Cell Surface Glycoconjugate Abnormalities and Corneal Epithelial Wound Healing in the *Pax6*$^{+/-}$ Mouse Model of Aniridia-Related Keratopathy

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Purpose. Congenital aniridia due to heterozygosity for *Pax6* is associated with ocular surface disease, including keratopathy. This study investigated how defects in glycoconjugate component of the cell surface of *Pax6*$^{+/-}$ could cause the abnormal cellular migration phenotypes associated with the disease.

Methods. Immunohistochemistry, lectin-based histochemistry, conventional staining techniques, and proteomic assays were performed on eyes and cultured corneal epithelial cells from wild-type and *Pax6*$^{+/-}$ littermates. Wild-type cells were manipulated in culture to replicate the glycoconjugate abnormalities found in *Pax6* heterozygotes and determine the consequences for wound healing.

Results. Multiple glycoconjugate defects were found in *Pax6*-mutant cells. Lectin cytochemistry of corneal epithelial cells suggested a partial failure of glycoprotein trafficking. Blocking cell surface carbohydrate moieties in wild-type corneal cells caused wound-healing delays similar to those seen in untreated *Pax6*$^{+/-}$ cells.

Conclusions. Alterations to the cell surface glycoconjugate signature of *Pax6*$^{+/-}$ corneal epithelia restrict the ability of cells to initiate migration in response to wounding. This underlies the observed wound-healing delay in cultured *Pax6*$^{+/-}$ epithelia. (*Invest Ophthalmol Vis Sci.* 2006;47:5276–5282) DOI:10.1167/iovs.06-0581

When an epithelium is wounded, a complex series of signaling pathways are initiated in cells radiating from the wound site that promote immediate cell migration into the wound.[1–4] Epithelial proliferation is initiated within a few hours, and stem cells or stemlike cells within the epithelium may be activated.[5]

Heterozygous deficiency in human *PAX6* leads to ocular surface disease characterized by corneal opacification (aniridia-related keratopathy [ARK]), and the *Pax6*$^{+/-}$ mouse models all the morphologic defects of human ARK.[6–9] In vitro, *Pax6*$^{+/-}$ corneal epithelial cells do not respond to wounding within the first 1 to 2 hours, in contrast to *Pax6*$^{+/+}$ cells that start to migrate into the wound within a few minutes.[10] However, when *Pax6*$^{+/-}$ cells overcome their block, they migrate as fast as wild-type, and so there is no intrinsic defect in cell migration, only in their ability to respond quickly to wounding.

ARK may be due to corneal epithelial fragility combined with an abnormal wound-healing response.[11] Impaired re-epithelialization after wounding increases the risk of infection, exacerbates inflammation, and undermines normal stromal remodeling.[12] In vivo studies have suggested that changes in the adhesive properties of mutant cells may underlie many of the observed morphologic abnormalities, and Pax6 controls expression of cell adhesion molecules, most of which are glycoproteins.[8,13–15]

Before *Pax6* was identified as the mutant gene in small-eye (*Pax6*$^{Sey/+}$) mice, Pritchard[16] and Pritchard et al.[17] biochemically identified multiple glycoconjugate abnormalities in *Pax6*$^{+/-}$ eyes and suggested that the defective gene may be involved in synthesis or processing of glycoproteins.

Glycosaminoglycans (GAGs) are linear polymers of amino sugar uronic acid disaccharides, usually covalently attached to a small protein core as components of proteoglycans, which may be anchored to the plasma membrane or released into the extracellular matrix (ECM).[18] GAG families are classed according to the disaccharides from which they are polymerized: chondroitin/dermatan sulfate, keratan sulfate, heparin/heparan sulfate, or hyaluronic acid.[19] GAGs are necessary for cell adhesion and directed cell migration.[19,20] They act as coreceptors for growth factor ligands in the ECM or on the cell surface.[20–22] Sulfated GAG chains are regulators of cell–cell and cell–ECM interactions.[23,24] Mutations in keratocan, a keratan sulfate proteoglycan, lead to cornea plana, whereas repair of damaged adult corneal epithelium is retarded in lumican (a keratan sulfate proteoglycan) and syndecan-1 (a heparan sulfate proteoglycan)–deficient mice (Gala PH et al. *IOVS* 2000; 41:ARVO Abstract 4846).[25–27]

Methods

Mice and Cell Culture

All animal procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. *Pax6*$^{+/Sey\text{-}Neu}$ (*Pax6*$^{+/-}$) mice[28] were maintained on the CBA/Ca genetic background by heterozygous mating, and corneas were dissected from *Pax6*$^{+/+}$ and *Pax6*$^{+/-}$ littermates 8 to 10 weeks old.

Studies were performed using primary corneal explant isolated and cultured according to Hazlett et al.[29] Explants were removed after 10 days and cultures were used 3 to 5 days later. *Pax6*$^{+/-}$ cultures express Pax6 at a level that is approximately 60% that of wild-type cells.[10]

Lectin Histochemistry and Cytochemistry

Lectin histochemistry was performed according to Buse and Seifert[30] using lectins that were known to bind to eye tissues. FITC-conjugated lectins were obtained from EY-Laboratories (San Mateo, CA) or Sigma-Aldrich (Poole, UK). Eyes were fixed in 4% paraformaldehyde (PFA) for 2 hours, and processed to wax. Rehydrated 7-μm sections were washed in lectin buffer (6.06 g/L Tris, 8.7 g/L NaCl, 0.203 g/L

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TABLE 1. Binding Specificity of Lectins Used in This Study

Lectin	Abbreviation	Primary Specificity (Secondary Specificity)
Canavalia ensiformis	ConA	α-D-Mannose, α-D-glucose (α-D-*N*-acetylglucosamine)
Triticum vulgaris	WGA	β-*N*-acetylglucosamine(1–4)glucose. (acetylneuramic acid)
Lens culinaris	LCA	Mannose
Maclura pomifera	MPA	α-D-Galactose (α-galactose (1–3) *N*-acetylgalactosamine)
Limulus polyphemus	LPA	Acetylneuramic acid
Dolichus biflorus	DBA	α-*N*-Acetylgalactosamine

$MgCl_2.6H_2O$, 0.111 g/L $CaCl_2$ [pH 7.6]), incubated in 50 mg/mL FITC-lectin in lectin buffer for 45 minutes at room temperature, washed in PBS, and mounted. Negative controls were performed by preincubating the lectin in a 1 M solution of the appropriate blocking monosaccharide (Table 1).

Cell cultures were washed three times for 5 minutes each in PBS and fixed in 4% PFA for 10 minutes and were washed in PBS and in lectin buffer two times for 5 minutes each, before the addition of the FITC-lectin.

Wholemount staining of corneas was performed by fixing eyes, 4% PFA, for 2 hours, washing overnight in PBS, then dissecting corneas. After three 5-minute washes in TBS, corneas were incubated in 1% pepsin, 10 mM HCl, 12 to 5 minutes at 37°C, neutralized in 0.1 M borate buffer (pH 8.5), washed three times in TBS then incubated with FITC-tagged lectin as just described. After washing, basal corneal epithelial cells were examined by confocal microscope in a plane parallel to the basement membrane.

Di-iron Amino Staining

Cells were washed two times for 3 minutes each in PBS, fixed in 4% PFA for 10 minutes, and washed in PBS, three times for 5 minutes each. The diamine solution was made up by mixing 120 mg *N*,*N*-dimethyl-*meta*-phenylenediamine-dihydrochloride and 20 mg *N*,*N*-dimethyl-*para*-phenylenediamine-dihydrochloride (Sigma-Aldrich) together in 50 mL distilled water, adding 1.4 mL 60% ferric chloride solution (BDH), and mixing. The solution was added to cells for 16 hours followed by washing with water. Sulfated mucins were stained dark purple-brown.

Rate of Wound-Healing in Epithelial Cultures

Linear scratch wounds 200 to 400 μm wide were introduced into confluent $Pax6^{+/+}$ and $Pax6^{+/-}$ monolayers in 35-mm dishes using a fire-polished glass pipette. The distance across each wound in four places was measured with calibrated eyepiece graticules every hour for 6 hours or until the wounds were completely closed. The cells were incubated at 37°C, 5% CO_2 at all times. The rate of wound healing was expressed in micrometers per hour.

Pretreatment with soluble agents to disrupt cell surface glycoconjugates was as follows:

Chondroitinase ABC (Sigma-Aldrich): 0.5 U/mL added directly to the culture medium for 1 hour at 37°C before wounding. At wounding, another 0.5 U/mL was added.

Chondroitin-6-sulfate (Sigma-Aldrich): 30 mg/mL added to the medium 1 hour before wounding.

Sodium chlorate: 30 mM sodium chlorate (or sodium sulfate as a control) added to culture medium of the cells 3 to 5 days before wounding. The medium was changed every day with addition of fresh 30 mM chlorate or sulfate ions.

Lectins: The cells were grown in normal culture medium containing 50 μg/mL lectin for at least 30 minutes before wounding. Because ConA could potentially bind glucose in the medium and sequester it from use by the cells, the ConA-treated cells were grown in medium with the addition of 8.7 mM fructose. Control experiments showed that added fructose by itself had no effect on the rate of culture growth.

Immunocytochemistry

Cultures were fixed in 4% PFA, permeabilized in methanol for 10 minutes, −20°C, rinsed three times with PBS, before blocking for 30 minutes (0.3% BSA, 4% normal goat serum, and PBS). Cells were incubated with primary antibody (1:150 in blocking buffer; GM130; AbCam, Cambridge, UK) overnight at 4°C. After washes in PBS, secondary antibody (rhodamine-goat anti-mouse, 1:1000 in blocking buffer) was added for 1 hour.

Proteomic Analysis of Corneal Tissues

Corneal epithelial cell cultures were lysed 1:1 (wt/vol) in 0.01 M Tris (pH 7.4), 1 mM EDTA, 8 M urea, 0.05 M dithiothreitol [DTT], 10% (vol/vol) glycerol, 5% (vol/vol) NP40, and 6% (wt/vol) ampholytes (pH 3–10) according to standardized techniques, at the Aberdeen Proteomic Facility. Two-dimensional gel analysis was performed, with separation in the first dimension according to isoelectric point and in the second dimension by PAGE according to apparent molecular weight. Two-dimensional gels were blotted and probed with horseradish peroxidase (HRP)–conjugated lectin MPA.

RESULTS

Lectin Histochemistry in *Pax6* Mutant Eyes

Eyes were obtained from $Pax6^{+/+}$ and $Pax6^{+/-}$ littermates 8 to 10 weeks old and subjected to fluorescence-tagged lectin histochemistry, by using six lectins with different targets, summarized in Table 1.

In general, patterns of lectin staining in $Pax6^{+/-}$ eyes were very similar to wild-type. MPA bound strongly to the apical layers of the corneal epithelium (Fig. 1A) and more weakly to the basal epithelium, stroma, and endothelium. Whereas MPA strongly labeled Descemet's layer in the wild-types, this was not the case in $Pax6^{+/-}$ (Figs. 1A, 1B). Cytoplasmic ConA staining was visible throughout all corneas (Figs. 1C, 1D). WGA labeling occurred in a basal–apical gradient in the corneal stromas of wild-types, and was uniformly strong in $Pax6^{+/-}$ stromas (Figs. 1E, 1F). LCA showed cytoplasmic or cell-surface staining throughout all corneas (Figs. 1G, 1H). Two lectins, DBA and PNA, produced no specific staining anywhere in the eye. The data suggest that glycoconjugate differences between adult wild-type and $Pax6^{+/-}$ eyes are subtle, although some of these differences occur in the corneal stroma and endothelium, neither of which express significant levels of Pax6.[31]

Glycoconjugate Mislocalization in Corneal Epithelial Cells

A component of the epithelial fragility observed in $Pax6^{+/-}$ corneas may be the cellular mislocalization of plasma membrane-associated glycoproteins. To identify such problems in heterozygous cells, we investigated the intracellular distribution of glycoproteins in mouse corneal epithelial cell cultures.

Corneal epithelial cells were fixed and labeled with fluorescence-tagged lectins as above. As previously, DBA and PNA did

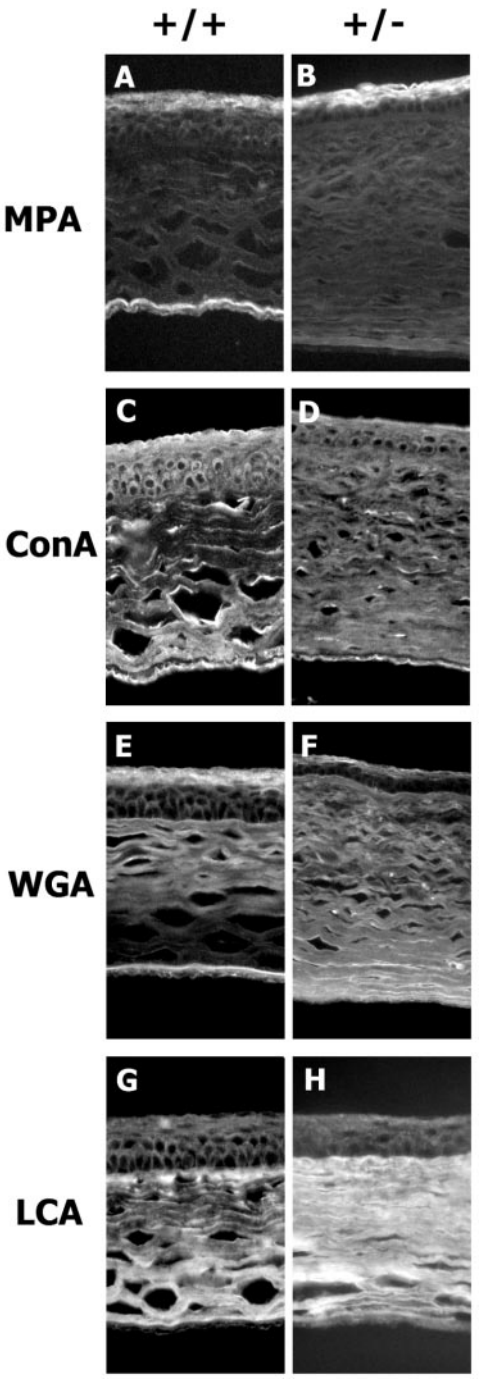

FIGURE 1. Lectin staining of wild-type and *Pax6*$^{+/-}$ littermate adult corneas. The epithelium is uppermost. Results from four lectins are shown (abbreviated as in Table 1). e: corneal epithelium; s: corneal stroma. *Arrowheads*: Descemet's membrane. Scale bar, 50 μm.

not produce significant staining. Staining patterns seen with the other four lectins were very surprising, however (Fig. 2). Although there were significant differences between the staining patterns of the four lectins, in all cases, widespread cell-surface labeling of *Pax6*$^{+/+}$ corneal epithelial cells was seen (Figs. 2A, 2C, 2E, 2G), as expected. In contrast, labeling of *Pax6*$^{+/-}$ cells was primarily intracellular (Figs. 2B, 2D, 2F, 2H). Staining tended to be concentrated in the perinuclear region of the cytoplasm, though weaker cell-surface labeling was still normally visible.

It was hypothesized that glycoproteins targeted to the cell surface were not trafficking correctly in *Pax6*$^{+/-}$ cells. This idea was tested by coimmunolabeling of the Golgi marker GM130 with each lectin (Fig. 3). There was strong, but not total, colocalization of lectin staining with the Golgi apparatus in *Pax6*$^{+/-}$ corneal epithelial cells (Figs. 3A–H). In wild-type cells, the colocalization was much poorer (Figs. 3I, 3J). The different patterns of glycoprotein localization were also seen in vivo from wholemount staining of fresh-fixed corneas analyzed by confocal microscopy (Fig. 4). In vivo there was considerable heterogeneity in lectin labeling patterns of the basal corneal epithelial cells, an unexpected finding that may be related to cell cycle or differentiation status. In general, however, perinuclear lectin labeling was seen in heterozygous cells.

To get an approximate estimation of how many glycoproteins are bound by the corneal epithelium, we isolated total protein from the wild-type corneal epithelial cell cultures. Two-dimensional polyacrylamide gels were run, blotted, and probed with biotin-labeled MPA (Fig. 5). Although the blots were generally dirty with high background (a consistent problem with using lectins in Western blot analysis) approximately 50 protein spots were detectable, probably corresponding to $<$50 proteins.

Wound Healing in the Presence of Lectins

Pax6$^{+/-}$ corneal epithelial cells show a 2-hour delayed response to wounding.[10] Because the molecular pathways un-

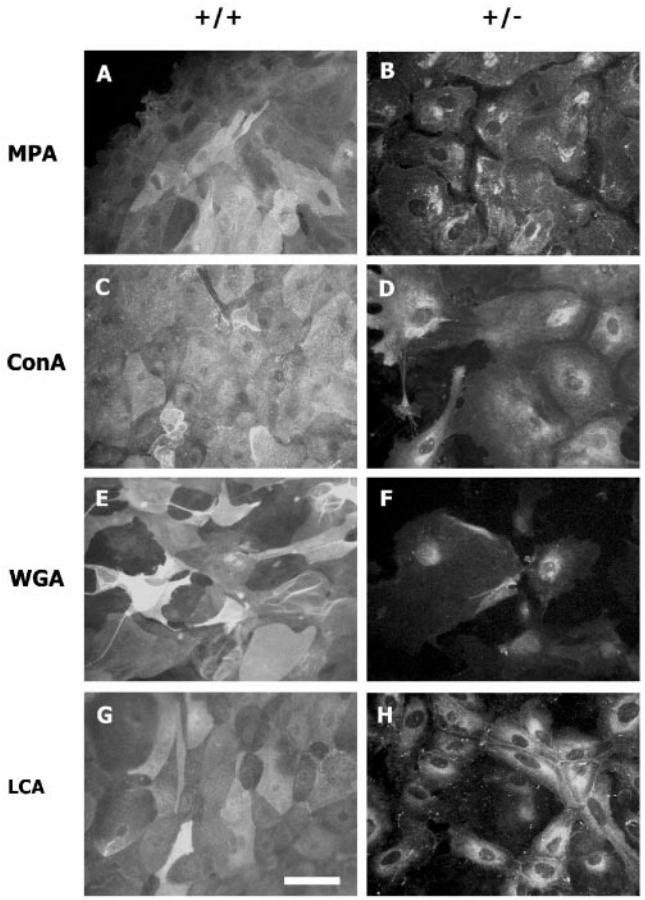

FIGURE 2. Lectin labeling of mouse primary corneal epithelial cell cultures. Cells in *Pax6*$^{+/-}$ cultures sometimes appeared larger and flatter than *Pax6*$^{+/+}$, though this was not consistent and was not quantified. (**A**, **C**, **E**, **G**) *Pax6*$^{+/+}$. (**B**, **D**, **F**, **H**) *Pax6*$^{+/-}$ littermates. Four lectins used (abbreviations as in Table 1). Scale bar, 50 μm.

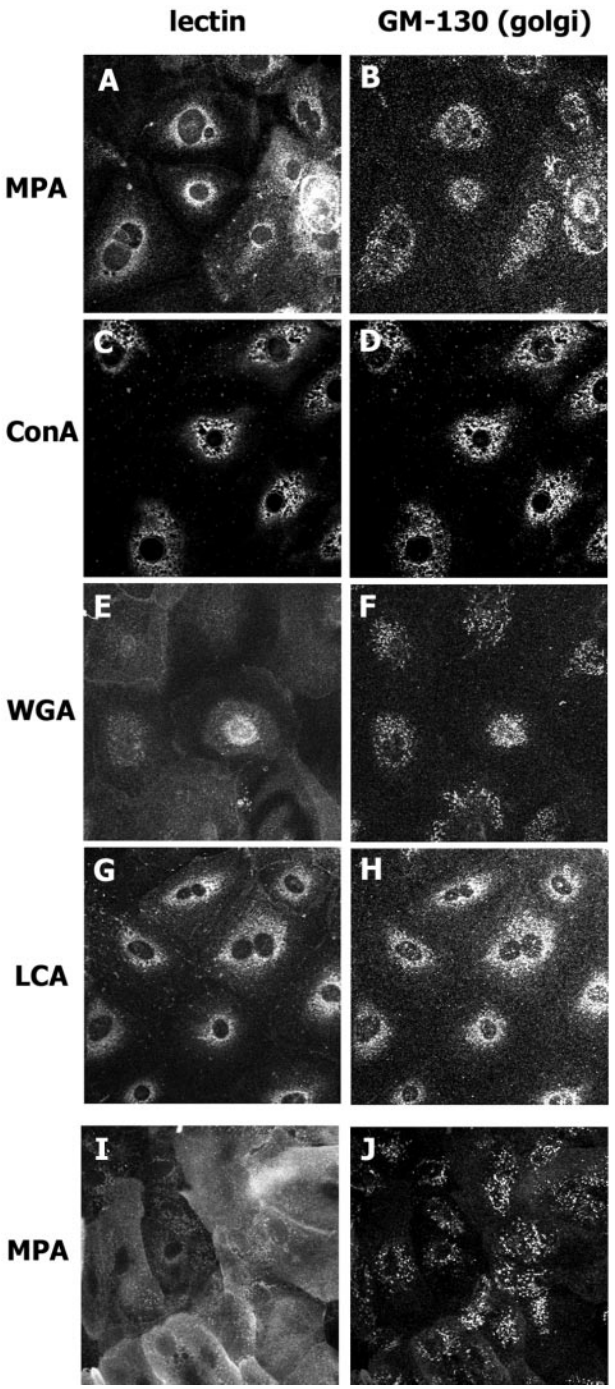

FIGURE 3. Localization of lectin staining to the Golgi apparatus in *Pax6*-heterozygous corneal epithelial cells. Lectin staining in left column (four lectins used, abbreviations as in Table 1). Golgi staining using GM-130 antibody in right column. (**A–H**) $Pax6^{+/-}$. (**I**, **J**) $Pax6^{+/+}$ cells stained with MPA for comparison.

derlying the wound healing response require correct cell surface expression of growth factor receptors and cell adhesion molecules, we considered that inhibition of the action of these cell surface glycoproteins using lectins would recapitulate the $Pax6^{+/-}$ wound-healing defect in wild-type cells.

$Pax6^{+/+}$ or $Pax6^{+/-}$ corneal epithelial cells were cultured in either control medium or media containing 50 μg/mL of ConA, WGA, or DBA for 30 minutes. ConA and WGA were chosen, because they have been shown to be localized to the

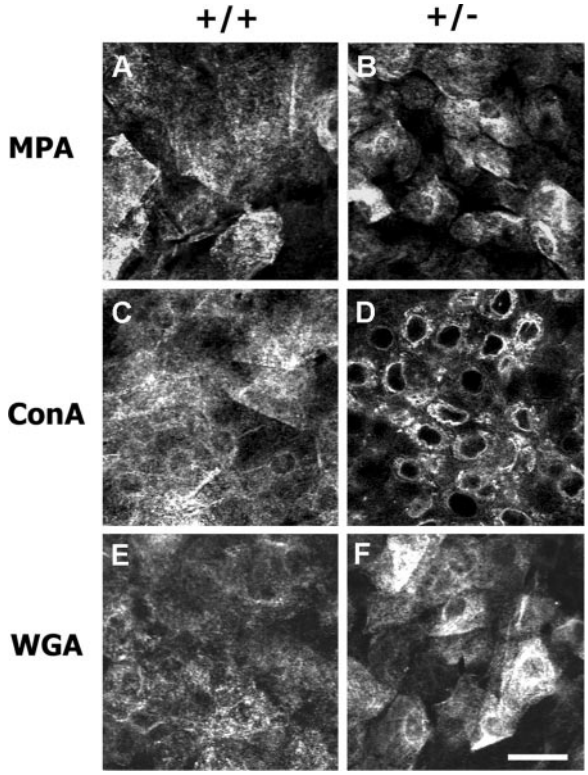

FIGURE 4. Localization of lectin staining to corneal epithelial cells in vivo. Confocal images after wholemount lectin labeling with (**A**, **B**) MPA, (**C**, **D**) Con A, and (**E**, **F**) WGA. Scale bar, 20 μm.

leading edge of healing rat corneal epithelia.[32] A single linear scratch of up to 200 μm was made in the epithelial sheet, and the rate of wound healing was measured at 37°C. Wild-type cells started to heal almost immediately (*first 2 hours*: rate of wound healing = 30.4 ± 5.1 μm/hour; n = 26; and *2 to 6 hours after wounding*: rate of wound healing = 29.8 ± 2.7 μm/hour; n = 26). In contrast, $Pax6^{+/-}$ cells hardly migrated at all within the first 2 hours of wounding, but from 2 to 6 hours after wounding, migrated at a rate that was not significantly different (t-test: $P = 0.086$) from wild-type (*first 2 hours*: rate of wound healing = 5.9 ± 2.1 μm/hour; *2 to 6 hours after wounding*: rate of wound healing = 36.7 ± 3.1 μm/hour; n = 26). Addition of ConA or WGA to the medium of wild-type cells reduced the immediate wound-healing cell migration to levels not significantly different from untreated $Pax6^{+/-}$ cells (ConA = 4.2 ± 1.5 μm/hour, n = 12; WGA = 5.6 ± 3.2 μm/hour, n = 8). The migration of ConA-treated cells remained significantly slower than untreated wild-type cells in

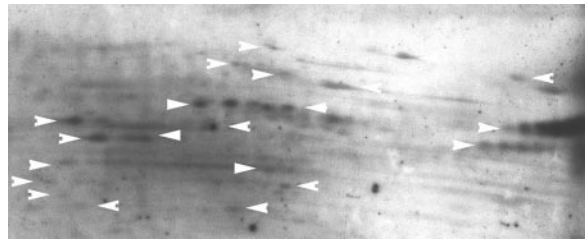

FIGURE 5. Two-dimensional gel of $Pax6^{+/+}$ corneal epithelial cell cultures probed with MPA. Although there is a lot of background, clusters of high-abundance spots are visible. Approximately, 50 proteins were visible on the gel, those that will be taken forward for identification are labeled (*white arrowheads*).

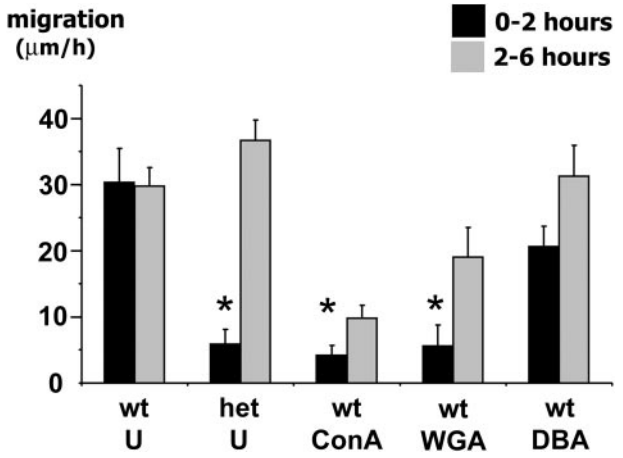

FIGURE 6. Wound healing in corneal epithelial cultures. Wound healing rates are divided into two phases, initial 0 to 2 hours and postinitial 2 to 6 hours, for untreated control (U) *Pax6*$^{+/-}$ and *Pax6*$^{+/+}$ cells (n = 15 and 26, respectively), and for *Pax6*$^{+/+}$ cells in the presence of lectins Con A (n = 12), WGA (n = 8), and DBA (n = 8). *Statistical significance, $P < 0.01$ compared with untreated wild-type controls.

the next 4 hours (9.8 ± 2.0 μm/hour, n = 12; t-test: $P < 0.0001$). WGA-treated cells started to migrate more quickly once they had overcome their first 2-hour block, although this was of marginal significance (19.1 ± 4.5 μm/hour; n = 8; t-test: $P = 0.030$). The migration of WGA-treated wild-type cells 2 to 6 hours after wounding was not significantly different from that of either untreated wild-type cells (t-test: $P = 0.062$) or ConA-treated cells (t-test: $P = 0.089$). These data are summarized in Figure 6.

The rate of migration of DBA-treated wild-type cells in the first 2 hours was smaller (20.6 ± 31 μm/hour; n = 8) than that of untreated cells, but this was not significant by t-test ($P = 0.11$), whereas the rate of migration in hours 2 to 6 was identical with wild-type (31.3 ± 4.7 μm/hour; n = 8; $P = 0.80$). This correlates with the very poor binding of DBA to cultured corneal epithelial cells.

Glycosaminoglycan Deficiency in *Pax6*$^{+/-}$ Corneal Epithelial Cells

Di-iron-amino (DIA) staining was performed on *Pax6*$^{+/+}$ and *Pax6*$^{+/-}$ corneal epithelial cells taken from adult littermates and cultured for 10 days. DIA staining recognizes the sulfated ester bond present in sulfated GAGs.

Strong DIA staining, was observed in *Pax6*$^{+/+}$ cultures, but its was very much weaker, sometime undetectable in *Pax6*$^{+/-}$ (Figs. 7A, 7B). Propidium iodide staining of cell nuclei showed little difference in cell density between wild-type and *Pax6*$^{+/-}$ (Figs. 7C, 7D).

The GAG component of wild-type corneal epithelial cultures was depleted by addition of sodium chlorate to the culture medium for 72 hours. Chlorate ions inhibit formation of sulfated GAGs by competing with sulfate ions in the synthesis of phosphadenosine 5′-phosphosulfate, the sulfate donor that mediates polysaccharide sulfation by sulfotransferases.[33] Addition of 30 mM sodium chlorate to tissue culture medium substantially eliminates GAGs in cultured cells within 44 hours.[33] DIA staining in chlorate-treated cultures was reduced compared with 30 mM sodium sulfate-treated controls, indicating depletion but not total elimination of cell-surface GAGs (Figs. 7E, 7F), but chlorate-treated cultures showed no obvious abnormalities (data not shown).

Wild-type cells were treated with sodium chlorate, 0.5 U/mL chondroitinase ABC (to deplete chondroitin sulfate proteoglycans) or 10 μg/mL chondroitin 6-sulfate. They were then wounded as before, and the rate of healing compared with the untreated and sulfate-treated control cells. Chlorate treatment significantly reduced the rate of wound-induced cell migration, but chondroitinase ABC had no effect, suggesting that although GAGs are important for normal cell migration, chondroitin sulfate GAGs are dispensable (Fig. 8). The rate of wound healing in chlorate-treated cultures was not significantly faster in hours 2 to 6 after wounding than in the first 2 hours (t-test: $P = 0.38$), and remained significantly slower than in the untreated controls ($P < 0.0001$). This result suggests that GAGs are necessary for normal cell migration. Addition of chondroitin 6-sulfate to the medium increased the mean rate of wound healing in the first 2 hours, but the trend was not significant ($P = 0.07$).

DISCUSSION

We have developed an in vitro assay for which the dynamics of corneal epithelial repair are similar to those found in other organ cultures or in vivo.[34] Pax6 upregulates metalloproteinase-9 in wounded corneas, and *Pax6*$^{+/-}$ corneas do heal.[35–37] However, corneal epithelial migration is modulated by cyto-

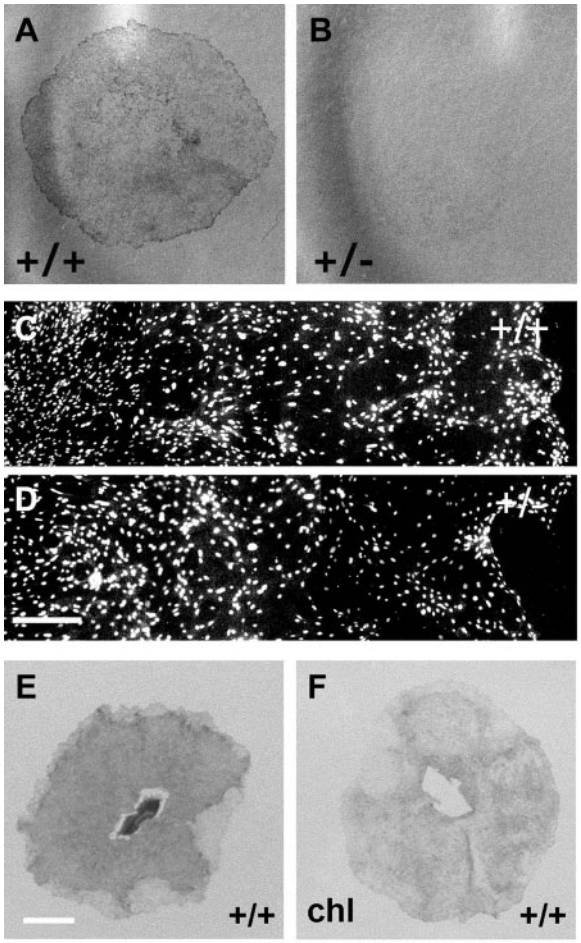

FIGURE 7. Glycosaminoglycans in primary mouse corneal epithelial cultures. (**A**, **B**) DIA staining of wild-type and *Pax6*$^{+/-}$ corneal epithelial cultures, respectively. Dark staining of GAGs is very faint in *Pax6*$^{+/-}$. (**C**, **D**) Propidium iodide staining of wild-type and *Pax6*$^{+/-}$ cultures, showing approximately equal cell density. (**E**, **F**) Wild-type corneal epithelial cultures either (**E**) controls or (**F**) grown in 30 mM sodium chlorate and stained with DIA. Scale bars: (**D**) 500 μm; (**E**) 3 mm.

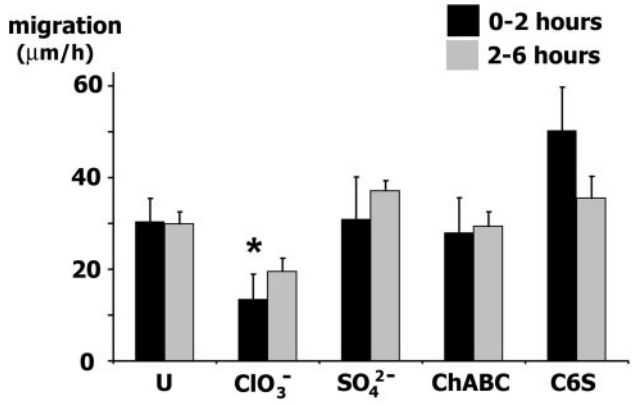

FIGURE 8. Corneal wound healing is modulated by glycosaminoglycans. Wound-healing rates are divided into two phases: initial, 0 to 2 hours; and postinitial, 2 to 6 hours, for untreated control (U) *Pax6*$^{+/+}$ cells ($n = 26$) and for *Pax6*$^{+/+}$ cells in the presence of 30 mM sodium chlorate ($n = 31$), 30 mM sodium sulfate ($n = 21$), 0.5 U/mL chondroitinase ABC (ChABC) ($n = 15$), and 30 mg/mL chondroitin 6-sulfate (C6S) ($n = 16$). *Statistical significance, $P < 0.01$ compared with untreated wild-type controls.

kines and growth factors released by the corneal stroma and other ocular tissues.[38,39] In the *Pax6*$^{+/-}$ cornea, the stromal surface becomes inflamed and irregular. These are factors that may alter wound healing, but are difficult to control for. Experiments on intact corneas provide useful clinical data relating to the management of diseased corneas, but are less informative about the primary defects in corneal epithelial cells that lead to disease in the first instance. It was to identify these defects in *Pax6*$^{+/-}$ cells in the absence of stromal modulation and in control media that this investigation was started. This strategy identified a primary wound-healing defect in *Pax6*$^{+/-}$ corneal epithelia, which show a reduced wound-induced wave of calcium signaling[10] leading to a 2-hour delay in the migratory response of cells to fill the wound. Over the lifetime of an individual, chronic minor wounding, exacerbated by epithelial fragility[8] may lead to corneal stromal opacification.

Recently, Ramaesh et al.[35] showed that in ex-vivo whole eye culture, *Pax6*$^{+/-}$ corneas healed *faster* than wild-type over a 6-hour period. As described above, in that system the corneal epithelial migration is modulated by the presence of an inflamed stroma, which would be expected to accelerate healing. The first 2 hours of healing was not studied by Ramaesh et al., and another important difference between the experiments is the size of the wounds. In their study, the wounds were very large (1000–1200 μm), encompassing up to half the cornea. Larger wounds healed faster than smaller wounds (presumably because larger wounds were closer to the limbus) and extrapolation from Figure 2 in Ramaesh et al. suggests that wounds smaller than 500 μm diameter in *Pax6*$^{+/-}$ epithelia would heal more slowly than in wild-type. Because wounds in our experiment were rarely more than 200 μm, there is therefore no immediate contradiction, although there the situation appears to be more complicated than that presented by either paper.

Aberrant lectin staining in *Pax6*$^{+/-}$ cells colocalized with the Golgi apparatus (Fig. 3), supporting the suggestion that glycoprotein trafficking may be disrupted in *Pax6*$^{+/-}$ cells.[16] The observed heterogeneity of cell morphology and lectin labeling in wild-type and *Pax6*$^{+/-}$ epithelial cells in culture and in vivo complicates the picture, and further work must be performed. However, this represents a new field of research in the link between Pax6, a transcription factor, and the expression of cell surface proteins that mediate the disease phenotype. We blotted 2D gels of corneal epithelia with MPA and labeled up to 50 proteins, suggesting that the glycoprotein mislocalization observed in *Pax6*$^{+/-}$ cells is a global trafficking problem. It also suggests that even proteins whose gene transcription levels are not affected by Pax6 may contribute to the mutant phenotype because they do not reach their intended target in the plasma membrane or extracellular matrix.

The apparent downregulation of GAGs in heterozygous cells was hypothesized to impede their wound response. It was found that GAG-depleted wild-type corneal epithelial cells were viable in long-term culture, but that after wounding, their initial migratory response was reduced to a level significantly less than untreated controls.

WGA and ConA were used to block cell surface glycoconjugates in wild-type cells and inhibited wound healing. It is possible that there is just one adhesion molecule or growth factor receptor, conjugated both to available β-*N*-acetylglucosamine(1-4)glucose moieties and α-D-mannose and/or α-D-glucose, that is required for wound healing and is mistargeted in *Pax6*$^{+/-}$ cells. We believe, however, that it is more likely that Pax6 exerts its effects in a pleiotropic manner through control of multiple components of the cell surface glycoconjugate signature.

In this study, we used a top-down approach to study the cell-surface deficiencies that underlie the *Pax6*$^{+/-}$ mutant phenotype. It is suggested therefore that in addition to the known roles of Pax6 in controlling the transcription of cell adhesion molecules[40] failure of trafficking of proteins whose transcription is itself not regulated by Pax6 may equally contribute to the cell adhesion and other defects exhibited by *Pax6*$^{+/-}$ cells.

References

1. Jacinto A, Martinez-Arias A, Martin P. Mechanisms of epithelial fusion and repair. *Nat Cell Biol*. 2001;3:E117–E123.
2. Thoft RA, Friend J. The X, Y, Z hypothesis of corneal epithelial maintenance. *Invest Ophthalmol Vis Sci*. 1983;24:1442–1443.
3. Woolley K, Martin P. Conserved mechanisms of repair: from damaged single cells to wounds in multicellular tissues. *Bioessays*. 2000;22:911–919.
4. Martin P. Wound healing-aiming for perfect skin regeneration. *Science*. 1997;276:75–81.
5. Cotsarelis G, Cheng SZ, Dong G, Sun TT, Lavker RM. Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells. *Cell*. 1989;57:201–209.
6. Ramaesh T, Collinson JM, Ramaesh K, Kaufman MH, West JD, Dhillon B. Corneal abnormalities in *Pax6*$^{+/-}$ small eye mice mimic human aniridia-related keratopathy. *Invest Ophthalmol Vis Sci*. 2003;44:1871–1878.
7. Baulmann DC, Ohlmann A, Flugel-Koch C, Goswami S, Cvekl A, Tamm ER. Pax6 heterozygous eyes show defects in chamber angle differentiation that are associated with a wide spectrum of other anterior eye segment abnormalities. *Mech Dev*. 2002;118:3–17.
8. Davis J, Duncan MK, Robison WG Jr, Piatigorsky J. Requirement for Pax6 in corneal morphogenesis: a role in adhesion. *J Cell Sci*. 2003;116:2157–2167.
9. Collinson JM, Chanas SA, Hill RE, West JD. Corneal development, limbal stem cell function, and corneal epithelial cell migration in the *Pax6*$^{+/-}$ mouse. *Invest Ophthalmol Vis Sci*. 2004;45:1101–1108.
10. Leiper LJ, Walczysko P, Kucerova K, et al. The roles of calcium signaling and ERK1/2 phosphorylation in a *Pax6*$^{+/-}$ mouse model of epithelial wound-healing delay. *BMC Biol.* 2006;4:27.
11. Ramaesh K, Ramaesh T, Dutton GN, Dhillon B. Evolving concepts on the pathogenic mechanisms of aniridia related keratopathy. *Int J Biochem Cell Biol*. 2005;37:547–557.
12. Ueno M, Lyons BL, Burzenski LM, et al. Accelerated wound healing of alkali-burned corneas in MRL mice is associated with a reduced inflammatory signature. *Invest Ophthalmol Vis Sci*. 2005;46:4097–4106.

13. Collinson JM, Hill RE, West JD. Different roles for *Pax6* in the optic vesicle and facial epithelia mediate early morphogenesis of the murine eye. *Development*. 2000;127:945–956.
14. Duncan MK, Kozmik Z, Cveklova K, Piatigorsky J, Cvekl A. Overexpression of Pax6(5a) in lens fiber cells results in cataract and upregulation of $\alpha5\beta1$ integrin expression. *J Cell Sci*. 2000;113:3173–3185.
15. Zaniolo K, Leclerc S, Cvekl A, et al. Expression of the alpha4 integrin subunit gene promoter is modulated by the transcription factor Pax6 in corneal epithelial cells. *Invest Ophthalmol Vis Sci*. 2004;45:1692–1704.
16. Pritchard DJ. A biochemical investigation of a dominant mutation associated with cellular invasiveness. PhD Thesis. Edinburgh: University of Edinburgh; 1973.
17. Pritchard DJ, Clayton RM, Cunningham WL. Abnormal lens capsule carbohydrate associated with the dominant gene "small-eye" in the mouse. *Exp Eye Res*. 1974;19:335–340.
18. Varki A, Cummings R, Esko J, Freeze H, Hart G, Marth J. *Essentials of Glycobiology.* Cold Spring Harbor, NY: Cold Spring Harbor Press; 1999.
19. Ruoslahti E, Yamaguchi Y. Proteoglycans as modulators of growth factor activities. *Cell*. 1991;64:867–869.
20. Bernfield M, Götte M, Park PW, et al. Functions of cell surface heparan sulphate proteoglycans. *Annu Rev Biochem*. 1999;68:729–777.
21. Corless CL, Mendoza A, Collins T, Lawler J. Colocalization of thrombospondin and syndecan during murine development. *Dev Dyn*. 1992;193:346–358.
22. Chamberlain CG, McAvoy JC. Fibre differentiation and polarity in the mammalian lens: a key role for FGF. *Prog Retin Eye Res*. 1997;16:443–478.
23. Perrimon N, Bernfield M. Specificities of heparan sulphate proteoglycans in developmental processes. *Nature*. 2000;404:725–728.
24. Davies JA, Fisher CE, Barnett MW. Glycosaminoglycans in the study of mammalian organ development. *Biochem Soc Trans*. 2001;29:166–171.
25. Pellegata NS, Dieguez-Lucena JL, Joensuu T, et al. Mutations in KERA, encoding keratocan, cause cornea plana. *Nat Genet*. 2000;25:91–95.
26. Saunders S, Jalkanen M, O'Farrell S, Bernfield M. Molecular cloning of syndecan, an integral membrane glycoprotein. *J Cell Biol*. 1989;108:1547–1556.
27. Saika S, Shiraishi A, Saika S, et al. Role of lumican in the corneal epithelium during wound healing. *J Biol Chem.* 2000;275:2607–2612.
28. Hill RE, Favor J, Hogan BL, et al. Mouse small eye results from mutations in a paired-like homeobox-containing gene. *Nature*. 1991;354:522–525.
29. Hazlett L, Masinick S, Mezger B, Barrett R, Kurpakus M, Garrett M. Ultrastructural, immunohistological and biochemical characterization of cultured mouse corneal epithelial cells. *Ophthalmic Res*. 1996;28:50–56.
30. Buse E, Seifert H. Glycoconjugate expression during early mouse oculogenesis. *Histochem J*. 1998;30:819–827.
31. Koroma BM, Yang JM, Sundin OH. The Pax-6 homeobox gene is expressed throughout the corneal and conjunctival epithelia. *Invest Ophthalmol Vis Sci*. 1998;38:108–120.
32. Gipson IK, Riddle CV, Kiorpes TC, Spurr SJ. Lectin binding to cell surfaces: comparisons between normal and migrating corneal epithelium. *Dev Biol*. 1983;96:337–345.
33. Davies J, Lyon M, Gallagher J, Garrod D. Sulphated proteoglycan is required for collecting duct growth and branching but not nephron formation during kidney development. *Development*. 1995;121:1507–1517.
34. Song B, Zhao M, Forrester J, McCaig C. Nerve regeneration and wound healing are stimulated and directed by an endogenous electrical field in vivo. *J Cell Sci*. 2004;117:4681–4690.
35. Ramaesh T, Ramaesh K, Leask R, et al. Increased apoptosis and abnormal wound healing responses in the $Pax6^{+/-}$ mouse cornea. *Invest Ophthalmol Vis Sci*. 2006;41:1911–1917.
36. Sivak JM, Mohan R, Rinehart WB, Xu PX, Maas RL, Fini ME. Pax-6 expression and activity are induced in the reepithelializing cornea and control activity of the transcriptional promoter for matrix metalloproteinase gelatinase B. *Dev Biol*. 2000;222:41–54.
37. Sivak JM, West-Mays JA, Yee A, Williams T, Fini ME. Transcription Factors Pax6 and AP-2α interact to coordinate corneal epithelial repair by controlling expression of matrix metalloproteinase gelatinase B. *Mol Cell Biol*. 2004;24:245–257.
38. Wilson SE, Mohan RR, Mohan RR, Ambrosio R Jr, Hong J, Lee J. The corneal wound healing response: cytokine-mediated interaction of the epithelium, stroma, and inflammatory cells. *Prog Retin Eye Res*. 2001;20:625–637.
39. Wilson SE, Netto M, Ambrosio R Jr. Corneal cells: chatty in development, homeostasis, wound healing, and disease. *Am J Ophthalmol*. 2003;136:530–536.
40. Simpson TI, Price DJ. Pax6; a pleiotropic player in development. *BioEssays*. 2002;24:1041–1051.