Comparison of Proliferative Capacity of Genetically-Engineered Pig and Human Corneal Endothelial Cells

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Key Words

Corneal endothelial cells · Endothelial keratoplasty · Pig, genetically-engineered · Proliferative capacity · Xenotransplantation

Abstract

Purpose: The possibility of providing cultured corneal endothelial cells (CECs) for clinical transplantation has gained much attention. However, the worldwide need for human (h) donor corneas far exceeds supply. The pig (p) might provide an alternative source. The aim of this study was to compare the proliferative capacity of CECs from wild-type (WT) pigs, genetically-engineered (GE) pigs, and humans. **Methods:** The following CECs were cultured: hCECs from donors (i) ≤ 36 years (young), (ii) ≥ 49 years (old), and WT pCECs from (iii) neonatal (< 5 days), (iv) young (< 2 months), and (v) old (> 20 months) pigs, and CECs from young (vi) GE pigs (GTKO/CD46 and GTKO/CD46/CD55). Proliferative capacity of CECs was assessed by direct cell counting over 15 days of culture and by BrdU assay. Cell viability during culture was assessed by annexin V staining. The MTT assay assessed cell metabolic activity. **Results:** There was significantly lower proliferative capacity of old CECs than of young CECs ($p < 0.01$) in both pigs and humans. There was no significant difference

in proliferative capacity/metabolic activity between young pCECs and young hCECs. However, there was a significantly higher percentage of cell death in hCECs compared to pCECs during culture ($p < 0.01$). Young GE pCECs showed similar proliferative capacity/cell viability/metabolic activity to young WT pCECs. **Conclusions:** Because of the greater availability of young pigs and the excellent proliferative capacity of cultured GE pCECs, GE pigs could provide a source of CECs for clinical transplantation.

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Introduction

The corneal endothelium plays an essential role in the maintenance of corneal transparency. Human (h) corneal endothelial cells (CECs) are essentially nonregenerative in vivo [1]. The loss of CECs from intraocular surgery (e.g., cataract surgery), dystrophy, or trauma triggers compensatory enlargement of the remaining CECs. Since a minimum CEC density of 500 cells/mm² is necessary to actively pump fluid from the stroma to the anterior chamber in the eye, loss of CECs often results in irreversible corneal endothelial dysfunction. For many years, penetrating (full-thickness) keratoplasty was the only ef-

fective therapy to restore the CECs, but it is not without complication [2]. In recent years, new surgical procedures have been developed that replace the endothelium without corneal trephination and suturing; these include endothelial keratoplasty, e.g., posterior lamellar keratoplasty, deep lamellar endothelial keratoplasty, Descemet stripping with endothelial keratoplasty, Descemet stripping automated endothelial keratoplasty, and Descemet membrane endothelial keratoplasty [3, 4]. Irrespective of the selected keratoplasty procedure, fresh donor corneas are required to obtain the high density CECs that are necessary ($>2,500$ cells/mm²) [5]. The corneal endothelium is extremely fragile and flaccid, thus difficult to transfer without damage.

Although hCECs do not have proliferative capacity *in vivo* and are arrested in the G1 phase of the cell cycle [6], they do have proliferative capacity *in vitro* [1]. The replacement of CECs with cultured CECs would provide a major advance in the treatment of corneal blindness [3, 7–9].

However, primary culture of hCECs is difficult, and a change in the morphology to a fibroblastic-like state cannot always be prevented. Furthermore, it is important to note that the worldwide need for human donor corneas far exceeds supply [10]. Pig (p) corneas might provide an alternative source. Corneas from specific pathogen-free pigs would avoid the potential problem of transfer of pathogenic microorganisms to the human recipient. Furthermore, stored pig corneas could be shipped to any location worldwide, and thus only a few specialized pig breeding facilities would be required. Because the cornea is an immune-privileged tissue, the evidence is that its fate as a xenograft will be significantly better than that of organ xenografts [11, 12].

Our recent *in vitro* studies have demonstrated that the human cellular and humoral immune responses to CECs from genetically-engineered (GE) pigs are no more vigorous than those to hCECs [13]. The transplantation (Tx) of cultured GE pCECs will be one potential therapeutic approach to restore the corneal endothelium. However, there have been no reports on whether the proliferative capacity of pCECs, especially GE pCECs, is similar to that of hCECs.

The aim of the present study was to compare the *in vitro* proliferative capacity of pCECs with that of hCECs. Furthermore, the *in vitro* proliferative capacity of GE pCECs was compared with that of wild-type (WT) pCECs. The present study therefore forms the basis for an innovative treatment utilizing cultured GE pCECs for Tx to correct corneal endothelial dysfunction.

Table 1. Pig corneas utilized in this study

Pig	Age	Corneas n	Mean CEC count cells/mm ² $\pm$ SEM
Neonatal			
WT	4–5 days	8	5,959 $\pm$ 436
Young age	6–8 weeks		
WT		4	3,809 $\pm$ 201
GE		6	3,768 $\pm$ 159
Old age			
WT	20–25 months	6	1,896 $\pm$ 186

SEM = Standard error of the mean; GE = genetically-engineered: GTKO/CD46 and GTKO/CD46/CD55.

Materials and Methods

Pig Corneas

Corneas were obtained from outbred large white WT pigs (Wally Whippo, Enon Valley, Pa., USA) and from GE pigs (Revivicor, Blacksburg, Va., USA; table 1). WT pig corneas were from neonatal (4–5 days), young (6–8 weeks), and old (20–24 months) pigs (table 1). GE pig corneas were obtained from α 1,3-galactosyltransferase gene-knockout (GTKO) pigs (that do not express the Gal α 1,3Gal antigen that is the major target for human anti-pig antibodies) transgenic for the human complement-regulatory protein CD46 (GTKO/CD46 pigs) [13, 14] and from GTKO/CD46/CD55 pigs [H. Hara, unpublished]. The density of pCECs was evaluated *ex vivo* using Confoscan 3 (NIDEK, Fremont, Calif., USA) [H. Hara, unpublished].

All animal care procedures were in accordance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health.

Human Corneas

Corneas from deceased human subjects were provided by the Pittsburgh Center for Organ Recovery and Education with the approval of the University of Pittsburgh Committee for Oversight of Research Involving the Dead, and in accordance with the guidelines of the Declaration of Helsinki for research involving the use of human tissues. All human corneas (ages 18–66 years) were considered to be unsuitable for Tx because of (i) lack of a blood sample from the donor to conduct serology tests, (ii) defects of the epithelium or stroma within the optical zone, (iii) stromal infiltrates, (iv) guttata, which is one of the characteristics of corneal dystrophy, (v) an unacceptably long period (>12 h) between time of death and time of corneal excision and storage, (vi) low CEC densities, (vii) sepsis or ocular infection, or (viii) donor chemotherapy. All human corneas were stored in Optisol-GS (Bausch & Lomb, Rochester, N.Y., USA) at 4°C until cell culture. Human corneas were divided into two groups (young ≤ 36 years, old ≥ 49 years) according to age (tables 2, 3).

Table 2. Details of corneas from young (≤ 36 years old) human donors

Donor No.	Age, years	Sex	Days ^a	Time ^b	CEC density cells/mm ²	Cause of death
1	18	M	1	14 h 27 min	2,548	motor vehicle accident
2	19	M	2	17 h 55 min	2,907	toxicology
3	20	M	1	18 h 30 min	3,670	toxicology
4	20	M	6	14 h 20 min	3,493	asphyxia due to hanging
5	21	M	4	17 h 39 min	3,764	seizure disorder
6	31	M	1	2 h 48 min	3,386	head trauma
7	35	M	2	13 h 6 min	3,052	intracranial hemorrhage
8	35	M	5	26 h 15 min	2,597	head trauma
9	36	M	2	14 h 11 min	3,434	subdural hematoma
Mean $\pm$ SEM	26.1 $\pm$ 2.6		2.7 $\pm$ 0.6	15 h 28 min $\pm$ 2 h 3 min	3,206 $\pm$ 150	

^a Period of time from death to culture of the corneal cells.^b Period of time from death to corneal excision and storage.**Table 3.** Details of corneas from old (>49 years old) human donors

Donor No.	Age, years	Sex	Days ^a	Time ^b	CEC density cells/mm ²	Cause of death
1	47	M	1	12.6	2,420	acute cardiac event
2	49	F	5	11.26	3,449	suspected drug overdose
3	49	M	3	13.10	3,014	acute cardiac event
4	58	F	3	17	2,321	acute cardiac event
5	65	M	2	15.49	4,478	acute cardiac event
6	66	F	3	11.30	3,920	anoxia
Mean $\pm$ SEM	57.4 $\pm$ 3.7		3.2 $\pm$ 0.5	13.9 $\pm$ 1.8	3,436 $\pm$ 370	

^a Period of time from death to culture of the corneal cells.^b Period of time from death to corneal excision and storage.

Corneal Endothelial Cell Culture

pCECs and hCECs were cultured as previously described [13]; pCECs were isolated from fresh corneas. In contrast, primary cultures of hCECs were initiated within 6 days after preservation in Optisol-GS (tables 2, 3). After primary culture, the culture medium for both pCECs and hCECs was replaced with Opti-MEM I reduced-serum medium (Invitrogen, Carlsbad, Calif., USA) containing calcium chloride (100 μ g/ml, Sigma-Aldrich, St. Louis, Mo., USA), 0.08% chondroitin sulfate (Sigma), ascorbic acid (20 μ g/ml, Sigma), 8% heat-inactivated fetal bovine serum (Invitrogen), antibiotic-antimycotic (Invitrogen), endothelial growth factor (30 μ g/ml, BD Biosciences, San Diego, Calif., USA), and human epidermal growth factor (10 ng/ml, Biomedical Technologies, Stoughton, Mass., USA).

Evaluation of Proliferative Capacity of CECs

Cell Numbers. After primary culture, CECs (passage 1–3) were seeded at 5,000 cells in collagen-I-coated 12-well tissue culture

plates (BD) and were cultured for 15 days. CECs were harvested and the cell number was counted every third day. The culture medium in the remaining wells was changed every third day. On each day the mean of triplicate results was expressed as a proliferation curve and compared with the rate of proliferation of each type of CEC.

Calculation of Rate of Proliferation (Doubling Time). To assess the rate of proliferation, the doubling time was calculated using the total number of CECs at 3 days and 6 days [15]. At each time-point the results from three separate wells were averaged. The doubling time was calculated as follows:

$$\text{Doubling time} = t/10 (\log D_2 - D_1),$$

where D1 is the number of CECs on day 3, D2 is the number of CECs on day 6, and t is the time interval between D1 and D2 (in this case 72 h).

Cell Cycling. DNA synthesis was measured by staining of 5-bromo-2'-deoxyuridine (BrdU) using BrdU flow kit (BD). The

60–80% confluent cultured CECs were exposed to medium containing 10 μ M BrdU for 30 min. The cells were then washed to remove unbound BrdU and fixed with Cytofix/Cytoperm buffer containing 4% paraformaldehyde solution (BD) for at least 10 min. The cells were permeabilized with Cytoperm Plus Buffer (BD) for 20 min, and incubated with DNAase (BD) for 60 min at 37°C. Subsequently, the cells were incubated with 1:20 diluted FITC-conjugated anti-BrdU monoclonal antibody (BD) for 40 min at room temperature. The unbound monoclonal antibodies were washed out and cellular DNA was counterstained with 7-amino-actinomycin D (7-AAD, BD). Cellular fluorescence was measured by a LSRFortessa flow cytometer (BD).

Cell Viability

Apoptotic cells were detected by flow cytometry using annexin V apoptosis detection kit I (BD). CECs were harvested after 70–80% confluence, washed with PBS (Invitrogen), and resuspended with freshly prepared 1 $\times$ binding buffer (BD). Then, 10⁵ cells per tube, including 200 μ l 1 $\times$ binding buffer, were incubated with 5 μ l phycoerythrin conjugated-annexin V (BD) for 30 min and 2.5 μ l 7-AAD for 20 min at room temperature. Annexin V-negative/7-AAD-negative cells were identified as live cells. Annexin V-positive/7-AAD-negative cells were identified as apoptotic, whereas annexin V-positive/7-AAD-positive cells were identified as necrotic or dead.

Metabolic Activity

The metabolic activity of the CECs was measured with the colorimetric 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) assay kit I (Roche Diagnostics, Indianapolis, Ind., USA). The harvested CECs were seeded in 96-well flat-bottom plates (Nunc, Rochester, N.Y., USA) that had been pre-coated with undiluted FNC Coating Mix (Athena Enzyme Systems, Baltimore, Md., USA), at a density of 4,000, 2,000, 1,000, and 500 cells/well. After 48-hour culture, 10 μ l MTT reagent (5 mg/ml) was added to each well and incubated for 4 h at 37°C. Solubilization solution (100 μ l of 10% sodium dodecyl sulfate containing 0.01 M HCl) was added to each well, and incubated overnight in the culture incubator (37°C, 5% CO₂). The optical density for each sample was read with a Victor 3 Multi-label Plate Reader (PerkinElmer, Waltham, Mass., USA) at 570 nm, with 660 nm used as a reference. It was necessary to correct the results because the total number of CECs in each well affected absorbance. As a parallel experiment, 4,000 CECs/well were seeded in a 96-well plate and cultured for 3 days, after which CECs were harvested and counted. Relative absorbance was calculated as follows:

Absolute absorbance $\times$ 4,000 cells/absolute cell number after 3 days culture.

Storage of Corneas

To investigate the effect of storage of the cornea, excised WT pig corneas (n = 4) were stored in Optisol for 3–5 days before primary CEC culture. Proliferative capacity, cell viability, and metabolic activity of the CECs were compared with those of CECs from the second cornea of the same donor which had not been stored (freshly cultured).

Statistical Analyses

Data obtained from two groups were compared using the Student t test, and comparisons among multiple groups were performed using the Kruskal-Wallis one-way analysis using GraphPad Prism version 4 (Graphpad Software, San Diego, Calif., USA). Values are presented as mean $\pm$ SEM. Differences were considered to be significant at $p < 0.05$.

Results

The Number of CECs in Pigs Decreased Significantly with Age

The number of CECs in pigs of different ages – WT (neonatal, young, and old) and GE (young) – were counted by Confoscan 3 (table 1). The mean number of CECs from WT neonatal and young pigs was 5,959 and 3,809 cells/mm², respectively, whereas from old pigs the mean number was 1,895 cells/mm². Young GE pigs (3,767 cells/mm²) showed similar CEC density to young WT pigs. The density of CECs is known to decrease significantly during life in humans [16, 17]. As in humans, these results indicated that the number of pCECs decline with age; there was a significant correlation between the density of CECs and age ($R^2 = 0.68$, $p < 0.01$).

Human corneas were selected with at least >2,000 cells/mm² for primary culture. Mean age of young corneas was 27.4 years (mean CEC number = 3,120/mm²), and of old corneas 55.7 years (mean CEC number 3,267/mm²) (tables 2, 3). There was no significant correlation between the number of CECs and age ($R^2 = 0.03$, $p = 0.51$).

Morphology of Cultured pCECs and hCECs

CECs from neonatal (fig. 1a) and young (fig. 1b) pigs showed typical polygonal morphology, but old pCECs had a round appearance (fig. 1c). Neonatal and young pCECs were smaller than old pCECs. Young hCECs also showed the typical homogeneous polygonal morphology (fig. 1d). In contrast, old hCEC showed larger cell size and heterogeneous morphology (fig. 1e).

Young CECs Have a Significantly Greater Proliferative Capacity than Old CECs

pCECs and hCECs of varying ages were cultured under identical conditions. Proliferation of pCECs and hCECs increased steeply after 3 days in culture, the number of CECs reaching a peak after 12–15 days (fig. 2a, b). The maximum number of pCECs and hCECs from young subjects was similar (480,000–580,000 cells/well or 1,200–1,500 cells/mm²; table 4). In contrast, the maximum number of pCECs and hCECs from old subjects was

Fig. 1. Morphology of pCECs and hCECs after primary culture to confluence. Micrographs of neonatal (5 days, **a**), young (2 months, **b**), and old (24 months, **c**) pCECs, and of young (19 years, **d**), and old (56 years, **e**) hCECs. Magnification $\times 200$.

approximately 250,000–300,000 cells/well (700–800 cells/mm²; table 4). (It is important to note that 100% confluence of old CECs was not reached even after culture for 15 days; not shown.)

Doubling time has been measured as an estimate of cells or tumor growth in experimental and clinical situations [18, 19]. In both pCECs and hCECs, there was a significantly longer doubling time in old than in young CECs, suggesting that young CECs have a more potent proliferative capacity (fig. 2c).

An evaluation of the activity of DNA synthesis in terms of the cell cycle provides an estimate of the proliferative capacity of cells [20]. The cell cycle status and DNA synthetic activity of cells can be determined by analyzing the correlation of total DNA and the level of incorporated BrdU by flow cytometry [21]. Staining of BrdU combined with 7-AAD provides the percentage of

Table 4. Maximum densities of CECs during culture

Type of CECs	CEC density ^a cells/mm ²	Versus old (same species)	Versus young human
Pig			
Neonatal	1,538 $\pm$ 120	p < 0.01	NS
Young	1,285 $\pm$ 113	p < 0.05	NS
Old	801 $\pm$ 69		p < 0.05
Human			
Young	1,474 $\pm$ 186	p < 0.01	
Old	702 $\pm$ 41		p < 0.01
NS = Not significant.			
^a Mean $\pm$ SEM.

Fig. 2. Comparison of proliferative capacity between pCECs and hCECs. The proliferative capacities of neonatal, young, and old pCECs (**a**), and young and old hCECs (**b**) were compared by direct cell counting for 15 days. The maximum numbers of neonatal and young pCECs were measured after 12 days of culture, and the maximum number of old pCECs was at 9 days, which was significantly different from neonatal and young pCECs ($p < 0.01$), (** $p < 0.01$ neonatal pCECs vs. young or old pCECs, $^{\dagger\dagger} p < 0.01$ neonatal or young pCECs vs. old pCECs). After 6 days of culture, there were significantly fewer old hCECs than young hCECs (** $p < 0.01$). The maximum number of young hCECs was at 9 days, in contrast to old hCECs, which was at 15 days ($p < 0.01$ vs. young hCECs). **c** The doubling time of pCECs and hCECs was measured (see 'Methods'). There was a significantly more rapid doubling time of neonatal and young CECs than of old CECs in both pigs and humans (** $p < 0.01$).

Fig. 3. Similar DNA synthesis in young pCECs and young hCEC, but less cell death in pCECs during culture. **a** A representative figure for cell cycle analysis using the BrdU assay by flow cytometry. Double staining with 7-AAD and BrdU demonstrated G0/G1 phase (7-AAD low and BrdU negative), S phase (7-AAD positive and BrdU positive), G2/M phase (7-AAD high and BrdU negative), and dead cells (7-AAD negative and BrdU negative). **b** Representative figures and statistical analysis of the percentage of CECs in S phase (**c**) and G2/M phase (**d**), and dead cells (**e**) (showing neonatal, young, and old pCECs, and young and old hCECs).

G0/G1, S, G2/M phase and dead cells were quantified by gating 7-AAD and BrdU. **c** The percentage of cells in the S phase was significantly lower in old CECs from both pigs and humans ($p < 0.01$). The S phase of old pCECs was also significantly lower than that of young hCEC ($p < 0.01$). **d** In the G2/M phase, there was no significant difference between each group of pCECs and hCECs. **e** During culture, there was a significantly higher incidence of cell death in hCECs compared to pCECs ($p < 0.01$). There was a significantly higher percentage of dead cells in old hCECs than in young hCECs ($p < 0.01$), (* $p < 0.05$, ** $p < 0.01$).

Fig. 4. During culture, there was a significantly lower incidence of apoptosis and necrosis in pCECs than in hCECs. **a** Representative figures of annexin V staining in young pCECs and hCECs. CECs were stained with annexin V and 7-AAD after 70–80% confluence. **b** During culture, there were significantly higher percentages of apoptosis and necrosis in hCECs than in pCECs, (* $p < 0.05$).

cells in a specific phase of the cell cycle, including G0/G1, S, and G2/M phases (fig. 3a). The S phase is the phase of DNA synthesis. The percentage of cells in the S phase was significantly lower in old pCECs and hCECs than in young CECs ($p < 0.01$, fig. 3b, c). These results correlated with cell counts (fig. 1). In contrast to the S phase, there was no significant difference in the G2/M phase between pCECs and hCECs of different ages (fig. 3d).

These results indicated that the proliferative capacity of neonatal and young pCECs was similar to that of young hCECs. However, both young and old hCECs showed a significantly higher incidence of cell death during culture compared to pCECs ($p < 0.01$; fig. 3b, e). In addition, old hCECs showed a higher incidence of cell death during culture compared to young hCECs ($p < 0.01$; fig. 3e). There was no significant difference in the

incidence of cell death during culture among pCECs of different ages.

Less Viability of hCECs during Culture Compared to pCECs

To further confirm whether hCECs showed a greater susceptibility to cell death during culture, the number of apoptotic cells was investigated. The incidence of both apoptosis (annexin V-positive/7-AAD-negative) and necrosis (annexin V-positive/7-AAD-positive) in hCECs was significantly greater than in pCECs (fig. 4a, b).

Similar Metabolic Activity between Pig and Human CECs

To investigate whether the reduced proliferative capacity of old CECs was associated with less metabolic ac-

Fig. 5. GE pCECs have similar characteristics to WT pCECs. Proliferation curve (**a**), doubling time (**b**), DNA synthesis (S phase) and cell viability (**c**), and metabolic activity (**d**) were compared between young WT ($n = 4$) and young GE ($n = 6$) pCECs. **a** Proliferation capacities of young WT and young GE pCECs were similar, except at 15 days, (* $p < 0.05$). **b** There was no difference in dou-

bling time between young WT and GE pCECs. **c** The BrdU assay demonstrated no difference in the percentage of cells in the S phase and in the number of dead cells between WT and GE pCECs. **d** There was no difference in metabolic activity between WT and GE pCECs.

tivity, the MTT assay was carried out after 3 days of culture. Since proliferation of both pCECs and hCECs increased steeply after 3 days in culture, measurement at 3 days provided an estimate of metabolic activity before proliferation increased. There was no significant difference in metabolic activity between pCECs and hCECs, or between young and old CECs (data not shown), suggesting that, during the early period of culture, any differences in proliferative capacity of CECs were not related to metabolic activity.

GE pCECs Had Similar Proliferative Capacity, Cell Viability, and Metabolic Activity as WT pCECs

There was no significant difference in cell numbers (fig. 5a), doubling time (fig. 5b), DNA synthesis, cell viability (fig. 5c), of metabolic activity (fig. 5d) between GE and WT pCECs.

Storage of CECs Had No Effect on Proliferative Capacity, Cell Viability, or Metabolic Activity

Human corneas had been stored in Optisol until primary culture, and this may have affected proliferative ca-

capacity and cell viability. To investigate this possibility, freshly cultured young pCECs were compared to stored young pCECs. There was no significant difference in proliferative capacity, cell viability, or metabolic activity between stored and fresh pCECs (data not shown).

Discussion

Corneal Tx has advanced rapidly during the past decade, with a shift in paradigm from full-thickness penetrating keratoplasty to partial-thickness (component) corneal Tx. Such partial-thickness Tx replaces the diseased tissue, but not the entire cornea. In particular, endothelial keratoplasty has received more attention in the clinic because it has clear advantages over penetrating keratoplasty, such as faster recovery of visual acuity, increased tectonic strength, and lower risk of intraoperative complications [22]. However, the availability of human corneas for endothelial keratoplasty is reduced by (i) a long delay after donor death before excision of the corneas, or (ii) technical difficulties because CEC damage has occurred during the handling of these fragile donor corneas. Even under the best conditions for Tx, subsequent graft failure from immunologic rejection or nonimmunologic endothelial decompensation may still occur [5].

Recently, the *in vitro* culture of hCECs has been considered as an alternative method for replacing damaged corneal endothelium [9]. Clinical observations suggest that transfer of CECs into the recipient anterior chamber might be sufficient to restore corneal transparency through endothelial cell migration [23]. The concept of using a carrier to implant *ex vivo* cultured CECs into the recipient anterior chamber may become an increasingly realistic approach [8, 24–26]. Hence, the ability to culture hCECs *in vitro* with relative consistency has stimulated research into the development of a suitable delivery system in the form of a synthetic or biological carrier. Tx of cultured corneal endothelium has been attempted in animal models to establish a new intervention for corneal endothelial dysfunction [8, 26].

In addition, a Rho-associated kinase inhibitor promotes cultured monkey CEC proliferation both *in vitro* and *in vivo* [27, 28]. However, there are still concerns regarding the use of human corneas to produce cultured CECs for Tx. These include difficulties in hCEC culture, the questionable proliferative capacity of old hCECs, immunological rejection and/or nonimmunological CEC damage, particularly in high-risk patients (highly sensitized to donor HLA antigens, or with an inflamed cor-

neal bed), and the worldwide shortage of suitable human corneas.

Pigs could provide an alternative source of CECs for Tx. The use of CECs from GE pigs offers resistance to the human immune responses [10, 11, 13]. As with hCECs, pCECs cannot proliferate *in vivo* [29], but can do so *in vitro* [13]. However, it was important to investigate whether pCECs have a similar or greater proliferative capacity *in vitro* than hCECs, and to determine whether old pigs can be used as sources for cultured CECs for Tx. The proliferative capacity of young (<39 years) hCECs was greater than that of old (>49 years) hCECs. This observation has also been reported by others [30], and indicates that young hCECs are more suitable for CEC Tx than old hCECs.

We used neonatal CECs (5–7 days) and young (6–8 weeks) and old (20–24 months) pigs for our study. Young pCECs demonstrated greater proliferative capacity than old pCECs. Although old pigs (>20 months) are approximately equivalent in age to a 25-year-old human, old pCECs showed significantly less proliferative capacity than young hCECs (<39 years), suggesting that hCECs may have a greater proliferative capacity at the equivalent age. We could not compare young pCECs (<2 months), which are approximately equivalent to a 3-year-old human, because of the unavailability of human corneas from young children. However, young pCECs showed a similar proliferative capacity to hCECs from young adults (<36 years).

These results indicated that human and pig corneas from young subjects might be an ideal source for *in vitro* expansion of CECs to provide cultured corneal endothelial sheets. However, young human corneas are difficult to obtain because the majority of high-quality corneas from young deceased humans are used in the clinic for corneal Tx. Corneas from healthy young donors are fewer than those from older donors, and, furthermore, most human corneas that become available for CEC culture are considered unsuitable for clinical corneal Tx (e.g., because of a prolonged period between donor death and corneal excision and storage, or low CEC density) and therefore will be of poorer quality.

In addition, there was significantly less cell death during culture in pCECs compared to hCECs. In particular, a higher percentage of cell death was found in old hCECs compared to young hCECs. This may contribute to difficulty in culturing hCECs. In addition, susceptibility to cell death in hCECs during culture might lead to an increased incidence of CEC loss after Tx from immunological and/or nonimmunological mechanisms.

Although collagen type I has been widely used for cell culture, including the culture of human vascular endo-

thelial cells [14], it may not be ideal for the culture of hCECs. However, if the cells are already being grown in a 'coated' plate or flask to support cell attachment, we believe the most important factor for cell culture is the medium. Plates with different 'coatings' have been used, such as chondroitin sulfate [31], FNC Coating Mix [19], and collagen type IV [32]. A preliminary study in our laboratory indicated no significant difference in CEC proliferation capacity and morphology during culture in collagen type I- or FNC-coated plates (data not shown). In addition, a recent report suggests that matrix-coating may not be mandatory for the culture of hCECs [33]. Therefore, coating plates with collagen type I would appear to have little effect on CEC proliferation capacity.

We have previously demonstrated the significant expression of Gal α 1,3Gal on cultured WT pCECs, and up-regulation of this antigen when cells were activated, which resulted in increased complement-mediated cytotoxicity by pooled human sera [13]. It would therefore be beneficial, if not essential, to use CECs from GTKO pigs to reduce the human humoral immune response. We demonstrated that human humoral and cellular immune responses to GTKO/CD46 pCECs were significantly reduced compared to WT pCECs [13]. GTKO/CD46/CD55 pigs have now been produced by Revivicor, and CECs from these pigs are resistant to complement-dependent lysis by pooled human sera [Hara et al., unpublished]. The hCD4⁺ T cell responses to nonactivated GTKO/CD46 pCECs are similar to those to hCECs, although they are increased when CECs are activated. The hCD4⁺ T cell response to activated GTKO/CD46 pCECs is significantly greater than to hCECs. Pigs transgenic for class II transactivator knock-down [34] and pCTLA4-Ig transgenic pigs [35, 36] may reduce the hCD4⁺ T cell immune response.

Further studies are required before cultured GE pCECs could be transplanted successfully into primates. These include (i) the phenotypic character of the cultured CECs after several passages, (ii) the expression pattern

of function-related proteins, e.g., ZO-1 and Na⁺/K⁺-ATPase, and (iii) functional tests of cultured CECs using *in vivo* corneal endothelial dysfunction models [37].

In conclusion, the clinical application of cultured pCECs for Tx would provide a new approach to the treatment of corneal endothelial dysfunction. Our studies suggest the feasibility of providing corneal endothelial sheets derived from young GE pCECs for clinical Tx because (i) young pCECs have a similar proliferative capacity to young hCECs, (ii) pCECs show less cell death during culture compared to hCECs, and thus we believe are easier to culture compared to hCECs, (iii) there is a greater availability of corneas from young pigs than young humans, (iv) GE pCECs are more resistant to the human immune response, and (v) a single GE pig cornea could provide enough cultured CECs for several patients. Optimistically, we believe that GE pigs will open new avenues by which researchers can use cultured GE pCECs to treat corneal blindness.

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Disclosure Statement

D.L.A. is an employee of Revivicor, Inc., and owns stock in the company.

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