Predicative Factors for Corneal Endothelial Cell Migration

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PURPOSE. To characterize the effects of Descemet’s stripping, Rho-associated protein kinase inhibitor Y-27632, and donor age on endothelial migration in human corneas maintained in ex vivo culture.

METHODS. Twenty-eight cadaveric human corneas underwent ex vivo culture in either standard or Y-27632-supplemented culture medium for 14 days. The posterior surface of each cornea was manipulated to create two types of wounds: scratched wound—corneal endothelial cells (CECs) were denuded from the Descemet’s membrane (DM) to leave behind a bare but intact DM; and peeled wound—both the DM and overlying CECs were stripped to leave behind bare corneal stroma. Endothelial migration was assessed via Trypan blue staining. Morphologic traits of CECs were assessed via Alizarin red microscopy and scanning electron microscopy.

RESULTS. The CECs migrated preferentially over scratched wounds compared with peeled wounds. Y-27632 supplementation accelerated endothelial migration over scratched wounds. Endothelial migration decreased with advanced donor age for both wound types, regardless of exposure to Y-27632. Y-27632 supplementation resulted in a less rapid decline in endothelial migration for donors older than 50 years of age for scratched surfaces. Greater cell density and hexagonality was observed over scratched wounds compared with peeled wounds, regardless of Y-27632 supplementation.

CONCLUSIONS. The presence of an intact DM, Y-27632 supplementation, and young donor age are factors that promote endothelial migration in an ex vivo human cornea culture model. The negative effect of age on endothelial migration can be mitigated by the presence of an intact DM and Y-27632 supplementation.

Keywords: cornea, endothelium, culture, Y-27632, Descemet’s

The posterior surface of a healthy adult cornea is lined by a monolayer of corneal endothelial cells (CECs) arranged in a regular hexagonal array with a density of approximately 2000–2500 cells/mm2.2 Corneal endothelial cells exert homeostatic control of corneal hydration by allowing passive diffusion of fluids into the corneal stroma and actively pumping excess fluid back into the anterior chamber.2

Current clinical and laboratory evidence indicates that CECs within the human eye are locked in the G1 phase of cell cycle and are incapable of active cellular proliferation.3–5 When the cornea is exposed to stresses such as dissipated phacoemulsification energy during cataract extraction, CECs may be damaged or permanently lost from the CEC monolayer.6 The resultant discontinuity within the CEC monolayer is compensated by expansion and migration of neighboring healthy CECs in order to maintain its functional integrity.7 When CEC density falls below a critical threshold of approximately 500 cells/mm2, the monolayer of CECs will no longer be able to exert sufficient homeostatic control to regulate corneal hydration.6 Corneal decompensation (bullous keratopathy) characterized by clinical signs such as stromal edema, epithelial edema, and Descemet’s membrane (DM) folds may ensue, leading to pain and compromise of visual acuity.8

At present, the only effective treatment for severe corneal endotheliopathy is corneal transplantation.9 However, all forms of keratoplasty are associated with postoperative endothelial cell losses.10–12 In the first year following Descemet’s membrane endothelial keratoplasty, Descemet’s stripping automated endothelial keratoplasty, and penetrating keratoplasty, endothelial cell losses have been reported as 35%,11 14.9%,12 and 16.5%,10 respectively. By the third postoperative year, endothelial cell losses will have increased to approximately 47%,11 29.7%,13,14 and 55%,10 respectively. The progression of endothelial cell loss may eventually lead to graft failure and repeat graft surgeries in some patients.15 Current global demand for donor corneal tissue remains well in excess of what may be supplied by eye banks, with corneal graft failure and the need for repeat graft procedures accounting for up to 22.4% of tissue demand.16 As such, there is a need to explore alternative therapeutic options that may either reduce the need for repeat corneal grafts by enhancing the survival of grafted corneal endothelial cells or, in a bolder approach, reduce the reliance on such large amounts of donor material in the treatment of corneal endothelial diseases. In particular, significant progress has been made in the field of corneal endothelial cell engineering...
over the last decade. The successful development of in vitro techniques to stimulate proliferation and migration of cultured CECs has led to attempts to translate these results clinically.19

Fuchs’ endothelial dystrophy (FED) is a primary cornea endotheliopathy characterized by guttae excrescences on the DM, accompanied by the presence of dysfunctional CECs. These guttae are aberrant collections of banded DM that occur in variable sizes and distribution densities, most commonly found within the interpalpebral fissure zone. Disease progression is characterized by an increase in the size and density of guttae and concomitant reductions in the population of healthy CECs. In contrast, pseudophakic bullous keratopathy (PBK) is an acquired corneal endothelial disorder that may occur following intraocular cataract extraction surgery. Various causative factors such as excessive phacoemulsification energy and mechanical trauma to the corneal endothelium during cataract extraction surgery have been implicated in causing stress-induced apoptosis of CECs. Although PBK is characterized by diffuse damage to the corneal endothelial layer, a peripheral rim of healthy CECs and DM is usually retained in corneas affected by FED. As such, it has been postulated that this reservoir of healthy CECs in FED corneas can potentially be exploited to repopulate the centrally diseased corneal endothelium either by migration or proliferation, on exposure to externally applied stimulants.

Topical Rho-associated protein kinase inhibitors (ROCK inhibitors) such as AR-12286, AMA0076, and Y27632 have been studied extensively, with proven efficacy in the treatment of primary open angle glaucoma and stimulation of CEC proliferation and migration under the appropriate culture conditions in vitro. More recently, an attempt to reproduce this effect in vivo by treatment of FED patients with topical Y27632 was also met with reasonable success. Patients with endothelial decompensation and corneal edema secondary to FED were first treated by transcorneal freezing to remove the diseased central endothelial cells, followed by intensive topical Y27632 treatment postoperatively. The efficacy of Y27632 in promoting recovery of the CEC monolayer was evidenced by significant reductions in corneal edema among three of four patients, with the eventual restoration of corneal clarity and achievement of 20/20 vision in one patient.

Although Y27632 has been shown to be efficacious in the treatment of FED, we believe that this therapeutic effect may not be universal and that it is likely to be strongly dependent on FED disease severity. Specifically, we hypothesized that confluent guttae found in advanced FED may hinder endothelial recovery by acting as physical barriers to the migration of CECs, thus limiting the success of topical Y27632 treatment in these patients. We further postulated that removal of the diseased DM and confluent guttae, prior to induction of treatment by topical Y27632, may improve outcomes. However, the practicality of this strategy is premised on successful migration of CECs from the DM onto bare posterior corneal stroma following removal of the diseased DM. The biologic feasibility of this phenomenon is uncertain and must be further investigated.

This study was designed to investigate the migration of human CECs within an ex vivo human cornea culture model, with the primary aim being to delineate the differences in CEC growth dynamics over DM-stripped, bare posterior corneal stroma (peeled wound) compared with that over structurally normal but CEC-denuded DM (scratched wound). The effects of Y27632 supplementation and donor age on corneal endothelial migration were also investigated.

**Materials and Methods**

**Ethics Statement**

The following protocols conformed to the tenets of the Declaration of Helsinki, and written consent was acquired from the next of kin of all deceased donors regarding eye donation for research. This study was approved by the institutional review board of the Singapore Eye Research Institute/Singapore National Eye Centre.

**Tissue Procurement**

A total of 28 cadaveric research-grade human corneas were procured from the Lions Eye Institute for Transplant and Research, Inc. (Tampa, FL, USA) and transported to our laboratory via air freight for the purpose of this study. Detailed donor information is available in Supplementary Table S1. All specimens were preserved in Optisol solution (Bausch & Lomb, Rochester, NY, USA) and encased within a specialized container en-route. Upon arrival at the laboratory, all cornea buttons were incubated in a wash buffer solution for three cycles, each lasting 5 minutes. The wash buffer comprised a mixture of Opti-MEM, endothelial serum-free media, and calcium/magnesium-supplemented PBS in a 1:1:2 ratio (all components from Life Technologies, Thermo Fisher Scientific Corporation, Carlsbad, CA, USA), containing gentamicin (25 μg/mL), amphotericin B (1 μg/mL), and 1× penicillin and streptomycin. Experimentation on the corneas was initiated immediately after the third cycle. There was a mean delay of 8.4 ± 2.4 days between time of donor death and initiation of experimentation.

**Wound Creation**

The primary aim of this study was to investigate the differences in CEC growth dynamics over DM-stripped, bare posterior corneal stroma (peeled wound) compared with that over structurally normal but CEC-denuded DM (scratched wound). As such, the posterior surface of each cornea was manipulated to produce both peeled and scratched wounds at predesignated sites prior to initiation of ex vivo CEC culture. Two methods of cornea manipulation were used: the schematic technique and representative outcome of method 1 are shown in Figures 1A and 1B, respectively, and those for method 2 are shown in Figures 1C and 1D.

**Method 1.** The outline of a circle measuring 5 mm in diameter was marked on the cornea endothelial surface with a trephine. Care was taken to apply minimal pressure to the cornea surface, in order to prevent injury to the DM or underlying corneal stroma. A pair of forceps was used to peel off the DM from half of the 5-mm circle in a piecemeal manner (peeled area), whereas the smooth, rounded shaft of a Sinskey hook was used to gently denude endothelial cells without damaging the DM in an adjacent quadrant within the 5-mm circle (scratched area). Normal endothelium and DM architecture were preserved in the remaining quadrant. All manipulation was performed under direct visualization with a Nikon SMZ2500 stereomicroscope (Nikon Instruments, Tokyo, Japan). Trypan blue (Life Technologies, Thermo Fisher Scientific Corporation) was used to assist visualization of DM, endothelial cells, and underlying stroma. A total of 14 corneas were processed as such for experiment 1 of this study.

**Method 2.** The outlines of two circles, each measuring 3 mm in diameter, were marked on the cornea endothelial surface with the aid of a trephine. A gap of approximately 2 mm was left in between these circles. Scratched wounds were created in a manner identical to that described for method 1. Peeled wounds in experiment 2 were created by a continuous
curvilinear descemetorhexis (CCD). The CCD involved the initial creation of a DM tear at the center of the circle, followed by extension of the tear in a continuous and curvilinear manner, up to the point where a complete circle of DM had been peeled off. A total of 14 corneas were processed in this manner for experiment 2 of this study.

Ex Vivo Culture

Each cornea was incubated in growth medium throughout the study for a total duration of 14 days. The formulation of the culture medium, also known as F99,35 is composed of a 1:1 mix of Ham’s F12 and M199 cell culture medium, supplemented with 5% fetal bovine serum, basic fibroblast growth factor (10 ng/mL), 1× penicillin/streptomycin, ascorbic acid (20 μg/mL), and 1× insulin/transferrin/selenium supplement (all components from Life Technologies, Thermo Fisher Scientific Corporation). Culture medium was refreshed every other day. All corneas were incubated in a controlled environment at 37°C with carbon dioxide concentration set at 5%.

Y-27632 Supplementation

The ex vivo cultures of selected corneas were subjected to treatment with the Rho-associated protein kinase inhibitor Y-27632 (Miltenyi Biotec GmbH, Cologne, Germany). In these corneas, a final concentration of 10 μM Y-27632 was used, in accordance with the optimal concentration for stimulation of in vitro human CEC proliferation found in previous reports.18,36 It should be noted that all corneas in experiment 1 originated from different donors, and they were randomly selected for Y-27632 supplementation. Conversely, the 14 corneas used in experiment 2 originated from seven donor-matched pairs, with one member of each pair randomly assigned to receive Y-27632 supplementation. Freshly prepared Y-27632 was added to the culture medium at each medium change (i.e., every other day).

Study Design: Experiment 1 Versus Experiment 2

In this study, we evaluated the capacities of human CECs to recover from an ex vivo model of corneal injury. Experiment 1 was a preliminary study, designed primarily to explore the effects of wound type on CEC migration. A pair of wounds (scratched and peeled) was created in each cornea using method 1. However, the creation of a peeled wound profile via the piecemeal approach of DM stripping (method 1) often resulted in an irregularly shaped descemetorhexis with an uneven residual stromal bed. The effect of wound type on CEC migration was determined by making pairwise comparisons (scratched versus peeled) for each of the 14 corneas. A total of five corneas were cultured in Y-27632–supplemented media, and endothelial migration was compared with that experienced by the remaining nine corneas that were not exposed to Y-27632. However, as the subjects in the two groups (Y-27632–supplemented versus non–Y-27632–supplemented) were not donor matched, the confounding effects of interdonor variability could not be avoided when making comparisons between these two groups.

Experiment 2 was designed in such a manner as to overcome some of the difficulties encountered in experiment 1. Creation of peeled wounds via the continuous curvilinear descemetorhexis approach (method 2) resulted in more consistent wounds profiles with much smoother residual stromal beds in contrast to the piecemeal approach (method 1). Seven donor-matched pairs of donor corneas were procured for experiment 2; a cornea from each pair was cultured in Y-27632–supplemented medium, whereas the contralateral cornea was cultured in standard, non–Y-27632–supplemented medium. The effect of Y-27632 supplementation on CEC migration could thus be determined via pairwise comparisons (Y-27632–supplemented versus non–Y-27632–supplemented) in a donor-matched fashion, which eliminated interdonor variability.
Imaging and Image Processing

Specimens were imaged immediately following initial manipulation and every other day until day 14 for both experiments. A 1:2 mixture of 0.4% Trypan blue solution (Sigma-Aldrich Corp., Singapore) to wash buffer (buffered Trypan blue solution) was reconstituted and used to assist visualization during creation of scratched and peeled wounds. Following 30 seconds of immersion in Trypan blue solution, both peeled and scratched wounds stained blue, whereas areas with an intact CEC monolayer did not exhibit any Trypan blue uptake and appeared transparent. Peeled wounds were also readily distinguished from scratched wounds by the presence of distinct descemetorhexis margins (Fig. 2). Images were obtained using the Nikon SMZ1500 stereomicroscope and Nikon DSFi 1-L2 high-definition color camera (Nikon Instruments), with backlit illumination provided by a halogen light source located beneath the microscope stage. A translucent white filter was placed beneath each cornea specimen during imaging so that the unstained areas of the cornea appeared white instead of transparent, which enhanced image contrast and improved accuracy of edge detection. All images were obtained at a digital resolution of five megapixels, with optical image magnifications of between 7.5× and 10×. Automatic white balancing was performed by the camera software prior to acquisition of each image. All images were analyzed with ImageJ (National Institutes of Health, Bethesda, MD, USA), with margins of the advancing CEC rims marked out manually. Maximum endothelial recovery referred to the maximum area of the wound that achieved negative staining (i.e., appeared white) by the end of the experiment, measured as percentage of the total wound area. Endothelial recovery rate was calculated as maximum endothelial recovery divided by the total number of days taken to achieve the recovery, measured as...
percentage recovery per day. Rate of endothelial migration and maximum area of endothelial migration were measured for all corneas with this method.

**Alizarin Red Microscopy**

A pair of donor-matched corneas was randomly selected for Trypan blue/Alizarin red staining following 14 days of ex vivo culture. Each of these corneas was first stained for 3 minutes in the buffered Trypan blue solution. The excess was decanted, and the cornea was immediately stained in freshly prepared filtered Alizarin red solution (0.5% in H2O, pH 4.5) (Sigma-Aldrich Corp.). The specimen was then washed for 60 seconds in a wash buffer, prior to wet mounting and examination with a Zeiss Axioplan 2 microscope (Zeiss, Oberkochen, Germany).

**Scanning Electron Microscopy**

Selected corneas were imaged by scanning electron microscopy. Specimens were first immersed overnight at 4°C in a fixative solution consisting of 2% glutaraldehyde in PBS (pH 7.4; Electron Microscopy Sciences, Hatfield, PA, USA), washed twice in PBS for 5 minutes each, and stained with 1% osmium tetroxide at room temperature for 1 hour prior to final fixation. Serial ethanol dehydrations were performed at ethanol dilutions of 25%, 50%, 75%, 95%, and 100% for 10 minutes each, with the last step repeated three times. This was followed by drying in a critical point dryer (BALTEC, Balzers, Liechtenstein) and mounting onto a scanning electron microscope (SEM) stub using carbon adhesive tabs. The specimen was then sputter-coated with a layer of gold (BALTEC) for 110 seconds prior to examination with a scanning electron microscope (Quanta 650FEG; FEI, Hillsboro, OR, USA).

**Statistical Analysis**

SPSS (version 22.0; SPSS, Inc., Chicago, IL, USA) was used for data analysis; *t* tests with Bonferroni corrections for multiple comparisons were applied to detect intergroup differences in endothelial migration. Analysis of covariance was used to determine the effect of donor age on endothelial migration in experiment 2. Unless otherwise stated, all numeric data obtained were expressed as mean ± SD or mean (95% confidence interval), and results were deemed to be statistically significant when *P* ≤ 0.05 was achieved.

**RESULTS**

**Trypan Blue Staining**

Representative images of a pair of young (donor age, 25 years old; corneas 19 and 20) and old corneas (donor age, 50 years old; corneas 19 and 20) are shown in Figure 3. Corresponding numerical values are shown in Table 1.

**Table 1. Effects of Wound Type and Y-27632 on Endothelial Migration (Experiment 2)**

<table>
<thead>
<tr>
<th>Wound Type</th>
<th>Negative Control</th>
<th>Y-27632</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum endothelial migration (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scratched</td>
<td>76.9 (42.1 to 111.6)</td>
<td>92.3 (79.2 to 105.3)</td>
<td>0.364</td>
</tr>
<tr>
<td>Peeled</td>
<td>24.4 (−2.29 to 51.1)</td>
<td>21.6 (4.1 to 39.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>P</td>
<td>0.032</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endothelial migration rate (%/day)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scratched</td>
<td>13.1 (6.82 to 19.3)</td>
<td>14.9 (11.5 to 18.3)</td>
<td>0.390</td>
</tr>
<tr>
<td>Peeled</td>
<td>1.74 (−0.16 to 3.65)</td>
<td>1.54 (0.29 to 2.79)</td>
<td>0.735</td>
</tr>
<tr>
<td>P</td>
<td>0.008</td>
<td></td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Numerical values corresponding to data points in Figure 3. Values represented as mean (95% confidence interval). *P* values obtained via paired *t* tests with Bonferroni correction for multiple comparisons. Negative control indicates corneas cultured in basal culture media; +Y-27632 indicates corneas cultured in Y-27632 supplemented culture media.
old; corneas 15 and 16) at days 3, 9, and 14 of experiment 2 are presented in Figure 2. Areas that stained blue represent either exposed posterior corneal stroma (peeled wounds) or exposed DM (scratched wounds). Peeled wounds were easily differentiated from scratched wounds by their desmectomorhexis margins. Following ex vivo culture, the presence of a layer of regenerated CECs over the peeled or scratched wounds inhibited Trypan blue staining. As such, the dimensions of the positively staining areas were observed to decrease with time, the extent (maximum % surface area of endothelial migration) and rate (% endothelial migration per day) of which were measured and subject to statistical analyses as detailed in the following sections.

Experiment 1

A total of 14 unpaired corneas were studied in experiment 1, with 5 corneas (35.7% of total) assigned to receive Y-27632 supplementation (Supplementary Table S1). The mean age and endothelial cell density of Y-27632-supplemented corneas was 55.4 ± 19.2 years old and 2435 ± 150 cells/mm², respectively, whereas that of non-Y-27632–supplemented corneas was 43.3 ± 21.5 years old (P = 0.308) and 2799 ± 448 cells/mm² (P = 0.109), respectively. Of the Y-27632-treated eyes, 40% were from male donors, compared with 67% among non-Y-27632–treated eyes (P = 0.334). There was a delay of 9.4 ± 1.8 days between donor death and initiation of cell culture in Y-27632–supplemented eyes and 7.2 ± 2.0 days in nonsupplemented eyes (P = 0.695).

The endothelial migration characteristics for all study groups in experiment 1 are summarized in Supplementary Figure S1. The general observation was that of faster and more extensive migration over scratched wounds compared with peeled wounds, regardless of Y-27632 supplementation. Specifically, in cultures not supplemented with Y-27632, scratched wounds showed greater endothelial migration rates (P < 0.001) and greater maximum endothelial migration (P < 0.001) compared with peeled wounds. With Y-27632 supplementation, scratched wounds also exhibited significantly faster endothelial migration rates compared with peeled areas (P = 0.008), but no significant differences were observed between scratched and peeled wounds in terms of maximum endothelial migration (P = 0.144).

Although Y-27632 supplementation resulted in significant increments of endothelial migration rates for scratched wounds compared with corneas cultured in non–Y-27632–supplemented medium (P = 0.016), a similar effect was not observed for peeled wounds (P = 0.350). The addition of Y-27632 also did not result in any significant improvements in maximum endothelial migration area for either scratched (P = 0.262) or peeled (P = 0.558) wounds. It should be noted that the small sample size or the effect of donor tissue age may have contributed toward the large confidence intervals associated with these measurements.

Experiment 2

A total of seven pairs of corneas were studied in experiment 2. Each member of a pair was randomly assigned to receive Y-27632 supplementation (Supplementary Table S1). The average donor age of corneas in each group was 45.6 ± 24.0 years old, with 57% of corneas derived from male donors. Cell culture was initiated within 8.9 ± 2.6 days of donor death. The mean endothelial cell density was 2784 ± 322 cells/mm². Endothelial migration characteristics for all study groups in experiment 2 are summarized in Figures 3 and 4. When cultured in non–Y-27632–supplemented medium, scratched wounds experienced greater maximum endothelial migration (P = 0.032) and endothelial migration rates (P = 0.008) compared with peeled wounds (Fig. 3; Table 1). These differences were accentuated under Y-27632 stimulation (P < 0.001 for both maximum endothelial migration and endothelial migration rates).

Analysis of covariance was subsequently performed to examine the effects of interdonor age variations on endothelial migration and to detect interactions between donor age and Y-27632 supplementation in regard to their effects on endothelial migration. Increasing donor age was associated with decreased maximum endothelial migration and decreased endothelial migration rates for both scratched and peeled wounds, regardless of Y-27632 supplementation (P < 0.001 for all models; Fig. 4). Significant interactions were found between donor age and Y-27632 supplementation in regard to maximum endothelial migration (P < 0.001) and endothelial migration rate (P = 0.005) over scratched wounds. Although young donors achieved complete and rapid endothelial migration over scratched wounds regardless of Y-27632 supplementation, sharp reductions in migration were encountered with increasing age (P < 0.001). The addition of Y-27632 resulted in a less rapid decline in maximum endothelial migration and endothelial migration rate over scratched wounds for donors 50 years of age and older (P = 0.003). No significant interactions were observed between donor age and Y-27632 supplementation for maximum peeled endothelial migration or peeled endothelial migration rate (P = 0.513).

Premature Culture Failure

Premature culture failure (PCF) was defined as complete and irreversible endothelial cell loss that occurred prior to completion of 14 days of follow-up in this study. This was characterized by complete Trypan blue uptake of the cornea's posterior surface (Fig. 2; cornea 16, day 9). Several corneas experienced PCF in experiment 1 and were excluded from the study (results not shown). In experiment 2, a total of three cornea pairs experienced PCF (Supplementary Table S2). Within these cornea pairs, all three corneas that were not exposed to Y-27632 succumbed to PCF, with an average mean survival duration of 3.67 ± 1.15 days (n = 3). When exposed to Y-27632, only two of the three donor-matched pairs experienced PCF. More importantly, their mean survival duration was 6 ± 1.41 days (n = 2).

Microscopic Studies

Alizarin red microscopy and scanning electron microscopy images are shown in Figure 5 and Table 2. Measurements of cell density, percentage hexagonality, cell circularity, and cell size were assessed based on images of Alizarin red staining. Cell density and hexagonality were higher in scratched wounds compared with peeled wounds for both Y-27632–supplemented and non–Y-27632–supplemented media (P < 0.001 for all). Y-27632 stimulation resulted in higher cell density over scratched wounds (P = 0.012) compared with non–Y-27632–supplemented medium. Under Y-27632 supplementation, CECs over peeled wounds were less circular than those scored over scratched wounds (P = 0.008). CECs over both peeled and scratched wounds appeared to be similarly circular when cultured in non–Y-27632–supplemented medium (P = 0.287). There were no statistically significant differences in cell sizes between scratched and peeled wounds for both Y-27632–supplemented (P = 0.089) and non–Y-27632–supplemented (P = 0.060) media.
DISCUSSION

In experiment 1, we found that scratched wounds experienced faster and more extensive endothelial migration compared with peeled wounds, regardless of Y-27632 supplementation. The results of experiment 1 were also suggestive that Y-27632 accelerated endothelial migration rates over scratched wounds ($P = 0.016$). While the mean values of maximum endothelial migration were higher in Y-27632–supplemented eyes compared with negative controls, these differences were not statistically significant ($P = 0.144$). We believe that the lack of statistical significance was due to the large confidence interval in the peeled group, which could be attributed to (1) small sample size and (2) inconsistent peeled wound profiles and uneven residual stromal beds obtained with the piecemeal method of DM stripping in experiment 1.

In order to validate these findings in experiment 1, we repeated the study using donor-matched cornea pairs in experiment 2 to eliminate interdonor variability. Peeled wounds were also created using the improved method of CCD, which resulted in consistent peeled wound profiles and improved stromal bed smoothness. Results from experiment 2 confirmed that CECs could migrate ex vivo but with a preference of a scratched surface instead of a peeled surface, regardless of Y-27632 supplementation. This suggested that an intact DM was required to facilitate the migration of CECs, which is not surprising in view of extensive evidence from in vitro culture studies that have shown that the presence of an
FIGURE 5. Alizarin red microscopy and scanning electron microscopy images obtained at day 14 of experiment 2. Cell density and hexagonality were higher in scratched wounds compared with peeled wounds regardless of Y-27632 supplementation (P < 0.001 for all). Among scratched wounds, Y-27632 supplementation resulted in higher cell density (P = 0.012); this effect was absent among peeled wounds. When supplemented with Y-27632, cells over peeled wounds were less circular than those over scratched wounds (P = 0.008). Scale Bars: 100 μm. Corresponding numerical values are shown in Table 2.

TABLE 2. Endothelium Morphologic Characteristics (Experiment 2)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Control</th>
<th>Scratched</th>
<th>Peeled</th>
<th>P_{scratched-peeled}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell density (cells/mm²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y-27632 +ve</td>
<td>2364 (2265–2462)</td>
<td>1161 (1099–1222)</td>
<td>685 (643–727)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>P_{Y-27632}</td>
<td>0.012</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% hexagonal cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y-27632 +ve</td>
<td>54.4 (46.9–61.9)</td>
<td>24.2 (21.2–27.2)</td>
<td>1.68 (0.29–3.12)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>P_{Y-27632}</td>
<td>0.871</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell circularity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y-27632 +ve</td>
<td>0.80 (0.77–0.83)</td>
<td>0.80 (0.76–0.85)</td>
<td>0.69 (0.64–0.73)</td>
<td>0.008</td>
</tr>
<tr>
<td>P_{Y-27632}</td>
<td>0.201</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell size (μm²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y-27632 +ve</td>
<td>410 (379–442)</td>
<td>1135 (909–1361)</td>
<td>1340 (1154–1526)</td>
<td>0.089</td>
</tr>
<tr>
<td>P_{Y-27632}</td>
<td>0.175</td>
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</table>

Numerical values corresponding to images in Figure 5. Endothelial cell counts and cell morphology were assessed based on Alizarin red microscopy images. Values are expressed as mean (95% confidence interval). P values were obtained by paired-sample t tests, with Bonferroni correction for multiple comparisons.
appropriate extracellular matrix facilitated corneal endothelial cell migration and proliferation.\textsuperscript{57–61} Additionally, the intact CEC monolayer that surrounded a CEC-depleted region provided a reservoir of healthy CECs from which cellular migration may be initiated to rapidly restore endothelial integrity.

CEGs over peeled wounds were much less densely packed and less hexagonal than those over scratched wounds, regardless of Y-27632 supplementation. Y-27632 supplementation was also found to increase CEC density over scratched wounds. Although no significant differences were detected between wound types in regard to the size of CECs, CEGs over peeled wounds were less circular than those over scratched wounds, with this difference accentuated by Y-27632 stimulation. Given the clinical observations of cellular pleomorphism and loss of cellular hexagonality associated with in vivo CEC migration and enlargement following acute endothelial cell loss,\textsuperscript{62} this disparity in CEC circularity may simply be a reflection of the exaggerated promigratory response mounted by CECs secondary to the potent stimulating effects of Y-27632. Additionally, the forcible migration of CECs over an anatomically unfavorable substrate (bare posterior corneal stroma: peeled wound) under the influence of Y-27632 may possibly have induced greater cellular pleomorphism than that demonstrated by CECs migrating across smooth, CEC-denuded DM (scratched wound).

Advanced donor age was associated with decreased endothelial migration for both scratched and peeled wounds. Additionally, donor age and Y-27632 supplementation were found to interact significantly in regard to their effects on endothelial migration over scratched wounds. Specifically, the stimulatory effect of Y-27632 supplementation on CEC migration over scratched wounds was most significant among elderly donors who were 50 years of age or older. In contrast, all subjects younger than 50 years of age demonstrated rapid and complete endothelial migration over scratched wounds regardless of Y-27632 supplementation. These findings suggest that young CECs probably retain the potential to migrate and possibly proliferate via the conservation and subsequent reactivation of pathways in a manner that may not be biologically possible in older CECs in the absence of external stimuli. Supplementation with Y-27632 likely reactivates these proproliferative and promigratory pathways, which mitigates the otherwise rapid decline in endothelial migration potential associated with increasing age.

Endothelial migration over peeled areas of most corneas was noted to be slow and incomplete even after 14 days of ex vivo culture. However, this was not an unexpected observation, in view of the multitude of clinical reports that indicate that the repopulation of corneal endothelial cells occurs over a relatively long period of time.\textsuperscript{42–45} In fact, the prolonged duration of migration of corneal clarity and restoration of functional visual acuity likely limits the clinical feasibility of such an approach. While it has been conclusively proven in this study that endothelial migration is slower in peeled wounds compared with scratched wounds, it would have been ideal to observe peeled wounds for longer durations in order to ascertain their maximum endothelial migration potentials and to accurately determine the influence of factors such as age and Y-27632 supplementation on endothelial migration. However, we found that the corneal endothelium was generally not amenable to being maintained in ex vivo culture for an extended duration. Beyond 14 days of ex vivo culture, most corneas began to exhibit patchy defects over both wounded and nonwounded (control) segments of the endothelium, which progressed to complete endothelial failure at approximately the third to fourth week of ex vivo culture (Soh Yu Qiang, Gary Peh, Jod S Mehta, unpublished data, 2015). In fact, such defects were noted as early as within 3–5 days from the initiation of ex vivo culture among several donor corneas 50 years of age and older. This may be due to procurement issues and processing of the tissue, or the use of tissue initially stored in Optisol before transfer to our laboratory.

Although the majority of the corneas were successfully cultured for at least 14 days, several corneas from experiment 1, as well as from experiment 2, were found to undergo PCF. We were able to further characterize PCF when comparisons were made using paired corneas. Here, mean donor age in the group of corneas that experienced PCF was significantly higher than those that did not experience PCF (72.4 ± 13.1 vs. 41.0 ± 18.9 years old). As such, we postulate that mechanisms that confer a pro-survival, anti-apoptotic advantage in younger cells are likely to be less active or possibly dysfunctional in older, senescent cells. Interestingly, intrapair comparisons indicated that the member of each pair that was cultured in Y-27632-supplemented medium survived for a longer duration in contrast to each matching contralateral cornea cultured in nonsupplemented medium (Supplementary Table S2). While this was not a statistically significant finding (P = 0.138), the small number of observations may have contributed toward a lack of statistical power to detect the pro-survival effect of Y-27632. Nonetheless, our observation was in keeping with a recent study that indicated the pro-survival effect of Y-27632 on CECs passed in vitro\textsuperscript{18} and other published literature on the pro-survival effects of Rho-associated protein kinase inhibitors among other cell types such as renal cells,\textsuperscript{44} cardiac myocytes,\textsuperscript{45} and retinal photoreceptor cells.\textsuperscript{46}

Unlike the healthy corneas used in this study (mean endothelial cell count, 2714 ± 371 cells/mm\textsuperscript{2}), FED is characterized by endothelial cell loss and the presence of numerous guttae on the DM. Not only do these guttate excrescences potentially impair CEC proliferation and migration, they also contribute toward intraocular light scatter, which leads to symptoms of glare.\textsuperscript{47} As such, removal of the entire diseased DM, or at least a region of DM where the guttae are most dense, would be desirable in any form of therapy. It would have been ideal to repeat these studies in corneas from patients with FED in order to investigate our initial hypothesis that Y-27632 supplementation without Descemet’s stripping may not result in universally favorable effects in regard to corneal endothelial migration, especially among patients with severe FED. However, the procurement of such corneas is sporadic and against standard eye banking protocols, which preclude the harvesting of cadaveric corneas affected by endothelial pathologies, and even more difficult from younger patients, hence making such comparisons as in our study systematically difficult. Additionally, the effects of Descemet’s stripping, Y-27632, and donor age on endothelial migration were investigated in an ex vivo culture model in our study. Given the considerable differences in biochemical compositions between culture media and aqueous humor, the in vivo migratory behavior of CECs may differ from that observed in our study.

In conclusion, this study showed that it was possible for remaining CECs to re-establish over areas of intact DM that were endothelial cell deficient (scratched wounds), and the Rho-associated protein kinase inhibitor Y-27632 could enhance endothelial migration, especially in older corneas that were 50 years of age and older. Migration of CECs was also seen to occur over areas of the posterior corneal surface from which DM had been removed (peeled wounds), but at a much slower rate, especially in older corneas. Finally, the use of Y-27632 was not found to significantly enhance endothelial
migration over peeled wounds within the time frame of this study.

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