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THE ROLE OF ELECTRICAL SIGNALS IN MURINE CORNEAL WOUND RE-EPITHELIALISATION

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Abstract

Ion flow from intact tissue into epithelial wound sites results in lateral electric currents that may represent a major driver of wound healing cell migration. Use of applied electric fields to promote wound healing is the basis of Medicare-approved electric stimulation therapy. This study investigated the roles for electric fields in wound re-epithelialisation, using the *Pax6*^{+/-} mouse model of the human ocular surface abnormality aniridic keratopathy (in which wound healing and corneal epithelial cell migration are disrupted). Both wild-type and *Pax6*^{+/-} corneal epithelial cells showed increased migration speeds in response to applied electric fields in vitro. However, only *Pax6*^{+/+} cells demonstrated directional galvanotaxis towards the cathode, with activation of pSrc signalling, polarised to the leading edges of cells. In vivo, the epithelial wound site normally represents a cathode, but 43% of *Pax6*^{+/-} corneas exhibited reversed endogenous wound-induced currents (the wound was an anode). These corneas healed at the same rate as wild-type. Surprisingly, epithelial migration did not correlate with direction or magnitude of endogenous currents for wild-type or mutant corneas. Furthermore, during healing in vivo, no polarisation of pSrc was observed. We found little evidence that Src-dependent mechanisms of cell migration, observed in response to applied EFs in vitro, normally exist in vivo. It is concluded that endogenous electric fields do not drive long-term directionality of sustained healing migration in this mouse corneal epithelial model. Ion flow from wounds may nevertheless represent an important component of wound signalling initiation.

Keywords

Galvanotaxis; Aniridic Keratopathy; electric field; cell migration; wound healing; Pax6; bioelectricity; cornea

INTRODUCTION

Failure of wound healing causes economic and personal harm to patients and society. Wound repair, for example in response to skin injury, may typically require the coordinated activity of multiple cell and tissue types over a period of days or weeks (Shaw and Martin 2004). Fundamental to the process however is successful re-epithelialisation, which restores

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barrier function and seals the wound against further environmental damage or pathogens. Lateral wound electric fields (EFs) (from surrounding intact tissue into the wound) have been demonstrated in wounded skin and corneas, and the fact that epithelial cell migration can be directed in vitro by application of lateral EFs suggested that endogenous electric currents may drive wound healing (Barker, et al., 1982, Chiang, et al., 1992, Foulds and Barker, 1983, Kloth, 2005, McCaig, et al., 2005, Nuccitelli, 2003, Zhao, et al., 2006). Endogenous electric currents arise as a consequence of asymmetric ion flow across tissues. For example the stratified corneal epithelium expresses active sodium transporters (Na^+/K^+ ATPases) producing inward sodium flow apically-basally, and actively transports chloride basally-apically, from the stroma to the tear film (Reid, et al., 2005). This separation of charges establishes a transepithelial potential difference (PD) of up to +40 mV, internally positive. Wounding breaches the electrical resistance and the PD drops to zero at the wound, establishing a laterally oriented EF extending up to a millimetre away from the wound (Chiang, et al., 1992), with the cathode at the wound site. Wound-induced electric currents can be measured empirically using a self-referencing vibrating microelectrode (Reid, et al., 2007) and the cathodal migration of healing cells in vivo can be recapitulated in vitro when rat, bovine, human and rabbit corneal epithelial cells migrate cathodally in applied electric fields of physiological strength or higher (Zhao, et al., 2006, Nishimura, et al., 1996, Sta Iglesia and Vanable, 1998, Zhao, et al., 1996a, Zhao, et al., 1996b). Pharmacological manipulation of endogenous ion flow to modulate the strength of the EF correlates with changes in the migration rate of cells in response to wounding (Reid, et al., 2005, Sta Iglesia and Vanable, 1998, Song, et al., 2002, Song, et al., 2004). The secondary messenger signals and cytoskeletal modification pathways by which cells interpret and respond to electric fields are substantially understood and include phosphatidylinositol-3-OH kinase (PI3K)-Akt pathways, small GTPases, and epidermal growth factor receptor signalling (Zhao et al., 2006; reviewed in McCaig et al., 2005) – the same pathways that are used to interpret, for example, chemotropic guidance cues. Activated (phosphorylated) Src tyrosine kinase signalling, which has been localised to the cut edge of wounded keratinocytes in vitro (Yamada et al., 2000), has also been shown to be polarised to the leading (cathodal) side of keratinocytes migrating in applied electric fields (Zhao et al., 2006). Inhibition of Src activity slows in vitro wound healing by cultured keratinocytes (Yamada et al., 2000).

Application of electric currents across chronic wounds and persistent ulcers (electrical stimulation therapy) is widespread and was approved for Medicare insurance coverage in 2002 (Kloth, 2005, Centers for Medicare and Medicaid Services (CMS), 2002) However standard protocols vary between studies, success is not guaranteed and the mechanism by which it is effective is still poorly known (Balakatounis and Angoules, 2008). Electrical stimulation therapy and in vitro studies show that EFs *can* modulate or drive migration, but understanding whether endogenous EFs are normally of significant importance and how EFs modulate healing is central to the optimisation of therapeutic strategies. This is especially important for interpretation of data from clinical trials using applied EFs to accelerate healing and regeneration (Shapiro, et al., 2005).

Wound healing may be impaired by genetic factors or disease (e.g. diabetes) and it is for pathological conditions that electric stimulation therapy may be most useful. Mice that are heterozygous for the transcription factor Pax6 are potentially informative in this respect. Pax6 mutation leads to chronic ocular surface degeneration characterised as aniridia-related keratopathy or (aniridic keratopathy) (ARK) (Ramaesh, et al., 2005). Pax6 is expressed in the corneal epithelium throughout life and Pax6^{+/-} mice have corneal epithelial cell migration defects (Collinson et al., 2004; Koroma et al., 1997). Epithelial monolayers cultured from Pax6^{+/-} corneal explants in vitro have a wound healing delay that is attributable to impaired calcium signalling at the wound site (Leiper, et al., 2006) thus linking ion flow with the wound response in a pathological model. Furthermore, in a whole

eye culture model, *Pax6*^{+/-} corneas maintained in basal medium without any serum or growth factor support show a reduction in wound healing re-epithelialisation compared to wild-type (Ou et al., 2008; Dorà et al., 2008).

Whereas the therapeutic relevance of EFs is robust, the importance of endogenous electric fields in directing cell migration has not been universally accepted, at least in part because of a lack of an in vivo genetic model of disrupted electrical activity. We now have that model: the corneal epithelium of the *Pax6*^{+/-} mouse, in which we report here that endogenous wound induced currents are abnormally low, or reversed. We have used this new model to investigate the importance of endogenous electric fields for guiding wound re-epithelialisation. Although results cannot be uncritically extrapolated to electric stimulation therapy of skin, the cornea offers several advantages for investigation of this type because the cornea is avascular (therefore does not form a clot), transparent (allowing imaging), and morphologically simple. Although corneal epithelial wounding induces underlying stromal vascularisation and keratocyte apoptosis, epithelial migration is rapid and the contribution of other tissues to immediate re-epithelialisation is relatively modest (Ramaesh et al., 2006). We have therefore used the mouse cornea to undertake direct investigation of factors controlling epithelial migration.

METHODS

The *Pax6*^{Sey-Neu} phenotypic null was maintained on a CBA/Ca background. *Pax6*^{+/-Sey-Neu} mice (*Pax6*^{+/-}) were mated to wild-type (WT). WT and *Pax6*^{+/-} littermates were used for comparisons. All experiments were performed under UK Home Office Licence and University of Aberdeen Ethical Review permissions

Current measurement

Eyes of *Pax6*^{+/-} and *Pax6*^{+/+} littermates were enucleated and placed into cold artificial tear solution (ATS) (BSS Sterile Irrigating Solution; Alcon Laboratories Inc., Fort Worth, TX, USA) containing (mM): 122.18 NaCl, 5.1 KCl, 1.05 CaCl₂, 0.98 MgCl₂, 2.96 Na₂HPO₄, 25 NaHCO₃, 5.11 D-Glucose, 0.3 glutathione disulfide, pH 6.85. Corneal epithelial wounding was performed by ophthalmological scalpel (Medical Sterile Products, Rincon, Puerto Rico). Vibrating probe measurements were made as described in (Reid, et al., 2007). In wounded corneas, measurements were made at the wound centre and within 50 µm of the cut edges, 10–20 minutes after wounding when wound induced current is maximal (Reid et al., 2005). Two wound edge measurements were taken per cornea, at opposite sides of the wound, and the mean wound edge current calculated.

Ion substitution—For ion substitution experiments, custom-made tear solutions with absence of major ions were used. Sodium-free ATS contained (mM): 2.14 KCl, 1.05 CaCl₂.2H₂O, 0.98 MgCl₂, 2.96 KH₂PO₄, 25 choline bicarbonate, 5.11 D-glucose, 0.3 glutathione disulfide, 125.4 choline chloride pH 6.85. Chloride-free solution contained (mM) 122.18 NaOH, 5.1 KOH, 1.05 Ca(NO₃)₂.4H₂O, 0.98 MgSO₄, 2.96 Na₂HPO₄, 25 NaHCO₃, 5.11 D-glucose, 0.3 glutathione disulfide, 131.34 methanesulfonic acid pH 6.85. Calcium-free solution contained (mM): 122.18 NaCl, 5.1 KCl, 0.98 MgCl₂, 2.96 Na₂HPO₄, 25 NaHCO₃, 5.11 D-Glucose, 0.3 glutathione disulfide, 2.1 mM choline chloride. Potassium-free solution contained (mM): 127.28 NaCl, 1.05 CaCl₂.2H₂O, 0.98 MgCl₂, 2.96 Na₂HPO₄, 25 NaHCO₃, 5.11 D-glucose, 0.3 glutathione disulfide. Immediately after wounding, electric current was measured in normal ATS, then the cornea was rapidly washed out in ATS missing one of the major ions described above, and the change in current again measured immediately. All experiments were repeated in the opposite order by performing the initial measurement in ATS lacking a major ion and the change in current

when complete ATS was washed through measured to confirm that this did not itself affect the magnitude of the current change. Replacing ATS with culture medium (see below), with or without serum, did not lead to changes in the wound-induced current, suggesting that the current did not depend critically on the composition or growth factor status of the environment, providing all major ions were present.

Wound healing experiments

Wound healing in whole eye culture was performed as described under Home Office licence in (Ou, et al., 2008), debriding the epithelium within a central circular wound made using a 0.8 mm diameter trephine. Eyes were enucleated and placed into 1 ml of complete culture medium (made up as follows: 15 ml Keratinocyte Basal Medium (Cambrex, UK) 19 ml DMEM:F12, 5 ml fetal bovine serum (12.5 % v/v), 125 μ M 2- β -Mercaptoethanol, 25 mM HEPES) for 24 h at 37°C, 5% CO₂. For in vivo wounding, mice (> 8 weeks old) were temporarily anesthetized and 1.5 mm diameter wounds were created. Wound healing rate was measured as described in Dorà et al. (2008) by photographing the ocular surface at a known scale after 0, 5 and 24 hours and calculating the mean diameter of the wound at each timepoint. After the required period of healing, eyes were fixed with 4% paraformaldehyde for immunohistochemistry or lysed for western blotting.

Western blotting

Tissues were lysed in 5% SDS, 1:50 protease and 1:100 phosphatase inhibitor cocktails (P2714 & P5726; Sigma Aldrich) and stored at -20°C until required. SDS-PAGE was performed in 12% polyacrylamide gels with 10% SDS and proteins were blotted onto nitrocellulose. Membranes were blocked in Tris-buffered saline (TBS) with 0.3% Tween-20, 10% skimmed milk, and incubated in primary antibody (mouse anti-Src, Cell Signaling Technology #2110; rabbit anti-pTyr416 Src, Cell Signaling Technology #2101; HRP-conjugated anti- β -actin, Sigma) diluted in 2% bovine serum albumin (BSA), 0.05% Tween-20, TBS overnight at 4°C and washed. HRP conjugated species-specific secondary antibody was applied for 1 hour before washing in TBS, 0.05% Tween-20, with detection using the enhanced chemiluminescence (ECL) kit (Amersham).

Immunohistochemistry

Whole corneas were fixed in 4% paraformaldehyde for 2 hours, washed in phosphate-buffered saline (PBS), and permeabilised by digestion in 1% pepsin, 10 mM HCl, 10 minutes, 37°C, followed by a 10 minute wash in 0.1 M sodium borate, pH8.5, room temperature, and 2 washes in PBS. Antigen retrieval was performed in 1% sodium dodecylsulfate, PBS, 5 minutes followed by 5 $\times$ 15 minute washes in PBS. Corneas were incubated in blocking buffer (PBS, 0.3% BSA, 0.1% Triton X-100, 2% normal goat serum, 2% donkey serum) 2 hours, followed by overnight incubation, with rotation at 4°C in primary antibody (anti-Src or anti-pSrc as above) diluted 1:150 in blocking buffer. After 4, 30 minute washed in PBS at room temperature, secondary antibody (Alexa-594 conjugated donkey anti-rabbit IgG, Alexa-488 conjugated goat anti-mouse IgG₁: Molecular Probes, Invitrogen) was added for 3 hours at room temperature with rotation, followed by 4 washes in PBS and mounting for fluorescence microscopy.

Cell monolayers were treated similarly, except that after 20 minute fixation in PFA, permeabilisation was by immersion for 10 minutes in methanol at -20°C, with no pepsin or SDS incubation.

In vitro applied electric field

Primary culture of murine corneal epithelial cell monolayers were prepared as described in Hazlett, et al. (1996) and cultured in complete culture medium with 10% fetal bovine serum. Each epithelial cell culture was derived from a different, single cornea, with no mixing of cells from different corneas. Cells were cultured on tissue culture plastic in order not to influence the experiments by imposing an ECM on the cells. Monolayers were used in preference to single cells in order to more accurately recapitulate the movement of cell sheets that occurs during re-epithelialisation, and were subjected to linear applied EFs applied as described previously (Song, et al., 2007). Cells received uniform field strength of 200 mV/mm, duration 2 hours and were tracked by time-lapse microscopy. Data were collected using Simple PCI software and analysed in ImageJ. The migration of each cell was recorded and the total distance travelled (the track of the cell) measured over time to give mean migration speed in $\mu\text{m/h}$ (Tt). The straight line linking the positions at the start and end of field application of a cell gives the total displacement of the cell. The angle of this displacement was measured relative to direction of the cathode (θ) and expressed as a cosine ($\cos\theta$) with $\cos\theta = 1$ representing a net migration directly to the cathode, $\cos\theta = 0$ representing migration perpendicular to the cathode-anode axis and $\cos\theta = -1$ representing migration to the anode. Dx, a value of displacement directly towards the cathode in μm was noted. Mean values of Tt, $\cos\theta$ and Dx were calculated for several hundred cells in culture under each experimental or control condition.

RESULTS

Migration of mouse corneal epithelium cells in applied electric fields in vitro

Bovine, rat and rabbit corneal epithelial cells migrate cathodally in applied electric fields of around 100 – 500 mV/mm. The galvanotactic migration is serum-dependent (Zhao et al., 1996). It was necessary to determine whether murine corneal epithelial cells could respond to EFs because this had not previously been done. Monolayers of *Pax6*^{+/+} and *Pax6*^{+/-} mouse corneal epithelial cells (each monolayer was derived from only one cornea) were cultured in vitro in complete culture medium with fetal calf serum (Hazlett et al., 1996) and monitored for 60–90 min of pre-field migration before an EF of 200 mV/mm was applied. Migration was tracked by time lapse microscopy. Mean trajectory speed (Tt) was calculated by measuring the total (tortuous) track length of cell migration. Mean direction of migration was expressed both as the mean absolute straight line displacement towards the cathode (Dx) and also as the mean cosine of the angle between the cells' displacement direction and the cathodal direction ($\cos\theta$). Mean positive Dx or $\cos\theta$ indicates cathodal migration.

It was shown for the first time that mouse corneal epithelial cells respond and migrate cathodally to applied EFs. Migration of wild-type cells in no EF on tissue culture plastic was random ($\cos\theta = 0.06 \pm 0.03$; Dx: $0.22 \pm 0.26 \mu\text{m}$, $n = 199$). An EF of 200 mV/mm for two hours biased mean movement of *Pax6*^{+/+} cells (measured as an angle θ relative to the vector between the cell and the cathode, and as an absolute displacement Dx along that vector) significantly towards the cathode ($\cos\theta = 0.266 \pm 0.028$; Dx: $6.07 \pm 0.71 \mu\text{m}$, $n = 579$. t -test $P = 0.0002$). Application of the field also increased the speed of cell migration by 42% (Tt: $11.47 \pm 0.35 \mu\text{m/hour}$) compared to random migration in no field ($8.07 \pm 0.36 \mu\text{m/hour}$; $n = 199$; t -test: $P < 0.0001$).

Pax6^{+/-} cells also responded to an applied EF. Migration speed (Tt) increased 66% from $7.50 \pm 0.25 \mu\text{m/hour}$ ($n = 419$) to $12.34 \pm 0.82 \mu\text{m/hour}$ ($n = 498$; t -test: $P < 0.0001$) upon application of the field, but the directionality of the response was marginal – some cells migrated apparently randomly or anodally, but those that moved cathodally migrated further on average. Mean $\cos\theta$ (-0.016 ± 0.033) was not significantly different from zero but there

was a small but significant bias of displacement of cells to the cathode: (Dx) was 1.63 ± 0.82 μm , significantly different from random migration in no field (t -test: $P = 0.024$).

Src localisation and activation in corneal epithelial monolayers in applied EFs

Upregulated activation of Src tyrosine kinases has previously been shown to be important for epithelial healing in vitro, and pSrc localises asymmetrically to the leading edge of galvanotaxic keratinocytes (Yamada et al., 2000; Zhao, et al., 2006). We investigated whether defects in Src signalling could underlie the reduced electrotactic response in *Pax6*^{+/-} corneal epithelial cells. We confirmed by immunohistochemistry on cells after 120 minutes in the EF that pSrc-kinase activity was polarized in the direction of migration in *Pax6*^{+/+} cells (highest levels at the leading edge, towards the cathode) (Figure 1A,B,E). *Pax6*^{+/-} cells showed little if any polarization of pSrc (Figure 1C, D), correlating with their reduced cathodal migration response. Phosphorylated epidermal growth factor receptor (pEGFR), RhoA and PI3-kinase were not polarised and did not show any changes in cell localization after electrotaxis in wild-type or *Pax6*^{+/-} (data not shown).

pSrc was also upregulated in *Pax6*^{+/+} cells but not in *Pax6*^{+/-} cells after application of an EF, expressed as a ratio of phospho-Src/total-Src (pSrc/tSrc) after quantitative densitometry of western blot (Figure 1F, Table 1). In addition the absolute levels of tSrc were higher in WT than *Pax6*^{+/-} (Fig. 1F).

Incubating WT cells in the specific Src tyrosine kinase inhibitor PP1 (20 μM) (Xu, et al., 2006) 3 h prior to application of the field eliminated the directedness of electrotactic response ($\cos\theta = 0.0089 \pm 0.0462$, $n = 226$ cells), and also significantly reduced their speed in EF (Tt of *Pax6*^{+/+} in EF with PP1 7.98 ± 0.23 $\mu\text{m}/\text{hour}$; t -test: $P < 0.0001$ cf. untreated *Pax6*^{+/+} in EF 11.47 ± 0.35 $\mu\text{m}/\text{hour}$ as above). Thus, inhibition of Src activity totally eliminates directionality of the cell migration response to an applied EF and reduces cell migration speed to approximately that of untreated cells migrating randomly in no field (8.07 ± 0.36 $\mu\text{m}/\text{hour}$ as above).

In conclusion, application of EFs to corneal epithelial cells in vitro caused mean biased cathodal migration, which required upregulation and cellular polarisation of pSrc and suggested that WT and *Pax6*^{+/-} corneal epithelial cells may respond to endogenous fields during wound healing. This was tested.

Wound mediated current of *Pax6*^{+/-} and *Pax6*^{+/+} corneal epithelium

When the corneal epithelium is lesioned, an induced wound lateral current is established with the wound acting as a cathode (Figure 2A). On the basis of data above, we hypothesised that *Pax6*^{+/-} epithelial cells should be able to respond to the wound induced current, but that their cathodal (healing) migration may not be as robust as wild-type. The profile of endogenous electric currents in corneal epithelial tissue was measured using a vibrating microelectrode (Reid, et al., 2007). Unwounded corneal epithelia of *Pax6*^{+/+} eyes displayed a very small mean outward current (from the interior of the eye to the tear film) of $0.3 \mu\text{A}/\text{cm}^2$ ($n = 5$), consistent with previous measurements of the rat cornea in Reid et al. (2005). Unwounded *Pax6*^{+/-} epithelia showed a mean outward current that was smaller, but not significantly different, from wild-type ($0.026 \mu\text{A}/\text{cm}^2$, $n = 11$, t -test: $P = 0.101$).

Wounding the epithelium always produced an outward current (from the intact tissue into the wound) in *Pax6*^{+/+} corneas ($3.8 \mu\text{A}/\text{cm}^2$; $n = 18$; Figure 2B) measured as described in the Methods section within 50 μm of the cut edges of the wounds. This is the first measurement reported for mouse corneas and is smaller than values measured for more highly stratified bovine corneas (Chiang, et al., 1992) but equivalent to values published previously in rats (Reid, et al., 2005, Reid, et al., 2007, Song, et al., 2007). *Pax6*^{+/-} corneas

showed significantly lower mean value of the wound-induced current ($0.714 \mu\text{A}/\text{cm}^2$; $n = 30$; t -test: $P < 0.0001$; Figure 2B). Interestingly in 13/30 $Pax6^{+/-}$ eyes the current was directed inward (the wrong way). If only the mean of those 17/30 $Pax6^{+/-}$ epithelia that showed an outward wound-induced current (henceforth $Pax6^{+/-}_o$) was considered this was still significantly smaller than that of wild-types ($2.044 \mu\text{A}/\text{cm}^2$; t -test: $P = 0.005$, Figure 2B). Reid et al. (2005) previously showed that the wound edge currents in rat corneas were significantly greater than currents measured at the wound centre whereas in this study there was no significant difference ($n = 17$; t -test $P = 0.32$; Supplementary Fig. 1). The apparent discrepancy is presumably because of the difference in wound size (3.5 mm in Reid et al. (2005) and $800 \mu\text{m}$ here). Subsequent analyses therefore only describe the wound-edge currents.

Ion substitution experiments were used to characterise the contribution of sodium and chloride to the net current in $Pax6^{+/+}$ and $Pax6^{+/-}$ eyes. Artificial Tear Solution (ATS - see the Methods Section) lacking one of the major ion components which carry the electrical current was used for vibrating probe measurement to determine the differences in current composition between $Pax6^{+/+}$ and $Pax6^{+/-}$ corneal epithelium. In unwounded epithelia no significant difference was found between the changes in ion flow of $Pax6^{+/+}$ and $Pax6^{+/-}$ intact epithelia in Na^+ -free, Cl^- -free or Ca^{2+} -free ATS. Values are expressed as the change in current ($\Delta\text{current}$) after ion depletion, positive values indicate increased current, negative values indicate reduced current (or a more negative current for $Pax6^{+/-}_i$). Upon sodium substitution (by choline) in unwounded epithelia, intact $Pax6^{+/+}$ and $Pax6^{+/-}$ corneas responded similarly, $\Delta\text{current}$ ($Pax6^{+/+}$) = $-0.327 \mu\text{A}/\text{cm}^2$, $Pax6^{+/-}$ = $-0.667 \mu\text{A}/\text{cm}^2$, $n = 5$, t -test, $P = 0.23$; chloride replacement $\Delta\text{current}$ ($Pax6^{+/+}$) = $0.0125 \mu\text{A}/\text{cm}^2$, $Pax6^{+/-}$ = $0.146 \mu\text{A}/\text{cm}^2$, $n = 6$, t -test, $P = 0.18$; calcium substitution by choline, $\Delta\text{current}$ ($Pax6^{+/+}$) = $0.136 \mu\text{A}/\text{cm}^2$, $Pax6^{+/-}$ = $-0.034 \mu\text{A}/\text{cm}^2$, $n = 5$, t -test, $P = 0.07$.

In wounded epithelia sodium substitution with choline caused a significantly larger decrease of the current in $Pax6^{+/-}$ eyes ($\Delta\text{current} = -2.527 \mu\text{A}/\text{cm}^2$, $n = 15$) compared to the decrease in $Pax6^{+/+}$ eyes ($\Delta\text{current} = -0.237 \mu\text{A}/\text{cm}^2$, $n = 13$; t -test: $P = 0.0006$) (Figure 2C). This significant decrease of the current in $Pax6^{+/-}$ eyes suggested hyperactivation of the sodium pumps and channels. Use of chloride-free ATS caused the wound-induced current to increase with no significant differences between $Pax6^{+/+}$ ($\Delta\text{current} = 0.912 \mu\text{A}/\text{cm}^2$ increase) and $Pax6^{+/-}$ ($\Delta\text{current} = 0.229 \mu\text{A}/\text{cm}^2$), $n = 8$; t -test: $P = 0.13$. Potassium substitution also did not show any significant difference between $Pax6^{+/+}$ ($\Delta\text{current} = -1.184 \mu\text{A}/\text{cm}^2$) and $Pax6^{+/-}$ ($\Delta\text{current} = -0.429 \mu\text{A}/\text{cm}^2$, $n = 4$, t -test: $P = 0.42$). We previously showed defective calcium signalling in $Pax6^{+/-}$ cells (Leiper, et al., 2006). Nevertheless calcium substitution did not produce any significant differences in wound-induced current between $Pax6^{+/+}$ and $Pax6^{+/-}$ ($\Delta\text{current} = 0.476 \mu\text{A}/\text{cm}^2$ and $0.296 \mu\text{A}/\text{cm}^2$ respectively; $n = 6$; t -test: $P = 0.37$) (Fig. 2C). In conclusion, the wound induced currents in $Pax6^{+/-}$ corneal epithelia rely more heavily on hyperactivity of sodium transport, compared to wild-type. We previously showed that $Pax6^{+/-}$ corneas are partly permeable to Lucifer Yellow dye, which suggests they may not fully maintain barrier function (Ou, et al., 2008). Hyperactivation of sodium channels may be a cellular response to compensate for the leakiness of the $Pax6^{+/-}$ mutant epithelium and re-establish a trans-corneal potential difference that more closely matches wild-type.

A genetic test of roles of EFs in wound healing

If endogenous electric fields were the primary drivers of direction or speed of cell migration during wound healing, we would predict that those $Pax6^{+/-}$ corneas with a reverse-directed (inward), wound current (henceforth $Pax6^{+/-}_i$) would fail to heal normally. Our previous work has suggested that in the absence of fetal calf serum, $Pax6^{+/-}$ corneal epithelia in ex-vivo cultures heal more slowly than wild-type (Dorà et al., 2008; Ou et al., 2008) but that

Pax6^{+/-} wound healing can be improved by addition of EGF. However optimal electrotactic response of corneal epithelial cells in culture requires serum (Zhao et al., 1996) so, after confirming that addition of serum does not affect the endogenous current in culture (see Methods) we measured the wound-induced current and wound healing rate in wild-type and *Pax6*^{+/-} mouse corneas for the first time to determine whether the two values were correlated.

Circular wounds were made in corneal epithelia of *Pax6*^{+/+} and *Pax6*^{+/-} littermates, the wound-induced electric current was measured, and the wounds were then allowed to heal in whole eyes ex-vivo in presence of 12.5% serum. Wound diameter was measured at 0, 5 and 24 hours post-wounding. Interestingly, under these conditions all epithelia healed at approximately equal rates irrespective of genotype or (for *Pax6*^{+/-}) the direction of the wound-induced electric current (Figure 2D). Leading edge migration was: *Pax6*^{+/+} 17.42 ± 2.65 μm/h, *Pax6*^{+/-}_i 16.38 ± 1.29 μm/h, *Pax6*^{+/-}_o 13.98 ± 1.34 μm/h, n = 8 for each, ANOVA: *P* = 0.411). This suggests that the magnitude and direction of endogenous EFs does not guide corneal epithelial cell migration. We have confirmed also that *Pax6*^{+/-} corneal epithelia heal normally over 12 hours in vivo suggesting that this result is not an artefact of culture conditions (PW, JMC, in prep).

Electric current versus wound healing rate

Wound-induced current changes over time (Reid, et al., 2005) (Supplementary Figure 2). If the magnitude of the endogenous EF was driving the speed of cell migration then it should be possible to correlate the two, as suggested by Reid et al. (2005) and Song et al. (2002). For the first time, we tested directly whether this was the case by measuring, in a longitudinal study, the changing profiles of wound induced electric current and speed of cell migration concurrently after wounding *Pax6*^{+/+} and *Pax6*^{+/-} eyes. The sizes of the wound and the electric current were measured at 0 h, 2 h, 4 h and 6 h, after which eyes were cultured up to 24 h. In *Pax6*^{+/+} eyes the mean wound-induced current at wounding of 3.2 ± 0.33 μA/cm² (n = 8) decreased over 6 h and reached 0.9 ± 0.28 μA/cm² (Figure 3A). In *Pax6*^{+/-} eyes (*Pax6*^{+/-}_o and *Pax6*^{+/-}_i) the mean wound-mediated current was lower: -0.35 ± 0.63 μA/cm² (n = 8) and not significantly different (1.23 ± 0.24 μA/cm²) 6 h post-injury (*t*-test: *P* = 0.20; n = 8 of each genotype) (Figure 3B). Wound healing rates remained constant, in both genotypes even though the wound-induced current varied, and again there was no significant difference in the rate of wound healing between wild-types and *Pax6*^{+/-}. For each 2 hour time-point (0 to 2 h, 2 to 4 h, 4 to 6 h) for each eye (of both genotypes), the magnitude of the wound-induced electric current was plotted against migration rate during that 2 hour period on scatter graphs (Figure 3C, D). These revealed no significant relationship (*P* > 0.05) between magnitude of the wound induced current and rate of wound healing, for wild-type or *Pax6*^{+/-} corneal epithelia. This suggests there is little or no significant contribution of endogenous electric current to directedness of migration in healing *Pax6*^{+/+} or *Pax6*^{+/-} corneal epithelia.

No polarisation of Src-family kinases after wounding corneal epithelia

We hypothesised that the failure of cells to respond directionally to endogenous EFs may lie at the level of Src activation. Corneal epithelial wounds were performed on 20 adult *Pax6*^{+/+} and 20 *Pax6*^{+/-} mice. Eyes of both genotypes always healed in vivo, with no additional Src activation observable by western blot, 80, 120 or 240 hours after wounding (Figure 4A, Table 2).

It was hypothesised that if pSrc upregulation was restricted to cells at or near the wound edge, then this may not be detectable at the level of Western blot analysis. Dissection of wounded corneal buttons into an inner near-wound button and an outer perilimbal annulus

also suggested no upregulation of pSrc around the wound (data not shown), but the same formal possibility of upregulation in a small number of cells at the migrating leading edge remained. Src localisation after wounding was therefore investigated by immunohistochemistry, 20 minutes, 1 and 4 hours after wounding, in wild-type and *Pax6*^{+/-} corneas (n = 4–8 of each genotype at each time point). At all timepoints, for both genotypes, there was no evidence of additional Src activation at the wound edge (comparing cells at the wound edge with cells at the edge of the cornea away from the wound) (Figure 4B–I). Further, no polarisation of pSrc was observed during cell migration after wounding. Src protein was detected uniformly at the edges of cells, both close to and away from the wound edge. This held true even at 20 minutes post-wounding when empirically we have determined that the wound current would be approaching a maximum (Reid et al., 2005; this study). Although wound current could not be measured in vivo because of the requirement to submerge the eye, eyes from >20 *Pax6*^{+/-} mice were used, and no variability was observed that would correlate with the direction of the wound current. Hence, although Src polarisation is seen in *Pax6*^{+/+} cells after 2 h exposure to an applied EF in vitro, eliciting an endogenous EF by wounding in vivo does not lead to a similar polarisation of pSrc. This represents a fundamental difference between response of cells to their endogenous EFs and to applied EFs.

DISCUSSION

It is well established that cells can migrate in response to applied electric fields (McCaig, et al., 2005). In this paper we have used the first genetic model of reversed endogenous EFs, the *Pax6*^{+/-} corneal epithelium. Around 43% of *Pax6*^{+/-} eyes were identified to have reversed current, secondary to abnormal Na⁺ transport across the epithelium. Pharmacological manipulation of sodium transport can reduce the endogenous wound induced current (Reid, et al., 2007, Sta Iglesia and Vanable, 1998, Song, et al., 2004). Our measurements provided an opportunity to test the importance of endogenous electrical cues without having to apply drugs: wound healing rate was found not to change significantly, regardless of the strength or direction of wound-mediated endogenous current. Although the effects were most marked for *Pax6*^{+/-} eyes, concurrent time-lapse EF measurement and migration speed analysis confirmed the same result for *Pax6*^{+/+} corneas. Hence, even in wild-type corneas the magnitude of the endogenous current was not a good predictor of the immediate rate of cell migration. We were able (data presented in Figure 3) to discount the possibility that there is a double defect in *Pax6*^{+/-} corneas: i.e. that magnitude of the endogenous current does direct migration speed, but that in addition to exhibiting a reversed wound current, *Pax6*^{+/-} corneas also exhibit a reversed (anodal) directional response to currents and that the result of this is that healing happens normally. If this were the case we would expect a v-shaped response in Figure 3D (centred on the origin of the scattergraph), but this was not the case. Indeed in both Figure 3C and 3D, some of the highest cell migration speeds occurred at the lowest measured currents. An alternative possibility is that the magnitude of the induced current is not proportional to migration speed, and that even the smallest currents recorded here, e.g. < 0.5 $\mu\text{A}/\text{cm}^2$ are enough to maintain cell migration, or that the role of endogenous currents is primarily to kick-start directional migration in the immediate aftermath of wounding. We consider it very possible that endogenous currents are important for planar polarisation of the cell immediately after wounding, and return to this point in the final section.

In light of our previous data that suggested a re-epithelialisation delay in *Pax6*^{+/-} corneal epithelia cultured ex vivo without serum (Ou et al., 2008; Dorà et al., 2008), it is clear that *Pax6*^{+/-} corneas are particularly reliant on externally supplied growth factors and that application of EGF or serum can restore wound healing. Other independent groups have now shown that under in vivo and in vitro conditions, *Pax6*^{+/-} corneal epithelial healing

proceeds reliably (e.g. Sivak et al., 2004; Ramaesh et al., 2006), and although the wound healing rate can be modulated by growth factors we conclude that there are no conditions under which *Pax6*^{+/-} corneal epithelia appear to show reverse or no wound-induced migration.

We have shown that cells can and do migrate against their endogenous fields. However, wild-type corneal epithelial cells from several species reliably migrate cathodally in response to applied electric fields in vitro, confirming the potent ability of EFs to direct cell migration. A voltage of 200 mV/mm has previously been considered physiological as it is roughly equal in magnitude to that measured in living tissue (Barker, et al., 1982). The resistivity of the corneal epithelium has never been directly measured but extrapolating from published data of other tissues (Faes, et al., 1999) is likely to be between 200 and 1000 Ωcm. Even allowing that directly measured wound induced currents (of around 4–50 μA/cm² in different systems) probably underestimate the actual wound edge current (Chiang, et al., 1992) it seems unlikely that the endogenous wound-induced fields exceed 10 mV/mm in the corneal epithelium. This suggests that applied therapeutic electric fields may be an order of magnitude greater than the endogenous field in some systems.

No correlation was found between the direction of migration of *Pax6*^{+/-} cells in applied EFs and the direction (*Pax6*^{+/-} i or *Pax6*^{+/-} o) of the wound induced current. Each cultured monolayer was derived from a single cornea and the migration of *Pax6*^{+/-} cells from a single culture in applied EFs was heterogeneous. The reduction of *Pax6*^{+/-} directed cell migration in applied fields correlated with failure to activate and polarise pSrc to the leading edge of the cells. Whereas Src activity was required for both increased migration speed and directedness in applied EFs, observations of *Pax6*^{+/-} cells, which increase their migration speed in response to an EF but show much poorer directionality, suggested that it is the polarisation of Src activity that mediates the directional EF response (Zhao, et al., 2006).

In contrast, we did not observe widespread activation or polarisation of Src in healing mouse corneal epithelia of any genotype in vivo. We cannot at present eliminate the possibility that a transient upregulation or polarisation of Src occurs rapidly after wounding and that we have missed it in our analyses. However at present the data suggest a hypothesis that the role of electric fields is context dependent at the level of Src signalling. Further work will determine if this likely to be an important mediator of the pharmacological response of cells to electric-field based therapy.

The roles of electric fields and the mechanism of electrical stimulation therapy

Our data permit a hypothesis for synthesis of studies investigating the roles of EFs for promoting wound re-epithelialisation. First, we suggest that the cathodal migration of epithelial cells in applied EFs in vitro, demonstrated in many studies and which underlies much of the science previously performed in this area represents an artificial polarisation of cellular signalling mechanisms such as pSrc by the imposed field. This polarisation is not recapitulated in vivo in the cornea, and we show that the directedness of endogenous wound induced current does not significantly direct epithelial cell migration. This assertion is the natural conclusion of previous studies that have shown that applying a reversed or alternating field to epithelial lesions in the skin can still produce an enhanced healing response (Kloth, 2005, Sta Iglesia and Vanable, 1998, Balakatounis and Angoules, 2008, Ojingwa and Isseroff, 2003). One overt characteristic of the wound-induced electric currents is that they are instant upon wounding and result from ion flow from injured tissue. The start of the flow and the concurrent establishment of an electric field coincide with and possibly influence the first signalling events after injury, which is intracellular calcium release from internal stores. This, in turn, activates MAP kinase signalling pathways that underlie the migratory healing response (Leiper, et al., 2006, Sung, et al., 2003, Yang, et al., 2004). In

light of our data it is now most likely that in vivo it is the ion flow itself, not necessarily its direction or indeed magnitude, that potentiates cell migration through initiating upregulation and rapid reorganisation and planar polarisation of the cellular organelles, cytoskeleton and cell surface components required for migration (Pu and Zhao, 2005). This would explain why applied EFs increase the rate of cell migration in *Pax6*^{+/-} cells irrespective of the failure of the directional response. It also suggests that applied electrical stimulation phenocopies or augments the ion flow that normally occurs after wounding and stimulates or maintains epithelial cells in a physiological wound-healing response mode. There are potentially many non-directional processes that could be upregulated by electrical stimulation, such as growth factor release, which would accelerate healing. It will be necessary to replicate these experiments in a more complex system such as the skin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Polarisation and upregulation of activated pSrc in wild-type corneal epithelial cells in response to applied exogenous EF in vitro

Immunostaining of Tyr-416-phosphorylated (activated) Src in corneal epithelial monolayers in vitro. **(A)** Wild-type cells, no EF. A, fluorescence image; A*, phase contrast, A** fluorescence overlay on phase contrast (enlarged). No polarisation is visible. Scale bar, 50 μ m. **(B)** Wild-type cells, applied field 200 mV/mm with cathode to top-left of the image. pSrc is polarised to the leading cathodal edge of the cells. **(C)** *Pax6*^{+/-} cells, no EF. Cell surface labelling is visible, but no polarisation. **(D)** *Pax6*^{+/-} cells, applied field 200 mV/mm with cathode to top of field. pSrc polarisation is much reduced or absent, compared to wild-type. **(E)** Quantification of pSrc polarisation in randomly selected wild-type cells exposed to applied EF and visualised in panel 1B. In ImageJ a line was drawn along the long axes of the cells and staining intensity (from 0 = black to 1 = maximum intensity) plotted using the Plot Profile function with the cathode to the right. Profiles from 8 cells were overlain to show graphically the accumulation of pSrc at the cathode. **(F)** Western blot of total and activated Src (tSrc and pSrc) from wild-type and *Pax6*^{+/-} monolayer cultures in control conditions or during exposure to electric field (EF). The experiment was repeated 4 times and the densitometry is presented in Table 1.

Figure 2. Endogenous wound-induced electric currents and healing rates in the cornea

(A) When a stratified epithelium such as in the vertebrate cornea is wounded, this short-circuits the apical-basal potential difference and instantaneously creates a standing electrical gradient (red arrow), with the wound site acting as a cathode. There is net inward transport of cations and net outward transport of anions in intact tissue, and free back-diffusion is prevented by tight junctions between superficial ('wing') epithelial cells (yellow). Ions such as calcium are released directly from damaged cells at the wound site (green arrows), which initiates the immediate wound response signalling pathways. The circuit is closed by return active transport of ions through intact tissue away from the wound site. (B) Wound induced electric current, expressed as mean $\mu\text{A}/\text{cm}^2$ recorded from the corneal epithelia of wt (black bar) and *Pax6*^{+/-} (dark grey bar) mice. Positive values indicate mean outward current (from the intact tissue into the wound). *Pax6*^{+/-} samples could be subdivided into those with an inward current (*Pax6*^{+/-i} - light grey bar) and those with an outward current (*Pax6*^{+/-o} - white). Standard errors of the means are shown. (C) Changes in wound-induced current upon ion depletion presented as the difference between current measured in ATS solution and each ion-free solution: negative values represent a decrease in the current and vice versa (i.e. a reduction in outward current or a more pronounced inward current). Only sodium depletion caused a significant difference between genotypes (see text).

(D) Amount of healing (expressed in μm advance of the epithelial sheet) 5 and 24 hours after wounding for wt (black bar), *Pax6*^{+/-i} (light grey) and *Pax6*^{+/-o} (white). Healing rate was unaffected by direction or mean magnitude of the endogenous wound induced current.

Figure 3. Changes in wound-induced current and healing rate over time

(A) Mean wound induced current at 0–6 hours after wounding for wt and *Pax6*^{+/-} corneas and (B) mean wound healing rate at intervals from 0–24 hours after wounding. Wound healing rates remain relatively constant in spite of large changes in the endogenous current. (C, D) Wound-healing rate plotted against wound-induced current for wt (C) and *Pax6*^{+/-} (D) corneas. Each point represents the data for one cornea during one time period 0–2, 2–4 or 4–6 hours post-wounding. There is no clear correlation for either genotype. (C: correlation coefficient $r = 0.135$. D: correlation coefficient $r = -0.138$).

Figure 4. Western blot and immunohistochemical analysis of total and activated Src after wounding in vivo

(A) Representative western blot of Tyr-416-phosphorylated (activated) Src (pSrc) in wild-type and *Pax6*^{+/-} corneas, either unwounded or 4 hours after wounding in vivo, against levels of total-Src and β-actin. Wounding produces no global upregulation for either genotype, although restricted upregulation in a small number of cells around the wound may be missed by this technique. (B–I) Flat-mount immunohistochemistry against (B,C, F–I) pSrc or (D,E) tSrc for (B–G) wild-type or (H,I) *Pax6*^{+/-} wounded corneal epithelia after 20 minutes (B, C) or 4 hours (D–I) of healing, when cell migration is active. Images are taken (C,E,G,I) at the wound edge or (B,D,F,H) at corneal periphery, away from the wound. No polarisation of pSrc is visible even at 20 minutes after wounding, when the induced EF should be maximal. These are unmanipulated conventional fluorescence images and all pSrc images are exposed identically for direct comparison. The background staining in 4E is non-specific stromal staining sometimes observed with this particular secondary antibody. Panels

B and C, D,E and F,G, H and I are images from different areas of the same corneas. Scale bar, 40 μm .

Table 1

Src activation response of $Pax6^{+/+}$ and $Pax6^{+/-}$ cells in applied electric fields in vitro.

	Conditions of experiments and ratios counted	Densitometry	n
A	No field [(pSrc/tSrc) $_{Pax6^{+/+}}$]/[(pSrc/tSrc) $_{Pax6^{+/-}}$]	1.18 ± 0.25	3
B	No field [(pSrc/ β -act) $_{Pax6^{+/+}}$]/[(pSrc/ β -act) $_{Pax6^{+/-}}$]	1.34 ± 0.22	3
C	$Pax6^{+/+}$ [(pSrc/tSrc) $_{\text{field}}$]/[(pSrc/tSrc) $_{\text{no field}}$]	1.78 ± 0.28 ,	4
D	$Pax6^{+/-}$ [(pSrc/tSrc) $_{\text{field}}$]/[(pSrc/tSrc) $_{\text{no field}}$]	1.17 ± 0.41	3

$Pax6^{+/+}$ and $Pax6^{+/-}$ corneal epithelial cells were cultured for 2 hours in presence or absence of an applied electric field of 200 mV/mm, then probed by western blot for phospho-Src, total Src and β -actin. A. shows that in absence of a field the pSrc/tSrc ratio was approximately equal in $Pax6^{+/+}$ and $Pax6^{+/-}$, but compared to β -actin (B), pSrc levels were slightly higher in wild-type. C shows that pSrc/tSrc ratios increased significantly for $Pax6^{+/+}$ cells in an applied EF, in contrast to $Pax6^{+/-}$ (D) for which no significant rise was observed. n represents number of western blots, for which 3–4 cultures were pooled into each. Densitometry reading shows mean and standard error of the ratios measured from independent gels.

Table 2*In vivo* Src response of corneal epithelia to wounding in vivo

Genotype	Ratio wound:non-wound of Src activation	Densitometry	n
<i>Pax6</i> ^{+/+}	[(pSrc/tSrc) _{wound}]/[(pSrc/tSrc) _{control}]	1.04 ± 0.09	8
<i>Pax6</i> ^{+/-}	[(pSrc/tSrc) _{wound}]/[(pSrc/tSrc) _{control}]	1.01 ± 0.06	5

Pax6^{+/+} and *Pax6*^{+/-} corneas were given epithelial wounds, levels of pSrc and tSrc measured by densitometry of western blots and the pSrc/tSrc ratio calculated and compared to control unwounded corneas. Wounding of either genotype did not increase the level of Src phosphorylation. n represents number of eyes assayed. Densitometry shows mean ratios and standard error of independent western blots.