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Contact-mediated control of radial migration of corneal epithelial cells

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Purpose: Patients with a heterozygous mutation in the gene encoding the transcription factor, PAX6, have a degenerative corneal opacity associated with failure of normal radial epithelial cell migration across the corneal surface and a reported wound healing defect. This study investigated the guidance mechanisms that drive the directed migration of corneal epithelial cells.

Methods: In vivo corneal epithelial wounding was performed in adult wild-type and *Pax6*$^{+/-}$ mice, and the healing migration rates were compared. To investigate the control of the cell migration direction, primary corneal epithelial cells from wild-type and *Pax6*$^{+/-}$ mice were plated on grooved quartz substrates, and alignment relative to the grooves was assayed. A reconstructed corneal culture system was developed in which dissociated wild-type and genetically mutant corneal epithelial cells could be cultured on a de-epithelialized corneal stroma or basement membrane and their migration assayed with time-lapse microscopy.

Results: The *Pax6*$^{+/-}$ cells efficiently re-epithelialized corneal wounds in vivo but had mild slowing of healing migration compared to the wild-type. Cells aligned parallel to quartz grooves in vitro, but the *Pax6*$^{+/-}$ cells were less robustly oriented than the wild-type. In the reconstructed corneal culture system, corneal epithelial cells continued to migrate radially, showing that the cells are guided by contact-mediated cues from the basement membrane. Recombining wild-type and *Pax6* mutant corneal epithelial cells with wild-type and *Pax6* mutant corneal stroma showed that normal *Pax6* dosage was required autonomously in the epithelial cells for directed migration. Integrin-mediated attachment to the substrate, and intracellular PI3Kγ activity, were required for migration. Pharmacological inhibition of cAMP signaling randomized migration tracks in reconstructed corneas.

Conclusions: Striking patterns of centripetal migration of corneal epithelial cells observed in vivo are driven by contact-mediated cues operating through an intracellular cAMP pathway, and failure to read these cues underlies the migration defects that accompany corneal degeneration in patients with mutations in *PAX6*.

Understanding the control of directional cell migration is fundamental to our understanding of embryogenesis, tissue maintenance, and repair. In adult vertebrate epithelia that are constantly turning over and are maintained by stem cells, there has to be a balance between cell loss, cell proliferation, and migration of cells to sites of injury or abrasion [1]. One such system is the adult vertebrate corneal epithelium. The vertebrate cornea is a morphologically simple structure, continuous with the sclera on the outer surface of the eye [2]. A hypocellular collagenous corneal stroma forms the bulk of the cornea, resting on an inner endothelial monolayer. The outer surface of the cornea is covered by a stratified epithelium with barrier function. Cells are lost from superficial layers of the corneal epithelium and are replaced by cell proliferation that occurs only in the basal layer [3,4]. In addition, a population of stem cells exists in the epithelium around the edge of the cornea (the limbal epithelium, contiguous with the corneal and conjunctival epithelia)—limbal epithelial stem cells (LESCs) [5,6]. LESCs normally divide slowly to produce proliferative basal corneal epithelial cells (transit amplifying cells), and multiple lines of evidence show that during adult life there is long-distance centripetal migration of basal epithelial cells from the limbus to the center of the cornea (reviewed [4]). In addition, the corneal epithelium shows an efficient wound healing response, in which cells migrate rapidly to fill gaps left by scratching. Failure of wound healing response and/or stem cell deficiency has been suggested as a causal factor in the development of corneal opacity following injury or disease, one example being aniridia-related keratopathy (ARK, also known as aniridic keratopathy) [7] caused by heterozygous mutation in the gene encoding the transcription factor PAX6 [8].

Migration of corneal epithelial cells across the cornea has been visualized by mosaically labeling mouse corneal epithelial cells with dyes or transgenically with β-galactosidase and fluorescent labels [9-16]. The migration rate in mice has been estimated to represent migration of 17–26 μm/day in a

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strikingly organized radial pattern, interpreted as streams of cells migrating to the center of the corneal epithelium from clonal patches of labeled LESCs at the periphery [10]. Radial corneal stripes are also visualized in some human conditions [17-19].

The corneal epithelium is an excellent model of long distance cell migration in vivo. Although much is known about the molecular control of directional epithelial cell migration in general, the specific drivers of corneal epithelial centripetal movement are still unknown. Several mechanisms have been proposed, for example: a) population pressure from the periphery due to production of new cells by LESCs [18,20,21]; b) biased cell loss, with increased rates of desquamation at the corneal center [22,23]; (3) chemotaxis (either attraction to a central signal or repulsion from the periphery) [9]; and (4) mechanotransduction, the response to substrate-based mechanical cues [24]. Endogenous electric currents due to ion flow across the corneal epithelia have also been proposed as guidance cues for migration [25], although we showed in wound situations that the rate of corneal epithelial healing correlates with neither the magnitude nor the direction of the endogenous electric field [26]. Recently, we showed that functional planar cell polarity signaling was required for normal centripetal migration of corneal epithelial cells in vivo [27].

Contact-mediated or thigmotactic guidance could potentially direct corneal epithelial cells. It has previously been shown that corneal epithelial cells can align parallel to grooves and migrate directionally along grooved surfaces with feature dimensions of as little as 70 nm, through a mechanism that requires regulation of small GTPase activity [28-30]. The corneal stroma underlying the epithelium consists of a hypocellular highly regular crystalline lattice of collagen fibrils and proteoglycans essential for transparency [31] and could potentially form a physical grid for cell migration, at least in species such as mice that do not have a significant anterior limiting lamina [32]. In addition, the basement membrane upon which corneal epithelial cells rest is a structured three-dimensional network of fibrillar extracellular matrix components that include type IV collagen and laminin 5, which are secreted in part by the corneal epithelial cells themselves [33-36]. Corneal epithelial cells are profoundly influenced by extracellular matrix (ECM)–epithelium interactions, including the production of differentiation markers and collagen [37]. Unprocessed laminin 5, which promotes cell adhesion and migration of human corneal epithelial cells via interaction with β1 integrin has been shown to be produced by leading epidermal keratinocytes during wound healing, over which the lagging cells migrate [38,39]. Although the basement membrane of the corneal epithelium can certainly modulate cell migration, it is not known whether the basement membrane of the corneal epithelium also provides the types of directional cues that corneal epithelial cells can use.

Although the guidance cues driving directed in vivo migration of corneal epithelial cells remain unknown, disrupted radial striping patterns in reporter transgenic mice with mosaic expression of *LacZ* that are also heterozygous for *Pax6* suggests that this gene is involved [12,40]. *Pax6* is expressed in the corneal epithelium from the start of development and throughout adult life [41]. Whether normal dosage of the gene is required for generation of directional cues or an epithelial response to external directional cues is unknown. In vitro at least, $Pax6^{+/-}$ corneal epithelial cells can heal faster, more slowly or at the same speed as wild-type, depending on the size of the wound and the growth factor content of the culture media [42-44], which suggests the need for a more detailed in vivo analysis but also suggests that *Pax6* dosage is not critical for the directionality of wound healing migration. This study investigated the molecular basis of the directional response of corneal epithelial cells to contact-mediated directional cues, showing for the first time that centripetal migration of corneal epithelial cells is guided by contact-mediated cues from the basement membrane through a cyclic-AMP-dependent mechanism and that PAX6 is required specifically for the interpretation of, and response to, these cues.

METHODS

Mouse maintenance: $Pax6^{Sey\text{-}Neu/+}$ mice ($Pax6^{+/-}$), heterozygous for an inactivating mutation in *Pax6* [45], were maintained on the CBA/Ca genetic background. $Pax6^{+/-}$ x $Pax6^{+/+}$ matings were set up, and adult $Pax6^{+/-}$ and $Pax6^{+/+}$ littermates were taken for tissue as adults 8–15 weeks old. $PI3K\gamma^{-/-}$ mice were maintained on the C57BL/6 genetic background as a homozygous stock. A C57BL/6 stock was maintained separately for control tissue. All experiments were approved by the University of Aberdeen Ethical Review Committee and performed under license of the Animals (Scientific Procedures) Act 1986 and in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research.

In vivo corneal epithelial wounding: Mice, 8–15 weeks old, were anesthetized by intraperitoneal injection of 1.5 mg ketamine hydrochloride and 0.2 mg medetomidine hydrochloride per 10 g body mass under veterinary advice. For each mouse, a central circular (1.0 mm diameter) corneal epithelial wound was made using a trephine blade without penetrating the underlying stroma, and the epithelial cells within the wound boundary were removed by scraping with

an ophthalmological scalpel blade. Anaesthesia was immediately reversed using Antisedan (atipamezole hydrochloride, 0.014 mg/10 g subcutaneous; Pfizer Animal Health, Exton, PA) to facilitate normal blinking and tear production. At appropriate times post-wounding, the mice were killed, and the eyes were enucleated, fixed with paraformaldehyde, and incubated with Hoechst nuclear stain to measure the size of the wound under a fluorescent microscope. The wound diameter was measured six times in different orientations using the ImageJ linear tool, and the mean of these six diameters was calculated.

Corneal epithelial cell preparation and culture: A protocol modified from Kawakita et al. [46] was used for isolation of primary mouse corneal epithelial cells. Briefly, the mice were killed and the eyes enucleated. The corneas were dissected from the eye without limbal or conjunctival tissue and incubated with 15 mg/ml Dispase II, 18 mg/ml sorbitol in supplemental hormonal epithelial medium (SHEM; 'DMEM/F-12, GlutaMAX™ Supplement; Cat. No. 31331. Gibco, Paisley, UK), 0.5% dimethyl sulfoxide (DMSO), 10 ng/ml epidermal growth factor (Sigma, Dorset, UK), 0.5 µg/ml hydrocortisone (Sigma), 0.1 mg/ml cholera toxin (Sigma), 5% fetal bovine serum (Gibco), 50 µg/ml gentamicin (Gibco), 1.25 µg/ml amphotericin B (Sigma)) at 4 °C for 18 h. The corneal epithelial sheets were peeled off gently, rinsed with PBS (1X; 136 mM NaCl, 2.6 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4), and incubated with 0.05% trypsin (Gibco) at 37 °C for 5 min and triturated mechanically by gentle pipetting to single cells. The cells were centrifuged briefly, resuspended in SHEM, and used immediately without further growth or passage. A degree of dedifferentiation may be inevitable in culture conditions, but we and other researchers [46] have shown that corneal epithelial cells isolated this way expressed high levels of cytokeratin-12, the definitive marker of mature corneal epithelial terminal differentiation.

Preparation of grooved quartz slides for alignment and migration analyses: Grooved substrata were previously prepared by the Department of Electronics and Engineering, Glasgow University, using electron beam lithography on fused quartz microscope slides as previously described [47]. The slides had three 5 mm × 5 mm squares of parallel grooves; each was 130 nm deep and 1, 2, or 4 µm wide. Corneal epithelial cells have previously been shown to align and migrate parallel to these grooved surfaces [30]. The groove areas were separated by areas of flat slide that provided an internal control environment. Slides were sterilized in concentrated nitric acid overnight followed by three rinses with sterile PBS and 10 min ultraviolet (UV) exposure. Cells were plated on the quartz slides in droplets of medium, left for 3 h to attach, and then flooded with SHEM. After 24 h culture, at 37 °C, 5% CO_2, images were taken of the cells on the grooves, and for all cells the angle between the long axis of the cell and the grooves was measured. The mean angles were calculated separately for the wild-type and *Pax6*$^{+/-}$ cells on 1, 2, and 4 µm grooves.

Preparation of corneal stromal beds for migration analyses (reconstructed corneal culture): Adult mice 8–15 weeks old were killed by cervical dislocation and their eyes enucleated. The corneas were dissected and the corneal epithelium removed either by scraping or using Dispase II as described. Microscopic examination confirmed that all epithelial cells had been removed. The denuded corneas were placed, endothelial side down, on a 60 mm tissue culture dish without medium for 5 min to adhere.

Corneal epithelial cells prepared as above were pipetted onto prepared corneal stroma and left 1 h at 37 °C to adhere, before the culture dish was flooded with SHEM. The migration of the cells was monitored using a Leica (Wetzlar, Germany) DMIRIIB time-lapse microscopy system with phase contrast optics, acquiring images every 12 min for 24 h at 37 °C in SHEM to which 20 mM HEPES, pH 7.4 (Sigma), was added.

Cell tracking on corneal stroma: Time-lapse sequences from multiple fields at known coordinates within the flatmounted corneal stroma/epithelial cell interface were imported into ImageJ, and cell migration was tracked manually over 24 h. Cells were chosen for analysis if they were attached to the substrate and migrated smoothly (without jerking movement that indicated weak attachment) with visible lamellipodia and without touching any artifactual landmark such as a crease or cut in the corneal stromal bed. The direction of migration of each cell in relation to the radial track directly to the mathematical center of the cornea was measured. The angles toward the center of the cornea were measured using a Microsoft Excel Macro from the values obtained from the tracking in ImageJ. The angle between the line going through the center of the cornea and the position of the cell and the line going through the previous position of the cell and the present position of the cell was computed, that is, the “angle mistake” of the cell against an ideal trajectory to or from the corneal center. The mean angle mistake for the particular cell was calculated from all the positions taken by the cell during its course.

Random migration was assayed by tracking the migration of corneal epithelial cells in a circular tissue culture dish and measuring their migration angles as above in relation to the center of the dish. This confirmed that the mean error of the random migration was 45° as expected. To standardize

between the experiments and to clarify the presentation of the data, for each experiment, the mean angle of error for the cells migrating in the reconstructed cornea was divided by the mean error measured for random migration on the tissue culture plastic to produce a migration index under each experimental condition that varied between 0 (perfect radial migration with no error) and 1 (random migration).

RESULTS

In vivo epithelial wound healing in Pax6$^{+/-}$ corneas: To determine how the rate of corneal epithelial wound healing compares to normal migration in the uninjured epithelium and whether *Pax6*$^{+/-}$ cells can migrate normally in vivo, wild-type and *Pax6*$^{+/-}$ corneal epithelia were wounded in vivo. Previous in vitro data suggested that normal PAX6 dosage in the first 2 h post-wounding was critical for initiation of cell migration [43]. In the present study, in vivo the wound edge underwent a mean retraction during the first 2 h, and there was no difference between the wild-type and *Pax6*$^{+/-}$ epithelia (Figure 1). In general, healing of *Pax6*$^{+/-}$ epithelia tracked that of wild-type quite closely, with a statistically significant difference only after 21 h of healing (mean ± standard error of the mean wild-type healing = 31±1 µm/h; mean *Pax6*$^{+/-}$ = 25±2 µm/h; *t* test: p = 0.003; n = 12 eyes each genotype). Over the last 12 h of healing (i.e., while directed healing migration was in progress), these means were approximately 55 µm/h for wild-type and 43 µm/h for *Pax6*$^{+/-}$, which is in the range previously reported for in vivo rabbit corneal wound healing [48] but faster than normal centripetal migration in the uninjured corneal epithelium of 17–26 µm/day. It was concluded in vivo that healing of *Pax6*$^{+/-}$ corneal epithelia was only mildly defective and that qualitatively *Pax6*$^{+/-}$ cells were competent to migrate rapidly and directionally in vivo. Further experiments were therefore performed to determine the guidance cues driving epithelial cell orientation and migration in the uninjured corneal epithelium and to determine whether the *Pax6*$^{+/-}$ cells were competent to respond to those cues.

Alignment on quartz grooves: Human and bovine corneal epithelial cells have previously been shown to orient parallel to grooves that are 1, 2 or 4 µm wide and 130 nm deep in quartz microslides coated with poly-L-lysine (see the Methods section) [30]. To determine whether mouse corneal epithelial cells behave similarly, and whether *Pax6* mutant cells can respond to the grooves, dissociated corneal epithelial cells from *Pax6*$^{+/+}$ and *Pax6*$^{+/-}$ mice were isolated and plated separately on 2 µm quartz microgrooves. The cells were allowed to orient overnight. After that, the orientation of the longest axis of several hundred cells relative to the grooves was measured (Figure 2A). The experiment was repeated five times. We have previously shown that isolated cultured corneal epithelial cells retain *Pax6* expression and that PAX6 protein is present at about 60–70% wild-type levels in cultured *Pax6*$^{+/-}$ corneal epithelial cells [43]. The *Pax6*$^{+/+}$ and *Pax6*$^{+/-}$ cells were found to orient parallel to the grooves, statistically significantly more than expected by random, for grooves of all three widths (Table 1). However, the *Pax6*$^{+/+}$ cells were found to orient statistically significantly better than the *Pax6*$^{+/-}$ cells (Table 1).

Some cell migration (defined as the cells that adhered fully to the quartz slide and traveled more than the distance of one cell body during 24 h in culture) was observed, for the *Pax6*$^{+/+}$ and *Pax6*$^{+/-}$ cultures. Time-lapse tracking of individual cells showed that they migrated back and forth, generally parallel to the grooves (Figure 2B). However, in contrast to our previous experiments using human corneal epithelial cells [27], the percentage of cells migrating was low: 3.1% (58/1,853 cells) for *Pax6*$^{+/+}$ and only 0.9% (9/1,053 cells) for *Pax6*$^{+/-}$. It is possible that this reflects reduced adhesion of *Pax6*$^{+/-}$ cells to the substrate [49]. The data suggested that the migration of *Pax6*$^{+/-}$ cells was less robustly oriented parallel to the grooves than that of *Pax6*$^{+/+}$ cells, but because such a small proportion of cells was migrating, no numerical analysis was attempted.

Migration on corneal stroma: Having confirmed that corneal epithelial cells align to contact-mediated substratum cues and that *Pax6*$^{+/-}$ cells demonstrate a less efficient response, experiments were performed to determine whether corneal epithelial cells could receive contact-mediated cues from the corneal stroma or basement membrane in vivo. Wild-type mouse corneas were obtained and denuded by scraping such that all epithelial cells were removed but the basement membrane was intact [50,51]. Primary mouse corneal epithelial cells were then seeded onto these denuded corneas in vitro. The cells were allowed to attach for 1 h after which their migration was monitored for a further 24 h with time-lapse microscopy. Up to 70% of cells migrated on the corneal stromal bed. Cells were observed to migrate on roughly radial tracks either toward (centripetally) or away from the center of the cornea. Sixty-one cells were tracked in the initial experiments, and approximately half (33 out of 61, i.e., 54%) of the cells initially migrated toward and half (28 out of 61) away from the center of the cornea. The cells often doubled back on themselves. These preliminary data were consistent with the possibility that cells receive radial guidance cues from the basement membrane but in the culture system did not receive the appropriate cue to bias their migration centripetally instead of centrifugally.

To investigate cell migration further and to determine whether *Pax6*$^{+/-}$ cells showed a defect in their migration, the cell migration trajectory angles in relation to the direction toward the center of the cornea were evaluated (see Methods). Corneal epithelial cells from adult wild-type and *Pax6*$^{+/-}$ mice were plated onto denuded wild-type corneas and their subsequent migration tracked in relation to the center of the cornea and compared to random migration of corneal epithelial cells on cell culture plastic in relation to the center of the circular culture well. Few if, any, cells migrated perfectly radially on the corneal substrate. All cells showed a tendency to jostle and follow a wandering path. However, when compared to random migration on the tissue culture plastic, the migration of the *Pax6*$^{+/+}$ cells was found to be non-random, with a radial bias. Directionality was expressed as a migration index (see the Methods section) on a scale of 0 (perfectly radial migration with no deviation) to 1 (random migration with no directional bias). The migration index of *Pax6*$^{+/+}$ cells on denuded corneas was 0.770±0.042, n = 86 cells, three experiments, compared to random migration on the tissue culture plastic of 1±0.042, n = 164 cells. In contrast, the migration of *Pax6*$^{+/-}$ cells on wild-type denuded corneas was not statistically significantly different from random migration (migration index 0.919±0.071, n = 56 cells, three experiments; Kruskal–Wallis test, p = 0.0051, followed by Dunn's post-tests, p<0.01 for *Pax6*$^{+/+}$ versus random and p>0.05 for *Pax6*$^{+/-}$ versus random; Figure 3A).

The migration speed of the *Pax6*$^{+/+}$ cells on the stroma was found to be not statistically significantly different from *Pax6*$^{+/-}$. At approximately 10–12.5 µm/h, this was equivalent

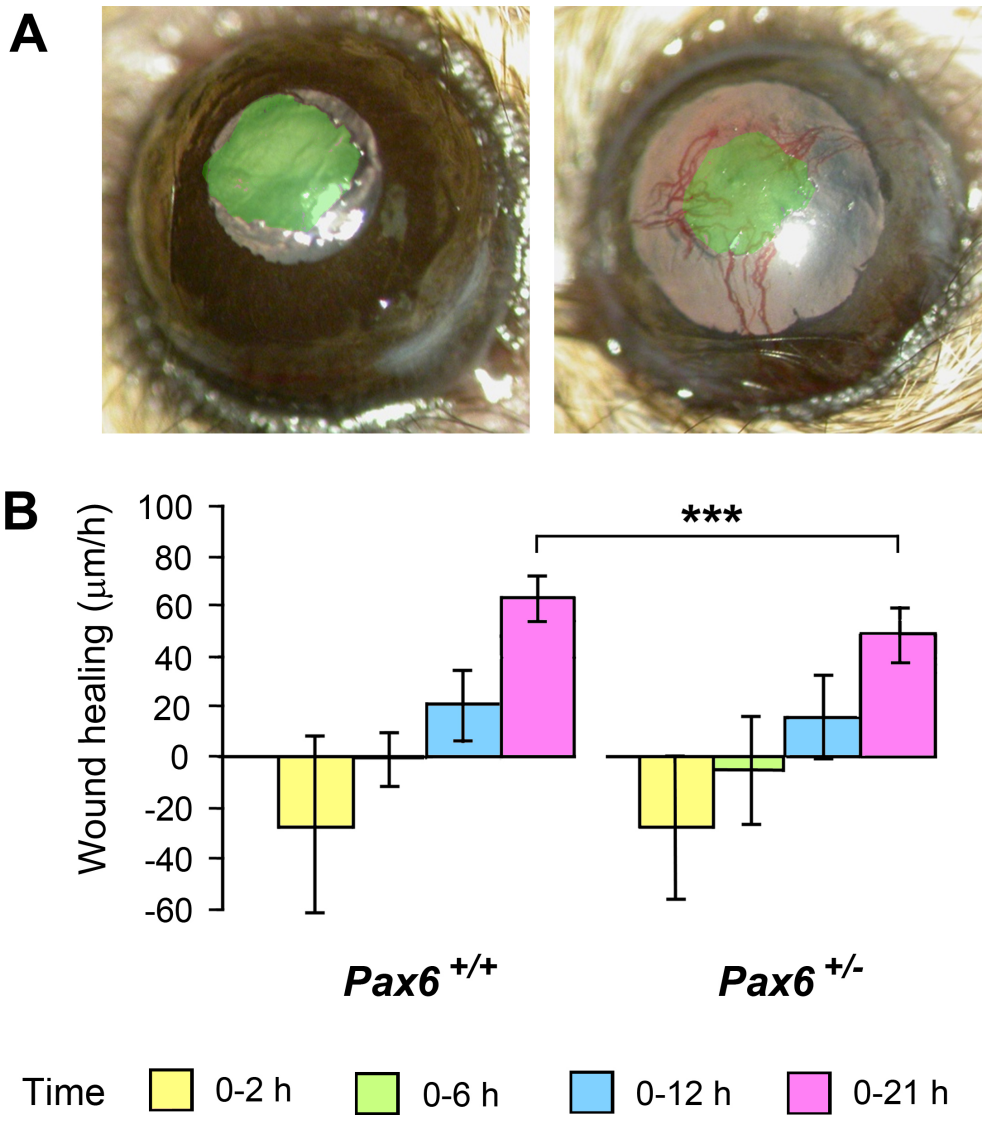

Figure 1. Corneal epithelial wound healing in vivo in mice. **A**: Images of wild-type (left) and *Pax6*$^{+/-}$ (right) adult mouse eyes shortly after corneal epithelial wounding. The wound areas of the approximately 1 mm diameter wounds have been highlighted for visualization. The boundary of the 1 mm diameter circular epithelial wound is visible. Vascularization of the cornea is a variable feature of the chronic wound healing phenotype of *Pax6*$^{+/-}$ mice (discussed in the text). **B**: Rates of epithelial wound healing in wild-type (left) and *Pax6*$^{+/-}$ (right) adult mice. Mean rates of migration are given over 0–2 h, 0–6 h, 0–12 h, and 0–21 h. Means ± standard deviation are shown. Number of wounds are 0–2 h, *Pax6*$^{+/+}$ = 10, *Pax6*$^{+/-}$ = 8; 0–6 h, *Pax6*$^{+/+}$ = 2, *Pax6*$^{+/-}$ = 2; 0–12 h, *Pax6*$^{+/+}$ = 8, *Pax6*$^{+/-}$ = 6; and 0–21 h, *Pax6*$^{+/+}$ = 12, *Pax6*$^{+/-}$ = 12. The wound healing profiles, with an initial retraction followed by re-epithelialization, are similar, but the migration of the *Pax6*$^{+/-}$ corneal epithelial cells is statistically significantly slower than in the wild-type in the latter healing phases.

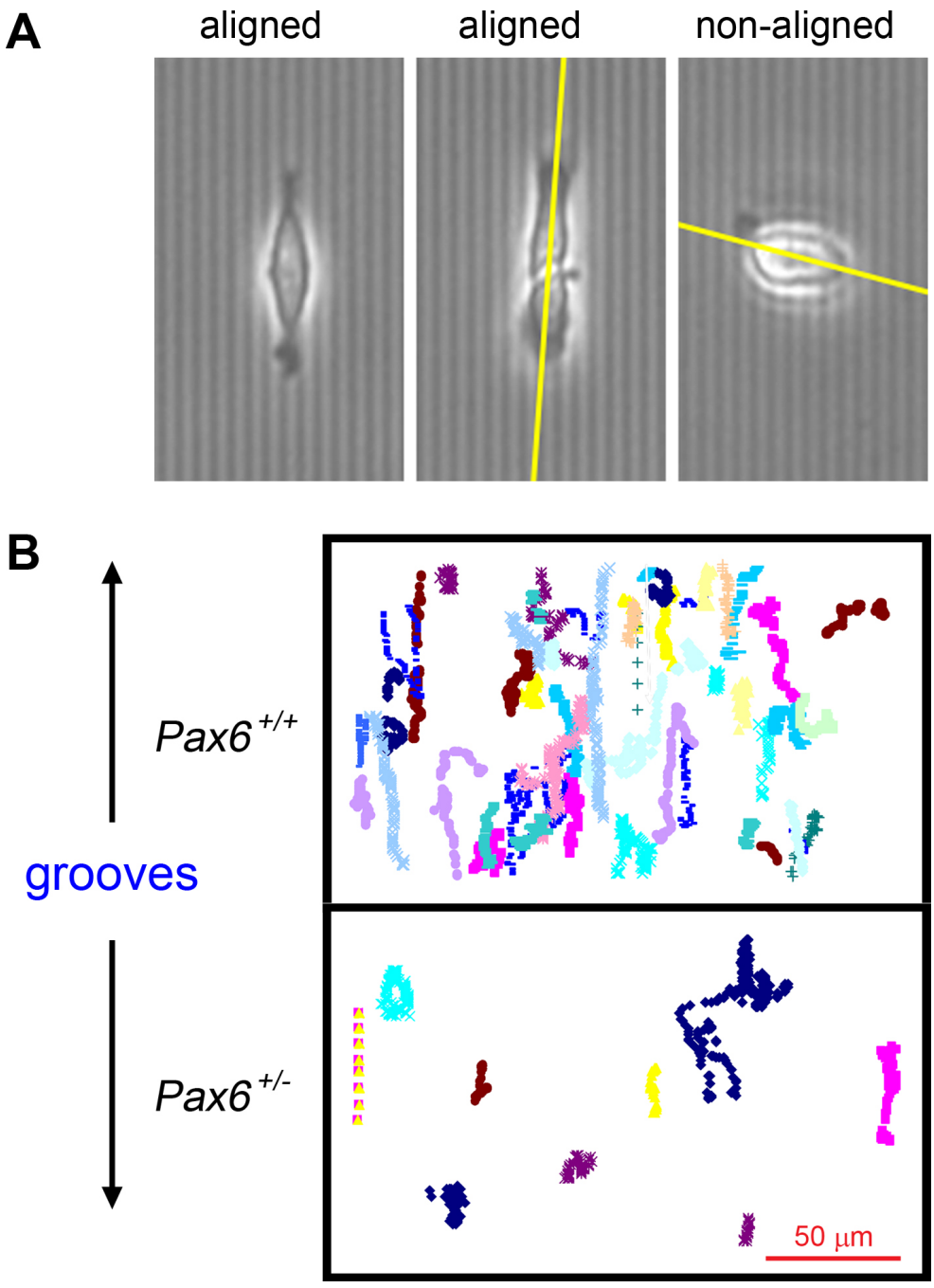

Figure 2. Alignment and migration of mouse corneal epithelial cells on grooved quartz substrata. **A**: Images of aligned (left and middle) and non-aligned (right) primary mouse corneal epithelial cells on 2 µm grooved quartz surfaces. Yellow lines indicate the long axis of the cells. See Table 1 for quantification. **B**: Tracks of cells migrating on 2 µm quartz grooves. Scale bar represents 50 µm.

to the migration of *Pax6*$^{+/+}$ cells on tissue culture plastic coated with laminin and faster than those on poly-L-lysine (approximately 5 µm/h; Figure 4).

These data suggested that corneal epithelial cells receive guidance cues from the basement membrane but that *Pax6*$^{+/-}$ corneal epithelial cells are unable to sense or respond to these cues. However, the migration, even of wild-type cells, was imperfectly directed. To determine whether, notwithstanding individual errors, the mean trajectory of the cell population was toward the corneal center, the tracks of all cells were projected onto a single map of each cornea, lines drawn connecting the start and endpoint of each cell, and the mean, most likely, point of convergence was plotted. This analysis confirmed that, on average, cells were moving toward or away from the center of the cornea (Figure 3B).

Table 1. Orientation of *Pax6*+/+ and *Pax6*+/− corneal epithelial cells on grooved quartz surfaces.

Groove width		1 µm		2 µm		4 µm	
Genotype		*Pax6*$^{+/-}$	*Pax6*$^{+/+}$	*Pax6*$^{+/-}$	*Pax6*$^{+/+}$	*Pax6*$^{+/-}$	*Pax6*$^{+/+}$
N (cells; 5 replicates)		384	322	384	399	304	369
Orientation of cells with respect to grooves	0–30°	274	275	289	338	213	308
	30–60°	71	28	59	41	53	40
	60–90°	39	19	36	20	38	21
Percentage of cells parallel (0–30°) to grooves		71.4	85.4	75.3	85.7	70.1	83.5
D-test versus random distribution for 0–30° (5 experiments)		***	***	***	***	***	***
D-test *Pax6*$^{+/+}$ versus *Pax6*$^{+/-}$ for 0–30° (5 experiments)		***		***		***	

***=p<0.001

The experiment was repeated using the extracellular protease, Dispase II, to denude *Pax6*$^{+/+}$ corneas before plating primary mouse corneal epithelial cells onto the bare stroma. In contrast to scraping, Dispase II removes the basement membrane [52], such that epithelial cells could be plated directly on the ribbon-like collagen network of the stroma. When this was performed, the cells adhered on the underlying stroma but did not migrate.

The failure of the *Pax6*$^{+/-}$ corneal epithelial cells to migrate radially on a wild-type basement membrane substratum, together with the reduced response on grooved quartz surfaces suggested that failure of the normal pattern of radial migration of *Pax6*$^{+/-}$ corneal epithelial cells in vivo was more likely due to failure of the epithelial cells to respond to guidance cues instead of a failure of the cues themselves. To test this, the ability of the *Pax6*$^{+/-}$ corneal stroma to drive centripetal migration was examined. It was found that wild-type epithelial cells migrated just as well on *Pax6*$^{+/-}$ corneas as on wild-type (migration index on *Pax6*$^{+/+}$ stroma: 0.838±0.0390, n = 156 cells, three experiments; migration index on *Pax6*$^{+/-}$ stroma: 0.800±0.038, n = 134 cells, three experiments; Figure 3). (The post-hoc Tukey-Kramer tests: *Pax6*$^{+/+}$ and *Pax6*$^{+/-}$ versus random migration gave p<0.05 and p<0.01, respectively; *Pax6*$^{+/-}$ versus *Pax6*$^{+/+}$ was not statistically significant).

Integrin β1 is not required for directionality: Integrin-mediated attachment of the cell to the extracellular matrix is a key determinant of keratinocyte cell migration. Functional integrins are dimers of α and β subunits; multiple isoforms of both exist, with the specificity of extracellular matrix protein binding determined by the combination of α and β subunits dimerized. For example, during wound healing, leading-edge keratinocytes bind dermal collagen using α2β1 integrin, and laminin 5 using α3β1 and α6β4 integrins, whereas α5β1 integrin binds fibronectin (reviewed in [39]). The key role of the β1 subunit is demonstrated by the observation that using the P4C10 antibody to block β1 integrin abrogates keratinocyte adhesion to laminin 5 and prevents wound closure by dermal keratinocytes in vitro [39,53]. To determine whether β1 integrin function is required for directed cell migration on the corneal stroma, an excess (5 µg/ml) of the P4C10 blocking antibody was added to the cultures of the wild-type mouse corneal epithelial cells on the wild-type stroma de-epithelialized by scraping as before. Time-lapse tracking of cell migration showed that the directionality of the cell migration was not adversely affected compared to control the wild-type cells (migration index 0.731±0.070), and not statistically significantly different from wild-type cells without blocking antibody (Figure 3). However, the percentage of cells that successfully migrated more than one cell body width dropped from 65.6% ± 4.03 (controls) to 22.0% ± 2.39 (β1 integrin-blocked; *t* test: $p = 2.7 \times 10^{-7}$; Figure 5). The smaller proportion of migrating cells may be an indication of reduced adhesion.

Directionality is mediated through cAMP-dependent pathways: Directional migration of keratinocytes in response to chemical gradients (chemotaxis) or electric fields (galvanotaxis) is mediated at least in part by G-protein coupled receptors (GPCRs) acting through phosphatidylinositol-3-OH kinase (PI3K) mediated activation of Akt/protein kinase B and cyclic-adenosine monophosphate (cAMP) dependent activation of cAMP-dependent protein kinase A (PKA)

[54,55]. Asymmetric accumulation of phosphatidylinositol-(3,4,5) triphosphate (PIP3), at the leading edge of the cells, synthesized by the activity of PI3K, is one of the earliest and key drivers of keratinocyte migration [56]. Crucial to this activity is PI3Kγ, a class I PI3K that is activated by Ras-GTP downstream of the heterotrimeric G protein Gβγ subunit. For example, neutrophils from mice in which the gene coding for PI3Kγ has been deleted fail to migrate directionally in response to chemoattractants such as interleukin-8 (Il-8) [57-59].

To determine whether PI3Kγ was required for contact-mediated directional migration on the corneal stroma, corneal epithelial cells were isolated from *PI3Kγ*$^{-/-}$ adult mice on the C57BL/6 genetic background and cultured on corneas that had been de-epithelialized by scraping, from *PI3Kγ*$^{-/-}$ and wild-type C57BL/6 adult mice. The *PI3Kγ*$^{-/-}$ corneal epithelial cells were found not to migrate on either the C57BL/6 wild-type stroma or on the *PI3Kγ*$^{-/-}$ stroma. The percentage of cells migrating more than one cell body width was only 1.98% ± 0.97 (*PI3Kγ*$^{-/-}$ epithelial cells on *PI3Kγ*$^{-/-}$ stroma) and 1.61% ± 0.65 (*PI3Kγ*$^{-/-}$ epithelial cells on *PI3Kγ*$^{+/+}$ C57BL/6 stroma; Figure 5). Because of the low number of cells migrating, no attempt was made to assess directionality. It was apparent that in this system, PI3Kγ is absolutely required for cell migration.

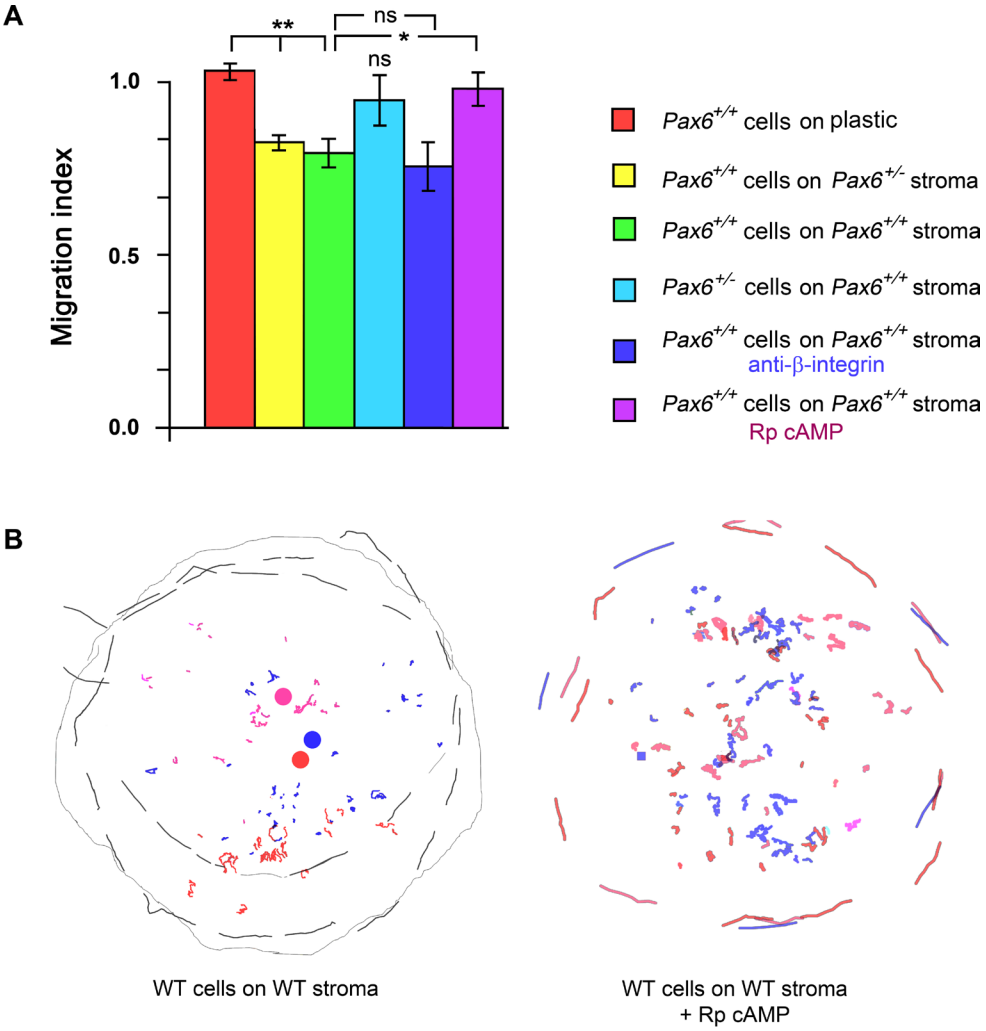

Figure 3. Migration trajectories of corneal epithelial cells in reconstructed corneal cultures. **A**: Migration index of non-random migration of corneal epithelial cells plated onto corneal basement membranes that had been de-epithelialized by scraping. The migration index is obtained by dividing the mean angle of cell migration by the radial direction to the corneal center under the experimental conditions described by the mean angle of cell migration of control cells on a tissue culture dish. Therefore, a migration index of 1 represents random migration; a migration index of 0 represents perfect radial migration toward the center of the cornea. Abbreviations: ns, nonsignificant. * represents $p<0.05$; ** represents $p<0.01$. All cells made migration errors, but wild-type mouse epithelial cells showed non-random migration biased radially when plated onto wild-type corneas. Conversely, *Pax6*$^{+/-}$ corneal epithelial cells plated onto wild-type stroma and wild-type cells treated with RP cAMPS to inhibit cAMP-mediated intracellular signaling migrated randomly. **B**: Migration tracks of wild-type mouse primary corneal epithelial cells on de-epithelialized wild-type corneas. Composite from three overlaid corneas from three separate experiments (pink, red, blue). For each cornea. the point of most likely convergence (i.e., with minimum mean migration error across all cells migrating on that cornea) is indicated (solid circles). **C**: Migration tracks of wild-type primary mouse corneal epithelial cells on de-epithelialized wild-type corneas with 40 µM RP cAMPS added to the culture medium. Composite from three overlaid corneas from three separate experiments (pink, red, blue).

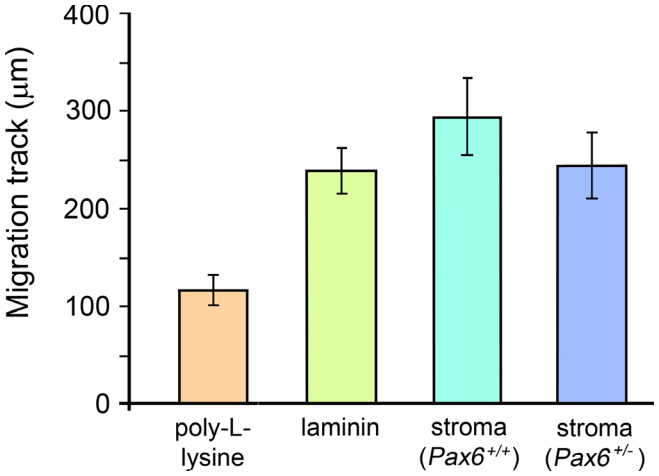

Figure 4. Migration speed of mouse corneal epithelial cells on different substrates. Tracks of cells were measured with time-lapse microscopy over 24 h culture and the mean total distance migrated ± standard error of the mean (SEM) is presented. From left to right: wild-type cells on tissue culture plastic coated with poly-L-lysine; wild-type cells on tissue culture plastic coated with laminin; wild-type cells on wild-type corneas de-epithelialized by scraping; *Pax6*$^{+/-}$ cells on wild-type corneas de-epithelialized by scraping.

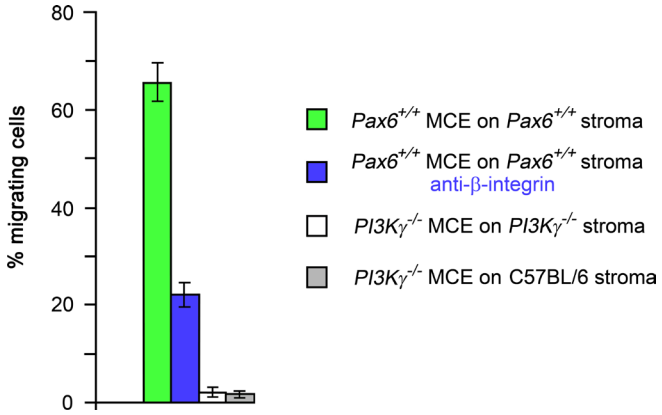

Figure 5. Failure of *PI3Kγ*$^{-/-}$ cells to migrate in reconstructed cornea system. Under conditions when 65% of wild-type cells plated onto wild-type corneas de-epithelialized by scraping successfully migrate, only 20% of cells do so in the presence of β1 integrin blocking antibody. Almost no *PI3Kγ*$^{-/-}$ cells migrate, whether plated onto wild-type or *PI3Kγ*$^{-/-}$ corneas. Abbreviation: MCE; mouse corneal epithelial cells.

Because knocking out PI3Kγ was uninformative for determining the role of a GPCR-PI3K-Akt pathway in driving the contact-mediated migration of corneal epithelial cells, cAMP was manipulated pharmacologically. The cAMP pathways were blocked using 40 µM Rp-8-Br-cAMP (RP-cAMPS) in cultures of wild-type epithelial cells plated onto wild-type corneal stroma, and de-epithelialized as above by scraping. RP-cAMPS acts as a potent and specific competitive inhibitor of the cAMP-induced activation of cAMP-dependent PKA, by blocking the cAMP-induced conformational transition of PKA [60].

This experimental treatment did not prevent migration, but randomized orientation of the trajectories (migration index 0.950±0.04, three experiments), not statistically significantly different from random migration (Figure 3). It was concluded that whereas PI3Kγ was required for corneal epithelial cell migration on the basement membrane, directionality was controlled by a cAMP-dependent pathway.

DISCUSSION

The cornea has long been used as a model system for the study of the interaction between epithelial cells and the extracellular matrix (reviewed in [37]). Experiments on corneal epithelial cells established the basis of epithelial–extracellular matrix interactions in the activation of focal adhesion kinase and PI3K that underlies directional cell migration [28,61]. The current study has shown that corneal epithelial cells receive directional cues from their own basement membrane and that this may explain their in vivo behavior of radial centripetal migration from the edge of the cornea to the middle. In the culture system employed here, epithelial cells still made trajectory mistakes, changed direction, and migrated back and forth, few if any on a perfectly radial track. Nevertheless, it was shown that migration was non-random and had a radial bias, and the cells converged, on average, on a point near the center of the cornea. Integrins and PI3K were shown to facilitate migration, but the directionality was mediated through cAMP pathways.

Guidance cues: Chemical or topographic?: Corneal epithelial cells are exquisitely sensitive to substrate topography, and it is well established that interactions between these cells and their substrate control multiple cell behaviors, including cell adhesion, alignment, migration, differentiation, and proliferation [28,29,62]. Scraping the corneal epithelium left the basement membrane intact with no trace of cell debris apparent, and care was taken to de-epithelialize the cornea using multiple randomly oriented strokes with an ophthalmological scalpel to ensure that no non-random pattern of abrasion was imposed on the basement membrane. However, it was considered possible that the basement membrane of the de-epithelialized cornea may retain a topographical or chemical "memory" or the previous in vivo radial migration that had occurred throughout adult life and that this was guiding the epithelial cells in culture. Although this would not invalidate the conclusions of this study, the observation that wild-type corneal epithelial cells continued to migrate radially when plated onto a *Pax6*$^{+/-}$ denuded cornea argues

against the possibility. Previous data have shown that radial corneal epithelial cell migration is disrupted or abolished in *Pax6*$^{+/-}$ mice [40] so it would be expected that the de-epithelialized *Pax6*$^{+/-}$ cornea would have little or no memory of previous radial migration that could guide wild-type cells. Together with the complementary observation that *Pax6*$^{+/-}$ epithelial cells do not migrate radially, even on a wild-type stroma, the data suggest that the cues directing mean radial cell migration of corneal epithelial cells are an inherent and immediate property of the corneal stroma, independent of previous epithelial cell behavior.

What these cues are remains to be determined. Dissection and culture of the corneal stromal bed remove the tissue from limbal vasculature and are unlikely to maintain a gradient of any soluble chemoattractant or repellent. A similar argument applies to the disrupted corneal epithelial cells, and in addition, any endogenous electrical cue would require the integrity of the epithelium to be maintained [25], which is not the case here. It is possible to postulate a gradient of any insoluble chemical that acts as guidance cue, for example, deposition of an adhesive extracellular matrix molecule increasing toward the center of the cornea that facilitates migration of epithelial cells up the adhesion gradient. Similar to that, it is possible to suggest durotaxis, the migration of epithelial cells up a gradient of increasing substrate rigidity toward the center of the cornea (from a more compliant to a less compliant substrate) [24,63]. In the case of an adhesion gradient, however, it is difficult to explain why cells migrate on mean radial tracks but go backward and forward, often doubling back on each other, up and down the postulated gradient. Foster et al. [24] showed that the nuclear localization of the Yes-associated protein (Yap) increased in corneal epithelial cells cultured on stiffer substrates and that Yap was primarily nuclear in basal corneal epithelial cells and cytoplasmic in basal limbal epithelial cells. This is strong evidence that there is a biologically relevant step change or steep gradient of substrate compliance at the limbal/corneal epithelial boundary that could form the mechanotransduction basis for centripetal migration. Each corneal epithelial cell has thousands of potential contact points on the basement membrane, the exact number determined by the density and pore size of the ECM, that act as points of focal adhesion and can align the cells [28,58]. The ECM change in substrate compliance at the edge of the cornea could provide asymmetric organization of focal adhesion that would facilitate directed migration. It is possible therefore that although in the reconstructed corneal system cells move backward and forward on radial tracks, in vivo the bias for centripetal, instead of centrifugal, migration is provided by the change of substrate compliance at the limbal–corneal boundary.

One possibility is that the basement membrane or corneal stroma has a structured topography that the epithelial cells can read to guide their migration. The aligned behavior and back-and-forth migration of corneal epithelial cells on grooved quartz surfaces described in this study, together with the less efficient response of *Pax6*$^{+/-}$ cells, recapitulates the behavior in the reconstructed corneal culture system and suggests a thigmotactic response to the microstructure of the surface of the de-epithelialized cornea. That cells behaved similarly on grooved surfaces irrespective of the width of the grooves suggests that the exact topographic dimensions of the surface are not qualitatively important for this aspect of their behavior. Scanning electron microscopy shows that the surface of the corneal epithelial basement membrane is a porous, richly interwoven network of fibrillary ECM proteins with topographical features in the nanometer scale and pore sizes in the 21–191 nm range [28,34]. Thus, the basement membrane has structure, but no report of overt asymmetry that could guide cells on radial tracks. In contrast, the collagen network of the corneal stroma is highly structured [64]. Corneal epithelial cells do not sit directly on the stroma, and it would be necessary to speculate that the stromal collagen topography is projected through the basement membrane. Whereas this might be possible in mice, it is unlikely to be the case in other species with a significant anterior limiting lamina (Bowman's layer), the layer of disorganized collagen fibrils directly under the basement membrane in species such as human that nevertheless also exhibit radial epithelial cell migration. It seems that the most likely explanation is that epithelial cells are responding to cryptic topographic or chiral cues in the cells' own basement membrane.

The role of cAMP in polarized cell behavior: Multiple growth factors and signaling pathways are known to control corneal epithelial cell migration and wound healing. Ligand-bound receptor tyrosine kinases such as fibroblast growth factor receptors (FGFRs) and epidermal growth factor receptor, among others, activate Src, mitogen-activated protein (MAP)-kinase, and Akt-mediated secondary messenger signaling pathways that are essential for normal directional migration in response to wounding [65]. Much research has focused on the molecular basis of the directional asymmetry of corneal epithelial cells, for example, through asymmetric localization of EGFR, Src-kinases, PI3K, and PTEN [25,56]. β-adrenergic signaling through cyclic AMP is also central [55]. G-coupled protein receptor signaling-mediated activation of adenylate cyclase leads to a rise in intracellular cAMP and subsequent activation of PKA [66]. One of the targets of PKA is phosphorylation of the cAMP response element binding protein (CREB), which controls the expression of multiple genes with a conserved cAMP response element

regulatory motif [67,68]. PKA has multiple targets, and its activation has profound effects on the organization of the cytoskeleton [69,70]. In particular, cAMP/PKA activation has been shown to be necessary for efficient cell migration, through mechanisms that include filopodia formation, actin polarization and activation of small GTPases [69,71]. β1-integrin-mediated adhesion to the extracellular migration is a key activator of the cAMP/PKA pathway driving the migration of fibroblasts, and integrin-based adhesion has been shown to modulate the response of corneal epithelial cells to topographic cues [72,73]. The role of cAMP signaling in the corneal epithelium is poorly understood [74]. cAMP signaling through CREB can be activated during squamous metaplasia of the corneal epithelium, suggesting a role in stress-induced receptor activation consistent with a role in the response to contact-mediated guidance cues [75]. Adrenergic receptors regulate the cAMP/PKA pathway and have been implicated in controlling the directed migration of corneal keratinocytes [55,76]. Corneal epithelial cells also express serotonin (5-HT7) receptors that signal through cAMP/PKA, but the role of serotonin in corneal epithelial maintenance is not known [74,77]. Alternative modes of cAMP-mediated signaling include regulation of cyclic nucleotide-gated ion channels [78] and activation of cAMP-binding popeye domain-containing proteins that also interact with epithelial channels [79]. Although ion channel polarization is important for epithelial homeostasis and is probably important for directed migration in applied electric fields [25], it seems unlikely to be responsible for the phenotype observed in the corneal reconstruction culture, in which inhibition of cAMP signaling caused loss of cell directionality without affecting the proportion of cells migrating or migration speed. We favor an alternative model in which the directional interpretation of integrin-mediated attachment to guidance cues in the basement membrane requires cAMP/PKA activity, whereas loss of integrin or PI3Kγ activity prevents cell migration by direct action on adhesion and motility.

The cellular basis of aniridia-related keratopathy: Pax6 is expressed in the developing lens, cornea, iris, ciliary body, and retina, with expression maintained throughout life in the lens and corneal epithelial cells and the neural retinal ganglion cells and amacrine cells [41]. PAX6 is a transcription factor that directly or indirectly controls the expression of many hundreds of downstream genes in the eye and is absolutely required for eye development [45,80]. Heterozygosity for *PAX6* in humans leads to multiple eye problems, including iris hypoplasia or absence (aniridia) cataract, glaucoma, retinal degeneration, and progressive corneal opacity (ARK) [8,81]. ARK is characterized by corneal epithelial fragility, encroachment of the conjunctival epithelium onto the cornea, vascularization, inflammation, and scarring of the underlying corneal stroma, leading to degenerative corneal opacity and blindness, usually during late childhood into early adulthood. The symptoms of ARK show many similarities with those of known LESC deficiencies caused by injury or disease, and clinically, ARK is treated as an LESC deficiency, with some success [7]. The *Pax6*$^{+/-}$ mouse is a remarkably good model of the human disease and shows a phenotype that recapitulates all the symptoms of ARK [82]. However, evidence of an LESC deficiency in *Pax6*$^{+/-}$ mice is sparse. In chimeric mice, mixtures of wild-type and *Pax6*$^{+/-}$ cells, clones of mutant LESCs were not as large as would be expected based on the global composition of the chimera, but *Pax6*$^{+/-}$ LESCs were present and functional, producing streams of cells that migrated into the cornea and showed only a mild defect in their ability to reach the center of the cornea [40]. In contrast, most of the features of ARK can be explained in terms of problems with the corneal epithelial cells themselves, a triple whammy of increased epithelial fragility, increased oxidative stress, and reduced metabolic capacity to deal with oxidative stress leads to a perpetual wound-healing physiologic state of the *Pax6*$^{+/-}$ epithelium that underlies eventual degeneration [83].

The observation that centripetal migration of corneal epithelial cells is disrupted in adult *Pax6*$^{+/-}$ mice [40] could be due to failure of normal guidance cues, failure of epithelial cells to respond to guidance cues, or constant divergence of normal radial migration to fill minor wounds (or any combination of the three). This study has addressed these possibilities. Regarding the contact-mediated guidance cues to which epithelial cells are exposed, the fact that wild-type epithelial cells can migrate normally on a *Pax6*$^{+/-}$ stroma suggests that normal guidance cues have not failed. In contrast, the failure of *Pax6*$^{+/-}$ cells to align properly when exposed to grooved quartz surfaces, and failure to migrate radially on the wild-type corneal stroma, demonstrates the second possibility, that mutant cells fail to respond to normal guidance cues. It seems likely that this could underlie at least some, maybe all, of the migration defects observed in vivo. However, based on Ou et al.'s data [83] and clinical observations of chronic wounding in patients with ARK, it is likely that the last possibility, a wounding overload, also contributes to the disease phenotype. In patients with *PAX6*$^{+/-}$, the cornea is fragile, and surgery can lead to poor healing, precipitating degeneration [81]. As stated in the Introduction, in vitro analyses of corneal epithelial wound healing have suggested delayed wound healing, normal wound healing, or faster wound healing, dependent on the culture system. Sivak et al. [84] suggested that in vivo, *Pax6*$^{+/-}$ corneal epithelia may heal faster than normal, without showing quantitative data. In this study, we

performed the first quantitative analysis of *Pax6*$^{+/-}$ corneal epithelia healing rate in vivo. In the first 2 h after wounding, the epithelium retracted, potentially due to mechanical consequences of injury, before healing rapidly at about 55 µm/h (wild-type) or 43 µm/h (*Pax6*$^{+/-}$). Thus, although the in vivo data confirm that there is a wound healing defect in *Pax6*$^{+/-}$ corneal epithelia, it is mild and affects only the later stages of wound healing, possibly due to failure of cell proliferation following an initially normal reorientation and migration phase. There is no reason to believe that *Pax6*$^{+/-}$ corneal epithelia cannot re-epithelialize acute wounds in time to prevent pathogen entry or inflammation. It seems more likely that the chronic effect of epithelial abrasion in corneas during life, combined with minor quantitative defects in stem cell activity, directional cell migration, and wound healing, leads to the degenerative corneal opacity in *Pax6* mutants.

Corneal regeneration and wound healing: The corneal epithelium is an unusual system in that the stem cells that maintain it are physically separate from the tissue they maintain [5]. Because of this, the long distance, extremely regular, directed epithelial cell migration is perhaps not a general model of cell movement in other uninjured tissues such as the skin. However, there is intense interest in the biomechanical engineering of wound repair in other tissues and the development of artificial corneas, for which understanding of how cell–ECM interactions guide cell migration is essential [85]. The robust contact-mediated directionality shown in the corneal epithelium is a paradigm of the behavior that must be recapitulated by artificial biomaterials. Understanding the biology of the corneal epithelium is essential to tissue engineering for clinical regeneration and repair.

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