Increased Apoptosis and Abnormal Wound-Healing Responses in the Heterozygous *Pax6*$^{+/-}$ Mouse Cornea

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Purpose. Corneal wound healing involves a cascade of interactions between the epithelium and stroma. Pax6 is upregulated, and early events include epithelial cell migration and apoptosis of superficial keratocytes. The mouse heterozygous *Pax6* (*Pax6*$^{+/-}$) corneal phenotype mimics human aniridia-related keratopathy (ARK), and some aspects of wound healing have been shown to be abnormal, including matrix metalloproteinase (MMP)-9 expression. The purpose of this study was to test whether the *Pax6*$^{+/-}$ genotype affects corneal wound-healing responses, including stromal cell apoptosis, epithelial cell migration rate, and MMP secretion in culture.

Method. *Pax6*$^{+/-}$ and wild-type (*Pax6*$^{+/+}$) mice were killed and their corneas wounded by epithelial debridement. Whole eyes were cultured in organ culture and corneal epithelial healing rates and keratocyte apoptosis were quantified by topical fluorescein staining and TUNEL, respectively. Dissociated corneal epithelial cells from *Pax6*$^{+/-}$ and wild-type mice were cultured, and the activities of secreted MMP-9 were determined by zymography.

Results. Wound-healing rates during the first 6 hours were significantly faster for larger wounds and for *Pax6*$^{+/-}$ corneas. Compared with wild-type, wounded *Pax6*$^{+/-}$ eyes showed significantly more stromal cell apoptosis, and cultured *Pax6*$^{+/-}$ corneal epithelial cells produced lower MMP-9 activity.

Conclusions. The cumulative effect of abnormal wound-healing responses, characterized by increased stromal cell apoptosis and reduced levels of MMP-9 secretion may contribute to the corneal changes in the *Pax6*$^{+/-}$ mice. Possible contributions of elevated stromal cell apoptosis and other abnormal wound-healing responses to ARK are discussed. (*Invest Ophthalmol Vis Sci.* 2006;47:1911–1917) DOI:10.1167/iovs.05-1028

The *Pax6* gene encodes a transcription factor that is essential for normal development of the vertebrate eye.[1,2] It is downregulated in many adult eye tissues but is expressed in the adult corneal epithelium[3] and may be involved in its maintenance. Human PAX6-deficiency results in aniridia, an inherited panocular condition, characterized by complete or partial hypoplasia of the iris and often associated with corneal changes.[4,5] The term aniridia-related keratopathy (ARK) includes all the corneal changes observed in aniridic individuals. Corneal abnormalities of the heterozygous *Pax6*$^{Sey\text{-}Neu/+}$ mice are similar to those of the corneal changes in patients with aniridia[6] and these mice provide an excellent model for the study of corneal abnormalities in human aniridia.

Although, the corneal changes in aniridia have been presumed to be due to limbal stem cell deficiency,[7] recent studies of heterozygous *Pax6* (*Pax6*$^{+/-}$) mice and mouse chimeras suggest that other factors may play a role.[8–10] The mechanisms underlying progressive corneal disease in aniridia appear multifactorial and include abnormal corneal epithelial differentiation leading to fragility of epithelial cells,[8,10] abnormal epithelial migration,[9] and reduction in cell adhesion molecules in the *Pax6* heterozygous state,[8] rendering the cells susceptible to natural shearing forces. Recent observations on the *Pax6*$^{+/-}$ mouse model of aniridia also show that the proliferation and proliferative potential of the corneal and limbal epithelial cells may not be primarily impaired.[8,10] Furthermore, clinical observations suggest that abnormal wound-healing responses in the *PAX6*$^{+/-}$ corneas may cause corneal scarring in aniridic patients.[11] These observations lead to the hypothesis that abnormal wound-healing responses may also contribute to ARK.

Corneal wound healing is a complex process that is coordinated and regulated by autocrine and paracrine interactions of growth factors, cytokines, and enzymes produced by the epithelial cells, the stromal cells, and the lacrimal glands.[12–15] The interactions of corneal epithelial cells with components of the stromal extracellular matrix (ECM) are also important factors.[16] Insufficient or excessive levels of these molecules could be of major significance in impaired wound healing, which ultimately leads to the development of chronic ocular diseases.

The wound-healing response varies, depending on the type of injury and the tissues involved. In an epithelial scrape injury, the denuded surface is covered with fibronectin, onto which the adjacent epithelial cells migrate to cover the defect,[17–19] then proliferate to form the stratified epithelium.[20] The earliest stromal event after an epithelial injury is keratocyte apoptosis,[21,22] and these cells are replaced by proliferation and migration of keratocytes adjacent to the acellular zone.[23] Fibronectin and other ECM components are later removed by enzymatic degradation, and the basement membrane undergoes remodeling[24,25] which is associated with upregulated expression of matrix metalloproteinases (MMPs).

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MMP-2 (gelatinase-A; 72 kDa) and MMP-9 (gelatinase-B; 92 kDa) belong to the family of MMPs that participate in degrading and remodeling of ECM in various physiological and pathologic conditions, including corneal wound healing.[26–28] MMP-2 is expressed by stromal keratocytes,[29] upregulated during wound healing but not regulated by *Pax6*. Migrating basal epithelial cells express MMP-9, and there is a relatively quick peak in this activity after corneal wounding.[26,30] Pax6 controls the activity of the promoter for MMP-9.[31]

The purpose of this study was to investigate the corneal wound-healing responses in the heterozygous *Pax6* mouse model of aniridia. We hypothesize that Pax6 deficiency results in abnormal wound-healing responses, which may contribute to ARK. The corneal abnormalities in aniridia may be due to abnormal epithelial migration, altered keratocyte apoptotic response to epithelial injury, and/or impaired expression of MMPs, and so we investigated whether any of these was affected in $Pax6^{+/-}$ mice. Our results showed that all three wound-healing responses differed in $Pax6^{+/-}$ and wild-type corneas.

While our study was in progress, Sivak et al.[32] reported that reduced Pax6 dosage in heterozygous $Pax6^{+/-}$ mice resulted in loss of MMP-9 expression at the migrating epithelial front during wound healing. They also mentioned that corneal epithelial migration during wound healing was faster than normal in $Pax6^{+/-}$ mice, but did not present any experimental results. Our investigations of corneal wound healing of $Pax6^{+/-}$ and wild-type mice differ from this study in several respects. We studied wound healing in vitro and have demonstrated quantitative differences for epithelial cell migration and stromal apoptosis as well as MMP-9 expression.

Materials and Methods

Mice

Animals were handled according to the guidelines in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Heterozygous $Pax6^{+/Sey\text{-}Neu}$ ($Pax6^{+/-}$) mice, and wild-type ($Pax6^{+/+}$) littermates were produced from crosses between wild-type (C57BL × CBA/Ca)F1 females and $Pax6^{+/Sey\text{-}Neu}$ males on a CBA/Ca genetic background. Both the wild-type and heterozygous *Pax6* mice, aged 11 to 12 weeks, were killed by cervical dislocation, and the eyes were immediately wounded and then removed.

Whole-Eye Organ Culture Model

A trephine was used to mark a 1-mm diameter circular wound area in the center of the cornea, under a dissecting microscope. The corneal epithelium was debrided with an Algerbrush corneal rust ring remover with a 0.5 mm burr (Altomed, Tyne-and-Wear, UK). The whole eye was dissected, rinsed in warm PBS containing 1.0% antibiotic and antimycotic solution, and placed in culture wells (the corneas facing up) on membrane filters (0.6 μm, DTTP; Isopore; Millipore, Durham, UK). Eyes were incubated in culture medium containing minimum essential medium (Sigma-Aldrich, Poole, UK), 10% fetal bovine solution, 1% antibiotic/antimycotic solution, and 2 mM glutamine for various time points (depending on the experiment), at 37°C and 5% CO_2.

Isolation and Culture of Mouse Corneal Epithelial Cells

For corneal epithelial cell cultures, the culture medium was prepared as described in Hazlett et al.[33] Wild-type and heterozygous *Pax6* mice were killed by cervical dislocation. Eyes were enucleated, and the corneas were dissected and placed in 1% antibiotic/antimycotic solution for 10 minutes and then rinsed three times with PBS. Corneas were transferred into dispase II and incubated at 37°C for 1 hour. The epithelial layer was separated mechanically under a dissecting microscope; the epithelial sheet was cut into small pieces, transferred into trypsin-EDTA solution, and incubated for 10 minutes. The epithelial cells were dispersed by gentle pipetting; 2×10^4 cells were seeded in 48-well plates with the culture medium, and incubated. After 40 hours (when cells were in small colonies but not confluent), the cells were washed twice with warm PBS, and serum-free medium was added to each well and incubated for 24 hours. Conditioned medium was collected and centrifuged, and the supernatant stored at −20°C until the MMP assay.

Rate of Epithelial Healing in Whole-Eye Organ Cultures

Immediately after wounding, the epithelial lesions of both the wild-type and heterozygous *Pax6* eyes were topically stained with fluorescein (fluorescein sodium 1.0% wt/vol, diluted 1:1 in saline; Chauvin Pharmaceuticals, Ltd., Romford, UK) and photographed under blue light with a digital camera on a dissecting microscope (M5A; Wild, Heerbrugg, Switzerland). The eyes were then rinsed in warm PBS and allowed to heal in tissue culture conditions. After six hours, the epithelial lesions were stained with fluorescein, and photographs were obtained at the same magnification as immediately after wounding. Some of the eyes from both the wild-type and heterozygous *Pax6* mice were fixed in formalin, and the sections of the eyes were stained with hematoxylin and eosin. Using an Image Tool for Windows, ver. 3.00 (University of Texas Health Sciences Center, San Antonio [UTHSCSA]), the circumference of the wound margin of each eye was traced and the measurements of wound area were obtained for each eye immediately after wounding and 6 hours after wounding. The rate of epithelial healing (in square millimeters per hour) was calculated for the 6-hour period as (initial wound area − final wound area)/6. The diameter and radius of the wounds immediately after wounding and after 6 hours were calculated from the wound area measurements (assuming a circular wound) and the rate of epithelial healing (millimeter per hour) was obtained in relation to wound radius.

Assessment of Apoptosis

Wounded and unwounded eyes were incubated in the whole-eye organ culture system for 4 hours. The eyes were fixed in 4% paraformaldehyde (PFA) overnight, and after the lenses were removed, the specimens were embedded in paraffin wax. Seven-micrometer-thick sections extending transversely across the central wounded and unwounded corneas of the eyes were mounted on poly-L-lysine–coated slides. An apoptosis kit (In Situ Cell Death Detection Kit, Fluorescein; Roche Molecular Biomedicals, Mannheim, Germany) was used to label apoptotic cells in tissue sections. Deparaffinized sections were rehydrated through graded alcohols and washed with PBS. Tissue sections were treated with proteinase K (80 μg/mL) for 20 minutes at room temperature. Slides were washed twice with PBS. For positive controls, sections were incubated with DNase 1, grade 1 (50 U/mL DNase 1 in 50 mM Tris-HCl [pH 7.5], 1 mM $MgCl_2$, 1 mg/mL BSA; Roche Molecular Biomedicals) for 30 minutes at 37°C, before labeling procedures. TUNEL (terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick-end labeling) labeling was performed according to manufacturer's instructions. Slides were then washed with PBS and mounted in antifade medium with propidium iodide (Vectashield; Vector Laboratories, Burlingame, CA). Samples were visualized by fluorescence microscopy, and the images were obtained for quantitative analysis.

By using an illustrator image tool software program (Adobe Systems, Mountain View, CA), a grid, which divided the full thickness of the cornea into three different layers—the anterior, middle, and the posterior—was placed on the image of the wounded region of the cornea (see Fig. 3E). This grid could be moved across the cornea, thereby, the entire stromal region beneath epithelial scrape was subjected to quantitative analysis for each image. The number of positive and negative cells was counted in each segment separately from two randomly selected sections for each eye. The percentage of positive

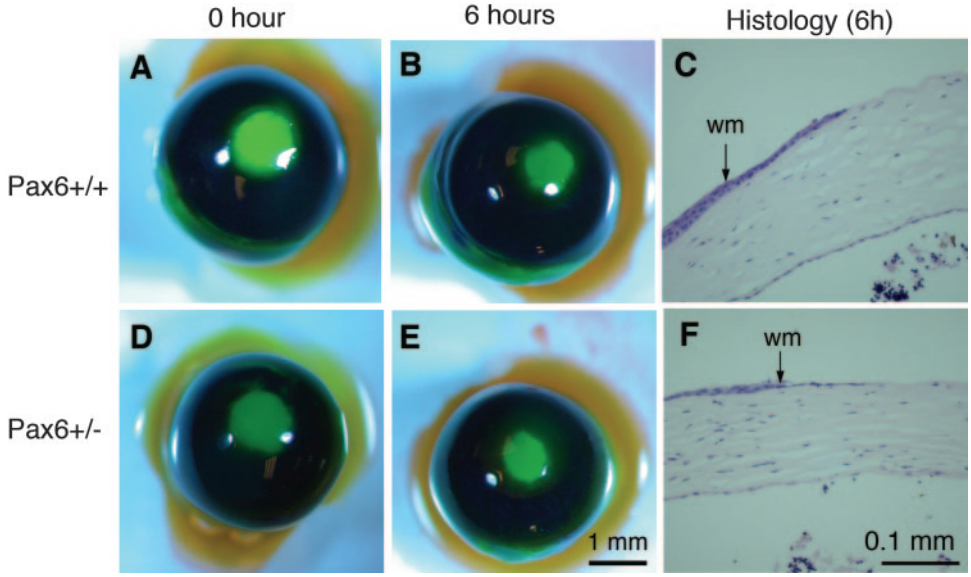

FIGURE 1. Corneal epithelial wound healing. (**A**, **B**, **D**, **E**) Representative images of wild-type and heterozygous *Pax6* eyes immediately after and 6 hours after wounding. The denuded surface of corneal regions were topically stained with fluorescein. Histology of wild-type and heterozygous *Pax6* eyes after 6 hours in culture (**C**, **F**), showing the migrating epithelial cells from the wound margin (wm) onto the wounded surface. Note that the migrating epithelial cells were extremely flattened in the *Pax6*$^{+/-}$ eye.

cells was obtained for each segment for statistical analysis. All percentages were transformed before statistical analysis using the arcsine transformation to stabilize the variance. The full (transformed) data set was analyzed by a three-way analysis of variance including effects for region (A, B, and C), layer (1, 2, and 3), and genotype (wild-type and *Pax6*$^{+/-}$) and all interactions.

MMP Zymography

Samples of conditioned media from the corneal epithelial cell cultures were brought to room temperature. From each sample, a volume of 7.5 μL was mixed with 7.5 μL of sample application buffer and subjected to gelatin zymography as described in Riley et al.[34] The bands of gelatinolytic activity detected by zymography were captured (GS-700 Imaging Densitometer; Bio-Rad, Hercules, CA) and the optical densities of the bands (expressed as uncalibrated optical density, uOD) were obtained using a computer-assisted image analysis system (Bio-Rad). Serial dilution of samples gives a linear change in the optical density measured. All sample measurements were taken in the linear (i.e., nonsaturated) range. The optical densities were compared by Student's *t*-test, and $P < 0.05$ was considered to be significant.

RESULTS

Corneal Epithelial Healing in Whole-Eye Organ Cultures

Reduction in wound size was observed in all the eyes after allowing them to heal in the culture system for 6 hours (Fig. 1A, 1B, 1D, 1E). In histologic sections of the healing corneas, the migrating epithelial sheet appeared as a monolayer on the wound surface in both genotypes (Figs. 1C, 1F). The *Pax6*$^{+/-}$ corneal epithelium consisted of three to four flattened cells, as reported previously for in vivo specimens,[6] and the migrating cells were extremely flattened (Fig. 1F).

Although, care was taken to keep the epithelial wound size constant (1 mm diameter) in all the eyes, some of the *Pax6*$^{+/-}$ samples had larger initial wounds, because the *Pax6*$^{+/-}$ corneal epithelium was fragile and easily detached from the surface. Therefore, some larger wounds were generated in the wild-type eyes to balance the groups. The mean two-dimensional healing rate (in square millimeters per hour) was calculated for the 6-hour period for the wild-type ($n = 25$) and *Pax6*$^{+/-}$ ($n = 22$) eyes. This showed linear trends of an increasing healing rate with increasing initial wound area for both genotypes (Fig. 2A) but the slopes differed. A linear regression model was fitted, allowing for separate regression lines for the two genotypes. The significance of the fit of the lines and the differences between slopes and intercepts were assessed by analysis of variance (F-tests). The overall fit of the two separate regression lines was significant ($P < 0.001$), and there were significant differences between the slopes ($P < 0.025$) and intercepts of the lines ($P < 0.001$), reflecting differences between genotypes.

The rate of change in the wound area with time is likely to be greater for larger wounds because they will have more cells at the wound's edge. To take account of this, the radius of the wound was calculated, and the analysis repeated for linear healing rate (in millimeters/hour), which relates more directly to the rate of movement of the epithelial cell sheet. Larger wounds still healed more quickly (Fig. 2B), and linear trends of increasing healing rate with increasing initial wound radius were significant for both genotypes ($P < 0.01$). Again the slope was steeper for *Pax6*$^{+/-}$ eyes (although this was not quite significant, $P < 0.06$) and the intercepts differed significantly ($P < 0.01$), implying differences between the genotypes. The analyses showed that the average healing rate was higher in *Pax6*$^{+/-}$ heterozygotes for wounds of ~0.5-mm radius or larger (area ≥ 0.7 mm^2) and that both the linear and the two-dimensional healing rates increased faster for *Pax6*$^{+/-}$ eyes as the initial wound radius increased.

Apoptotic Cells in the Stroma after Wounding in Whole-Eye Organ Cultures

Seven wounded and two unwounded eyes from each of the wild-type and the heterozygous *Pax6* mice were incubated in the whole-eye organ culture system for 4 hours. TUNEL-positive cells were detected in the stroma of all wounded corneas of both genotypes, whereas the unwounded corneas were devoid of positive cells (Figs. 3A–D). After they were wounded, the *Pax6*$^{+/-}$ corneas had more positive cells than did the wild-type. Although the positive cells were mostly located in the anterior stroma of the wild-type eyes, they were also detected in the deeper stroma of the *Pax6*$^{+/-}$ eyes (Figs. 3C, 3D).

Figure 3E shows the different layers and the regions of the stroma beneath the wound, in which the percentages of TUNEL-positive cells were obtained. The total number of stromal cells beneath the epithelial wound was higher for *Pax6*$^{+/-}$

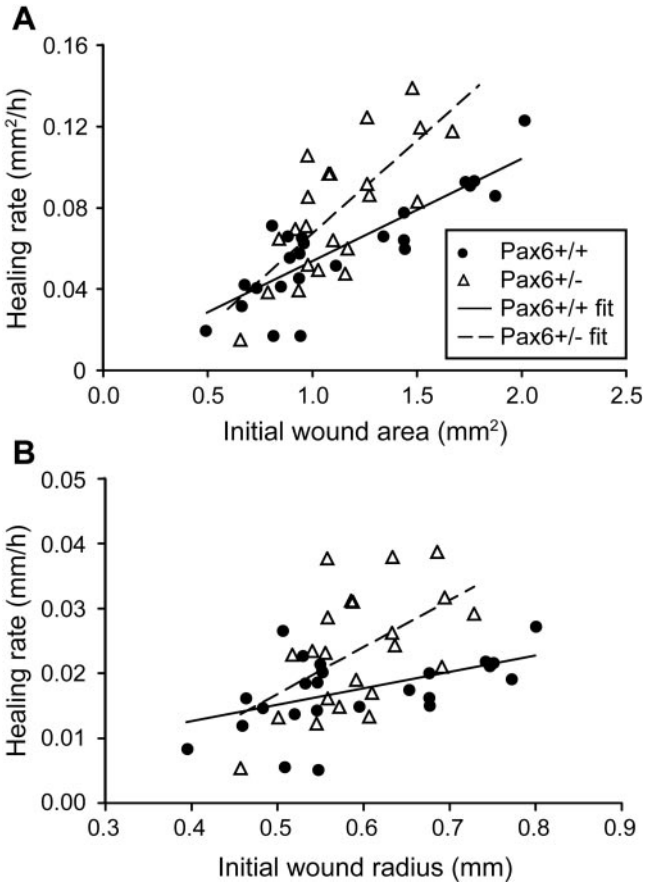

FIGURE 2. The relationship between the epithelial healing rate and the initial wound size in wild-type and heterozygous *Pax6* eyes. The healing rate increased with increasing initial wound area (**A**) and initial wound radius (**B**) for both genotypes and the increase in healing rate was faster for $Pax6^{+/-}$ eyes than wild-type. A linear regression model was fitted. For the wound area, the regression line for wild type was: $y = 0.0028 + 0.050x$, and the regression line for $Pax6^{+/-}$ was: $y = -0.025 + 0.092x$. For the wound radius, the regression line for wild type was: $y = 0.0024 + 0.025x$ and the regression line for $Pax6^{+/-}$ was: $y = -0.020 + 0.072x$.

eyes (mean ± SEM: 145.50 ± 9.99) than the wild-type (89.71 ± 3.74). The histogram (Fig. 3F) shows the mean percentage of positive cells in these locations. It clearly demonstrates that $Pax6^{+/-}$ corneas contained many TUNEL-positive cells, and these were not confined to the superficial part of the stroma. Highly significant differences were found individually between genotypes ($P < 0.001$) and among layers ($P < 0.001$) but not among regions ($P = 0.94$). The two-factor interactions were highly significant for genotype by layer ($P < 0.001$) and significant for region by genotype ($P < 0.05$). These results clearly show that the percentage of TUNEL-positive cells was less for the wild-type stroma in all layers. Significant levels of apoptosis occurred in the deeper layers of wounded $Pax6^{+/-}$ corneas, but there was much less in wild-type corneas. There was a slight but statistically significant tendency for wounded $Pax6^{+/-}$ corneas to have a higher percentage of apoptosis in region B (the central region). This difference may not be biologically significant because the trend was for slightly less apoptosis in region B of wounded wild-type corneas.

The $Pax6^{+/-}$ corneal stromas were significantly thinner than the wild-type stromas (118.1 ± 8.5 μm vs. 163.1 ± 5.2 μm) so the middle of the stroma (layer 2) would usually be closer to the surface (in absolute terms) in $Pax6^{+/-}$ mice than in wild-type mice. This difference could not entirely account for the much higher frequency of TUNEL-positive cells in layer 2 in $Pax6^{+/-}$ corneas, because the frequency of apoptosis was higher in $Pax6^{+/-}$ heterozygotes for all three layers. However, to confirm that $Pax6^{+/-}$ corneas had more apoptotic stromal cells in deeper layers than wild-type corneas, the frequency of TUNEL-positive cells was reanalyzed in the top and second 30-μm layers of region B. The top 30-μm of the stroma contained more TUNEL-positive cells in $Pax6^{+/-}$ corneas than in wild-type corneas (65.2% ± 6.1% vs. 48.5% ± 5.8%) and, more important, TUNEL-positive cells were still abundant in the deeper layer (30–60 μm) of region B. Overall, 54.4% ± 4.8% of cells in the 30- to 60-μm layer were TUNEL-positive cells in $Pax6^{+/-}$ corneas, but none was seen in wild-type corneas, and the difference was significant ($P = 0.0008$ by Mann-Whitney test).

MMP Secretion by Wild-Type and $Pax6^{+/-}$ Corneal Epithelial Cells

The conditioned medium from cultured corneal epithelial cells exhibited bands at 92-kDa (corresponding to MMP-9). The wild-type samples produced strong bands of activity, but bands from $Pax6^{+/-}$ cells were mostly weak and varied among samples (Fig. 4A). The quantitative analysis showed that the band optical density (mean ± SEM) was greater for wild-type cells than for $Pax6^{+/-}$ cells (23.11 ± 1.3 uOD vs. 14.75 ± 3.1 uOD; $n = 8$ in each case) and this was of borderline significance by *t*-test ($P = 0.05$). Although equal numbers of cells were seeded in each well, the number of cells in some of the wells containing the $Pax6^{+/-}$ epithelial cells appeared fewer at the time the medium was collected. After the medium was removed, the adherent cell nuclei were stained with hematoxylin and the number of cells was counted in five different areas. The mean cell count for $Pax6^{+/-}$ cultures was generally lower and more variable. This may reflect differences in cell adhesion.[8] A second gelatin zymogram was produced, where samples were loaded by normalizing the cell number (the volume of the culture medium was adjusted according to the average cell number of each well) for each sample (Fig. 4B). Quantitative analysis of this zymogram again revealed a significant reduction in the density (mean ± SEM) of the 92-kDa MMP-9 bands for the $Pax6^{+/-}$ samples compared with wild-type (11.75 ± 1.7 uOD vs. 18.27 ± 0.83 uOD; $n = 10$ in each case) by *t*-test ($P = 0.004$). Bargagna-Mohan et al.[35] showed that MMP-9 production was downregulated directly by high cell density, but our zymographic analyses clearly demonstrated that equivalent numbers of $Pax6^{+/-}$ epithelial cells secreted significantly less MMP-9, even if they were growing at a lower density than the wild-type epithelial cells.

DISCUSSION

Corneal epithelial healing involves epithelial cell migration, adhesion, proliferation, and stratification.[20] MMP-9 is induced in the epithelial cells adjacent to the wound,[30,32] and apoptosis is induced in stromal cells.[21] This is followed by a combination of stromal cell proliferation, migration, and matrix remodeling events that are an attempt to restore the function of the cornea. These events are regulated in a precise manner and any derangement of the regulation may fail to restore the clarity of the cornea. Pax6 is upregulated during corneal wound healing, and the process is abnormal in $Pax6^{+/-}$ heterozygotes. Abnormal corneal epithelial responses, have been reported recently in an in vivo study of $Pax6^{+/-}$ mice,[32] but no experimental evidence has been reported for faster wound healing or increased stromal cell apoptosis in $Pax6^{+/-}$ heterozygotes.

In corneal wound healing, factors released from the tear film, vasculature, inflammatory cells, and neuronal factors par-

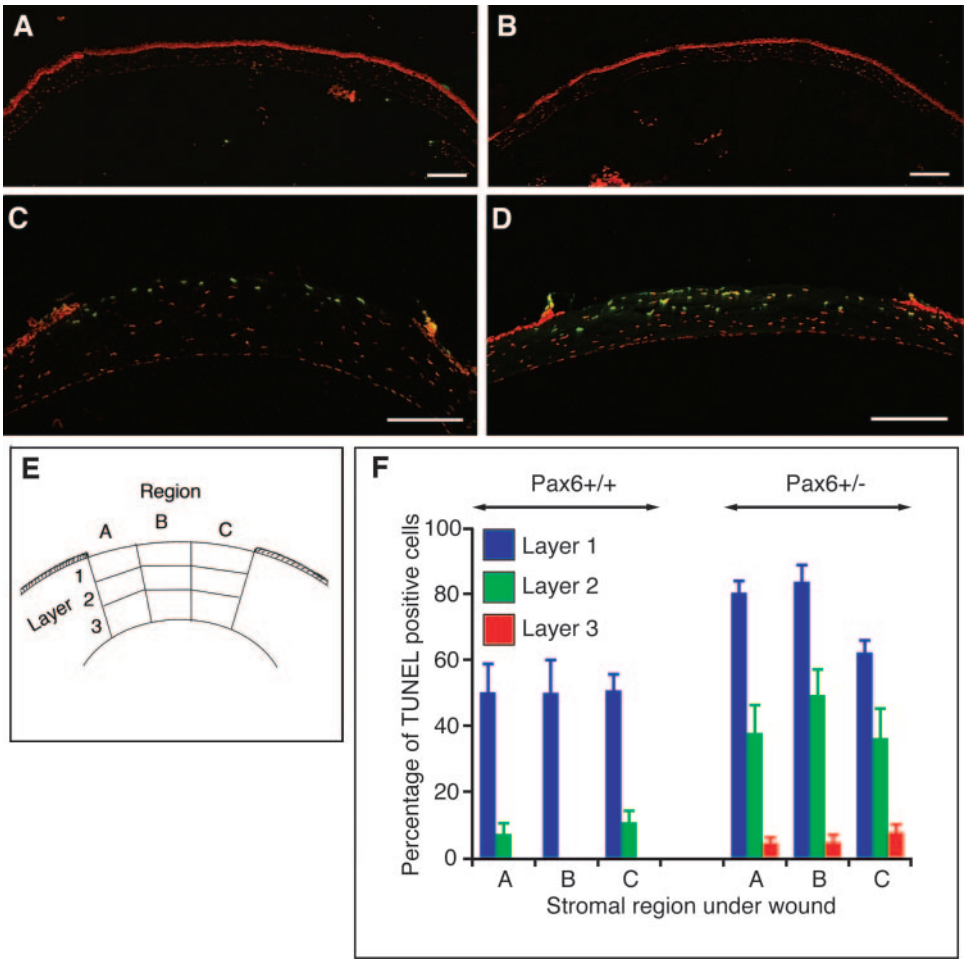

FIGURE 3. TUNEL labeling of stromal cell apoptosis in wild-type and heterozygous *Pax6* mouse eyes. TUNEL-positive cells were not detected in the unwounded corneas of (**A**) wild-type or (**B**) heterozygous *Pax6* corneas. Positive cells were detected mostly in the superficial stroma of the (**C**) wild-type corneas but in the (**D**) heterozygous *Pax6* corneas, there were more positive cells, some of which were located deeper in the stroma. (**A**) and (**B**) show most of the cornea and are at a lower magnification than (**C**) and (**D**). (**E**) Diagram showing division of the stroma into different regions and layers for estimation of percentage of TUNEL-positive cells. (**F**) Histogram showing the distribution of percentages of TUNEL-positive cells in wild-type and heterozygous *Pax6* mouse corneas. Scale bar, 200 μm.

ticipate in the responses to injury. A previous study shows that the *Pax6*$^{Sey\text{-}Neu/+}$ mouse corneal stroma often becomes vascularized and infiltrated with inflammatory cells.[6] The in vitro corneal wound-healing model we used has the advantage of eliminating vascular components, such as platelets, which are a major source of TGF-β and other cytokines that mediate wound healing. Therefore, this in vitro wound-healing model provided an optimal system for the study of the epithelial and stromal wound-healing response and its relationship to low levels of Pax6 in the cornea.

Rate of Corneal Epithelial Healing

We have demonstrated that the healing rate was faster for larger wounds for both genotypes, which is consistent with a previous study on rabbit corneal wound healing.[36] We also showed that wound healing was faster for *Pax6*$^{+/-}$ than wild-type corneas, and this difference was greater for larger wounds.

One of the first responses to epithelial debridement is detachment of individual epithelial cells from the remaining epithelium as a result of a loss of adhesion to adjacent cells.[37] Active movement of epithelial cells is also important at the initial stage of epithelial healing, and mitosis plays a role at later stages. *Pax6*$^{+/-}$ corneal epithelial cells show reduced levels of adhesion[8] and increased rates of proliferation compared with wild type cells.[8,10] Reduced cellular adhesion could be mediated partly by affects on levels of desmoglein, β-catenin, γ-catenin, and keratin-12[8] and increased proliferation could be mediated through effects on epidermal growth factor (EGF).[38] Decreased adhesion and increased proliferation may both fa-

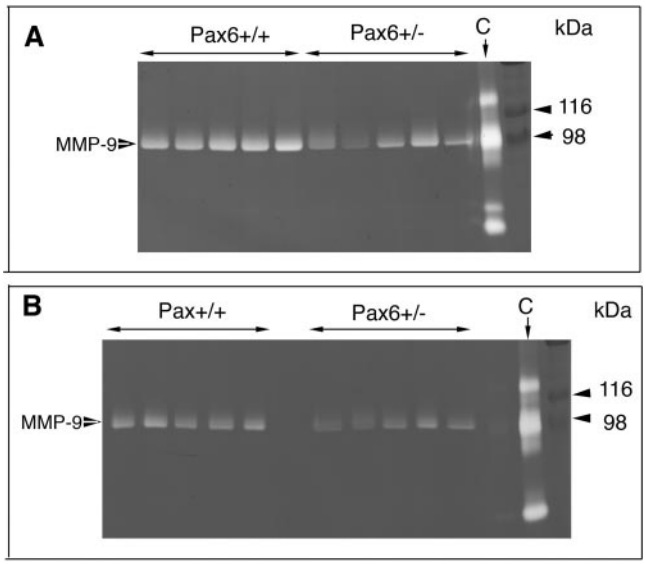

FIGURE 4. Gelatin zymograms of conditioned medium from cultured corneal epithelial cells. Conditioned medium from the corneal epithelial cells exhibited bands at 92 kDa, corresponding to MMP-9. In zymogram (**A**), equal volumes (7.5 μL) of samples were loaded in each lane; in zymogram (**B**), samples were loaded according to the number of cells attached to the culture wells after the medium had been collected. C, control sample, human amniotic fluid, which contains MMP-9. *Right*: protein markers and the corresponding molecular weights.

cilitate faster healing rates in this genotype, but differences in proliferation may be less important in the short-term response that we studied. Although the initial healing rate appeared faster in the *Pax6*$^{+/-}$ corneas, the restoration of epithelial barrier function may be affected if these cells are less able to reestablish cell–cell and cell–matrix adhesions at later stages of epithelial wound healing.

Stromal Cell Apoptosis

The TUNEL analysis showed that wounding caused more stromal cell apoptosis in heterozygous *Pax6* than wild-type corneas. It is possible that some of the apoptotic stromal cells were inflammatory cells and/or vascular cells (which are often present in the *Pax6*$^{+/-}$ stroma) it seems likely that most were keratocytes. It is known that epithelial scrape injury causes apoptosis of keratocytes[21] but it is not known whether this can cause apoptosis of inflammatory cells. Keratocyte apoptosis is an important determinant of wound healing associated with corneal surgery[39] and may be triggered by the release of cytokines, such as interleukin (IL)-1, Fas ligand, and bone morphogenic protein 2/4, from the injured epithelium.[21,39–41] The present study demonstrates, for the first time, that stromal cell apoptosis is increased in *Pax6*$^{+/-}$ mouse corneas after epithelial debridement.

The observation that the level of IL-1α was increased in migrating corneal epithelial cells after epithelial debridement in MMP-9-deficient mice[42] raises the possibility that the increase in stromal cell apoptosis in heterozygous *Pax6* mice could be partly attributable to a similar IL-1α increase, mediated by decreased MMP-9 secretion (discussed later). Alternatively, *Pax6*$^{+/-}$ keratocytes could be intrinsically more sensitive to proapoptotic signals. For example, cultured keratocytes from keratoconus, a corneal dystrophy, have more IL-1 receptors,[43,44] suggesting a greater sensitivity to IL-1. Although the exact mechanism by which Pax6 deficiency causes increased stromal cell apoptosis remains unclear, the discovery that stromal cell apoptosis is increased in heterozygous *Pax6* mice after corneal wounding is likely to be relevant to the clinical treatment of patients with aniridia and ARK (discussed later).

Secretion of MMP-9 by Corneal Epithelial Cells

The conditioned medium from the corneal epithelial cell cultures routinely produced the 92-kDa MMP-9 band and, as predicted, the activity was significantly reduced from the cells derived from the *Pax6*$^{+/-}$ corneas. Reduced levels of MMP-9 in the injured corneal epithelium has been associated with faster epithelial healing rate,[42] and so the faster healing rate in the *Pax6*$^{+/-}$ mice, in both our whole-eye organ cultures and in vivo,[32] could be related to reduced levels of MMP-9 secreted by the *Pax6*$^{+/-}$ corneal epithelial cells.

Abnormal Wound-Healing Responses in *Pax6*$^{+/-}$ Corneas and ARK

Corneal changes in ARK include a thin and fragile corneal epithelium containing goblet cells, aberrant basement membrane, defective Bowman's layer and abnormal stroma with infiltration of inflammatory cells and neovascularization.[4,5,7,45] The corneal changes are progressive as the cornea appears normal and transparent during infancy and childhood, but begins to show changes that are marked by the ingrowth of blood vessels from the limbal region into the peripheral cornea and subsequent appearance of goblet cells in the corneal epithelium during early teens.[7,45] These changes can eventually culminate in opacification of the corneal stroma that leads to visual loss. There is clinical evidence that wound healing may be abnormal in patients with aniridia,[11] and it seems likely that abnormal wound healing contributes to the features of ARK. If so, the risk of inducing abnormal scarring should be borne in mind when considering surgical intervention to treat corneal opacities in patients with aniridia.

Both increased stromal apoptosis and reduced MMP-9 secretion during *Pax6*$^{+/-}$ wound healing could contribute to the corneal deterioration associated with ARK. Excessive apoptosis may result in tissue damage, contribute to degenerative corneal diseases,[46,47] and alter the wound-healing responses. Significant local depletion of keratocytes may cause activation of adjacent keratocytes, and subsequent generation of fibroblasts with repair-phenotype, or myofibroblasts, which could result in fibrotic wound healing and scar formation in the cornea,[48] rather than regenerative wound healing. Extracellular matrices provide structural organization to the cornea, and their specific composition contributes to the corneal transparency. The corneal matrices undergo constant slow remodeling during health, and rapid remodeling during repair.[27,28,48] Remodeling is mediated by matrix metalloproteinases,[26,49] including MMP-9, which is secreted by migrating basal epithelial cells after corneal wounding.[30,32] Reduced levels of MMP-9 in the healing corneal epithelial cells as a result of Pax6-deficiency could result in accumulation of fibrin, inflammatory cells, and extracellular matrix, which disturb the normal collagen-matrix relationship and the transparency of the cornea. These factors along with other cytokines, growth factors, and proteolytic enzymes may tilt the balance toward angiogenesis in the cornea, which eventually leads to the corneal opacities in ARK.

Other factors may contribute to abnormal corneal wound healing in *Pax6*$^{+/-}$ mice. A recent study of the bovine cornea identified putative stromal progenitor cells, which expressed Pax6.[50] During corneal wound healing, stromal progenitor cells are likely to provide the replacement adult keratocytes, which secrete transparent extracellular matrix. If Pax6 depletion in heterozygous *Pax6* mice impaired the function of stromal progenitor cells, this may promote the activation of keratocytes, and lead them to assume a fibrotic phenotype and secrete a nontransparent extracellular matrix.

In conclusion, our in vitro study has identified three aspects of corneal wound healing that differ between *Pax6*$^{+/-}$ and wild-type mice. We confirmed that *Pax6*$^{+/-}$ corneal epithelial cells secrete lower levels of MMP-9, provided the first experimental evidence that the corneal epithelial healing rate is faster in *Pax6*$^{+/-}$ mice, and showed that more stromal cell apoptosis occurs after wounding in *Pax6*$^{+/-}$ heterozygotes. We suggest that the pathogenic mechanisms of corneal changes seen in aniridia are attributable, in part, to abnormal corneal scarring resulting from excessive apoptotic response and delayed and deficient extracellular matrix metabolism that aggravate the corneal changes.

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