

Differences Between Niche Cells and Limbal Stromal Cells in Maintenance of Corneal Limbal Stem Cells

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PURPOSE. To investigate the differing characteristics of limbal niche cells (LNCs) and limbal stromal cells (LSCs) in the maintenance of limbal epithelial stem/progenitor cells in the cornea.

METHODS. Limbal niche cells were obtained from direct dissection of the human corneal limbus, and LSCs were obtained from explant cultures of limbal stromal tissues under the same culture conditions. The resulting cultures were examined for their ability to support the growth of limbal stem/progenitor cells in colony-forming capacity, stratified epithelial cell sheet formation, maintenance of limbal epithelial stem/progenitor cell characteristics, and gene expression levels of factors that supported the limbal epithelial stem/progenitor cells.

RESULTS. The colony-forming efficiency of limbal epithelial stem/progenitor cells in the LNC group ($6.57 \pm 1.54\%$) was significantly higher than that in the LSC group ($1.43 \pm 0.47\%$). The epithelial cell sheets in the LNC group stratified into four or five layers compared with two or three stratified layers in the LSC group. Staining of both the colonies and the epithelial cell sheets in the LNC group showed a higher intensity of the limbal stem cell marker $\Delta Np63$ than in the LSC group. Moreover, reverse transcription polymerase chain reaction analysis revealed that compared with the common expression of *EGF* and so on, the LNCs showed a higher expression level of E-cadherin and a lower expression level of neurotrophin-3 (*NT3*) than the LSCs.

CONCLUSIONS. LNCs have a different role compared to LSCs in their ability to support epithelial stem/progenitor cells and epithelial cellular sheet formation.

Keywords: limbal stem cells, niche cells, stromal cells, colony forming efficiency

Increasing evidence indicates that interactions with the microenvironment are essential for maintaining and activating adult stem cells, such as intestinal,¹ hair follicle,² and neural stem cells.³ The stem cell niche is a special microenvironment composed of cellular and extracellular components, including specialized extracellular matrix (ECM) and secreted molecules. The interactive crosstalk between the surrounding cells, ECM, and soluble signals is critical for stem cell homeostasis or activation. Identification and characterization of adult stem cell niches are helpful for understanding niche regulation of stem cell self-renewal and fate decisions and have substantial potential for clinical application of stem cells in regenerative medicine.⁴

Corneal epithelium is believed to be maintained by limbal stem/progenitor cells located in the anatomical palisades of Vogt and to reside within a niche called limbal crypts in humans.⁵ The stem/progenitor cells in the limbal basal layer exhibit a slow-cycling label-retaining property⁶ and high proliferative potential, and preferentially express putative epithelial stem cell markers including $\Delta Np63\alpha$,^{7,8} ABCG2,⁸ $\beta 1$ -integrin,^{9,10} and N-cadherin¹¹; however, there are no differentiated corneal epithelium markers such as keratin 3/12 and connexin 43.^{9,10} The neighboring cells in the limbal niche include melanocytes,¹² antigen-presenting Langerhans cells,¹³ suppressor T-lymphocytes,¹³ and recently identified

limbal niche cells (LNCs).¹⁴ Moreover, the crypt structure extending into limbal stroma without Bowman's membrane in the limbal area suggests that limbal stem cells might interact closely with cells in the underlying limbal stroma, where a highly cellular and distinct vascular supply surrounds the limbal crypts.¹⁵

Limbal niche cells that are closely associated anatomically with limbal basal cells have also been isolated and characterized recently as maintaining limbal stem/progenitor cells via stromal cell-derived factor and its receptor CXCR4 (SDF-1-CXCR4) signaling.^{16–18} Previous reports have shown that LSCs located in limbal stroma can also support proliferation of limbal stem/progenitor cells^{19,20} and even induce differentiation of hair follicle or embryonic stem cells into corneal epithelial-like cells,^{21,22} which suggests that the LSCs may be another important cellular population in the limbal stem cell microenvironment. Because of the close anatomical location and the common capacity to support limbal stem/progenitor cells, these two types of cells have often been confused in the research. Distinguishing LNCs and LSCs has great significance for further understanding of the structure and function of the limbal niche. However, there have been no direct reports on the differences between these two cellular populations. In the current study, we isolated LNCs and LSCs, identified differences in gene expression, the ability to support colony forming and

TABLE. Primers Used in Reverse Transcription-PCR

Gene	Direction	Primer Sequence (5'→3')	Product Size, bp
<i>Vim</i>	Forward:	GGCTCAGATTTCAGGAACAGC	327
	Reverse:	GCTTCAACGGCAAAGTTCTC	
<i>K3</i>	Forward:	CATCATTGCTGAAGTTGGTGC	291
	Reverse:	TCTTGGAGCTTGGCATTGG	
<i>K4</i>	Forward:	TTTGTGGTCTTAAAGAAGGACGTG	643
	Reverse:	TGGCATTCTCCAGACATTCTGTAC	
<i>GAPDH</i>	Forward:	TCCAGAACATCATCCCTGCCTCTA	255
	Reverse:	TGTTGAAGTCAGAGGAGACCACCTG	
<i>NT3</i>	Forward:	TACGCGGAGCATAAGAGTCAC	332
	Reverse:	GGCACACACACAGGACGTGTC	
<i>E-cad</i>	Forward:	CGACCCAAACCAAGAATCTA	172
	Reverse:	AGGCTGTTGCCTTCCTACAGA	
<i>EGF</i>	Forward:	AATGTGAGATGGGTGTCCAGTGT	236
	Reverse:	AGGGCAAATCAGTCCTGGTTCAGA	
<i>FGF2</i>	Forward:	AAGAGCGACCCTCACATCAAGCTA	236
	Reverse:	TACTGCCAGTTCGTTTCAGTGC	
<i>EPR</i>	Forward:	AGGAGGATGGAGATGCTCTG	498
	Reverse:	TCAGACTTGCGGCAACTCTG	
<i>HGF</i>	Forward:	GCCTGAAAGATATCCCGACA	523
	Reverse:	TTCCATGTTCTTGTCCCAACA	
<i>KGF</i>	Forward:	AGGCTCAAGTTGCACCAGGCA	495
	Reverse:	TGTGTGTCGCTCAGGGCTGGA	
<i>NGF</i>	Forward:	GAGGTGCATAGCGTAATGTCCA	233
	Reverse:	TCCACAGTAATGTTGCGGGTCT	
<i>GDNF</i>	Forward:	GCCCTTCGCGTTGAGCAGTGAC	343
	Reverse:	GTCGTACGTTGTCTCAGCTGC	
<i>BDNF</i>	Forward:	AACAATAAGGACGCAGACTT	222
	Reverse:	TGCAGTCTTTTGTCTGCCG	
<i>N-cad</i>	Forward:	CACCCAACATGTTTACAATCAACAATGAGAC	444
	Reverse:	CTGCAGCAACAGTAAGGACAAACATCCTATT	
<i>IPO13</i>	Forward:	CCCTGAGGCACCTACTGT	478
	Reverse:	AGGGTCCAGGTTTACTCTTT	

Vim, vimentin; *K3*, cytokeratin 3; *K4*, cytokeratin 4; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *NT3*, neurotrophin-3; *E-cad*, E-cadherin; *EGF*, epidermal fibroblast growth factor; *FGF2*, fibroblast growth factor 2; *EPR*, epi-regulin; *HGF*, hepatocyte growth factor; *KGF*, keratinocyte growth factor; *NGF*, nerve growth factor; *GDNF*, glial cell-derived neurotrophic factor; *BDNF*, brain-derived neurotrophic factor; *N-cad*, N-cadherin; *IPO13*, importin 13.

stratified epithelial cellular sheet formation, and the maintenance of limbal epithelial stem/progenitor cellular characteristics using the two cells as feeder layers.

METHODS

Isolation of Limbal Epithelial Cells, Niche Cells, and Stromal Cells

All human corneas ($n = 6$) were obtained from the Northwest Lions Eye Bank (Seattle, WA) and handled according to the Declaration of Helsinki. Limbal niche cells and LSCs isolated from the same cornea were used for comparison. We performed a comparison of the feeder cells three times in the current study. After the central cornea was removed for penetrating keratoplasty, the limbal tissues were treated with 2.4 U/mL dispase (BD Biosciences, Bedford, MA) for 1 hour at 37°C and 0.02% EDTA solution (Nacalai Tesque, Kyoto, Japan) for 2 minutes at room temperature. The limbal cells were scraped mechanically and incubated with 0.25% trypsin-EDTA (Invitrogen, Grand Island, NY) for 15 minutes at 37°C. To culture primary limbal epithelial cells, the scraped cells were suspended in keratinocyte culture medium (KCM) composed of Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM/F12, 3:1), 5% fetal bovine serum (FBS; Invitrogen), 1 nM cholera toxin (Calbiochem, La Jolla, CA), 2 nM triiodothy-

ronine (Takeda, Osaka, Japan), 0.4 µg/mL hydrocortisone (Kowa, Tokyo, Japan), 1% insulin-transferrin-selenium supplement (Invitrogen), 10 ng/mL human recombinant epidermal growth factor (EGF; Sigma-Aldrich, St. Louis, MO), 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen). To culture LNCs, the scraped limbal cells were suspended in KCM and cultured for 10 to 14 days until outgrowth of fibroblastic cells around the epithelial cellular colony. All epithelial cells were scraped off for selective purification of fibroblastic niche cells. To culture LSCs, limbal explants after the removal of anterior stroma (containing residual epithelium) and posterior stroma (containing corneal endothelium) were incubated in the DMEM supplement with 10% FBS until fibroblast outgrowth. Both LNCs and LSCs were passaged and cultured in 10% FBS-DMEM at 37°C in 5% CO₂, and the medium was changed every 3 days.

Preparation of Feeder Layers

To examine the different sources of support for limbal stem/progenitor cell proliferation among NIH/3T3 cells (ATCC CRL-1658), LNCs, and LSCs, the three cell types (the second passage for LNCs and LSCs) were mitotically inactivated by incubation with 8 µg/mL mitomycin C (MMC; Kyowa Hakko, Tokyo, Japan) for 2 hours at 37°C. To determine the optimal concentration for the LNC and LSC feeder layer, 2×10^4 , 1.5×10^4 , 1.0×10^4 , 0.8×10^4 , and 0.5×10^4 cells/cm² of feeder cells

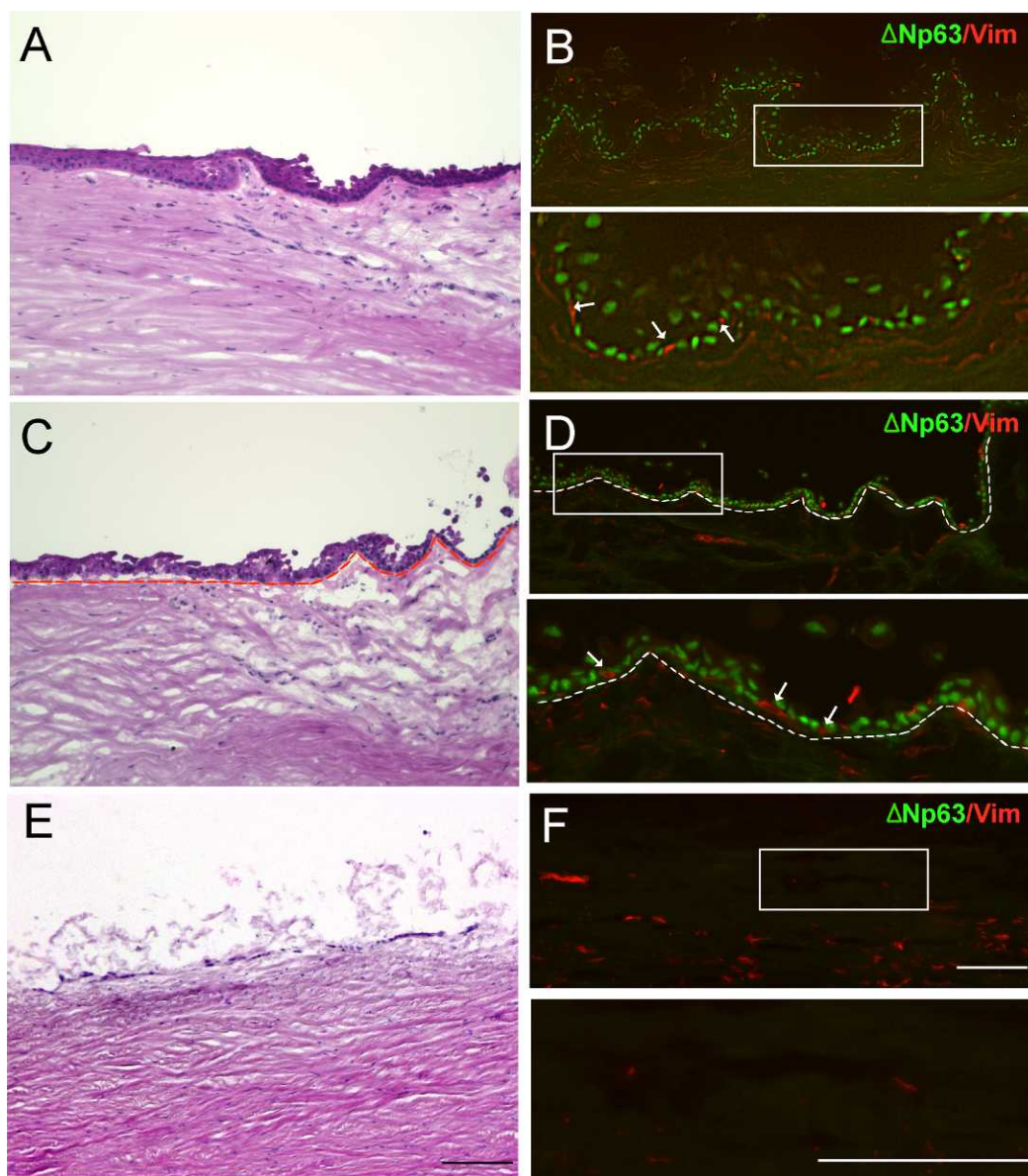


FIGURE 1. Identification of limbal stem cells, niche cells, and stromal cells in situ. (A) Normal limbal tissue maintained the typical stratified epithelium and underlying high cellularity in limbal stroma. (B) p63+ limbal epithelial stem/progenitor cells and vimentin+ limbal mesenchymal cells (arrows) coexisted in the basal epithelial layer of the limbal crypts (rectangle outlined in white shown in a high-magnification view at bottom). The limbal epithelium was loosened from the stroma after dispase treatment only (C); it contained both p63+ limbal epithelial cells and vimentin+ mesenchymal cells (arrows). (D) Loosened cells above dotted line were collected by scraping. After dispase treatment and scraping, the limbal tissue contained few p63+ epithelial cells or vimentin+ mesenchymal cells in the basal epithelial layer (E, F). Scale bars: 100 μ m or 300 μ m. Vim, vimentin.

were plated in six-well plates for limbal stem cell colony-forming assay. Since the colony-forming efficiency in the 0.8×10^4 cells/cm² group was the highest, we chose this concentration for preparation of the LNC and LSC layer in subsequent experiments. The cells were washed thoroughly and reseeded into six-well plates or type I collagen gel (Collagen Gel Culturing Kit; Nitta Gelatin, Osaka, Japan) coated with Transwell (Corning, Cambridge, MA) at 0.8×10^4 cells/cm² (NIH/3T3 cells were prepared at 2×10^4 cells/cm²) as the feeder layers.

Colony-Forming Assay

Primary limbal epithelial cells were seeded at a density of 1000 cells per well on six-well plates on various MMC-treated feeder

layers and incubated for 10 to 13 days as previously described.¹¹ The colonies were fixed with 10% neutral buffered formalin and stained with 1% rhodamine B (Wako, Osaka, Japan). The colony-forming efficiency (CFE) was calculated as the percentage of colonies formed divided by the total number of viable cells seeded.

Preparation of Limbal Epithelial Cell Sheets

Primary limbal epithelial cells were inoculated on the feeder cells containing collagen gel-coated Transwell (Corning) inserts at 1 to 2×10^5 cells per insert. The cells were submerged in the KCM for 12 days and then exposed to air for 6 days to promote epithelial stratification. The medium was changed every 2 days.

Reverse Transcription-PCR and Quantitative PCR

Total RNA was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Complementary DNAs were synthesized from total RNA using the First-strand cDNA Synthesis System (OriGene, Rockville, MD) for polymerase chain reaction (PCR) and quantitative PCR. The PCR procedure was as follows: initial denaturation at 94°C for 5 minutes, denaturation at 94°C for 30 seconds, extension at 60°C for 30 seconds and at 72°C for 30 seconds, and a final extension of 10 minutes at 72°C for a total of 35 cycles. The PCR products were run on a 2% agarose gel and scanned using an ultraviolet gel doc (Bio-Rad Laboratories, Inc., Hercules, CA). The specific primers for PCR are listed in the Table. Expression of the various genes was normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. Quantitative PCR was carried out using Taqman probes and the Applied Biosystems 7900 HT sequence detection system instrument (both from Applied Biosystems, Foster City, CA). The primers for $\Delta Np63$ (Hs00978339_m1), *keratin 3* (Hs00365080_m1), and *GAPDH* (Hs99999905_m1) were obtained from Applied Biosystems. The cycling conditions were 10 seconds at 95°C followed by 45 two-step cycles (15 seconds at 95°C and 1 minute at 60°C). The quantification data were analyzed with Sequence Detection System (SDS) software (Applied Biosystems) using GAPDH as an internal control. The final results were expressed as the average of three experiments.

Histology and Immunofluorescence Staining

For the staining of limbal cell suspensions after dispase treatment and scraping, the samples were prepared for cytospin onto a slide using Cytofuge (StatSpin Technologies, Norwood, MA) at 150g for 5 minutes at a density of 6.0×10^4 cells per chamber. The cytospin preparation was dried for 5 minutes, followed by fixation with 4% paraformaldehyde for 15 minutes for subsequent immunofluorescence staining.

The donor limbal tissues obtained before and after dispase treatment and the harvested cellular sheets were cryosectioned to 6- to 8- μ m thickness, followed by fixation with either acetone at -20°C for hematoxylin and eosin (H&E) staining, or 4% paraformaldehyde at 4°C for 30 minutes for subsequent immunofluorescence staining.

For immunofluorescence staining, the fixed samples were blocked in 4% nonfat milk and 0.3% Triton X-100 in phosphate-buffered saline (PBS) for 1 hour at room temperature. The samples were incubated overnight at 4°C with the following primary antibodies: anti-keratin 3 (1:100; Progen Biotechnik, Heidelberg, Germany), anti-keratin 12 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), anti-p63 (4A4; 1:100; Santa Cruz Biotechnology), and anti-vimentin (1:100; Bioss, Woburn, MA). After washing with PBS, the samples were incubated for 1 hour with Alexa 568 or FITC-conjugated secondary antibody (1:200; Invitrogen) and finally counterstained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) and viewed under a Zeiss fluorescence microscope (Axiovert 200M; Carl Zeiss Jena GmbH, Jena, Germany). The same concentration of corresponding normal, nonspecific IgG was used as a negative control. Hematoxylin and eosin staining was performed according to standard protocols for histologic examination. Epithelial sheet immunofluorescence staining signal was quantified using ImageJ software (National Institutes of Health, Bethesda, MD). A total of three immunofluorescence images from the same sample were quantified, and the cell/nuclear number was calculated. Relative fluorescence intensity was calculated as the result of total fluorescence intensity divided

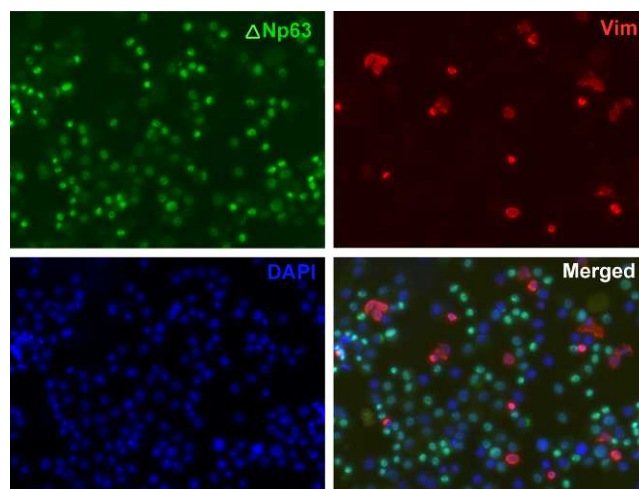


FIGURE 2. Identification of limbal stem cells and niche cells in cytospun limbal cell suspensions after dispase treatment and scraping. The immunostaining showed p63+ limbal stem/progenitor cells and vimentin+ and p63- niche cells collected after dispase treatment and scraping.

by the number of cells. The final results were expressed as the average of three experiments.

Statistics

Statistical analysis was performed using SPSS 16.0 software (SPSS, Inc., Chicago, IL). The data were analyzed using a one-way ANOVA (group). $P < 0.05$ was considered statistically significant.

RESULTS

Identification of Limbal Stem Cells, Niche Cells, and Stromal Cells In Situ

To identify the cellular components of limbal niche, we firstly performed H&E staining for normal limbal tissues. The results showed the typical stratified epithelium and underlying high cellularity in the limbal stromal area (Fig. 1A). The limbal stem/progenitor cells (p63+ cells in Fig. 1B) were distributed in the basal layer of the limbal epithelium, while vimentin+ mesenchymal cells were located close to the basal epithelial cells in the limbal crypts (Fig. 1B, arrows). However, the limbal epithelium was loosened from the limbal stroma after dispase treatment (Fig. 1C). The loosened limbal cellular sheet contained not only p63+ epithelial cells but also vimentin+ p63- cells (Fig. 1D, arrows). Loosened cells (p63+ limbal stem cells and vimentin+ p63- niche cells, above the dotted line) were collected by scraping off for selective purification of LNCs (Figs. 1C, 1D). The two types of cells (p63+ limbal stem cells and closely associated vimentin+ niche cells) could not be found in the limbal tissue after mechanical scraping, while some vimentin+ stromal cells were still found in the remaining deeper limbal stroma (Figs. 1E, 1F). We also checked for the coexistence of limbal stem cells and niche cells in the cytospun limbal cell suspensions after dispase treatment and scraping. The immunostaining showed that p63+ limbal stem/progenitor cells and vimentin+ and p63- niche cells were collected after dispase treatment and scraping (Fig. 2).

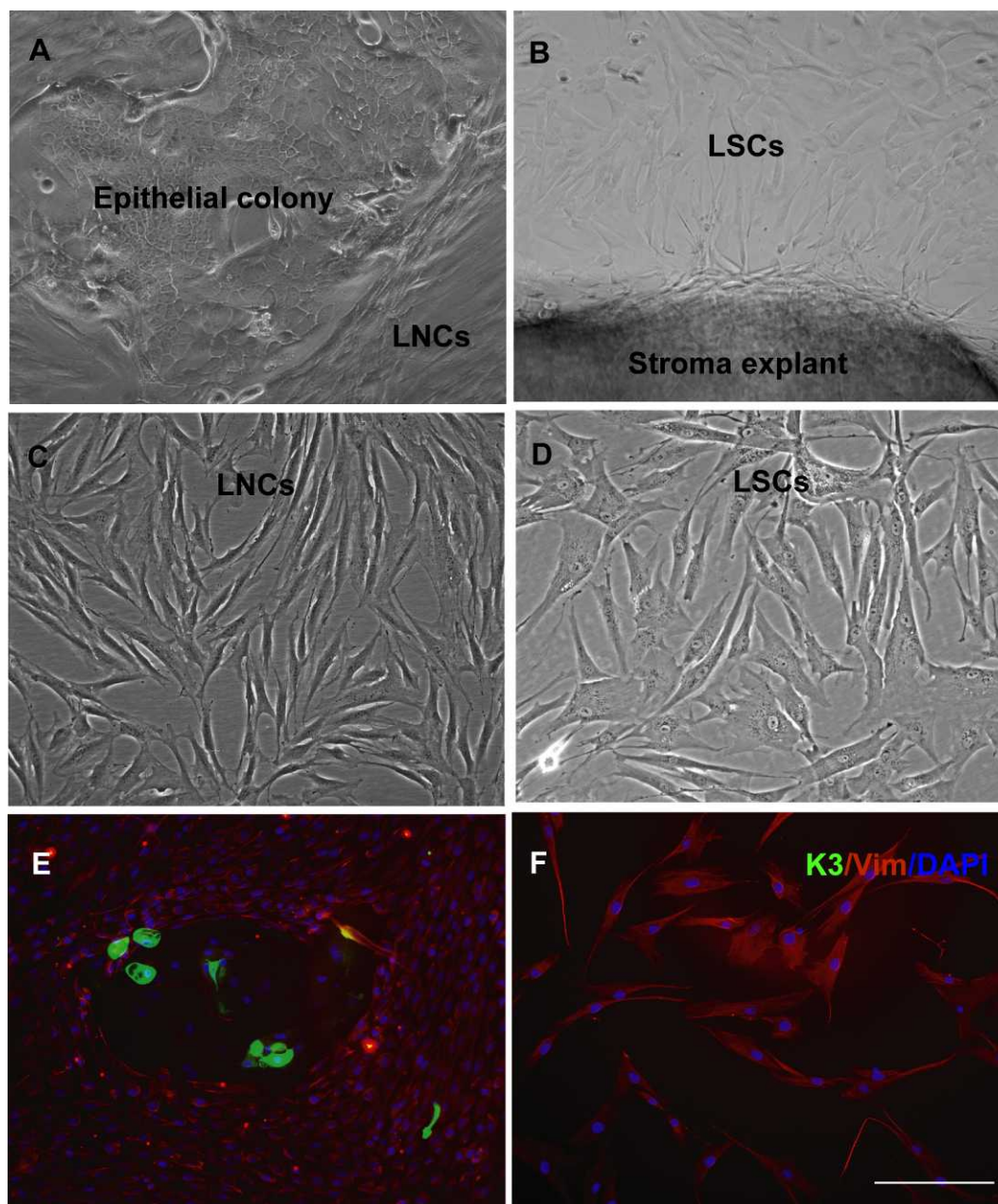


FIGURE 3. Isolation and culture of LNCs and LSCs. Limbal cellular suspensions were collected after dispase and scraping and cultured in KCM without feeder cells for 10 to 14 days. Typical epithelial colonies are surrounded by fibroblast-like cells (A), referred to as LNCs, and were passaged after scraping off the epithelial colonies (C). Immunofluorescent staining showed that the K3 epithelial colonies were enclosed by vimentin+ cells (E). LSCs were cultured with limbal stromal explants from the tissues after dispase treatment and scraping. The stromal cells migrated from explants after 3 to 5 days (B), were passaged (D), and were identified as vimentin+ fibroblasts (F). Scale bar: 200 μ m.

Isolation and Culture of LNCs and LSCs

To culture the LNCs, the limbal cellular suspensions were collected after treatment with dispase and scraping and inoculated on the cellular culture plate in KCM without feeder cells. Typical epithelial colonies were found after 7-day culture; they were always surrounded by fibroblast-like cells when the culture was extended to 10 to 14 days (Fig. 3A). The fibroblast-like cells (referred to as LNCs) were trypsinized and passaged after the epithelial colonies were scraped off (Fig. 3C). However, explant culture was used to isolate the LSCs after treatment with dispase and scraping. Only the middle part of the limbal stroma was used in order to prevent possible contamination of the niche cells near the basal epithelial cells.

The LSCs migrated from the limbal stroma explants after 3 to 5 days (Fig. 3B) and were passaged for identification and further experiments (Fig. 3D). In addition, immunofluorescence staining showed epithelial colonies (keratin 3+) surrounded by fibroblast-like cells (vimentin+) in the culture of the LNCs (Fig. 3E), while there were only vimentin+ fibroblasts in the culture of the LSCs (Fig. 3F).

Colony-Forming Assay and Stem Cell Properties

To examine the possible differences in the three cellular types—LNCs, LSCs, and NIH/3T3 fibroblasts—on clonal expansion of limbal epithelial stem/progenitor cells, the

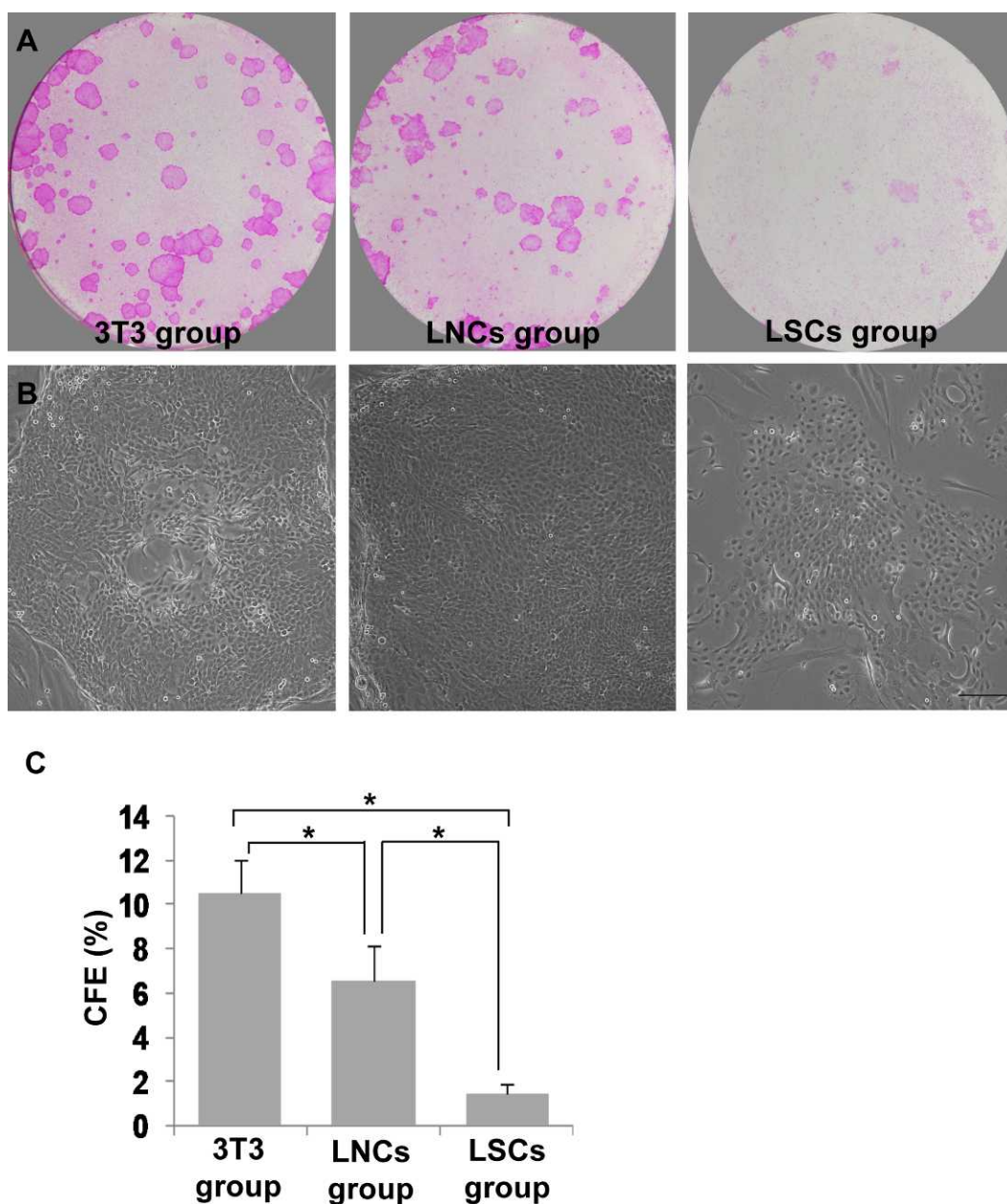


FIGURE 4. Colony-forming assay. Primary limbal epithelial cells form typical cellular colonies on the LNCs, LSCs, and NIH/3T3 fibroblast feeder layers after 10 to 13 days in culture. Coculture with LSCs yielded fewer colonies than with either LNCs or NIH/3T3 cells (A). Compared with LNCs, the central cells of the epithelial colonies on the NIH/3T3 feeder layer show significantly differentiated epithelial morphology, and the colonies on the LSCs layer show medium size, a wrinkled outline, and highly irregular shape (B). Quantitative CFE analysis showed that LNCs and NIH/3T3 cells significantly increased the clonal growth of limbal epithelial cells compared with LSCs (C). Scale bar: 200 μ m. * $P < 0.05$.

colony-forming assay was performed with the three cell types as feeder layers. The primary limbal epithelial cells formed typical cellular colonies on the three feeder cell types after culture for 10 to 13 days; however, coculture with LSCs yielded fewer colonies than with either LNCs or NIH/3T3 cells (Fig. 4A). Compared with the LNCs, the central cells of the epithelial colonies on the NIH/3T3 feeder layer showed substantially differentiated epithelial morphology, while the colonies on the LSC layer were of medium size and had a wrinkled outline and highly irregular shape (Fig. 4B). A quantitative analysis of the colony density showed that LNCs (CFE, $6.57 \pm 1.54\%$) and NIH/3T3 cells (CFE, $10.57 \pm 1.46\%$) significantly ($P < 0.05$) increased the clonal growth of the limbal epithelial cells compared with the LSCs (CFE, $1.43 \pm 0.47\%$; Fig. 4C).

Moreover, immunofluorescence staining suggested that coculturing of limbal epithelial cells with NIH/3T3 feeder cells showed higher positive staining for the differentiated corneal epithelial cellular marker keratin 3 (Fig. 5A), while coculturing with LNCs resulted in higher expression of limbal stem/progenitor cellular marker Δ Np63 (Fig. 5B) and mRNA transcripts (Figs. 5C, 5D).

Characterization of Cultured Limbal Epithelial Cell Sheets

Limbal niche cells, LSCs, and NIH/3T3 cells supported the stratification of limbal epithelial cells after 3-week culture. Morphologic observation showed that the NIH/3T3 cells and

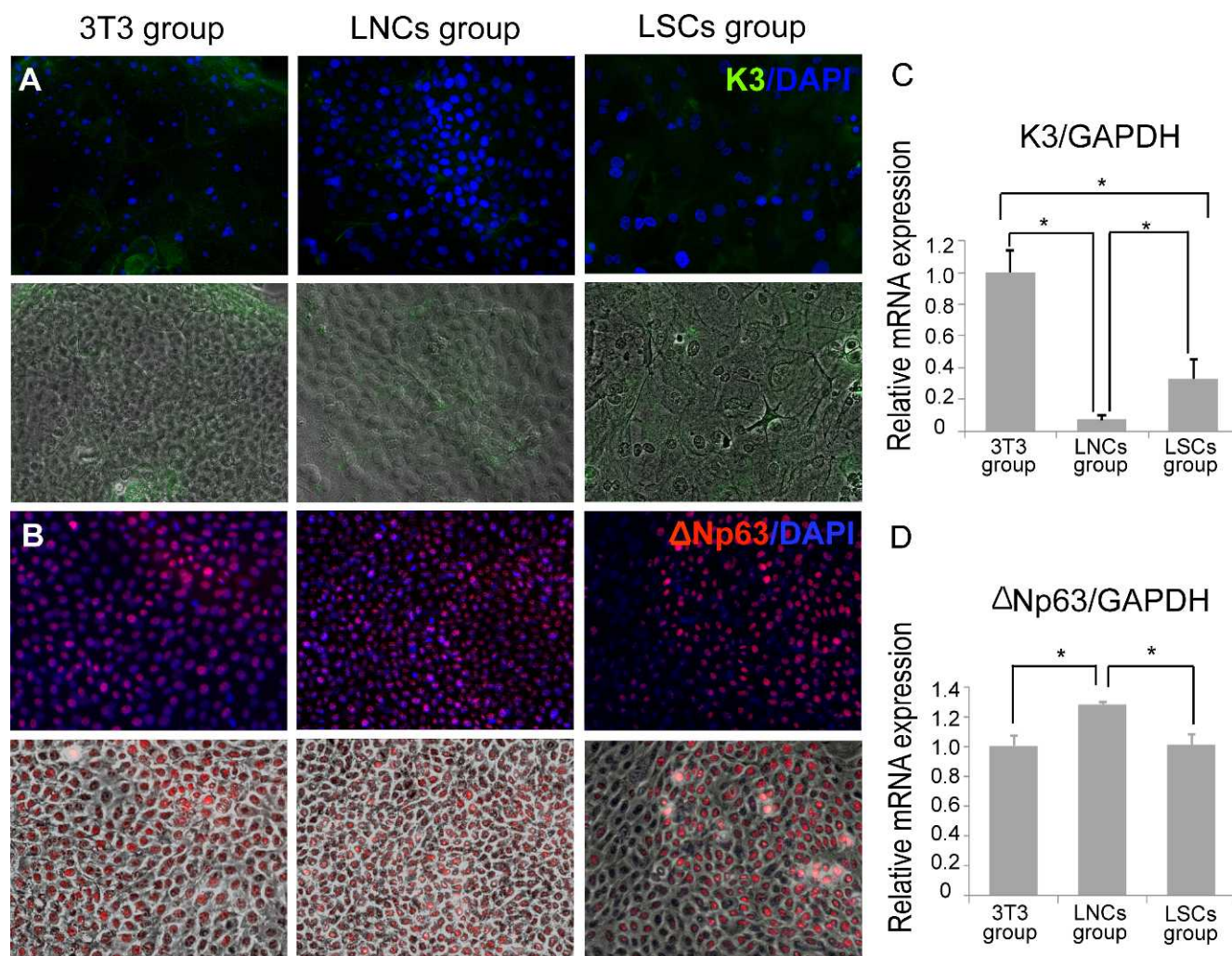


FIGURE 5. Characterization of limbal epithelial stem/progenitor colony. Coculture of limbal epithelial cells with NIH/3T3 feeder cells showed higher positive staining for differentiated corneal epithelial cellular marker K3 (A). Coculture with LNCs resulted in higher expression of limbal stem/progenitor cellular marker Δ Np63 (B) and quantitative analysis of mRNA transcripts confirmed this result (C, D). Scale bar: 200 μ m. * $P < 0.05$.

LNCs supported formation of an epithelial cellular sheet of four to five stratified layers, while the LSCs supported an epithelial cellular sheet of only two or three stratified layers (Fig. 6A). Immunofluorescence staining showed that the epithelial cellular sheet cocultured with NIH/3T3 feeder cells had higher positive staining for keratin 3 (Figs. 6B, 6E), and the limbal stem/progenitor cellular marker Δ Np63 stained only in basal cells, while coculturing with LNCs resulted in higher expression of Δ Np63 in basal and wing cells (Figs. 6C, 6F). Keratin 12 staining did not show substantial differences among the three cellular sheets (Figs. 6D, 6E).

Different Expression Profiles Between LNCs and LSCs

Reverse transcriptase-PCR analysis showed that cultured LNCs and LSCs expressed vimentin but not corneal epithelial cellular marker keratin 3 (K3) or conjunctival cellular marker keratin 4 (K4), suggesting that these two cells were not contaminated by corneal epithelial cells or conjunctival cells (Fig. 7A). We also checked the expression levels of several genes related to the support of limbal stem/progenitor cells according to previous studies.^{23–26} The results showed LNC and LSC expression of epidermal fibroblast growth factor (EGF), fibroblast growth

factor 2 (FGF2), epiregulin (EPR), hepatocyte growth factor (HGF), keratinocyte growth factor (KGF), nerve growth factor (NGF), glial cell-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), N-cadherin, and importin 13. However, LNCs exhibited significantly lower expression of neurotrophin-3 (NT3) and significantly higher expression of E-cadherin than did the LSCs (Fig. 7B).

DISCUSSION

In the current study, we have reported a novel method for the isolation of LNCs and compared the differences between LNCs and stromal cells with regard to supporting limbal stem/progenitor cells. The results showed that the LNCs had significantly greater capacity to support limbal epithelial stem/progenitor cells than the LSCs, including the expression of limbal epithelial stem/progenitor cellular markers, the capacity for colony forming, and stratification of epithelial cellular sheets. In addition, compared with the common expression of EGF, FGF2, EPR, HGF, KGF, NGF, GDNF, BDNF, N-cadherin, and importin 13, the LNCs showed a higher expression level of E-cadherin and a lower expression level of

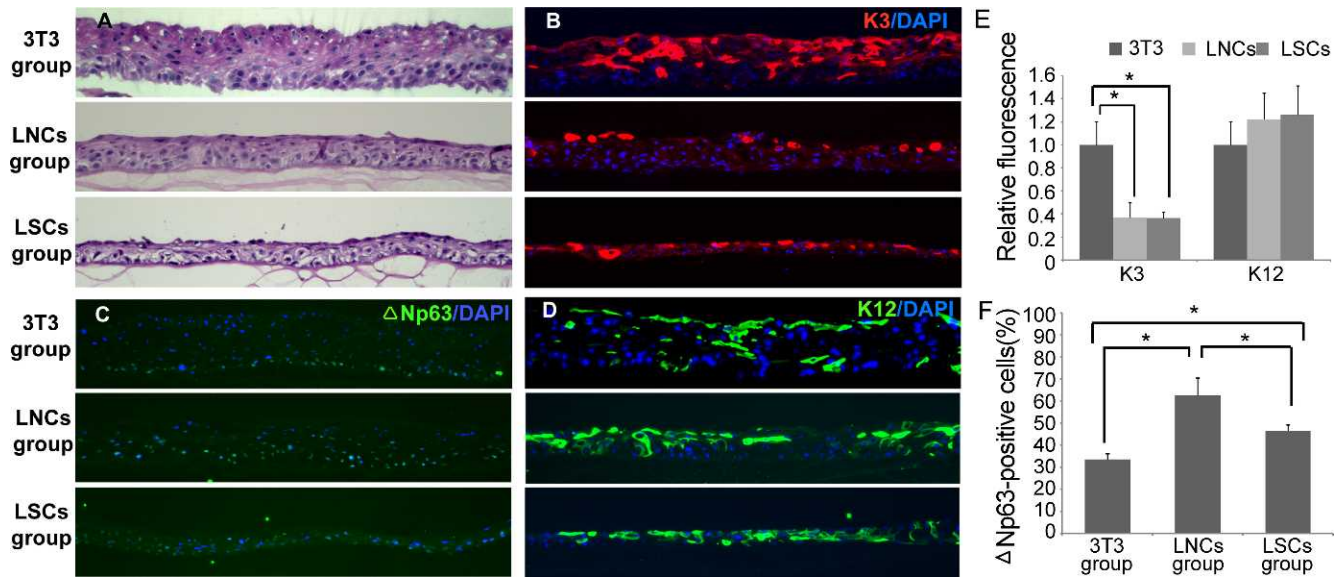


FIGURE 6. Characterization of cultured limbal epithelial cellular sheets. LNCs, LSCs, and NIH/3T3 cells supported the stratification of limbal epithelial cells after 3-week culture. NIH/3T3 feeder cells and LNCs support formation of four or five layers of epithelium, while LSCs support only two or three stratified layers (A). K3 staining (B), ΔNp63 staining (C), and keratin 12 (K12) staining (D) were detected in cultured epithelial cell sheets. Semiquantitative analysis of marker intensity revealed no difference in level of K12 in all groups, but the level of K3 was higher in the NIH/3T3 group (E). The percentage of ΔNp63-positive cells in the cell sheets cultured with LNCs was significantly higher than that in cell sheets cultured with LSCs and NIH/3T3 cells (F). Scale bar: 200 μm. **P* < 0.05.

NT3 than did the LSCs, which may be involved in the promotion of limbal epithelial stem/progenitor cells.

Corneal epithelial stem cells have been believed to be in the palisades of Vogt at the corneoscleral limbus.^{5,27} Under the basement membrane of the limbal epithelial cells, the cells in the limbal stroma are heterogeneous and poorly defined.¹⁴ Recently, Chen et al.¹⁶ and Xie et al.^{17,18} identified a novel cell type, referred to as LNCs, residing in the limbal basal epithelial layer and characterized their maintenance of limbal stem/progenitor cells. When culturing limbal epithelial stem/progenitor cells, we found more colonies if the limbal tissues were scraped intensely after dispase treatment. We also confirmed that there were few vimentin+ cells in the basal layer of the limbal epithelium after both treatment with dispase and scraping, which suggested that additional scraping can remove more basal epithelial cells and niche cells compared with dispase treatment only. Actually, immunofluorescence staining of limbal cell suspensions prepared by cytopsin confirmed that p63+ limbal stem/progenitor cells and closely

associated vimentin+ mesenchymal cells were collected together after dispase treatment and scraping. We found that vimentin+ fibroblast-like cells proliferated around the limbal epithelial cellular colonies when cultured in KCM as previously used to culture limbal epithelial cells on 3T3 feeder cells. Considering the tight interactions between the limbal stem cells, the fibroblast-like cells surrounding the epithelial cellular colonies may represent LNCs. In addition, to compare the LNCs and LSCs, we sectioned only the middle layer of the limbal stroma to exclude possible contamination of niche cells, since the two cell types showed similar morphology and even gene expression.^{28,17} However, the removal of anterior stromal cells may eliminate the most efficacious stromal cells adjacent to limbal basal cells, which may reduce the supporting potential of LSCs for limbal stem cells. It should be mentioned that because the medium we used contained serum, which may promote differentiation of LNCs or stromal cells,²⁹ we used only the second passages of cells to prepare the feeder layer.

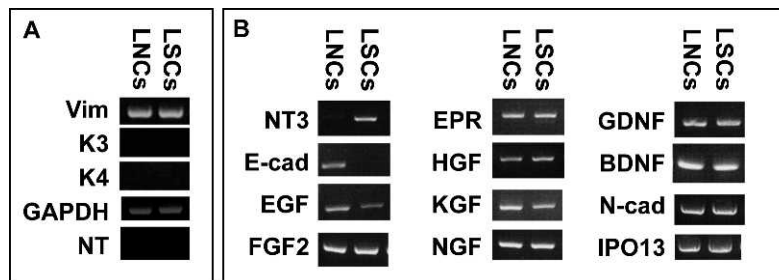


FIGURE 7. Different expression profiles between LNCs and LSCs. LNCs and LSCs are not contaminated by conjunctival or corneal epithelial cells based on the positive expression of vimentin and negative expression of K3 and K4 (A). The two cell types had similar levels of EGF, FGF2, EPR, HGF, KGF, NGF, GDNF, BDNF, N-cadherin, and importin 13. However, LNCs exhibited significantly lower expression of NT3 and significantly higher expression of E-cadherin than the LSCs (B). *Vim*, vimentin; *K3*, cytokeratin 3; *K4*, cytokeratin 4; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *NT*, no reverse transcriptase; *NT3*, neurotrophin-3; *E-cad*, E-cadherin; *EGF*, epidermal fibroblast growth factor; *FGF2*, fibroblast growth factor 2; *EPR*, epiregulin; *HGF*, hepatocyte growth factor; *KGF*, keratinocyte growth factor; *NGF*, nerve growth factor; *GDNF*, glial cell-derived neurotrophic factor; *BDNF*, brain-derived neurotrophic factor; *N-cad*, N-cadherin; *IPO13*, importin 13.

Regarding the support of limbal epithelial stem/progenitor cells, our results were similar to previous findings^{16,17} in that LNCs had significantly greater capacity to support limbal epithelial stem/progenitor cells than did the LSCs, including expression of limbal epithelial stem/progenitor cellular markers, the ability to form colonies, and stratification of the epithelial cellular sheet. In the current study, LNCs supported the formation of four or five stratified epithelial cellular sheets, while LSCs supported only two or three stratified layers. It is interesting that cell sheets in LNC group expressed K3 mainly in the superficial cell layers. The clonal cells and epithelial cellular sheet cocultured with the LNCs showed significantly lower K3 expression than the cells on the NIH/3T3 feeder layers. More importantly, immunofluorescence staining and mRNA transcripts of the epithelial colony showed that the expression of Δ Np63 in epithelial stem/progenitor cells cocultured with LNCs was higher than with LSCs and NIH/3T3 feeder cells. Immunofluorescence staining of the epithelial cellular sheet showed that epithelial stem/progenitor cells cocultured with LNCs contained more Δ Np63-positive cells in the basal and wing cells than those cocultured with LSCs and NIH/3T3 cells. This suggested that LNCs can maintain stem/progenitor cells in expansion more efficiently than LSCs and NIH/3T3 cells. Therefore, we believe that LNCs both supported the expansion of epithelial cells and maintained the undifferentiated characteristics of limbal stem/progenitor cells in vitro.

In our experiment we digested and incubated the LNCs and LSCs in 10% FBS-DMEM medium, which is not suitable for corneal epithelial cell growth but benefits mesenchymal cell growth. Isolated cells showed fibroblast cell morphology, as well as negative expression of K3 and K4, to make sure that LNCs and LSCs were not contaminated by corneal epithelial cells or conjunctival cells. In addition, reverse transcriptase-PCR analysis showed both LNC and LSC expression of various growth factors (EGF, FGF2, EPR, HGF, KGF, NGF, GDNF, and BDNF) and the potential corneal epithelial stem/progenitor cell marker N-cadherin¹¹ and importin 13.³⁰ However, LNCs expressed a higher level of E-cadherin and a lower level of NT3 compared with LSCs. Growth factors play an important role in the maintenance and normal wound healing of the corneal epithelium³¹; among these, most are predominantly expressed by fibroblasts and affect the proliferation and differentiation of the surrounding epithelial cells in a paracrine manner through their cognate receptors.^{23–26} Our results confirmed that both LNCs and LSCs secrete various growth factors. Although LSCs expressed higher levels of NT3 than did LNCs, NT3 is considered to have a weak effect on limbal stem/progenitor cells because its receptor TrkC is weakly expressed by limbal epithelial stem/progenitor cells.³² Our findings also verified this. Cadherin mediates cell–cell adhesion and plays a major role in embryonic development, tissue formation, and cellular growth and differentiation.³³ Previous studies had found that N-cadherin (neuronal cadherin) plays an important role in the interactions between hematopoietic/limbal stem/progenitor cells with their niche cells.^{11,34} N-cadherin in limbal epithelial stem/progenitor cells likely acts as an anchor molecule for their attachment to N-cadherin+ niche cells.¹¹ Limbal niche cells located in the basal layer of the limbal epithelium express N-cadherin, suggesting that they might have direct contact with epithelial stem/progenitor cells in the limbus via N-cadherin. E-cadherin (epithelial cadherin), which is located on the lateral surfaces of most epithelial cells, plays an important role in transformation of mesenchyme to epithelium in embryos³⁵ and plays an essential role in the collective directional migration of large epithelial sheets as mediate wound epithelium healing.³⁶ It is noteworthy that LNCs express higher levels of E-cadherin than LSCs, which

might be why LNCs had a greater capacity to support the limbal epithelial stem/progenitor cells than the LSCs. It should be mentioned that the LNCs have the potential to different when cultured in serum-containing medium for a long time. According to a previous report, a modified embryonic stem cell medium can maintain the undifferentiated state and the expression of embryonic stem cell markers of LNCs,¹⁷ so further study should be performed to compare LNCs and LSCs under the embryonic stem cell medium condition.

In conclusion, this study provided a novel method to isolate LNCs, described the differences between LNCs and LSCs in their ability to support epithelial stem/progenitor cells and epithelial cellular sheet formation, and compared the different gene expression levels. The results showed that isolated LNCs might be an optimal cellular type to treat limbal stem cellular deficiency.

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