Requirement for Pax6 in corneal morphogenesis: a role in adhesion

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Summary

The Pax6 transcription factor functions early during embryogenesis to control key steps in brain, pancreas, olfactory and ocular system development. A requirement for Pax6 in proper formation of lens, iris and retina is well documented. By examining the corneas of heterozygous Small eye (SEY) mice, this report shows that Pax6 is also necessary for normal corneal morphogenesis. In particular, the epithelial component of the postnatal and adult SEY (+/–) cornea is thinner owing to a reduction in the number of cell layers, despite a tenfold increase in the proliferative index and no change in TUNEL labeling. Ultrastructural views revealed large gaps between corneal epithelial cells and a change in the appearance of desmosomes, suggesting that adhesion abnormalities contribute to the corneal phenotype of SEY (+/–) mice. Western blot analysis and immunofluorescence showed equivalent amounts and normal localization of E-cadherin in SEY (+/–) corneas, and the actin cytoskeleton appeared normal as judged by phalloidin staining. By contrast, the levels of desmoglein, β-catenin and γ-catenin were reduced in the SEY (+/–) cornea. In addition, the amount of keratin-12 mRNA and protein, the major intermediate filament, was reduced in SEY (+/–) corneal epithelium as shown by in situ hybridization and immunohistochemistry. Finally, the SEY (+/–) corneal epithelium adheres less well than wild-type when challenged with gentle rubbing using a microsponge. In conclusion, our results indicate that cellular adhesion is compromised in the SEY (+/–) corneal epithelium and suggests a role for Pax6 in the proper generation and maintenance of the adult cornea.

Key words: Pax6, Cornea, Adhesion, SEY mice, Development

Introduction

The process of vision begins by the passage and bending of light rays through the cornea, a tissue specialized for that purpose by its transparent and refractive properties. The adult cornea is a lamellar tissue consisting of a stratified surface epithelial layer, a collagenous stroma sparsely populated by keratocytes and an inner endothelial layer. Embryonically, a region of surface ectoderm defined by the lens placode gives rise to the corneal epithelium as well as to the lens, whereas the corneal stroma and endothelium develop from cells originating in the neural crest (Haustein, 1983; Pei and Rhodin, 1970; Pei and Rhodin, 1971; Trainor and Tam, 1995). In the mouse, the corneal epithelium continues to mature after birth: at two weeks of age, the epithelium expands from two cell layers to a self-renewing, stratified epithelium comprising eight to ten cell layers (Hay, 1979; Zieske and Wasson, 1993). Cells in the stratified epithelium are distinguished by their position, characteristic morphology and expression profiles of various biochemical markers (Klyce and Beuerman, 1988).

The causal factors that orchestrate corneal morphogenesis and continued turnover of the adult stratified epithelium have not been elucidated. Here, we have explored the role of Pax6 in corneal development. *Pax6* is a member of the *Pax* gene transcription factor family (Walther and Gruss, 1991; Walther et al., 1991). Heterozygous mutations in *Pax6* result in a spectrum of eye abnormalities in humans, including aniridia, Peter's anomaly and autosomal dominant keratitis (Glaser et al., 1990; Glaser et al., 1992; Hanson et al., 1994), and a distinct small eye syndrome in the Small eye (SEY) mouse and rat (Hill et al., 1991; Matsuo et al., 1993). SEY mice also have defects in parts of the central nervous system (Callaerts et al., 1997; Glaser et al., 1994; Tremblay and Gruss, 1994). In mice, $Pax6^{Sey}$, $Pax6^{SeyNeu}$ and $Pax6^{Coop}$ represent three SEY strains with different point mutations in *Pax6* whereas $Pax6^{SeyDey}$ and $Pax6^{SeyH}$ strains carry *Pax6* gene deletions (Favor et al., 1988; Hill et al., 1991; Hogan et al., 1986; Lyon et al., 1979; Lyon et al., 2000; Schmahl et al., 1993; Theiler et al., 1978; Theiler et al., 1980). Homozygous SEY mice lack eyes and olfactory structures and die at birth (Grindley et al., 1995; Hogan et al., 1986). The semidominant heterozygous phenotypes from different SEY strains of mice show comparable developmental ocular abnormalities, including defects in eye size, lens and retina, although the severity of the phenotypes can vary (Callaerts et al., 1997; Hill et al., 1991). Cataracts, glaucoma and corneal opacities can develop in mutant SEY mice after birth (Lyon et al., 2000; Theiler et al., 1978). Phenotypic variability is observed within a single SEY strain (Hogan et al., 1986; Theiler et al., 1978), even between two eyes of the same mouse, and might result from a stringent requirement that Pax6 activity be present at specific levels at precise times during development (Hill et al., 1991; Schedl et al., 1996; van

Raamsdonk and Tilghman, 2000). Recent functional studies of Pax6 using transgenic technology have revealed specific requirements for Pax6 during lens and retinal development (Ashery-Padan and Gruss, 2001). Moreover, Pax6(5a), one of the two major Pax6 isoforms, is required postnatally to establish various aspects of a normal iris, lens, retina and cornea (Singh et al., 2002).

The expression pattern of *Pax6* coincides well with the phenotypes observed in SEY mice. *Pax6* is expressed at mouse embryonic day 8 in expanded regions of surface and neural ectoderm (Walther and Gruss, 1991). Subsequently, expression is restricted to lens and olfactory placodes, forebrain, hindbrain, neural tube and optic vesicle (Grindley et al., 1995; Walther and Gruss, 1991). At mid-gestation, Pax6 protein is detected in most cells of the neural retina and all parts of the eye derived from surface ectoderm, including the lens pit, lens vesicle, lens and cornea (Davis and Reed, 1996). At six weeks of age, Pax6 is restricted to a subset of retinal neurons, the lens, cornea, conjunctiva, iris and ciliary body (Davis and Reed, 1996; Koroma et al., 1997), consistent with a function in the adult as well as in the embryonic eye.

Some downstream molecular targets mediating the effects of Pax6 activity in the eye have been identified. The expression of several transcription factors in developing lens and retina is linked to Pax6 (Ashery-Padan et al., 2000; Foerst-Potts and Sadler, 1997; Grindley et al., 1995; Marquardt et al., 2001; Sakai et al., 2001; Xu et al., 1997). Pax6 acts as a positive and negative regulator of crystallin gene expression (Chambers et al., 1995; Cvekl et al., 1994; Cvekl et al., 1995; Duncan et al., 1998; Gopal-Srivastava et al., 1996; Richardson et al., 1995). Pax6 has been shown to activate the corneal gene promoters for *keratin 12* (*K12*) (Liu et al., 1999; Shiraishi et al., 1998) and *gelatinase B* (Sivak et al., 2000). Recent microarray analysis identified many genes that were expressed differently when *Pax6* was misexpressed in the eye (Chauhan et al., 2002). Of particular significance to this study, overexpression of Pax6(5a) in transgenic mice resulted in an increase in lens expression of α5 and β1 integrins, paxillin, and $p120^{ctn}$ (Duncan et al., 2000), whereas a reduction in N-cadherin expression was found in cells of the developing lens in the SEY (+/–) mouse (van Raamsdonk and Tilghman, 2000). Here, we show that two strains of SEY mice have abnormal corneal morphology, especially in the epithelium. Furthermore, we present evidence supporting the idea that alterations in cell adhesion contribute to the mutant corneal phenotype. Importantly, our results suggest that Pax6 is required not only for embryonic development but also for the postnatal (PN) development and maintenance of the adult cornea.

Materials and Methods

Mouse strains

Two strains of mice, representing two different mutant alleles of *Pax6*, were analysed. Identical data were obtained with both mutants unless otherwise specified. The $Pax6^{SeyDey}$ strain in a C3H background was purchased from Jackson Laboratories and maintained as a small colony. A second strain of mice harboring the $Pax6^{SeyNeu}$ allele was a generous gift from Y. Furuda (M. D. Anderson Cancer Center, Houston, TX). A morphological assessment of the enucleated eyes was used to establish the genotype of each mouse. Six-week-old C57BL/6 mice were used for Pax6 immunohistochemistry.

Histology

Whole eyes were immersion fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C, washed in PBS and then in saline for 30 minutes each, dehydrated through a series of ethanol washes, cleared in xylene and embedded in paraffin. Prior to staining, 10 μm sections were deparaffinized, rehydrated using a graded series of ethanol washes and rinsed in distilled water for 15 minutes. Eye sections were stained in Gill's hematoxylin and eosin as described (Luna, 1968).

Electron microscopy

Whole eyes were removed, slit at the limbus, fixed for 24 hours or more at room temperature in a solution of 2.5% glutaraldehyde and 6% sucrose buffered to pH 7.2 with 50 mM sodium cacodylate. Small (0.5×1 mm) portions of the central cornea were processed for electron microscopy by dehydration in an ethanol series and embedding in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate, then examined and photographed using a JEM-100CX electron microscope (JEOL, Peabody, MA).

Immunohistochemistry

Fresh-frozen eye sections (10 μm) were collected on Superfrost/Plus slides (Fisher, Pittsburgh, PA) and stored at –80°C until further use. All steps were carried out at room temperature except where noted otherwise. Tissue sections were fixed in 4% paraformaldehyde in PBS for 10 minutes and processed as reported previously (Davis and Reed, 1996). For fluorescent microscopy, the sections were incubated with rhodamine (TRITC)-conjugated AffiniPure $F(ab')_2$ Fragment Donkey anti-mouse or anti-rabbit (Jackson ImmunoResearch Laboratories, West Grove, PA) according to the manufacturer's instructions. Slides were counterstained with DAPI as described (Davis and Reed, 1996), coverslipped using Aqua-Poly/Mount (Polysciences, Warrington, PA) and photographed using a Zeiss Axioplan2 microscope with Spot camera.

The following antibodies were used: anti-Pax6 (1:200; a gift from R. Reed, Johns Hopkins University, Baltimore, MD); anti-N-terminal and anti-C-terminal K12 (1 μg ml^{-1}; a gift from W. Kao, University of Cincinnati, Cincinnati, OH); cytokeratin 4 clone 6B10 (1:10, ICN Biomedicals, Aurora, OH); anti-laminin (1:50; Sigma, St Louis, MO); anti-ZO-1 and anti-E-cadherin (1 μg ml^{-1} each; Zymed Laboratories, San Francisco, CA); anti-α5, α6, β1 and β4 integrins (0.5 μg ml^{-1} each; BD PharMingen, San Diego, CA); anti-epidermal growth factor receptor (EGFR) and anti-ErbB2 (1:200 each; Santa Cruz Biotechnology, Santa Cruz, CA).

Phalloidin staining

F-actin was visualized using rhodamine phalloidin (Molecular Probes, Eugene, OR) as previously described (Xu et al., 2000).

Apoptosis assay

Apoptosis was analysed via in situ labeling of DNA fragments using an ApopTag Plus Fluorescein kit (Intergen, Purchase, NY) according to the manufacturer's instructions for cryosections. A negative control slide was prepared by omitting the deoxynucleotidyl transferase in the reaction mix. A positive control was prepared by pretreating with DNase I (Roche Molecular Biochemicals, Indianapolis, IN) for 5 minutes at room temperature prior to the addition of the reaction mix.

BrdU labeling

24 hours prior to sacrifice, wild-type and heterozygous SeyDey mice were given an intraperitoneal injection of BrdU (10 mM stock solution used at 1 ml per 100 g body weight). Fresh, frozen eye sections were prepared, fixed and washed in PBS as described above. The sections (10 μm) were incubated in 2 N HCl in 0.5% Triton X-100 for 30 minutes, followed by a second incubation of fresh 2 N HCl in 0.5% Triton X-100 containing 1 mg ml^{-1} pepsin (Sigma P7012, St Louis, MO) for 10 minutes. The slides were washed with PBS three times for 10 minutes each, blocked in 10% normal rabbit serum in a humidified chamber and then incubated with a rat monoclonal anti-BrdU antibody (Ab) (1:100; Accurate Chemical and Scientific, Westbury, NY) overnight at 4°C. Complete processing of the sections was achieved using a rat Vectastain Elite ABC kit and diaminobenzidine as described previously (Davis and Reed, 1996).

Western blot analysis

Mice were euthanized and whole eyes were enucleated and placed in chilled PBS on ice. Using a dissection microscope, corneas were isolated from the rest of the eye by introducing a small opening at the limbus with fine forceps and then separating the cornea from the conjunctiva by pulling on either side of the opening with fine forceps. The corneas were trimmed free of any remaining non-corneal tissues with a scalpel blade, frozen on dry ice and stored at –80°C until further use. Tissues were solubilized in 150 mM NaCl, 50 mM Tris, pH 7.4, 0.5% NP-40, 0.5% sodium deoxycholate, 5 mM EDTA, 0.25% SDS, pepstatin, leupeptin, PMSF and aprotinin. Protein concentration was determined using the Bio-Rad Protein assay (Bio-Rad, Hercules, CA). PAGE was performed using precast gels, buffers and 2× Tris-glycine SDS sample buffer containing 50 mM dithiothreitol followed by transfer to PVDF membrane in Tris-glycine transfer buffer according to the manufacturer's recommendation (Novex: Invitrogen, Carlsbad, CA). The membrane was stained with Ponceau Red (where applicable), blocked in 5% nonfat dry milk in Tris-buffered saline (Blotto) for 1 hour at room temperature and incubated with primary Ab in Blotto at 4°C overnight. The membrane was washed with 1× TBS; 0.1% Tween 20, three times for 15 minutes each. The blot was incubated with a horseradish peroxidase-conjugated, secondary Ab (1:10,000 in Blotto, Amersham Pharmacia Biotech, Piscataway, NJ) for 30 minutes. After washing, the immunoreactive complex was visualized using ECL Plus (Amersham Pharmacia Biotech, Piscataway, NJ). The following primary antibodies were used: anti-desmoglein, anti-paxillin, anti-β-catenin and anti-γ-catenin (1:1000, 1:10,000, 1:2000, 1:500, respectively; D28120, P13520, C26220, C19220, Transduction Laboratories, Lexington, KY), anti-E-cadherin (2 μg ml^{-1}; 13-1900, Zymed Laboratories, San Francisco, CA) and anti-tubulin (1:500; T3526, Sigma, St Louis, MO).

In situ hybridization

Fresh, frozen, 10 μm eye sections were fixed in 4% paraformaldehyde, treated with proteinase K (0.2 μg ml^{-1} PBS) for 8-10 minutes and processed for in situ hybridization as described previously (Borchelt et al., 1996). Riboprobes were synthesized using a DIG RNA Labeling Kit (Sp6/T7) (Roche Molecular Biochemicals, Indianapolis, IN) with linearized, proteinase-K-treated, full-length plasmid cDNA templates for mouse K12. Hybridizations were carried out at 55°C using 200 ng sense or antisense riboprobe per ml hybridization buffer. Detection of digoxigenin-labeled hybridization was achieved using an alkaline phosphatase (AP)-conjugated anti-digoxigenin Ab at a 1:500 dilution followed by the addition of nitroblue tetrazolium salt and bromo-4-chloro-3-indolylphosphate toluidinium salt. The reaction was allowed to proceed until a purple color was visible (~2 hours), at which time reactions for both the sense and antisense riboprobes were terminated.

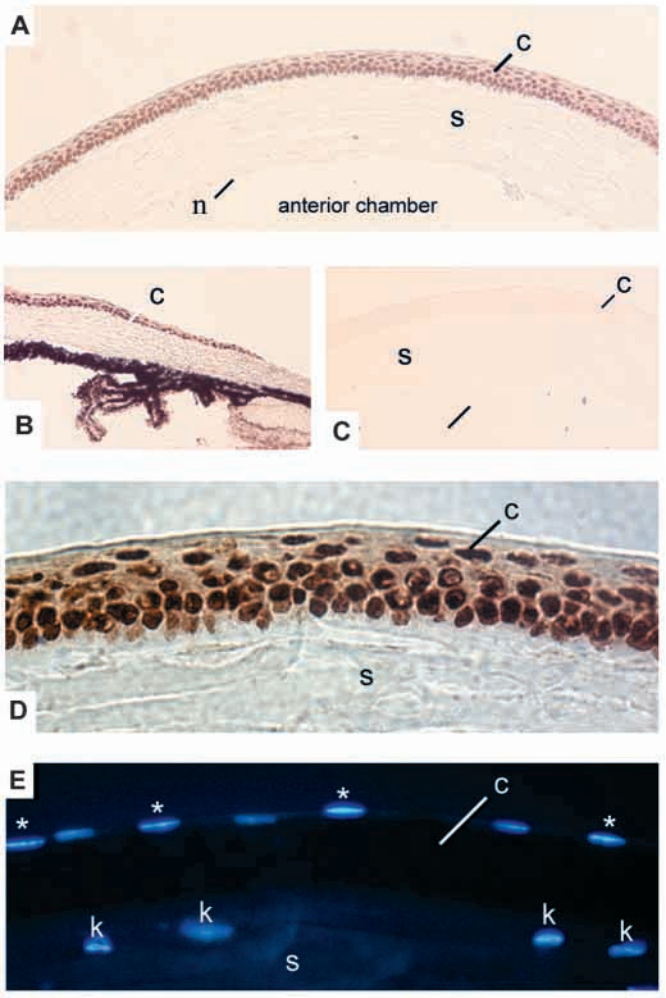

Fig. 1. *Pax6* expression in C57Bl/6 mouse cornea. Localization of Pax6 protein to the corneal epithelium, but not the stroma or endothelium, is shown by bright-field microscopy using an antibody against Pax6 on cryosections from the central (A) and peripheral (B) corneal regions of a 6-week-old wild-type mouse. No primary antibody used (C) serves as a control. Higher magnification of central cornea (D) showed nuclear localization of Pax6, indicated by the brown immunoreactive product. DAPI counterstaining of nuclei (E) (on the same section as in D) revealed that most corneal epithelial cells produce Pax6, indicated by the quenching of DAPI fluorescence by the brown Pax6-dependent immunoreactive product. Epithelial cells in the most anterior layer of the cornea, however, fluoresce brightly (asterisks), indicating that Pax6 is reduced in this cell layer. Abbreviations: c, corneal epithelium; k, keratocyte; n, endothelium; s, stroma.

Cornea fragility assay

The fragility of the corneas was assessed as described previously (Kao et al., 1996). Briefly, mice were euthanized and the cornea of the right eye was immediately brushed gently three times with a PBS-saturated microsponge (K20-5010, Katena Products, Denville, NJ). The left eye was not brushed. Several drops of fluorescein (2% in PBS; F-6377, Sigma, St Louis, MO) were applied to both eyes for 1 minute and then the eyes were washed repeatedly with PBS. The eyes were examined within 3 minutes to avoid diffusion of the fluorescein and photographed using a Zeiss Stemi SV11 fitted a Spot camera with a GFP filter (485/20 excitation).

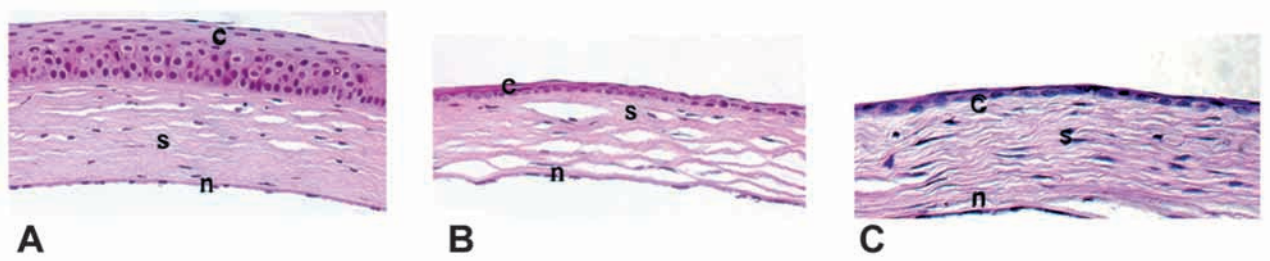

Fig. 2. Morphology of corneas from adult SEY mice. Corneas were visualized by bright-field microscopy using hematoxylin and eosin. Same-magnification views of corneas from wild-type (A), *Pax6*^{SeyDey} (+/–) (B) and *Pax6*^{SeyNeu} (+/–) (C) adult (6 weeks) mice showed severe reductions in corneal epithelial thickness in the SEY (+/–) mice. Abbreviations: c, corneal epithelium; n, endothelium; s, stroma.

Results

Pax6 localizes to the epithelial cells of the adult mouse cornea

Pax6 protein was localized to the nuclei of epithelial, but not stromal or endothelial cells of the adult cornea using an antibody directed against Pax6 (Fig. 1A-C). These results confirm those published previously (Koroma et al., 1997). In addition, we discovered that, although Pax6 protein is present in most cells of the central and peripheral epithelium (Fig. 1B), it is reduced or absent in the most superficial cellular layer of the corneal epithelium (Fig. 1D). Owing to the quenching of the fluorescent nuclear counterstain DAPI by the peroxidase-generated immunoreactive product, the nuclei fluoresce only faintly in the cells where Pax6 is present (Fig. 1E). The brilliance of the DAPI-stained nuclei of the superficial layer combined with the absence of immunoreactive signal (which is harder to discern in the flattened cell layer) indicate that Pax6 protein is reduced as cells move to the apical surface of the epithelium.

Postnatal and adult SEY (+/–) mouse corneas are abnormal

Histological analysis of adult eye sections revealed that corneas from SEY (+/–) mice were defective when compared with wild-type siblings. The corneal epithelium was eight to ten cell layers thick in adult wild-type mice (Fig. 2A), independent of the strain, compared with 1-7 cell layers in the SEY (+/–) mouse. Extreme reductions in epithelial thickness are shown for adult *Pax6*^{SeyDey} (Fig. 2B) and *Pax6*^{SeyNeu} (Fig. 2C) mice. The reduction in corneal epithelial thickness occurred across the entire corneal surface and varied from mouse to mouse, even between eyes from the same animal, but was always reduced relative to wild-type corneas. Additionally, the characteristic morphology of individual cell layers of the corneal epithelium was altered in the SEY (+/–) epithelium. In general, the basal cells were more rounded and the cell layers were not packed as tightly as the wild-type counterparts.

We confirmed that cells in the corneal epithelial region were bona fide corneal epithelial cells based on their expression of K12 mRNA and protein, a marker for corneal but not conjunctival epithelial cells (Liu et al., 1994; Moyer et al., 1996). A robust hybridization signal was observed for *K12* mRNA in wild-type corneal epithelium using an antisense (Fig. 3A) but not a sense (Fig. 3B) riboprobe. A variable *K12* hybridization signal, often reduced, was observed in most SEY (+/–) corneal epithelium (Fig. 3E,H). Similarly, an immunoreactive signal of variable intensity relative to the wild type (Fig. 3C) was also observed in the corneal epithelium of SEY (+/–) mice (Fig. 3F,G,I,J) using two different antibodies raised against K12. Specific reactivity of the N-terminal K12 antibody with corneal but not conjunctival epithelium is shown in Fig. 3K. Immunoreactivity of anti-K12 antibody with stromal fibroblasts is nonspecific (W. Kao, personal communication) and disappears at lower dilutions of antibody (data not shown). Thus, the expression of *K12* mRNA and protein using three independent means of detection indicate that these cells retain features that are characteristic of corneal epithelial cells. K12 staining was not detected in corneas with reductions in corneal epithelial thickness to one or two cell layers; instead, these cell layers were positive for keratin 4 (K4), a conjunctival epithelium-specific keratin (Kurpakus et al., 1994) (data not shown). Thicker epithelia were negative for K4 staining (data not shown). No goblet cells were observed in the two strains of SEY mice used in this study.

Other regions of the SEY (+/–) cornea were abnormal. Immunostaining using anti-laminin antibodies revealed that the basement membrane was intact. However, large aggregates of laminin also appeared in the anterior region of the SEY (+/–), but not the wild type, corneal stroma (Fig. 4). In ~20% of the SEY (+/–) corneas, the stroma showed deviations in thickness and a general disorganization (Fig. 2). Like the wild-type corneal endothelium, the SEY (+/–) corneal endothelium was intact as a monolayer as evaluated by both light (Fig. 2) and electron (data not shown) microscopy.

One feature of squamous epithelial cell stratification is the establishment of cell layers in an orderly fashion. A time course of stratification in the wild-type C57BL/6 mouse showed that the corneal epithelium is two cell layers thick at birth and develops postnatally to its adult size (6 weeks) of eight to ten cell layers as revealed by hematoxylin and eosin staining (data not shown). Between PN14 and PN21, the epithelium expands by four to six cell layers, however, neither the PN14 nor the PN21 SEY (+/–) corneal epithelium was as stratified as a same-age, wild type epithelium (data not shown). These results are consistent with our findings in the adult SEY (+/–) cornea and suggest that the process of establishing or maintaining the corneal epithelium with an age-appropriate number of cell layers is impaired as early as the second week after birth in the SEY (+/–) mouse.

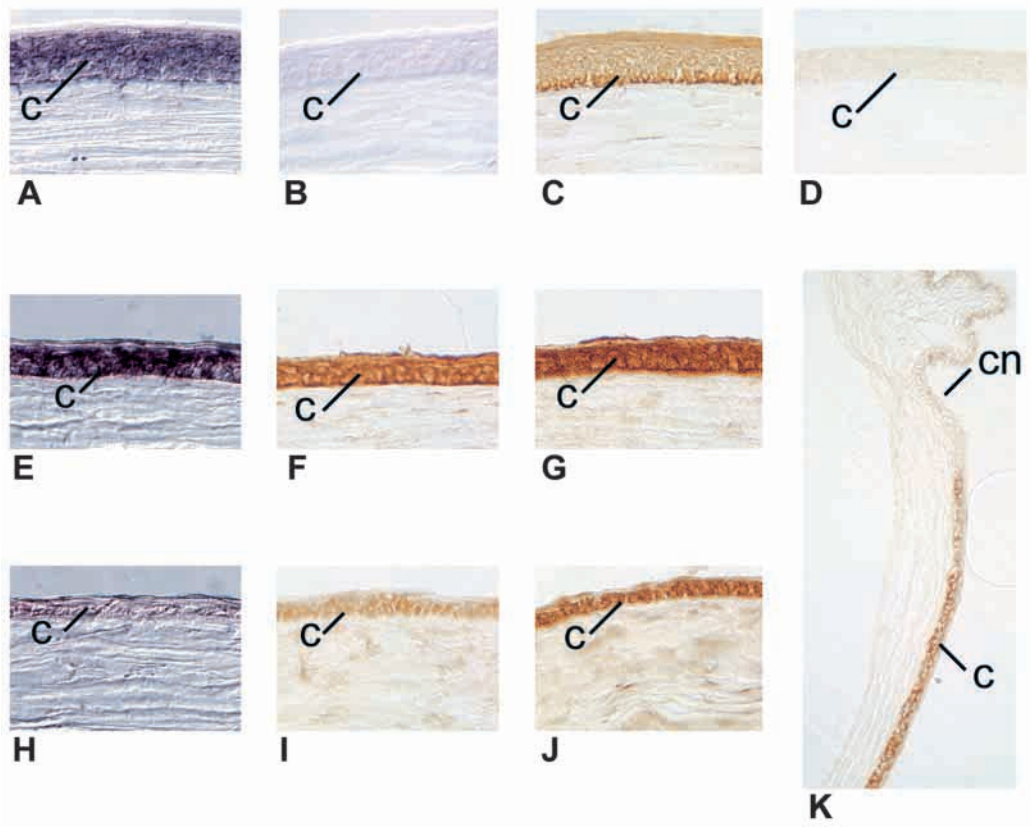

Fig. 3. Keratin 12 (K12) in corneas from wild-type and SEY (+/–) mice. *K12* mRNA was detected by in situ hybridization in the corneal epithelium of wild-type (A,K) and SEY (+/–) (E,H) mice using an antisense riboprobe. A control using a sense probe is shown for a wild-type mouse (B). K12 protein was detected by immunostaining with an anti-N-terminal K12 antibody in wild-type (C) and SEY (+/–) (F,I) corneal epithelium and with an anti-C-terminal K12 antibody in SEY (+/–) (G,J). No primary antibody used (D) serves as a control. The specificity of the N-terminal K12 antibody for corneal but not conjunctival epithelium is shown in K. Abbreviations: c, corneal epithelium; cn, conjunctival epithelium.

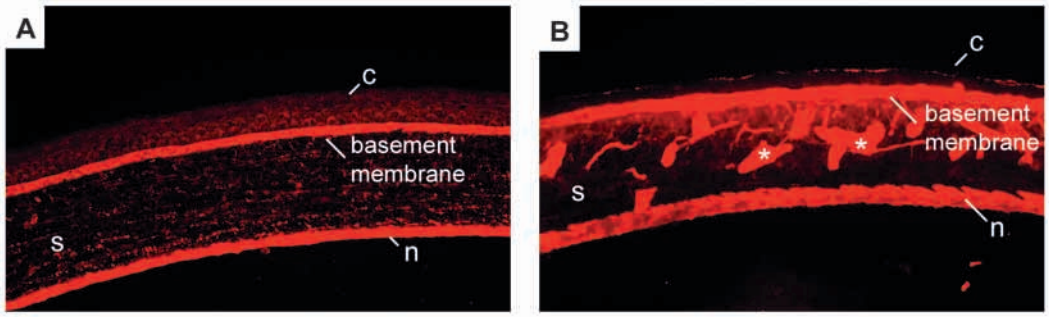

Fig. 4. Localization of laminin in SEY (+/–) mouse corneas. Laminin was detected by immunofluorescence in the basement membrane underlying the corneal epithelium and the endothelium of wild-type (A) and mutant (+/–) *Pax6*^{SeyDey} (B) corneas. The dim fluorescence present in wild-type epithelial cells was absent in mutant cells, and large aggregates of immunofluorescence (asterisks in B) occasionally appeared in the stroma of mutant corneas. Similar staining was observed in the *Pax6*^{SeyNeu} (+/–) cornea (data not shown). Abbreviations: c, epithelium; s, stroma; n, endothelium.

Changes in proliferative or apoptotic rates do not account for reduced corneal epithelium thickness in the SEY (+/–) mouse

Several avenues of investigation were taken to ascertain whether the SEY corneal epithelium phenotype results from changes in the growth status of the epithelial cells. First, we determined whether a reduction in cell proliferation, as measured by DNA synthesis, could account for the abnormal corneal phenotype. Mice were harvested 24 hours following an intraperitoneal injection of BrdU and the eyes were processed for immunohistochemistry using an anti-BrdU antibody. The number of BrdU-labeled cells was divided by the total number of epithelial cells to determine the proliferative index (PI). Surprisingly, the PI of the *Pax6*^{SeyDey} (+/–) epithelium was greater than that of wild-type siblings at 6 weeks of age (Fig. 5). A PI of 45% in *Pax6*^{SeyDey} (+/–) compared with 4% in wild-type epithelium indicated that more cells have entered S phase in the mutant (+/–) epithelium. Similar PIs were calculated for PN21 *Pax6*^{SeyDey} (+/–) and wild-type corneas (data not shown).

The possibility that a reduction in the layers of the cornea was due to an increase in cell turnover caused by apoptosis was analysed by TUNEL labeling of the corneal epithelium in PN21 and adult mice. There was no difference in the number of TUNEL-positive cells between wild-type and SEY (+/–) corneas at either age; only zero to two labeled cells were observed per section, in contrast to the positive control slide, in which all nuclei were labeled following pretreatment with DNase (data not shown).

The roles of EGFR and ErbB2, two growth-factor receptors previously shown to mediate corneal epithelial cell proliferation (Savage and Cohen, 1973; Xie et al., 1999), were examined by immunohistochemistry. The pattern of

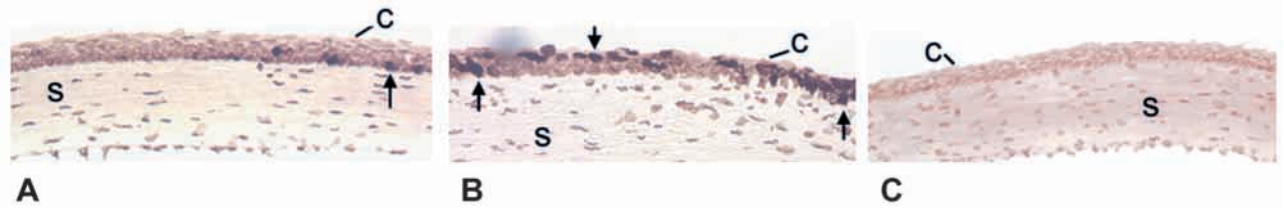

Fig. 5. DNA synthesis in wild-type and *Pax6*^{SeyDey} (+/–) mouse corneas. Mice were sacrificed 24 hours after an injection of BrdU and corneas were processed for immunohistochemistry using an anti-BrdU antibody. Approximately ten times more BrdU-positive cells (black signal indicated by arrows) are detected in 6-week-old SEY (+/–) mouse corneal epithelium (B) than in wild type (A) corneal epithelium. BrdU-positive cells are generally located on the basal side of the wild-type epithelium, whereas they are distributed uniformly in the SEY (+/–) epithelium. The brown color is observed in a control section of PN21 wild-type cornea (C), where no anti-BrdU antibody was used and is considered to be background. Abbreviations: c, epithelium; s, stroma.

immunostaining for ErbB2 appeared similar in the SEY (+/–) (Fig. 6B) and wild-type (Fig. 6A) corneal epithelial cells. However, the staining pattern for EGFR differed between SEY (+/–) (Fig. 6D) and wild-type (Fig. 6C) corneas: large aggregates of immunoreactive product for EGFR were observed in the nuclei of the SEY (+/–) but not the wild-type cells at 6 weeks of age. Expression analysis of EGFR and ErbB2 by semiquantitative reverse-transcription PCR, showed no significant difference between the levels of mRNA expression in the SEY (+/–) and wild-type corneas (data not shown).

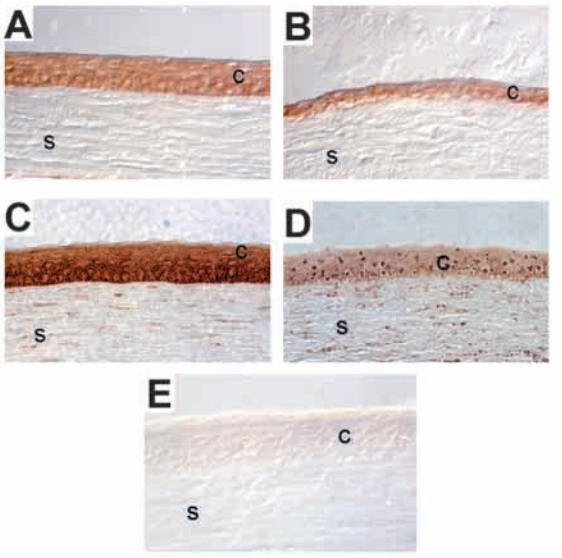

Fig. 6. Corneal epithelial localization of ErbB2 and epidermal-growth-factor receptor (EGFR). Wild-type (A,C) and SEY (+/–) (B,D) corneas of 6-week-old mice were incubated with antibodies against ErbB2 (A,B) or EGFR (C,D). Immunostaining patterns for ErbB2 were similar in the wild-type and mutant cornea. However, there is a change in EGFR localization as revealed by a strong, diffuse staining pattern in the wild type versus the pronounced aggregation of immunoreactive product in the nuclei of SEY cells. As a control, a section of wild-type adult cornea (E) was processed exactly as above except that no primary antibody was used. Abbreviations: c, epithelium; s, stroma.

Defects in cellular adhesion contribute to reduced thickness of the SEY (+/–) cornea epithelium

Electron microscopy revealed that the close adhesion of the cell layers normally present in the corneal epithelium was absent in the SEY (+/–) epithelium (Fig. 7). Large gaps appeared between cells that remain attached to each other through larger than normal desmosomal complexes (Fig. 7B,D). These abnormalities were restricted to the suprabasal, middle and superficial layers of the epithelium (data not shown). The SEY (+/–) corneal cells also appeared swollen.

To investigate the nature of the defect in adhesion, we examined the levels and/or locations of several molecules that are known to be important in the two major classes of anchoring junctions. Western blot analysis showed equivalent levels of E-cadherin (the transmembrane core of epithelial adherens junctions) in total corneal extracts from wild-type and SEY (+/–) mice (Fig. 8A). Moreover, there was no difference between the plasma membrane locations of E-cadherin in wild-type (Fig. 8G) and SEY (+/–) (Fig. 8H) corneal epithelial cells by immunofluorescence. By contrast, the level of desmoglein, the major cadherin found in desmosomes, was reduced eight times in SEY (+/–) relative to wild-type corneas (Fig. 8B). The amounts of β- and γ-catenin, additional components of anchoring junctions, were also reduced, about two times, in mutant SEY (+/–) cornea (Fig. 8C,D, respectively). Equal loading of soluble corneal extracts was confirmed by staining duplicate lanes on the blot with Ponceau Red (Fig. 8F). Most protein bands show equivalent intensity except for the 54 kDa band (corresponding to aldehyde dehydrogenase class 3, an abundant corneal epithelial protein), which is also reduced in the SEY (+/–) cornea (J. Davis and J. Piatigorsky, unpublished). Abundant, equivalent amounts of transketolase at 68 kDa indicate that the extracts were derived primarily from corneal rather than conjunctival epithelium, which has low basal levels of transketolase (Guo et al., 1997).

Although there were no visible gaps in the basal and suprabasal cell layers, the presence of molecules mediating adherence to the extracellular matrix via hemidesmosomes and focal adhesions was examined. Localization of the α5, α6, β1 and β4 integrin subunits using specific antibodies revealed similar, basement membrane staining in the corneas of wild-type and SEY (+/–) mice (data not shown). Western blot analysis showed that the amount of paxillin, a protein associated with focal adhesions, was similar in SEY (+/+) and SEY (+/–) corneas, except for a small upwards shift in electrophoretic migration in paxillin from the SEY (+/–) cornea (Fig. 8E), a possible post-translational modification.

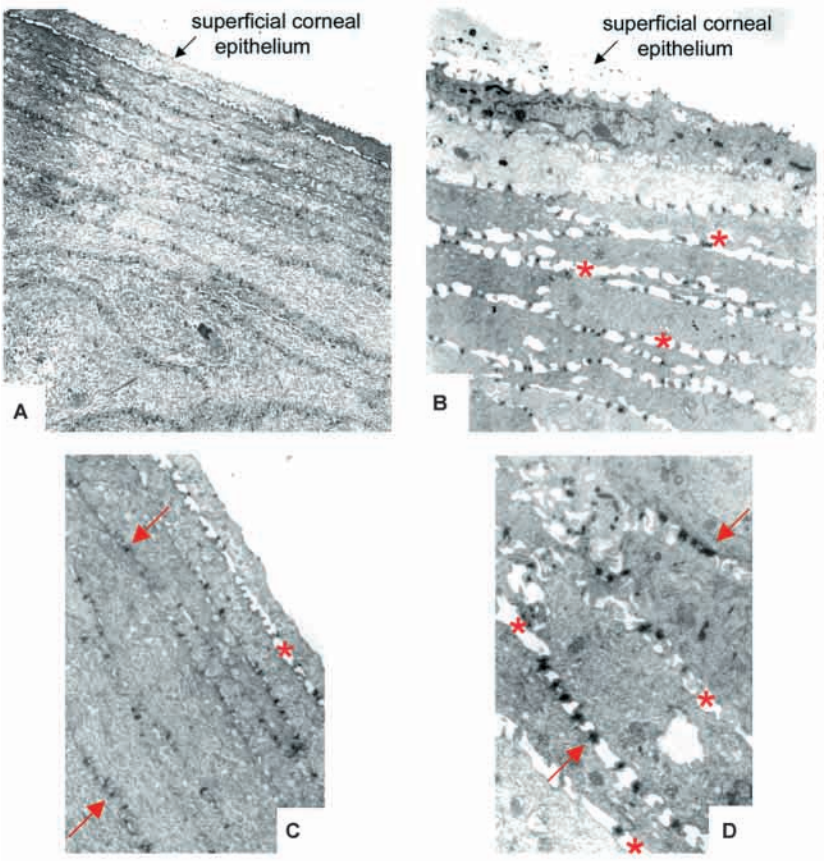

Fig. 7. Ultrastructure of wild-type and SEY (+/–) corneal epithelium. 6-week-old wild-type (A,C) and SEY (+/–) (B,D) corneal epithelium are shown at 9900× (A,B) and 15,000× (C,D) magnification. Large gaps (asterisks in B,D) were present between cells from the suprabasal to superficial layers in SEY (+/–) epithelium. By contrast, notice the tightly adhered cell layers in the wild-type epithelium; gaps (asterisk in C) are present only in the most superficial layer of the wild-type epithelium. Furthermore, the number of individual desmosomal complexes (the electron-dense structures; arrows in C,D), are reduced, but the size of these complexes is increased in the SEY (+/–) compared with the wild-type epithelium.

Wild-type and SEY (+/–) corneas fluoresced with a similar pattern after staining with rhodamine-conjugated phalloidin, showing that the actin cytoskeleton was intact (Fig. 9A,B, respectively). By contrast, the intermediate filament K12 mRNA and protein was reduced in the corneal epithelium in >90% of SEY (+/–) compared with wild-type mice (Fig. 3). These results, taken together, suggest that desmosomes and the intermediate filaments to which they attach are abnormal and contribute to a loss of adhesion in the SEY (+/–) corneal epithelium.

Finally, we tested whether the epithelial cells of SEY (+/–) corneas adhere more loosely than those of the wild-type corneas. Gentle rubbing with a microsponge saturated with PBS removed the corneal epithelium of SEY (+/–) mice but equivalent rubbing did not affect the corneal surface of wild-type mice (Fig. 10). The bright staining in the SEY (+/–) relative to the wild-type cornea represents an epithelial defect. Fluorescein staining occurs when there is a loss of cells at the epithelial surface. Epithelial cell loss in SEY (+/–) eyes after rubbing was confirmed by light microscopy (data

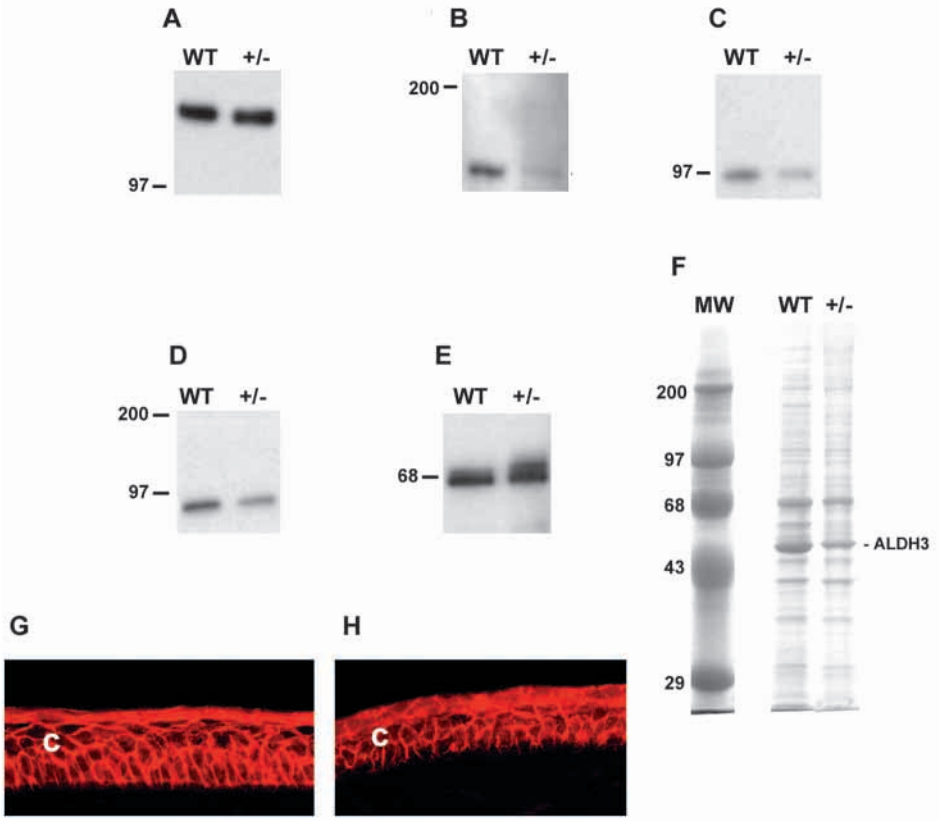

Fig. 8. Production of adhesion-related proteins in the cornea. Western blot analysis of E-cadherin (A), desmoglein (B), β-catenin (C), γ-catenin (D) and paxillin (E) derived from wild-type and SEY (+/–) corneal extracts from 6-week-old mice. Levels of desmoglein and β- and γ-catenin are reduced approximately eight, two and two times, respectively. A Ponceau-stained protein blot (F) from wild-type and SEY (+/–) corneal extracts shows equivalent amounts of protein. Immunolocalization of E-cadherin shows similar cell membrane staining in wild-type (G) and SEY (+/–) corneas (H) corneas. Abbreviations: ALDH3, aldehyde dehydrogenase 3; c, epithelium; MW, molecular weight markers; WT, wild type.

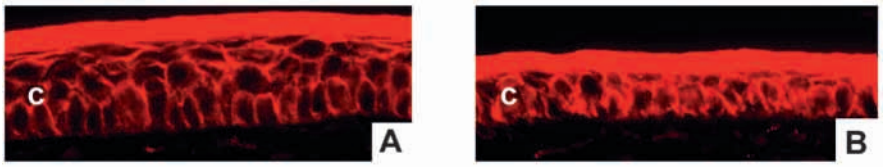

Fig. 9. Cytoskeletal marker in wild-type and SEY corneas. The actin cytoskeleton was similar in wild-type (A) and SEY (+/–) (B) corneal epithelium of 6-week-old mice as revealed by rhodamine-phalloidin. Abbreviations: c, epithelium; s, stroma.

not shown). Even without rubbing, fluorescein staining often revealed diffuse, punctate defects in the corneas of SEY (+/–) mice (Fig. 10C).

Discussion

This study reveals that the corneal epithelium, a site of *Pax6* expression, is abnormal in two strains of mice heterozygous for *Pax6* and is consistent with a strong role for Pax6 in corneal morphogenesis. Our data indicate that cellular adhesion is compromised in the SEY (+/–) corneal epithelium. An increase in the proliferative index was observed in the *Pax6*^{SeyDey} (+/–) corneal epithelium, raising the possibility that Pax6 functions, directly or indirectly, in the regulation of this phase of the corneal epithelial cell cycle. The variability observed in corneal thickness and K12 mRNA and protein levels is consistent with previous studies showing that *Pax6* has gene-dosage-dependent actions (Estivill-Torrus et al., 2001; Glaser et al., 1994; Hogan et al., 1986; Schedl et al., 1996; van Raamsdonk and Tilghman, 2000; Vasioukhin et al., 2001).

Our data show that cell-cell junctions, known to be important and plentiful in epithelial cells (Albert and Jakobiec, 1994), are altered in SEY (+/–) corneal epithelia. Desmosomes in the SEY (+/–) cornea formed larger complexes than their wild-type counterparts. Coincident with changes in the desmosomal ultrastructure was the widening of intercellular spaces between cells. These defects were most apparent in the superficial, middle and suprabasal layers of the SEY (+/–) corneal epithelium, consistent with the finding that desmosomes are denser in the superficial layers of the corneal epithelium (Hogan et al., 1971). A similar phenotype was observed in the skin of transgenic mice overexpressing a mutant form of *Dsg3* (Allen et al., 1996). Our results are also compatible with clinical symptoms in patients with phemphigus vulgaris, who suffer from losses in cell adhesion in squamous epithelia including ocular tissue as a result of raising autoantibodies against Dsg3 (Allen et al., 1996; Koch et al., 1997; Smolin and Thoft, 1994).

Concomitant with the ultrastructural changes in the desmosomes, we also found that the level of Dsg, an adhesion molecule that forms the transmembrane core of desmosomes, was reduced in the SEY (+/–) cornea (Angst, 2001). The amounts of individual desmosomal proteins appears to be crucial, because desmocolin1 (Chidgey et al., 2001) or Dsg 3 (Koch et al., 1997) null mice form ‘normal-looking’ desmosomes but their skin eventually loses adhesion and falls off.

In contrast to desmosomal cadherins, the levels of E-cadherin (the transmembrane core of adherens junctions) and its localization to the plasma membrane were similar in wild-type and mutant SEY corneas. However, two other structural components of adherens junctions, β- and γ-catenin, were reduced in SEY (+/–) corneal cells. γ-Catenin has been shown

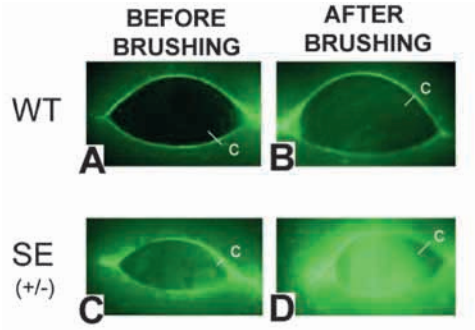

Fig. 10. Corneal epithelial defects after brushing the corneal surface with a microsponge. Wild-type (A,B) and SEY (+/–) (C,D) eyes from 6-week-old mice were untreated (A,C) or treated (B,D) by brushing the corneal surface with a microsponge. The eyes were then stained with fluorescein, washed and photographed. After brushing, intense, diffuse fluorescein staining was observed in the SEY (+/–) cornea (D), in contrast to that observed in the wild-type cornea (B). Abbreviation: c, cornea.

to increase adhesion in vitro (Marcozzi et al., 1998) and in vivo (Bierkamp et al., 1999). It is possible, therefore, that the decrease in catenins in SEY (+/–) cornea further reduces the ability of the remaining junctions to provide strong intercellular adhesion.

A decrease in K12, the major intermediate filament-forming keratin of mouse corneal epithelial cells (Liu et al., 1993; Liu et al., 1994), might also contribute to loss of adhesion. The anchoring of the intermediate filaments to the desmosomes produces supracellular scaffolding that is essential in maintaining epithelial tissue integrity (Kivela and Uusitalo, 1998; Kowalczyk et al., 1999; Smith and Fuchs, 1998; Steinberg and McNutt, 1999; Troyanovsky and Leube, 1998; Vasioukhin and Fuchs, 2001). Gentle rubbing of the corneal epithelium produced mild erosion of the SEY mutant similar to that observed in *K12* homozygous-null mice (Kao et al., 1996).

A role for Pax6 in cell adhesion has been suggested by previous studies (Brunjes et al., 1998; Chapouton et al., 1999; Collinson et al., 2000; Dohrmann et al., 2000; Estivill-Torrus et al., 2001; Gotz et al., 1996; Grindley et al., 1997; Mastick et al., 1997; Matsuo et al., 1993; Quinn et al., 1996; St-Onge et al., 1997; Stoykova et al., 1996; Stoykova et al., 1997; Warren et al., 1999). Transcriptional targets of Pax6 include genes controlling cell-cell interactions (Chalepakis et al., 1994; Duncan et al., 2000; Edelman and Jones, 1995; Holst et al., 1997; Meech et al., 1999). We propose that Pax6 has a role in normal turnover of the corneal epithelium by maintaining, directly or indirectly, various factors that contribute to adhesion. In connection with this, we find that Pax6 is not detected in the outermost cell layer of the corneal epithelium (Fig. 1), coincident with the appearance of gaps between this

layer and the layer below it (Fig. 7). Based on the current study, a decrease in Pax6 would lead to a reduction in adhesion conducive to the natural sloughing of the most superficial layer of the cornea.

The most plausible explanation for an increased proliferative index in the *Pax6*^{SeyDey} (+/–) corneal epithelium is that the barrier has been perturbed because of the loss of cells from the upper layers, a condition known to induce cell proliferation (Chung et al., 1999; Cotsarelis et al., 1989; Zieske, 2000). Further, the EGFR is found localized as large aggregates in the cytoplasm and nucleus following corneal wounding (Zieske et al., 2000), as was observed in the SEY (+/–) corneal epithelial cells. Because the *Pax6* gene and flanking chromosomal regions are deleted in the *Pax6*^{SeyDey} strain, it is also possible that the proliferative changes noted here are due to genes other than *Pax6*.

The persistence of *K12* gene expression in most SEY corneas in our investigation indicates that the SEY (+/–) epithelial cells are authentic corneal epithelial cells. Nevertheless, a few corneas produced K4 in the corneal epithelial region of the ocular surface, but only if there was a severe reduction in the epithelium (to one or two cell layers), suggesting that conjunctivalization, a response associated with a deficiency in limbal stem cells and known to occur in human aniridia patients, might also occur in the small-eye syndrome of the mouse (Daniels et al., 2001; Dua and Azuara-Blanco, 2000; Moyer et al., 1996). In the present study, several reduced-thickness epithelia were immunoreactive for K4 on the top and K12 on the bottom cell layer (data not shown), suggesting that a dynamic process of corneal epithelial loss and conjunctival epithelium resurfacing might take place in some cases. These results warrant further investigation into a role for Pax6 in limbal stem-cell number and the corneal infiltration of conjunctival cells in the SEY mouse model.

Eye development depends on the coordinated interaction of many tissues. The inductive action of lens on corneal development obscures an understanding of whether Pax6 has a primary effect in the cornea, independent of its effect in the lens (Beebe and Coats, 2000; Genis-Galvez, 1966; Genis-Galvez et al., 1967; Grainger, 1992; Kidson et al., 1999; Reneker et al., 2000; Zinn, 1970). Future experiments analysing mice overexpressing *Pax6* specifically in the corneal epithelium beginning at eye opening will hopefully provide an answer to whether Pax6 has a direct, primary role in the generation and maintenance of the adult, stratified corneal epithelium.

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