Disruption of Mouse Corneal Epithelial Differentiation by Conditional Inactivation of Pnn

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Purpose. To investigate the specific role of Pinin (Pnn) in the development of anterior eye segment in mice.

Methods. Conditional inactivation of Pnn in the developing surface eye ectoderm and lens was achieved by creating mice carrying a Pnn null and a floxed Pnn allele as well as a Pax6-Cre-GFP (Le-Cre) transgene. The resultant Pnn conditional knockout mice were examined by histologic and immuno-histologic approaches.

Results. Pax6-Cre-mediated deletion of Pnn resulted in severe malformation of lens placode-derived tissues including cornea and lens. Pnn mutant corneal epithelium displayed the loss of corneal epithelial identity and appeared epidermis-like, down-regulating corneal keratins (K12) and ectopically expressing epidermal keratins (K10 and K14). This squamous metaplasia of Pnn mutant corneal epithelium closely correlated with significantly elevated β-catenin activity and Tcf4 level. In addition, Pnn inactivation also led to misregulated level of p68 RNA helicase in mutant corneal epithelium.

Conclusions. These data indicate that Pnn plays an essential role in modulating and/or orchestrating the activities of major developmental factors of anterior eye segments. (Invest Ophthalmol Vis Sci. 2010;51:1927–1934) DOI:10.1167/iosv.09-4591

A smooth, intact, and healthy corneal epithelium is requisite for normal vision. The stratified squamous non-keratinizing epithelium of the cornea accounts for approximately 80% of the refractive power of the anterior eye. In addition, this epithelium is at the interface with the external environment; thus, it must resist pressure and provide a barrier to fluid loss and pathogen entrance, all while maintaining maximal transparency.

The specific identity and differentiated qualities of the corneal epithelium afford many properties that are requisite for its role at the anterior surface of the eye. Indeed, there exist numerous examples of ocular surface diseases whereby the corneal epithelial quality or differentiative qualities are lost, and significant anterior eye physiological perturbations and dramatic vision loss result. Many of these pathologic states result in the transformation of the bone fide corneal epithelium to a keratinized epithelium, which is a vastly inadequate epithelium for the maintenance of the stable tear film, corneal surface barrier, and avascular corneal stroma. Thus, elucidation of the molecular details pertaining to establishment and maintenance of the corneal epithelia is central to the design of new therapies to impact the maintenance and repair of the corneal epithelial phenotype and the corneal epithelial barrier.

It is now becoming apparent that the maintenance of the specific cell phenotype and physiology of the anterior eye remains dependent on sustained regulation of some of the same molecular pathways and mechanisms that play such important roles in development. However, a relatively small number of genes encoding transcription factors have been shown to be central to the development of the anterior eye. Yet, these transcription factors and/or cofactors need to coordinate an incredibly complex array of interactions and communications between the tissues comprising the anterior eye. One most extensively studied gene, Pax6 (paired box homeotic gene 6) is expressed in all cell types of anterior eye lineage and clearly central to the timing and coordination of events during the formation of the anterior eye. Additionally, in recent years, substantial effort has been made in a search for other crucial factors of anterior eye development. These studies significantly advanced our knowledge on the progression of anterior eye diseases, such as squamous metaplasia, as well as on the differentiation of ocular surface ectoderm. However, it is still most clear that further identification of additional elements that are essential for providing tight and precise regulation during eye development will ensure our best chance to develop the most effective therapies for such devastating eye diseases.

Pinin (Pnn/DRS/mema), a 140 kDa phosphoprotein associated with and localized in the nuclear speckles, has been shown to participate in the transcriptional regulation and RNA processing through its physical interaction with many transcriptional and splicing factors. We have previously shown that Pnn is essential for sustaining cell-cell adhesion and maintaining epithelia. The expression of exogenous Pnn in transformed cells drove these cells to the epithelial phenotype and reversed anchorage independent growth, while interfering with Pnn (eg, siRNA) in epithelial cell cultures caused the cells to disassociate and change growth qualities. Interestingly, Pnn hypomorphic mice (that exhibit general 90% knockdown in Pnn, resulting from inclusion of neo-cassette) displayed perinatal mortality with a wide range of developmental defects, including cardiac outflow tract and craniofacial malformation, and anterior eye dysgenesis (JHJ, unpublished data, 2007). Our most recent data indicate that Pnn plays a crucial role in the establishment and maintenance of epithelia throughout the body (JHJ, unpublished data, 2009). In vivo conditional inactivation of Pnn resulted in dramatic phenotypes in pulmonary and small intestinal epithelium, where viability and growth properties of the mutant epithelial cells were similar to controls, yet epithelial differentiation and morphogenetic programs were dramatically altered. These studies revealed Pnn to be involved with the maintenance of the epithelial differentiation and the specific epithelial phenotype.
Here we report the Pax6-Cre (Le-Cre)-mediated deletion of Pnn in mice. The specific loss of Pnn in the ocular surface ectoderm resulted in severe malformation of lens placode-derived tissues including cornea and lens. Most interestingly, deletion of Pnn in the corneal epithelium resulted in the loss of corneal epithelial identity. The mutant corneal epithelia appeared epidermis-like, downregulated corneal keratins (K12), and turned on epidermal keratins (K10 and K14). This metaplasia of Pnn mutant corneal epithelium closely correlated with elevated β-catenin activity and misregulated p68 levels. Together, these data indicate that Pnn is essential for modulating and/or orchestrating the activities of major developmental factors of anterior eye segments.

**MATERIALS AND METHODS**

**Experimental Animals**

Specific methods for the generation of Pnn-floxed conditional mice were previously reported.\(^{19}\) Pnn conditional allele (2f) contains two loxP sites flanking exons 3 to 8 and Pnn knock out allele (1f) contains one remaining loxP site with exons 3 to 8 deleted. The Pax6-Cre (Le-Cre) mouse line was kindly provided by Ed Levine (University of Utah) and Peter Gruss (Max-Planck Institute of Biophysical Chemistry). For timed matings, the presence of a vaginal plug was checked in the morning (Embryonic day 0.5; E0.5). All mice were housed in pathogen-free conditions, and animal procedures were adherent to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the Institutional Animal Care and Use Committee at University of Florida.

**Histology, Immunohistochemistry, and β-galactosidase Staining**

Histologic and immunohistochemical analyses were performed by standard methods as previously described.\(^{19,21}\) Commercially available antibodies used for immunostaining were goat polyclonal keratin 12 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); p68 (Abcam, Cambridge, MA); rabbit polyclonal keratin 10, Pax6 (Covance, Emeryville, CA); β-catenin (Cell Signaling, Danvers, MA); rabbit monoclonal Tcf4 (Cell Signaling); mouse monoclonal keratin 14 (Biocare Medical, Concord, CA); and active β-catenin (Millipore Co., Billerica, MA). Whole-mount β-galactosidase staining of embryonic intestines was performed as previously described\(^{19}\) with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Fisher, Pittsburgh, PA).

**Semi-quantitative RT-PCR**

Total RNA was isolated from corneas of postnatal day (PN)21 mice with reagent (Trizol; Invitrogen Co., Carlsbad, CA) and treated with RNa-se free DNase I. 1 µg of total RNA was reverse transcribed (SuperScript III First-Strand Synthesis kit; Invitrogen Co.) using oligo-dT primers. Subsequent PCR steps were performed (GoTaq Flexi DNA Polymerase; Promega Co., Madison, WI) according to manufacturer’s specifications. Primer sequences and PCR conditions are available on request.

**Brdu Incorporation and TUNEL Assay**

To detect cells in S-phase of mitosis, timed-pregnant females were injected intraperitoneally with BrdU (30 µg/gm body weight) in sterile PBS. Two hours after injection, female mice were killed and embryos were collected. BrdU detection was carried out (Vectastain Elite ABC anti-rat Kit; Vector Laboratories, Burlingame, CA) and rat anti-BrdU monoclonal antibody (Accurate Chemicals, Westbury, NY). TUNEL assay was performed (In Situ Cell Death Detection Kit; Roche, Indianapolis, IN) as per manufacturer’s protocol.

**RESULTS**

**Disruption in Anterior Eye Segment Development in Pnn Mutant Mice**

Conditional inactivation of Pnn in the developing presumptive anterior eye tissues, including surface eye ectoderm and lens, was achieved by creating mice carrying a Pnn null (1f) and a floxed Pnn alleles (2f),\(^{19}\) as well as a Pax6-Cre-GFP (Le-Cre) transgene.\(^{27}\) The resultant Pnn conditional knockout mice (Pax6-Cre;Pnn\(^{2f/1f}\)) hereafter termed Pnn mutant) were viable and exhibited comparable body size and weight to the control.
littermates until the weaning age (3 weeks). As shown in Figures 1A–1C, successful Cre-mediated recombination in lens placode derivatives was confirmed by a Cre-dependent lacZ reporter transgene (R26R). Semi-quantitative RT-PCR assays demonstrated decreased level of Pnn transcripts in mutant eyes (Fig. 1D). During embryonic eye development, Pnn mutant displayed severe corneal disorganization and lens degeneration (Figs. 1E–1J). All mutant eyes were microphthalmic and displayed irregular shape of pupil (Fig. 1F). Histologic analyses of embryonic day (E)14.5 mutant eyes revealed increased cellularity and uncharacteristic cellular arrangement in mutant corneal stroma (Figs. 1H, 1I). Mutant eyes also showed abnormal accumulation of mesenchymal and vascular cells in the posterior area of the cornea (Fig. 1J). While these data clearly demonstrate that Pnn is indispensable for corneal development, there was no considerable increase in apoptotic cell death or obvious decrease in cell proliferation in mutant cornea. Pnn-depleted corneal epithelial cells showed labeling, after a 2-hour pulse of BrdU, at comparable levels to the controls (Figs. 2A–2D) and seemed to normally express proliferation markers, such as Cyclin D1 and Ki67 (data not shown). TUNEL assays showed no significant change in mutant cornea throughout the embryonic and early postnatal stages (Figs. 2E, 2F). In addition, X-gal (R26R) staining-positive Pnn-null corneal epithelial cells were readily observed in mutant cornea even at PN21 (Fig. 2H), suggesting that Pnn depletion does not result in general cellular proliferation arrest or cell death, but rather may lead to alterations in critical programs required for the differentiation and maintenance of the corneal epithelium.

**Squamous Metaplasia of Pnn Mutant Corneal Epithelium**

At postnatal stages, Pnn mutant eyes displayed an abnormal expression pattern of cytokeratins. Expression of cornea-specific Keratin, K12, was markedly reduced in mutant corneal epithelium at PN21 (Figs. 3B, 3G). Instead, mutant corneal epithelium exhibited strong expression of epidermal keratins, K10 and K14 (Figs. 3C–3F, 3G), which are well-characterized feature of the squamous metaplasia of the corneal epithelium. Consistently, transcript level of Aquaporin-5 (Aqp5), a member of water channel family which is highly expressed in corneal epithelium, was also significantly decreased in mutant cornea (Fig. 3G), confirming the loss of specific corneal epithelial identity.

K12 has been shown to be first expressed in developing corneal epithelium at E15.5. However, in Pnn mutant corneal epithelium, K12 expression was repressed at all stages examined. As shown in Figures 4A–4D, while E16.5 control corneal epithelium strongly expresses K12, mutant epithelial cells did not show K12 expression. Immunofluorescent stainings also showed coexpression of K12 and GFP (from Pax6-Cre-GFP transgene) in control corneal epithelium, whereas GFP-positive mutant corneal epithelial cells are negative for K12 immunoreactivity (Figs. 4C, 4D).

Downregulation of Pax6, a master regulator of eye development, was shown to be closely associated with decreased K12 expression in numerous pathologic conditions of ocular surface. Thus, to determine whether abolished K12 expression...
in mutant corneal epithelium was due to the decline in Pax6 expression, the level of Pax6 was examined. Interestingly, immunohistochemical analyses revealed that the expression of Pax6 in mutant corneal epithelium remained at the comparable level to control corneal epithelium throughout embryonic development (Figs. 4E, 4F). It was at PN1 when the level of Pax6 was noticeably reduced in mutant cornea (Figs. 4G, 4H), suggesting that changes in specific keratin expression precede the downregulation of Pax6 in Pnn mutant corneal epithelium, and thus Pax6 may not be directly involved in the misregulation of

**Figure 3.** Altered expression of corneal epithelial marker genes in mutant corneal epithelium. (A, B) Immunohistochemical staining (red) of cornea-specific keratin K12 shows the reduction of K12 expression in Pnn mutant corneal epithelium. On the other hand, control corneal epithelial cells shows specific expression of K12. (C, D) Strong expression of epidermal keratin K14 is detected in all layers of mutant corneal epithelia. In control littermates, corneal epithelial cells express undetectable level of K14, while conjunctival epithelial cells show positive K14 immunoreactivity. (E, F) Another epidermal keratin K10 is also expressed in suprabasal cells of mutant corneal epithelium. All insets are higher magnification images of each boxed area in (B), (D), and (F). (G) Semi-quantitative RT-PCR assays show downregulation of corneal epithelial marker genes and upregulation of epidermal markers in mutant cornea at PN21. Original magnification: (A–F) 50X. (B, D, F, insets) 200X.

**Figure 4.** Earlier downregulation of K12 than Pax6 in Pnn mutant corneal epithelium. (A, B) Mutant cornea displays significantly decreased expression of K12 (arrowheads in B) at E16.5. (C, D) Immunofluorescence assays also reveal clear downregulation of K12 in mutant. While control corneal epithelium shows coexpression of K12 (Red) and GFP (from Pax6-Cre-GFP transgene), mutant cells only exhibit GFP signals. (E, F) Immunostaining analysis of Pax6 shows similar level of Pax6 expression in control and mutant corneal epithelium at E16.5. (G, H) Pax6 immunoreactivity is considerably reduced in Pnn mutant cornea (arrowheads in H) at PN1. CEp, corneal epithelium; CS, corneal stroma; L, lens. Original magnification: (A, B, E–H) 400X. (C, D) 200X.
Increased Levels of β-catenin and Tcf4 in Pnn Mutant Corneal Epithelium

Previously, we observed sustained activation of Wnt signaling (TCF/Lef activity and β-catenin accumulation) associated with the disruption of epithelial differentiation under the reduction19 or absence of Pnn expression (our unpublished data, 2009). Thus, as a potential explanation for the squamous metaplasia of Pnn mutant corneal epithelium, we postulated that upregulation of Wnt signaling activity might be involved in the process. aberrant, ectopic, and/or sustained activation of the Wnt pathway has been frequently reported to block epithelial differentiation and, in some cases, facilitate metaplasia of the specific epithelial types to other types. To test this hypothesis, we examined cellular β-catenin level in mutant cornea. Intriguingly, Pax6-Cre–mediated deletion of Pnn resulted in the dramatic increase in intracellular and nuclear β-catenin within corneal epithelial cells. While control eyes exhibited a distinct pattern of β-catenin expression (high level of β-catenin in conjunctiva and marginal level in cornea), Pnn mutant displayed drastically increased corneal β-catenin level, which is comparable to that of conjunctiva at PN21 (Figs. 5A–5D). Supporting this observation, immunostaining analyses with another antibody, which specifically detects ser37/thr41-hypo- phosphorylated-β-catenin (active β-catenin), also revealed considerably increased level of active β-catenin in mutant corneal epithelial cells (Figs. 5E–5H). Furthermore, we observed dramatic increase in Tcf4, a major Wnt downstream transcription factor, in the basal cells of mutant corneal epithelium (Figs. 5I–5L). These observations suggest that Pnn-depletion results...
in the abnormal upregulation of Wnt activity in mutant cornea, and also that Pnn-deficiency-dependent Wnt activation may play a key role in the disruption of corneal differentiation and maintenance.

Misregulation of p68 Level in Pnn Mutant Cornea

Although we propose abnormal Wnt activation as one of the possible mechanisms for the epithelial metaplasia of Pnn mutant cornea, the exact molecular mechanism to activate Wnt pathway in the absence of Pnn remains unclear. To identify the potential molecular switch for the Wnt activation, we examined the expression profiles of candidate or Pnn-interacting proteins. Within the immunoisolated nuclear Pnn-complex, we identified p68 RNA helicase (our unpublished data, 2005). This was indeed an exciting discovery, because p68 has been recently implicated in Wnt signaling through its association with β-catenin.32 The expression pattern of p68 itself during corneal development was quite amazing in the way that it is highly expressed until E15.5 (Fig. 6A) and becomes almost completely silent at E16.5 and afterward (Figs. 6C, 6E). To our surprise, mutant cornea failed to silence p68 expression at E16.5 and its expression remained very high even at PN21 (Figs. 6D, 6F). Yang et al. have shown that p68 (Ddx5) promotes β-catenin accumulation and nuclear transllocation.32 It is therefore tempting to speculate that misregulation of p68 subsequent to Pnn deletion leads to Wnt/β-catenin activation and alteration of epithelial differentiation, epithelial dysplasia, and corneal pathologies.

**DISCUSSION**

The early studies from Pnn hypomorphic mice implicated Pnn with recently implicated in Wnt signaling through its association with p68 RNA helicase (our unpublished data, 2005). In this study, we more precisely demonstrate specific requirement for Pnn in the corneal epithelial differentiation by using conditional gene inactivation approach. Absence of Pnn in developing ocular surface epithelial cells specifically affected corneal epithelial differentiation, resulting in the squamous metaplasia of corneal epithelium. The Pnn-null corneal epithelium, which is derived from the Cre-expressing cells based on both GFP expression from the Pax6-Cre-GFP transgene (Fig. 4D) and X-gal-positive signals (Fig. 2H), was apparently healthy, but was simply the wrong epithelium for the cornea. Intriguingly, this defect intimately correlated with significantly elevated β-catenin and Tcf4 level, as well as misregulated expression of p68 RNA helicase.

While it is tempting to speculate that Pnn may impact the differentiation program of the corneal epithelium through altering Pax6 expression, our initial analyses of specifics in timing of the gene expression change suggest that changes in specific keratin expression may precede the downregulation of Pax6 in Pnn mutant. Indeed, several other recent studies also reported Pax6-independent abnormalities of anterior eye segments.11–15 Although a much more detailed analysis is required before drawing any conclusions of putative pathways leading to the observed epithelial phenotypic changes, because immunofluorescence and immunohistochemistry analyses are far from quantitative, our current data indicate that Pnn seems to exert its effect on corneal differentiation in a Pax6-independent manner during embryonic stages. However, since downregulation of Pax6 has been shown to be closely linked to squamous metaplasia of the corneal epithelium in human pa-
tients with severe ocular surface diseases,31 an eventual loss of Pax6 expression in Pnn mutant corneal epithelial cells at PN1 may suggest the possible involvement of Pax6 downregulation in further development and/or maintenance of squamous metaplasia of Pnn mutant corneal epithelium.

The precise orchestration of epithelial morphogenesis during eye development requires an exquisite degree of precision of varied signaling pathways to define where tissues of the eye form and how individual cells within the eye differentiate. Local regulation of Wnt/β-catenin signaling activity has been shown to be essential for determining cell fate decisions within the ocular surface ectoderm33 and ectopic activation of Wnt/β-catenin signaling within the ocular surface ectoderm blocks lens placode induction and lacrimal gland outgrowth.34 Wnt/β-catenin signaling is initiated by binding Wnt ligands to cell surface receptors (consisting of Frizzled and the LRPS/6 subunit). The subsequent receptor activation leads to stabilization of β-catenin, which then translocates into the nucleus to activate target gene expression. We have observed the dramatic increase in both intracellular and nuclear β-catenin (consistent with elevated Wnt activity) in Pnn-depleted cells in vitro and in vivo. Thus, we speculate that elevation of Wnt activity results in the blockage of the correct epithelial differentiation program and in turn reprogramming of the corneal epithelial cell fate. Mouse corneal epithelia made null for Wnt inhibitor, Dkk2, exhibit a loss of typical corneal epithelial phenotype, with a loss of keratin 12 (corneal keratin) and subsequent increase in keratin 10 (epidermal keratin).35,36 Similarly, culturing rabbit corneal epithelia on dermis induced a dedifferentiation and transdifferentiation to epidermis, presumably due to Wnt ligand activation derived from dermis.37,38 Finally, Kao and colleagues have demonstrated that forced expression of ectopic stabilized mutant β-catenin in corneal epithelial cells results in the loss of corneal epithelial phenotype and the subsequent metaplasia to epidermal-like epithelium (Liu CY, et al. IOVS 2009;50:ARVO E-Abstract 2594). These β-catenin expressing corneal epithelia may eventually exhibit epithelial hyperplasia and invasion of the corneal stroma. While the extent to which the Wnt pathway may be involved in normal corneal development per se remains uncertain, it appears the Wnt activation is very relevant to epithelial wound healing and instances of loss of epithelial phenotype. It will be of great relevance to determine the role that ectopic Wnt activation plays in the epithelial metaplasia seen subsequent to Pnn inactivation in the corneal epithelium.

We have focused specifically on one protein with which Pnn is in complex; RNA helicase p68 (Ddx5), a member of the Dead Box RNA helicases.39–42 It is now clear that several members of this family are multifunctional and also have significant roles in transcriptional regulation. p68 is considered one of the prototypic dead box proteins together with its binding partner p72. The finding that p68 co-purifies with Pnn along with other spliceosomal proteins suggests a role for this protein in RNA splicing. In addition, Oncomine meta-analyses have suggested that p68 and p72 may be co-regulated with Pnn.43 Intriguingly, similar to Pnn, p68 has been reported to act as a bridge between transcription factors/co-activators, such as CBP/p300 and RNA POL II. In our present study, we showed that the deletion of Pnn in the corneal epithelium results in a dramatic increase in p68 level, concurrent with the increase of nuclear β-catenin. Yang and colleagues have demonstrated that phosphorylation of p68 under platelet-derived growth factor (PDGF) stimulation mediated epithelial-mesenchymal transition (EMT).32,44 They showed that PDGF treatment led to phosphorylation of p68, which promotes β-catenin nuclear translocation via a Wnt–ligand-independent pathway. The phosphorylation of β-catenin nuclear translocation via blocking phosphorylation of β-catenin by GSK-3β and displacing Axin from β-catenin.32 Thus, we postulate that increased p68 level in Pnn mutant corneal epithelium may, at least in part, account for the observed ectopic upregulation of β-catenin activity.

On the other hand, p68, generally known to show ubiquitous expression pattern in virtually all cell types, has been shown to be growth- and developmentally-regulated to correlate with epithelial differentiation.39–41 Several studies suggest that p68 is involved in cellular differentiation. It interacts with Runx2 to regulate osteoblast differentiation, and it has been suggested that it contributes to both keratinocyte proliferation/differentiation and control of gene expression.31,45 Furthermore, we now show tight spatiotemporal regulation of p68 level during corneal epithelial differentiation for the first time. Although the exact mechanism of downregulation of p68 during corneal epithelial differentiation (which occurs specifically between E15.5 and E16.5 in mice) has not been determined, its specific relationship to Pnn and its upregulation in the Pnn null corneal epithelia are of significant interest and relevance to how the deletion of Pnn in the corneal epithelium translates to such a dramatic phenotype.

In summary, our findings clearly demonstrate that Pnn is critical for ensuring normal differentiation of corneal epithelium. Conditional Pnn inactivation in developing corneal epithelium results in squamous metaplasia accompanied with significantly increased β-catenin activity and p68 level. Future studies are warranted to determine the molecular mechanisms that account for observed alterations of corneal epithelial differentiation in Pnn mutant eyes.

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References


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