

# A Role for Notch Signaling in Human Corneal Epithelial Cell Differentiation and Proliferation

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**PURPOSE.** To identify the role of Notch signaling in the human corneal epithelium.

**METHODS.** Localization of Notch1, Notch2, Delta1, and Jagged1 in the human corneal epithelium was observed with the use of indirect immunofluorescence microscopy. Gene and protein expression of Notch receptors and ligands in human corneal epithelial cells was determined by RT-PCR and Western blot analysis, respectively. The effects of Notch inhibition (by  $\gamma$ -secretase inhibition) and activation (by recombinant Jagged1) on epithelial cell proliferation (Ki67) and differentiation (CK3) were analyzed after Western blotting and immunocytochemistry.

**RESULTS.** Immunofluorescent labeling localized Notch1 and Notch2 to suprabasal epithelial cell layers, whereas Delta1 and Jagged1 were observed throughout the corneal epithelium. Notch1, Notch2, Delta1, and Jagged1 genes and proteins were expressed in human corneal epithelial cells.  $\gamma$ -Secretase inhibition resulted in decreased Notch1 and Notch2 expression, with an accompanying decrease in Ki67 and increased CK3 expression. The activation of Notch by Jagged1 resulted in the upregulation of active forms of Notch1 and 2 proteins ( $P < 0.05$ ), with a concurrent increase in Ki67 ( $P < 0.05$ ) and a decrease in CK3 ( $P < 0.05$ ) expression. Interestingly,  $\gamma$ -secretase inhibition in a three-dimensional, stratified corneal epithelium equivalent had no effect on Ki67 or CK3 expression. In contrast, Jagged1 activation resulted in decreased CK3 expression ( $P < 0.05$ ), though neither Notch activation nor inhibition affected cell proliferation in the 3D tissue equivalent.

**CONCLUSIONS.** Notch family members and ligands are expressed in the human corneal epithelium and appear to play pivotal roles in corneal epithelial cell differentiation. (*Invest Ophthalmol Vis Sci.* 2007;48:3576–3585) DOI:10.1167/iovs.06-1373

The Notch signaling pathway controls diverse cellular processes, including differentiation, proliferation, apoptosis, cell-cell adhesion, and migration events through local cell-cell interactions.<sup>1–3</sup> In higher vertebrates, multiple Notch ho-

mologues have been identified, including Notch1 to Notch4 in rodents and humans.<sup>4–7</sup> Notch1 and Notch2 have the highest homology with each other, whereas Notch3 and Notch4 slightly diverge structurally from Notch1 and Notch2 in extracellular and intracellular domains. The intracellular domain of Notch is known to act as a transcriptional activator and is often referred to as activated Notch.<sup>1–3</sup>

Multiple ligands for the Notch receptors are identified in mammals (Jagged1/Serrate1, Jagged2/Serrate2, Delta1, Delta3, and Delta4). Delta1, Jagged1, and Jagged2 have been characterized as ligands for Notch1, Notch2, and Notch3 receptors.<sup>8–12</sup> The Notch receptor undergoes a ligand-dependent extracellular cleavage that releases the extracellular domain, leaving a membrane-tethered intracellular domain.<sup>13</sup> Subsequent intramembrane cleavage by  $\gamma$ -secretase<sup>1,14–17</sup> results in the translocation of the Notch intracellular domain to the nucleus, where it influences the decision of cell fate.<sup>1,14–17</sup> It is generally believed that Jagged and Delta act as transmembrane proteins that interact with Notch receptors expressed on adjacent cells.<sup>2</sup>

Maintenance of corneal structure is crucial for its physiological functions in biodefense and as a refractive tissue.<sup>18</sup> The corneal epithelium is dependent on its constant renewal through slow-cycling stem cells at the limbus and the centripetal migration of transient amplifying cells.<sup>19</sup> Cell proliferation, differentiation, and stratification are essential to this process. However, precise mechanisms that regulate these processes have yet to be fully elucidated, and cell interaction is an important component.

In this study, we have demonstrated the presence of Notch receptors and their ligands in the human corneal epithelium. Furthermore, we have shown that the Notch signaling pathway regulates cell differentiation in the human corneal epithelium.

## METHODS

### Source of Tissue

Human donor corneoscleral disks and eyes (within 24 hours of cadaver time) were obtained from the UK Corneal Transplant Service Eye Bank (Bristol, UK). Mean age of donors was  $62 \pm 19$  years. Human donor tissue was used in accordance with the tenets of the Declaration of Helsinki.

### Immunolocalization of Notch Receptors and Their Ligands in Human Corneas

Corneas were snap frozen in liquid nitrogen-cooled isopentane and embedded in optimum cutting temperature (OCT) compound. Cryostat sections ( $7 \mu\text{m}$ ) were prepared, fixed for 15 minutes at room temperature with 4% paraformaldehyde, and permeabilized for 15 minutes in 0.2% Triton X-100. After three washes with PBS, the sections were incubated for 2 hours with primary antibodies (diluted 1:100 in PBS) directed against Notch1, Notch2, Delta1, and Jagged1. After three 10-minute washes in PBS, the sections were incubated for

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TABLE 1. Primer Sequences for RT-PCR

Gene	Primer Sequence	Annealing Temperature (°C)	Product Amplified (bp)
<i>Notch1</i>	F: 5'-GACATCACGGATCATATGGA-3'	50	666
	R: 5'-CTCGATTGACCATTCAAAC-3'		
<i>Notch2</i>	F: 5'-CCAGAATGGAGGTTCCCTGTA-3'	52	377
	R: 5'-GTACCGAGGCCATCAACACA-3'		
<i>Notch3</i>	F: 5'-CACTGAAGGCTCGTTCCA-3'	50	202
	R: 5'-GGTTGCTCTGGCATTCA-3'		
<i>Notch4</i>	F: 5'-AGCCGATAAAGATGCCCA-3'	50	687
	R: 5'-ACCCAGTCAAGTTGAGG-3'		
<i>Delta1</i>	F: 5'-AGACGGAGACCATGAACAAC-3'	52	382
	R: 5'-TCCTGGGATATGACGTACAC-3'		
<i>Delta3</i>	F: 5'-GTGAATGCCGATGCCCTAGAG	54	256
	R: 5'-GGTCCATCTGCACATGTCAC-3'		
<i>Delta4</i>	F: 5'-TGACCACTTCGGCCACTATG-3'	50	620
	R: 5'-AGTTGGAGCCGGTGAAGTTG-3'		
<i>Jagged1</i>	F: 5'-AGTCACTGGCAGGTTGTAG-3'	54	227
	R: 5'-TCGCTGTATCTGTCCACCTG-3'		
<i>Jagged2</i>	F: 5'-GATTGGCGGCTATTACTGTG-3'	52	600
	R: 5'-AGGCAGTCGTCAATGTTCTC-3'		
<i>β-actin</i>	F: 5'-CATCACCATTGGCAATGAGC-3'	58	284
	R: 5'-CGATCCACACGGAGTACTTG-3'		

F, forward; R, reverse; bp, base pairs.

2 hours in appropriate (1:1000 in PBS) secondary antibodies (Alexa Fluor 488; Molecular Probes, Eugene, OR). After another three washes with PBS, the sections were mounted in mounting medium (Hydro-mount; BDH, Poole, UK) containing bis-benzamide (Hoechst 33342; 1 in 500 dilution of 1-mg/mL stock solution). Substitution of the primary antibodies with same-species serum or elimination of primary antibody served as negative controls. The sections were visualized, and images were captured as described.

### Isolation and Culture of Human Corneal Epithelial Cells

Corneoscleral rims were cut into 1- to 2-mm explants that were placed epithelial-side down in six-well culture plates. The explants were maintained at 37°C in a growth medium consisting of a 1:1 mixture of Dulbecco modified Eagle medium (DMEM)/Ham F-10 medium supplemented with 5% FCS, 5% donor horse serum, 0.5% dimethyl sulfoxide

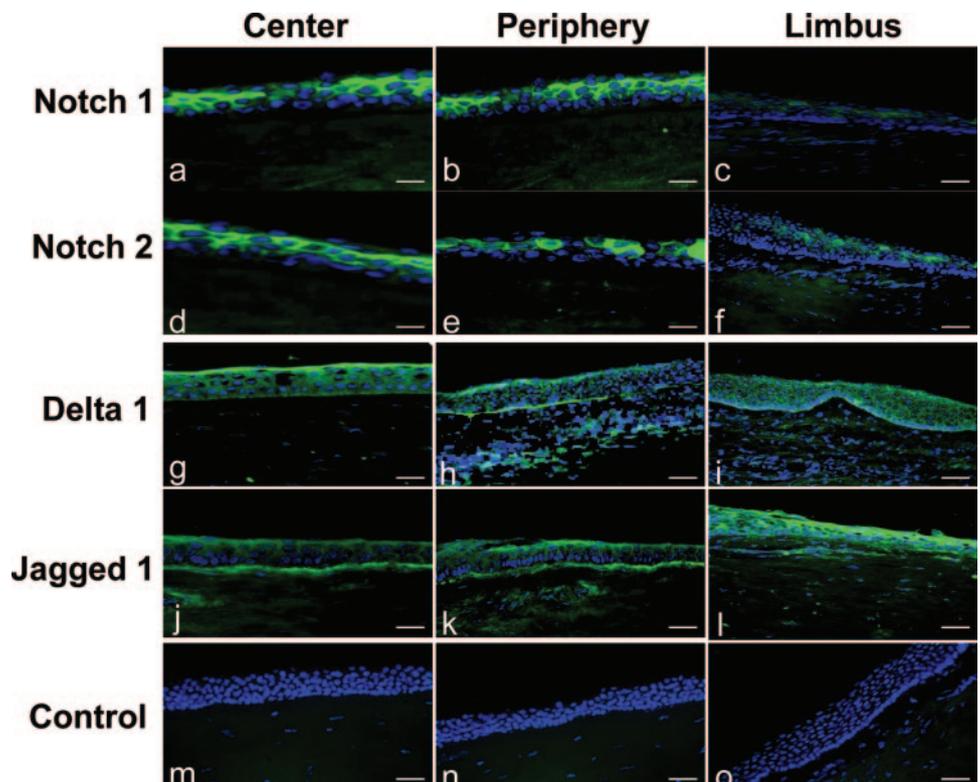


FIGURE 1. Immunolocalization of Notch1, Notch2, Delta1, and Jagged1 in human cornea. (a-f) Notch1 and Notch2 were immunolocalized to suprabasal cell layers of the corneal epithelium but were not detected in superficial surface cells or basal cell layers of the central, peripheral, or limbal region. (g-l) Delta1 and Jagged1 were expressed throughout all cell layers of the central, peripheral, and limbal epithelium. (m-o) Immunoreactivity was not detected in control corneal sections, processed with same species serum or PBS, in place of primary antibodies. Blue: Hoechst nuclear localization. Bar, 50  $\mu$ m.

(DMSO), 5  $\mu\text{g}/\text{mL}$  insulin, 2.5  $\mu\text{g}/\text{mL}$  fungizone, 0.1  $\mu\text{g}/\text{mL}$  cholera toxin, 100  $\mu\text{g}/\text{mL}$  streptomycin, 100  $\mu\text{g}/\text{mL}$  kanamycin, 100 U/mL benzyl penicillin, and 2 mM glutamine. Growth medium was changed every 2 to 3 days, and explants were removed after epithelial cell outgrowth. Primary epithelial cells were used in all experiments. The purity of epithelial cell cultures was confirmed by positive immunostaining for the epithelial cell differentiation marker cytokeratin 3 (CK3).<sup>20</sup>

### RT-PCR Analysis of Notch Receptors and Ligands

Total RNA was isolated from cultured epithelial cells using phenol-guanidine isothiocyanate reagent (TRIzol; Invitrogen, Glasgow, UK) and was subjected to RT-PCR (First-Strand Synthesis Kit; Abgene, Epsom, UK) and PCR (ReddyMix; Abgene) according to the manufacturer's instructions. RNA concentration was determined spectrophotometrically, and an equal quantity of total RNA was used for each sample. PCR primers for Notch receptors (Notch1-Notch4) and their ligands (Delta1, Delta3, Delta4, Jagged1, Jagged2) were designed according to the gene sequences obtained from GenBank. Primer sequences, required annealing temperatures, and sizes of expected PCR products are detailed in Table 1. cDNA was amplified (PCR Master Mix; Applied Biosystems, Foster City, CA). Each cycle consisted of 20 seconds at 94°C, 30 seconds at the defined temperature (Table 1), 60 seconds at 72°C, and a final extension at 72°C for 7 minutes.  $\beta$ -Actin was used as a positive control. PCR products were electrophoresed on a 1.2% agarose gel containing ethidium bromide and visualized under UV light.

### Western Blot Analysis of Notch Receptors and Ligands, Ki67, and CK3 Expression

Corneal epithelial cells were lysed by shaking at 4°C for 30 minutes in RIPA buffer (50 mM Tris-HCl, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM  $\text{Na}_2\text{VO}_4$ , and NaF) containing protease inhibitors (1  $\mu\text{g}/\text{mL}$  each of aprotinin, leupeptin, pepstatin, EDTA, phenyl methyl sulfonyl fluoride [PMSF]). Remnants were detached using a cell scraper, and lysates were transferred to Eppendorf tubes and centrifuged at 12000g for 15 minutes at 4°C. The supernatant was transferred to a new microfuge tube, mixed with sample buffer (12 mM Tris-HCl, 96 mM glycine, 10% SDS, 1% 2-mercaptoethanol, and 0.1% bromophenol blue, pH 6.8), and boiled for 5 minutes. Total protein was determined by the BCA protein assay (Pierce, Rockford, IL), and equal protein concentrations were loaded onto an 8% polyacrylamide gel using a 5% stacking gel. Samples, containing equal protein loadings, were subjected to SDS-PAGE. Resolved proteins were transferred to a nitrocellulose membrane and were probed with anti-human Notch1 (1:200), Notch2 (1:200), Jagged1 (1:200), Delta1 (1:200; all from Santa Cruz Biotechnology, Autogen-BioClear, UK), rabbit anti-Ki67 (1:100; DakoCytomation, Glostrup, Denmark), and monoclonal anti-CK3 (1:200; MP Biomedicals, Inc, Aurora, OH) antibodies for 2 hours at room temperature, followed by incubation for 1 hour in appropriate secondary antibodies conjugated with horseradish peroxidase (HRP; 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA). The enhanced chemiluminescence (ECL) system (Santa Cruz) was used to develop immunopositive bands.

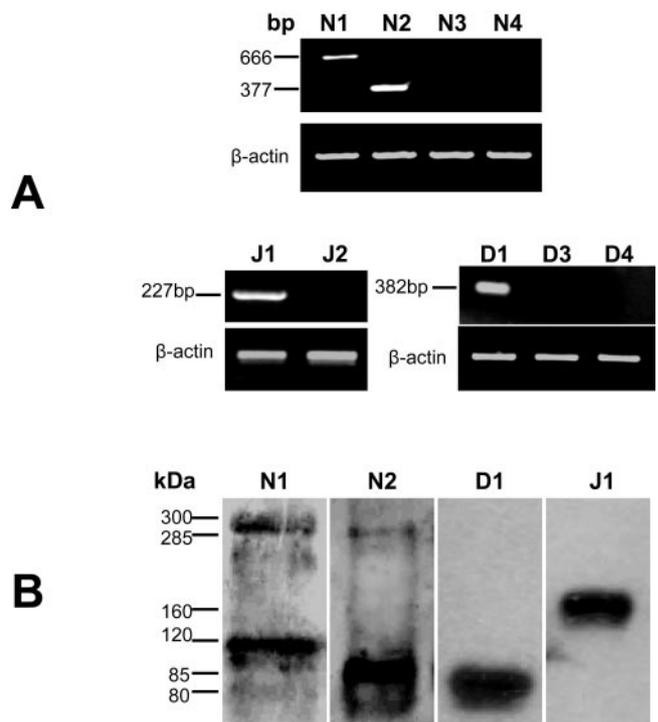
### Effect of Inhibition and Activation of Notch on Corneal Epithelial Cell Differentiation and Proliferation

$\gamma$ -Secretase inhibitor (LY L685458, Sigma-Aldrich, Poole, UK) and recombinant Jagged1 (rJagged1; R&D Systems, Minneapolis, MN) as an activator were used to evaluate the function of Notch signaling in the regulation of epithelial cell differentiation and proliferation. Subconfluent epithelial cells (800,000 cells/well) were incubated in serum-free medium for 1 hour, then incubated in medium containing either  $\gamma$ -secretase inhibitor (25  $\mu\text{M}$  or 50  $\mu\text{M}$ ) for 4 hours or Jagged1 (5  $\mu\text{M}$  or 10  $\mu\text{g}/\text{mL}$ ) for 2 days. Concentrations of  $\gamma$ -secretase inhibitor and

Jagged1 were chosen because of their previously reported respective inhibitory and stimulatory effects on the Notch pathway.<sup>9,21</sup> Control wells were incubated in medium containing either 0.2% dimethyl sulfoxide (DMSO) or 1% PBS. The expressions of Notch1, Notch2, Ki67, and CK3 were semiquantified after scanning densitometry (Expression 1680 scanner; Epson, Long Beach, CA) of immunopositive blots using imaging software (Image; Scion Corporation, Frederick, MD).

### Immunolocalization of Notch Receptors and Ligands in Corneal Epithelial Cells

Human corneal epithelial cells were cultured on coverslips, precoated with attachment factor (TCS CellWorks, Buckingham, UK), until subconfluence was reached. Then the cells were treated with  $\gamma$ -secretase inhibitor or Jagged1 in triplicate wells as described. After fixation in 1% paraformaldehyde (PFA) for 20 minutes and two washes in PBS, the cells were permeabilized by 0.2% Triton-X-100 for 20 minutes. Non-specific binding sites were blocked by 5% milk in PBS for 30 minutes, after which the cells were incubated with Notch1 (1:100), Notch2 (1:100), Ki67 (1:50), and CK3 (1:100) antibodies for 2 hours at room temperature; this was followed by three washes in PBS and a 2-hour incubation in Alexa Fluor 488 or 594 secondary antibodies (each at 1:1000 dilution in PBS, Molecular Probes, UK). After three more washes with PBS, the cells were mounted in a water-based mounting medium (Hydromount; National Diagnostics, Atlanta, GA) containing bis-benzamide (Hoechst 33342, 1 in 500 dilution of 1 mg/mL stock



**FIGURE 2.** Expression of Notch family members in corneal epithelial cells. (A) Total RNA was isolated from cultured human corneal epithelial cells. RT-PCR was performed and PCR products were electrophoresed in a 1.2% agarose gel containing ethidium bromide and visualized under UV light. Notch1 (N1), Notch2 (N2), Delta1 (D1), and Jagged1 (J1) mRNA were expressed by human corneal epithelial cells. However, Notch3 (N3), Notch4 (N4), Delta3 (D3), Delta4 (D4), and Jagged2 (J2) mRNA were not detected.  $\beta$ -Actin was amplified as a positive control. (B) Protein expression of Notch1, Notch2, Delta1, and Jagged1 were identified in corneal epithelial cell lysates. Full-length and truncated isoforms of Notch1 and Notch2 were identified.

solution). Substitution of the primary antibodies with same-species nonimmune serum served as negative control. Experiments, in triplicate, were repeated at least twice. Images were captured digitally in five random microscope fields from each sample with the appropriate software (QFluoro; Leica Microsystem AG, Wetzlar, Germany) under a wide-field microscope (DMRAZ; Leica).

The percentage of positively labeled cells was analyzed with image analysis software (Image Pro-Plus; Image Solutions, UK) by an observer who was masked to treatment group allocation; particular care was taken to identify nuclear and membrane-associated nonnuclear labeling. The total number of cells was determined by counting the Hoechst-labeled nuclei. Hoechst-labeled nuclei were then removed from the image, and the number of cells, with positively labeled cell membranes, including cell borders, was determined. The number of immunopositive-labeled nuclei was quantified in a similar fashion.

### Notch Signaling in Corneal Epithelial Cell Stratification

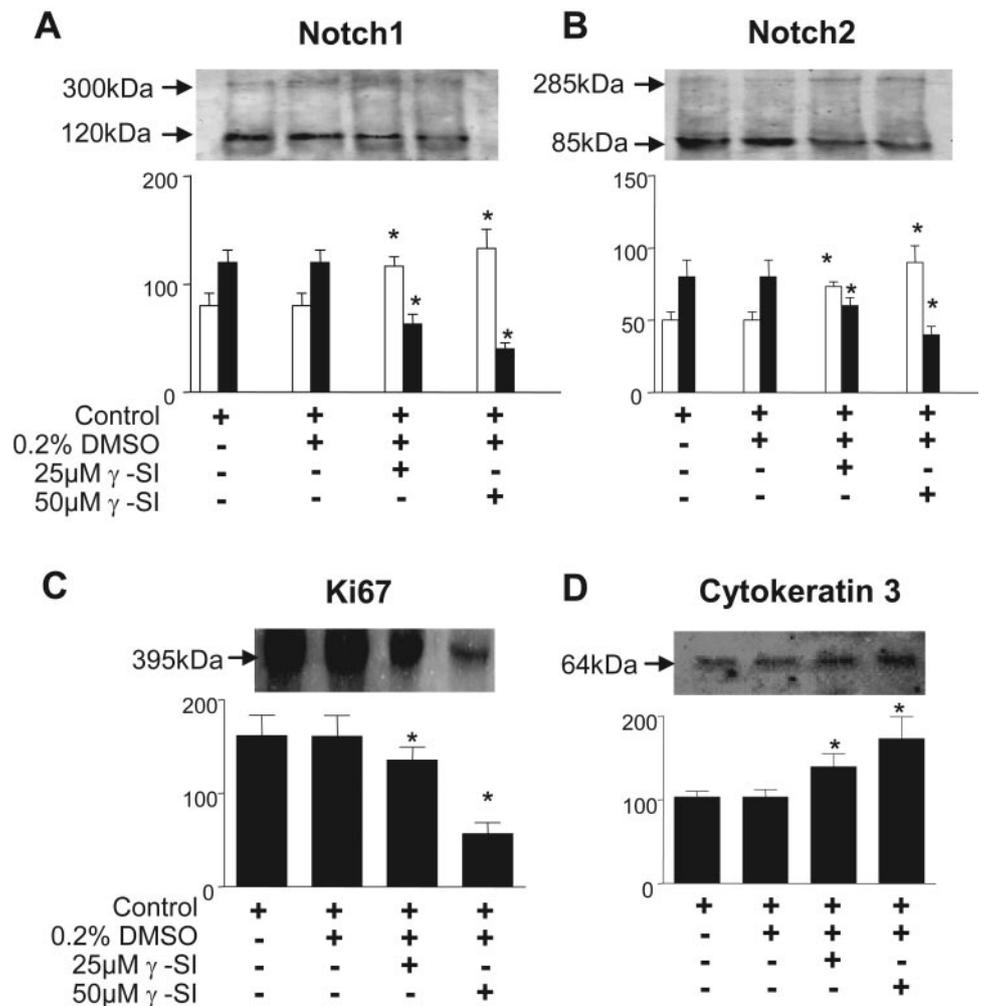
Corneal epithelial cells were cultured according to the air-lift stratification method described by Koizumi et al.<sup>22-24</sup> In brief, corneoscleral rims were cut into six pieces, and the posterior endothelial-stromal explant was excised and discarded. The remaining epithelial-stromal explant was placed, epithelial side down, onto the amniotic membrane (AM) in a culture insert, with  $3.0 \times 10^4$  cells/cm<sup>2</sup> mitomycin-treated 3T3 fibroblasts (NIH 3T3 fibroblast cell line, European collection) in the lower chamber. Each explant was cultured while submerged in

SHEM media (DMEM/F-12 1:1 mixture, FBS 10%, insulin 5 mg/mL, cholera toxin 0.1 nM, epidermal growth factor 10 ng/mL, and penicillin-streptomycin 50 IU/mL) to promote epithelial cell outgrowth. At 7 days, epithelial cell cultures were incubated in SHEM containing 0.2% FBS for 4 hours at 37°C. The wells were rinsed with PBS twice and were cultured in control media (SHEM ± 0.2% DMSO), 10 µg/mL rJagged1 in SHEM, or 50 µM γ-secretase inhibitor in SHEM + 0.2% DMSO for another 1, 2, or 3 weeks, with cultures air lifted in the final (fourth week) by reducing the level of media to expose the epithelial cell surface to air, thus promoting epithelial stratification.

Cultures were snap-frozen in liquid nitrogen-cooled isopentane and were cryosectioned as described before. They were subjected to immunofluorescent localization of Notch1, Notch2, Ki67, and CK3, as described. The number of cells, labeled positively for Ki67, CK3, or Hoechst, was analyzed, by a masked observer (Image Pro-Plus; Image Solutions). The percentage of Ki67- and CK3-labeled cells was expressed as a percentage of Hoechst-labeled nuclei. To examine corneal epithelial morphology, sections were stained using a standard histologic stain, hematoxylin and eosin.

### Statistical Analysis

All experiments were repeated at least twice, and the results were expressed as mean ± SEM. Statistical analysis was performed using one-way ANOVA or Kruskal-Wallis, dependent on distribution of data.  $P < 0.05$  was considered statistically significant.



**FIGURE 3.** γ-Secretase inhibition of Notch1 and Notch2 in corneal epithelial cells. Cultured corneal epithelial cells were incubated with γ-secretase inhibitor (25 µM or 50 µM γ-SI) or control media, with or without vehicle (0.2% DMSO), for 4 hours. Lysates were resolved by SDS-PAGE, and immunopositive bands were semiquantified by scanning laser densitometry. (A) Notch1 (full-length: N1-300, white; NICD: N1-120, black). (B) Notch2 (full-length: N2-285, white; NICD: N2-85, black). (C) Ki67. (D) CK3. Data are expressed as mean ± SEM. \* $P < 0.05$ .

## RESULTS

### Immunolocalization of the Expression of Notch Receptors and Their Ligands in Human Cornea

The spatial distribution of Notch receptors and their ligands in human corneas was identified after immunolocalization of Notch1, Notch2, Delta1, and Jagged1. Notch1 and Notch2 were localized to suprabasal cells in central, peripheral, and limbal regions of the cornea (Figs. 1a-f) but were absent from the basal cell layer in all regions. The surface superficial epithelial cells also appeared to lack Notch labeling. Delta1 and Jagged1 were localized throughout all cell layers in the central, peripheral, and limbal epithelium (Figs. 1g-l). Immunoreactivity was not detected in negative control slides (Figs. 1m-o).

### Gene and Protein Expression of Notch Receptors and Ligands in Corneal Epithelial Cells

RT-PCR identified expression of the genes for the Notch receptors Notch1 and Notch2 and their ligands Delta1 and Jagged1 in cultured human corneal epithelial cells (Fig. 2A). Gene expression of Notch3, Notch4, Delta3, Delta4, and Jagged2 was not detected (Fig. 2A).

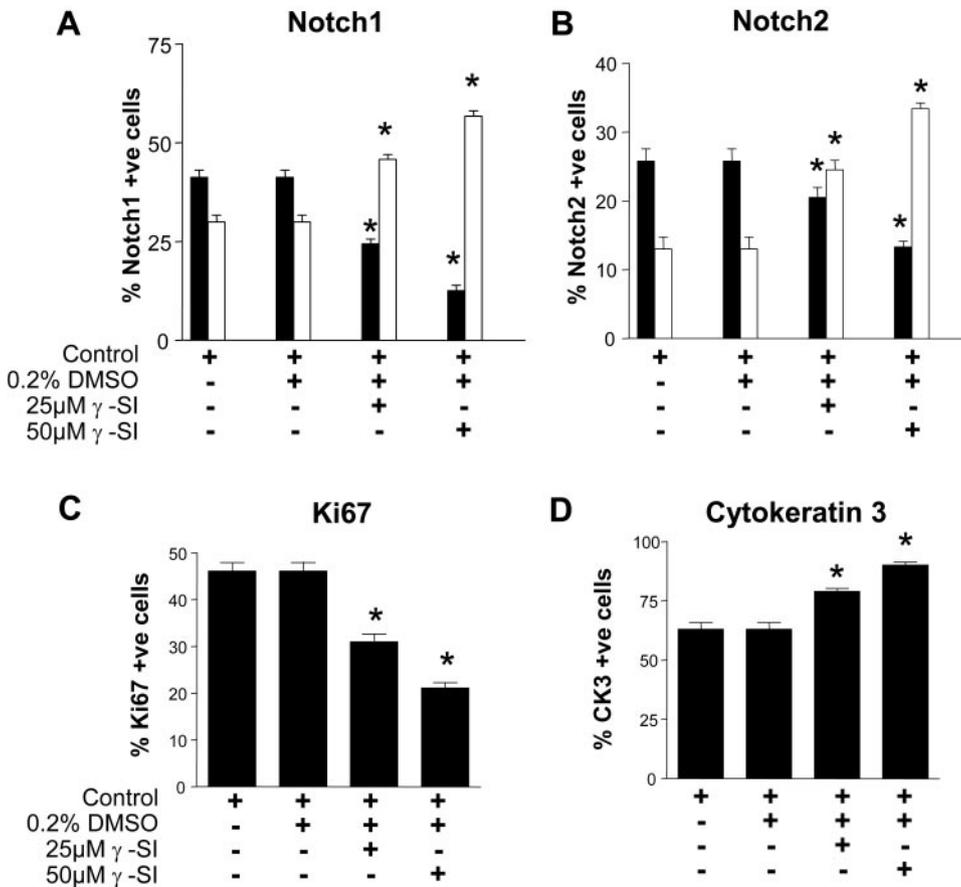
Protein expression was confirmed by Western blotting. The active Notch intracellular domain (NICD) and full-length isoforms of Notch1 and Notch2 and ligands Delta 1 and Jagged 1 were identified in primary passage-cultured epithelial cell lysates according to their molecular weights after immunoblotting, as demonstrated in Figure 2B: Notch1 NICD (Notch1-120, 120 kDa), full-length Notch1 (Notch1-300, 300 kDa), Notch2 NICD (Notch2-85, 85 kDa), full-length Notch2

(Notch2-285, 285 kDa), Delta1 (80 kDa), and Jagged1 (160 kDa).

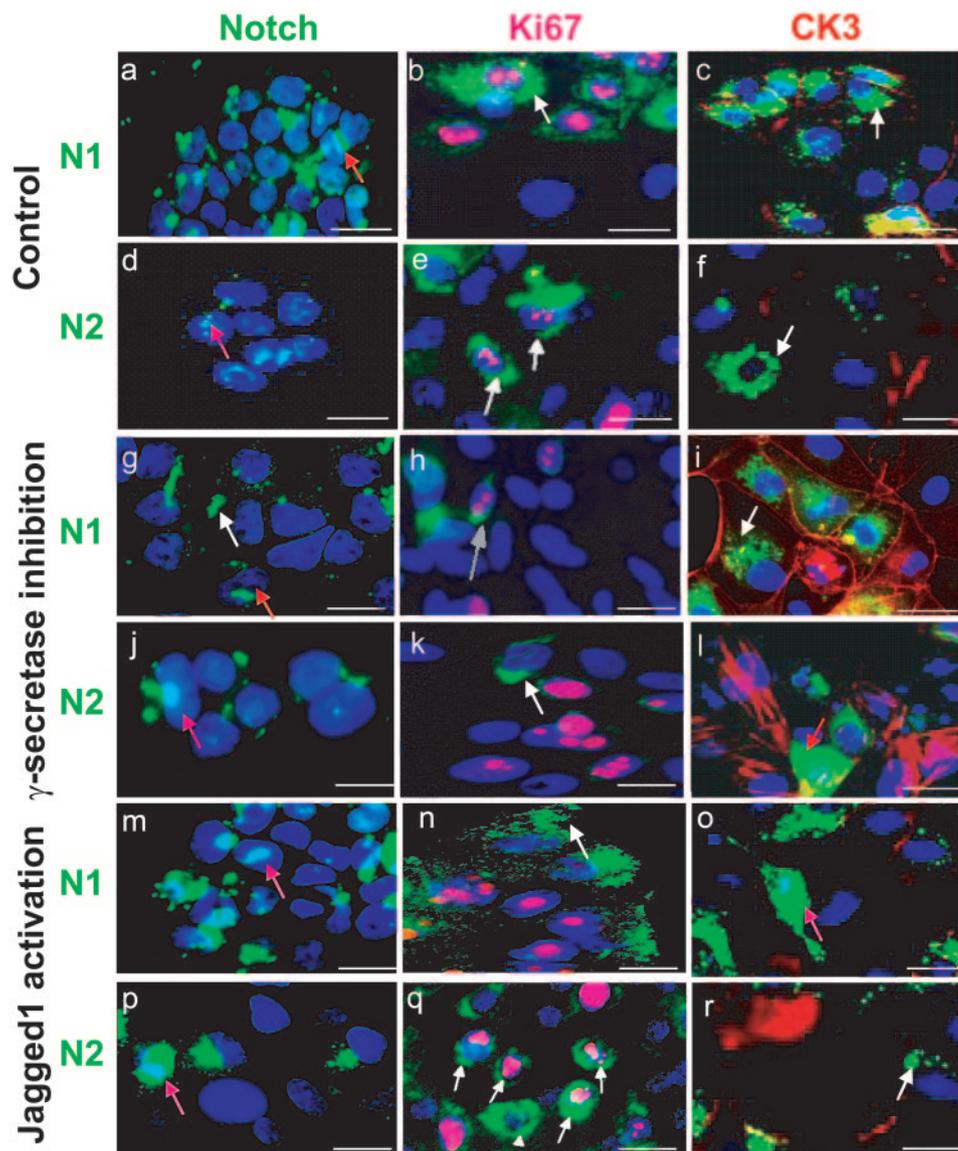
### Inhibition of Notch Signaling Decreased Epithelial Cell Proliferation and Increased Cell Differentiation

Exposure of cultured corneal epithelial cells to a  $\gamma$ -secretase inhibitor, which blocks cleavage of the intracellular domain of the Notch receptor, resulted in decreased expression of active NICD Notch1-120 (Fig. 3A) and Notch2-85 (Fig. 3B) and increased the expression of full-length Notch1-300 (Fig. 3A) and Notch2-285 (Fig. 3B) proteins. Alterations in protein expression appeared to be dose dependent such that levels of expression were significantly altered with a concentration of  $\gamma$ -secretase inhibitor from 0 to 25  $\mu$ M to 50  $\mu$ M. These differences were statistically significant at 25  $\mu$ M ( $P < 0.05$ ) and 50  $\mu$ M ( $P < 0.05$ )  $\gamma$ -secretase inhibitor treatment. The  $\gamma$ -secretase inhibitor also resulted in significantly decreased expression of Ki67 (Fig. 3C;  $P < 0.05$ ), a marker of actively cycling cells,<sup>25</sup> and increased expression of the cornea-specific epithelial differentiation marker CK3<sup>26</sup> (Fig. 3D;  $P < 0.05$ ). These effects were greatest after treatment with 50  $\mu$ M  $\gamma$ -secretase inhibitor.

Consistent with these findings,  $\gamma$ -secretase inhibition resulted in a dose-dependent decrease in the percentage of cells showing positive cell nuclei-associated Notch1 and Notch2 immunoreactivity (Figs. 4A, 4B, 5g, 5j) from  $41.3\% \pm 3.1\%$  to  $12.5\% \pm 2.4\%$  and  $25.8\% \pm 3.1\%$  to  $13.3\% \pm 3.5\%$ , respectively ( $P < 0.05$ ). A concurrent increase in cell membrane-associated labeling (Figs. 4A, 4B, 5g, 5j) from  $29.9\% \pm 3.1\%$  to  $56.7\%$



**FIGURE 4.**  $\gamma$ -Secretase inhibition of Notch1 and Notch2. Immunolocalization of Ki67 and CK3 in corneal epithelial cells. Cultured corneal epithelial cells were challenged with 25  $\mu$ M or 50  $\mu$ M  $\gamma$ -secretase inhibitor ( $\gamma$ -SI) for 4 hours. Percentage cell nuclei (*black*) expressing Notch1 (A) and Notch2 (B) decreased, with a concurrent increase in percentage membrane-associated (*white*) Notch1 (A) and Notch2 (B) cell immunoreactivity. A decrease in Ki67-positive cells (C) and an increase in CK3-positive cells (D) were also observed. Control cells were incubated in media or media containing vehicle (0.2% DMSO). \* $P < 0.05$ .



**FIGURE 5.** Effect of Notch inhibition (50  $\mu$ M  $\gamma$ SD) and activation (10  $\mu$ g/mL Jagged1) on cellular expression of Notch1, Notch2, Ki67, and CK3. Corneal epithelial cells incubated with  $\gamma$ -secretase inhibitor or Jagged1. (a–f) Control cells (untreated media, with or without vehicle). (g–l)  $\gamma$ -Secretase inhibitor-treated cells. (m–r) Jagged1-treated cells. *Green*: Notch1/Notch2; *pink*: Ki67; *red*: CK3; *blue*: Hoechst nuclear localization. *White arrows*: Cell membrane-associated Notch immunoreactivity. *Red arrows*: Notch immunoreactivity cell nuclei. Bars, 50  $\mu$ m.

$\pm 2.4\%$  cells (Notch1) and  $13.0\% \pm 3.1\%$  to  $33.4\% \pm 3.5\%$  (Notch2) was also identified ( $P < 0.05$ ). An accompanying decrease in the percentage of cells expressing the proliferation marker Ki67 from  $46.1\% \pm 3.1\%$  to  $21.2\% \pm 4.5\%$  (Figs. 4C, 5h, 5k) and increased CK3 immunoreactivity (Figs. 4D, 5i, 5l) from  $63.0\% \pm 6.8\%$  to  $90.1\% \pm 6.8\%$  ( $P < 0.05$ ) was also noted.

#### Activation of Notch Signaling Increased Epithelial Cell Proliferation and Decreased Differentiation

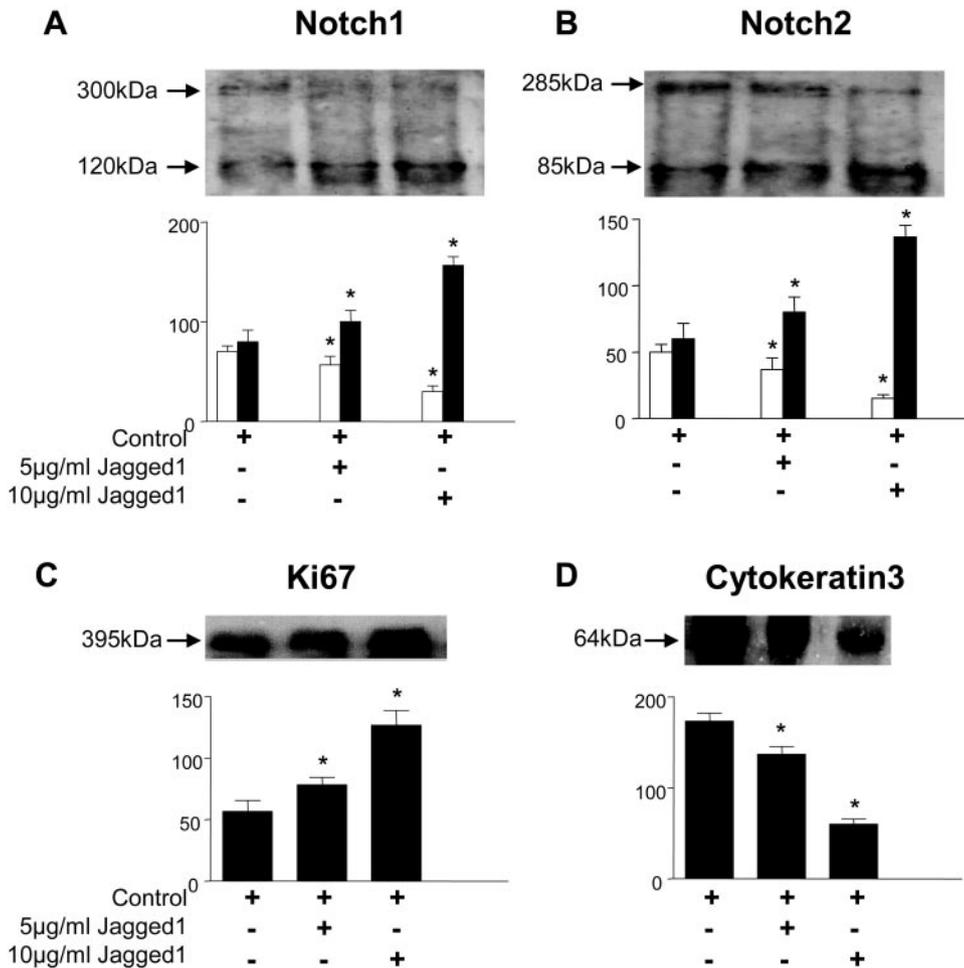
Incubation of cultured corneal epithelial cells with rJagged1 resulted in increased expression of active isoforms Notch1-120 and Notch2-85, with a concurrent decrease in the expression of full-length isoforms Notch1-300 and Notch2-85, respectively (Figs. 6A, 6B). These changes in Notch expression were accompanied by a significant increase in Ki67 (Fig. 6C) and decrease in CK3 (Fig. 6D). All effects were dose dependent, and all differences in expression were statistically significant after 5  $\mu$ g/mL ( $P < 0.05$ ) and 10  $\mu$ g/mL ( $P < 0.05$ ) Jagged1 treatment.

After Jagged1 activation, dose-dependent increased expression of cell nuclei-related Notch1 immunoreactivity, from  $41.3\% \pm 3.1\%$  to  $82.6\% \pm 5.1\%$  cells ( $P < 0.05$ ; Figs.

5m, 7A), and of Notch2 immunoreactivity, from  $25.8\% \pm 3.1\%$  to  $63.8\% \pm 3.1\%$  cells ( $P < 0.05$ ; Figs. 5p, 7B) was observed. In addition, concomitant decreases in cell membrane-associated labeling expression of Notch1 (Fig. 7A), from  $29.9\% \pm 3.1\%$  to  $6.7\% \pm 4.4\%$  cells ( $P < 0.05$ ), and of Notch2 (Fig. 7B), from  $13.0\% \pm 3.1\%$  to  $3.7\% \pm 3.5\%$  cells ( $P < 0.05$ ), were determined. This was noted to coincide with increased cell Ki67 positivity (Figs. 5n, 5q, 7C), from  $46.7\% \pm 3.5\%$  to  $81.5\% \pm 7.1\%$  cells ( $P < 0.05$ ) and with decreased CK3 immunoreactivity (Figs. 5o, 5r, 7D) from  $63.0\% \pm 6.8\%$  to  $18.1\% \pm 6.5\%$  cells ( $P < 0.05$ ).

#### Notch Signaling in Corneal Epithelial Stratification

Morphologic appearance of human corneal epithelial cells during the air-lift stratification cultures indicated that with normal treatment, corneal epithelial cells formed five to six layers of stratified epithelium by 4 weeks (Fig. 8A1). Inhibition of Notch resulted in an increased number of epithelial cell layers (5–6 to 7–8; Figs. 8A2, 8B), with no change in the percentage of cells expressing the proliferative marker Ki67 and differentiation marker cytokeratin3 (Figs. 8C, 8D). By week 4, basal epithelial



**FIGURE 6.** Jagged1 activation of Notch1 and Notch2 in corneal epithelial cells. Cultured corneal epithelial cells were incubated in 5 or 10 µg/mL Jagged1 or control media for 2 days. Lysates were resolved by SDS-PAGE, and immunopositive bands were semiquantified by scanning laser densitometry. (A) Notch1 (full-length: N1-300, *white*; NICD: N1-120, *black*). (B) Notch2 (full-length: N2-285, *white*; NICD: N2-85, *black*). (C) Ki67. (D) CK3. Data are expressed as mean ± SEM. \* $P < 0.05$ .

cells were flatter and superficial cells appeared to slough off more easily from the surface layers (Fig. 8A2).

Notch activation resulted in reductions in epithelial cell layers (5–6 to 3–4; Figs. 8A3, 8B) and in cyokeratin3 expression (Fig. 8D;  $P < 0.05$ ) compared with control cultures. No change in the percentage of Ki67 cells was observed compared with controls (Fig. 8C). By week 4, the epithelial layer was thinner than control, and the cell nuclei appeared slightly larger (Fig. 8A3), suggesting fewer differentiated epithelial cells.

## DISCUSSION

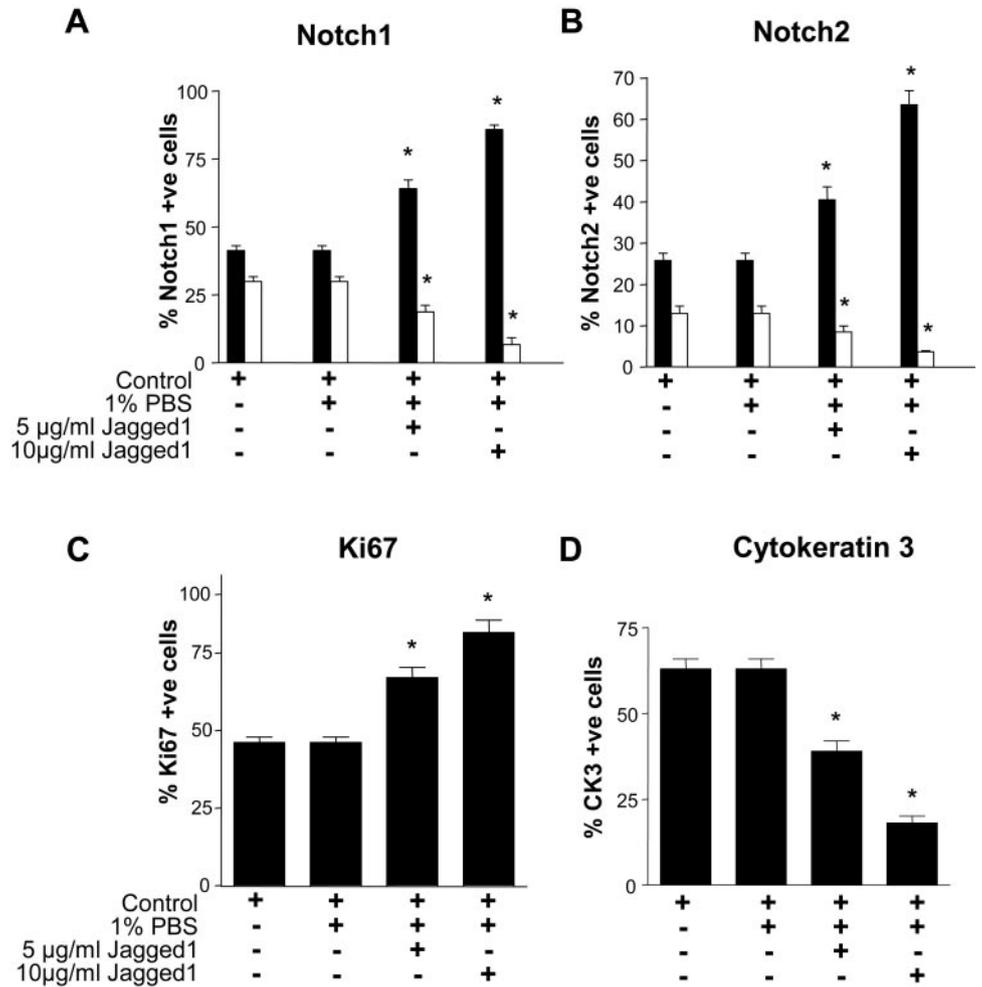
Corneal epithelial cells turn over approximately every 7 to 14 days, continuously maintaining the stratified structure of the epithelium. Defining the mechanisms responsible for the differentiation and self-renewal of corneal epithelial cells is a prerequisite to achieving complete understanding of the process of corneal epithelial cell homeostasis and wound healing. One possible regulator is Notch signaling, which regulates cell fate in many different tissues in a wide range of organisms, including the nervous system, vascular system, hematopoietic system, somites, muscle, skin, and pancreas.<sup>2,24</sup>

This study has identified the presence of receptors and ligands known to play roles in Notch signaling in adult human corneal epithelium. Two Notch receptors (Notch1 and Notch2) and two Notch ligands (Delta1 and Jagged1) were identified in adult human corneal epithelial cells at the gene

and protein levels, indicating that Notch signaling is likely to be involved in the orchestration of adult human corneal epithelial homeostasis. Notch1 and Notch2 were localized throughout the corneal epithelium in suprabasal cell layers.

In the Notch downstream pathway,  $\gamma$ -secretase cleaves the membrane-tethered Notch fragment within its transmembrane domain,<sup>14,15,24</sup> resulting in the release of the intracellular domain (NICD) and translocation into the nucleus to participate in the transcriptional activation of target genes<sup>8,25</sup> and subsequent downstream events. Notch signaling plays different roles in different tissues and within the same tissue at different developmental stages.<sup>2</sup> In most cases, Notch signaling blocks a primary differentiation fate in a cell and forces the cell to remain in an undifferentiated state, and it can regulate terminal differentiation.<sup>2,26–29</sup> However, recent evidence shows that Notch signaling can promote differentiation in some circumstances.<sup>25,30</sup>

$\gamma$ -Secretase inhibition of Notch cleavage prevents Notch signaling.<sup>14,30</sup> Consistent with this finding, our results demonstrated decreased expression of the active NICD isoforms of Notch1 and Notch2 in human corneal epithelial cells, with a concomitant increase in full-length, membrane-bound isoforms. Functional changes in epithelial cells after  $\gamma$ -secretase inhibition were significantly reduced cell proliferation and increased cyokeratin 3 (CK3) expression. These results have two implications. One is that  $\gamma$ -secretase exists in human corneal epithelial cells and is involved in the mediation of Notch signaling, and the other is that  $\gamma$ -secretase



**FIGURE 7.** Jagged1 activation of Notch1 and Notch2. Immunolocalization of Ki67 and CK3 in corneal epithelial cells. Cultured corneal epithelial cells were challenged with different concentrations of Jagged1 (5 and 10  $\mu$ g/mL) for 2 days. The percentage of cell nuclei (black) expressing (A) Notch1 and (B) Notch2 increased, with a decrease in the percentage of cells expressing membrane-associated (white) (A) Notch1 and (B) Notch2 immunoreactivity. An increase in (C) Ki67-positive cells and a decrease in (D) CK3-positive cells were also observed. Control cells were incubated in media or media containing vehicle. \* $P < 0.05$ .

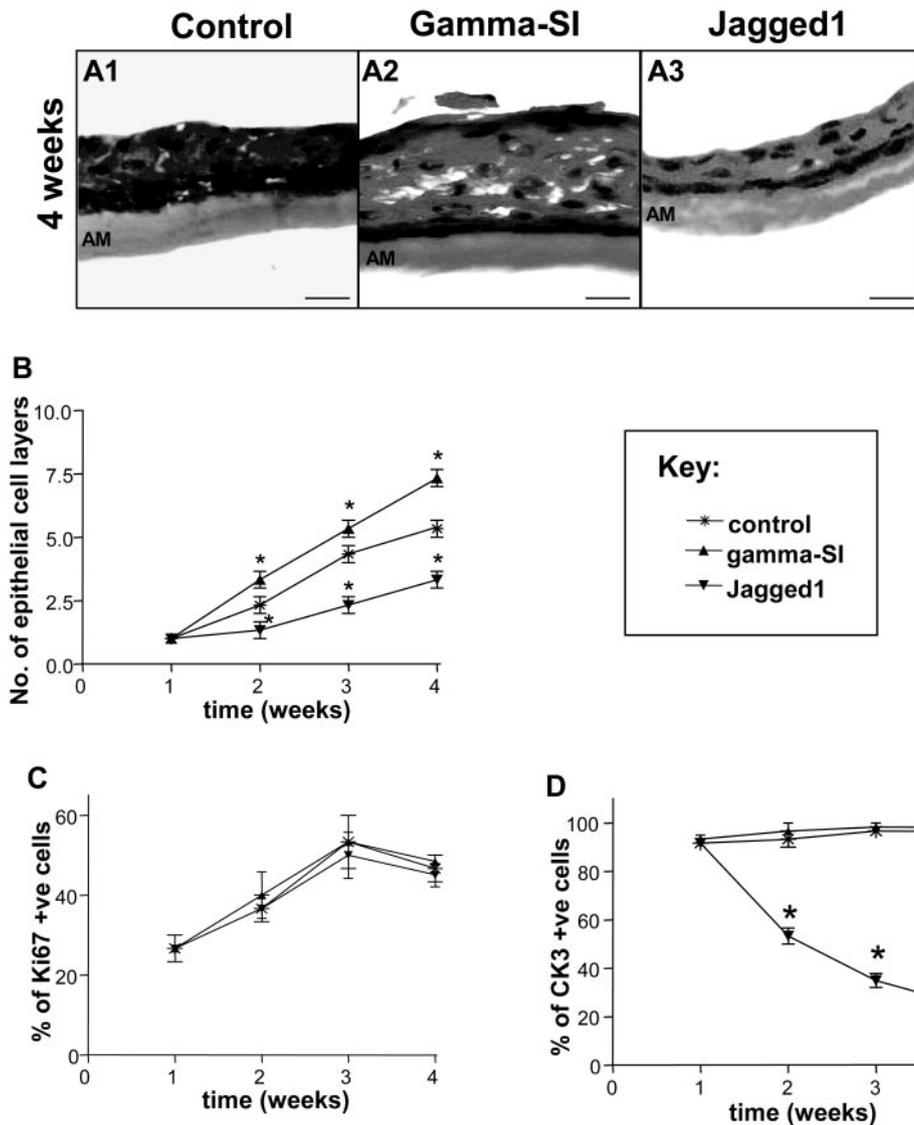
inhibition represses cell proliferation and promotes the differentiation of corneal epithelial cells in a manner consistent with the downregulation of Notch signaling. In contrast, Notch activation in human corneal epithelial cells by Jagged1, an established activator of the Notch downstream pathway,<sup>2,9</sup> resulted in upregulation of the active isoforms of Notch1 and 2, accompanied by an increase in epithelial cell proliferation and a decrease in CK3 expression. These results confirmed that Notch1 and Notch2 could be activated by Jagged1 in corneal epithelial cells and that Jagged1 activation of Notch could play a role in cell proliferation and epithelial differentiation.

It should be noted that  $\gamma$ -secretase cleaves other substrates, including CD44 and E-cadherin, which are present in the cornea, and the putative substrates amyloid precursor protein (APP)<sup>31-33</sup> and low-density lipoprotein receptor-related protein (LRP).<sup>34</sup> Thus, functional changes in epithelial cell homeostasis after  $\gamma$ -secretase inhibition in corneal epithelial cells may be a result of factors other than Notch. However, the presence of the Notch1 and Notch2 receptors and the ligands Delta1 and Jagged2, combined with the antagonistic effects of Jagged1 activation and  $\gamma$ -secretase inhibition on Notch1 and 2 isoform expression, cell proliferation, and differentiation in human corneal epithelial cells, strongly supports a role for Notch signaling in corneal epithelial cell homeostasis.

The involvement of Notch1 and Notch2 in the regulation of human corneal epithelial cell homeostasis was further elucidated and characterized in vitro in a three-dimensional epithe-

lial stratification model previously described by Connon et al.<sup>35</sup> Notch signaling had a significant effect on corneal epithelial differentiation but had no effect on cell proliferation. Notch activation appeared to suppress the generation of differentiated epithelial cells, whereas inhibition of Notch signaling resulted in increased epithelial cell layers with no change in expression of the differentiation marker cytokeratin3. Interestingly, the cultures in the latter consisted of cells more likely to slough off from the multilayered epithelium. The different proliferative effects of Notch in the two-dimensional and three-dimensional in vitro studies suggest that the functional Notch signaling pathway may be affected by its interaction with other signaling pathways, as has been shown in the in vivo state.<sup>2,36</sup> In addition, signals—whether cell or matrix derived—in the three-dimensional stratification model might have been stronger than those of the Notch ligand or inhibitor such that other factors promoted cell proliferation to establish a multilayered epithelium, similar to that in a wound healing response.

This study supports a role for Notch signaling in the regulation of epithelial cell differentiation in corneal homeostasis, though its importance in the control of epithelial cell proliferation is controversial. Other researchers have revealed that Notch signaling, in addition to its well-established effects on cell differentiation, is implicated in cell proliferation<sup>2,37-39</sup> and apoptotic events,<sup>2</sup> though the elements mediating the nonautonomous effect of Notch on cell proliferation are unknown. It is likely that the proliferative effect of Notch signaling in



**FIGURE 8.** Effect of Notch inhibition (50  $\mu$ M  $\gamma$ -SI) and activation (10  $\mu$ g/mL Jagged1) on corneal epithelial cell stratification. (A1–A3) Corneal epithelial air-lift cultures after 4 weeks of culture. A1, control media; A2,  $\gamma$ -secretase inhibition; A3, Jagged1 activation. AM, amniotic membrane. Bars, 50  $\mu$ m. (B) Graph depicting number of cell layers at different time points, after different treatments. (C) Ki67 and (D) CK3 expression in stratified corneal epithelium after Notch activation/inhibition in air-lift cultures: After  $\gamma$ -secretase inhibitor was added, a significant increase in the number of epithelial cell layers was observed (A2, B) compared with control cultures (A1, B; \* $P < 0.05$ ). However,  $\gamma$ -secretase inhibition had no significant effect on percentage (C) Ki67 or (D) CK3 immunopositive cells. Jagged 1 treatment resulted in a significant decrease in epithelial cell layers (A1, A3, B;  $P < 0.05$ ), accompanied by a significant decline in percentage CK3-positive cells at weeks 2, 3, and 4 (D;  $P < 0.05$ ). (C) No change in the percentage Ki67 cells was observed compared with controls.

different tissues may be the result of a synergistic effect between Notch and other proteins.<sup>2</sup>

A balance between cell proliferation and differentiation is essential for maintaining corneal epithelial homeostasis. The existence of Notch receptors and their ligands in the differentiated, nonproliferating suprabasal cell layers throughout the human corneal epithelium implicates Notch signaling in corneal epithelium differentiation and suggests preventing cell entry into the cell cycle. Indeed, previous studies have indicated that Notch1 acts as a tumor suppressor in mammalian skin.<sup>40</sup>

The lack of Notch receptor expression in the basal cell layers of the cornea and limbus, where transient amplifying cells appear, disputes a role for Notch in the *in vivo* induction of cell proliferation. The mechanism for acquiring the proliferative function of Notch may be reprogrammed or inhibited, or it may be related to the specific location of basal cells and their contact with basement membrane. It may be that *in vitro*, the removal of cells from the *in vivo* state isolates the cells from the cell-cell or cell-matrix contacts that prevent entry into the cell cycle.

Similar protein expression patterns of Notch1 have been found in other epithelia, including the human epidermis, and

in mucosae of the oral cavity, esophagus, ectocervix, and vagina.<sup>41</sup> In such structures, it is thought that the exclusion of Notch1 from the dead external surface cells implies a function of Notch in regulating epithelial maturation. In fact, this may be the case in the human corneal epithelium, with Notch in suprabasal cells regulating epithelial differentiation such that terminal differentiation is inhibited. Indeed, Notch1 inactivation by tissue-specific gene targeting has demonstrated that such inactivation in the mouse cornea induces keratinization.<sup>40</sup>

Overall, the expression of Notch receptors and ligands in the human corneal epithelium and the functional associations among the expression of active and inactive Notch isoforms and cell status shown in this study suggest Notch signaling has a pivotal role in the maintenance of corneal epithelial cell homeostasis.

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