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Anatomical location and culture of equine corneal epithelial stem cells

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Abstract

Objective To identify morphologically the locations of equine corneal epithelial stem cells (CESCs) and to culture these cells.

Animals studied We studied the eyes of 12 adult thoroughbred horses.

Procedures Eye tissues were immunostained for two positive stem cell markers (p63, CK14) and one negative marker (CK3) to identify the locations of CESCs, so we could compare their immunostaining patterns with those of human stem cells previously reported. We compared the proliferation rates and morphological features of epithelial cells isolated from the corneal limbus and central cornea.

Results Undifferentiated cells expressing the same immunostaining pattern as human CESCs were present in the equine corneal limbus. Cultured epithelial cells isolated from the limbus expressed the same immunostaining pattern that CESCs show histologically, but cells isolated from the central cornea did not proliferate and could not be evaluated.

Conclusions Equine CESCs were localized in the epithelial basal layer of the corneal limbus, where melanocytes reside. They could be cultured without loss of their undifferentiated nature. When collecting such stem cells, it may be useful to harvest and culture corneal epithelial tissues in the limbus where melanocytes serve as an indicator of the collecting area.

Key Words: corneal epithelial cells, corneal epithelium, horse, limbus, localization of corneal epithelial stem cells, stem cell

INTRODUCTION

Racehorses have a high incidence of corneal injuries because their proximity to each other when racing can result in trauma. Over 3000 Japanese racehorses experienced corneal disorders in the 12 years from 1997 to 2008.[1] Most of these cases were superficial traumatic ulcers caused by injury from materials on the track surface kicked up by other horses during races. Some horses with these injuries take a long time to heal and return to racing.[1] Others end their careers early because of reduced vision due to scars and opacity in the cornea, even if they do not become blind.

There are a number of therapeutic approaches to such medically uncontrollable corneal disorders in horses, including amniotic membrane transplantation and penetrating keratoplasty.[2–4] Targeted lamellar keratoplasty was reported recently as being a successful therapy.[5] However, most of these treatments use allotransplantation, which has associated problems with graft rejection and difficulties in maintaining a stable supply of grafts.[4,6,7]

In humans, cultivated corneal epithelial transplantation has been used recently in the treatment for intractable corneal disorders, such as those associated with Stevens–Johnson syndrome.[8–12] This method of transplantation is minimally invasive for donor eyes, because grafts can be prepared from small tissue fragments containing stem cells. Therefore, even for human patients whose corneal epithelial stem cells (CESCs) are extensively and severely damaged, grafts for autotransplantation can still be prepared as long as even small amounts of stem cells remain. Furthermore, not only corneal epithelium but also oral mucosal epithelium could be sufficient as sources of stem cells.[8–17] Cultivated corneal epithelial transplantation can

simultaneously solve many of the problems associated with allotransplantation, such as posttransplantation rejection and the shortage of donors.[3,4,17,18] It is also known that human CESCs reside in the basal region of the limbus and are involved in the renewal and regeneration of the corneal epithelium.[17,19,20]

To our knowledge, there have been no reports of the localization of equine CESCs or of cultivated corneal epithelial transplantation in horses. As the tissue repair process in equine corneal disorders is similar to that in humans, we can infer that equine CESCs and their niche should be present in the equine corneal limbus and that, as in humans, they are involved in the maintenance of corneal repair and homeostasis.[21–23]

The objective of this study was to identify morphologically the location of equine CESCs based on the assumption that they reside in the basal epithelial layer of the corneal limbus, as in humans. A further objective was to obtain CESCs from among the equine limbal epithelial cells (ELECs) on the basis of this hypothesis and culture them.

MATERIALS AND METHODS

Cross-reactivity of primary anti-human antibodies with horse

To immunohistochemically identify equine CESCs, we selected three stem cell markers, p63, cytokeratin (CK) 14, and CK3. The p63 and CK14 are positive markers for general stem cells in humans, and CK3, a corneal epithelial differentiation–associated marker, is a negative marker for CESCs.[8,10,24–28] Although anti-human p63 and CK14 antibodies are known to cross-react with horse epithelium, there are no reports of cross-reactivity of anti-human CK3 antibody.[29,30] Therefore, we confirmed that anti-human CK3 antibody would also cross-react with equine corneal epithelium by immunoblotting.

We used frozen human corneal tissues supplied by SightLife Eye Bank (Seattle, WA, USA) and frozen equine corneal tissues collected from an adult thoroughbred horse for samples. These frozen tissues were pulverized by mixer mill (MM200; Retsch GmbH, Hann, Germany) under freezing conditions. Next, total proteins from each samples were extracted in manufactured SDS sample buffer (B7709S; New England BioLabs, Ipswich, MA, USA) with protease inhibitors (Complete Mini Protease Inhibitor Cocktail Tablets; Roche Diagnostics GmbH, Mannheim, Germany), followed by centrifugation at 4 °C, 12 000 × ***g*** for 5 min to collect the supernatant which was boiled at 100 °C for 5 min.[29] Each protein was separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane. Immunoblots were blocked with 2.5% casein and incubated with anti-human CK3 antibody (AE5; Progen Biotechnik GmbH, Heidelberg, Germany) (1:400), followed by alkaline phosphatase–conjugated anti-mouse IgG secondary antibody (1:100). Then, the electrophoretic membrane was colorized by nitro blue tetrazolium (NBT/BCIP solution kit; Nakarai Tesque, Kyoto, Japan) to enhance visualization.

Localization of CESCs in the equine corneal limbus

Eyeballs were collected from adult thoroughbred horses that were being killed in a slaughterhouse and were immediately transported to our laboratory in phosphate-buffered saline. After confirming that the tissues were free of disease by gross macroscopic examination, we dissected the eyeballs transversely and fixed them overnight in methacarn fixative (methanol–chloroform–acetic acid at a volume ratio of 6:3:1). These steps were completed within 1 h after death. The trimmed and fixed eyeballs were embedded in paraffin blocks and sectioned at 4-μm thickness. We immunohistochemically identified the locations of three proteins in the corneal limbus: p63, cytokeratin (CK) 14, and CK3. Before immunostaining, sections were pretreated with 'target retrieval solution' (pH 9; Dako, Glostrup, Denmark) for p63 and 6 *M* urea for CK14 and CK3. Following retrieval of antigens, sections were prepared with 0.5% periodic acid for blocking of endogenous peroxidase activity and 0.5% casein to block nonspecific binding. Then, sections were reacted with the each primary antibody, anti-p63 antibody (4A4; Dako, Glostrup, Denmark) (1:250), anti-CK14 antibody (LL002; Novocastra Laboratories Ltd, Newcastle, UK) (1:40), and anti-CK3 antibody (AE5; Progen Biotechnik GmbH, Heidelberg, Germany) (1:50). As secondary antibodies, fluorescein isothiocyanate–conjugated polyclonal rabbit anti-mouse immunoglobulins (Dako, Glostrup, Denmark) (1:40) were used. Finally, after nuclear counterstaining with propidium iodide, we observed sections using confocal laser scanning microscopy (LSM510; Carl Zeiss GmbH, Jena, Germany).

Isolation and culture of equine corneal epithelial cells

Corneal epithelial tissues were harvested from the horses as described previously ($n = 7$) and from adult thoroughbred horses euthanized for reasons other than ocular disease at the Equine Research Institute of the Japan Racing Association ($n = 5$). The tissue was divided into 2 groups: Tissue harvested from the corneal limbus and containing ELECs (group L, $n = 12$) and tissue harvested from the central cornea and containing no ELECs (group C, $n = 12$). A 50-mm^2 piece of corneal epithelium was collected from each of the limbi and central corneas of the same eye of each horse at the same time. In group L, after removal of the conjunctiva from the corneal limbus with corneal scissors, we performed a lamellar keratectomy of the limbal epithelium, stripping a piece 1 mm wide by 50 mm long with an ophthalmic scalpel. In group C, we performed a lamellar keratectomy of the central epithelium, stripping a piece 5 mm wide by 10 mm long with an ophthalmic scalpel. Corneal epithelial cells (CECs) were isolated from these tissues using dispase II

(1000 U/mL GD81070; Sanko Junyaku Co., Ltd.; now Eidia Co., Ltd., Tokyo, Japan) and a 0.25% trypsin EDTA solution (Gibco, Grand Island, NY, USA). The isolated cells were plated onto a 35-mm-diameter culture plate (which had been preseeded with feeder cells) at a density of 2×10^4 cells/well. They were then cocultured with standard supplemented hormone epithelial medium (SHEM), consisting of Dulbecco's modified Eagle's medium (D6429; Sigma, St. Louis, MO, USA), Ham's F12 (N6658; Sigma), fetal bovine serum (Equitech-Bio, Kerrville, TX, USA), penicillin–streptomycin (Gibco, Grand Island, NY, USA), insulin (Wako, Osaka, Japan), cholera toxin (227041; Calbiochem, Darmstadt, Germany), gentamicin (Gibco, Grand Island, NY, USA), hydrocortisone (Wako, Osaka, Japan), dimethylsulfoxide (D2650; Sigma), and epidermal growth factor (Invitrogen, Grand Island, NY, USA).[9,19,31] Plating was completed within 6 h after death. The SHEM medium was exchanged three times per week throughout the culture period. The proliferation rate and morphological features of the cultured cells were observed and recorded until confluence occurred.

To prepare the feeder cells, we plated NIH/3T3 mouse fibroblasts treated with 8 μg/mL mitomycin-C at a density of 2×10^5 cells/well and incubated them at 37 °C for 1 h, then cultured them for 24 h at 37 °C in 5% CO_2–95% air.

To identify the immunohistochemical staining patterns of the cultured cells, we additionally plated the cells at 1.66×10^4 cells/well onto 2-well chamber slides (20 mm × 20 mm) preseeded with feeder cells and then cocultured them in standard SHEM medium as above.[9,19,31] When confluence was achieved (day 22), the cells were immunostained for p63 (1:125), CK14 (1:40), and CK3 (1:50) as described previously.

RESULTS

Cross-reactivity of primary anti-human antibodies with horse

Both horse and human samples reacted with anti-human CK3 antibody to produce a band of approximately the same size (Fig. 1).

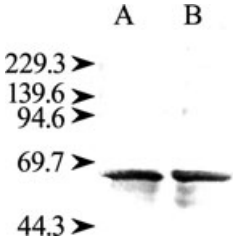

Figure 1. Immunoblotting of corneal epithelium with anti-CK3 antibody (AE5). Lane A, human; B, horse. Markers are about the same molecular weight.

Localization of CESCs in the equine corneal limbus

In sections stained for p63, the nuclei of 2–3 layers of corneal epithelial basal cells located apically from the basolateral side stained positive, consistent with the region where melanocytes are present in the corneal limbus, (Fig. 2b,B).

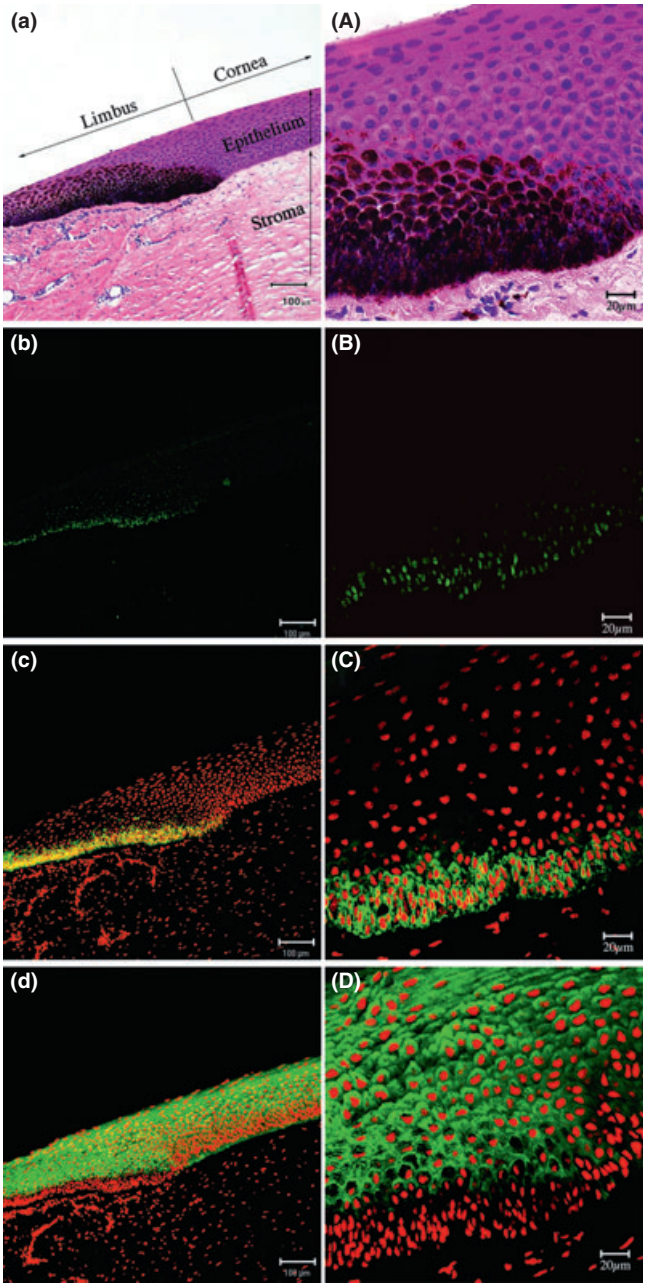

Figure 2. Distribution of cells expressing stem cell markers in the corneal limbus. (Capital letters indicate higher magnification of images labeled with lower-case letters.) Stains: (a, A) hematoxylin and eosin, (b, B) anti-p63, (c, C) anti-CK14, and (d, D) anti-CK3. Nuclei were costained with propidium iodide (red in c, C, d, D). p63 expression was evident in the nuclei of the basal epithelium in the limbus (green in b, B). CK14 expression was evident in the cytoplasm of the basal epithelium in the limbus (green in c, C). CK3 expression was evident in other epithelium, but not in the basal epithelium in the limbus (green in d, D).

In sections stained for CK14, again the cytoplasms of 3–4 layers of corneal epithelial basal cells located apically from the basolateral side were uniformly densely stained, consistent with the region where melanocytes are present in the corneal limbus (Fig. 2c,C). In sections stained for CK3, the cytoplasms of all CECs except for 4–6 layers of basal cells in the region where melanocytes are present in the corneal limbus were densely stained. The staining was strongest in the cells in the interlayers and tended to be weaker in the cells on the apical side as well as in the basal layer (Fig. 2d,D).

Isolation and culture of equine CECs

We collected 2.01 ± 0.67 (SD) × 10^6 nucleated cells from the 50-mm^2 tissue samples from group L and 0.730 ± 0.034 × 10^6 nucleated cells from group C samples. Group L cells formed colonies several days after plating in 10 of the 12 samples, and confluence on the culture plate occurred at day 21.7 ± 4.4 (SD) (Table 1). In the remaining 2 samples, proliferation stopped after colonies were formed. The cells in group C formed a few colonies, but confluence was never reached in any of the 12 samples.

The colonies of group L were approximately circular and were composed of cells of relatively small and uniform size (Fig. 3a). They developed closely apposed polygonal cells with a cobblestone-like morphology characteristic of epithelial cells (Fig. 3c). The colonies of group C had a distorted shape and were composed of larger cells that were inconsistent in size (Fig 3b,d).

Immunohistochemistry could only be performed on group L cells, which consisted of ELECs. Cultured ELECs stained for p63 showed staining in the nuclei of almost all of the cells, tending to be stronger in smaller cells (Fig. 4b). The cytoplasms of nearly all cells stained for CK14 were uniformly densely stained (Fig. 4c). Only a few large cells showed faint staining for CK3, and most were not stained (Fig. 4d).

Table 1. Days required for corneal epithelial cells isolated from each group to reach confluence (35-mm culture plate; seeding density, 2 × 10^4)

Horse No.	Group L	Group C
1	27	–
2	16	–
3	30	–
4	18	–
5	22	–
6	20	–
7	25	–
8	16	–
9	–	–
10	21	–
11	–	–
12	22	–
Mean ± SD (tissues/total)	21.7 ± 4.4 (10/12)	ND (0/12)

–: Confluen ce was not reached.

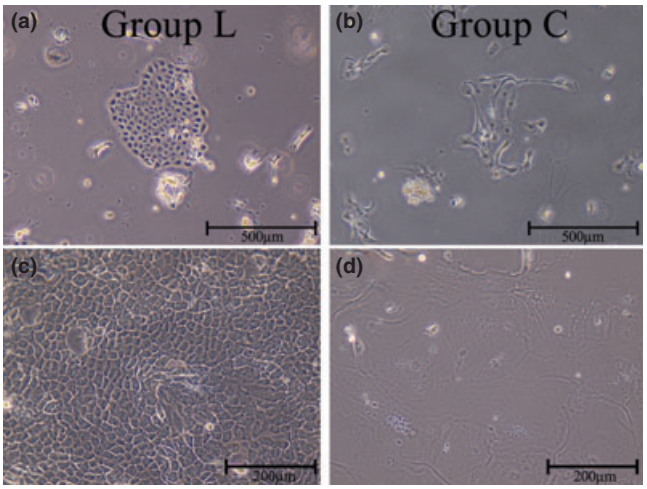

Figure 3. Colony characteristics of cells isolated and cultured from groups L and C, and cell morphologies. (a) Colonies of group L at day 7. (b) Colonies of group C at day 9. (c) Cell morphology of group L at day 16. (d) Cell morphology of group C at day 16.

DISCUSSION

To collect CESCs for the preparation of autografts for cultivated corneal epithelial transplantation in horses, we first needed to identify their location in the cornea. We hypothesized that they reside in the basal layer of the limbic epithelium, at the junction between the cornea and sclera as in human eyes. In cross-sectional samples that included the sclera, limbus, and cornea, we successfully located them by immunostaining with three stem cell markers.

Human CESCs are characterized by their expression of markers such as p63, CK14, CK19, ABCG2, and integrin α9, and the absence of corneal epithelial differentiation–associated markers such as CK3 and CK12.[10,25] Moreover, they are slow-cycling cells.[32] p63, which we used as a positive marker, is a nuclear transcription factor within the p53 family. p63 is deeply involved in the proliferative potential of human keratinocytes, being expressed in the basal cells of various human epithelial tissues, and is a stem cell marker.[13,14,24–26,31,33] CK14 is useful for identifying epithelial progenitor cells with basal cell activity and is also a stem cell marker.[10] In contrast, CK3 is not expressed in the basal layers of the limbal epithelium but is expressed in the mature corneal epithelium; therefore, the absence of CK3 is consistent with cells that may be CESCs.[10,34] Among these stem cell markers, anti-human p63 and CK14 antibodies were previously known to cross-react with equine epithelium, and we confirmed that CK3 antibody cross-reacts with equine corneal epithelium in this study. So, it may be useful to identify equine CESC using these three stem cell markers as is done in humans.

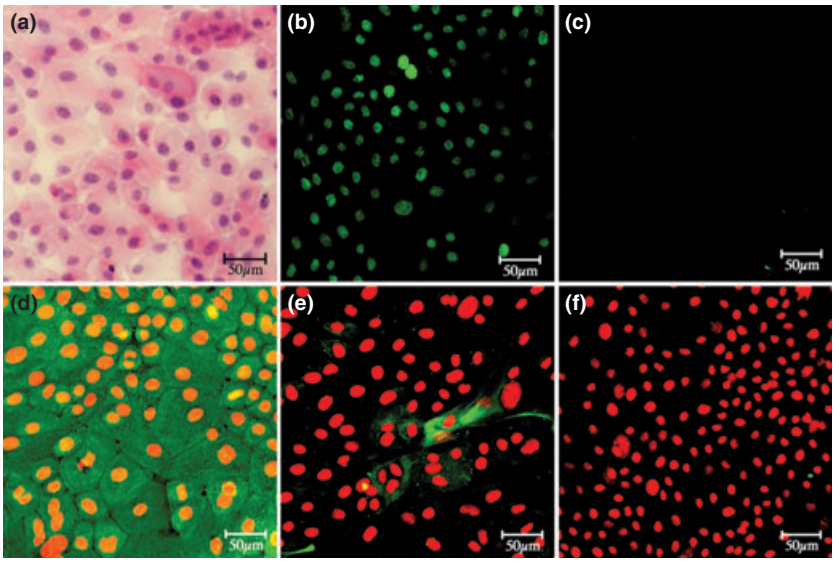

Figure 4. Immunohistochemistry of cultured ELECs (day 22). (a–c) Single stains; (d–f) nuclei were costained with propidium iodide (red). Stains: (a) hematoxylin and eosin, (b) anti-p63 (green), (c) no stain (negative control), (d) anti-CK14 (green), (e) anti-CK3 (green), (f) negative control. p63 expression was confirmed in the nuclei of almost all cultured ELECs. Cytoplasms of all cultured ELECs uniformly expressed CK14 densely. A few large cells faintly expressed CK3, but most did not.

Equine corneal immunostaining showed positive expression of p63 and CK14 in the epithelial basal layer but negative expression of CK3, consistent with the region of the limbus where melanocytes reside. This expression pattern is similar to that of epithelial basal cells of the corneal limbus, which are considered in humans to be CESCs, thus indicating that CESCs are also distributed in the epithelial basal layer of the equine corneal limbus.[10] Moreover, CESCs expressing these markers are not seen in any regions other than the limbus, suggesting that the distribution of CESCs in horses is more similar to that of humans or rats than to that of mice.[19,35–38] Specifically, the ELECs contain equine CESCs and undifferentiated cells close to those stem cells, whereas the CECs in the central region consist of advanced differentiated cells closer to terminal differentiated cells. In horses, as in humans, we can infer that tissue repair and homeostasis are maintained as CESCs included among the ELECs migrate toward the central cornea while differentiating into terminal differentiated cells.

In humans, the basal layer of corneal epithelium, in which CESCs are thought to reside, contains a wrinkled structure referred to as the palisades of Vogt (POV).[39] In people with pigmented skin, the POV are easily visible under a magnifying loupe because of the deposition of melanin pigment. Identification of the POV structure macroscopically is considered to be a simple and convenient clinical indicator of the presence or absence of CESCs.[39] On the other hand, in the horse eyeball, which lacks the POV, the distribution of CESCs expressing p63 and CK14 and not expressing CK3 corresponds approximately to the region containing melanocytes in the limbal corneal epithelium, suggesting that ELECs that include stem cells can be efficaciously harvested using melanocytes as a location indicator.

The CECs in group L showed high proliferative activity in 10 of 12 tissue samples, whereas in group C, which did not contain ELECs, proliferation stopped in all samples. This indicates a clear difference in proliferative potential and colony characteristics between the two groups. It is well known that small keratinocytes are capable of active proliferation and that proliferation becomes less active as the cell becomes larger.[40] Furthermore, depending on differences in colony-forming ability during single-cell culture of keratinocytes and in the frequency of terminal colonies, there are three different clonal types: holoclone, meroclone and paraclone.[26,41] Differentiation advances from the holoclone, which has higher proliferation potential and is closest to stem cells, to the meroclone and then to the paraclone.[41,42] This classification leads us to infer that the small size and active proliferation potential of the cells isolated from group L, which consisted of ELECs, correspond to the features of holoclones. In contrast, the large size and lower proliferation activity of the cells isolated from the central cornea correspond to the features of paraclones or meroclones, which are at a more advanced stage of differentiation. These inferences suggest that the ELECs isolated from the corneal limbus included stem cells in large numbers.

To corroborate our inferences, we examined the expression patterns of the stem cell markers in cultured ELECs. In cells stained for p63, the nuclei of almost all of the cultured ELECs were stained, but small cells tended to be more densely stained than large cells, suggesting that the small cells had greater proliferative activity. In cells stained for CK14, the cytoplasms of almost all of the cultured ELECs were uniformly densely stained. In cells stained for CK3, apart from a few large cells, no cultured ELECs were stained. These expression patterns were the same as those of CESCs localized in the corneal limbal tissue, demonstrating that the cells grown from the ELECs were equine CESCs. This result suggests that culture of ELECs isolated from the equine corneal limbus allows the ELECs to proliferate while maintaining the

undifferentiated state of the CESCs included among the ELECs.

We were able to grow ELECs to cover a 35-mm culture dish from cells harvested from the corneal limbus and plated at only 2×10^4 cells/well. In the future, for clinical application of cultivated corneal epithelial transplantation and to allow for processing losses, it should be possible to prepare a cultured cell sheet for transplantation even if only 1-mm^2 of limbal epithelial tissue remains in the donor.

In conclusion, we have shown that CESCs required for the preparation of cultured equine CEC sheets are localized in the epithelial basal layer of the corneal limbus where melanocytes reside. Moreover, we showed that ELECs including undifferentiated CESCs can be isolated by harvesting corneal epithelial tissue identified in association with melanocytes. We further found that by culturing the isolated ELECs, the CESCs among them could be cultured without loss of their undifferentiated state.

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