Rat Limbal Niche Cells Prevent Epithelial Stem/Progenitor Cells From Differentiation and Proliferation by Inhibiting Notch Signaling Pathway In Vitro

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Submitted: August 29, 2016
Accepted: April 13, 2017


PURPOSE. Limbal niche cells (LNCs) play a pivotal role in regulating limbal epithelial stem/progenitor cells (LESCs). This study aimed to investigate whether Notch signaling is involved in LNCs’ regulation of LESCs.

METHODS. Rat limbus was digested by dispase and collagenase, respectively. Limbal niche cells were isolated by serial passage of collagenase-digested cells on coated Matrigel in a modified embryonic stem cell medium (MESCM). Dispase-isolated cells, with or without LNCs, were seeded on three-dimensional (3D) Matrigel. The effects of LNCs, Notch inhibition (by N-(N-[3,5-difluorophenacetyl]-lalanyl)-S-phenylglycine t-butyl ester [DAPT] or Notch1-siRNA) and activation (by Jagged1) on LESCs were analyzed using quantitative RT-PCR, immunostaining, and Western blot.

RESULTS. Dispase isolated pan cytokeratin (PCK)+ limbal epithelial cells (LECs). Collagenase isolated subjacent native LNCs, which were purified and expanded with expression of Oct4, Rex1, Nanog, SSEA4, N-cadherin, and CD34. Limbal niche cells reunited with p63a+ LESCs to form clusters and prevented their differentiation on 3D Matrigel. Notch signaling was unactivated in rat corneal and limbal epithelium in vivo, but activated in cultured LECs in vitro. Limbal niche cells inhibited the Notch signaling of LECs in culture. Notch inhibition (by DAPT or Notch1-siRNA) increased p63a expression and decreased CK12 expression in LECs to the level of LNCs’ effects. Notch inhibition by DAPT also decreased Ki67 expression in LECs to the level of LNCs’ effects.

CONCLUSIONS. Rat LNCs prevent LESCs from differentiation and proliferation primarily via inhibiting the Notch signaling in vitro. Manipulating the Notch signaling pathway may help to preserve LESCs for corneal epithelial tissue engineering.

Keywords: limbus, niche cells, stem cells, differentiation, notch, three-dimensional culture
Stem cells (SCs) are undifferentiated cells that have the unique potential to self-renew, multiply differentiate, and maintain homeostasis. Growing evidence supports the idea that adult SCs are regulated by their niche1,2 (i.e., a specialized microenvironment consists of adjacent cellular and extracellular matrix components). Limbal epithelial stem/progenitor cells (LESCs) are exclusively located in the basal layer of the limbal epithelium2 and the limbal niche is believed to reside in the underlying limbal stroma subjacent to the epithelial basement membrane.4 Limbal niche cells (LNCs) are dominant cellular constituents in the limbal niche, and possess phenotypic characteristics of mesenchymal SCs and embryonic SCs,5

大鼠角膜缘微环境细胞通过抑制 Notch 信号通路体外阻止角膜缘上皮干细胞分化和增殖

目的：角膜缘微环境细胞(limbal niche cells, LNCs)在角膜缘上皮干细胞(limbal epithelial stem/progenitor cells, LESC)的调控中发挥重要作用。本研究旨在探索 Notch 信号在 LNCs 对 LESC 的调控中所起的作用。

方法：分别使用中性蛋白酶和胶原酶消化大鼠角膜缘组织。在改良的胚胎干细胞培养基中，将胶原酶来源的细胞在包被 Matrigel 上连续传代，从而获得 LNCs。将中性蛋白酶来源的细胞单独种植，或与 LNCs 混合后种植于 3D Matrigel 进行培养，使用 RT-PCR、免疫染色和免疫印迹法检测 LNCs Notch 抑制(使用 DAPT、Notch1-siRNA)或 Notch 激活(使用 Jagged1)对 LESC 的影响。

结果：使用中性蛋白酶消化角膜缘得到 PCK+角膜缘上皮细胞(limbal epithelium cells, LECs)。将胶原酶消化的细胞连续传代得到纯化的 LNCs。LNCs 表达 Oct4, Rex1, Sox2, Nanog, SSEA4, N-cadherin 和 CD34 等标志物。LNCs 与 p63α+LESCs 在 3D Matrigel 上结合形成 cluster 样生长，且 LNCs 阻止 LESC 的分化。

Notch 信号在大鼠角膜上皮和角膜缘上皮以未激活的形式表达，经过体外培养后，LECs 的 Notch 信号被激活。在其培养体系(LECs+LNCs)中，LNCs 抑制 Notch 信号在 LECs 的表达。LECs 的 Notch 信号被抑制后(使用 DAPT 或 Notch1-siRNA)，p63α表达增加，CK12 表达降低，p63α 和 CK12 在 LECs+LNCs 和 Notch 抑制的 LECs 中表达相同。LECs 的 Notch 信号抑制(使用 DAPT)也降低了 Ki67 的表达，Ki67 在 LECs+LNCs 和 Notch 抑制的 LECs 中表达相同。

结论：大鼠 LNCs 主要通过抑制 Notch 信号在体外阻止 LESC 的分化和增殖，对 LESC 进行 Notch 信号的调控有望应用于角膜上皮的组织工程。
which can be stably maintained in three-dimensional (3D) Matrigel. In a prior study, we demonstrated that the function of LESCs depends on their physical association with native niche cells, and that the embryonic SC markers expressed by LNCs are critical to prevent differentiation of LESCs. However, the intrinsic mechanism that coordinates the manner in which LNCs regulate LESCs remains unclear. Direct cell–cell contact and ligand-receptor interaction mediated through Notch signaling are known to maintain SCs in various niches. In bone marrow, Notch signaling is mediated through Notch receptors and ligands. Collagenase-isolated cells and LNCs from different species can be stably maintained in three-dimensional (3D) Matrigel. In a prior study, we demonstrated that the function of LESCs depends on their physical association with native niche cells. In this study, rat LNCs were successfully isolated and cocultured with rat limbal epithelial cells (LESCs) using a previously reported 3D Matrigel. Our study found that rat LNCs inhibited Notch signaling in LESCs. Inhibition of Notch signaling using Notch inhibitor N-(N-[3-5-difluorophenacetyl]-lalanyl)-S-phenylglycine t-butyl ester (DAPT) and Notch1-small interfering (siRNA) both prevented LESCs from differentiating by sequential addition. These findings substantiate the regulatory effect of rat LNCs on LESCs and highlight the importance of the Notch signaling pathway in the process.

**Materials and Methods**

**Animals**

Adult male Sprague-Dawley rats (weighing 180–220 g) were obtained from the Laboratory Animal Center of Tongji Medical College of Huazhong University of Science & Technology (Wuhan, China). The design and implementation of animal-related activities were adherent to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Only rats with clean and transparent corneas were used in the study.

**Isolation of Limbal Sheets and Clusters**

Limbal sheets and clusters were isolated by enzymatic digestion with dispase II and collagenase A according to the subsequently described steps. After the rats were killed, their whole eyeballs were removed and rinsed three times with Hank's balanced salt solution containing 50 μg/mL gentamicin and 1.25 μg/mL amphotericin B. Corneoscleral rims (1 mm within and beyond the limbus) were obtained by removing the central cornea, conjunctiva, sclera, iris, trabecular meshwork, and endothelium. Each corneoscleral rim was cut into six equal segments. Intact limbal sheets were isolated by digestion at 37°C for 30 minutes with 10 mg/mL dispase II in modified embryonic stem cell medium (MESC). In parallel, some limbal segments were digested with 1 mg/mL collagenase A in MESC at 37°C for 3 hours to generate clusters. Modified embryonic stem cell medium is made of Dulbecco’s modified Eagle’s medium (DMEM/F12;1:1; HyClone, Logan, UT, USA) supplemented with 10% knockout serum, 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL sodium selenite, 4 ng/mL bFGF 10 ng/mL human leukemia inhibitory factor (hILF), 50 μg/mL gentamicin, and 1.25 μg/mL amphotericin B. Limbal sheets and clusters were further digested with 0.25% trypsin and 1 mM EDTA (T/E) at 37°C for 10 minutes to yield individual cells. Additionally, corneal stromal cells (CSCs) were isolated via collagenase A digestion after removing the epithelium and endothelium.

**Coated and 3D Matrigel Culture and Treatment**

Coated and 3D Matrigel were prepared in plastic dishes by adding 50 μL of 5% diluted Matrigel (BD, Franklin Lakes, NJ, USA) and 200 μL of 50% diluted Matrigel (all in DMEM/F12) per centimeter squared, respectively, and then by incubation at 37°C for 1 hour before use. Dispase-isolated cells were seeded on 3D Matrigel at a density of 12 × 10^5/cm^2 in MESC. Collagenase-isolated cells were expanded in MESC on coated Matrigel. Passage 3 (P3) expanded cells or CSCs were prelabeled with red fluorescent nanocrystals, mixed at a 1:4 ratio with dispase-isolated cells, and seeded at the same density on 3D Matrigel. To inhibit or activate the Notch signaling, 15 μM of γ-secretase inhibitor DAPT or 10 μM of Jagged1 was added in MESC on day 0. In some cultures, Notch1-siRNA (5’-CAACAUAGCUACCUUGCUAdTdT-3’) was transfected into dispase-isolated cells to knock down Notch1 expression. Cells seeded on 3D Matrigel were harvested on Day 7 by digestion of Matrigel in 10 mg/mL dispase II at 37°C for 2.5 hours. Some of the harvested cells were rendered into single cells by T/E. Limbal clusters and cells were imaged with an inverted microscope (OLYMPUS DP26; Olympus Tokyo, Japan). All materials used for cell isolation and culture are listed in Supplementary Table S1.

**Immunofluorescence Staining**

Limbal sheets or clusters obtained by dispase or collagenase digestion, respectively, were cryosectioned to 6 μm prior to fixation. Paraffin-embedded rat corneas were cut into 4-μm sections and used for immunofluorescence staining to localize the Notch receptors and ligands. Collagenase-isolated cells and cells obtained on Day 7 were prepared for cytoxin at 980 rpm for 10 minutes. Steps for immunofluorescence staining were followed. Permeabilized and blocked samples were sequentially incubated with specific primary antibodies and corresponding secondary antibodies. Incubation with PBS in place of primary antibodies was used as a negative control. Nuclear counterstaining was achieved using DAPI before samples were photographed under a fluorescence microscope (OLYMPUS BX53) or a confocal microscope (Zeiss LSM 700; Carl Zeiss, Inc., Thornwood, NY, USA).

**Quantitative Real-Time Polymerase Chain Reaction**

Total RNA was isolated using TRIzol reagent and reverse transcribed to cDNA using ReverTra Ace qPCR RT Kit (TOYOBO, Osaka, Japan). Each 20-μL reaction contained 4 μL cDNA, 10 μL SYBR Green supermix, and 0.4 μL of each of the corresponding forward and reverse primers (see Supplementary Table S2). The following thermal cycler program was used: 2 minutes at 50°C, 10 minutes at 95°C for initial activation, followed by 40 cycles of 30 seconds at 95°C, and 30 seconds at 60°C for primer annealing and extension. The relative gene expression data was assessed by the comparative cycle threshold (CT) method and normalized to β-actin as an internal control.

**Western Blot Analysis**

Protein in cells harvested on Day 7 was extracted using RIPA buffer supplemented with protease inhibitors and phosphatase. Lysates were denatured and separated using SDS-PAGE on 5% to 12% Bis-Trismini gels. Protein bands were transferred to a polyvinylidene fluoride (PVDF) membrane, which was then blocked with 5% (wt/vol) fat-free milk followed by sequential incubation with specific primary antibodies and respective secondary antibodies using glyceraldehyde-3-phosphate dehy-
As reported previously, these Vim+ LECs, but also adjacent Vim+ mesenchymal cells, resulted in a cluster of cells that consisted of not only PCK+-LECs, but also adjacent PCK+/Vim+stromal cells. Scale bars: 20 μm (A, C); 100 μm (B).

Drotenase (GAPDH) as the loading control. Immunoreactive proteins were detected with a chemiluminescence reagent. Detailed information about antibodies for immunostaining and Western blot is listed in Supplementary Table S3.

**Statistics**

Data were presented as means ± SD. Differences between the two groups were evaluated using unpaired two-tailed Student’s t-test. Significant differences between the two groups were indicated by asterisks (*P < 0.05; **P < 0.01).

**RESULTS**

**Cluster Growth Formed by Reunion of Rat LESC**

Anatomically, corneal epithelial stem/progenitor cells reside in the limbus. Similar to a human limbal epithelial sheet, an intact rat limbal epithelial sheet was mechanically separated after digestion with dispase. We noted that the dispase-isolated sheet consisted predominantly of pan-cytokeratin(PCK)+ LECs and few PCK–/Vim– mesenchymal cells (Fig. 1A). Nonetheless, the resulting cell clusters by collagenase digestion (Fig. 1B) consisted of not only the PCK+/Vim– LECs, but also adjacent PCK–/Vim+ mesenchymal cells (Fig. 1C). As reported previously, these Vim+ mesenchymal cells can be purified by expanding on coated Matrigel up to P3 (Fig. 2A) and can be identified as LNCs for the expression of embryonic SC markers and other SC markers, including Oct4, Rex1, Sox2, Nanog, SSEA4, N-cadherin, and CD34 after seeding to 3D Matrigel (Figs. 2B–H).

Unlike human LECs, rat LECs alone failed to form clusters on 3D Matrigel for 7 days (Fig. 3A). Rat LNCs (P3) gathered and exhibited asterism-shaped growth (Fig. 3B). As previously reported, LESCsonly and with native LNCs to yield sphere growth that exhibited a corneal fate in the 3D Matrigel culture model. When we mixed rat LECs with LNCs at the ratio of 4:1, clusters formed (Fig. 3C) and consisted of LECs and LNCs prelabeled by red nanocrystals (Fig. 3D).

Immunostaining on Day 7 clusters showed that all the PCK+ epithelial cells were p63α+ (i.e., an epithelial progenitor marker) (Fig. 3E), indicating their progenitor status. Limbal epithelial cells mixed with CSCs at a 4:1 ratio also yielded similar clusters on 3D Matrigel (data not shown). Collectively, we confirmed that rat LESCswere reunited with LNCs or CSCs to yield cluster growth.

**LNCs Prevent LESC From Differentiation**

To determine whether the aforementioned cluster reunion with LNCs or CSCs affect the LESC differentiation, the expression of SC and differentiation markers was analyzed by quantitative RTPCR. Compared with LECs as a control, the LESC+LNCs group expressed a 2.2-fold increase of p63α transcript (n = 6, P < 0.01) and a 0.5-fold decrease of CK12 transcript (n = 6, P < 0.05), but a 0.4-fold decrease of p63β transcript (n = 6, P < 0.01; Fig. 4A). The expression difference was also confirmed at the protein level by quantification analysis of immunostaining (Figs. 4B, 4C). Compared with LECs, the addition of LNCs increased the percentage of p63α+ cells from 87.4 ± 6.0% to 96.4 ± 2.2% (n = 6, P < 0.05), and decreased the percentage of CK12+ cells from 69.3 ± 6.0% to 20.3 ± 1.9% (n = 6, P < 0.01). Mixing LECs with CSCs decreased the percentage of p63α+ cells to 50.8 ± 8.9% (n = 6, P < 0.01; Fig. 4C). Therefore, we concluded that rat LNCs, rather than CSCs, prevented LESC from differentiation.
Limbal Niche Cells Support Stem Cells by Inhibiting Notch

Expression of Notch Ligands and Receptors in Rat Cornea and Limbus

The Notch signaling is known to be involved in SCs niche regulation through direct cell–cell contact. To determine whether this signaling is involved in modulating between LNCs and LESCs, the immunoreactivity of Notch receptors and ligands on cross-sectioned rat cornea and limbus was confirmed. Immunostaining showed that Notch receptors and ligands were expressed in the membrane and cytoplasm, not in the nucleus of rat corneal and limbal epithelium (Fig. 5A). The Notch receptor Notch1 was strongly expressed in limbal epithelium, and weakly expressed in corneal epithelium, while the expression of Notch ligands (Delta1, Jagged1) was stronger in corneal epithelium than in limbal epithelium. Weak Notch2 staining was found in limbal epithelium with no difference from corneal epithelium (data not shown). The positive membrane and cytoplasmic staining for Notch1 and Jagged1 was observed in the underlying limbal stroma subjacent to basement membrane (Fig. 5A, white arrowheads). The nuclear translocation of the Notch intracellular domain (NICD) indicates the activation of Notch signaling. A NICD-specific antibody was used and the absence of nuclear expression was confirmed in cross-sectioned samples (Data not shown). Immunostaining also showed the membrane and cytoplasmic expression of Notch1, Delta1, and Jagged1 in dispase-isolated LECs and P3 expanded LNCs (Fig. 5B). Thus, we confirmed that Notch family was expressed in rat limbus and cornea, but Notch signaling was unactivated in vivo.

LNCs Inhibited Notch Signaling of LESCs in Vitro

Although Notch signaling is unactivated in vivo, its effect in vitro is unknown. To investigate whether Notch signaling was involved in LESCs regulation by rat LNCs in vitro, we examined the expression of NICD and Hes1, a major downstream target. On Day 7, cytospin preparation of the resultant cells showed that some PCK+ epithelial cells were NICD+ and Hes1+ in nuclei (Fig. 6A). Further quantification analysis revealed that the addition of LNCs decreased the percentage of NICD+ epithelial cells in PCK+ epithelial cells from 93.0 ± 3.0% to 67.9 ± 2.0% (n = 6, P < 0.05; Fig. 6B), and decreased the percentage of Hes1+ epithelial cells in PCK+ epithelial cells from 70.0 ± 2.5% to 17.9 ± 2.0% (n = 6, P < 0.01; Fig. 6B). Compared with the control group, LECs+LNCs expressed a significant downregulation of 0.3-fold Hes1 transcript (n = 6, P < 0.01; Fig. 6C). The addition of CSCs did not change the expression of NICD and Hes1 in the nuclei of LECs (n = 6, both P > 0.05; Figs. 6B, 6C). Based on these collective data, we concluded that LNCs, rather than CSCs, inhibited Notch signaling of LESCs in vitro.

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**Figure 3.** Clusters formed from reunion of rat LESCs and LNCs. Dispase-isolated rat LECs failed to form clusters on 3D Matrigel for 7 days (A). Rat LNCs (P3) gathered and exhibited asterism-shaped growth (B). Limbal epithelial cells and LNCs (LECs+LNCs) reunited and grew in a cluster-shape manner (C). These clusters (Day 7) were composed of epithelial cells and LNCs prelabeled by red nanocrystals (D). Immunofluorescence staining showed that all the PCK+ epithelial cells in the cluster expressed p63α (E). Scale bars: 200 μm (A-C); 100 μm (D, E).

**Figure 4.** Limbal niche cells prevent LESCs from differentiation. Quantitative analysis by RT-PCR revealed that the addition of LNCs increased p63α transcript (n = 6; \( *P < 0.01 \)) and decreased CK12 transcript (n = 6; \( *P < 0.01 \)); the addition of CSCs didn’t change CK12 transcript (n = 6, P > 0.05), but decreased p63α transcript (n = 6; \( *P < 0.01 \)) (A). Immunostaining of single cells obtained on Day 7 showed the p63α and CK12 expression in LECs (B). Compared with LECs, LECs+LNCs exhibited a higher percentage of p63α+ epithelial cells (n = 6; \( *P < 0.05 \)), and a lower percentage of CK12+ epithelial cells (n = 6; \( *P < 0.01 \)). The addition of CSCs decreased the percentage of p63α+ epithelial cells (n = 6; \( *P < 0.01 \)), but didn’t change the percentage of CK12+ epithelial cells (n = 6, P > 0.05) (C). Data are expressed as mean ± SD. Scale bars: 20 μm (B).
Inhibition of Notch Signaling in LESCs Prevents Their Differentiation

The above inhibition of Notch signaling in LESCs by LNCs prompted us to examine whether Notch inhibition prevented LESCs from differentiation. We chose DAPT and Jagged1 to inhibit and activate Notch signaling, respectively. The results showed that DAPT effectively decreased NICD nuclear expression and Hes1 transcript in LECs, and Jagged1 increased their expression. Notch inhibition by DAPT increased p63α transcript by 1.7-fold and decreased CK12 transcript by 0.6-fold, and Notch activation by Jagged1 decreased p63α transcript by 0.5-fold and increased CK12 transcript by 1.5-fold. The addition of LNCs had similar effects on p63α and CK12 expression in LECs. These results indicated that Notch inhibition by DAPT prevented LESCs from differentiation and Notch activation by Jagged1 promoted their differentiation.

To further confirm the role of Notch signaling in LESCs differentiation, we also used Notch1-siRNA transfection, which decreased the Notch1 transcript by 70% in LECs. Western blot followed by densitometry showed a 1.9-fold increase of p63α expression and a 0.4-fold decrease of CK12 expression compared with the control group. Both Notch1-siRNA and DAPT had similar effect on LESCs differentiation. Collectively, these data confirmed that inhibition of Notch signaling in LESCs prevented their differentiation.

Inhibition of Notch Signaling in LESCs Prevents Their Proliferation

Because Notch signaling has been proven to be a key regulator in SC proliferation, we examined Ki67 in different cultures. Compared with LECs, Notch inhibition by DAPT decreased Ki67 transcript by 0.3-fold, and Notch activation by Jagged1 increased Ki67 transcript by 1.6-fold. The addition of LNCs had similar effects on Ki67 expression in LECs.

**FIGURE 5.** Expression of Notch ligands and receptors in rat cornea and limbus. The Notch receptor (Notch1) was strongly expressed in limbal epithelium and weakly expressed in corneal epithelium, while the expression of Notch ligands (Delta1, Jagged1) was stronger in corneal epithelium than in limbal epithelium. Positive staining for Notch1 and Jagged1 was observed in subjacent limbal stroma (white arrowheads). Immunoreactivity was not detected in negative control slides (A). Immunofluorescence staining confirmed the expression of Notch1, Delta1, and Jagged1 in LECs and P3 expanded LNCs (B). Scale bars: 50 μm (A); 20 μm (B).

**FIGURE 6.** Limbal niche cells inhibited the Notch signaling of LESCs in vitro. Immunostaining of single cells obtained on Day 7 cultures showed that some PCK+ epithelial cells were nuclear expressing of NICD and Hes1 (A). Further quantitative analysis revealed that LECs+LNCs exhibited lower percentages of positive epithelial cells stained for NICD and Hes1 (n = 6; *P < 0.05 and n = 6; **P < 0.01). The addition of CSCs did not change the percentages of NICD+ and Hes1+ epithelial cells (n = 6, both P > 0.05) (B). Limbal epithelial cells +LNCs exhibited a lower mRNA expression of Hes1 compared with that in LECs (n = 6; *P < 0.01), and the addition of CSCs didn’t change the mRNA expression of Hes1 (n = 6, P > 0.05) (C). Each column with a bar represents the mean ± SD. Scale bar: 20 μm (A).
addition of LNCs had a similar effect on Ki67 expression to that of DAPT (α = 3, *P < 0.05; Fig. 8A). Further quantification analysis of Ki67 expression in different immunostained cells (Fig. 8B) confirmed that DAPT decreased the percentage of Ki67+ cells from 15.0 ± 1.0% to 9.7 ± 4.1% (α = 6, *P < 0.05; Fig. 8C), while Jagged1 increased this percentage to 22.9 ± 6.0% (α = 3, **P < 0.01; Fig. 8D). No significant difference was noted in the group treated with DAPT when compared with the group addition of LNCs (α = 6, *P > 0.05; Fig. 8C). These results suggested that inhibition of the Notch signaling pathway in LESCs prevented their proliferation.

FIGURE 8. Inhibition of Notch signaling in LESCs prevents their proliferation. Quantitative RT-PCR revealed that DAPT decreased Ki67 expression in LECs (α = 3, **P < 0.01); Jagged1 increased Ki67 expression in LECs (α = 3, *P < 0.05). The addition of LNCs to LECs also decreased the Ki67 transcript to a level similar to that observed in DAPT-treated LECs (α = 3, **P < 0.01) (A). Immunostaining of single cells obtained on Day 7 showed the nuclear expression of Ki67 in different cultures (B). Further quantification analysis demonstrated that DAPT decreased the percentage of Ki67+ cells (α = 6, *P < 0.05), while Jagged1 increased the percentage of Ki67+ cells (α = 5, **P < 0.01). The addition of LNCs to LECs also decreased the percentage of Ki67+ cells to a level similar to that observed in DAPT-treated LECs (α = 6, **P < 0.01) (C). Each column with a bar represents the mean ± SD. Scale bar: 20 μm (B).

Sprague-Dawley rats were chosen for the localization of epithelial SCS in the limbus,4,13,15 In our prior study,10 the native LNCs isolated from human collagenase-isolated clusters reunited with LESCs to generate sphere growth. Because collagenase digestion preserves the basement membrane, one may argue whether or not this reunion owes to independent binding to some basement membrane components. In the present study, dispase-isolated LESCs and purified LNCs also reunited to form clusters (Figs. 3C–E). This finding supports that the reunion is independent from the basement membrane. The reunion with LNCs prevented LESCs from differentiation evidenced by higher p63α and lower CK12 expression in LECs+LNCs (Fig. 4), which is consistent with a previous report that the reunion between LESCs and LNCs in immobilized heavy chain-hyaluronic acid/pentraxin 3 (HC-HA/PTX3) exhibits inhibition of corneal epithelial lineage commitment/differentiation.30 Although a similar reunion was formed by LECs mixing with CSCs, CSCs decreased p63α expression in LECs (Fig. 4). This finding was similar to a prior report showing that the corneal stroma promoted epithelial differentiation.31

The Notch family was expressed in adult mammal corneas,13,52 however, Notch signaling was unactivated in vivo (Fig. 5). Activation of Notch signaling in transgenic mice did not alter corneal epithelial cell proliferation and differentiation in vivo.53 However, the results that LNGs decreased NICD and Hes1 expression in LESCs (Fig. 6) indicate the involvement of Notch signaling in limbal epithelium regulation in vitro. In addition to the activation of Notch signaling through receptor-ligand interactions (trans-interactions), ligands have been found to interact with Notch receptors autonomously (cis-interactions) leading to Notch inhibition.34,55 Cis-inhibition may play a role in inhibiting Notch signaling in LESCs. The mechanism underlying Notch signaling inhibition in LESCs by LNCs remains unclear and requires further study. Notch...
inhibition (by Notch1-siRNA or DAPT) had a similar effect to LNCS in preventing LESCs from differentiation (Fig. 7). Therefore, we concluded that LNCS prevented LESCs from differentiation mainly, although not entirely, by inhibiting Notch signaling in vitro.

Our result is different from a prior study, which demonstrated that inhibition of Notch signaling promoted epithelial cell differentiation evaluated by a single nonspecific corneal differentiation marker.13 Aside from this, we agree on the role of Notch in LESCs proliferation.13 The difference may result from the different SC and differentiation markers used between the two studies. Previous studies have also reported that Notch signaling activation promotes adult SC differentiation in diverse tissues. In human meibomian gland epithelial, the level of activated Notch1 strongly increased with differentiation.37 Notch activation causes maturation and differentiation in human keratinocytes.38 Notch activation is also required for hepatic progenitor cells differentiation into cholangiocytes in cholestatic liver fibrosis.39

Recently, SC-based tissue engineering has emerged as a promising avenue for the treatment of some corneal diseases.40,41 The association between LNCS and maintenance of the undifferentiated state of LESCs can be deployed as a strategy for ex vivo investigation. Manipulating the Notch pathway may help to preserve LESCs in vitro for corneal epithelial tissue engineering.

Acknowledgments

The authors thank Xi Han for her insightful comments regarding this manuscript.

Supported by grants from National Natural Science Foundation of China (Grants: 81300736 and 81370993; Beijing, China).

Disclosure: J. Li; None; S.-Y. Chen; None; X.-Y. Zhao; None; M.-C. Zhang; None; H.-T. Xie; None

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