The limbus is anatomically located between the cornea and the conjunctiva on the ocular surface. The basal layer of the limbal epithelium is enriched with a special cell population, named limbal epithelial stem cells (LSCs) [1]. The cornea contains a stratified squamous epithelium that turns over rapidly. Renewal of the corneal epithelium is supported by the transient amplifying cells (TACs) generated from asymmetric division of LSCs [1,2]. LSC deficiency may arise following injuries including chemical or thermal burns and through diseases such as aniridia, chronic infection (e.g., trachoma and mycotic keratitis), and Stevens–Johnson syndrome. Partial or total loss or dysfunction of LSCs (clinically termed LSC deficiency) leads to corneal neovascularization, recurrent erosions, stromal scarring, and ulceration, thus causing vision loss.

Currently, transplantation of the ex vivo expanded limbal epithelial sheet has become the most widely used therapy for LSC deficiency [3]. This ex vivo expanded limbal epithelial sheet generally involves placing a small limbal biopsy removed from either the patient or a donor on transplantable
carriers such as the denuded human amniotic membrane to support limbal cells migrating out from the biopsy and outgrowth to form a limbal-like epithelial sheet [4,5]. The failure of limbal transplantation often arises from the depletion of LSCs in culture [6-10]. Continuous culture of LSCs leads to gradual loss of cell proliferation and induction of terminal differentiation [8-10]. A recent study indicated that a better prognosis after limbal transplantation is associated with cultures in which LSCs constituted more than 3% of the total number of clonogenic cells [7]. Evidence suggests that epithelial–mesenchymal transition (EMT) may be involved in LSC senescence. When rabbit limbal explants were cultured at the air–medium interface to induce corneal epithelium formation, LSC intrastromal invasion also occurred due to a mechanism involving EMT, which led to the LSC population decrease [11]. This EMT-mediated loss of LSCs is also shown in ex vivo expansion of human LSCs on the intact amniotic membrane [12]. Prolonged culture of a murine corneal/limbal epithelial progenitor at low density in a serum-free medium leads to irreversible EMT [13]. EMT is a transdifferentiation process in which markers of epithelial cells, such as cytokeratins and E-cadherin, are gradually replaced by mesenchymal markers, such as alpha smooth muscle actin (α-SMA) and vimentin [14]. Transforming growth factor (TGF)-β is a major EMT inducer [15]. TGFβ binds TGFβ receptor type II (TGFβRII) inducing the association of TGFβRII with TGFβRI. TGFβRI is then phosphorylated by TGFβRII. Hereafter, the intracellular signaling transducer Smad2 and Smad3 are phosphorylated by TGFβRI, form complexes with Smad4, and translocate into the nucleus, where they activate transcription of the target genes [16]. However, Smad7 is a negative regulator of TGFβ intracellular signaling by recruiting protein phosphatase to dephosphorylate TGFβRII [17,18]. LSCs express two types of TGFβ (TGFβ1 and TGFβ2), and the levels of spontaneous EMT that occur in culture can be partially blocked with a TGFβ1-neutralizing antibody [11,13], supporting the notion that blocking the TGFβ signaling pathway benefits the expansion of LSCs in vitro. Here, the irreversible EMT is at last in part triggered by Smad-mediated TGFβ signaling [19]. As an inhibitory cytokine, TGFβ1 suppresses the growth of cultured LSCs induced by epidermal growth factor or acidic fibroblast growth factor [19]. The TGFβ-induced EMT has been proposed to be the mechanism responsible for LSC loss in continuous culture. However, the discrepancy of TGFβ-induced EMT between freshly isolated LSCs and serially passaged cells is still unclear. It is also uncertain whether Smad7 can suppress EMT development during LSC expansion in culture. Identifying the intrinsic molecules in LSCs that modulate TGFβ activation can increase our understanding of the mechanism by which EMT develops and can also lead to an improved culture technique for LSC expansion for therapeutic applications.

Previous reports indicated that Notch-mediated cell-to-cell signaling is vital in maintaining stem cell living, including the regulation of stem cell maintenance, cell differentiation, and cellular homeostasis [20]. Notch proteins are transmembrane receptors (Notch 1–4). Notch signaling is initiated by the interaction between the ligand (delta/jagged family) on one cell and the receptor on a neighboring cell [20]. After ligand binding, γ-secretase-dependent proteolytic cleavage of the Notch receptor is triggered to release the Notch intracellular domain (NICD) to the nucleus and then target genes such as hairy and enhancer of split (Hes1; OMIM: 139605 and Hes5; OMIM: 607348) are induced [21]. Recent evidence suggested Notch signaling is involved in maintaining the undifferentiated status of LSCs. Notch-1 protein diminished in dividing cells in culture [22] and in the proliferating limbal epithelium in response to corneal wounding [23]. Activated Notch signaling in cardiac myocytes has proved to be crucial for inhibiting TGFβ-mediated cardiac fibrosis induced by pressure overload [24]. However, whether Notch activation in LSCs may also suppress TGFβ-mediated EMT remains elusive.

The gradual loss of proliferation potential of LSCs and TGFβ-facilitated EMT in late stage cell culture implies a mechanism that may be exercised in early stage LSC culture to shield the cells from the effect of TGFβ. We therefore looked into early stage LSC culture for such a mechanism and found Notch activation may assume this role. Notch activation marker Hes1 and TGFβ signaling inhibitor Smad7 are highly expressed in early LSC culture. Treatment with Notch inhibitor N-(N-[3,5-difluorophenacetyl]-l-alanyl)-S-phenylglycine t-buty1 ester (DAPT) reduced Smad7 expression and caused LSCs to become susceptible to TGFβ-mediated cell proliferation arrest and EMT. These findings suggest Notch signaling may inhibit EMT by upregulating Smad7.

**METHODS**

**Materials:** HEPES-buffered Dulbecco’s modified Eagle’s medium (DMEM), Ham’s/F12 medium, trypsin-EDTA, fetal bovine serum (FBS), antibiotic-antimycotic solutions, and trypsin were purchased from Invitrogen (Carlsbad, CA). DAPT, IWR-1, cyclopamine, hydrocortisone, dimethyl sulfoxide (DMSO), insulin-transferrin-sodium selenite (ITSE) media supplement, mitomycin C (MMC), bovine serum albumin (BSA), 5-bromo-2′-deoxyuridine (BrdU), Triton X-100, Hoechst 33,258 dye, and formalin were all from Sigma-Aldrich (St. Louis, MO). Dispase II and epidermal growth factor (EGF) were obtained from Roche (Indianapolis,
**Immunocytochemistry for BrdU-LSCs**

**Isolation and culture of limbal epithelial stem cells:** LSCs were isolated from 6-month-old New Zealand white rabbits and used for cell-suspension culture by modifying a previously described method [25]. The experiments were approved by the Mackay Memorial Hospital Review Board for Animal Investigation. Briefly, rabbit limbal tissues were washed in PBS (1X; 137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4) containing 50 μg/ml gentamicin. After the iris, and excessive sclera, was carefully removed, the limbal rings were exposed to dispase II (1.2 IU/ml in Hanks’ balanced salt solution free of Mg²⁺ and Ca²⁺) at 4 ℃ for 16 h. The loosened epithelial sheets were removed with a cell scraper and separated into single cells by treating with 0.5 ml trypsin (0.25% and 0.01% EDTA) for 15 min at 37 ℃ with gentle shaking. Cells were transferred to 9 ml of 10% FBS/DMEM/F-12 medium and then collected by centrifugation (400 g for 5 min).

Approximately 1×10⁵ cells were seeded on each well of a six-well plate and incubated with a DMEM/F-12 basal medium (10 mM HEPES, 5 ng/ml human EGF, 1% ITSE, 1% antibiotic-antimicrobial solutions, 0.5% DMSO, and 0.5 μg/ml hydrocortisone) supplemented with 10% FBS for 2 days, and then shifted to serum-free DMEM/F-12 basal medium for 3 more days until they reached confluence (passage 0; P0). Cultures were incubated at 37 ℃ under 5% CO₂. LSCs were cocultured with MMC-treated NIH-3T3 fibroblast feeder cells located within Transwell (0.4 μm pore, BD Biosciences, Bedford, MA) [25]. For passage, near-confluent cells were harvested with 0.25% trypsin, and then 1 × 10⁶ subcultured cells were cultured in the DMEM/F-12 basal medium, and sub-cultured three times at 4-day intervals (P1 to P3).

**Western blot analysis:** Cell lysis, fractionation, and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) were performed as previously described [26]. For rat corneal and limbal tissue preparation, the rat limbal rings were exposed to dispase II (1.2 IU/ml in Hanks’ balanced salt solution free of Mg²⁺ and Ca²⁺) at 4 ℃ for 16 h, and then the loosened limbal epithelial sheets were obtained using a cell scraper. Subsequently, the intact corneal epithelial sheet was harvested and dissolved in sample buffer. A total of 20 μg of protein was loaded per well and separated on SDS-PAGE. Samples were probed with anti-∆Np63α antibody (fold dilution, 1:1,000; BioLegend, San Diego, CA), anti-Bmi-1 antibody (1:1,000; Millipore, Bedford, MA), anti-ABCG2 antibody (1:1,000; Abcam, Cambridge, MA), anti-Smad7 antibody (1:1,000; GeneTex, San Antonio, Tex), anti-Hes1 antibody (1:1,000; GeneTex), anti-NICD antibody (1:1,000; Cell Signaling Technology Inc., Beverly, MA), anti-phospho-Smad2 antibody (1:1,000; S465/S467, Upstate Biotechnology, Lake Placid, NY), anti-phospho-Smad3 antibody (1:1,000; S423/S425, R&D Systems, Minneapolis, MN), anti-Smad2 antibody (1:1,000; Abcam), Smad3 (1:1,000; Zymed Laboratories, San Francisco, CA), anti-TGFβRI antibody (1:1,000; GeneTex), anti-TGFβRII antibody (1:1,000; GeneTex), anti-Smad2 antibody (1:1,000; GeneTex), or anti-TGFβRII antibody (1:1,000; GeneTex). β-actin (1:1,000, Sigma-Aldrich) were used to verify equal loading of protein. Proteins of interest were detected using the appropriate immunoglobulin G-horseradish peroxidase (IgG-HRP) secondary antibody (Santa Cruz Biotechnology) and enhanced chemiluminescence (ECL) reagent (Amer sham, Arlington Heights, IL). X-ray films were scanned on a model GS-700 imaging densitometer (Bio-Rad Laboratories, Hercules, CA) and analyzed using Labworks 4.0 software. For quantification, blots of at least three independent experiments were used. Values were normalized to β-actin content and expressed as a percentage or fold of control.

**Bromodeoxyuridine labeling:** 2 × 10⁵ LSCs were seeded on a slide coated with FNC solution (Athena Enzyme Systems, Baltimore, MD) and incubated in culture medium with or without 10 ng/ml TGFβ1 for 1 day. BrdU (final, 10 μM) was added to the culture for 6 h. After being fixed with 4% paraformaldehyde, the cells were exposed to cold methanol for 2 min, and then treated with 1 N HCl at room temperature (RT) for 1 h before immunofluorescence was performed.

**Immunofluorescence:** Immunocytochemistry for BrdU labeling of proliferative LSCs was performed as in our previous study [25]. Deparaffinized mouse eye sections were blocked with 10% goat serum and 5% BSA in PBS containing 0.1% Tween-20 for 1 h at RT. The eye sections were double-stained with antibodies to rabbit polyclonal anti-∆Np63α antibody (1:100; BioLegend) and monoclonal anti-mouse Hes1 antibody (1:100; ab87395, Abcam) or monoclonal anti-mouse Smad7 antibody (1:100; sc-365846; Santa Cruz Biotechnology) at 37 ℃ for 2 h. The sections were then incubated with rhodamine-conjugated donkey anti-mouse IgG antibody and fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG antibody (1:500; Santa Cruz Biotechnology) for 1 h at RT. Nuclei were monitored by counterstaining with Hoechst 33,258 for 7 min. Images were captured using a Zeiss epifluorescence microscope (Oberkochen, Germany) with a charge-coupled device (CCD) camera and photographs taken using the Axiovert software.

**RNA extraction and quantitative real-time PCR:** Experiments were performed as previously described [27]. The sequence of specific PCR primers was rabbit Ki67 sense, 5'-ATG TTC CAT TTC ATC AGC CA-3'; antisense, 5'-TTT AAA TCG
CTC CTC CAT CC-3′ (accession number: XM_002723010; PCR product: 95 bp), rabbit Hes1 sense, 5′-AAT AAA TGA AAG CCT GAG CCA-3′; antisense, 5′-GGT CAT CTC CAG GAT ATC GG-3′ (XM_002716517; 106 bp), rabbit Smad7 sense, 5′-CCT TCT GCT GTG CAA AGT GT-3′; antisense, 5′-GAT TCA CAG CAA CAC AGC CT-3′ (XM_002713526; 78 bp), and rabbit GAPDH sense, 5′-TCT GGC AAA GTG GAT GTT GT-3′; antisense, 5′-GTG GGT GGA ATC ATA CTG GA-3′ (NM_001082253; 87 bp).

Statistics: Results are expressed as the mean±standard error of the mean (SEM). One-way ANOVA was used for statistical comparisons. p<0.05 was considered significant.

RESULTS

Differential effects of transforming growth factor-beta on epithelial–mesenchymal transition induction in limbal epithelial stem cells before and after subculture:

The extent of TGFβ1-induced EMT on early isolated LSCs (P0) and first passage LSCs (P1) was assayed with western blotting of mesenchymal markers including α-SMA and vimentin. Exposure of P1 cells to 5–20 ng/ml TGFβ1 for 48 h increased the protein levels of α-SMA and vimentin in a dose-dependent manner (Figure 1A). However, TGFβ1 considerably decreased the protein levels of LSC marker ABCG2 and ∆Np63α compared with the untreated cells. Importantly, western blotting results further revealed that P0 cells treated with TGFβ1 caused only a moderate induction of mesenchymal markers, compared with P1 cells (Figure 1B,C; α-SMA: 2.2±0.15 versus 4.3±0.29-fold; vimentin: 1.4±0.13 versus 4.7±0.42-fold). Likewise, P0 cells were more resistant to TGFβ1-induced reduction of LSC markers compared to P1 cells (ABCG2: 83.8±7.6% versus 22.3±4.5%; ∆Np63α: 89.5±3.4% versus 17.3±3.1%). These results indicate that EMT induced by exogenous TGFβ1 is more pronounced in P1 cells than P0 cells.

Differential effects of transforming growth factor-beta on suppressing limbal epithelial stem cell proliferation before and after subculture: To determine the effect of TGFβ1 on

Figure 1. The effects of transforming growth factor-beta on mesenchymal marker and limbal epithelial stem cell marker expression in cultured limbal epithelial stem cells. A: Dosage effect on P1 limbal epithelial stem cells (LSCs). P1 cells were treated with 2–20 ng/mL transforming growth factor-beta (TGFβ1) for 48 h, and then processed for western blot analysis. B: P0 and P1 LSCs were treated with 10 ng/ml TGFβ1 for 48 h, and proteins were detected with western blot analysis with antibodies as indicated. Representative blots (A and B) and densitometric analysis with standard deviation (SD; C) of four independent experiments are shown. Untreated P0 cells (UT) set as onefold or 100%. *p<0.005 versus TGFβ1-treated P1 cells. **p<0.01 versus TGFβ1-treated P1 cells.
the cell proliferation of LSCs (labeled with ΔNp63α), LSCs were pulse-labeled with BrdU (6 h; Figure 2A) and then analyzed with immunostaining. The proliferating ratio for P0 cells was higher than that of P1 cells (16.5±2.1% versus 11.8±1.7%; Figure 2B, white columns), indicating that P0 cells have higher proliferative potential than P1 cells under the cell culture condition. Notably, TGFβ1 diminished cell proliferation of P0 and P1 LSCs by a factor of 1.5 and 6.7, respectively, which indicates that P0 cells are more resistant to TGFβ1-mediated growth arrest. This result was confirmed with qPCR analysis of the Ki67 transcript, a proliferation-associated nuclear antigen. The Ki67 transcript was down-regulated almost fourfold in the P1 cells, whereas the level of the Ki67 transcript in the P0 cells remained relatively the same in the presence or absence of TGFβ1 (about 1.4-fold; Figure 2C).

**Expression of Hes1 and Smad7 in limbal epithelial stem cells dramatically decreased after subculture:** Western blot analysis of protein extracts from P0 cells confirmed the expression of LSC markers including ΔNp63α, Bmi-1, Hes1, and β-catenin, whereas protein expression gradually decreased following the serial passages (Figure 3A). We also noted that all LSC marker protein expression within the P3 cells was obviously reduced compared to that of the P0–P2 cells, suggesting that the P3 cells entered senescence. Next, the protein expression of TGFβ signaling components including TGFβ1, TGFβ receptor I/II (RI and RII), and Smad2/3 at serial passaged LSCs was determined. Western blotting results revealed the expression of the TGFβ signaling components was relatively similar among LSCs throughout the P0 to P2 passages. This suggests that LSC might sustain a TGFβ autocrine in culture. Importantly, the level Smad7 protein, a TGFβ signaling antagonist, was closely related to how many times LSCs were subjected to subculturing; the expression of the Smad7 protein was the highest at P0, but decreased at P1 and almost disappeared at P2 (Figure 3A,B). We also observed that the expression of Hes1, a major Notch signaling target, was very similar to that of Smad7. Collectively, the decrease in Smad7 protein in the passaged cells may benefit TGFβ1 signaling transduction, thus facilitating induction of EMT in later passage cultures.

**Notch signaling activation is essential for maintaining Smad7 expression:** Wnt/β-catenin, hedgehog, and Notch signaling pathways are known to participate in maintaining stem cells. The involvement of these signaling pathways in Smad7 expression was evaluated with inhibitors, including IWR-1 (inhibitor of Wnt/β-catenin signaling), cyclopamine (inhibitor of hedgehog signaling), and DAPT (inhibitor of Notch signaling).
signaling). As depicted in Figure 4A, western blot analysis of the cell extracts from the P0 cells treated with DAPT (10 μM, 48 h) revealed that Smad7 protein expression was attenuated, whereas treatment with IWR-1 and cyclopamine did not change the protein level of Smad7. To further confirm that DAPT affects Notch signaling, we examined the levels of the Hes1 protein. Western blot revealed that DAPT effectively blocked Hes1 expression. In contrast, DAPT (10 μM) had no significant effect on the expression of TGFβ, TGFβ RI/RII, or Smad2/3 (data not published). Western blot result also confirmed that the Smad7 protein level was significantly downregulated at DAPT concentrations above 5 μM (Figure 4B). qPCR showed that Hes1 and Smad7 mRNA were downregulated with the DAPT treatment, compared with the untreated P0 cells (8.3-fold and 6.5-fold, respectively; Figure 4C). Collectively, these results suggest that Smad7 is a downstream target of Notch signaling.

**Smad7 expression in mouse limbal epithelium:** We investigated whether Smad7 is also expressed by LSCs in vivo. Immunofluorescent staining of corneal limbal tissue revealed Smad7 was intensively expressed in the cytoplasm of certain basal cells and a few suprabasal cells of the limbal epithelium, but not in the entire corneal epithelium (Figure 5A). Immunofluorescent staining also showed that Hes1 was predominantly expressed in the limbal basal epithelium. Preimmune serum did not possess immunoreactivity toward Hes1 and Smad7 (data not shown). The accumulation of Smad7 and Hes1 proteins in the limbal epithelium (marked by ΔNp63α) devoid of the corneal epithelium was also evaluated with western blotting (Figure 5B). The results confirmed that levels of Smad7 and Hes1 proteins in limbal epithelium were significantly higher than that in the full corneal epithelium.

**Notch signaling is an intrinsic defense mechanism to against transforming growth factor-beta-induced epithelial–mesenchymal transition and growth arrest in limbal epithelial stem cells:** TGFβ1-induced phosphorylation of Smad 2/3 is an essential step leading to induction of EMT components [28,29]. As illustrated in Figure 6A, TGFβ1 induced the time-dependent phosphorylation of Smad2 and Smad3 in the P1 cells. The peak phosphorylation of Smad2 and
Smad3 occurred between 30 and 40 min and 20 and 40 min, respectively. There was no obvious change in total Smad2 or Smad3. It has been demonstrated that Smad7 can block TGFβ-induced Smad2/3 phosphorylation by forming stable complexes with activated TGFβ RI [17,18]. Therefore, we investigated the importance of Smad7 in protecting LSCs from TGFβ1-induced EMT. First, we examined whether Smad7 expressed at P0 cells could affect TGFβ1-induced Smad2/3 phosphorylation. DAPT pretreatment (10 μM, 48 h) was used to block Smad7 expression before TGFβ1 treatment for 30 or 40 min. As depicted in the right-hand panels of Figure 6A, as predicted, phosphorylation of Smad2 and Smad3 in the DAPT-pretreated P0 cells was markedly higher than the DMSO vehicle-pretreated P0 cells, suggesting Smad7 plays an important role in suppressing Smad2/3 phosphorylation and following nuclear transcription regulation in LSCs. Western blot analysis also demonstrated that the DAPT pretreatment facilitates TGFβ1 effects on induction of mesenchymal markers, compared to vehicle pretreatment at P0 cells (Figure 6B; α-SMA: 3.8±0.78-fold versus 1.7±0.13; vimentin: 2.2±0.46-fold versus 1.4±0.13). DAPT treatment alone had no significant effect on altering the basal levels of the α-SMA and vimentin proteins, compared with those receiving DMSO treatment alone (Figure 6B). In addition, the TGFβ1-mediated inhibition effect on cell proliferation marker Ki67 became prominent with the DAPT pretreatment (Figure 6C; 69.3±10.1 versus 44.3±9.5%; DMSO vehicle alone set as 100%), indicating that DAPT pretreatment may sensitize P0 cells to TGFβ1-induced growth arrest.

Figure 4. Suppression of Notch activation prevents Smad7 expression. A: P0 limbal epithelial stem cells (LSCs) were treated with 10 μM of 4-(1,3,3a,4,7,7a-Hexahydro-1,3-dioxo-4,7-methano-2H-isoinol-2-yl)-N-8-quinolinyl-Benzamide (IWR-1), cyclopamine, or N-(N-[3,5-difluorophenacetyl]-l-alanyl)-S-phenylglycine t-butyl ester (DAPT) for 48 h. Cell extracts were then harvested for western blotting. A representative picture of three independent experiments is shown. B: DAPT dose-dependently suppresses Smad7 expression. Representative blots (below panels) and densitometric analysis with standard deviation (SD; upper columns) of three independent experiments are shown. *p<0.001 versus untreated cells. C: Hes1 and Smad7 mRNA in P0 LSCs assayed with quantitative real-time PCR (qPCR). The cycle threshold (Ct) value of the Hes1 or Smad7 PCR product and control mRNA (GAPDH) were used to calculate the relative quantities of mRNA between the samples. Data represent three independent experiments. *p<0.005 versus untreated cells. **p<0.002 versus untreated cells.
Next, we investigated whether the level of the Smad7 protein in the P1 cells could be induced by the Notch ligand Jagged-1. Exposure of P1 cells to 1 μM or greater Jagged-1 for 12 h increased the NICD to approximately seven-fold compared with the untreated cells (Figure 7A). In addition, P1 LSCs treated with Jagged-1 for further 24 h demonstrated a significant increase in Hes1 and Smad7 protein levels. Reciprocally, DAPT pretreatment (1 h) suppressed the Jagged-1-mediated induction of NICD, thus underscoring the role of Notch signaling in upregulation of Smad7. To examine whether Jagged-1 can also protect LSCs from TGFβ-mediated growth suppression, the Ki67 transcript levels were assessed with qPCR. As shown in Figure 7B, pretreatment with Jagged-1 for 24 h before TGFβ1 exposure suppressed TGFβ-mediated growth suppression of the P0 and P1 cells. In addition, the western blotting results revealed that Jagged-1 pretreatment displayed an inhibitory effect on TGFβ1-mediated induction of mesenchymal markers in the P0 and P1 cells compared to TGFβ1-treated alone (Figure 7C). Taken together, these results indicate that one Notch signaling effect on LSCs is responsible for sustaining Smad7 expression against TGFβ signaling.

**DISCUSSION**

LSC expansion ex vivo to generate limbal equivalent is a widely accepted approach for reconstructing the ocular surface in patients with severe LSC deficiency. LSCs retain TGFβ autocrine that triggers the formation of EMT, which has been regarded as a risk factor for limbal transplantation [13]. The influence of other intrinsic signaling on the activation of TGFβ signaling in LSCs has not been well documented. In this study, blockage of Notch signaling with the DAPT inhibitor provides evidence that Notch signaling contributes to preventing mesenchymal marker expression induced by exogenous TGFβ1. Moreover, we demonstrated for the first time that Smad7, a TGFβ signaling negative regulator, was present in the highest amount in early isolated LSCs and dramatically diminished after cell passages or were treated with DAPT. Our results strongly imply that Smad7 is a Notch downstream target, and the physiologic role of
Smad7 is critical for protecting LSCs from spontaneous EMT formation.

The physiologic role of Notch signaling in the limbal epithelium remains largely unclear. Hes1 is a major Notch signaling target and is usually used as an indicator of Notch activation. Exogenously inhibiting Notch signaling by DAPT significantly suppressed Hes1 expression in the P0 cells, supporting the proposition that Hes1 expression in LSCs is at least in part regulated by Notch signaling. In addition, a recent study reported that Hes1 is expressed predominantly in the basal epithelial layer of the mouse limbus, but is not detected in the differentiated corneal epithelial cells [30]. By assaying murine ocular specimens, our study confirmed that Hes1 is higher in the limbal epithelium than in the corneal epithelium. In this regard, Hes1 expression plays a critical role in maintaining LSC stemness [30]. In the present study, we identified that Smad7 is another Notch effector target in LSCs and might play an important role in antagonizing EMT. Recent studies also found that Notch signaling in different types of cells display distinct roles in regulating EMT. For example, activation of Notch signaling in alveolar epithelial cells and corneal endothelial cells has been demonstrated to enhance TGFβ-induced mesenchymal marker gene expression as demonstrated partly by the cooperation between Notch signaling and TGFβ, which is blocked by DAPT [31,32]. Whether the level of Smad7 is positively correlated with Notch activation in these cells remains unknown and requires further investigation.

Figure 6. Suppressing Smad7 protein expression with Notch inhibitor N-(N-[3,5-difluorophenacetyl]-l-alanyl)-S-phenylglycine t-butyl ester facilitates epithelial–mesenchymal transition and growth arrest induced by transforming growth factor-beta. A: Western blot analysis of Smad 2/3 phosphorylation. Left planes: Transforming growth factor-beta (TGFβ1) time-dependently induces Smad2/3 phosphorylation in P1 cells. Right planes: P1 cells were treated with 10 ng/ml TGFβ1 for 30 and 40 min as positive control. P0 and P1 cells were pretreated with 10 µM N-(N-[3,5-difluorophenacetyl]-l-alanyl)-S-phenylglycine t-butyl ester (DAPT) or dimethyl sulfoxide (DMSO) vehicle for 48 h and then stimulated with TGFβ1 for 30 and 40 min, respectively. Cells were harvested and subjected to western blot analysis with phosphospecific antibodies of Smad2 and Smad3. The total protein levels of Smad2 and Smad3 were estimated by reprobing the membranes with total Smad2 and Smad3 antibody shown in the panels below. B: P0 LSCs pretreated with DAPT facilitates mesenchymal marker expression induced by TGFβ1. Representative blots (left panels) and densitometric analysis with standard deviation (SD; right columns) of three independent experiments are shown. *p<0.05 versus DMSO/TGFβ1-treated cells. C: P0 LSCs pretreated with DAPT facilitates growth arrest induced by TGFβ1. Cells were pretreated with DMSO or DAPT for 48 h and then treated with TGFβ1 for further 24 h. Subsequently, total RNA was extracted for determining Ki67 mRNA with quantitative real-time PCR (qPCR) assay as described in Figure 2C. Data represent three independent experiments. *p<0.002 versus DMSO/TGFβ1-treated cells.
TGFβ receptor type 1 and 2 (RI and RII) are two major TGFβ receptors expressed in LSCs in vivo [33]. Our in vitro data revealed that the protein levels of TGFβ signaling components including TGFβ1, TGFβR I/II, and Smad2/3 are expressed in relatively stable amounts during continuous passage of LSCs. This finding is in line with the previous notion that Smad2/3 plays an essential role in TGFβ-induced EMT and growth arrest [28,29]. Accordingly, LSCs might maintain a TGFβ autocrine loop in vitro. In this regard, a recent study demonstrated that TGFβ1-neutralizing antibody partially inhibits α-SMA expression in cultured LSCs [13], further supporting the proposition that TGFβ autocrine is functional in LSCs. We found that exogenous TGFβ1-induced EMT and growth arrest are only slightly active in P0 LSCs, but become prominent in the later passage P1 cells. These observations lead to the hypothesis that LSC is under constant pressure for EMT and a mechanism exists that resists EMT at least in the early stage of LSC culture. This hypothesis is supported by the observation that when a DAPT inhibitor was used to block Smad7 expression in the P0 LSCs, the P0 cells became susceptible to TGFβ1-induced EMT formation. This
result supports the previous notion that Smad7 plays a crucial role in antagonizing TGFβ signaling.

We found that Smad7 expression in fresh isolated LSCs declined rapidly during cell passages. It seems that Notch activity and Smad 7 expression are associated, but how Smad 7 expression is regulated remained elusive. Smad 7 is reportedly induced by interferon-gamma through Janus kinase 1/ signal transducers and activators of transcription 1 (JAK1/ STAT1) signaling and by tumor necrosis factor (TNF)-alpha and interleukin-1beta through the nuclear factor kappa B (NF-κB) [34-36]. Interestingly, it has also been shown that Jagged-Notch pathway induces NF-κB and JAK/STAT signaling to trigger inflammatory responses in cultured astrocytes [37]. Whether Notch may induce this signaling in LSCs to upregulate Smad7 remains to be determined. Recent studies have shown that Smad7 transcription is suppressed by transcription regulators including metastasis associated 1 (MTA1) and Ski/Sno corepressor complexes [38-40]. However, it has also been shown that Smad7 protein degradation is mainly controlled by E3 ubiquitin ligases, including Smurf2, Arkadia, and Cbl-b [41-43]. Further research is necessary to delineate the involvement of the signaling mechanisms regulating Smad7 in early LSC culture.

In conclusion, the decrease in spontaneous EMT during LSC culture expansion is an important subject in understanding the role in antagonizing TGFβ signaling.

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