 Slug Is Upregulated during Wound Healing and Regulates Cellular Phenotypes in Corneal Epithelial Cells

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PURPOSE. The involvement of the epithelial mesenchymal transition (EMT) in the process of corneal wound healing remains largely unclear. The purpose of the present study was to gain insight into Slug expression and corneal wound healing.

METHODS. Slug expression during wound healing in the murine cornea was evaluated using fluorescence staining in vivo. Slug or Snail was stably introduced into human corneal epithelial cells (HCECs). These stable transfectants were evaluated for the induction of the EMT, cellular growth, migration activity, and expression changes in differentiation-related molecules.

RESULTS. Slug, but not Snail, was clearly expressed in the nuclei of corneal epithelial cells in basal lesion of the corneal epithelium during wound healing in vivo. The overexpression of Slug or Snail induced an EMT-like cellular morphology and cadherin switching in HCECs, indicating that these transcription factors were able to mediate the typical EMT in HCECs. The overexpression of Slug or Snail suppressed cellular proliferation but enhanced the migration activity. Furthermore, ABCG2, TP63, and keratin 19, which are known as stemness-related molecules, were downregulated in these transfectants.

CONCLUSIONS. It was found that Slug is upregulated during corneal wound healing in vivo. The overexpression of Slug mediated a change in the cellular phenotype affecting proliferation, migration, and expression levels of differentiation-related molecules. This is the first evidence that Slug is regulated during the process of corneal wound healing in the corneal epithelium in vivo, providing a novel insight into the EMT and Slug expression in corneal wound healing. (Invest Ophthalmol Vis Sci. 2012;53:751–756) DOI:10.1167/iovs.11-8222

The epithelial mesenchymal transition (EMT) is an important biologic event that was first defined as a process resulting in the transformation of epithelial cells into mesenchyme-like fibroblastic cells during gastrulation and somatogenesis in embryonic development.1 The Snail family of zinc finger transcription factors, Snail/SNAI1 and Slug/SNAI2, are highly conserved transcription repressors implicated in embryonic development and tumorigenesis and are considered to be key EMT-inducible transcription factors.2 Several studies have shown that EMT is also associated with wound healing, tissue regeneration, and organ fibrosis in physiologic and pathologic states.3,4 Regarding the wound healing of skin tissue, keratinocytes at wound margins recapitulate part of the EMT process, including migratory activity and reduced cell–cell adhesion.5,6 Meanwhile, Slug plays an important role in maintaining keratinocyte differentiation and homeostasis.7 A microarray analysis comparing the basal levels of gene expression in unperturbed epidermal samples from wild-type and Slug-null mice revealed that over 200 genes related to epidermal differentiation, proliferation, apoptosis, adhesion, and motility, as well as angiogenesis and responses to environmental stimuli, can be detected.8 Slug controls a key role of stem-cell function in mouse and human, suggesting that similar mechanisms are involved in cancer stem-cell properties in oncology.9 Corneal injury is a significant clinical problem that reduces the quality of vision,10 therefore, the process of corneal wound healing has been intensively investigated. The cellular and biologic process of corneal wound healing is considered to undergo the following phases: the migration of superficial cells to cover the surface of the epithelial defect area, cellular proliferation, and differentiation for reconstruction of the stratified layers.11 In the corneal epithelium, involvement of the EMT and its regulator Slug in the process of corneal wound healing remains largely unclear. Slug reportedly modulates corneal epithelial cell migration in canine eyes.12 Recently, we have demonstrated that transforming growth factor beta (TGF-β) induces EMT with the sustained upregulation of Snail and Slug through Smad and non-Smad pathways in human corneal epithelial cell (HCEC) culture in vitro.13 The results strongly suggested that the EMT process is actually induced in corneal epithelial cells and may be involved in the wound healing of corneal injuries. Based on these findings, we examined the expression of Slug during corneal epithelial wound healing in vivo and investigated the biologic role of its expression in an HCEC line.

METHODS

Wound Healing Model in Animal Study

Adult male BALB/c mice (Japan Clea, Tokyo, Japan) were used in this experiment. Animal experiments were performed in compliance with the Rules and Regulations of the Animal Care and Use Committee of the Kinki University Faculty of Medicine and were conducted in accordance with...
the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The mice were given a general anesthesia by the intraperitoneal injection of 50 mg/kg sodium pentobarbital (Kyoritsu Pharmaceutical, Tokyo, Japan) and topical 0.4% oxybuprocaine hydrochloride eye drops (Santen Pharmaceutical, Osaka, Japan) before scraping. The central corneal epithelium (5 mm in diameter) was scraped using a straight microsurgical blade (Merocel; MANI Inc., Tochigi, Japan) under a stereomicroscope (Nikon, Tokyo, Japan). The mice were euthanized at 18 or 24 hours after scraping. Other nonoperated mice were euthanized as a control. The eyes were enucleated and fixed in a commercial rapid fixative solution (Super Fix; Kurabo Industries, Osaka, Japan) and embedded in paraffin before thin sectioning (4 μm).

**Immunohistochemistry**

The sections were deparaffinized and incubated for 20 minutes at 120°C with a commercial reagent (Target Retrieval Solution ×10; Dako, Glostrup, Denmark). Then, the sections were cooled down for 30 minutes at room temperature (RT), washed three times with TNT washing buffer (0.1 M Tris-HCl, pH 7.5, containing 0.15 M NaCl and 0.05% Tween 20) and incubated for 30 minutes with a mixture of methanol (200 mL) and H2O2 (1 mL) at RT. After being washed three times with TNT washing buffer, the sections were exposed to TMB blocking buffer (0.1 M Tris-HCl, pH 7.5, containing 0.15 M NaCl, 0.5% blocking reagent (PerkinElmer, Waltham, MA)) for 1 hour at RT, then incubated with anti-Snail antibody (1:200 dilution in TMB; Cell Signaling Technology [CST], Danvers, MA) at 4°C overnight. After being washed three times with TNT washing buffer, the sections were incubated for 30 minutes at RT with anti-rabbit IgG-HRP (Histofine Simple Stain; Nichirei Biosciences, Tokyo, Japan). The signals were visualized using a commercial fluorescence system (TSA Plus; PerkinElmer) according to the manufacturer’s instructions. Slides were mounted in commercial mounting medium containing 4’,6-diamidino-2-phenylindole (DAPI) (Vectorshiled; Vector Laboratories, Burlingame, CA). The sections were observed using an immunofluorescence microscope (IX71-PAFM; Olympus, Tokyo, Japan).

**Cell Line and Culture Conditions**

The SV40-immortalized HCEC line was used in this study.12 HCECs were cultured in Dulbecco’s modified Eagle’s medium/nutrient mixture F12 (DMEM/F12; Gibco-BRL, Grand Island, NY) supplemented with 15% fetal bovine serum (FBS; Gibco-BRL) and 40 μg/mL of gentamicin (Sigma, St. Louis, MO) at 37°C in a humidified incubator with 5% CO2.

**Plasmid Construction, Viral Production, and Stable Transfectants**

The cDNA fragments encoding human full-length Snail and Slug were isolated using PCR and a commercial product to detect DNA extracts (PrimeSTAR HS DNA Polymerase; Takara Bio Inc., Otsu, Japan). The isolated using PCR and a commercial product to detect DNA extracts (MedProbe; Medprobe Systems, Gaithersburg, MD) according to the manufacturer’s recommendations. The following primers were used: Snail, forward 5′-GGA GAA TTC ACC GCC ATG CCG CAG TCT TTC CTC-3′ and reverse 5′-AGG GAG CCT CGA GGG TCA GGG GAA TCC CTC-3′; Snail, forward 5′-GGG GAA TTC GCC GGC ATG CCT CCG GCC TCC TTC TCG GTC-3′ and reverse 5′-CC CTC CAG GGT CAC TCA GTG TGC TAC AAT GCA TCC GCC-3′, respectively. The methods used in this section have been previously described.13 The stable transfectants expressing enhanced green fluorescent protein (EGFP) only or EGFP and Snail in HCECs were designated as HCEC/EGFP, HCEC/Snail, and HCEC/Slug. These stable cells were established from bulk transfected cells and not from cloned single cells.

**Fluorescence Imaging**

The methods used in this section have been previously described.14 The following antibodies were used: anti-Snail (diluted 1:250; Santa Cruz Biotechnology, Santa Cruz, CA), anti-Slug (diluted 1:250; CST), anti-vimentin (diluted 1:500; CST), anti-E-cadherin (diluted 1:500; Invitrogen, San Diego, CA), and secondary green fluorescence dye-conjugated goat anti-mouse IgG antibody (Alexa Fluor 488; Invitrogen).

**Real-Time Reverse-Transcription PCR**

The real-time RT-PCR method has been previously described.15 The following primers were used: Snail, forward 5′-TCT GAG CCC TGG CTG CTA CAA-3′ and reverse 5′-ACA TCT GAG TGG TGG ATG AGG TG-3′; Snail, forward 5′-ATG CAT ATT CGG ACC ACC ACA TTA C-3′ and reverse 5′-AGA TTT GAC CTG CTG GCA AAT GCT C-3′; Ecadherin, forward 5′-TTA AAC TCC TGG CCT CCA GGA ATC-3′ and reverse 5′-TCT TAT CAT GGG CAA AGC AAC TG-3′; N-cadherin, forward 5′-CGA ATG GAT GAA AGA AGG CCC ACC ATC-3′ and reverse 5′-AGA GCC ACT GCC TTC ATA GTC AA-3′; vimentin, forward 5′-TGA CGG GAG AAG GGT GTA GGG GAG A-3′ and reverse 5′-TAG CAG CTT CAA CCG CAA AGT TC-3′; ABCG2, forward 5′-GCA ACC ATC AAT TCA GCT GAA GA-3′ and reverse 5′-GAA ACA CAA CAC TTT GCT GCT GCA A-3′; TP63, forward 5′-GCA TTT TGG TCG TAA GGC GCT G-3′ and reverse 5′-TTC TGG AAA TCC CAC TAT CCC AAG-3′; KRT19, forward 5′-AGA GGT GAA GAT CCG CCA GGT CTG-3′ and reverse 5′-ACA ATC CGG TAC TTC TCA ATG GTG-3′; Intollarin, forward 5′-GGG AAT TAT TGT CCA GTG GCA AC-3′ and reverse 5′-AGG ATC CCA AGA CCT CCA GGA TTT A-3′; GJA1, forward 5′-CCC TAA GGA TGC TGC GTA GTC G-3′ and reverse 5′-GCA GGG ACT TAA GGA CAA TCC TC-3′; Nestin, forward 5′-CTC CAA GAA TGG AGG CTG TAG GAA A-3′ and reverse 5′-CCT ATG AGA TGG AGC CAG GA-3′; ITGB4, forward 5′-GGA AGT ACT GTG CCT GCT GCA A-3′ and reverse 5′-TCC CGC AGC ATG TAG TGG TC-3′; ITG42, forward 5′-AGA CAT CAT CAT ACA GGA GGC A-3′ and reverse 5′-TGG GTG GTA TGC CGT AAT GTA CAC AAA-3′; and GAPD, forward 5′-GCA CGG TCA AGG CTG AGA AC-3′ and reverse 5′-ATG GTG GTG AAG ACC GCA GT-3′. GAPD was used to normalize the expression levels in the subsequent quantitative analyses.

**Western Blot and Cell Proliferation Assay**

A Western blot analysis was performed as described previously.16 The antibodies used in this study were anti-Snail, anti-Slug, anti-vimentin, anti-ABCG2, anti-TP63, anti-β-actin, and HRP-conjugated secondary antibody (CST) as well as anti-E-cadherin and anti-N-cadherin (Invitrogen). The experiment was performed in duplicate. The cell proliferation was evaluated using a colorimetric assay (MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]; Kurabo Industries), as described previously.16 The experiment was performed in triplicate. The data were quantified by automated densitometry (Multigauge ver. 3.0; Fujifilm, Tokyo, Japan). The densitometric data were normalized by β-actin and the average was shown above the Western blot as a ratio of control sample.

**In Vitro Migration Assay**

The migration assay was performed using the Boyden-chamber method with chemotaxis cells (Kurabo Industries), as described previously.14 The experiment was performed in triplicate.

**Statistical Analysis**

Statistical analyses were performed using commercial spreadsheet software (Excel; Microsoft, Redmond, WA) to calculate the SD and to test for statistically significant differences between the samples using a Student’s t-test. A value of P < 0.05 was considered statistically significant.

**Results**

Slug Was Clearly Expressed in Basal Lesions of the Corneal Epithelium during Wound Healing In Vivo

We previously demonstrated that TGF-β induces the sustained upregulation of Snail and Slug through Smad and non-Smad pathways and induces EMT in HCECs.13 To date, however, no
data have been reported on the protein expression of Snail or Slug in corneal epithelial cells during wound healing in vivo. Slug was not expressed in the corneal epithelium under the unwounded resting state, whereas it was markedly expressed in the nuclei of corneal epithelial cells in basles of the corneal epithelium at 18 and 24 hours after wound generation (Fig. 1). Slug expression was decreased at 48 hours, but low-level expression was observed at least at 72 hours (data not shown). Therefore, we speculate that expression of slug may return to baseline on several days after wound generation. Snail expression was not detected (data not shown). These results strongly suggest that Slug expression is involved in the process of wound healing. We therefore examined the biologic roles of Slug overexpression using HCECs.

**Overexpression of Slug or Snail Mediates EMT in HCECs**

Stable HCEC cell lines overexpressing EGFP, EGFP and Snail, and EGFP and Slug were established and designated as HCEC/EGFP, HCEC/Snail, and HCEC/Slug, respectively. From a morphologic aspect, the EMT is characterized by an increase in scattering and an elongation of the cell shape. The phase-contrast and fluorescent microscopy findings demonstrated that the overexpression of Slug or Snail mediates cell scattering and an elongation of the cell shape (Fig. 2A). The data showed that Slug or Snail mediates EMT-like morphologic changes in HCECs. The cellular distribution of Snail or Slug was examined using immunofluorescence analysis. Both Slug and Snail protein were detected in the nuclei (Fig. 2B).

Next, we examined EMT-inducible molecules. The mRNA expression levels of Slug or Snail were markedly upregulated in HCEC/Snail and HCEC/Slug cells, as expected (Fig. 3A). Cadherin switching, the downregulation of E-cadherin and the upregulation of N-cadherin, and the upregulation of vimentin were observed in HCEC/Snail and HCEC/Slug cells. Western blotting showed the downregulation of E-cadherin and the upregulation of N-cadherin and vimentin (Fig. 3B). The results were consistent with the results of realtime RT-PCR. Phalloidin/DAPI staining for the visualization of intracellular F-actin

**FIGURE 1.** Slug expression in corneal epithelium during wound healing in vivo. The mice were given a general anesthesia and the central corneal epithelium (3 mm in diameter) was scraped using a straight microsurgery blade under a stereomicroscope. Slug expression was evaluated on the corneal tissue of unwounded animals or 18 or 24 hours after wounding. The expression levels were examined using fluorescent immunostaining. Yellow arrows, wounded area; green, Slug, blue, DAPI.

**FIGURE 2.** Overexpression of Slug or Snail mediates morphologic changes in HCECs. (A) Morphologic changes were evaluated using phase-contrast microscopy (top panels) and fluorescence microscopy (bottom panels). Stable HCEC cell lines overexpressing enhanced green fluorescent protein (EGFP), EGFP and Snail, and EGFP and Slug were established and designated as HCEC/EGFP, HCEC/Snail, and HCEC/Slug, respectively. Arrows: the cells that have a morphologic change. (B) Overexpression of Slug or Snail was observed in the nuclei using fluorescence immunostaining for Snail (left) or Slug (right).
enabled the rearrangement of the cytoskeletal organization from cell-cell borders into stress fibers in response to the overexpression of *Slug* or *Snail* (Fig. 3C). The downregulation of E-cadherin in the cellular membrane and the upregulation of vimentin in the cytoplasm were observed in HCEC/Snail and HCEC/Slug cells using an immunofluorescence analysis (Fig. 3C). Collectively, the overexpression of *Slug* or *Snail* mediates the typical EMT in HCECs.

**Overexpression of Slug or Snail Suppresses Cellular Proliferation and Enhanced Migration in HCECs**

We examined whether the overexpression of *Slug* or *Snail* affected the cellular proliferation in HCECs using an MTT assay. Interestingly, Snail and Slug significantly suppressed the cellular proliferation with a 49% reduction and a 32% reduction at 72 hours, respectively (Fig. 4A). In addition, a migration assay using the Boyden-chamber method revealed that the overexpression of *Slug* or *Snail* significantly enhanced cellular migration in both cell lines (Fig. 4B). Overexpression of *Snail* or *Slug* enhanced the wound healing (data not shown). These results showing the decreased proliferation and increased invasion of *Snail* transfectants were consistent with a recent report.17

**Overexpression of Slug or Snail Regulated the Expression of Differentiation-Related Molecules**

Since the corneal epithelium is a highly differentiated and unique tissue among all the bodily tissues, changes in the cellular differentiation of corneal epithelial cells during wound healing are of interest. In addition, a recent study has demonstrated that the induction of the EMT in human mammary epithelial cells results in the acquisition of mesenchymal traits and the expression of stem-cell markers.18 Therefore, we investigated whether *Slug* or *Snail* expression regulates the expression of differentiation-related molecules including the ATP-binding cassette, subfamily G, member 2 (*ABCG2*), tumor protein p63 (*TP63*), and keratin 19 (*KRT19*), which are regarded as stemness markers in the corneal epithelium.19–21 Genes involved in differentiation were also examined, including gap junction protein, alpha 1 (*GJA1*); also known as connexin 43), Nestin, integrin, beta 4 (*ITGB4*), integrin, alpha 2 (*ITGA2*), and Involucrin. The overexpression of *Slug* or *Snail* significantly downregulated the mRNA levels of the stemness markers *ABCG2*, *TP63*, and *KRT19* (Fig. 5A). *GJA1* and *Nestin* were upregulated by Snail or Slug induction; however, *ITGB4*, *ITGA2*, and *Involucrin* were not upregulated, indicating that *Snail* or *Slug* induces *ABCG2*, *TP63*, and *KRT19* downregulation but that its involvement in differentiation is not clear and
needs further study. The suppression of ABCG2 in HCEC/Slug cells and that of TP63 in HCEC/Slug and HCEC/Snail cells were observed using Western blotting (Fig. 5B).

**DISCUSSION**

We have shown that Slug expression was overexpressed during wound healing in murine corneal epithelium. These data strongly suggest that the EMT is induced in corneal epithelial cells during wound healing and is involved in the process. TGF-β signaling is activated in the cornea after wounding,22,23 and the expression levels of TGF-β in the tear film are dramatically increased after a photorefractive keratectomy.24 In addition, our previous data showed that TGF-β induces the sustained upregulation of Slug and Snail in HCECs.13 Combining this previously acquired evidence and the present data, we hypothesized that wounding stimulates TGF-β signaling in corneal epithelial cells, mediating Slug expression and the EMT during wound healing, although further investigation is necessary.

Slug expression in the resting state in uninjured normal cornea was scarcely detected but was markedly upregulated during wound healing. Since the overexpression of Slug or Snail enhanced cell migration ability (Fig. 4B), the upregulation of Slug may contribute to wound healing with increasing cell migration. Meanwhile, we found that Slug overexpression inhibited cellular proliferation in HCECs (Fig. 4A). The forced expression of Slug in prostate cancer cells led to the inhibition of proliferation with G1 cell-cycle arrest in cancer cells.25 Moreover, two of the HepG2 clones overexpressing Snail (i.e., S7 and S15) had a scattered fibroblastic morphology and acquired a higher motility than that of parental HepG2. The proportion of the G0/G1 phase in the S7 and S15 clones was higher than that of parental HepG2.26 Based on these studies, the Slug-mediated inhibition of cellular proliferation in corneal epithelial cells is thought to induce G0/G1 phase arrest.

A direct link between the EMT and the gain in epithelial stem-cell properties has been proposed.18 Unexpectedly, our present data showed that the overexpression of Slug or Snail directly downregulated the expression levels of stemness-related molecules in corneal epithelial cells. Consistent with our results, Slug directly downregulated TP63 transcription in human squamous cell carcinoma.27 On the other hand, the corneal differentiation-related gene Nestin, a specific marker for neural cells expressed only in the corneal epithelial cells but not in limbal basal cells,28 was markedly upregulated, whereas Involucrin, a structural protein expressed in differentiated human keratinocytes, was markedly downregulated. The heterodimeric transmembrane glycoprotein ITGA2 was also downregulated by the overexpression of Slug or Snail. These results suggest that the overexpression of Slug or Snail in corneal epithelial cells confers a heterogeneous effect on the gene expression of stemness and differentiation markers. Because EMT is a cellular process with a dramatic remodeling of the cytoskeleton and a losing of cell–cell contacts,29,30 the process of EMT may affect these gene expressions, whereas Snail or Slug may directly modulate the gene expressions as target genes. Further investigation of the EMT in corneal epithelial cells is warranted.

In conclusion, we found that the overexpression of Slug was observed during healing in corneas in vivo. The overexpression of Slug or Snail mediated the EMT, inhibited proliferation, enhanced cellular migration, and downregulated stemness-related markers in HCECs. These findings indicated that the EMT may be involved in wound healing in corneal epithelial cells.

**Acknowledgments**

The authors thank Shinji Kurashimo, Yoshitaka Horiuchi, Katsumi Okumoto, and Tomoko Kitayama for their technical assistance.
References


