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

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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 niche\(^1\)\(^,\)\(^2\) (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 epithelium\(^3\) 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.\(^5\)

**Methods**: Limbal epithelial stem/progenitor cells (LESCs) were isolated and cultured as described, with the addition of Matrigel. LESC cultures were treated with DAPT or Notch1-siRNA. The effects of Notch pathway inhibition on LNCs were assessed using RT-PCR, immunofluorescence microscopy, and Western blotting.

**Results**: Treatment with DAPT or Notch1-siRNA led to a significant increase in the proportion of LNCs expressing Notch target genes. These findings suggest that Notch signaling is crucial for the maintenance and function of LNCs.

**Conclusions**: The data presented here provide compelling evidence for the role of Notch signaling in the regulation of LNCs, and suggest potential therapeutic strategies for the treatment of limbal stem cell deficiency.
which can be stably maintained in three-dimensional (3D) Matrigel.\textsuperscript{6,7} In a prior study, we demonstrated that the function of LESCs depends on their physical association with native niche cells,\textsuperscript{8} and that the embryonic SC markers expressed by LNCs are critical to prevent differentiation of LESCs.\textsuperscript{7} 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.\textsuperscript{9,10} In bone marrow, Notch signaling mediated through Notch receptors and ligands is essential for self-renewal and repopulation of hematopoietic SCs.\textsuperscript{11} In skeletal muscles, Notch signaling controls the homing of satellite cells, stimulating them to contribute to their own microenvironment and to adhere to myofibers.\textsuperscript{12} The Notch family has been reported to affect corneal epithelial cell differentiation.\textsuperscript{13} However, it remains unclear whether Notch signaling is involved in LESCs’ regulation by their native limbal niche.

In this study, rat LNCs were successfully isolated and cocultured with rat limbal epithelial cells (LECs) using a previously reported 3D Matrigel.\textsuperscript{5} Our study found that rat LNCs inhibited Notch signaling in LESCs. Inhibition of Notch signaling using Notch inhibitor N-[N-(3-[5-difuorophenacetyl]-lalanyl)-S-phenylglycine t-butyl ester (DAPT) and Notch1-small interfering (si)RNA both prevented LESCs from differentiated into GSLCs. 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’-CACACUACAGCUCCGUAdTdT-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 cytospin 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-Tris mini 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-
drogenase (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 LESCs and LNCs

Anatomically, corneal epithelial stem/progenitor cells reside in human and rat limbus,3,14,15 similar to a human limbal epithelial sheet,16 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+ LECs and few PCK+/C0 mesenchymal cells (Fig. 1C). In contrast, digestion with collagenase resulted in a cluster of cells (B) that consisted of not only PCK+ LECs, but also adjacent Vim+ stromal cells (C). Scale bars: 20 μm (A, C); 100 μm (B).

Figure 1. Isolation of rat limbal sheets and clusters using dispase and collagenase. Dispace digestion removed the entire epithelial sheet (A), which consisted predominantly of PCK+ LECs and few Vim− mesenchymal cells (A). In contrast, digestion with collagenase resulted in a cluster of cells (B) that consisted of not only PCK+ LECs, but also adjacent Vim+ stromal cells (C). Scale bars: 20 μm (A, C); 100 μm (B).

Figure 2. Phenotypic characterization of P3 expanded limbal mesenchymal cells. The collagenase-isolated stromal cells were purified by expanding on coated Matrigel in MESCM up to P3 (A), and were identified as rat LNCs for their embryonic SC and other SC phenotype (B-H). Scale bars: 20 μm.

Unlike human LECs,8 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,7 LESCs reunited 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 p63a+ (i.e., an epithelial progenitor marker17) (Fig. 3E), and the expression difference was also confirmed at the protein level by quantification analysis of immunostaining. The p63a transcript (n = 6, P < 0.01) and a 0.4-fold decrease of p63a transcript (n = 6, P < 0.05), but a 0.4-fold decrease of CK12 transcript (n = 6, P < 0.05), but a 0.4-fold decrease of CK12 transcript (n = 6, P < 0.01). The expression difference was also confirmed at the protein level by quantification analysis of immunostaining (Figs. 4B, 4C). Compared with LECs alone (n = 6, P > 0.05), but a 0.4-fold decrease of p63a transcript (n = 6, P < 0.01). 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 p63a+ cells from 87.4 ± 4.6% 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 p63a+ cells to 50.8 ± 8.9% (n = 6, P < 0.01; Fig. 4C).

LNCs Prevent LESCs From Differentiation

To determine whether the aforementioned cluster reunion with LNCs or CSCs affect the LESCs differentiation, the expression of SC and differentiation markers was analyzed by quantitative RT-PCR. Compared with LECs as a control, the LESCs+LNCs group expressed a 2.2-fold increase of p63a 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 p63a 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 p63a+ cells from 87.4 ± 4.6% 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 p63a+ cells to 50.8 ± 8.9% (n = 6, P < 0.01; Fig. 4C).

Therefore, we concluded that rat LNCs, rather than CSCs, prevented LESCs from differentiation.
Limbal Niche Cells Support Stem Cells by Inhibiting Notch

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, 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% (*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% (**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 LESCs (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.

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. Rat LNCs (P5) 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 p63+ (E). Scale bars: 200 μm (A–C); 100 μm (D, E).

**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 (P5) 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 p63+ (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 p63+ 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 p63+ transcript (n = 6; **P < 0.01) (A). Immunostaining of single cells obtained on Day 7 showed the p63+ and CK12 expression in LECs (B). Compared with LECs, LECs+LNCs exhibited a higher percentage of p63+ 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 p63+ 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. 23,24 The results showed that DAPT effectively decreased NICD nuclear expression (n = 6, both P < 0.05; Fig. 7A) and Hes1 transcript in LECs (n = 3, both P < 0.05; Fig. 7B), and Jagged1 increased their expression (Figs. 7A, 7B). Notch inhibition by DAPT increased p63α transcript by 1.7-fold and decreased CK12 transcript by 0.6-fold (n = 6, both P < 0.01; Figs. 7C, 7D). Notch activation by Jagged1 decreased p63α transcript by 0.5-fold and increased CK12 transcript by 1.5-fold (n = 6, both P < 0.01; Figs. 7C, 7D). The addition of LNCs had similar effects on p63α and CK12 expression in LECs to those of DAPT addition (n = 6, both P > 0.05; Figs. 7C, 7D). 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 (data not shown). 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 (Fig. 7E). Both Notch1-siRNA and DAPT had similar effect on LESCs differentiation with that of LNCs (Fig. 7E). 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, 25–27 we examined Ki67 (a proliferation marker28) in different cultures. Compared with LECs, Notch inhibition by DAPT decreased Ki67 transcript by 0.3-fold (n = 3, P < 0.01; Fig. 8A) and Notch activation by Jagged1 increased Ki67 transcript by 1.6-fold (n = 3, P < 0.05; Fig. 8A). The

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 (n = 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% (n = 6; P < 0.05; Fig. 8C), while Jagged1 increased the percentage to 22.9 ± 0.9% (n = 6; P < 0.05; Fig. 8C). No significant difference was noted in the group treated with DAPT when compared with the group addition of LNCs (n = 6; P > 0.05; Fig. 8C). These results suggested that inhibition of the Notch signaling pathway in LESCs prevented their proliferation.

**DISCUSSION**

Due to the scarcity of human corneal donors in many countries or regions, an alternative research model using animal sources is necessary for understanding LESCs’ regulation. The Notch family was expressed in adult mammal corneas, however, Notch signaling was unactivated in vivo. In our prior study, 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 LESCs+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. 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.

The Notch family was expressed in adult mammal corneas, however, Notch signaling was unactivated in vivo. Inactivation of Notch signaling in transgenic mice did not alter corneal epithelial cell proliferation and differentiation in vivo. However, the results that LNCs 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. 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.

Sprague-Dawley rats were chosen for the localization of epithelial SCs in the limbus.

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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. 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 Notch1 strongly increased with differentiation. Notch activation causes maturation and differentiation in human keratinocytes. Notch activation is also required for hepatic progenitor cells differentiation into cholangiocytes in cholestatic liver fibrosis.

Recently, SC-based tissue engineering has emerged as a promising avenue for the treatment of some corneal diseases. 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.

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