Keratocyte Density and Recovery of Subbasal Nerves After Penetrating Keratoplasty and in Late Endothelial Failure

Sanjay V. Patel, MD; Jay C. Erie, MD; Jay W. McLaren, PhD; William M. Bourne, MD

Objective: To determine central keratocyte and subbasal nerve densities in clear and failed grafts after penetrating keratoplasty.

Methods: Clear grafts and grafts with late endothelial failure (LEF) were examined using confocal microscopy 1 to 31 years after penetrating keratoplasty. Keratocyte density, number of keratocytes in a full-thickness column of stroma, and subbasal nerve density were determined from images. Comparisons were made with normal corneas.

Results: The mean±SD keratocyte density in clear grafts (22101±3799 cells/mm³) was lower than that in normal corneas (26610±3683 cells/mm³; \( P < .001 \)) but did not differ from that in grafts with LEF (21268±3298 cells/mm³; \( P = .47 \)). The mean±SD number of keratocytes in clear grafts (10325±1708 cells) was lower than that in normal corneas (11466±1503 cells; \( P < .001 \)) but did not differ from that in grafts with LEF (10778±1760 cells; \( P = .39 \)). Median subbasal nerve density in clear grafts (150 µm/mm²) was lower than that in normal corneas (7025 µm/mm²; \( P < .001 \)), and nerve recovery correlated with time after surgery (\( r = 0.36; P < .001 \)).

Conclusions: Keratocyte density and number are decreased in penetrating grafts compared with normal corneas. Subbasal nerve density does not recover to normal through 3 decades.

Arch Ophthalmol. 2007;125(12):1693-1698

Penetrating keratoplasty (PK) involves the replacement of host stroma and endothelium by donor tissue and requires transection of all host corneal nerves. Changes in the endothelium after PK are well documented and include a faster-than-normal rate of cell loss and decreased permeability. Endothelial cell loss results in nonimmunologic endothelial failure, which is a leading cause of graft failure that we term late endothelial failure (LEF).

The etiology of the stromal translucency in LEF is not known, but changes in keratocyte density and function might increase corneal backscatter. Little is known about the role of keratocytes and corneal nerves after PK. Confocal microscopy enables visualization of stromal keratocytes and corneal nerve fiber bundles in vivo, and we devised methods for quantifying keratocyte density and subbasal nerve fiber density from confocal images of corneas. In a preliminary study that used this method, we found that keratocyte density was decreased in clear penetrating grafts compared with normal corneas. In the present study, we expand this series of clear penetrating grafts and also report keratocyte and subbasal nerve densities in grafts with LEF.
CONFOCAL MICROSCOPY

A Tandem Scanning confocal microscope (Tandem Scanning Corp, Reston, Virginia) was used to examine corneas in vivo as described previously. Briefly, after instillation of topical anesthetic, a drop of an optical coupling medium was placed on the objective, and the objective was adjusted to provide an en face view of the central cornea. The patient fixated on a target with the contralateral eye to minimize eye movements. Digital images of the central cornea were recorded with the optical section advancing through the full-thickness cornea. Each image represented a coronal section of cornea that was approximately 475 × 350 µm (horizontal × vertical). A “through-focus” series of images of 1 cornea constituted 1 “scan” and consisted of fewer than 300 video frames, depending on the thickness of the cornea. Two to 4 scans were acquired per eye.

KERATOCYTE DENSITY

The best-quality scan without motion artifact was selected for each cornea. For density measurement, the corneal stroma was divided into 5 layers: the anterior, middle, and posterior thirds of the stroma, and the anterior and posterior thirds were further subdivided into 2 unequal layers so that the anterior 10% and the posterior 10% of the stroma were represented. Two images without motion artifact were selected from each layer; for the anterior 10% layer, 1 of the 2 images was always the most anterior image containing keratocytes. Images were analyzed using a custom automated program, which objectively identified bright objects (presumed to represent kerocyte nuclei) and calculated keratocyte density.

SUBBASAL NERVE DENSITY

Subbasal nerve fiber bundles are visible at the basal aspect of the basal epithelial cell layer in confocal microscopy images of normal corneas. All confocal scans for each penetrating graft were reviewed by a single observer (J.C.E.), who was masked to the clinical status and the postoperative age of the graft. The total length of all visible subbasal nerve fiber bundles and their branches longer than 50 µm in each scan was measured using a custom program. Each nerve fiber bundle was measured only once, but if its length extended across several adjacent images, the total length was measured as if it were projected onto 1 image. Subbasal nerve density for each scan was calculated as the total length of nerve divided by the area of the image (0.187 mm²), and the subbasal nerve density for each cornea was the mean of the densities in all usable scans for that examination.

DATA ANALYSIS

Because central corneal thickness increases with time after PK, we calculated the total number of keratocytes in a full-thickness column of central stroma with 1 mm² of frontal surface area; for brevity, we refer to this variable as “number of keratocytes.” Full-thickness keratocyte density was calculated as a weighted mean by dividing the number of keratocytes by the central stromal thickness, which was also measured using confocal microscopy. Mean keratocyte density of full-thickness stroma, keratocyte density for each layer of stroma, number of keratocytes, stromal thickness, and subbasal nerve density were compared between clear grafts and controls and between clear grafts and grafts with LEF. Differences were examined using unpaired t tests if the data were distributed normally or Wilcoxon rank sum tests if the data were not distributed normally. P < .05 was considered statistically significant. Correlations between keratocyte density or subbasal nerve density and time after keratoplasty were assessed using Pearson cor-

### Table 1. Preoperative Diagnoses in Clear Penetrating Grafts and Grafts With Late Endothelial Failure

<table>
<thead>
<tr>
<th>Preoperative Diagnosis</th>
<th>Clear Penetrating Grafts, No. (n=99)</th>
<th>Grafts With Late Endothelial Failure, No. (n=21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Keratoconus</td>
<td>52</td>
<td>13</td>
</tr>
<tr>
<td>Fuchs dystrophy</td>
<td>33</td>
<td>5</td>
</tr>
<tr>
<td>Pseudophakic corneal edema</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Aphakic corneal edema</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Traumatic scar</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Other</td>
<td>4</td>
<td>1c</td>
</tr>
</tbody>
</table>

*a Quantitative analysis of confocal microscopy images was possible in only 12 eyes (10 with keratoconus, 1 with Fuchs dystrophy, and 1 with pseudophakic corneal edema).

*b Includes 1 eye each with herpes simplex keratitis, interstitial keratitis, granular dystrophy, and central cloudy dystrophy of François.

*c Failed graft; original diagnosis was keratoconus.

### Table 2. Keratocyte Density and Number of Keratocytes in Normal Corneas, Clear Penetrating Grafts, and Grafts With LEF

<table>
<thead>
<tr>
<th></th>
<th>Normal (Nonsurgically Treated) Corneas (n=63)</th>
<th>Clear Penetrating Grafts (n=99)</th>
<th>Grafts With LEF (n=12)</th>
<th>MDD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full-thickness keratocyte density, weighted mean±SD, cells/mm²</td>
<td>26 610±3683</td>
<td>22 101±3799b</td>
<td>21 268±3298b</td>
<td>3250</td>
</tr>
<tr>
<td>Keratocyte density by depth of stroma, mean±SD, cells/mm²</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anterior 0%-10%</td>
<td>42 513±8544</td>
<td>33 598±7967c</td>
<td>34 586±7791c</td>
<td>6900</td>
</tr>
<tr>
<td>11%-33%</td>
<td>28 092±4918</td>
<td>24 855±5289b</td>
<td>26 170±5883d</td>
<td>4650</td>
</tr>
<tr>
<td>34%-66%</td>
<td>23 614±4744</td>
<td>21 441±5129</td>
<td>19 215±5618c</td>
<td>4500</td>
</tr>
<tr>
<td>67%-90%</td>
<td>23 943±4561</td>
<td>17 281±5580b</td>
<td>15 372±4187c</td>
<td>4750</td>
</tr>
<tr>
<td>Posterior, 91%-100%</td>
<td>24 141±4704</td>
<td>17 654±6089b</td>
<td>17 985±666c</td>
<td>5350</td>
</tr>
<tr>
<td>No. of keratocytes in full-thickness central stroma with frontal area of 1 mm², mean±SD, cells</td>
<td>11 466±1503</td>
<td>10 325±1708b</td>
<td>10 778±1760d</td>
<td>1490</td>
</tr>
</tbody>
</table>

Abbreviations: LEF, late endothelial failure; MDD, minimum detectable difference between clear grafts and grafts with LEF (α=.05; β=.20).

a No significant differences existed between clear grafts and grafts with LEF.

b P < .001 vs normal (unpaired t tests).

c P < .01 vs normal (unpaired t tests).

d P > .05 vs normal (unpaired t tests). The MDD for keratocyte density in the 11% to 33% region of stroma was 4550 cells/mm² and for number of keratocytes was 1400 cells (α=.05 and β=.20 for both analyses).
relation coefficients if the data were distributed normally or Spearman tests if the data were not distributed normally. A paired analysis was performed on clear grafts that were examined twice 4 years or more apart and 5 years or more after keratoplasty. The annual rate of keratocyte loss (percentage lost per year) was calculated from the number of keratocytes at each examination and the interval between examinations by assuming that the number of keratocytes decreased as a simple first-order loss. Generalized estimating equation models were used to adjust for potential correlation between fellow eyes of the same patient. The generalized estimating equation model results are not reported because they did not alter any of the conclusions.

**RESULTS**

**CLEAR GRAFTS AFTER PK**

Mean keratocyte density of the full-thickness stroma was 17% lower in clear grafts than in controls ($P < .001$) (Table 2). Keratocyte density in each layer of stroma was also lower in clear grafts compared with controls (Table 2 and Figure 1). The number of keratocytes in clear grafts was 10% lower than that in controls ($P < .001$) (Table 2). The central stroma of clear grafts (mean±SD, 470±46 µm) was thicker than that of controls (433±36 µm; $P < .001$). Median subbasal nerve density was lower in clear grafts (150 µm/mm², range 0-5846 µm/mm²) (n=94) than in controls (7025 µm/mm²; range, 2371-12,448 µm/mm²; $P < .001$) (n=76); nerves often regenerated in a random and disordered pattern (Figure 2). No subbasal nerves were detected in 45 clear grafts (48%). Median subbasal nerve density in clear grafts for keratoconus (897 µm/mm²; range, 0-5846 µm/mm²) (n=31) was lower than in controls for Fuchs dystrophy (377 µm/mm²; range, 0-2981 µm/mm²; $P = .04$) (n=31).

Mean keratocyte density correlated weakly with time after surgery ($r = -0.20; P = .05$) (n=99), whereas there was no correlation between the number of keratocytes and time after surgery ($r = -0.04; P = .68$) (n=99) (Table 3 and eFigure 1; available at: http://www.archophthalmol.com). Stromal thickness and recovery of subbasal nerve density were correlated with time after surgery ($r = 0.26$; $P = .009$ [n=99] and $r = 0.36; P < .001$ [n=94], respectively) (Table 3 and eFigure 2; available at: http://www.archophthalmol.com).

Sixteen clear grafts of 14 eyes were examined on 2 occasions separated by a mean±SD of 5.0±0.6 years (range, 4-6 years). Time after keratoplasty for the first and second examinations was 17.6±4.8 years (range, 5-24 years) and 22.6±5.2 years (range, 9-30 years), respectively. Mean keratocyte density for the full-thickness stroma was decreased 13% at the later examination compared with the earlier examination ($P = .02$) (Table 4). Keratocyte density decreased only in the middle and posterior thirds of the stroma between the first and second examinations (Table 4 and Figure 3). The number of keratocytes was decreased 13% at the second examination compared with the first examination ($P = .03$) (Table 4). The mean±SD rate of loss of keratocytes was 2.9%±5.0% per year. Mean±SD central stromal thickness did not differ between the first (461±40 µm) and second (473±61 µm) examinations ($P = .48$); the minimum detectable difference was 50 µm ($\alpha = .05; \beta = .20$) (n=16).

**GRAFTS WITH LEF**

Keratocyte density could be measured in only 12 grafts with LEF (12 patients); images of the remaining grafts with LEF

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**Table 3. Correlations With Time After Keratoplasty in Clear Penetrating Grafts**

<table>
<thead>
<tr>
<th></th>
<th>r</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full-thickness keratocyte density</td>
<td>-0.20</td>
<td>.05</td>
</tr>
<tr>
<td>Keratocyte density by depth of stroma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anterior</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0%-10%</td>
<td>0.35</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>11%-33%</td>
<td>-0.09</td>
<td>.37</td>
</tr>
<tr>
<td>34%-66%</td>
<td>-0.27</td>
<td>.008</td>
</tr>
<tr>
<td>67%-90%</td>
<td>-0.33</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Posterior, 91%-100%</td>
<td>-0.14</td>
<td>.18</td>
</tr>
<tr>
<td>No. of keratocytes in full-thickness central stroma with frontal area of 1 mm²</td>
<td>-0.04</td>
<td>.68</td>
</tr>
<tr>
<td>Subbasal nerve density (n=94)</td>
<td>0.36</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

aThere were 99 grafts for all tests unless stated otherwise.
were hazy from stromal edema, or an interdigitating network of keratocyte processes in the anterior stroma prevented identification of individual keratocytes (eFigure 3; available at http://www.archophthalmol.com). Keratocyte density and number of keratocytes did not differ between grafts with LEF and clear grafts (Table 2 and Figure 1). The central stroma in grafts with LEF was thicker (mean ± SD, 508 ± 48 µm) than in clear grafts (470 ± 46 µm; P = .008). Median subbasal nerve density in grafts with LEF (340 µm/mm²) (n = 11) did not differ from that in clear grafts (150 µm/mm²; P = .95) (n = 94).

Keratocyte density and subbasal nerve fiber bundle density after PK were significantly lower than those in normal corneas measured using confocal microscopy in vivo. Although keratocytes after PK were lost at a faster-than-normal rate,7 we did not detect a difference between keratocyte density in clear grafts vs grafts with LEF. Subbasal nerve fiber bundles did not seem to regenerate to any clinically significant extent through 30 years after PK.

Keratocyte density is highest in the most anterior layers of normal corneal stroma, and density declines with age at a rate similar to normal corneal endothelial and trabecular meshwork cells.7 Given that central corneal thickness increases with time after keratoplasty1,2 and that the stroma of penetrating grafts was thicker than normal in the present study, it is not surprising that keratocyte density in clear grafts was decreased compared with normal because of redistribution of cells over a larger volume of stroma. However, the number of keratocytes was also reduced in penetrating grafts compared with normal, contributing to decreased keratocyte density. The paired analysis showed that the number of keratocytes in the central stroma continued to decrease many years after keratoplasty at a rate of 2.9% ± 5.0% per year. Keratocyte density also decreased during the 5-year period, but only in the middle and posterior thirds of the cornea. This decrease in density might be explained by greater loss of keratocytes from the middle and posterior stroma than from the anterior stroma, more swelling in the middle and posterior stroma than in the anterior stroma,21 or both. Swelling could not, however, explain the overall loss of keratocytes from the full-thickness column of stroma. We did not detect an increase in stromal thickness in the 5 years between repeated examinations, although with this sample size, we could not detect differences smaller than 50 µm. The rate of decrease of keratocytes of 2.9% per year in the paired analysis was not supported by the cross-sectional analysis (eFigure 1) and was much higher than the rate of endothelial cell loss in the second decade after keratoplasty.1,2 We calculated image contrast from the first and second confocal examinations in the paired analysis and found that contrast was significantly lower at the second examination compared with the first examination. However, even after adjusting keratocyte density for variations in contrast, keratocyte density and number of keratocytes were still 10% lower at the second examination compared with the first examination. We are unaware of other studies that quantified keratocyte loss after PK, but it is conceivable that keratocytes are lost at a faster rate than endothelial cells and that keratocyte loss is higher in certain subgroups after PK.

Donor keratocytes are lost by apoptosis and necrosis during tissue preservation,22 but continued keratocyte losses several years after transplantation have not been previously documented. Keratocyte loss in penetrating

**Table 4. Keratocyte Density at Repeated Examinations of Clear Penetrating Graftsa**

<table>
<thead>
<tr>
<th></th>
<th>First Examination (n=16)</th>
<th>Second Examination (n=16)</th>
<th>P Value (MDD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Keratocyte density by depth of stroma, mean±SD, cells/mm³</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anterior</td>
<td>21 631±3605</td>
<td>18 840±5792</td>
<td>.02</td>
</tr>
<tr>
<td>0%-10%</td>
<td>34 096±8464</td>
<td>33 548±8393</td>
<td>.84 (7750)</td>
</tr>
<tr>
<td>11%-33%</td>
<td>23 864±5329</td>
<td>21 674±7219</td>
<td>.22 (5150)</td>
</tr>
<tr>
<td>34%-66%</td>
<td>21 472±4889</td>
<td>16 688±5075</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>67%-90%</td>
<td>16 169±3771</td>
<td>13 085±4963</td>
<td>.02</td>
</tr>
<tr>
<td>Posterior, 91%-100%</td>
<td>18 503±5811</td>
<td>13 143±5335</td>
<td>.009</td>
</tr>
<tr>
<td>No. of keratocytes in full-thickness central stroma with frontal area of 1 mm², cells</td>
<td>9959±1797</td>
<td>8749±2260</td>
<td>.03</td>
</tr>
</tbody>
</table>

Abbreviation: MDD, minimum detectable difference between the first and second examinations (paired analysis: a = .05 and b = .20; n=16).

aData are included for 16 grafts of 14 patients examined twice 4 or more years apart and 5 or more years after penetrating keratoplasty. The first and second examinations were a mean±SD of 17.6±4.8 years (range, 5-24 years) and 22.6±5.2 years (range, 9-30 years) after keratoplasty, respectively.

**Figure 3.** In clear grafts examined twice a mean±SD of 17.6±4.8 years and 22.6±5.2 years after keratoplasty, keratocyte density decreased in the middle and posterior thirds of the stroma between examinations.
grafts might be caused by starvation from decreased endothelial permeability, chronic apoptosis, or other undefined mechanisms. Although we could not show a difference in the number of keratocytes between grafts with LEF and clear grafts, this study had limited power to detect small differences and was cross-sectional and not prospective in design; the sample may also have been biased because only 12 of 21 grafts with LEF had images that could be analyzed quantitatively. Nevertheless, even if a small difference were to exist between keratocyte density in grafts with LEF and clear grafts, endothelial cell loss seems to be more critical to the development of LEF.

In clear penetrating grafts, it is unclear whether transplanted keratocytes survive for decades or whether they are replaced by host keratocytes that migrate from the periphery. Donor keratocytes survive in clear grafts for at least a year after keratoplasty in rabbits and for as long as 4 years after keratoplasty in failed grafts in humans. Keratocytes also have migratory potential and can repopulate epikeratopathia lenticularis, anterior lamellar grafts, and acellular collagen implants. The present study was not designed to determine whether host keratocytes migrate into the donor, but if that were the case, the results indicate that the rate of repopulation would be inadequate to maintain or increase the number of keratocytes in the corneal stroma.

Alterations in stromal transparency after PK could result from keratocyte dysfunction with or without decreased keratocyte density. The transparency of keratocytes seems to be related to the expression of intracellular crystallins, and decreased crystallin expression has been associated with repair cell phenotypes and increased backscatter from cells. We frequently noted keratocyte activation in grafts with LEF (eFigure 3), and long-term keratocyte activation after PK might result in increased corneal backscatter, which would clinically manifest as stromal opacity.

Decreased keratocyte density after PK has been found by other investigators using different confocal microscopes. Hollingsworth et al used a scanning slit confocal microscope and found that anterior and posterior keratocyte densities were decreased but stable during the first year after keratoplasty compared with normal corneas, similar to our prospective data during the first year after PK. Niederer et al used a laser scanning confocal microscope and found that keratocyte density was decreased in the anterior, middle, and posterior thirds of penetrating grafts several years after keratoplasty. The confocal microscopes used in the 2 latter studies are limited in their ability to measure the depth of confocal images accurately, and, therefore, volumetric keratocyte density and the absolute number of keratocytes were not calculated, preventing direct comparison with the present study. However, the confocal microscopy images in the studies by Hollingsworth et al and by Niederer et al are of higher contrast compared with images from the Tandem Scanning confocal microscope, and identifying objects as keratocytes is easier than with our system. Nevertheless, Mikek et al also used a scanning slit confocal microscope with high-contrast images and found no difference in keratocyte density after PK compared with normal corneas. Because interpretation of the low-contrast images from our confocal microscope may be subjective, we used an automated program to identify keratocytes objectively and calculate keratocyte density.

Subbasal nerve fiber bundles are transected during PK, and regeneration of nerve fiber bundles in the donor is slow and incomplete, even 3 decades after surgery. These cross-sectional data suggest an increase in subbasal nerve density with time after keratoplasty, but the increase was not clinically significant in the context of normal subbasal nerve density. Slow and limited subbasal nerve regeneration after PK in humans has been previously demonstrated using confocal microscopy and acetylcholinesterase staining ex vivo. Corneal sensitivity was not measured in the present study, but sensitivity is known to remain significantly reduced, if not absent, for decades after PK. Tervo et al showed that epithelial innervation was reestablished after PK in humans, but stromal nerve regeneration was essentially absent, and they suggested that the discontinuity of Schwann cell channels at the graft-host junction impaired stromal nerve regeneration. Indeed, the hypothesis of Tervo et al is supported by evidence of significant stromal nerve regeneration after close apposition of limbal incisions in rabbits and also by recovery of normal subbasal nerve density by 2 and 3 years after photorefractive keratectomy and laser in situ keratomileusis, respectively. The reason for higher subbasal nerve density in grafts for keratoconus compared with grafts for Fuchs dystrophy is not known, but the present results confirm the findings of Niederer et al.

Whether corneal nerves are required to sustain keratocyte density and function is uncertain, but anterior keratocyte losses have been noted after keratorefractive surgery, in which there is an extended period of denervation postoperatively. No specific physiologic relationships between nerves and keratocytes have been established, but, anatomically, human corneal nerve fibers invaginate the cytoplasm of keratocytes, suggesting that keratocytes might receive trophic factors from corneal nerves, and, therefore, chronic denervation in grafts might contribute to keratocyte loss.

Further studies are necessary to determine the mechanisms of keratocyte loss after PK and the relationships among corneal nerves, keratocytes, endothelial cells, and stromal transparency. Preventing loss of stromal transparency could improve graft longevity. Furthermore, similar physiologic processes might affect the transparency of the host stroma after posterior lamellar keratoplasty, which is currently popular among corneal surgeons, and could affect the long-term success of these procedures.

Submitted for Publication: June 7, 2007; final revision received July 17, 2007; accepted July 23, 2007.

Correspondence: Sanjay V. Patel, MD, Department of Ophthalmology, Mayo Clinic College of Medicine, 200 First St SW, Rochester, MN 55905 (patel.sanjay@mayo.edu).

Financial Disclosure: None reported.

Funding/Sponsor: This study was supported by grant EY02037 from the National Institutes of Health, by Re-
search to Prevent Blindness Inc (Dr Patel as Olga Keith Wiess scholar and an unrestricted grant to the Depart-
ment of Ophthalmology, Mayo Clinic), and by the Mayo Foundation.

Previous Presentations: This study was presented in part at the Annual Meeting of the Association for Research
in Vision and Ophthalmology; May 8, 2007; Fort Lau-
derdale, Florida; and at the Annual Meeting of the Ameri-
can Ophthalmological Society; May 22, 2007; White Sul-
phur Springs, West Virginia.

Additional Information: The eFigures are available at http:

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**eFigure 1.** Cross-sectional analysis of 99 clear penetrating grafts revealed no correlation between the number of keratocytes in the full-thickness central stroma and time after surgery ($r=-0.04; P=.68$).

**eFigure 2.** Cross-sectional analysis of 94 clear penetrating grafts suggested that subbasal nerve fiber bundle density increased with time after surgery ($r=0.36; P<.001$) but remained markedly decreased compared with normal corneas (median, 7025 µm/mm$^2$; $n=76$).

**eFigure 3.** Confocal microscopic image of anterior keratocytes in a penetrating graft with late endothelial failure shows visible cell processes, suggesting keratocyte activation.