ERRATUM

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The final sentence of the acknowledgements should read: 'This research was supported by the Intramural Research Programs of the National Institute of Child Health and Human Development and of the National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health.'

We apologise to the authors and readers for this mistake.

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An error in this paper was not corrected before the article went to press.

Morasso and Westphal are co-senior authors on this work, rather than equal contributors.

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Dkk2 plays an essential role in the corneal fate of the ocular surface epithelium

Mahua Mukhopadhyay[1], Marat Gorivodsky[1], Svetlana Shtrom[1], Alexander Grinberg[1], Christoph Niehrs[2], Maria I. Morasso[3,*] and Heiner Westphal[1,*,†]

The Dkk family of secreted cysteine-rich proteins regulates Wnt/β-catenin signaling by interacting with the Wnt co-receptor Lrp5/6. Here, we show that Dkk2-mediated repression of the Wnt/β-catenin pathway is essential to promote differentiation of the corneal epithelial progenitor cells into a non-keratinizing stratified epithelium. Complete transformation of the corneal epithelium into a stratified epithelium that expresses epidermal-specific differentiation markers and develops appendages such as hair follicles is achieved in the absence of the *Dkk2* gene function. We show that Dkk2 is a key regulator of the corneal versus epidermal fate of the ocular surface epithelium.

KEY WORDS: Mouse, Dkk2, Cornea, Epidermis, Differentiation, Wnt/β-catenin

INTRODUCTION

Wnt proteins are implicated in diverse developmental processes during embryonic patterning, including anteroposterior axis formation, generation of cell polarity and specification of cell fate (reviewed by Logan and Nusse, 2004). Cell proliferation is commonly regulated by Wnt signaling, and Wnt knockout phenotypes can often be explained by a loss of cell proliferation. For example, tissue outgrowth is impaired in limb buds lacking Wnt5a (Yamaguchi et al., 1999), and expansion of the CNS fails in Wnt1 mutants (Megason and McMahon, 2002). Interestingly, the cell cycle regulators Myc and cyclin D1 are direct Wnt signaling targets in colon cancer cells (reviewed by Morin, 1999). Wnt signaling also affects cell adhesion, where formation of an epithelial bud during hair follicle development requires the repression of E-cadherin transcription. Inputs from both Wnt, which stabilizes β-catenin, and from the BMP inhibitor Noggin, which induces Lef1 expression, directly repress the E-cadherin promoter (Jamora et al., 2003). Proper regulation of Wnt signaling is not only essential for embryogenesis but also for postnatal development and tissue homeostasis. This is illustrated by mutations in the Wnt co-receptor gene *Lrp5* that lead to familial osteoporosis, high bone density syndromes and ocular disorders (reviewed by He et al., 2004). Wnt signaling also regulates stem-cell self-renewal and differentiation in a range of epithelia, and the inappropriate activation of these pathways contributes to epithelial cancers (reviewed by Watt, 2004). Thus, investigating Wnt signaling pathways is crucial for understanding development and disease.

A variety of secreted Wnt inhibitors have been identified that regulate Wnt signaling by binding either to Wnts or to their receptors. Inhibitors that directly bind Wnts, such as sFRP (Rattner et al., 1997), WIF (Hsieh et al., 1999) and Cerberus (Piccolo et al., 1999) are structurally similar to the extracellular domains of Frizzled (Fz) family of Wnt receptors. Cerberus is a multifunctional inhibitor of Bmp, Nodal and Wnt signals (Piccolo et al., 1999). These Wnt inhibitors have been implicated in repression of the canonical Wnt/β-catenin signaling pathway.

The *Dickkopf* (Dkk) family of secreted cysteine rich proteins represent another class of Wnt inhibitors that regulate Wnt pathways by interacting with the Wnt co-receptor LRP5/6 and with the Kremen class of transmembrane proteins (He et al., 2004; Mao and Niehrs, 2003). In *Xenopus* and mammals, Dkks are differentially expressed in various neural, mesenchymal tissues (Hashimoto et al., 2000; Monaghan et al., 1999) and in hindbrain-derived neural crest populations, suggesting that these ligand proteins are involved in diverse inductive processes. *Dkk1* is a Wnt antagonist whose roles in head induction and limb development are well established (Glinka et al., 1998; Mukhopadhyay et al., 2001). To investigate the function of the mammalian *Dkk2* gene in development we have generated *Dkk2* knockout mice through homologous recombination. Our study uncovers an essential role for the Dkk2 gene in ocular surface epithelial fate determination.

MATERIALS AND METHODS

Generation of *Dkk2*$^{-/-}$ mutant mice

Following our previously published strategies (Mukhopadhyay et al., 2001), the targeting vector was constructed by replacing the entire first exon of *Dkk2* with a PGK-NEO cassette, preserving 2.9 kb (5′) and 2.5 kb (3′) of flanking homologous sequences and using the thymidine kinase gene for double selection. The targeting vector was linearized at a unique *Not*I site before transfection of ES cells. Transfected ES cells were subjected to double selection. Targeted disruption of the *Dkk2* gene via homologous recombination was confirmed by Southern blot analysis. Two independently derived recombinant ES cell lines were injected into blastocysts to generate chimeric mice. Chimeric males were mated with C57BL/6 females to produce *Dkk2*$^{+/-}$ animals, which were intercrossed to produce offspring for analysis.

Genotyping

The genotyping was performed by polymerase chain reaction using the following primers.

Dkk2 wild-type allele primer set: left primer, 5′-GGT CTC CTG GGT GAC CAA ACC TCT CCT AA-3′; right primer, 5′-GAG GCC TTG GCT AGC CTT TCA AGT CAG-3′.

Mutant allele primer set: left primer, 5′-GGT CTC CTG GGT GAC CAA ACC TCT CCT AA-3′; right primer, 5′-GTA GAA TTG ACC TGC AGG GGC CCT CGA-3′.

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Histology and immunohistochemistry

Embryos were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin and sectioned at 5 μm. Sections were either stained with Hematoxylin and Eosin (H&E) or Toluidine Blue or periodic acid-Schiff base for histological analyses. Immunohistochemistry was performed on paraffin sections collected from E15, E17, P2, P5 and P10 eyes. Paraffin was removed by a series of xylene and ethanol rinses. We quenched endogenous peroxidase activity by incubating the slides in 2.5% hydrogen peroxide in methanol for 30 minutes. Antigen retrieval was carried out by boiling slides in a 5 mM sodium citrate buffer (pH 6.0). We incubated the sections with primary antibodies diluted with PBS containing 2% normal serum. The antibodies used are as follows: anti-K12 (Santa Cruz Biotechnology), anti-K1, anti-filaggrin and anti-Pax6 (Covance). After primary antibody incubation, sections were washed in PBS, incubated with biotinylated secondary antibodies (Vector Laboratories) and processed with a Vectastain ABC kit (Vector Laboratories). The sections were developed by using AEC as chromogen (Zymed), counterstained with Hematoxylin, and mounted with Aqua PolyMount (Polysciences). For alkaline phosphatase-conjugated secondary antibodies, BM-purple blue was used as chromogen (Roche). For immunofluorescence, P2, P10 and P15 eyes were embedded in OCT compound (Tissue Tek) and 10 μm frozen sections were cut. Sections were stained with commercially available anti-Dkk2 antibody (Santa Cruz Biotechnology) and rhodamine-conjugated secondary antibodies (Molecular Probes).

β-Galactosidase expression analysis

β-Galactosidase expression was visualized by X-gal staining of whole eyes using a standard protocol (Hogan et al., 1994). Stained eyes were embedded in paraffin, sectioned and photographed.

RNA isolation and RT-PCR analysis

Total RNA was isolated from dissected corneas using the 'S.N.A.P. total RNA isolation kit' from Invitrogen (Carlsbad, CA). RNA quantification was performed using RiboGreen RNA Quantification reagent and a kit from Molecular Probes (Eugene, OR). For RT-PCR we used the 'Cloned AMV first-strand synthesis kit' from Invitrogen. The sequences of *Shh* primers used for RT-PCR analysis are as follows: left primer, 5′-CCA TTA GCC TAC AAG CAG TTT ATT CCC-3′ and right primer, 5′-TTG GAT TCA TAG TAG ACC CAG TCG AAA-3′. *Lef1* primers are: left primer, 5′-ACT CTG GCT ACA TAA TGA TGC CCA ATA-3′ and right primer, 5′-AGG TGT TAC AAT AGC TGG ATG AGG GAT-3′. A β-actin control primer-set from Invitrogen was used for quantification.

RESULTS

Corneal phenotype in *Dkk2*$^{-/-}$ mice

Dkk2 mutation was generated via homologous recombination in embryonic stem (ES) cells (Fig. 1A,B). Homozygous mutant mice are viable and fertile but are characterized by a conspicuous eye phenotype that affects the cornea. The mutant cornea appears normal at birth, and defects in corneal surface morphology appear by P5 after birth. Adult mutant mice show normal skin and pelage development, but are smaller in size than their wild-type littermates owing to a bone density defect that is addressed in studies described elsewhere (Li et al., 2005).

The outer surface of the eye is covered by the cornea, which consists of an external stratified non-keratinizing epithelial cell layer, a neural crest-derived collagenous stroma and a single cell layer endothelium (Fig. 1E). By contrast, the outer ocular surface of adult *Dkk2*$^{-/-}$ mice appears opaque and we noticed hair growth. Histological analysis revealed the presence of sebaceous glands as well as conjunctiva-specific goblet cells (Fig. 1F-H). The goblet cells localized preferentially to the periphery of the cornea, suggesting invasion of conjunctival tissues as seen in several corneal diseases (Puangsricharern and Tseng, 1995) and in *Pax6*$^{+/-}$ heterozygote mice (Ramaesh et al., 2003). We also observed presence of pigment

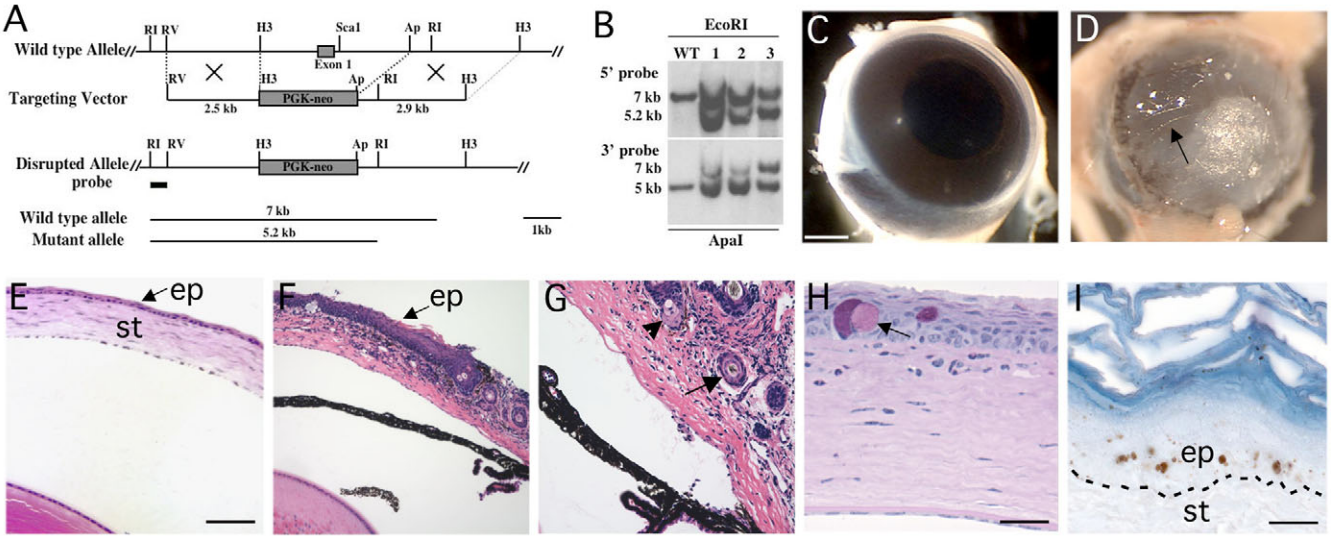

Fig. 1. Gene targeting at the *Dkk2* locus and the cornea phenotype of *Dkk2*$^{-/-}$ mutant mice. (**A**) Partial restriction maps of the wild-type *Dkk2* locus, the targeting vector and the disrupted *Dkk2* allele. Exon 1 and part of the intron 1 of the *Dkk2* allele were replaced with the neomycin (neo) cassette. The expected size of *Eco*RI-generated fragments are 7 and 5.2 kb. RI, *Eco*RI; RV, *Eco*RV; H3, *Hin*dIII; Ap, *Apa*I. (**B**) Southern blot analysis: *Eco*RI-digested genomic DNA isolated from wild-type and three targeted ES cell lines (lanes 1-3) generated 7 kb and 5.2 kb bands, respectively, when detected with a 5′ external genomic probe. *Apa*I-digested genomic DNA generated 7 kb and 5 kb bands for wild-type and mutant alleles, respectively, when detected with a 3′ external genomic probe. (**C**,**D**) Corneal phenotype. An opaque plaque and hairs (arrow) are visible on the ocular surface of a *Dkk2*$^{-/-}$ mutant (D), while an age-matched wild-type control eye contains a transparent cornea (C). (**E-G**) Paraffin sections of eyes stained with Hematoxylin/Eosin, indicating epithelial layer (ep) and the stroma (st). (E) Wild type. Corneal epithelial hyperplasia is evident in the *Dkk2*$^{-/-}$ eye (F). (G) Higher magnification view of F; skin appendages such as hair follicles (arrow) and sebaceous glands (arrowhead) are indicated. (**H**) Periodic acid-Schiff base staining of the *Dkk2*$^{-/-}$ cornea demonstrates presence of goblet cells (arrow). (**I**) Ectopic pigment-granules are present in the epithelial basal cells of the mutant cornea. The border between the epithelial (ep) and stromal (st) layers of a mutant cornea is indicated (broken line). Scale bars: in C, 1 mm for C,D; in E, 200 μm for E,F; 50 μm in H; 35 μm in I.

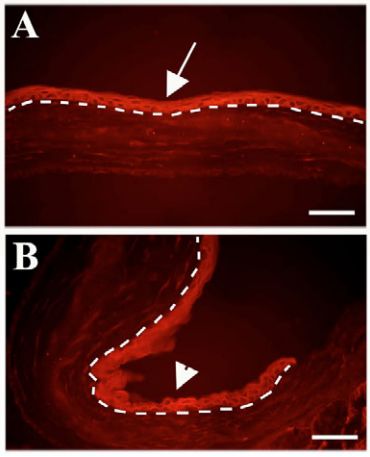

Fig. 2. Expression of the Dkk2 protein in adult cornea. (**A**,**B**) Paraffin sections from wild-type cornea are stained with anti-Dkk2 antibody. Expression of the Dkk2 protein is localized to the epithelial layers in the cornea (A, arrow) and the limbus (B, arrowhead). The epithelial and stromal border of the cornea and the limbus are indicated (broken lines). Scale bars: 100 μm.

granules in cells of the basal epithelial layer of the mutant cornea (Fig. 1I), reminiscent of similar granules in epidermal keratinocytes. These results suggest that the ocular surface epithelium of *Dkk2*$^{-/-}$ eyes contain cell types normally found in skin.

Expression pattern of *Dkk2*

The onset of *Dkk2* gene expression in the mouse embryo has been observed at embryonic day E9.5 in neural epithelial cells of the ventral diencephalon and in the otic vesicle and by E11.5 *Dkk2* expression extends to mesenchymal cells condensing around the eye and the optic stalk, and notably to the developing cornea (Monaghan et al., 1999). *Dkk2* expression in the cornea is maintained throughout development (Ang et al., 2004). Stratification of the corneal epithelia takes place during the first 2 weeks after birth, and it is in corneal cells and in the limbus that *Dkk2* is prominently expressed (Fig. 2). Its expression is localized to the epithelial layers of the cornea and the limbus. The limbal epithelium at the border between cornea and sclera is considered the site of the progenitors that are required for the maintenance of the corneal epithelium (Cotsarelis et al., 1989; Pellegrini et al., 1999). Thus, the expression pattern of Dkk2 is consistent with the development of the observed phenotype in the null mice.

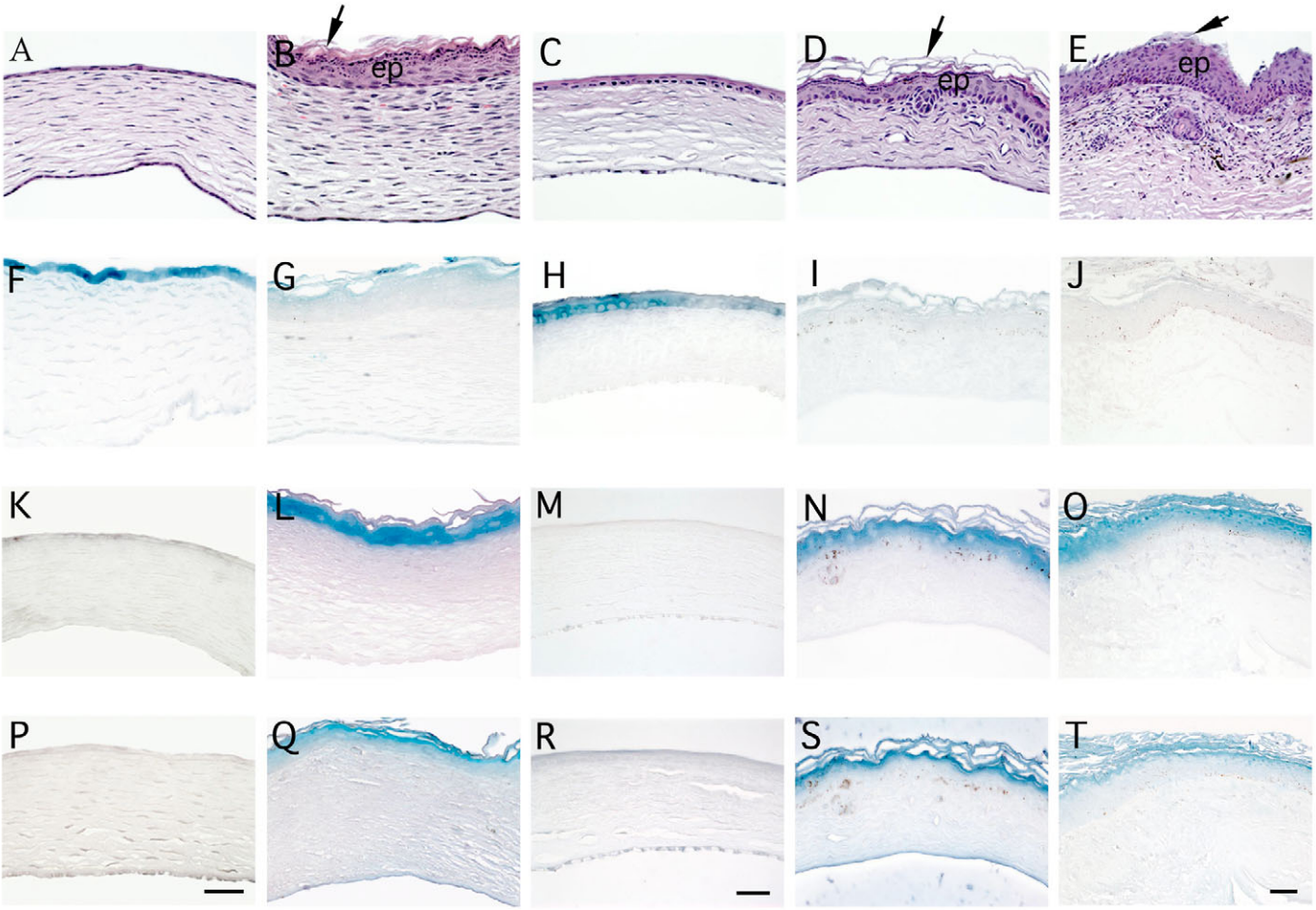

Fig. 3. Expression of epithelial keratinocyte markers in wild-type and *Dkk2*$^{-/-}$ corneas. Hematoxylin/Eosin staining (A-E) and expression of epithelial markers (F-J,K-O,P-T) in wild-type and mutant corneas from P5 (A,B,F,G,K,L,P,Q), P10 (C,D,H,I,M,N,R,S) and 6-month-old (E,J,O,T) mice. (**A-E**) Hyperplasia of the corneal epithelial layer (ep) is apparent in the mutant corneas at all stages. The more superficial cells of the mutant cornea form interwoven and partially desquamated scaly layers (arrows). (**F-J**) K12 expression is present in wild-type corneas (F,H), but not in mutant corneas (G,I,J) at all postnatal stages. Conversely, K1 (**K-O**) and filaggrin (**P-T**) expression is induced in mutant corneas (L,N,O,Q,S,T) but not in wild-type corneas (K,M,P,R). Scale bars: 50 μm.

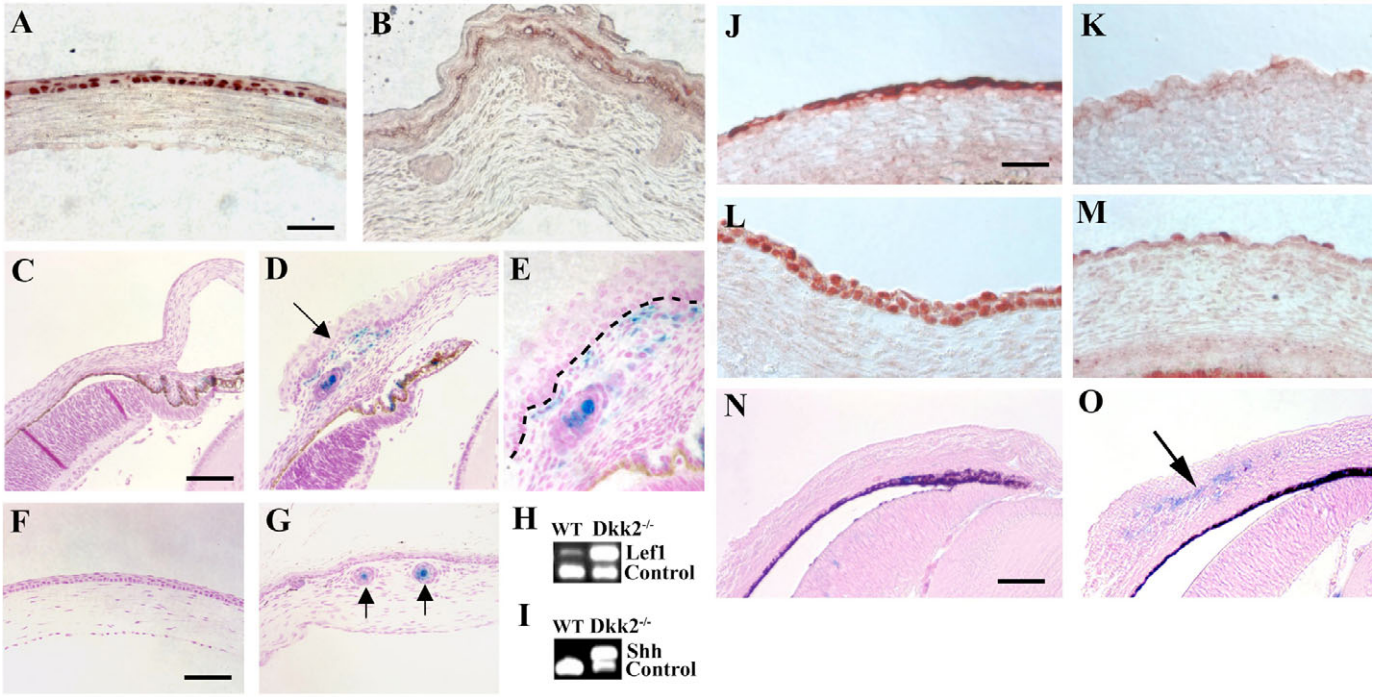

Fig. 4. Molecular mechanism of *Dkk2* gene function. (**A**,**B**) At P10, Pax6 protein is detected in the nuclei of the epithelial basal cells of the wild-type cornea (A) but is absent or excluded from the nuclei of the mutant cornea (B). (**C-G**) X-gal staining on paraffin sections taken from wild-type and *Dkk2*$^{-/-}$ corneas of mice carrying a β-galactosidase (*lacZ*) gene under the control of β-catenin-responsive elements. At P2, significant *lacZ* expression is detected in the limbus of the mutant cornea (D), but not in the wild-type cornea (C). (E) Higher magnification view of D, showing localization of the X-gal staining in the stroma of the limbus. The limbal epithelial-stromal border is indicated. At P15, *lacZ* expression is absent in the central region of a wild-type cornea (F), but detected in a few hair follicles located in the central cornea of a mutant eye (G). (**H**,**I**) RT-PCR analysis. *Lef1* mRNA expression is significantly upregulated in mutant corneas compared with wild-type corneas; *Shh* mRNA expression is induced in mutant corneas and absent in wild-type corneas. (**J-M**) Immunostaining and (**N**,**O**) X-gal staining on paraffin-embedded sections from E15.5 corneas. K12 expression is present (J) in wild-type cornea but absent (K) in a *Dkk2*$^{-/-}$ cornea. Pax6 expression is present in a wild-type cornea (L), but absent in a mutant cornea (M). TOPGAL expression is induced in the limbal stroma region of a *Dkk2*$^{-/-}$ cornea (O), but not in wild-type cornea (N). Scale bars: in A, 50 μm for A,B; in C, 100 μm for C,D; in F, 100 μm for F,G; in J, 50 μm for J-M; in N, 150 μm for N,O.

Expression of epidermal keratinocyte markers in the ocular surface epithelium of *Dkk2*$^{-/-}$ mice

The ocular surface epithelia shares basic features with the epidermal stratified epithelia. Initial differentiation in the corneal and epidermal cells is associated with a switch from basal keratin5/keratin14 to the tissue-specific K3/K12 and K1/K10 keratins, respectively (Chaloin-Dufau et al., 1993; Wolosin et al., 2004). In the keratinizing epidermis, the intermediate filament-associated protein filaggrin (FIL) is a marker for terminal differentiation and a precursor of the cornified envelope (Candi et al., 2005). Keratin and FIL immunostaining (Fig. 3F-T), along with Hematoxylin/Eosin staining (Fig. 3A-E) has generated useful information concerning the lineage origin of the epithelial cells. Fig. 3 shows that the adult *Dkk2*$^{-/-}$ cornea stains for K1 (Fig. 3L,N,O) and FIL (Fig. 3Q,S,T) but lacks the corneal epithelial marker K12 (Fig. 3G,I,J). Thus, the adult mutant ocular surface expresses proteins characteristic of epidermis. As it also contains hair follicles and sebaceous glands, i.e. typical epidermal appendages, we conclude that the *Dkk2* mutant cornea was transformed into a stratified epidermis.

Molecular mechanism of *Dkk2* gene function

Epithelial cells of the cornea derive from ectoderm that expresses *Pax6*, a homeobox gene of profound importance for eye development and maintenance of the cornea (Ramaesh et al., 2003; Davis et al., 2003). In the cornea of mice heterozygous for a *Pax6* null mutation, there is decreased expression of keratin K12 and, in addition, conjunctival invasion (Ramaesh et al., 2003; Davis et al., 2003). We found that *Pax6* gene expression is absent in the ocular surface of the *Dkk2* mutant (Fig. 4B), whereas the nuclei of corneal epithelial basal cells of wild-type corneas show Pax6 immunostaining (Fig. 4A). Therefore, the observed cornea-to-skin fate change in the *Dkk2*-null mutant eye occurs in the absence of Pax6 expression.

Wnt and sonic hedgehog (Shh) signaling pathways play key roles in the complex series of events that determine the ectodermal appendages that appear in the epidermis (Millar, 2003; St-Jacques et al., 1998; Huelsken et al., 2001). We examined β-catenin activity in the *Dkk2*-null mutant eye using the TOPGAL transgene in which *lacZ* expression is controlled by a β-catenin-responsive promoter sequence (DasGupta and Fuchs, 1999). Upregulation of the Wnt/β-catenin pathway was noticed first shortly after birth (P2) in the form of β-gal staining in the limbal mesenchyme (stroma) (Fig. 4D,E). At later stages of development, we observed occasional *lacZ* signals in hair follicles located in regions corresponding to the stromal layer of the central cornea (Fig. 4G). No β-gal staining was detected in the wild-type corneas (Fig. 4C,F).

Additional evidence for a Wnt inhibitory role of Dkk2 in the mammalian eye came from results of our RT-PCR analysis that shows upregulation of the Wnt downstream gene *Lef1* in the cornea

of *Dkk2* mutant mice (Fig. 4H). Wnt upregulation in the *Dkk2* null mutant cornea also coincides with enhanced *Shh* expression (Fig. 4I). Shh plays a major role at discrete stages of hair follicle morphogenesis, and Wnt and Shh pathways appear to interact during follicle development (St-Jacques et al., 1998). The appearance of hair follicles in the mutant cornea is consistent with these findings.

We next examined corneas from embryonic stages to determine the temporal requirement for *Dkk2* gene function in cornea development. Expression of the cornea-specific keratinocyte marker K12 is first induced in the developing cornea at embryonic day 15.5. We observed a punctate staining pattern of the K12 gene in wild-type corneal epithelium at this stage (Fig. 4J), while mutant corneas were devoid of any K12 staining (Fig. 4K). In the developing cornea, Pax6 acts as the upstream regulator of cyokeratin-K12 gene expression (Ramaesh et al., 2003). In the mutant corneal epithelium Pax6 expression was absent at E15.5 (compare Fig. 4L with 4M). Moreover, its expression was already affected at E14.5 (data not shown), a day before K12 expression is normally induced in wild-type corneas. The absence of Pax6 expression in mutant corneas may well be a consequence of increased Wnt signaling. In support of this notion, we observed an induction of TOPGAL reporter expression in the limbal stroma region of the mutant cornea but not in the wild-type cornea at E15.5 (compare Fig. 4N with 4O). These data suggest that proper development of the corneal epithelium requires suppression of Wnt pathways in the limbus rather than in the central cornea. As the Dkk2 protein is expressed in the outer epithelial layer of the cornea, it stands to reason that this Wnt regulator acts non-autonomously by inhibiting signaling in the adjacent stroma.

DISCUSSION

The striking corneal phenotype of the *Dkk2*$^{-/-}$ mutant pinpoints the role of this Wnt inhibitor in corneal epithelial fate determination. In answer to increased Wnt signaling, progenitor cells change fate and give rise to a stratified skin and ectodermal appendages in place of the cornea. We show that cornea-specific gene expression is suppressed and epidermal markers are induced. This is reminiscent of grafting experiments demonstrating that adult mouse cornea juxtaposed with embryonic dermis can form cutaneous appendages (Ferraris et al., 2000). Furthermore, our experiments indicate that suppression of cornea-specific gene expression is preceded by downregulation of Pax6 expression in the developing corneal epithelium. This agrees with recent findings that transdifferentiation of corneal epithelium into epidermis involves downregulation of Pax6 expression in basal corneal epithelia (Pearton et al., 2005).

The development of epidermal appendages, such as hair follicles, requires reciprocal interactions between the surface epithelium and the mesenchyme (reviewed by Millar, 2003). Wnt signaling has been implicated in the initiation of hair follicle development. In the mouse, ectopic expression of *Dkk1* in the skin completely blocks development of skin appendages (Andl et al., 2002). Using an activated form of β-catenin, Silva-Vargas et al. showed that adult epidermis can be reprogrammed to form interfollicular epidermis, sebaceous glands or new hair follicles, depending on the strength of the β-catenin signaling (Silva-Vargas et al., 2005). In the present study, we show that the activation of β-catenin in the mutant limbal mesenchyme, owing to lack of Dkk2 expression, is concomitant with the formation of a stratified epidermis and derived ectodermal appendages. All together, these data suggest that Dkk2 expression in the corneal/limbal epithelium is required to modulate Wnt activity in the limbal stroma region, and that this is a required step in the pathway that leads to corneal epithelium formation and prevents the development of a stratified epidermis and skin appendages.

At present, we know little about the nature of cells that are the initial targets of Dkk2-mediated epithelial transformation. It is very likely, that they reside in the limbus because it is here, in the transitional zone between the transparent cornea and the white conjunctiva that we observe strong Wnt signaling in the mutant. Some of the steps in the profound change of differentiation programs that bring about the epidermal components of the *Dkk2*-null mutant ocular surface are suggested by two recent reports. Nicolas et al. (Nicolas et al., 2003) report that conditional ablation of the *Notch1* gene function leads to epidermal hyperplasia and extensive hyperplasia and keratinization of the corneal epithelial cells through activation of Wnt pathways, though hair follicles were not observed. The second study by Pearton et al. (Pearton et al., 2005), described transdifferentiation of corneal epithelium into epidermis in a setting where central rabbit cornea is abutting embryonic mouse dermis. The authors show data that are compatible with a model whereby the change is brought about by a stepwise process involving a dedifferentiation of basal epithelial cells in the cornea, followed by induction of hair follicles and subsequent formation of interfollicular epidermis. In this process, Wnts are thought to be responsible for the downregulation of Pax6 and to interact with Noggin to induce hair. Although limbus tissue was excluded from this in vitro experiment, we may nonetheless hypothesize that a similar series of events is triggered by Wnt signals emanating from the limbus of the newborn *Dkk2*-null mutant eye.

Our findings provide direct genetic evidence that Dkk2 controls the integrity of the cornea. In its absence, an increase of Wnt signaling in the limbus triggers epidermal and suppresses corneal pathways of differentiation.

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