Comparison of the Proliferative Capacity of Human Corneal Endothelial Cells from the Central and Peripheral Areas

Kenji Konomi, Cheng Zhu, Deshea Harris, and Nancy C. Joyce

Purpose. To compare the relative proliferative capacity between human corneal endothelial cells (HCECs) cultured from the central and peripheral areas of the cornea.

Methods. Human corneas were divided into two groups based on donor age (younger group, ≤30 years of age; older group, ≥50 years of age). Corneas were trephined, and Descemet's membrane with HCECs was stripped from the central (0–6.75 mm) and peripheral (6.75–9.5 mm) areas. HCECs were then isolated from Descemet's membrane and cultivated. An equal number of passage-1 endothelial cells from each area were seeded, and the number of cells was determined at various times after seeding. Doubling times of cells from each area were compared. The antibody against minichromosome maintenance-2 (MCM2) protein was tested for replication competence.

Results. Morphologically, HCECs from the central area were similar to cells from the peripheral area. The doubling time (in hours) of HCECs from the central area was 35.20 in the younger group ($n = 4$) and 54.54 in the older group ($n = 4$) and from the peripheral area, 29.37 in the younger group and 46.23 in the older group. There was no significant difference (younger: $P = 0.515$; older: $P = 0.222$) between the central and peripheral area in each age group. MCM2-positive cells were consistently observed in cultures from the central, as well as peripheral, area. There was no significant difference (younger: $P = 0.929$; older: $P = 0.613$) in the percentage of MCM2-positive cells between these two areas in either age group. Even though there was no significant difference, there was a tendency toward increased doubling time and decreased percentage of MCM2 in the central area of the older group.

Conclusions. These results indicate that corneal endothelial cells from both the central and peripheral areas retain potential proliferative capacity. (*Invest Ophthalmol Vis Sci.* 2005;46:4086–4091) DOI:10.1167/iovs.05-0245

Corneal endothelial cells form a single layer behind the cornea and play a crucial role in maintaining corneal transparency, by performing barrier and pump functions.[1–3] Human corneal endothelial cells (HCECs) are normally nonproliferative in vivo, and this results in an age-related decrease in cell density.[4–6] HCECs are thought to compensate for cell loss by migration and cell enlargement instead of by proliferation.[7,8] In several pathologic conditions, and as a result of trauma or previous corneal transplantation, there can be more severe cell loss that will lead to corneal endothelial decompensation and finally result in corneal edema.

Studies from this laboratory have demonstrated that HCECs retain proliferative capacity, even though they are nonproliferative in vivo.[9,10] By various techniques, HCECs have been successfully isolated and cultured.[11–15] One recent study demonstrated that HCECs from younger donors (<30 years of age) proliferate more readily than those from older donors (>50 years of age),[16] but in that study, no comparison was made of the proliferative capacity of HCECs from the central versus peripheral area. Bednarz et al.[17] have demonstrated that cells from the central area (0–6.5 mm diameter) do not proliferate, whereas those in the peripheral region (6.5–9.0 mm) do. In addition, studies by Schimmelpfennig[18] and Amann et al.[19] showed that the cell density of HCECs in the central area is less than that in the peripheral area. Together, these findings have led to the hypothesis that HCECs in the central area do not have proliferative capacity, whereas, cells in the peripheral area do, providing an explanation for the difference in cell density in these areas.

In the present study, we investigated the difference in proliferative capacity and competence for replication between HCECs cultured from the central and peripheral areas of the cornea, by comparing the relative doubling time and the expression of minichromosome maintenance-2 (MCM2) protein, a marker of replication competence.[20–23]

Materials and Methods

Serum-free medium (OptiMEM-1), Medium 199 (M199), Hanks' balanced salt solution (HBSS), gentamicin, and trypsin-EDTA were purchased from Invitrogen-Life Technologies (Carlsbad, CA). Nerve growth factor (NGF; from mouse submaxillary glands), and bovine pituitary extract were from Biomedical Technologies (Stoughton, MA). Epidermal growth factor (EGF; from mouse submaxillary glands) was obtained from Upstate Biotechnologies (Lake Placid, NY); fetal bovine serum (FBS) from Hyclone (Logan, UT); ascorbic acid, chondroitin sulfate, calcium chloride, 0.02% EDTA solution (EDTA disodium salt), and antibiotic-antimycotic solution from Sigma-Aldrich (St. Louis, MO); cell attachment reagent (FNC Coating Mix) from Biological Research Faculty and Facility, Inc. (BRFF; Ijamsville, MD); Barron donor cornea punches (6.75 and 9.5 mm) from Katena Products, Inc. (Denville, NJ); mouse anti-MCM2 IgG from BD-Pharmingen (San Diego, CA); fluorescein (FITC)-conjugated donkey anti-mouse IgG from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA); and mounting medium with propidium iodide (Vectashield) from Vector Laboratories, Inc.; Burlingame, CA).

Isolation and Growth of Human Corneal Endothelial Cells

Donor corneas were obtained from National Disease Research Interchange (NDRI; Philadelphia, PA), and stored in preservative (Optisol-

From the Schepens Eye Research Institute and Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts.

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Corresponding author: Nancy C. Joyce, Schepens Eye Research Institute, 20 Staniford Street, Boston, MA 02114; njoyce@vision.eri.harvard.edu.

TABLE 1. Donor Information

Age	Days*	Time†	Cause of Death
16	5	9 h 8 min	Gastrointestinal bleeding
25	5	15 h 34 min	Pneumonia
29	4	3 h 5 min	Trauma
30	4	6 h 43 min	Pulmonary fibrosis
55	3	14 h	Head trauma
64	3	11 h	Motor vehicle accident
65	3	8 h 4 min	Cardiovascular disease
73	5	7 h	Intracranial hemorrhage

* Days from death to culture.
† Death to preservation time.

GS; Chiron, Vision, Irvine, CA) at 4°C. Handling of donor information by the source eye bank, NDRI, and this laboratory adhered to the tenets of the Declaration of Helsinki in protecting donor confidentiality. Table 1 provides important information regarding the corneas used for endothelial culture. All corneas received from NDRI were considered to be unsuitable for transplantation. Donor ages ranged from 16 to 73 years. Exclusion criteria for donor corneas were the same as previously reported[24] except for one criterion. Corneas with endothelial densities less than 2000/mm^2 were excluded. HCECs were isolated and cultured according to published protocols.[15,16] Corneas were removed from the preservative (Optisol-GS) and washed three times with M199 containing 50 μg/mL gentamicin and 1:100 diluted antibiotic/antimycotic solution before being placed in a Barron donor cornea punch. Corneas were cut with trephines at 9.5- and 6.75-mm diameters, rinsed with M199, and placed endothelial side up in a Petri dish. Descemet's membrane and endothelium were carefully stripped from the central (0–6.75 mm) and peripheral (6.75–9.5 mm) areas. The central and peripheral tissue was washed three times with M199 and then incubated in culture medium overnight to stabilize the endothelial cells before culture. The tissues strips were then incubated in 0.02% EDTA solution at 37°C for 1 hour to loosen cell–cell junctions. HCECs were isolated from Descemet's membrane by forcing the tissue and medium multiple times through the narrow opening of a flame-polished pipette. HCECs were pelleted and resuspended in a serum-free medium (Opti-MEM-1) also containing 8% FBS, 5 ng/mL EGF, 20 ng/mL NGF, 100 μg/mL pituitary extract, 20 μg/mL ascorbic acid, 200 μg/mL calcium chloride, 0.08% chondroitin sulfate, 50 μg/mL gentamicin, and antibiotic-antimycotic solution diluted 1:100. Isolated cells from each area (0–6.75 and 6.75–9.5 mm) were plated separately in one well of a 12-well tissue culture plate that had been precoated with undiluted cell attachment reagent (FNC Coating Mix). Cells were then incubated at 37°C in a 5% carbon dioxide, humidified atmosphere. Medium was changed every other day. After primary cultures reached confluence, cells were subcultured at a 1:2 split ratio. When passage-1 cells were confluent, cells were recovered from the wells with 0.05%:0.02% trypsin-EDTA solution and seeded at 10,000 cells per well in a 24-well tissue culture plate for cell counting. A microscope (model TS100; Nikon, Melville, NY) with a digital camera (Coolpix 995; Nikon) was used to take phase-contrast images of confluent passage-2 cells to document morphology. The remaining rim of corneal tissue (>9.5 mm from the center) was fixed with 10% formaldehyde, sectioned, stained with hematoxylin and eosin, and examined by light microscopy, to document the position of the trabecular meshwork.

Calculation of Population Doubling Time

Passage-1 endothelial cells isolated from the central or peripheral areas were seeded into 24-well plates at 10,000 cells per well and cultured for up to 3 weeks in the culture medium described earlier, containing 8% FBS, but without EGF, NGF, or pituitary extract. At various times after plating, these passage-2 cells were trypsinized and counted under a microscope (Eclipse TS1000; Nikon), with a hemacytometer (Fisher Scientific. Pittsburgh, PA). Cells from each well were counted three times. Three separate wells were counted per time point. Results were averaged, and the standard deviation was calculated. Population doubling time [DT = $(t_1 - t_2) \log 2 / \log N_2 - \log N_1$] was calculated from the log phase of each growth curve. Statistical significance was determined by Student's unpaired *t*-test. $P < 0.05$ was considered significant.

Immunocytochemical Localization

Cultured passage-1 cells were seeded in four-well chamber slides (Nalge Nunc International, Naperville, IL) that had been precoated with FNC Coating Mix and grown in culture medium containing only 8% FBS. Immunocytochemical staining for MCM2 was performed on subconfluent (days 4–6) and confluent (days 30–31) cells. Established protocols were used for fixation, blocking, and antibody incubation steps.[16] Briefly, anti-MCM2 was used at a 1:100 dilution. FITC-conjugated donkey anti-mouse IgG (diluted 1:200) was used as a secondary antibody. Secondary antibody alone acted as a negative control for all immunolocalization studies. Coverslips were mounted in medium containing propidium iodide (PI) to stain all nuclei. Positive staining of cultured cells was visualized on a fluorescence microscope (Eclipse E-800; Nikon) equipped with a digital camera (Spot; Diagnostic Imaging, Sterling Heights, MI). Five images were taken per culture chamber, with a ×40 objective lens. An image-analysis software program (Image J 1.62; available by ftp at zippy.nimh.nih.gov/ or at http://rsb.info.nih.gov/nih-image; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD) was used to count total PI-stained nuclei and total MCM2-positive nuclei. Differences in the counts were analyzed with Student's unpaired *t*-test. $P < 0.05$ was considered significant.

RESULTS

Morphology of HCECs Cultured from Central and Peripheral Areas

The diagram in Figure 1A shows the position of the trephine cuts made in the corneal tissue relative to the center of the cornea. As indicated earlier, Descemet's membrane with the associated endothelial cells was dissected from the central (0.0–6.75 mm) and peripheral (6.75–9.5 mm rim) areas. The total area for these two regions was very similar (central area, 35.8 mm^2, peripheral area, 35.1 mm^2). HCECs from each area were cultured and tested for their relative proliferative capacity, as described below. The outer rim of the corneal tissue remaining after the 9.5-mm trephination was examined by light microscopy to determine whether cells from the trabecular meshwork might have contaminated the tissue taken from the peripheral region. As seen from the representative hematoxylin and eosin (H&E)-stained image in Figure 1B, there was still a small amount of Descemet's membrane with its associated endothelial cells remaining outside the 9.5-mm trephined area. This provides evidence that cells dissected from the peripheral rim were corneal endothelial cells and that cultures were not contaminated with trabecular meshwork cells.

The morphology of endothelial cells cultured from the central and peripheral areas was compared. For these studies, passage-1 cells isolated from the central and peripheral areas were seeded at 10,000 cells per well, grown to confluence, and then examined by phase–contrast microscopy. Figure 2 presents representative images of confluent cells cultured from a 30- and a 73-year-old donor. A consistent finding was that, in cultures derived from a single donor, there was no obvious difference in cell size or shape between the central and peripheral areas (compare Fig. 2A with 2B and 2C with 2D). In contrast, cell size differed with donor age. Confluent cells from younger donors (≤30 years old) were consistently smaller than those from older donors (≥50 years old), regardless of the area they were dissected from (compare Figs. 2A, 2B with Figs. 2C, 2D). A similar age-related difference in cell size was demonstrated in a previous report from this laboratory.[24] Of impor-

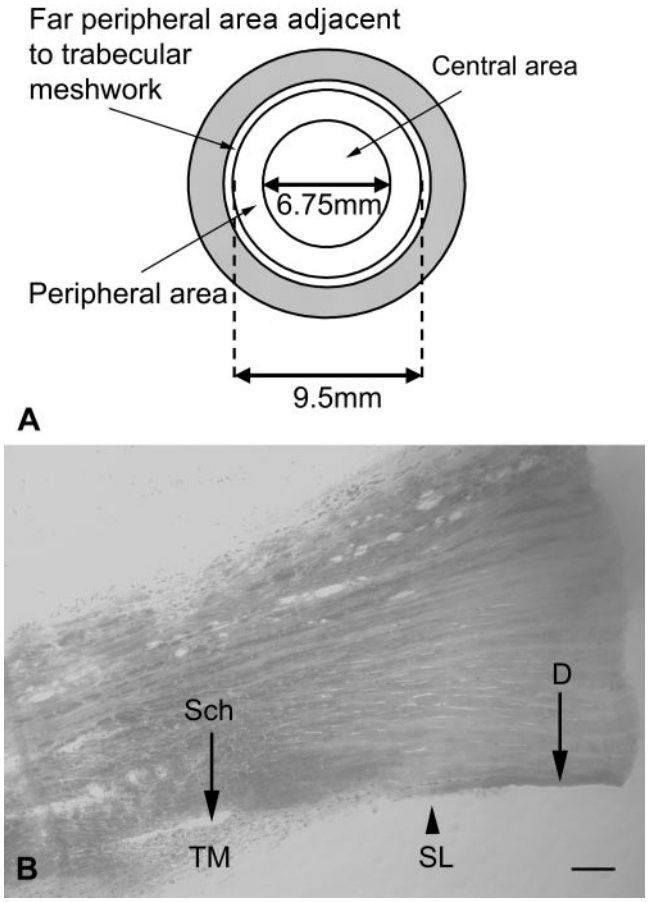

FIGURE 1. (**A**) Scheme of the trephined central and peripheral areas of the cornea used for these studies. (**B**) Transverse section of far peripheral corneal tissue not used in the study. TM, trabecular meshwork; D, Descemet's membrane; Sch, Schlemm's canal; SL, Schwalbe's line. Scale bar, 100 μm.

tance is the fact that there was no case in which cells from the central area did not grow.

Growth Characteristics of Central and Peripheral Cells

Growth curves of central and peripheral cells from younger donors are shown in Figure 3A and those from older donors are in Figure 3B. The bar graph in Figure 4 compares the population-doubling time of HCECs cultured from the central and peripheral areas of each donor cornea. Mean population-doubling times are presented in Table 2. HCECs cultured from the central and peripheral areas of individual donor corneas produced almost similar growth curves. In general, cells from younger donors yielded higher peak cell counts at the plateau phase of growth than did cells from older donors. No significant difference ($P = 0.515$) in mean population-doubling time was observed between central and peripheral HCECs cultured from younger donors. Cells cultured from the central and peripheral areas of older donors also showed no statistically significant difference ($P = 0.222$) in mean population-doubling time. In contrast, a significant difference was observed in the mean population-doubling time of central cells between young and older donors ($P = 0.043$), indicating an age-related increase in cell cycle time. Although no statistically significant difference ($P = 0.057$) in mean population-doubling time was observed between peripheral cells cultured from young and older donors, there was a tendency for the cells from older donors to divide at a slower rate.

MCM2 Staining

Micrographs in Figure 5 show representative examples of positive nuclear staining for the replication-competence marker, MCM2, in subconfluent cells cultured from both the central and peripheral areas. The average percent of MCM2-positive cells for each area and age-group was calculated during the log-phase of growth and results are shown in Figure 6. Cells from both the central area (younger group, 63.0% ± 10.1%; older group, 39.0% ± 12.1%) and peripheral area (younger

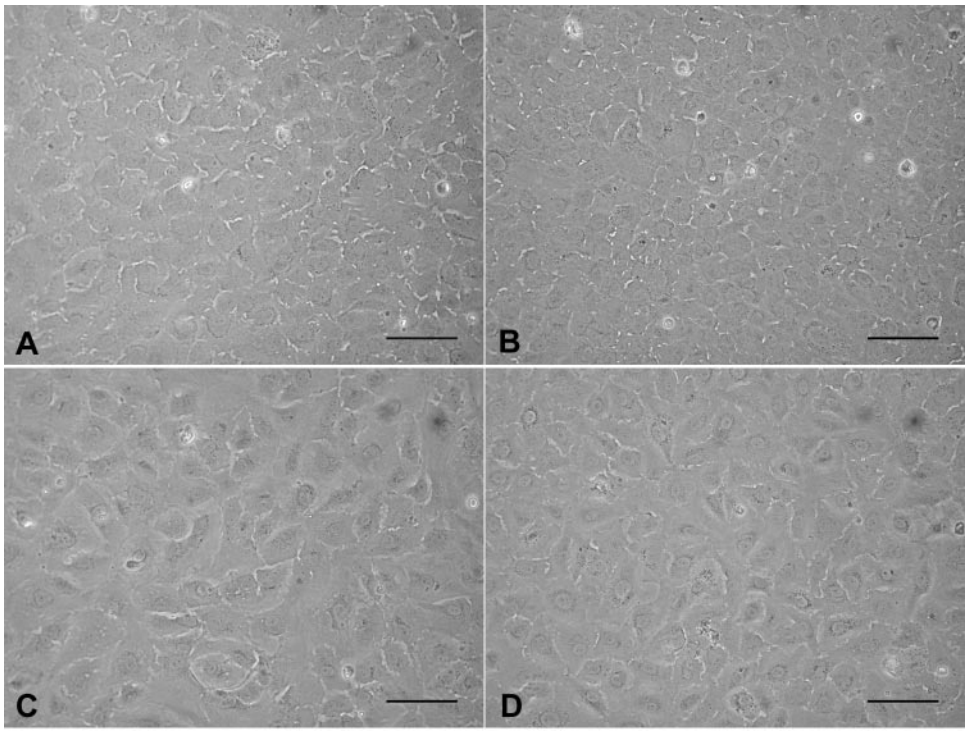

FIGURE 2. Phase-contrast images of confluent passage-2 HCECs. (**A**, **B**) 30-year-old donor; (**C**, **D**) 73-year-old donor. Cells were cultured from the (**A**, **C**) central and peripheral (**B**, **D**) areas and were fully confluent and generally assumed a polygonal shape. Cells cultured from the younger donor appeared smaller than those from the older donor. Scale bar, 100 μm.

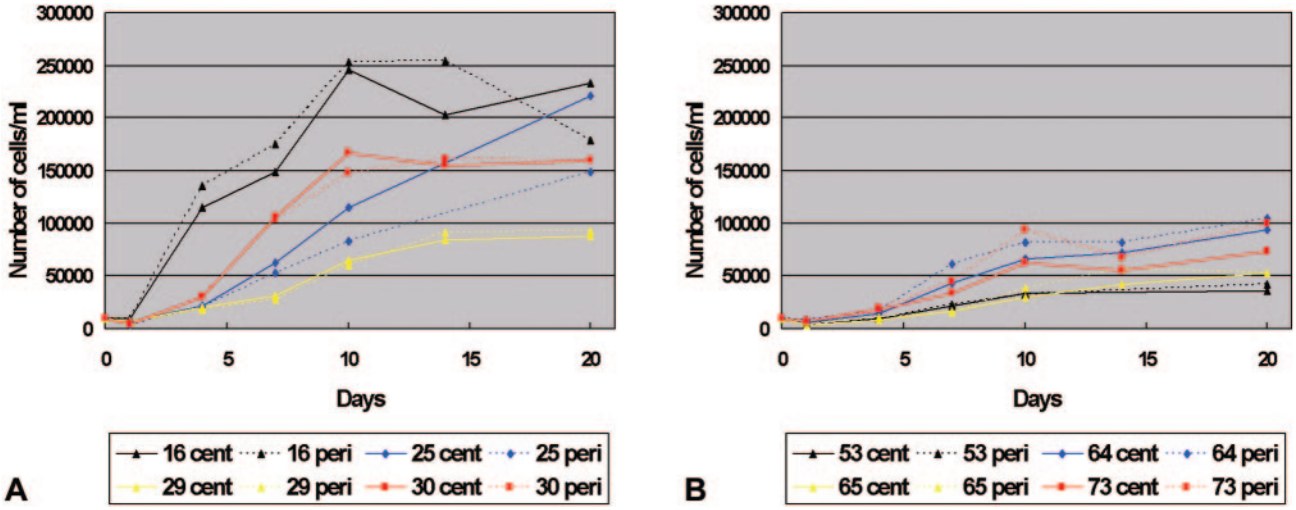

FIGURE 3. Growth curves of HCECs cultured from the central and peripheral areas in (**A**) younger ($n = 4$) and (**B**) older ($n = 4$) donors. Passage-1 cells were seeded at low density and counted in triplicate at each time point. Data show the average cell count for each time point. Standard deviations have not been added, to simplify the graph.

group, 62.4% ± 8.4%; older group, 43.6% ± 12.8%) showed positive staining, indicating that HCECs in both areas of the cornea are competent to replicate their DNA. There was no significant difference observed in the relative percentage of MCM2-positive cells between the central and peripheral areas within either age group (younger group, $P = 0.929$; older group, $P = 0.613$). Statistically significant differences were observed in the percentage of positive cells in subconfluent cultures obtained from the central ($P = 0.023$) and peripheral ($P = 0.05$) areas of the younger group compared with those of the older group. As expected, the percentage of MCM2-positive cells was considerably reduced (<2%) in quiescent, confluent cells, regardless of specific area or donor age (data not shown).

DISCUSSION

Results from direct cell counts and calculation of population-doubling time showed successful culture of HCECs, not only from the peripheral region, but also from the central region of the endothelium, indicating that HCECs from both areas are capable of dividing. HCECs cultured from the central and peripheral regions of a single donor grew in a similar manner, indicating that, under our culture conditions, there was no significant difference in the overall kinetics of cell cycle progression based on the relative position of the cells within the endothelium. Previous studies from this laboratory cultured cells isolated from the entire endothelium and demonstrated a reproducible, age-related difference in proliferative response.[16,24] The current studies have extended those observations by finding that relative differences in proliferative response more closely correlate with age than with position of cells within the endothelium.

MCM2 is a component of the origin recognition complex (ORC). This complex contains polypeptides that bind at specific DNA sequences and determines where replication will be initiated.[25] MCM proteins associate with the ORC during the G_1-phase, making chromatin competent (licensed) for replication, and then dissociate from the complex during the S-phase.[26] MCM2 is not expressed in the G_0-phase (resting) cells, or in cells that have entered replicative senescence or are terminally differentiated.[20] This makes MCM2 a sensitive marker for replication competence.[21,22] Although Ki67 has been used successfully by this laboratory to detect actively cycling cells,[10] we used MCM2 immunostaining in this study to detect all cells that were competent in replicating DNA, providing a sensitive method to detect any difference in potential

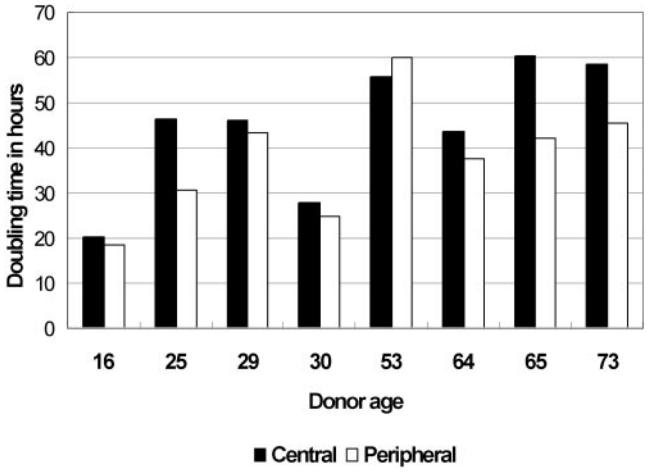

FIGURE 4. Population-doubling time for passage-2 HCECs cultured from the central and peripheral areas of all donor corneas. Doubling time was calculated from the log phase of each growth curve by the equation shown in the Materials and Methods section.

TABLE 2. Mean Population-Doubling Time

	Younger (≤30 Years, $n = 4$)		Older (≥50 Years, $n = 4$)	
	Central	**Peripheral**	**Central**	**Peripheral**
Mean	35.20	29.37	54.54	46.23
SD	13.19	10.51	7.40	9.72
Min	20.32	18.61	43.76	37.51
Max	46.48	43.27	60.17	59.99

Data are expressed in hours. Comparisons of central and peripheral areas in the younger group: $P = 0.515$; central and peripheral areas in the older group: $P = 0.222$; central area between the young and older donors: $P = 0.043$; and peripheral area between young and older donors: $P = 0.057$.

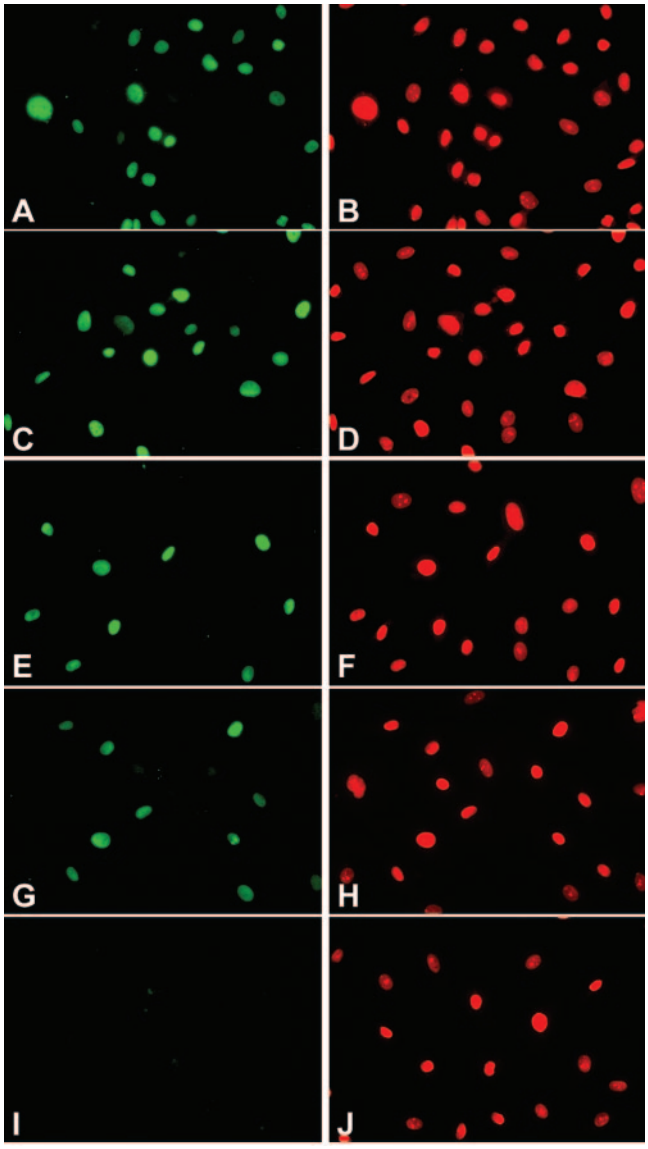

FIGURE 5. Representative images of subconfluent cultures immunostained for MCM2. (**A**, **C**, **E**, **G**, **I**) *Green* fluorescence (FITC) localized MCM2 in the nuclei, and (**B**, **D**, **F**, **H**, **J**) *red* fluorescence (PI) showed all nuclei within the same area. Cells were from the (**A**, **B**) central and (**C**, **D**) peripheral areas of a 30-year-old donor or the (**E**, **F**) central and (**G**, **H**) peripheral areas of a 73-year-old donor. (**I**, **J**) Negative control (secondary antibody only). Cells from both the central and peripheral areas stained positively for MCM2. Original magnification, ×40.

ability to divide that may exist between cells cultured from the central and peripheral regions. Results obtained using this method were very similar to those obtained by direct cell counting.

Together, these results indicate that HCECs from both the central and peripheral areas within a single cornea are capable of dividing. A consistent finding was that donor age had a greater influence on proliferative capacity than relative position. It should be noted that, although no significant difference was observed in the relative proliferative capacity of central and peripheral cells from older donors, there was a tendency for fewer cells within the central region to divide. The findings obtained in these studies differ from those reported by Bednarz et al.,[17] who found that HCECs cultured from the central area (0–6.5 mm) exhibited little to no mitotic activity, whereas cells cultured from the peripheral area (6.5–9.0 mm) were able to divide. There are several possible reasons for such a discrepancy in these findings. One may be the specific methods used for cell culture. A second may be the nature of the growth factors used in the culture medium. Our medium contained serum free-medium (OptiMEM-1) supplemented with 8% FBS, 5 ng/mL EGF, 20 ng/mL NGF, and 100 μg/mL pituitary extract, whereas the medium used by Bednarz et al. contained 7.5% FCS, 7.5% newborn calf serum, 20 μg/mL insulin, and 1 ng/mL bFGF. A third may be the viability of the donor cornea. Our experience has been that, not only age and endothelial cell density, but also death-to-preservation time, preservation period, health history of the donor, and specific cause of death can affect the ability to culture HCECs successfully.[24]

In the present study, we used two different-sized trephines (6.75 and 9.5 mm) to separate the endothelium into central and peripheral areas, but also tried to use larger trephines (7 and 10.0 mm) to recover more cells from each area for culture and to obtain cells from the far peripheral area. However, there was no clear boundary between the trabecular meshwork and the endothelial cell layer,[27] and the peripheral tissue obtained using the 10.0-mm trephine sometimes included trabecular meshwork cells. We also tried to separate the cornea into three pieces to yield central, paracentral, and peripheral cells as defined by Amann et al.,[19] to compare the proliferative capacities more precisely; however, in our experience, it was difficult to dissect the tissue precisely and obtain a sufficient number of cells for culture, because increased handling of the tissue during the dissections caused significant cell loss.

Studies conducted by Schimmelpfennig[18] and Amann et al.[19] showed that the density of HCECs in the peripheral area was higher than in the central area. The reason for this difference is not known, but it has been suggested that the higher density may be due to the presence of progenitor cells that slowly divide to produce a continuous population of cells that could migrate centrally to help maintain cell density in the central area.[19] The current studies do not directly address the question of the existence of these highly proliferative cells, in that we did not examine cells from the far peripheral region and did not serially passage cells from the central and peripheral areas to compare their relative proliferative lifespan.

In this study, we used a culture model system to evaluate the difference in proliferative capacity between HCECs cultured from the central and peripheral areas. It is possible that this model may be selective, in that only healthy cells can attach to the tissue culture plate and proliferate. In other words, we cannot successfully culture cells that are replicative senescent or truly senescent. This selective potential may affect our results. For example, in Figure 4, the doubling time of

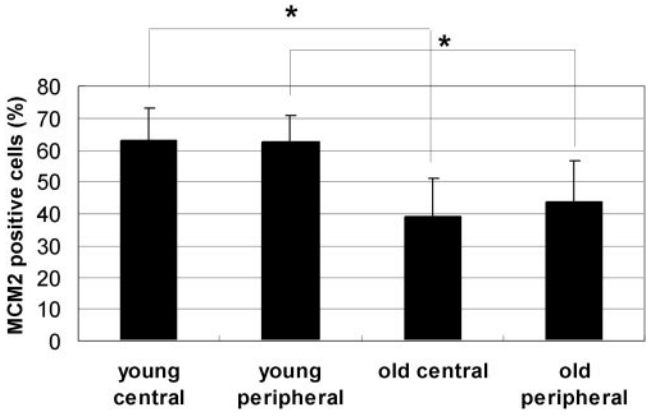

FIGURE 6. Average percentage of MCM2-positive HCECs cultured from the central and peripheral areas of each donor age group ($n = 4$ per age-group; $^*P < 0.05$).

cells from the central area tended to be longer than that from the peripheral area in seven of eight donors, even though not all differences were statistically significant. Therefore, we cannot deny the possibility that there is a difference in proliferative capacity between the central and peripheral areas. An ex vivo wound model was also used to test for MCM2 staining. We found that cells in the central area were positively stained for MCM2 in this model, providing additional support for our observation of replication-competent cells in central cornea (data not shown). Additional studies are being conducted to determine the proliferative status of HCECs in this ex vivo wound model.

In summary, HCECs from both the central and peripheral areas are capable of cell division in vitro in response to serum. The morphology and proliferation rate of HCECs cultured from the central area were similar to those from the peripheral area. These results indicate that corneal endothelium from the central, as well as peripheral, areas retain potential proliferative capacity. Further investigations are needed to determine whether this capacity could be used to induce proliferation to increase corneal endothelial cell density in vivo.

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