The Biological Character of Donor Corneal Endothelial Cells Influences Endothelial Cell Density Post Successful Corneal Transplantation

Koji Kitazawa, MD, PhD,1,2 Munetoyo Toda, PhD,3 Morio Ueno, MD,1 Asako Uehara,1 Chie Sotozono, MD, PhD,1,2 Shigeru Kinoshita, MD, PhD2,3

1 Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan

2 Baptist Eye Institute, Kyoto, Japan.

3 Department of Frontier Medical Science and Technology for Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan.

*Author Contributions: Dr. Kitazawa and Dr. Toda contributed equally to this article.


Financial Support: This research was partially supported by JSPS KAKENHI Grant Number JP20H03844 and the Japan Agency for Medical Research and Development (AMED) (Grant Number: 15bm0504002h0005) for translational research on the clinical application of regenerative medicine using cultured corneal endothelial cells.

Conflict of Interest: No conflicting relationship exists for any author.

Running head: Influence of the biological maturity of corneal endothelium
Correspondence and Address for Reprints: Shigeru Kinoshita, MD, PhD, Department of Frontier Medical Science and Technology for Ophthalmology, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Hirokoji-agaru, Kawaramachi-dori, Kamigyo-ku, Kyoto 602-0841, Japan; Tel.: +81-75-251-5772, Fax: +81-75-251-5938, E-mail: shigeruk@koto.kpu-m.ac.jp

Keywords:
Corneal endothelial cell density, Endothelial cell loss, Cultured corneal endothelial cells, DSAEK, Corneal transplantation.

Abbreviations and Acronyms:
BK = bullous keratopathy; CECs = corneal endothelial cells; CST = cell-state transition; DMEK = Descemet's membrane endothelial keratoplasty; ECD = endothelial cell density; ECL = endothelial cell loss; FACS = fluorescence activated cell sorting; FECD = Fuchs endothelial corneal dystrophy; HCEC = human CEC; IOL = intraocular lens; IOP = intraocular pressure; OXPHOS = oxidative phosphorylation; PK = penetrating keratoplasty; Q-Q = quantile-quantile
Abstract

Purpose: Corneal endothelial cell density (ECD) gradually decreases after corneal transplantation by unknown biological, biophysical or immunological mechanism. To assess the association between donor corneal endothelial cell (CEC) maturity in culture and postoperative endothelial cell loss (ECL) after successful corneal transplantation.

Design: Prospective cohort study.

Participants: This cohort study was conducted at Baptist Eye Institute, Kyoto, Japan, between October 2014 and October 2016. It included 68 patients with a 36-month follow-up period who had undergone successful Descemet’s stripping automated endothelial keratoplasty (DSAEK) or penetrating keratoplasty (PK).

Methods: Human CECs (HCECs) from remaining peripheral donor corneas were cultured and evaluated for maturity by surface markers (CD166+, CD44−/dull, CD24−, and CD105−) using fluorescence activated cell sorting (FACS). Postoperative ECD was assessed according to the mature-differentiated HCEC contents: high maturity group: >70%, middle maturity group: 10–70%, low maturity group: <10%. The successful rate of ECD maintained at 1,500 cells/mm² at 36-months postoperative was analyzed using the log-rank test.

Main Outcome Measures: ECD and ECL at 36-months postoperative.

Results: The 68 included patients (Mean [SD] age 68.1 [13.6] years, 47.1% female, 52.9% DSAEK). The high, middle, and low maturity groups included 17, 32, and 19 eyes. At 36-months postoperative, the mean (SD) ECD significantly decreased to 911 (388) cells/mm² by 66% in the low maturity group, compared to 1,604 (436) by 40% and 1,424 (613) cells/mm² by 50% in the high and middle maturity groups (P < 0.001 and P = 0.007, respectively) and the low maturity group significantly failed to maintain ECD at 1,500 cells/mm² at 36-months.
postoperative ($P < 0.001$). Additional ECD analysis for DSAEK patients alone displayed a
significant failure to maintain ECD at 1,500 cells/mm$^2$ at 36-months postoperative ($P < 0.001$).

**Conclusions:** The high content of mature-differentiated HCECs expressed in culture by the
donor peripheral cornea was coincident well with low endothelial cell loss, suggesting that a high
maturity CEC content predicts long-term graft survival. Understanding the molecular mechanism
for maintaining HCEC maturity could elucidate the mechanism of ECL after corneal
transplantation and aid in developing effective interventions.
Introduction

The development of corneal endothelial transplantation, such as Descemet's stripping automated endothelial keratoplasty (DSAEK) and Descemet's membrane endothelial keratoplasty (DMEK), has dramatically improved visual prognosis after corneal transplantation. The mid-term graft survival rate has also improved due to a reduction in allograft rejection compared with conventional full-thickness corneal transplantation. However, corneal endothelial cell density (ECD) still decreases substantially and continuously with years following DSAEK, DMEK, and penetrating keratoplasty (PK), even if no apparent complications such as allograft rejection occur after transplantation. For example, Lass et al. reported that the ECD at 5 years post-DSAEK declined by over 70% of the baseline ECD. Furthermore, the Cornea Donor Study and other reports have demonstrated continuous yearly decreases in ECD after corneal transplantation, with graft survival or endothelial cell loss (ECL) associated with factors that included donor ECD, as well as donor age, sex, preservation time, and donor size. In addition, Yamaguchi et al. proposed that iris damage was a risk factor for early corneal endothelial decompensation due to the elevation of inflammatory cytokines in the anterior chamber. However, no clear reasons can yet explain why transplanted donor corneal endothelial cells (CECs) show this continuous decrease in ECD as compared to cataract surgery, etc., and that some cases after transplantation eventually fall into chronic graft failure without obvious allograft rejection.

Of particular interest is that approximately 10% of donor corneal endothelium shows over 2,000 cells/mm² after 5 years post penetrating keratoplasty without any association of donor factors or host disease, implying unsolved biological aspects of the donor cornea may be involved in this event. In addition, there is evidence showing that donor corneas contained some dead CECs that were not related to storage period or donor age. These suggest that the viability
and longevity of CECs may vary among individual donor corneas. Based upon these pieces of evidence, we hypothesize that a biological pre-commitment of cell longevity already exists in the donor corneas before transplantation, though we cannot detect such factors using known biological markers or clinical parameters including donor ECD.

In the present study, to explore the possibility mentioned above, we used one biological cell character obtained from our basic experiment of human CEC (HCEC) culture for HCEC injection therapy, which is the proportion of CD166+, CD44−/dull, CD24−, and CD105− in the cultured HCECs at confluency. They are mature-differentiated cells without cell-state transition (CST) and disposed to mitochondria-dependent oxidative phosphorylation (OXPHOS). We surmise from our previous experiments that high proportion of HCECs with CD166+, CD44−/dull, CD24−, and CD105− possibly possess longer longevity with good mitochondrial function than those with its low proportion.15,16 Based upon this hypothesis, we investigated the relationship between the postoperative ECD after successful corneal transplantation and the biological character mentioned above, using cultured HCECs from the peripheral cornea of the same donor used for corneal transplantation; possibly indicating the healthiness and longevity of the donor CECs (Fig 1).

Methods

Patients

This cohort study of consecutive patients who underwent DSAEK or PK was conducted at the Baptist Eye Institute, Kyoto, Japan, and was approved by the institutional review board (Approval #ERB-C 1006). Prior to surgery, written informed consent was obtained from all subjects in accordance with the tenets set forth in the Declaration of Helsinki. The study included
68 patients who underwent DSAEK or PK at the Baptist Eye Institute between October 2014 and October 2016. All surgeries were performed by one expert corneal surgeon (S.K.). The indications were corneal stromal opacity in 21 eyes, glaucoma-related bullous keratopathy (BK) in 17 eyes, pseudophakic/aphakic BK in 15 eyes, Fuchs endothelial corneal dystrophy (FECD) in 6 eyes, keratoconus in 4 eyes, and other-type BK in 5 eyes (i.e., iridocorneal endothelial syndrome in 1 eye, pseudoexfoliation keratopathy in 1 eye, and unknown causes in 3 eyes). None of the patients experienced graft failure throughout the 36-month-postoperative follow-up period.

**Cell Culture of Donor CECs**

The HCECs obtained from the peripheral rims of 68 human donor corneas were individually cultured according to published protocols, with some modifications. Briefly, the Descemet's membranes with the CECs at the peripheral rims were stripped from donor corneas without contamination of trabecular meshwork tissues and digested at 37°C with 1 mg/mL collagenase A (Roche Diagnostics GmbH, Mannheim, Germany) for 2 hours. The HCECs obtained from a single donor corneal peripheral rim were seeded in a single well of a Type-I collagen-coated 24-well plate (Corning®, Inc., Corning, NY). The culture medium was prepared according to published protocols. Briefly, basal medium was prepared with Opti-MEM® I (Thermo Fisher Scientific, Inc., Waltham, MA), 8% fetal bovine serum, 5 ng/mL epidermal growth factor (EGF; Thermo Fisher Scientific), 20 μg/mL ascorbic acid (Sigma-Aldrich, St. Louis, MO), 200 mg/L calcium chloride (Sigma-Aldrich), 0.08% chondroitin sulfate (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan), 10 μM Y-27632 (Fujifilm Wako Pure Chemical Corporation), 10 μM SB203580 (Cayman Chemical Co., Ann Arbor, MI) and 50 μg/mL gentamicin. The HCECs at passage 0 were cultured at 37°C in a humidified 5% CO₂ atmosphere with the change of
culture medium twice weekly. After reaching confluence at 5 weeks, the cultured cells were
passaged at the cell density of 800 cells/mm², continuing each culture using the same media for 5
weeks until confluence at passage (P) 1 (P1). The cultured donor CECs at P1 confluency was
used for the subsequent flow cytometric analysis. Those cells at P0 confluency were not be able
to use for analysis due to the shortage of cell amount.

**Flow Cytometry Analysis of the Cultured Donor CECs**

Cultured donor HCECs at P1 confluency were collected from the culture dish by treatment with
10 × TrypLE™ Select (Thermo Fisher Scientific) at 37°C for 12 minutes. The cells were then
suspended at a concentration of 4 × 10⁶ cells/mL in fluorescence activated cell sorting (FACS)
buffer (phosphate buffered saline containing 1% bovine serum albumin and 0.05% sodium
azide). An equal volume of antibody solution was added and incubated at 4°C for 2 hours. The
antibodies were the following: E-conjugated anti-human CD166 mAb, PerCP-Cy 5.5 conjugated
anti-human CD24 mAb, PE-Cy 7-conjugated anti-human CD44 mAb (all from BD Biosciences,
Franklin Lakes, NJ), and APC-conjugated anti-human CD105 mAb (Thermo Fisher Scientific).

After washing with FACS buffer, the cultured HCECs were analyzed with a FACSCanto™ II
Flow Cytometry Analyzer System (BD Biosciences). The content of mature-differentiated
HCECs (CD166⁺, CD44ᵈᵒᵘˡˡ, CD24⁻, and CD105⁻) was measured by FACS. The cultured HCECs
were classified into the following 3 groups: 1) high maturity group: a >70% content of mature-
differentiated HCECs, 2) middle maturity group: a 10 to 70% content of mature-differentiated
HCECs, and 4) low maturity group: a <10% content of mature-differentiated HCECs, based
upon the top 25%, the middle 50%, and the bottom 25% of the entire donors, respectively, as the
overall ECD, as well as the ECL, at 36-months postoperative was normally distributed (Kolmogorov-Smirnov test; \( P > 0.100 \)) (Supplemental Figs 1A, 1B, 1C, and 1D).

### Surgical Technique

The donor corneas in this study were obtained from CorneaGen™ Eye Bank (Seattle, WA). All DSAEK flaps used for implantation were prepared by CorneaGen™ prior to shipping to Japan. The patients underwent general anesthesia (or retrobulbar anesthesia if they had any previous history of respiratory, heart, or kidney problems). The host cornea was trephined using a Hessburg-Barron Vacuum Trephine (Katena Products, Inc., Denville, NJ) or a Moria One® Single-Use Adjustable Vacuum Trephine (Moria, Inc., Doylestown, PA) for PK, and the donor cornea was then cut with a Barron Vacuum Donor Cornea Punch (Katena Products) or a Moria One® Corneal Vacuum Punch (Moria) for PK and DSAEK. The techniques used for all DSAEK and PK surgeries were as previously described.\(^\text{19, 20}\) Briefly, for DSAEK, the Descemet’s membrane at the central posterior cornea was removed using a reverse Sinskey hook (Bausch & Lomb Inc., Rochester, NY), the prepared DSAEK flap was inserted into the anterior chamber using a Busin glide, and air was then injected into the anterior chamber in order to sufficiently increase the intraocular pressure (IOP) to ensure firm attachment of the graft to the host cornea. For PK, after trephination, the donor corneal graft was fixed to the host eye with eight interrupted sutures, followed by a continuous suture. Cataract extraction was performed by phacoemulsification and aspiration, followed by intraocular lens (IOL) implantation or transscleral suture of IOLs with 10-0 polypropylene if necessary.

### Postoperative Management
As we previously reported, following corneal transplantation, each patient received a systemic dose of 4 mg betamethasone for 2 days, followed by 1 mg betamethasone for 5 days, together with topical application of 0.3% gatifloxacin and 0.1% betamethasone eye drops 4 times daily.

An adequate systemic dose (i.e., 125 mg) of methylprednisolone was administered immediately prior to surgery. The topical 0.1% betamethasone eye drops were continued for 6 postoperative months for DSAEK patients and PK patients, and then tapered to 0.1% fluorometholone eye drops 2- to 4-times daily.

**Clinical Evaluation**

In order to investigate the relationship between the postoperative ECD and the biological indicator in maturity of donor CECs, ECD in each patient was measured every 6 months with a non-contact specular microscope (EM-3000; Tomey Corporation, Nagoya, Japan) and the biological quality of the donor cornea was evaluated by HCEC culture (Fig 1). Donor CECs were cultured from the peripheral area of the donor transplant remaining after puncture removal, according to our previously reported method, and HCEC morphology and maturity were determined according to the expression pattern of surface markers at 5 weeks after the P1 culture. CD166+, CD44-/dull, CD24-, and CD105- cultured donor CECs, with a high maturity, were considered to represent healthy in vivo donor CECs, as described in our previous paper. In this study, the donor characteristics included donor age, sex, trephination size, cause of death, donor-cell preservation time, and the number of days post-mortem of the donor cornea. Donor cause of death was classified into the following 2 groups: 1) acute (e.g., heart disease, cerebrovascular disease, or acute respiratory failure [e.g., asphyxia]) and 2) chronic (e.g., a malignant tumor or chronic liver disease).
Statistical Analysis

Statistical analyses were performed using Prism 9 v. 9.2.0.283 software (GraphPad Software, San Diego, CA). The ECD graphs are presented as the median ± 25 to 75 percent tile. Normality assumption for samples was examined with Kolmogorov-Smirnov test and normal quantile-quantile (Q-Q) plot. Dunn's multiple comparison test analyzed the ECD and ECL following PK or DSAEK among the maturity groups at each assessment. According to the maturity of the donor cornea, the postoperative ECD over time was analyzed with the mixed effects model for repeated measures. Each patient was determined as a random effect, and time and maturity were determined as fixed effects. According to the donor maturity, the statistical significance was analyzed with a log-rank test in cases maintained at more than 1,000 cells/mm², 1,500 cells/mm², and 2,000 cells/mm² at 36-months postoperative. The Spearman rank correlation coefficient test examined the correlation between the maturity of donor HCECs and postoperative ECL. Differences in recipient characteristics, donor characteristics corresponding to each recipient, and recipient and postoperative ECDs among the three groups were analyzed using the Kruskal-Wallis test or the chi-squared test with Bonferroni correction. A P-value of < 0.05 was considered statistically significant.

Results

Maturity of the Cultured Donor CECs

Phase-contrast microscopy revealed a non-fibroblastic phenotype with a characteristic polygonal shape and monolayer in the maturity groups; however, HCECs in the low maturity group showed the contamination of fibrotic phenotype including endothelial-mesenchymal transition and/or
senescence cells, and were more variable in size than in the high maturity group and middle
maturity group. These morphological phenotypes suggested a substantial number of non-
functional CECs existed. Assessment of the content of mature-differentiated HCECs in the
cultured donor CECs by FACS revealed 17 eyes in the high maturity group, 32 eyes in the
middle maturity group, and 19 eyes in the low maturity group (Table 1, Supplemental Figs 2A
and 2B).

Examination of the variable donor factors that could affect the quality of the donor-eye
CECs, including donor age, donor sex, donor ECD, trephination size, cause of death, elapsed
time from death to preservation, and the number of days post-mortem of the donor cornea,
revealed no significant differences among the 3 groups (Table 1). In addition, the recipient
characteristics, including age, sex, primary indication, and surgical procedure, were examined
according to the maturity of the donor CECs. No significant differences were found among the 3
groups in regard to any of the clinical factors (Table 1). Moreover, the overall ECD/ECL was
normally distributed (Kolmogorov-Smirnov test; \( P > 0.100 \)) (Supplemental Figs 1A, 1B, 1C, and
1D), but the proportion of mature CECs displayed the right skew distribution, indicating a non-
ormal distribution, as shown in the normal Q-Q plot (Kolmogorov-Smirnov test; \( P = 0.007 \)),
(Supplemental Figs 3A and 3B).

**Postoperative ECD and ECL According to the Maturation of Donor CECs**

The postoperative ECD and the proportion of mature-differentiated HCECs in the donor corneas
were examined for a potential association. The overall mean (SD) ECDs at baseline and at 6-,
12-, 24-, and 36-months postoperative in 68 eyes was 2,770 (282), 2,137 (477), 1,926 (502),
1,636 (592), and 1,325 (577) cells/mm$^2$, respectively. No allograft rejection occurred in this
study, and all of the grafts retained their transparency throughout the follow-up period.

The mean (SD) ECD at baseline and at 6-, 12-, 24-, and 36-months postoperative was 2,695
(194), 2,268 (200), 2,034 (286), 1,878 (437), and 1,604 (436) cells/mm$^2$, respectively, in the high
maturity group, 2,832 (336), 2,157 (524), 1,999 (535), 1,770 (579), and 1,424 (613) cells/mm$^2$,
respectively, in the middle maturity group, and 2,734 (236), 2,000 (531), 1,711 (542), 1,216
(530), and 911 (388) cells/mm$^2$, respectively, in the low maturity group (Table 2). At 6-months
postoperative, the ECD in each group was found to have declined sharply from that at baseline.

Thereafter, the ECD decrease became slow and gradual in the high and middle maturity groups,
whereas a steady decline remained in the low maturity group. A comparison of the 3 groups
revealed that there was a significant decrease of ECD in the low maturity group at 24- and 36-
months postoperative (Kruskal-Wallis test, $P = 0.003$ and $P < 0.001$, respectively), and a
significantly lower ECD in the low maturity group than in the high maturity group at 24- and 36-
months postoperative (Dunn’s multiple comparison test, $P = 0.007$ and $P < 0.001$, respectively)
(Fig 2 and Table 2) and the middle maturity group at 24- and 36-months postoperative (Dunn’s
multiple comparison test, $P = 0.011$ and $P = 0.007$, respectively). ECL in the low maturity group
displayed a significant decrease of 53% at 24-months postoperative and 66% at 36-months
postoperatively, compared to the 24- and 36-month postoperative ECL of 38% and 50%,
respectively, in the middle maturity group and 30% and 40%, respectively, in the high maturity
group (Kruskal-Wallis test, $P = 0.006$ and $P < 0.001$, respectively) (Table 2). In addition, the
mixed effects model revealed that all 3 groups displayed a significant decrease in ECD ($P <
0.001$) throughout the time of the examination period. In this context, there was a significant
difference in ECD change over time among all 3 maturity groups ($P < 0.001$) (Fig 2). Generally,
the middle maturity group tended to show an intermediate level of ECL and ECD change between the high maturity and low maturity groups.

**Postoperative ECD and the Maturity of Donor CECs**

A high retention of ECD throughout the postoperative follow-up period is a clinically useful hallmark of a successful corneal transplantation. ECD that was maintained at a density of more than 1,000 cells/mm², 1,500 cells/mm² and 2,000 cells/mm² at 36-months postoperative was analyzed with the Kaplan–Meier survival curve according to the maturity of the donor CECs. Unlike the high maturity group, the low maturity group significantly failed to maintain ECD at 1,500 cells/mm² at 36 months post corneal transplantation, which is thought to be one of the hallmark indicators of long-term graft survival (Fig 3A). The high maturity group was found to be more likely to maintain an ECD above 1,500 cells/mm² for over 3 postoperative years (log-rank test; \( P < 0.001 \)), as well as an ECD of 2,000 cells/mm² and 1,000 cells/mm² (log-rank test; \( P = 0.021 \) and \( P = 0.001 \), respectively) (Fig 3A and Supplemental Figs 4A and 4B).

**Postoperative ECD in DSAEK Patients and the Maturity of Cultured Donor CECs**

The patients in this present study underwent two different surgical procedures, DSAEK and PK, which can influence, to some extent, the postoperative ECD validation due to the primary indication and the amount of surgical invasion. Thus, in order to minimize the amount of surgical bias, ECD and ECL were analyzed in the DSAEK patients alone. The overall mean (SD) ECD following DAESK at baseline and at 6-, 12-, 24-, and 36-months preoperative was 2,792 (272), 2,039 (548), 1,813 (488), 1,566 (564), and 1,290 (499) cells/mm², respectively, and the mean (SD) ECL was 27 (17), 35 (17), 46 (20), 53 (18)%, respectively (Supplemental Figs 5A and 5B),
whereas following PK, the mean (SD) ECD was 2,746 (300), 2,241 (368), 2,049 (494), 1,713 (622), and 1,344 (657) cells/mm\(^2\), respectively, and the mean (SD) ECL was 18 (10), 23 (15), 38 (23), 51 (24)\%, respectively (Supplemental Figs 5C and 5D).

Previous reports have indicated that short-term ECL is higher post DSAEK than post PK,\(^{21}\) even though the ECL was found to be comparable for both DSAEK and PK by 10-years postoperative.\(^{22}\) In fact, there was a greater decline of ECD at 6-months post DSAEK due to predicted surgical trauma, and the ECL was much lower in DSAEK patients than in PK patients until 24-months postoperative. The maturity of the donor CECs was assessed further for DSAEK patients alone, as the recipients of DSAEK are considered to have a better controlled background than the recipients of PK.

In regard to the donor factors and the recipient backgrounds in this present study, no significant differences were found among the 3 groups. The mean (SD) ECDs at 6-, 12-, 24-, and 36-months postoperative was 2.811 (235), 2.293 (97), 1,954 (263), 1,949 (293), and 1,639 (373) cells/mm\(^2\), respectively, in the high maturity group, 2,833 (320), 2,079 (611), 1,854 (572), 1,638 (613), and 1,373 (541) cells/mm\(^2\), respectively, in the middle maturity group, and 2,722 (204), 1,877 (543), 1,683 (443), 1,278 (473), and 1,026 (381) cells/mm\(^2\), respectively, in the low maturity group (Supplemental Figs 6 and Table 2). The ECD at 24- and 36-months postoperative was significantly higher in the high maturity group than in the low maturity group (Dunn’s multiple comparison test, \(P = 0.030\) and \(P = 0.025\), respectively), and the trend for postoperative ECD in each group was similar to that shown in Figure 2 (Supplemental Fig 6). When compared to the ECDs of the high and middle maturity groups, the ECD in the low maturity group continued to decline, and a greater decrease of ECD was found in the low maturity group compared to the high maturity group, which remained throughout the follow-up period.
Kaplan–Meier survival curve analysis revealed that the low maturity group displayed early failure to maintain ECD at $1,500 \text{ cells/mm}^2$, and that the survival rate was significantly higher in correlation with the maturity of the donor HCECs (log-rank test, $P = 0.005$) as well as what was observed in cases with an ECD maintained at $2,000$ and $1,000$ cells/mm$^2$ ($P = 0.027$ and $P = 0.029$, respectively) (Fig 3B and Supplemental Figs 7A and 7B).

**Correlation Between Maturity of Donor CECs and ECL Post Corneal Transplantation**

The association between the maturity of the CECs and postoperative ECL was analyzed with the spearman rank test. The postoperative ECL clearly declined in direct relation to the proportion of mature CECs ($r = -0.365$, 95% confidence interval [CI]; $-0.560$ to $-0.132$, $P = 0.002$) (Supplemental Fig 8A). The correlation analysis in DSAEK alone showed similar results ($r = -0.290$, 95% CI; $-0.572$ to $0.05$, $P = 0.086$) (Supplemental Fig 8B). In fact, the histogram bar displayed that in accordance with the cell maturity, the high maturity group included more patients with a lower ECL and the low maturity group included more patients with a higher ECL, while the ECL in the middle maturity group was in the middle (Supplemental Figs 9A, 9B, and 9C).

**Discussion**

The results of this present study indicate that the donor peripheral CECs that display a high proportion of mature-differentiated cultured HCECs at P1 (high maturity group) correspond well to a higher postoperative ECD at the center of the transplanted donor cornea in the mid-term postoperative period when compared with donors who contain a low proportion of mature-differentiated cultured HCECs (low maturity group). In fact, the high maturity group showed a
slow decrease in ECD when compared with the steeper decline seen in the low maturity group, and displayed a large number of patients with an ECD of over 1,500 cells/mm² at 36-months post DSAEK and PK. In our novel cultured HCEC-injection therapy, the proportion of mature-differentiated HCECs is used as a biological marker of quality control. In other words, there is accumulating evidence that a higher proportion of mature-differentiated cells in culture results in better quality cell products. For example, one clinical trial that injected reasonably high-maturity cultured HCECs into the anterior chamber for the treatment of BK reported cell survival that extended over a 5-year follow-up period. Furthermore, and as shown in our previous report, when a cell subpopulation consisting of over 90% mature-differentiated cells is used for cultured HCEC-injection therapy, it results in a better ECD and low ECD attrition over a period of 3 postoperative years. Moreover, we previously reported that the high-maturity HCECs that possess unique characteristics of cell-surface markers such as CD166⁺, CD44⁺/dull, CD24⁺, CD26⁻, and CD105⁻ were capable of growth, even when seeded at a low cell density in the culture dish, whereas low-maturity HCECs exhibited poor proliferative behavior even though they stained positive for ZO-1 and Na⁺/K⁺/ATPase, two well-known markers of normal HCECs. Furthermore, mature-differentiated HCECs have displayed a reasonable metabolic activity that can maintain their longevity and functions. As shown in Supplemental Figure 1, high-maturity HCECs maintain their hexagonal shape and are smaller in size than low-maturity HCECs, thus suggesting that high-maturity HCECs avoid epithelial-mesenchymal transition or a senescence phenotype undergoing cell death. In fact, we previously reported that the biological status of mature-differentiated HCECs is controlled through the intracellular signaling pathway by positive regulation of p53 and miR34a and negative regulation of c-Myc, thus resulting in maintaining a healthy OXPHOS metabolism. Taken as a whole, the above evidence may
explain why the high maturity group of HCECs was found to be associated with the extended longevity of graft survival post transplantation.

Previous reports have demonstrated that donor age,\textsuperscript{4,29} donor sex\textsuperscript{5}, and corneal preservation time\textsuperscript{8,30,31} were associated with graft survival and ECL. It is true that sex differences can affect the longevity of the CECs, even though that factor was not found to be statistically significant in this present study. In previous studies, it has been reported that the female donor factor has a positive effect on ECD during the postoperative follow-up period.\textsuperscript{5,6} However, findings in other reports have demonstrated that female sex is known to be a risk for FECD, and that estrogen metabolites cause oxidative stress, thus leading to CEC apoptosis.\textsuperscript{32,33} Hence, it remains controversial as to whether or not female sex has a positive effect on CECs post transplantation.

In this study, we highlighted evidence that the biological maturity of cultured HCECs from donor corneas is also a potential predictor for graft survival post transplantation, even though the differences in surgical procedures should be taken into account. Overall, our data and close analysis of the patients after undergoing DSAEK showed similar ECD trends among the groups, and no donor factors were related to the maturity of HCECs. Our present findings seem to suggest that both the primary indication and the specific surgical procedure used has no influence on the postoperative ECD. We previously reported the presence of dead cells in donor corneal endothelium preserved in storage medium, and presented our finding that the number of those cells declined after incubation.\textsuperscript{14} Those findings suggest that an endothelial cell count alone is not sufficient for a proper judgment of the quality of donor corneas and may ultimately result in cell death following keratoplasty. Considering these findings, cell maturity in culture would appear to be an independent donor factor that could be used to predict longer HCEC survival.
post transplantation. This biological character may be influenced by either the intrinsic factor of
the donor CEC itself or the specific method used for donor cornea storage, possibly due to the
oxidative stress environment.

Postoperative ECL can be affected by baseline recipient characteristics as well as donor
factors. Unlike FECD, both pseudophakic/aphakic BK and glaucoma are reportedly associated
with an increased risk of graft failure.\textsuperscript{34, 35} Several previous reports have shown that the graft
survival rate at 5-years postoperative declines to below 50% in DSAEK or DMEK patients with
previous history of glaucoma,\textsuperscript{36-38} and the potential effect of the recipient host environment due
to the primary indication for surgery should be addressed. However, in the present study, 4 of the
5 glaucoma patients (80%) in the high maturity group were found to have maintained an ECD of
more than 1,000 cells/mm\textsuperscript{2} at 36-months postoperative, whereas over that same postoperative
period, ECD was maintained in only 40% of the glaucoma patients in the low maturity group
even though there was no significant difference between the two groups due to underpowering.

The mechanism underlying ECL remains unclear, and it appears to be unrelated to donor age,
sex, trephination size, cause of death, elapsed time from death to preservation, and the number of
days post-mortem of the donor cornea. Thus, it appears that in future studies it would be
beneficial to try and elucidate the biomarkers associated with the high/low maturity of
endothelial cells, as they can be assessed non-invasively.

\textbf{Limitations}

It should be noted that in this present study, the evaluation of donor CECs was performed with
cell culture, so some technical experimental bias may have occurred. However, we adhered to
the previously published protocols for HCEC culture applied to clinical trials,\textsuperscript{17} which should
have minimized this bias to a large degree. Moreover, an additional argument could be raised in regard to the difference in the region of the cornea in which CECs were obtained; i.e., the central vs. peripheral region. However, from our experience, there has been no difference in cell growth in CECs obtained from those two regions. Another limitation in this study was that the low maturity group included 12 cases that contained none of mature HCECs, in which of cases did not even grow the CECs in culture and the sample population displayed right-skewed distribution. However, that did not seem to influence the present results showing the lower ECD and higher ECL in the low maturity group post surgery. In addition, the previously reported evidence that the injection of cultured HCECs with high maturity survived throughout a 5-year follow-up period\textsuperscript{23} also supports the present findings. Another possible limitation is that the baseline recipient characteristics varied. Although the number of cases analyzed in this study was limited, the ECD trend after DSAEK for the treatment of BK showed that the HCECs had a more prolonged survival in the high maturity group than in the low maturity group, thus suggesting that the present findings were reasonable.

Conclusion

Donor corneas with higher-maturity HCECs in culture were found to contribute to higher postoperative ECD and lowered postoperative ECL, thus suggesting that a high content of mature HCECs in culture can result in an extended longevity and healthiness of primary in vivo HCECs, which could be a predictive indicator of long-term graft survival. A better understanding of the molecular mechanism underlying the maintenance of HCEC maturity will hopefully lead to the elucidation of the mechanism of ECL after successful corneal transplantation and the successful development of effective interventions.
Acknowledgements

This research was partially supported by JSPS KAKENHI Grant Number JP20H03844 and the Japan Agency for Medical Research and Development (AMED) (Grant Number: 15bm0504002h0005) for translational research on the clinical application of regenerative medicine using cultured corneal endothelial cells.
References


Figure Legends

Figure 1. Schematic figure of the biological evaluation of the donor corneas. The relationship between the postoperative endothelial cell density (ECD) and the maturity of cultured corneal endothelial cells (CECs) from the peripheral region of the donor cornea was investigated.

Figure 2. Postoperative endothelial cell density (ECD) over time in patients transplanted with donor corneal grafts consisting of corneal endothelial cells (CECs) of differing maturity. Overall, the ECD in the groups included Descemet’s stripping automated endothelial keratoplasty and penetrating keratoplasty patients according to the maturity of the donor CECs. The upper and lower edges of each box represent the interquartile range (25th–75th percentile). The line inside each box is the median. The upper bar indicates the maximum value and the lower bar indicates the minimum value. *** Indicates $P < 0.001$, ** indicates $P < 0.01$, and * indicates $P < 0.05$.

Figure 3. Kaplan–Meier survival curve graph of the cases in which an endothelial cell density (ECD) of 1,500 cells/mm$^2$ was maintained post transplantation of donor corneal grafts consisting of corneal endothelial cells of differing maturity. A, Overall, the group includes Descemet’s stripping automated endothelial keratoplasty (DSAEK) and penetrating keratoplasty patients. The survival rate indicates the cases in which an ECD of more than 1,500 cells/mm$^2$ was maintained throughout the postoperative follow-up period. The log-rank test was used for statistical analysis. B, Subgroup that included DSAEK patients only.
Table 1. Characteristics of donor and recipient according to maturity of cultured corneal endothelial cells (CECs)

<table>
<thead>
<tr>
<th></th>
<th>High maturity</th>
<th>Middle maturity</th>
<th>Low maturity</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Donor</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, mean (SD), y</td>
<td>60.5 (8.0)</td>
<td>61.1 (10.1)</td>
<td>61.7 (9.6)</td>
<td>0.731</td>
</tr>
<tr>
<td>Range</td>
<td>42-71</td>
<td>23-74</td>
<td>30-73</td>
<td></td>
</tr>
<tr>
<td>Female, No. (%)</td>
<td>12 (71)</td>
<td>12 (38)</td>
<td>8 (42)</td>
<td>0.080</td>
</tr>
<tr>
<td>ECD, mean (SD), cells/mm²</td>
<td>2695 (194)</td>
<td>2832 (336)</td>
<td>2734 (236)</td>
<td>0.403</td>
</tr>
<tr>
<td>Range</td>
<td>2505-3094</td>
<td>2506-3543</td>
<td>2503-3296</td>
<td></td>
</tr>
<tr>
<td>Cause of death (Acute/Chronic)</td>
<td>12/5</td>
<td>22/10</td>
<td>11/8</td>
<td>0.666</td>
</tr>
<tr>
<td>Death to preservation mean (SD), min</td>
<td>817 (343)</td>
<td>808 (310)</td>
<td>709 (398)</td>
<td>0.426</td>
</tr>
<tr>
<td>Range</td>
<td>345-1424</td>
<td>375-1427</td>
<td>210-1430</td>
<td></td>
</tr>
<tr>
<td>Post-mortem days mean (SD), day</td>
<td>6.0 (0.8)</td>
<td>6.0 (0.9)</td>
<td>6.4 (1.1)</td>
<td>0.248</td>
</tr>
<tr>
<td>Range</td>
<td>5-8</td>
<td>4-7</td>
<td>4-9</td>
<td></td>
</tr>
<tr>
<td>Trephination sizes (SD), mm</td>
<td>7.90 (0.21)</td>
<td>7.94 (0.25)</td>
<td>7.92 (0.40)</td>
<td>0.730</td>
</tr>
<tr>
<td>Range</td>
<td>7.5-8.5</td>
<td>7.25-8.5</td>
<td>7.0-9.0</td>
<td></td>
</tr>
</tbody>
</table>

**Recipient**
## Table

<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, mean (SD), y</strong></td>
<td>62.5 (16.8)</td>
<td>69.6 (12.5)</td>
<td>70.5 (11.3)</td>
<td>0.343</td>
</tr>
<tr>
<td><strong>Range</strong></td>
<td>26-85</td>
<td>44-94</td>
<td>49-88</td>
<td></td>
</tr>
<tr>
<td><strong>Female, No. (%)</strong></td>
<td>10 (59)</td>
<td>13 (41)</td>
<td>9 (47)</td>
<td>0.483</td>
</tr>
<tr>
<td><strong>Primary indication, No. (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corneal opacity</td>
<td>5 (29)</td>
<td>12 (38)</td>
<td>4 (21)</td>
<td>0.470</td>
</tr>
<tr>
<td>Glaucoma-related BK</td>
<td>5 (29)</td>
<td>7 (22)</td>
<td>5 (26)</td>
<td>0.837</td>
</tr>
<tr>
<td>PBK/ABK</td>
<td>3 (18)</td>
<td>7 (22)</td>
<td>5 (26)</td>
<td>0.824</td>
</tr>
<tr>
<td>FECD</td>
<td>1 (6)</td>
<td>3 (9)</td>
<td>2 (11)</td>
<td>0.878</td>
</tr>
<tr>
<td>KC</td>
<td>2 (12)</td>
<td>2 (6)</td>
<td>0 (0)</td>
<td>0.329</td>
</tr>
<tr>
<td>Other BK</td>
<td>1 (6)</td>
<td>1 (3)</td>
<td>3 (16)</td>
<td>0.242</td>
</tr>
<tr>
<td><strong>Surgical procedure, No. (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSAEK</td>
<td>4 (24)</td>
<td>13 (41)</td>
<td>8 (42)</td>
<td>0.429</td>
</tr>
<tr>
<td>DSAEK+IOL</td>
<td>3 (18)</td>
<td>4 (13)</td>
<td>4 (21)</td>
<td>0.716</td>
</tr>
<tr>
<td>PK</td>
<td>6 (35)</td>
<td>9 (28)</td>
<td>2 (11)</td>
<td>0.202</td>
</tr>
<tr>
<td>PK+IOL</td>
<td>4 (24)</td>
<td>6 (19)</td>
<td>5 (26)</td>
<td>0.811</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>17</td>
<td>32</td>
<td>19</td>
<td></td>
</tr>
</tbody>
</table>

ECD: endothelial cell density, BK: bullous keratopathy, PBK: pseudophakic BK, ABK: aphakic BK, FECD: Fuchs endothelial corneal dystrophy, KC: keratoconus, DSAEK: Descemet’s stripping automated endothelial keratoplasty, IOL: intraocular lens, PK: penetrating keratoplasty Statistical analysis for multiple comparison was performed with Kruskal-Wallis test.
Table 2. Endothelial cell density (ECD) and endothelial cell loss (ECL) over time after successful corneal transplantation

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean ECD (SD), cells/mm²</th>
<th>Mean ECL (SD), %</th>
<th>ECD, P value</th>
<th>ECL, P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High maturity</td>
<td>Middle maturity</td>
<td>Low maturity</td>
<td>High maturity</td>
</tr>
<tr>
<td>Overall groups</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>2695 (194)</td>
<td>2832 (336)</td>
<td>2734 (236)</td>
<td></td>
</tr>
<tr>
<td>6 m</td>
<td>2268 (200)</td>
<td>2157 (524)</td>
<td>2000 (531)</td>
<td>17 (9)</td>
</tr>
<tr>
<td>12 m</td>
<td>2034 (286)</td>
<td>1999 (535)</td>
<td>1711 (542)</td>
<td>24 (13)</td>
</tr>
<tr>
<td>24 m</td>
<td>1878 (437)</td>
<td>1770 (579)</td>
<td>1226 (530)</td>
<td>30 (18)</td>
</tr>
<tr>
<td>36 m</td>
<td>1604 (436)</td>
<td>1424 (613)</td>
<td>911 (388)</td>
<td>40 (18)</td>
</tr>
<tr>
<td>Subgroup (DSAEK)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>2811 (235)</td>
<td>2833 (320)</td>
<td>2722 (204)</td>
<td></td>
</tr>
<tr>
<td>6 m</td>
<td>2293 (97)</td>
<td>2079 (611)</td>
<td>1877 (543)</td>
<td>20 (7)</td>
</tr>
<tr>
<td>12 m</td>
<td>1954 (263)</td>
<td>1854 (572)</td>
<td>1683 (443)</td>
<td>31 (12)</td>
</tr>
<tr>
<td>24 m</td>
<td>1949 (293)</td>
<td>1638 (613)</td>
<td>1278 (473)</td>
<td>31 (12)</td>
</tr>
<tr>
<td>36 m</td>
<td>1639 (373)</td>
<td>1373 (541)</td>
<td>1026 (381)</td>
<td>41 (15)</td>
</tr>
</tbody>
</table>

DSAEK: Descemet’s stripping automated endothelial keratoplasty, ECD: endothelial cell density, ECL: endothelial cell loss, SD: standard deviation, Statistical analysis for multiple comparison was performed with Kruskal-Wallis test.
Figure 1

Donor Cornea

Donor Center
- Corneal Transplantation
- Evaluation by Specular Microscopy

Donor Periphery
- Cell Culture
- Evaluation by Cell Character
Figure 2

Endothelial cell density (cells/mm²) vs. Time (Months) for ECD-36M.

- **High Maturity**
- **Middle Maturity**
- **Low Maturity**

Significant differences indicated by asterisks:
- *: p < 0.05
- **: p < 0.01
- ***: p < 0.001
Figure 3

A. Overall

ECD > 1500 cells/mm²

- High Maturity
- Middle Maturity
- Low Maturity

Probability of Survival vs. Time (Months)

$p < 0.001$

B. DSAEK alone

ECD > 1500 cells/mm²

- High Maturity
- Middle Maturity
- Low Maturity

Probability of Survival vs. Time (Months)

$p = 0.005$
PRÉCIS

This prospective study revealed a relationship between postoperative ECL and the maturity of cultured HCECs from the donor corneas. The postoperative ECD decreased more gradually in the group with high HCEC maturity than low maturity.