% penicillin-streptomycin (Gibco-BRL) for 48 h, and then replaced with DMEM containing 10% fetal bovine serum without antibiotics.

Immunohistofluorescence staining

Tissue sections (5 µm) were deparaffinized, rehydrated in a graded ethanol series and subjected to antigen retrieval in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) with boiling for 30 min. Corneal sections were then blocked with 3% bovine serum albumin in PBS containing 0.05% NP-40 for 1 h at room temperature, then incubated overnight at 4°C with primary antibodies diluted in the same buffer. After three washes in PBS containing 0.1% Tween 20 (PBST), slides were incubated at room temperature for 1 h with Alexa Fluor 488- and/or Alexa Fluor 555-conjugated secondary antibodies (Life Technologies) and 1 µg/ml DAPI as a nuclear counterstain. Following incubation, the slides were washed with PBST and mounted with Mowiol (Sanofi-Aventis). Sections were examined and photographed using a Zeiss Axio Observer Z1 microscope equipped with an AxioCam Mrm camera. For data acquisition, we used Axiovision 4.6 software (Zeiss). Antibodies used are listed in supplementary material Table S2.

Western blotting analysis

Human HTCE cells, human HTK immortalized cells (Jester et al., 2003) or corneal stroma fibroblast primary cells were lysed in lysis buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% deoxycholate, 1× protease inhibitor cocktail (Sigma P8340)]. Frozen tissue was homogenized in RIPA buffer containing phosphatase and protease inhibitors. Protein lysates (20 µg) from each sample were separated on a 4–20% linear gradient Tris-HCl denaturing polyacrylamide Ready Gel (Bio-Rad) and transferred to PVDF membrane (Whatman). Primary antibodies were incubated in 5% nonfat dried milk in TBST (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20) at 4°C overnight. After three washes in PBST, Alexa Fluor 488- and/or Alexa Fluor 555-conjugated secondary antibodies (Life Technologies) were incubated at room temperature for 1 h. Following three washes in PBST, samples were examined and photographed using a VersaDoc 4000MP imaging system (Bio-Rad). Antibodies are listed in supplementary material Table S2.

Cytokine expression screening and quantitative real-time PCR (qRT-PCR)

Mouse corneal stroma fibroblast primary cells were infected with adenovirus *Ad-Cre-GFP* for 4 days to delete the *Ctnnb1* gene. Cells were infected with Ad-GFP as a control. Total RNA (10 µg) was isolated from virus-infected cells using TRIzol RNA isolation reagents (Life Technologies) then annealed to random primers and reverse transcribed with an avian reverse transcriptase (RT) kit (Promega) according to the manufacturer's instructions. cDNA was subjected to RT-PCR-based gene expression screening using a mouse cytokine primer library (RealTimePrimers, #MCA-II). qRT-PCR was performed using the CFX96 real-time system on a C1000 thermal cycler (Bio-Rad). After the initial step at 95°C for 3 min, 40 cycles were performed at 95°C for 15 s, 62°C for 15 s, 72°C for 20 s. The cycle threshold values were used to calculate the normalized expression of genes of interest against *Gapdh* using QGene software. RT-qPCR primer sets are listed in supplementary material Table S3.

Chromatin immunoprecipitation (ChIP) assay

Murine NIH3T3 cells (ATCC® CRL-1658™) were co-transfected with 20 µg *pcDNA3-β-catenin* plasmid (Kolligs et al., 1999) and *pcDNA/Myc-TCF4* (Korinek et al., 1997) using GeneJammer transfection reagent (Invitrogen). 48 h post-transfection, cells were cross-fixed with 1% formaldehyde at 37°C for 10 min and subjected to ChIP assay with antibodies against TCF4 (Cell Signaling, 2569) using the ChIP Assay Kit (Millipore, 17-295) following the manufacturer's instructions. Purified DNA post-ChIP was used as the template in PCR to verify the interaction

between DNA and protein. Primer sets are listed in supplementary material Table S3.

Promoter-luciferase assay

HTK cells seeded in six-well plates at 80% confluence were transiently transfected using GeneJammer transfection reagent with a mixture of three different plasmids: (1) *prhL-TK* (Promega), which served as the internal control; (2) *pGL3.0-Basic* vector (Promega) or recombinant plasmid harboring the mouse *Bmp4* promoter *pGL3.0-mBmp4pr* (Yasunaga et al., 2011); (3) the cDNA expression plasmid *pcDNA3-β-catenin* (Kolligs et al., 1999) or *pcDNA/Myc-TCF4* (Korinek et al., 1997). 48 h post-transfection, cells were collected in 1× passive lysis buffer (Promega). The luciferase assay was conducted using the Dual Luciferase Reporter (DLR) Assay System (Promega) according to the manufacturer's recommendations. Luminescence was measured using a Glomax multidetection system (Promega).

Statistical analysis

Two-tailed Student's *t*-test was performed in Excel (Microsoft). *P*<0.05 was considered statistically significant. All quantification data are presented as mean±s.e.m.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Y.Z. and C.-Y.L. designed the study and wrote the manuscript. Y.Z. and S.Z. performed most of the *in vivo* experiments and data analysis. L.-K.Y. prepared the TEM images. Y.Y., M.C. and M.Y. performed the *in vitro* experiments. W.W.-Y.K. modified the manuscript.

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Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.125393/-/DC1>

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