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ORIGINAL ARTICLE

# Upregulation of Slug expression by cyclosporine A contributes to the pathogenesis of gingival overgrowth

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**KEYWORDS**
cyclosporine A;
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Slug

*Background/Purpose:* Gingival overgrowth occurs as a side effect of systemic medication with immunosuppressant cyclosporine A (CsA). Slug, a master regulator of epithelial–mesenchymal transition, is dramatically upregulated in a variety of fibrotic diseases. The aim of this study is to investigate the role of epithelial–mesenchymal transition marker Slug in the pathogenesis of CsA-induced gingival overgrowth.
*Methods:* Clinically healthy gingiva and CsA-induced gingival overgrowth specimens were analyzed by immunohistochemistry. The effect of CsA on normal human gingival fibroblasts (HGFs) was used to elucidate whether Slug expression could be affected by CsA by real-time reverse transcription-polymerase chain reaction and western blot. Cell proliferation in CsA-treated HGFs with Slug lentiviral-mediated shRNAi knockdown was evaluated by tetrazolium bromide reduction assay.
*Results:* Slug expression was higher in CsA-induced gingival overgrowth specimens than in clinical healthy gingiva ($p < 0.05$). Slug expression was significantly higher in CsA-induced gingival overgrowth specimens with higher levels of inflammatory infiltrates ($p < 0.05$). CsA was found to increase Slug transcript and protein expression in HGFs in a dose-dependent manner ($p < 0.05$). In addition, knockdown of Slug significantly suppressed CsA-induced cell proliferation in HGFs ($p < 0.05$).

Conflicts of interest: The authors have no conflicts of interest relevant to this article.
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*Conclusion:* Taken together, upregulation of Slug in HGFs stimulated by CsA may play an important role in the pathogenesis of CsA-induced gingival overgrowth.
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## Introduction

Gingival overgrowth is a common side effect of the chronic use of immunosuppressive drug cyclosporine A (CsA). Etiological factors causing and underlying gingival overgrowth have been reviewed, and it was determined that local, systemic, and genetic factors may also contribute to its development and progression.[1] Histological change of gingival overgrowth is characterized by fibrotic or expanded connective tissues as well as by an enlarged gingival epithelium with different degrees of inflammation.[2] CsA-induced gingival overgrowth may result in dysregulation of connective tissue hemostasis, which interferes with fibroblast proliferation[3] with an increased accumulation of extracellular matrix.[4–10] However, pathologic mechanisms of CsA-induced gingival overgrowth still need to be further clarified.

Epithelial–mesenchymal transition (EMT), a transdifferentiation program that converts adherent epithelial cells into individual migratory cells, is involved in embryonic development and disease development including drug-induced gingival overgrowth.[11,12] Slug is a well-characterized member of the Snail superfamily, and human Slug has been demonstrated recently, which functions as a transcriptional repressor by specifically binding to the E-box motif. Slug is a key regulator of the EMT of mesodermal cells.[13] Several experimental and clinical studies have revealed that overexpression of Slug leads to a loss of E-cadherin expression and has also been implicated in pathological alterations of the epithelial-to-mesenchymal cell phenotype. Recently, Slug has been found to be overexpressed in keloid[14] and Crohn's disease-associated intestinal fibrosis.[15] However, Slug-mediated molecular mechanisms in CsA-induced gingival overgrowth still remains to be elucidated.

Recently, chlorhexidine mouthwash[16] and epigallocatechin-3-gallate[17] were found to be the adjuvants for the amelioration or prevention of CsA-induced gingival overgrowth. In this study, we explore a possible role of Slug in the pathogenesis of CsA-induced gingival overgrowth. Slug expression was demonstrated to play a major role in the pathogenesis of CsA-induced gingival overgrowth. In addition, Slug may serve as a new therapeutic target for the treatment of CsA-induced gingival overgrowth in the future.

## Methods

### Immunohistochemistry

Following approval by the Institutional Review Board of the Chung Shan Medical University Hospital, Taichung, Taiwan, surgically removed gingival tissues were fixed with 10% buffered formalin overnight; the specimens were dehydrated in an ascending series of graded alcohols and embedded in paraffin. Sections (5 μm) were stained with the monoclonal anti-Slug antibody (sc-15391; Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:100 dilution) using a standard avidin–biotin–peroxidase complex method. The compound 3-amino-9-ethylcarbazole (DAKO, Carpinteria, CA, USA) was then used as the substrate for localizing antibody binding. Negative controls included serial sections from which either primary or secondary antibodies were excluded. The preparations were counterstained with hematoxylin, mounted with Permount (Merck, Darmstadt, Germany), and examined by light microscopy. In addition, one section from each CsA-induced gingival overgrowth specimen was stained with hematoxylin and eosin to evaluate the magnitude of inflammation at the histological level, as described previously.[5,6]

### Cell cultures

Five healthy individuals were selected from the crown lengthening procedure for this study. HGFs were cultured using an explant technique, as described previously.[4,8]

### Slug expression analysis

HGFs were arrested in $G_0$ by serum deprivation according to our previous experiments.[7] Nearly confluent monolayers of HGFs were washed with a serum-free medium and immediately thereafter exposed, at indicated incubation times, to 0 ng/mL, 100 ng/mL, 200 ng/mL, 500 ng/mL, and 1000 ng/mL CsA (Sigma-Aldrich, St Louis, MO, USA). Cell lysates were collected at 48 hours for real-time reverse transcription-polymerase chain reaction (RT-PCR) and western blot assays. Cultures without fetal calf serum (FCS) were used as negative control.

### Real-time RT-PCR

Total RNA of cells was purified using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to our previous publication.[12] The following Slug primers (NM_003068) were designed: forward: GTGATTATTTCCCCGTATCTCTAT; reverse: CAATGGCATGGGGGTCTGAAAG. The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) housekeeping gene was amplified as a reference standard. The following *GAPDH* primers (NM_002046) were designed: *GAPDH* (forward): CATCATCCCTGCCTCTACTG; *GAPDH* (reverse): GCCTGCTTCACCACCTTC.

## Western blot

Extraction of proteins from cells and immunoblotting analysis were performed as described previously.[8,9]

## Slug knockdown in CsA-treated HGFs by lentiviral-mediated shRNAi

The pLV-RNAi vector was purchased from Biosettia Inc. (San Diego, CA, USA). The method of cloning the double-stranded shRNA sequence is described according to our recent publication.[12] Lentiviral vectors expressing short hairpin RNA (shRNA) that targets human Slug (sh-Slug-1: 5′-AAAAGCATACCACAAATGCAATATTGGATCCAATATTGCATTTGTGGTATGC-3′; sh-Slug-2: 5′-AAAAGCCCAGTCTAGAAAATCTTTTGGATCCAAAAGATTTTCTAGACTGGGC-3′) were synthesized and cloned into pLVRNAi to generate a lentiviral expression vector. The shRNA that targets luciferase (sh-Luc: 5′-CCGGACTTACGCTGAGTACTTCGAACTCGAGTTCGAAGTACTCAGCGTA-3′) was utilized for an experimental control.

## Cell proliferation

HGFs placed in 96-well plates were washed with phosphate-buffered saline, and the medium was replaced with Dulbecco's modified Eagle's medium (DMEM) for serum starvation overnight. After treatment with 200 ng/mL CsA for 48 hours, cell proliferation was tested using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay kit (Sigma-Aldrich) as described previously.[12]

## Statistical analysis

Three replicates of each experiment were performed for each test. All assays were repeated three times to ensure reproducibility. The correlation between Slug expression and the grade of inflammation in CsA-induced gingival overgrowth specimens was analyzed using Fisher's exact test. The significance of the results obtained from control and CsA-treated HGFs was statistically analyzed by one-way analysis of variance. Differences between the treatments were analyzed by Duncan's test. A $p$ value $< 0.05$ was considered to be statistically significant.

## Results

Slug staining in gingival tissue was stronger in the CsA-induced gingival overgrowth group than in the clinical healthy gingival group ($p < 0.05$). Figure 1A represents gingival tissue obtained from the clinical healthy gingival group with faint Slug expression. In the CsA-induced gingival overgrowth group, Slug expression was observed mainly in the cytoplasm of fibroblasts, epithelial cells, and inflammatory cells (Figure 1B).

As shown in Table 1, rank orders of cells positively stained by Slug in the CsA-induced gingival overgrowth group were as follows: inflammatory cells (100%) > fibroblasts (84.6%) > epithelial cells (30.8%). Slug positive staining in the clinical healthy gingival group was occasionally seen in fibroblasts and epithelial cells (Table 1). No expression of Slug was detected in the negative control gingival specimens.

Slug expression in CsA-induced gingival overgrowth specimens with low or high levels of inflammation is listed in Table 2. A significantly greater Slug expression was noted in CsA-induced gingival overgrowth tissues with high levels of inflammation, as analyzed using Fisher's exact test ($p = 0.038$).

HGFs were treated with CsA, and the levels of Slug transcript and protein were measured by RT-PCR and western blot. As shown in Figure 2, CsA was found to increase Slug transcript in HGFs in a dose-dependent manner

**Table 1** Localization and number of cells expressing Slug in four clinical healthy gingival specimens and 13 cyclosporine A-induced gingival overgrowth specimens.

| | Epithelial cells | Fibroblasts | Inflammatory cells |
|---|---|---|---|
| Clinical healthy gingival specimens | 1 (25) | 1 (25) | 0 (0%) |
| CsA-induced gingival overgrowth specimens | 4 (30.8) | 11 (84.6) | 13 (100) |

Data are presented as $n$ (%).
CsA = cyclosporine A.

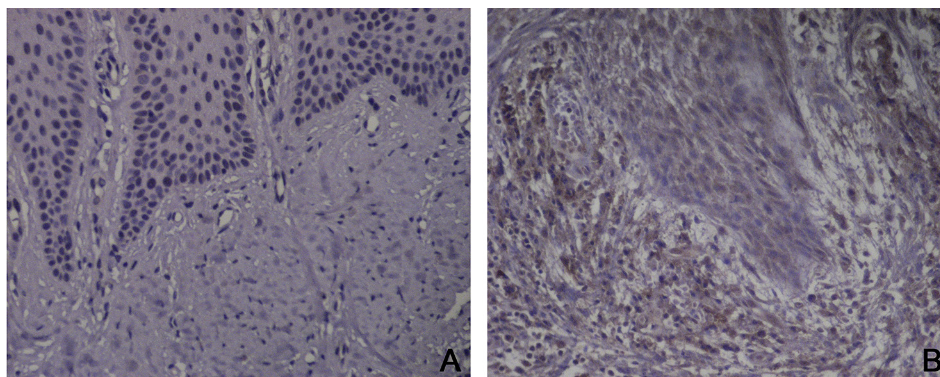


**Figure 1** (A) Very faint immunoreactivity of Slug is observed in normal human gingival tissue (200×). (B) Strong immunostaining for Slug is noted in the CsA-induced gingival overgrowth specimen (200×). CsA = cyclosporine A.

**Table 2** Results of Slug expression and the grade of inflammation in CsA-induced gingival overgrowth tissues.[a]

| | Inflammation high | Inflammation low |
|---|---|---|
| Slug high | 10 | 0 |
| Slug low | 1 | 2 |

CsA = cyclosporine A.
[a] A significantly greater Slug expression was noted in CsA-induced gingival overgrowth tissues with high levels of inflammation as compared with tissues with low levels of inflammatory cell infiltrates by Fisher's exact test ($p = 0.038$).

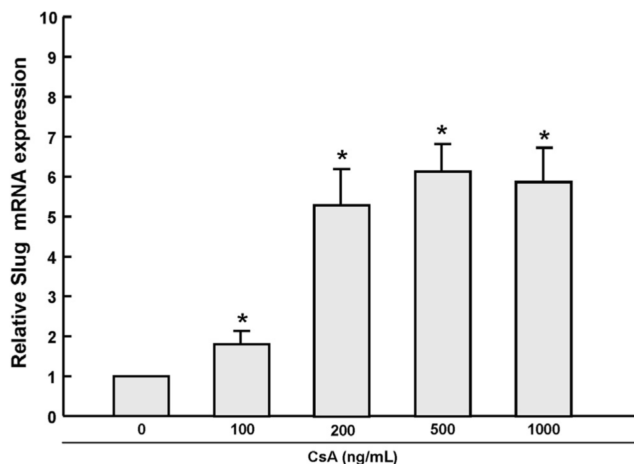


**Figure 2** Slug mRNA expression is examined by RT-PCR. HGFs were exposed for 48 hours in a medium containing various concentrations of CsA as indicated. The relative Slug mRNA expression represents the mean ± SD. * Significant difference from control values with $p < 0.05$. CsA = cyclosporine A; HGF = human gingival fibroblast; RT-PCR = reverse transcription-polymerase chain reaction; SD = standard deviation.

($p < 0.05$). In addition, CsA was found to upregulate Slug protein expression in a dose-dependent manner ($p < 0.05$; Figure 3A). Using the AlphaImager 2000 (Alpha Innotech Corp., San Leandro, CA, USA), the levels of the Slug protein were found to increase about 1.6-, 3.2-, 3.0-, and 2.8-fold after exposure to 100 ng/mL, 200 ng/mL, 500 ng/mL, and 1000 ng/mL CsA, respectively (Figure 3B).

To investigate the role of Slug in CsA-induced gingival overgrowth, downregulation of Slug in CsA-treated HGFs was achieved by viral transduction with the lentiviral vector expressing shRNA targeting Slug. In addition, the lentiviral vector expressing sh-Luc was used as a control. RT-PCR and western blot were used to confirm the downregulation of Slug expression by its shRNA. RT-PCR demonstrated that lentivirus expressing both sh-Slug-1 and sh-Slug-2 markedly reduced the level of CsA-induced Slug expression in HGFs (Figure 4A). Western blot also demonstrated that the knockdown of Slug could reduce Slug expression in CsA-stimulated HGFs (Figure 4B).

In functional assays, we constructed lentivirus vector with Slug shRNA and infected HGFs. As shown in Figure 5, MTT results showed that Slug knockdown significantly reduced cell proliferation of HGFs stimulated with 200 ng/mL CsA ($p < 0.05$).

## Discussion

Previously, it was reported that EMT likely occurs in drug-induced gingival overgrowth including being induced by CsA with diminished E-cadherin and elevated levels of FSP-1 and fibronectin.[11] Slug is a transcriptional repressor of E-cadherin, and its upregulation occurs in EMT. In this study, to the best of our knowledge, we first found that Slug expression is upregulated in CsA-induced gingival overgrowth specimens compared with clinical healthy gingival tissues. Slug was detected in fibroblasts, epithelial cells, and inflammatory cells. Taken together, our results suggest that EMT is a biological process that may contribute to CsA-induced gingival overgrowth via the Slug pathway.

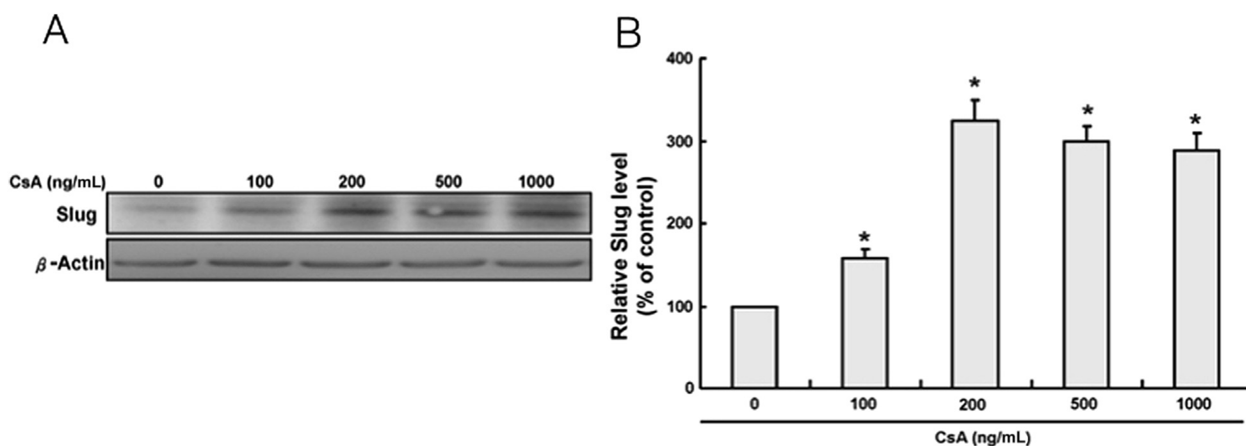


**Figure 3** CsA dose dependently induced Slug expression in HGFs. (A) HGFs were serum starved and treated with indicated concentrations of CsA for a further 48 hours in a serum-free medium. The expression of Slug was detected by western blot; β-actin was used as a protein loading control. (B) Protein levels of Slug stimulated by CsA in HGFs were measured with a densitometer. The relative level of Slug protein expression was normalized against β-actin signal, and the control was set as 1.0. Optical density values represent the mean ± SD. * Significant difference from control values with $p < 0.05$. CsA = cyclosporine A; HGF = human gingival fibroblast; SD = standard deviation.

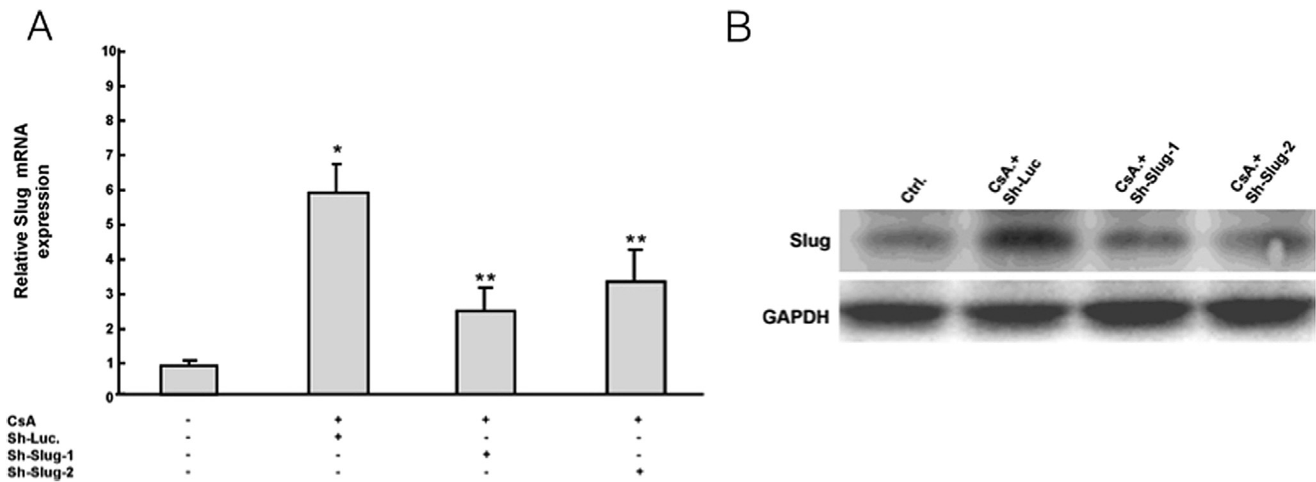


**Figure 4** The silencing effect of Slug shRNA in CsA-treated HGFs was validated transcriptionally by RT-PCR and western blot. Single-cell suspension of HGFs was transduced with sh-Luc or sh-Slug lentivirus, individually or concurrently, and treated with or without CsA (200 ng/mL) as indicated. (A) Result of RT-PCR. The relative Slug mRNA expression represents the mean $\pm$ SD. (B) Western blotting also demonstrated that knockdown of Slug significantly reduced Slug expression in CsA-stimulated HGFs. GAPDH was used as a protein loading control. *$p < 0.05$, sh-Luc + CsA group versus control group. **$p < 0.05$, sh-Slug-1 + CsA or sh-Slug-2 + CsA versus sh-Luc + CsA group. CsA = cyclosporine A; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; HGF = human gingival fibroblast; RT-PCR = reverse transcription-polymerase chain reaction; SD = standard deviation.

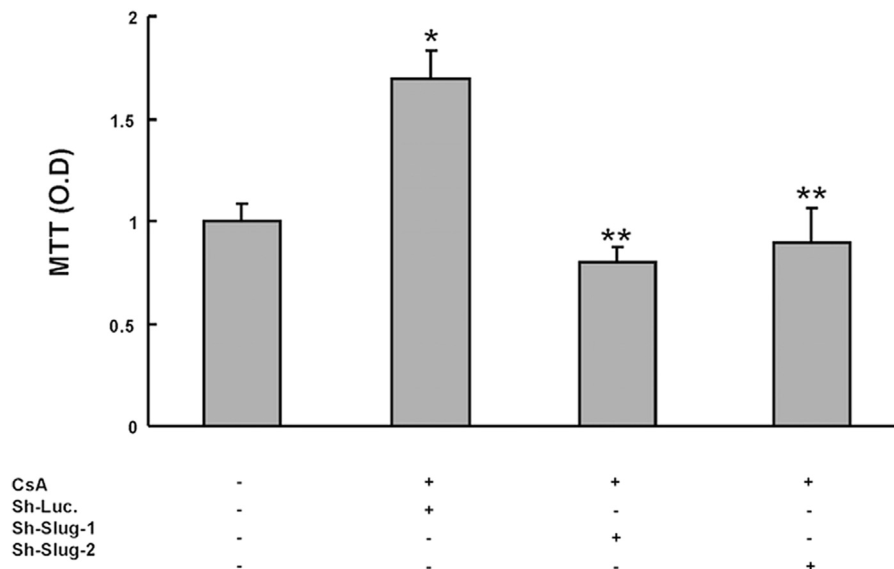


**Figure 5** Silencing of Slug expression suppresses 200 ng/mL CsA-stimulated cell proliferation capabilities of HGFs. Cell viability of the control or Slug-knockdown CsA-treated HGFs was evaluated by an MTT assay. Cells were exposed for 48 hours in a medium containing various concentrations of CsA as indicated. *$p < 0.05$, sh-Luc + CsA group versus control group. **$p < 0.05$, sh-Slug-1 + CsA or sh-Slug-2 + CsA versus sh-Luc + CsA group. CsA = cyclosporine A; HGF = human gingival fibroblast; MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

In this study, the expression of Slug was also shown to increase with the grade of inflammation in CsA-induced gingival overgrowth specimens. The reason for this finding is not clear. It has been reported that bacterial plaque and the resulting gingival inflammation are factors that promote gingival overgrowth significantly.[18] Inflammatory mediators are believed to contribute to the pathogenesis of periodontal diseases. Tumor necrosis factor-$\alpha$ was found to upregulate Slug protein expression in human breast carcinoma cell line MCF-7 cells.[19] Recently, interleukin-1$\beta$ was also shown to increase Slug expression in human oral squamous cell carcinoma cell line TW2.6 cells.[20] Therefore, inflammatory cytokines secreted by infiltrated inflammatory cells and fibroblasts might provide an inflammatory microenvironment to promote EMT in the pathogenesis of CsA-induced gingival overgrowth.

Transforming growth factor-$\beta$1 (TGF-$\beta$1) is a key regulator of the biochemical mechanisms associated with the pathogenesis of CsA-induced gingival overgrowth.[21] The Snail family of transcriptional repressors (Snail and Slug) is

a key downstream target of TGF-β1 that regulates transcriptional E-cadherin repression.[22] In addition, Slug mRNA and protein levels were found to be significantly upregulated by TGF-β1 in primary human gingival epithelial cells.[11] Therefore, the CsA-induced Slug mRNA and protein expression in this study could be the effects of CsA-induced TGF-β1 expression. Therefore, the interaction between TGF-β1 and Slug is worthy of further investigation.

Fibroblasts are the principal cell type residing in connective tissue and are the cells responsible for the formation and turnover of the extracellular matrix. Fibroblast function is, in turn, regulated by bioactive molecules acting in local tissue environment. To the best of our knowledge, this study was the first to report that CsA upregulated Slug mRNA and protein expression in HGFs. In addition, RT-PCR and western blot confirmed that lentivirus expressing both sh-Slug-1 and sh-Slug-2 markedly reduced the expression level of CsA-induced Slug transcript and protein expression in HGFs. Previously, CsA was found to increase the expression of Slug protein in human choriocarcinoma cell line JEG-3 cells.[23] CsA was also found to increase Slug gene and protein expression in human HK2 renal tubular cells.[24] Taken together, induction of Slug expression by CsA is not cell type specific. These findings suggest that one of the pathogenic mechanisms of CsA-induced gingival overgrowth may be the induction of Slug expression by resident cells in response to CsA challenge.

Increased fibroblast proliferation by CsA in HGFs has been characterized by the development and progression of CsA-induced gingival overgrowth.[21] In this study, knockdown of Slug was first found to suppress CsA-stimulated cell proliferation ability in HGFs. Similar results were reported in the nonsmall cell lung cancer cell line A549 that knockdown of Slug expression could suppress lung cancer cell proliferation.[25] Furthermore, the present *in vitro* findings suggest that the downregulation of Slug may represent a novel targeted therapy for potential antifibrosis in CsA-induced gingival overgrowth.

In conclusion, to our knowledge, this is the first systematic attempt to evaluate the role of Slug expression in CsA-induced gingival overgrowth in humans both *in vivo* and *in vitro*. We have demonstrated that Slug is elevated in CsA-induced gingival overgrowth compared with clinical healthy gingival tissues. Data from our *in vitro* experiments showed that CsA was capable of stimulating Slug mRNA and protein expression in HGFs. Knockdown of Slug expression significantly suppressed HGFs cell proliferation. The downregulation of Slug may represent a novel targeted therapy for CsA-induced gingival overgrowth.

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