Isolation of adult progenitor cells with neuronal potential from rabbit corneal epithelial cells in serum- and feeder layer-free culture conditions

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Purpose: To isolate progenitor cells from rabbit corneal epithelial cells (CEC) in serum- and feeder layer-free culture conditions and to compare the self-renewal capacity of corneal epithelial progenitor cells obtained from the central and limbal regions of the cornea.

Methods: Tissue samples of New Zealand white rabbit corneas were dissected from the limbal and central regions to obtain CEC for sphere-forming culture, in which the cells formed spheres in serum-free medium containing growth factors. The number of primary and secondary sphere colonies and the size of the primary spheres were compared between the limbal and central regions. To promote differentiation, isolated sphere colonies were plated in dishes coated with poly-L-lysine (PLL)/laminin. The expression of epithelial, neural, and mesenchymal mRNAs was examined in the sphere colonies and their progeny by immunocytochemistry and/or the reverse transcription–polymerase chain reaction (RT–PCR). Adherent differentiated cells from the sphere colonies were also examined morphologically.

Results: Primary spheres were isolated from both the limbal and central regions of the cornea. The rate of primary sphere formation by CEC from the limbal region (55.6 ± 10.6/10,000 cells) was significantly higher than that by cells from the central cornea (43.1 ± 7.2/10,000 cells, $p=0.0028$), but there was no significant difference in the size of primary spheres derived from both regions. The self-renewal capacity of cells from the limbal region was higher than that of cells from the central region, as evidenced by the significantly higher secondary sphere formation rate of limbal cells (38.7 ± 8.5/10,000 cells) in comparison with that for central cells (31.3 ± 5.7/10,000 cells, $p=0.013$). The primary sphere colonies expressed bromodeoxyuridine (BrdU), a 63-kDa protein (p63), p75 neurotrophin receptor (p75^{NTR}), and nestin, whereas their progeny expressed cytokeratin 3, cytokeratin 12, vimentin, α-smooth muscle actin, microtubule-associated protein 2, and neuron-specific enolase on immunocytochemical analysis. These markers were confirmed by RT–PCR.

Conclusions: Our findings indicate that limbal CEC contain more progenitor cells with a stronger self-renewal capacity than cells from the central region. These progenitor cells differentiate into the epithelial lineage, and can also produce neuronal protein.

The corneal epithelium (CE) is a nonkeratinized epithelium composed of multiple layers of cells with self-renewal capacity. Corneal epithelial stem cells are thought to be localized in the basal cell layer of the limbus and are believed to correspond to transient amplifying cells and terminally differentiated cells in the central CE [1-4]. Wound healing at the central CE occurs via centripetal migration and growth of stem cells from the periphery [5-9]. Over the past few years, a considerable number of workers have undertaken a molecular and histological analysis of corneal epithelial stem cells [10-16]. Kruse and Tseng [17] demonstrated that limbal stem cells could be differentiated into transient amplifying cells. Du et al. [18] reconstructed rabbit corneal surface using cultured human limbal cells on amniotic membrane. Yoshida et al. [19] demostrated neural crest-derived, multipotent stem cells exist in the adult cornea. However, little attention has been paid to attempting the selective isolation of stem cells or progenitor cells from the CE. Although corneal stem cells expressing neuronal markers were recently isolated from rodents [20], almost all of the differentiated cells derived from rodent corneal spheres show characteristics similar to those of fibroblast-like cells [21]. Thus, the separation of true epithelial spheres that retain the potential to differentiate into the corneal epithelial lineage has not been achieved yet because of the difficulty in isolating corneal epithelial cells (CEC) from mouse or rat stroma without contamination by fibroblasts. Because rabbit and human corneas are far larger than those of rats or mice, these

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may be more suitable for the isolation and characterization of corneal epithelial stem or progenitor cells.

In this study, we achieved the first isolation of adult progenitor cells from rabbit CE in serum- and feeder layer-free culture conditions. We also investigated the difference in self-renewal capacity between CEC from the central and limbal regions and whether the isolated cells could differentiate into multiple lineages.

METHODS

Isolation of corneal epithelial cells: All animals were treated in accordance with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research and with the protocols approved by the Committee for Animal Research at the University of Tokyo Graduate School of Medicine. Rabbits were obtained from Saitama Experimental Animals Inc., Japan.

Primary cultures were established from 12-week-old male New Zealand white rabbits weighing an average of 2.4 kg. The basal medium for culture was DMEM/F12 (1:1; Sigma-Aldrich, St. Louis, MO) supplemented with 2% B27 (Invitrogen, San Diego, CA), 20 ng/ml of epidermal growth factor (EGF; Sigma-Aldrich), and 20 ng/ml of basic fibroblast growth factor (bFGF; Sigma-Aldrich), as described previously [22]. Anesthesia was induced by intramuscular injection of ketamine hydrochloride (60 mg/kg; Sankyo, Tokyo, Japan) and xylazine hydrochloride (10 mg/kg; Bayer, Leverkusen, Germany). After disinfection and sterile draping of the orbital region, a surgical blade was used to carefully dissect small tissue pieces from the limbal region. These tissue pieces measured approximately 1 mm×2 mm and were 100 µm thick with intact epithelium. To compare the sphere formation rate between the limbal cornea and central cornea, a sample of epithelium was excised from the central 6.0-mm region of the cornea. Each tissue sample was washed three times with sterile saline, and then immersed for 5 min in saline containing 10% povidone-iodine (Meiji, Tokyo, Japan) and 50 µg/ml gentamicin (Sigma-Aldrich). After further rinsing with saline, the limbal epithelial tissues were cut into small pieces and incubated in basal medium containing 0.02% collagenase (Sigma-Aldrich) overnight at 37 °C. After washing with Ca^{2+}- Mg^{2+}-free phosphate-buffered saline (PBS; Sigma-Aldrich), the tissue pieces were incubated in 0.2% EDTA at 37 °C for 5 min and were dissociated into single cells by trituration with a fire-polished Pasteur pipette. After centrifugation at 800× g for 5 min, the cells were resuspended in basal medium. Isolated limbal epithelial cells were counted with a Coulter counter and their viability was confirmed to be >90% by trypan blue staining (Wako Pure Chemical Industries, Osaka, Japan).

Isolation of sphere colonies from corneal epithelial cells: Primary culture was done according to the neurosphere assay [23]. Basal medium containing methylcellulose gel matrix

(1.5%; Wako Pure Chemical Industries) was employed to prevent reaggregation of the cells, as described previously [24]. Plating was done for floating culture at a density of 10.0 viable cells/µl (40,000 cells/well) in uncoated 60-mm culture dishes (BD Biosciences, San Jose, CA). Under these conditions, reaggregation did not occur and most (or all) of the sphere colonies were derived from single cells [24,25]. Culture was done in a humidified incubator with an atmosphere of 5% CO_2.

After seven days, cell clusters (i.e., sphere colonies) were detected. The number of primary spheres was counted after 7 days of culture. To distinguish growing spheres from dying cell clusters, only clusters with a diameter >50 µm were counted. For passaging, primary spheres (day 7) were treated with 0.5% EDTA and dissociated into single cells, which were plated in 24-well culture plates at a density of 10.0 cells/µl. Then culture was done for a further 7 days in basal medium containing methylcellulose gel matrix. To measure the diameter of the sphere colonies, cultures were observed under an inverted phase-contrast microscope (Nikon ELWD 0.3, Tokyo, Japan) with a 10× objective lens, and the images were analyzed by employing the NIH image program developed at the USA National Institutes of Health and available on the Internet.

Differentiation of sphere colonies: To assess the multipotentiality of isolated sphere colonies, individual primary spheres (day 7) were transferred to 13-mm glass coverslips coated with 50 µg/ml poly-L-lysine (PLL; Sigma-Aldrich) and 10 µg/ml fibronectin (BD Biosciences, Billerica, MA) in separate wells, as described previously [26]. To promote differentiation, 1% fetal bovine serum (FBS) was added to the basal medium to form differentiation medium and culture was continued for another 7 days.

Immunohistochemistry of spheres for bromodeoxyuridine (BrdU): Expression of BrdU in the sphere colonies was determined by immunocytochemistry. The 7-day primary spheres were incubated overnight with 10 µM/ml BrdU (Sigma-Aldrich). After fixing in methanol (Wako Pure Chemical Industries) at 4 °C for 5 min and treatment with 2 M HCl (Wako Pure Chemical Industries) in PBS at room temperature for 60 min, the cells from sphere colonies were stained with FITC-conjugated anti-BrdU antibody at room temperature for 60 min in the dark. After washing with PBS, fluorescence was observed under a fluorescence microscope (model BH2-RFL-T3 and BX50; Olympus, Tokyo, Japan).

Immunocytochemistry of spheres and their progeny: Immunocytochemical analysis was performed on 7-day spheres and their progeny after 7 days of adherent culture on glass coverslips. Cells were fixed with 4% paraformaldehyde (Wako Pure Chemical Industries) in PBS for 10 min. After washing in PBS, the cells were incubated for 30 min with 4% BSA (BSA; Sigma-Aldrich) in PBS containing 0.3% Triton X-100 (BSA/PBST; Rohm & Haas, Philadelphia, PA) to

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TABLE 1. PRIMERS USED FOR POLYMERASE CHAIN REACTION.

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Gene	Forward primer	Reverse primer	Size (bp)
p63	5′-GCCACCTGGACGTATTCCAC-3′	5′-CATACTGGGCATGGCTGTTCC-3′	259
nestin	5′-TTGAGAC(A/T)CCTGTG(C/A)CAGCCT-3′	5′-CTCTAGAC(T/C)CAC(T/C)GGATTCT-3′	383–387
ketarin-3	5′-GCAGCAGCAGGATGAGCTG-3′	5′-GTTGAGGGTCTTGATCTG-3′	482
keratin-12	5′-GAGCTGGCCTACATGAAG-3′	5′-TTGCTGGACTGAAGCTGCTC-3′	250
G3PDH	5′-CATCACCATCTTCCAGGAGC-3′	5′-ACAATGCCGAAGTGGTCGTG-3′	294

block nonspecific binding. Then the cells were incubated for 2 h at room temperature with specific primary antibodies diluted in BSA/PBST. The following antibodies were used: mouse anti-cytokeratin 3 monoclonal antibody (1:2,000; AE-5; Progen Biotechnik GMBH, Heidelberg, Germany), goat anti-cytokeratin 12 polyclonal antibody (1:2,000; L-20; Santa Cruz Biotech, Santa Cruz, CA), mouse monoclonal anti-p63 antibody (1:400; Imgenex, San Diego, CA), mouse monoclonal anti-nestin antibody (1:400; BD Biosciences), mouse monoclonal anti-nerve growth factor (NGF) receptor p75[NTR] antibody (1:400; DAKO, Kyoto, Japan), Cy3-conjugated mouse anti-α-smooth muscle actin (SMA) mAb (1:400; Sigma-Aldrich), mouse monoclonal anti-nestin antibody (1:400; BD Biosciences), mouse monoclonal anti-microtubule-associated protein 2 antibody (MAP2, 1:400; Chemicon, Temecula, CA), mouse monoclonal anti- neuron specific enolase antibody (NSE, 1:400; DAKO), and mouse monoclonal anti-BrdU/fluorescein antibody (1:100; Roche Diagnostics, Basel, Switzerland). As a negative control, mouse IgG (1:1,000, Sigma-Aldrich) was used instead of the primary antibody. After incubation with these primary antibodies, cells were incubated for 30 min with fluorescence-labeled anti-mouse IgG or anti-goat IgG (Alexa Fluor, 1:2,000; Molecular Probes, Eugene, OR) as the secondary antibody. After washing with PBS, fluorescence was observed under a fluorescence microscope.

Extraction of total RNA and RT–PCR: Total RNA was isolated from primary sphere colonies, the adherent progeny of sphere colonies, and rabbit corneal epithelial cells before culture with a kit (Isogen; Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions, after which RT–PCR was done to investigate the expression of nestin, keratin-3, and glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) as a housekeeping gene. Then the isolated total RNA was treated with RNase-free DNase I (Stratagene, La Jolla, CA) for 30 min, and cDNA was formed by using Super Script II (Invitrogen) as the reverse transcriptase. T12VN primer (at a concentration of 25 ng/µl) was used to make the 1st strand. As the negative control, RT–PCR was performed in the absence of reverse transcriptase. The PCR buffer contained 1.5 mM $MgCl_2$ with 0.2 mM of each dNTP (Applied Biosystems, Foster City, CA), 0.2 mM of each primer, and 25 units/l of Amp Taq Gold (Applied Biosystems). After an initial 9 min of denaturation at 95 °C, amplification was performed for 30 cycles (30 s at 94 °C, 30 s at 60 °C, and 45 s at 72 °C), followed by a final 7 min of elongation using a thermal cycler (I-Cycler; Bio Lad Laboratories, Hercules, CA). The oligonucleotide primers for RT–PCR were based on the sequences of *p63*, nestin, ketarin-3, keratin-12, and *G3PDH*. The primer pairs and produce size are shown in Table 1. Products were separated on 1% agarose gel and then visualized by staining with ethidium bromide.

Statistics: Student's unpaired *t*-test was used to compare mean values. All analyses were done with the Stat View statistical software package (Abacus Concepts, Berkeley, CA) and $p < 0.05$ was considered to indicate significance.

RESULTS

Isolation of sphere colonies from single cells: We adapted the neurosphere-forming assay that was originally devised to enrich neural stem cells and other progenitors [20,21,24,25, 27-32] for isolation of adult stem cells from rabbit corneal limbal and central epithelium (Figure 1A). CEC were disaggregated into single cells and plated in uncoated wells with basal medium containing methylcellulose gel matrix to prevent reaggregation at a density of 10 viable cells/µl, as described elsewhere [24,25]. Under these conditions, sphere colonies are derived from proliferation and are not formed by reaggregation of dissociated cells [24,25]. Almost complete disaggregation into single cells was confirmed by counting the percentage of single cells, double cells, and triple cells, which demonstrated that more than 99% of the cells were single. Primary spheres were isolated from CEC derived from both the limbal and central regions. Photographs of representative spheres obtained from the limbal and central regions are shown in Figure 1B,C. When the number of sphere colonies obtained from the CEC was counted, there was a significantly greater number of spheres (55.6±10.6, mean ±standard deviation) obtained from the limbal region than from the central region (43.1±7.2) per 10,000 plated cells (Figure 2A). There were no statistically significant differences in the size of primary spheres from the two regions after 3, 5, and 7 days (Figure 2B).

Secondary sphere formation from primary spheres: To evaluate the self-renewal capacity of CEC, primary spheres were passaged under the same culture conditions as CEC. Secondary spheres were generated from dissociated primary spheres obtained from the limbal or central CEC. Photographs of representative secondary spheres are shown in Figure 3A.

The number of secondary spheres per 10,000 cells was significantly higher for primary spheres from the limbal region than from the central region (38.7±8.5 versus 31.3±5.7, respectively, p=0.013, unpaired *t*-test; Figure 3B).

Immunocytochemistry and RT–PCR of spheres: Nuclear protein p63 was recently proposed as a progenitor cell marker that can be used to identify epidermal stem cells [33]. Nestin is expressed by immature neural progenitor cells in multipotential sphere colonies derived from the brain [34], skin [22], inner ear [35], retina [36], corneal stroma [27], and endothelium [28-32]. To examine the potential of sphere colonies, primary spheres were immunostained for p63 and nestin as stem/progenitor cell markers. Additionally, p75 neurotrophin receptor (p75NTR), the nerve growth factor receptor, was used as a marker of epidermal basal progenitor cells [11]. Most cells in the spheres were immunoreactive for p63, p75[NTR], nestin, and BrDU (Figure 4A,B). The expression

of p63 and nestin was also confirmed by the detection of mRNA (Figure 4C). Spheres derived from both the limbal and central regions showed the same patterns of immunostaining and mRNA expression.

Immunocytochemistry of the progeny of individual spheres: To investigate whether the progeny of the spheres possessed the characteristics of mesenchymal or neural lineage cells, single spheres (day 10 of culture) were transferred onto poly L lysine/fibronectin-coated glass coverslips in 24-well plates, and then were cultured in differentiation medium containing 1% fetal bovine serum (FBS). After 7 days, many cells migrated out from the spheres. A small population of these migrating cells was p63- and nestin-positive, while most of the cells were positive for cytokeratin 3, a differentiated epithelial cell marker. The cells were also positive for MAP2 and NSE, indicating that the cells showed neuronal differentiation under these conditions (Figure 4D). Thus, culture of CEC generated p63- and nestin-positive progenitor cells that gave rise to epidermal and neuronal cells, suggesting the bipotency of these progenitor cells.
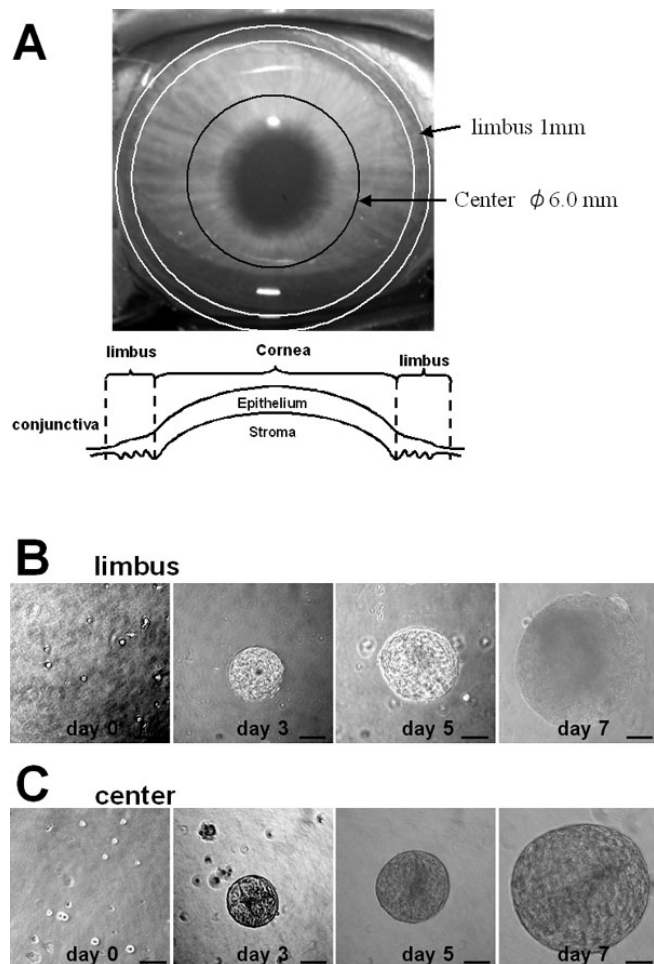

Figure 1. Sphere-forming culture of rabbit corneal epithelium. **A**: Anterior view of a rabbit cornea and a diagram of the corneal epithelium. **B**, **C**: Rabbit corneal epithelial cells from the limbus or central cornea formed spheres. Growth of a representative single sphere is shown until 7 day.
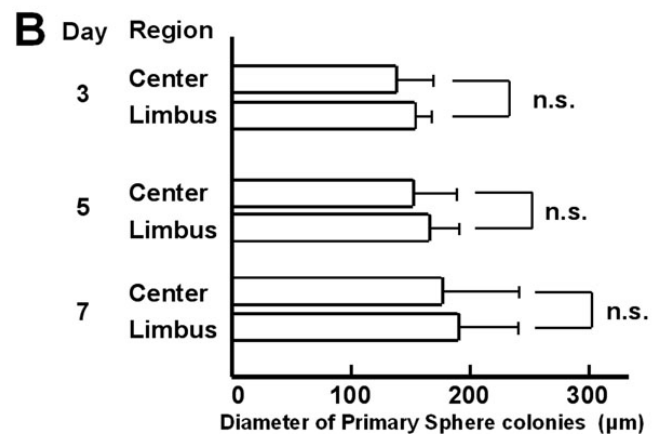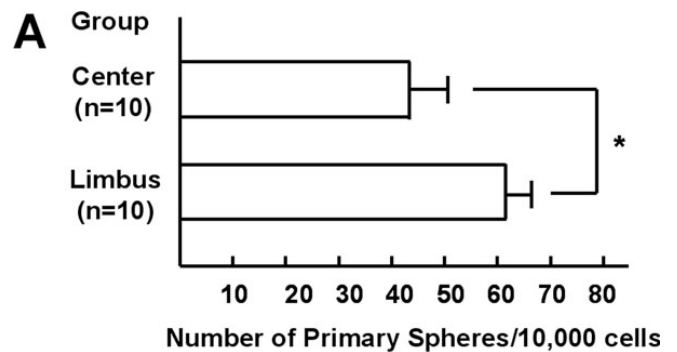

Figure 2. Comparison of primary sphere formation by rabbit CEC from the periphery and center of the cornea. **A**: The number of spheres obtained from limbal CEC (n=10) was significantly higher than that from central CEC (n=10) after 7 days of culture. The experiment was repeated twice and representative data are shown. The asterisk indicates a p=0.0028 (unpaired *t*-test). **B**: The mean sphere size exceeded 250 μm on day 7.

DISCUSSION

Considerable attention has been directed toward understanding the role of stem cells or progenitor cells in corneal development. Kruse and Tseng [17] demonstrated the differentiation of isolated corneal stem cells to transient amplifying cells. Yoshida et al. [19,21] isolated corneal precursors from the mouse cornea by sphere-forming assay. They found that the phenotype of mouse keratocytes can be maintained in vitro for more than 12 passages by the serum-free sphere culturing technique [21] and that neural crest-derived, multipotent stem cells exist in the adult cornea [19]. However, there have been few reports about the harvesting of stem cells or progenitor cells from the cornea by sphere-forming culture, and the only research concerning the isolation of tissue-specific stem cells from the corneal limbal epithelium by this method has been performed in rodents [20]. In our previous study, CEC isolated from the human corneal limbus did not form spheres in floating culture and became adherent to uncoated culture dishes [37]. This suggests that rabbit CEC may be fundamentally different from human cells. In addition, we have previously reported on the isolation of corneal progenitor cells from the corneal stroma [27] and endothelium by sphere-forming culture [28-32]. We found that the cells isolated from adult rabbit corneal epithelium were directed toward the ectodermal lineages and expressed markers for epidermal, and neural cells. To our knowledge, this is the first report about differentiation of progenitor cells isolated from the limbal region or the central cornea into epidermal and neural cells by floating culture.

In this study, we isolated progenitor cells from rabbit CEC by the sphere-forming culture method established by Toma et al. [22]. Spheres derived from rabbit CEC had a strong proliferative capacity as shown by BrdU staining and were positive for the epidermal progenitor cell marker p63, the epidermal basal progenitor cell marker p75NTR, and the neuron stem cell marker nestin. Self-renewing potential was indicated by the ability of the progeny of individual spheres to generate secondary spheres. Regarding the self-renewal capacity of these cells, we could not exclude the possibility that these progenitor cells did not have a uniform renewal capacity, with all of the growth coming from a rare subset of cells in the spheres. Additional studies will be required to determine whether or not these progenitor cells are adult stem cells.

It is also noteworthy that the spheres were positive for CEC markers (cytokeratins 3 and 12), and a neuronal marker (MAP2), indicating that epithelial and neuronal differentiation occurred in the spheres under serum-free culture conditions. Moreover, the progeny of the spheres also expressed epithelial and neuronal markers. The neurotrophin receptor p75NTR is most highly expressed in the progenitor cells of the corneal limbal epithelium [38]. Grueterich et al. [11] reported that p75NTR was localized in the suprabasal limbal epithelium and entire corneal epithelium, but was not detected in the limbal basal epithelium, suggesting that p75NTR can be considered a differentiated progenitor marker of corneal epithelium. These findings indicate that the spheres contained lineage-uncommitted bipotent progenitor cells.

Various studies have already been conducted on corneal epithelial stem cells by diverse methods [10-16,39]. A common limitation of studies on these cells has been the stem cell isolation procedure. Zhao et al. [20] offered a model for characterizing the neural potential of corneal stem cells by the sphere-forming assay. However, they failed to show that CEC stem cells can differentiate into the authentic epithelial lineage. In this study, we proposed the first successful isolation procedure for progenitor cells that mainly generated CECs positive for cytokeratins 3 and 12. Thus, precursors obtained from the corneal epithelium may be more appropriate than multipotential stem cells for tissue regeneration or cell transplantation, because such precursors should efficiently differentiate to produce their tissue of origin.

We demonstrated that the number of spheres derived from the limbal epithelium was significantly higher than that obtained from the central corneal epithelium, while there was no significant difference in the size of spheres from the limbal and central areas. The expression of differentiation markers such as a specific corneal marker (cytokeratin 3) was
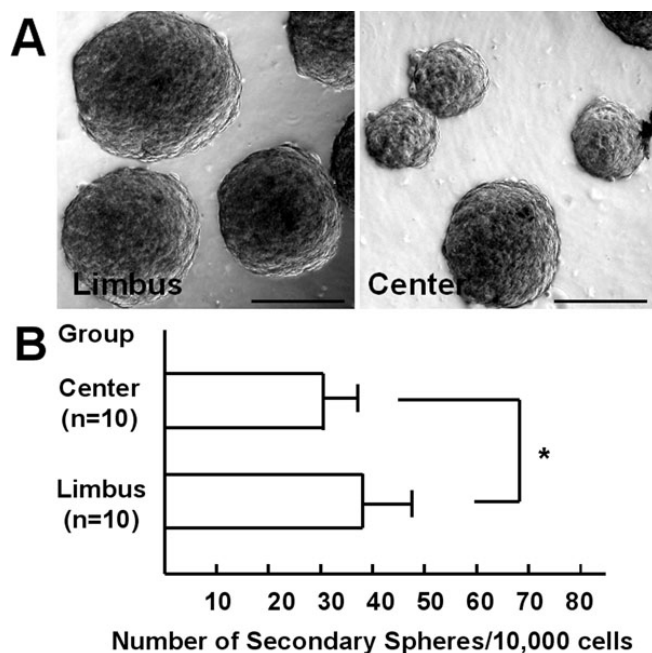

Figure 3. Formation of secondary spheres. **A**: Secondary spheres were generated from dissociated primary limbal or central spheres. **B**: The replating efficiency for formation of secondary spheres was higher when the cells were derived from the limbal cornea than from the central cornea (p=0.013, unpaired *t*-test).
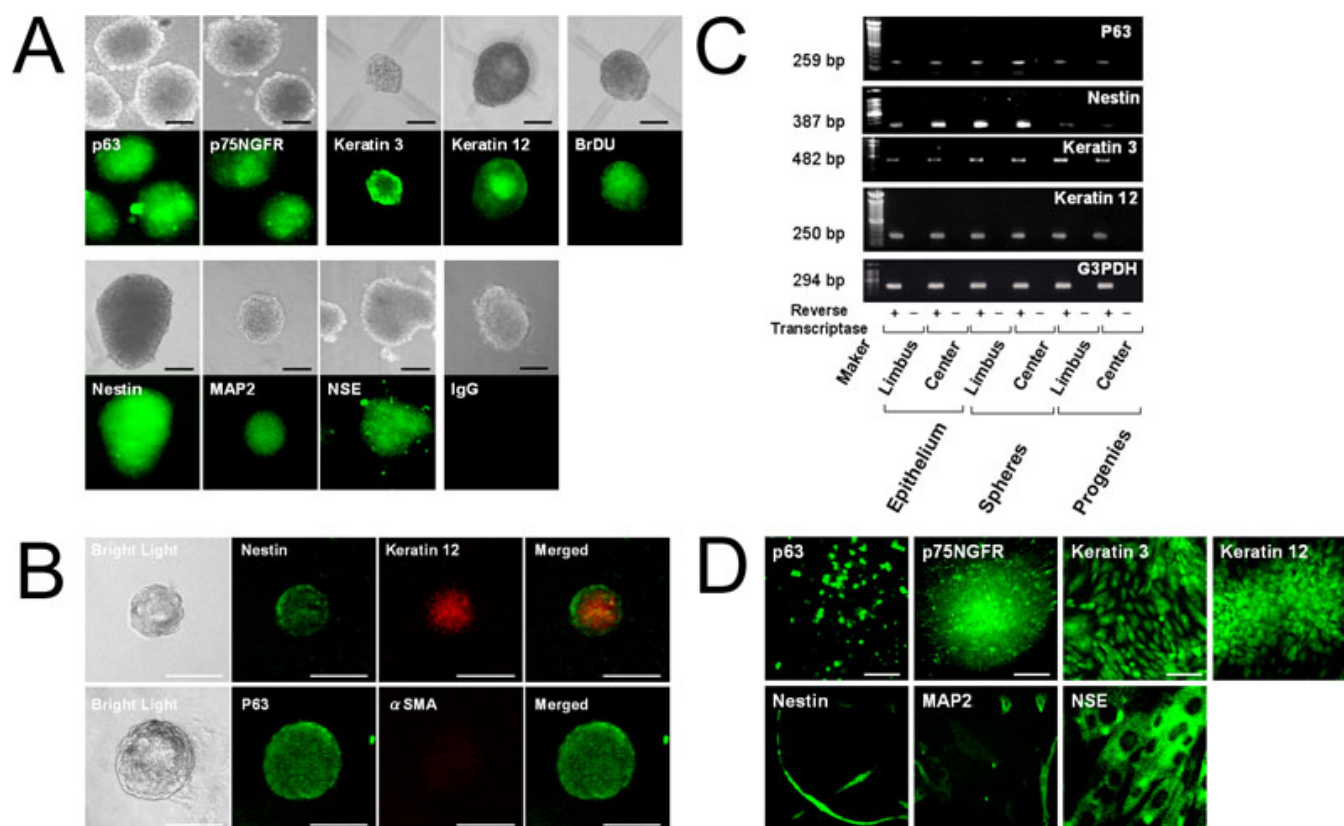
Figure 4. Analysis of spheres and their progeny. **A**: Immunocytochemical analysis of sphere colonies on day 7. Bright-field images and immunostaining of spheres for p63 (an epidermal stem/progenitor cell marker), p75[NTR] (an epidermal basal progenitor cell marker), cytokeratins 3 and 12 (differentiated epithelial cell markers, nestin (a neural stem cell marker), microtubule-associated protein 2 (MAP2: a differentiated neuronal cell marker), and neuron-specific enolase (NSE: a differentiated neuronal cell marker). Each colony has been labeled by BrdU. As a control, IgG was used instead of the primary antibody. Scale bar=100 μm. **B**: Double immunocytochemical staining of a sphere colony. The spheres are double immunostained by nestin and cytokeratin 12 or by p63 and alpha smooth muscle actin (αSMA). Scale bar=100 μm. **C**: RT–PCR of corneal epithelial tissues, spheres, and their progeny. Genes for *P63*, keratin 3, keratin 12, and nestin are present in corneal epithelial tissues, spheres, and their progeny derived from the limbal or central regions, but not in total RNA processed without reverse-transcription. **D**: Immunocytochemical analysis of differentiated cells obtained from spheres. Cells migrating out of the spheres express both cytokeratin 3 and cytokeratin 12 (differentiated epithelial cell markers). These cells are also positive for MAP2, and NSE. Scale bar=100 μm.

markedly higher in spheres derived from the central cornea compared with spheres from the limbal cornea (data not shown), indicating that cells composing spheres derived from the central corneal epithelium were more prone to undergo differentiation. These findings imply that limbal epithelium contains more stem or progenitor cells than the central epithelium and that spheres derived from limbal CEC proliferate while maintaining a more immature state in comparison with CEC from the central cornea. Conversely, even the transient amplifying cells or progenitor cells of the rabbit central cornea may have a finite proliferative potential and may differentiate into the epithelial and neuronal lineages. The present findings obtained by sphere-forming culture are consistent with the concept that the corneal limbus is rich in stem cells [7-9].

In summary, we isolated adult progenitor cells from rabbit corneal epithelium by sphere-forming culture and compared the relative abundance and self-renewal capacity of CEC progenitor cells obtained form the central and limbal regions of the cornea. Our findings demonstrated that both limbal and central CEC contain a significant number of progenitor cells, although the limbal region of the rabbit cornea has a higher density of progenitor cells with a stronger self-renewal capacity than the central region. Future our study will focus on the transplantation of autologous isolated sphere-forming progenitors. Proliferating sphere-forming cells derived from CEC may be useful for the treatment of corneal diseases associated with limbal stem cell deficiency.

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