STAT3 Regulates the Proliferation and Differentiation of Rabbit Limbal Epithelial Cells via a ΔNp63-Dependent Mechanism

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PURPOSE. To explore the roles of STAT3 in the regulation of ΔNp63-dependent proliferation and differentiation of rabbit limbal keratinocytes.

METHODS. siRNAs were designed to specifically suppress the expression of STAT3 and ΔNp63, and their effects on limbal epithelial cell proliferation and differentiation were examined. Ectopically expressed ΔNp63 was used to compensate for the decreased endogenous ΔNp63. Immunoblot was used to examine the expressions of STAT3, ΔNp63, K3, integrin β1, and involucrin.

RESULTS. Limbal tissue expresses higher level of phosphorylated and nuclear translocated STAT3 compared with that of the cornea. Knockdown of STAT3 expression reduces the expression of ΔNp63, inhibits the expansion of limbal epithelial outgrowth, suppresses the expression of integrin β1, and promotes the expression of involucrin.

CONCLUSIONS. STAT3 enhances the proliferation of limbal keratinocytes through a ΔNp63-dependent mechanism. Suppression of this pathway inhibits cell proliferation with a concomitant increase of cell differentiation. (Invest Ophthalmol Vis Sci. 2011;52:4685–4693) DOI:10.1167/iovs.10-6103

The ocular epithelium consists of corneal, limbal, and conjunctival epithelial cells that are derived from two distinct phenotypic cell lineages, namely, the cornea and conjunctiva.1–2 An overwhelming body of evidence has firmly suggested that limbus is the location of corneal epithelial stem cells.3–6 The fact that limbal epithelial stem cell bear several inherent properties of adult stem cells is well established in that they (1) constitute a small population of relatively undifferentiated cells; (2) have a high proliferative potential and unlimited self-renewal ability; (3) are slow-cycling or label-retaining cells; (4) undergo asymmetric cell division; and (5) reside in a specialized niche.5 In severe ocular surface disorders, limbal epithelial stem cells are extensively damaged with a consequent failure in the supply of corneal keratinocytes, leading eventually to corneal inflammation (erosion, ulceration, or even perforation) and conjunctivalization (opacification and vascularization), and hence visual impairment.12–14

The nuclear transcription factor p63, a member of the p53 family, was found to be specifically expressed in the basal cells of many human epithelia15,16 and was reckoned to be a specific marker for human corneal epithelial stem cell.17 Six p63 isoforms have been reported, and ΔNp63α has been shown to be the major isoform expressed in the basal layer of limbal epithelium.18,19 Recently we showed that the suppression of ΔNp63 expression inhibits proliferation of rabbit limbal keratinocytes cultured on human amniotic membrane.20 Moreover, ΔNp63 has been shown to be a marker of both resting and activated limbal epithelial stem cells.21 Collectively these results implied that p63 may be crucial in regulating the proliferation and differentiation of limbal epithelial stem cell.

The proliferation and differentiation of stem cell are regulated by several signaling pathways.22 Among them, transcription factor signal transducer and activator of transcription 3 (STAT3) has been proved to play a pivotal role in modulating self-renewal of the embryonic23 and hematopoietic stem cells.24 Recently we showed that ΔNp63 autoregulates its own expression via the activation of STAT3 pathway in human nasopharyngeal carcinoma cells.25 In a more recent report using human liver carcinoma cells, we found that expression of ΔNp63 exerts a positive regulatory effect (autoregulation) on the activity of ΔNp63 promoter through activation, phosphorylation, dimerization, and nuclear translocation of STAT3.26 Taken together, these observations clearly suggested that STAT3 plays important roles in the expression of ΔNp63 in tumor cells. However, whether similar regulatory mechanisms exist in limbal epithelial stem cell has yet to be elucidated.

In the present study, we aimed to delineate the role of STAT3 in the regulation of ΔNp63 expression, limbal epithelial cell proliferation, and differentiation by using limbal explant cultures with or without STAT3 silencing. Our results indicate that in limbal epithelial outgrowth, STAT3 is localized mostly in the nucleus and is correlated with a higher ΔNp63 expression. Knockdown of ΔNp63 expression by siRNA suppresses the expansion of limbal epithelial outgrowth. Moreover, knockdown of STAT3 downregulates ΔNp63 expression and suppresses limbal epithelial outgrowth. Interestingly, such growth suppression is rescued by ectopically expressed ΔNp63.

MATERIALS AND METHODS

Materials

Dulbecco’s modified Eagle’s medium (DMEM), Ham’s F-12 nutrition, trypsin-EDTA, fetal bovine serum (FBS), and dispase II were purchased.
from Invitrogen-Gibco (Gaithersburg, MD). Dimethyl sulfoxide (DMSO) and bovine insulin were from Sigma-Aldrich (St. Louis, MO). Mouse receptor grade epidermal growth factor (EGF) was from Upstate Biotech (Lake Placid, NY). All plastic cell culture wares were from Corning Costar Co. (Corning, NY).

The primary antibodies used were K3 (AE5) monoclonal antibody (against rabbit K33; Chemicon, Temecula, CA; 1:1000 dilution), K19 (E6) monoclonal antibody (against rat, cross-react with rabbit27; Abcam Ltd, Cambridge, UK; 1:1000 dilution), p63 (4AA) monoclonal antibody (against human TAP63 and ∆Np63, cross-react with rabbit26; Chemicon; 1:1000 dilution), TAP63 (specific for TAP63 and ∆Np63 (specific for ∆Np65) polyclonal antibodies (against human, cross-react with rabbit26; Biologend, San Diego, CA; 1:1000 dilution), K14 (L1002) monoclonal antibody (against human, cross-react with rabbit26; Chemicon; 1:1000 dilution), integrin β1 (MB1.2) monoclonal antibody (against mouse, cross-react with rabbit26; Chemicon; 1:1000 dilution) STAT3 (124H6), and phospho-STAT3 (32E) monoclonal antibodies (against mouse, cross-reactivity and antibody-sensitivity to rabbit were confirmed with STAT3 polyclonal antibody [no. 9132] in cultured limbal keratinocyte; Cell Signaling, Beverly, MA; all at 1:1000 dilution), involucrin (SY5) monoclonal antibody (against human, cross-react with owl, monkey, canine, gorilla, pig, and rat; NeoMarkers, Fremont, CA; 1:100 dilution), glyceralddehyde-3-phosphate dehydrogenase (G6P) monoclonal antibody (against rabbit GAPDH; Chemicon; 1:20,000 dilution), Lamin B1 polyclonal antibody (against mouse, cross-react with human, mouse, rat, Indian Muntjac, and Xenopus; Abcam, Cambridge, MA; 1:2000 dilution).

Isolation of Rabbit Limbal Tissue and Limbal Explant Culture
Limbal biopsy was performed on the healthy eyes from New Zealand White rabbits (2–2.5 kg). Animals were housed and treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and according to an experimental procedures approved by the committee for Animal Research of the Chang Gung University. The eyelid was sterilized with povidone iodine and limbal tissue containing epithelial cells and part of corneal stroma was separated from the limbal margin and excised from the superficial corneal stroma by lamellar keratectomy. The limbal explants were cultured as previously described with minor modifications.14,28 Briefly, the limbal tissue (1 mm x 2 mm) was implanted with the epithelial side up onto plastic dish and maintained in a growth medium containing DMEM/Ham’s F12 (1:1, 10 mM Hepes buffered) supplemented with 5% FBS, 5% CO2 and were maintained for 2 to 3 weeks. The medium was replaced with the same medium every 2 days.

siRNA Transfection
The rabbit siRNA sequence was modified from the human siRNA based on the sequence information obtained from the National RNAi Core Facility siRNA database (Academia Sinica, Taipei, Taiwan) and was confirmed with desired rabbit cDNA (http://www.genome.ucsc.edu). The sense rabbit siRNA sequences were obtained from MWG-Biotech AG (Ebersberg, Germany), including STAT3 siRNA, 5'-AGUCAGGUUGCGUGUCAAA-3', 30 ∆Np63 siRNA, 5'-CUCAUAGGCGACUCAUU-3', 32 p63 siRNA, 5'-CCUGACGUGCGCCGUA-3', 33 and control nonsilencing siRNA, 5'-UUCCUGCCAGGUGCACGU-3'. Limbal explant cultures in growth medium containing outgrowth cells were incubated with reduced serum medium (OPTI-MEM; Invitrogen, Carlsbad, CA) on day 7 for 12 hours. The cells were then transfected with desired siRNA (50 nM Lipofectamine 2000; Invitrogen) on day 8 for 6 hours. On day 14, the cells were transfected once as above described.

Construction and Infection of Recombinant Adenovirus Vectors
The recombinant adenovirus vectors were constructed as described previously.29 The vectors, designated Ad-∆Np63 and Ad-GFP, were propagated in AD-293 cells, collected until the appearance of cytopathic effect, and purified by four rounds of freezing and thawing. A recombinant adenovirus carrying GFP alone was used as a control. The limbal explant cultures containing outgrowth cells were transfected by Ad-∆Np63 or Ad-GFP. Briefly, the viral stock was diluted into 0.5 mL reduced serum medium (OPTI-MEM) and added to the medium preincubated explant culture on day 8 for 6 hours. The transfection was repeated once on day 14, and the explant was further cultured in the growth medium. On day 21, the explant cultures were stained with trypan blue, photographed, and the area of the epithelial outgrowth was measured by image analysis software (Image-Pro Plus; Media Cybernetics, Silver Spring, MD).

In Vitro Cell Migration Assay
Cell migration was measured by the scratch wound method.32 Briefly, limbal epithelial cells were harvested from limbal explant cultures on day 11. The cells were plated and grown in 60-mm dishes in growth medium until confluent. The confluent cultures were incubated with reduced serum medium (OPTI-MEM) for 12 hours and were transfected with desired siRNA (50 nM) for 6 hours as described above. After transfection, the cells were further incubated in the growth medium for 36 hours. Cultures were then wounded by gently pressing a sterilized razor through the epithelial sheet into the plastic dish to mark the origin and drawing the razor on one side through the monolayer to remove the cells. After the cells were wounded, the medium was replaced with the same medium to remove the floating cells. Keratinocyte migration was permitted for up to 6 hours and stopped by fixation with 4% paraformaldehyde. The area of cells across the origin line into the wounded side was measured (Image-Pro Plus).

Total Protein Extraction
Cornea tissue was punched out by 6.0-mm Barron vacuum cornea punches (Katenà, Denville, NJ) after limbal tissue was dissected and removed as described above. The tissue and limbal epithelial outgrowth culture were washed with ice cold 1× PBS. The epithelial layer of corneal and limbal tissue was scrapped into 10× volume (approximately 0.5 mL) of tissue protein extraction reagent (T-PER; Pierce, Rockford, IL). The reagent contains 25 mM bicine and 150 mM sodium chloride (pH 7.6) and was supplemented with 10 mM NaF, 10 mM sodium orthovanadate, and 1× protease inhibitor cocktail (Sigma-Aldrich, Milwaukee, WI). The limbal epithelial outgrowth was scrapped into 0.1–0.5 mL (depending on outgrowth area) of the reagent by plastic cell scrapers. The suspension was transferred to Eppendorf vials on ice and sonicated to break the cells. After centrifugation for 15 minutes at 4°C, the supernatant was pooled and designated as the total protein extract. Protein concentration was determined using a protein assay kit (Bio-Rad, Hercules, CA).

Preparation of Cytosolic and Nuclear Protein Fractions
The epithelial layer of the limbal tissue or limbal epithelial outgrowth was washed with cold PBS twice, incubated in 10× volume of a cytosolic extraction buffer (10 mM HEPES, pHi 7.9, 10 mM KCl, 10 mM EDTA, 1 mM dithiothreitol, 0.4% [octylphenoxethanol, and protease inhibitors) on ice for 10 minutes. The epithelial layer of the limbal tissue or limbal epithelial outgrowth was scraped, and the cell clumps were disrupted by repetitive pipetting. The suspension was then centrifuged at 14,000 g for 3 minutes at 4°C. The supernatants were pooled and designated as the cytosolic protein fraction. The pellet was resuspended in 150 μL of a nuclear extraction buffer containing 20 mM HEPES, pHi 7.9, 200 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, and protease inhibitors and was vortexed at full speed for 10 seconds. The sample was allowed to sit on ice for 2 hours, shaken once every 20 minutes, and centrifuged at 14,000 g for 5 minutes at 4°C. The supernatant was collected and designated as the nuclear protein fraction.
Avidin Biotin Conjugated-ΔNp63 Promoter (DNA) (ABCD) Pull-Down Assay

The nuclear protein extract (200 μg) was precleared with streptavidin-conjugated magnetic beads (Promega, Madison, WI) in 0.5 ml of ABCD binding buffer (20 mM Hepes, pH 8.0, 1 mM EDTA, 100 mM NaCl, 0.5% Nonidet P-40, 1 mM dithiothreitol, 10 mM NaF, 10 mM sodium orthovanadate, 1 mM PMSE, and 1% cocktail protease inhibitor; Sigma–Aldrich) for 30 minutes at 4°C. The magnetic beads were pelleted by magnet for 1 minute at 4°C. The supernatant was precleared with or without hSIE oligonucleotides (20 mmol), a high-affinity consensus STAT3 binding element (5′-CTTCATTTCGCTAAATTCCCTAAGCT-3′ and 5′-AGCITTTAGGATTCGAGGGAATGA-3′) at room temperature for 20 minutes. The hSIE was used to confirm the binding specificity of the STAT3 to its DNA binding site. The supernatant was further incubated with 10 μg of poly(dI–dC) (Pharmacia Biotech, Piscataway, NJ) and 100 pmol of biotinylated annealed probes at room temp for 20 minutes. The annealed probes used were the PCR product, amplified from biotinylated ΔNp63 specific primer (5′-GGGACACATATTACGAGATTCTCCTAT-3′) and GL2 primer (5′-CTTATTTTTGTCCTTCC-3′) from the pGL3 vector containing human ΔNp63 promoter.25 The DNA-protein complex was precipitated with magnetic beads for 30 minutes, washed three times in ABCD binding buffer, eluted by boiling in 2× SDS loading buffer, analyzed by SDS-PAGE, and visualized by Western blotting using anti-STAT3 (Cell Signaling) antibody.

Analysis of ΔNp63 Promoter Luciferase Activity

The recombinant human ΔNp63–823 luciferase reporter vector (pGL3–823-Luc) and STAT3 binding site deleted vector (pGL3–823ΔSTAT3-Luc) were constructed according to our previous study.26 The pGL3-Basic vector was used as a control. Primary rabbit limbal epithelial cells were seeded in 12-well tissue culture plates at 2 × 10^4 per well. The cultures were incubated with reduced serum medium (OPTI-MEM) for 12 hours and were transfected with desired luciferase vectors, siRNAs and β-galactosidase vector (0.5 μg), for 6 hours. After transfection, the cells were further incubated for 48 hours in the growth medium. The cells were washed twice with ice cold PBS, and luciferase and β-galactosidase assays were performed using luciferase and β-galactosidase assay reagents, respectively (Promega) per the manufacturer’s protocols. Luciferase activity was read using FB12 luminometer (Berthold Detection Systems, Pforzheim, Germany). The β-galactosidase values were used to normalize the luciferase activity.

Analysis of STAT3 Binding Site of the ΔNp63 Promoter

The matrix sites were identified within the ΔNp63 promoter (Matinspector; Genomatix, Munich, Germany). Position weight matrices (matrix) were used in motif-finding algorithms to identify the cis-regulatory motifs. To predict the putative transcription factor binding sites, Matrix Family Library Version 8.2 (Jan. 2010) was used, and the selected groups were General Core Promoter Elements and Vertebrates (core/matrix sim 0.75/Optimized). The human genome database was based on the February 2009 human reference sequence (GRCh37) produced on the draft assembly (Broad Institute/NCBI project 12,819, AAGW00000000), Apr. 2009).

Confirmation of the In Vivo STAT3 Binding to ΔNp63 Promoter

A chromatin immunoprecipitation (ChIP) assay was performed to confirm the binding of STAT3 to human and rabbit ΔNp63 promoters (EZ-CHIP assay kit; Upstate Biotechnology, Waltham, MA) according to the manufacturer’s instructions. The lamin and corneal tissues were sonicated (10 seconds pulses × 5 at 50% output power, Microson XL) to generate chromatin sizes between 200 and 1000 bp. The protein-DNA complex is immunoprecipitated using STAT3 antibody. Normal rabbit IgG (IgG2) antibodies (Millipore) served as negative control. Cross-links of eluted immune complexes were reversed by heating at 65°C for 4 hours. After purification, PCR was used to analyze the immunoprecipitated DNA (Go Taq Green Master Mix; Promega). The primers were used were 5′-AAACACCTAAACAGATTG-3′ and 5′-GGTTGGAAGATTCTCTAAGTCT-3′, which recognize proximal rabbit ΔNp63 promoter from −435 to −142 (relative to the transcriptional start site). The primers recognize rabbit ΔNp63 exon14 from +104,582 to +104,752 (5′-CCCACTTGTCCCTACGG-3′ and 5′-TGATAGCAGTGCTGCTGTG-3′) were also used to serve as negative control. The products of PCR reaction were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining.

Immunoblotting

Samples containing equal amounts of protein were loaded per lane, fractionated on 10% SDS-polyacrylamide gel, and transferred onto membrane (Immobilon-P membrane; Millipore, Bedford, MA). Membranes were immunoblotted with desired primary antibodies overnight at 4°C and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech, Freiburg, Germany) at 1:10,000 dilution. The immunoreactive protein bands were visualized by chemiluminescence.

RESULTS

Activation of STAT3 in the Limbal Tissue

Phosphorylation and nuclear localization is an essential first step for a transcription factor to exert its function. Thus, the phosphorylation and the relative abundance of the cytosolic and nuclear STAT3 are indicative of its transcriptional activity.34 As shown in Figure 1A, the expression levels and the phosphorylation status of STAT3 were conspicuously higher in the limbal than in the corneal tissues. When the cytosolic and nuclear STAT3 was further examined, the difference between limbus and cornea was even more obvious (Fig. 1B). The result suggested that limbal epithelium contains much higher STAT3 activity then the cornea epithelium.

Regulation of ΔNp63 Expression by STAT3

The p63 isoforms expressed in limbal tissue and by limbal epithelial outgrowth were examined by Western blot using


![Figure 1](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933461/ on 06/01/2017)
STAT3 binds to the ΔNp63 promoter

The genomic sequence upstream to the exon 3' of the rabbit ΔNp63 is shown in Figure 3A. Only 369 bp sequence was known in this region based on the rabbit genomic database (Broad Institute, oryCun2, Apr. 2009), and the STAT3 binding site was not present in this region. Because rabbit sequence is 90% similar to human in this region, we therefore used the human ΔNp63 exon 3' upstream sequence (698 bp, from transcription start site) for the protein pull-down experiment.

A putative STAT3-responsive element has been identified in the human ΔNp63 promoter region.26 To confirm that STAT3 binds to the ΔNp63 promoter in limbal epithelial cells, we performed an avidin biotin conjugated-ΔNp63 promoter (DNA) (ABCD) pull-down assay. The nuclear extracts of the limbal epithelial outgrowth express undetectable level of ΔNp63. We next examined whether STAT3 regulates ΔNp63 expression by limbal epithelial cell. We transfected the STAT3 siRNA into the limbal epithelial outgrowth to knock down the endogenous STAT3 expression and examined its possible effect on ΔNp63 expression. The cell extracts prepared from cells with or without STAT3 silencing were then blotted for ΔNp63 expression using ΔNp63-specific antibody. As shown in Figure 3B, the expression of ΔNp63 was suppressed by STAT3 silencing. In contrast, when both ΔNp63 and TAp63 were silenced by si-p65(DBD), the expression level of STAT3 was not affected.

STAT3 binds to ΔNp63 promoter

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To ascertain the use of human ΔNp63 promoter construct is valid, we examined the human ΔNp63 promoter reporter activity in primary rabbit limbal epithelial cells. The human ΔNp63–823 luciferase reporter vector (pGL3–823-Luc) or STAT3 binding site deleted vector (pGL3–823ΔSTAT3-Luc) was transfected into primary rabbit limbal epithelial cells, with si-non or si-STAT3 co-transfection. The result showed that in cells transfected with STAT3-DN or si-STAT3, the ΔNp63 reporter activity was greatly reduced (Fig. 3C). This result indicated that STAT3 binding onto human ΔNp63 promoter sequence is functional and site specific in rabbit limbal epithelial cells.

An examination of the rabbit ΔNp63 upstream promoter region in the rabbit genome database (Broad/oryCun2) sug-
A

Input | Pulled
--- | ---
--- | ---
hSIE | - | + | - | +
Pull down by ΔNp63 promoter | - | - | + | +
STAT3 | ![STAT3 bands](image)

B

C

STAT3 binding site

| Human | -629 | ATTCCTATTTCCCCTATATAATATAGGA | -603 |
| Rabbit | -719 | TGGCTTTTTCCCCTATTTCAATAGCC | -693 |

D

E

ΔNp63

-433/-142

+104582/+104751

anti-STAT3 rabbit IgG Input anti-STAT3 rabbit IgG Input

-433/-142

104582/104751

Limbus epithelium Cornea epithelium

**Figure 3.**
gested a putative STAT3 promoter binding site at −711 to −704 (Fig. 3D). Comparison of the rabbit ΔNp63 promoter sequence (−719 to 0) with the corresponding human genomic sequence revealed that there is a 82% similarity, suggesting that the regulation machinery of ΔNp63 expression is probably highly conserved between the two species.

A chromatin immunoprecipitation assay was performed to examine the binding of STAT3 to ΔNp63 rabbit promoter. STAT3 was found to bind to the proximal region of ΔNp63 promoter (nearly −433 to −142) in limbal epithelium but not that of the cornea (Fig. 3E). The result clearly showed that STAT3 specifically bound to ΔNp63 promoter in the rabbit limbal epithelium.

**Knockdown of STAT3 or ΔNp63 Expression Suppresses Limbal Epithelial Outgrowth**

To examine if the regulation of ΔNp63 expression by STAT3 affected the proliferation of the limbal epithelial cells, we used STAT3 siRNA to knock down endogenous STAT3 expression and examined the rate of limbal epithelial outgrowth from the limbal explants. As shown in Figure 4, the epithelial outgrowth was suppressed by siRNAs to ΔNp63, p63(DBD) (against DNA binding domain of ΔNp63 and TAp63), or STAT3. A close to 92% growth suppression was obtained by si-p63(DBD), followed in order by si-ΔNp63 (78% inhibition) and si-STAT3 (62% inhibition) when measured on day 21. Because limbal epithelial outgrowth expressed undetectable TAp63 in our hands (Fig. 2A), the more effective inhibition by si-p63(DBD) was probably due to a more effective silencing of ΔNp63 expression (Fig. 5). The growth suppression by si-STAT3 was partially reversed when the cells were cotransfected with a ΔNp63-expressing vector, ad-ΔNp63α (Fig 4, lower right panel). The silencing efficacies of the siRNAs (50 nM each) were examined and are shown in Figure 5. The expression of ΔNp63 was suppressed the most by si-p63(DBD), followed in order by si-ΔNp63 and si-STAT3. Figure 5 also showed the overexpression of ectopic ΔNp63 by ad-ΔNp63α transfection (the rightmost lane).

**Effect of siRNA on Cell Migration**

To see if the suppression of epithelial outgrowth by siRNA was correlated with suppression of cell migration, we used limbal epithelial cell monolayer culture and the razor wound method to evaluate cell migration. Figure 6 shows that the migratory activity of the limbal keratinocytes was not affected by anyone of the siRNA transfection.

**Silencing of STAT3 or ΔNp63 Expression Promotes Limbal Keratinocyte Differentiation**

To examine the possible effect of STAT3 or ΔNp63 expression on limbal keratinocyte differentiation, we compared the relative abundance of K3, involucrin, and integrin β1 in limbal epithelial outgrowth without or with siRNA transfection. K3 has been considered a specific differentiation marker of the corneal epithelium1 and involucrin has been referred to as an
early marker for terminal differentiation of epidermal keratinocytes. K14, K19, and integrin β1 were known as undifferentiation markers and were reported to be expressed in limbal epithelium. These markers were detected in rabbit limbus, but only integrin β1 was also detectable in cornea and limbal epithelial outgrowth (Fig. 7A). Therefore, the expression of K14, K19 was not further studied. Figure 7B showed that K3 was highly expressed in all experimental groups regardless of STAT3 or ΔNp63 expression. Involutrin expression was conspicuously enhanced on transfection with si-ΔNp63, si-p63(DBD), or si-STAT3 and was partially reverted by the ectopic expression of ΔNp63 in si-STAT3-transfected cells. In contrast, integrin β1 expression was suppressed when transfection with si-p63(DBD) or si-STAT3 and was only slightly reverted by the ectopic expression of ΔNp63.

**DISCUSSION**

The transcription factor p63 has long been reported to be highly expressed in the basal layer of limbal epithelium and has been shown to be important in regulating the proliferation and differentiation of limbal epithelial stem cells. However, how p63 expression is regulated in limbal epithelial cells has yet to be elucidated. In the present study, we showed that limbal epithelium expressed a much higher level of activated STAT3 than corneal epithelium. In cultured limbal epithelial outgrowth, STAT3 was also highly expressed, and it regulated ΔNp63 expression by direct binding to ΔNp63 promoter. The function of STAT3 as a transcription factor has been well elucidated. STAT3 is activated through tyrosine phosphorylation and nuclear translocation. The protein expression of STAT3 has been shown to be regulated by the transcription factor TRPS1 (which belongs to the GATA family), which binds to STAT3 promoter and represses its expression. Interestingly, GATA3 was shown to be essential for skin differentiation, and we have observed that GATA3 was highly expressed in cornea and was less expressed in limbus (our unpublished data), suggesting that the STAT3 expression in corneal epithelium could be suppressed by its higher GATA3 content. In a recent report, we showed that keratinocyte growth factor (KGF) upregulated ΔNp63 expression by limbal epithelial cells in culture. However, an earlier report by Liang et al. showed that KGF could not activate Jak/STAT3 pathways in corneal epithelial cells. Moreover, STAT3 has been shown to be activated by a number of cytokines including leukemia inhibitory factor, EGF, and interleukin-6 in epithelial carcinoma, embryonic stem cell, and limbal keratinocyte, suggesting the existence of multiple pathways governing STAT3 activity.

Data presented in the present study clearly indicated that silencing of STAT3 or ΔNp63 inhibited the epithelial outgrowth of the limbal explants. The inhibitory effect by STAT3 silencing appeared to be mediated through downregulation of the ΔNp63 expression. This conclusion was supported by the observation that knockdown of STAT3 by si-STAT3 downregulated ΔNp63 expression, and the inhibition of epithelial outgrowth by si-STAT3 was partially reverted by cotransfection of ad-ΔNp63. In the si-STAT3 and ad-ΔNp63 cotransfected cells, the ectopically expressed ΔNp63 was higher than the control cells (Fig. 5). It was therefore intriguing that the reversion on growth inhibition by si-STAT3 was only partial. It strongly suggested that STAT3-mediated limbal epithelial cell proliferation acts through multiple pathways, and the ΔNp63 pathway was one of them. Increased STAT3 activity has been shown to promote the expression of cell growth regulators such as cyclin D1, c-fos, and c-myc. In addition, ΔNp63 has been shown to upregulate transcription of the cell-cycle related genes p21, 14–3-3σ, and GADD45α. Thus, the higher expression levels of nuclear STAT3 and ΔNp63 in limbal epithelium strongly suggested that STAT3/ΔNp63 pathway plays important roles in the regulation of limbal keratinocyte proliferation and differentiation.

In an established rabbit corneal epithelial cell line, RCE1, keratin pairs K5/K14, K6/K16, and K3/K12 have been shown to mimic the stage-dependent differentiation of the primary cultures of the rabbit corneal keratinocytes. In the present study, limbal explants were allowed to culture on a plastic dish for 21 days. Presumably, the outgrowth should have undergone differentiation and were K14 and K19 negative and K3 positive (Fig. 7). ΔNp63 has been proposed to be a direct enhancer of K14 expression, nevertheless, we were unable to detect K14 expression in ΔNp63-positive limbal epithelial outgrowth, or in the adeno-ΔNp63α transfected group (data not shown). The expression level of K3 remained high regardless of the expression status of STAT3 or ΔNp63. The structure of K3 promoter has been reported; however, its upstream regulator has yet to be elucidated. Our results indicated

![Figure 7](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933461/)
that STAT3 and ΔNp63 are not involved in the regulation of K3 expression during limbal keratinocyte differentiation.

In contrast to K3, the expression of involucrin was increased, and the expression of integrin β1 was suppressed significantly when ΔNp63 or STAT3 was silenced (Fig. 7B). The result suggested that the STAT3/ΔNp63 pathway plays a role in maintaining the undifferentiation phenotype of limbal keratinocyte. ΔNp63 expression has been shown to be essential for epithelial development and differentiation.53–55 and activation of STAT3 has also been shown to play a role in the regulation of epithelial differentiation.56–57 Therefore, our results are consistent with earlier reports on various epithelia that have linked STAT3 activation and ΔNp63 expression to the regulation of proliferation and differentiation of the limbal epithelial cells.

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