A Comparison of Methods for Isolation of Limbal Niche Cells: Maintenance of Limbal Epithelial Stem/Progenitor Cells

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PURPOSE. Limbal niche cells (LNCs) play a vital role in the maintenance of limbal epithelial stem/progenitor cells (LESCs). Four methods have been reported to isolate and expand LNCs: digestion by collagenase alone (C-LNC), collagenase following dispase removal of the limbal epithelium (DC-LNC), dissection of dispase-isolated limbal epithelial sheets (D-LNC), and explant cultures of limbal stromal tissues (Ex-LNC). This study aimed to isolate LNCs using those four methods and to compare their capacity to maintain LESCs.

METHODS. LNCs were isolated from the rat corneal limbus by the following methods: C-LNC, DC-LNC, D-LNC, and Ex-LNC. Quantitative real-time PCR and immunofluorescence staining were used to analyze the expression of embryonic stem cell (ESC) markers. The ability to maintain LESCs was assessed on the basis of colony-forming capacity and the expression of progenitor, proliferation, and differentiation markers in three-dimensional (3D) Matrigel and Transwell systems. Notch signaling of LESCs supported by different LNCs in Transwell inserts was analyzed by quantitative real-time PCR.

RESULTS. DC-LNCs exhibited lower expression of CK12 during isolation and expansion. Among P4-expanded LNCs, DC-LNCs expressed significantly higher levels of Sox2, Oct4, Nanog, and N-cadherin than C-LNCs, D-LNCs, and Ex-LNCs. Compared with other LNCs, DC-LNCs were more effective in maintaining LESCs with higher holoclone-forming efficiency, greater expression of ΔNp63α and Ki67, and lower expression of CK12. DC-LNCs were also more capable of downregulating Notch signaling of LESCs.

CONCLUSIONS. DC-LNCs were more effective in expressing ESC markers and maintaining LESCs compared to other LNCs. This study identifies an optimal method for the isolation of LNCs in tissue engineering and ocular surface reconstruction.

Keywords: limbus, niche cells, stem cells, coculture, notch

Limbal epithelial stem/progenitor cells (LESCs), present exclusively in the limbal basal epithelium,[1,2] are poorly differentiated cells that have unlimited potential to self-renew.[3,4] When the central cornea is traumatically wounded, LESCs proliferate and migrate centripetally toward the central cornea for cell replacement.[5,6] The quiescence, self-renewal, and final differentiated state of LESCs are regulated by the limbal niche,[7] a specialized anatomical microenvironment proposed to be composed of extracellular matrix, limbal vasculature, adjacent supporting cells (i.e., subjacent mesenchymal cells, melanocytes, Langerhans cells, and vascular cells), and secreted factors.[8–10]

Limbal niche cells (LNCs) are assumed to be mesenchymal cells closely associated anatomically with LESCs in the limbal niche, and they heterogeneously express both mesenchymal and putative embryonic stem cell (ESC) markers.[11,12] As native microenvironment components of the limbal niche, LNCs have the unique advantage of maintaining LESCs in an undifferentiated state.[13] Evidence indicates that the expression of ESC markers is critical for LNCs to support LESCs.[14] In a prior study, we demonstrated that LNCs prevent LESCs from differentiating predominantly via inhibiting the Notch signaling pathway.[15] Hitherto, four methods have been reported to isolate and expand LNCs: digestion by collagenase alone (C-LNC),[11,14] use of collagenase following dispase removal of the limbal epithelium (DC-LNC),[16,17] dissection of dispase-isolated limbal epithelial sheets (D-LNC),[18,19] and explant cultures of limbal stromal tissues (Ex-LNC).[18–20] However, the optimal LNC isolation method to determine their expression of ESC markers and maintenance of LESCs has remained elusive. In this investigation, we isolated and expanded LNCs using the four aforementioned methods, compared the expression level of ESC markers, and evaluated their ability to maintain LESCs in colony-forming assays and three-dimensional (3D) Matrigel and Transwell systems (Corning Inc., Corning, NY, USA).

MATERIALS AND METHODS

Animals

Eighty 8-week-old male Sprague–Dawley rats (weighing 150–200 g) were provided by the Animal Research Committee of the Huazhong University of Science and Technology (Wuhan, China). Animal-related activities in this investigation adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. This study was approved by the Institutional Animal Care and Use Committee at Tongji Medical College, Huazhong University of Science and Technology (IACUC no. S2351, 2018; Supplementary Table S1).

Cell Isolation and Culturing

Isolation and culturing of LNCs were performed as subsequently described. After enucleation, rat eyeballs were separated into the limbus and central cornea by a 3.5-mm-diameter trephine. Careful removal of excess sclera, conjunctiva, iris, and endothelium followed. Corneoscleral rims were then clipped 1 mm within and beyond the anatomic limbus and cut into six sections. C-LNC clusters were isolated with 1 mg/mL collagenase A, which digested corneoscleral segments at 37°C for 4 hours. DC-LNC clusters were obtained using 10 mg/mL Dispase II at 37°C for 20 minutes, a step that was performed to remove the limbal epithelium before the remaining stroma was digested by collagenase A. Limbal epithelial sheets of D-LNC clusters were dissected mechanically after digestion with Dispase II at 37°C for 20 minutes. Ex-LNCs were expanded from explant cultures of limbal stromal tissues after removal of the limbal epithelium. The C-LNC, DC-LNC, and D-LNC clusters were further digested with 0.25% trypsin and 1-mM EDTA (T/E) at 37°C for 15 minutes to yield single cells. They were then suspended at a density of $1 \times 10^4/\text{cm}^2$ into six-well plastic culture plates coated with 5% Matrigel in modified embryonic stem cell medium (MESCM). MESCM is composed of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12, 1:1; Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% knockout serum, 5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL sodium selenite, 4 ng/mL basic fibroblast growth factor, 10 ng/mL human leukemia inhibitory factor (hLIF), 50 µg/mL gentamicin, and 1.25 µg/mL amphotericin B. The four types of LNCs were passaged at a ratio of 1:4 until passage 4 (P4) to prevent contamination of the corneal epithelial cells and to compare the expression of ESC markers Sox2,[21] Oct4,[22] and Nanog[23] and putative stem cell marker N-cadherin.[24]

To compare the capacity to maintain LESCs, the four kinds of P4 LNCs were processed for coculture with passage 0 (P0) limbal epithelial cells (LECs). Limbal epithelial sheets were removed by Dispase II at 37°C for 20 minutes and then incubated in T/E at 37°C for 15 minutes to obtain single LECs for further experiments.

Colony-Forming Assay

The feeder layer was first prepared by treating C-LNCs, DC-LNCs, D-LNCs, and Ex-LNCs with 4 µg/mL mitomycin C at 37°C for 2 hours before seeding at a density of 1×10^5 cells per six-well plate. Primary P0 LECs were then inoculated at a density of 2000 cells per six-well plate on various LNC feeder layers in supplemented hormonal epithelial medium (SHEM) for 12 days. SHEM is made of DMEM/F12 (1:1) supplemented with 5% fetal bovine serum, 2.5 µg/mL insulin, 2.5 µg/mL transferrin, 2.5 ng/mL sodium selenite, 0.45 µg/mL hydrocortisone, 20 ng/mL human epidermal growth factor, 10 ng/mL hLIF, 50 µg/mL gentamicin, and 1.25 µg/mL amphotericin B. Epithelial colonies were classified into holoclone, meroclone, and paraclone based on the criteria for skin keratinocytes.[25] Rhodamine B was used to stain the clonal growth, and colony-forming efficiency was calculated by dividing the percentage of clone number by the total number of seeded LECs.

Coculturing in 3D Matrigel

The 3D Matrigel was prepared with 250 µL of 50% diluted Matrigel (in DMEM) per chamber of a 24-well plastic plate, which was then incubated at 37°C for 1 hour. Single C-LNCs, DC-LNCs, D-LNCs, and Ex-LNCs expanded up to P4 were each mixed with P0 LECs at a ratio of 1:4 and inoculated at a total density of $5 \times 10^6/\text{cm}^2$ on 3D Matrigel before coculturing in MESCM for 7 days. The mixtures composed of LNCs and LECs were harvested by Dispase II digestion at 37°C for 4 hours and incubated with T/E at 37°C for 15 minutes to yield single cells. Subsequent experiments were conducted to detect the expression of corneal epithelial marker CK12[26] and epithelial stem cell marker ΔNp63α.[27]

Coculturing in the Transwell System

In the Transwell system, primary P0 LECs were seeded at a density of $5 \times 10^4/\text{cm}^2$ into Transwell inserts coated with 5% Matrigel, with P4-expanded C-LNCs, DC-LNCs, D-LNCs, and Ex-LNCs serving as bottom feeder layers. LECs supported by different LNCs were all cocultured in SHEM for 7 days. Airlift culture was performed for another 2 weeks to promote limbal epithelial stratification. Immunofluorescence staining of CK12, ΔNp63α, and Ki67 (a proliferation marker)[28] was performed for the stratified epithelium supported by different LNCs. Transcript levels for Notch family members Delta1, Notch1, and Hes1 in LECs cocultured with various LNCs were detected later on. All materials used in cell isolation and culturing are listed in Supplementary Table S2.

Hematoxylin and Eosin Staining

The paraffin sections of stratified limbal epithelium were first stained with hematoxylin for 8 minutes and then soaked in 1% acid and 1% ammonia. After 10 minutes of dehydration with 70% and 90% alcohol, the sections were finally stained with acidic eosin solution for 3 minutes.

Immunofluorescence Staining

Slides of tissues, spheres, and single cells were fixed with 4% paraformaldehyde for 15 minutes, saturated with 0.5% Triton X-100 in PBS for 60 minutes and blocked with 2% BSA for 60 minutes. The slides were incubated with primary antibodies (overnight at 4°C) and their respective secondary antibodies (1 hour at room temperature). The nucleus was stained with 4′,6-diamidino-2-phenylindole for 5 minutes. Fluorescence images were captured by a laser confocal fluorescence microscope (LSM700; Carl Zeiss Microscopy, White Plains, NY, USA). All antibodies used in this investigation are listed in Supplementary Table S3.

Quantitative Real-Time PCR

Total RNA was extracted by TRIzol Reagant (Thermo Fisher Scientific) and reverse-transcribed to cDNA using a reverse transcription kit (GeneCopoeia, Rockville, MD, USA). The quantitative real-time PCR amplification procedure began with initial pre-denaturation at 50°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 10 minutes, annealing at 95°C for 30 seconds, and extension at 60°C for 30 seconds. The relative gene expression was analyzed by the comparative cycle threshold method; β-actin was used as an internal reference. All quantitative real-time PCR experiments were repeated three times. Detailed information on primer sequences is provided in Supplementary Table S4.

Statistics

All data are shown as mean ± SD and analyzed using one-way ANOVA (group) by SPSS Statistics 16.0 (IBM, Armonk, NY, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Unique Expression of Limbus

To identify the anatomical position of LESCs and LNCs in the limbal niche, we performed immunofluorescence staining of the limbus and central cornea. CK12 was expressed in the stratified epithelium of the limbus and central cornea. Pax6 was stained in the whole layers of the central corneal epithelium and basal layer of the limbal epithelium but not in the underlying stroma. LESCs expressing ΔNp63α were located in the basal membrane of the limbal epithelium. Furthermore, LNCs were identified as vimentin+ mesenchymal cells closely associated anatomically with LESCs in the limbus (Fig. 1A). Also, primary P0 LECs were initially stained as CK12+, containing several ΔNp63α+ LESCs (Fig. 1B).

Isolation and Purification of C-LNCs, DC-LNCs, D-LNCs, and Ex-LNCs

To isolate C-LNCs, DC-LNCs, D-LNCs, and Ex-LNCs, limbal segments were digested using four different methods. In the primary culture of D-LNCs, abundant rounded epithelial cells were suspended on days 1 to 2, typical epithelial colonies formed on day 4, and fibroblast-like LNCs were observed around the epithelial colonies on day 7 (Fig. 2A). In the explant culture of Ex-LNCs, limbal stromal tissues adhered to dishes on day 0; LNCs began to migrate from limbal stroma explants on day 3, gradually increased on day 5, and entered a logarithmic growth phase on day 8 (Fig. 2B). C-LNCs, DC-LNCs, D-LNCs, and Ex-LNCs were passaged at a ratio of 1:4 until P4. Morphological observation indicated that rounded epithelial cells were significantly less in DC-LNCs compared to other LNCs during the process of isolation and expansion (Fig. 2C).

To verify the epithelial and mesenchymal constituents in the culture process of C-LNCs, DC-LNCs, D-LNCs, and Ex-LNCs, we detected the expression of CK12 and vimentin. During the passage of C-LNCs, DC-LNCs, D-LNCs, and Ex-LNCs, CK12+ epithelial cells decreased and vimentin+ mesenchymal cells dominated gradually. The disappearance of CK12 in P3 indicated the purification of these four types of LNCs by expanding in MESCM (Fig. 3A). In P1 LNCs, DC-LNCs and Ex-LNCs exhibited negative expression of CK12 (Fig. 3B).

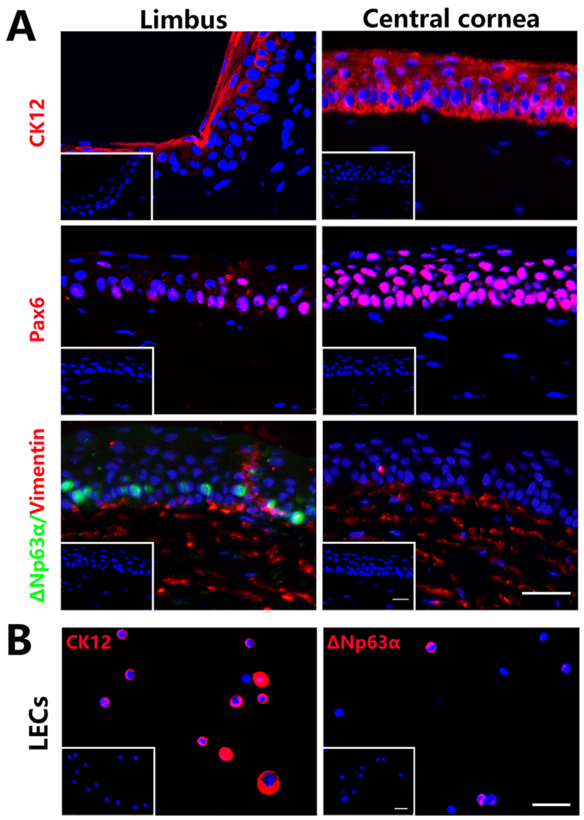

FIGURE 1. Unique expression of the limbus. (**A**) Immunofluorescence staining of CK12, Pax6, ΔNp63α, and vimentin in the limbus and central cornea. (**B**) Initial phenotypic signature of LECs before coculture. Boxes in the *left bottom* show the immunofluorescence controls. *Scale bars*: 50 μm.

ESC Marker Expression of C-LNCs, DC-LNCs, D-LNCs, and Ex-LNCs

Given that the expression of ESC markers in LNCs is essential for their niche function,[14] we processed LNCs that had expanded up to P4 to compare ESC marker expression. Quantitative real-time PCR showed significantly higher transcript levels of Sox2, Oct4, Nanog, and N-cadherin in DC-LNCs than those in C-LNCs, D-LNCs, and Ex-LNCs ($n = 3$, all $P < 0.01$) (Fig. 4A). The expression difference was further confirmed by quantification analysis of immunofluorescence staining (Figs. 4B, 4C). The results showed that DC-LNCs exhibited significantly higher expression of Sox2, Oct4, Nanog, and N-cadherin than other LNCs ($n = 4$, all $P < 0.01$) (Fig. 4C). In contrast, the expression of Sox2, Oct4, Nanog, and N-cadherin was lower in D-LNCs ($n = 4$, all $P < 0.01$) (Fig. 4C). Collectively, DC-LNCs expressed significantly more ESC markers overall.

Maintenance of LESCs in Colony-Forming Assays

To assess the capacity of LNCs to support LESCs in forming epithelial colonies, we conducted colony-forming assays. The results of rhodamine B staining showed that LESCs cocultured with DC-LNCs yielded bigger clones compared

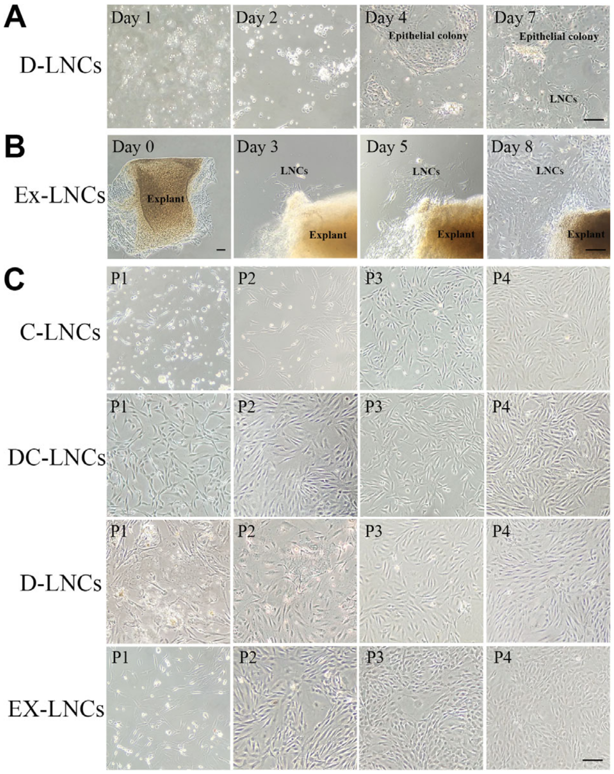

Figure 2. Morphological characterization of C-LNCs, DC-LNCs, D-LNCs, and Ex-LNCs. (**A**) Abundant epithelial cells appeared in P0 D-LNCs. (**B**) The migration process of Ex-LNCs from limbal stromal explants. (**C**) Serial passage of the four kinds of LNCs from P1 to P4. *Scale bars*: 50 µm.

to those cocultured with other LNCs (Fig. 5A). Morphological observation revealed that the clones were divided into holoclones, meroclones, and paraclones (Fig. 5B). The quantification analysis of clonal growth demonstrated that LESCs supported by DC-LNCs generated more holoclones than those supported by C-LNCs ($n = 3$, $^{**}P < 0.01$), D-LNCs ($n = 3$, $^{**}P < 0.01$), or Ex-LNCs ($n = 3$, $^{**}P < 0.01$) (Fig. 5C). Collectively, these results indicate that DC-LNCs had a stronger ability to support LESCs in clonal growth compared to the other LNCs.

Maintenance of LESCs in 3D Matrigel Culture

To evaluate the capacity of LNCs to maintain the undifferentiated characteristics of LESCs, we mixed P4-expanded LNCs with P0 LECs in 3D Matrigel and detected the expression of progenitor and differentiation markers. Visually, spheres formed by LECs alone were smaller than those consisting of LECs and LNCs. Moreover, spheres containing DC-LNCs were much larger compared to those containing C-LNCs, D-LNCs, and Ex-LNCs (Fig. 6A). Double immunostaining showed the components of CK12+ LECs and vimentin+ LNCs in LEC + DC-LNC spheres, and the expression of ΔNp63α indicated the progenitor status of LECs (Fig. 6B). Supplementary Videos S1 and S2 provide 3D reconstruction images of spheres in the DC-LNC group. The addition of LNCs decreased the transcript level of CK12 and increased that of ΔNp63α in LECs (Fig. 6C). The transcript of ΔNp63α in the DC-LNC group was upregulated 1.9-fold more than in the C-LNC group ($n = 3$, $P < 0.01$), 3.9-fold more than in the D-LNC group ($n = 3$, $P < 0.01$), and 2.7-fold more than in the Ex-LNC group ($n = 3$, $P < 0.01$) (Fig. 6C). Immunofluorescence staining analysis confirmed the downregulation of CK12 and upregulation of ΔNp63α in LECs after reunion with LNCs (Figs. 6D, 6E). The expression of CK12 in the DC-LNC group was significantly lower than in the other groups ($n = 4$, all $P < 0.01$) (Fig. 6D). Furthermore, the percentage of ΔNp63α+ cells in the DC-LNC group increased to 45.4% ± 3.3% compared to 20.3% ± 2.1% in the C-LNC group ($n = 4$, $P < 0.01$), 13.9% ± 2.1% in the D-LNC group ($n = 4$, $P < 0.01$), 16.6% ± 1.4% in the Ex-LNC group ($n = 4$, $P < 0.01$), and 1.4% ± 0.8% in the control group ($n = 4$, $P < 0.01$) (Fig. 6D). These results suggest that DC-LNCs were more capable of maintaining LESCs in 3D Matrigel than were the other LNCs.

Maintenance of LESCs in Transwell System

To examine the ability of LNCs to support the formation of stratified corneal epithelium, we cocultured the four kinds of P4 LNCs and P0 LECs in the Transwell system. The results of hematoxylin and eosin staining showed that LECs could be airlifted to induce stratification to form multilayered epithelium by C-LNCs, DC-LNCs, D-LNCs, and Ex-LNCs. Compared with other LNCs, DC-LNCs supported LECs in forming epithelial sheets of three to four stratified layers. Immunofluorescence staining of the stratified epithelium indicated that CK12 was expressed in all layers, Ki67 was stained in basal cells, and ΔNp63α was labeled in basal-to-superficial layers (Fig. 7A). Immunostaining analysis demonstrated that the expression of Ki67 and ΔNp63α in LECs supported by DC-LNCs was significantly higher than in the C-LNC, D-LNC, and Ex-LNC groups (Figs. 7B, 7C). The expression level of Ki67 in the DC-LNC group was upregulated 2.9-fold more than in the C-LNC group ($n = 4$, $P < 0.01$), 5.1-fold more than in the D-LNC group ($n = 4$, $P < 0.01$), and 3.2-fold more than in the Ex-LNC group ($n = 4$, $P < 0.01$) (Fig. 7B). Furthermore, the percentage of ΔNp63α+ cells in the DC-LNC group was 2.7-fold higher than in the C-LNC group ($n = 4$, $P < 0.01$), 6.7-fold higher than in the D-LNC group ($n = 4$, $P < 0.01$), and 1.4-fold higher than in the Ex-LNC group ($n = 4$, $P < 0.01$) (Fig. 7C). In contrast, LECs cocultured with D-LNCs showed lower expressions of Ki67 ($n = 4$, $P < 0.05$ for the C-LNC group, $P < 0.01$ for the DC-LNC group) (Fig. 7B) and ΔNp63α ($n = 4$, all $P < 0.01$) (Fig. 7C). Therefore, we concluded that DC-LNCs have a unique advantage in maintaining the proliferative and undifferentiated characteristics of LESCs in the Transwell system.

Notch Signaling in LESCs Cocultured with C-LNCs, DC-LNCs, D-LNCs, and Ex-LNCs

It has been proven that LNCs prevent the differentiation of LESCs by inhibiting the Notch signaling pathway,[15] and we detected Notch signaling in LECs supported by various LNCs in the Transwell system. LNCs decreased the transcript level of Notch family members in LECs. The transcript of Delta1 (a Notch ligand) in LECs was downregulated 6.5-fold by DC-LNCs ($n = 3$, $P < 0.01$), 3.3-fold by Ex-LNCs ($n = 3$, $P < 0.01$), 1.3-fold by C-LNCs ($n = 3$, $P < 0.01$), and 1.1-fold by D-LNCs ($n = 3$, $P < 0.01$). Likewise, the expression of Notch receptor Notch1 in LECs was decreased 3.8-fold by DC-LNCs ($n = 3$, $P < 0.01$), 3.0-fold by Ex-LNCs ($n = 3$, $P < 0.01$), 1.5-fold by C-LNCs ($n = 3$, $P < 0.01$), and 1.1-fold by D-LNCs

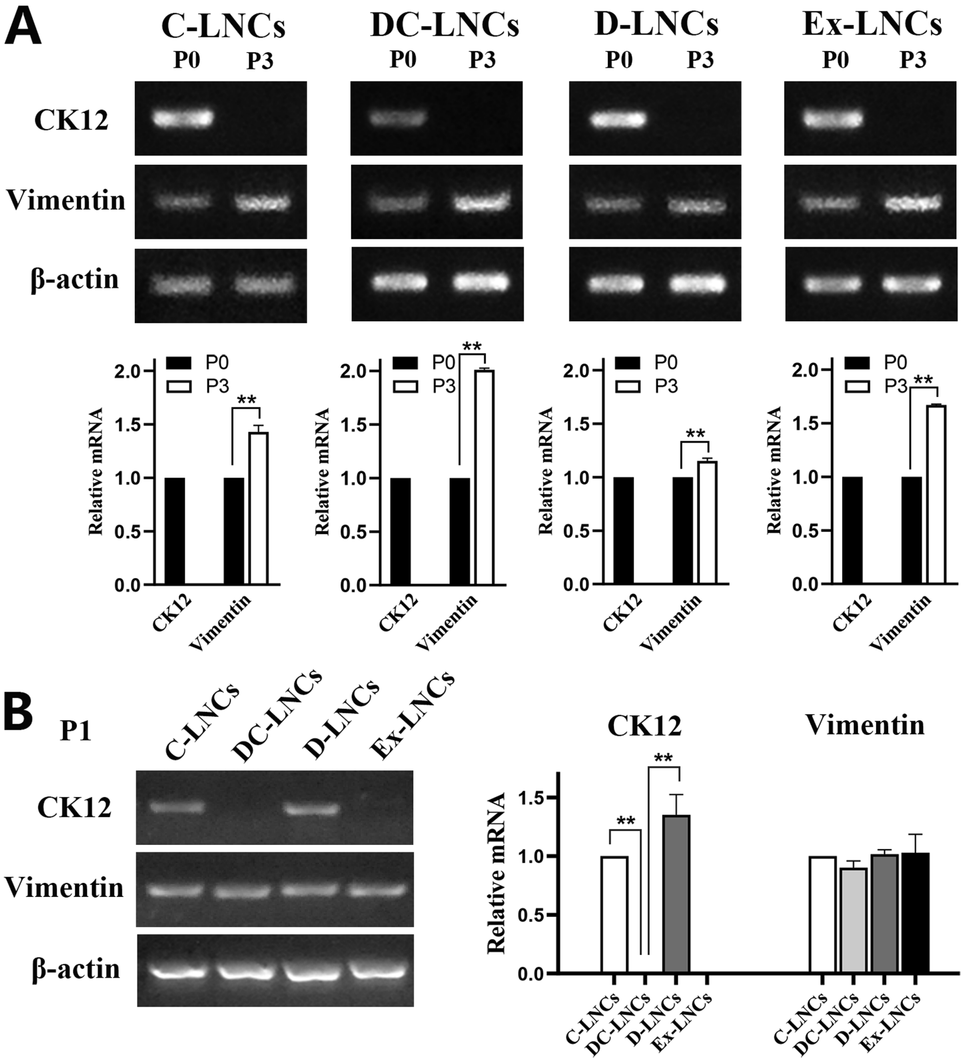

Figure 3. Purification of C-LNCs, DC-LNCs, D-LNCs, and Ex-LNCs. (**A**) The transcript levels of CK12 and vimentin in P0 and P3 LNCs. (**B**) In P1 LNCs, DC-LNCs and Ex-LNCs exhibited negative expression of CK12.

($n = 3$, $P < 0.05$). Compared with LECs alone, LECs supported by DC-LNCs expressed a 3.9-fold downregulation of Hes1 (a major downstream target) transcript ($n = 3$, $P < 0.01$), with 3.2-fold downregulation in the Ex-LNC group ($n = 3$, $P < 0.01$) and 1.4-fold downregulation in the C-LNC group ($n = 3$, $P < 0.01$) (Fig. 8). These data collectively indicate that DC-LNCs imposed a significant inhibitory effect on Notch signaling in LESCs.

DISCUSSION

In this investigation, we evaluated a range of different methods (C-LNC, DC-LNC, D-LNC, and Ex-LNC) to optimize the isolation process of LNCs for corneal tissue engineering. We observed that DC-LNCs had a greater capacity to express ESC markers and maintain LESCs compared to C-LNCs, D-LNCs, and Ex-LNCs. This was further confirmed by the inhibition of Notch signaling.

Cumulative evidence suggests that LNCs play a significant role in regulating the state of LESCs,[13,29] as expression of ESC markers in LNCs is crucial for their niche function.[14] Previous studies have shown that dispase cleaves the basement membrane[30] and yields substantial epithelial colonies[31] but removes only a few subjacent mesenchymal cells.[11] In addition, explant culture of limbal stroma tissues has also been reported in the isolation of LNCs[32] and shown to be more efficient than the D-LNC method.[19,33] In the studies of Chen et al.[11] and Xie et al.,[14] collagenase cleaved the interstitial stroma, not the basement membrane, and isolated clusters containing more LNCs compared to dispase. Furthermore, Li et al.[16] demonstrated that the DC-LNC method enriched the isolation of LNCs, yielding clusters composed of approximately 95% mesenchymal cells and 5% epithelial cells compared to clusters consisting of roughly 20% mesenchymal cells and 80% epithelial cells in the C-LNC method. Consistent with their findings, our results showed fewer epithelial components in DC-LNCs compared to other LNCs. We observed that the expression of ESC markers in DC-LNCs was significantly higher than in the other three groups. Given the powerful potential of mesenchymal cells to support epithelial progenitors in the limbal niche,[34] we hypothesize that epithelial constituents "deplete" the stem cell characteristics of mesenchymal constituents in vitro. This might explain why DC-LNCs express more ESC markers

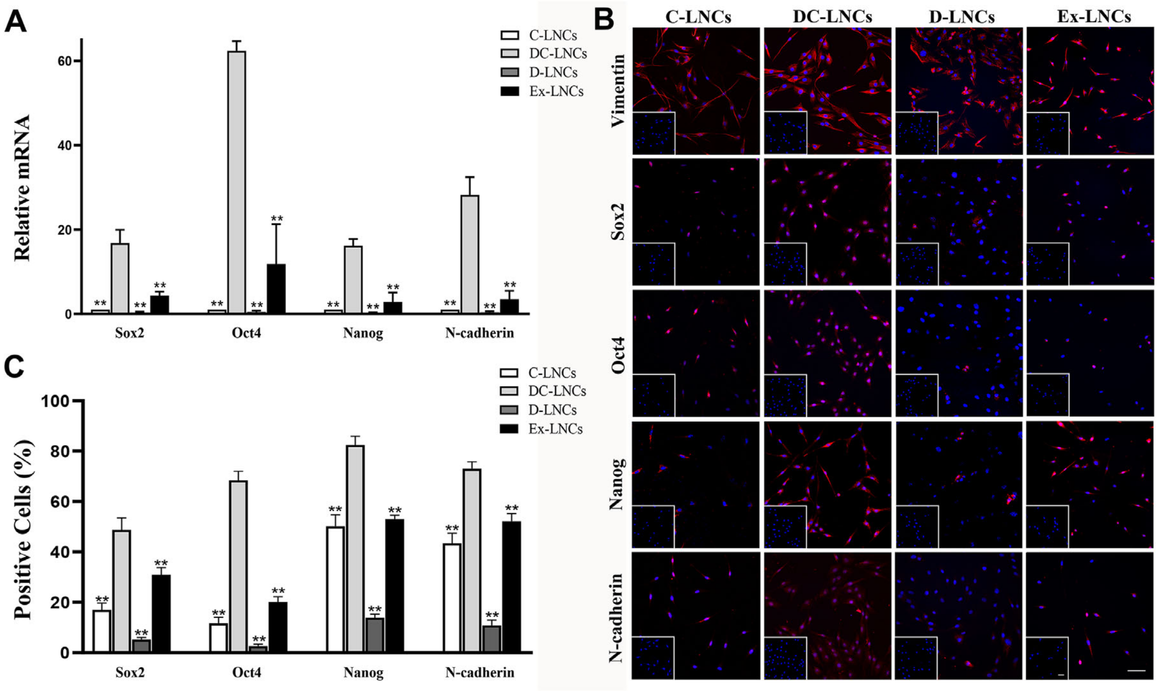

FIGURE 4. ESC marker expression of C-LNCs, DC-LNCs, D-LNCs, and Ex-LNCs. (**A**) Higher transcript levels of Sox2, Oct4, Nanog, and N-cadherin were observed in DC-LNCs than in C-LNCs, D-LNCs, and Ex-LNCs ($n = 3$, $^{**}P < 0.01$). (**B**) Immunofluorescence staining of vimentin, Sox2, Oct4, Nanog, and N-cadherin in the four types of LNCs. (**C**) A higher positive percentage of Sox2, Oct4, Nanog, and N-cadherin was observed in DC-LNCs than in other LNCs ($n = 4$, $^{**}P < 0.01$). Boxes in the *left bottom* show the immunofluorescence controls. *Scale bars*: 50 µm.

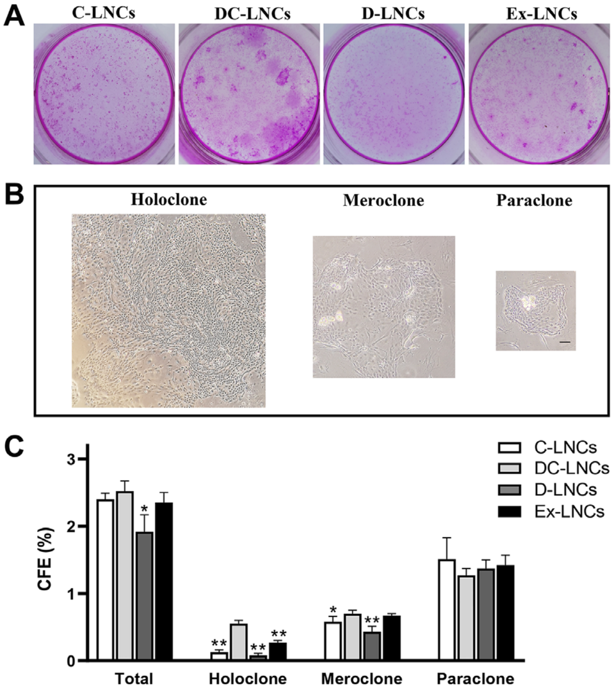

FIGURE 5. Comparison of capacity to support LESCs in forming colonies. (**A**) Rhodamine B staining of epithelial colonies supported by C-LNCs, DC-LNCs, D-LNCs, and Ex-LNCs. (**B**) Morphological characterization of holoclone, meroclone, and paraclone. (**C**) Colony-forming efficiency of total clone, holoclone, meroclone, and paraclone in the four groups. *Scale bar*: 50 µm.

than do the other LNCs, which will be further investigated by us in the future.

As LNCs have the capacity to maintain LESCs in vitro, colony-forming assays and 3D Matrigel and Transwell cocultures are used extensively in many investigations.[18,35,36] Colony-forming assays have remained an important tool for assessing the status of LESCs, as they clearly reveal the self-renewable potential and proliferative characteristics of a single stem cell.[11,14] Growing evidence emphasizes the importance of a 3D cellular environment in determining cell fate. 3D Matrigel restores the close association between LESCs and LNCs in vitro and thus helps prevent corneal epithelial differentiation compared to coated and two-dimensional Matrigel.[14,37] In addition, the contactless support in the Transwell system facilitates assessing the ability to maintain LESCs among the different LNCs. Furthermore, airlifting in the Transwell system promotes proliferation, migration, and stratification of corneal epithelial cells,[38] which might provide an experimental basis for transplantable corneal epithelial sheets in clinical use. The higher holoclone-forming efficiency in the DC-LNC group demonstrated the outstanding capacity of DC-LNCs to support LESCs in clonal growth. The results from 3D Matrigel coculture indicate downregulation of CK12 and upregulation of ΔNp63α in LECs cocultured with DC-LNCs compared to the C-LNC, D-LNC, and Ex-LNC groups. Similarly, the results in the Transwell system further confirmed upregulation of Ki67 and ΔNp63α in LECs cocultured with DC-LNCs. These results collectively suggest that LNCs generated by the DC-LNC method were more successful in maintaining the undifferentiated and proliferative characteristics of LESCs. Also, the parallel connection between stemness and proliferation in

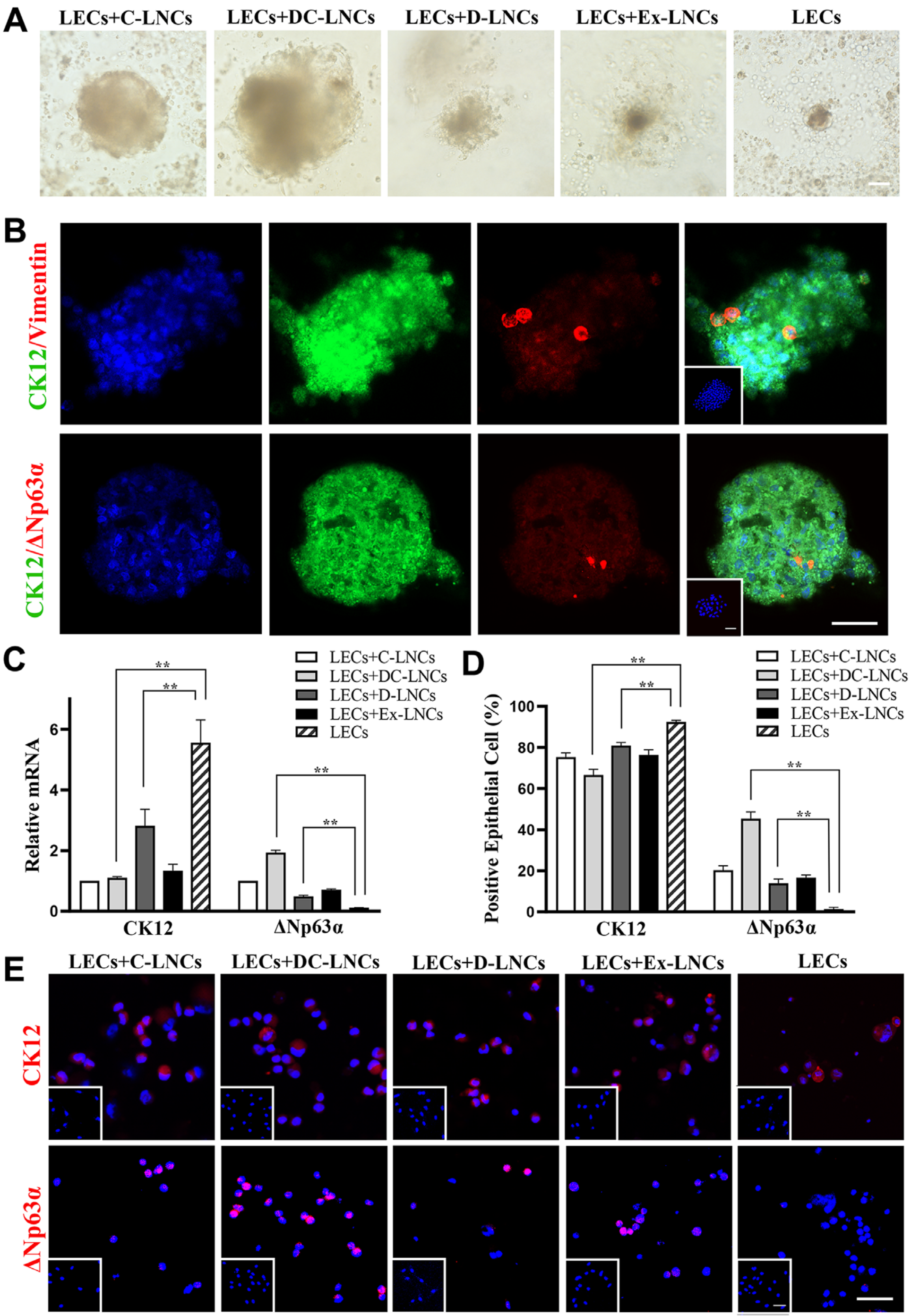

FIGURE 6. Comparison of capacity to maintain LESCs in 3D Matrigel. (**A**) Mix of P0 LECs with P4 C-LNCs, DC-LNCs, D-LNCs, and Ex-LNCs in 3D Matrigel. (**B**) Double immunostaining of CK12 and vimentin or ΔNp63*α* in LEC + DC-LNC spheres. (**C**) Transcript level of CK12 and ΔNp63*α* in LECs before and after reunion with various LNCs. (**D**) Immunofluorescence staining analysis of CK12 and ΔNp63*α* in LECs before and after the addition of different LNCs. (**E**) Immunostaining of CK12 and ΔNp63*α* in single cells obtained on day 7. Boxes in the *left bottom* show the immunofluorescence controls. *Scale bars*: 50 µm.

LESCs could be attributed to the culture medium (SHEM) we used in the Transwell system, which contained the cytokines (i.e., insulin, transferrin, sodium selenite, human epidermal growth factor, and human leukemia inhibitory factor) that are supposed to promote the proliferation and stratification of LESCs. Because the proliferative capacity and percentage of p63+ cells in cultivated epithelial grafts are closely associated with successful transplantation,[39–41] DC-LNCs present an exciting prospect for use in corneal tissue engineering applications.

Prior studies have suggested that LNCs prevent LESCs from differentiating primarily by inhibiting the Notch signaling pathway.[15,42] Our results indicate that DC-LNCs inhibit the Notch signaling of LESCs specifically via inhibition of

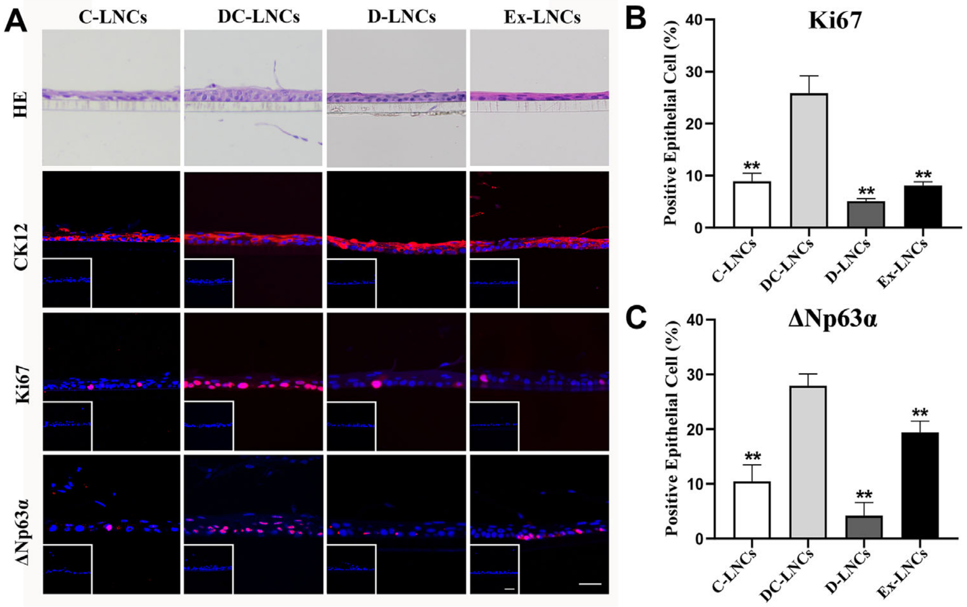

Figure 7. Comparison of capacity to maintain LESCs in Transwell system. (**A**) Hematoxylin and eosin and immunofluorescence staining of CK12, Ki67, and ΔNp63α in stratified LECs induced by C-LNCs, DC-LNCs, D-LNCs, and Ex-LNCs. (**B**, **C**) Significantly higher expression of Ki67 and ΔNp63α was observed in the DC-LNC group than in the C-LNC, DC-LNC, D-LNC, and Ex-LNC groups ($n = 4$, $^{**}P < 0.01$). Boxes in the *left bottom* show the immunofluorescence controls. *Scale bars:* 50 µm.

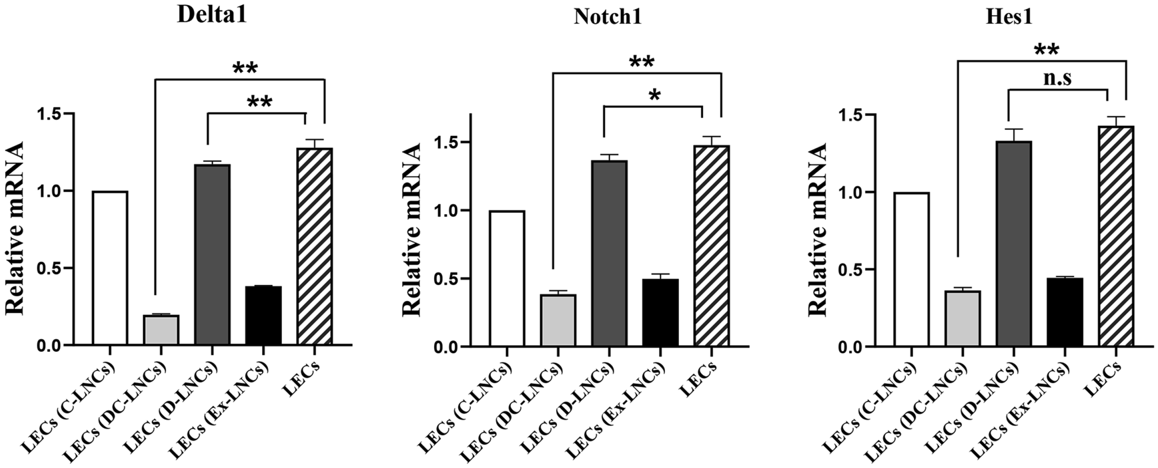

Figure 8. Notch signaling in LESCs cocultured with C-LNCs, DC-LNCs, D-LNCs, and Ex-LNCs. The transcript levels of Notch family members Delta1, Notch1, and Hes1 in LECs supported by C-LNCs, DC-LNCs, D-LNCs, and Ex-LNCs in Transwell inserts. Single LECs served as control.

the Notch ligand through a process called *cis*-inhibition,[43] which works through ligand–receptor interactions, playing a pivotal role in the regulation of Notch signaling.[44,45] Based on these data, we concluded that DC-LNCs prevent LESCs from differentiating predominantly through *cis*-inhibition of the Notch signaling.

Our results differ from a prior study, however, which described that dispase-isolated cells have a greater capacity to maintain LESCs than do Ex-LNCs.[18] This difference might be ascribed to the different culture media used in the isolation and expansion of LNCs. The investigation by Li et al.[18] demonstrated that LNCs could be isolated by dispase digestion and expanded in keratinocyte culture medium (KCM) containing 5% fetal bovine serum. In our experiment, the culture medium we used was MESCM containing 10% knockout serum replacement, which differs from KCM. Previous studies have reported that LNCs tend to differentiate and irreversibly lose the ESC characteristic phenotype when cultured in serum-supplemented medium for a long time.[46] We found that the expression of ESCs and proliferative markers of LNCs was better preserved in MESCM than in serum-supplemented medium.[14] Furthermore, a prior study comparing seven different LNC-culture media types demonstrated that a medium containing knockout serum replacement produced a cell phenotype closest to a pluripotent stem cell.[47] We speculate that a comparison of methods used to isolate LNCs might be most appropriate when culturing in MESCM.

CONCLUSIONS

This investigation identified an optimal approach to isolating LNCs by evaluating four previously reported methods. Our study found that DC-LNCs had fewer epithelial components in the process of isolation. We also observed that DC-LNCs exhibited a significantly greater capacity to express ESC markers and maintain LESCs in an undifferentiated state compared to the other LNCs. This was further evidenced by the inhibition of Notch signaling. Our investigation could promote standardization of the isolation process of LNCs for tissue engineering and provide an exciting prospect for ocular surface reconstruction.

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SUPPLEMENTARY MATERIAL

SUPPLEMENTARY VIDEO S1. 3D reconstruction of LEC + DC-LNC sphere stained with CK12 and vimentin.

SUPPLEMENTARY VIDEO S2. 3D reconstruction of LEC + DC-LNC sphere stained with CK12 and ΔNp63α.