Limbal Niche Cells and Three-Dimensional Matrigel-Induced Dedifferentiation of Mature Corneal Epithelial Cells

Hui Zhu,[1] Wei Wang,[1] Yongyao Tan,[1] Guanyu Su,[1] Lingjuan Xu,[1] Meng lin Jiang,[1] Shen Li,[1] Yaa-Jyuhn James Meir,[3] Yunming Wang,[2] Guigang Li,[1] and Huamin Zhou[2]

[1]Department of Ophthalmology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei Province, China
[2]State Key Laboratory of Material Processing and Die & Mould Technology, School of Materials Science and Engineering, Huazhong University of Science & Technology, Wuhan, Hubei Province, China
[3]Department of Biomedical Sciences, College of Medicine, Chang Gung University, Linkou, Taiwan

Correspondence: Guigang Li, Department of Ophthalmology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei Province 430030, China; guigli@163.com.
Yunming Wang, State Key Laboratory of Material Processing and Die & Mould Technology, School of Materials Science and Engineering, Huazhong University of Science & Technology, Wuhan, Hubei Province 430074, China; wang653@hust.edu.cn.
Yaa-Jyuhn James Meir, Department of Biomedical Sciences, College of Medicine, Chang Gung University, Taoyuan 333, Taiwan; hercats1@gmail.com.
Huamin Zhou, State Key Laboratory of Material Processing and Die & Mould Technology, School of Materials Science and Engineering, Huazhong University of Science & Technology, Wuhan, Hubei Province 430074, China; hmzhou@hust.edu.cn.

Hui Zhu and Wei Wang contributed equally to this work.

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Purpose. To investigate the phenotypic changes of mature corneal epithelial cells (MCECs) that cocultured with limbal niche cells (LNCs) in three-dimensional Matrigel (3D Matrigel) in vitro.

Methods. MCECs were isolated from central corneas, and limbal epithelial progenitor cells (LEPCs) were isolated from limbal segments with Dispase II. LNCs were isolated and cultured from limbal niche using the collagenase A digestion method and identified with PCK/VIM/CD90/CD105/SCF/PDGFRβ. MCECs were cultured on 3D Matrigel (50%, v/v) with or without LNCs for 10 days. Expression of CK12 and p63α and clone formation test were used to compare the progenitor phenotypic changes for MCECs before and after induction using LEPCs as control.

Results. Homogeneous LNCs were isolated and identified as spindle shape and adherent to a plastic surface coated with 5% Matrigel. Double immunostaining of the fourth-passage LNCs was uniformly PCK$^-$/VIM$^+$/CD90$^+$/CD105$^+$/SCF$^+$/PDGFRβ^+. Reverse transcription and quantitative real-time polymerase chain reaction (RT-qPCR) revealed the decrease of PCK expression from the second passage and elevation of Vim, CD90, CD105, SCF, and PDGFRβ transcripts from the third passage, and the transcription level of Vim, CD90, CD105, SCF, and PDGFRβ was elevated statistically in the fourth passage compared to the first passage ($P < 0.01$). Both immunofluorescence (IF) staining for cross section and cytospin cells demonstrated that MCECs expressed higher CK12 while lower p63α than LEPCs ($P < 0.01$). Sphere growth formation was noticed as early as 24 hours in the MCEC + LNC group, 48 hours in the LEPC group, and 72 hours in the MCEC group. The diameters of the spheres were the biggest in the MCEC + LNC group (182.24 $\pm$ 57.91 µm), smaller in the LEPC group (125.71 $\pm$ 41.20 µm), and smallest in the MCEC group (109.39 $\pm$ 34.85 µm) by the end of the 10-day culture ($P < 0.01$). Double immunostaining with CK12/p63α showed that cells in the sphere formed from MCECs expressed CK12 but not p63α; in contrast, some cells in the MCEC + LNC group expressed CK12, but most of them expressed p63α. RT-qPCR revealed a significant reduction of CK12 transcript but elevation of p63α, Oct4, Nanog, Sox2, and SSEA4 ($P < 0.05$). Holoclone composed of cubic epithelial cells could be generated in the MCEC + LNC group but not in the other two groups.

Conclusions. The data shows that human MCEC cell phenotype could be induced to the dedifferentiation stage when cocultured with LNCs in 3D Matrigel that simulated the microenvironment of limbal stem cells *in vitro*.

Keywords: limbal niche cell, mature corneal epithelial cell, dedifferentiation

Corneal disease is the second major cause of blindness worldwide, attributing to 3.2% of global blindness.[1] Limbal stem cell deficiency (LSCD) is one of the most common debilitating eye disorders that causes corneal blindness. The limbus forms a border between the corneal and conjunctival epithelium, and cornea epithelial stem cells, traditionally named limbal stem cells (LSCs), locate in limbal palisades of Vogt, are ready to self-renew, and give birth to transiently expanded cells (TACs), which proliferate and differentiate into mature corneal epithelial cells (MCECs)

and maintain an intact cornea epithelial layer that protects the cornea from injury and infection.[2] Normally, the quiescent state, self-renewal, proliferation, and terminal differentiation of LSCs are well regulated by the limbal microenvironment.[3] However, LSCs could be destroyed by various etiologies, which can result in a delay of corneal epithelial regeneration, overgrowth of conjunctival-derived epithelial cells, corneal stromal neovascularization, and corneal opacity.[4,5] Literature has confirmed that patients with unilateral LSCD could be cured by autologous LSC transplantation (LSCT),[6] but patients with binocular LSCD have lost their autologous LSCs and thus could have only allograft LSCT or autologous substitute cell transplantation.[7] The major disadvantage of allogenic LSCT is immune rejection from the recipient, while autologous substitute cells, such as oral mucosal epithelial cells, have a higher possibility of angiogenic differentiation and the uncertainty of long-term transplantation effects,[8–13] and thus more strategies for autologous cell reverse differentiation into LSCs are expected in the future.

Regeneration of injured tissues is usually associated with cell fate plasticity, and reverse differentiation, also called dedifferentiation, has been reported in more and more tissue regeneration environments, which provide strategies to restore autologous stem cells in vivo.[14] As early as 2003, Del Rio-Tsonis and Tsonis[15] had found that in adults, mature cells could dedifferentiate and become proliferative while maintaining their original lineage commitment and forming cells loyal to the fate of the original progenitor cells. In recent years, studies using lineage tracing technology have proven the phenomenon of original cell fate changes caused by injury in many mammalian tissues, such as the reverse differentiation of epithelial cells under pathologic conditions.[11] In 2013, Tata et al.[16] demonstrated that after ablation of airway epithelial stem cells, the luminal secretory cells had dedifferentiated into basal stem cells, which not only showed indistinguishable morphology from stem cells and expressed stem cell markers (CK5, p63) but also could proliferate and differentiate to produce new mature epithelial cells and repair damaged epithelium. Moreover, single secretory cells cultured in the three-dimensional (3D) Matrigel environment in vitro could dedifferentiate into multipotent stem cells in vitro. In 2018, Nasser et al.[17] discovered that following deletion of LSCs without destruction of the limbal stroma niche, dedifferentiation of corneal epithelial cells into $K15^+$ stem cells was induced and restored the limbus function, while deletion of LSCs together with the niche abolished $K15^+$ recovery and resulted in severe LSCD, suggesting that dedifferentiation of corneal epithelial cells into $K15^+$ LSCs requires an intact limbal stroma niche. Thus, it could be established that corneal epithelial cells including TACs and MCECs could be induced to dedifferentiation back to LSCs by the natural limbal stroma niche in vivo, but it is not known whether MCECs could also be induced to dedifferentiation back to LSCs by the limbal niche microenvironment in vitro.

The components of the LSC niche[15] include extracellular Matrigel, melanocytes,[18] stromal cells, blood vessels, nerves, and so on. The isolation and expansion of the microenvironment cells have important scientific significance for understanding LSC niche regulation, playing a crucial role for the in vitro reconstruction of the LSC niche.

In 2012, Xie et al.[19] first isolated and expanded limbal niche cells (LNCs) from the limbus niche of the cornea, which express vimentin and other embryonic stem cell markers such as Oct4, Sox2, and Nanog but not PCK. Li et al.[20,21] further investigated LNCs and proved them to be a group of multipotent stem cells that could give birth to blood vessel endothelial progenitor cells and mesenchymal stem cells. Through in vitro and in vivo experiments, Li et al.[22] showed that LNCs were a more powerful resource than bone marrow mesenchymal stem cells (BMMSCs) to prevent LSCD in an alkali burn rabbit model, and the power for such a protective effect may be due to increased activation of SCF signaling.

Three-dimensional Matrigel and a modified ESC medium (MESCM) culture condition could induce the reverse differentiation of mature cells into stem cells.[23] In our previous experiments, serial passages on 5% coated Matrigel resulted in rapid expansion of LNCs, of which the expression of embryonic stem cell markers (Oct4, Sox2, Nanog, Rex1, SSEA4) and vascular progenitor cell markers (flk-1, CD34, CD31) reduced gradually, but those markers could be restored after culture in 3D Matrigel in MESCM.[19,21] Limbal epithelial progenitor cells (LEPCs) cocultured with LNCs in 3D Matrigel and MESCM for 10 days could generate spheres, and the epithelial cells expressed a more epithelial stem cell marker (p63α) and a less corneal-specific differentiation mature marker (CK12).[19–21]

In the current research, we found that when MCECs and LNCs were cocultured in 3D Matrigel, it was possible to induce them to dedifferentiate into LSCs. This new discovery may bring a new strategy for the source of autologous LSCs, which may be a kind of potential novel therapy for LSCD in the future. This study also suggests that 3D Matrigel plus LNCs are effective in simulating the LSC microenvironment in vitro.

Materials and Methods

Cell Isolation and Culturing

Corneas from donors 50 to 60 years old were obtained from the Red Cross Eye Bank of Wuhan City, Tongji Hospital (Hu Bei, China) and managed in accordance with the Declaration of Helsinki and approved by the Tongji Ethics Committee. The records of replicates in different-age donors are provided in the Supplementary Table S1.

Human LEPCs were isolated as reported,[19] and limbal explants were digested with 10 mg/mL Dispase II (Roche, Indianapolis, IN, USA) at 4°C for 16 hours to generate intact limbal epithelial sheets. Human MCECs were isolated from the central cornea using the same method as LEPCs to obtain intact epithelial sheets. Central and limbal epithelial sheets were prepared for a 6-µm cryosection to be examined with immunofluorescence (IF) staining or further digested with 0.25% trypsin and 1 mM EDTA (T/E) at 37°C for 15 minutes to yield single epithelial cells.

Human LNCs were isolated and cultured as previously prescribed.[19,21] Limbal explants were digested with 2 mg/mL collagenase A at 37°C for 8 to 10 hours to generate clusters containing the entire limbal epithelial sheet with subjacent stromal cells. The clusters were further digested with T/E at 37°C for 15 minutes to obtain single cells before being seeded at a density of 1×10^4 per cm^2 in 6-well plates coated with 5% Matrigel for 2 hours before use in MESCM. MESCM is made of Dulbecco's modified Eagle's medium supplemented with 10% knockout serum, 5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL selenium, 50 µg/mL gentamicin, 1.25 µg/mL amphotericin B, 10 ng/mL leukemia inhibitory factor (LIF), and 4 ng/mL basic fibroblast growth factor (b-FGF). Upon 80% to 90% confluence, LNCs were passaged serially at a density of 5×10^3 per cm^2. The fourth-passage LNCs underwent cytospin for IF, and the fourth- to sixth-passage LNCs

were used in the following experiments. All materials used for cell isolation and culturing are listed in Supplementary Table S2.

Cell Culture in 3D Matrigel

As reported previously,[21] 3D Matrigel was prepared by adding 150 µL of 50% Matrigel (diluted in MESCM) per chamber of an 8-well chamber slide following incubation at 37°C for 30 minutes. MCECs were seeded at a total density of 4×10^4 in each well in 3D Matrigel in the MCEC group. MCECs were seeded at a total density of 4×10^4 in each well together with 1×10^4 LNCs to get a 4:1 MCEC/LNC cell ratio in MCEC + LNC group. LEPCs were seeded at a total density of 4×10^4 in each well in 3D Matrigel in the LEPC group. Cells were viewed and photo recorded every 24 hours, and medium changed every 3 days during a 10-day culture period. The sphere growth formation times were recorded and the dimeters of the spheres were measured using ImageJ (National Institutes of Health, Bethesda, MD, USA) software. The resultant sphere growth was collected by digestion of Matrigel with 10 mg/mL Dispase II at 37°C for 2 hours, and then the spheres were digested further with T/E at 37°C for 15 minutes to yield single cells.

IF Staining

Single cells and spheres were prepared for cytospin using Cytofuge at 1000 rpm for 8 minutes at a cell density of 1×10^3 each slide (StatSpin, Inc., Norwood, MA, USA). For immunofluorescence staining, the samples were fixed with 4% paraformaldehyde, permeated with 0.2% Triton X-100 in PBS for 15 minutes, and blocked with 2% BSA in PBS for 1 hour before being incubated with primary antibodies overnight at 4°C. After the uncombined primary antibodies were washed away with PBS, corresponding secondary antibodies were incubated for 1 hour using appropriate isotype-matched nonspecific IgG antibodies as controls. The nucleus was counterstained with DAPI before being viewed and analyzed with a Zeiss LSM 700 confocal microscope (LSM700; Carl Zeiss AG, Oberkochen, Germany). The quantitation was done by ImageJ (National Institutes of Health) software. Detailed information about primary and secondary antibodies and agents used for IF is listed in Supplementary Table S3.

Reverse Transcription and Quantitative Real-Time Polymerase Chain Reaction

Total RNAs were extracted by the RNeasy Mini RNA Isolation Kit (Qiagen, Valencia, CA, USA). A total of 1 to 2 µg total RNA was reverse-transcribed to cDNA by the High Capacity cDNA Transcription Kit (Applied Biosystems, Foster City, CA, USA). Reverse transcription and quantitative real-time polymerase chain reaction (RT-qPCR) was carried out in a 20-µL solution containing cDNA, TaqMan Gene Expression Assay Mix, and universal PCR Master Mix (Applied Biosystems). The results were normalized by an internal control (i.e., glyceraldehyde-3-phosphate dehydrogenase). All assays were performed in triplicate for each primer set. The relative gene expression was analyzed by the comparative CT method ($\Delta\Delta C_T$). All TagMan Gene Expression Assays with probe sequences are listed in Supplementary Table S4.

Fibroblast CFU Assay on 3T3 Feeder Layer

To determine the holoclone formation rate (fibroblast CFU [CFU-F]) for MCECs before and after culture in 3D Matrigel with or without LNCs, 1×10^3 cells were seeded on mitomycin (MMC)–processed 3T3 cells as reported in the protocol, and the number and dimeters of the epithelial holoclone formed in each group were recorded and compared between groups after 12 days of culturing.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, USA) and ImageJ (National Institutes of Health) software. All assays were performed in triplicate, each with a minimum of three donors. The data were reported as means ± SD and compared using the appropriate version of Student's unpaired *t*-test. Test results were reported as two-tailed *P* values, where $P < 0.05$ was considered statistically significant.

Results

Homogeneous LNCs Cultured on Coated Matrigel in MESCM

To prepare homogeneous LNCs from human cornea for this study, we repeated the experiments of isolating and expanding LNCs using the Collagenase A method as Xie et al.[19] and ourselves[21] have reported previously. Without a doubt, clusters could be separated from the limbus segment after 10 hours of digestion with 1 mg/mL Collagenase A (Fig. 1A, clusters), then further digested with T/E to get single cells and seeded at a density of 1×10^5 per cm^2 and serially passaged on a plastic surface coated with 5% Matrigel in MESCM. After 5 days of culture, spindle cells proliferated among small cubic epithelial cells in P0 (Supplementary Fig. S1, P0). Spindle LNCs and some cubic epithelial cells could be seen in the first passage (Fig. 1A, P1), and cubic epithelial cells were reduced dramatically in the second passage (Fig. 1A, P2). Homogeneous spindle LNCs could be warranted from the third passage (Fig. 1A, P3) and more confidently in the latter passages (Fig. 1A, P4). Double immunostaining of fourth-passage LNCs prepared with cytospin showed that such cells were uniformly $PCK^-/Vim^+/CD90^+/CD105^+/SCF^+/PDGFR\beta^+$ (Fig. 1B). Nuclei were counterstained by DAPI (blue) (scale bar = 50 µm in A clusters, while 20 µm in other figures). RT-qPCR revealed the decrease of PCK expression from the second passage with elevation of Vim, CD90, CD105, SCF, and PDGFRβ transcripts from the third passage, and the transcription level of Vim, CD90, CD105, SCF, and PDGFRβ was elevated dramatically in the fourth passage compared to the first passage ($P < 0.01$; Fig. 1C).

MCECs Express More CK12 but Less p63α Than That of LEPCs

To identify the phenotype baseline of MCECs before coculture with LNCs, we used LEPCs as control group. Following the reported protocol,[19–21] central or limbus segments were treated with 10 mg/mL Dispase II for 10 hours in 4 °C, which could digest the basement membrane of the epithelial cells and separate an intact epithelial layer from the stroma (Fig. 2A, left column). Such epithelial layers were either prepared for frozen cross section (Fig. 2A, middle column) or further separated into single cells by T/E digestion. Both cross sections and single cells were double immunostained with CK12/p63α. MCECs from the central cornea uniformly expressed CK12 but not p63α (Fig. 2A,

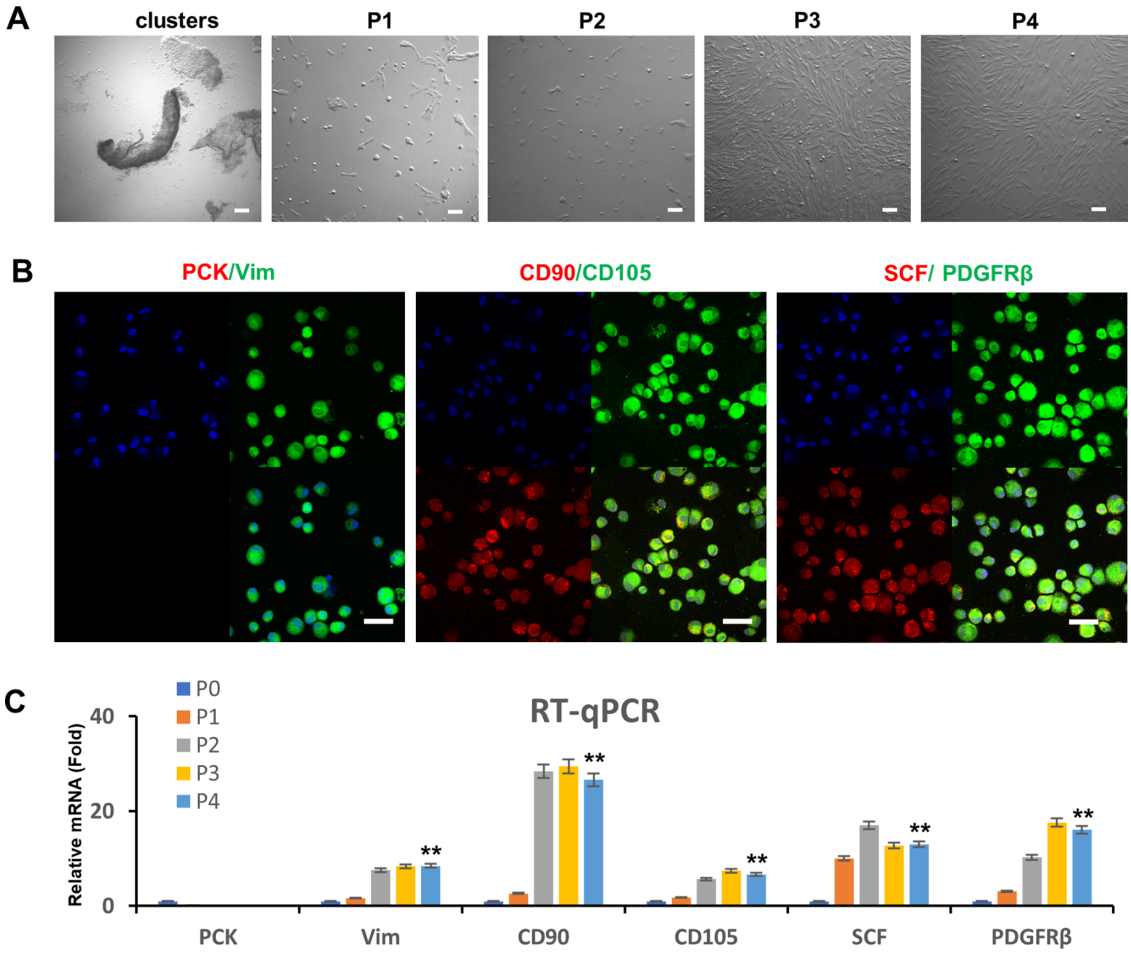

Figure 1. Homogeneous LNCs cultured on coated Matrigel in MESCM from the third passage. Clusters could be separated from the limbus segment after 10 hours of digestion with 1 mg/mL collagenase in 37°C (**A**, clusters), then further digested with T/E to get single cells and seeded at a density of 1×10^5 per cm^2 and serially passaged on coated Matrigel in MESCM. After a 5-day culture, spindle cells and some cubic epithelial cells existed in the first passage (**A**, P1), and epithelial cells reduced dramatically in the second passage (**A**, P2). Homogeneous spindle LNCs could be warranted from the third passage (**A**, P3) and more confidently in the latter passages (**A**, P4). Double immunostaining of fourth-passage LNCs prepared with cytospin shows that such cells were uniformly $PCK^-/VIM^+/CD90^+/CD105^+/SCF^+/PDGFR\beta^+$ (**B**). Nuclei were counterstained by DAPI (blue). Scale bar = 50 µm in A clusters, while 20 µm in other figures. RT-qPCR revealed the decrease of PCK expression from the second passage and elevation of Vim, CD90, CD105, SCF, and PDGFRβ transcripts from the third passage. The transcription level of Vim, CD90, CD105, SCF, and PDGFRβ was elevated dramatically in the fourth passage compared to the first passage (**C**) ($P < 0.01$).

upper row). In contrast, LEPCs from the limbus expressed a lower level of CK12 but higher p63α than MCECs, with $CK12^-/p63\alpha^+$ progenitor cells predominately localized in the basal layer (Fig. 2A, bottom row). Nuclei were counterstained by DAPI (blue). Statistics of the positive rate of CK12 and p63α revealed the higher CK12 but lower p63α level in MCECs than in LEPCs (Fig. 2B, $P < 0.01$). Another stem cell marker, K15, was also identified. Double immunostaining with K15 (green) and p63α (red) revealed that MCECs from the central cornea expressed neither K15 nor p63α (upper row), whereas LEPCs from the limbus expressed both K15 and p63α (bottom row, white arrow) (Supplementary Fig. S2).

MCECs or LEPCs Alone Form Smaller Spheres Than MCECs Cocultured With LNCs on 3D Matrigel

As reported in the previous study, sphere growth by single cells obtained by trypsin and EDTA from collagenase-isolated clusters emerged in a density-dependent manner in 3D Matrigel with a close association with LNCs, which indicated that LNCs enhance the ability of epithelial clonal growth.[21] However, it is not known whether MCECs alone or cocultured with LNCs in 3D Matrigel could generate sphere growth. To test the influence of 3D Matrigel and LNC coculture on the epithelial cell phenotype, MCECs alone (MCEC group), LEPCs alone (LEPC group), or MCECs together with LNCs (MCEC + LNC group) were cultured in 3D Matrigel for 10 days (Fig. 3A, D1). Sphere growth formation was noticed as early as 24 hours in the MCEC + LNC group, 48 hours in the LEPC group, and 72 hours in the MCEC group (Fig. 3A, D3). The diameters of the spheres were the biggest in the MCEC + LNC group (182.24 ± 57.91 µm), smaller in the LEPC group (125.71 ± 41.20 µm), and smallest in the MCEC group (109.39 ± 34.85 µm) by the end of the 10-day culture (Fig. 3A, D10). The diameters of the spheres in the MCEC + LNC group were significantly larger than that of the other two groups ($P < 0.01$; Fig. 3B). Moreover, the number of spheres in the MCEC group, LEPC group, and MCEC + LNC group decreased from the beginning (day 1), which may

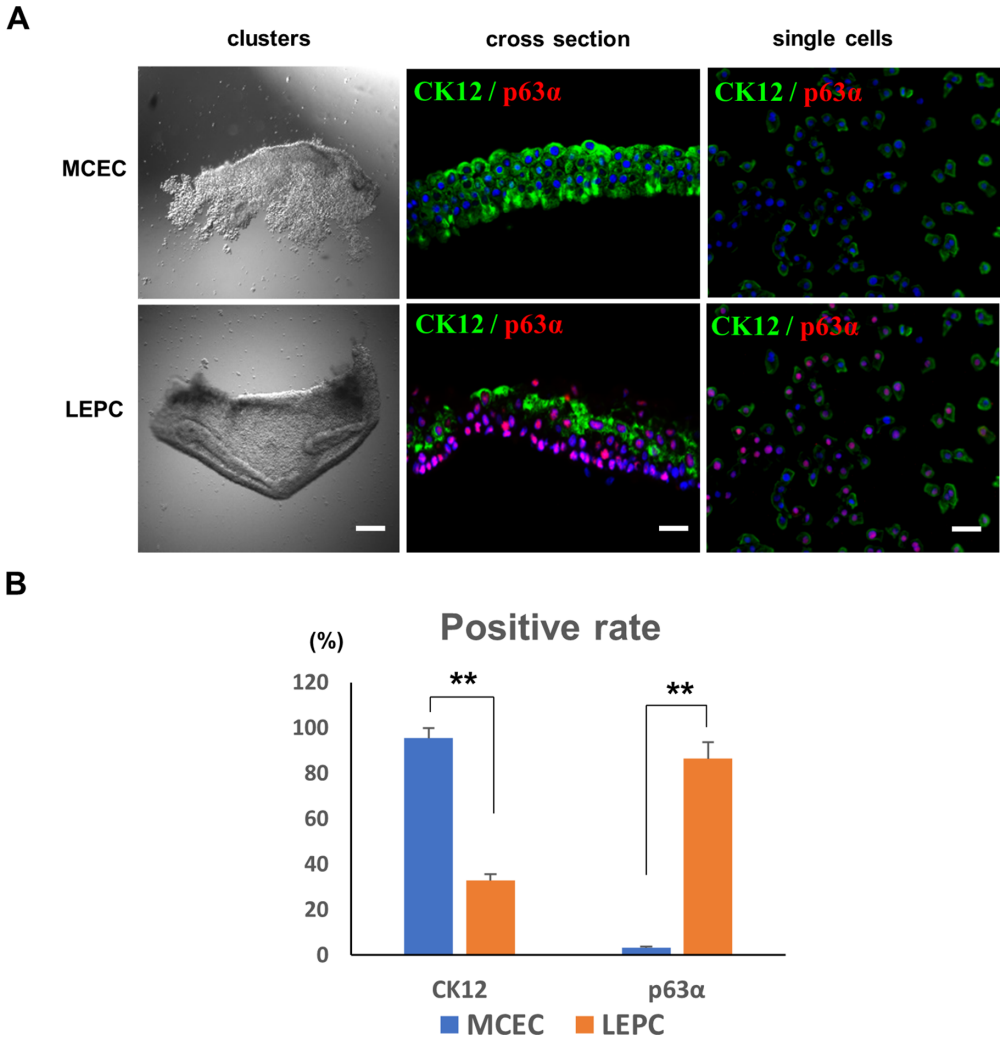

Figure 2. MCECs express more CK12 while less p63α than that of LEPCs. An intact epithelial layer of central or limbus segments (**A**, left column, scale bar = 100 µm) and frozen cross section (**A**, middle column, scale bar = 20 µm). MCECs from the central cornea uniformly express CK12 but not p63α (**A**, upper row). In contrast, LEPCs from the limbus lower the level of CK12 but have a higher p63α than MCECs, with $CK12^{-}/p63\alpha^{+}$ progenitor cells predominately localized in the basal layer (A, bottom row). Nuclei were counterstained by DAPI (blue). Scale bar = 100 µm in clusters, 20 µm in cross section and single cells. Statistics of the positive rate of CK12 and p63α compared between these two group cells revealed the higher CK12 but lower p63 level in MCECs than that in LEPCs (**B**, $^{**}P < 0.01$).

be because of cell aggregation. Nevertheless, the number of spheres in MCEC and LEPC groups was significantly lower than that in MCEC + LNC group on day 1, day 3, and day 10, respectively (Supplementary Fig. S3).

Epithelial Cells from MCEC + LNC Spheres Express More p63α Genes and Form More Epithelial Holoclones Than That from MCECs or LEPCs on 3T3 Feeder Layer

After a 10-day culture in 3D Matrigel, the resultant sphere growth was collected by digestion of Matrigel with 10 mg/mL Dispase II at 37°C for 2 hours, and the spheres underwent cytospin for IF examination. Double immunostaining with CK12/p63α showed that cells in the sphere formed from MCECs expressed CK12 but not p63α; in contrast, some cells in the MCEC + LNC group expressed CK12, but most of them expressed p63α (Fig. 4A, scale bar = 50µm). RT-qPCR revealed a significant reduction of CK12 transcript but elevation of p63α, Oct4, Nanog, Sox2, and SSEA4 (Fig. 4B, $^{**}P < 0.01$, $^{*}P < 0.05$). The spheres were separated into single cells and seeded in 3T3 feeder layers pretreated with MMC at a density of 1×10^3 per 6 wells; holoclone made of cubic epithelial cells could be generated in the MCEC + LNC group but not in the other two groups (Fig. 4C, right figure is an enlargement of the left one, scale bar = 50 µm). PCK^{+}/Vim^{-} and PCK^{-}/Vim^{+} cells could be seen in spheres after a 10-day culture, suggesting that spheres were composed of epithelial cells (PCK^{+}/Vim^{-}) and LNCs (PCK^{-}/Vim^{+}), MCECs, and LNCs could be reunited in 3D Matrigel (Supplementary Fig. S4).

Discussion

It goes without saying that compared to allogenic limbal stem cell transplantation, autologous stem cell transplantation could avoid the risk of immune rejection and represents the future direction of limbal stem cell transplantation. Unfortunately, patients with severe LSCD lose their own LSCs. To get autologous LSCs for the treatment of LSCD,

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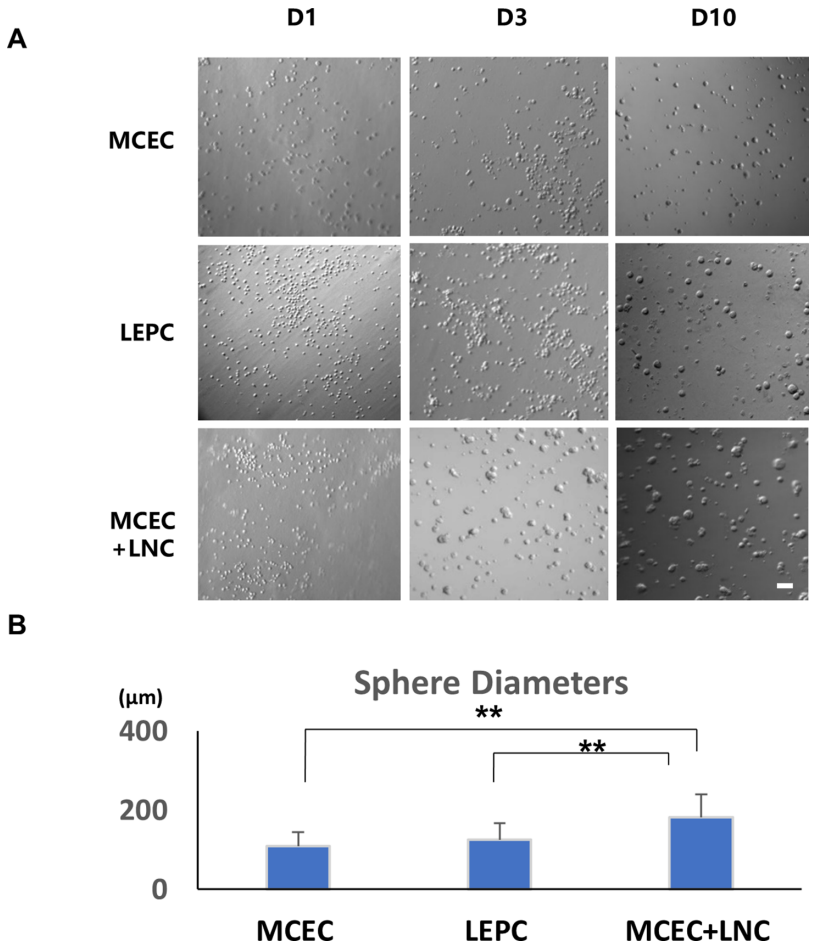

Figure 3. MCECs or LEPCs alone form smaller spheres than MCECs cocultured with LNCs on 3D Matrigel. MCECs alone, LEPCs alone, and MCECs together with LNCs were seeded at 3D Matrigel, respectively (**A**, D1). Sphere growth formation was noticed as early as 24 hours in the MCEC + LNC group, 48 hours in the LEPC group, and 72 hours in the MCEC group (**A**, D3). The diameters of the spheres were the biggest in the MCEC + LNC group, smaller in the LEPC group, and smallest in the MCEC group by the end of the 10-day culture (**A**, D10). The diameters of the spheres in the MCEC + LNC group were significantly larger than that of the other two groups (**B**, $^{**}P < 0.01$).

several strategies had been reported to induce substrate cells toward LSCs, including oral mucosal epithelial cells, hair follicle epithelial stem cells, mesenchymal stem cells, and so on. The most critical step in this work is to simulate and reconstruct the limbal stem cell microenvironment, and the isolation and culture of LNCs from the limbal stem cell niche is one of the most important developments recently.

Previously, Xie et al.[19] and our experiments[20,21] have shown that LEPCs cocultured with LNCs on 3D Matrigel result in sphere growth, and such a coculture prevents the differentiation of LEPCs toward mature cells, indicated by a reduction of CK12 and the promotion of p63α and CEBPδ, as well as elevated holoclone formation efficiency on the 3T3 feeder layer. We speculated that this phenomenon indicating that LNCs and 3D Matrigel in MESCM simulate the niche of LSCs is helpful to maintain the stemness of LSCs in vitro, but in the later experiments and inspired by the concept that the stem cell niche could induce the dedifferentiation of mature cells, we hypothesized that during the coculture period, what happened is not only the maintenance of the LSC stemness but also may be the induction of MCECs back into LSCs. This study tests the rationality of this hypothesis.

The first step is to get homogeneous LNCs in vitro. Although Xiao et al.[24] demonstrated recently that D + C (Dispase + Collagenase A) is optimal to isolate and expand LNCs from rats, our previous experiments indicated that D + C digestion of limbus segments has the advantage of getting homogeneous LNCs earlier than that of Collagenase A alone (P2 vs. P4)[21] and the same ability to support LEPCs in 3D Matrigel in MESCM. We have further verified that the Collagenase A method, first reported by Xie et al.[19] in 2012, has the highest success rate from a single human cornea, and thus we have applied for an patent in China on how to isolate and identify LNCs from human cornea. The results show that clusters could be separated from the limbus segment after 10 hours of digestion with 1 mg/mL Collagenase A (Fig. 1A, clusters), which consisted of not only the entire PCK^+ limbal epithelial cells but also the adjacent PCK^-/Vim^+ mesenchymal cells (MCs). Therefore, there were $PCK^+/P63\alpha^+$/stem cells (SC) $marker^+$ (N-cadherin, Nestin, and Oct4) cells in the digested cells. At the same time, small $PCK^-/p63\alpha^-/Vim^+$ cells that express Oct4, Nanog, SSEA4, Sox2, Rex1, N-cadherin, and CD34 represent limbal native niche cells (NCs).[19] Moreover, the cluster does not include limbal stroma, so that limbal NCs expressing SC markers are adjacent to limbal basal epithelial cells. In addition, PCK^+ cells were rapidly eliminated after the passage on coated Matrigel, as evidenced by the disappearance of p63 and

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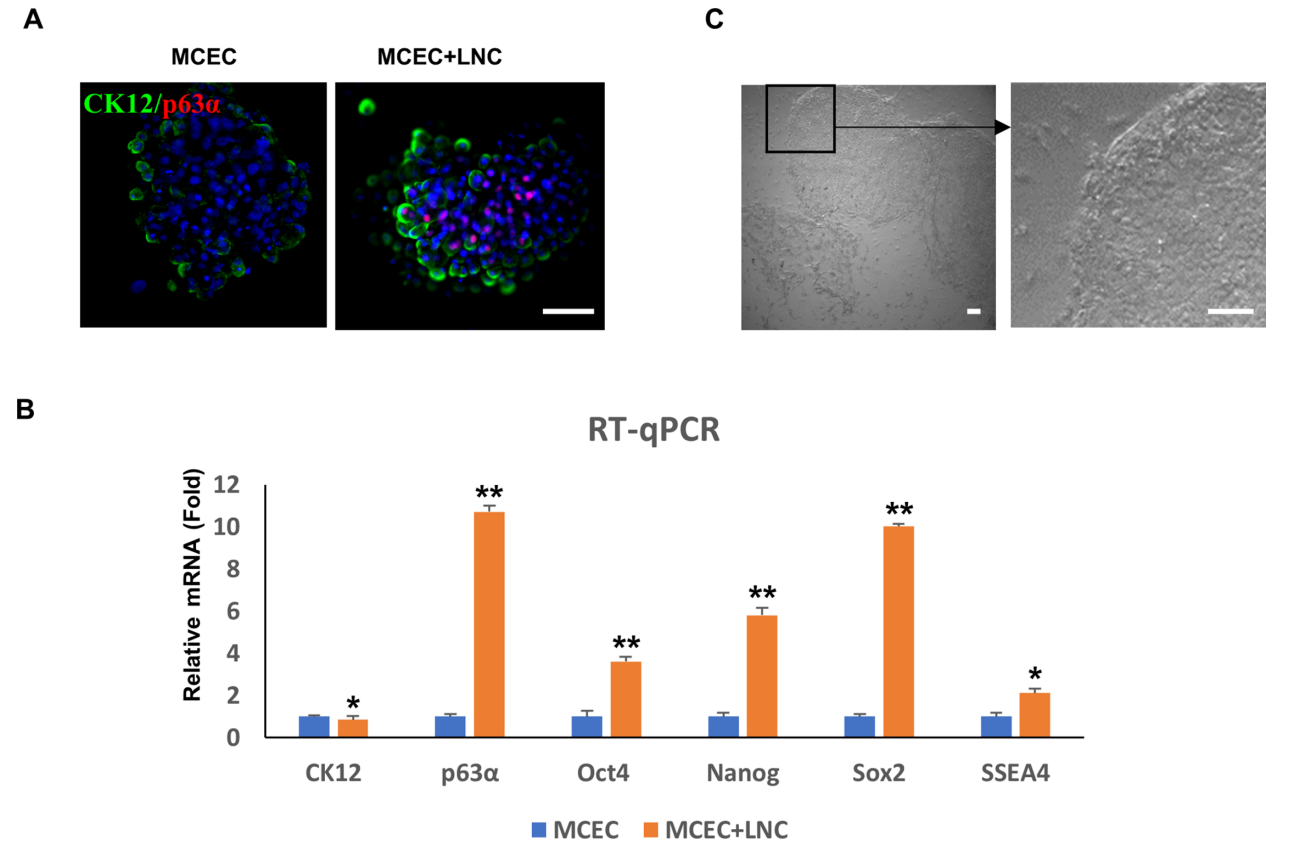

Figure 4. Epithelial cells from MCEC + LNC spheres express more p63α and form more epithelial holoclones than that from MCECs or LEPCs on the 3T3 feeder layer. Double immunostaining with CK12/p63α shows that cells in the sphere formed from MCECs express CK12 but not p63α; in contrast, some cells in the MCEC + LNC group express CK12, but most of the cells in the MCEC + LNC group express p63α (**A**, scale bar = 50 µm). RT-qPCR revealed a significant reduction of CK12 transcript but elevation of p63α, Oct4, Nanog, Sox2, and SSEA4 (**B**, $^{**}P < 0.01$, $^{*}P < 0.05$). Holoclone made of cubic epithelial cells on 3T3 feeder layers could be generated in the MCEC + LNC group but not in the other two groups (**C**, the right figure is an enlargement of part of the left one, scale bar = 50 µm).

CK12. Spindle cells proliferated among small round cells in P0 (Supplementary Fig. S1, P0). After a 5-day culture, spindle LNCs and some cubic epithelial cells could be seen in the first passage (Fig. 1A, P1), and cubic epithelial cells were reduced dramatically in the second passage (Fig. 1A, P2). As a result, homogeneous spindle LNCs could be warranted from the third passage (Fig. 1A, P3) and more confidently in the latter passages (Fig. 1A, P4). Such cells were uniformly $PCK^{-}/VIM^{+}/ CD90^{+}/CD105^{+}/SCF^{+}/PDGFR\beta^{+}$ (Fig. 1B).

The second step is to mimic the LSC niche in vitro. Adult tissue-derived epithelial organoids require generic niche factors such as laminin-rich extracellular matrix (Matrigel),[23] and 3D Matrigel culturing is one of the best ways to simulate the microenvironment of stem cells in vivo, because it simulates the natural state for cell morphology, proliferation, differentiation, and material transportation.[25] Tata et al.[16] reported that YFP^{+} secretory cells cultured in the 3D Matrigel could be induced to reverse differentiation in vitro. Nasser et al.[17] also reported that MCECs could be induced back to LSCs to rebuild the LSC pool in vivo in a mouse model. It is not known whether or not the simulated LSC niche in vitro could induce the dedifferentiation of MCECs.

Espana et al.[26] used the rabbit model to prove that when corneal epithelial tissue was combined with living limbal matrix, corneal epithelial cells would lose mature phenotype (CK3), suggesting that the limbal matrix microenvironment could induce the reverse differentiation of corneal epithelial cells to LSCs. Both corneal stromal cells and BMMSCs could be induced into limbal epithelial progenitor cells in a 3D environment, and the corneal stromal cells are more efficient than BMMSC.[27,28]

As reported in the previous study, sphere growth generated with single cells obtained by trypsin and EDTA from collagenase-isolated clusters emerged in a density-dependent manner in 3D Matrigel.[21] In this study, data show that time needed for sphere growth formation in the MCEC group was longer than that in the MCEC+ LNC group, and the spheres formed in the MCEC+ LNC group were the largest in diameter (Fig. 3B). Moreover, the MCEC + LNC group generated more spheres than both the MCEC and LEPC groups (Supplementary Fig. S3). A previous study[21] has demonstrated that when Dispase-isolated limbal epithelium cells were mixed with LNCs, more and relatively large spheres were produced in 3D Matrigel compared with that formed by Dispase-isolated corneal epithelium cells, and the expression of p63 increased, while CK12 decreased, which indicated that reunion of LNCs in MESCM inhibits corneal epithelial differentiation and promotes the sphere growth of limbal epithelial progenitor cells.

The ability to form sphere growth in 3D Matrigel is regarded as a characteristic of neural stem cells.[29] In this study, data show that time needed for sphere growth formation in the MCEC group was longer than that in the MCEC+

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LNC group, and the spheres formed in the MCEC+ LNC group were the largest in diameter, indicating a better stem cell phenotype exhibited by the MCEC + LNC group cells.

We cocultured LNCs with MCECs in 3D Matrigel in MESCM for 10 days, and the results showed that epithelial cells in spheres expressed less CK12 and more p63α compared with MCECs. For the first time, we demonstrated that LNCs could decrease the expression of CK12 and increase the expression of p63α in MCECs, which indicated a tendency for MCECs to dedifferentiate into stem cells in the 3D Matrigel environment when cocultured with LNCs. A previous study[19] showed that Dispase-isolated epithelial cells cocultured with LNCs could form spheres consisting of both cells on day 10. Therefore, we also demonstrated that MCECs cocultured with LNCs could form spheres consisting of both cells on day 10, where PCK^+ represented epithelial cells and Vim^+ represented LNCs (Supplementary Fig. S4). Besides, this finding is in line with prior studies showing that basement membrane components improve proliferation and differentiation capacity of human BMMSCs[27,28,30] and that Matrigel helps retain the undifferentiated state of human embryonic stem cells.[31] Therefore, reunion of LNCs with MCECs may affect the phenotype of MCECs compared with the MCEC alone or LEPC group in 3D Matrigel.

In conclusion, our study revealed for the first time that MCECs obtained from central corneas lost mature cornea epithelial markers and gained more epithelial stem cell markers after 10 days of coculture with LNCs in 3D Matrigel in vitro. MCECs have the potential of dedifferentiation to cornea epithelial stem cells, which could be a novel source of LSCs for an alternative stem cell therapy for the treatment of LSCD. The mechanism and power for the LSC niche mimicked by LNCs + 3D Matrigel in vitro could give light on how to induce other autologous cells into LSCs and be used to treat LSCD without worrying about immune rejection.

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