A Hierarchy of Endothelial Colony-Forming Cell Activity Displayed by Bovine Corneal Endothelial Cells

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PURPOSE. To test the hypothesis that the robust expansion of bovine corneal endothelial cells (BCECs) in vitro is due to the presence of individual endothelial cells with various levels of proliferative potential.

METHODS. BCECs and bovine vascular endothelial cells (ECs) derived from aorta, coronary artery, and pulmonary artery were cultivated in optimized medium. These cell populations were confirmed by morphologic features, functional assays, and gene expression profiles. Moreover, ECs were plated in a single-cell clonogenic assay to evaluate colony-forming ability.

RESULTS. Both corneal and vascular ECs were confirmed to be pure populations of endothelium uncontaminated with hematopoietic cells. A complete hierarchy of endothelial colony-forming cells (ECFCs) was identified in BCECs by a single-cell clonogenic assay. The distribution of the various types of ECFCs was similar to the control ECs removed from the systemic vessels.

CONCLUSIONS. Cultured BCECs display clonal proliferative properties similar to those of vascular ECs. (*Invest Ophthalmol Vis Sci.* 2010;51:3943–3949) DOI:10.1167/iov.09-4970

Corneal endothelium is formed by migration and proliferation of neural crest-derived precursor cells in the posterior aspect of the cornea where the endothelial cells play a barrier-bump function to maintain corneal clarity. Corneal endothelial cells (CECs) fail to proliferate in vivo in response to injury, disease, or aging and are arrested in the G₁ phase of the cell cycle.¹ However, CECs possess replicative potential that can be revealed on in vitro endothelial cell (EC) culture and/or application of a stress such as mechanical wounding or ethylene diamine tetraacetic acid (EDTA) treatment to disrupt cell-cell interactions within the endothelial monolayer on the ex vivo cultured cornea.² With the use of in vitro culture approaches, recent studies have demonstrated that the proliferation of CECs varies with the age of the donor (cells derived

from younger donors divide more than those from older donors) and varies from the central (low proliferative potential) to the peripheral (high proliferative potential) cornea.^{2,3} These obvious differences in the temporal and spatial distribution of proliferative potential within the mammalian cornea raise an interesting question of whether all corneal endothelial cells possess the same inherent replicative capacity or whether this property is heterogeneously distributed within cell subsets.

Vascular ECs form a monolayer that lines the interior surface of blood vessels and provides important barrier and anticoagulant properties. Vascular ECs are derived from mesodermal precursor cells and play important roles in maintaining tissue-specific vascular homeostasis throughout human development and aging. We have discovered novel methods of examining clonal proliferative behavior of circulating and vascular ECs and have identified a hierarchy of endothelial colony-forming cell (ECFC) activity, ranging from high proliferative potential ECFCs (HPP-ECFCs) to nondividing mature ECs.^{4,5} Human circulating ECFCs display phenotypical and functional properties that are also present in ECs derived from human blood vessels.⁵ Differences in the distribution of ECFCs within the microvasculature and macrovasculature ECs have been identified in some organs,⁶ and there are reported differences in the clonal proliferative properties of ECFCs isolated from umbilical cord blood and adult peripheral blood.^{4,7} Nonetheless, circulating and resident ECFCs form human blood vessels de novo when subcutaneously implanted into immunodeficient mice, and these vessels participate in carrying blood as a part of the host murine systemic circulation.^{7–9} Therefore, these studies suggest that ECFCs represent stem/progenitor cells for the endothelial lineage, at least in the systemic circulation.

CECs differ from vascular ECs with respect to developmental origin, anatomic localization, and physical function.^{1,10,11} Whereas systemic vascular endothelium slowly proliferates throughout life,^{12–14} CECs fail to proliferate in situ and merely expand in size to accommodate areas of CEC loss due to injury or senescence.¹ This feature is well recognized in the corneas of elderly subjects in whom the CEC density may be significantly lower than that in the newborn infant cornea. Although many papers have been published over the past 30 years to explain some of the cellular and molecular mechanisms that regulate proliferative potential in CECs,¹⁵ to our knowledge, no one has used clonal analytical techniques to examine individual CEC behavior. In this report, we examined the clonal proliferative properties of the CECs compared with the behavior displayed by ECs derived from several systemic vessels (known to possess clonal proliferative properties). We report that bovine (B)CECs display a complete hierarchy of ECFC behavior that is similar to the distribution of ECFC activity

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present in ECs isolated from bovine aorta, coronary artery, and pulmonary artery. HPP-ECFCs in corneal endothelium can be replated into at least secondary colonies and retain high levels of telomerase activity similar to HPP-ECFCs derived from resident endothelium in blood vessels. These novel data provide new insights into the complexity of the regulation of CEC proliferative potential, suggesting that in the adult bovine cornea, a subset of CECs with robust proliferative potential resides among numerous other CECs with limited proliferative potential.

MATERIALS AND METHODS

Isolation of Bovine Peripheral Blood Mononuclear Cells

Blood (50–100 mL) was collected from a local abattoir, diluted, layered onto cell separation gradient (Histopaque 1119; Sigma-Aldrich, St. Louis, MO), and centrifuged for 30 minutes at 1800 rpm at room temperature (Beckman Coulter, Fullerton, CA). Low-density bovine peripheral blood mononuclear cells (MNCs) were collected and washed three times with Dulbecco's phosphate-buffered saline (DPBS; Invitrogen, Grand Island, NY). Subsequently, the MNCs were resuspended in DPBS with 2% fetal bovine serum (FBS; Hyclone, Logan, UT) for direct analysis by fluorescence-activated cell sorting (FACS; BD Biosciences, San Diego, CA).

Culture of Bovine Vessel Wall-Derived ECs

Bovine aortic endothelial cells (BAECs), bovine coronary arterial endothelial cells (BCAECs), and bovine pulmonary arterial endothelial cells (BPAECs) were purchased from Lonza (Walkersville, MD). They were plated onto type I rat tail collagen (50 μ g/mL; BD Biosciences, Bedford MA)-precoated tissue culture flasks and cultured in endothelial cell growth medium EGM-MV (Lonza) supplemented with 1.5% antibiotic-antimycotic (Invitrogen) at 37°C in 5% CO₂ in a humidified incubator.

Isolation and Culture of BCECs

Bovine eyes were obtained from a local abattoir several hours after death. The isolation of BCECs was performed as previously described.¹⁶ BCECs from an 18- and a 24-month-old mixed-breed steer were isolated and identified as BCEC18 and BCEC24, respectively. These CECs were plated in tissue culture flasks filled with DMEM supplemented with 5% FBS and 1.5% antibiotic-antimycotic. BCECs were grown to form a confluent monolayer with a typical hexagonal appearance. They were then released from the culture dish (TryPLE; Invitrogen) and replated in 75-cm² tissue culture flasks for further passage.

Immunophenotyping of ECs

Early-passage (2–4) bovine vascular ECs and BCECs, as well as bovine blood MNCs, were stained with a mouse monoclonal antibody against bovine CD45 conjugated to fluorescein isothiocyanate (FITC; Serotec, Oxford, UK), FITC-conjugated *Lycopersicon esculentum* (tomato) lectin (LEL), and *Griffonia simplicifolia* I lectin (GSL I; Vector Laboratories, Burlingame, CA) or mouse isotype control antibody for 30 minutes at 4°C, washed three times, and analyzed by flow cytometry (FACS Calibur; BD Biosciences) for cell surface expression, as previously described.¹⁷

Ingestion of 488-Conjugated Acetylated Low-Density Lipoprotein (488-AcLDL)

Early-passage (2–4) bovine vascular ECs and BCECs were incubated with 10 μ g/mL of 488-AcLDL (Invitrogen) in the medium for 8 hours at 37°C. The cells were washed three times, costained with 1.5 μ g/mL of the nuclear stain, 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma), and examined by inspection through an inverted fluorescence microscope (Carl Zeiss Meditec, Inc., Thornwood, NY) at 40 $\times$ magnification.

Tube Formation Assay

Early-passage (2–4) bovine vascular ECs and BCECs were seeded onto 96-well tissue culture plates precoated with 30 μ L synthetic basement matrix (Matrigel; BD Biosciences) at a cell density of 10,000 to 30,000 cells per well. The cells were observed every 2 hours with an inverted microscope for the formation of capillary-like structures.

Reverse Transcription-Polymerase Chain Reaction

Total cellular RNA was extracted (TRIzol; Invitrogen) in a single-step method, as described by the manufacturer. RT reactions were performed with polymerase (SuperScript First-Strand DNA Synthesis; Invitrogen). PCR was conducted according to the manufacturer's instructions (Go Tap Flexi DNA Polymerase; Promega, Madison, WI). The primer sequences used are shown in Table 1. The PCR cycle was 94°C, 5 minutes; 94°C 30 seconds; 53°C, 57°C, or 59°C (depending on the primer), 30 seconds; 72°C, 45 seconds; and 32 cycles with a final 72°C 7-minute cycle. PCR products were added to wells in a 2% agarose/ethidium bromide gel, exposed to electrophoresis current, and migrating bands were photographed under UV light.

Single-Cell Clonogenic Assay

Early-passage (2–4) bovine vascular ECs and BCECs were sorted with a (FacsVantage Sorter; BD Biosciences) and deposited, one cell per well, into 96-well plates precoated with type I rat tail collagen in 200 μ L of medium. The cells were cultured at 37°C in 5% CO₂ in a humidified

TABLE 1. Primer Sequences used in Study

Gene	Forward	Reverse	T _m (°C)	Product Size (bp)
<i>CD36</i>	AACCACTTTTCATCAGACCCG	ACGTGTCATCCTCAGTTCCA	55	475
<i>Enolase 1</i>	GCAGGAGAAGATCGACAAGC	GTAACCTCTGCTCCGATGC	57	310
<i>Enolase 2</i>	GGACTTGGATGGGACTGAGA	GCGTCCTTGCCATACTTGTC	57	327
<i>eNOS</i>	TGAGCAGCTGCTGAGCCAGG	CAGCTCGCTCTCTCGGAGGT	57	209
<i>Factor VIII</i>	ACTGCCTCATCCCACTTACG	GGGGTCTAGAGCATTCACCA	57	327
<i>GAPDH</i>	GGTGAAGTCCGAGTGAACG	GGGTCAATGATGGGACGA	59	117
<i>LDLR</i>	ACAACCCCGTGTACCAGAAG	AGGGTCAGGGGAGAAAGTGT	57	195
<i>N-Cadherin</i>	AGCAACTGCAATGGGAAAAG	GATGGGAGGGATAACCCAGT	55	461
<i>PECAM 1</i>	ATGTGCTGCTTCACAACGTC	TGAATTCAGCGTCACAAAA	53	345
<i>S100B</i>	GGTGACAAGCACAAAGCTGAA	CAGTGGTAATCATGGCAACG	55	184
<i>VE-Cadherin</i>	GAGTGTGGACCCCAAGAAGA	GCTGGTACACGACAGAAGCA	57	359
<i>Vimentin</i>	CTTCGCCAACTACATCGACA	GGATTCCACTTTACGCTCCA	55	340
<i>ZO 1</i>	GAATCCGATGTGGGTGATTC	GCAGGTTTCTCTTGGAGCTG	57	310

FIGURE 1. The morphology of cultured bovine vessel wall-derived ECs and BCECs. Bovine vascular ECs derived from aorta (BAECs), coronary artery (BCAECs), and pulmonary artery (BPAECs) displayed a spindle-shaped morphology and BCECs displayed a hexagonal morphology within the cell monolayer. Magnification, $\times 10$; scale bar, 100 μm .

incubator. The medium was changed every 5 days. After 14 days of culture, the cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) in DPBS for 30 minutes at room temperature, washed twice, stained with 1.5 $\mu\text{g}/\text{mL}$ DAPI, and examined for determination of the number of ECs. The wells containing two or more cells were identified as positive for proliferation under a fluorescence microscope at $10\times$ magnification. Culture wells containing fewer than 50 cells were counted by direct visual inspection with a fluorescence microscope at $10\times$ magnification. For the wells with more than 50 cells, colonies were imaged, and the number of cells was quantified with the Image J 1.36v program (NIH Image or ImageJ software developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>).

Sphere-Forming Assay

Sphere forming assays were performed as previous described with some modifications.^{18,19} Early-passage (1–2) BCECs were released from the culture dish (TryPLE; Invitrogen) and then suspended into single cells in the culture medium. The cells were seeded at a density of 50 cells/ μL on plastic dishes as hanging drops (10 μL each), to allow spheroid formation by cell aggregation. The cells then were cultured at 37°C in 5% CO_2 in a humidified incubator for 3 days. Under this condition, the suspended cells contributed to formation of a single spheroid per drop of a defined size (diameter, $>100 \mu\text{m}$).

Telomerase Assay

Telomerase activity was measured by the telomeric amplification protocol (TRAP) with a kit (TRAPeze; Chemicon, Temecula, CA). Cell lysate from 1000 cells was used in each assay. HeLa cell extract served

as a positive control, and lysis buffer alone served as a negative control. The PCR products were exposed to an electrophoretic current on a 12.5% nondenaturing polyacrylamide gel and visualized by SYBR gold staining (Molecular Probes, Eugene, OR).

Statistical Analysis

Results are expressed as the mean $\pm$ SEM for the study variables. Data were compared by ANOVA, and significant differences were set at $P < 0.05$ (InStat software; GraphPad Software Inc., La Jolla, CA).

RESULTS

Characterization of BCECs and Bovine Vascular ECs

Primary cultured BCEC18s and BCEC24s displayed a typical hexagonal morphology (similar to the endothelial morphology in the intact cornea by direct visualization) at confluence. In comparison, bovine ECs from aorta, coronary artery, and pulmonary artery exhibited more morphologic heterogeneity, with spindle-shaped and large oval cells (Fig. 1). These differences in BCEC and vascular EC morphology persisted from 3 to 20 passages.

None of the ECs expressed CD45, whereas the peripheral blood low-density MNCs (leukocytes) expressed high levels of CD45 (Fig. 2A) demonstrating absence of blood cell contamination of the endothelial samples. Both corneal and vascular ECs were able to bind LEL, whereas peripheral blood MNCs did not display such an ability (Fig. 2A). In addition, all cells

FIGURE 2. Phenotypic and functional characterization of bovine vessel wall-derived ECs and BCECs. (A) Immunophenotyping of bovine peripheral blood MNCs and the monolayers derived from BAECs, BCAECs, BPAECs, and BCECs, determined by fluorescence flow cytometry. BAECs, BCAECs, BPAECs, and BCECs bound to the lectins GSL I and LEL but did not express the common leukocyte antigen CD45. *Green*: negative control cells. (B) Incorporation of 488-AcLDL in bovine vessel wall-derived ECs and corneal ECs. BAECs, BCAECs, and BPAECs were able to ingest 488-AcLDL (*green*), but BCECs failed to bind the material. *Blue*: DAPI-stained nuclei. (C) LDLR and CD36 expression in bovine vascular and corneal ECs identified by RT-PCR. LDLR was expressed in all types of ECs, whereas CD36 was detectable only in bovine vascular ECs. (D) Formation of capillary-like structures when bovine vascular ECs and BCECs were plated in synthetic basement membrane. Three independent experiments showed similar results. (B, D) Magnification, $\times 40$; scale bar, 100 μm .

FIGURE 3. RT-PCR analysis of gene expression in bovine vessel wall-derived ECs and bovine corneal ECs. PECAM1, VE-cadherin, and eNOS were specifically expressed in bovine vascular ECs but not in BCECs. *S100B* transcripts were detectable in BCECs but not in vessel wall-derived ECs. Enolase2 was also detected in BCECs with variable expression level. Three independent experiments showed similar results.

examined were able to bind GSL I (Fig. 2A). Actually, BCECs and the various bovine vascular ECs had the same lectin binding pattern for all 30 lectins tested (Supplementary Fig. S1, <http://www.iovs.org/cgi/content/full/51/8/3943/DC1>). Thus, BCECs share some cell surface molecule expression with bovine vessel wall-derived ECs, but there was no evidence that any of the ECs expressed the most common hematopoietic antigen CD45.

Ingestion of AcLDL is a feature displayed by many ECs and, not surprisingly, all bovine vessel wall-derived ECs readily ingested the lipoprotein complex (Fig. 2B). In contrast, BCECs were unable to ingest AcLDL (Fig. 2B). The LDL receptor (LDLR) and scavenger receptors, such as CD36, mediated native and modified LDL-ingestion. We examined LDLR and CD36 expression in bovine vascular and corneal ECs, and LDLR transcripts were detectable in all types of ECs, whereas CD36 transcripts were present only in vascular ECs (Fig. 2C). Capillary-like tube formation is another feature displayed by essentially all vascular ECs when examined in vitro. BCECs, similar to all the bovine vascular ECs, readily formed capillary-like structures when plated on synthetic basement membrane (Fig. 2D). Thus, the BCEC and vascular EC populations displayed properties consistent with pure uncontaminated cultured endothelial cells.

Gene Expression of BCECs Compared with Bovine Vascular ECs

To further analyze the ECs isolated from the cornea and blood vessels, we isolated cellular RNA and examined the expression of certain mRNA transcripts that have been published as commonly expressed in vascular ECs (Fig. 3).^{17,20,21} BCECs did not express vascular endothelial-cadherin (VE-Cadherin), nitric oxide synthase 3 (NOS3, or eNOS), or platelet endothelial cell adhesion molecule 1 (PECAM1) which are expressed in bovine vascular ECs. Although as yet there has been no single specific genetic marker for ECs identified, we scanned the published literature^{1,22-24} and identified numerous genes, including *S100B*, enolase2, N-cadherin, vimentin, *ZO-1*, and factor VIII, that are reportedly expressed preferentially in ECs. *S100B* mRNA was present in BCECs and BPAECs, but absent in BAECs and BCAECs. Neuron-specific enolase 2 mRNA was present in BCEC18s and BCAECs and in three experiments, detectable in

BCEC24s but at a low level of detection. N-cadherin mRNA was present in all ECs except BAECs. Furthermore, vimentin, enolase1, *ZO1*, and factor VIII mRNAs were detectable in all ECs. Thus, BCECs displayed a gene expression profile that differed somewhat from bovine vessel wall-derived ECs; however, no single gene product was BCEC specific. Whether these differences truly represent tissue specific differences in gene expression or are related to the in vitro culture conditions (known to modulate gene expression) needs further investigation. In sum, the BCECs and vessel wall-derived ECs expressed gene products consistent with uncontaminated cultured endothelial cells.

Hierarchical Organization of the Proliferative Potential in BCECs and Bovine Vascular ECs

We have defined a hierarchy of ECFCs present in both circulating and vessel wall-derived ECs in human subjects and other vertebrates.⁴⁻⁶ Bovine vessel-derived ECs and BCECs possessed a similar hierarchy of cells with various levels of proliferative potential. Some ECs did not divide and some divided and gave rise to various sized colonies of endothelium (Fig. 4). Significantly more single BAECs divided at least once during 14

FIGURE 4. Quantitation of the clonogenic and proliferative potential of single endothelial cells derived from bovine vascular endothelium and bovine corneal endothelium. (A) The percentage of single BAECs, BCAECs, BPAECs, and BCECs that divided at least once after 14 days in culture. There were significantly fewer BCECs undergoing division compared with bovine vessel wall-derived ECs. (B) The distribution of different sizes of colonies derived from plated single ECs in an individual well after 14 days of culture. There was a significantly higher percentage of HPP-ECFC than LPP-ECFC colonies and endothelial clusters in BCEC24s and BAEC samples than was observed in the BCAEC and BPAEC samples. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by parametric ANOVA ($n = 3$).

days of culture compared with BCECs (BAECs versus BCEC18s versus BCEC24s was $65.67\% \pm 2.52\%$ vs. $32.63\% \pm 1.22\%$ vs. $41.40\% \pm 5.24\%$). However, there was no significant difference in the frequency of dividing cells among BAECs, BCAECs ($48.00\% \pm 4.67\%$), and BPAECs ($46.93\% \pm 7.46\%$) or among BCAECs, BPAECs, and BCECs (Fig. 4A). Surprisingly, $39.82\% \pm 2.71\%$ of the individual BCEC18s and $43.25\% \pm 3.49\%$ of the individual BCEC24s that divided gave rise to well-circumscribed colonies containing more than 10,000 progeny in the 2-weeks assay. This frequency was significantly higher than that measured for individually plated BAECs ($33.67\% \pm 4.22\%$). Differences in the ECFC distribution were observed in BCEC18s versus BCEC24s (at the same passage number), suggesting that some variability may be observed between donors with respect to this kind of distribution analysis.

To examine whether any BCECs or bovine vascular ECs are HPP-ECFCs, the progeny of primary ECFC colonies containing more than 10,000 cells were isolated from culture and replated at a single-cell level. After another 14 days of culture, the individually replated cells generated all sizes of colonies including some colonies containing more than 10,000 cells (data not shown). Thus, a complete hierarchy of ECFCs was identified in BCECs and bovine vascular ECs, and these ECFC-derived progeny comprised nondividing, mature ECs, endothelial clusters (2–50 cells/colony), low proliferative potential-ECFCs (LPP-ECFCs, 51–2000 cells/colony), and HPP-ECFCs (2001 or greater cells/colony), with secondary colony HPP-ECFC potential.

High Levels of Telomerase Activity in BCECs and Bovine Vascular ECs

We have reported that human cord blood HPP-ECFCs have high levels of telomerase activity.⁴ The progeny of HPP-ECFCs isolated from BCECs displayed telomerase activity similar to that of the progeny of HPP-ECFCs derived from BAECs, BCAECs, and BPAECs (Fig. 5). Thus, HPP-ECFCs in both BCECs and bovine vascular ECs expressed quantifiable levels of telomerase activity, suggesting a potential mechanism through which proliferative potential is retained within the subset of ECs that have proliferative potential.

Sphere-Forming Ability Displayed by BCECs

Human and rabbit CECs have been reported to form sphere colonies in vitro.^{25,26} Sphere formation has often been used as a surrogate assay to reflect the presence and frequency of stem and/or progenitor cells for the lineage under investigation.^{27–30} In the present study, low-passage cultured BCECs were able to form in vitro spheres (Fig. 6A) with excellent efficiency of

FIGURE 5. Telomerase activity of HPP-ECFCs derived from bovine vessel wall-derived ECs and BCECs. The presence and intensity of a ladder of PCR products with six base increments indicates the level of telomerase activity in the cells. Thus, the progeny of HPP-ECFCs isolated from BCECs displayed telomerase activity similar to that of the progeny of HPP-ECFCs derived from BAECs, BCAECs, and BPAECs. P, telomerase activity in the Hela cells (positive control); N, negative control; IC, 36-bp internal control sample provided by the manufacturer. Three independent experiments showed similar results.

FIGURE 6. The formation of a sphere colony by bovine corneal endothelial cells. (A) Representative photograph of a sphere derived from plated BCECs. (B) The distribution of different-sized colonies derived from single BCECs dissociated from corneal endothelial sphere colonies in an individual well after 14 days of culture. The complete hierarchy of ECFC is present in BCECs residing in the spheres. Scale bar, 100 μ m.

53 ± 10 spheres per 100,000 cells after 3 days of culture. To investigate the spheres for evidence of the clonal proliferative potential of the ECs, 3-day-old primary spheres were dissociated, and the cells were plated in a single-cell clonogenic assay. Of the 3000 sphere-derived, single BCECs plated, only $9.58\% \pm 6.09\%$ survived as single cells. However, $90.43\% \pm 6.56\%$ of the surviving cells demonstrated the ability to divide at least once and form ECFC-derived colonies of various sizes. We noted that the complete hierarchy of ECFCs was identified in sphere-derived BCECs (Fig. 6B), and the distribution of proliferative potential was similar to that in primary cultured BCECs (Fig. 4B). Thus, these corneal endothelial spheroids are nondividing mature ECs, endothelial clusters, LPP-ECFCs, and HPP-ECFCs. Therefore, although most BCECs that formed spheres in vitro failed to survive at a single-cell level, those surviving cells retained a distribution of proliferative potential similar to that of the BCECs used to establish the in vitro spheres.

DISCUSSION

It is well known that CECs are apparently restrained from proliferating in vivo, but they retain proliferative potential and can be expanded in vitro. The present study is the first to demonstrate that not all CECs display the same ability to proliferate at a clonal level. In fact, we define a hierarchy of ECFCs in BCECs based on their proliferative potential using a single-cell clonogenic assay. We report that the distribution of ECFCs derived from single BCECs was quite similar to the distribution observed for bovine vascular endothelium from aorta, coronary artery, and pulmonary artery (known to display clonal prolif-

erative behavior) and is consistent with circulating and resident vascular ECs in adult human subjects.^{4,5,7}

The proliferative activity of corneal endothelium *in vitro* has been observed under a variety of *in vitro* conditions,^{1,2,31,32} indicating that at least some CECs inherently possess high proliferative capacity. Most recently, Mimura and Joyce³ reported that peripheral CECs display more proliferative capacity than those more centrally located. Moreover, Mimura et al.²⁵ and Yamagami et al.³³ demonstrated in a sphere-forming assay that there is a greater number of CEC precursor cells in the periphery than in the center and concluded that the higher frequency of sphere-forming ability in the periphery must be related to the retention of precursor cells. However, these important studies did not use a single-cell clonogenic assay, which can quantitatively and stringently determine the proliferative potential of individual ECs, to test whether CECs harbor different populations that could be distinguished by their clonogenic potential.

We have provided evidence that corneal endothelium possesses the complete hierarchy of ECFCs and that corneal endothelial HPP-ECFCs can give rise to all subsequent stages of ECFC development. Thus, HPP-ECFCs display properties of corneal endothelial progenitor cells, as they have the most proliferative capacity and can be cultured *in vitro* in the absence of stromal cells. However, it should be realized that the BCECs used to demonstrate the clonogenic ability in the present study were cultured cells instead of primarily isolated cells. Most freshly isolated CECs, once removed from Descemet's membrane, are lost when plated *in vitro*.^{34,35} The cells that survive and expand in culture are those with the most robust proliferative ability, and thus the culture is enriched for ECFCs. Thus, our single-cell analysis most likely overrepresents the actual frequency of these cells *in vivo*.

Although a sphere-forming assay has been extensively used to quantitatively measure stem cell frequency in many fields,²⁷⁻³⁰ recent studies have indicated that not all cells capable of forming a sphere meet the criteria to be stem cells.³⁶ Moreover, the *in vitro* conditions to form spheres often permits the highly motile spheres to merge, which argues against the notion that these spheroid structures arise from a single stem cell.³⁶ Therefore, use of a sphere-forming assay may not permit accurate determination of which individual cells display proliferative potential. In this study, we used a single-cell clonogenic assay to provide direct evidence that CEC precursors exist in corneal endothelium and that HPP-ECFCs display the property of corneal endothelial progenitor cells.

In this study, we tried to generate corneal endothelial spheroids from plated BCECs (passage 1 or 2), rather than from freshly isolated CECs. We noticed that without addition of FBS to the culture medium, isolated cells aggregated but did not form spheres. Furthermore, when plated in the floating culture method as previously described,^{25,26,33} the BCECs frequently became free of the sphere and reattached to the culture substrate. Thus, we had to use a hanging-drop method of sphere formation that was modified from previous publications.^{18,19} Under these culture conditions, BCECs were able to form numerous spheres when 500 cells were deposited per drop of medium. The frequency, however, was much lower than that reported in a human CEC sphere-forming assay, in which 257 ± 83 spheres were generated from 50,000 cells after 10 days of culture.²⁶ This low yield may be due to obvious differences in culture conditions, to species differences, or to the use of freshly isolated instead of plated CECs. It is of interest that BCECs residing in the spheres displayed the complete hierarchy of ECFCs (Fig. 6B) when examined clonally, and the distribution of proliferative potential was similar to cultured BCECs that emerged from resident corneal endothelium at first plating (Fig. 4B). On the basis of the findings, we propose that

a single-cell clonogenic assay is an alternative method of stringently and directly quantitating resident corneal endothelial progenitor cells.

CECs differ from vascular ECs in many ways, including the fact that CECs are neural crest derivatives, do not line a vascular structure, and do not divide *in vivo* to replace senescent, injured, or diseased cells.^{1,10,11} Thus, these two types of ECs would be expected to exhibit different gene expression and functional properties. We report that VE-cadherin, PECAM1, and eNOS, which are specific markers associated with bovine vascular ECs, are not expressed in BCECs at the transcriptional level (Fig. 3), although VE-cadherin was reported to be expressed in murine CECs.³⁷ The disparity of VE-cadherin expression may be due to the difference between these two species. Although incorporation of AcLDL is an important property of vascular ECs, CECs were reported to ingest native circulating LDL,^{38,39} but not chemically modified lipoproteins such as AcLDL. Our data, consistent with data from these previous studies, demonstrated that CECs cannot incorporate AcLDL, which may result from the absence of expression of the scavenger receptor CD36. Thus, the populations studied exhibited properties consistent with those of populations in prior published work, displayed the typical morphologic and biochemical properties of CECs and various vascular ECs, and suggest that the proliferative behavior displayed by the CECs and vascular ECs does not result from a biased method of culture or a mixture of cells of one population with cells of another.

In summary, we have identified an entire hierarchy of ECFCs present in BCECs as well as in BAECS, BCAECs, and BPAECs. The present study provides a new conceptual framework for defining corneal endothelial progenitor cells. The identification of corneal HPP-ECFCs may contribute to regenerative medicine in corneal transplantation if tools for the prospective isolation, expansion, and storage of these cells can be developed.

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