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# Corneal Endothelial Tissue Bioengineering Using Cultured Human Corneal Endothelial Precursor Cells

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Additional information is available at the end of the chapter

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## 1. Introduction

The cornea is composed of a multilayered epithelium, Bowman's membrane, stroma, Descemet's membrane, and endothelium. The corneal endothelium (CE) is a single layer of hexagonal cells that separates the corneal stroma from the aqueous humor of the anterior chamber. Transparency of the cornea is maintained by regulation of stromal hydration through the barrier and pump functions of the CE, and corneal transplantation has long been used to treat corneal endothelial defects. In fact, more than half of the patients who undergo full-thickness corneal transplantation have impairment of visual acuity due to corneal endothelial problems alone and have a normal corneal epithelium (Cosar et al., 2002; Mannis et al., 1981; Rapuano et al., 1990). Corneal transplantation requires a fresh human cornea, but there is a worldwide shortage of donors (Barboza et al., 2007; Cao et al., 2006; Shimazaki et al., 2004; Tuppini et al., 2007).

Stem cells or progenitor cells are defined by a capacity for self-renewal and the ability to generate different types of cells (multipotentiality) that are involved in the formation of mature tissues. In contrast, precursor cells are unipotential cells with limited proliferative capacity. Regenerative stem cells or precursors can be detected by the sphere-forming assay in various adult tissues, including the central nervous system (Nunes et al., 2003), bone marrow (Krause et al., 2001), skin (Kawase et al., 2004; Toma et al., 2001), retina (Coles et al., 2004), corneal epithelium (Mimura et al., 2010a; Yokoo et al., 2008), corneal stroma (Amano et al., 2006; Mimura, 2008a, 2008b; Uchida et al., 2005; Yamagami et al., 2007), and corneal endothelium (Amano et al., 2006; Mimura, 2005a, 2005b, 2005c, 2007, 2010b; Yamagami, 2006, 2007; Yokoo et al., 2005).

Despite the successful isolation and characterization of stem cells from various tissues, relatively few animal studies have been done to investigate the efficacy of stem cell transplantation. A three-dimensional carrier that maintains cell-to-cell interactions is indispensable for tissue engineering using stem cells, but the resulting structural complexity does not allow us to easily perform investigations of stem cell transplantation.

We have isolated precursors with the propensity to develop into corneal endothelial-like cells from the CE of human donor corneas (Yokoo et al., 2005). We have also demonstrated that cultured human corneal endothelial cells (HCECs) and rabbit CE-derived precursors are an effective cell source for treating corneal endothelial defects in a rabbit model (Mimura 2005a, 2005b). Because the number of corneal endothelial cell (CEC) precursors that can be isolated from a native cornea is insufficient for corneal transplantation, establishment of a method for the mass production of precursor cells is required before CEC transplantation can be employed clinically.

In this chapter, we introduce our recent work in the fields of regenerative medicine and tissue engineering for the CE using bipotential precursor cells. We isolated precursors with the propensity to develop into CECs from human CE, and we investigated the distribution and proliferative capacity of precursor cells derived from the central and peripheral regions of the cornea by the sphere-forming assay. We also tested the effect of injecting human corneal endothelial spheres anterior chamber (instead of full-thickness corneal transplantation) in a rabbit model of bullous keratopathy, a condition associated with corneal endothelial defects.

## **2. Origin and development of the Corneal Endothelium**

Neural crest cells, from which the CE is derived (Bahn et al., 1984; Johnston et al., 1979), migrate and differentiate in two waves during corneal development (Liu et al., 1998; Meier et al., 1982). In the first wave, the corneal epithelium is formed by periocular mesenchymal cells of neural crest origin and it synthesizes the primary stroma, after which neural crest cells migrate to the margin of the optic cup and then migrate between the lens and corneal epithelium to contribute to development of the CE and the trabecular meshwork. In the second wave, neural crest cells invade the primary stroma and differentiate into corneal keratocytes.

## **3. Isolation of sphere colonies from human Corneal Endothelium**

### **3.1. Primary sphere-forming assay**

This study was conducted in accordance with the Declaration of Helsinki. Corneas were obtained from the Central Florida Lions Eye Tissue Bank and the Rocky Mountain Lions' Eye Bank at 4 to 10 days after death. The age of the donors was 41 to 78 years. The CE and Descemet's membrane were peeled away in a sheet from the periphery to the center of the inner surface of the cornea with fine forceps, as described previously (Sakai et al., 2002). To

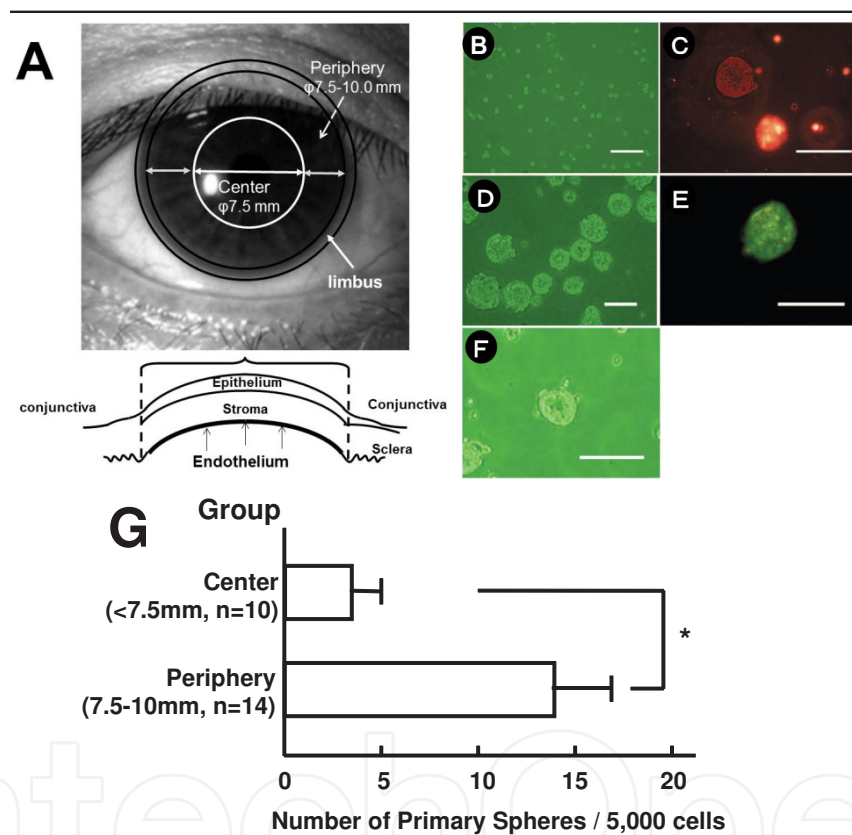
avoid the inclusion of posterior stromal tissue, we only used endothelium that was smoothly peeled off together with Descemet's membrane. The harvested CE was incubated at 37°C for 3 hours in basal medium containing 0.02% collagenase (Sigma-Aldrich, St. Louis, MO). This was followed by incubation in 0.2% ethylenediaminetetraacetic acid (EDTA) at 37°C for 5 minutes, and then dissociation into single cells by trituration with a fire-polished Pasteur pipette. The viability of the isolated CECs was >90%, as shown by trypan blue staining (Wako Pure Chemical Industries, Osaka, Japan). After addition of a trypsin inhibitor (Invitrogen-Gibco), the cells were resuspended in basal medium and the number of cells was counted (Coulter counter; Beckman-Coulter, Hialeah, FL). Neither cytokeratin-3 nor cytokeratin-12 expression was detected, indicating that the cells thus obtained were all CECs without contamination by other corneal cell types.

Half of the cells were labeled with a fluorescent cell tracker (CM-DiI; C-7000; Molecular Probes, Eugene, OR), as described elsewhere (Mimura et al. 2004), to examine sphere formation by reaggregation. DiI-labeled cells and unlabeled cells were mixed and seeded at a density of 1 cell/ $\mu$ L (250 cells/cm<sup>2</sup>), 10 cells/ $\mu$ L (2,500 cells/cm<sup>2</sup>), 30 cells/ $\mu$ L (7,500 cells/cm<sup>2</sup>), or 50 cells/ $\mu$ L (12,500 cells/cm<sup>2</sup>) on 60-mm uncoated dishes containing 5 mL of medium for floating culture (Reynolds & Weiss, 1992, 1996) (Fig. 1B). No spheres were generated in the cultures with only 1 viable cell/ $\mu$ L, but numerous spheres were formed at 30 and 50 cells/ $\mu$ L, with some arising from reaggregation as indicated by DiI staining. Spheres were completely DiI-positive or DiI-negative when culture was performed at 10 cells/ $\mu$ L (Fig. 1C), indicating that these spheres were derived from proliferation and not from reaggregation of the dissociated cells.

Incubation was done in a humidified incubator under an atmosphere of 5% CO<sub>2</sub>, with 40 ng/mL basic fibroblast growth factor (bFGF) and 20 ng/mL epidermal growth factor (EGF) being added to the medium every other day. To investigate whether the isolated cells were contaminated with corneal epithelial cells, expression of epithelial markers such as keratins K3 and K12 (Irvine et al., 1997; Moll et al., 1982) was assessed by the reverse transcription-polymerase chain reaction (RT-PCR) before the start of culture. Then primary culture was performed and the existence of fibroblast-like cells was investigated to assess contamination by stromal cells. CECs were isolated without contamination by corneal epithelial cells, as demonstrated by RT-PCR analysis of corneal epithelial markers (K3 and K12 genes), as well as the characteristic hexagonal shape of the cells in primary culture (data not shown). Almost complete disaggregation into single cells was achieved, since counting of single, double, and triple cells showed that 99% of all cells were single (Fig. 1B).

After incubation for 5 days, small floating spheres formed. These spheres grew larger after 10 days, while the nonproliferating cells died and were eliminated (Fig. 1D). After 10 days, we only counted cell clusters with a diameter of at least 50  $\mu$ m, in order to distinguish growing spheres from dying ones. To verify that the increase of colony size was actually due to cell proliferation, we added the thymidine analogue BrdU to cultures at 24 hours before fixation. Then the spheres were stained with an FITC-conjugated anti-BrdU antibody (1:100; Roche Diagnostics, Basel, Switzerland) at room temperature (RT) for 60 minutes in the dark. We found that BrdU labeled most of the cells in each sphere on day 10 (Fig. 1E), indicating that the spheres contained proliferating cells. These results suggested that the sphere colonies arose from single

isolated HCECs and that the sphere-forming cells possess the capacity to proliferate. When the number of spheres obtained was counted after 10 days of culture, we found that  $257 \pm 83$  spheres (mean  $\pm$  SD,  $n=8$ ) were generated per dish (50,000 cells). In a typical case,  $2.5 \pm 104$  cells were isolated from a 10-mm piece of corneal tissue, generating approximately 130 spheres after 10 days. These spheres had a diameter of  $88.3 \pm 15.9 \mu\text{m}$  (mean  $\pm$  SD,  $n=35$ ). The replating efficiency showed a dramatic decline between primary and secondary sphere colonies. When the primary spheres were trypsinized and incubated in serum-free floating culture, secondary colonies were generated (Fig. 1F) at a level of approximately  $15 \pm 1$  ( $n=3$ ) per dish of 10,000 cells. This suggests that HCECs have the capacity for self-renewal and formation of sphere colonies, but this capacity is limited.



**Figure 1.** Sphere formation from donor human corneal endothelium. (A) Anterior view of a human cornea and a diagram of the corneal epithelium and stroma. Stromal keratocytes were isolated from specimens obtained from both the peripheral cornea (7.5-10.0 mm in diameter) and the central cornea (7.5 mm in diameter). (B-F) Sphere formation by human corneal endothelial cells (HCECs). After disaggregation into single cells, HCECs were plated at a density of 10 viable cells/ $\mu\text{L}$  in basal medium (B). More than 99% of the cells were single cells on day 0. (C) Spheres were completely Dil-positive or Dil-negative after culture at a density of 10 viable cells/ $\mu\text{L}$ . (D) The mean ( $\pm$ SD) sphere diameter was  $88.3 \pm 15.9 \mu\text{m}$  on day 10. (E) Each sphere colony was labeled with BrdU on day 10. (F) Secondary spheres generated after the dissociation of primary spheres. The replating efficiency was much lower than that of the primary spheres. Scale bar= $100 \mu\text{m}$ . (G) The number of primary spheres obtained was compared between the peripheral and central regions of the cornea. The number of sphere colonies obtained from the peripheral cornea ( $n=14$ ) after 10 days of culture was significantly higher than that obtained from the central cornea ( $n=10$ ) (unpaired  $t$ -test). This experiment was repeated 3 times using different donor corneas, and representative data are shown as the mean  $\pm$  SD. \* $P<0.0001$ . These figures were modified from Yokoo et al. (2005) and Yamagami (2007) with permission.



### 3.2. Distribution of sphere colonies derived from human Corneal Endothelial cells

HCECs were obtained from the central cornea (up to 7.5 mm from the center) and the peripheral cornea (from 7.5 to 10 mm) (Fig. 1A). As a result, the number of primary sphere colonies per 5,000 cells (mean  $\pm$  SD) was significantly higher when peripheral HCECs were used ( $13.6 \pm 3.5$  spheres/5,000 cells) than when central HCECs were used ( $3.3 \pm 1.6$  spheres/5,000 cells) (Fig. 1G). The rate of sphere formation by HCECs from the peripheral cornea was approximately 4 times that for HCECs from the central cornea in repeated experiments (data not shown).

It has generally been accepted that human CE does not proliferate after birth, but our findings and some previous reports suggest that the CE may undergo slow proliferation *in vivo*. In 2003, Amann et al. demonstrated that paracentral and peripheral HCECs exist at a higher density than central HCECs by specular microscopy and histological observation of donor corneas. The presence of slowly proliferating HCEC precursors in the peripheral cornea could explain this higher cell density at the periphery. Otherwise, the cell density should be uniform throughout the corneal endothelium, because it tends to equalize over time. Another suggestive point is the outcome of Sato's method of anterior-posterior refractive surgery that involves making multiple peripheral and midperipheral incisions in the endothelium and stromal layer from the anterior chamber to treat myopia (Kanai et al., 1982; Kawano et al., 2003). This type of radial keratotomy performed via the anterior chamber leads to a decrease of HCECs many years later, possibly as a result of the corneal incisions causing more rapid cell loss than would occur with normal aging (Kanai et al., 1982; Kawano et al., 2003). It is possible that direct damage to HCEC precursors slows their proliferation, so that replacement of CECs decreases. The third point to consider is the outcome of corneal transplantation for various conditions associated with damage to the cornea, such as bullous keratopathy, keratoconus, and corneal leukoma. In hosts who retain their peripheral CE, such as patients with keratoconus, the grafts survive for much longer than in hosts with loss of the peripheral endothelium, such as patients with bullous keratopathy (Boisjoly et al., 1993; Williams et al., 1992; Yamagami et al., 1996). Keratoconus patients are typically younger than those with bullous keratopathy, so it could be suggested that their peripheral endothelium has greater proliferative potential because of this age difference, but differentiation of CEC precursors from the host cornea augmenting viable cells from the graft may be another reason for the longer survival of grafts after transplantation for keratoconus compared with bullous keratopathy. Therefore, when full-thickness corneal transplantation is done, a larger graft may be preferred for eyes with bullous keratopathy because it can supply more HCEC precursors, whereas a smaller graft may allow the optimum use of host-derived HCEC precursors in patients with keratoconus.

### 3.3. Characterization of primary spheres derived from human Corneal Endothelium

Immunocytochemical analysis of 10-day spheres was performed as follows. The spheres were fixed with methanol (Wako Pure Chemical Industries) in phosphate-buffered saline (PBS) for 10 minutes, washed in PBS, and incubated for 30 minutes with 3% bovine serum albumin (BSA) in PBS containing 0.3% Triton X20 (BSA/PBST) to block nonspecific staining. Then, the spheres were incubated for 2 hours at RT with the following specific primary antibodies diluted in

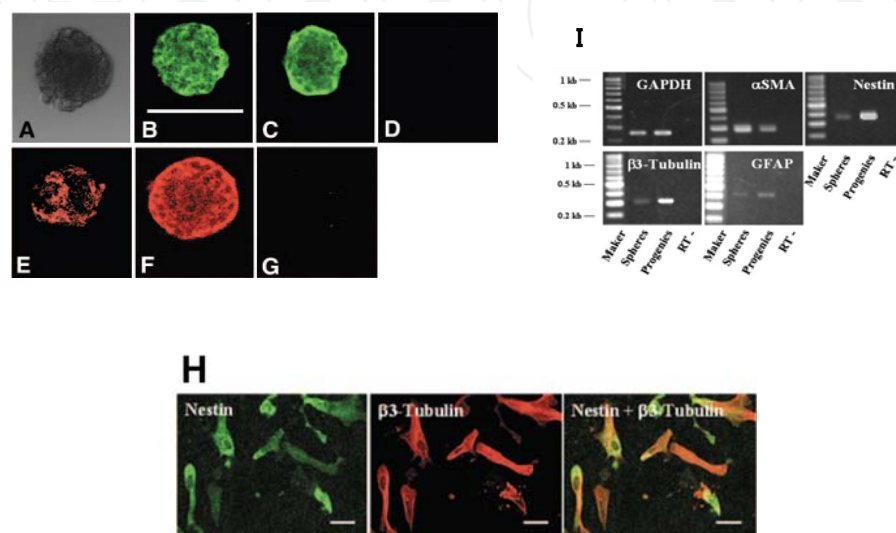
BSA/PBST: mouse anti-vimentin monoclonal antibody (mAb) (1:300; Dako, Glostrup, Denmark), mouse anti-nestin mAb (1:200; BD PharMingen, San Diego, CA), rabbit anti-p75 neurotrophin receptor (p75 NTR) polyclonal antibody (pAb) (1:200; Promega Corp., Tokyo, Japan), mouse anti-neurofilament 145 mAb (NFM, 1:400; Chemicon, Temecula, CA), rabbit anti- $\beta$ -tubulin pAb (1:2000; Covance Research Products, Denver, PA), rabbit anti-glial fibrillary acidic protein (GFAP) pAb (1:400; Dako), mouse anti-O4 mAb (1:10; Chemicon), rabbit anti-peripherin pAb (1:100; Chemicon), and mouse anti- $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) mAb (1:200; Sigma-Aldrich). As a control, mouse IgG (1:1000; Sigma-Aldrich) or normal rabbit serum (1:1000; Dako) was used instead of the primary antibody. After the spheres were washed in PBS, incubation was done for 1 hour at RT with the appropriate secondary antibody diluted in BSA/PBST. The secondary antibodies were fluorescent-labeled goat anti-mouse IgG (Alexa Fluor 488, 1:200; Molecular Probes) and fluorescent-labeled goat anti-rabbit IgG (Alexa Fluor 594, 1:400; Molecular Probes). Nuclei were counterstained with Hoechst 33342 (1:2000; Molecular Probes). After another wash in PBS, the spheres were examined under a laser scanning confocal microscope (Fluoview; Olympus, Tokyo, Japan). When anti-O4 or anti-p75NTR mAb was used, the permeabilization step was omitted.

Figure 2A shows a bright-field image of a typical sphere colony. Spheres derived from HCECs were not stained by nonimmune mouse IgG (Fig. 2D) or normal rabbit serum (Fig. 2G). Nestin has been used as a marker for the detection of immature neural progenitor cells in multipotential sphere colonies derived from the brain (Gage, 2000), skin (Toma et al., 2001), inner ear (Li et al., 2003), retina (Trobepe et al., 2000), corneal epithelium (Mimura et al., 2010a; Yokoo et al., 2008), corneal stroma (Amano et al., 2006; Mimura 2008a, 2008b; Uchida et al., 2005; Yamagami et al., 2007), and CE (Amano et al., 2006; Mimura 2005a, 2005b, 2005c, 2007, 2010b; Yokoo et al., 2005, Yamagami, 2006, 2007). Expression of  $\alpha$ -SMA (a marker of mesenchymal myofibroblasts) and expression of p75 NTR (a marker of neural crest stem cells) was also investigated by immunocytochemistry because HCECs are derived from the neural crest. Cells in the spheres showed immunoreactivity for nestin (Fig. 2B) and for  $\alpha$ -SMA (Fig. 2C), but not for p75 NTR (data not shown). Next, the spheres were immunostained for various neural markers. As a result, spheres were found to be positive for an immature neuronal marker ( $\beta$ -tubulin, Fig. 2E) and an astroglial marker (GFAP, Fig. 2F), but not a mature neuronal marker (NFM), an oligodendroglial marker (O4), or a peripheral nerve neuronal marker (peripherin; data not shown). These findings indicated that spheres isolated from human donor CE contain bipotential precursors that are capable of undergoing differentiation into mesenchymal cells and neuronal cells.

### 3.4. Secondary sphere formation

To further evaluate the proliferative capacity of HCECs, cells from the primary spheres were passaged under the same conditions as those used for the initial sphere culture. On day 10, primary spheres were treated with 0.05% trypsin/0.02% EDTA and dissociated into single cells, which were added to 24-well culture plates at a density of 10 cells/ $\mu$ L in medium containing primary culture supernatant. These cells were then incubated for a further 10 days in basal medium.

Secondary spheres were generated from the dissociated primary spheres, but the yield of secondary sphere colonies was lower than after primary culture. Although self-renewal potential was indicated by the ability of cells from individual primary spheres to form secondary spheres, this potential was limited, as evidenced by the failure of sphere formation at the third passage. These results indicated that the precursor cells had a limited proliferative capacity. Photographs of representative secondary spheres are shown in Figure 1F.



**Figure 2.** Immunocytochemistry (A-H) and RT-PCR analysis (I) of sphere colonies and their progeny. (A) Bright-field image of a typical sphere colony. (B) Immunostaining of the entire sphere on day 10 identifies cells expressing nestin, a marker of immature cells. (C-G) Spheres show immunostaining for a mesenchymal myofibroblast marker ( $\alpha$ -SMA, C), an immature neuronal marker ( $\beta$ 3-tubulin, E), and an astroglial cell marker (GFAP, F), indicating that both mesenchymal and neuronal differentiation have occurred. Sphere colonies derived from HCECs are not stained by nonimmunized mouse IgG (D) or normal rabbit serum (G). Differentiated cells derived from primary spheres are double immunostained by nestin and  $\beta$ 3-tubulin, indicating that the colonies contain immature (undifferentiated) cells. (I) RT-PCR analysis of cells from spheres and their progeny. GAPDH gene expression is detected in the sphere colonies and their progeny (30 cycles), but not when reverse transcription is omitted. Nestin,  $\alpha$ -SMA,  $\beta$ 3-tubulin, and GFAP genes are detected in both spheres and their progeny, but not when total RNA is processed without reverse transcription (35 cycles). Scale bars=100 $\mu$  m (A-G) or 200 $\mu$  m (H). Figures are modified from Yokoo et al. (2005) with permission.

### 3.5. Differentiation of sphere colonies

Individual primary spheres (day 10) were transferred to 13 mm glass coverslips coated with 50  $\mu$ g/ml poly-L-lysine (PLL) and 10  $\mu$ g/ml fibronectin (BD Biosciences, Billerica, MA) in separate wells, as described previously (Reynolds & Weiss, 1992). To promote differentiation, 1% fetal bovine serum (FBS) was added to the basal medium, and culture was continued for another 7 days. Immunocytochemical examination of spheres and their progeny was performed after 7 days of adherent culture on glass coverslips.

To investigate whether sphere progeny possessed the characteristics of mesenchymal or neural cells, single spheres (day 10) were transferred onto PLL/laminin-coated glass coverslips in medium containing 1% or 15% FBS or onto bovine ECM-coated culture plates in medium containing 15% FBS. Spheres remained adherent to the PLL/laminin-coated glass coverslips,



but cells migrated out from the spheres grown on glass coverslips coated with bovine ECM alone. After 7 days, some of the cells that had migrated from the spheres showed double immunostaining for nestin and  $\beta$ 3-tubulin (Fig. 2H), as has been reported for human scalp tag-derived cells (Toma et al., 2001). However, there was no staining of cells migrating out of the spheres for  $\alpha$ -SMA, p75NTR, NFM, peripherin, GFAP, or O4.

RT-PCR was performed to examine the expression of genes governing the proteins detected by immunocytochemistry in the spheres and their progeny (Fig. 2I). GAPDH mRNA was detected in both spheres and progeny, but not in the control assay without the RT reaction. Expression of nestin,  $\beta$ 3-tubulin, GFAP, and  $\alpha$ -SMA mRNA was detected in the spheres and adherent progeny after 35 PCR cycles. However, mRNAs for NFM, p75NTR, and peripherin were not found under any cycling conditions. Nestin and  $\beta$ 3-tubulin mRNAs were also detected in HCECs from primary culture.

These findings indicated that spheres isolated from human CE contain bipotential precursors, yielding progeny that display the morphologic characteristics of HCECs. Taken together, these results suggest that precursors from the CE remain close to the tissue of origin and undergo differentiation into CECs. Because precursors should ideally differentiate efficiently to produce their tissue of origin, precursors obtained from the CE may be more appropriate for tissue regeneration or cell transplantation than those derived from the multipotential stem cells.

## 4. Isolation of precursors from cultured human Corneal Endothelial cells

### 4.1. Culture of human Corneal Endothelial cells

As mentioned in sections 3.1-3.4, we have isolated precursor cells from human donor corneas (Yamagami et al. 2007; Yokoo et al., 2005). However, the number of precursors that can be isolated from a cornea is insufficient for corneal endothelial regeneration, so establishment of a mass production method for precursor cells is needed before clinical application can be attempted. Accordingly, we isolated spheres from cultured HCECs and investigated whether the cells of these spheres had CE-like functions. We also tested the effect of injecting these spheres into the anterior chamber (instead of full-thickness corneal transplantation) in a rabbit model of bullous keratopathy, representing a state in which corneal endothelial defects exist.

Several groups have established HCEC culture techniques (Chen et al., 2001; Engelmann & Friedl 1989; Miyata et al., 2001; Yue et al. 1989). Various growth factors have been reported to influence the proliferation of cells cultured from human CE, including fibroblast growth factor (Chen et al., 2001; Engelmann 1988, 1989, 1995; Yue et al. 1989; Samples et al., 1991), epidermal growth factor (Chen et al., 2001; Samples et al., 1991; Schultz et al., 1992; Yue et al. 1989), nerve growth factor (Chen et al., 2001), and endothelial cell growth supplement (Blake et al., 1997; Yue et al. 1989). In addition, cell attachment and growth can be supported by seeding cells onto an artificial matrix, such as chondroitin sulfate or laminin (Engelmann et al., 1988), laminin-5 (Yamaguchi et al., 2011), extracellular matrix

secreted by bovine corneal endothelial cells (Blake et al., 1997; Miyata et al., 2001), or fibronectin/type I collagen coating mix (Joyce & Zhu, 2004).

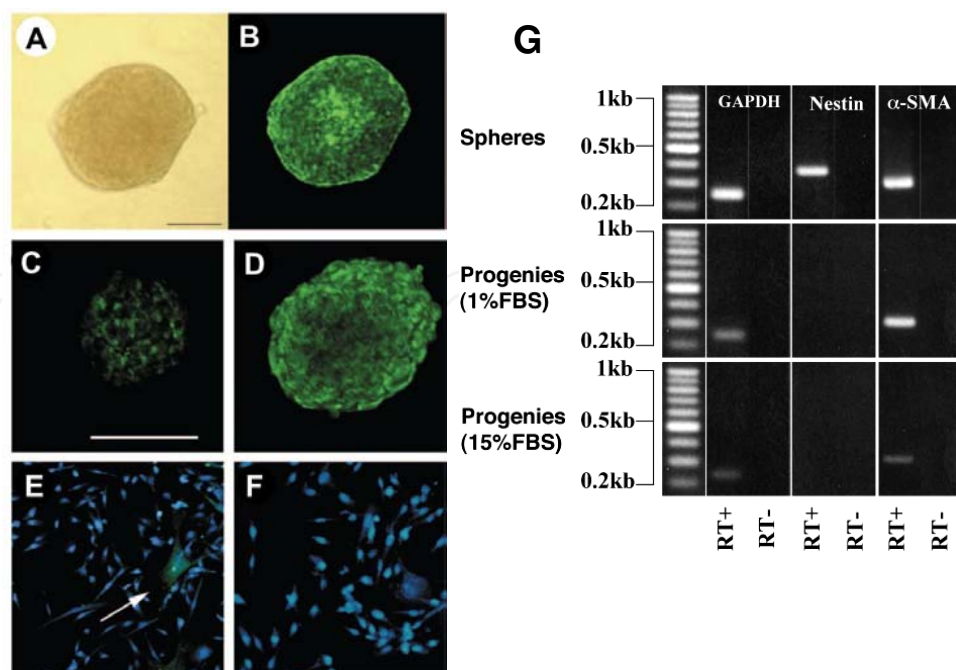
In our studies, HCECs were isolated and cultured according to the published protocols of Joyce and our laboratory with some modifications (Chen et al., 2001; Joyce & Zhu, 2004; Miyata et al., 2001). Briefly, Descemet's membrane was carefully dissected with the intact CE. After centrifugation, membrane strips were incubated in 0.02% EDTA solution at 37°C for 1 hour to loosen intercellular junctions. Then isolated cells were plated in 6-well tissue culture plates that had been precoated with undiluted fibronectin/type I collagen coating mix, and incubation was done at 37°C under a humidified atmosphere with 5% CO<sub>2</sub>. After primary cultures reached confluence, cells were subcultured at a 1:4 ratio, and cells from the 4th to 6th passages were used.

#### 4.2. Isolation and characterization of sphere colonies

Cells from the 4th or 5th passages were used in this study. HCECs were incubated in 0.2% EDTA at 37°C for 5 minutes and then were dissociated into single cells by pipetting with a flame-polished Pasteur pipette. The viability of the isolated HCECs was >90% as shown by trypan blue staining. The sphere-forming assay was used for primary culture (Reynolds & Weiss, 1992). Cells were plated at a density of 10 viable cells/μL (40,000 cells per well or 1,420 cells/cm<sup>2</sup>) in the uncoated wells of 60-mm culture dishes. The basal medium was Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with B27, epidermal growth factor (EGF, 20 ng/mL), and basic fibroblast growth factor (bFGF, 20 ng/mL). A methylcellulose gel matrix (1.5%; Wako) was added to the medium to prevent reaggregation of the cells (Gritti et al., 1999; Kawase et al., 2004). To distinguish growing spheres from dying cell clusters, only spheres with a diameter of more than 50 μm were counted. For passaging, primary spheres were harvested on day 7 and treated with 0.5% EDTA for dissociation into single cells, which were plated in 24-well culture plates at a density of 10 cells/μL. Then culture was continued for another 7 days in basal medium containing the methylcellulose gel matrix.

Spheres formed after 7 days of culture (Fig. 3A), while nonproliferating cells were eliminated. Many of the cells in each sphere were BrdU-positive (Fig. 3B), indicating that such cells were proliferating. These findings suggested that the spheres had developed from single HCECs and that the sphere-forming cells displayed proliferative activity. The number of sphere colonies obtained after 7 days of culture was  $44 \pm 10$  per 10,000 cells (mean  $\pm$  SD). Replating of primary spheres to generate secondary sphere colonies was less efficient, indicating that the cells only had limited self-renewal capacity.

On immunostaining, the spheres were positive for nestin (Fig. 3C), which is a marker of immature cells (Lendahl et al., 1990), and for  $\alpha$ -SMA (Fig. 3D), a mesenchymal myofibroblast marker. We previously demonstrated that primary spheres derived from human donor CE express  $\beta$ -III tubulin and GFAP, a mature glial cell marker, as well as nestin and  $\alpha$ -SMA (Fig. 2), but  $\beta$ -III tubulin and GFAP were negative in the spheres derived from cultured HCECs.



**Figure 3.** Immunocytochemistry (A-F) and RT-PCR analysis (G) of sphere colonies derived from cultured HCECs were their progeny. Cultured HCECs were disaggregated into single cells and plated at a density of 10 viable cells/ $\mu$ L in basal medium containing a methylcellulose gel matrix to prevent reaggregation. (A) A representative day 7 sphere. (B) Cells in a sphere colony labeled by BrdU on day 7. A total of  $44 \pm 10$  primary spheres were generated per 10,000 cells (mean  $\pm$  SD). Scale bar=50  $\mu$ m. (C-F) A day 7 sphere shows staining for nestin (C) and  $\alpha$ -SMA (D). Less than 5% of the sphere progeny cells were stained by the mesenchymal cell marker  $\alpha$ -SMA (E, arrow). There is no staining by control IgG (F). Scale bar=100  $\mu$ m. (G) RT-PCR of spheres and progeny. cDNA was obtained from spheres and from their progeny cultured in 1% FBS or 15% FBS. GAPDH was detected in all samples, except those reacted without reverse transcriptase. Nestin mRNA expression was detected in cultured spheres, but not in their progeny cultured in either 1% or 15% FBS. Both the spheres and progeny were positive for  $\alpha$ -SMA mRNA. Figures are modified from Mimura et al. (2005b) with permission.

#### 4.3. Differentiation of sphere colonies

Individual primary spheres (day 7) were transferred to 13-mm glass coverslips coated with 50  $\mu$ g/mL PLL and 10  $\mu$ g/mL fibronectin in separate wells (Mimura et al., 2005a). To promote differentiation, 1% or 15% FBS was added to the basal medium, after which culture was continued for another 7 days.

Then the spheres were transferred to PLL/fibronectin-coated glass coverslips in 24-well plates and were cultured in a differentiation medium containing 1% or 15% fetal bovine serum (FBS). After 7 days, many cells were found to have migrated out of the spheres. Fewer than 5% of these cells were  $\alpha$ -SMA-positive (Fig. 3E), whether cultured with 1% or 15% FBS. All of these cells were negative for control IgG (Fig. 3F) and for the differentiated epithelial cell marker cytokeratin 3, as well as for nestin,  $\beta$ -III tubulin, and GFAP (not shown). These findings indicated that a single sphere colony could give rise to a small population of mesenchymal cells under clonogenic conditions. Expression of nestin and  $\alpha$ -SMA by the spheres, as well as  $\alpha$ -SMA expression by their progeny, was confirmed using RT-PCR (Fig. 3G). Positivity for  $\beta$ -III tubulin mRNA was only detected in cultures with 1% FBS.

Spheres derived from donor CE expressed an immature cell marker (nestin), an immature neuronal marker ( $\beta$ -III tubulin), and a mature glial cell marker (GFAP), while their progeny expressed  $\beta$ -III tubulin and nestin, but not GFAP. In contrast, the spheres and progeny obtained from cultured HCECs did not express neuronal markers and showed decreased expression of immature cell markers. These findings suggested that the precursors were close in nature to the original tissue and underwent differentiation during culture. Thus, precursors obtained from cultured HCECs may be a more appropriate cell source than cells from donor CE, because precursors that efficiently differentiate into the tissue of origin are ideal for tissue regeneration or cell transplantation.

#### 4.4. Assessing the pump function of cells derived from spheres

The pump function of four collagen sheets seeded with cells derived from HCEC spheres was measured in an Ussing chamber, as reported previously with some modifications (Wigham, 1981, 2000; Hodson & Wigham 1983). The collagen sheets were obtained from the Nippi Biomatrix Research Institute (Tokyo, Japan). Cells from HCEC spheres were suspended at  $5.0 \times 10^6$  cells in 1.5 mL of culture medium and transferred to circular collagen sheets (10 mm in diameter). Each sheet was placed in one well of a 24-well plate, and the plate was centrifuged at 1,000 rpm (176 g) for 10 minutes to enhance cell attachment. Then the sheets were incubated in culture medium for 2 days, after which nonadherent cells and debris were removed (Fig. 4A). Human donor corneas with the epithelium removed mechanically (n=4), plain collagen sheets (n=4), or HCEC-coated collagen sheets (n=4) were mounted in the Ussing chamber.

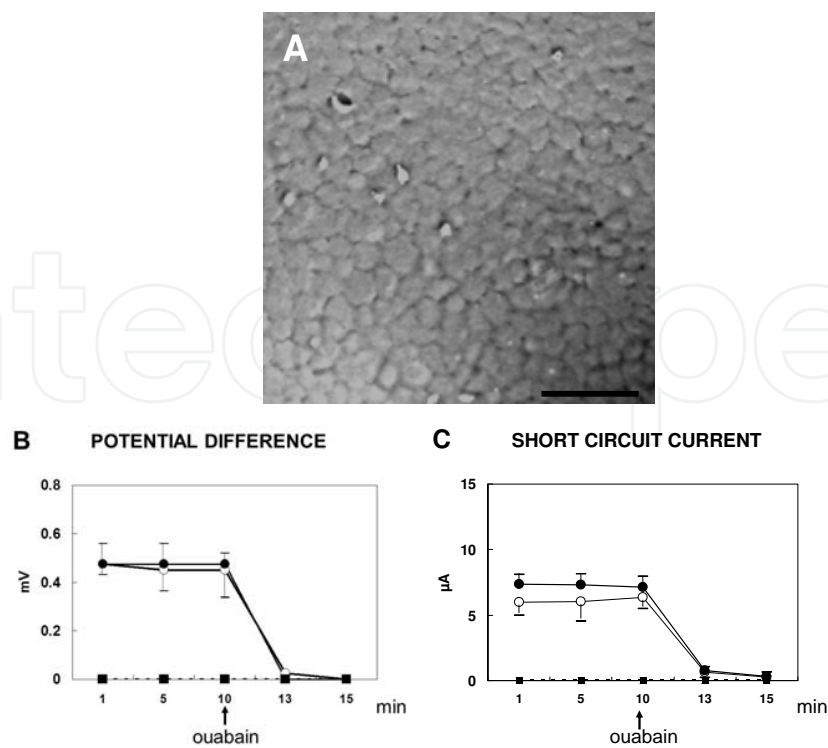
Changes of the potential difference (Fig. 4B) and short circuit current (Fig. 4C) were compared between human donor corneas without epithelium and HCEC-coated collagen sheets constructed with cells from spheres. The average potential difference and short circuit current of the HCEC-coated sheets ranged from 81% to 100% at 1, 5, and 10 minutes, corresponding to the results for normal human donor corneas denuded of epithelium. These findings suggested that the cultured HCEC spheres could generate CE-like cells with adequate transport activity.

#### 4.5. Migration and proliferation of spheres on rabbit descemet's membrane

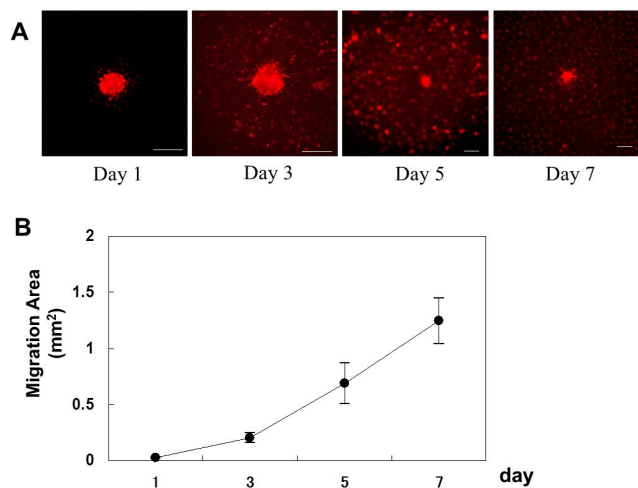
Animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Primary HCEC spheres (cultured for 7 days) were labeled with a fluorescent cell tracker (CM-DiI). After the endothelium was gently scraped off four freshly excised rabbit corneas with a sterile cotton swab, HCEC spheres were applied to the posterior surface of each cornea. Then the corneas were placed in 24-well plates and maintained in culture medium for 7 days. HCECs that migrated onto the corneas were detected under a fluorescence microscope, and the area occupied by fluorescent cells migrating from the spheres was measured with the NIH image program (n=10).

Figure 5A shows cells migrating from DiI-labeled spheres on days 1-7. The mean area covered by migrating cells per sphere reached  $1.2 \pm 0.2 \text{ mm}^2$  on day 7 (Fig. 5B).





**Figure 4.** Morphology (A) and transport activity (B, C) of cells from cultured HCEC spheres, modified from Mimura et al. (2005b) with permission. (A) Confluent cells cultured in DMEM containing 10% FBS show the characteristic hexagonal shape of corneal endothelial cells. Changes of the potential difference (B) and short circuit current (C) for human donor corneas without epithelium and HCEC-coated collagen sheets (mean  $\pm$  SD). The mean potential difference and short circuit current after 1, 5, and 10 minutes ranged from 81% to 100% for the HCEC-coated sheets, similar to the results for human corneas denuded of epithelium, indicating that the HCEC-like cells generated in culture had adequate transport activity. When  $\text{Na}^+ - \text{K}^+$  ATPase inhibitor ouabain was added to the chamber, the potential difference decreased to 0 mV and the short circuit current declined to 0  $\mu$ A in all cases.



**Figure 5.** Migration of sphere-derived cells during culture for 7 days, modified from Mimura et al. (2005b) with permission. DiI-labeled spheres were seeded onto the denuded Descemet's membranes of rabbit corneas and cultured for 1 week in a humidified incubator. (A) Representative photographs of cell migration around an adherent DiI-labeled sphere. Scale bar=100  $\mu$ m. (B) Mean area occupied by cells migrating from the spheres on each day (n=10).



## 5. Treatment of bullous keratopathy with precursors derived from cultured spheres

### 5.1. Cryoinjury and injection of spheres into the anterior chamber

Animals were handled in accordance with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research. New Zealand White rabbits (weighing 2.0–2.4 kg,  $n=24$ ) were anesthetized with an intramuscular injection of ketamine hydrochloride (60 mg/kg; Sankyo, Tokyo, Japan) and xylazine (10 mg/kg; Bayer, Leverkusen, Germany). To detach the CE from Descemet's membrane, a brass dowel cooled in liquid nitrogen was touched onto the cornea nine times (at the center and at eight peripheral sites). This procedure was repeated twice. Then the anterior chamber was washed three times with PBS through a 1.5-mm paracentesis.

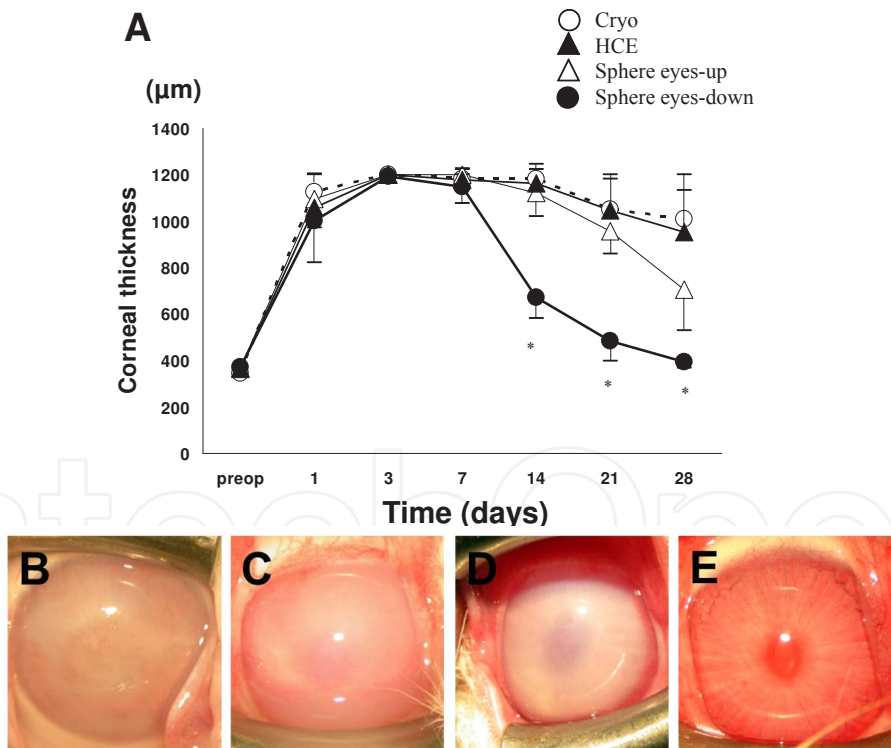
To estimate the number of spheres needed to cover the inner surface of the cornea (Descemet's membrane), DiI-labeled spheres were seeded onto the denuded Descemet's membrane of freshly excised rabbit corneas and the mean area covered per sphere was found to be  $1.2 \pm 0.2$  mm<sup>2</sup> on day 7 (Fig. 5B). Therefore, it was calculated that 75 spheres were needed to cover a cornea. To allow for loss of spheres that failed to adhere, 150 DiI-labeled HCEC spheres or  $1.0 \times 10^7$  HCECs were injected into the anterior chamber of the right eye after cryoinjury. Then the rabbits were maintained in the eyes-down position (Descemet's membrane down) for 24 hours to allow attachment (sphere eyes-down group,  $n=6$ ). Cryoinjury alone (cryo group,  $n=6$ ), injection of cultured HCECs with the eyes-down position being maintained for 24 hours (HCEC group,  $n=6$ ), and injection of spheres in the eyes-up position (sphere eyes-up group,  $n=6$ ) were also tested as controls. However, injection of cultured HCECs or injecting spheres in the eyes-up position did not reduce corneal edema in our preliminary study (Mimura T, unpublished observation, 2003), so these controls were not used in the present study. Each eye was inspected 2 or 3 times a week and was photographed on postoperative days 7, 14, 21, and 28. Central corneal thickness was measured with an ultrasonic pachymeter having a range of 0 to 1,200  $\mu$ m (Tomey, Nagoya, Japan) and intraocular pressure was determined with a pneumatic tonometer (model 30 Classic; Mentor O & O, Norwell, MA) at 1, 3, 7, 14, 21, and 28 days after surgery. The average of three readings was obtained each time. One-way analysis of variance and Scheffe's multiple comparison test were used to compare mean values.

### 5.2. Findings after surgery

Our previous studies had suggested that cultured HCEC precursors have a limited self-renewal capacity and mainly differentiate into HCEC-like cells. Then we investigated the use of precursors derived from cultured HCECs in a rabbit model of corneal endothelial damage. In the cryo and HCEC groups, the mean corneal thickness ranged from  $953 \pm 182$  to  $1,200 \pm 0$   $\mu$ m (mean  $\pm$  SD), as shown in Figure 6A. The mean ( $\pm$ SD) corneal thickness of the sphere eyes-up group ( $704 \pm 174$   $\mu$ m) was significantly less than that of the cryo group ( $1,011 \pm 190$   $\mu$ m;  $P=0.006$ ) and the HCEC group ( $953 \pm 182$   $\mu$ m;  $P=0.022$ ) after 28 days of observation, but the corneas were still edematous in the eyes-up group (Fig. 6A). In contrast, the corneal thickness decreased rapidly in the sphere eyes-down group, and the corneas were significantly thinner

than in the other three groups after 14 ( $672 \pm 90 \mu\text{m}$ ), 21 ( $483 \pm 84 \mu\text{m}$ ), and 28 ( $394 \pm 26 \mu\text{m}$ ) days ( $P=0.006$ ; Fig. 6A). Representative anterior segment photographs from the cryo group (Fig. 6B), HCEC group (Fig. 6C), and sphere eyes-up group (Fig. 6D) show that the corneas of rabbits from these groups were edematous and displayed stromal opacity. In contrast, corneas from the sphere eyes-down group corneas became clear and the anterior chamber was easily visualized (Fig. 6E). No apparent inflammatory reactions suggesting rejection were observed by slit lamp microscopy throughout the postoperative period. On day 14, the intraocular pressure of the sphere eyes-up group was significantly higher than that of the cryo group ( $P=0.013$ ). However, there was no increase of intraocular pressure (a possible side effect) on any other day in any group (Table 1).

Injection of spheres in the eyes-down position, but not injection of differentiated cultured HCECs or injection of spheres in the eyes-up position, restored endothelial function and decreased corneal edema in this rabbit model of bullous keratopathy model. These findings suggest that injection of spheres derived from cultured HCECs and maintenance of an eyes-down position for 24 hours may be a potential treatment strategy for corneal endothelial defects that is less invasive compared with conventional full-thickness corneal transplantation.



**Figure 6.** Changes of corneal thickness and other findings in a rabbit model of bullous keratopathy, modified from Mimura et al. (2005b) with permission. (A) Mean corneal thickness decreases gradually in the sphere eyes-down group (closed circles,  $n=6$ ). It is significantly less than in the cryo group (open circles,  $n=6$ ), HCEC group (closed triangles,  $n=6$ ), and sphere eyes-up group (open triangles,  $n=6$ ) on days 14, 21, and 28 ( $*P<0.001$  by one-way analysis of variance and Scheffe's multiple comparison test). (B–E) Representative photographs of corneas from each group. The cornea is opaque in the cryo group (B), HCEC group (C), and sphere eyes-up group (D), and the anterior chamber is not well visualized. In contrast, there is no corneal opacity in the sphere eyes-down group (E).

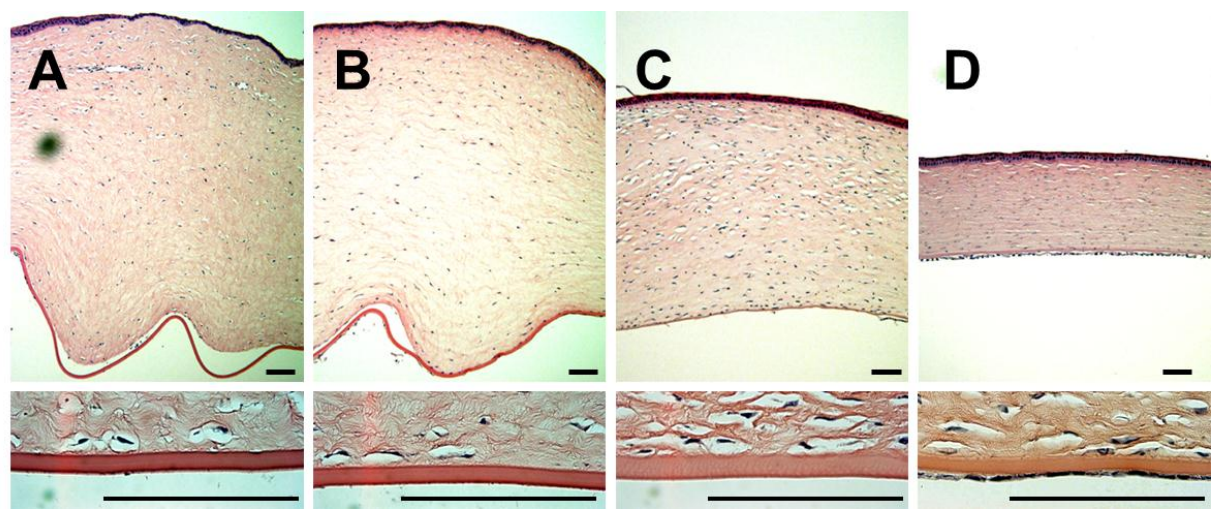
Group	Days after Surgery				
	0	7	14	21	28
Cryo	12.7 ± 1.9	10.8 ± 1.8	10.5 ± 4.3*	11.0 ± 1.6	10.2 ± 1.5
HCEC	11.8 ± 1.5	11.6 ± 2.8	12.3 ± 4.5	11.8 ± 2.2	11.5 ± 2.8
Sphere (eyes-up)	13.2 ± 2.0	9.9 ± 1.9	17.2 ± 4.0*	13.3 ± 3.4	10.5 ± 3.1
Sphere (eyes-down)	13.5 ± 3.2	8.7 ± 2.1	14.8 ± 2.2	13.7 ± 1.7	11.7 ± 1.9

**Table 1.** Intraocular pressure in each group after surgery (mm Hg). Data represent the mean ± SD for six rabbits.  
 \* $P=0.013$  by one-way analysis of variance and Scheffe's multiple comparison test.

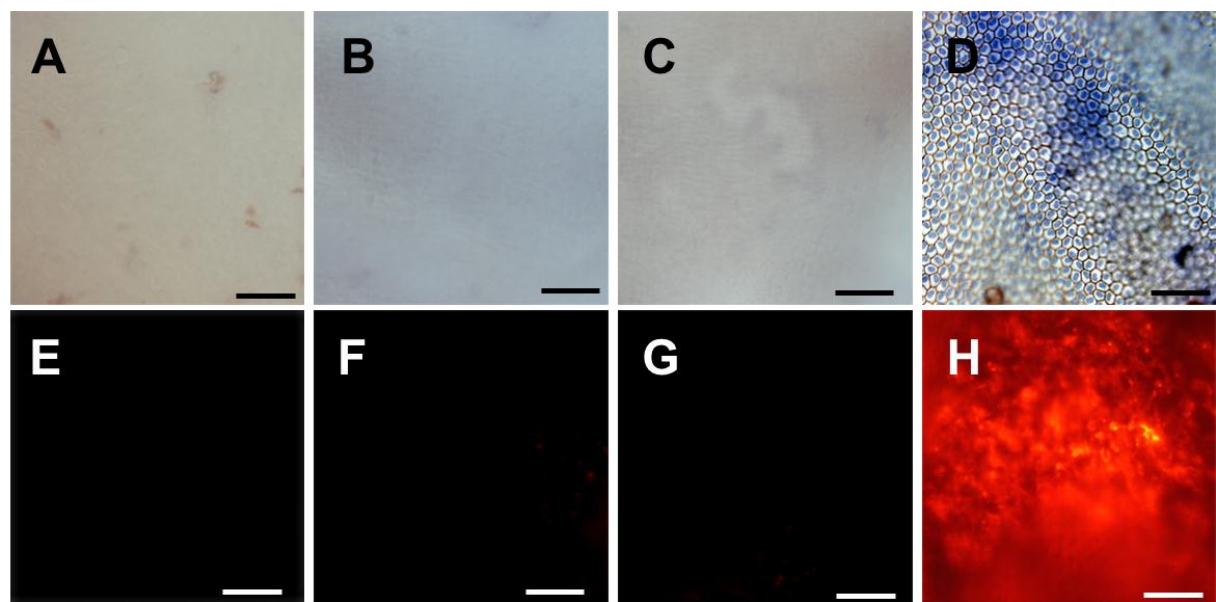
### 5.3. Histologic findings

Examination of hematoxylin & eosin-stained sections revealed that corneas from the cryo group (Fig. 7A), HCEC group (Fig. 7B), and sphere eyes-up group (Fig. 7C) were thickened and no cells could be detected on Descemet's membrane. In contrast, a monolayer of cells had formed on Descemet's membrane in the sphere eyes-down group, and there was no edema and no mononuclear cell infiltration of the posterior stroma (Fig. 7D). In the cryo group (Figs. 8A, 8E), HCEC group (Figs. 8B, 8F), and sphere eyes-up group (Figs. 8C, 8G), no HCECs (Figs. 8A–C) with positive staining for DiI (Figs. 8E–G) were found on Descemet's membrane at the central cornea in flat mount preparations. In contrast, HCEC-like hexagonal cells were detected at this site in the sphere eyes-down group (Fig. 8D). These cells were DiI-positive (Fig. 8H), indicating that they had originated from the injected spheres and not from the host. In the sphere eyes-down group, DiI-negative cells were present in the peripheral cornea, but all cells in the central and paracentral (8 mm in diameter) cornea were DiI-positive. The density of HCECs in the six grafts of the sphere eyes-down group at 28 days after surgery ranged from 2,625 to 2,875 cells/mm<sup>2</sup>, with a mean (±SD) value of 2,781 ± 92 cells/mm<sup>2</sup>. Before surgery, the density of endothelial cells in the rabbit cornea was from 3,300 to 3,500 cells/mm<sup>2</sup>. In the sphere eyes-down group, very few DiI-positive cells were detected in the inferior trabecular meshwork or on the iris, whereas a number of DiI-positive cells were attached at these sites in the HCEC group and the sphere eyes-up groups (data not shown).

Cells adherent to the inner surface of the cornea (Descemet's membrane) were DiI-positive in the sphere eyes-down group, indicating that these were HCECs derived from the injected spheres and not residual host cells. In addition, DiI-positive cells were rarely detected in the trabecular meshwork or on the surface of the iris, so the spheres mainly attached to and spread over the cornea in the eyes-down group. These results suggested that sphere-derived HCECs could restore corneal hydration after sphere transplantation.



**Figure 7.** Histologic findings, modified from Mimura et al. (2005b) with permission. In the cryo group (A), HCEC group (B), and sphere eyes-up group (C), stromal edema is prominent and no cells are present on Descemet's membrane at the central cornea. In contrast, a monolayer of cells can be detected on Descemet's membrane in the sphere eyes-down group (D). There is no mononuclear cell infiltration near Descemet's membrane, suggesting no rejection of the xenogeneic cells (D). Scale bar=100  $\mu$ m, hematoxylin & eosin stain.



**Figure 8.** Flat mount preparations with phase-contrast (A–D) and fluorescence (E–H) microscopy, modified from Mimura et al. (2005b) with permission. At the central cornea, there are no cells on Descemet's membrane in the cryo group (A, E), HCEC group (B, F), and sphere eyes-up group (C, G). In contrast, HCEC-like hexagonal cells are present in the sphere eyes-down group (D). These cells are also Dil-positive (H). Scale bar=100  $\mu$ m.

### 5.4. Advantages of transplanting CE precursors

For regenerative medicine, amplification of stem cells is required to treat each tissue or organ. Although much attention has been paid to maintaining the undifferentiated nature



("stemness") of stem cells and promoting their amplification, the molecular mechanisms of stem cell replication and differentiation are still not fully understood. In comparison with amplification of adult stem cells, cultured cells can be used more easily to produce tissue-committed precursors by the sphere-forming assay, as demonstrated in our studies. Similar techniques to produce abundant precursors should be tested for various tissues as a method of obtaining cells for regenerative medicine.

Transplantation of HCEC precursors into the anterior chamber has several advantages over penetrating keratoplasty with a full-thickness donor cornea. For example, complications associated with open-sky surgery (expulsive hemorrhage and the risk of wound dehiscence) are essentially eliminated. In addition, several postoperative complications, such as irregular astigmatism, wound leakage, corneal infection, neovascularization, and persistent epithelial defects, can be avoided when using the combined approach. After conventional full-thickness human corneal allografting with local and/or systemic immunosuppressants, the leading cause of failure is graft rejection (Price et al., 1991; Wilson & Kaufman, 1990). Although there was no apparent inflammatory reaction histologically, we cannot deny the possibility of allograft rejection over the long term because nonadherent cells should migrate out of the anterior chamber. It is noteworthy that injection of HCEC precursors did not improve bullous keratopathy created by scraping off endothelial cells in rabbits (data not shown). This may be because cryoinjury to the cornea, but not endothelial cell scraping, promoted the proliferation and migration of HCEC s that led to recovery of corneal clarity.

## 6. Conclusion

We demonstrated that the endothelium from the peripheral region of the human cornea contains a higher density of precursors with strong proliferative capacity compared to the central endothelium. These HCEC precursors are able to differentiate into both mesenchymal and neural cells. We have also established a method for mass production by isolation of precursors from cultured HCECs using the sphere-forming assay. Transplantation of spheres into the anterior chamber and short-term maintenance of the eyes-down position was shown to be a simple and effective treatment strategy in our rabbit model of bullous keratopathy. This method of managing corneal endothelial defects may have the potential to replace conventional full-thickness corneal grafting and compensate for the worldwide shortage of donor corneas.

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